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Biomedical Chemistry: Current Trends and Developments

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Managing Editor: Anna Rulka

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Preface

Biomedical Chemistry focuses on creating molecules that help advance the understanding and treatment of diseases. Basic chemical ideas and determination of disease etiology are approached by developing techniques to ensure optimum interaction between drugs and human cells.

This book provides readers with an understanding of how fundamental chemical concepts are used to combat some diseases. The authors explain the interdisciplinary nexus of chemistry with biology, physics, pharmacy and medicine. The results of chemical research can be applied to understand chemical processes in cells and in the body, and new methods for drug transportation.

Biomedical Chemistry: Current Trends and Developments is an excellent resource for students and researchers in health-related fields with frontier topics in medicinal and pharmaceutical chemistry, organic chemistry and biochemistry.

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Section 1: **Chemical Principles in Drug Design and Discovery**

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1.1 Functional Groups of Biomolecules and their Reactions

Abstract: This chapter starts with a general introduction on some concepts needed to understand the reactivity of organic functional groups. Elementary reaction mechanisms are then presented according to their functionality with relevant biological examples. These reactions explain the vast majority of transformations involving biomolecules. Finally, two examples on the application of the presented concepts are given, namely the metabolism of fatty acids and reactivity of penicillin. Both of these examples call for various types of reactions showing the diversity and simplicity of biological transformations when analysed step by step.

1.1.1 Functional Groups in Biological Systems

The main definition of a functional group in organic chemistry books is as a chemically reactive group of atoms within a molecule that contribute to its characteristic reactivity. Functionality is usually regarded as *"implying the presence of heteroatoms and/or unsaturation, but it would not be helpful to attempt to define precisely the limits of application of the term"* (IUPAC, Commission on Nomenclature of Organic Chemistry, 1993).

Functional group reactivity may be changed by the presence of other neighbouring functional groups but usually behaves uniformly in every molecule where it can be found. There are several common functional groups that are related to families of organic compounds according to their structural features. However, from those functional groups only a few are found in biological systems (Table 1.1.1). The types of bonding found in these functional groups may be explained by the existence of various hybrid atomic orbitals of the carbon atom created from combination of the one *2s* and the three *2p* orbitals (Table 1.1.2).

Table 1.1.1: Common functional groups present in biomolecules. In parentheses are the names of the families of compounds, where the group has the highest priority in the compound.

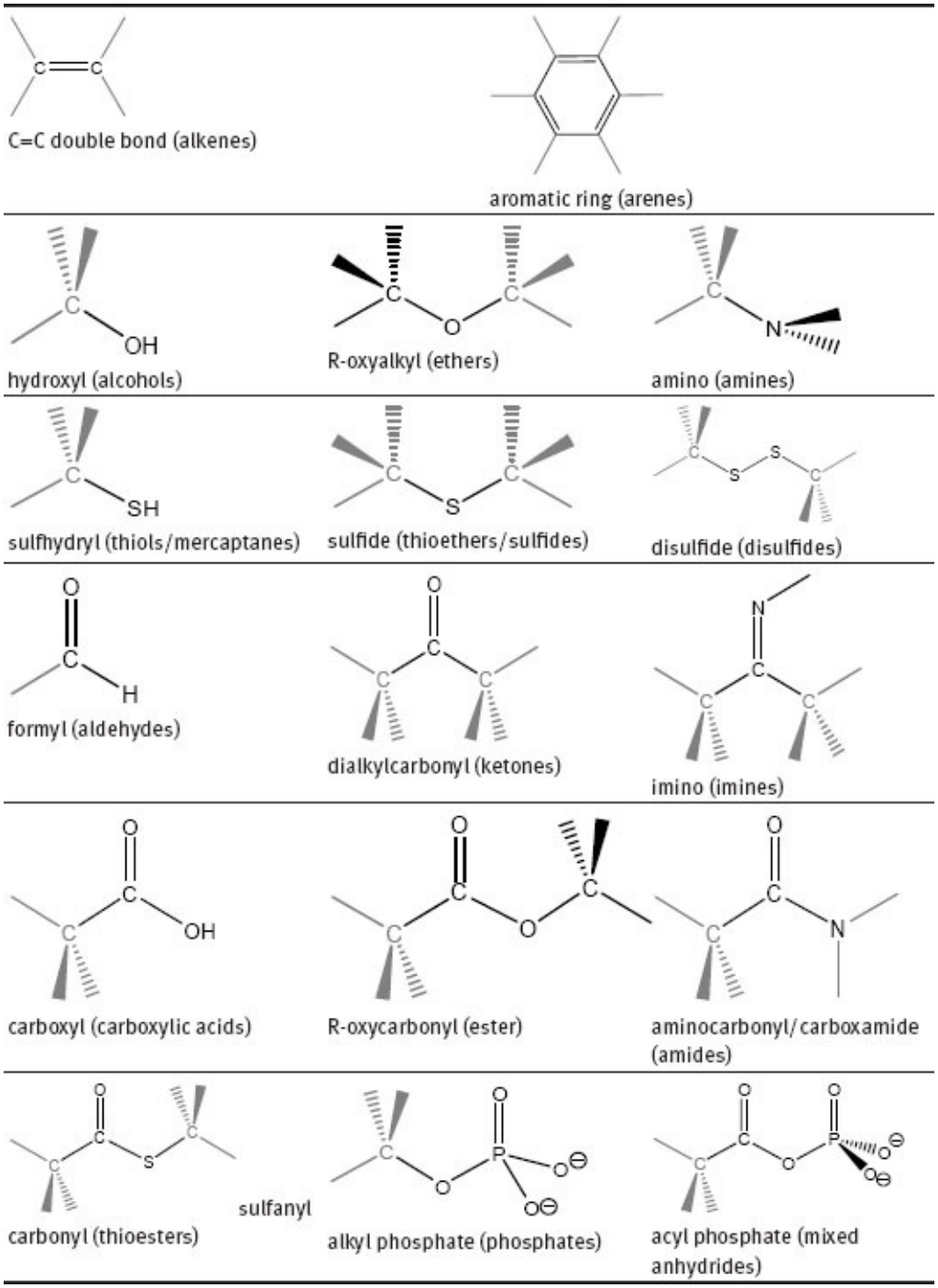
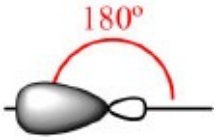


Table 1.1.2: Possible orbital combinations in the carbon atom, their spatial configuration and geometry with inter-bond angles.

Combination	Hybrid result	Geometry
1 x 2*s* + 3 x 2*p*	4 x sp^3	

		
1 x 2*s* + 2 x 2*p*	3 x sp^2	tetrahedral 
1 x 2*s* + 2 x 2*p*	2 x *sp*	trigonal planar  linear

All of the hybrid orbitals may be used to form *σ* (sigma) molecular orbitals by fusion with *s* or with other hybrid orbitals from other atoms (according to Molecular Orbital Theory). If there are remaining *2p* orbitals in the carbon atoms (in the case of sp^2 and *sp* hybrid orbitals) they are used to form *π* (pi) molecular orbitals by lateral combination with other adjacent *2p* orbitals (Fig. 1.1.1). A simple bond is formed with a single *σ*-bond; the double bond is formed with a *σ*-bond and a *π*-bond; the triple bond is formed with a *σ*-bond and two *π*-bonds.

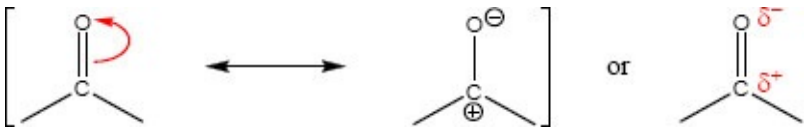
Figure 1.1.1: A simplified representation of the spatial distribution of two adjacent p orbitals (a) and their combination into a π bond (b) between two sp^2 carbon atoms. The π-bonds in a triple bond would be along orthogonal planes to each other.

These hybridisations have several consequences, such as the electron density of a *π*-bond lying above and below the plane of the bonding atoms (Fig. 1.1.1), resulting in greater exposure for a reaction. Simultaneously, with the increasing *s* character of the hybrid orbital:

– the formed bond length decreases;

– the polarity of a C-H bond increases;
– breaking a bond between carbon and a more electronegative atom is more difficult (*e.g.* the C-O bond in isopropanol is easier to cleave than the C-O bond in isopropenol).

The electronegativity of an element can also be an important factor to explain some functional groups reactivity. For instance, alcohols, ethers, amines, thiols, sulfides, disulfides and phosphates (Table 1.1.1) all have a carbon forming a single bond with a more electronegative atom, causing the carbon to bear a partial positive charge (δ^+). These modifications affect both the σ- and π-bonds, although in the case of some π-bonds the resonance effect should also be considered. The carbonyl group can be classically treated as a resonance hybrid represented by two resonance structures (Scheme 1.1.1), which contributes to the reactivity of the compounds that have this functional group (aldehydes, ketones, carboxylic acids, esters, thioesters, amides, acyl phosphates). Resonance is possible whenever movement of electrons are allowed within the same molecule without movement of atoms.



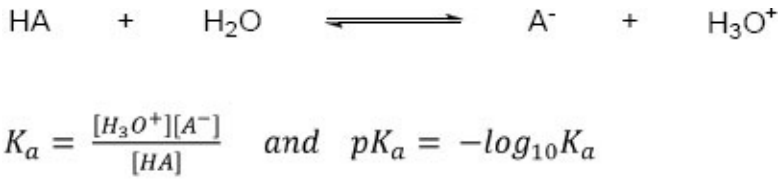
Scheme 1.1.1: Carbonyl group resonance structures and equivalent notation, explicitly showing the bond polarity with partial charges.

1.1.2 Acids and Bases Versus Electrophiles and Nucleophiles

Acids and bases are very important in biological transformations, as most require some form of acid or basic catalysis to occur. The simplest acid-base theory is the Brønsted-Lowry theory, which states that acids are molecules that donate protons (hydrogen ions, H^+) and bases/alkalis are molecules that accept protons. For example, a carboxylic acid can donate a proton to a base, such as an amine, in a reversible proton-transfer reaction (Scheme 1.1.2).

Scheme 1.1.2: Example of a proton transfer reaction. This specific reaction explains why it is difficult for condensations to happen directly between an amine and a carboxylic acid, as the non-ionic forms of these molecules are more reactive (Chapter 1.1.4.5).

Acids can differ in their ability to donate protons, being classified as strong or weak according to the extent of deprotonation. A strong acid will have a stable conjugate base (or weak conjugate base), resulting in ready donation of a proton. Table 1.1.3 lists the acidity of some typical functional groups (water and ammonium acidity are also given for comparison). The acidity is measured by the acidity constant, K_a or by its pK_a (Scheme 1.1.3), where a stronger acid has a smaller pK_a and a weaker acid has a larger pK_a. The same approach can be applied to bases and their strength.



Scheme 1.1.3: Acidity constant and pK_a.

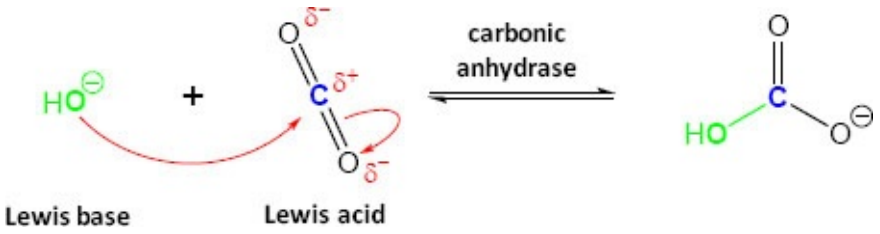
The problem with the Brönsted/Lowry definition is that it only covers the compounds that donate or accept protons. The more general and widely used model is the Lewis definition. A Lewis acid is a molecule that accepts a pair of electrons and a Lewis base is a molecule that donates a pair of electrons. To accept electrons, a Lewis acid must have a vacant low-energy orbital. As a consequence, many species, including H^+ itself, metal cations such as Mg^{2+} and Zn^{2+}, and neutral species such as boron trifluoride (BF_3) and carbon dioxide (CO_2) are Lewis acids.

Lewis acids and bases are involved in many biological reactions, such as the transformation of carbon dioxide into hydrogen carbonate (Scheme 1.1.4). Lewis bases use unshared electrons to form new bonds with other atoms and are usually referred as nucleophiles (“nucleus-loving”, versus Lewis acids as electrophiles, “electron-loving”). The terms “electrophile” and “nucleophile” are commonly used in organic

and bioorganic transformations. Electrophiles are either positively charged or neutral and have at least one positively polarised, electron-poor atom. Conversely, nucleophiles are either negatively charged or neutral and have a lone pair of electrons that can be donated.

Table 1.1.3: Relative acidity strengths of some functional groups and common reactants.

Functional group	Example	pK_a
carboxylic acid	CH_3COH (O)	4.76
ammonium	NH_4^+	9.26
alkylthiol	CH_3SH	10.3
alkylammonium ion	$CH_3NH_3^+$	10.66
β-keto ester	$CH_3CCH_2COCH_3$ (O, O)	10.6
water	H_2O	15.74
alcohol	CH_3CH_2OH	16.0
ketone	CH_3CCH_3 (O)	19.3
thioester	CH_3CSCH_3 (O)	21
ester	CH_3COCH_3 (O)	25



Scheme 1.1.4: Example of a biochemical reaction involving a Lewis acid and a Lewis base. Reversible conversion of carbon dioxide to hydrogen carbonate can occur rapidly in the active site of a carbonic anhydrase, the equilibrium being regulated by factors such as pH.

1.1.3 Stereoisomerism and Chirality

Stereoisomers having the spatial orientation of bonds as their unique

difference, maintaining the same composition of atoms and bonds.

1.1.3.1 *Cis/trans* Isomerism

Cis/trans stereoisomerism exists in alkenes (double bonds) and saturated rings (cycloalkanes), the latter because the π-bonds and σ–bonds of saturated rings cannot rotate freely. As a consequence, a group in one side of the double bond or saturated ring cannot change sides without breaking bonds. This causes different properties in compounds with opposite configurations. A double bond presents a *cis*-configuration if equal substituents are on the same side and a *trans*-configuration if they are on opposite sides (Fig. 1.1.2). This type of isomerism is particularly relevant for the physical properties of compounds, with differences being greater if polar groups are present. Reactivity is also affected, as *cis* compounds tend to have larger strain on the bonds making them easier to break.

Although the *cis*/*trans* designation is more common, it is worth of mentioning that *Z*/*E* designation is also used in some cases. The general designation of *Z*/*E* isomerism for stereoisomers follows simple rules of priority established by Cahn, Ingold and Prelog which allow unequivocal classification of each isomer. In this nomenclature, substituents with equal priority on the same side of the double bond or saturated ring give the *Z*-isomer, whereas in opposite sides give the *E*-isomer. Nevertheless, it is important to highlight that in biological systems the vinylic systems present, almost always, hydrogen atoms attached to the vinylic carbons.

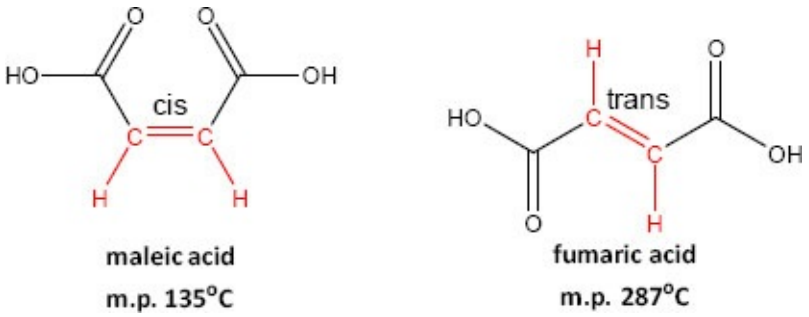


Figure 1.1.2: Maleic and fumaric acid structures with their respective melting points, highlighting the importance of cis-trans isomerism for physical properties.

1.1.3.2 Chirality and Enantiomerism

A compound is defined as **chiral** if it does not possess any planes of symmetry. This implies that two chiral molecules, constructed in such a way that one is the reflection of the other upon a plane, are not superimposable. In this case, these two molecules are classified as **enantiomers**. One can refer to carbons as asymmetrical or chiral whenever all of its substituents are unique, and are also referred to as **stereogenic centers** (stereocenters), as they can cause stereoisomerism in the molecule.

A molecule can have more than one stereocenter, such as 1,2-dimethylcyclopropane with two asymmetric carbons. Molecules that differ in the configuration of one or more (but not all) stereocenters, whilst being stereoisomers but not enantiomers, are called **diastereomers**. Having that in mind, it follows that any of the *trans*-1,2-dimethylcyclopropane enantiomers is a diastereomer of *cis*-1,2-dimethylcyclopropane, since it is equivalent to switching only one of the methyl groups. The *cis*-stereoisomer does not have enantiomers because it has a plane of symmetry. Structures such as *cis*-1,2-dimethylcyclopropane are called a *meso* structures.

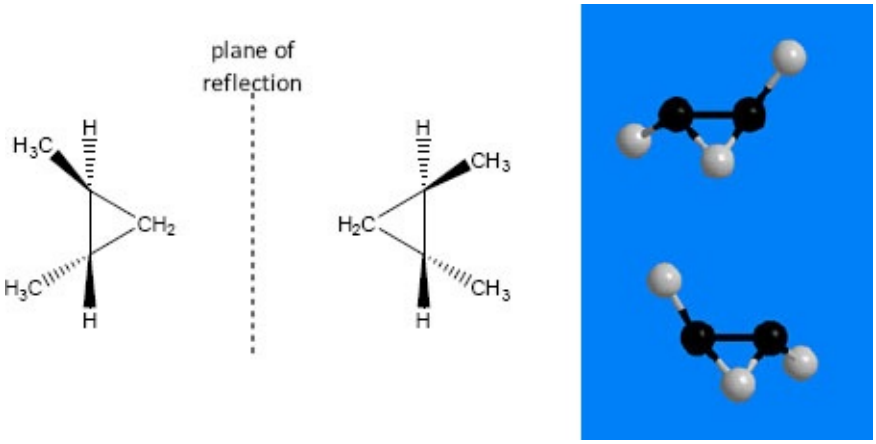


Figure 1.1.3: trans-1,2-Dimethylcyclopropane is an example of a molecule with enantiomers. The 3D representation (with hydrogen atoms omitted) shows that the enantiomers do not overlap (the asymmetric carbons are in black).

It is worthy to note that enantiomers only differ by reflection, so their physical properties are exactly the same because the steric hindrances and dipolar moments of each molecule are the same. However, one physical property that distinguishes each enantiomer is its **optical**

rotation: when illuminated, as a solid or in solution, chiral molecules rotate the plane of polarization of light transmitting through it. One of the enantiomers rotates this plane clockwise by some amount dependant on concentration and path length of the light through the sample. The other enantiomer will rotate light by the same amount but anticlockwise. **Racemic mixtures** (racemates) are mixtures with same amount of both enantiomers of a compound and have a null optical rotation.

Biological systems are highly **stereospecific** – in general, only one stereoisomer is reactive towards an enzyme or a certain receptor. As a result, racemate resolution is of extreme importance for drug synthesis, particularly when one enantiomers has a negative effect. This problem is often solved by two different approaches:

1. Enzymatic resolution: the racemate is transformed in such a way that the resulting product may be then cleaved by an enzyme (*e.g.* lyase). Because of the stereospecificity of the enzyme, only one of the compounds is cleaved, enabling separation of cleaved and uncleaved product.

2. Diastereomeric resolution: the racemate is made to react with a specific, enantiomerically pure reagent (*e.g.* L-tartaric acid, to form an ester). The obtained products are diastereomers, which unlike enantiomers possess different properties and are therefore separable. After separation, the reverse reaction is performed to return the original enantiomers.

1.1.4 Common Mechanisms in Biological Chemistry

Reactions that occur in living organisms follow the same rules of those occurring in the laboratory. The solvent, temperature and almost certainly the catalyst can be different, but the fundamental reaction mechanisms are the same. So conveniently, common organic reaction

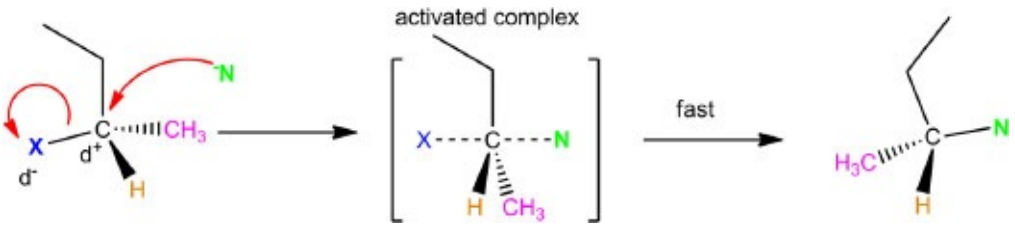
mechanisms can be used to understand the equivalent biological transformations.

1.1.4.1 Nucleophilic Substitution Reactions

Nucleophilic substitution reactions occur when a group attached to an sp^3 carbon is substituted for a more nucleophilic one. These reactions may follow two similar mechanisms – bimolecular and unimolecular – but with very different implications for biological systems in terms of stereochemistry.

1.1.4.1.1 S_N2 – Bimolecular Nucleophilic Substitution

This type of mechanism (Scheme 1.1.5) is called a bimolecular nucleophilic substitution (S_N2) since the determining step involves the reaction of two species, the nucleophile and the substrate (electrophile species).

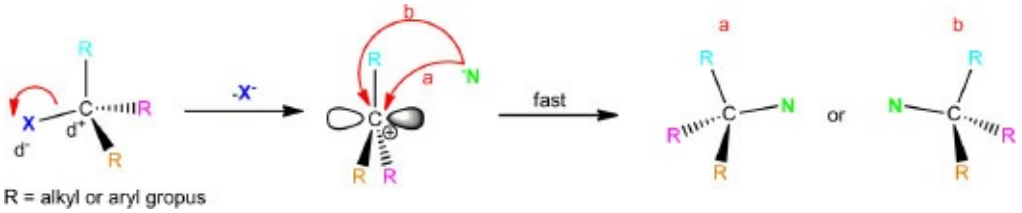


Scheme 1.1.5: General reaction for an S_N2 reaction mechanism.

The following happens in one concerted step: a nucleophile (N) attacks the carbon atom as a more electronegative group (X) leaves, with inversion of stereochemistry through an unstable transition state. Note that N can be a neutral protic nucleophile that deprotonates after the substitution. This type of mechanism is typical of primary alkyl halides substitutions. One reaction often performed in a laboratory is an *O*-methylation using methyl iodide. The oxygen atom (of an alcohol, for example) acts as nucleophile substituting the iodide which is a very good leaving group.

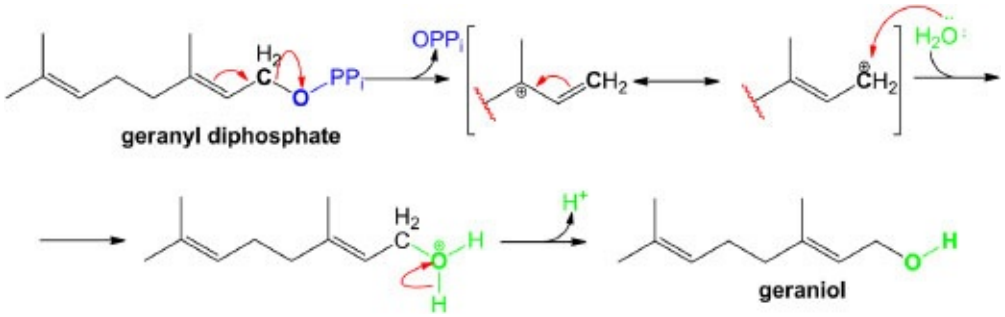
1.1.4.1.2 S_N1 – Unimolecular Nucleophilic Substitution Reactions

Unimolecular nucleophilic substitutions (S_N1) occur when a carbocation intermediate is stable enough to be transiently formed. In this case, the rate determining step involves reaction of only one species: the substrate where the substitution will take place (Scheme 1.1.6).



Scheme 1.1.6: General reaction for a S_N1 reaction mechanism.

A carbocation may be easily formed on tertiary carbons because the carbocation is stabilized through **inductive effect** by vicinal carbons (R = alkyl or aryl groups). With this in mind, the more electronegative moiety is able to **heterolytically** cleave its bond to the carbon atom. Since the carbocation is planar, there is no preference for the nucleophile on which side to attack, resulting in a mixture of enantiomers if the product in question in chiral. In enzymes, this does not happen because the active sites are chiral themselves, restricting addition to only one side. This type of mechanism is typical of tertiary alkyl halides substitutions and allylic phosphates. For example, geranyl diphosphate cleaves at the C-O bond and the corresponding allylic carbocation, well stabilized by **resonance**, is attacked by water which deprotonates to produce geraniol (Scheme 1.1.7).

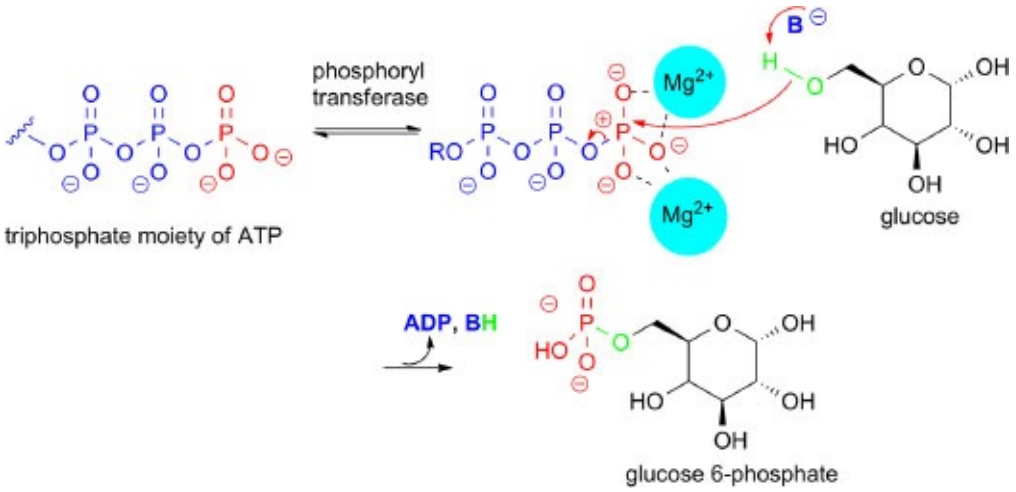


Scheme 1.1.7: S_N1 reaction mechanism of geranyl diphosphate forming geraniol.

1.1.4.1.3 Phosphate Group Transfer – the Grey Area of Nucleophilic Substitutions in Biological Systems

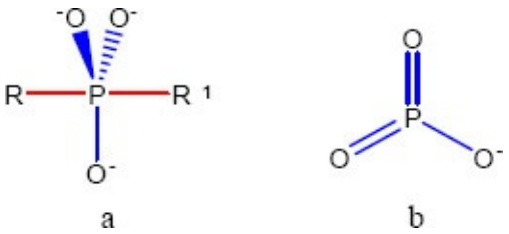
Nucleophilic substitutions are not restrained to carbon atoms.

Phosphate and acyl group transfer reactions, key pieces in metabolic pathways, are also nucleophilic substitution reactions although with some differences. In the phosphorylation of glucose a phosphate group is transferred from ATP to glucose with a phosphorus atom undergoing a nucleophilic substitution (Scheme 1.1.8).



Scheme 1.1.8: Glucose phosphorylation.

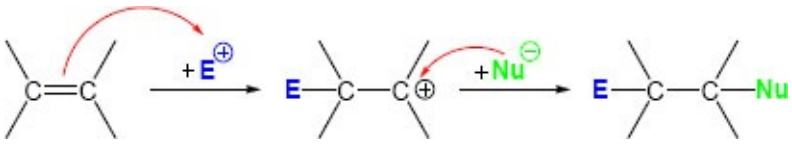
In this reaction, the phosphorus electrophilicity is reinforced trough chelation with two Mg^{2+} ions present in the phosphoryl transferase enzyme. In the complex, the most representative resonance structure places a positive formal charge in the P atom and negative charges on the O atoms. This makes the tetrahedral phosphate easy to be attacked by the oxygen atom from the 6-hydroxyl group in glucose, while a base captures the released proton. The mechanism shown in Scheme 1.1.8 is a simplified version of what has been observed. The preferred reaction path is not well defined in biological systems, since it is highly dependent on the nature of the nucleophile and the enzyme scaffold. Intermediates may or may not be involved. Either a pentavalent trigonal bipyramidal (associative intermediate) or a metaphosphate (dissociative intermediate) intermediate may be formed (Scheme 1.1.9). A concerted reaction mechanism has also been observed, where the substitution is done smoothly in one step (*i.e.* S_N2 like).



Scheme 1.1.9: a) Phosphorane intermediate – analogue of the activated complex – in red are the axial positions (collinear) and in blue are the equatorial positions (coplanar); b) metaphosphate (planar, stabilised by resonance) – analogue of a carbocation.

1.1.4.2 Electrophilic Addition Reactions

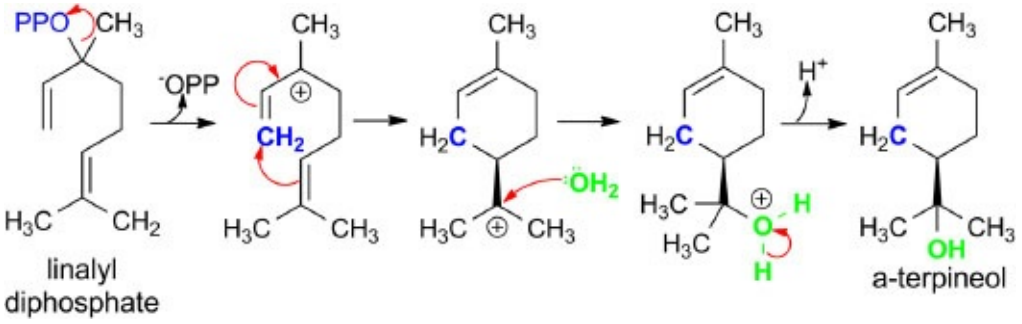
Electrophilic reagents can react with compounds that are electron rich in certain exposed regions, from which alkenes are a typical example. In these systems, the π bond results from overlapping of p orbitals and provides regions of increased electron density above and below the plane of the molecule. π electrons are more loosely bound than those of a σ-bond so they can interact more easily with a positively charged electrophilic species, forming a new σ-bond and a carbocation (Scheme 1.1.10). This in turn rapidly reacts with a nucleophile to form another σ-bond. In this case, the nucleophile is either an anion or a neutral moiety with free pairs of electron that will become neutral again eliminating a group or an atom.



Scheme 1.1.10: Electrophilic addition reaction mechanism.

1.1.4.2.1 Synthesis of α-Terpineol – Intramolecular Addition

In the following example we can see the simplified (without enzyme interactions) biosynthetic mechanism of α-terpineol from linalyl diphosphate, which occurs through an electrophilic addition (Scheme 1.1.11).



Scheme 1.1.11: Synthesis of α-terpineol from linalyl diphosphate.

It should be noted that in this intramolecular addition, the electrophile is generated via diphosphate ion (PPO^-) elimination. The diphosphate ion leaves easily because it is itself a stable anion and the generated carbocation is also stable, as it is an allylic carbocation stabilized by resonance. The delocalized positive charge turns the terminal carbon into a strong electrophile capable of adding to the double bond; the deficiency of electrons in the electrophile does not always coincide with the atom that will be attacked. Biological examples of electrophilic reactions occur frequently in metabolic processes, such as in the β-oxidation pathway of the fatty acid metabolism (Chapter 1.1.5).

1.1.4.3 Aromatic Substitutions

In simple terms, **aromaticity** may be described as a chemical property that arises from delocalization of electrons in a ring. These electrons may be provided by conjugated unsaturation, lone pair electrons or even orbitals; the system becomes aromatic when agreeing with the Huckel rule, which are state that the number of the total conjugated electrons must be 4n+2 electrons. It is a particularly strong form of resonance stabilization making aromatic compounds more stable than expected otherwise. The orbital alignment required for aromatic stabilization turns the aromatic (aryl) moieties planar (Figs. 1.1.4 and 1.1.5). Because of the intermediate character of its bonds (Fig. 1.1.4) the reactivity of aromatic compounds is not identical to the reactivity of other unsaturated compounds.

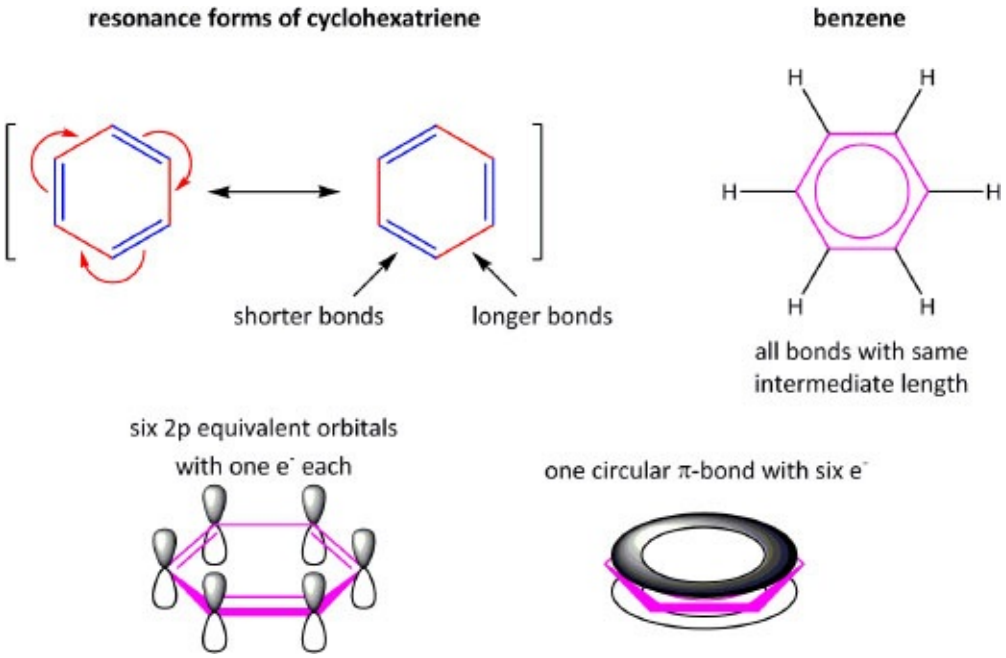


Figure 1.1.4: Clarification of the aromatic stabilization of benzene. Hypothetically, cyclohexatriene would have two different types of bonds: simple and double bonds, but spectroscopic data show that all bonds are equivalent. The bonds in benzene have an intermediate character between a simple and a double bond with the electrons delocalized (density evenly distributed).

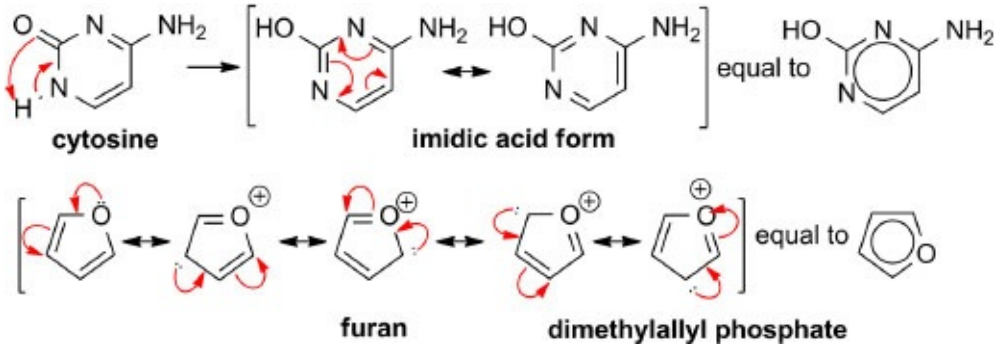
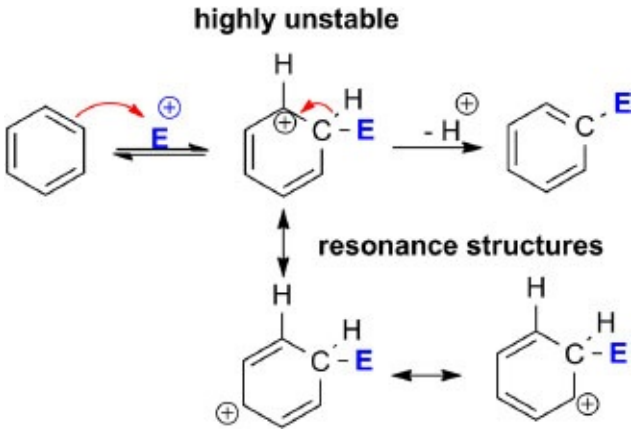


Figure 1.1.5: Cytosine is an example of a biomolecule exhibiting aromaticity in its imidic acid form. Furan is an example of a heteroaromatic compound where a lone pair of electrons of the heteroatom is delocalized into the ring to allow aromaticity. The Fig. explicitly shows all the resonance forms in the rings of the compounds.

1.1.4.3.1 Electrophilic Aromatic Substitution

A strong electrophile may capture electrons from the ring, forming a very unstable intermediate. Since the loss of aromaticity is energetically unfavourable, a nucleophile does not add to the cation. Instead, the ring eliminates a proton (Scheme 1.1.12).

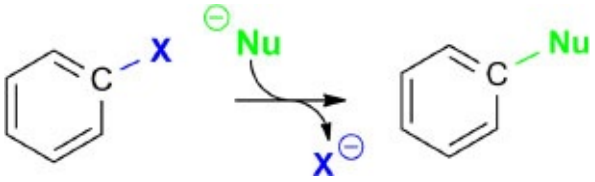


Scheme 1.1.12: Mechanism of an electrophilic aromatic substitution reaction.

The reactivity of the aromatic ring is enhanced if electron donating (hydroxyl, amino and alkoxyl) groups are attached to the ring, increasing the electron density and making the attack to the electrophile easier.

1.1.4.3.2 Nucleophilic Aromatic Substitutions

Nucleophilic aromatic substitutions are also possible when electron withdrawing groups are attached to the ring (*e.g.* nitro group and carbonyl moieties) allowing it to accommodate a carbanion, even though aromatic rings are already dense in electrons. The leaving group also needs to be quite electronegative to leave easily and drive the reaction forward (Scheme 1.1.13). The exact mechanism varies dependently on the leaving group having some parallels to normal nucleophilic substitution.

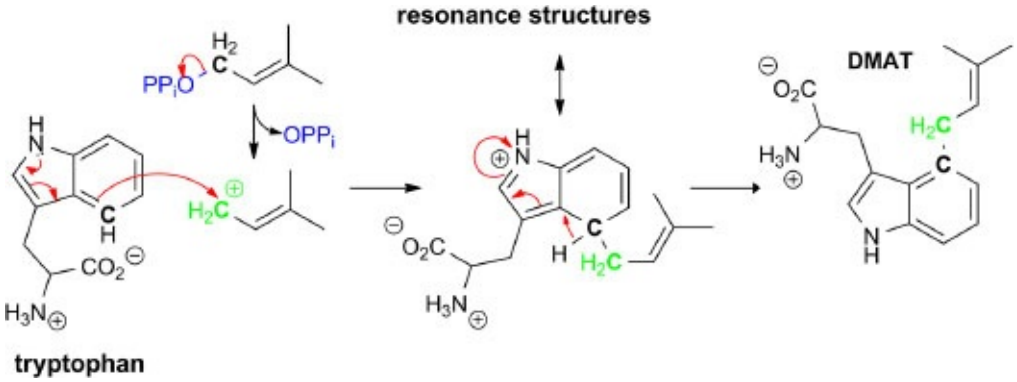


Scheme 1.1.13: General reaction of a nucleophilic aromatic substitution.

1.4.3.3 Hallucinogen Synthesis – Aromatic Substitution on Fungi

Ergot fungi produce a variety of alkaloids often with strong hallucinogenic effects upon consumption. The first pathway-specific step in their synthesis is the alkylation of tryptophan by dimethylallyl diphosphate obtaining the dimethylallyl tryptophan (DMAT) (Scheme

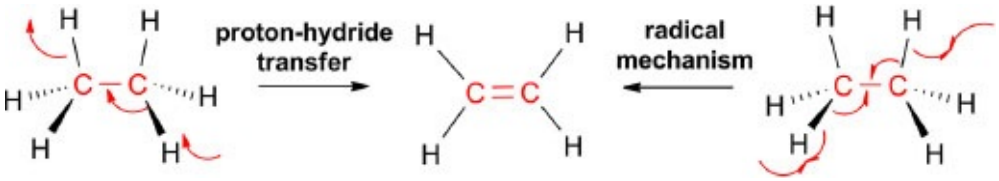
1.1.14). This step consists of an electrophilic aromatic substitution, catalysed by DMAT synthase.



Scheme 1.1.14: Reaction mechanism for the synthesis of DMAT from tryptophan and dimethylallyl phosphate.

1.4.4 Eliminations Reactions

An elimination reaction is a type of reaction upon which there is a net elimination of a molecule from another. To clarify this point, look at the example shown in Scheme 1.1.15.



Scheme 1.1.15: Dehydrogenation of ethane to form ethene through two different mechanisms: proton-hydride transfer and a radical mechanism, both leading to the elimination of what is equivalent to a hydrogen molecule (the arrows without origin represent electron transfers to/from other molecules).

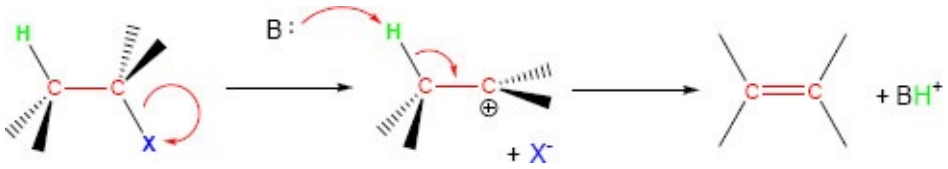
A **dehydrogenation** reaction involves the net elimination of a hydrogen molecule, but does not necessarily release a hydrogen molecule. It may proceed through abstraction of a proton connected to one of the carbons followed by transfer of a hydride from the other. It may also follow a radical mechanism by hydrogen atom abstraction from both carbons (Scheme 1.1.15). In a dehydrogenation, the elimination is oxidative because the oxidation state of each carbon atoms goes from -3 to -2, a net molecular change of +2 (Chapter 1.1.4.8).

In mechanistic terms, non-oxidative eliminations may occur in

various steps having its variations similarly to the nucleophilic substitutions. In all of the following cases, the reactions shown are [1,2]-eliminations, meaning that the reaction implicates electron movement between two vicinal carbons. Other types of eliminations may occur involving more distant carbon atoms and heteroatoms – [1,3] in decarboxylations or [1,4] in aldol condensations (Chapter 1.1.4.6).

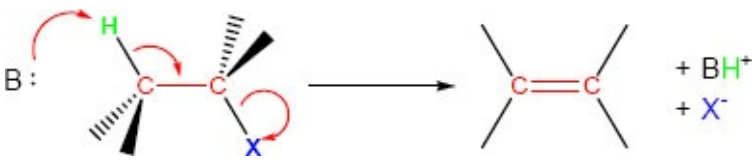
1.1.4.4.1 E1 – Unimolecular Elimination

An E1 reaction mechanism occurs when a relatively stable carbocation may be formed by elimination of a stable anion (X^-), such as a chloride ion (Cl^-) for a good leaving group. The only rate determining (slow) step is the dissociation of the leaving group to form a carbocation (hence a unimolecular reaction). A base (B) captures the proton released from one carbon away of the carbocation formed in the E1 reaction mechanism. It is worthy to note that the E1 mechanism competes with the S_N1 mechanism since the nucleophile (B) may react directly at the halogenated carbon (substitution) or at the neighbouring hydrogen atom (elimination). Steric hindrance both at the base and at the carbocation, as well as stronger bases promote elimination.



Scheme 1.1.16: General E1 reaction mechanism.

1.1.4.4.2 E2 – Bimolecular Elimination

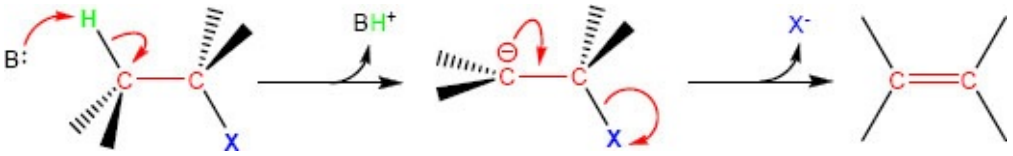


Scheme 1.1.17: General E2 reaction mechanism.

E2 mechanisms occur through a concerted transfer of a set of electron pairs from the base to the more electronegative group (X), the latter leaving as its anion and a vicinal proton is transferred to the base (Scheme 1.1.17). This mechanism is preferred if no stable ionic

intermediate can be formed. The double bond is formed in a step involving the two reagents, thus the elimination mechanism is bimolecular. It is noteworthy that the E2 mechanism competes with the S_N2 mechanism, as both reactions involve a base and an electronegative leaving group. More steric hindrance and stronger bases favour an E2 mechanism over S_N2.

1.1.4.4.3 E1cB – Unimolecular Elimination through Conjugate Base

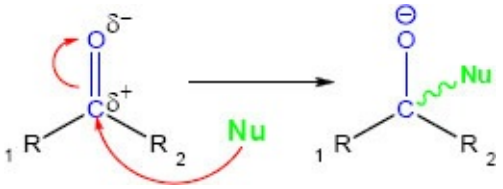


Scheme 1.1.18: General E1cB reaction mechanism.

E1cB mechanism is the symmetric version of the E1 mechanism. First, the proton is removed from the main molecule to form its conjugate base, a carbanion, which promotes the elimination of the electrophilic group. This mechanism is preferred whenever a carbanion intermediate is stabilized, in most of the cases by resonance (Scheme 1.1.18). This mechanism is particularly relevant in biological transformation, as it is by far the most frequent elimination mechanism because of the high occurrence of carbonyl compounds which form relatively stable carbanions.

1.1.4.5 Nucleophilic Carbonyl Addition Reactions

This type of reaction happens between a nucleophile and a carbonyl group where a pair of electrons from the nucleophile is transferred to the carbonyl carbon (Scheme 1.1.19).

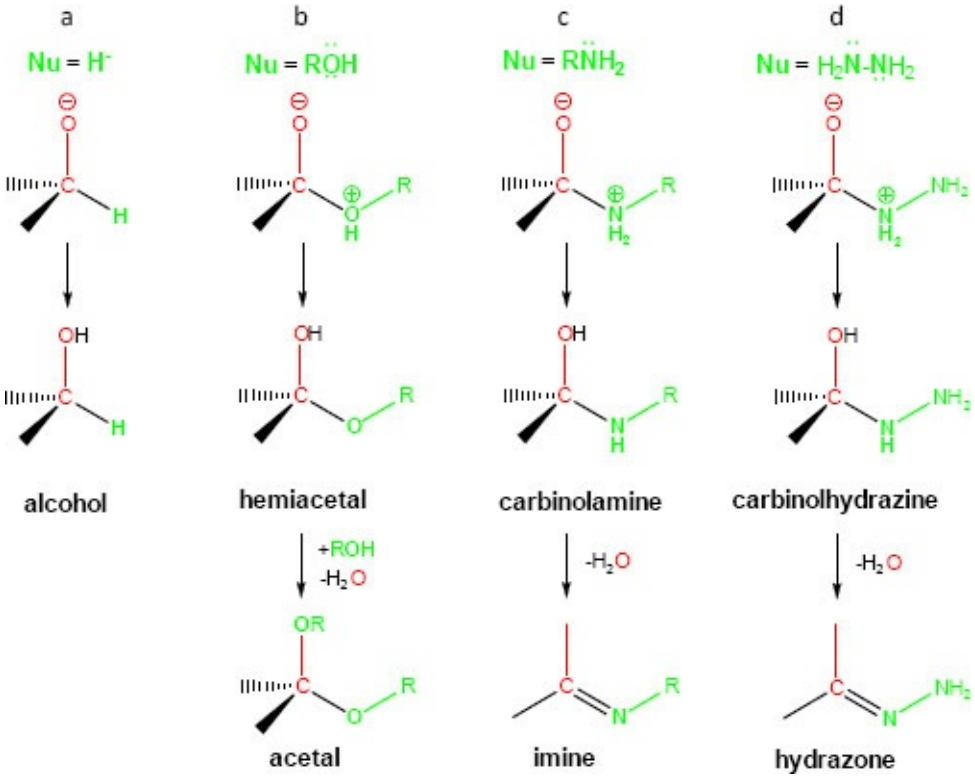


Scheme 1.1.19: General simplified scheme for a nucleophilic addition reaction

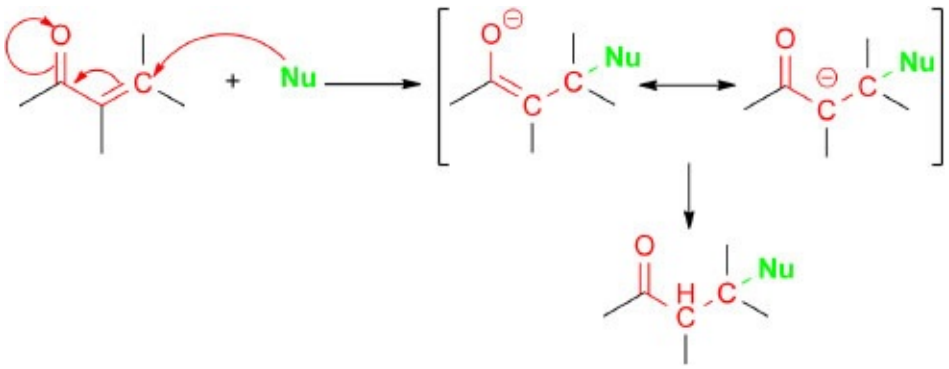
If the nucleophile is neutral and gives a pair of electrons then it will acquire a positive charge, which in turn is compensated by a

deprotonation of the introduced group (as in the formation of imines; Scheme 1.1.20c). If the nucleophile is anionic, no positive charge is generated. In both cases, a negative charge is generated at the most electronegative atom, the carbonyl oxygen, which is usually neutralized through protonation (Scheme 1.1.20). Scheme 1.1.20 illustrates some of typical nucleophilic addition reactions of different nucleophiles to aldehydes and ketones.

All the reactions depicted in Scheme 1.1.20 are called “direct” or [1,2]-additions. A special case of nucleophilic addition reactions is the Michael or [1,4]-conjugate addition. This reaction is quite frequent in biochemical pathways and consists of a nucleophile addition to the β–position of an α,β-unsaturated carbonyl system (Scheme 1.1.21).



Scheme 1.1.20: Nucleophilic addition reactions: a) with a hydride ion as nucleophile; b) with an alcohol as nucleophile; c) with an amine as nucleophile; and d) with a hydrazine as nucleophile.

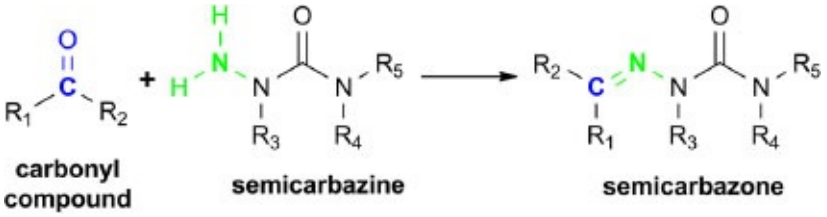


Scheme 1.1.21: General scheme for a Michael or [1,4]-conjugate addition reaction.

As with electrophilic addition reactions, the nucleophile may be added to any of the p orbitals, leading to the formation of enantiomer mixtures (in the case of α,β-unsaturated ketones, monosubstituted at β-position or disubstituted with two different groups). However, enzymes can be enantioselective and produce only one enantiomer.

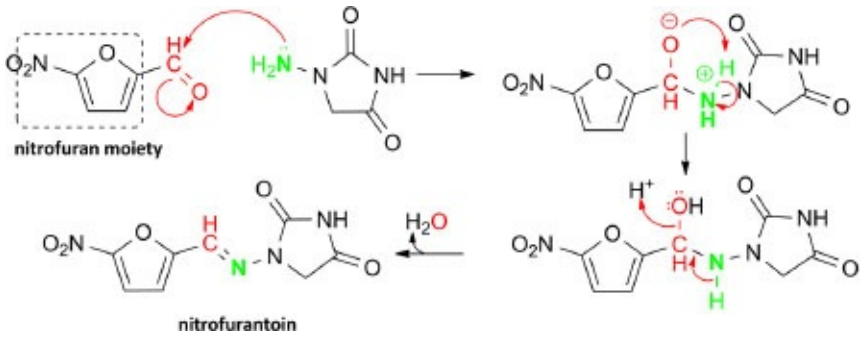
1.1.4.5.1 Nitrofurantoin – a Semicarbazone

Semicarbazones (Scheme 1.1.22) are a family of compounds classified as imine derivatives, originating from the action of semicarbazines on aldehydes or ketones (instead of amines). The $-NH_2$ group of a semicarbazide is akin to a primary amino group. Thus, the reaction mechanism is the same as with other nucleophilic carbonyl addition reactions.



Scheme 1.1.22: General scheme for a semicarbazone synthesis.

There are some semicarbazones with pharmacological interest, such as nitrofurantoin (non-systematic name). Nitrofurantoin is a nitrofuran-based antibiotic considered an essential medicine by the World Health Organization. Scheme 1.1.23 presents the mechanism of the nitrofurantoin synthesis from 5-nitrofuran-2-carbaldehyde and 1-aminoimidazolidine-2,4-dione.



Scheme 1.1.23: Synthesis of Nitrofurantoin from 5-nitrofuran-2-carbaldehyde and 1-aminoimidazolidine-2,4-dione.

The reaction steps are the same as in any other nucleophilic carbonyl addition reaction followed by **dehydration**:

1. nucleophilic attack of the nucleophilic amino moiety to the electrophilic carbonyl carbon;

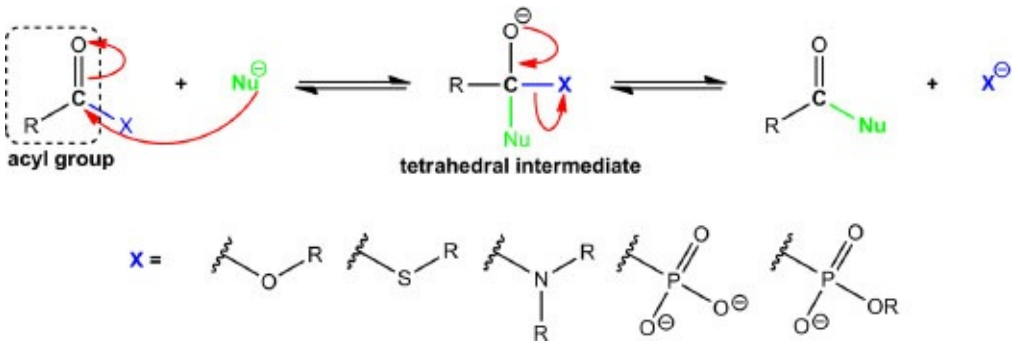
2. deprotonation of the positive nitrogen atom and protonation of the negative oxygen atom;

3. elimination of water by protonation of the hydroxyl group and deprotonation at the nitrogen atom.

This synthetic route as a whole is a condensation reaction: two molecules produce a larger molecule with the loss of a small molecule, in this case water. In fact, imine and hydrazone synthesis are condensations too, but were introduced as additions (see above) since the condensation product is often readily generated from the unstable addition product unless very strict reaction conditions are used.

1.1.1.4.6 Acyl Substitution Reactions

Acyl substitutions are another class of reactions involving carbonyl groups (Scheme 1.1.24). An acyl substitution reaction is favoured over a simple addition whenever an electronegative group is attached to the carbonyl group. This property is a main feature presented by carboxylic acids and their derivatives.



Scheme 1.1.24: General mechanism for an acyl substitution reaction.

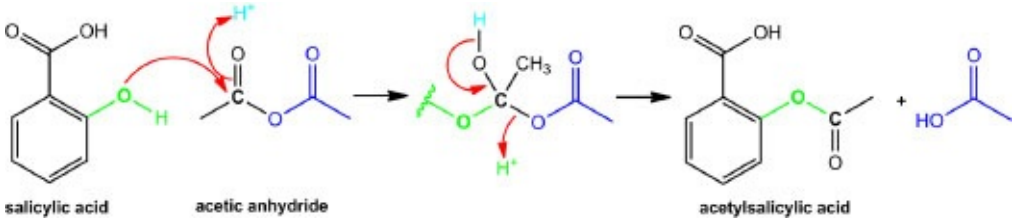
The first step of this mechanism is shared with nucleophilic addition reactions and consists in the nucleophilic addition to the carbonyl group. The second step is a regeneration of the carbonyl group with elimination of the anion. It is important to note that the whole reaction is reversible unless some product stabilization is provided, such as if the leaving group is a stable anion, which is unlikely to be able to attack the carbonyl again. Acyl phosphates are considered activated analogues of carboxylic acids, as the leaving phosphate is a very stable anion and it is unlikely that a better leaving group is attached to a carbonyl group. This shifts the equilibrium towards product formation. On the other hand, a simple carboxylic acid would have the hydroxyl as leaving group which is a strong nucleophile capable of adding onto the carbonyl again. This is the reason why esters, thioesters and acyl phosphates play a major role in promoting substitution reactions within biological systems. In the laboratory environment it is more common the use of carboxylic acid anhydrides and acyl chlorides or bromides. The resulting anions (carboxylates and halogens, respectively) are very weak bases promoting the completeness of the reaction. Because of that, these compounds are very sensitive to hydrolysis thus rendered useless in biological systems.

1.1.4.6.1 Aspirin – Esterifications and Transesterifications

Aspirin, acetylsalicylic acid or, by its systematic name, 2-acetoxybenzoic acid, is a nonsteroidal anti-inflammatory drug that inhibits the formation of prostaglandins and thromboxanes by inactivating cyclooxygenases (COXs). It is synthesized by **esterification** of salicylic acid with acetic anhydride (Scheme 1.1.25). One of its action pathways

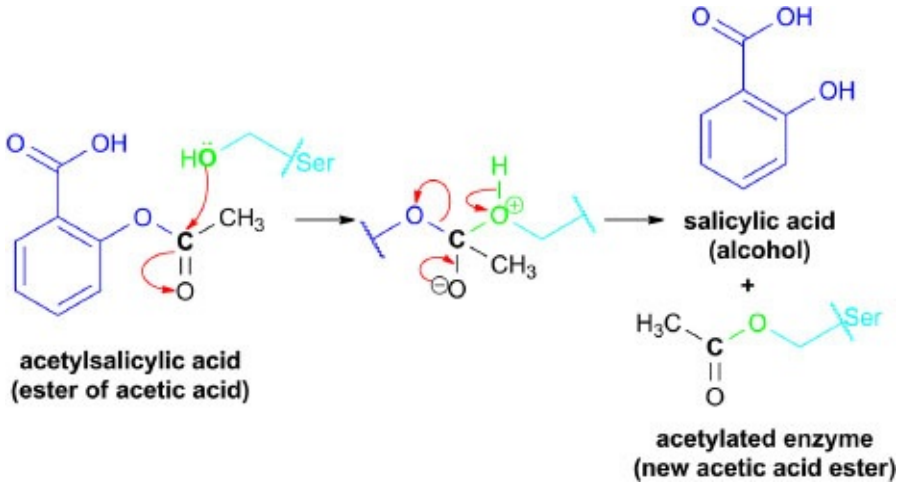
involves the acetylation of a serine residue in the enzyme through a **transesterification** reaction (Scheme 1.1.26).

Esterification is an acyl substitution reaction where an acyl group is transferred to the oxygen atom of the alcohol. In the synthesis of aspirin, acetic anhydride is used for efficiency reasons as it is a much better acetylating agent than acetic acid (Scheme 1.1.25).



Scheme 1.1.25: Simplified mechanism for the synthesis of aspirin from salicylic acid and acetic anhydride. This reaction is normally performed under acid catalysis with sulfuric or phosphoric acids.

Transesterifications are acyl substitution reactions where the nucleophile is an alcohol and the electrophile is an ester. Essentially, the alkoxyl moiety in the ester is substituted by another. In the case of aspirin, the alcohol/nucleophile is the side chain of the serine residue in the COX enzyme which substitutes the alcohol moiety of the salicylic acid (Scheme 1.1.26).



Scheme 1.1.26: Simplified mechanism of the transesterification reaction of aspirin with the serine residue.

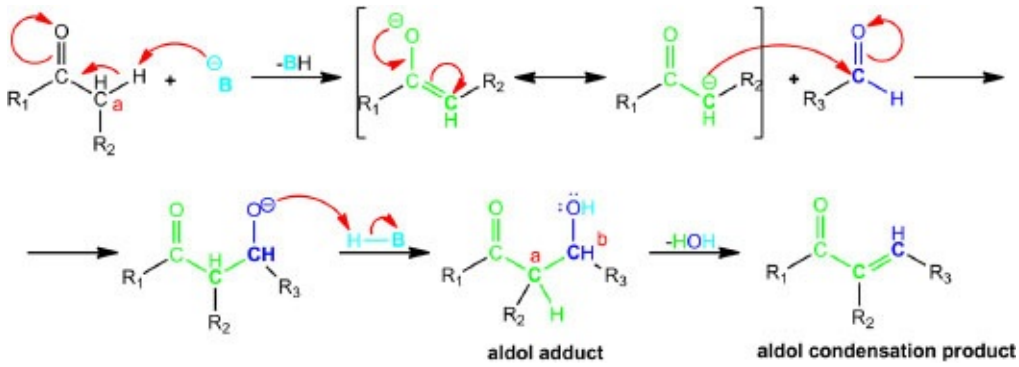
1.1.1.4.7 Carbonyl Condensation Reactions

As we have seen before, a condensation reaction joins two molecules

together to form a bigger one and liberates a small one (*e.g.* water, methanol, acetic acid). Carbonyl condensations occur with two carbonyl compounds. This type of reaction is a very important one, adding to the chemical versatility that carbonyl groups grant to a system. Carbonyl condensations are essentially nucleophilic substitutions that allow easy carbon-carbon bond formation in relatively mild conditions (certainly within the reach of an enzyme), adding chain formation and polymerization to the list of enzyme-catalysed reactions.

1.1.4.7.1 Aldol Reaction

An aldol reaction involves carbonyl compounds such as aldehydes or ketones in which at least one species has an α-proton (Scheme 1.1.27). Although it is not formally a condensation reaction unless dehydration happens, it is often named as aldol condensation in biochemical fields. The "proper" aldol condensation product though is the result of a dehydration of the formed aldol.

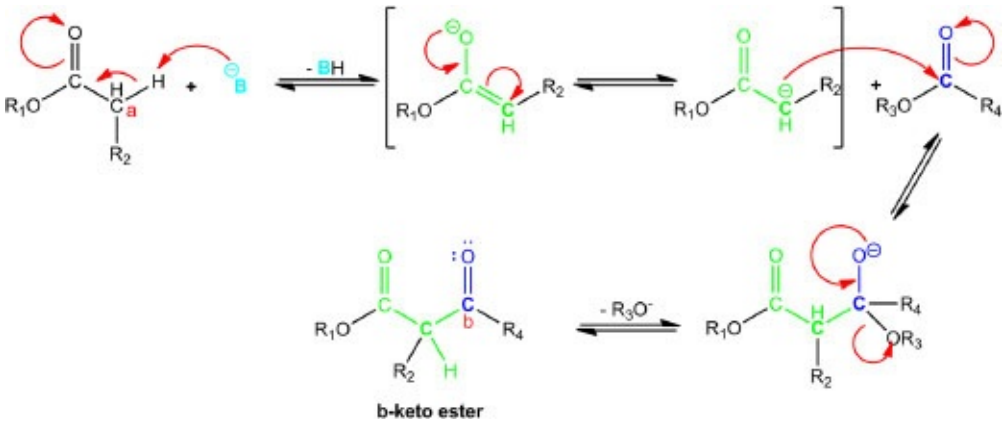


Scheme 1.1.27: General mechanism for an aldol condensation reaction. The aldol adduct suffers a dehydration for which the mechanism is not specified since the precise step sequence may vary according to catalyst used.

First of all, the enolate is generated from a ketone or an aldehyde by an α-deprotonation with a base. The enolate is a strong nucleophile and a **nucleophilic addition** occurs unto an aldehyde (or ketone) acting as electrophile. The resultant anion, the aldolate, is protonated to produce the aldol, a β-hydroxy-aldehyde or ketone. The aldol may then eliminate a water molecule yielding an α,β-unsaturated aldehyde or ketone (Scheme 1.1.27).

1.1.4.7.2 Claisen Condensation

Claisen condensation is the base-catalysed condensation reaction of an ester with another carbonyl compound taking place through the mechanism depicted in Scheme 1.1.28.

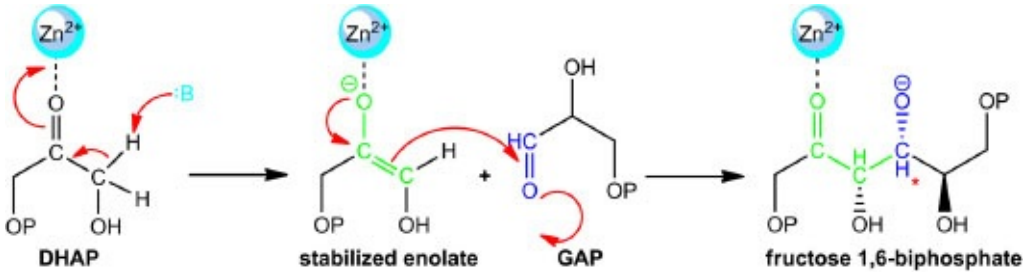


Scheme 1.1.28: General mechanism for a Claisen condensation reaction.

Firstly, the enolate is generated from an ester by α-deprotonation with a base. The enolate performs an **acyl substitution** followed by elimination of the alcoxyl group, giving a β-keto ester (Scheme 1.1.28).

1.1.4.7.3 Aldolases – Stabilization Strategies

In order to better perform aldol additions, enzymes create stabilized intermediate forms that provide a lower energy reaction path (Scheme 1.1.29). In the case of class II fructose-biphosphate aldolases, a zinc(II) ion is used to further polarize the carbonyl C=O bond of dihydroxyacetone phosphate (DHAP). The latter deprotonates easily, as the enolate intermediate is stabilized by the zinc ion. The enolate then attacks the carbonyl carbon of the glyceraldehyde 3-phosphate (GA3P or GAP), producing the addition product fructose biphosphate. This reaction is completely stereospecific as with all enzyme catalysed reactions this statement in parenthesis should be in the description of Scheme 1.1.29. The reverse reaction (hydrolysis) is also catalysed by another enzyme.



Scheme 1.1.29: Mechanism of an aldol addition in a class I fructose-biphosphate aldolase.

Class I fructose-biphosphate aldolases have another stabilization mechanism that does not resemble a regular aldol reaction, though the final product is the same (Scheme 1.1.30). The mechanism occurs in the following abbreviated steps:

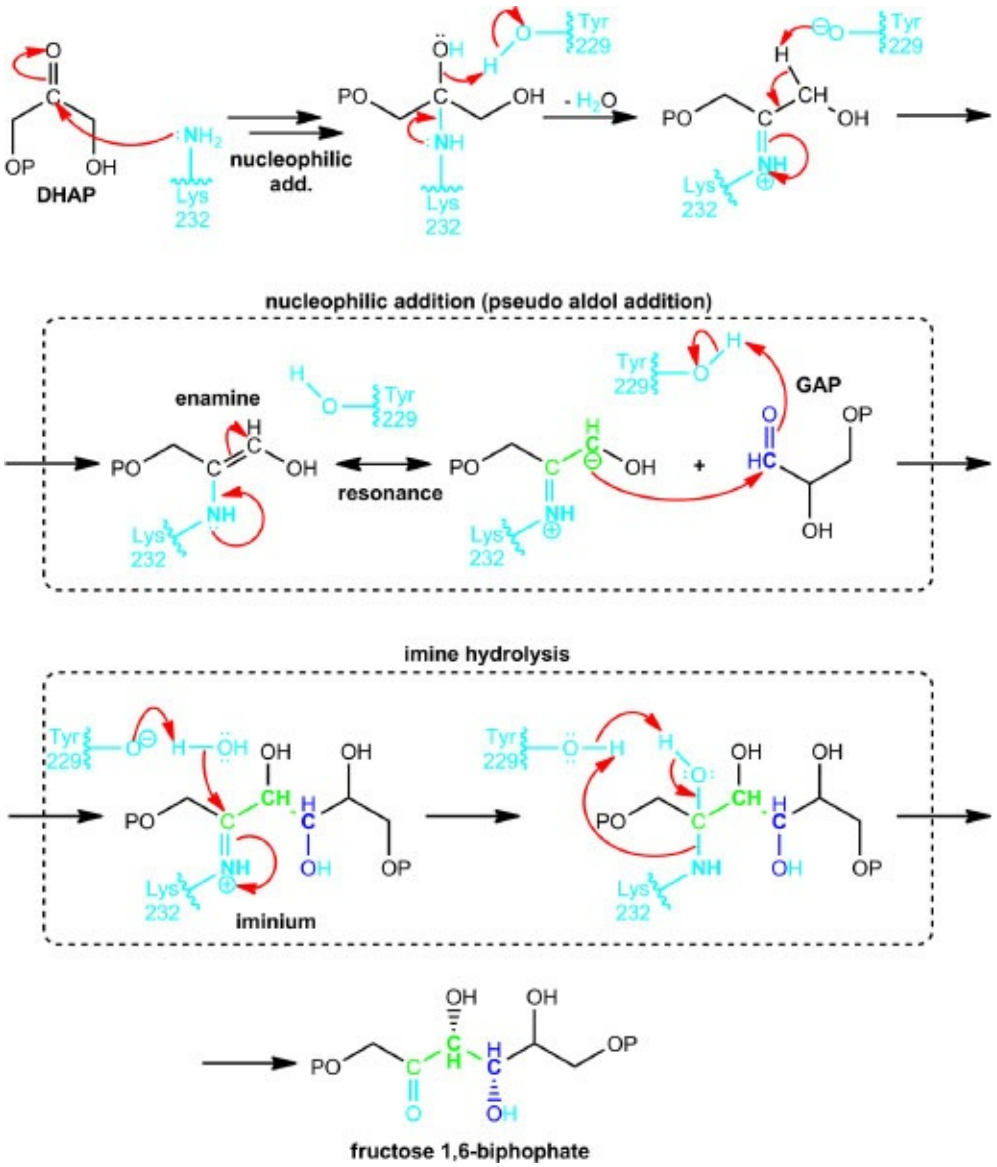
1. A carbinolamine is formed by nucleophilic addition of a lysine residue to DHAP

2. The carbinolamine eliminates water through acid/base catalysis, forming an enamine

3. The enamine acts as an enol (through analogous resonance structures) and adds to GAP

4. Water is added to the resulting iminium ion (hydrolysis is the reverse of the imine synthesis)

5. The carbonyl and lysine residue are regenerated from the new carbinolamine.



Scheme 1.1.30: Simplified mechanism of an aldol addition in a class II fructose-biphosphate aldolase.

1.1.4.8 Oxidations and Reductions

Oxidation and reduction reactions, or simply **redox** reactions, are a complex class of reactions with diverse mechanisms.

The procedure for determining the oxidation state of a carbon atom is similar to other compounds (Fig. 1.1.6):

1. For each bond to less electronegative atoms, such as a hydrogen atoms, count as -1

2. For each bond to more electronegative atoms, such as oxygen atoms, count as +

3. Bonds between carbon atoms do not affect the oxidation state, unlike other elements.

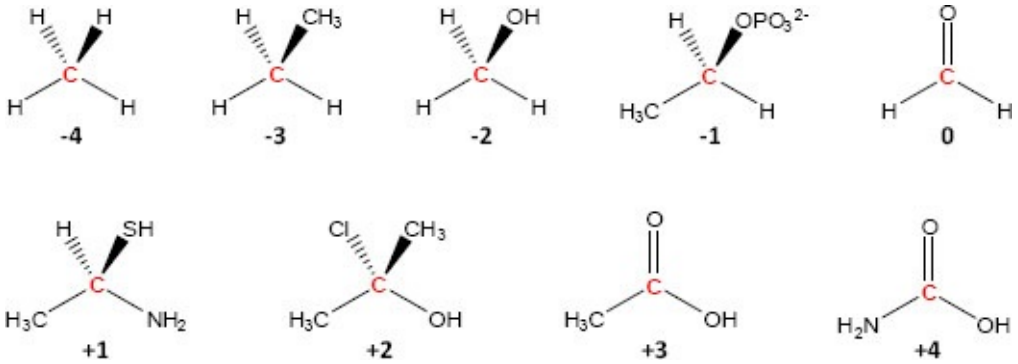
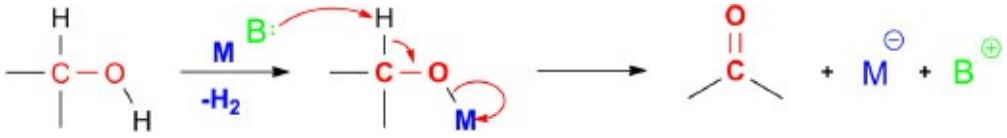


Figure 1.1.6: Compounds with the oxidation state of the highlighted carbon indicated below each structure. In order: methane, ethane, methanol, ethyl phosphate, formaldehyde, (R)-1-aminoethane-1-thiol, 1,1-dichloroethan-1-ol, acetic acid and carbamic acid.

The simplest redox reaction in a biological system is the oxidation of an alcohol to a carbonyl compound. In a laboratory, a metal in a high oxidation state is usually used as oxidant, where it attaches to the oxygen of the alcohol then acts as a leaving group with an E2-like mechanism (Scheme 1.1.31).



Scheme 1.1.31: General mechanism for the oxidation of an alcohol to a carbonyl compound with a metal or its complex (M). Note that the M leaves in a lower oxidation state.

In the case of an aldehyde, the carbonyl group may be oxidized to carboxyl by nucleophilic attack of water generating a hydrated aldehyde, in which one of the hydroxyl groups is then oxidized to carbonyl yielding the carboxyl group.

In biological systems however, the majority of the hydroxyl/carbonyl redox reactions involve the coenzyme NAD^+ (oxidized nicotinamide adenine dinucleotide) or $NADP^+$ (oxidized nicotinamide adenine dinucleotide phosphate) in lieu of a metal catalyst (Fig. 1.1.7). The

oxidation reaction occurs in just one step: as a base captures the proton attached to the oxygen atom, a **hydride** is transferred to NAD^+ and the carbonyl group is generated (Scheme 1.1.32). The hydride transfer is a simple conjugate nucleophilic addition, as the ion is a strong nucleophile. The reduction reaction is simply the reverse of the oxidation process (Scheme 1.1.33).

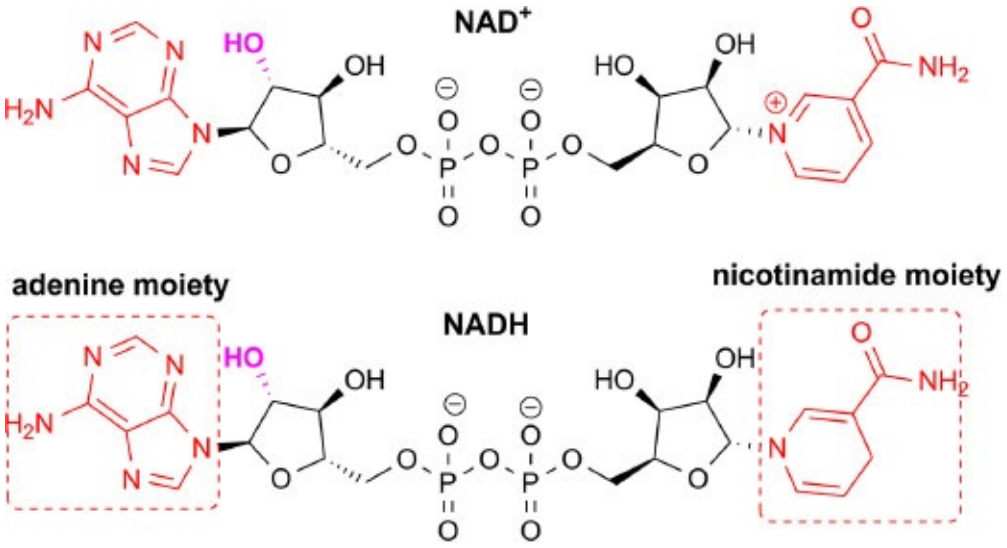
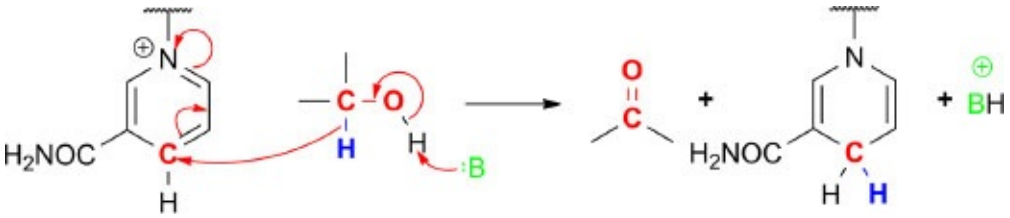
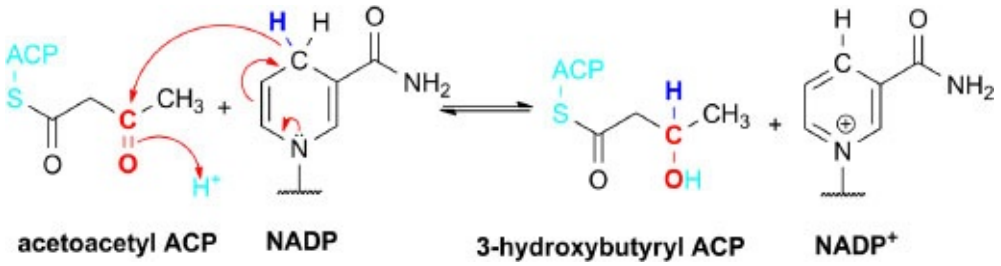


Figure 1.1.7: Structure of NAD^+ and NADH. The structures of $NADP^+$ and NADPH are identical with exception of the highlighted hydroxyl group (in magenta), which is replaced by a phosphate group.



Scheme 1.1.32: Mechanism of an alcohol oxidation by NAD^+ or $NADP^+$ – the base may be provided by residues of an enzyme.

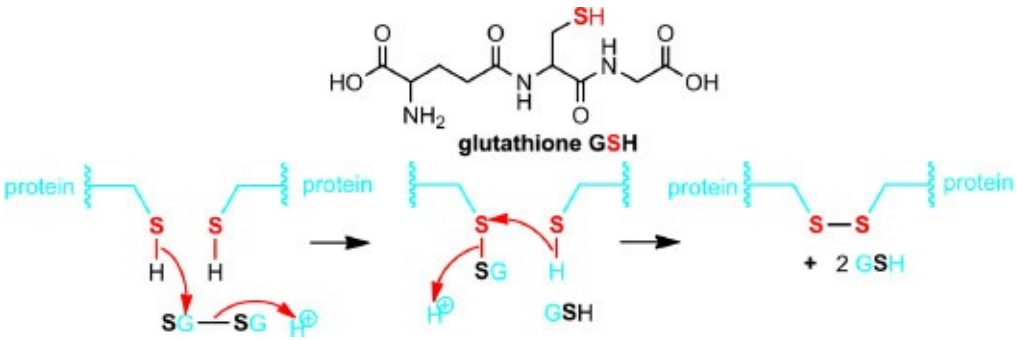


Scheme 1.1.33: Mechanism of an alcohol reduction of a carbonyl group by NAD or NADP, in this case of an acetylated acyl carrier protein (ACP), an important part in the fatty acid synthesis.

1.1.4.8.1 Disulfide Bridges – Oxidized Thiols

Disulfide bridges are essential in a protein to allow stable structural scaffolds. They are S–S bonds between cysteine residues (Scheme 1.1.34). Note that the sulfur loses a bond to a hydrogen and gains one to another sulfur atom, so the formation of this linkage is a redox reaction with thiols being oxidized. The oxidant (to be reduced) is a glutathione dimer (GSSG), which consists in two glutathione molecules connected by a disulfide bridge (Scheme 1.1.35).

Scheme 1.1.34: Disulfide bridge formation.



Scheme 1.1.35: Mechanism of disulphide linkage formation by a glutathione dimer, releasing two reduced glutathione molecules.

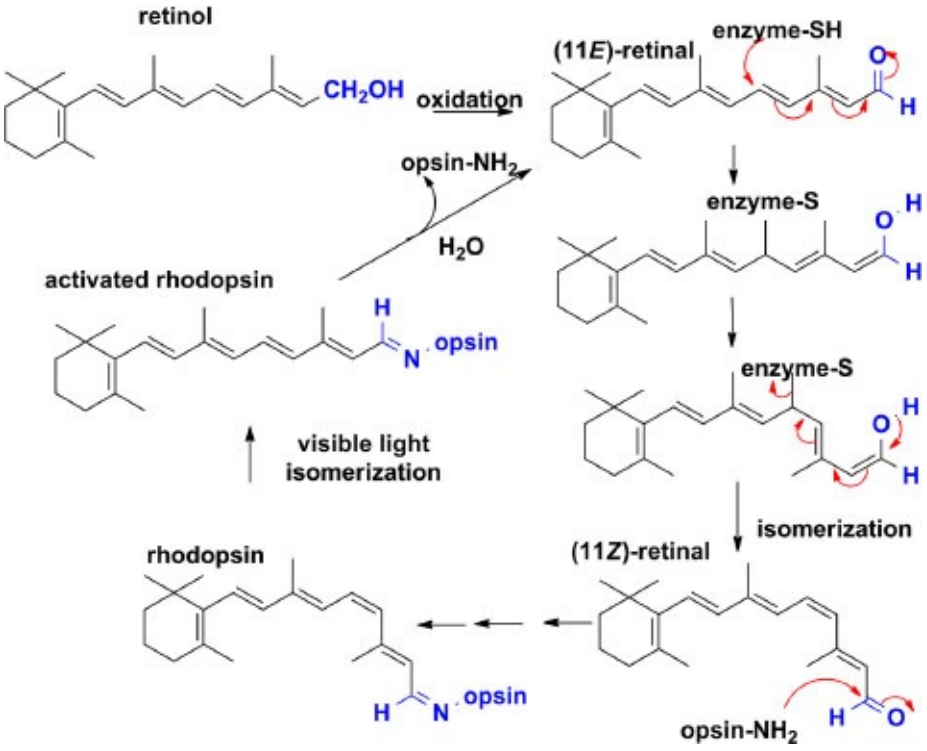
1.1.5 The Organic Mechanisms of Biological Transformations

Previously, we highlighted that common organic reaction mechanisms can be used to understand the biosynthetic pathways. Herein some illustrative examples are presented.

1.1.5.1*Cis/trans*-Isomers Interconversion in the Vision Pathway

It is common knowledge that vitamin A, retinol (Scheme 1.1.36), plays an important role in our vision. Although the sequence of reactions and detailed transformations are not in the scope of this chapter, it is remarkable that a simple oxidation and change in configuration is ultimately responsible for a complex process such as vision. Cis/trans

isomerase enzymes, through a cysteine residue, are responsible for this isomerization. The next step is Schiff base (imine) formation through the reaction of retinal with a lysine residue of the protein opsin to produce rhodopsin, which isomerizes upon absorption of visible light. This change in geometry causes an electrical signal that is sent to the brain. Finally, the hydrolysis of the imine linkage regenerates the opsin protein and (11*E*)-retinal (Scheme 1.1.36).



Scheme 1.1.36: The chemistry of vision.

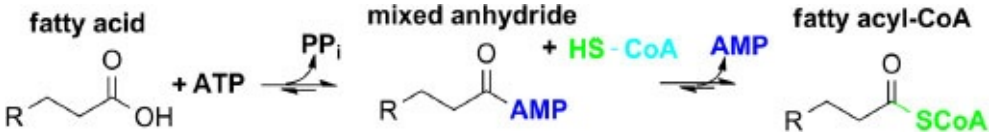
1.1.5.2 Metabolism of Fatty Acids – β-Oxidation Pathway

The catabolism of fatty acids (saturated or unsaturated) starts with chemical activation by esterification with coenzyme A (Scheme 1.1.37), with the following steps occurring in an acyl-CoA synthase:

1. The carboxylate acts as a nucleophile to attack the double P–O bond in ATP;

2. The diphosphate group leaves, resulting in an activated acid in the form of a mixed anhydride (acyl AMP). (PP_i is a good leaving group as it is stable and not nucleophilic)

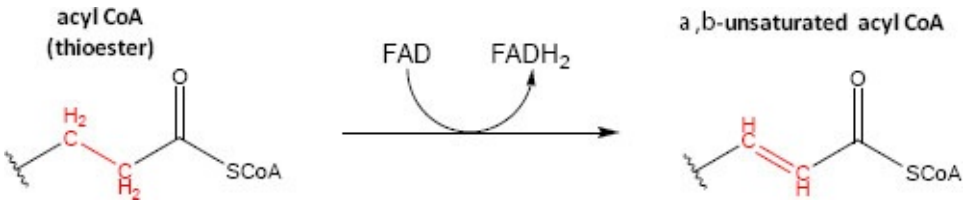
3. Another acyl substitution is performed on the mixed anhydride by the thiol moiety in CoA (a moderate nucleophile). Again, this is facilitated because AMP is a good leaving group.



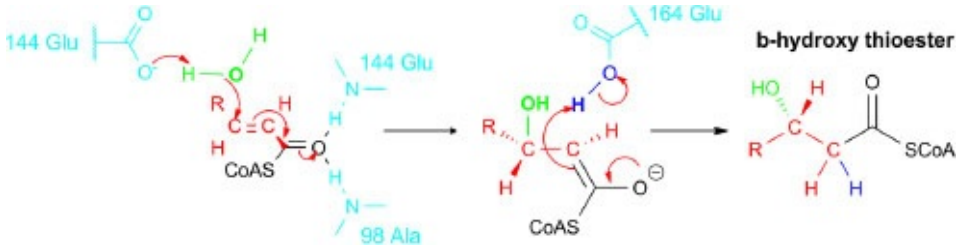
Scheme 1.1.37: Activation of a fatty acid via esterification.

With the thioester (activated fatty acid) formed, β-oxidation may now occur through a sequence of dehydrogenation, hydration and dehydrogenation reactions:

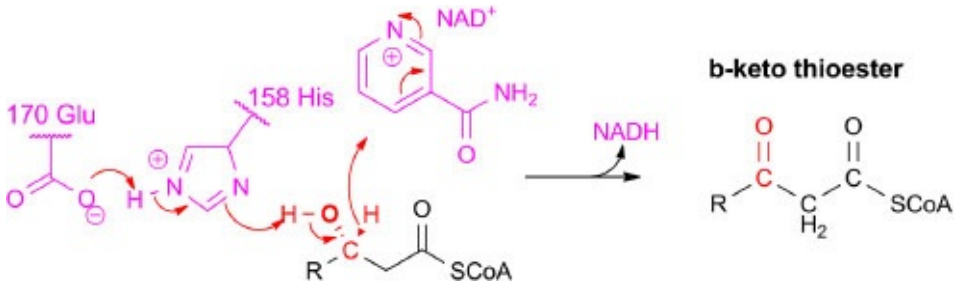
1. The Cα-Cβ single bond is oxidised to a double bond, with flavin adenine nucleotide (FAD) as the oxidant. This step occurs in a family of acyl-CoA dehydrogenases where the products are $FADH_2$ (reduced FAD) and α,β-unsaturated acyl-CoA. The mechanistic details of this step are not yet fully resolved, though it is known that one of the α protons may be first attacked by 376-Glu and a β-hydride is abstracted from the fatty acid by FAD.



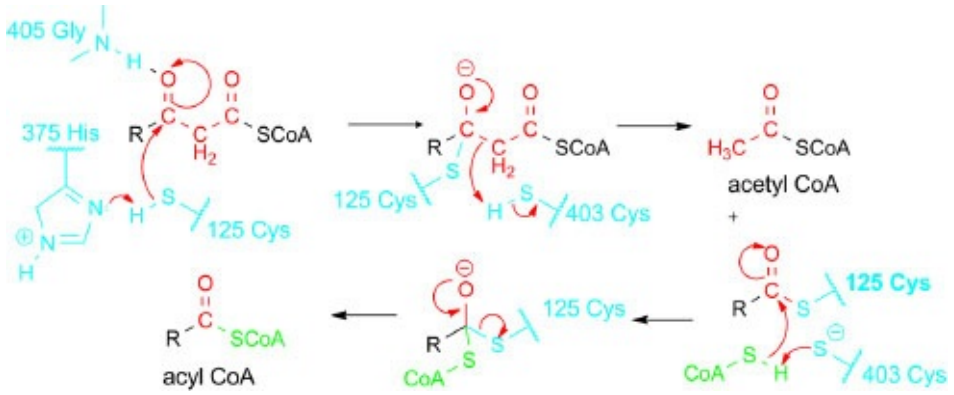
2. Enoyl-CoA hydratase performs a nucleophilic addition of water to the unsaturated double bond. The hydroxyl (nucleophile) is added exclusively in the β-position to the carbonyl since the enzyme is stereospecific.



3. The formed hydroxymethylene group is oxidised to a carbonyl group with a β-hydroxyacyl-CoA dehydrogenase. This reaction yields an NADH ion, formed from the coenzyme NAD^+ acting as the oxidant, and the β-ketoester.



4. A retro-Claisen reaction cleaves the β-ketothioester yielding two thioesters, producing the initial acyl-CoA shortened by two carbons and acetyl-CoA (this proceeds to the citric acid cycle, ultimately being oxidized to CO_2).



The pathway is repeated until all of the fatty acid is oxidised. If, during the cleavage process, a *cis*-oriented unsaturation is encountered, the stereochemistry of the double bond is switched to *trans* by an isomerase and the reaction sequence continues as for saturated fatty acids, since the enoyl-CoA hydratase is stereospecific for a *trans*-configuration.

1.1.5.3 Penicillin – a Strong Acylating Agent

Historically, penicillin antibiotics are one of the most important classes of antibiotics and are worth studying since many different reactions in its synthesis and mode of action are involved. The essential feature in penicillin class antibiotic's structure is shown in Fig. 1.1.8. Penicillins are part of the bigger class of β-lactam antibiotics, named because the β-lactam ring is the active site of the compound. A lactam is a cyclic amide, and the β means there are 2 carbons in between the O and N atoms in their structure.

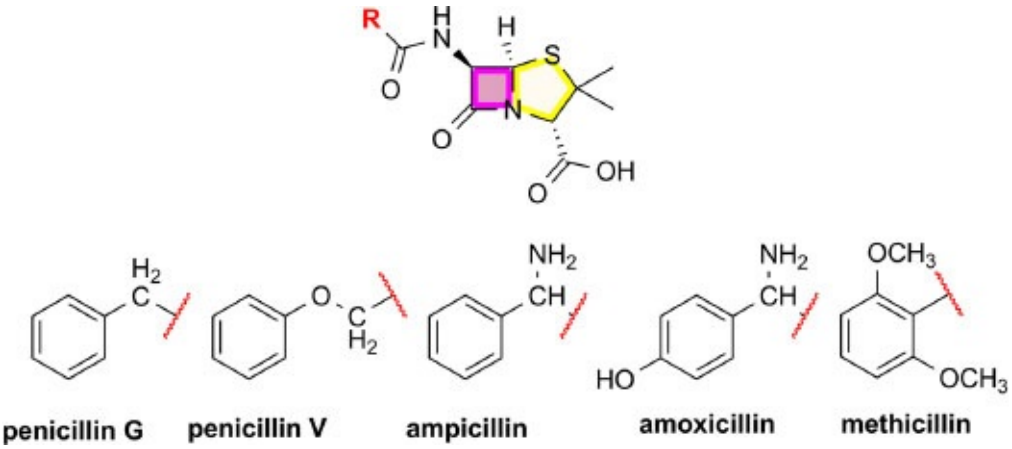
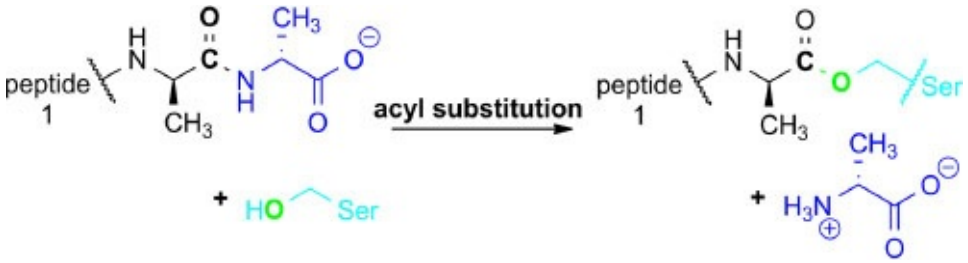


Figure 1.1.8: On top, the penicillin core structure is depicted. The β-lactam ring is highlighted in magenta, and in yellow the thiazolidine ring. R is a variable group and the structures on the bottom are those of the named antibiotics in this group. There are many other known possible groups that improve certain properties (absorption, hydrolysis, resistance, etc.).

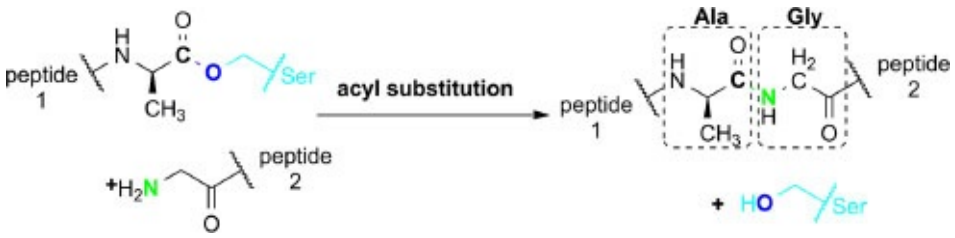
1.1.5.3.1 Transpeptidase Mechanism

Penicillin acts by irreversibly inhibiting the enzyme transpeptidase, which catalyses the formation of a peptide bond between an alanine and a terminal glycine of different polypeptides present in the cell wall. This halts the fabrication of new peptide crosslinks in the peptidoglycan layer, which degrades and eventually leads to cytolysis. The following steps constitute the catalysis mechanism of transpeptidase:

1. One peptide chain is linked to the enzyme via an acyl substitution reaction. The nucleophile is the hydroxyl from a serine residue of the transpeptidase and the leaving group is the *C*-terminal of alanine.

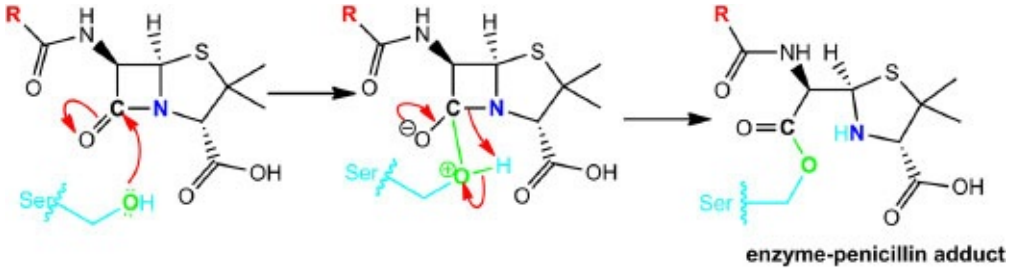


2. Another acyl substitution reaction involves a second peptide chain that displaces the link to the hydroxyl of a serine residue resulting in the two peptide chains being linked.



1.1.5.3.2 Transpeptidase Inhibition

As seen above, the core transformation in the transpeptidase reaction is an acyl substitution reaction. Penicillin (Scheme 1.1.38) is similar to the normal transpeptidase substrates, so it mimics the substrate and binds irreversibly to the enzyme active site. What makes penicillin so effective is that the β-lactam ring is under considerable strain, making the reaction irreversible. The thiazolidine ring further increases the strain by distorting the bonds and removing resonance stabilization. The β-lactam in anionic form is also protected from hydrolysis so the absorption is more efficient.



Scheme 1.1.38: Mechanism of the acyl substitution reaction occurring with penicillin and the serine residue from transpeptidase.

1.1.5.3.3. Penicillin Biosynthesis

Penicillin biosynthesis begins with the formation of the tripeptide L-δ-(α-aminoadipoyl)-L-cysteinyl-D-valine (ACV) (Scheme 1.1.39) by

condensation of three amino acids catalysed by ACV synthase. ACV is then processed by isopenicillin-N synthase, an enzyme of the oxyreductase family, with the following mechanistic steps (Scheme 1.1.40):

1. Attachment of the cysteine thiol moiety by displacing a water ligand

2. Oxidation of Fe(II) to Fe(III) by molecular oxygen, creating a radical species

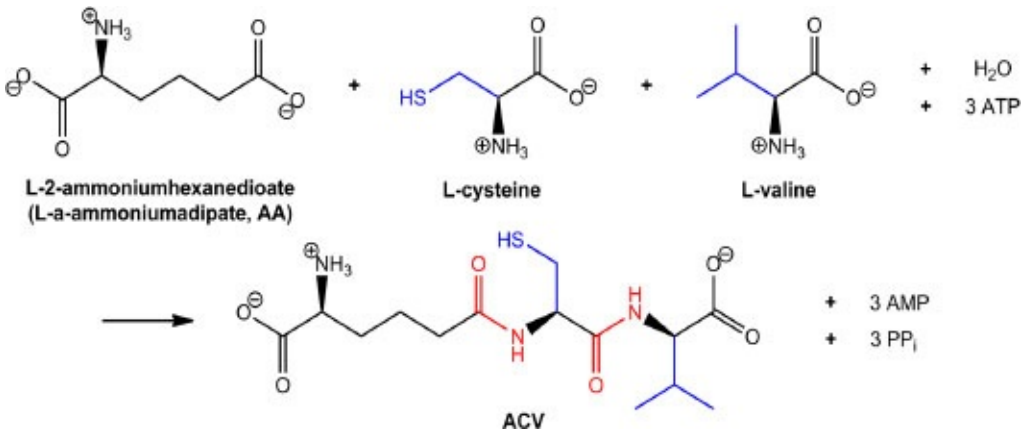
3. Intramolecular hydrogen transfer, oxidation of the thiol to thioaldehyde (extremely reactive due higher dipole moment than that of an aldehyde) and reduction of Fe(III) to Fe(II)

4. Amide deprotonation by the hydroperoxide ligand and nucleophilic addition of the nitrogen to the thiol with oxidation of Fe(II) to Fe(IV). This transformation results in the lactam ring formation

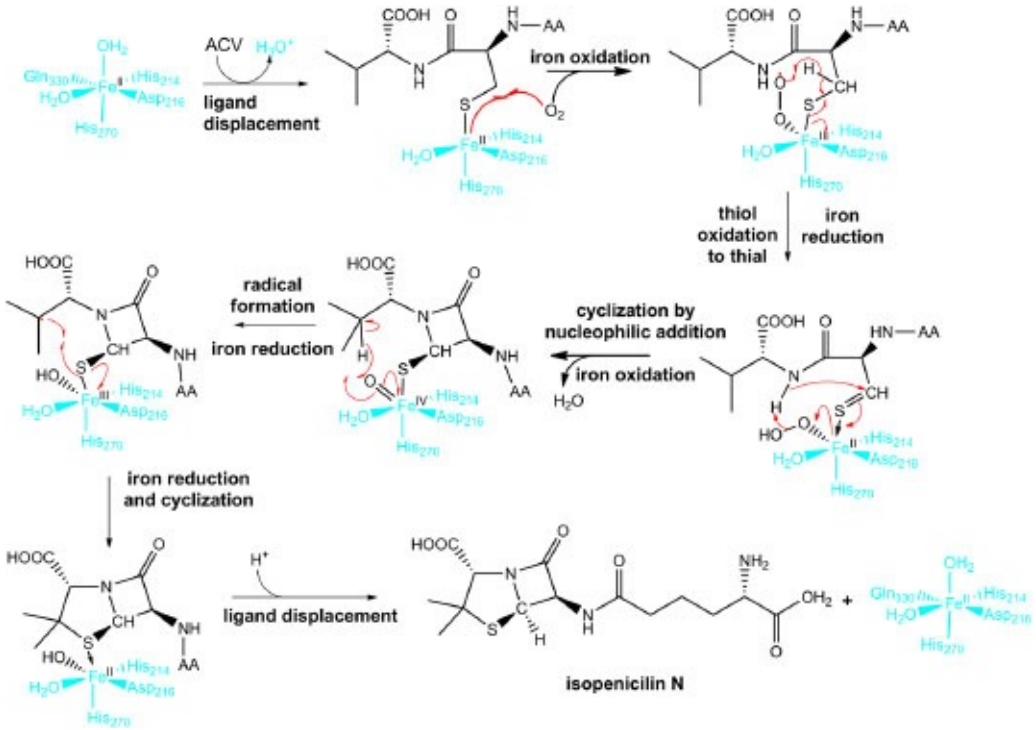
5. Radical formation by hydrogen abstraction by the oxide (turns into an hydroxide ligand) with reduction of Fe(IV) to Fe(III)

6. Radical attack of the sulfur atom, closing the thiazolidine ring and reducing Fe(III) to Fe(II)

7. Displacement of the sulfur by water to restore the enzyme active site and release isopenicillin-N.

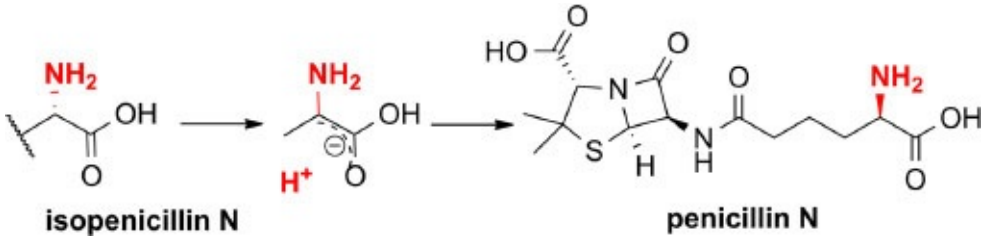


Scheme 1.1.39: Equation for the synthesis of ACV.



Scheme 1.1.40: Mechanism for the synthesis of isopenicillin-N by the action of isopenicillin-N synthase.

The isopenicillin-N may then proceed to be epimerised by isopenicillin-N epimerase (Scheme 1.1.41). This enzyme acts by α-deprotonation of the amino adipoyl moiety followed by protonation on the opposite side of the plane of the formed carboanion.



Scheme 1.1.41: Reaction scheme of the epimerisation of isopenicillin-N.

Substitutions of the side chain can also be performed by penicillin acylase (PA) to give other types of penicillin. PA catalyses the hydrolysis and synthesis of the amide bond to some side chain. The use of this enzyme *in vitro* enables the formation of semi-synthetic penicillin.

1.1.5.4 NAD^+ – a Classical Coenzyme

An enzyme cannot catalyse oxidation reactions unless a coenzyme is present. In some sense, the enzyme's role is to hold the substrate and coenzyme together to facilitate the oxidation reaction. One of the most commonly used coenzymes is nicotinamide adenine dinucleotide (NAD^+) (Fig. 1.1.9). There is evidence that sirtuins are proteins related to several diseases and are NAD^+ dependent.

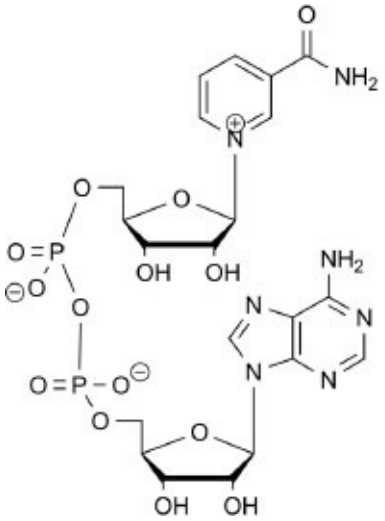
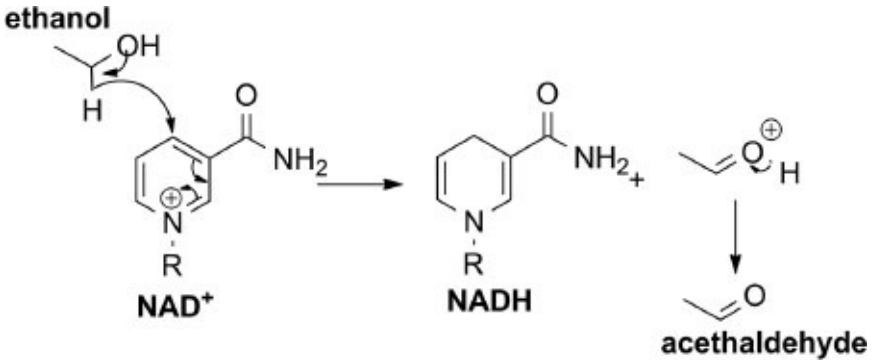


Figure 1.1.9: Structure of nicotinamide adenine dinucleotide (NAD^+).

The most known intervention of coenzyme NAD^+ is its role in the ethanol metabolism, where it acts as a common hydride acceptor (Scheme 1.1.42).



Scheme 1.1.42: Mechanism of ethanol oxidation in cells.

1.1.5.5. FAD – a More Versatile Coenzyme

Flavin adenine dinucleotide (FAD) (Fig. 1.1.10) is another coenzyme used in oxidation reactions and is normally associated tightly to a protein, usually designated as a *flavoprotein*. For example, in chorismate synthase, FAD is linked through the flavin ring $C_{(2)}$=O to one protein histidine residue (His^{106}) (Scheme 1.1.44). FAD is considered a more versatile coenzyme than NAD^+, because it can participate in oxidation reactions by several different mechanisms. For example, oxidation of dihydrolipoate involves a nucleophilic attack on C-4a whereas oxidation of an amino acid involves a nucleophilic attack on the N-5 position (Scheme 1.1.43).

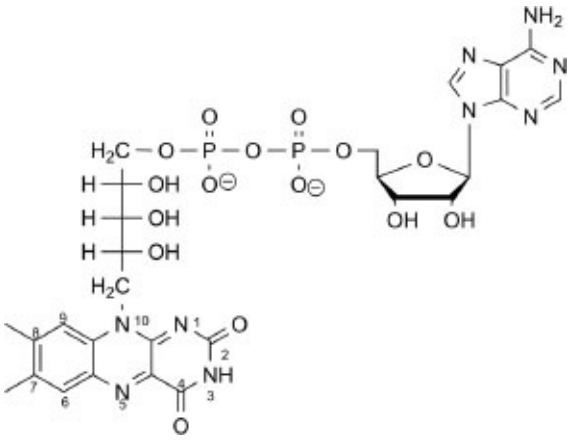
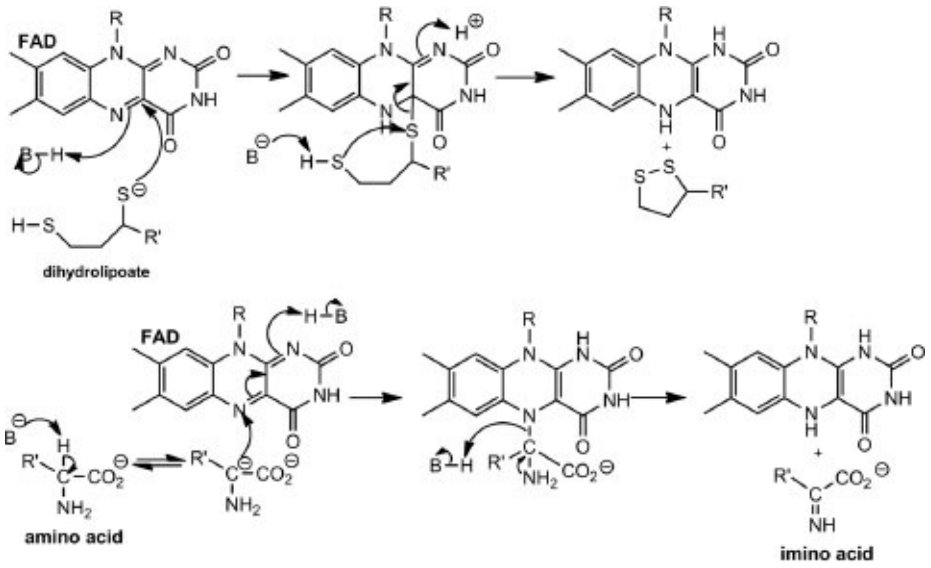
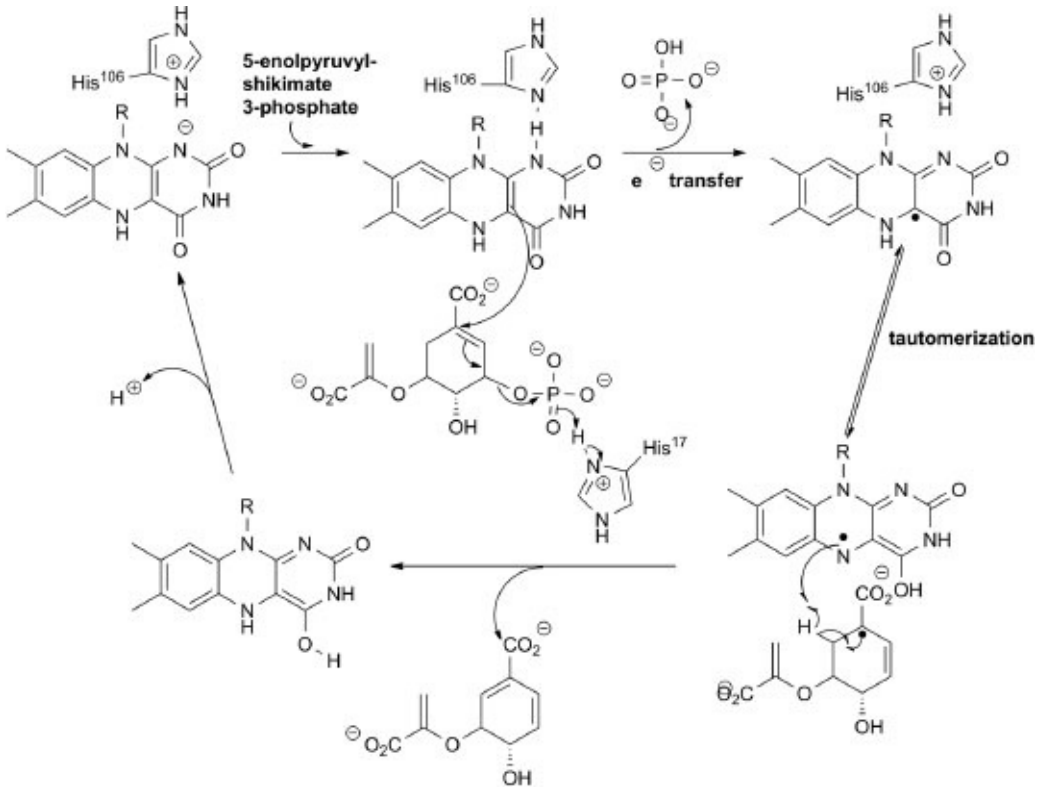


Figure 1.1.10: Structure of flavin adenine dinucleotide (FAD).



Scheme 1.1.43: Examples of FAD versatility in oxidation reactions.

Furthermore, FAD oxidation mechanisms are controversial because in some proposals, such as the chorismate synthase reaction (Scheme 1.1.44), the authors suggest the involvement of ionic and radical structures.



Scheme 1.1.44: Chorismate synthase reaction.

1.1.5.6 Biotin and Carboxylation Reactions

Biotin-dependent enzymes are common in living organisms and are involved in carboxylation reactions. Biotinylation occurs by the addition of a biotin molecule to a specific lysine residue (Fig. 1.1.11).

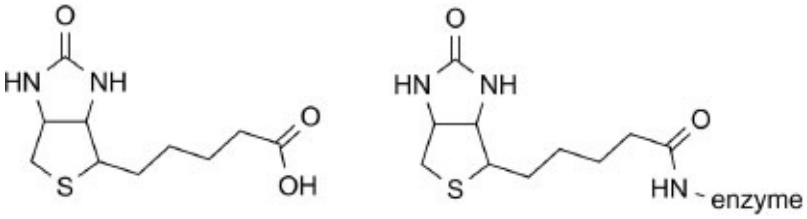
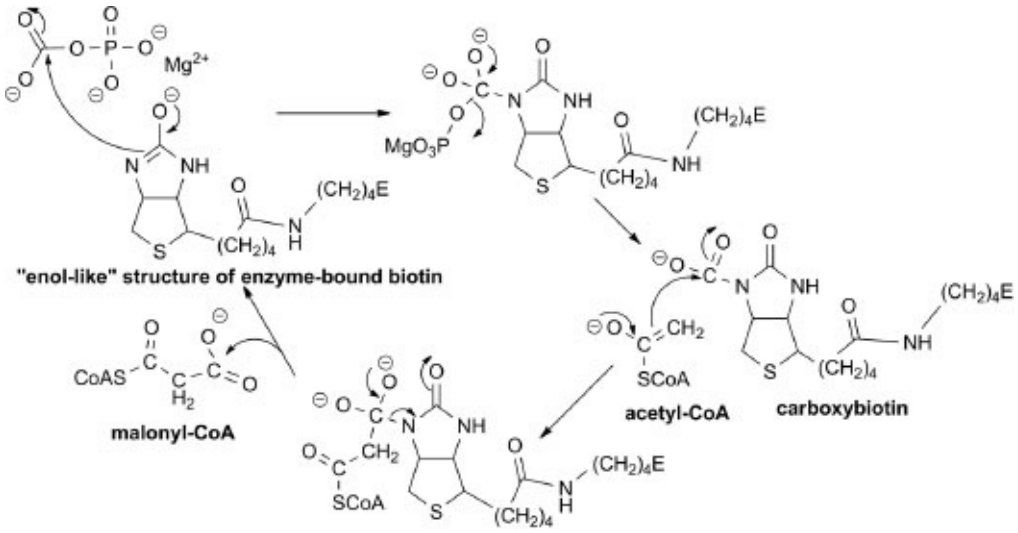


Figure 1.1.11: Biotin and enzyme-bound biotin.

The carboxylation catalysed by biotin-dependent enzymes use carbonate (HCO_3^-) as source of the carboxyl group, ATP to activate it and Mg^{2+} to decrease the overall negative charge. The mechanism involves a nucleophilic attack of the biotin moiety on the activated carbonate, resulting in the formation of carboxybiotin (Scheme 1.1.45). Nucleophilic attack by the substrate on carboxybiotin results in the transfer of the carboxyl group from biotin to the substrate, as shown in Scheme 1.1.45 with acetyl-CoA.



Scheme 1.1.45: Acetyl-CoA carboxylation mechanism.

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Francisca Lopes, Maria M. M. Santos and Rui Moreira*

1.2 Designing Covalent Inhibitors: A Medicinal Chemistry Challenge

1.2.1 Introduction

The design of enzyme inhibitors is one of the most attractive research topics in Medicinal Chemistry. In particular, covalent inhibitors provide the opportunity of combining concepts of chemical reactivity and mechanisms of organic reactions with the structural features required for optimal molecular recognition in order to obtain the appropriate reactivity and selectivity profile towards the desired enzyme target. Typically, these inhibitors present an electrophilic functionality capable of reacting irreversibly with catalytic amino acid residues containing a nucleophilic group (e.g. serine, threonine and cysteine) (Powers, 2002; Santos, 2007). In spite of its tremendous potential (Robertson, 2007; Potashman, 2009), designing selective covalent inhibitors remains a challenging task as the electrophilic groups present in many inhibitor structures can also react with other macromolecules leading to deleterious (off-target) events, or can be scavenged by ubiquitous low-molecular-weight nucleophiles such as glutathione leading to sub-optimal drug concentration at the site of action (Johansson, 2012). However, there are several examples of covalent inhibitors that are widely used drugs, including acetyl salicylic acid (the active ingredient of Aspirin), orlistat (anti-obesity drug) and ampicillin (antibiotic) (Fig. 1.2.1). Many of these drugs were not originally designed as irreversible inhibitors and their exact mechanism of action was often discovered afterwards. A recent example is the case of clopidogrel (antiplatelet agent), which was found to require activation by cytochrome P450 in the liver to generate an active metabolite containing a free thiol capable of reacting with a cysteine residue of adenosine 5´-diphosphate (ADP) receptor to form a covalent disulfide adduct (Fig. 1.2.1). Overall, nearly 30% of the enzymes that are inhibited by marketed drugs are irreversibly

inhibited via covalent modification, which highlights the therapeutic potential of covalent inhibitors (Robertson, 2005; Robertson, 2007; Johansson, 2012).

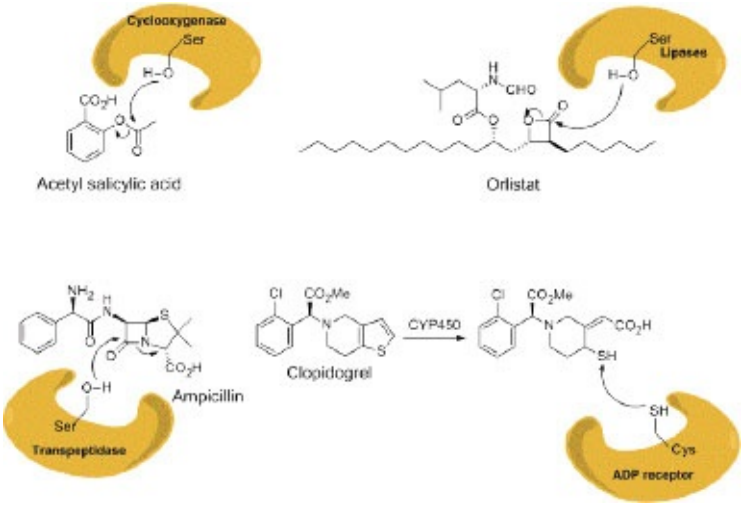
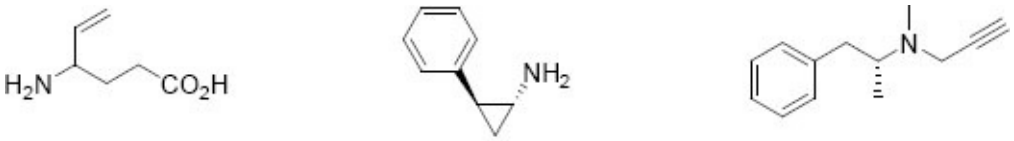


Figure 1.2.1: Drugs acting as covalent inhibitors including their protein target(s) with active-site nucleophiles. Arrows indicate the reaction of the enzyme's nucleophilic residue with the electrophilic site at the drug or its metabolite resulting in covalent modification of the target.

1.2.2 Designing Safer Covalent Inhibitors

Several approaches have been adopted by researchers in academia and pharmaceutical companies to overcome the liabilities associated with covalent inhibitors. A widely adopted strategy to develop highly selective enzyme inhibitors has been the design of mechanism-based or suicide inhibitors (Powers, 2002; Lucas, 2013). These are substrate analogs containing a poor electrophile moiety that are activated by the catalytic machinery of the target enzyme to generate a more electrophilic species capable of reacting with a nucleophile in the active site, leading to irreversible inhibition of the enzyme. This challenging approach has led to the discovery of suicide inhibitors (Fig. 1.2.2), which are in clinical use such as vigabatrin, an anticonvulsivant agent designed to irreversibly inhibit the GABA transaminase. Other suicide inhibitors such as tranylcypromine (monoamine oxidase inhibitor, antidepressant) and selegiline (monoamine oxidase inhibitor, antiparkinsonian) had their mechanism of action discovered serendipitously.

A second widely used strategy is to modulate the reactivity of the electrophilic site in the inhibitor and to optimize the molecular recognition towards the target enzyme. This approach was successfully used to develop selective Michael acceptors inhibitors towards cysteine proteases expressed by several viruses and parasites that are crucial for the development and infectiveness of these infectious agents. Molecular hybridization, where two different pharmacophores are joined through a linker (Meunier, 2008), has also emerged as a useful tool in medicinal chemistry to modulate the reactivity and improve selectivity of toxic compounds, including covalent irreversible inhibitors. These approaches will be dealt in more detail in the following sections.



Vigabatrin Tranylcypromine Selegiline

Figure 1.2.2: Mechanism-based covalent inhibitors. For detailed description on their mechanism of action see Silverman, 1992.

Fragment-based methods have been developed to rapidly discover selective irreversible covalent inhibitors of cysteine proteases with tempered reactivity. Typically, an initial assessment of the intrinsic reactivity of a panel of low-molecular weight Michael acceptors is performed using papain as a model cysteine-dependent enzyme (Santos, 2007), allowing the selection of the most effective warhead (Kathman, 2014). In this way, a fragment with the most specific binding affinity, rather than the most reactive fragment, might be identified for future optimization.

More recently, the concept of reversible covalent inhibitors has emerged as a powerful approach to avoid toxicity issues often associated to the formation of irreversible covalent adducts with off-targets. Although frequently designed to inactivate conserved, catalytically essential cysteines, covalent inhibitors can also achieve maximal selectivity among related targets by exploiting the intrinsic nucleophilicity of poorly conserved, solvent-exposed non-catalytic cysteines (Singh, 2011). Elegant work developed by Taunton and collaborators paved the way to establish the chemical basis for

designing reversible, cysteine-targeted covalent inhibitors. Based on a report that revealed that simple thiols reacted instantaneously with 2-cyanoacrylates at physiological pH, although the corresponding products could not be isolated or structurally characterized (Pritchard, 1968), Taunton hypothesized that this scaffold underwent Michael-type conjugate addition via a rapid-equilibrium process (Serafimova, 2012). Using a simple model reaction of different Michael acceptors with β-mercaptoethanol (BME) monitored by NMR, the group showed that, while compounds with a single electron-withdrawing group (e.g. 3-phenyl acrylonitrile, Fig. 1.2.3A) led to a stable adduct, their counterparts with two electron-withdrawing groups (e.g. ethyl 3-phenyl-2-cyanoacrylate, Fig. 1.2.3A) formed an adduct that rapidly reverted to the starting material upon ten-fold dilution with phosphate saline buffer (PBS). Combining this result with the predicted binding orientation of the pyrrolopyrimidine scaffold, similar to the irreversible fluoromethylketone-based inhibitor developed previously by the same group for the C-terminal domain of the p90 ribosomal protein S6 kinase RSK2, led to the discovery of the first reversible covalent kinase inhibitor (Fig. 1.2.3B) (Serafimova, 2012). Additional scaffolds were discovered *de novo* using a fragment-based ligand approach, where a library of low molecular-weight cyanoacrylamides was screened against several kinase assays (Fig. 1.2.3B) (Miller, 2013).

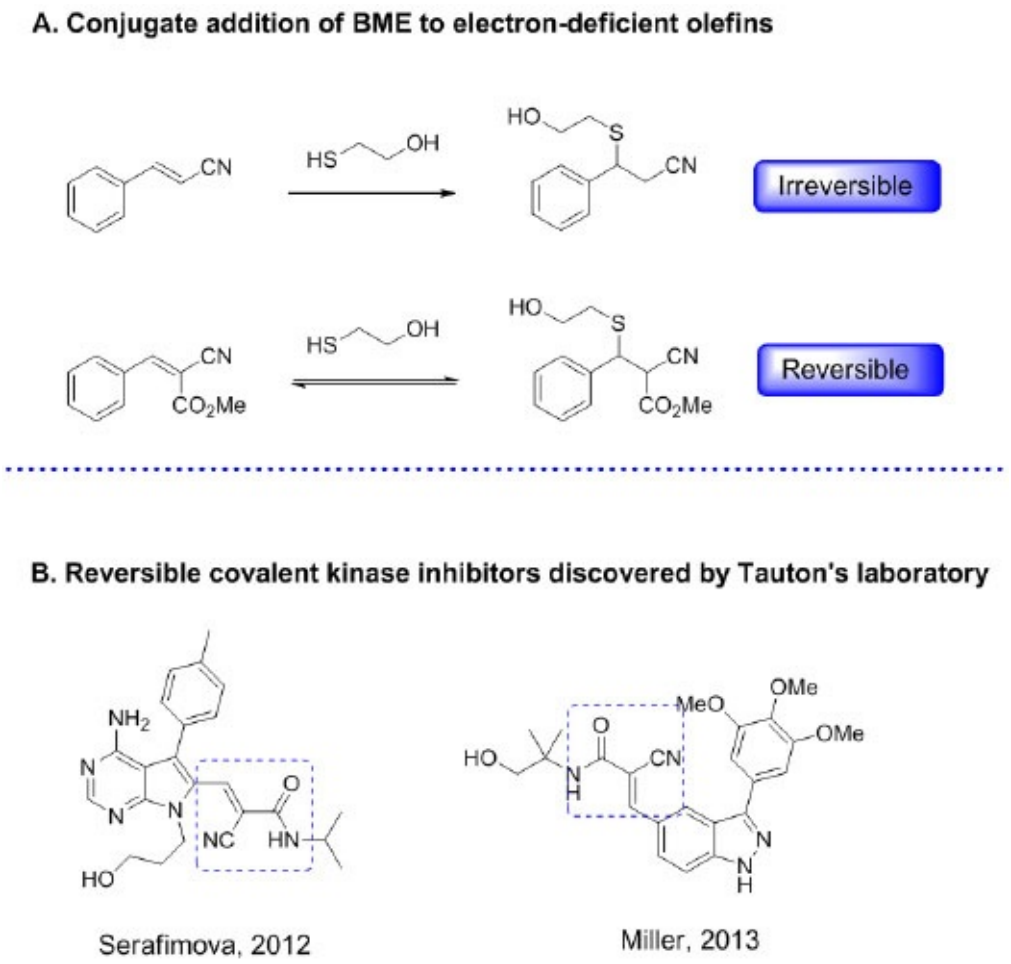


Figure 1.2.3: A) Thiol reactivity of electron-deficient olefins; B) Reversible covalent inhibitors that selectively target the non-catalytic cysteine-436 present in the C-terminal domain of the p90 ribosomal protein S6 kinase RSK2.

The reversibility of thiol addition to electron-deficient olefins relates to the propensity of the resulting adduct to undergo β-elimination via an E1cB mechanism (Fig. 1.2.4). A kinetic study to determine the β-elimination rates of BME from the adduct highlighted the structural features required to design reversible covalent inhibitors (Krishnan, 2014). Remarkably, the rates were shown to correlate inversely with the computed proton affinity of the corresponding carbanions, suggesting that the acidity of the proton at the α-position of the adduct provides the driving-force for the β-elimination (Fig. 1.2.4). In this way, a feasible method is now available to fine-tune the intrinsic reversibility of the thiol-Michael reaction in a predictable way.

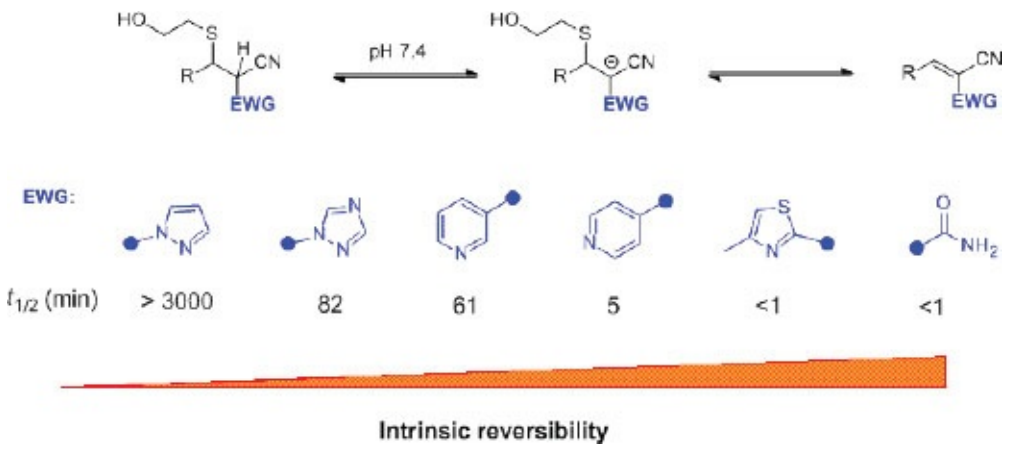


Figure 1.2.4: β-Elimination half-lives for the adducts from electron-deficient acrylonitriles and β-mercaptoethanol (BME) – adapted from Krishnan, 2014.

1.2.3 Case Study 1: Michael Acceptors to Treat Infectious Diseases

Electrophilic compounds that act as irreversible enzyme inhibitors are generally considered unsuitable drugs by medicinal chemists because the covalent binding might lead to off target effects, and many compounds are metabolically unstable (Wilson, 2013). However, this mode of action is quite common in approved drugs and biologically active molecules that inhibit enzymes (Johansson, 2012). In fact, several electrophilic compounds containing a Michael acceptor were described as potent cysteine protease inhibitors (Powers, 2002; Santos, 2007). Typically, Michael acceptor cysteine protease inhibitors have a peptide component (or mimic) that binds to subsites of the cysteine protease target. These compounds have considerable potential utility for therapeutic intervention in a variety of diseases, such as malaria, Chagas' disease and the common cold.

Peptidyl Michael acceptor inhibitors were introduced by Hanzlik and co-workers as specific irreversible inhibitors of the cysteine protease papain (Hanzlik, 1984; Thompson, 1986; Liu, 1992). Following that work, several vinyl sulfones and α,β-unsaturated carbonyl derivatives have been developed as highly potent inhibitors for many other cysteine proteases (Fig. 1.2.5) (Olson, 1999; Roush, 2001; Kumar, 2012; Graczyk, 1999; Ekici, 2006; Glória, 2011; Dragovich, 2003; Tan, 2013).

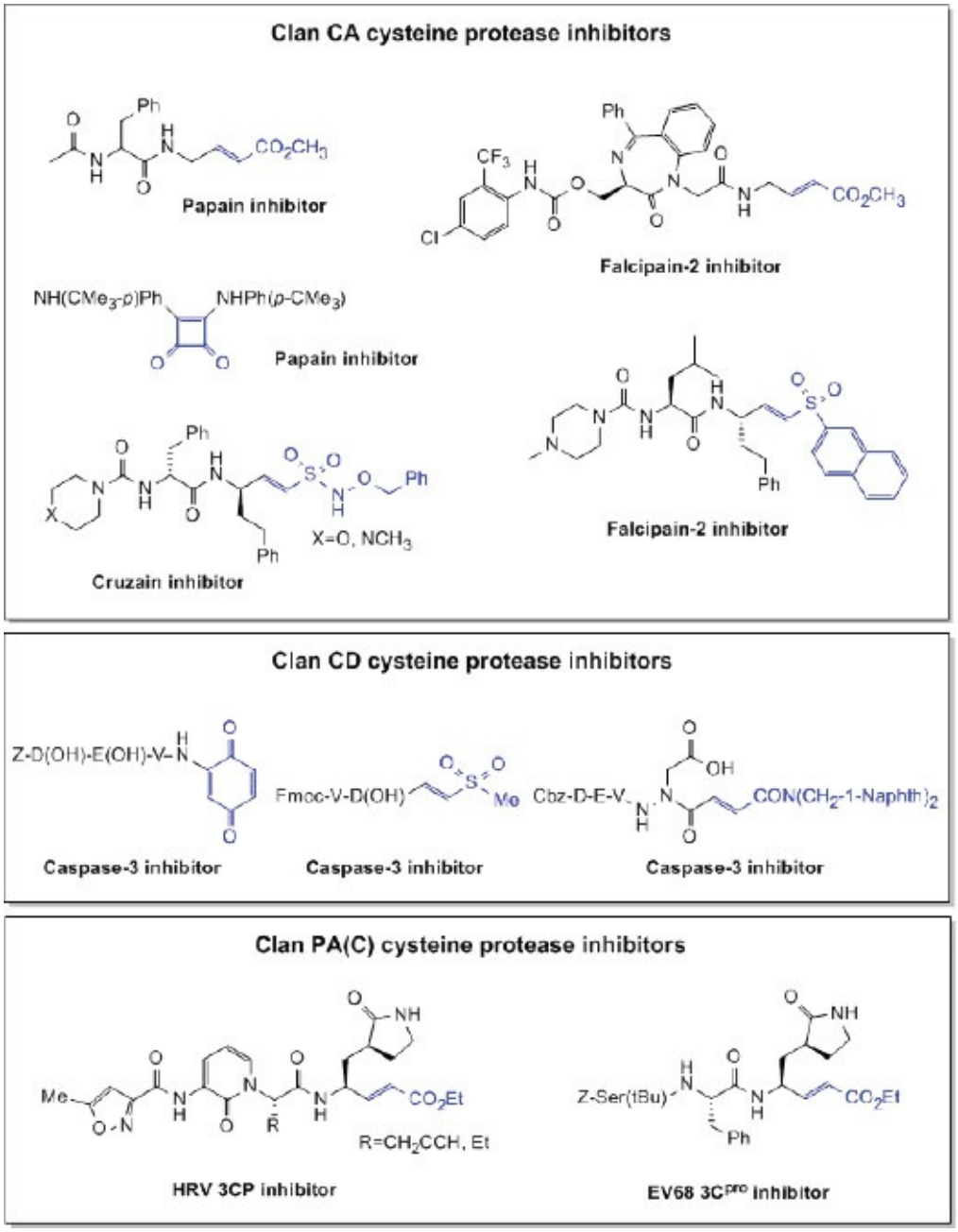


Figure 1.2.5: Selected examples of Michael acceptors that act as cysteine protease inhibitors.

These compounds inhibit cysteine proteases by forming covalent bonds with the active site thiol of cysteine proteases. In general, they are stable, and need the catalytic machinery of the cysteine proteases for activation. Of all the compounds developed, two Michael acceptor cysteine protease inhibitors, K-777 and Ruprintrivir (Fig. 1.2.6), have entered clinical trials.

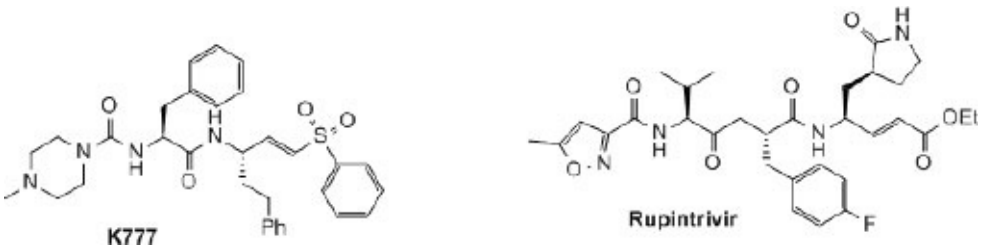


Figure 1.2.6: Cruzain inhibitor **K777** and human rhinovirus 3C protease inhibitor **Rupintrivir**.

1.2.3.1 K777 Inhibitor

Cruzain, a cysteine protease that belongs to clan CA, is a key protease required for the survival of *Trypanosoma cruzi*, the ethiologic agent of Chagas' disease (McGrath, 1995; Wilkinson, 2009). For that reason, a promising group of drug leads for Chagas' disease is cysteine protease inhibitors targeting cruzain. In 1998, McKerrow's group reported a dipeptide vinyl sulfone, K-777 (Fig. 1.2.6), that rescued mice from a lethal *Trypanosoma cruzi* infection by inhibition of cruzain (Engel, 1998). Proof of mechanism came from the crystal structure of inhibitor K777 bound to cruzain, where the catalytic cysteine residue (Cys25) is covalently linked to the β-carbon of the vinylsulfone (Fig. 1.2.7). K777 was shown to be safe and efficacious in animal models of acute and chronic Chagas disease (Doyle, 2007; Barr, 2005). In particular, this vinyl sulfone protected Beagle dogs from cardiac damage during infection by *T. cruzi* (Barr, 2005). The results of preclinical trials showed that K777 was non-mutagenic, well-tolerated, and demonstrated efficacy in models of acute and chronic Chagas' disease in both mice and dogs (Kerr, 2009). On the basis of these results the inhibitor entered clinical trials, but in 2013 the clinical assays were stopped due to tolerability findings at low dose in primates and dogs (http://www.dndi.org/diseases-projects/portfolio/k777.html).

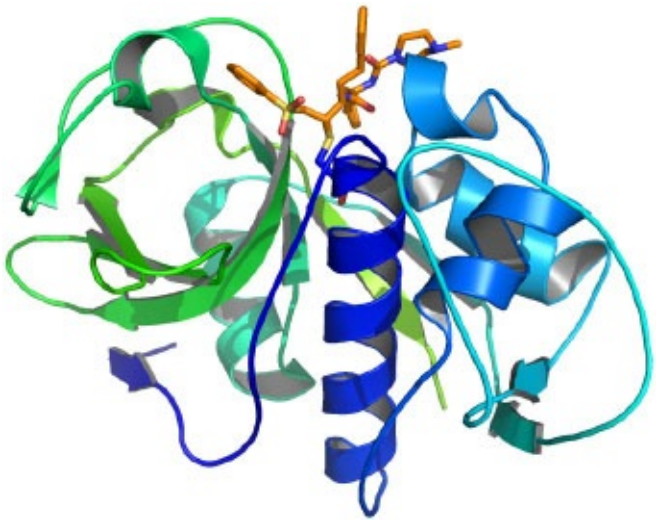


Figure 1.2.7: The crystal structure of inhibitor **K777** bound to cruzain (pdb 2OZ2). The insert shows the interaction of K777 with the primary recognition site (S_2) and secondary recognition sites (S_1 and S_3) of cruzain.

1.2.3.2 Rupintrivir (AG7088)

The human rhinovirus 3C protease (3CP) belongs to clan PA(C). Because the rhinoviral protease (3CP) is specific to rhinovirus, inhibitors of this protease should have few side effects in humans. Several compounds containing a Michael acceptor moiety were described as potent Human rhinovirus (HRV) 3C protease inhibitors (Santos, 2007). Specifically, Rupintrivir (also known as Ruprintrivir or AG7088) was developed by Agouron Pharmaceuticals to treat HRV infections (Fig. 1.2.6). Rupintrivir is an α,β-unsaturated carbonyl that irreversibly inhibits the HRV 3C protease and shows potent, broad-spectrum anti-HRV activity *in vitro* (Hayden, 2003). The phase I and phase II results showed good safety and pharmacokinetic profiles. Although it successfully completed the initial Phase II trials (Hayden, 2003), due to lack of efficacy in natural infection studies, further development of Rupintrivir was stopped in PhaseII/III trials (http://www.drugdevelopment-technology.com/projects/ag7088/).

1.2.4 Case Study 2: From Covalent Inhibitors to Hybrid Drugs

The design of hybrid compounds is a widely used strategy to improve

activity or to overcome the emergence of resistance to treatments infectious diseases. It has been successfully used to optimize the targeting of reactive cysteine protease covalent inhibitors in the field of parasitic diseases.

Falcipain-2 (FP-2) and falcipain-3 (FP-3) are cysteine proteases, located in the digestive vacuole of the malaria parasite and involved in the digestion of host hemoglobin essential for the survival of *Plasmodium falciparum*, the causing agent of the most lethal form of malaria. Rosenthal and co-workers demonstrated that inhibition of these enzymes allowed the cure of murine models of the disease and thus they constitute a validated target for its treatment. Generally, the inhibitors of falcipains have a backbone recognition element coupled with an electrophilic warhead such as aldehydes, fluoro methyl ketones, vinyl esters, vinyl sulfones (Fig. 1.2.6), and chalcones that can react with the catalytic cysteine residue (Shenai, 2003). However, the intrinsic reactivity of many of these warheads has prompted the development of hybrid compounds in order to modulate reactivity, improve selectivity and to avoid the emergence of resistant strains. Several hybrid compounds were prepared combining endoperoxides with different inhibitors of hemoglobin digestion. The goal was to bring together two drugs that can act in the digestive vacuole by two different mechanisms of action and taking advantage of the iron (II) coming from the digestion of the host hemoglobin (Meunier, 2008).

1.2.4.1 Hybrid Compounds Containing an Electrophilic Warhead

In one of the first studies of hybrid compounds containing an electrophilic warhead, Capela et al. reported the synthesis of hybrids comprising artemisinin and dipeptidyl vinyl sulfones (Fig. 1.2.8) (Capela, 2009). These were designed to act in the parasite food vacuole – where host hemoglobin is decomposed to provide nutrients – via endoperoxide activation by Fe(II) and falcipain inhibition. All compounds were very potent against the *P. falciparum* W2 strain, with IC_{50} values in the low nanomolar range. Some of the compounds showed also superior activity than chloroquine or artemisinin, the golden standards to treat malaria, against four additional resistant *P. falciparum*

strains with different phenotypes. However the hybrid compounds were only moderately active against FP-2 in the low micromolar range. The authors ascribed the antiplasmodial activity of the compounds to the size of the artemisinin moiety inside the active site of falcipain, which might preclude optimal binding to the enzyme.

Using the same rationale, Oliveira et al. synthesized 1,2,4,5-tetraoxane-based hybrid molecules (Fig. 1.2.8) containing dipeptidyl vinyl sulfone partners to deliver the FP-2 inhibitor once activated in the parasitic food vacuole (Oliveira, 2013). These compounds showed excellent antimalarial activity against both chloroquine-sensitive and chloroquine-resistant strains of *P. falciparum*. In spite of the low *in vitro* inhibitory activity against FP-2, the hybrid compounds inhibited hemoglobin digestion inside the parasites at low nanomolar concentrations, which was confirmed by the microscopic observation of swelling of the parasite digestive vacuoles. This result is consistent with a Fe(II)-triggered process inside the parasite that leads to the delivery of a potent vinyl sulfone (Fig. 1.2.9). Importantly, the authors were able to detect the intact parent vinyl sulfone in infected erythrocytes 48h post-treatment with the hybrid compounds, which strongly suggests that the vinyl sulfone inhibitor remained within infected erythrocytes long after the fast-acting endoperoxide moiety had exerted its effects. These results indicate that the intrinsic activity of the vinyl sulfone partner can be masked, suggesting that a tetraoxane-based delivery system offers the potential to attenuate the off-target effects of known drugs (e.g. irreversible enzyme inhibitors such as Michael acceptors).

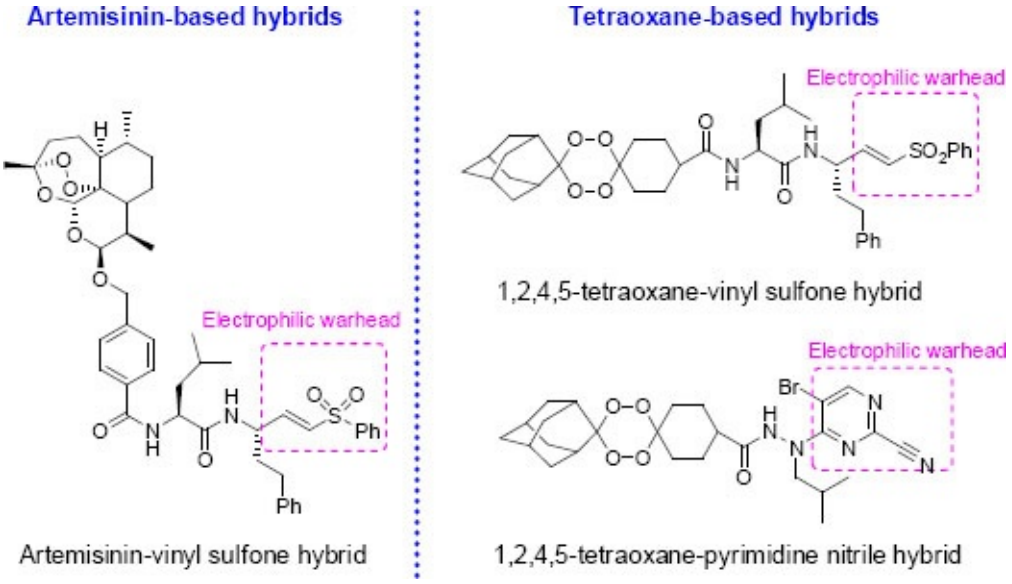


Figure 1.2.8: Structures of endoperoxide-based hybrids containing an electrophilic warhead for cysteine protease inhibition.

The hybrid approach was extended to tetraoxane derivatives designed to deliver FP-2 inhibitors based on peptidomimetic pyrimidine nitriles. Nitriles inhibit cysteine proteases by forming a reversible thioimidate intermediate resulting from the nucleophilic attack of the catalytic cysteine residue (Ehmke, 2011; Ehmke, 2012). Several heterocyclic and peptidomimetic compounds containing a nitrile warhead displayed excellent inhibitory activity against falcipain-2 and against cultured *P. falciparum* (Ehmke, 2011; Coterón, 2010). The pyrimidine nitrile tetraoxane hybrids (Fig. 1.2.8) displayed potent nanomolar activity against three strains of *P. falciparum* and FP-2, combined with low cytotoxicity. These hybrid compounds showed also significant effects on liver stage model *in vitro* using *P. berghei* infecting Huh-7 human hepatome cells and one derivative also showed *in vivo* efficacy when compared with a non-treated control group (Oliveira, 2014). As with their vinyl sulfone counterparts, the pyrimidine nitrile tetraoxane hybrids were shown to deliver the corresponding peptidomimetic pyrimidine nitrile inside the parasite upon activation by Fe(II).

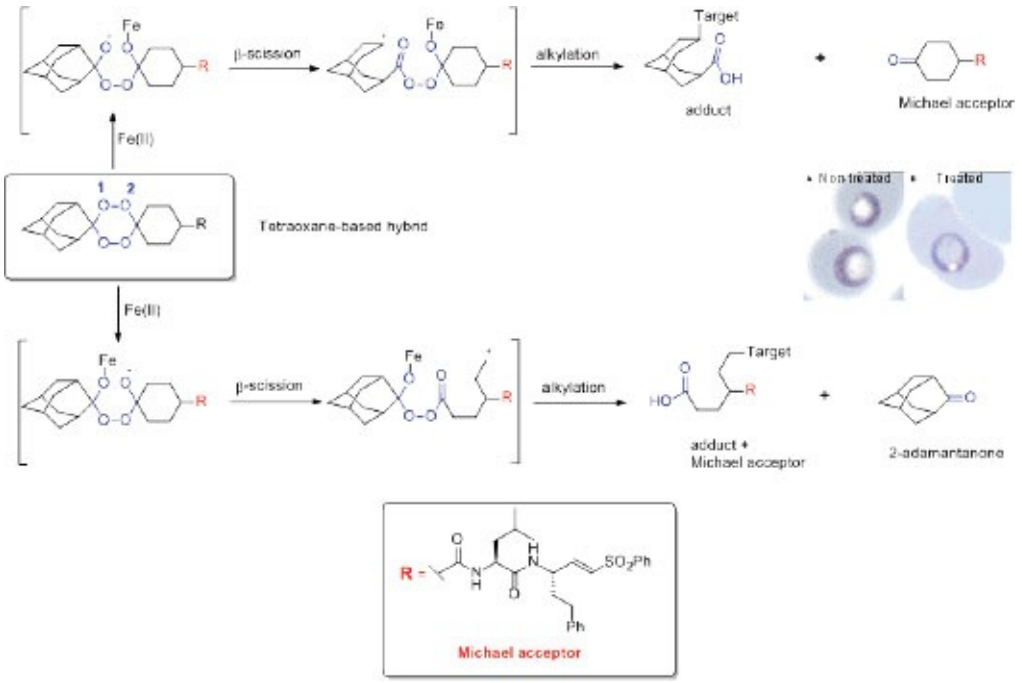


Figure 1.2.9: Tetraoxane-vinylsulfone hybrid compounds and their activation by Fe(II) from host hemoglobin in infected erythrocytes (Oliveira, 2013). Insert: delivery of the Michael acceptor (vinyl sulfone) *via* iron binding to oxygen-2 leads to the swelling of the parasitic digestive vacuole.

1.2.4.2 Hybrid Compounds Containing a Masked Electrophilic Warhead

Based on Roche's antimalarial arteflene, O'Neill reported a prodrug approach consisting of an endoperoxide containing cysteine protease inhibitors in a latent form. After entering the ferrous-rich food vacuole of the malaria parasite, the molecule will be fragmented by iron (II) releasing various antiparasiticidal elements (Fig. 1.2.10). These compounds exhibit significant antimalarial activity, in the low nanomolar range, and they can act by two different mechanisms, cytotoxicity exerted by the C-radicals species formed by the activation of the peroxide, and inhibiting falcipains due to the effect of the carbonyl inhibitor released in the activation process (O'Neill, 2004). They demonstrated that in the presence of Fe(II) the compounds liberated a chalcone and additional potential parasitocidal chemical species. Furthermore, they proved, by LC-MS analysis of pro-drug exposed parasites, that these systems are capable of liberating a chalcone in the living parasites (O'Neill, 2004).

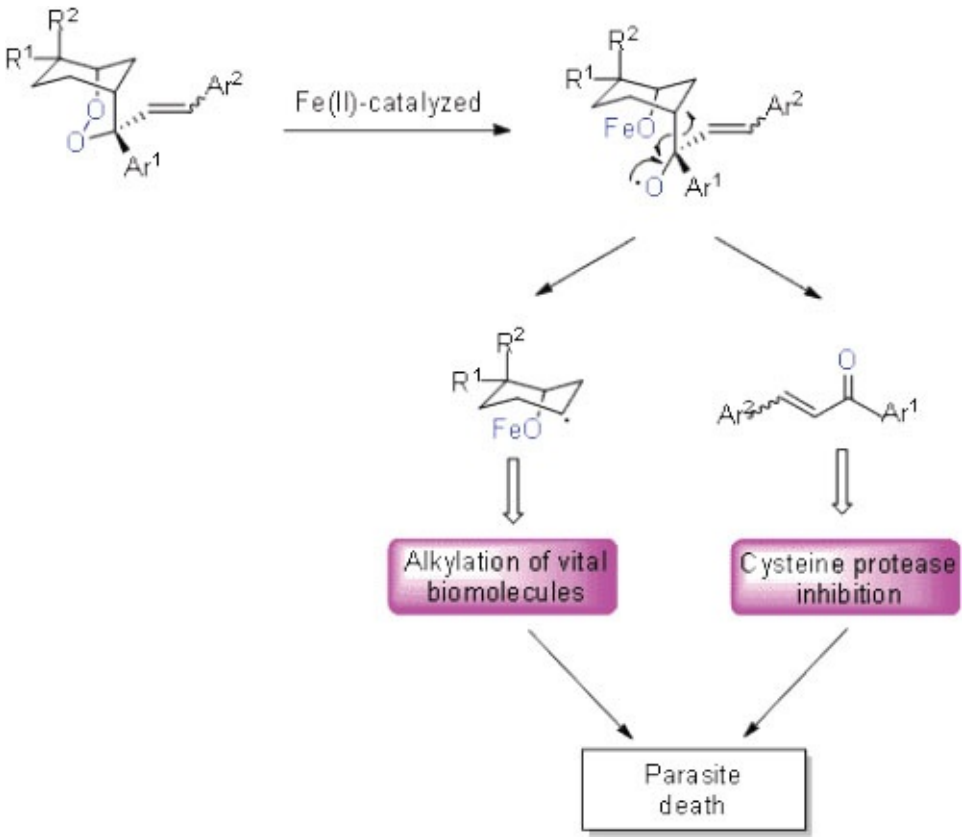


Figure 1.2.10: Fragmentation of arteflene-related peroxide to secondary carbon centered radical species and protease inhibitor, active against falcipain-2 from *P. falciparum*. Adapted from O'Neill, 2004.

The same group extended the concept to a new type of hybrids combining a 1,2,4-trioxolane moiety with peptidic cysteine protease inhibitors. They demonstrated that in the presence of Fe(II) the 1,2,4-trioxolane scaffold decomposes into a potentially toxic carbon radical that alkylates the heme *in vitro*, and a Michael acceptor that acts as a cysteine protease inhibitor (Fig. 1.2.11). The most potent compound of the series presented an IC_{50} value in the low nanomolar region (35 nM) against the 3D7 strain of *P. falciparum*. Remarkably, although the same compound showed inhibitory activity of FP-2 in the submicromolar range (0.5 μM), the corresponding aldehyde released upon Fe(II) activation (Fig. 1.2.11) was shown to inhibit FP-2 with an IC_{50} value of 16 nM, thus confirming 1,2,4-trioxolane scaffold as a site-specific delivery system for electrophilic aldehydes and ketones (Gibbons, 2010).

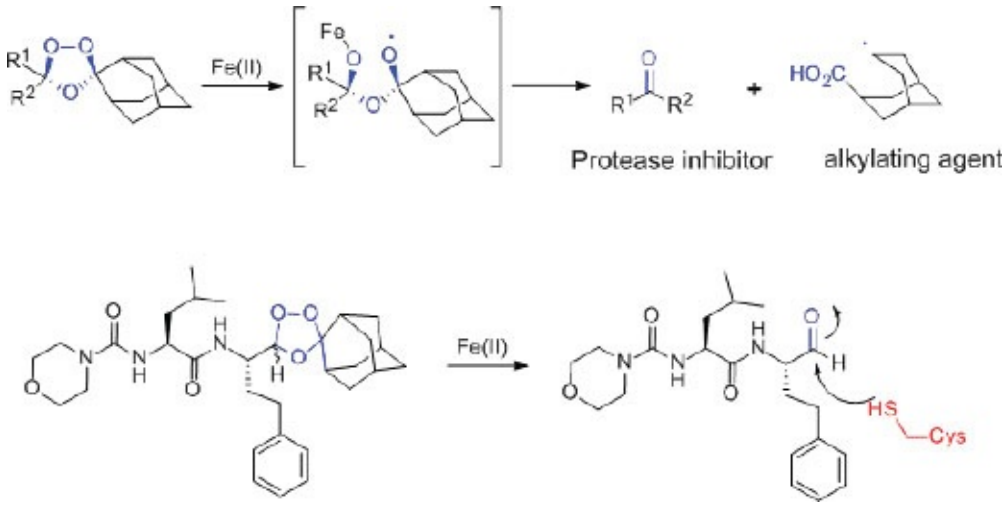


Figure 1.2.11: Fragmentation of 1,2,4-trioxolane to secondary carbon centred radical species and protease inhibitor, active against falcipain-2 from *P. falciparum*. Adapted from Gibbons, 2010.

1.2.5 Conclusions

Designing selective covalent inhibitors remains one of the most attractive areas of Medicinal Chemistry, where concepts of Organic Chemistry and Biochemistry are used to discover compounds that react preferentially with the active site of the target enzyme. The chemical basis to design irreversible and reversible covalent inhibitors is now well established, providing the medicinal chemist with a variety of solutions to modulate intrinsic reactivity and reduce the likelihood of toxicity issues. The chemical toolbox is further expanded with the concept of molecular hybridization to modulate the reactivity of electrophilic warheads or even to mask these chemical functionalities. This chemical toolbox is already delivering interesting drug candidates into the pipelines of pharmaceutical companies, small biotech and academic laboratories.

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Section 2: **Chemical Basis of Drug Action and Diseases**

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2.1 Pharmacokinetics and Bioanalysis to Improve Drug Development

Abbreviations

ADME – absorption, distribution, metabolism and excretion;

APCI – atmospheric pressure chemical ionization;

AUC – area under the drug concentration-time curve;

BA/BE – bioavailability/bioequivalence;

BCRP – breast cancer resistance protein;

Caco-2 – human colon adenocarcinoma cell line;

CL – clearance;

C_{max} – maximum concentration;

CV – coefficient of variation;

CYP – cytochrome P450;

DDD – drug discovery and development;

DDI – drug-drug interactions;

EMA – European Medicines Agency;

ESI – electrospray ionization;

FDA – Food and Drug Administration;

GC – gas chromatography;

HPLC – high performance liquid chromatography;

HTS – high-throughput screening;

ICH – International Conference on Harmonisation;

IS – internal standard

LC – liquid chromatography;

LLE – liquid-liquid extraction;

LLOQ – lower limit of quantification;

LOD – limit of detection

MDCK – Madin-Darby canine kidney cell line;

MIST – metabolites in safety testing;

MRM – multiple-reaction monitoring

MS – mass spectrometry;

MS/MS – tandem mass spectrometry;

NCEs – new chemical entities;

PAMPA – parallel artificial membrane permeability assay;

P_{app} – apparent permeability;

PepT1 – oligopeptide transporter;

QC – quality control;

Q-TOF – quadrupole coupled with time-of-flight mass analyzer;

RE – relative error;

RSD – relative standard deviation;

SIM – selective ion monitoring;

SPE – solid-phase extraction;

SRM – single-reaction monitoring;

$t_{1/2}$ – elimination half-life;

TDM – therapeutic drug monitoring;

t_{max} – time to reach the C_{max};

UGT – UDP glucuronosyltransferase;

UHPLC – ultra-high pressure liquid chromatography;

UV – ultraviolet;

Vss – apparent volume of distribution at steady state.

2.1.1 Introduction

The first decade of the 21st century was a challenging period for the pharmaceutical industries focused on drug research. Several changes in technology and market dynamics have occurred over the last years and they inexorably revolutionized the drug discovery and development (DDD) process. The tremendous progress in biomedical knowledge and the completion of the human genome sequencing project have accelerated the discovery of the molecular basis of human diseases and have allowed the identification of important new biological therapeutic targets. In addition, the synergistic interaction between rational drug design, recombinant biotechnology and combinatorial chemistry prompted a fast increment of the number of new chemical entities (NCEs) with molecular characteristics propitious to interact with specific biological targets (Ohlmeyer, 2010).

Despite this exponential increase in the number of NCEs, pharmaceutical industry is suffering from a lack of productivity considering the number of new drugs that have successfully reached the market (Keserü, 2009). According to recent news, it is estimated that only one in ten drug candidates that enter the clinical development phase will reach the market and the success rate for drugs to be approved considering all the therapeutic areas is only 11% (Tamimi, 2009).

The current climate of downward pressure on healthcare costs combined with the need of expensive technologies and greater information about drugs under development prompted pharmaceutical companies to develop paradigm-shifting strategies and tactics in order to cut drug development costs, fill the depleting product pipelines and reverse the trend of dropping productivity. A well-succeeded front-loading approach consisted on selecting, in the early drug discovery stage, the drug candidates that are not only pharmacologically active but also those having desirable pharmacokinetic properties. It is particularly disheartening when the *in vitro* potency is achieved, but the

lead compound fails *in vivo* because, for instance, it is so short-lived that its clinical benefits would be minimal. In fact, since the pharmaceutical industries embraced the early pharmacokinetic screening of drug candidates, the attrition rates of DDD were substantially improved and, therefore, it is currently straightforward to make a decision on the pharmacokinetic desirability of each drug candidate in the beginning of DDD processes and not only later in the non-clinical and clinical development stages (Saxena, 2008; Gallo, 2010). This demand expanded the investigation on pharmacokinetics into the initial phases of drug discovery and several *in silico*, *in vitro* and *in vivo* models have been developed and improved to optimize the pharmacokinetic behavior of drug candidates at different stages of DDD. Moreover, pharmacokinetic principles are also applied during the post approval period of a drug, particularly for therapeutic drug monitoring (which has recently been defined as therapeutic drug management, TDM), evaluation of line-extension medicinal products and generic formulations, particularly through bioavailability/bioequivalence (BA/BE) studies.

Taking into account these new attempts, a close involvement between pharmacokinetics and bioanalysis (defined as the determination of drug concentrations on biological specimens) became required in order to obtain data with a high quality level and allow an adequate interpretation and ultimately rational decision-making. On the other hand, the increasing number of NCEs that must be investigated and the application of pharmacokinetic screening at initial phases of DDD increases the number of samples that must be analyzed and, consequently, new bioanalytical techniques with higher sensitivity, selectivity and speed have emerged. Indeed, decades ago, high performance liquid chromatography (HPLC) coupled with ultraviolet (UV) or fluorescence detection and gas chromatography (GC) with mass spectrometry (MS) ensured the acquisition of plasma concentration data to define drug exposure in animal tests and clinical studies. In current practices, liquid chromatography (LC) coupled to MS or tandem mass spectrometry (MS/MS) are becoming essential due to their higher throughput capacity to identify and quantify the parent drugs and metabolites in biological matrices, enabling fast and effective decision-making. Furthermore, new strategies for sample preparation have also

been thoroughly developed to support the high-throughput analysis of samples, including automated online sample preparation with instrumental analysis.

It is important to acknowledge that although the high-throughput strategy is well established in all DDD phases, it has different emphases and, consequently, each stage of DDD places different requirements on the bioanalytical assays used to provide information. Briefly, in the initial phases of DDD, straightforward, fast, efficient and high-throughput analytical methods are needed without requiring a fully regulated validation. As the drug candidate moves to development stages, the quality of bioanalytical methods must be improved and their accuracy, precision and reliability are regulated by governmental requisites.

Thus, in this chapter the role of pharmacokinetics in each stage of the DDD process and the general characteristics of the bioanalytical methods developed and employed to assess the pharmacokinetic properties of drug candidates will be discussed. Additionally, owing to the recent evolution of the international guidelines on bioanalytical method validation and its increasingly stricter sanctions on drug development and post-marketed studies, a section of this chapter is devoted to overview the most important regulatory guidelines. Moreover, due to the significant technological advances continuously reached in this field, recent bioanalytical strategies that promote greater speed and productivity in bioanalysis during the pharmacokinetic studies are also addressed.

2.1.2 Pharmacokinetics on Drug Discovery and Development Process

The pharmacokinetic principles applied during the process of DDD have undergone a revolution in the last decades, as it was understood that the efficacy of any successful drug depends not only on its pharmacological potency but also on its absorption, distribution, metabolism and excretion (ADME) characteristics.

Over the time, it became clear that drug candidates with low and/or

high variable bioavailability and/or short half-life in animal studies were frequently prevented to progress to clinical trials. Furthermore, poor pharmacokinetic properties of new drug candidates in phase I clinical trials accounted for their clinical failure, while the incidence of severe (toxic) adverse side effects resulting from drug metabolism and drug-drug interactions (DDI) observed during phase II and III clinical trials may lead to the termination of potential new drugs or the withdrawal of marketed drugs. Although ADME studies are undeniably important throughout the stages of (non)-clinical drug development, some of the aforementioned failures could have been avoided if the pharmacokinetic deficits of the drug candidate were identified earlier in the discovery phase. The recognition that understanding the ADME processes of test compounds would potentially prevent their unsuccessful progression into clinical development phases expanded the ADME/pharmacokinetics interest from its traditional role as non-clinical safety support towards the early stage of drug discovery. Today, ADME/ pharmacokinetic studies are considered essential from drug discovery to phase IV clinical development and their main goals on each phase are summarized in Table 2.1.1. To attain these objectives, a range of *in silico*, *in vitro* and *in vivo* approaches have been developed and optimized as it will be further explained.

2.1.2.1 ADME/Pharmacokinetic Evaluation on Early Drug Discovery Phases

As projects advance from the beginning of drug discovery (hit identification) to the last stage of drug discovery, which is named lead characterization and mostly performed *in vivo*, the required throughput (number of candidates to be considered) declines but assay predictability (content of information) increases proportionally. Most importantly, during the early and middle stages of drug discovery (including hit identification, lead identification and optimization), high-throughput screening (HTS) assays are utilized, allowing to test hundreds of compounds to be tested per day (Pereira, 2007; Carlson, 2008; Di, 2008; Wan, 2009).

In current practice, even before NCEs are synthesized, *in silico*

mathematical models have been employed to predict the pharmacokinetic attributes of compounds, preventing the synthesis and screening of compounds with obvious liabilities. Based on mathematical and statistical relationships between the physicochemical and molecular characteristics of the NCEs and their pharmacokinetic properties, recent *in silico* models are commercialized as software that predict pharmacokinetic parameters: Meteor®, MetabolExpert® and MetaSite® are examples of commercial metabolic fate/stability predictors; GastroPlus® and IDEA® perform pharmacokinetic simulations of the rate and extent of absorption in gastrointestinal tract (Parrott, 2002; Kuentz, 2006). Although *in silico* models have higher throughput ADME screening ability, saving more time and effort than *in vitro* and *in vivo* models, they can hardly be accurate enough to replace real circumstances (Lavè, 2007; Burton, 2010; Gallo, 2010).

Thus, during the phases prior to lead characterization where the throughput is high, the potential pharmacokinetic features are mainly addressed by *in vitro* tools. On the other hand, in the last phase of drug discovery, more comprehensive *in vitro* and *in vivo* models may provide a definitive assessment of overall drug disposition of the best compounds previously screened. Table 2.1.2 summarizes the major *in vitro* model systems most frequently employed for ADME/pharmacokinetic analysis in the early stages of drug discovery programs and their respective objectives. At the initial stages of drug discovery, ADME screening is conducted in a high-throughput mode and includes, but it is not limited to, determinations of apparent permeability [using parallel artificial membrane permeability assays (PAMPA) and/or cell lines such as human colon adenocarcinoma (Caco-2) or Madin-Darby canine kidney cells (MDCK)], plasma protein binding, human or animal liver microsomal stability, and identification of the reactive metabolites. Most of these *in vitro* models are generally amenable to miniaturization and automation, improving the evaluation capacity of HTS assays (Mayr, 2009). For ADME/pharmacokinetic analysis in the late stage of drug discovery, major methodologies include protein binding (as well as blood– plasma partitioning and plasma stability), hepatocyte stability, metabolic enzyme phenotyping and human cytochrome P450 enzyme (CYP) inhibition/induction.

Table 2.1.1: The role of pharmacokinetics in different stages of drug discovery and development process (Panchagnula, 2000; Chien, 2005; Bhogal, 2008; Zhang, 2012).

Stages	**Objectives**	**Purpose of pharmacokinetic studies**
Drug Discovery	The global aim is to choose the optimum candidates considering their pharmacokinetic characteristics in connection with their intrinsic activity and potency and particularly:	
	Predict *in vitro* ADME of NCEs; Understand the mechanisms underlying the ADME of NCEs;	Screen NCEs according to their absorption; Identify the main elimination pathways and metabolic enzymes involved; Foresee the plasma protein binding of NCEs; Predict their potential to develop drug-drug interactions;
	Determine NCE bioavailability in rodent species. Predict pharmacokinetic parameters.	*In vivo* fast exposure and pharmacokinetic screen of the NCEs.
Pre-clinical Development	The global aim is to evaluate, in laboratory animal models, the pharmacokinetics and toxicokinetics of the most promising compounds found on the drug discovery stage and:	
	Characterize the pharmacokinetic profile of the parent compound and its main metabolites; Demonstrate biologic activity and safety in laboratory animal models; Investigate the food effect on pharmacokinetics. Select the best formulation for drug exposure and provide confidence that the predicted drug effects are achievable via the chosen route of administration; Establish safe and/or	Determination of compound full exposure in animals; Determine absolute and relative bioavailability; Evaluate dose proportionality; Assess the margin of safety based on efficacy concentration and exposure data from toxicokinetic studies; Determine the best formulation for drug exposure; Predict compound pharmacokinetics in humans; Integration of pharmacokinetics and pharmacodynamics data to understand the pharmacology in animals from different species;

	efficient starting doses and dosing regimens for clinical trials; Determine whether a drug is deemed to be suitable for administration to humans on the basis of an acceptable risk assessment.	
Phase I	Investigate whether the compound is safe and well tolerated in humans; Assess pharmacokinetics and pharmacodynamics in humans and investigate the pharmacokinetic attributes of the compound; Evaluate if the compound should move on to further development phases; Establish dosing range and dosage regimens.	Tolerability and safety over a range of doses; Measurement of pharmacokinetic parameters using single and multiple doses; Establishing whether plasma concentration increases in proportion to the dose administered in humans; Evaluate the influence of age, gender, genetic characteristics and food on drug pharmacokinetics in humans.
Phase II	Demonstrate efficacy in the intended population; Identify attributes of the compound in target population compared to the existing therapy; Identify the metabolites and evaluation of their contribution to the biological profile of the compound; Evaluate sex, food and polymorphism influence in drug therapeutic and safety profiles.	Quantitative determination of the distribution of a compound in bodily fluids and tissues; Investigate absolute and relative bioavailability; Establishing a clear dose-response relationship; Assessment of the influence of gender and food on pharmacokinetic profiles; Assessment of the influence of genetics in drug metabolizing enzymes on pharmacokinetic profiles.
Phase III	Demonstrate safety and efficacy for clinical use; Studies at patient	Determination of the pharmacokinetic profile in the target population; Determination of the pharmacokinetic profile

	subgroups at potential risk to adjust dose regimens; Confirm dose/exposure response relationship in target population and subpopulations.	in subgroups of target population; Determination of the pharmacokinetic profile of the final formulation of the drug candidate; Determination of a clear dose-response relationship in target population and subpopulations.
Phase IV	Dosage form improvements; Change drug formulation (modified release preparations, line extensions).	Determination of the pharmacokinetic profile of new formulations; Determination of the pharmacokinetic profile of modified drugs designed to extend patent life; Determination of eventual influence of other drugs on the pharmacokinetic profile;

ADME, absorption, distribution, metabolism and excretion; NCE, new chemical entity

Independently of the precise nature of the screening technology, all methodologies must generally follow five criteria when designed and implemented during the discovery program (Fretland, 2010; Volpe, 2010):

Table 2.1.2: Major *in vitro* model systems used to investigate the absorption, distribution and metabolism of new chemical entities in initial phases of drug discovery.

In vitro model	General characteristics	Applications
Absorption *in vitro models*		
PAMPA	HTS assay rapidly performed in 96-well plates; P_{app} measurement through a hydrophobic filter impregnated with lipids; Compound quantification by UV/VIS spectrophotometry; Good predictability and easily to be automated.	Screen NCEs in accordance to their absorption ability by transcellular passive diffusion; Prediction of human intestinal fraction absorbed.
Caco-2 cell line	Cells have human origin and encompass many characteristics of intestinal epithelium; Cells are grown on semi-	Screen NCEs in accordance to their permeability through Caco-2 monolayer; Evaluation of absorption and prediction of human intestinal

	porous filters in 6-, 12-, 24- or 96-well plates; P_{app} is determined from apical to basolateral sides and *vice-versa*. Compound quantification by HPLC.	permeability; Understand mechanisms underlying intestinal absorption; Identification of NCEs that are substrates or inhibitors of the intestinal efflux transporter, P-glycoprotein. Identify efflux transporters involved in compounds intestinal absorption. Toxicity studies.
Ussing chamber	Uses intestinal membrane from mice or rats; Segments of small Intestine are mounted in the chamber with oxygenated buffer surrounding it for approximately 3 h. P_{app} is determined from apical to basolateral sides and *vice-versa*. Compound quantification by HPLC with simple sample preparation for bioanalysis.	Screen NCEs in accordance to their permeability through animal intestinal membrane; Evaluation of absorption and understand the mechanisms underlying intestinal absorption Identify efflux transporters involved in compounds intestinal absorption. Evaluation of specific transport mechanisms, enhancing strategies on a mechanistic basis; Evaluation of toxicity of compounds.
Transporter *in vitro* models		
Caco-2	Caco-2 cells undergo enterocytic differentiation and become polarized in culture (usually for 21 days), resembling human intestinal epithelium in transporter expression and tight junction formation. Bi-directional studies and compound quantification by HPLC with simple sample preparation for bioanalysis. Determination of efflux coefficient.	Evaluate the efflux transport via P-glycoprotein and BCRP. Identification of P-glycoprotein substrates and inhibitors in drug discovery. Evaluation of drug-drug interaction potential.

Transfected MDCK cell line	MDCK are previously transfected with P-glycoprotein gene to over-express the transporter. 5 days of incubation is usually required to initiate the studies. Bi-directional studies and compound quantification by HPLC with simple sample preparation for bioanalysis. Determination of efflux coefficient.	Evaluate the efflux transport via P-glycoprotein. Identification of P-glycoprotein substrates and inhibitors in drug discovery. Evaluation of drug-drug interaction potential.
Membrane vesicles	Membrane vesicles prepared from organs (such as liver, kidney and intestine) that naturally express high concentrations of transporters or from transfected cell lines (such as MDCK) that over-express a single transporter. This technique allows separate preparations of the blood side of cell membranes (liver- sinusoidal, kidney and intestine basolateral) and luminal side of cell membranes (liver-bile canalicular, the intestine and kidney-brush-border).	Assess the transporter mechanisms in liver, kidney and intestine.
Protein binding *in vitro models*		
Ultrafiltration	A semi-permeable membrane separates an upper chamber where drug protein solution is placed from a lower chamber. Centrifugation (approximately 2000 *x g*) is applied to separate the bound and unbound fractions due to the high molecular size of compound-protein complex which cannot pass through the membrane. The free drug concentration in	The simplest and fastest method for determining the concentration of the drug that is not binding to the plasma proteins during: - Drug discovery; - Clinical therapeutic drug monitoring; - Clinical pharmacodynamic and pharmacokinetic studies.

	the lower chamber and the total drug concentration prior to ultrafiltration are determined and then used to determine the extent of protein binding. Commercially available 96-well ultrafiltration apparatus. HPLC is frequently employed.	
Equilibrium Dialysis	Equilibrium dialysis is based on the principle of the diffusion of solutes along a concentration gradient across a semi-permeable membrane. The protein solution containing drug is placed in one chamber while buffer is placed in the opposing chamber. The unbound drug passes through the membrane and at equilibrium, the unbound drug will be at equal concentrations on both sides of the membrane while the bound drug will remain in the protein chamber. Equilibration times are long (typically 12–48 h). HPLC is used to quantify the free and total drug concentrations.	Frequently used in drug discovery in order to determine the percentage of drug binding to plasma proteins maintaining a true equilibrium during the whole experiment.
Metabolic *in vitro* models		
Expressed enzymes	Expressed enzymes are concentrated enzymes with high activity toward a specific substrate, they are often used as "bioreactors" to generate metabolites. Using the generated metabolites that are collected as chromatographic fractions, it is possible to test for their pharmacological activity.	Conduct reaction-phenotyping for drug candidates and identify non-CYP microsomal or cytosolic enzymes often involved in drug metabolism. Identify the relative contribution of each CYP isoform to the total hepatic clearance. Identify the involvement of CYPs in metabolic pathways.

		Predict drug-drug interaction potential.
Microsomes	They are the most widely used subcellular fractions for drug metabolism studies, with the advantages of being inexpensive and easy to handle while containing the major drug metabolism enzymes, e.g., CYPs and UGTs. It is necessary to supplement the subcellular fractions with enzymes cofactors to initiate the various enzymatic reactions. By including or excluding certain co-factors, one can pinpoint the involvement of certain metabolic pathways for a given drug candidate. Compounds are quantified after predefined times of incubation by HPLC. Both reversible inhibition and time-dependent inhibition studies provide information on the possibility of the drug candidate being a perpetrator of drug-drug interaction for a co-administered drug.	Determine intrinsic clearance, a useful parameter for facilitating the screening process for stable compounds and for establishing an in vitro correlation between animals and humans. Identification of the main metabolites. Assess extra-hepatic metabolism and further strengthen the in vitro prediction of total body clearance by using microsomes from various tissues. Microsomal assays are the default assays for metabolism and DDI studies at the drug discovery stage.
Cell culture and cell lines	Primary cultures of hepatocytes: They carry enzymes and co-factors at physiological concentrations and provide a drug metabolism environment that closely mimics the *in vivo* conditions; however they have a limited life span and differentiation ability. Immortalized human hepatic tumor cell lines (HepG2 or HepaRG) They present lower cost and higher ease of storage than	Identification of the metabolites generated. Predict drug clearance; Predict DDI potential of drug candidates. 'Gold standard' for conducting CYP induction studies before (non)-clinical investigations.

	primary culture of hepatocytes, making them more suitable as a HTS methodology. However as they exhibit some morphological changes regarding *in vivo* models that must be considered during the analysis of the results. Compounds are quantified after predefined times of incubation by HPLC.	
Three-dimensional hepatic models	Different cells-types besides hepatic cells are seeded in the same scaffold and may include fibroblasts, Kupffer cells, vascular and biliary duct endothelial cells in order to maintain the liver-cells homeostasis. Although allow better cell-cell matrix interactions, resembling better *in vivo* functions and morphology than cell culture/lines, CYP expression may vary according to experimental conditions.	Predict hepatic clearance; Mainly used to predict drug hepatic toxicity.

BCRP, breast cancer resistance protein; Caco-2, human colon adenocarcinoma cell line; CYP, cytochrome P450; DDI, drug-drug interaction; HPLC, high performance liquid chromatography; HTS, high throughput screening assay; MDCK, Madin-Darby canine kidney cell line; PAMPA, parallel artificial membrane permeability assay; P_{app}, apparent permeability; UGT, UDP glucuronosyltransferase.

- *Relevance*: the result of the screen should have good concordance with the corresponding *in vivo* properties of the drug, requiring validation of the screen with standard compounds of known animal or human performance;
- *Effectiveness*: the cut-off criterion should eliminate a substantial fraction of compounds without eliminating every compound, otherwise the screen would merely add a useless extra step that delays the discovery process;

- *Speed*: the experimental procedure must be fast enough to keep pace with the input rate of new compounds from chemistry;
- *Robustness*: the experimental procedure must be applicable to a wide variety of chemical structures;
- *Accuracy and reproducibility*: any screening procedure has an inevitable characteristic error rate, because it is usually necessary to sacrifice some accuracy or precision to achieve the desired speed. Thus, when a large number of compounds is carried through a particular screen model, some of the compounds will be classified incorrectly. False positives are tolerable, but false negatives may be lost forever if the failure eliminates them from further testing.

It is also essential to regularly validate the *in vitro* models against *in vivo* data to ensure that decisions based on *in vitro* results are sufficiently predictive (Burton, 2010).

Once exhibiting these characteristics, *in vitro* assays contribute to understanding the underlying mechanisms of drug absorption and disposition, which are invaluable to establishing structure-activity relationships to guide subsequent chemical synthesis of new drugs, avoiding potential obstacles in drug development. It is also important to highlight that *in vitro* data are in current practice recognized as good predictors of *in vivo* end-points. Even the Food and Drug Administration (FDA) accepts the importance of *in vitro* data, as it can inclusively avoid certain clinical studies (FDA, 2000, 2003, 2006). For instance, since a negative result in CYP induction for *in vitro* human-based studies using primary hepatocyte cultures always seems to lead to a negative induction in human clinical studies, FDA typically waives clinical DDI studies if the drug candidate is tested as negative in a human *in vitro* CYP induction study.

However, the relative simplicity of *in vitro* systems for studying ADME/pharmacokinetics in comparison to real *in vivo* drug behavior means that absolute correlations between *in vitro* and *in vivo* findings are often difficult to establish routinely, particularly for humans. For this reason, *in vitro* screens are frequently viewed as a means to rank compounds for further study rather than for outright rejection, so the *in vivo* pharmacokinetic studies performed in animals during lead

characterization remain crucial after *in vitro* evaluation.

Without requiring full pharmacokinetic characterization, the *in vivo* studies performed at this stage ensure that lead compounds have appropriate pharmacokinetic properties to be evaluated in non-clinical pharmacology and safety studies (Table 2.1.1). In fact, when combined with *in vitro* results, they quickly assess the bioavailability of a compound, giving a good indication of the suitability for advancement to pre-clinical and clinical trials. The current trend is to use rodents (mice and rats) as first animal species to test drug exposure because they are less expensive and require smaller amount of test compound. These *in vivo* pharmacokinetic studies in rodents include single oral and intravenous pharmacokinetic studies and provide information such as drug oral bioavailability, distribution, clearance and duration of exposure for a drug and its metabolites. Thus, they can help to identify some limitations of a new chemical series regarding their ADME characteristics, such as low absorption or high clearance, leading to undesirable pharmacokinetic profiles. Subsequently, *in vitro* models such as Caco-2 can be used to optimize absorption of compounds from the same chemical series. The microsomal stability assay can also be applied to select more stable compounds.

Although a continuous demand for *in vivo* pharmacokinetic studies remains at discovery stages, most of these studies are still conducted in a traditional, low-throughput manner in many pharmaceutical companies. Furthermore, compound quantities as well as practical and ethical issues preclude *in vivo* studies performed at a sufficient rate to investigate the high number of new drug candidates (Balani, 2005). Conventional pharmacokinetic studies include the intravenous and oral administration of each compound to groups of 6–8 animals with blood samples taken at 6–12 different points of time (Alves, 2008), requiring a high number of animals, time and intensive bioanalysis. Consequently, in order to make timely and meaningful contributions in early discovery programs, *in vivo* pharmacokinetic analysis has been efficiently modified and accomplished through recent strategies such as pooling of plasma samples and fewer sampling time points per study, reducing the number of samples analyzed per each compound (Table 2.1.3). Interestingly, similar pooling strategies have also been applied in order

to determine brain concentrations and estimate the concentration ratios in relation to plasma levels (Amore, 2010).

2.1.2.2 Pharmacokinetic Evaluation on Drug Development Phases

The most promising compounds found in discovery stages are then evaluated in at least two relevant animal species to obtain pharmacodynamic, pharmacokinetic, and toxicological information. The animal species are chosen according to the previous *in vitro* analysis.

The extensive pharmacokinetic studies performed at this stage include single and multiple dose administration with several ascending doses, metabolic profiling and evaluation of drug potential to induce/inhibit drug-metabolizing enzymes. Major objectives of these studies are depicted in Table 2.1.1. Compartmental and noncompartmental methods are used to determine multiple pharmacokinetic parameters, including the maximum concentration (C_{max}), time to reach the C_{max} (t_{max}), plasma drug concentration-time curve (AUC), apparent volume of distribution at steady state (Vss), clearance (CL), elimination half-life ($t_{1/2}$) and absolute or relative bioavailability, providing characterization of the pharmacokinetic profiles for a test compound.

Table 2.1.3: Comparison between the strategies employed to increase the throughput of *in vivo* pharmacokinetic studies during drug discovery stages (Ward, 2001; Cheung, 2005; Li, 2013).

Strategy	General characteristics	Advantages	Disadvantages	Application
Cassette dossing (or *N*-in-one dossing)	Co-administration of several compounds (≈ 5–10) to a single animal; Collection of plasma samples over the study time course; Measurement of concentrations of each compound in a single run by high precision bioanalytical methods.	Allows obtaining complete pharmacokinetic profiles for each compound in each animal. Provides a faster assessment of *in vivo* exposure levels; Requires fewer animals and bioanalysis resources.	Compounds must be compatible within the same dosing solution that is administered; Difficulties in preparing a convenient formulation of multiple compounds; Co-administration of NCEs may increase significantly the potential to develop DDI; Complexity in bioanalysis of multiple analytes in the same run.	Triage compounds based on their oral exposure.
Snapshot Pharmacokinetic Study	Compounds are dosed individually as in conventional pharmacokinetic studies; Oral administration; Decrease the number of samples analyzed per compound, usually using only two animals per compound and collecting samples up to 5 h (4 time points). Sample pooling (also named cassette analysis) consists on combining equal volumes of plasma from the same time point from each animal; Sample pooling can be done with samples of animals dosed individually with different compounds.	Reduced time analysis; Savings in number of animals and analytical resources in relation to rapid pharmacokinetic studies; No DDI interference; Complete pharmacokinetic profiles for each compound.	No inter-animal variability data available; Only truncated $AUC_{0\text{-}5h}$ is obtained; Sample dilution and higher LLOQ; Complex bioanalysis and longer bioanalytical method development time if samples from animals dosed with different compounds are pooled; Difficult to adapt to mice due to the small sample volume.	Triage compounds based on their oral exposure; Investigate structure-activity relationships.
Rapid Pharmacokinetic study	Identical to cassette analysis but six animals are usually used per compound and samples are collected up to 24 h (6 time points). The six samples from the same collection time are pooled; Oral and intravenous administration.	No DDI interference; Compounds are analyzed separately; Amendable for automation; Suitable to obtain full set of pharmacokinetic parameters; Probable to reveal no sample dilution and low LLOQ.	No inter-animal variability data available; Labor intensive in-life portion; No savings on in-life dosing, sampling or number of animals.	To get full pharmacokinetic parameters : CL, Vss, C_{max}, AUC, $t_{1/2}$ and F.

AUC, area under the curve; CL, systemic clearance; C_{max}, maximum concentration; DDI, drug-drug interaction; F, systemic bioavailability; LLOQ, lower limit of quantification; NCEs, new chemical entities; $t_{1/2}$, half-life time; Vss, apparent volume of distribution at steady state

In parallel, toxicity tests are always performed in this stage and include genotoxicity, safety pharmacology in all biological systems, reproductive toxicology in male and female animals and long-term carcinogenicity. In this context, toxicokinetics, which is defined as the generation of pharmacokinetic data under the conditions of the toxicity studies themselves, is required as an integral component of non-clinical toxicity studies (Guideline ICH S3A). The primary objective of toxicokinetics is to describe the systemic exposure achieved in animals and its relationship to the dose level and the time course of the toxicity study. These objectives are mainly achieved by measuring the plasma (or whole blood or serum) concentrations of the parent compound and/or metabolite(s) through time and determining the plasma (or whole blood or serum) AUC and C_{max}. Subsequently, toxicokinetics relates the exposure achieved in toxicity studies with the toxicological

findings to assess the relevance of these results on human clinical safety. Toxicokinetics is thus recognized as an integral part of non-clinical testing programs that enhances the value of the toxicological data generated, not only to understand the toxicity results but also to predict the risk and safety in humans. Together with bioavailability evaluation, metabolite profile determination and plasma drug levels associated with toxicity, toxicokinetics determines the choice of the no observed adverse effect level, which is essential to define the human equivalent dose and the maximum recommended starting dose in first-in-human clinical trials (FDA, 2005).

At non-clinical development, disposition studies are also performed using radiolabeled compounds administered to animals to provide useful mass balance data, first-pass metabolism from vascular and portal vein cannulation animal models, biliary excretion conducted on bile-duct cannulated animals and tissue distribution, where the organs can be removed and drug quantified in order to evaluate whether drug accumulation may be involved. In all these studies, identification and quantification of drug (radiolabeled or otherwise) and main metabolites is required, implying the application of bioanalytical techniques adequately developed and validated for that purpose.

Clinical development initiates with the first administration of the drug to humans and all the stages are strictly based on specific guidelines from the International Conference on Harmonisation (ICH), European Medicines Agency (EMA) and FDA (ICH, 1997; EMA, 1998 2008; FDA, 2008a; Milton, 2009). The main goal of phase I clinical trials is to investigate whether the compound is safe and well tolerated in humans (Table 2.1.1). Pharmacokinetic properties must also be evaluated and the development of the drug can be stopped if its elimination half-life is found to be too short, too long or if it has poor bioavailability. Phase I clinical trials usually start with single sub-therapeutic dose studies which are escalated gradually followed by multiple dose studies and if the drug is not well tolerated, it is dropped from development. These closely-monitored trials help define the safe dosing range and whether the compound should move on to further development phases (Duff, 2007). Thus, from the pharmacokinetic point of view, absorption and elimination phases of the plasma concentration-

time curve must be fully characterized and all metabolites must be fully resolved, identified and quantified, demanding analytical techniques with enough sensitivity and selectivity to quantify the small amounts present in biological samples. Indeed, regulatory guidance documents have highlighted not only the importance of the identification and structure elucidation of drug metabolites, but also their early quantification in clinical development in order to address three important requirements: determination of whether metabolites contribute significantly to the overall pharmacology of a drug; assessment of safety coverage of metabolites and understanding the DDI potential of the drug and its metabolites (FDA, 2008b; 2012; Zhu, 2009; Frederick, 2010; EMA, 2012). The general key factors to be considered for these metabolites are relative quantity (major or minor metabolites), pharmacological activity, likelihood to be formed via reactive intermediates and whether they result from phase I or phase II metabolic reactions.

Thus, the FDA guidance on Safety Testing of Drug Metabolites (FDA, 2008b) defines a major metabolite as that which AUC value is higher than 10% of the AUC of the parent drug at steady-state conditions. The DDI guidance also proposes that human metabolites present at ≥ 25% of the parent drug's exposure (given by AUC) should be investigated *in vitro* for their DDI potential (EMA, 2012; FDA, 2012).

Considering Table 2.1.1, the pharmacokinetic analysis in phase II clinical trials is employed to assess the dose/exposure response and dose ranging studies are carried out to establish the effective doses for phase III clinical trials. On the other hand, phase III clinical trials can involve up to several thousands of patients to create an adequate database for assessing the efficacy and safety profile of a compound and to enable accurate drug labeling. Pharmacokinetic analysis is particularized for special sub-populations, including patients with impaired renal or hepatic functions (EMA, 2004a, 2005) as well as pediatric and elderly populations (EMA, 2004b). Hundreds of sites around the world participate in the study to get a large and diverse group of patients, implying a vast number of samples to be handled and analyzed.

Even after receiving approval to be marketed, a drug must continue to be evaluated by an appropriate pharmacovigilance system. Indeed, as a much larger number of patients begin to use the drug, companies must continue to monitor it carefully in order to evaluate long-term safety and the effect of the new drug in specific subgroups of patients.

Importantly, although several new drugs are brought to the market and help fight many diseases, ADME processes of a drug may differ among individuals, resulting in differences in the relationship of drug plasma concentrations and dosage administered. Since inter-individual differences may occur due to differences in age, weight, genetic polymorphism, co-morbid diseases and DDI, drug posology regimen is frequently individualized and TDM plays a very important role in this personalized medicine field. Its major objective is to ensure that drug concentrations are within a defined optimal therapeutic range to maximize drug efficacy, minimize drug toxicity and reduce therapeutic costs. In practice, it involves the measurement of drug concentrations in plasma or serum samples, and the interpretation of these results in an attempt to adjust the dose to that particular patient. Not all drugs are candidates for TDM, as it must meet the following criteria: (1) a narrow therapeutic range; (2) significant inter-individual variability in systemic exposure at a given dose; (3) a clear relationship between blood exposure and clinical effect; and (4) a validated bioanalytical method to measure drug concentration in plasma or serum.

2.1.3 Bioanalysis and Validation Requirements on DDD

As described in the previous section, in an attempt to diminish the attrition rate of DDD process, the pharmaceutical industry has put more emphasis on pharmacokinetic evaluation in the beginning of NCEs discovery. Consequently, bioanalysis emerged as an essential support tool to characterize ADME/pharmacokinetics of NCEs through all stages of DDD and not only in the development ones. Around the world, bioanalysis shares the main goal of quantifying drugs (and/or metabolites) in biological matrices, ensuring that high-quality data for valid scientific decision making is obtained. For that, several initiatives

are taken in day-to-day laboratory practices, such as various kinds of validation, inclusion of independent quality control (QC) samples or calibration curves mimicking real samples as close as possible. Considering the distinct objectives and techniques employed in each stage of DDD to characterize ADME/pharmacokinetics, it is evident that bioanalysis must be adapted to each particular case, requiring different sample preparation and analytical methodologies which do not need the same quality validation results.

Table 2.1.4 suggests the general characteristics of bioanalytical methods and the validation requirements for each DDD stage. In opposition to the regulated bioanalysis, which is focused on the rigorous development and validation of highly specific bioanalytical methods for a selected drug candidate in a particular biological fluid, the most striking feature of discovery bioanalysis is its breadth and diversity of *in vitro* and *in vivo* studies. Consequently, a high number of sample matrices are generated and a high number of different NCEs are simultaneously measured. As a result, the throughput, capacity and turnaround requirements are generally much higher in discovery phases in order to make timely decisions and process the elevated number of samples. At this stage, early screening tests conducted *in vitro* and *in vivo* can be performed following either good laboratory practices or not, with no real need to input significant resources in an attempt of developing a rugged and fully validated method. Instead, only some parameters, such as accuracy, precision and selectivity must be contained under certain well-accepted boundaries to guarantee that the results can be used for critical decision making. Thus, at drug discovery stages, the ideal bioanalytical assay should be simple, straightforward, rapid, largely applicable, efficient and allow significant throughput in order to be a successful screening tool capable of quantifying several NCEs subjected to the *in vitro* and *in vivo* tests performed in initial screening experiments.

Table 2.1.4: Bioanalysis and method validation requirements suggested during drug discovery and development.

Stage	Characteristics	Objectives	Validation
Drug Discovery	• Very fast • High-throughput • Straightforward • Moderate quality data	• Absorption screening (*n*-octanol/water, PAMPA, Caco-2 cells) • Metabolic stability screening (microsomes, hepatocytes) • *In vitro* cytochrome P450 inhibitory screening (microsomes, human recombinant enzymes) • Plasma protein binding (ultrafiltration, equilibrium dialysis) • *In vivo* pharmacokinetic screening (cassette dosing, pooling samples)	• Early study of detection and chromatographic conditions • Stability assays of stock solution • Former sample extraction procedure • Selectivity (no sample endogenous interferences, no interference of co-administered leads in cassette dosing and/or cassette analysis) • Calibration curve (low number of standards, n = 3-5) • Preliminary quantification range • Reasonable precision and accuracy
Preclinical Drug Development	• Fast • Moderate-throughput • High quality data	• Pharmacokinetic characterization in rodents, dogs, monkeys (single and multiple dose studies, absolute bioavailability) • Route-dependent pharmacokinetic disposition (intravenous/oral) • Toxicokinetics characterization of parent drug and its metabolites in toxicology species (dose/exposure levels, estimating the first dose to human exposure) • *In vivo* drug-drug interaction potential	• Sample extraction procedure optimization • Selectivity (no sample endogenous interferences, no interference of parent drug/metabolites) • Internal standard selection • Defined range of standard calibration curve • LLOQ and ULOQ establishment • Intra and interday precision and accuracy assessment (3-5 validation runs) • Effect of dilution on precision and accuracy • Extraction recovery assessment (parent drug/metabolites and internal standard) • Stability experiments to cover sample handling and storage conditions
Clinical Drug Development	• As fast as possible • Low-throughput • Very high quality data	• Pharmacokinetics in healthy humans, target patient population and special populations (paediatric, geriatric, hepatic and renal impairment) • Food effect and relative bioavailability (solution/suspension vs solid dosage form) • Drug-drug interactions with agents commonly co-prescribed or with narrow safety window	• Finalization of extraction, chromatographic and detection conditions (based on anticipated concentrations following the lowest dose in humans) • Full validation is required: selectivity, linearity, LLOQ, LOD, accuracy, precision, recovery, parent drug/metabolites stability experiments • Verification of robustness/ruggedness of the assay

Caco-2, human colon adenocarcinoma cell line; LLOQ, lower limit of quantification; LOD, limit of detection; PAMPA, parallel artificial membrane permeability assay; ULOQ, upper limit of quantification.

As the drug candidate enters to the development stages, matrix complexity increases as well as the rigor needed to accurately quantify the NCEs in biological samples and then estimate the pharmacokinetic parameters. The throughput of the method may decrease, but the quality of data must be higher and validation should be formalized and mandated as per the required norms. The most widely accepted guidelines for validation of bioanalytical methods, particularly in the pharmaceutical and medical sciences, are the Technical Requirements for Registration of Pharmaceuticals for Human Use guideline Q2(R1) issued by the ICH (2005), the Guidance for Industry, Bioanalytical Method Validation by the FDA (2001) and the Guideline on Bioanalytical Method Validation recently issued by the European Medicines Agency (EMA) in 2011. Typical validation parameters and the requirements of each guideline are presented in Table 2.1.5. Selectivity is referred to as the ability of a bioanalytical method to unequivocally differentiate and quantify the analytes of interest in the presence of other sample components. Endogenous matrix components, metabolites, and

decomposition products or even co-prescribed drugs include the most common interferences. Thus, it is necessary to establish that the chromatographic signal produced is only due to the analyte and not as a result of the co-elution of other compounds. A bioanalytical method is considered to be selective if the lack of response in the blank biological matrix at the retention time of the analytes is demonstrated at the lower limit of quantification (LLOQ). The determination of method sensitivity, linearity, precision and accuracy is required by the three guidelines and usually performed at three concentration levels in several replicates (typically three to five). Linearity should be demonstrated within the defined calibration range with the quality of the calibration curve as an aspect of the greatest importance in bioanalytical method validation. In fact, as Almeida et al. (2002) stated, the quality of bioanalytical data is highly dependent on the quality of the calibration curve used to extrapolate the analyte concentrations in unknown samples. The calibration curve is the relationship between instrumental response and the concentrations of the analyte and it must be generated for each analyte, using a sufficient number of calibration standards (Table 2.1.5). Precision, which expresses the closeness degree among individual measures of an analyte when the same procedure is applied repeatedly to multiple aliquots of a homogenous matrix, is frequently performed on the same day (intra-day precision or repeatability) and over different days (intermediate precision or inter-day precision), both expressed by the relative standard deviation (RSD), which is the absolute value of coefficient of variation (CV). Accuracy, which describes the closeness between the mean experimental data obtained by the method and the corresponding nominal concentration, is also determined intra- and inter-daily and is expressed by the relative error (RE), as a percentage, which is the ratio between experimental and nominal concentrations, or by bias, which is the deviation from the nominal value as a percentage. Matrix effects, carry-over and dilution integrity have been recently integrated in the guideline issued by EMA as well as stability evaluation. From a practical point of view, these parameters can be estimated from the analysis of QC samples, which represent the future real samples.

Table 2.1.5: The ICH, FDA and EMA validation parameters and respective limits instituted.

VALIDATION PARAMETER	ICH	FDA	Limits	EMA	Limits
Selectivity	V	6 blank samples	No interferences	6 blank samples	No interferences
LLOQ	V	6 replicates	Precision ≤ 20% Accuracy ± 20%	6 replicates	Precision ≤ 20% Accuracy ± 20%
LOD	V	NR		NR	
ULOQ	NR	NR		Intra and inter-day (n = 6)	Precision ≤ 20% Accuracy ± 20%
Calibration curve linearity	5 calibration concentration levels	6-8 calibration concentration levels	Accuracy ± 15%[a]	≥ 6 calibration concentration levels	Accuracy ± 15%[a]
Range	V	NR		Defined between LLOQ and ULOQ	
Accuracy (Bias %)	3 concentration levels 3 replicates for each one	3 concentration levels 5 replicates for each one	Accuracy ± 15%	4 concentration levels[b] 5 replicates for each one	Accuracy ± 15%[c]
Precision (RSD or CV %)	3 concentration levels 3 replicates for each one	3 concentration levels 5 replicates for each one	Precision ≤ 15%	4 concentration levels[b] 5 replicates for each one *With-in run and between-run*	Precision ≤ 15%
Recovery (%)	NR	3 replicates	Precise and consistent	NR	
Carry over effect	NR	NR		Blank sample analysis following the high concentration standard	Interference should be ≤ 20% of LLOQ and 5% for internal standard
Dilution Integrity	NR	NR		Matrix spiked with analyte concentration > ULOQ and diluted with blank matrix 5 replicates per dilution factor	Precision ≤ 15% Accuracy ± 15%
Matrix Effects (%)	NR	NR		6 lots of blank matrix	Precision ≤ 15%
Robustness	V	NR		NR	
Stability	NR	Stability of stock and working solution of standards, Stability of the analyte in matrix includes: long-term storage, short-term temperature stability, post-preparative stability, freeze–thaw cycle.		Stability of stock and working solution of standards, Stability of the analyte in matrix includes: long-term storage, short-term stability at room temperature or sample processing temperature, post-preparative stability at room temperature or under the storage conditions to be used during the study (dry extract or in the injection phase), freeze–thaw cycle. Mean concentration at each level within ± 15% of nominal concentration.	

[a] Limits defined for all calibration standards with exception of LLOQ (in LLOQ ± 20%);

[b] One must correspond to the LLOQ

[c] At LLOQ ± 20%

V, Parameter required but its limits are not referenced; LLOQ, Lower limit of quantification; LOD, Limit of detection; NR, Not required; RSD, relative standard deviation (corresponds to the absolute value of coefficient of variation); ULOQ, Upper limit of quantification.

During pre-clinical development, bioanalytical data may integrate the regulatory submission process and influence clinical trials design, and, therefore, the method may differ from the original assay of the discovery phase. Frequently, an internal standard (IS) is added to samples in order to compensate errors that can occur during sample preparation and chromatographic analysis, ensuring the reliability of analyte quantification. Validation experiments should be performed maintaining constant IS concentration, where the chromatographic response is given by analyte/IS peak area or height ratio. A stable isotope-labeled derivative of the parent compound is increasingly being

used as IS. However, when not possible, a compound structurally similar to the analyte and sharing identical behaviors during the entire bioanalytical protocol is used.

It is noteworthy that various animal species and matrices are frequently investigated within pre-clinical studies, requiring significant effort to fully validate all the assays if they are independently developed to each species/matrix. In these situations, a partial validation or cross validation may be sufficient to manage time and resources more efficiently at this stage. Using cross validation, the analyst will rely on the ability of the full validation protocol established in the matrix of one species to predict the concentrations of NCE in a similar matrix of other species. However, the scope of partial validation must be justified and certain parameters must be assessed separately in the sample type that is being subjected to cross validation (FDA, 2001; EMA, 2011), including stability and selectivity. In essence, although a significant shortcut of the procedures is undertaken, it cannot compromise either the data quality or the data integrity.

As the molecule advances into clinical trials, the assays developed to analyze human samples need to be more sensitive, rugged and robust to some small variations in matrices. Particularly in phase I clinical trials, high sensitivity is demanded to ensure that the lowest effective doses can be identified. In fact, if the samples are from first-time-into-humans studies, resolution is more important than throughput since the number of samples is low but the fate of the compound is unknown, requiring complete resolution of the analyte peak from all eventual matrices interferences. Additionally, stability must be evaluated again because matrices are distinct from those analyzed in pre-clinical stages. Similarly, it is also required to evaluate the method selectivity, also considering the metabolites formed *in vivo* and drugs probably co-prescribed to the patients in order to avoid their interference with the drug candidate under investigation. Accordingly, it is desirable to develop flexible and robust assays that enable minor alterations in chromatographic conditions to circumvent eventual interfering peaks if necessary.

In current practice, MS detection is employed in the analysis of the

majority of pharmacokinetic samples from clinical studies, and the issues of selectivity and specificity should not pose a major concern for the majority of NCEs at this stage. However, other elements associated with LC/MS assays have to be assessed, including the matrix effect, ion suppressing effect and, in the event of selective ion monitoring (SIM), the generation of a common *m/z* ion for quantification of multiple peaks (Srinivas 2008). It is also important to emphasize that the number of samples increases significantly in the later phases II/III and IV clinical trials and high-throughput, although desirable, may not be the best choice as compared to improving analytical efficiency. At these stages, advantages can be found in automating the bioanalytical process using fast, sensitive and reproducible chromatography with detection and data-capturing capabilities that lead with high amount of data. In the next section, some strategies within this scope will be outlined.

2.1.4 Bioanalysis & Pharmacokinetics, a Synergistic Partnership on DDD

In the world of DDD, speed is essential to ensure a competitive position and success in the highly-competitive life sciences marketplace. Therefore, it is important to employ innovative thinking in all areas of DDD and identify laboratory technologies/methodologies that reduce the cycle time from milestone to milestone in conformation with all the requirements laid out for a regulated environment. To address this challenge, besides the advances observed in the HTS *in vitro* and *in vivo* methodologies broached in *section 1.2*, high-throughput bioanalysis has been continuously designed and refined to improve the speed, quality and efficiency necessary to support the increasing number of samples from ADME/pharmacokinetic studies. Table 2.1.6 represents the flexibility in today's number and types of hyphenated bioanalytical techniques, providing several recent bioanalytical techniques employed to investigate the pharmacokinetics of NCEs in all stages of DDD programs. Study objectives, sample preparation and validation parameters evaluated are also summarized therein.

As expected, samples obtained from *in vitro* and *in vivo* are

predominantly analyzed by LC (Table 2.1.6). Indeed, chromatography is critical to the success of any bioanalytical assay as it allows the separation of the analytes from endogenous substances and metabolites. Resolution of the peaks correspondent to analytes and metabolites is essential to prevent an overestimation of the concentration of parent compound and subsequently incorrect determination of pharmacokinetic parameters and drug exposure. Even using highly selective MS or MS/MS detection, a prior chromatographic separation (although simpler than those required with other detection methods) is essential because the co-elution of endogenous or exogenous compounds may cause ion suppression or ion enhancement, both detrimental to the development of a sensitive method. Reversed-phase LC is the chromatographic technique most widely employed in bioanalysis of drugs and their metabolites during DDD due to its application to small molecules, which are separated by their different affinity to the hydrophobic stationary phase in relation to the mobile phase. However, compounds with low octanol-water partition coefficients and the majority of common metabolites may hamper the development of a reliable reversed-phase LC method, as they present a very short or non-existent retention time. Thus, the demand for analytical laboratories to increase sample throughput provided the impetus for HPLC column manufacturers to introduce new stationary phases and column geometries in order to increase speed and sensitivity. In this context, monolithic columns were created and have been employed with fast gradients and high flow rates for the direct analysis of several pharmaceutical compounds in biological samples (Zhou, 2005; Huang, 2006), but these columns have not been very frequently employed during DDD. Indeed, ultra-high pressure liquid chromatography (UHPLC) seems to be the most important achievement in LC evolution over the last decade and its application is increasing in the pharmaceutical field (Table 2.1.6). This technology retains the practicality and principles of traditional HPLC system but it is capable of providing liquid flow at pressures higher than 10,000 psi and columns packed with small particles (< 2 μm) that can withstand these pressures (Guillarme, 2013; Rodriguez-Aller, 2013; Fekete, 2014; Nishi, 2014). It is well-known that the theoretical plate height is proportional to the particle size, so using particle sizes down to 1.7 μm decreases theoretical

plate height and consequently produces a significant gain in efficiency that does not diminish at increased flow rates or linear velocities (Gilpin, 2008). UHPLC is becoming increasingly popular (Table 2.1.6) because it enables high speed analysis, superior resolution, increased sensitivity and reduced overall cost per analysis, ensuring that higher quality information is gained and laboratory productivity is optimized using sub-2 µm particle technology. However, certain practical concerns may need improvement, including sample introduction, reproducibility and the possible formation of temperature gradients within UHPLC columns at such high pressures. Moreover, extremely narrow sample plugs are required to minimize sample volume effect on peak broadening.

Table 2.1.6: Examples of pharmacokinetic investigations performed during DDD, the bioanalytical conditions employed and respective parameters that were validated.

Study type	Objectives	Sample preparation	Chromatographic conditions	Parameters validated	Ref.
Acyloxy(alkyl) ester based amino acid linked prodrugs of GOCarb					
In vitro metabolic stability in intestine	Estimate the half-life time of the prodrugs in Caco-2 cell homogenates Verify the conversion to the parent drug (GOCarb).	Samples filtration before HPLC analysis.	**HPLC-PDA** C_{18} reversed-phase column (5 µm, 4.6 × 250 mm) Mobile phase: water and ACN contained 0.1% TFA Flow rate: 1 mL min^{-1}	NR	Gupta, 2013
In vitro intestinal permeability	Evaluate the epithelial transport across Caco-2 monolayers; Identify the most permeable prodrug candidate.	Samples filtered in 96-well filter plates before analysis.	**LC-MS** C_{18} column (5 µm, 2.1 × 50 mm) with guard column. Mobile phase: water and ACN, containing 0.1% formic acid Flow rate: 0.2 mL min^{-1} Positive ESI mode.	NR	
In vivo mice pharmacokinetic studies	Oral (10 mg kg^{-1}) and intravenous administration (1 mg kg^{-1}) to Swiss Webster mice. Determine C_{max}, t_{max}, AUC and F of the prodrug and active metabolite (GOCarb).	NR	**LC-MS/MS** C_{18} (5 µm, 150 × 2.1 mm) column Mobile phase: 0.1% formic acid/ACN Flow rate: 0.2 mL min^{-1}. Data acquired by MRM in ESI positive mode.	NR	
BMS 690514					
In vitro absorption study	Evaluation of the P_{app} through Caco-2 cell monolayers.	NR	**LC-UV** PDA detector.	NR	Marathe, 2010
In vitro metabolic studies	Investigation of the oxidative metabolism in - liver microsomes and suspensions of hepatocytes isolated from mouse, rat, dog, monkey and humans. - specific CYP isoforms in cDNA-expressed human CYP enzymes.	NR	For microsomes studies **LC-MS/MS** ODS-AQ S-5 120 Å (2.0 × 250 mm) column Mobile phase: (A) 95% water and 5% ACN (v/v) containing ammonium acetate (pH 5.0) (B) 100% ACN. Flow rate: 0.2 mL min^{-1} Hepatocytes **LC–UV-radiometric-MS^n** ODS-AQ S-5 120 Å (4.6 × 150 mm) column. Mobile phase: (A) water, CAN, 10mM ammonium acetate, pH 5.0 (B) 100% ACN Flow rate of 1.0 mL min^{-1}	NR	

In vitro equilibrium dialysis *In vivo* preclinical pharmacokinetic studies	Determination of the extent of plasma protein binding in several species. Analysis of the systemic, urine and brian pharmacokinetics in mice, rats, dogs and monkeys after administration of a single intravenous or oral dose.	PP with acidified acetonitrile containing IS and direct analysis of the supernatant. Brain was homogenized with water and pretreated similarly to plasma.	**LC-MS/MS** C_{18} (2.1 × 50 mm, 5µm) Mobile phase: (A) 10 mM ammonium formate, 0.1% formic acid and 0.2% ACN in water (B) ACN with 0.1% formic acid Flow rate: 0.3 mL min^{-1}. Data acquisition by SRM transition	Linearity; Accuracy.	Marathe, 2010
In vivo disposition and biotransformation studies	Describe the *in vivo* biotransformation of brivanib after a single oral dose of [14C]brivanib alaninate to rats, monkeys and humans. Identification of the metabolites and elucidation of their structure.	Blood collection to K2EDTA tubes and glycine buffer, followed of centrifugation to obtain plasma. SPE extraction with a 96-well SPE plate. IS: Stable isotope-labeled brivanib	<u>Quantification of brivanib in plasma</u> **LC-MS/MS** C_{18} HPLC column (2.1 × 150 mm, 5 µm) Mobile Phase: (A) 0.1% formic acid in water; (B) ACN Quadrupole linear ion trap mass spectrometer operated in positive ESI mode. Detection achieved by MRM.	NR	Gong, 2011
α_v-β_3 bone integrin antagonist					
In vitro ultrafiltration	Evaluate the binding plasma proteins. Perform a HTS ultrafiltration method employing the 96-well plate.	Extraction with Cyclone HTLC column (50 × 0.5 mm, 60 µm) and the mobile phase composed of ACN and 2 mM ammonium formate. IS: derivative of α_v-β_3 bone integrin antagonist	**LC-ESI/MS/MS** C_{18} column (30 × 2.1 mm, 3 µm) Mobile phase: 80% ACN and 20% 2mM ammonium formate (pH 3.0). Triple quadrupole mass spectrometer performed in the positive ESI mode. Detection by SRM scan mode.	Validation according to international guidelines.	Zhang, 2006
Cytarabine and 5'-amino acid esters prodrugs					
In vitro Caco-2	Determination of P_{app} through Caco-2 monolayer; Identify the most permeable prodrug candidate and evaluate if it is a substrate of the PepT1 or if it inhibits PepT1.	NR	**HPLC-UV** C_{18} column (5 µm, 200 × 4.6 µm) Mobile phase: NaH_2PO4 buffer solution (5 mM): methanol:formic acid (70:30:0.1%) Flow rate:1.0 mL min^{-1} Wavelength: 272 nm.	Linearity; Recovery, Accuracy; Precision.	Sun, 2008
In vivo rat pharmacokinetic dose response	Determine the absolute bioavailability in rats after oral administration (5, 15, 30 mg kg^{-1}) and compare it to that of the oral aqueous solution of cytarabine (8 mg kg^{-1}). Determination of the concentrations of the prodrug, cytarabine and its metabolite (ara-U) in plasma.	Blood samples were placed into heparinized tubes and centrifuged to obtain plasma. IS: lamivudine <u>*For Cytarabine and prodrug extraction*</u> Plasma pretreatment by SPE cation-exchange SPE cartridges. <u>*For Ara-U extraction*</u> PP with ACN. IS: isoniazid	**HPLC-MS/MS** C_{18} column (50 × 2.1 mm, 1.7 µm). Quantification by MRM <u>*Analysis of Cytarabine and prodrug:*</u> Mobile phase: Water containing 2% methanol and methanol containing 0.1% formic acid. ESI source set in positive ionization. <u>*Analysis of Ara-U*</u> Mobile phase: Water and ACN; ESI source set in negative ionization for Ara-U and positive mode for IS.	Linearity; Recovery, Accuracy; Precision.	
CYP selective inhibitors					
In vitro CYP metabolism studies in human liver microsomes	Evaluate the effect of CYP inhibitors on each isoenzyme activity using a specific substrate probe cocktail in human liver microsomes.	PP with ice-cold ACN and direct analysis of the supernatant.	**UHPLC-QTOF-MS** C_{18} column (100 × 2.1 mm; 2.5 µm) Mobile phase: (A) water with 0.1% formic acid; (B) ACN with 0.1% formic acid Flow rate: 400 µL min^{-1} Q-TOF-MS system was operated in wide-pass quadrupole mode	Linearity; Inter and intra-day precision; Matrix effect.	Spaggiari, 2014
In vitro direct and metabolism-dependent CYP inhibition studies	Evaluate the inhibition potential of test compounds on 8 human CYP enzymes.	PP with ice-cold ACN; Supernatant was put in 96-well plates for analysis. IS: Carbamazepine	**LC-/MS/MS** C_{18} column (150 × 4.6mm, 5 µm) protected by a C_{18} guard column (2.0 × 4.0 mm) Mobile phase: (A) 0.1% v/v formic acid in water; (B) 0.1% v/v formic acid in acN. Flow rate: 0.7 mL min^{-1} API MS/MS operated in the positive and negative ion mode Quantitation was performed by MRM.	Linearity; Accuracy and precision.	Lee, 2013

CYP substrate probes

In vitro cocktail CYP inhibition studies	Evaluate the influence of NCEs in several CYP isoforms, using specific substrates and human liver microsomes. Quantify the main metabolite of each substrate probe in the presence and absence of the NCE.	PP with ACN. Plates centrifugation and direct injection of the supernatant previously diluted. IS: labetalol.	**HPLC-MS/MS** RP-Amide column (2.1 × 50 mm, 5.0 µm) Mobile phase: (A) Aqueous 0.1% acetic acid and 25:75 (v/v) methanol:ACN (B) 0.1% acetic acid. Flow rate: 0.6 mL min^{-1} API Triple Quadropole LC-MS used in the positive ESI mode. SRM to identify each probe substrate, their metabolites and IS.	NR	Otten, 2012
Clinical pharmacokinetic study	Simultaneous quantification of five CYP substrate probes and their main metabolites in plasma. Administration of the CYP substrate cocktail to healthy volunteers and quantify the parent drugs and main metabolites.	Blood centrifugation to obtain plasma. Dual liquid-liquid extraction with ethyl acetate. Organic layers evaporation followed of reconstitution with methanol. IS: Propranolol	**HPLC-MS/MS** C_{18} column (100 × 2.1 mm, 3.5 µm) Mobile phase: (A) 0.1% formic acid in water (B) 0.1% formic acid in acetonitrile Flow rate: 0.2 mL min^{-1} Linear ion trap mass spectrometer triple quadrupole mass spectrometer equipped with a turbo ion spray source. ESI positive ion mode Quantification by MRM.	Full validation according to FDA guideline.	Oh, 2012

Didanosine and peptidomimetic prodrugs of didanosine

In vitro Caco-2	Determination of P_{app} through Caco-2 monolayer; Identify the most permeable prodrug candidate and very if: - it is a substrate of the oligopeptide transporter (PepT1); - influence the uptake of glycylsarcosine.	Sample direct injection No IS	**HPLC-UV** C_{18} column (5 µm, 200 × 4.6 µm) Mobile phase: mixture (25:75) of methanol 0.05 M and KH_2PO_4 buffer solution Flow rate:1.0 mL min^{-1}, Wavelength: 250 nm.	NR	Yan, 2011
In vivo rat pharmacokinetic dose response	Determine the absolute bioavailability of the previously selected prodrug candidate in rats after oral administration (5, 15, 30 mg kg^{-1}). Similar studies performed co-administering the prodrug with glycylsarcosine.	Blood samples were placed into heparinized tubes and centrifuged to obtain plasma. Plasma pretreatment by SPE using HLB cartridges (1 cc, 30 mg) IS: lamivudine	**UHPLC-MS/MS** Hydrophilic interaction column (50 × 2.1 mm, 1.7 µm) Mobile Phase: water containing 0.1% formic acid and methanol (85:15, v/v) ESI source set in positive ionization. Quantification by MRM.	NR	

Eslicarbazepine acetate, carbamazepine, and other derivatives

In vitro mice intestine permeability	Determine the P_{app} through CD-1 mice intestine membrane mounted in Ussing chamber; Identify substrates of P-glycoprotein.	Sample centrifugation prior analysis.	LiChroCART Purospher Star (C_{18}, 3 µm, 55 × 4 mm; Merck KGaA) column. Mobile Phase: water–methanol–acetonitrile (64:30:6, v/v/v) UV: 235 nm	Partial Validation: Linearity; Intra- and inter-day precision and accuracy.	Fortuna, 2012
In vivo pharmacokinetic studies	*In vivo* pharmacokinetic characterization of the test compounds and their corresponding metabolites after oral administration to mice. Calculate C_{max}, t_{max}, AUC, $t_{1/2}$ and MRT in plasma and brain.	**Plasma:** Blood samples were collected to heparinized tubes and centrifuged to obtain plasma. **Brain:** Brain tissue was homogenized in a 0.1 M sodium phosphate buffer pH 5 (4 mL g^{-1}) and centrifuged. Plasma (300 µL) and brain homogenate (1 mL) were pretreated by SPE using HLB cartridges (30 mg, 1 mL). Eluents were evaporated to dryness and the dry residues were analyzed after reconstitution.	Chiral column LiChroCART 250-4 ChiraDex. UV: 235 nm Mobile phase: water and methanol <u>For CBZ, OXC, ESL, *S*-Lic, *R*-Lic and CBZ-E and main metabolites:</u> Flow rate: 0.9 mL min^{-1} IS: Chloramphenicol <u>For BIA 2-059, BIA2-024, BIA 2-265 and their main metabolites:</u> Flow rate: 0.7 mL min^{-1} IS.: CBZ-10,11-E poxide <u>For *trans*-diol:</u> Mobile Phase: water-methanol-ACN Flow rate: 1.0 mL min^{-1} IS.: 10,11-dihydrocarbamazepine	Full validated according to International guidelines.	Fortuna, 2013

Eslicarbazepine acetate

Clinical Studies	Investigate the effect of gender, food and hepatic failure in the biodisposition of eslicarbazepine after oral administration of eslicarbazepine acetate.	Blood samples were collected into tubes containing lithium heparin and centrifuged to obtain plasma. **Plasma pretreatment** with Schleicher and C_{18}/100 mg 96-well SPE plate. Final extract was reconstituted in water:methanol. IS: 10,11-dihydrocarbamazepine	**LC-MS** LichroCART 250-4 ChiraDex analytical column (β-cyclodextrin, 5 µm), a LichroCART 4-4 ChiraDex guard column (β-cyclodextrin, 5 µm). Mobile Phase: (A) 0.2 mM sodium acetate (B) 0.2 mM sodium acetate, MeOH. ESI source set in positive ion mode.	Linearity; Precision and accuracy (with 3 QCs)	Falcão, 2007; Almeida, 2008

Gabapentine enacarbil

In vitro metabolic stability	Evaluation of the rate conversion of gabapentine enacarbil to gabapentine, quantifying both coumpds after several times of incubation in plasma, intestinal S9, liver S9, lung S9.	NR	**HPLC-MS/MS** C_8 (150 × 4.6 mm; 5 µm) Mobile phase: (A) 0.1% formic acid in water (B) 0.1% formic acid in ACN Flow rate: 800 µL min^{-1} Detector was an API MS/MS Data acquisition using the MRM mode.	Linearity; Intraday precision; Intraday accuracy.	Cundy, 2004
In vitro inhibition of CYP isoenzymes	In a 96-well format, analyze the influence of gabapentine enacarbil on the metabolism of CYP substrate probes using baculosomes...				
In vitro metabolism by CYP isoforms in human liver S9 fraction	Identify the CYP enzymes involved in the metabolism of gabapentine enacarbil using specific inhibitors of each isoform.				
In vitro ultrafiltration	Determine the plasma protein binding of gabapentine enacarbil.				
In vitro transcelular transport	Determination of the Papp of gabapentine enacarbil and gabapentine through Caco-2 and MDCK cell monolayers.				
Randomized, double-blind, placebo-controlled design.	Investigate the pharmacokinetics and tolerability after immediate-release capsules of gabapentin enacarbil oral administration at 5 ascending single doses and compared with commercialized gabapentine capsules.	Blood samples were collected into tubes containing K2EDTA and centrifuged to obtain plasma. Plasma samples were quenched with methanol immediately before analysis. Urine samples had no preparation before analysis	**LC/MS/MS** C_{18} HPLC column (50 × 4.6 mm) Mobile phase: (A) water/ACN/formic acid (95:5:1); (B) ACN/water/formic acid (95:5:1). Flow rate: 2.0 mL min^{-1} Detector was an API MS/MS and detection performed with MRM.	Full validation according to international guidelines.	Cundy, 2004
Randomized crossover	Evaluate the pharmacokinetics and tolerability after extended-release tablets of gabapentin enacarbil had been orally administered.				
Randomized-sequenced double blind, placebo-controlled crossover clinical study	Investigate in healthy volunteers the pharmacokinetics and tolerability of gabapentin enacarbil orally administered as extended release tablets as a single oral dose.	Blood was collected into tubes containing dipotassium ethylenediaminetetra-acetic acid and centrifuged to obtain plasma. Two 1-mL aliquots were immediately transferred using a pipette to Nalgene tubes and quenched with 3 mL of methanol. IS for gabapentine: L-4-chlorophenylalanine IS for gabapentin enacarbil: Leu-gabapentin phenylacetamide.	**LC-MS/MS** Hydro-RP, Phenomenex (50 × 4.6 mm; 4 µm) Mobile phase: (A) 0.1% formic acid in water (B) 0.1% formic acid in ACN Flow rate: 1200 µL min^{-1} API MS detector with the positive-ion MRM mode.	Full validation according to international guidelines.	Lal, 2009
Randomized, open-label, crossover study	Evaluate the effect of food on the tolerability and pharmacokinetics of gabapentin enacarbil administered as extended release tablets.				Lal, 2010
Open label single dose pharmacokinetic study	Describe a population pharmacokinetic analysis of gabapentin enacarbil in patients with varying degrees of renal function.				Lal, 2013
Randomized, double-Blind, placebo- and Active-controlled, crossover	Quantification of plasma levels of gabapentin several times after administration of gabapentin enacarbil.			Full validation according to international guidelines.	Chen, 2012

Glimepiride

Bioequivalence study	Quantify glimepiride in plasma samples from ongoing development of an immediate-release formulation.	Addition of *o*-phosphoric acid (2.5%) to plasma samples which are loaded onto HLB 96 well cartridges.	**LC-MS/MS** C_{18} column (100 × 3 mm, 3.0 µm) Mobile phase was ACN and 2 mM ammonium format, pH 3.5 with formic acid Flow rate: 0.5 mL min^{-1} Triple quadrupole mass spectrometer equipped with an ESI ion source in positive ionization SRM mode.	Full validation according to international guidelines	Kundlik, 2012

HM781-36B, a new anticancer drug

In vitro metabolism in liver	Profile and identify the metabolites in microsomes and recombinant CYPs.	Centrifugation and supernatant evaporation to dryness. Analysis of the residue after reconstitution.	**LC-MS/MS** C_{18} column (150 × 2.1 mm, 3.5 µm) Mobile phase: ammonium acetate (10 mM, pH 3.6)/ACN Flow rate: 0.2 mL min^{-1} Use of MS, MS^2 and MS^3.	NR	Kim, 2013
In vivo metabolic study	Profile and identify the metabolites in plasma, urine and feces after oral administration to dogs. Determination of AUC, t_{max}, C_{max} and $t_{1/2}$.	Blood samples were collected into heparin tubes and centrifuged to obtain plasma. Feces samples were homogenized after freeze-drying. Liquid-liquid extraction with 0.05% formic acid and ethyl acetate. Supernatant evaporation and injection of the residue after reconstitution.	**UHPLC/Q-TOF MS** UHPLC analytical conditions similar to those used to identify the metabolites; Q-TOF mass spectrometer equipped with an ESI source working in positive mode. MS/MS experiments performed using the MS^1.	NR	
In vivo systemic pharmacokinetic study	Determination of jugular plasma concentrations after oral administration to dogs. Determination of C_{max}, t_{max}, AUC_{0-t} and $t_{1/2}$.	Blood samples were collected into heparin tubes and centrifuged to obtain plasma. IS: HM60781	**LC-MS/MS-MRM** Phenylhexyl column (50 × 2.0 mm, 5.0 µm) Mobile phase: 80% methanol/20% ammonium acetate buffer, 10 mM, pH 3.6. MRM mode used for quantification	Full validated according to FDA guideline.	
In vitro equilibrium dialysis	Determination of drug binding to plasma proteins and brain tissue using a 96-well system.				
In vivo cassete dosing study	Evaluate systemic and brain pharmacokinetics of the 12 test compounds after intravenous bolus injection to ICR mice (2.5 mg kg^{-1}).				
In vivo pharmacokinetic evaluation	Determination of C_{max}, t_{max}, AUC, $t_{1/2}$, F and Cl after oral (10 mg kg^{-1}) or intravenous (3 mg kg^{-1}) administrations	Blood was collected into EDTA tubes and centrifuged to obtain plasma. PP with ACN containing the IS.	**LC–MS/MS-MRM** NR	Linearity	

Psoralenoside, isopsoralenoside, psoralen and isopsoralen from *Psoralea corylifolia* extract

In vivo pharmacokinetic study	Calculate C_{max}, t_{max}, AUC, MRT and Cl after *Psoralea corylifolia* extract or benzofuran glycoside fraction oral administration to Wistar rats.	Blood samples collection into heparinized tubes and centrifuged to obtain plasma. PP with methanol; water addition and injection of the supernatant. IS: sophoricoside	**UHPLC-MS/MS** C_{18} (2.1 × 50 mm,1.7 µm) Mobile phase: (A) methanol (B) 0.1% formic acid aqueous Flow rate: 0.2 mL min^{-1} Triple quadrupole mass spectrometry Data acquisition by MRM in ESI positive mode.	Full validation according to FDA guidelines	Wang, 2014

Rohitukine					
In vitro hepatic and intestinal metabolism	Stability evaluation in rat intestinal and hepatic microsomes; Prediction of the first pass effect on drug bioavailability;	PP with methanol and direct analysis of the supernatant.	**HPLC-MS/MS** C_{18} column (5 µm, 4.6 × 150 mm) column, preceded with a guard column. Mobile Phase: 10 mM ammonium acetate (pH 4): methanol Flow rate: 0.6 mL min^{-1} ESI source set in positive ionization. Quantification by MRM	NR	Chhonker, 2014
In vivo pharmacokinetic evaluation	Calculate C_{max}, t_{max}, AUC, Vd, $t_{1/2}$, Cl and F after oral (50 mg kg^{-1}) and intravenous (5 mg kg^{-1}) administrations.	Blood samples collected into micro-centrifuge tubes containing heparin and centrifuged to obtain plasma. Plasma pretreatment by SPE (1 mL, C_{18}) cartridges. Evaporation of eluents and analysis of the dry residue reconstituted in methanol. IS: Phenacetin		Full validation, according to US FDA guideline.	
15 derivatives of Sulfonamide with antitumor activity					
In vitro metabolic stability and elucidation of metabolites structures	All test compounds were incubated for 2 h with human liver microssomes in order to: - quantify the remaining parent compound; - estimate their metabolic stability; - separate and identify the metabolites generated.	Centrifugation and direct injection of the resulting supernatant.	**LC-MS/MS** C_{18} column (3.0 × 100 mm, 2.7 µm) Mobile Phase: (A) 0.1% formic acid in water (B) 0.1% formic acid in ACN Flow rate: 0.25 mL min^{-1} MS detection with ESI positive mode. MS/MS detection with Q-TOF analyzer to collect the fragmentation spectra of biotransformation products.	NR	Belka, 2014
Tricin-amino acid derivatives as prodrugs					
In vitro cell permeability evaluation	Investigate the P_{app} through MDCK cell monomlayers. Quantification of tricin concentrations initially in apical solution and at the end in the basolateral solution.	Direct injection of apical and basolateral solutions.	**UV/Vis Spectrometry at 330 nm**	NR	Ninomiya, 2011
In vivo systemic pharmacokinetic studies	Determination of C_{max}, t_{max}, AUC, $t_{1/2}$,Vd and Cl of tricin after a single oral dose of prodrugs (100, 300 or 1000 mg kg^{-1}) to Crl:CD rats.	Blood centrifugation to obtain plasma. Liquid-liquid extraction with acetone; water addition to the organic layer, centrifugation and inject ion of the supernatant.	**HPLC-UV** C_{18} column (250 × 4.6 mm, 5 µm) Mobile phase: ammonium acetate buffer (0.1 M, pH 5.1) and methanol plus EDTA. Flow-rate: 1 mL min^{-1}. Tricin concentration in plasma was determined by an IS method.	Linearity; Inter-and intra-day precision (with 3QCs); Stability.	Ninomiya, 2011; Cai 2003

ACN, acetonitrile; APCI, atmospheric pressure chemical ionization; API, atmospheric pressure ionization; Ara-U, 1-ß-D-arabinofuranosyluracil; AUC, area under the curve; BBB, blood-brain barrier; BCRP, breast cancer resistance protein; CE, collision energy; Cl, systemic clearance; DAD, diode array detection; DP, declustering potential; F, absolute bioavailability; FDA, food and drug administration; GOCarb, guanidine oseltamivir carboxylate; HPLC, high performance liquid chromatography; IS, internal standard; MRM, multiple reaction monitoring; K_2 EDTA, dipotassium salt of ethylenediaminetetraacetic acid; MRT, mean residence time; NR, not reported; PDA, photodiode array; PP, protein precipitation; QC, quality control samples; SPE, solid phase extraction; TFA, Trifluoroacetic acid; $t_{1/2}$, half-life time; UHPLC, ultra-high-performance liquid chromatography; UV, ultraviolet; Vd, volume of distribution;

Currently, LC/MS and LC/MS/MS take the bulk of the work during pharmacokinetic evaluation in DDD programs (Table 2.1.6), particularly due to the inherent specificity and sensitivity of these techniques, which dramatically increase throughput for the quantitative determination of drugs and metabolites in biological matrices. In addition, these methods reduce the need of exhaustive chromatographic separations prior to

detection, reducing analysis time. Even though both LC/MS and LC/MS/MS are very expensive and not available to all research laboratories, bioanalysts have been devoted to improve MS and MS/MS hardware and software, not only to identify and quantify metabolites in a variety of *in vitro* and *in vivo* assays during all stages of DDD, but also to allow users to benefit from lower LLOQs and ease of use. In fact, reduced LLOQ values are essential in order to guarantee strong characterization during the elimination phase of a compound. Besides their high sensitivity and specificity, MS and MS/MS detections are based on a combination of the unique parent and fragment masses of each compound, eliminating the need for baseline separation and achieving fast analyses. Prior to MS detection, analyte ionization is required and can be achieved by electrospray ionization (ESI), atmospheric pressure ionization (API) or atmospheric pressure chemical ionization (APCI) modes. MS detection of ionized analytes has been carried out in a linear scan mode in which a range of *m/z* values is constantly monitored. In a more selective and sensitive mode called SIM, one specific *m/z* value (or more) is selected for the monitoring. Single-reaction monitoring (SRM) or multiple-reaction monitoring (MRM) modes can also be used: in SRM mode, a specific product ion of a specific parent ion is detected, producing very simple plots (usually containing only a single peak). MRM delivers a unique fragment ion that can be monitored and quantified in a very complicated matrix, making the MRM plot ideal for sensitive and specific quantifications. Commonly, several kinds of tandem mass spectrometers including the triple stage quadrupole, the three-dimensional ion-trap, linear ion-traps/quadrupole coupled with time-of-flight (Q-TOF) and hybrid triple quadrupole linear ion trap mass spectrometers have been used in DDD.

Nevertheless, classical detection modes such as UV and fluorescence, although less employed in current practice than MS or MS/MS detections, continue to be applied in the early *in vitro* bioavailability or metabolic stability screening and remain valuable in cases where sensitivity is not paramount or where LC/MS is not economically viable.

2.1.4.1 Bioanalytic Support of *In Vitro* ADME Studies

Advances in techniques for chemical synthesis allow the synthesis of hundreds to thousands compounds every month. As described in Chapter 2.1.2, several high-throughput *in vitro* assays have been extensively used in early discovery to select the NCEs most likely to have favorable pharmacokinetic parameters. The early ADME screening of a larger number of samples is therefore required, which in turn calls for high-throughput bioanalytical approaches. Although most publications on *in vitro* studies hardly pay attention to the bioanalytical aspects involved in obtaining data, it becomes clear considering the examples depicted in Table 2.1.6 that bioanalytical techniques used to support these investigations commonly employ HPLC-UV, LC-MS or LC-MS/MS.

LC-UV techniques are mainly used to collect data from *in vitro* absorption methods, which aim at investigating the *in vitro* transport of drugs through intestinal or other cell layers, predicting the *in vivo* transport of a drug through the intestinal membrane and the involvement of certain transporter proteins, particularly the efflux transporter P-glycoprotein and the oligopeptide transporter (PepT1). The most common *in vitro* systems are the colorectal carcinoma cell line (Caco-2) and other cell lines, like the MDCK (Volpe, 2011). The Ussing chamber technique has also been employed, incorporating a healthy intestinal membrane. In both techniques, after a specific incubation time, samples are taken from both sides of the monolayers for bioanalytical determination of the drug concentration. Most of these *in vitro* techniques are calibrated within the laboratory using a set of commercial drugs with known human absorption fraction, which are plotted against the experimentally obtained apparent permeability (Fortuna, 2012). In our laboratory, we have developed an Ussing chamber technique employing mouse jejunum segments in order to predict the human intestinal absorption fraction and the involvement of P-glycoprotein in drug absorption. The technique was initially validated with reference compounds and then applied to compare the absorption of nine derivatives of carbamazepine, a classical antiepileptic drug (Fortuna, 2012). An HPLC-UV technique was successfully employed in order to quantify each compound in our samples. However, a major drawback is one frequently ascribed to the use of HPLC-UV in these analyses, which is that for poorly permeable drugs, the LLOQ achieved

is often not compatible with the amount of drug present in the samples. At this point, some companies are opting to develop LC-MS techniques or automated LC-MS/MS approaches to circumvent this limitation (Table 2.1.6).

Nevertheless, the major application of LC-MS and LC-MS/MS during *in vitro* ADME analysis regards drug metabolism. In fact, due to the publication of the FDA guidelines on metabolites in safety testing (MIST) and DDI, metabolite profiling and screening for metabolites in plasma samples have been recently included as part of new drug discovery stages, implying the development of bioanalytical techniques that quickly identify and quantify the parent drug and its metabolites. Information generated from these studies could therefore enable the scientist to predict earlier the major metabolite exposure in animals and humans, allowing a better strategy to be developed. For instance, for compounds with extensive metabolism, identification of metabolites may help medicinal chemists to modify the NCE to block the sites of metabolism. On the other hand, active metabolites may have better pharmacokinetic profiles than the dosed NCE and may become a new lead compound.

Moreover, the DDI guidance of EMA recommends the identification of the enzymes involved in the formation and elimination pathway of metabolites that contribute to more than 50% of the *in vivo* pharmacological effect (EMA, 2012). Since CYP enzymes are the most frequently involved in drugs metabolism, the identification of the specific isoforms involved in the metabolism of new compounds at early *in vitro* stages become crucial, specifically whether the drug candidate is metabolized by single or multiple enzyme isoforms and whether highly polymorphic enzymes, such as CYP2D6 and CYP2C19, contribute to their metabolic clearance. In addition, these *in vitro* techniques (mainly employing microsomes and hepatocyte cell lines) can also be useful in identifying drug candidates that are inhibitors or inducers of CYP isoforms, and can therefore predict their DDI potential. For these investigations, CYP probe substrates must be used and their respective metabolites must be quantified in the presence and absence of the drug candidate. Thus, it is not surprising that significant efforts have been devoted to develop rapid chromatographic techniques that

simultaneously quantify CYP probe substrates/metabolites. At this level, LC-MS and LC-MS/MS are undeniably involved in the characterization and quantification of the metabolites and have been most frequently used to support these *in vitro* studies (Table 2.1.6). To increase throughput, many probes have been applied to assess multiple CYP activities simultaneously within a single experiment. This strategy, named the *cocktail approach*, is starting to be employed *in vitro* to predict the clinical impact of genetic polymorphism to CYP-based DDI (Rhodes, 2011; Kozakai 2012; Pillai 2013). Traditional analytical methods using LC coupled with UV, radiometric, fluorescence and luminescence detections have some limitations including a lack of substrate specificity, solvent assay sensitivity and analytical interferences. Thus, LC-MS and LC-MS/MS offer advantages over these classical methods such as high specificity and sensitivity to investigate several enzyme-specific substrate probes and selectively quantify each specific metabolite. The current methodologies employing the cocktail approach to assess *in vitro* the activity of human CYPs can be found in the recent review published by Spaggiari et al. (2014).

According to Table 2.1.6, ion trap mass spectrometers have been used to a large extent to support *in vitro* metabolic studies as a qualitative tool, but in comparison with classical quadrupole MS/MS, the quantitative features of ion trap mass spectrometers remain less used. In fact, due to its small footprint and high sensitivity, the ion trap was considered somewhat of an alternative to the triple quadrupole MS for quantitative high-throughput analysis. However, the generation of MS/MS data requires a relatively long duty cycle, limiting the numbers of analytes that can be quantified simultaneously. Furthermore, the LC-Q-TOF MS technique has been widely applied to obtain high-resolution mass spectral data of precursor and product ions due to its high-throughput analysis, specificity, selectivity and resolution power. The enhancement of its selectivity is attractive to reduce the problems such as matrix suppression and metabolite interferences. It is also of great value in the interpretation and elucidation of the chemical structure of the metabolites of each NCE. As a result, the best strategy uses an ion trap to obtain the structural information by sequential MS^n experiments and MRM screening could be subsequently used to perform quantitation

for the drug and metabolites at trace levels, not only *in vitro* but also *in vivo* (Table 2.1.6). For further confirmation of metabolites, the LC-Q-TOF-MS/MS technique can be applied for accurate mass measurement of the protonated molecules and their fragment ions.

It must be emphasized that LC-MS/MS is also frequently employed to quantify the drug fraction unbound to plasma proteins during early screening studies (Table 2.1.6). Indeed, the plasma protein binding of a drug can dramatically affect the circulating concentration of the compound as well as its ability to be distributed and/or accumulated through different compartments of the body. It is well accepted that only the unbound drug is available to cross membrane barriers, be distributed to tissues, and exert pharmacological and/or toxicological effects. *In vitro* equilibrium dialysis and ultrafiltration are the techniques most frequently employed in initial phases of DDD (Table 2.1.2). Devices based on multi-well formats have been recently developed (Fung 2003; Zhang 2006; Plumb 2008; van Liempd, 2011; Zamek-Gliszczynski, 2011) and employed to investigate NCEs (Table 2.1.6). Briefly, they consist of 48 or 96-well Teflon base plates with a semi-permeable membrane that allows the passage only of the unbound drug. When equilibrium between both membrane sides is reached in the equilibrium dialysis technique, the total drug concentration is determined in the plasma compartment while the free drug concentration is determined in buffer compartment in order to calculate the percentage of drug bound to plasma proteins. On the other hand, ultrafiltration is currently emerging as a faster and simpler alternative to equilibrium dialysis once the centrifugation applied (approximately 2000 × g) accelerates the passage of the unbound drug through the membrane (Fung 2003; Zhang 2006). Particularly because of the low free drug levels that can be achieved in the buffer compartment or in the filtrate, both *in vitro* techniques require the application of LC-MS/MS methods to accurately quantify the drugs (Table 2.1.6). Both MRM (Zamek-Gliszczynski, 2011) and SRM (van Liempd, 2011) detection modes seem to be successful. Sample preparation prior to chromatographic analysis is very simple, and relies on protein precipitation with acetonitrile (van Liempd, 2011, Zamek-Gliszczynski, 2011) or a mixture of acetonitrile/0.1% formic acid aqueous solution (90/10, v/v) (Plumb, 2008) followed by centrifugation. It is

important to highlight that the bioanalytic methodologies employed to quantify the drugs were at least partially validated in order to confirm their acceptability for each specific compound (Table 2.1.6).

2.1.4.2 Bioanalytic Support of *In Vivo* ADME/Pharmacokinetic Studies

As depicted in Table 2.1.6, most of the *in vitro* techniques implemented to evaluate the ADME of NCEs are complemented with non-clinical pharmacokinetic studies performed in rodent animals (mice or rats). As expected, the major objectives include the determination and evaluation of the extent and rate of absorption (AUC, C_{max} and t_{max}), distribution (Vd), clearance and duration of exposure ($t_{1/2}$) of a drug and its metabolites after oral and/or iv administration. UV, MS and MS/MS are the major detection methods used for the quantification of parent drug in biological matrices, but MS and MS/MS have been more frequently used and seem to be preferable for relating metabolite response with that of the parent drug due to higher accuracy and sensitivity. In fact, identification and structure elucidation of the metabolites previously obtained *in vitro* must be confirmed in non-clinical and then in clinical studies. Similarly, the monitoring of metabolite exposure in non-clinical species and humans are also mandatory and therefore widely investigated in current practice (Table 2.1.6) (Zhu *et al.*, 2009). These results are crucial since, according to the FDA guidelines on MIST and DDI, the relative exposure of metabolite can be obtained from the metabolite profiling of time-averaged AUC-pooled human plasma samples from multiple ascending dose studies.

It is interesting to note that similar chromatographic conditions can be employed to investigate the pharmacokinetics of NCEs both *in vitro* and *in vivo*, although detection may vary depending on the study objectives and the DDD stage. For instance, Fortuna et al. (2012, 2013) employed a validated HPLC-UV technique to firstly determine the *in vitro* apparent permeability of nine derivatives of carbamazepine through mice intestine and secondly characterize the plasma and brain pharmacokinetics after oral administration to mice. To attain those objectives, a C_{18} column was used (Table 2.1.6) and the parent

compounds and main metabolites were extracted from biological samples by a solid-phase extraction (SPE) procedure. The same chiral column and similar SPE protocols were employed to analyze plasma samples from clinical studies (Falcão 2008; Almeida 2008) but with MS/MS detection instead of UV. Moreover, Lv et al. (2013) developed an effective screening strategy to select new agents for brain tumor chemotherapy from a series of low molecular weight anticancer agents [ON123x] by combining several *in vitro* studies. These studies aimed to evaluate compound metabolic stability in mouse and human liver microsomes, predict their BBB permeability using MDCK-MDR1 cell monolayers and estimate their binding to plasma proteins and brain tissue. *In vivo* cassette dosing studies were then conducted in mice for the 12 compounds, permitting the examination of *in vitro*/*in vivo* relationships to confirm the suitability of the *in vitro* assays. The same bioanalytical technique was employed for the analysis of all *in vitro* and *in vivo* samples, utilizing a C_{18}-reversed-phase column and MS/MS detection after a simple protein precipitation to clean-up the samples.

It is noteworthy that the majority of the *in vivo* studies which quantify parent drug and/or metabolites in biological samples require more demanding and complex sample preparation protocols than *in vitro* techniques, resorting to validated analytical techniques which guarantee the acquisition of precise and accurate data (Table 2.1.6). Indeed, it is well known that besides chromatographic separation, sample preparation is essential to reduce the effect of the endogenous and exogenous compounds that exist in biological samples and to concentrate the analytes to consequently enhance the method sensitivity.

In current practice, SPE seems to be the most widespread used procedure to extract drugs and metabolites from biological samples (Table 2.1.6), probably because it is versatile, very efficient and easily automated. Similarly to HPLC column packing materials, SPE cartridges utilize a wide range of silica-based and polymer-based sorbents and the extraction procedure follows the generic chromatographic protocol. A novel 96-well SPE plate format is being widely employed, conferring a unique design that makes the use of the sorbents higher efficiency and allows elution of target compounds with small quantities of solvent. This

HTS sample preparation procedure is useful in initial phases of DDD but also in clinical trials including BA/BE studies, since high number of samples must be analyzed as fast as possible (Falcão 2007; Almeida 2008; Xie, 2010; Boer 2012; Kundlik, 2012). Particularly the 96-well HLB μElution SPE plate is composed of a 2 mg high-capacity SPE sorbent (divinylbenzene/N-vinylpyrrolidone polymer), exhibiting excellent wetting properties and strong hydrophobic retention. Due to these properties, the sorbent can be run to dryness without loss of recovery, which is a major drawback of SPE sorbents based on silica-C_{18}. Moreover it allows elution of target compounds with only 25 μL of solvent and the high capacity of the sorbent also tends itself to a concentration step (up to 15 times) with no evaporation or reconstitution, which is a potentially critical factor in low dose scenarios (Mallet, 2003; Yang 2005; Murphy, 2007; Lindegardh, 2008). On the other hand, the liquid-liquid extraction (LLE) is also used due to its simplicity, ability to provide clean extracts and the lower costs required. The extraction efficiency of LLE is related with the partition coefficient that is controlled by the characteristics of the extraction solvent (e.g., viscosity, surface tension, solubility in water, *etc*). In general, organic solvents with low miscibility in water and are preferred for LLE. However, selecting the right solvents (and their respective quantities) for sample partitioning makes the development of LLE methods laborious. Once optimized, the application of liquid-handling workstations allows semi-automated operations and therefore few LLE performed in 96-well format have been employed for high-throughput analysis (Chapter 12). Furthermore, 96-well LLE procedures may require large volumes of extraction solvent, limiting their application during DDD programs. Instead, the automated LLE combined with LC-MS/MS has been increasingly used in recent years (Eerkes, 2003; Xue 2004) for the analysis of pharmaceutical moieties based on advantages in efficiency, cost, and throughput. It is important to bear in mind that plasma protein precipitation, dilution and shoot approaches can also be used, but only to prepare biological samples that are simple and mainly composed of water, such as urine, cerebrospinal fluid, saliva and tears.

2.1.5 Conclusions

Current discovery and development programs of new drugs promote pharmacokinetic analysis with *in vitro* techniques from the earliest stages and throughout preclinical and clinical stages. Thus, the quantification of the parent compound and its main metabolites, as well as the identification of the major metabolites in buffers and biological samples become essential and may determine the success of a developing drug. The introduction of advanced analytical technologies with improved sensitivity and selectivity has opened new frontiers in obtaining pharmacokinetic information with higher accuracy and speed. Conventional HPLC techniques particularly when coupled to mass spectrometry detection (MS or MS/MS) have been successfully employed over the last decade for drug metabolism and clinical investigations. Bioanalysis should not be performed blindly and the analytical methods need to be subject to appropriate method validation depending on the DDD stages. The development and validation of sound bioanalytical methods to support the DDD process is paramount to produce high-quality data, contributing for a reliable interpretation of pharmacokinetics.

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Ma-Li Wong, Martin Lewis and Julio Licinio*

2.2 Translational Research in Endocrinology and Neuroimmunology Applied to Depression

2.2.1 Major Depressive Disorder

Major depressive disorder (MDD) is a complex chronic illness of gene environment-interactions with enormous medical, social and economic impact. It affects 1.4 million Australians yearly, having the highest non-fatal disease burden and being the nation's leading cause of disability (Tempier, 2009). MDD is a major cause of suicide, which accounts for the third highest fatal disease burden in Australian men (Tempier, 2009). MDD has considerable morbidity and mortality (Wong & Licinio, 2001, 2004). Currently, the point prevalence of this disorder ranges around 4–7% and the lifetime prevalence estimate is about 15-20% (Kessler, 2005; Kessler, 1994). In Australia, depression is the most common mood disorder, affecting 6.2% of the population (Lohoff, 2010), it is the largest single cause of nonfatal disease burden and the leading cause of disability (Vos, 2004). By the year 2020, MDD will become the second most important contributor to the global burden of disease (Lopez & Murray, 1998). However, MDD has been one of the oldest medical mysteries, described since Hippocrates (460-37 BC) as melancholia (μελαγεολια). It has been extensively studied in the past forty years; however, we continue to understand little about its fundamental biology (Kessler, 2005; Kessler, 1994; Licinio & Wong, 2011) and the genetic factors conferring susceptibility to this disorder (Sullivan, 2012).

The following findings provide persuasive evidence for a role of the stress system in the pathophysiology of MDD: a) antidepressants directly down regulate the hypothalamic-pituitary-adrenal (HPA) axis function; b) antagonism of corticotropin-releasing hormone (CRH) reduces neuroendocrine, autonomic, and behavioural responses to stress in primates, and c) increased cerebrospinal fluid (CSF) concentrations of noradrenaline are increased around the clock, including in sleep, which

imply that dysregulation of a stress-related system is primary and not simply a consequence of depressed mood (Wong, 2000; Wong & Licinio, 2001). Thus, chronic uncontrollable stress may promote the onset of MDD and a shift towards environment withdrawal (Clark & Beck, 2010).

2.2.2 The Stress Response

A series of physiological and behavioral stereotyped responses, which have been evolutionary developed to promote survival, characterize our reaction to danger. That reaction, popularly known as the "fight-or-flight response", is better reflected by the term "fight, flight, freeze or fawn response" and includes increased heart rate and defensive/offensive behaviors, which are modulated by an extensive and complex circuitry that prepares the cardiovascular, musculoskeletal, endocrine, metabolic and immune systems to deal with threating events, and inhibits certain physiological functions, such as reproduction, sleep, foraging/digestion and growth, which are dispensable until the cessation of the short-term acute stress (Cahill & McGaugh, 1998; LeDoux, 1995). The core stress system includes the CRH, the locus ceruleus-norepinephrine (LC-NE), and the immune systems. This core system detects and monitors the intensity and duration of the stress response, promotes arousal, modulates the limbic system and the cortical functions in order to favor survival.

While the stress response to acute, short-term stress is physiological and adaptive to promote survival during threatening situations, the persistent stress response to long-term chronic stress is generally maladaptive and harmful, as it dysregulates the stereotyped stress response (McEwen, 1998).

2.2.2.1 The CRH System and the Stress Response

Four related ligands (CRH, urocortin I, II and III), two receptors (CRHR1 and 2), and a binding protein (CRHBP) that acts as an endogenous antagonist have been recognized in this system (Aubry, 2013; Heinrichs, 1997). For the purpose of this chapter, we will focus on the CRH and the

CRHR1, as both are widely distributed in the brain, and CRH has high affinity to CRHR1 and poor affinity to CRHR2.

CRH is the key hypothalamic factor in the HPA axis, it stimulates the pituitary gland to release adrenocorticotropin hormone (ACTH) and indirectly regulates glucocorticoids secretion and its production in the adrenal cortex. Besides its critical role in the HPA axis, CRH plays multiple additional roles in the stress response; it is implicated in coordinating the behavioral, neuroendocrine, autonomic and neurovegetative aspects of the stress response; it is relevant in functions such as the activation the LC-NE system, the sympathetic nervous system, catecholamine synthesis in the adrenal cortex, fear-related behavior, and the inhibition of exploratory behavior, food consumption, and growth and reproduction functions (Chrousos & Gold, 1992; Gold, 1988a, 1988b). CRH also modulates anxiety behavior, as corroborated by findings in studies of mice lacking CRHR1, which display decreased anxiety (Smith, 1998) and of CRHR1 antagonists, such as Antalarmin, which is a non-peptide selective CRHR1 antagonist. Antalarmin administration results in inhibition of anxiety-like responses induced by stress and the promotion and establishment of conditioned fear (Deak, 1999; Habib, 2000). Antarlamin also appeared to prevent increases in circulating ACTH, NE, epinephrine, and cortisol levels (Habib, 2000).

2.2.2.2 The Locus Ceruleus Norepinephrine (LC-NE) System and Other Central Nervous System (CNS) Structures that Modulate the Stress System

Located in the brainstem, the LC is a homogenous nucleus with the largest number of noradrenergic neurons in the brain that innervates the entire neuroaxis. LC neurons have synchronous spontaneous activity that provides a mechanism for large-scale NE release across the CNS in response to stimuli (Berridge & Waterhouse, 2003). The LC acts as the brain's alarm system, after a stressful challenge there is strong activation of this system that provides a mechanism by which external and internal stimuli induce arousal and vigilance. Multiple stressors that activate the HPA axis, also engage the LC-NE system, and induce immediate-early gene expression and NE release (Cullinan, 1995). The

LC-NE system stimulates the HPA axis and the sympathetic nervous system while hindering neurovegetative functions and the parasympathetic nervous system (reviewed in Aston-Jones, 1996).

The LC promotes survival during a crisis by favoring fast and simpler responses through inhibition of frontal cortex functions (Arnsten, 2000) and via the amygdala and other CNS structures that encode aversive memories.

LC neurons discharge in 2 types of modes: tonic and phasic to modulate attention and behavior (Aston-Jones & Cohen, 2005). The effects of CRH or stressors not only increases LC activity but also modify LC discharge toward the tonic mode; this shift in discharge style is attenuated by CRH antagonism or by opiate antagonism and the combined removal of CRH and opioid actions rendered the LC activity resistant to the effects of the stressor (Curtis, 2012). Stress-induced LC activation increasing arousal but could also facilitate behavioral flexibility during threatening situations (Snyder, 2012).

The amygdala can also modulate hypothalamic CRH release and autonomic centers in the brainstem to respectively increase HPA axis and LC-NE system activities while inhibiting the prefrontal cortex (Cahill & McGaugh, 1998). Neurons in the hypothalamus, LC and amygdala contain multiple feed-forward connections that can promote a robust and persistent stress response.

The dorsolateral prefrontal cortex is implicated in cognition and attention, and the ventromedial prefrontal cortex regulates affect, neuroendocrine, and autonomic activities [reviewed in (Arnsten, 2000; Fuster, 2000, 2001; Smith & Jonides, 1999)]. Cortical lesions in humans and rodents cause exaggerated autonomic and endocrine responses; thus, there is a reciprocal inhibitory relationship between the prefrontal cortex inhibiting the stress system, in which they can inhibit the activity of each other (Fuster, 2000, 2001; Smith & Jonides, 1999), and activation of the prefrontal cortex and consequent restraint of the stress and the sympathetic nervous systems promote flexibility in cognition and affect (Smith & Jonides, 1999).

2.2.2.3 The Immune System

Stress affects many central and peripheral systems in the body, and the immune system is critical for the promotion of survival; therefore, it has key roles during the stress response and immune functions can be enhanced or suppressed by stressors. Endocrine and cytokine mediators modulate the immune function during short-term acute stress, which can modulate immune responsiveness encompassing humoral and cellular aspects of both innate and adaptive immune responses.

The humoral changes related to stress response to psychological stressors, such as the Trier Social Stress Test, include significant increases in the concentration of cytokines, including interleukin-6 (IL-6) and IL-1ß (Altemus, 2001; Aschbacher, 2012; Pace, 2006; Prather, 2013; Puterman, 2014; Steptoe, 2007). Cytokines are small proteins released by the immune system, typically under inflammatory situations. These increased circulating cytokine levels may enhance the immune system during acute stress, contribute to survival, and are also related to emotional states. Peripheral IL-6 levels during stress reaction are related to the experience of anger; it is likely that an angry individual will engage in an aggressive confrontation and sustain injuries and an enhanced immune response will help promote wound healing (Puterman, 2014).

It is now understood that peripheral cytokines modulate brain functions during physiological conditions, where they can regulate neuronal processes, including stress, inflammatory challenges, sickness behaviour, feeding, sleep, learning and memory (Vitkovic, 2000a; 2000b), and that the communication between the brain and the immune system is bidirectional. Our lab has contributed to the understanding of many of the central aspects of cytokine response. We described the expression of cytokines and immunomediators in the brain during baseline and after inflammatory challenges (Licinio, 1991, 1992; Wong, 1997; 1996; Wong & Licinio, 1994); during systemic inflammation, there is a high expression of central IL-1ß. Contrary to the potent systemic counter regulatory anti-inflammatory response, in the brain the expression of counter regulatory cytokines, such as IL-1 receptor antagonist and IL-10, is much lower, which supports that differential regulation of anti-inflammatory cytokines in the CNS and periphery (Wong, 1997).

Acute stress also orchestrates a massive redistribution of immune cells in the body, which is consistent with the critical role the immune system has on survival; therefore, functions such as wound healing and immune surveillance (Dhabhar, 2012) would be enhanced during acute stress. Stress responsive hormones such as NE, corticosterone and epinephrine (EPI) influence many different subsets of immune cells. NE and EPI are released early in the stress response; while, NE increases leucocyte numbers, mobilizing immune cells, including neutrophils, monocytes, and lymphocytes to enter the blood; EPI mobilizes neutrophils and monocytes into the blood, but directs lymphocytes to leave the circulation to specific tissues, such as skin. Corticosterone is then released and mobilizes immune cells to leave the blood towards tissues.

Acute restraint stress increases the numbers of T cells, such as memory and effector helper cells, in sentinel lymphnodes (Dhabhar & Viswanathan, 2005). Stress-induced increments in T cell memory may stimulate the increase of infiltrating lymphocytes and macrophage detected on antigen re-exposure several months later and this process is driven by increased levels of CD4+ T helper (Th) cells type 1 (Th1) cytokines IL-2, interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α).

2.2.3 The Effect of Chronic Stress and MDD in Dysregulating the Core Stress System

In chronic or long-term stress the physiological stress response continues long after the stressor has ended, resulting in a prolonged exposure to stress hormones and stress-related reactions, or is activated repeatedly as a result of continued exposure to the stressor. Chronic activation of the HPA axis and sympathetic nervous system are common in MDD, melancholic subtype; circulating levels of cortisol are elevated and it is commonly accepted that the central CRH level is increased (or inappropriately non-suppressed for the level of hypercortisolism). Elevations of the cerebrospinal fluid (CSF) 24-hour levels of NE and plasma cortisol indicate that there is a persistent stress-system

dysfunction in MDD, melancholic subtype, which is not conditional on the conscious awareness of stress (Wong, 2000). Circadian measures of CSF NE and CSF CRH and peripheral cortisol suggest mutually reinforcing bidirectional relationships between brain NE and CRH pathways that promotes increased central noradrenergic, adrenomedullary, and adrenocortical secretion, which promote a state of hyperarousal and anxiety found in MDD (Gold, 2005; Wong, 2000).

In MDD, atypical subtype, patients display fatigue, lethargy, hypersomnia, and hyperphagia, which are related to suppression of the stress system mediators (Chrousos & Gold, 1992; Gold, 1988a, 1988b). Patients with atypical features have been reported to be eucortisolemic and have low CSF CRH levels (Geracioti, 1997; Gold, 1995). However, detecting decrement in HPA axis is challenging. In response to synthetic CRH, these patients show delays in peripheral ACTH response and very attenuated cortisol responses (Schulte, 1984).

We have reported the involvement of the *CRHR1* gene in antidepressant response; anxious patients homozygous for the GAG haplotype of *CRHR1* gene had a better antidepressant response (Licinio, 2004); as pre-clinical studies suggested that antidepressants suppress CRH gene expression (Brady, 1992; 1991; Reul, 1993) and clinical studies reported that antidepressants reduce CSF CRH concentrations (De Bellis, 1993; Heuser, 1998; Veith, 1993), downregulation of CRH activity could be a common pathway for the therapeutic actions of antidepressants.

Chronic stress can also result in dysregulation with activation or suppression of the immune system; clinical and pre-clinical research support a role of immune system dysregulation in MDD with activation of the cellular and humoral immune responses (Kronfol & Remick, 2000; Licinio & Wong, 1999). Increased circulating lymphocytes and monocytes have been described (Herbert & Cohen, 1993; Rothermundt, 2001; Seidel, 1996). Increased circulating IL-6 levels have been described in MDD and these levels were significantly correlated with self-reported mood ratings (Alesci, 2005). Pro-inflammatory cytokines, such as IL-1ß and IL-6, stimulate the HPA axis (Plata-Salaman, 1991). Stress-induced depressive-like behaviours also increase peripheral IL-6 levels in rodents (Himmerich, 2013), and recently brain IL-6 has been implicated in

facilitating cognitive flexibility in the orbitofrontal cortex (Donegan, 2014). A depressive syndrome can be associated with cytokine administration, typically during IFN-α treatment of hepatitis C or malignant melanoma (Bonaccorso, 2000; Dieperink, 2004; Hauser, 2002; Kraus, 2003; Schaefer, 2004), the psychopathological presentation can include changes in mood or cognition, psychomotor retardation, fatigability, sleep disturbances and decreased appetite (Capuron & Miller, 2004; Loftis, 2006; Raison, 2006).

Dysregulation of the T-cell arm of the immune system has corroborated the role of immunomediators in depression (Kronfol & Remick, 2000; Pavon, 2006). Evidence supporting both the predominance of T-cells Th1 or Th2 have increased and the balance of these two subtypes of cytokine-producing T cells Th1/Th2 regulates the immune response to pathogens. These two main lineages of cells differentiate from naïve CD4+ T lymphocytes and have distinct cytokine profiles. Th1 patterns are related to increased inflammation and autoimmune conditions, and the production of IFN-γ, TNF-α or IL-1; conversely, Th2 patterns are related to allergic responses and asthma, and the production of IL-6, IL-10 or IL-13 (Kimball, 2005; Pavon, 2006; Scott, 2007).

Data from our lab supports that there is an imbalance of Th1/Th2 activities with a predominance of Th1 in MDD; we found increased chemokine CXCL10/IP10 levels, which decreased with antidepressant treatment, and decreased IL-13 levels; although no distinct Th1 or Th2 cytokine patterns was found in our MDD patients (Wong, 2008). Nevertheless, different types of MDD may exhibit divergent immune profiles, and decreased Th1 activation has been described in melancholic depressed patients, which is accompanied by a marked HPA axis activation. Non-melancholic MDD patients show increased inflammation features with elevated monocyte count and levels of IL-1ß and α2-macroglobulin (Kaestner, 2005; Rothermundt, 2001). Post-partum depression also shows a predominance of Th1 activation (Maes, 2002; Ostensen, 2005), and symptoms of anxiety and depression in the early post-partum period are related to increased levels of pro-inflammatory cytokine IL-8 (Maes, 2002).

In the brain, stress-induced increased glucocorticoids enhances immune function, promotes microglia proliferation and activation, and a loss of astrocytes number and volume (Czeh, 2006). Increased concentrations of pro-inflammatory mediators can lead to hippocampal dendritic atrophy and neuronal death (Sapolsky, 1985; Woolley, 1990).

Overall, antidepressants reduce Th1 cytokines production *in vitro*, promoting a shift towards Th2 patterns (Leonard, 2001). For instance, imipramine, clomipramine, fluoxetine, sertraline and venlafaxine amongst other antidepressants, significantly decrease the IFNγ/IL-10 ratio (Kubera, 2001; Maes, 1999; Szuster-Ciesielska, 2003). Amitriptylin and nortriptylin inhibit IL-1ß and TNF-α release in *in vitro* cultures of microglial and mixed glial cells (Obuchowicz, 2006). In MDD patients, decreases in circulating IL-6 levels and TNF-α (Basterzi, 2005; Frommberger, 1997; Narita, 2006; Sluzewska, 1995), and increases in anti-inflammatory Th3-cytokine transforming growth factor beta (TGF-ß) (Myint, 2005) have also been reported during antidepressant treatment.

2.2.4 Summary and Conclusions

Optimal performance of the CNS requires that the core stress system be maintained within a particular range, and emotional responses involve the integration of several areas of the brain. Superb collaboration of several CNS and peripheral systems in a sequentially coordinated manner are required for the optimal response of the stress system, which will ultimately determine the ability of an organism to survive under a threat. Dysregulation of the stress system is implicated in MDD; melancholia involves the hyperactivity of the stress system and atypical features are related to the down-regulation of the stress response; likewise, dysregulation of the immune response implicates the activation or suppression of humoral and cellular immune components. The core stress and immune systems interact with other CNS pathways that have also been reported to be dysregulated in MDD, including the serotonergic, glutamatergic, and kynurenine pathways (Muller & Schwarz, 2007).

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2.3 Understanding the Metabolic Syndrome Using a Biomedical Chemistry Profile

2.3.1 Introduction

For quite some time, it has been identified that high blood pressure, dyslipidemia [increased triglycerides and reduced high-density lipoprotein (HDL) cholesterol levels], impaired glucose homeostasis and abdominal obesity take place concurrently more than by random, supporting the existence of the *metabolic syndrome* (MetSyn). Additionally, hyperuricemia, a prothrombotic state, oxidative stress, chronic low-grade inflammation, increased levels of apolipoprotein-B and small dense low-density lipoprotein (LDL) cholesterol (contributing to atherogenic dyslipidemia), non-alcoholic fatty liver disease and/or non-alcoholic steatohepatitis, obstructive sleep apnea and/or polycystic ovarian disease (Fulop, 2006; Alberti, 2009; Roberts, 2009; Ma, 2012; Matsuda, 2013; Mule, 2014; Carson, 2015) are quite often present on the MetSyn, although not yet included in its current/actual definition. Taking this into consideration, it is not surprising that the MetSyn associates with an increased risk of type 2 diabetes mellitus (T2DM) and atherosclerotic cardiovascular disease (Fulop, 2006; Qiao, 2007; Carson, 2015).

Epidemiological and experimental evidence has demonstrated beneficial effects of dietary magnesium, calcium, potassium and bicarbonate on the MetSyn or some of its individual components (Luft, 1990; Van Leer, 1995; Schorr, 1996; Whelton, 1997; Franch, 2004; Rylander, 2004; Schoppen, 2004; Franzoni, 2005; Karppanen, 2005; Schoppen, 2005; He, 2006; Feldeisen, 2007; Schoppen, 2007; Champagne, 2008; Rylander, 2008; Volpe, 2008; Perez-Granados, 2010; Adeva, 2011; Rice, 2011; Lee, 2013).

Natural mineral-rich waters are good sources of highly absorbable and bioavailable minerals (such as calcium, magnesium and potassium)

and bicarbonate (Heaney, 1989; Bohmer, 2000; Kessler, 2000; Sabatier, 2002; Bacciottini, 2004; Kiss, 2004; Heaney, 2006; Karagulle, 2006; Petraccia, 2006; Karagulle, 2007; Sabatier, 2011). So, the particular composition of natural mineral-rich waters would be responsible for their favorable effects. In fact, the World Health Organization has recognized that mineral-rich drinking-waters may provide substantial contributions to total intakes of calcium and magnesium in some populations or population subgroups (Cotruvo, 2009). In line, two recent and exhaustive studies revealed that consumption of public drinking waters and bottled natural mineral waters is a relevant complementary source of calcium and magnesium in Spain (Vitoria, 2014; Maraver, 2015).

Beneficial effects of natural mineral-rich waters ingestion on MetSyn features, included or not in its definition, and MetSyn complications (Luft, 1990; Simunic, 1990; Schorr, 1996; Polushina, 1998; Polushina, 2002; Rylander, 2004; Schoppen, 2004; Schoppen, 2005; Schoppen, 2007; Benedetti, 2009; Botvineva, 2010; Perez-Granados, 2010; Santos, 2010; El-Seweidy, 2011) as well as on the MetSyn itself (Pereira, 2012a; Pereira, 2013; Pereira, 2014a; Pereira, 2014b; Pereira, 2014c; Pereira, 2015) have been published.

2.3.2 Natural Mineral-rich Waters and MetSyn

The consumption of sodium bicarbonate containing/mineral-rich waters decreased systolic blood pressure in mildly hypertensive men (3 L day^{-1}, 7 days) (Luft, 1990) and mean arterial blood pressure in elderly normotensive individuals (1.5 L day^{-1}, 4 weeks) (Schorr, 1996). The ingestion of sulfate, calcium, magnesium and bicarbonate-rich natural mineral water (at least 1 L day^{-1}, 4 weeks) reduced systolic and diastolic blood pressure in adults with borderline hypertension and low urinary magnesium and calcium excretion levels (effect perceived after 2 weeks of consumption and sustained until the end of the dietary protocol) (Rylander, 2004). Vaquero *et al.* showed that, in moderately hypercholesterolemic young adults, the ingestion of bicarbonated natural mineral water, rich in sodium, chloride and potassium, and with

a high bicarbonate to sodium ratio (1 L day^{-1}, 8 weeks), reduced systolic blood pressure (an effect already seen after 4 weeks), apolipoprotein-B, total-cholesterol and LDL-cholesterol fasting serum levels as well as total-cholesterol and LDL-cholesterol to HDL-cholesterol ratios (Perez-Granados, 2010). The same group showed, in healthy postmenopausal women, that a) the ingestion of the previous water (1 L day^{-1}, 2 months) increased HDL-cholesterol and decreased endothelial dysfunction markers, glucose, total-cholesterol and LDLcholesterol fasting serum levels as well as total-cholesterol and LDL-cholesterol to HDL-cholesterol ratios (Schoppen, 2004), and b) the consumption of sodium-rich bicarbonated mineral waters (0.5 L each) with a standard fat-rich meal increased insulin sensitivity [more distinctly in the women with higher homeostasis model assessment (HOMA) index values] and decreased lipemia (Schoppen, 2005; Schoppen, 2007). Both a decrease in lipid and protein oxidation products and an increment of total antioxidant capacity and total thiol plasma levels were observed in healthy individuals drinking a sulfurous mineral water (0.5 L day^{-1}, 2 weeks) (Benedetti, 2009).

Our group evaluated the effects of the ingestion of a Portuguese natural mineral-rich water, and some of the possible mechanisms involved, on metabolic function in a well-validated MetSyn animal model (Polizio, 2006; Rayssiguier, 2006; Oron-Herman, 2008): male Sprague-Dawley rats treated with 10% fructose in drinking water, for 8 weeks. Animals were randomly assigned into three groups with free access to food and a) tap water, b) 10% fructose in tap water or c) 10% fructose in Portuguese natural mineral-rich water. As expected, 10% fructose in tap water induced metabolic features characteristic of the MetSyn, such as increased plasma levels of triglycerides, insulin and leptin [with a strong tendency toward decreased insulin sensitivity index (Cacho, 2008)] and decreased plasma levels of magnesium as well as increased systolic blood pressure and heart rate. Fructose-induced effects in the redox state (liver), endoplasmic reticulum homeostasis (liver), glucocorticoid and insulin signalling pathways (liver and visceral and/or subcutaneous adipose tissue) and endothelial dysfunction markers expression (cavernous tissue) may have contributed to explain the induction of MetSyn; some compensatory mechanisms against

fructose-ingestion were also revealed. Importantly, the co-ingestion of the Portuguese natural mineral-rich water reduced and/or prevented most of the changes induced by fructose and, additionally, strengthened the compensatory mechanisms and induced *per se* protective pathways in response to stress (Pereira, 2012a; Pereira, 2013; Pereira, 2014a; Pereira, 2014b; Pereira, 2014c; Pereira, 2015). This Portuguese natural mineral-rich water also increased hepatic catechol-O-methyltransferase activity in healthy Wistar Han rats (Bastos, 2014). Its high-content of protective minerals, such as magnesium, calcium and potassium, as well as bicarbonate, and low chloride content may explain the favourable results obtained (Pereira, 2012a; Pereira, 2013; Pereira, 2014a; Pereira, 2014b; Pereira, 2014c; Pereira, 2015).

2.3.3 Magnesium and MetSyn/MetSyn Features – Associated Mechanisms

Chronic deficiency of magnesium (in animal models with low magnesium intake) is associated with hypertension and increased heart rate (and somewhat higher plasma corticosterone levels) as well as dyslipidemia, insulin resistance and oxidative stress (Caddell, 1991; Balon 1994; Laurant, 1999; Busserolles, 2003; Takaya, 2012).

Clinical and experimental studies point to magnesium intake/status being inversely associated with the risk of hypertension, T2DM and coronary heart disease. Additionally, magnesium intake may decrease triglycerides and increased HDLcholesterol circulating levels (Balon, 1994; Touyz, 2003; Takaya, 2004; Barbagallo, 2007; Belin, 2007; Abete, 2011; Heer, 2015).

Individuals with MetSyn (or with some of its individual components) frequently show reduced magnesium status and reduced magnesium intake as compared with non-MetSyn (or healthy) subjects (Barbagallo, 2007; Belin, 2007; Evangelopoulos, 2008; Abete, 2011; Heer, 2015). Interestingly, hypomagnesaemia has been associated with metabolic abnormalities characteristic of MetSyn in the absence of obesity and, conversely, normal circulating levels of magnesium seem to be protective against the development of metabolic complications in obese

individuals (Guerrero-Romero, 2013). Often, circulating and/or intracellular magnesium levels are reduced under insulin resistance/T2DM in (obese) children, adolescents and adults (Takaya, 2004; Huerta, 2005; Belin, 2007; Wells, 2008; Celik, 2011). Lecube *et al.* provided evidence that T2DM was the main factor accounting for the hypomagnesemia found in morbidly obese individuals. They observed that the percentage of morbidly obese individuals with serum magnesium concentration lower than 0.75 mmol L^{-1} was three fold higher in T2DM patients than in non-T2DM subjects. They also found that not only the degree of blood glucose control (when considering fasting plasma glucose and HbA1c levels) and serum magnesium concentration were significantly and negatively correlated but also fasting plasma glucose and HbA1c levels were, in multiple linear regression analysis, independently associated with serum magnesium concentration. Additionally, in the morbidly obese patient subgroup that went through bariatric surgery, serum magnesium levels increased in T2DM subjects in whom diabetes resolved; serum magnesium levels lasted unchanged in whom T2DM did not resolve (the same happened in non-T2DM obese subjects) (Lecube, 2012).

The mechanisms by which T2DM could lead to low serum magnesium levels remain to be fully understood. However, insulin resistance, hyperinsulinemia, hyperglycemia and/or glycosuria may negatively interfere with renal reabsorption of magnesium, contributing to hypomagnesemia (McNair, 1982; Djurhuus, 1995; Barbagallo, 2007; Belin, 2007; Lecube, 2012; Takaya, 2012). Insulin has a prime role in magnesium metabolism regulation and insulin resistance/inhibition of insulin-stimulated glucose uptake may decrease magnesium uptake by tissues and increase magnesium efflux from tissues (Takaya, 2004; Barbagallo, 2007; Belin, 2007).

Magnesium is a cofactor of several enzymes involved in insulin and glucose metabolism, among other processes (Takaya, 2004; Barbagallo, 2007; Belin, 2007; Guerrera, 2009; Takaya, 2012). Briefly, insulin binds to its receptor (IR) inducing its autophosphorylation on tyrosine residues and, subsequently, tyrosine residues phosphorylation of its substrates (IRS) and Src homology 2 domain containing transforming protein 1 (Shc). Phosphorylated IRS, particularly IRS1 and 2, activate

phosphatidylinositol 3-kinase (PI3K), which converts phosphatidylinositol (4,5)-bisphosphate into phosphatidylinositol (3,4,5)-trisphosphate (PIP3) on the plasma membrane. PIP3 recruits, binds and activates phosphatidylinositol-dependent protein kinase-1 (PDK1), which phosphorylates protein kinase B (Akt) contributing to its activation. Akt activation mediates insulin-induced glycogen and protein synthesis, gluconeogenesis inhibition and glucose transporter 4 (GLUT4) translocation to the plasma membrane (what increases glucose uptake by insulin sensitive tissues). Instead, phosphorylation of Shc by IR promotes a parallel signaling pathway, leading to the activation of serine/threonine kinases, such as MAPK-kinase (MEK-1/2) and extracellular receptor kinase (ERK), responsible for insulin-induced cell growth and differentiation (Cohen, 2006; Tsatsoulis, 2013). Interestingly, magnesium has a positive impact on tyrosine kinase activity at the IR level as well as on the translocation of the GLUT4 to the cellular membrane (Takaya, 2004; Barbagallo, 2007; Belin, 2007; Guerrera, 2009; Takaya, 2012). Moreover, magnesium deprivation, in a renal epithelial cell line (Madin-Darby canine kidney cells), inhibited cell proliferation and decreased ERK1/2 phosphorylation; re-addition of magnesium increased phosphorylated ERK1/2 levels. The use of a specific inhibitor of the MEK-ERK cascade inhibited this last effect, indicating that magnesium is involved in the regulation of the MEK-ERK cascade and cell proliferation, at least in this cell line (Ikari, 2010).

Magnesium deprivation may also increase glucocorticoid exposure, even during fetal development (Caddell, 1991; Laurant, 1999; Takaya, 2011a; Takaya, 2012), which is involved in insulin resistance/T2DM and dyslipidemia (Pereira, 2011; Pereira, 2012b; Pereira, 2012c; Paredes, 2014; van Raalte, 2014). Takaya *et al.* investigated the effects of feeding pregnant rats a very-low magnesium diet (0.003% magnesium) upon cytosine-guanine dinucleotides methylation in hepatic glucocorticoid genes of neonatal offspring *versus* controls (0.082% magnesium). Mean methylation of the 11β-hydroxysteroid dehydrogenase type 2 gene (Hsd11b2) promoter (11β-hydroxysteroid dehydrogenase type 2 inactivates tissue's glucocorticoids) in the magnesium-deficient offspring was three times higher than in controls, predicting higher hepatic intracellular glucocorticoid exposure (Takaya, 2011a).

Additionally, in female weanling Wistar/NIN rats, maternal and postnatal Mg deficiency played important (and different) roles in the programming of increased body adiposity, insulin resistance and impaired glucose tolerance and insulin secretion in rat offspring (Venu, 2005; Venu, 2008).

Magnesium deficiency is associated with vascular smooth muscle cells (SMC) proliferation, intimal thickening, thinning and fragmentation of elastic membranes, collagen accumulation and calcification as well as inflammation [increased interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α) and C-reactive protein (PCR) circulating levels], high rate of free-radical formation (which leads to an increased NO degradation by superoxide anions), increased susceptibility of lipoproteins to peroxidation and increased tissue lipid peroxidation. All these effects contribute to/have been associated with hypertriglyceridemia, pro-atherogenic alterations in lipoprotein metabolism, cardiovascular lipid deposition and/or the pathogenesis of vascular lesions following magnesium deficiency (Bussière, 1995; Gueux, 1995; Laurant, 1999; Busserolles, 2003; Rayssiguier, 2006; Rayssiguier, 2010).

Through calcium antagonism, magnesium promotes vascular relaxation and thus plays an important role on blood pressure control (Laurant, 1999; Rice, 2011). Conversely, magnesium deprivation activates the sympathetic nervous system which contributes to increased blood pressure and heart rate (Laurant, 1999; Rayssiguier, 2010). Aldosterone, due to both genomic and non-genomic effects, also controls systemic vascular resistance, blood pressure and heart rate (MacFadyen, 1997; Freel, 2004). In line, magnesium deficiency has been described to stimulate the synthesis and secretion of aldosterone, possibly by decreasing the antagonism to calcium influx in the zona glomerulosa of the adrenal glands (Laurant, 1999).

Magnesium supplementation during 4 weeks not only decreased fasting circulating C-peptide and insulin concentrations in healthy overweight/obese individuals but also altered whole blood gene expression [with negative regulation of 36 genes (including some involved in metabolic and inflammatory signaling pathways) and with

positive regulation of 24 genes (some of them involved in magnesium homeostasis)] (Chacko, 2011). Additionally, magnesium supplementation reduced liver damage induced by ethanol ingestion both in humans (Poikolainen, 2008) and in rats (Markiewicz-Gorka, 2011). Although magnesium deprivation alone may induce metabolic abnormalities similar to those observed in rodents under chronic high-fructose feeding (model of diet-induced MetSyn), upon magnesium deficiency high-fructose feeding metabolic actions are potentiated (Busserolles, 2003; Rayssiguier, 2006). In this context, magnesium supplementation of rats submitted to high-fructose feeding prevented and/or improved the alterations induced by fructose. In particular, it ameliorated insulin sensitivity [evaluated as homeostasis model assessment insulin resistance (HOMA-IR) and muscle glucose utilization] and fasting circulating lipid profile, and decreased blood pressure as well as fasting circulating glucose, insulin and lipid peroxidation marker levels (Balon, 1994; Olatunji, 2007).

In this regard, we have described that the Portuguese natural mineral-rich water mentioned above partially prevented the decrease of the hepatic magnesium content of fructose-fed Sprague-Dawley rats (Pereira, 2014a).

2.3.4 Calcium and MetSyn/MetSyn Features – Associated Mechanisms

Serum calcium levels are increased in individuals with MetSyn and positively correlated with serum triglycerides and blood pressure values (Park, 2012; Cho, 2011). Calcium and/or dairy products ingestion has been associated with decreased adipose tissue mass, lower blood pressure, better circulating lipid profile (increased HDL-cholesterol and decreased total-cholesterol and LDL-cholesterol levels as well as decreased total-cholesterol to HDL-cholesterol ratio and increased HDL-cholesterol to LDL-cholesterol ratio) and prevention/reduced incidence of insulin resistance, T2DM and MetSyn (Teegarden, 2006; van Meijl, 2008; Tremblay, 2009; Abete, 2011; Rice, 2011). The inconsistency between serum calcium levels and dietary calcium intake values with

MetSyn still needs to be clarified (Park, 2012; Cho, 2011).

The increase of fecal excretion of fat and inhibition of intestinal bile salts absorption (with the consequent increase of cholesterol conversion into bile acids in the liver) as well as the stimulation of lipolysis and inhibition of lipogenesis improve plasma lipid profile and decrease adipose tissue mass as a consequence of calcium intake (Abete, 2011; Rice, 2011). Regulation of parathyroid hormone and 1,25-dihydroxycholecalciferol levels by dietary calcium mediates its effects on fat mass and insulin signaling/action (Teegarden, 2006). There is also evidence that adequate calcium intake, by decreasing the concentration of 1,25-dihydroxycholecalciferol, decreased the uptake of calcium by vascular SMC and, so, impaired contraction and reduced peripheral resistance and blood pressure (Rice, 2011). Additionally, lowering of 1,25-dihydroxycholecalciferol associated with a calcium-induced decrease of reactive oxygen species, oxidative stress markers and pro-inflammatory markers (such as TNF-α, IL-6 and PCR) and increase of anti-inflammatory markers (such as adiponectin), in adipose tissue and/or blood in obese humans and/or obese mice (Zemel, 2008). Dietary calcium supplementation (started 120 days after birth and lasting for 2 months) in adult offspring rats programmed during lactation by maternal nicotine exposure restored insulin sensitivity, reversed the concentration of serum leptin as well as the percentage of both total body fat content and visceral fat mass, and decreased the mRNA expression of leptin in visceral adipose tissue *versus* the nicotine exposed/conditioned rat group. Dietary calcium supplementation of the programmed rats also increased the hepatic expression of sirtuin 1 (Sirt1) *versus* the control rat group (without nicotine exposure or calcium supplementation) (Nobre, 2011). Regarding Sirt1, growing evidence suggests that its deacetylase activity regulates glucose-lipid metabolism, glucose production, inflammation, oxidative stress, autophagy and mitochondrial function and biogenesis as well as adiponectin and insulin secretion. Positive effects of Sirt1 overexpression and Sirt1 activators have been described (Kitada, 2013a; Kitada, 2013b; Xu, 2013; Li, 2014).

With an opposite approach, female Wistar rats subjected to a very-low-calcium diet [(0.008% calcium) *versus* regular-calcium control diet-

fed rats (0,9% calcium)], for 2 weeks, showed lower fasting serum levels of adiponectin and higher HOMA-IR. Moreover, the mRNA expression of 11β-hydroxysteroid dehydrogenase type 1 (activates tissue's glucocorticoids) in the liver was up-regulated (with the same tendency for the hepatic glucocorticoid receptor), before the animals developed obesity or other evident features of MetSyn (Takaya, 2011b), amplifying the glucocorticoid exposure in this tissue.

2.3.5 Potassium and MetSyn/MetSyn Features – Associated Mechanisms

Dietary potassium is inversely associated with hypertension and potassium supplementation may improve and/or prevent hypertension (Whelton, 1997; Franzoni, 2005; Rice, 2011). Inhibition of pro-inflammatory events in vascular SMC, reduction of platelet aggregation and reduction of renal vascular resistance seem to mediate potassium favorable effects on blood pressure (Rice, 2011).

Potassium intake and serum potassium levels have also been negatively associated with MetSyn prevalence (Lee, 2013; Sun, 2014). Lower serum potassium levels, and to a minor degree lower dietary potassium intake levels, have been associated with an increased risk of diabetes (Chatterjee, 2011; Lee, 2013). Even a moderate depletion of serum potassium (without frank hypokalemia) is associated with glucose intolerance/insulin resistance and, hence, with an increased risk of T2DM, by reducing insulin secretion (Norbiato, 1984; Lee, 2013). Serum potassium levels are closely regulated by homeostatic mechanisms and depend on dietary potassium intake and potassium excretion (and its regulators) as well as on partitioning between intracellular and extracellular spaces (modulated by insulin, catecholamines and thyroid hormone) (Chatterjee, 2011). Renal potassium excretion is primarily controlled by sodium delivery to the distal nephron and urine flow, vasopressin levels, acid-base status (also hormone regulated, as above) and the renin-angiotensin-aldosterone system (Chatterjee, 2011). As a consequence of the strict control of serum potassium levels, dietary and serum potassium levels are not inevitably

associated (Chatterjee, 2011).

2.3.6 Bicarbonate and MetSyn/MetSyn Features – Associated Mechanisms

In fact, besides calcium, magnesium and potassium content on natural mineral-rich waters, hydrogen carbonate concentration also deserves attention. Hydrogen carbonate-rich mineral waters may decrease calcium and magnesium renal excretion (by increasing their renal reabsorption) and, so, contribute to minerals´ homeostasis in the body (Brandolini, 2005; Rylander, 2008). Water pH and water hydrogen carbonate content are particularly relevant when considering the increased acid load of the Western diet, mainly related to the: a) high-ingestion of proteins (especially from animal origin), since sulfur ions are formed during amino acids metabolism, as well as high-sodium chloride consumption, and b) low-ingestion of fresh fruit, vegetables, tubers, roots and nuts, that are net base producers. As a result, the consumption of a Western diet induces a chronic, low-grade metabolic acidosis that worsens with the decline of kidney function, for example with aging (Cordain, 2005; Rylander, 2008; Zhang, 2009; Adeva, 2011). In line, it was observed that renal sulfate excretion negatively correlates with urine pH and is higher in insulin resistance *versus* normal insulin sensitivity, highlighting an association among (animal) protein ingestion, endogenous acid production and insulin resistance. Furthermore, insulin resistance has been linked with metabolic acidosis markers (such as low urine pH and low serum bicarbonate levels) (Adeva, 2011).

Metabolic acidosis may induce insulin resistance by impairing the insulin signaling pathway through inhibition of PI3K activity and, consequently, of its downstream effectors, in the skeletal muscle (Franch, 2004; Adeva, 2011). This gives dietary acid load a solid role in anticipating the metabolic dysfunction and the cardiovascular risk of the healthy, overweight and obese individuals as well as diabetic, hypertensive and chronic kidney failure patients (Zhang, 2009; Adeva, 2011; Odermatt, 2011). Moreover, acidosis-induced inhibition of PI3K

activity blocked the antiproteolytic effect of insulin, which could be related to decreased lean body mass in chronic kidney failure patients (Franch, 2004). Metabolic acidosis increases both glucocorticoid secretion and plasma cortisol levels, which definitely contribute to insulin resistance, T2DM, MetSyn, blood pressure and inflammation. Contemporary acidogenic diet associates with cortisol excess, the latter being prevented by bicarbonate administration (Zhang, 2009; Adeva, 2011; Pereira, 2011; Pereira, 2012b; Pereira, 2012c).

In this context, once more, natural mineral-rich water consumption would be extremely beneficial.

2.3.7 Magnesium, Calcium, Potassium and Bicarbonate *versus* Sodium

As highlighted above, an adequate intake of magnesium, calcium or potassium has a favorable effect on metabolic regulation and/or blood pressure (Whelton, 1997; Geleijnse, 2005; Karppanen, 2005; Teegarden, 2006; Feldeisen, 2007; Olatunji, 2007; van Meijl, 2008; Cho, 2009; Tremblay, 2009; Chaudhary, 2010; Abete, 2011; Rice, 2011). However, their association, as occurs in the dietary approaches to stop hypertension (DASH diet), which includes, among others, magnesium, calcium and potassium-rich foods, seems more effective, particularly in blood pressure control (Vaskonen, 2003; Al-Solaiman, 2010). Furthermore, magnesium, calcium and potassium reduce sodium retention, which may contribute to their positive effects on blood pressure (Vaskonen, 2003; Rice, 2011). In some studies, sodium chloride is clearly associated with hypertension (Ziomber, 2008; Santos, 2010). This association depends on sodium being accompanied by chloride, as sodium *per se* (in the drinking solution) does not increase blood pressure (Ziomber, 2008; Santos, 2010). Possibly through sodium potassium ATPase inhibition (Blaustein, 2006), only sodium chloride seems to increase plasma volume and blood pressure (Kunes, 2004; Santos, 2010), although sodium salts, with chloride or other anions (with equimolar amounts of sodium), produce similar suppression of the renin-angiotensin axis (Luft, 1990; Santos, 2010).

In line with these findings, natural mineral-rich waters intake, with a high content of both sodium and bicarbonate, decrease blood pressure (Schorr, 1996; Perez-Granados, 2010) or has no effect on it (Schoppen, 2004; Santos, 2010).

2.3.8 Conclusion

As shown above, an adequate body homeostasis of magnesium, calcium, potassium and bicarbonate has a prime role in preventing and/or improving MetSyn, or some of its features. The ingestion of natural mineral-rich waters could contribute to an adequate supply of those dietary micronutrients, being most relevant in the context of the Western diet.

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2.4 Brain Neurochemistry and Cognitive Performance: Neurotransmitter Systems

In Biomedical Chemistry: Current Trends and Developments

Though there is no universal definition of cognition, it is widely accepted that it may encompass many different components including: concept formation, mental abstraction, language acquisition, text comprehension, higher linguistic abilities, inference, learning, symbolic reasoning, planning, decision making and metacognition (Metzger, 2011). However, this description may also extend to empathy, procedural memory, introspection and emotional components relevant to the cognitive process. Globally, cognition relies on a balanced function of several neural circuits and pathways modulated primarily by a range of neurotransmitters.

The present chapter reviews how altered neurochemical function may result in impaired cognition, with a focus on major classical neurotransmitters. Nonetheless, several other neurotransmitters, mainly small neuropeptides, but also purinergic elements and lipidic endocannabinoids among others, are now known to play relevant roles in cognitive processes. At the end of the present chapter we included a short review of two emerging areas of knowledge in the field of cognitive science: i) the role of gliotransmitteres, referring to the cross-talk between neurons and glial cells, which has previously been underestimated; and ii) the modulation of neurotransmission systems to achieve “neuroenhancement“ or cognitive potentiation.

2.4.1 Monoaminergic Neurotransmission and Cognition

Dopamine appears early in the embryonic CNS, and plays a key role in neural development. Interfering with the normal interaction between

dopamine and dopamine receptors, during developmental periods is long known to alter the standard embryonic development, disrupting neuronal proliferation, cell migration, neuronal maturation, pruning and proper connectivity.

Dopaminergic neurons are concentrated in three major cell groups: the substantia nigra (SN) the ventral tegmental area (VTA), and a cell group of dopamine-containing cells in the caudal extension of the SN (Fallon, 1978; 1985a; Deutch, 1988). The SN and VTA regions are strongly implicated in cognition, affective and behavioral disorders (Roeper, 2013), which is partially explained by a relevant overlap with the brain reward and limbic systems (Koob, 1988; 1992). The main efferent connections from the SN-VTA are well described and can be summarized in three groups: the mesostriatal or nigrostriatal projections, the mesolimbic projections and the mesocortical projections (sometimes referred together as mesocorticolimbic). The mesostriatal pathway originates largely from dopaminergic neurons in the ventral sheet of the SNc projecting into the dorsal striatum (Fallon, 1985b; 1988; Voorn, 1988). In the dorsal striatum, dopaminergic terminals synapse with GABAergic and cholinergic neurons, resulting in either excitatory or inhibitory effects depending on the pattern of dopaminergic receptors displayed at the synapse. Dopamine in the dorsal striatum plays an important role in controlling patterns of motor activity, reward-based learning and sequencing, which constitute a complete motor act and represent a cognitive function (Calabresi, 2007; Leisman, 2014). The striatum receives major glutamatergic inputs from the cortex and can control dopaminergic neurons in the SN-VTA through striatal feedback projections (Voorn, 1988; Robbins, 1992; Calabresi, 2000). The mesolimbic pathway arises predominantly from the dorsal sheet of the SNc, SNl and dorsal VTA (Fallon, 1995). These projections innervate, among other areas, the amygdala, and the nucleus accumbens (Fallon, 1985b; 1988), which plays a major role in the limbic-motor integration (Nicola, 2000), underlying complex adaptive behaviors involved in brain stimulation reward and self-administration of psychoactive drugs (Woodward, 1999; Schramm, 2002). In the nucleus accumbens, DA seems to modulate limbic hippocampal and amygdalar inputs (Mogenson, 1988). Dopamine in this brain region was also shown to be

involved in stimulus conditioned responsiveness (Bassareo, 2002) and locomotor activity. The mesocortical pathways of dopaminergic projections arise mainly from the VTA and medial SN, densely innervating the prefrontal, cingulated, suprarhinal and entorhinal cortices, and the dorsal and ventral hippocampus and the visual cortex. Dopamine was described to act in the prefrontal cortex (PFC), by modulating signals received from relevant thalamic afferents (Thierry, 1988). Under stressful situations, dopamine metabolism is increased in the PFC and, therefore, dopamine seems to be also relevant in plastic responses to cope with the stressors (Sullivan, 1998; 2004). Dopamine is also involved in the control of memory-guided behavior in the prefrontal cortex (Goldman-Rakic, 1992) and in cognitive tasks (Weinberger, 1988; Puig, 2014). It is noteworthy that these pathways are under a complex net of direct stimulation and feedback regulation within the dopaminergic system.

In the CNS, serotonin (5-hydroxytriptamine, 5-HT) is a key player in mood control. Alterations in 5-HT function have been implicated in several clinical states, including affective disorders, obsessive-compulsive disorders, schizophrenia, anxiety states, phobic disorders, eating disorders, migraine, and sleep disorders (Stockmeier, 2003; Albert, 2014). There is also evidence that 5-HT is involved in learning and memory processes (Meneses, 1999; Faulkner, 2014).

The majority of serotonergic neurons are located in the midline raphe nuclei (Tork, 1990). Serotonergic neurons in the raphe nuclei have extensive projections to virtually all areas of the brain and spinal cord (Tork, 1990), but the brain regions usually implicated in cognition and mood receive 5-HT projections mainly from the dorsal raphe nucleus (DRN) and median raphe nucleus (Halliday, 1995). The majority of the ascending 5-HT fibers originate at MRN or DRN, while a caudal cluster gives rise to almost all the descending 5-HT fibers (Steinbusch, 1981; Halliday, 1995). The early development of the 5-HT cell bodies and their extensive net of connections through all the brain, are indicative of the important role that serotonin plays in the development and functioning of the CNS. Diencephalic structures, the basal forebrain, septal regions, hippocampal formation and the cerebellum all receive ascending projections from the DRN and MRN (Halliday, 1995). These projections

are essentially non-overlapping and distribute to separate sites. Fibers from the DRN primarily project to the SNc, VTA, amygdala, striatum, lateral preoptic area, substantia innominata, nucleus accumbens and several regions of the cortex, while MRN fibers distribute mainly to midline and para-midline structures (Vertes, 1997; 1999). Direct synaptic connections between raphe serotonergic terminals and DA neurons were first demonstrated in the VTA (Herve, 1987), since then, a close relationship between the serotonergic and the dopaminergic systems has been well established.

Synthesis of dopamine in DA-neurons starts with the metabolization of L-tyrosine into 3,4-dihydroxiphenylalanine (L-DOPA) by TH (Nagatsu, 1964; 1998), followed by almost immediate conversion of L-DOPA into dopamine by L-aromatic amino acid decarboxilase (L-AADC) (Deutch, 1999). Dopamine is stored into vesicles in the presynaptic terminal of dopaminergic neurons, which not only prevents DA from degrading, but also delays its diffusion to the extracellular space. Dopamine is released into the synapse by calcium dependent exocytotic mechanisms. Once in the synaptic cleft, DA can interact with presynaptic autoreceptors (D_2-like receptors that include D_2, D_3 and D_4), regulating DA synthesis, release and neuronal firing-rate; or with postsynaptic receptors (D_1-like, that include D_1 and D_5), modulating the response of the postsynaptic neuron. The D_1 receptor is the most widespread receptor, the most expressed and exclusively postsynaptic (Vallone, 2000). The D_2 receptor is found mainly expressed by GABAergic neurons (Civelli, 1991). Importantly, these receptors are known to form heteromers, which can have relevant functions in the dopaminergic brain (Hasbi, 2011; Perreault, 2014). The dopamine transporter protein (DAT) seems to be targeted by a complex net of regulation mechanisms, which depend primarily on the extracellular levels of DA. This transporter is a major target for psychostimulant drugs, such as cocaine and amphetamines, which can easily block it or use it to enter the dopaminergic terminal, leading to increased extracellular levels of DA and increased oxidative stress (Reith, 1997; Chen, 2000). DAT increased levels are potentially dangerous to the neuron (due also to its ability to transport toxins). A positive correlation between the levels of DAT expression and

neuroinjury has been demonstrated (Miller, 1999). Storing of DA depends on a vesicular transporter protein, the vesicular monoamine transporter (VMAT). If left free on the cytoplasm, DA is rapidly inactivated by the mitochondrial enzyme MAO-A, simultaneously producing H_2O_2.

Serotonin is synthesized from the amino acid tryptophan that is hydroxylated into 5-hydroxytryptophan (5-HTP) by the rate-limiting enzyme tryptophan hydroxylase (TPH). The serotonin precursor, 5-HTP, is decarboxylated by the L-AADC originating the neurotransmitter 5-HT. Synthesis of serotonin depends mainly on the availability of tryptophan, which is present in high levels in the plasma and crosses the blood-brain barrier by active transport. Dietary levels of tryptophan may affect substantially the levels of serotonin in the brain. In the presynaptic terminal, 5-HT is stored in vesicles by a process identical to the one described for dopamine. Interaction of extracellular 5-HT with 5-HT autoreceptors regulates synthesis and release of 5-HT. Inactivation of released 5-HT is mainly attained by 5-HT reuptake through the membrane carrier-protein 5-HT transporter (SERT). Once inside the neuron, 5-HT can be reacumulated into vesicles, or inactivated by the mitochondrial enzyme MAO-B, that metabolizes 5-HT into 5-hydroxyindoleacetic acid (5-HIAA) simultaneously producing H_2O_2. Inhibition of 5-HT neuron firing activity is mediated through the somatodendritic 5-HT_{1A} autoreceptor, increasing the membrane hyperpolarization (Pineyro, 1999). Serotonin receptors represent the most complex family of neurotransmitter receptors. With the exception of the 5-HT_3 receptor, which is a ligand-gated ion channel, all 5-HT receptors belong to the G-protein-coupled receptor superfamily (Hoyer, 2002). Due to its role in antidepressant drug action, 5-HT receptors have been the target of intense research (Pineyro, 1999). These receptors have been divided, according to their primary function, in three main groups. The first group comprises the 5-HT_1-like receptors (5-HT_{1A}, 5-HT_{1B} and 5-HT_{1D}), which couple preferentially to $G_{i/o}$ proteins (pertussis toxin-sensitive) to inhibit cAMP formation (Hoyer, 2002). In the raphe nuclei, 5-HT_{1A} receptors are somatodendritic and act like autoreceptors inhibiting cell firing, while postsynaptic 5-HT_{1A} receptors, are present in

a number of limbic structures, particularly in the hippocampus (Hoyer, 2002). The 5-HT_{1A} receptor was associated with modulation of anxiety-related behaviors (Heisler, 1998) and also with locomotor activity (Carey, 2001). The 5-HT_{1B} receptors are expressed mainly in the basal ganglia, striatum, nucleus accumbens and frontal cortex, and are thought to act as terminal autoreceptors; in addition, they can also serve as terminal heteroreceptors, controlling the release of other neurotransmitters (Hoyer, 2002). The 5-HT_2-like receptor class comprises the 5-HT_{2A}, 5-HT_{2B} and 5-HT_{2C} (formerly 5-HT_{1C}) receptors, which couple preferentially to G proteins that employ protein kinase C, (Blaukat, 2000) to increase the hydrolysis of inositol phosphates and increase cytosolic calcium (Hoyer, 2002). In the CNS, the 5-HT_{2A} receptors are mainly located in the cortex, claustrum and basal ganglia, and are particularly important to the behavioral effects of drugs of abuse (Filip, 2001; Munzar, 2002; Porras, 2002). The 5-HT_{2B} and 5-HT_{2C} receptors are restricted to a few brain regions and were reported to be implicated in mediating hypo/hyperphagia, grooming frequency and anxiety behavior (Hoyer, 2002). The 5-HT_3 receptors are intrinsic ligand-gated channels, which trigger rapid depolarization due to transient inward current. The 5-HT_7 receptor, in accordance with its distribution in the limbic system and thalamocortical regions, plays a relevant role in the pathophysiology of affective and mood disorders (Vanhoenacker, 2000; Hoyer, 2002).

Accumulating evidence shows that optimal brain function relies on a complex equilibrium of neurotransmitters and respective receptors in well-organized circuitries. For most of these players, an inverted “U”-shaped curve is the best fit to describe the relation between either sub-optimal or supra-optimal levels and a specific behavioral performance (Cools, 2011; Yadid, 1998). For DA this seems to be particularly relevant. Balanced DA transmission is essential for correct modulation of the cognitive process. Deregulation of the DA function at the frontocortical level will impact on decision-making, judgment capacity, planning, working-memory, attention, cognitive flexibility, and impulsivity (Brozoski, 1979; Cai, 1997; Fischer, 2010). As such, the dopaminergic system is often targeted by pharmacologic approaches meant to improve the cognitive function in several diseases (but also in healthy

individuals). A common example is the prescription of methylphenidate, a DAT blocker, to attention-deficit hyperactivity disorder (ADHD) patients (Agay, 2010; Gamo, 2010). Likewise, most typical and atypical antipsychotic drugs display a significant affinity to dopaminergic (and serotonergic) receptors. Deficient decision-making, judgment capacity and impulse control are likely to result in increased risk-tanking and facilitate drug experimentation, binge intoxication and development of addictive behaviors (either substance or non-substance dependent).

Addiction to drugs refers to the loss of control over drug intake or the compulsivity to seek and consume drugs despite adverse effects. Addiction only occurs through repeated exposure and implies adaptations of several pathways at the molecular and cellular levels (Nestler, 2001). Behavioral adaptations in addiction result from the dysfunctional interaction of three key neural systems: an hyperactive/impulsive amygdala-striatum loop that promotes automatic and repetitive behaviors; an hypoactive/reflective PFC, impairing decision-making; and the insula-dependent decision-making processes related to uncertain risk/reward, which potentiates impulsivity and weakens the cognitive function (Evans, 2008; Noel, 2013). In addicted persons, the uncontrollable urge to obtain drugs and relapse is associated with a pathological balance between excitatory and inhibitory neurotransmission circuits, leading to persistent behavioral abnormalities (Winder, 2002).

Changes in DA balance are also implicated in the physiopathology of other psychiatric conditions, such as schizophrenia, and anxiety and mood disorders (Willner, 2005; Chaudhury, 2013; Tye, 2013). In schizophrenia, manifestation of the positive symptoms (hallucinations, delusions and psychosis) is a consequence of an hiperactive mesolimbic DA system, while hipofrontality is linked to reduced mesocortical DA function (Abi-Dargham, 2002). Although it is now accepted that DA deregulation in schizofrenia is secondary to the GABAergic dysfuntion, most therapeutic approaches target the DA and 5-HT receptors. The role of DA in anxiety disorders has long been acknowledged. Within mood disorders, DA is strongly implicated in depression-bipolar states, where the mania/depression cycling was associated with hyper/hypodopaminergic states (Cousins, 2009).

Loss of DA neurons in the SNc (and also in the VTA) and consequent reduction of dopaminergic innervation is a hallmark of Parkinson Disease (PD). As PD progresses these patients display several primary motor symptoms characteristic of decreased DA function within the basal ganglia, such as palsy (tremor), bradykinesia, muscular rigidity, plus postural instability at later stages (Moore, 2003; Samii, 2004). Cognitive decline can be seen in mild progressive stages, often accompanied by depression and anxiety (Riedel, 2010). Apathy, anhedonia, helplessness and impaired concentration were also reported. Loss of DA neurons results from either genetic or environmental causes, leading to formation of cytoplasmatic insoluble protein aggregates, resulting from mutated forms of different proteins that cannot be degraded by proteossomic activity. Although α-synuclein is the most studied of these mutated proteins, this not the most common form of mutation. These aggregates were associated with increased oxidative stress, mitochondrial dysfunction and cell death. It is noteworthy that recent studies suggest components of serotonergic, noradrenergic, cholinergic and glutamatergic disruption in PD patients (Bohnen, 2007; Kish, 2008).

Also of relevance for the present review is the role of DA in memory formation. Mesolimbic dopaminergic signaling was shown to act on memory encoding at the CA1 hippocampal region through D1 and D5 modulation of long term potentiation (LTP) (Swanson-Park, 1999) These mechanisms revealed the role VTA-mediated rewarding events in memory encoding and recalling, highlighting the relevance of DA and the reward system in associative learning, and the role of prediction and expectation in long-term memory (Rossato, 2009; Singer, 2009).

Serotonin also plays a key role in cognitive function. The relevance of the serotonergic system in learning and memory formation is well documented. While agonists on the 5-HT1 receptor were seen to impair memory retention, stimulation of the 5-HT7 receptors or blockage of 5-HT3, 5-HT4 and 5-HT6 receptors improves memory formation (Seyedabadi, 2014). Although, multiple serotonergic intracellular pathways are involved in neuronal plasticity and memory formation, 5-HT seems to act through modulation of cholinergic, dopaminergic, GABAergic or glutamatergic transmission. In accordance, in memory

impairing diseases, such as Alzheimer's disease (AD), where a profound alteration in the density of serotonergic receptors was shown, the action of serotonin seems to rely on the interplay with other neurotransmitters and second messengers relevant to memory formation.

2.4.2 Glutamate Neurotransmission and Cognition

Glutamate is a ubiquitous anionic amino acid that exists in all cell types, but in the brain it acts as a signaling molecule, being stored and released from the glutamatergic neurons subpopulation. Glutamate is considered the main excitatory neurotransmitter in the CNS, used by around half of the neurons in the brain (Fonnum, 1984; Liguz-Lecznar, 2007). Glutamate is derived from glutamine through enzymatic conversion involving phosphate-activated glutaminase (PAG) (Albrecht, 2007), and is stored in small synaptic vesicles at nerve terminals by the action of vesicular glutamate transporters 1 and 2 (VGLUT1 and 2). Following membrane depolarization and Ca^{2+} entry into cells, synaptic vesicles fuse with the plasma membrane and release the glutamate by exocytosis into the synaptic cleft. After being released, glutamate is transported back to the neuron or into the glial cells by the action of excitatory amino acid transporters (EAATs). In astrocytes, glutamate is converted to glutamine by glutamine synthase, and glutamine is transferred back to neurons, probably through the sequential action of amino acid system N and A transporters (Albrecht, 2007; Liguz-Lecznar, 2007; Lee, 2010). Once released from the presynaptic nerve terminal, glutamate binds to specific receptors in the postsynaptic membrane to conduct excitatory transmission. Pre-synaptic glutamate receptors act in the modulation of glutamate release. The effects of glutamate are mediated by activation of ionotropic or metabotropic receptors, which differ in their molecular, biochemical, physiological and pharmacological properties (Kew, 2005; Kim, 2001). The ionotropic glutamate receptors have been classified into three distinct subgroups, α.amino-3-hydroxy-5-methyl-4-isoxazolepropionic (AMPA), N-methyl-Daspartate (NMDA) and Kainate (KA) receptors (Dingledine, 1999; Mayer, 2004). AMPA and kainate receptors are responsible for most of the fast excitatory transmission in the vertebrate CNS. They are voltage-

independent ion channels and permeable to Na^+ and K^+, leading to a net depolarizing influx of cations upon activation by glutamate (Swanson, 1997). AMPA receptors are composed of four possible subunits, GluA1-4, which associate in different stoichiometries to form receptors with distinct properties (Greger, 2007). NMDA receptors are ligand-gated ion channels that exhibit strong voltage dependence owing to the blocking of the receptor channels at negative membrane potentials by extracellular magnesium. As a result, these receptors contribute little to the postsynaptic response during low-frequency synaptic activity. However, when the cell is depolarized, Mg^{2+} dissociates from its binding site within the NMDAR channel, allowing Ca^{2+} and Na^+ to enter the dendritic spine (Cull-Candy, 2001). Functional NMDA receptors are heterotrimeric complexes containing both GluN1 and GluN2 subunits (Prybylowski, 2004). Metabotropic glutamate receptors are coupled to G proteins (which in turn stimulate second messenger signaling pathways), and, as such, they mediate slower synaptic responses, occurring over seconds and minutes, rather than milliseconds as occurs for ionotropic glutamate receptors. There are three groups of mGluR, distinguished based on sequence homology, signal transduction mechanisms and agonist selectivity (Pin, 2002; Kim, 2008; Niswender, 2010).

Glutamatergic neurons play crucial roles in physiological mechanisms, such as synaptic plasticity mechanism like long-term potentiation (LTP) and long-term depression (LTD) underlying processes of learning and memory. However, disturbances in glutamatergic signaling contribute to the pathogenesis of several neurological disorders, such as ischemic stroke, schizophrenia, epilepsy and neurodegenerative disorders (Dong, 2009; Szydlowska, 2010). Neuronal excitotoxicity refers to the injury of neurons resulting from a prolonged exposure to glutamate and consequent overactivation of both ionotropic and metabotropic glutamate receptors (Choi, 1988; Kroemer, 2009). The associated sustained influx of ions into neurons, and particularly, calcium overload through NMDA receptors and calcium release from intracellular stores triggered by mGluRs is highly neurotoxic, leading to the activation of enzymes that will degrade membranes, proteins, and nucleic acids, which ultimately lead to cell death (Friedman, 2006;

Dong, 2009).

Stroke is a complex and devastating disease, constituting the second leading cause of death worldwide and the leading cause of acquired disability in adults (Brouns, 2009; Lloyd-Jones, 2009; Moskowitz, 2010). Stroke can be subdivided into ischemic and hemorrhagic (Doyle, 2008). Ischemic strokes are more frequent and are caused by a thrombosis or an embolic occlusion of a cerebral blood vessel, more frequently the middle and anterior cerebral arteries, or a general hypo-perfusion, all of which result in a constraint of blood flow to the brain, reducing the delivery of substrates, particularly oxygen and glucose, and ATP production (Dirnagl, 1999; Doyle, 2008; Lloyd-Jones, 2009). Because of its high metabolic activity, together with large concentrations of glutamate (Choi, 1992), the brain is particularly vulnerable to ischemic insults. The ischemic core is the irreversibly damaged tissue characterized by less than 20% of normal blood flow levels, reduced ATP levels and irreversible energetic failure (Lo, 2008b). Cells in the core are killed rapidly by proteolysis, lypolysis, bioenergetic failure and collapse of ion homeostasis (Doyle, 2008; Brouns, 2009). In the peripheral areas of stroke, between the normal brain and the damaged core, lies the ischemic penumbra or peri-infarct zone (Astrup, 1981). In this region, blood flow deficits are less severe, hardly sufficient to support basal ATP levels and normal ionic gradients (Moskowitz, 2010). Therefore, in the penumbra region the tissue is functionally impaired but potentially salvageable, and can be rescued by enhancing blood flow or interfering with the ischemic cascade (Lo, 2008b; 2008a; Brouns, 2009). With time, the infarct core expands into the ischemic penumbra, so, accurate detection of this tissue at risk can help to identify patients who might benefit from treatment with recombinant tissue plasminogen activator (rtPA), the only approved drug to treat acute stroke patients, by promoting clot lysis and reperfusion, therefore restore blood flow at an early time point (NINDS, 1995; Paciaroni, 2009). The decreased ATP production leads to the dysfunction of energy-dependent ion transport pumps, and to the consequent depolarization of neurons and glia (Katsura, 1994; Martin, 1994). The Na^+/K^+ ATPase at the plasma membrane of neurons maintains high K^+ and low Na^+ intracellular concentrations, that are essential for the propagation of action

potentials. After ischemia, the inhibition of ATP synthesis by mitochondria leads to a rapid ATP consumption, which causes a neuronal membrane depolarization with the release of K^+ and Na^+ entry into cells (Caplan, 2009). Energy failure also impedes the plasma membrane Ca^{2+} ATPase to maintain the very low calcium concentrations usually found within the cells (Doyle, 2008). This, together with the activation of voltage-dependent calcium channels, leads to the release of neurotransmitters, especially glutamate, which plays a critical role in the ischemic damage (Nicholls, 1990; Brouns, 2009). A large concentration gradient of glutamate is maintained across the plasma membrane by sodium-dependent glutamate transporters located at the plasma membrane, which maintain a high cytosolic glutamate concentration (approximately 10 mM) when compared to the synaptic concentration (in the micromolar range) (Hsu, 1998). Membrane depolarization during ischemia also induces a reversal of glutamate uptake carriers and enables glutamate to exit the cells along its concentration gradient, further increasing its accumulation in the extracellular space (Nicholls, 1990; Rossi, 2000). The extracellular accumulation of glutamate leads to excitotoxic stimulation of synaptic and extra-synaptic ionotropic and metabotropic glutamate receptors, which will result in neuronal dysfunction and death (Choi, 1988; Sims, 1995; Kroemer, 2009). In particular, the stimulation of AMPA and NMDA receptors causes an influx of Na^+, and NMDA receptors are also characterized by a high Ca^{2+} permeability and conductance properties (Dong, 2009). Activation of group I mGluR receptors, which includes mGluR1 and mGluR5, increases the intracellular inositol trisphospate (IP_3), thereby activating protein kinase C and releasing Ca^{2+} from neuronal stores (Pin, 2002; Friedman, 2006). Therefore, overactivation of NMDA receptors and mGluR contribute to a $[Ca^{2+}]_i$ overload. AMPA receptors are not normally calcium permeable due to the GluA2 subunit, but the downregulation of this subunit after ischemia increases the calcium permeability of these receptors, and thus allows AMPA receptors to contribute to delayed cell death (Liu, 2006; Peng, 2006).

Glutamatergic signaling and excitotoxic mechanisms play a role in chronic neurodegenerative disorders. Alzheimer's Disease is characterized by 3 neuropathological hallmarks: deposits of amyloid

beta (Aβ) peptides that form senile plaques, neurofibrillary tangles of hyperphosphorylated tau and neuronal loss (Parameshwaran, 2008). Several studies have demonstrated that excitatory synaptic transmission and plasticity are impaired in the hippocampus of AD, and particularly the glutamatergic system is altered leading to synaptic dysfunction and neurodegeneration (Parameshwaran, 2008). Several studies demonstrated that levels of VGLUTs are decreased, particularly VGLUT1, in AD patient's cortices, and that Aβ peptides accumulate preferentially in glutamatergic neurons, expressing VGLUT1/2 (Sokolow, 2012). Also, EAAT levels are reduced, indicating that glutamate reuptake from the synaptic cleft is compromised (Scott, 2011). Furthermore, glutamate synthase levels are reduced, increasing glutamate levels in astrocytes, which can be released to the synaptic cleft (Robinson, 2001). These effects may increase glutamate levels at the synapse, triggering excitotoxic mechanisms that will contribute to cell death in AD (Dong, 2009). It has been hypothesized that changes in AMPA receptors (AMPARs) number and function play an important role in AD pathogenesis. Various studies reported a downregulation of AMPARs by Aβ peptides at initial stages of the disease, probably related to a synaptic failure underlying initial cognitive impairment, prior to neuronal loss (Selkoe, 2002). This reduction of AMPARs levels contributes to loss of synaptic function at initial stages of the disease, and is related to caspase cleavage or endocytosis of the receptors triggered by Aβ peptides (Chan, 1999; Chang, 2006). Moreover, Aβ affects proteins related to AMPARs insertion and stabilization at the plasma membrane, such as post-synaptic density protein 95 (PSD-95) in a NMDA receptors (NMDARs) dependent mechanism (Roselli, 2005). Excitotoxic neuronal death triggered by excessive glutamate at the synapse and sustained Ca^{2+} influx through NMDARs is believed to be one of the major causes of neurodegeneration in AD (Harkany, 2000). Abnormal NMDARs upregulation contribute to elevated production of Aβ, resulting in increased glutamate levels and activation of NMDARs as disease progresses, indicating that NMDARs play a key role in Aβ induced neurotoxicity (Miguel-Hidalgo, 2002). It is also believed that a functional downregulation of NMDARs at initial stages of the disease is related to a compromised glutamatergic function. Aβ peptides reduce surface NMDARs levels at the synapse through endocytosis, which depresses the

glutamatergic function (Snyder, 2005). Moreover, Aβ facilitates LTD in a NMDA-dependent mechanism, indicating that in AD not only receptor levels, but also their function, is impaired and may be linked to reduced glutamatergic transmission associated with cognitive deficits (Kim, 2001).

Parkinson's disease (PD) main pathological hallmarks are the loss of dopaminergic neurons in the substantia nigra pars compacta (SNc) projecting to the corpus striatum, and the presence of cytoplasmic inclusions of protein aggregates, composed mainly of α-synuclein, and other proteins, called Lewi bodies (Betarbet, 2002). Several studies demonstrated that dopaminergic neurodegeneration in SNc is mainly associated with mitochondrial dysfunction and oxidative stress. However, it is widely accepted that glutamate excitotoxicity also plays an important role in PD pathology (Blandini, 2010). The dopaminergic denervation of the striatum associated with SNc neurodegeneration triggers changes in the basal ganglia circuitry which are associated with the motor neurological symptoms of the disease, namely tremors and rigidity (Blandini, 2000). Within the basal ganglia circuitry, glutamate neurotransmission occurs in the projections from cortical areas to the striatum and to the subthalamic nucleus (STN), and from the STN to the substantia nigra, therefore playing an important role in the mechanisms related to PD motor symptoms (Blandini, 2000). Also, glutamate excitotoxic effects may be relevant to the neurodegenerative processes occurring in PD. Dopaminergic neurons from SNc are particularly vulnerable to excitotoxicity triggered by a bioenergetic failure related to an impairment of mitochondrial function (Erecinska, 1990). Also, the presence of dopamine contributes to the sensititvity of SNc neurons to glutamate toxicity, since it may amplify glutamate signaling (Shimizu, 2003). The neurodegeneration of dopaminergic neurons in PD is associated with rearrangements in basal ganglia circuitry. In initial phases of the disease, STN glutamatergic activity may increase the dopaminergic activity of surviving neurons in SNc. However, through disease progression, persistent glutamatergic stimulation of dopaminergic neurons will be neurotoxic, further contributing to neurodegeneration (Shimo, 2009).

Schizophrenia is characterized by a dysfunction in cognitive function

that compromises daily activities, and brain regions involved in cognitive processes show glutamatergic impairment (Miller, 2000; Falkenberg, 2014; Plitman, 2014). The glutamatergic system is currently recognized to have a crucial role in the pathogenesis of schizophrenia (Coyle, 2012). It is proposed that hypofuncional NMDARs in inhibitory interneurons disinhibit pyramidal excitatory neurons, therefore increasing glutamate release (Moghaddam, 2012). The glutamate release will overstimulate non-NMDARs, particularly AMPARs, which will result in an excitotoxic effect related to structural features of the disease (Deutsch, 2001). Neuroanatomical changes were reported in patients, such as a loss of grey matter in several brain regions, like the cortex, thalamus and basal ganglia; whole brain volume reduction and white matter changes (Lawrie, 1998; Meyer-Lindenberg, 2011; Colibazzi, 2013). Various studies linked these neuroanatomical changes to neurodegeneration triggered by excitotoxic mechanisms, particularly related to decreased levels of glutamate in the thalamus, that will hypostimulate NMDARs in interneurons, leading to increased glutamate release in thalamocortical pathways (Theberge, 2007). Glutamate overstimulation of AMPARs will probably lead to excitotoxic increase of intracellular Ca^{2+} (Deutsch, 2001). Also, other studies implicated glutamate transporters EAATs in the glutamatergic dysfunction occurring in schizophrenia. Particularly, both EAAT1 and 3 where shown to be upregulated in schizophrenic patients, and the increase in EAAT3 may attenuate NMDARs function, leading to the hipo-NMDARs stimulation (Bauer, 2008; Rao, 2012).

Ataxia is a deregulation of limb movements and poor coordination of the limbs. The most common ataxias are cerebellar ataxias, which are caused by a dysfunction of the cerebellum. Spinocerebellar ataxia (SCA) is caused by an abnormal function of the part of the cerebellar cortex that receives input from the spinal cord. Usually, hereditary dominant forms of SCA are progressive and fatal neurodegenerative disorders. Seven SCAs are caused by expansion of CAG-repeats in particular genes, leading to long polyglutamine sequences in the proteins translated. These polyglutamine containing proteins aggregate and form misfolded protein deposites that constitute characteristic neuronal cytoplasmic or nuclear inclusions, which are hallmarks of SCAs and are associated with

toxicity and eventually neuronal death (Zoghbi, 2000; Ross, 2004). In a number of SCAs, disruption of dendritic Ca^{2+} spikes through stimulation of AMPARs in Purkinje cells, and subsequent downstream signaling, is associated with disease pathology (Carlson, 2009). For instance, in SCA1, mutant ataxin 1 disrupts motor function by affecting synaptic plasticity in Purkinje cells, associated with an increase in intracellular calcium levels through glutamate receptors (Clark, 1997; Serra, 2004). In SCA5, mutations in β-III spectrin contribute to the stabilization of EAAT4 at the cell surface, affecting glutamatergic signaling (Ikeda, 2006). In SCA6, there is an accumulation of mutant Ca^{2+} channels which leads to an increase in intracellular calcium (Gatchel, 2005).

Status epilepticus (SE) describes a persistent epileptic state during which epileptic seizures are unceasing and self-sustaining (Chen, 2007; Meldrum, 2007). Simultaneously with changes in inhibitory neurotransmission, AMPA and NMDARs subunits are recruited to the plasma membrane at synaptic sites, forming additional glutamate receptors, further increasing excitability (Wasterlain, 2009). For instance, GluN1 subunit translocates from extrasynaptic to synaptic sites, increasing the number of functional NMDARs at the synapse (Chen, 2007). Furthermore, changes in synaptic enzymes may also contribute to increased excitability, such as the autophosphorilation of Ca^{2+} calmodulin-dependent protein kinase II, which may increase glutamate release (Wasterlain, 1992). Seizures can also induce rapid changes in AMPARs subunit composition and function, particularly involving a decrease in GluA2 subunit, with subsequent increased Ca^{2+} permeability. This increases AMPARs mediated epileptogenesis in the hippocampus (Sanchez, 2001).

In addicted persons, the uncontrollable urge to obtain drugs and relapse is associated with a pathological function of excitatory transmission (Winder, 2002). Glutamatergic interconnections occur between amygdala, NAc and PFC (Cardinal, 2002). The NAc core receives glutamatergic input from the PFC. This pathway is associated with learned behaviors in response to stimuli-predicting relevant motivational events and is highly relevant for drug-seeking (Sellings, 2003). Increased glutamate release occurs in the NAc following drug reinstatement (McFarland, 2003). This is mainly due to adaptations in

glutamatergic signaling, promoting presynaptic glutamate and altering postsynaptic response to glutamate release. Increased release is involved in decreased presynaptic inhibitory regulation by mGluR2/3 autoreceptors (Baker, 2003). After withdrawal there is a reduction in extracellular glutamate due to loss of cystine-glutamate exchanger, which is responsible for the majority of glutamate in the synaptic cleft and maintains the tone for mGluR2/3 (Baker, 2003). Postsynaptic responses are associated with changes in intracellular signaling and trafficking of glutamate receptors to the membrane, such as the reduction of scaffolding proteins like PSD-95 and Homer (Ghasemzadeh, 2003; Yao, 2004). As an example, chronic methamphetamine (METH) involves excitotoxicity following increased glutamate release, which is thought to create oxidative stress and DA terminal degeneration, particularly in the striatum (Nash, 1992). The striatum receives glutamatergic input mainly from cortical terminals, and the corticostriatal pathway is regulated by basal ganglia circuits, particularly the GABAergic nigrothalamic and glutamatergic thalamocortical pathways (Gerfen, 1989). The striatal GABAergic projections terminate in the SN, which contains high density of DA neurons that project to the striatum (Trevitt, 2002). DA regulates GABAergic signaling in the SN and to the thalamus (Aceves, 1995; Timmerman, 1997). METH increases glutamate in the striatum through a polysynaptic pathway, characterized by an increase in striatonigral GABA transmission, which will decrease nigrothalamic GABAergic signaling, disinhibiting thalamocortical glutamatergic transmission, ultimately causing an increase in glutamatergic release in striatum via the corticostriatal pathway. The increase of glutamate in the striatum will contribute to the degeneration of DA terminals, leading to a long-term depletion of DA in this brain region (Mark, 2004). It was also shown that regulation of VGLUT1 expression and function via this polysynaptic pathway facilitates vesicular accumulation and glutamate release in the striatum after METH administration, contributing to a sustained increase in glutamatergic transmission in the corticostriatal pathway (Mark, 2007). The increase in glutamate release might lead to an overstimulation of NMDARs and consequent oxidative stress contributing to neuronal damage (Gunasekar, 1995).

Recent evidence also shows that glutamatergic inputs, especially those contacting GABAergic PV-positive interneurons, may be developmentally regulated by repeated exposure to cannabinoids, which can result in disruption of the glutamatergic facilitation of PV-positive interneuron function and underlie the cognitive impairments seen in young adults that chronically abuse these drugs. Overstimulation of the CB1 receptor over the adolescent developmental period seems to alter frontal circuits leading to a schizophrenic-like disorder (Caballero, 2012).

2.4.3 GABAergic Neurotransmission and Cognition

The amino acid γ-aminobutyric (GABA) is the main inhibitory neurotransmitter in the CNS. This neurotransmitter is synthesized by the enzyme glutamic acid descarboxylase (GAD) which catalyzes the decarboxylation of glutamate. Although the expression of GABA in the nervous system was first described in 1950 by Eugene Roberts and Jorge Awapara, it was only accepted as a neurotransmitter more than 10 years later (Roberts, 1950; Del Castillo, 1964). The difficulty in the identification of GABA as a neurotransmitter came from its enormous abundance in the vertebrate brain (which is about 1000 fold higher than monoamine transmitters), its simple structure and its role in the Krebs cycle, suggesting that it was likely more involved in metabolism than in intercellular signaling (Schuske, 2004). Cellular release of GABA may be mediated by several different mechanisms (Saransaari, 1992): (1) GABA can be released from neurons by exocytosis through synaptic vesicles, which is the most common mechanism of GABA release under physiological conditions; (2) it may simply leak through plasma membranes; (3) the plasma membrane GABA transporters-GAT may be reversed (due to changes in the electrochemical gradients); and (4) finally, ion channels in the membranes may also mediate GABA release despite the size of this molecule. The release of GABA in extra-synaptic sites activates non-synaptic GABAA receptors to generate tonic inhibitory currents. These synaptic and extra-synaptic modes of GABA action have been termed phasic and tonic effects, respectively, and control neuronal excitability in a different manner (Mody, 2004; Farrant,

2005). Inhibitory neurotransmission in the adult nervous system is primarily mediated by the exocytosis of synaptic vesicles containing GABA and glycine. GABAergic inhibition predominates in the brain, whereas both glycine and GABA act as the primary inhibitory neurotransmitter in the spinal cord and brainstem. To date, a single vesicular transporter was identified for the filling of synaptic vesicles at both GABAergic and glycinergic synapses; it is referred to as vesicular GABA transporter (VGAT) (McIntire, 1997) or vesicular inhibitory amino acid transporter (VIAAT) (Sagne, 1997). GABA exerts its effects through three types of receptors, named GABAA, GABAB and GABAC receptors. The different GABA receptor subtypes were originally characterized based on their pharmacological properties. GABAA receptors are ionotropic chloride channels, the activation of GABAA receptors allows the influx of chloride, hyperpolarizing the membrane and decreasing the excitability of the cell. The chloride homeostasis in neurons is determined by two major transporters, the Na^+-K^+-Cl^- co-transporter, NKCC1 (a Cl^- accumulator), and the K^+-Cl^- co-transporter KCC2 (a Cl-exporter) (Ben-Ari, 2002; Owens, 2002). During embryonic development and maturation, neurons downregulate NKCC1 expression and upregulate KCC2 expression, resulting in a lower $[Cl^-]i$ in most mature neurons. The fast inhibitory actions of GABA are mediated by the activation of GABAA receptors in the brain. A similar role is played by GABAC receptors in the retina. GABAB receptors are metabotropic receptors that address second messenger systems through the binding and activation of guanine nucleotide-binding proteins (G proteins) (Campbell, 1993). GABAB receptors predominantly couple to Giα- and Goα-type G proteins (Pinard, 2010). It is now well established that presynaptic GABAB receptors repress Ca^{2+} influx by inhibiting Ca^{2+} channels in a membrane-delimited manner via the Gβγ subunits. Postsynaptic GABAB receptors trigger the opening of K^+ channels, again through the Gβγ subunits (Bettler, 2006). This results in a hyperpolarization of the postsynaptic neuron (Luscher, 1997). Besides modulating ion channels through Gβγ, GABAB receptors activate and inhibit adenylyl cyclase via the Giα/Goα and Gβγ subunits. Presynaptic GABAB receptors are subdivided into those that control GABA release (autoreceptors) and those that inhibit all other neurotransmitter release

(heteroreceptors).

The activity of GABAergic synapses is important in the regulation of the overall activity of neuronal networks, refraining the effect of excitatory activity. In the cerebral cortex there are two main classes of neurons, pyramidal cells and interneurons, which use glutamate and GABA as main neurotransmitters, respectively. Interneurons comprise 20–30% of the 15 cortical neuronal population and are locally projecting cells that control and synchronize the output of pyramidal neurons. Increasing evidence suggests that disruption of the excitatory-inhibitory (E/I) balance maintained by pyramidal cells and interneurons is linked to the etiology of multiple neuropsychiatric and ischemic conditions (Obrenovitch, 2008; Wang, 2011).

Research in cerebral ischemia and excitotoxic neuronal damage has been mainly focused on the excitatory mediators and much less attention was given to the changes in GABAergic activity (Schwartz-Bloom, 2001). The release of GABA in the ischemic brain and the consequent activation of GABAA receptors may be neuroprotective through reduction of membrane depolarization. Although Cl^- entry through GABAA receptors in association with overactivation of glutamate receptors may further increase the influx of water and cell swelling, strategies to increase GABAergic neurotransmission, targeting both sides of the synapse, have been quite efficient in models of ischemia (Schwartz-Bloom, 2001). The impairment of GABAergic synaptic transmission in brain ischemia is partly due to a down-regulation of synaptic GABAA receptors, which may contribute to the ongoing neuronal excitability and possibly to neuronal death (Schwartz-Bloom, 2001). Furthermore, exposure of hippocampal slices to oxygen- and glucose-deprivation was shown to induce an early release of GABA by exocytosis, followed by a delayed phase of neurotransmitter release mediated by reversal of the plasma membrane transporter (Allen, 2004).

The balance between excitation and inhibition is an important mechanism in epilepsy, with inhibitory GABAergic regulation considered the main disorder in epilepsy (Schindler, 2008). Evidence shows that during the onset of the status epilepticus endocytosis of $GABA_A$ receptors takes place in the hippocampus, decreasing post-

synaptic inhibitory currents (Naylor, 2005a; 2005b). Glutamate and GABA transporters also affect the excitatory/inhibitory balance. Glutamate transporter activity could trigger GAT reversal activity in astrocytes, by elevating intracellular Na^+ levels. This glutamate/GABA exchange may act as a negative feedback to decrease excitation during seizures (Gadea, 2001; Heja, 2012; Wu, 2014). Neuronal network remodeling is also common in epilepsy, including loss of GABAergic interneurons and axon growth. The formation of new excitatory circuits may increase seizure intensity (Wu, 2014). Excitatory/inhibitory balance determination shows that inhibition superimposes on pyramidal neurons during initiation of seizure events, shifts towards excitation during seizures propagation, and eventually inhibition dominates again during seizures termination (Murase, 2014). The mechanism underlying such homeostatic changes involves increased glutamate signaling and alterations in GABAergic transmission, related to suppressed presynaptic inhibition and GABAergic vesicular depletion (Trevelyan, 2013).

Several evidences show that GABA plays an important role in the cognitive features of schizophrenia (Gonzalez-Burgos, 2011). Most domains of GABAergic signaling are altered in schizophrenic patients, such as in the expression of GAD 65 and 67, and GAT1 (Pierri, 1999; Hashimoto, 2008). Alterations in $GABA_A$ receptors were also described in schizophrenic patients (Fatemi, 2013), depending on the targeted brain region, GABAergic transmission can be either increased or decreased (Deidda, 2014). The imbalance of excitatory/inhibitory stimulus has also been linked to schizophrenia-related GABAergic dysfunction (Lewis, 2005). Hypofunction of NMDARs and increased D2 activity leads to hypostimulation of GABAergic neurons (Lewis, 2005). GABAergic excitatory/inhibitory imbalance and consequent discoordination between GABAergic and glutamatergic firing are critical to the cortical abnormal function and consequent cognitive deficits reported in schizophrenia (Sullivan, 2012).

In AD, dysfunction of the GABAergic system may also play a role in the cognitive impairment associated with this disease. It has been shown that AD patients display lower GABA levels in the brain and in cerebrospinal fluid (CSF) (Grouselle, 1998). Although the AD

pathogenesis is usually associated with glutamate release, altered GABAergic transmission also contributes to disrupting the excitatory/inhibitory balance and leads to impaired cognitive function in AD patients (Rissman, 2011). Several studies have shown that GABAergic neurons as well as GABAergic receptors appear to be resistant to neurodegeneration in AD, particularly in the hippocampus, despite the mild reduction reported for $GABA_A$ receptor subunits α1, α5 and β3 (Reinikainen, 1988; Marczynski, 1998). It was then proposed that Aβ decreases pyramidal cell firing, while in these neurons GABA receptors remain unaffected, which will decrease the activation of downstream GABAergic neurons resulting in decreased inhibition of excitatory neurons (Kamenetz, 2003). Increased Aβ may also elicit seizures in the cortex and hippocampus of AD, possibly related to enhanced GABA activity in the dentate gyrus and aberrant excitatory/inhibitory balance (Minkeviciene, 2009; Palop, 2010). It was proposed that Aβ decreases pyramidal cell firing, while in these neurons GABA receptors remain unaffected. This will decrease the activation of downstream GABAergic neurons, which will decrease the inhibition to the inhibitory neurons they innervate, with a resultant decreased inhibition of excitatory neurons (Kamenetz, Tomita et al. 2003).

In Parkinson's disease, the degeneration of dopaminergic neurons in the Substancia Nigra (SN) and consequent loss of dopamine (DA) leads to alteration in the neurotransmitters in the basal ganglia circuitry, particularly an imbalance in glutamate and GABA neurotransmission in the nigrostriatal pathway, resulting in an increased excitation in the SN promoting excitotoxicity and cell death (Ampe, Massie et al. 2007; Calabresi, Picconi et al. 2007; Walker, Moore et al. 2012). There are strong evidences showing a strong interaction between GABA, glutamate and dopamine in the basal ganglia. For instance, the DA input to the striatum affects glutamate and GABA transmission and DA modulates glutamate costicostriatal signalling to GABAergic medium spiny neurons (Kalivas and Duffy 1997; Surmeier, Ding et al. 2007).

Dysfunctional GABAergic transmission is also present in the altered function of the reward system that underlies the addictive behavior. Exposure to psychoactive substances may affect the GABAergic system either directly, as in the case of alcohol abuse, or indirectly as in chronic

exposure to psychostimulants or depressants. Acute exposure to alcohol increases the inhibitory effect of $GABA_A$ receptors, and enhances other inhibitory modulators, decreasing also the function of excitatory neurotransmitters. Prolonged drinking has the opposite effect, decreasing $GABA_A$ receptor function by decreasing receptor levels or altering protein composition. Behaviorally, acute alcohol consumption translates into a range of dose-dependent effects that scale from social disinhibition to impaired motor control and decision-making, followed by mild to severe ataxia and hippocampal dysfunction, and eventually ending in sedation, coma, cardiac arrest and possible death (Tabakoff, 1996; 2013; Gilpin, 2008; Koob, 2014). Chronic exposure to alcohol leads to compensatory adaptations in the reward circuitry, and to the development of alcohol related behaviors, such as tolerance, meaning that over time one will need a higher alcohol dose to obtain the same reward effect. When alcohol consumption is discontinued, several withdrawal effects occur, such as tremors, seizures, insomnia, and confusion. These effects may result from the hyperactive adaptations suffered under prolonged use that are no longer balanced by the alcohol inhibitory effects. The consequent increase in glutamatergic activity may lead to toxicity and cell death (Becker, 2014). Under chronic exposure to psychostimulants the GABAergic component of the reward system is also deeply affected, reduced GABAergic inhibition of the dopaminergic mesocorticolimbic pathway contributes to addiction by disrupting the frontal cortical circuits that regulate motivation, drive, and self-control, increasing the motivational salience of drug-associated stimuli (Volkow, 2002)

2.4.4 Gliotransmitters

The communication between neurons and astrocytes through the so-called gliotransmitters (such as glutamate, ATP, and D-serine) has been shown to modulate synaptic transmission and plasticity through several mechanisms (Araque, 2014). Research in the field of neuron-glia interactions has gained relevance over the last few years, revealing the involvement of astrocytes and microglia directly at the synaptic cleft. It is now clear that astrocytes and microglia play multiple roles in brain

circuitries, modulating signaling in many pathways. While recent studies show that some of this processes seems to be age-related and, therefore, transitory in nature (Sun, 2013), others were shown to directly modulate learning and behavior. Astrocyte signaling and gliotransmission were proposed to represent a highly evolved integrative interface in brain communication, coupling slow modulatory signaling from different sources with fast synaptic transmission, contributing to fine tuning the synaptic circuitry according to the environmental surroundings (Araque, 2014). Microglia, rather than just reacting to injury or infection, were shown to prune synapses by monitoring synaptic transmission (Schafer, 2013), refining neuronal circuitry and improving plasticity (Salter, 2014). In the hippocampus (and other brain regions), microglia is required for proper function of the glutamatergic synapses. Several studies have shown that local microglia-derived BDNF is essential for long term potentiation and depression, which represents a critical mechanism for memory and learning (Salter, 2014). In accordance, with increasing data revealing the role of microglia in many neuronal disorders, such as AD, PD, mood disorders, neurophatic pain or addictive behavior. Knowledge gained in this field is expected to contribute to the design of new therapeutic approaches in most of such conditions.

2.4.5 Cognitive Enhancement

Currently, a number of pharmacologic approaches traditionally used to treat medical conditions are used as cognitive enhancers. Medications for ADHD and narcolepsy targeting the monoaminergic systems, such as methylphenidate and modafinil, were found to provide acute enhancing effects to healthy individuals (Repantis, 2010). Other compounds, like donepezil, rivastigmine or galantamine are drugs that improve cognition targeting the cholinergic system. As discussed for dopamine, the effect of cholinergic enhancers is usually represented by a bell shaped curve, meaning that excessive doses may reduce the cognitive performance (Balsters, 2011). The long-term consequences of such drugs remain mostly unexplored and its regulation is yet to be addressed by policymakers and other stakeholders. Their use is particularly

widespread the United States mainly on college campuses, reaching a life-time use as high as 30%. Data regarding the prevalence of such practice in Europe is yet scarce, but seems to be lower than in the US (Franke, 2011; Ragan, 2013). While a sporadic use of such drugs is likely to be safe, repeated exposure may lead to deleterious consequences, possibly by reshaping the neuronal circuits of the PFC, and affecting the control of goal-directed behaviors, decision making and risk-assessment (Lee, 2009).

Neuromodulation methodologies employing invasive and non-invasive procedures can also be used as possible enhancers. Deep brain stimulation (DBS) is a reversible surgical procedure that implies the insertion of small electrodes in specific brain regions. DBS has been reported to improve memory functions, but simultaneous changes in personality were also reported (Glannon, 2009). Non-invasive brain stimulation is yet mostly experimental. Transcranial stimulation (electric or magnetic) has been shown to enhance several functions, such as sensorimotor skills, memory, attention, and problem solving (Nitsche, 2008). While possible benefits may last up to several months (Cohen Kadosh, 2010; Iuculano, 2014), recent evidence shows that enhancing one ability might temporarily decrease another (Iuculano, 2013).

Biofeedback, or neurofeedback, combines readings of brain activity in the form of EEG, with operant conditioning methodologies. This technique is based on the specificity of EEG patterns for different emotional states or cognitive tasks. Individuals are then trained to produce desired brain states by visual and auditory feedback, modulating in real-time their neural activity (Dornhege, 2006; Muller, 2008; Ziemann, 2008). These brain-computer interfaces are increasingly used for a wide range of enhancement purposes, such as meditation training, computer usability research, improved cognitive performance, creativity and impulse control. All these tools act through modulation of neurotransmitters' release, leading to acute and long term reshaping of the neural circuitries.

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Section 3: **Strategies to Develop New and Better Drugs**

Nuno Vale*

3.1 Amino Acids and Peptides in Medicine: Old or New Drugs?

3.1.1 Introduction

3.1.1.1 Amino acids: biological and chemical concepts

Amino acids (AA) are molecules containing an amine group, a carboxylic acid group and a side-chain that varies between different amino acids. AA are an important class of cell signalling molecules, involved in the regulation of gene expression and the protein phosphorylation cascade, as well precursors of hormone synthesis and low-molecular nitrogenous substances (Wu, 2009). The 20 natural AA are commonly found in proteins and are also referred to as alpha amino acids (Fig. 3.1.1). The α-amino acids differ in the nature of the side-chain (R group) attached to their α-carbon, which can vary in size from just one hydrogen atom in glycine to a large heterocyclic group in tryptophan. α-Amino acids are typically divided into three categories, based on the properties of their side-chain. The first category contains α-amino acids with relatively nonpolar R groups (glycine, alanine, valine, leucine, isoleucine, proline, phenylalanine, tryptophan, and methionine), the second contains α-amino acids with uncharged but polar R groups (serine, threonine, cysteine, tyrosine, asparagine, glutamine), and the third contains α-amino acids with charged R groups (aspartic acid, glutamic acid, lysine, arginine and histidine) (Vig, 2013).

Proteins are key players in many vital processes in living organisms. They transport substances, catalyze chemical reactions, pump ions or recognize signalling molecules. The complexity and variety of proteins is tremendous: in the human body alone, there are more than 100,000 different proteins at work. But almost all of them are made up of just twenty different AA. Only a few highly specialized proteins additionally contain selenocysteine (Sec, Fig. 3.1.2), the very rare 21st amino acid

discovered in 1986. Sec is identical to cysteine (Cys), except that it contains selenium instead of sulphur. Selenocysteine is recognized as the 21st amino acid in ribosome-mediated protein synthesis and its specific incorporation is directed by the UGA codon (Stadtman, 1996).

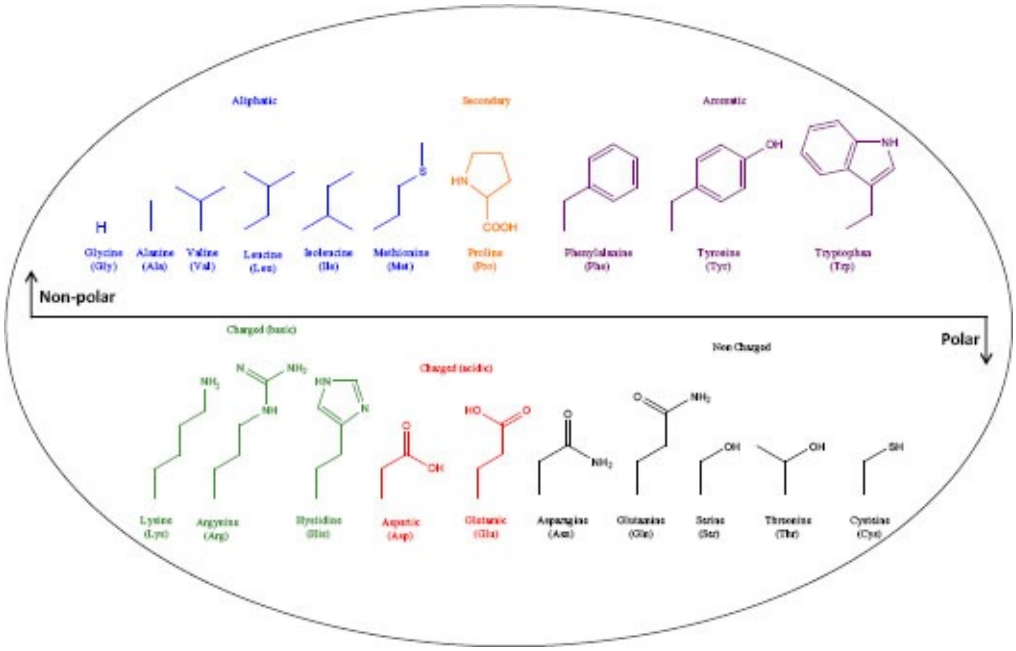
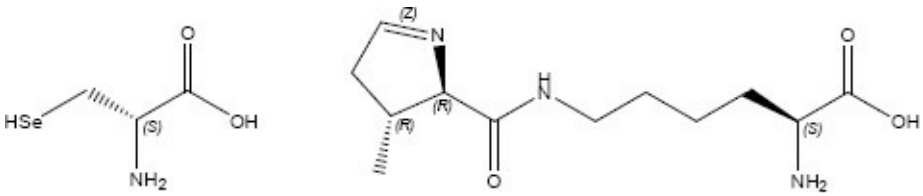


Figure 3.1.1: Structures of natural amino acids.



L-Selenocysteine (Sec)

L-Pyrrolysine (Pyl)

Figure 3.1.2: Structures of selenocysteine and pyrrolysine.

Selenium is in the same family below sulfur in the periodic table and the two elements share many similar chemical properties. Sec has a distinct functional advantage because the selenol group is more fully ionized than the thiol group of Cys at physiological pH (biosynthesis of Sec and de novo synthesis of Cys can be observed in Fig. 3.1.3). When Sec is replaced with Cys, the catalytic activity of a selenoenzyme is drastically reduced (Stadtman, 2000).

A big surprise was the discovery of a 22nd amino acid in methane-producing archaea of the family *Methanosarcinaceae* in 2002, pyrrolysine (Pyl, Fig. 3.1.2). It is genetically encoded in a similar manner

as that of selenocysteine and the other twenty amino acids. The archaea use this unusual amino acid in proteins required for energy conversion. Pyrrolysine is located in the catalytic center of the proteins carrying it and is essential for their function. The energy generation process of the archaebacteria would not work without pyrrolysine (Srinivasan, 2002). Biosynthesis of this amino acid is based on the radical *S*-adenosyl-L-methionine (SAM) protein PylB, which mediates a lysine mutase reaction and produces 3-methylornithine, which is then ligated to a second molecule of lysine by PylC before oxidation by PylD to produce pyrrolysine (Gaston, 2011). In addition, Sec and Pyl are often referred as the natural amino acids.

Among more than 300 AA in nature, only 20 of them (α-AA) serve as building blocks of proteins. However, represented in Fig. 3.1.4 are non-protein α-AA (e.g. ornithine, citrulline, and homo-cysteine) and non-α AA (e.g. taurine and β-alanine), which also play important roles in cell metabolism (Curis, 2007; Perta-Kajan, 2007; Manna, 2009).

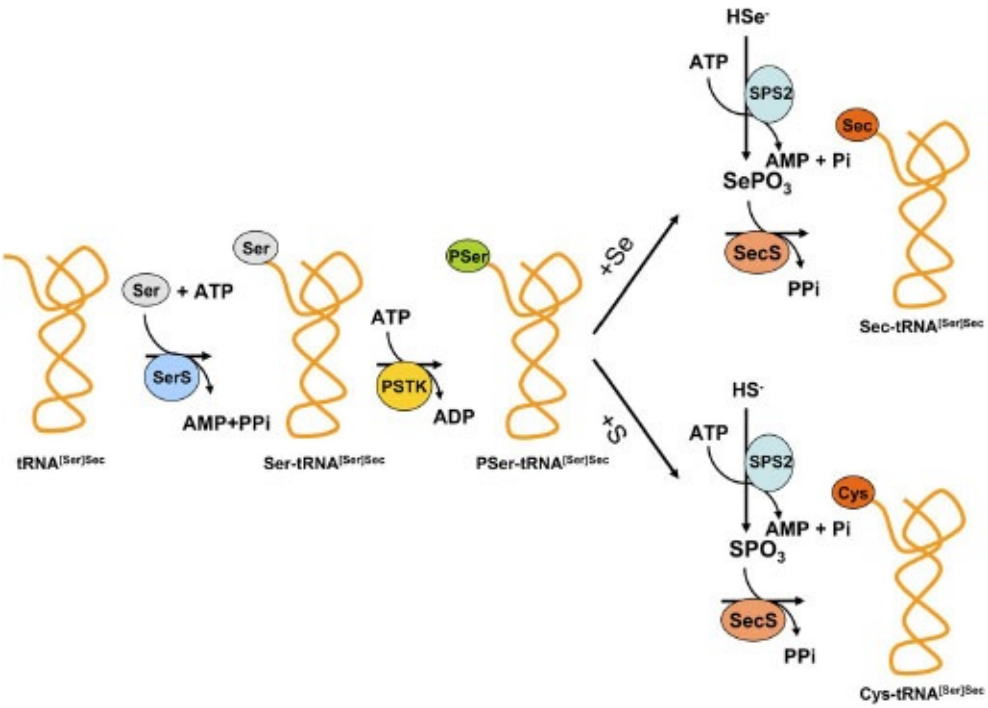


Figure 3.1.3: Biosynthesis of Sec and de novo synthesis of Cys. The complete synthesis of Sec on its tRNA in eukaryotes and archaea uses selenite and ATP are substrates for SPS2, yielding selenophosphate. This interacts with SecS and the intermediate, likely dehydroalanine, is used to generate SectRNA$^{[Ser]Sec}$ (shown on the right in the upper pathway). In the lower pathway, the de novo synthesis of Cys is shown in mammals, where sulfide and ATP are substrates for SPS2 and yield thiophosphate, which interacts with SecS and yields Cys-tRNA$^{[Ser]Se}$ from the SecS

intermediate (once again, likely dehydroalanine) (adapted from Turanov, 2011).

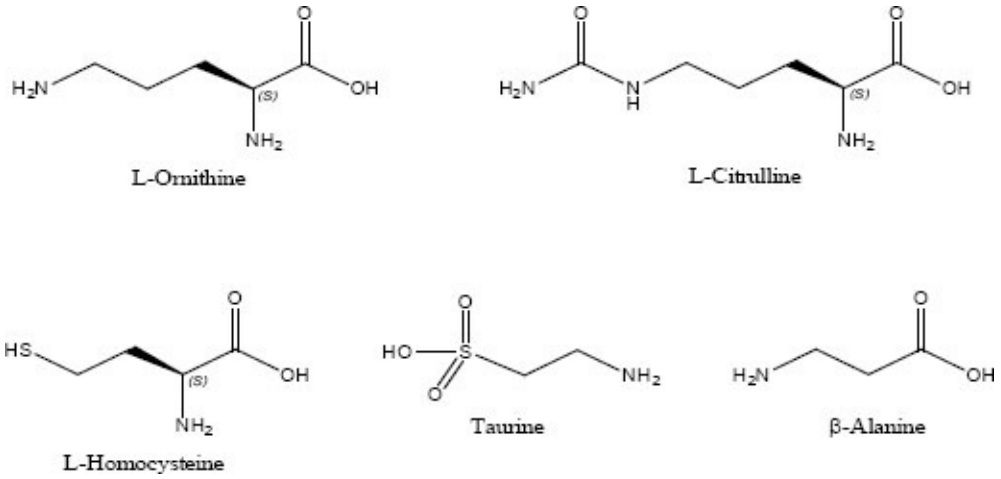


Figure 3.1.4: Structures of non-protein α-AA (ornithine, citrulline, homo-cysteine) and non-α-AA (taurine, β-alanine).

The catabolism of several AA exhibit a number of common characteristics throughout different organisms. Important metabolites of AA include ammonia, CO_2, long-chain and short-chain fatty acids, glucose, H_2S, ketone bodies, nitric oxide (NO), urea, uric acid, poly-amines, and other nitrogenous substances with enormous biological importance. Complete oxidation of AA carbons occurs only if their carbons are ultimately converted to acetyl-CoA, which is oxidized to CO_2 and H_2O via the Krebs cycle and the mitochondrial electron transport system (Wu, 2009).

Biotransformation of amino acids into bioactive molecules can be essential in biomedical considerations, such as the hydroxylation of Tyr into L-Dopa (hihydroxyphenylalanine) followed by another decarboxylation to produce dopamine in the brain (Youdin, 2006). Histamine is a small molecule derived from the decarboxylation of the amino acid histidine (Haas, 2008). Trp is processed to form serotonin, a neurotransmissor that is linked to depression at low levels (Oxenkrug, 2013). After the action of serine dehydratase, Ser is converted into aminoacrylate that tautomerizes to the imine which then hydrolyzes into pyruvate and ammonia (Birolo, 1995). Arg is hydrolysed to urea and ornithine by arginase before it can be used for the synthesis of polyamines or Pro (Grillo & Colombatto, 2004). Arg is also associated with Glu formation, as well His. Met reacts with ATP to form S-

adenosylmethionine (Lu & Mato, 2012). Deriving from Cys, taurine (2-aminoethanesulfonic acid) is the most abundant amino acid in mammals. Being widely distributed in the CNS, second only to glutamate in concentration, its concentration differs depending on the regions of the brain, brain activity and animal species studied. It also presents different functions, which have been studied for their potential in neurology as a trophic factor in brain development, regulating calcium transport, in the integrity of the eardrum, as an osmoregulator, neurotransmitter and neuromodulator as well as for its neuroprotective action (Wu & Prentice, 2010).

3.1.2 Amino Acids and Drug Development

All α-amino acids except glycine have a chiral α-carbon and can exist in two optical isomers, L-form and D-form. The L-form of amino acids occurs naturally and prodrugs utilizing these amino acids are generally activated by naturally occurring enzymes. Both L- and D-amino acid prodrugs tend to have very similar physicochemical properties but the latter is generally more stable to hydrolysis by naturally occurring enzymes (Vig, 2003). This property of amino acids are often utilized by medicinal chemists to develop stable amino acid prodrugs. In addition to the natural amino acids and their D-forms, extensive arrays of synthetic amino acids and di-/tri-peptides are commercially available for medicinal chemists as promoieties (Ma, 2003).

3.1.2.1 Rationale for Drug Design

Amino acids can be associated with drugs as a component of prodrug development. This association depends on the purpose of the prodrug, type of functional groups available on the parent drug, chemical and enzymatic conversion mechanisms of the prodrug to the parent drug and safety of the promoiety. Amino acids as promoieties ease manufacturing and offer several other advantages (Table 3.1.1; Vig, 2013).

Most amino acid prodrugs are either esters or amides, in which the α-

amine or α-carboxylic group of an amino acid is attached to parent functional groups (hydroxyl, amine, and carboxyl). α-Amine or α-carboxylic groups of amino acid can also be linked to the parent drug via carbonate and carbamate links. The use of amino acid side chains, which offer wealth of functional groups (e.g. amine, carboxylic acid, alcohol, thiol) as prodrug handles presents tremendous opportunities in prodrug design. While in most cases amino acids are directly conjugated to the parent drugs, bifunctional linkers have been used to further increase the structural diversity and types of parent drugs that could be linked to amino acids (Gupta, 2009; Cao, 2012).

Table 3.1.1: Advantages of using amino acids in drug development (adapted from Vig, 2013).

Advantage
• Large structural diversity
• Wide-range of functional groups for attachment to parent drug
• α-Amine and α-carboxylic group
• Well established prodrug chemistry
• Commercial availability
• Fewer safety concerns
• Substrates for various intestinal influx transporters
• Availability of commercially successful amino acid prodrugs
• Used to improve pharmaceutical properties of marketed drugs or difficult compounds
Rationale
• 20 common natural amino acids
• A number of other naturally occurring amino acids

- Extensive array of synthetic amino acids
- α-Amine and α-carboxylic groups
- Side chain functional groups e.g. amine, carboxylic acid, alcohol, and thiol
- Commonly used linkage chemistry between amino acid and parent including ester, amide, carbonate and carbamate bonds
- Large number of amino acid suppliers available
- Amino acids are building blocks for proteins and are generally regarded as safe
- Can potentially target carrier-mediated transporters for improved delivery across cell membranes
- Many amino acid prodrugs have been commercially developed and help patients
- Commercial and regulatory precedence in developing amino acid prodrugs
- Improved pharmaceutical properties of already marketed compounds (e.g. valacyclovir, valganciclovir) and new chemical entities (e.g. brivanib alaninate, LY354740)
- Proven to improve solubility, permeability, sustained release, targeting transporter, overcoming resistance.

Introducing an amino acid, either a natural or its derivative, to a parent drug usually increases the water solubility by several orders of magnitude through an ionized carboxylate anion or ammonium cation. Consequently, there are several amino acid ester prodrugs that are investigated as water soluble derivatives for oral administration (Chan, 1998; Mulholland, 2001; Gingrich, 2003; Marathe, 2009). Amino acid esters (Bundgaard, 1984) amino acid amides (Pochopin, 1995; Bradshaw, 2002; Mittal, 2007; Rasheed, 2011) or other amino acid examples like amidoximes (Kotthaus, 2011) or ureas (Hemenway, 2010) have been investigated for parenteral use, albeit to a lesser extent. In proline, the side-chain links to the α-amino group, meaning it is the only

amino acid containing a secondary amine at this position. The pKa of the α-amino group is typically in the range of 8.8 to 10.6 and the acidic α-carboxyl group has a pKa typically between 1.7 and 2.6. The dissociation constant of both the α-amino and α-carboxyl groups are affected by each other and the side chain. The α-amino group in its charged state is strongly electron withdrawing and makes the α-carboxyl group more acidic, thereby lowering its pKa as compared with the typical carboxylic acid pKa of approximately 4.5. At physiological pH, the predominant form of α-amino acids contains both a negative carboxylate and a positive ammonium group. This molecular state is known as a zwitterion and has minimum solubility at the isoelectric point (mid-point between 2 pKa). However, in most α-amino acid prodrugs either the carboxylic acid or amine group is linked to a parent drug, abolishing the zwitterionic form. Exceptions are α-amino acids that have an additional functional group in the side chain amenable to prodrug formation (e.g. -SH in Cys, -OH in Tyr, -NH_2 in Lys, -COOH in Asp and Glu) which consequently can maintain a double charge. Water solubility of α-amino acids themselves is largely a function of the polar or nonpolar nature of the side chain. An increase in hydrocarbon content of the side chain (R group) from Gly to Val or Leu decreases water solubility. Consequently, amino acids with shorter hydrocarbon side chains resulted in higher aqueous solubility versus amino acids with longer hydrocarbon side chains for a series of α-amino acid amide prodrugs of dapsone (Fig. 3.1.5, Pochopin, 1995).

Moreover, α-amino acids with uncharged but polar R groups are generally more soluble than the corresponding α-amino acids having nonpolar R groups. This trend is in line with the observations from several studies, where phenylalanine as a promoiety resulted in the least soluble prodrug in a series of amino acid prodrugs (Pochopin, 1995; Rautio, 1999). Among the α-amino acids with ionizable R groups, Lys renders relatively high water solubility over the wide pH range due to the ionized ε-amine regardless of prodrug type (e.g. ester or amide) (Pochopin, 1995; Nam, 2003).

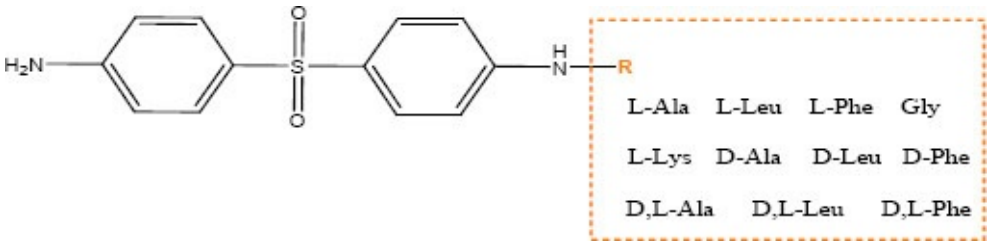


Figure 3.1.5: Structures of dapsone (R=H) and prodrugs with amino acids.

Amino acid prodrugs are expected to pose synthetic challenges similar to other prodrugs. Amino acid prodrug linkage chemistry is well-established, therefore many of the synthetic challenges will depend on the number and type of protecting groups required to mask the active groups on the amino acid and parent drug. Furthermore, all amino acids except glycine introduce a stereocenter to the prodrug. Like other prodrugs, amino acid prodrugs increase the molecular weight of the parent, requiring mindfulness of the increase in dose due to the "non-active" contribution of the prodrug relative to the bioavailability enhancement with prodrug (Vig, 2013).

3.1.2.2 Amino Acid Prodrug in Drug Delivery

Many drugs suffer from an extensive first-pass metabolism leading to drug inactivation and/or production of toxic metabolites, which makes them attractive targets for prodrug design. The classical prodrug approach, which involves enzyme-sensitive covalent linkage between the parent drug and a carrier moiety, is a well-established strategy to overcome bioavailability/toxicity issues. However, the development of prodrugs that can regenerate the parent drug through non-enzymatic pathways has emerged as an alternative approach in which prodrug activation is not influenced by inter- and intra-individual variability that affects enzymatic activity. As discussed above, amino acids are excellent promoieties to increase the aqueous solubility of the parent drug. To increase the dissolution rate further, amino acid prodrugs can be converted to their salt forms. In the following sections, we will present some examples of commercial as well as investigational amino acid prodrugs that have been designed to improve 1) oral bioavailability, 2) sustained drug delivery, 3) intravenous drug delivery, 4) to target drugs to their site of action and 5) improve enzymatic stability.

Prodrug strategies have arguably been successful for a number of clinically-used therapeutic agents. However, prodrug research encounters various challenges and requires additional work in preclinical and clinical settings, much of which can be attributed to understanding the bioconversion mechanisms of prodrugs. Many enzymes involved in prodrug activation are subject to interindividual variabilities in their activities. The main factors contributing to this variability are intrinsic, especially polymorphisms in the genes encoding the enzymes, but can also be extrinsic (i.e. interactions caused by other drugs and xenobiotics). Both intrinsic and extrinsic factors may cause insufficient or excessive conversion of the prodrugs into their active forms. Moreover, interspecies differences in enzyme activation represent another hurdle to the prediction of human disposition of certain prodrugs (Hutunen, 2011).

The enzymatic hydrolysis of amino acid esters and amides by various hydrolases (e.g. esterases and/or peptidases) is far more effective than chemical hydrolysis. The half-lives of prodrugs are usually several orders of magnitude shorter in blood or tissue homogenates than in aqueous solutions. Often the activating enzymes are unidentified. However, it has been suggested that the biphenyl hydrolase-like protein human valacyclovirase (VACVase) is at least partly responsible for hydrolysis of the prodrugs valacyclovir and valganciclovir (Fig. 3.1.6) and might be involved in the activation of other amino acid prodrugs as well (Kim, 2003; 2004; Lai, 2004). As the specificity of this enzyme resides mainly in the amino acid acyl promoiety (and to a lesser extent in the alcohol moiety of a parent drug) and that the α-amino group in the substrate is important for activity, the enzyme can be better defined as an α-amino acid ester prodrug-activating enzyme (Burnette, 1995; Lai, 2004). VACVase prefers small, hydrophobic (valine, proline) or aromatic side chains (phenylalanine) over the charged amino acids (lysine, aspartic acid). It has shown clear stereoselectivity for L-valine over D-valine prodrugs irrespective of the parent drug while exhibiting comparable hydrolytic activity toward D-phenylalanine and L-phenylalanine esters, as indicated in a study of various nucleoside analogue prodrugs (Kim, 2004). The success of valacyclovir and valganciclovir has proved that an L-valine prodrug approach is a very

efficient option to improve the oral bioavailability by utilizing amino acid transporters. Therefore, there are several other L-valyl ester prodrugs, including nucleoside analogues levovirin valinate, valopicitabine, and valtorcitabine currently undergoing clinical evaluation.

L-Valyl ester prodrugs are probably the first commercial examples of amino acid prodrugs that utilize intestinal transporters to increase the permeability and consequently poor oral bioavailability of their parent drugs. Valacyclovir (Valtrex®) was the pioneer of L-valyl ester prodrugs, which achieved 3-5-times higher oral bioavailability (> 60%) (Soul-Lawton, 1995) than that of its parent drug, acyclovir (10–20%) (de Miranda and Blum, 1983). Valacyclovir is absorbed by the PepT1 and $ATB^{0,+}$ transporters and is then rapidly hydrolyzed to acyclovir, predominantly by biphenyl hydrolase-like protein (valacyclovirase) (Kim, 2003; Hatanaka, 2004). Finally, acyclovir triphosphate acts as an antiherpetic agent by inhibiting viral DNA replication. Soon, after the discovery of valacyclovir, valganciclovir (Valcyte®) was designed. Valganciclovir is also absorbed by the PepT1 and $ATB^{0,+}$ transporters then bioactivated by valacyclovirase (Kim, 2003; Umapathy, 2004). The oral bioavailability of ganciclovir is approximately 60%, which is almost 10-times higher than that from oral ganciclovir (6–8%) (Jung & Dorr, 1999).

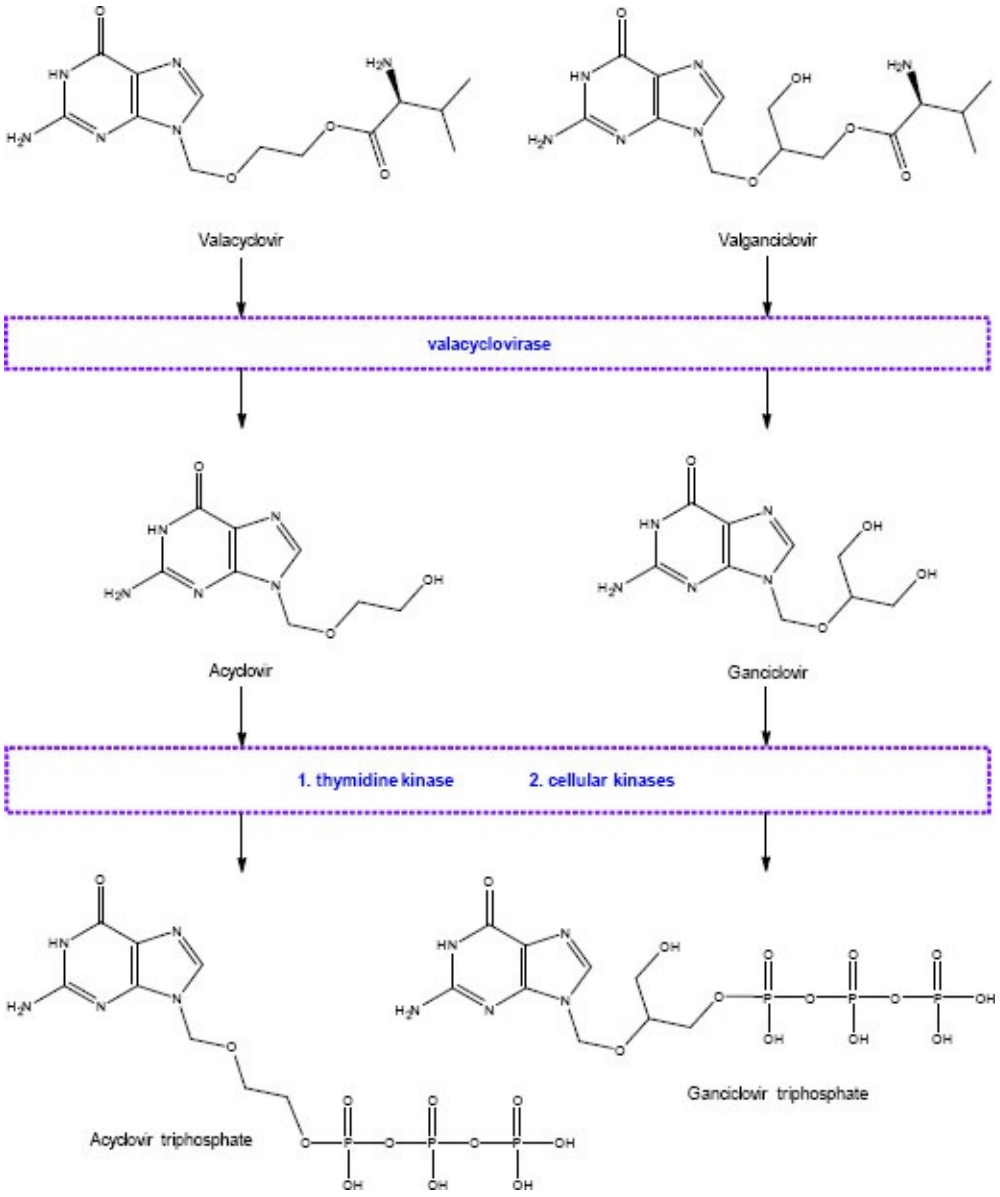


Figure 3.1.6: Bioconversion of valacyclovir and valganciclovir to their parent drugs by valacyclovirase and to their corresponding triphosphates, firstly by thymidine kinase and secondly by cellular kinases.

The successful results obtained for valacyclovir and valganciclovir, amino acid ester prodrugs of acyclovir and ganciclovir, respectively, has prompted the investigations of amino acids as promoieties for other agents. This success has been attributed to their enhanced intestinal transport via oligopeptide transporters. The work of Han prove that amino acid ester prodrugs significantly improve the cellular uptake of the parent drugs via peptide transport mechanism, though there is no peptide bond in their structures (Han, 1998). The fact that some cancer epithelial cells are rich in this type of transporter suggests their use for

the delivery of peptidomimetic anticancer agents (Gonzalez, 1998; Nakanishi, 2000; Vig, 2003). Anticancer drugs are primarily cytotoxic agents and exert their antitumor activity by interfering with some aspects of DNA replication, repair, translation or cell division. However, these agents are not “magic bullets”, as they do not destroy tumor cells while sparing the normal cells (Sinhababu, 1996).

The prodrug strategy is once again used with anticancer agents as a way to alleviate their toxicity. Different prodrug strategies have been employed to not only to improve solubility, transport and pharmacokinetic properties, but also to enable selective activation in target tissues. As a means to reduce the toxic effects of these agents, prodrugs designed for selective activation in target tissues are by far the most efficient and attractive option. However, an enzyme or transporter that is exclusively or preferentially expressed in these tissues is a prerequisite for selective targeting (Landowski, 2006; Sinhababu, 1996).

There are numerous studies of amino acid derivatives of the clinically-effective anticancer agents floxuridine (Vig, 2003) and gemcitabine (Song, 2005) concerning the activation of the prodrug. Unlike the desired rapid activation required for valacyclovir, extensive intestinal activation of floxuridine and gemcitabine prodrugs would lead to severe intestinal toxicity. In this regard, Song and Landowski demonstrated that amino acid ester prodrugs provided resistance to deamination of gemcitabine (Song, 2005) and to floxuridine cleavage (Landowski, 2005a), respectively.

The studies developed with the amino acid esters of fluxoridine were consistent with previous findings: 5’-L-valyl floxuridine was the most efficiently transported floxuridine prodrug, exhibiting the highest PEPT1-mediated transport and permeability across Caco-2 monolayers. The length and stereochemistry of the amino acid moiety side chain influences the transport efficiency of floxuridine prodrugs. The slightly more branched isoleucyl side chain reduced the transport of these prodrugs to half, but the branching at γ carbon (as in leucine) side chain decreases this transport even further. The permeability of 5’-monoester prodrugs of floxuridine across Caco-2 monolayers was significantly higher than that of the parent drug and also reflected a profound

promoiety dependency. Thus, the permeability of the 5'-L-valyl prodrug was roughly 2- and 5-fold higher than the permeability of 5'-L-isoleucyl and 5'-leucyl floxuridine prodrugs, respectively (Landowski, 2005a).

The metabolic conversion of floxuridine to 5-fluorouacil (5-FU) following systemic delivery has been shown to be detrimental to the therapeutic efficacy of floxuridine. The mechanism of action of these two drugs are well understood, where the toxicity of 5-FU is predominantly caused by 5-FU incorporation into RNA. Unlike 5-FU, floxuridine is specifically incorporated into DNA, which leads to the minimization of adverse effects. It is important to highlight that floxuridine has shown to inhibit cell proliferation 10- to 100-fold more than 5-FU. However, floxuridine is rapidly converted to 5-FU in many tissues (including the liver) by the enzyme thymidine phosphorylase (Tsume, 2008). As a consequence, higher doses of floxuridine are required to maintain clinical efficacy, which leads to greater toxicity. Therefore, protection of floxuridine prodrugs against this enzyme is essential to enhance therapeutic efficacy at low doses and obviate toxicity.

All amino acid ester prodrugs examined by Landowski and co-workers were stable to glycosidic bond cleavage by thymidine phosphorylase. It is therefore possible to conclude that modification of one or both of the free hydroxyl groups on the sugar moiety provide protection from glycosidic bond cleavages. The rate of conversion of the prodrugs to the parent drug after transport would determine floxuridine disposition and therapeutic action (Landowski, 2005a). As previously reported to structure, stereochemistry and the site of esterification of the amino acid promoiety affect the rates of activation of floxuridine prodrugs. Therefore, the wide range of variations for prodrug structure suggests that the hydrolysis rate can be tailored to produce a prodrug with the desired half-life (Landowski, 2005a; Vig, 2003).

The roughly 5- to 12-fold higher activity in Caco-2 cell homogenates compared with pH 7.4 buffer suggests the predominance of enzymatic bioconversion of the prodrugs. The results obtained for the leucyl ester prodrugs indicate that they would not be suitable candidates. In comparison, isoleucyl ester prodrugs of floxuridine are enzymatically

more stable than the valyl ester floxuridine prodrugs and the reference drug valacyclovir (Landowsky, 2005b). The combined results of the *in vitro* studies suggest that isoleucyl monoesters of floxuridine may be potentially promising candidates for improving oral bioavailability of floxuridine *in vivo*. The prodrugs given orally could improve the intestinal uptake of floxuridine as well as shield it from unwanted degradation (Landowsky, 2005b). Various dipeptides and peptidomimetics have also been tested to characterize the hPEPT1 transporter and improve its affinity, and mono amino acid ester prodrugs have been evaluated as hPEPT1 substrates (Landowsky, 2005a; 2005b; Song, 2005). Based on those reports, six amino acids were chosen to be N-terminal amino acids of the dipeptide and paired with three others to test the hypothesis that molecular sizes may structurally affect its ester bond stability (Tsume, 2008). In this study, dipeptide prodrugs appeared to be less stable in pH 7.4 buffers than the corresponding mono amino acid ester prodrugs. Since no mono amino acid ester prodrug degradation products were detected, it is quite likely that the dipeptide monoester prodrugs degrade through parallel pathways, as previously suggested for some anti-viral dipeptide prodrugs.

Although gemcitabine is clinically effective in the treatment of advanced or metastatic pancreatic cancer, it also exhibits various side effects which are attributed to its inability to distinguish between normal or target cells. It is known that gemcitabine exerts its antiproliferative activity via multiple mechanisms of action. It is initially phosphorylated intracellularly by deoxycytidine kinase and subsequently by nucleotide kinases to its active metabolites, gemcitabine diphosphate and gemcitabine triphosphate. The influence of gemcitabine on DNA synthesis has been strongly correlated with the gemcitabine triphospate intracellular concentration. However, extensive degradation of gemcitabine by cytidine deaminases to an inactive metabolite adversely affects gemcitabine activity (Huang, 1991; Song, 2005). To overcome this disadvantage, Song and co-workers reported the synthesis of amino acid ester prodrugs of gemcitabine and evaluated their affinity to oligopeptide transporterts (hPEPT1), which are overexpressed in the gastrointestinal tract and recently known to be expressed in tumor cells (Song, 2005; Vig, 2003). The promoieties used

in this study were the aliphatic amino acids L-valine, D-valine, and L-isoleucine, as well as the aromatic amino acids L-phenylalanine and D-phenylalanine. The results obtained were once again consistent with the previously mentioned findings with amino acid prodrugs. All gemcitabine prodrugs (Fig. 3.1.7) showed a greater affinity to the oligopeptide transporter compared with the parent drug and the preference for the 5'-monoester and L-configuration persist. The prodrugs rates of hydrolysis were also observed to be affected by the structure, stereochemistry and site of esterification of the promoiety (Song; 2005).

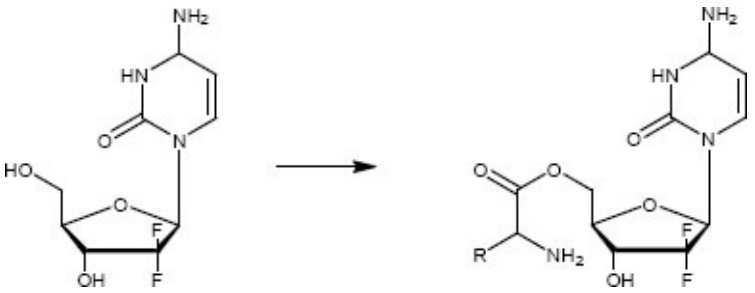


Figure 3.1.7: Amino acid-gemcitabine prodrug (R = L-Val, L-Ile, L-Phe, D-Val, D-Phe).

The chemical stability and rapid enzymatic bioconversion characteristic of the 5'-L-valyl-gemcitabine derivative suggests it potential in enhancing oral absorption of gemcitabine. On the other hand, the 5'-L-isoleucyl-gemcitabine showed a slow bioconversion in Caco-2 cells and in human plasma, as well as an unusual resistance to cytidine deaminase deactivation. This way a longer systemic circulation half-life is possible which may facilitate the targeting of cells over expressing hPEPT-1 transporter (Song, 2005).

Spontaneous cyclization of oligopeptides and prodrugs with amino acids in solution does not easily occur without the intervention of specific enzymes, with the exception of dipeptides that easily cyclize to piperazine-2,5-diones or diketopiperazines (DKP, **1**). The DKP scaffold is widely found in compounds of biological interest and could serve as a drug template with appropriately arrayed pharmacophores (Gomes, 2007). The major role of DKP in prodrug design falls in the domain of approach (ii): by linking adequate dipeptide carriers to a drug, a prodrug can be created which undergoes a strictly chemical cyclization-elimination process via intramolecular aminolysis of the dipeptide moiety to a DKP, with simultaneous departure of the free parent drug

(Fig. 3.1.8, Gomes, 2003).

Peptide derivatives of some drugs have been prepared and evaluated as prodrug candidates where prodrug activation processes involved DKP formation. One example are the peptide conjugates of the cytotoxic agent vinblastine, designed as potential prodrugs targeted at prostate cancer cells. *In vitro* and *in vivo* evaluation showed the best derivative was a conjugate bearing an octapeptide segment attached by an ester linkage to position 4 of vinblastine. This conjugate was found to undergo fast ($t_{1/2}$ = 12 min) and specific cleavage of the Gln-Ser peptide bond by prostate enzymes, and additional data from metabolism studies supported that the final spontaneous vinblastine release was driven by DKP formation from a dipeptidyl intermediate (Brady, 2002).

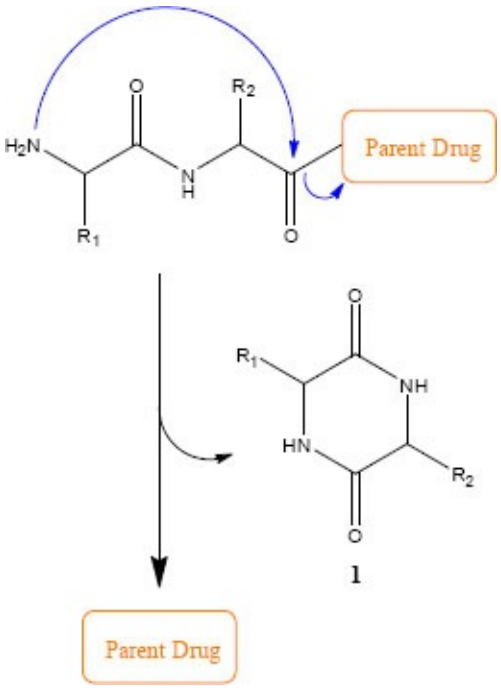


Figure 3.1.8: Prodrug intramolecular activation via DKP formation.

The formation of DKPs was reported as a major degradation pathway for simple alkyl esters (Larsen, 2004) and dipeptide *p*-nitroanilides (Goolcharran, 1998). Dipeptides have been thus proposed as drug carriers to deliver the parent drug through enzyme-independent processes, namely via DKP formation. Dipeptides are also readily accessible carriers that can be easily modified to optimize the rate of release of the parent drug (Shan, 1997). Some authors have therefore considered that dipeptides might play a crucial role as carriers for

hydroxyl-containing drugs. This hypothesis was developed in a systematic study of the reactivity of paracetamol dipeptide esters, as this phenolic drug represents an excellent leaving group in the course of intramolecular dipeptide ester aminolysis (Santos, 2005) and is known to originate hepatotoxic metabolites (Bertoloni, 2006). Dipeptide esters of paracetamol were found to be quantitatively hydrolysed to the parent drug and corresponding DKPs at physiological pH and temperature. Additional evidence that the rate of paracetamol release depended on the structure of the dipeptide carrier also supported an intramolecular pathway. Paracetamol esterification with dipeptides also led to significant decrease or even elimination of the drug's hepatotoxic effects on mice, reinforcing the great potential of dipeptide carriers in prodrug design (Santos, 2005). From what has been described, it is clear that a major drawback of dipeptide-based prodrugs is their susceptibility to non-specific peptidases. However, this problem can be easily solved by incorporating at least one non-natural amino acid. For instance, enzymatically stable dipeptides (e.g. containing α-aminoisobutyric acid or Sar) have been successfully employed in prodrugs of cytarabine (Wipf, 1996) and cyclosporine A (CsA) (Hamel, 2004). Dipeptide esters of CsA showed high thermodynamic stability, differential conversion rates under physiological conditions and strongly increased water solubility, offering a novel route for the design of CsA prodrugs (Hamel, 2004). Unnatural amino acids were also used to construct prodrugs of 5-fluorodeoxyuridine (FdU) to improve prodrug stability against protease/esterase action. Interestingly, these compounds were designed to be resistant to certain enzymes but to be susceptible to others. In fact, the antibacterial prodrugs of FdU developed by Wei and Pei are first activated *in vivo* by peptide deformylase, after which spontaneous intramolecular aminolysis of the resulting dipeptide carrier forms a DKP, with simultaneous release of FdU (Wei, 2000).

3.1.2.3. L-type Amino Acid Transporter

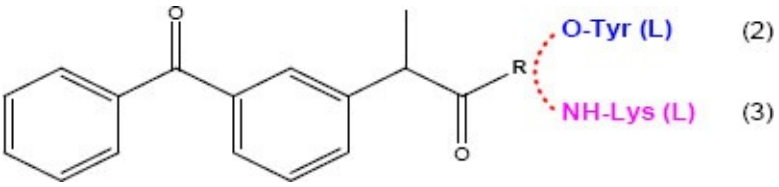
Although the classical approach to improve membrane permeability of polar drugs uses lipophilic derivatives to increase passive membrane penetration, the targeted prodrug approach uses transporters designed

for facilitating membrane transport of polar nutrients such as amino acids and peptides. There is direct and indirect evidence for the participation of carrier-mediated membrane transport mechanisms, where several hydrophilic compounds seem to be absorbed efficiently via specific transporters (Mizuma, 1992). Therefore, targeting specific membrane transporters is particularly important when prodrugs are polar or charged. From this point of view, use of intestinal epithelial transporters to facilitate the absorption of appropriately modified drugs seems to be an attractive strategy for improving the bioavailability of poorly absorbed drug molecules. Prodrugs can be designed to structurally resemble intestinal nutrients and to be absorbed by specific carrier proteins. In this case, prodrugs may have the additional advantage of producing nontoxic nutrient by products during conversion to the parent drug molecules. There have been many attempts to improve drug absorption targeting specific membrane transporters, including amino acid, peptide, and glucose transporters (Han & Amidon, 2000).

To exemplify the utility of amino acid prodrugs targeted to transporters expressed in the gastrointestinal tract, gabapentin and baclofen are structural analogs of GABA (gamma amino butyric acid) used for the treatment of various neurological disorders. Both gabapentin and baclofen have structural features of amino acids. They are both absorbed in the upper small intestine by a low-capacity solute transporter localized in the upper small intestine, possibly an L-type amino acid transporter (Vig, 2013). XP13512 and XP19986 are novel prodrugs of gabapentin and baclofen, respectively, designed to overcome pharmacokinetic limitations of the parent drugs. Transport of these prodrugs is mediated by monocarboxylate transporter type 1 (MCT-1) and sodium-dependentmultivitamin transporter (SMVT) (Cundy, 2004; Lal, 2009).

L-type amino acid transporter 1 (LAT1) is a sodium-independent heterodimeric transmembrane protein found in brain, testis and placenta. The levels of functional LAT1 are also significantly up-regulated in the surface of several human tumour cells, highlighting its essential role in cell growth and proliferation. LAT1 is responsible for transporting large neutral amino acids such as L-Leu, L-Trp, L-Ile and L-

Phe into cells (Kanai, 1998) but can also carry amino acid-derived drugs such as levodopa, gabapentin, melphalan and baclofen (Cornford, 1992; Kageyama, 2000; Wang & Welty, 1996). Furthermore, LAT1 has been demonstrated to be able to transport amino acid prodrugs, where the amino acids have been conjugated with drug molecules which are not LAT1 substrates as such (Killian, 2007; Walker, 1994). Recently, Gynther and co-workers described a feasible means to achieve carrier-mediated drug transport into the rat brain via the specific transporter by conjugating a model compound to L-Tyr (**2**) or L-Lys (**3**) with the hydrophilic drug ketoprofen, which is not a substrate for LAT1. The mechanism and kinetics of the brain uptake of the prodrug were determined with an in situ rat brain perfusion technique. The brain uptake of the prodrug was found to be concentration-dependent. These results showed that a prodrug approach can achieve uptake of drugs via LAT1 into the brain intracellular fluid. The distribution of the prodrug in the brain parenchyma and the site of parent drug release in the brain were also shown with *in vivo* and *in vitro* studies (Gynther, 2008; 2010).



Peptide transporters appear to be attractive targets in prodrug design for their high capacity, broad substrate specificity, strong expression in the intestinal epithelium and low occurrence of functional polymorphisms. Conjugating a specific amino acid with a drug can make it a substrate for PepT1 to enhance the absorption of the parent drug. Two excellent examples of marketed prodrugs that exploit carrier-mediated transport are valacyclovir (Valtrex; GlaxoSmithKline) and valganciclovir (Valcyte; Roche) (Cao, 2012). They are L-Val esters (i.e. valine as the promoiety) of acyclovir and ganciclovir, which both have limited and variable oral bioavailability owing to their high polarity. These amino acid prodrugs increased the intestinal permeation of their parent drugs by 3–10-fold, and their membrane transport was mediated predominantly through the di- and tripeptide transporter (hPepT1) expressed in intestinal epithelial cells (Han, 1998; Sugawara, 2000).

3.1.2.4. Variability of Amino Acid Application to Exclusive Drugs

Primaquine (PQ, **4**, Fig. 3.1.9) is the only generally available anti-malarial that prevents relapse in vivax and ovale malaria, and is the only potent gametocytocide in falciparum malaria. Primaquine becomes increasingly important as malaria-endemic countries move towards elimination, and although it is widely recommended, it is commonly not given to malaria patients because of haemolytic toxicity in subjects who are glucose-6-phosphate dehydrogenase (G6PD) deficient (gene frequency typically 3–30% in malaria endemic areas; > 180 different genetic variants) (Ashley, 2014). PQ is often associated with serious adverse effects because of its toxic metabolites (Vale, 2009). Blocking the terminal primary amine in PQ may represent a huge improvement in terms of PQ bioavailability, as it can significantly reduce the extent of PQ conversion into carboxyPQ, the principal metabolite. *N*-Acylation of anti-malarials with amino acids and oligopeptides has been used in several works aimed at improving drug transport into malaria-infected erythrocytes and over the last two decades, these modifications started to be also seen as a means to avoid premature PQ inactivation by oxidative deamination to carboxyPQ (Kirk, 2001). An earlier work describes the synthesis of *N*-cysteinyl-PQ that was subsequently coupled to carrier proteins, and both the anti-malarial activity *in vivo* and the acute lethal toxicity of the protein-drug conjugates were evaluated. The causal prophylactic activity of the lactosaminated serum albumin conjugate was two times higher than that of the free drug, whereas its acute lethal toxicity was at least 6.5-fold lower than that of PQ [mean lethal dose (LD50) > 85 mg of PQ base kg^{-1}]. This yielded a therapeutic index for the conjugate at least 12 times higher than that of the free parent drug (Hofsteenge, 1986).

Oligopeptide derivatives of PQ such as PQ-Lys-Leu-d-Val, PQ-Lys-Leu-Ala and PQ-Lys-Leu-l-Val have been prepared and tested for radical curative anti-malarial activity against *P. cynomolgi* in rhesus monkeys and blood-schizontocidal activity against *P. berghei* in mice. The d-Val-containing derivative was found to be less toxic and more active than both its l-Val counterpart and PQ, whereas the activity of the Ala-bearing compound was comparable to that of the l-Val derivative

(Philip, 1988). PQ *N*-acylation with amino acids or oligopeptides yields structures that are amenable to proteolytic degradation. Despite this bioreversibility being desirable in a prodrug, fast conversion into PQ will not overcome the problems associated with PQ-based therapies. Thus, additional protection of the peptide moiety in peptidyl-PQ compounds became a new goal so structures could be obtained with resistance to early proteolytic cleavage and bioreversibility at target tissues or cells to release the active molecule (Bundgaard and Moss, 1989). Two groups of tetrapeptides with general formula PQ-Y-Ala-Leu-X-NH_2 were also synthesized. In the first group, Leu, Tyr, Lys and Asp were used in the Y positions and Ala at X. In the second group, Ala, Tyr, Lys and Asp were used in X positions, while Y was Leu. The peptide derivatives were then coupled to polyacryl starch microparticles (via the *N*-terminal amino acid α-amino group), forming lysosomotropic drug carriers (Borissova, 1995).

Dipeptide derivatives of PQ (PQ-Arg-Phe, PQ-Arg-Lys and PQ-Ala-Phe) were also synthesized and tested against Chagas disease. PQ-Arg-Lys was seen to be active against *T. cruzi* development inside host cells, probably by interfering with the initial steps of trypomastigote-amastigote transformation. This derivative was more active than the other two, so it seems that specific cleavage has an important role in PQ release (Chung, 1997). Portela have also evaluated the dipeptide derivatives of PQ as novel transmission-blocking anti-malarials. In contrast with PQ, none of the compounds were able to block oocyst production in mosquitoes' midguts at 3.75 mg kg^{-1} but all were found to completely inhibit the formation of oocysts at 15 mg kg^{-1}, where *N*-acetylPQ is not active at this dose. As a whole, this work has demonstrated that acylation of the PQ's primary amino group effectively prevents the early conversion of PQ into carboxyPQ while confirming that the presence of a terminal amino group, as in the dipeptide derivatives of PQ, is essential for gametocytocidal activity (Portela, 1999). Gomes worked on the further transformation of the amino acid moiety linked to the PQ primary amine with the introduction of an imidazolidin-4-one ring at the amino acid's α-amino group (**5**, Fig. 3.1.9). Condensation of the *N*-aminoacyl derivatives of PQ with carbonyl compounds (ketones or aldehydes) was suggested by these authors as a

means to protect the amino acid moiety against premature proteolytic degradation (Gomes, 2004). Imidazolidin-4-ones were highly stable in human plasma, with a weak or null degree of PQ release after 3 days of incubation. Moreover, compound **5** hydrolyzed 50-100 times slower in aqueous buffer at physiological pH and *T* than the corresponding imidazolidin-4-ones derived from di- and penta-peptides (Araújo, 2005; Chambel, 2006). These imidazolidin-4-ones (**5**) were effective in preventing *P. berghei* malaria transmission from BalbC mice to *Anopheles stephensi* mosquitoes (Araújo, 2005; Vale, 2005). Most of these compounds were also active against *Pneumocystis carinii* above 10 μg mL^{-1}, which was correlated to their anti-*P. falciparum* blood-schizontocidal activity *in vitro* (Vale, 2008a).

Later, PQ dipeptide derivatives bearing an imidazolidin-4-one moiety at the *N*-terminus (**6**, Fig. 3.1.9) were synthesized and evaluated as potential transmission-blocking antimalarial prodrugs. All compounds were hydrolyzed to the parent dipeptide derivative of PQ in neutral and basic solutions, with half-lives ranging from 0.7 to 31 h at 37°C, depending on the nature of the substituents present in the imidazolidin-4-one moiety and in the C-terminal amino acid directly coupled to PQ. The imidazolidin-4-one derived from Ala-Ala-PQ and acetone reduced the transmission of the infection to mosquitoes more efficiently than PQ, as shown by the significant decrease in the number of oocysts in the midguts of the mosquitoes at 10 and 50 mmol kg^{-1} when compared to the control (Vale, 2009a).

A recent report from the same authors provides the synthesis and biological activity of N^1-aminoacyl derivatives of **5** (**7**, Fig. 3.1.9) as mimetics of PQProXaa structures (where Xaa represents a general amino acid). Such structures were again found to be remarkably stable, modestly active as a blood-schizontocidal against a CQ-resistant strain of *P. falciparum* and effective inhibitors of the sporogonic cycle of *P. berghei* in *A. stephensi* mosquitoes (Vale, 2008b).

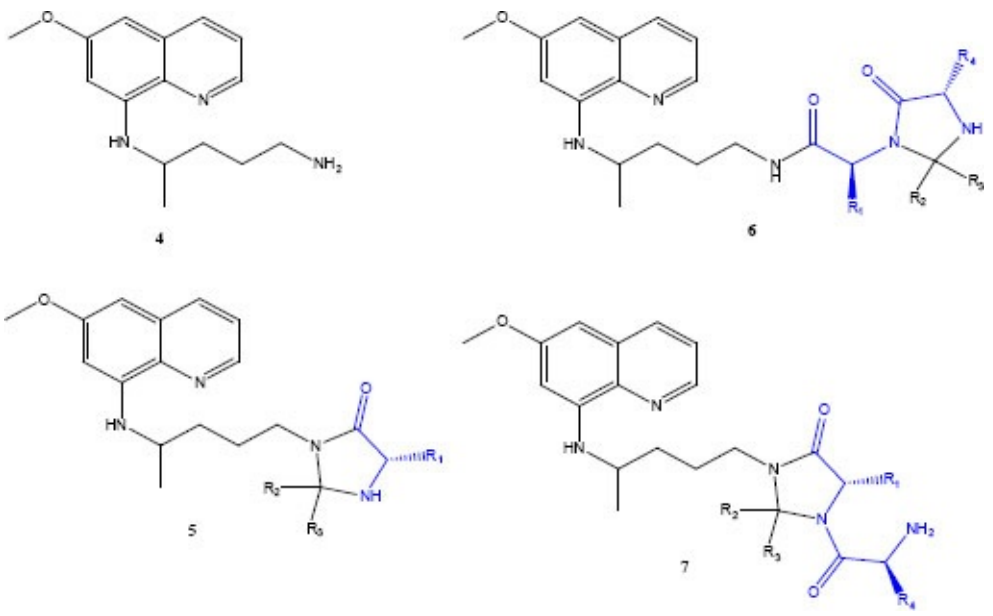


Figure 3.1.9: Structures do PQ and its imidazolidin-4-ones derived from L-amino acids and carbonyl compounds.

This research group incorporated the imidazolidin-4-one moiety into dipeptide derivatives of PQ (**7**, Fig. 3.1.9), both to introduce a terminal basic amino group reported as relevant for activity, and effectively suppress hydrolysis of the imidazolidin-4-one through acylation of the N^1 nitrogen atom (Vale, 2008b; 2008c). These peptidomimetic derivatives were active against a chloroquine-resistant *P. falciparum* strain and inhibited the development of the sporogonic cycle of *P. berghei*, affecting the appearance of oocysts in the midguts of the mosquitoes and were extremely stable, both in human plasma and in pH 7.4 buffer as a result of N^1-acylation (Vale, 2008b). All compounds were also active in many biological assays (*in vivo* transmission-blocking activity, *in vitro* tissue-schizontocidal activity on *P. berghei* infected hepatocytes and *in vitro* anti-*P. carinii* activity), with generally lower activity than the parent drug. However, these imidazoquines are stable compounds because of blockage of the aliphatic amine of PQ by insertion of the peptidomimetic carrier. Structure **7** compounds are not vulnerable to oxidative deamination, which is the main metabolic process behind the low oral bioavailability of PQ. On the other hand, the use of a peptidomimetic instead of a dipeptide carrier makes compound **7** stable to proteolytic degradation by action of amino- or endopeptidases (Vale, 2009b).

3.1.3 Peptides for Biomedicine

To fulfil their therapeutic function, most drug-like compounds must pass through the cellular membrane to enter a cell. Small molecules that obey Lipinski's rule of five (no more than 5 hydrogen bond donors, not more than 10 hydrogen bond acceptors, a molecular mass less than 500 Da and an octanol-water partition coefficient not greater than 5) have a reasonable chance of penetrating the cell membrane. However, an increasing number of therapeutics, such as biologics, cannot be considered 'small molecules' and these therapeutic are often unable to enter the cell unassisted (Liskamp, 2014). One of the vehicles used to facilitate the passage of these compounds into cells are peptides.

Peptides and proteins play a central role in numerous biological and physiological processes in living organisms: they are involved as hormones and neurotransmitters in intercellular communication, act as antibodies in the immune system to protect organisms against foreign invaders, and are also involved in the transport of various substances through biological membranes. Peptides have significant advantages over other small molecules in terms of specificity/affinity for targets and toxicity profiles, and over antibodies in terms of tissue penetration owing to their smaller size. A large number of peptide-based drugs are now being marketed and the number of candidates entering clinical evaluation in recent years is steadily increasing. Therefore, the synthesis of such structures has been a major focus of organic chemistry for over a century in order to improve the prospects for synthetic therapeutic peptides. In this section, the relevance of peptides in biomedicine will be discussed.

3.1.3.1 Antimicrobial Peptides (AMPs)

Parasitic diseases are most common in tropical regions with poverty and undeveloped health care systems. Many parasitic diseases have been out of focus on national and international agendas and increasing drug resistance combined a lack of vaccines in several countries make this very alarming. To combat this reality, it is imperative to understand the relationship between insect and host associated to parasitic disease.

Insects are widely distributed and developed in various ecological niches and serve as vectors of many parasitic diseases in humans and animals, suggesting outstanding strategies for defending against pathogens such as microorganisms by overcoming and adapting to different environmental conditions (Lowenberger, 2001). After infection, the parasites are presented to the host immune system and induce the production of defence compounds such as proteins and peptides. Several of these humoral response peptides exert antibacterial, antifungal, or antiviral properties (Bulet, 1999) and are known as small cationic peptides, host defense peptides or antimicrobial peptides (AMPs) (Bell, 2011).

AMPs mostly contain 15–45 amino acid residues and are generally cationic at physiological pH, often with an amphipathic character and encoded by separated genes. They form the first line of host defence against pathogenic infections and are a key component of the ancient innate immune system. AMPs have been identified in various species ranging from bacteria and frogs to mammals, including humans. In insects, AMPs are synthesized in the fat body, blood cells (hemocytes) or epithelia and are released into the hemolymph, the insect blood. In vertebrates, AMPs are present in amphibian skin secretions (Simmaco, 1999) and epithelia (Ganz & Weiss, 1997; Bals, 1998). In mammals, they were also observed in lymphocytes (Agerberth, 2000) and leukocytes (Sorensen, 1997). Because of their broad activity against microbes and expression triggered by various infections, AMPs are currently intensely examined as potential antiparasitic compounds (Vale, 2014).

In 2004, the antimicrobial peptide database (APD, http://aps.unmc.edu/AP/main.php) reported a significant number of AMPs that have been discovered at both the gene and protein levels (Wang Z & Wang G, 2003). The APD was later updated and expanded to allow users to search peptide families (bacteriocins, cyclotides, or defensins), peptide sources (fish, frogs or chicken), post-translationally modified peptides (amidation, oxidation, lipidation, glycosylation or D-amino acids), and peptide binding targets (membranes, proteins, DNA/RNA, LPS or sugars) (Wang, 2009).

3.1.3.1.1 AMPs: Mechanism of Action and Peptide Families

Several models have been proposed for the mechanism of action of AMPs on membrane permeabilization or insertion, and this mechanism depends on a combination of hydrophobic and electrostatic effects (Shai, 1999; Sanderson, 2005). The most prominent models are the carpet mechanism, the barrel-stave model, and the toroidal pore model. Overall, the mechanism of interaction of AMPs with membranes results either in the translocation of peptides into the cell, the formation of pores embedded in the membrane to enable a flux of ions and molecules, or in membrane permeabilization/disruption (Fig. 3.1.10).

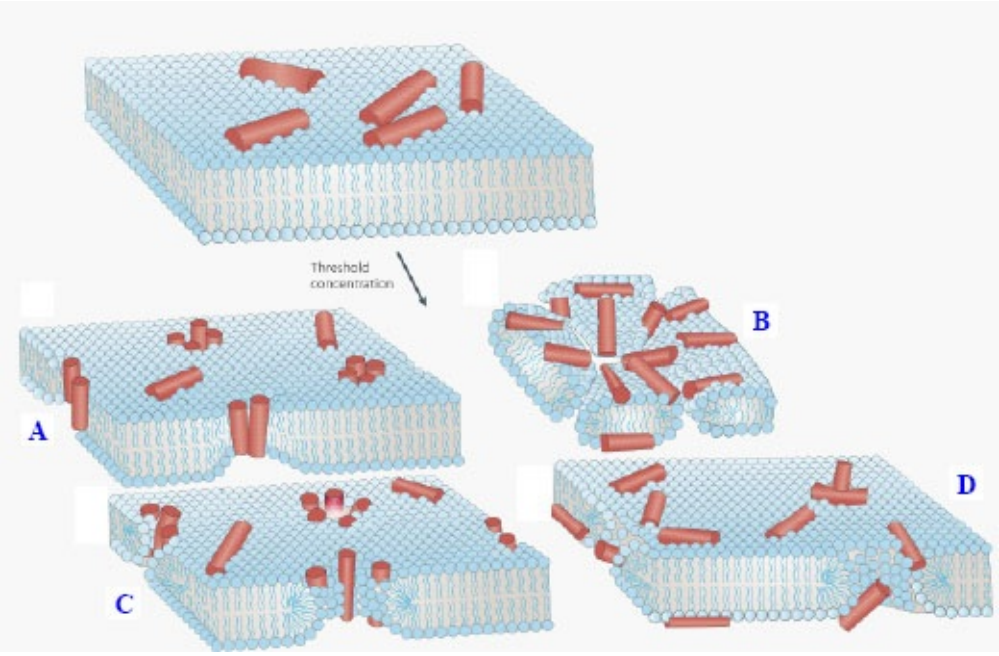


Figure 3.1.10: Proposed mechanisms of AMP-mediated membrane disruption. **A**) Barrel-stave pore. Peptides insert perpendicularly in the bilayer, associate and form a pore. The peptides line the pore lumen in a parallel direction relative to the phospholipid chains, which remain perpendicular to the bilayer plane. **B**) Carpet mechanism. Peptides adsorb parallel to the bilayer and, after reaching sufficient coverage, produce a detergent-like effect that disintegrates the membrane. **C**) Toroidal pore. As with the barrel-stave pore, peptides insert perpendicularly in the bilayer, but instead of packing parallel to the phospholipid chains, the peptides induce a local membrane curvature in such a way that the pore lumen is lined partly by peptides and partly by phospholipid head groups. **D**) Disordered toroidal pore. A recent modification to the toroidal pore model proposes that less-rigid peptide conformations and orientations are formed, where the pore lumen is lined by the phospholipid head groups (adapted from Melo, 2009).

The potency and range of the antimicrobial activity of AMPs vary

depending on various parameters, such as the amino acid composition of the structure and its stability under different environmental conditions, as well as on parameters of the target cell such as membrane composition and structural features (Fig. 3.1.11). As the factors that contribute to peptide-lipid interactions are beginning to be understood, many opportunities remain for the organic chemist to design new systems that address some of these issues.

Eukaryotic plasma membranes contain phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine and sphingomyelin, and are enriched in cholesterol in contrast to bacterial plasma membranes, which are often composed of one major lipid (Pretzel, 2013). Besides the negatively-charged phosphatidylserine, none of the aforementioned phospholipids has a net charge, meaning eukaryotic membranes are generally neutral. In contrast, prokaryotic membranes are enriched in negatively charged lipids such as in Gram-positive bacteria, which contain the negatively charged teichoic and teichuronic acids in their envelope (Yeaman & Yount, 2003). Combined with differences in the electrochemical gradients of cells from different organisms, differences in the membrane environment and the remodeling of the host cell membrane by intracellular parasites (Hsiao, 1991), these factors could explain why AMPs discriminate between microbial cells and host cells, or between parasites and mammalian cells (Pretzel, 2013; Yeaman & Yount, 2003).

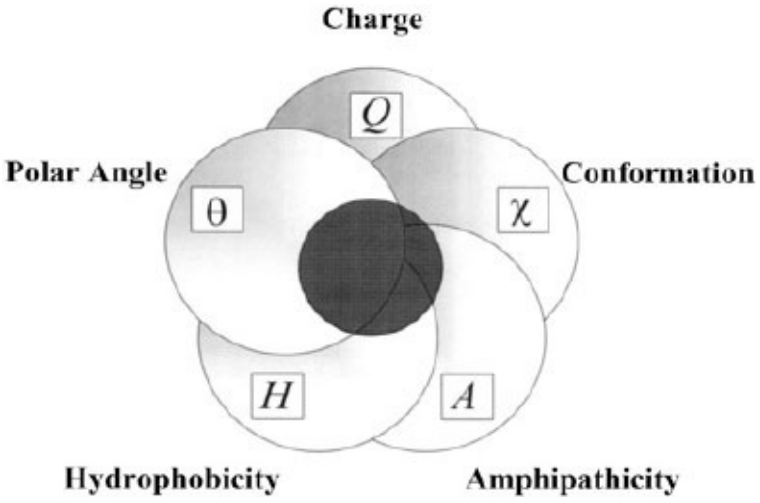


Figure 3.1.11: Interrelationship among structural determinants in antimicrobial peptides. Fundamental composition and amino acid sequence influence not only the biochemical properties of the peptide [e.g. charge (*Q*), amphipathicity (*A*), and hydrophobicity (*H*)], but also

govern their three-dimensional configuration [e.g. conformation (χ), polar angle (θ), and overall stereo geometry]. Changes in composition, sequence, and intramolecular bonds may profoundly affect the structure-activity relationships of antimicrobial peptides in solution, upon binding to target membranes, or during conformational phase transition to activated states. Therefore, optimal antimicrobial peptide efficacy lies in the relevant coordination of these relationships (shaded area) as they relate to microbial target versus host cells in a particular context of infection (adapted from Yeaman & Yount, 2003).

In current practice, it is known that AMPs not only target cell membranes but may have intracellular targets. The AMP buforin II kills *E. coli* without lysis of the cell membrane. Buforin II penetrates the cell membrane, accumulates inside the bacterial cell and binds to the DNA and RNA, which leads to cell death by inhibiting cellular functions (Park, 1998). Pleurocidin inhibits DNA and protein synthesis in *E. coli* without damaging the cytoplasmic membrane (Patrzykat, 2002). Other AMPs inhibit enzymatic activities inside the target cell such as phyrrhocoricin, apidaecin, and drosocin, which interact with the *E. coli* heat shock protein DnaK and inhibit protein folding (Kragol, 2001). Some AMPs are capable of inducing apoptotic cell death, including the breakdown of mitochondrial membrane potential and activation of caspase 3-like activity, such as in *Leishmania* (Kulkarni, 2006). AMPs may have different target sites and act in more than one mechanism to kill the same species, and different AMPs may act synergistically (Fieck, 2010).

According Boman, AMPs can be classified into three groups: a) linear, often α-helical peptides free of cysteine residues; b) peptides containing disulfide bridges, giving peptides a β-sheet structure; and c) peptides with an overrepresentation in certain amino acids, such as proline, arginine, tryptophan, or histidine (Boman, 2003).

3.1.3.1.2 α-Helical Peptides without Cys Residues

Cecropin-AMP families share a similar structure containing two α-helical domains linked by a flexible region, and the different cecropins from different organisms vary in their range of antimicrobial activity. The antibacterial activity of insect cecropins is based on the pore formation in bacterial membranes (Ekengen & Hultmark, 1999; Tanaka, 2008). A

positive surface charge of cholesterol present in the membrane bilayer decreases the channel formation potency of cecropins (Christensen, 1988). Cholesterol increases the thickness and condensation of membranes, so cecropins have little to no effect on eukaryotic cells, which contain a high amount of cholesterol in contrast to bacteria (Beevers & Dixon, 2010).

A second class of linear, cysteine-free peptides, the magainins, is exclusively found in amphibians. Magainin 1 and magainin 2 adopt an α-helical conformation in solution (Zasloff, 1987). Magainins are proposed to induce toroidal pores in bacterial membranes (Ludtke, 1996). The nonhemolytic feature of magainin 2 and its protocidal activity make it highly interesting for the examination of its activity against human parasites. Another family of amphibian AMPs is the dermaseptin superfamily. These AMPs exhibit a broad range of antimicrobial activity and some aggregate on the membrane surface in a carpet-like manner (Pouny, 1992; Raghuraman & Chattopadhyay, 2007). Melittin is an α-helical cationic peptide, composed of 26 amino acid residues in which the amino-terminal region is predominantly hydrophobic and the carboxy-terminal region is hydrophilic due to the presence of a stretch of positively charged amino acids (Raghuraman & Chattopadhyay, 2007), and the membrane permeabilization mechanism is proposed to result from pore formation according to the toroidal model (Yang, 2001). Cationic antimicrobial peptides are a class of small charged peptides, imparted by the presence of multiple Lys and Arg but with a substantial portion (50% or more) of hydrophobic residues. They are known for their broad-spectrum antimicrobial activity and, most recently, the ability to modulate the innate immune response (Powers & Hancock, 2003).

3.1.3.1.3 Peptides Containing Disulfide Bridges

Defensins are peptides from mammalian phagocytes that contain 6 Cys residues (or for some insect defensins, eight Cys) that stabilize the peptide structure by forming three intramolecular disulphide bridges (Selsted, 1985). The mechanism of action of these peptides is based on membrane permeabilization, probably by pore formation, and defensins are more active against negatively charged phospholipids (Lehrer, 1989;

Wimley, 1994). The penaeidin class of peptides consist of a proline-rich N-terminus and a C-terminus containing six Cys residues engaged in three disulfide bridges (Destoumieux, 2000). The Pro-rich domain of penaeidin class AMPs confers the target specificity and antimicrobial activity of penaeidin (Cuthbertson, 2004). The carboxyl Cys-rich domain consists of an amphipathic helix linked to the upstream and downstream coils by two disulfide bonds.

3.1.3.1.4 Peptides Rich in Pro, Gly, His, Arg and Trp Residues

This group includes AMPs such as apidaecins, short-chain Pro-rich peptides that may adopt a polyPro helical type II structure, possibly forming the structural basis to bind specific targets and confer antibacterial activity (Li, 2006). The mechanism of membrane action does not include the formation of pores but is energy-driven, resulting in a transporter-mediated model (Castle, 1999).

As for the Pro-rich antimicrobial peptide class, the Gly rich peptides have variable sizes and do not show clear sequence consensus, apart from the high proportion of Gly in their primary sequence. These peptides are in general longer than AMP from other classes and between 25 to 50% of their amino acids are glycine. They have disordered structure in water but tend to self-order when in contact with artificial membranes (Bruston, 2007). Attacins are a group of six glycine-rich AMPs and can be grouped into four basic (A-D) and two acidic (E-F) peptides, probably derived from two attacin genes (Yi, 2014). Attacin inhibits the synthesis of outer membrane proteins of *E. coli* by blocking the transcription of the respective genes (Carlsson, 1991). This is presumably achieved by an indirect mechanism, where attacin binds to lipopolysaccharides (which serve as a receptor for attacin) but does not enter the bacterial cell (Carlsson, 1998).

The archetypical Trp rich peptide is indolicin, which unlike the amphipathic alpha helical structure of the cecropin class of peptides, has a linear structure (no disulfide bridges) and no particular secondary structure in water. Some authors suggest that structural changes and strong membrane affinity are key to the antimicrobial activity of indolicin (Ladokhin & White, 2001). Trp-rich AMP sequences contain

more than 25% of Trp. This peptide has the ability to permeate bacterial membranes and, depending on its three dimensional shape, inhibits DNA synthesis by binding to its strand (Hsu, 2005).

His-rich amphipathic cationic peptides are peptides with 25% of their amino acids represented by His. They show a global cationic amphipathic helical structure. They trigger microorganism membrane disruption when the peptide adopts an alignment parallel to the membrane surface. However, pore formation is not essential for their high antimicrobial activity (Mason, 2009). Clavinin (van Kan, 2002) and daptomycin (Jeu & Fung, 2004) are two studied members of this antimicrobial peptide class.

Cell-penetrating Peptides: A Tool for Effective Delivery

The hydrophobic nature of cellular membranes protects cells from an influx of exogenous molecules, including bioactive molecules such as peptides, proteins, and oligonucleotides. New strategies have been developed to overcome these aspects, as microinjection, electroporation, and liposome and viral-based vectors. However, these methods have various drawbacks, including low efficiency, high toxicity, low bioavailability and poor specificity. An alternative strategy to traverse the phospholipid bilayer of the cell membrane emerged from two unexpected findings. In 1988, the HIV TAT transactivating factor was discovered (Frankel & Pabo, 1988) and a few years later, the *Drosophila* Antennapedia transcription factor (later named penetratin) was also discovered. TAT and penetratin served as the foundation for the development of a new type of molecular vector able to promote the delivery of a variety of cargos: cell-penetrating peptides (CPPs). A vast number of interdisciplinary studies report numerous applications for CPPs in the delivery of various cargos such as nucleic acids, polymers, liposomes, nanoparticles and low molecular weight drugs. The main characteristics of CPPs are low cytotoxicity, the ability to be taken up by a variety of cell types, dose-dependent efficiency and no restriction with respect to the size or type of cargo (Heitz, 2009). With a broad sequence variety and large differences in terms of physical chemical properties, CPPs can be linear, cyclical, cationic, hydrophobic, hydrophilic, amphipathic, non-amphipathic, random coiled, α-helical or β-sheets.

CPPs differ from most other peptides with respect to specific features that reflect various mechanisms used to enter the cell (Milletti, 2012).

Cell-penetrating peptides (CPPs) are relatively short peptides that consist of less than 40 amino acids, are able to enter cells by means of various mechanisms, including endocytosis, and are able to assist in the further intracellular delivery of covalently or noncovalently conjugated bioactive cargos (Heitz, 2009; Vale, 2016), as illustrated in Fig. 3.1.12.

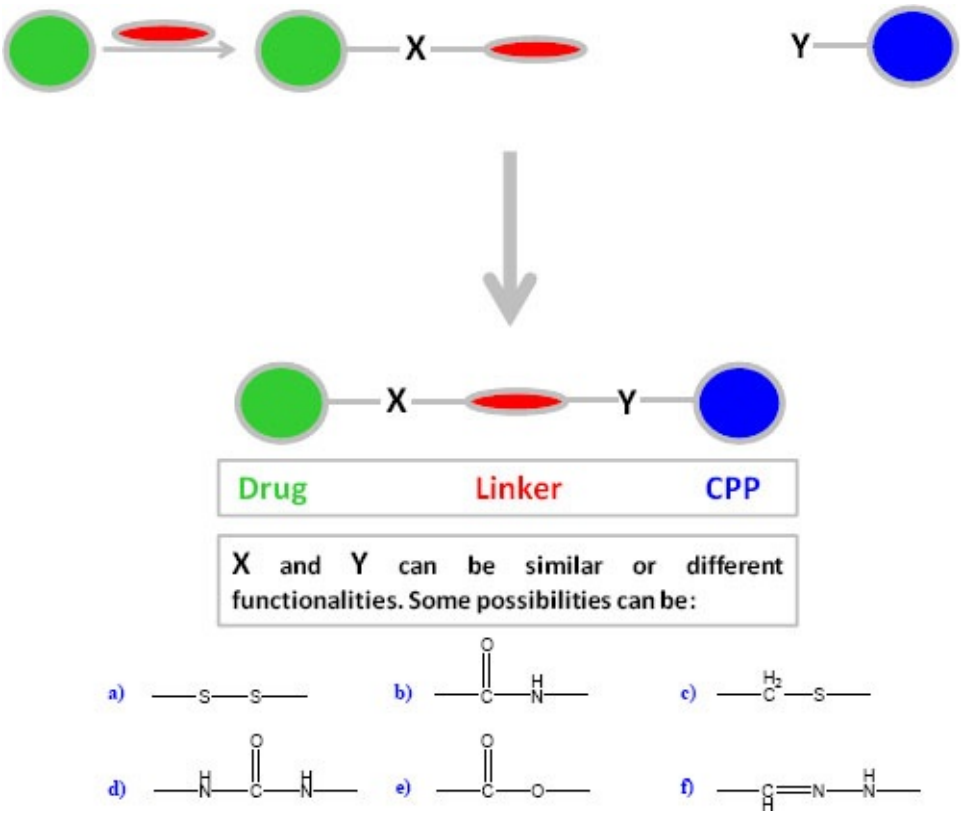


Figure 3.1.12: The structure of a drug-linker-peptide (CPP) conjugate developed in our research group. X and Y represent the common functional groups used to connect either the drug or the peptide to the linker. X may be similar to or different than Y. Here, the nature of the X bond and the drug peptide conjugation chemistry has been classified according to the nature of the X bond: a) disulfide bond, b) amide, c) thioether, d) carbamate ester, e) carboxylic acid ester or f) hydrazone bond.

Sequences of common CPPs are provided in Table 3.1.2. The common virtue of all CPPs is the ability to efficiently pass through cell membranes while carrying a wide variety of cargos inside cells (Fig. 3.1.13) (Capolovici, 2014). Interestingly, CPP sequences are known to vary considerably as seen by examining Table 3.1.2. There are, however, several similarities between the structural natures of these short

peptides. Almost every CPP sequence involves positively charged amino acids. In fact, a chain of arginines form one of the most widely used CPPs (Myrberg, 2008). The membranolytic properties of a given CPP can also be governed by its secondary structure, specifically its helicity. It has been shown that peptides with an α-helical region can enter cells more efficiently.

Table 3.1.2: Sequences of common CPPs.

Name	Sequence / (Refs)	Origin
TAT (48-60)	GRKKRRQRRRPPQ (Frankel and Pabo, 1988; Green and Loewentein, 1988)	Human immunodeficiency virus type 1 (HIV-1) TAT
Penetratin	RQIKIWFQNRRMKWKK (Derossi, 1994)	Drosophila Antennapedia homeodomain
MAP	KLALKLALKALKAALKLA[a] (Oehlke, 1998)	Amphipathic model peptide
Transportan/ TP10	GWTLNS/ AGYLLGKINLKALAALAKKIL[a] (Pooga, 1998; Soomets, 2000)	Galanin-Lys-mastoparan
VP22	NAKTRRHERRRKLAIER (Eliott & O'Hare, 1997)	Herpes simplex virus
Polyarginine	R_n,[a] n = 8,9 (Futaki, 2001)	Positively charged sequence
MPG	GALFLGFLGAAGSTMGA[b] (Morris, 1997)	Hydrophobic domain from the fusion sequence of HIV gp41 and NLS of SV40 T-antigen
Pep1	KETWWETWWTEWSQPKKKRKV[b] (Chaloin, 1998)	NLS from Simian Virus 40 large T antigen and reverse transcriptase of HIV-1
pVEC	LLIILRRRIRKQAHAHSK[a] (Säälik, 2004)	VE-cadherin
YTA2	YTAIAWVKAFIRKLRK[a]	MMP cleavage site as seeding sequence

	(Lindgreen, 2006)	
YTA4	IAWVKAFIRKLRKGPLG[a] (Lindgreen, 2006)	MMP cleavage site as seeding sequence
M918	MVTVLFRRLRIRRACGPPRVRV[a] (El-Andaloussi, 2007)	Tumor suppressor protein p14ARF
CADY	GLWRALWRLLRSLWRLLWRA[b] (Crombez, 2007)	Derived from PPTG1 peptide, W and charged amino acids

[a] C-terminal amide. [b] C-terminal cysteamide.

Initially, CPPs were composed of only natural amino acids but recently have included non-primary and even unnatural amino acids (Farrera-Sinfreu, 2005). Furthermore, when lysine residues are replaced with ornithine residues, the peptide becomes more resistant to cellular degradation (Ezzat, 2011; Angeles-Boza, 2010). Cargo delivery efficiency can also be improved by changing the structure of the peptides, such as modification into dendrimers (Angeles-Boza, 2010; Bode, 2014), cyclic peptides (Mandal, 2011; Northfield, 2014), and the often-used strategy of modifying the side chains of CPPs, such as in TP10 derivatives (Andaloussi, 2011; Oskolkov, 2011). Recently, Vale's research group developed new CPP-Gemcitabine conjugates for drug delivery in cancer therapy (Vale, 2016).

Further investigations into the structure and mechanisms of uptake of CPP-cargo complexes will be required to evaluate CPPs as potential delivery tools for biomolecules *in vitro* and *in vivo* models. However, considering evidence from numerous studies, CPPs have the potential to become a universal tool to carry therapeutic molecules across cellular membranes without a risk of toxicity or inflammatory reactions.

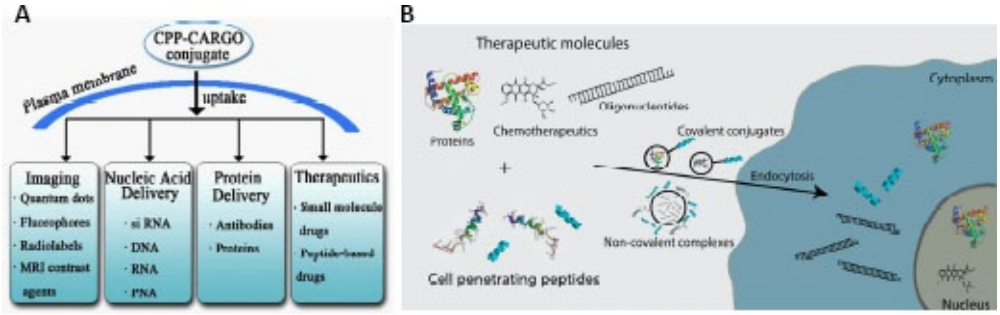


Figure 3.1.13: A) Applications of cell-penetrating peptides as molecular delivery vehicles. **B**) Intracellular delivery of CPP-cargo complexes (specific internalization pathways have not been fully elucidated and seem to depend on the specific nature of the CPP and the cargo attached to it). Adapted from (Capolovici, 2014; Wang, 2014).

3.1.3.3 Peptides: Scaffolding Materials in Tissue Engineering

3.1.3.3.1 Peptide-based Biopolymers

Peptides provide an extensive repertoire of signals that can be used to synthetically recreate complex biological processes required for tissue regeneration, such as enabling naturally occurring enzyme-mediated degradation, specific binding of biomolecules, or directing cell adhesion, proliferation and differentiation. Therefore, peptides represent attractive functional and structural building blocks to create versatile materials (Chan & Mooney, 2008; Smith, 2011).

The use of peptide therapeutics is expected to increase in the near future not only as the active ingredient, but also as new conjugated forms with polysaccharides or synthetic polymers to improve potency and specificity (Vlieghe, 2010). In this context, peptides can be used as targeting moieties, as carriers to provide transport across cellular membranes, and to modify the bioactivity of the original compound/material. In the field of biomaterials, peptides have also been extensively used as cell-instructive motifs with different roles, namely to promote cell-adhesion to otherwise non-adhesive polymers, as well as proliferation and differentiation (Collier & Segma, 2011; Wojtowicz, 2010). Finally, peptides by themselves are paving the way as new biomaterials. This is the case for a new class of materials named self-assembling peptides (SAPs), which self-assemble into nanofiber-like hydrogen bond networks under physiological concentrations of salt solutions and have found numerous applications as three-dimensional matrices in the biomedical field (Collier & Segma, 2011; Matson & Stupp, 2012).

3.1.3.3.2 Strategies to Create Scaffolds as Instructive Extracellular Microenvironments for Tissue Engineering - Peptide-conjugated

Polymers to Mimic Natural ECM

New generations of synthetic biomaterials are being developed not only to provide structural support for damaged tissues, but also to integrate with these tissues and ideally promote regeneration. The surface of biomaterials can be functionalized with specific factors that are capable of modulating cell behaviour to promote wound healing and tissue regeneration (Bellis, 2011). Synthetic ECMs replace many functions of the native ECM: organizing cells into a three-dimensional architecture, providing mechanical integrity to the new tissue and providing a hydrated space for the diffusion of nutrients and metabolites to and from the cell (Rowley, 1999).

Cell adhesion is not only critical for stimulating proper tissue development at implant/tissue interfaces, but also necessary for materials that serve as carriers for the delivery of reparative cells to wound sites. Furthermore, cell attachment to a biomaterial scaffold is an important early step in the generation of *in vitro*-engineered tissue substitutes. There are several molecular interactions that can mediate cell attachment, however much of the research in this area has focussed on utilizing pro-adhesive factors, such as adhesive peptides, that engage and activate integrin adhesion receptors on the cell surface. Integrins are heterodimeric transmembrane receptors that bind to proteins within the ECM including fibronectin, laminin, various collagens, and many other molecules (Bellis, 2011).

The most widely studied cell-adhesive peptide in the biomaterials field is the oligopeptide Arg-Gly-Asp (RGD), identified as the minimal essential cell-adhesion sequence in fibronectin (Ruoslahti & Pierschbacher, 1987; Cha, 2012). The incorporation of this motif as described initially by Rowley is highly effective at promoting the attachment of numerous cell types to a plethora of diverse materials (Rowley, 1999; Bellis 2011).

The RGD sequence can bind to multiple integrin species, and synthetic RGD peptides offer several advantages for biomaterials applications. Because integrin receptors recognize RGD as a primary sequence (although conformation of the peptide can modulate affinity), the functionality of RGD is usually maintained throughout the processing

and sterilization steps required for biomaterials synthesis, many of which cause protein denaturation. The use of RGD, as compared with native ECM proteins, also minimizes the risk of immune reactivity or pathogen transfer. Another benefit is that the synthesis of RGD peptides is relatively simple and inexpensive, which facilitates translation into the clinic (Bellis, 2011).

3.1.3.3.3 Peptide-based Biomaterials Responsive to Environmental Cues

The design and development of materials that react accordingly to the surrounding environment is expected to open up the potential of peptide-based materials (Mart, 2006). Peptides are ideally suited for this purpose because of the range of distinct physical properties available from the naturally occurring amino acids. This diversity allows for rational incorporation of non-covalent interactions including electrostatic (acidic and basic amino acids), hydrophobic, ππ-stacking (aromatic amino acids), hydrogen bonding (polar amino acids) as well as covalent (disulfide) bonds and steric contributions (strand directing amino acids). While individually these interactions are quite weak, collectively they can give rise to very stable structures. These interactions depend significantly in different ways on environmental conditions such as ionic strength, pH and temperature (Mart, 2006).

Enzyme responsiveness can be programmed into these materials by incorporation of peptide sequences that are known substrates for proteases, kinases, or phosphatases. The dynamic nature of these interactions allows the protein molecular organization to be altered in response to changes in the direct environment. The degradation rate of the scaffold is another critical factor that can control tissue morphogenesis. In this context, enzyme-sensitive hybrid hydrogels composed of synthetic or natural polymers and peptide/protein domains, which respond to specific cell-secreted proteases (particularly proteases) have been prepared using genetic engineering and/or chemical approaches (Lin & Anseth, 2009). The action of enzymes can be used as stimuli for triggering drug release and also to facilitate scaffold remodelling and replacement by resident and host cells, enabling the infiltration of blood vessels as well as controlling the

release of matrix-associated growth factors and morphogens to enhance tissue regeneration (Lin & Anseth, 2009; Chan & Mooney, 2008).

Most ECM proteins, including collagen, fibrin, fibronectin, and laminin have specific cleavage sites for degradation by enzymes, such as matrix metalloproteinases (MMPs), plasmin, and elastase. In this perspective, MMPs and plasmin are particularly interesting as they participate in ECM remodelling and degradation, having a key role in wound healing and tissue regeneration. In particular, plasmin can degrade and remodel the provisional fibrin matrix act generated at the onset of clot formation and can also activate latent growth factors or morphogens by specific cleavage events (Page-Mc Caw, 2007; Patterson & Hubbell, 2011; Aizawa, 2012; Fonseca, 2013). The cleavage site specificity of proteolytic enzymes can be determined by means of combinatorial libraries which provide information applicable to the design of inhibitors and to the identification of protein substrates (Turk, 2001; Chau, 2004). The development of efficient drug delivery systems with enhanced therapeutic efficiency is relevant in cases where a prolonged treatment is required. Biodegradable enzyme-sensitive hybrid polymers are susceptible to the action of specific proteases yielding small nontoxic metabolites that can be easily excreted through natural body mechanisms. Their biocompatibility and low immunogenicity make them suitable for repeated parenteral administration and allow the use of high molecular weight carriers to optimise pharmacokinetics and high polymer doses (Duro-Castano, 2014).

3.1.3.3.4 Self-assembling Peptides as Biomaterials

Biocompatible and bioactive small molecules, capable of self-assembly and degradation into predictable metabolites over time are ideal building blocks for scaffolds to regenerate tissues and organs. The high signaling capacity and therapeutic efficacy of peptide-based scaffolds is expected to open up the potential of self-assembling peptides as new therapeutic agents in regenerative medicine. Furthermore, these hydrogels are biocompatible since they are composed of simple naturally-occurring amino acids. There are several classes of self-assembling peptide-based materials, such as peptide amphiphiles,

Fmoc-peptides, self-complementary ionic peptides and hairpin peptides, among others (Matson & Stupp, 2012; Ischakov, 2013).

The peptide-based hydrogels are developed through molecular self-assembly of peptides and their derivatives present several advantages over other polymeric gelators. These hydrogels are formed without the use of potentially hazardous chemicals that could affect their biocompatibility. They also comprise nanofibrous networks which imitate the structural architecture of natural ECM. Finally, molecular self-assembling systems can also incorporate features that enhance the overall hydrogel properties (Johnstone, 2013). Short peptide building blocks are easy to manufacture in large quantities and can also be chemically decorated with biologically-active components, facilitating the design of hydrogels with improved targeting and *in vivo* stability (Zhou, 2009; Ischakov, 2013).

A large number of self-assembling materials responsive to biologically relevant stimuli, such as pH or temperature, are being developed based on small molecular building-blocks (Table 3.1.3). Aggeli and colleagues have designed short β-sheet forming peptides that self-assemble into a variety of nanostructures with tunable physical and chemical properties (Aggeli, 2003). Pochan and colleagues have developed β-hairpin peptides that fold to acquire an intramolecular amphiphilic structure to form self-assembled hydrogel networks (Pochan, 2003). Recent studies demonstrated that self-assembled peptides can be used to mimic the structure of natural ECM fibers and to trigger physiological processes in cells. The design of self-assembled scaffolds that can act as anchors to the cultured cells can be achieved by incorporation of short adhesive motifs into the fibers of the backbone of a synthetic ECM-like matrix (O'Leary, 2011; Fleischer & Dvir, 2013).

Table 3.1.3: Examples of self-assembling peptides.

Peptide Sequence / References	**Activity**
β-Hairpin peptides (Loo, 2013)	β-Hairpin secondary structures (two β-strands linked by a kink) can be rationally designed to self-assemble into ibrillary macromolecular scaffolds

Fmoc-peptides (Orbach, 2009)	Fluorenylmethoxy-carbonyl (Fmoc)-protected amino acids and dipeptides can form hydrogels. The Fmoc moiety is widely used as a protecting group in peptide synthesis and it was even reported that a number of Fmoc-amino acids show anti-inflammatory properties
β-sheet (Loo, 2013; Hauser & Zhang, 2010)	β-sheet peptides are ionic self-complementary, as a result of positive and negative side chains on one side of the β –sheet and hydrophobic side chains on the other. This motif causes the peptides to fold into β-sheet secondary structures with distinct hydrophobic and hydrophilic surfaces. The hydrophobic side forms a double sheet inside of a nanofiber and the hydrophilic side becomes the exterior of the nanofibers, which interacts with water molecules to form an extremely high water content hydrogel. The 4 ionic self-complementary peptides RADA16-I, RAD16-II, EAK-I and EAK16-II form stable β –sheet structures in water and undergo spontaneous assembly to form nanofiber scaffolds
α-helical coiled coil (Stephanopoulos, 2013)	α-helices result from the hydrogen bonding of a peptide backbone amide hydrogen to a backbone carbonyl four residues away. This motif results in a right-handed helical twist configuration. The formation of α-helices is determined by the amino acid sequence and is dependent on hydrophilic and hydrophobic amino acid residues organizing into a hydrophobic and a hydrophilic face. The gelation can be controlled using heat to provide a versatile scaffold for cell culture, especially when gel dissolution at elevated temperatures is desired. The α-helical peptide gels proved highly cytocompatible, and PC12 cells cultured in them were able to proliferate and differentiate
Peptide amphiphiles (Cui, 2010; Matson, 2011; Matson & Stupp, 2012)	Peptide amphiphiles are short peptide sequences attached to a hydrophobic tail, usually an alkyl chain. Peptide amphiphiles combine the structural features of amphiphilic surfactants with the functions of bioactive peptides and are known to assemble into a variety of nanostructures

3.1.3.4 Therapeutic Peptides and Market

The obstacles that remain for the development of peptides as therapeutics are significant. As in all drug development, *in vivo* efficacy might differ from promising *in vitro* models. The reason can be the result of interactions with many *in vivo* components in the circulation, gastrointestinal tract, dermis, relative clearance and affinity to target receptors (Lin & Lowman, 2003).

The main limitations generally attributed to therapeutic peptides are (i) oral bioavailability (injection is normally required), (ii) short half-life

because of rapid degradation by proteolytic enzymes of the digestive system and blood plasma, (iii) rapid removal from the circulation by the liver or kidneys, and (iv) high synthetic and production costs (Bray, 2003; Picherean & Allary, 2005).

However, therapeutic peptides are becoming increasingly attractive for the discovery and development of new generations of drugs. With the recent addition of new methodologies, peptides can be engineered to have long plasma half-lives and low immunogenicity (Salo, 2006), along with alternative routes of administration. Production of synthetic therapeutic peptides has become possible for the pharmaceutical industry with recent developments of SPPS, initially developed by Merrifield. SPPS is crucial in the early steps of preclinical research and in the production of peptide-based active pharmaceutical ingredients (APIs) (Bruckdorfer, 2004). SPPS is especially suited for medium-sized peptides (up to 80 amino acids residues), which comprise the majority of therapeutically-relevant peptides.

3.1.3.4.1 Chemical Strategies to Improve Peptide Biological Activity

To develop a peptide as a therapeutic agent, the crucial parameters to consider are its biological effect, pharmacokinetic profile and low immunogenicity. Various chemical strategies have been developed to try and overcome the limitations of peptides by increasing *in vivo* plasma residence time (Table 3.1.4) (Vlieghe, 2010).

The chemical optimization strategy of a therapeutic peptide is based on structure-activity relationship (Witt, 2001; Ladner, 2004; Wittand & Davis, 2006) with the aim of improving bioavailability, reducing elimination and biodegradation as well as increasing selectivity or affinity to its target or receptor. According to Lipinski's rule of five (Lipinski, 1997; 2000; 2004), completed by Veber analysis (Veber, 2002), peptides are poor candidates to move from the digestive tract to the circulatory system based on their physicochemical properties. Until a few years ago, therapeutic peptides were generally administered by subcutaneous, intramuscular or intravenous routes to circumvent the gut barrier. When administered orally, peptides have to face a strongly acidic gastric environment, high levels of intestinal proteolytic activity

and a high intestinal permeability barrier (Woodley, 1994).

Table 3.1.4: Lead peptide chemical optimization (adapted from Vlieghe, 2010).

Lead	Attribute
1	Blocking N- or C-terminal ends by N-acylation, N-pyroglutamate, C-amidation and so on, or addition of carbohydrate chains (glycosylation: glucose, xylose, hexose and so on) to increase plasma stability (notably, resistance towards exopeptidases)
2	Search for the minimum active sequence (MAS) from N- and/or C-terminal truncated analogues
3	Simplification and/or optimization of the structure after alanine scanning (Ala-scan) and/or D-scanning (D-scan) to eliminate potential sites of cleavage (notably by endopeptidases) and to determine important functional groups involved in the interaction with the target of interest
4	Isosteric, or not, amide bond replacement between two amino acids: NH-amide alkylation, the carbonyl function of the peptide bond can be replaced by CH_2 (reduced bond: -CH_2-NH-), C(S) (endothio-peptide, -C(S)-NH-) or PO_2H (phosphonamide, -P(O)OH-NH-). NH-amide bond can be exchanged by O (depsipeptide, -CO-O-), S (thioester, -CO-S-) or CH_2 (ketomethylene, -CO-CH_2-). The peptide bond can also be modified: retro-inverso bond (-NH-CO-), methyleneoxy bond (-CH_2-), thiomethylene bond (-CH_2-S-), carba bond (-CH_2-CH_2-), hydroxyethylene bond (-CHOH-CH_2-) and so on, to increase plasma stability of the peptide sequence (notably towards endopeptidases)
5	Cyclization of the peptide sequence (between side chains or ends of the peptide sequence: head to tail, N-backbone to N-backbone, end to N-backbone, end to side chain, side chain to N-backbone, side chain to side chain) through disulfides (disulfide-bond cyclization scan), lanthionine, dicarba, hydrazine or lactam bridges, to decrease the conformational flexibility of linear peptides and reduce hydrogen bonding, enhance membrane permeability, and most importantly to increase their stability against proteolysis by endo- and exopeptidases
6	Substitution of a natural amino acid residue by an unnatural amino acid (D-configuration), an N-methyl-α-amino acid, a non-proteogenic constrained amino acid or a β-amino acid, to increase plasma stability (e.g. resistance to endopeptidases) of the peptide and/or affinity (activity) for its target
7	Deletion of one or more consecutive amino acid(s) and combinatorial deletion with two or more positions omitted independently throughout the sequence

8	Significance of the N- and C-terminus
9	N-terminal esterification (phosphoester) or pegylation modifications to enhance plasma stability (e.g. resistance to exopeptidases) and to reduce immunogenicity. Pegylation is also designed to make the peptide larger (generally > 50 kDa) to retard excretion through the kidneys (renal clearance)

Alternative routes for the administration of peptide-based drugs have been improved in recent years and novel peptide delivery technologies have emerged, including controlled-release parental drug, mucosal route, oral route and transdermal route (Pettit & Gombotz, 1998). In spite of some limitations, synthetic therapeutic peptides present numerous advantages compared with their homologous compounds (proteins and antibodies) and with small organic molecules, thanks to progress in chemical synthesis and routes of administration (Vlieghe, 2010). In Table 3.1.5, the principal advantages of peptides over proteins/antibodies or over small organic molecules that make up traditional medicines are presented.

Table 3.1.5: Advantages of peptides over other drug candidates as proteins/antibodies or organic molecules (Loffet, 2002; Lien & Lowman, 2003; Witt & Davis, 2006; Vlieghe, 2010; McGregor, 2008; Hummel, 2006).

Proteins and antibodies	Organic molecules
✓ Peptides have the potential to penetrate further into tissues owing to their smaller size and charge	✓ Organic molecules often represent the smallest functional part of a protein, offering greater efficacy, selectivity and specificity
✓ Therapeutic peptides are generally less immunogenic than recombinant proteins and antibodies	✓ The degradation products of peptides are amino acids (minimizing the risks of systemic toxicity or drug-drug interactions
✓ Lower manufacturing costs (synthetic vs. recombinant production)	✓ Because of their short half-life, few peptides accumulate in tissues (reduction of risks of complications caused by their metabolites)
✓ Higher activity per unit mass	

- ✓ Lower royalty stack
- ✓ Greater stability
- ✓ Produced potential for interaction with the immune system
- ✓ Better organ or tumor penetration.

- ✓ Peptides derived from natural source are receptor agonists, and generally, small quantities of these peptides are necessary to activate the targeted receptors

3.1.3.4.2 Market

The USA and EU are the major markets for drugs of all kinds, so first approvals for peptide therapeutics have occurred primarily in one of these two regions. All 19 peptides approved in the USA during the period 2001 to 2012 were first approved in either the USA or EU. The notable expansion of peptide therapeutic development in the late 1990s and 2000s led to an unprecedented number of marketing approvals in 2012, and has provided a robust pipeline that should deliver numerous approvals during the remainder of the 2010 (Kaspar & Reichert, 2013).

In the US, annual sales of peptide drugs exceed 13 billion, representing 1.5% of drug sales globally. Protein drugs such as therapeutic antibodies also represent a larger share with the combined biopharmaceutical market valued at over 70 billion. In Europe, Germany and the UK account for 63% of the peptide therapeutic market with France, Italy, Scandinavia and Spain making up the rest of the major users (Hervé, 2008).

According to a review by Vlieghe, more than 60 synthetic therapeutic peptides (including those used for medical diagnostics or imaging) with a size < 50 amino acids, have reached the American, European and/or Japanese pharmaceutical markets through marketing authorization as APIs (Vlieghe, 2010).

The peptides at each phase of development are undergoing evaluation for a wide variety of indications (Fig. 3.1.14). The diversity of therapeutic areas (TAs) represented is substantially higher in Phase I and II compared with Phase III, which is at least in part because of the

greater number of peptides in early-stage compared with pivotal studies. The top two are metabolic disease and oncology for the Phase I and II peptides, whereas oncology and infectious disease are the top two TAs for peptides in Phase III (Kaspar & Reichert, 2013).

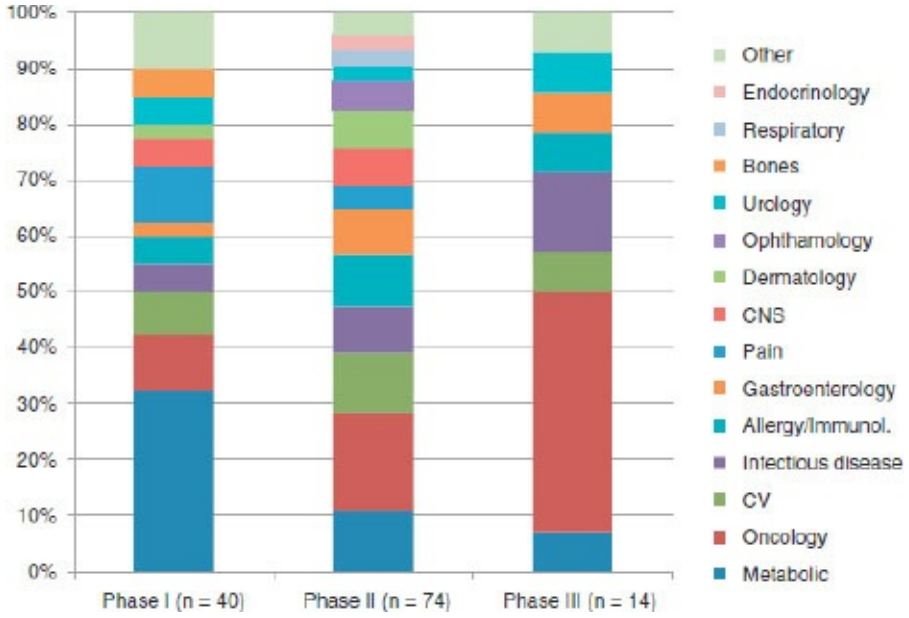


Figure 3.1.14: Therapeutic areas for peptides in clinical studies (on 15 February 2013; CNS-central nervous system; CV-cardiovascular; adapted from (Kaspar & Reichert, 2013).

The commercial value of peptide therapeutics that have been marketed for years is well-established, but could substantially increase as recently approved products and those on the horizon gain market share. Peptides will probably be used intensively in the near future for various applications in the treatment of CNS diseases, notably in the design of peptide regulators of protein activity, if they are able across the BBB by absorptive-mediated transport (Hervé, 2008; Pardridge, 2003). CMT- or RMT-based peptides (de Boer & Gaillard, 2007a; 2007b) developed for CNS drug targeting will also certainly contribute to the development of peptide-based prodrugs with facilitated access to the CNS. Also, in current trials conducted to treat cancer, gene disorders, and autoimmune diseases, CPP-based therapies are being used. Gene-targeted therapies have a huge potential to fight rare genomic diseases and will be associated to the usage of CPP to deliver therapeutic agents into cells (Figueiredo, 2014).

3.1.4 Conclusions

Many drugs see medicinal application denied due to physical, pharmacokinetic or pharmacodynamic properties. The use of different strategies to improve solubility, stability, permeability and targeting problems in drug discovery and development is crucial. The implementation of a prodrug approach in the early stages of drug discovery is a growing trend. Prodrugs are bioreversible derivatives of drug molecules that undergo an enzymatic and/or chemical transformation to release the parent drug. There are several promoieties that can be use in prodrug design, where the selection of a specific promoiety is essential. The ultimate goal is a chemically stable prodrug that does not generate toxic metabolites after bioconversion. Some of these works were based on amino acid applications. Amino acids are basic constituents of a cell structure and require specialized transport systems to cross the plasma membrane. Over the past few years, several amino acid transport systems have been identified and classified on the basis of their substrate affinity, dependence on sodium ions, energy and pH. The low toxicity of amino acids makes them attractive carriers for the development of prodrugs for poorly absorbed therapeutic agents. To make the prodrug a substrate for carrier mediated transport, an amino acid promoiety also balances the water solubility of the prodrug.

New synthetic strategies for limiting metabolism and alternative routes of administration have emerged in recent years and resulted in a large number of peptide-based drugs now being marketed. Peptide-based drug discovery could be a serious option for addressing as-of-yet unresolved problems. New peptide therapeutics are delivering significant commercial value in a broad range of indications including expanding markets such as diabetes and oncology, as well as orphan indications.

Developments in solid-phase peptide synthesis (SPPS) using Fmoc chemistry have led to larger peptide molecules and small proteins being accessible by chemical synthesis on scales of multiple kg. The great advantage of SPPS over solution-phase synthesis is the simplicity, readiness and efficiency of the synthetic process. In solution-phase synthesis, each synthetic intermediate has to be isolated and purified after every reactional step, which leads to a very expensive and time consuming process. Furthermore, it is not convenient to use a large

excess of reagents, since it significantly complicates the necessary purifications. Following a SPPS strategy, the synthesis is significantly less time consuming as there is no need to isolate the synthetic intermediates. The global yield also increases, since the whole process is performed in the same reaction vessel and the use of surplus reagents is admissible as they are later removed by washing and filtration of the peptidyl-resin contained in the vessel, which is usually attached to a vacuum filtration system.

The progression of peptide compounds into clinical therapy goes along with the increasing need for new therapeutic strategies due to the limitations of current biomaterials for engineering complex tissues. The chemical synthesis of peptides enables the conjugation of other small molecules or incorporation of non-natural amino acids by design. Conjugation of small molecules to a peptide opens up the possibility for greater chemical diversity, analogous to small-molecule medicinal chemistry approaches for developing high-affinity, high-specificity molecular recognition increasing the range of potential applications of these molecules in the growing area of tissue engineering.

Although the interaction of CPPs with cellular membranes often determines their uptake efficiency, CPPs are first and foremost delivery vectors. This means that they must efficiently deliver various cargo molecules to their designated location, whether it is in the cytoplasm or nucleus. CPPs were discovered 20 years ago based on the potency of several proteins to enter cells. In recent years CPPs have been associated with biodistribution and efficacy of oral biodrug delivery. Nevertheless, the recent progress of CPPs as new carriers for intracellular cargo delivery were focused in tumor cells, where only small quantity of drugs were used as cargo. We propose that new methodologies will be used to design and develop CPP-drug complexes, in that CPPs transport their cargo inside cells using endocytosis. Each modification will be carefully designed to avoid problems that could lead to low synthetic yields, poor solubility, aggregation or toxicity. The *in vivo* delivery of drugs (as cargo) requires a longer drug circulation time, which in turn requires a more stable complex between the CPP and its drug, as it introduces different hydrophilic groups to an already cell-permeable peptide. When using CPPs as a delivery system, one must consider that drug delivery must

often be highly specific.

Chemical strategies can improve the *in vivo* plasma residence time and, after that, introduce new peptides as drugs in the market. This way, the commercial value of peptides therapeutic will be well-established.

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James J. Chambers*

3.2 Targeting Calcium-mediated Excitotoxicity in the CNS

3.2.1 Introduction

At the heart of our ability to recognize, remember, and perform basic motor functions, is the highly coordinated placement of neuronal receptors at the interface between adjacent cells that control the flux of metal ions across the membrane (Kerchner & Nicoll, 2008; Ribrault, 2011; Wang, 2012). Glutamate is the neurotransmitter that mediates the majority of fast, excitatory neurotransmission in the central nervous system (Cotman & Monaghan, 1986). Vesicles of glutamate are released from the presynaptic cell when action potentials depolarize the membrane of synaptic boutons (terminals) and vesicles fuse with the membrane (Debelleroche & Bradford, 1977). Glutamate then diffuses across the synapse and activates postsynaptic glutamate receptors, initiating postsynaptic current flow via ion entry and depolarizing that cell, thus completing chemical synaptic communication (Moore & Buchanan, 1993). Despite decades of inquiry, we still do not fully understand the elegant electrical and chemical neuronal signals that are generated in a highly complex tangle of minuscule structures.

Communication between cells in the nervous system requires neurons to quickly alter their membrane potential, transiently entering a depolarized, excited state to pass action potentials along to neighboring neurons, and then rapid repolarization to be prepared for new incoming information. Over-excitation of neurons by various means maintains a depolarized state for longer than usual and can contribute to neurological disease. Some of these diseases, which are linked to neuronal over-activity, can also cause cell damage and death via a process called excitotoxicity. Excitotoxicity is thought to play a role in neuronal cell and network damage following stroke and traumatic brain injury and, quite possibly, in the progressive damage associated with a

number of congenital and sporadic neurodegenerative disorders such as Alzheimer's, stroke, Huntington's, Parkinson's, Amyotrophic Lateral Sclerosis (ALS), and Multiple Sclerosis (MS).

Receptors located at the neuronal membrane help regulate these fluctuations in membrane potential by controlling the opening and closing of transmembrane ion channels, which variously allow cations and anions to flow into and out of the neuron down their electrical and chemical concentration gradient. During typical communication at an excitatory synapse, the presynaptic cell releases glutamate, the primary excitatory neurotransmitter, after an influx of calcium that enters through voltage-gated calcium channels. Glutamate crosses the synaptic cleft by diffusion and binds to receptors on the postsynaptic cell. Some glutamate receptors are ionotropic ligand-gated channels that are found on the postsynaptic neuron. When an agonist such as glutamate binds, the associated ion channel opens, allowing monovalent cations, primarily sodium, to flow into the neuron. As the positive charge flows into the neuron, the membrane potential of that postsynaptic cell depolarizes, and if it is sufficiently depolarized, other receptors located nearby on the membrane will open as well, eventually allowing calcium to enter the postsynaptic cell.

Glutamate is the most prevalent excitatory neurotransmitter in the central nervous system (CNS) and glutamate receptors (GluRs) play a key role in both normal excitatory neurotransmission, and in modulating both normal and constructive plasticity as well as excitotoxic biochemistry, the main topic of this chapter (Cotman & Monaghan, 1986; Debelleroche & Bradford, 1977; Moore & Buchanan, 1993). There are two major types of GluRs that differ in the way they influence neuronal response to excitatory neurotransmission. The first are the metabotropic glutamate receptors (mGluRs) which, upon binding to glutamate, set off an intracellular biochemical cascade via second messenger signaling. While mGluRs are certainly targets of interest for negating the excitotoxic effects of over-stimulation, they are not the focus of this work, however, a well-written and expansive review of mGluRs and pharmacological agents was published recently (Williams & Dexter, 2014). Of more importance here, ionotropic glutamate receptors (iGluRs) mediate fast responses to glutamate release and also, as their

name implies, allow ions to enter or leave the cell via an intrinsic ion channel. iGluRs are typically heteromultimeric, integral membrane proteins that are composed of four subunits that form a transmembrane ion channel. This channel is allosterically connected to the agonist-binding site and upon binding to glutamate or an exogenous agonist, the channel portion opens and allows cations to pass into the cell which directly depolarize the membrane potential.

3.2.2 Glutamate and Glutamate Receptors

The postsynaptic iGluR composition has been found to mediate the ionic makeup of the current that results from glutamate binding. The iGluRs are subdivided into NMDA receptors and non-NMDA receptors with the latter being further divided into AMPA receptors and kainate receptors. The AMPA receptors, named after the synthetic agonist α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid, are located on the acceptor, or postsynaptic, dendrite where they bind neurotransmitters, causing a conformational change in the receptor protein structure that opens intrinsic sodium ion channels. AMPA receptors are responsible for the vast majority of excitatory neurotransmission and they open quickly in response to glutamate and close fast as well. The influx of sodium ions alters the electrical potential of the postsynaptic cell, producing a local signal that, after summation and integration at the neuron and then circuit level, ultimately results in cognition or action. Another family of iGluRs is the kainate receptors. These appear to be more related to AMPA receptors, but they are still the subject of ongoing research and their roles in either normal signaling or excitotoxicity has not been fully explored. If the cellular polarization is above a certain threshold, neighboring NMDA receptors, named after the synthetic agonist *N*-methyl-D-aspartate, fully or partially eject an ion channel-bound magnesium ion allowing calcium to flow into the cell. The entry of calcium then sets off myriad intracellular events, not least of which is activation of calcium-activated kinases and, eventually, synaptic potentiation (Lisman, 2012). A change in the communication strength between adjacent neurons arises from trafficking of receptors to or from the synaptic zone and it is presently thought that this molecular

trafficking plays a crucial role in synaptic plasticity, which, in turn, is believed to underlie memory formation and loss.

3.2.2.1 AMPA Receptors

AMPA receptors are assembled from subunits GluA1 through GluA4 and are typically comprised of heterotetramers, quite often containing GluA2 subunits. These receptors are known to play a critical role in long-term potentiation (LTP), one of the mechanisms thought to underlie memory formation as well as maladaptive and even neurodegenerative plasticity (Kang, 2009). Most AMPA receptors are not permeable to calcium ions, due to the presence of the GluA2 subunit. In the GluA2 subunit RNA, a key modification is made to translate a codon to arginine instead of a glutamine in the ion channel pore. The incorporation of arginine in the ion conduction pore results in a channel that does not allow divalent cations, such as calcium, to pass.

A subset of AMPA receptors that are currently of considerable interest in the neurobiology field and in particular the field of neurodegeneration are those that lack the GluA2 subunit. GluA2-containing channels are quite often calcium-impermeable due to an mRNA codon editing event (Araki, 2010; Isaac, 2007). The precise role of GluA2-lacking receptors in synaptic plasticity or, for that matter, normal synaptic signaling, is still the subject of ongoing debate in the field (Henley, 2011). While it is thought that most functional AMPA receptors contain an edited GluA2 subunit, there is now considerable evidence for a subpopulation of GluA2-lacking receptors that result in calcium-permeable AMPA receptors (Keller, 1992; McDermott, 2003; Szabo, 2012; Wright & Vissel, 2012). The enzyme Adenosine deaminase acting on RNA 2 (ADAR2) specifically mediates RNA editing of the glutamine/arginine (Q/R) site of GluA2 subunit of the AMPA receptors and it has been found that motor neurons expressing Q/R site-unedited GluA2 undergo slow death in conditional ADAR2 knockout mice (Hideyama, 2012). This line of research also found that unedited GluA2 mRNA was expressed in a large proportion of motor neurons from ALS patients while motor neurons from normal and disease control subjects expressed only edited GluA2 mRNA. ADAR2 was significantly down-regulated in all the motor

neurons of ALS patients, more extensively in those expressing Q/R site-unedited GluA2 mRNA than those expressing only Q/R site-edited GluA2 mRNA. These findings suggest that antagonism of calcium-permeable AMPA receptors could slow disease progression, a topic we will discuss later in this manuscript.

3.2.2.2 Kainate Receptors

Kainate receptors are also heterotetrameric ion channels assembled from subunits GluA5-7 and KA1-2 (also known as GluK1-2). The properties of kainate channels are similar to AMPARs in that they allow ion flux if glutamate activates the channel. Further, kainate receptors are mostly impermeable to calcium. One main difference between AMPA and kainate receptors is their subcellular location. While most AMPA receptors are located at the postsynaptic membrane, kainate receptors have been found on the presynaptic side as well as the postsynaptic side. It remains unclear if presynaptic kainate receptor stimulation by glutamate results in more or less vesicular release of glutamate into the synapse, though in the case of neuronal necrosis and ischemia, it very likely does. These are discussed later in this chapter. The function of postsynaptic kainate receptors is similar to that of AMPA receptors in that depolarization via glutamate-evoked kainate channel current can unplug the magnesium ion blocking NMDA receptors, thus leading to calcium entry into the postsynaptic spine.

3.2.2.3 NMDA Receptors

NMDA receptors are heterotetramers and are assembled from three major families of subunits. The NR1 subunit is ubiquitously expressed, there are four NR2 subunits (A-D), and two NR3 members (A and B). Most NMDA receptors that have been studied to date contain both NR1 and NR2 subunits and they share the common attributes of being activated by glutamate but normally being blocked by a magnesium ion that is removed upon strong stimulation of the postsynaptic spine. Besides glutamate, it was found that glycine was a required co-agonist to elicit NMDA channel opening.

NMDA receptors have slow gating kinetics, but once open, are highly permeable to both calcium and sodium. Because of the large ion permeability to calcium, this influx generates intracellular calcium waves or transients (also the term “calcium spikes” is popular in the literature) that set off myriad calcium-mediated biochemical cascades. The NMDA receptors are bound to postsynaptic cytoskeletal scaffolding proteins that are collectively known as the postsynaptic density. This scaffolding also supports kinases and other proteins that have downstream signaling roles, likely to receive the calcium transient as efficiently as possible. Under normal circumstances, glutamate is quickly removed from the synaptic cleft by excitatory amino acid transporters (EAAT) located on nearby glial cells. This ensures that only a limited amount of calcium will be able to enter the postsynaptic neuron.

3.2.3 The Role of Calcium in Normal Neuronal Biochemistry

NMDA receptors are opened when the neuronal membrane potential is depolarized to a sufficient level to expel the bound magnesium ion. Opening of these ionotropic receptors leads to a rise in postsynaptic calcium concentration, which has been linked to long-term potentiation (LTP) via protein kinase activation. It is thought that strong depolarization fully expels the magnesium plug, whereas milder depolarization partially displaces the magnesium, resulting in less access to calcium through NMDA receptors. This latter process is thought to underlie long-term depression (LTD), in which lower concentrations of postsynaptic calcium activates protein phosphatases and initiates partial dismantling of the synaptic connection.

The activated protein kinases that arise from the high calcium concentration phosphorylate a variety of postsynaptic targets including some AMPA receptors. These post-translational modifications have been shown to enhance single channel conduction through these channels, thereby potentiating the connection between these two neurons. Further, these kinases can phosphorylate the protein machinery that determines the number of postsynaptic AMPA receptors, thus opening

more “docking” sites for new AMPA receptors from nearby locations. In the end, the result of controlled calcium entry into a synaptic spine is more AMPA receptors that are more permeable to cations. Of course, protein phosphatases can act to reverse these potentiation modifications to return the synapses to the pre-LTP levels.

Besides direct activation of postsynaptic kinases, calcium entry can activate a second messenger cascade to turn on gene transcription of proteins such as CaMKII and PKAII leading to relatively high local concentrations of these kinases in the synaptic spine. Both of these kinases have been found to play a role in LTP processes, such as spine volume, addition of new AMPA receptors, and direct phosphorylation of ion channels. It is this local effect of LTP that allows for selective potentiation of single synapses and, because of this, each synaptic spine could be considered isolated from other postsynaptic zones as all of the changes in kinase activity are highly restricted to that local spine. This addressable and highly spatial system is likely related to long-term memory storage.

3.2.4 Excitotoxicity

A common molecular mechanism in both acute and chronic neurodegenerative diseases including stroke, Alzheimer’s, Parkinson’s, amyotrophic lateral sclerosis, multiple sclerosis, and others is the excitotoxic loss of dendritic synaptic spines and eventual neuronal death. Dendritic synaptic spines are the postsynaptic regions of a neuron that receive the chemical signal from the presynaptic cell, in this case via glutamate diffusion. These spines are essential to brain function, such as memory formation and related neural plasticity mechanisms. Pruning or loss of synaptic spines may occur through misdirected voltage- or ligand-gated ion channels. The excitotoxic effects of calcium have long been thought to be due to excess quantities of calcium, but evidence is accumulating that the route by which calcium enters the cell may be at least as important (Szydlowska & Tymianski, 2010). Recent research suggests that in Huntington’s disease, a neurodegenerative disorder, calcium entering neurons via

NMDA receptors located outside the synapse (extrasynaptic NMDA receptors) may initiate different and more damaging molecular cascades than calcium entering neurons via synaptically-located NMDA receptors (Hardingham & Bading, 2010).

3.2.5 The Role of Calcium in Excitotoxic Neuronal Biochemistry

Controlled increases in glutamatergic activity can result in increased postsynaptic calcium, which plays a critical role in neuronal processes supporting learning and memory. Over-activation of glutamate receptors, however, can lead to excitotoxic concentrations of calcium. This can lead to neuronal death and degeneration through a variety of molecular mechanisms. Intracellular calcium activates a number of enzymes that can have deleterious effects including phosphatases, proteases, lipases, caspases, and endonucleases and eventually the cell can undergo apoptosis through activation of caspases. Additionally, the NMDA receptor interacts directly with nitric oxide synthase, an enzyme sensitive to calcium influx that induces the production of toxic nitric oxide along with reactive oxygen species (Szydlowska & Tymianski, 2010).

One of the earliest findings of pathological excitotoxicity was the release of glutamate into extracellular space minutes after spinal cord injury by neurons that were at the site of injury. This non-controlled glutamate release can stimulate glutamate receptors, further stimulating the local cohort of neurons. In stroke patients, blood flow is reduced to inadequate levels and the concentration of extracellular glutamate rises, causing uncontrolled excitation of glutamate receptors. This excitatory insult is thought to be exacerbated by a lack of oxygen and glucose to power the machinery to remove the glutamate from the effected area.

The effect of the increased concentrations of glutamate in the extracellular space is that neurons are strongly depolarized to the point where the removal of the magnesium ion that normally blocks NMDA receptors occurs. This, of course, then results in uncontrolled calcium entry through the NMDA receptors into a previously unaffected neuron, thus propagating the cellular insult. In addition, it has been shown that

the NMDA receptors found on oligodendrocytes, the cells that provide myelin sheaths the axons in the CNS, are activated as well, thereby exacerbating excitotoxicity. Once in the cell, the increased calcium activates kinases, but also has an effect on mitochondria. In response to high calcium, the mitochondrial permeability transition pore is opened and it is presently thought that pore opening may allow the release of reactive oxygen species, further contributing to apoptosis and slowing or halting the local production of adenosine triphosphate (ATP). Lack of ATP quickly eliminates the electrochemical gradient across the membrane of some ions, which in turn shuts down glutamate transporters, the proteins that remove glutamate from extracellular space, to terminate glutamatergic signaling. The result of this excitotoxic cascade is the accumulation of glutamate in the extracellular milieu which can spread to neighboring neurons.

3.2.6 Diseases that are Potentially Exacerbated by Calcium-mediated Excitotoxicity

3.2.6.1 Amyotrophic Lateral Sclerosis (ALS)

Calcium dysregulation is a central factor in the incurable neurodegenerative disorder ALS (Grosskreutz, 2010). This disorder results in muscle atrophy followed by paralysis and eventual death of patients 3-5 years after the presentation of symptoms. The underlying cause of ALS is the selective degeneration of motor neurons in the spinal cord and brain stem as well as the motor cortex. Degeneration of these cells results in difficulty of movement, speech, and swallowing and progresses to problems with respiratory function. A dismaying psychological factor is that patients with ALS typically suffer no cognitive deficit and are thus fully cognizant of their own demise as the disease progresses inexorably toward death. Present numbers on the prevalence of ALS indicate that the adult lifetime risk for ALS is near 1 in 400, a number which is similar to that for multiple sclerosis (Benatar & Wuu, 2012).

The best known genetic factor for familial ALS, occurring in about

20% of the cases, are mutations in a gene that encodes the copper/zinc-dependent superoxide dismutase protein (SOD1). In more recent studies, further mutations have been found by using genome wide association studies of patients afflicted with ALS, however, in many of these cases, the underlying molecular pathology is not yet known (Renton, 2014). Sporadic ALS does not have such target mutations, however, there are a few examples of environmental exposures that can result in ALS or ALS-like neurodegeneration and new gene targets with incomplete penetrance (Turner, 2013). One of the striking characteristics of familial ALS as well as SOD1 mutant mouse models is the high degree of selectivity that the disease shows for motor neurons over all other cells in the body, even though the mutant form of SOD1 protein is in many other cell types in the patient. A possible mechanism for this selectivity is based on excitatory neurotransmitter-mediated neuropathology, in particular glutamate. It is presently believed that motor neurons are especially susceptible to excitotoxicity because they are known to receive strong glutamatergic input. Additionally, spinal motor neurons have recently been found to express calcium-permeable AMPA receptors on their surface. Multiple lines of neurobiological research have resulted in the current understanding that calcium-permeable AMPA receptors play crucial roles in synaptic signaling and plasticity in the CNS. An overabundance of these receptors, coupled with glutamatergic excitation, could overwhelm the calcium buffering capacity of a cell, resulting in metabolic/mitochondrial breakdown followed by cell death.

Presently, riluzole (Rilutek) is the only United States Food and Drug Administration approved therapeutic to treat ALS. The specific biological target of riluzole is controversial, but it is thought that the drug acts by reducing excitatory neurotransmission, resulting in the influx of less calcium into motor neurons slowing the progression of the disease. Unfortunately, riluzole is not a cure and only offers some of those suffering with ALS around 3 additional months of life.

3.2.6.2 Multiple Sclerosis (MS)

MS is an inflammatory disease of the myelin sheath that insulates neurons in the brain and spinal cord. The breakdown of this insulator

erodes the ability for neurons to communicate and results in a wide variety of symptoms in patients suffering from MS, ranging from physical disability to psychiatric and cognitive deficits. The progression of MS has been observed to occur either in isolated “attacks” and a slower degenerative progression. In each case, permanent damage is done as the disease advances, however, it appears that some functions can be relearned likely through network plasticity.

Presently, the molecular cause of MS is not fully elucidated but much evidence points to a failure of the immune system in which the myelin-producing cells are attacked. Glutamate-mediated excitotoxicity has also been implicated as a root cause. As discussed previously, the oligodendrocytes, the cells that provide the myelin sheath for neurons in the CNS, are also susceptible to excitotoxicity. In a recent study in an animal model of MS, glutamate receptor blockers were found to increase the survival of oligodendrocytes and prevent some of the molecular mechanism from over-stimulation (Suhs, 2014).

3.2.6.3 Alzheimer’s Disease (AD)

Characterized by progressive death and degeneration of neurons, Alzheimer’s disease leads to increasing memory loss and eventual dementia. A small percentage of cases (Ballard, 2011) have been found to be caused by a congenital defect and these cases are classified as Familial Alzheimer’s Disease (FAD). The majority of Alzheimer’s disease cases are classified as sporadic Alzheimer’s and there is presently no known direct cause. Two classic indications of the disease that can be observed in brain tissue of both FAD and sporadic Alzheimer’s sufferers are the presence of neurofibrillary tangles of hyperphosphorylated tau inside of neurons and plaques consisting primarily of extracellular aggregations of beta-amyloid (Aβ) peptide fragments.

Aβ clusters have been found to influence glutamate transmission in several ways, some of which could lead to excessively high concentrations of calcium and thus excitotoxicity. It was shown that the presence of Aβ impairs the ability of glutamate transporters to help remove glutamate from the extracellular area around the synapse, potentially resulting in excitotoxic effects (Li, 2009). Aβ clusters also

appear to increase the population of expressed NMDA receptors containing the NR2B subunit and the number of metabotropic mGluR5 receptors present at the synapse, which could also lead to excitotoxicity. Recent research has also suggested that extrasynaptic NMDA receptor activation could lead to an increase in Aβ production, unlike synaptic NMDA receptor activity. Further complicating matters is the fact that the different groups (group I and group II) of metabotropic glutamate receptors (mGluRs) have differing effects on Aβ production by neurons (Hu, 2012). Further, two observed effects of Aβ are not directly linked to excitotoxicity, but further implicate glutamatergic transmission in AD. When cultures of neurons that were collected from the hippocampus, an area of the brain critical to memory formation, were exposed to sub-nanomolar concentrations of Aβ, it was found to promote glutamate release in an activity dependent manner. While this may possibly be a normal function as it would support the cellular and molecular processes underlying learning and memory, other experiments on hippocampal neurons found that high nanomolar concentrations of Aβ clusters bind to the postsynaptic spines of glutamatergic synapses. This binding is associated with the internalization of AMPA receptors, which can lead to the loss of the synaptic spine, and thus that synaptic connection. This synaptic loss would reduce the ability of affected neurons to communicate with each other, and could impair the neuronal connections that support memory formation and maintenance. There is also some evidence that hyperphosphorylated tau may interfere with iGluR trafficking, which could also impair synaptic formation and maintenance (Hu, 2012).

3.2.6.4 Huntington's Disease (HD)

Huntington's disease is an inherited degenerative disorder that results in the death of cortical and striatal neurons, specifically certin GABA-releasing neurons known as GABAergic medium-sized spiny neurons (Mony, 2009). The loss of these neurons results in a range of physical, psychological, and cognitive symptoms. Involuntary spastic movement and difficulty initiating and controlling voluntary movement are common in the early stages of this progressive disorder. These

symptoms are often accompanied by cognitive decline. The cause of the disease is a mutation in the gene that encodes the huntington protein, which results in the production of a longer than normal sequence of glutamine residues at the N-terminus of the protein (Okamoto, 2009). Although the exact mechanism by which the mutation causes the progressive death of these neurons remains unknown, some evidence suggests that the mechanism may be a dysregulation in expression of NMDA receptors at synapses and, more importantly, expression of these receptors at extrasynaptic locations. When intracellular calcium concentrations are increased due to extrasynaptic NMDA receptor activity, the calcium appears to trigger different downstream cascades than calcium ions that enter the neuron through synaptic NMDA receptors. As discussed previously, controlled increases in calcium that result from synaptic activity can activate beneficial, synapse building pathways; equivalent increases through extrasynaptic NMDA receptor activity were shown to cause disruptions to mitochondrial membrane potential and cell death (Hardingham & Bading, 2010; Milnerwood, 2010). It has been suggested that weak glutamate antagonists such as memantine, or NR2B subunit specific antagonists, may provide a clinically relevant outcome if they can be designed to block extrasynaptic NMDA receptors without preventing normal synaptic NMDA receptor activity (Mony, 2009).

3.2.6.5 Stroke

During a stroke, the blood flow to part of the brain is disrupted either due to a blockage in a blood vessel feeding the brain (ischemic stroke) or due to a rupture in one of these vessels (hemorrhagic stroke). Neurons in the area of the brain that are supplied by the affected vessel are often deprived of the required supply of oxygen and glucose and are thus unable to continue operating the energy-demanding ion transporters that establish the negative membrane potential. With a loss of these pumps, the membrane begins to depolarize and when it has sufficiently depolarized to the point that magnesium plugs have been ejected from the NMDA receptors, calcium ions enter the neuron at the slightest exposure to glutamate. As already discussed, this calcium entry then

initiates excitotoxic molecular mechanisms (Lipton, 2006).

After a stroke, excess calcium is also able to enter neurons by a non-glutamate receptor pathway. The Transient Receptor Potential (TRP) channels allow calcium to enter neurons in response to ischemic-linked changes in extracellular levels of divalent cations, pH, and levels of reactive oxygen species. Further, the stored calcium that is normally localized to the endoplasmic reticulum or mitochondria may also be released into the cytoplasm following ischemia, further exacerbating the condition by increasing the concentration of calcium in the neuronal cytoplasm (Szydlowska & Tymianski, 2010).

A number of drugs have been developed specifically to treat excitotoxicity in the aftermath of stroke and, for the most part, they are aimed at preventing calcium entry into neurons or by targeting downstream molecules involved in the excitotoxic cascade, but clinical trials have been largely unsuccessful. These compounds include glutamate receptor antagonists, glutamate release blockers, nitric oxide synthase inhibitors, and free radical scavengers that are designed to prevent oxidative damage. Most of these agents that have been tested unfortunately have been ineffective or have had intolerable side effects precluding their use in the clinic (Lau & Tymianski, 2010).

An additional molecular factor that may increase the excitotoxic effects of stroke is an upregulation of calcium-permeable AMPA receptors. As described in the introduction to glutamate receptors previously, most AMPA receptors contain a GluA2 subunit that prevents calcium from passing through the ion channel. However, following some types of ischemic events, it has been found that the expression of GluA2 mRNA is reduced; suggesting that there could be an increased expression of calcium-permeable AMPA receptors (i.e. those that lack the GluA2 subunit) would result. An increase in these receptors offers yet another possible means for calcium to enter into neurons already suffering from hyperactivation, leading to abnormally long periods of depolarization and excessive calcium influx (Lau & Tymianski, 2010).

3.2.6.6 Parkinson's Disease

The progression of Parkinson's disease involves the loss of the dopaminergic neurons in the substantia nigra. These neurons help control muscle movement and as they degenerate, patients lose control of voluntary and spontaneous muscle movement presenting symptoms that include resting tremors, slowed movement, difficulty with movement, and balance problems. In addition, a number of cognitive symptoms typically develop as the disease progresses, including memory loss, depression, and eventually dementia. The mechanism behind the initial neuronal degeneration is only known in a small percentage of cases, termed familial Parkinson's, which has been linked to an inherited genetic mutation. However, like AD, most other cases are sporadic Parkinson's and there is no known cause. However, recent research has implicated glutamate excitotoxicity as a factor as the reduction in dopaminergic transmission leads to a loss of regulation of striatal neurons, resulting in an in increase in glutamatergic activity (Koutsilieri & Riederer, 2007; Meissner, 2011; Mony, 2009).

A logical method to prevent glutamate-mediated excitotoxicity is to prevent glutamate release and/or binding to the iGluRs. Recent experimental treatments have included drugs that do just this (Meissner, 2011). The results have interestingly demonstrated that, in animal models of Parkinson's, a shift in the subunit composition of the NMDA receptor is found, which is thought to be a potential excitotoxic mechanism. L-DOPA treatment was shown to alter NMDA receptor subunit composition as well, leading to L-DOPA induced dyskinesia. Unfortunately, though general NMDA receptor antagonists have been found to be successful in treating both Parkinsonian and dyskinesia symptoms in animal models of the disease, the side effects of these agents in humans preclude their use in the clinic. In an effort to circumvent these side effects, drug developers have focused on compounds targeted to the NR2B subunit of the NMDA receptor. One such compound, CP-101,606 (Traxoprodil) worked well for the treatment of both Parkinsonian and dyskinetic symptoms in animal models, but in human trials only appeared to treat dyskinetic symptoms (Koutsilieri & Riederer, 2007; Mony, 2009).

3.2.6.7 Traumatic Brain or Spinal Cord Injury

Intracranial injury, more commonly know as traumatic brain injury (TBI), occurs when a head injury results in damage to neuronal structure and integrity. There are two phases of TBI; the acute initial damage from the insult and the secondary injury that are caused by excitotoxicity. Much like the progressive neurodegenerative diseases already discussed and acute stroke, glutamate dysregulation leads to calcium influx into neurons close to the region of initial insult, which results in necrosis of neurons. TBI is complicated as there may or may not be blood flow alterations, hypoxia, swelling, and intracranial pressure. Presently, no pharmacological intervention is available to treat this secondary, excitotoxic cascade that results from TBI. Like other excitotoxic diseases, NMDA receptor antagonists showed promise in animal studies but failed to show efficacy in human clinical trials.

3.2.7 Why not Fully Block Calcium Entry via Pharmacological Agents?

Could it be as simple as blocking the glutamatergic transmission so as to cut off the link between over-stimulation by extracellular glutamate and calcium-induced excitotoxicity inside of downstream neurons? Based on the studies that have been performed, it appears that is not the case. One very possible problem that is presented, though likely underappreciated, when fully antagonizing these receptors is the self-regulating mechanism that synapses use, called homeostatic plasticity.

Homeostatic plasticity is the broad term used to describe all of the molecular plastic changes that a neuron uses to govern and adjust its own intrinsic excitability. One common underlying method that neurons use to make these adjustments is synaptic scaling (Thalhammer & Cingolani, 2014). That is, the neuron will adjust the properties of ion channels to meet a certain set point. This is a process that was first observed in neurons adjusting their excitatory response to glutamate release after chronic manipulations of their activity. *In vitro* experiments with neurons have demonstrated that chronic blockage of either NMDA receptors or AMPA receptors results in a process that looks like LTP (Lee & Chung, 2014). There have been reports of enhanced trafficking of

iGluRs to the surface of the cell and delivery to synaptic locations after treatments. Thus, we draw a parallel to these findings and suggest that full blockage of iGluRs could lead to homeostatic plasticity induction and this would be counterproductive to reducing excitotoxicity, since it would result in an increased expression of just the channels that clinicians are trying to modulate. We hypothesize that a partial antagonist will be effective in reducing spine loss and neuron death due to excitotoxic calcium influx. In fact, one of the few pharmacological agents that actually demonstrates some efficacy on slowing excitotoxic effects is memantine, a partial and weak antagonist of NMDA receptors (Gardoni & Di Luca, 2006).

3.2.8 Emerging Targets for Reducing Calcium-mediated Excitotoxicity

As described earlier in this chapter, calcium-permeable AMPA receptors are garnering much attention as they have been found to play a role now in both normal plasticity as well as neurodegenerative. Interestingly, in almost all studies of calcium-permeable AMPA receptors, one common ligand has been employed in many experiments (Meyer, 2012; Wen & Barth, 2012). This molecule is naphthylacetylspermine (NAS) and is a synthetic agent that was designed based on a number of natural toxins biosynthesized by certain spiders and wasps. All of these molecules are use-dependent, full antagonist, pore blockers of calcium-permeable AMPA receptors. Calcium-permeable AMPA receptors are inserted into the membrane after broad AMPA receptor antagonism in cultured neurons, suggesting a role for them in homeostatic plasticity and maintenance of synaptic communication (Beique, 2011). This latter point leads us to believe that partial antagonism may be more fruitful than simply blocking all CP-AMPARs for ALS treatment.

There is much experimental evidence that suggests CP-AMPARs are expressed on cultured motor neurons (Carriedo, 1996; Van Den Bosch & Robberecht, 2000; Van den Bosch, 2002). These specific receptors have been shown to be a calcium conduit that could be responsible for excitotoxic levels of calcium entering motor neurons and it is known

that blockage of these channels can prevent or delay cell death (Corona & Tapia, 2007; Van Damme, 2002; Van Den Bosch & Robberecht, 2000). In addition, the calcium-permeable AMPA receptor-mediated excitotoxicity can be delayed by chelation of calcium in models of ALS by application of BAPTA (Corona, 2007). Our present understanding of the molecular determinants of ALS progression all point to the involvement of calcium-permeable AMPA receptors and thus these ion channels warrant further study and targeting for new pharmacological agents. Of course, an alternative method to potentially reduce the expression of calcium-permeable AMPA receptors would be to restore the enzymatic activity of ADAR2, the enzyme responsible for editing the GluA2 RNA to make calcium-impermeable AMPA receptors.

3.2.9 Conclusions and Outlook

With an increasingly large aging population, the numbers of people afflicted with the diseases of excitotoxicity will continue to grow. Treatment of the effects of calcium-mediated excitotoxicity should be at the forefront of our collective efforts to treat these diseases. In too few cases, there are obvious genetic targets that can potentially be addressed with the new genome editing technology that is currently maturing. However, for the vast majority of sporadic cases of disease, the root cause is either undetected genomic mutations or environmental or developmental insults. For these categories, genome editing has no known target and thus, must be tackled at the site of molecular damage. With calcium-mediated excitotoxicity implicated in so many neurological diseases, the prize for the scientist who solves this problem will likely be substantial and society will be indebted to them.

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Rebecca Fransson, Christian Sköld and Anja Sandström*

3.3 Strategies for Conversion of Peptides to Peptidomimetic Drugs

3.3.1 Peptides as Starting Points in Drug Discovery

Due to their importance in many biological functions, bioactive peptides are interesting as starting points in drug discovery and as valuable research tools in initial investigations of the biological mechanisms of various diseases (Sewald, 2002). A compound intended as an oral therapeutic agent should display not only the desired effect (pharmacodynamics), but also satisfying pharmacokinetic criteria (Fig 3.3.1). Thus, adsorption, distribution, metabolism and excretion (ADME) properties play a critical role in defining a good drug candidate. However, due to the structural characteristics of peptides, they suffer from inherent drawbacks, such as low bioavailability, low metabolic stability, poor absorption from the gastrointestinal tract (GI), and poor brain exposure as a result of low permeability over the blood-brain barrier (BBB) (Humphrey, 1986; Veber, 1985).

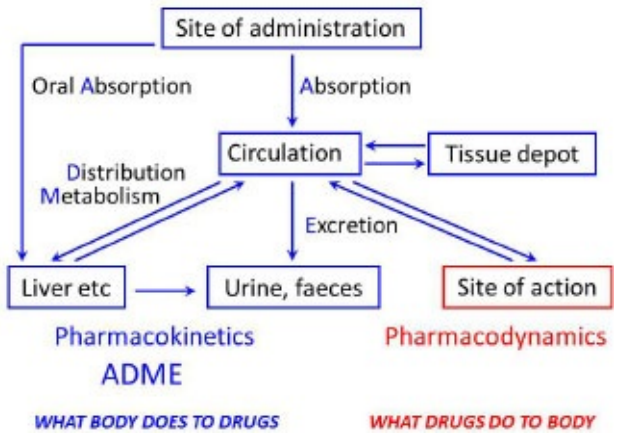


Figure 3.3.1: Drug distribution in the human body is determined by its ADME properties.

Furthermore, peptides have a large degree of conformational flexibility, and can fold into complex tertiary structures crucial for their molecular recognition and their ability to produce a biological response. In order to fully benefit from the potential of biologically active peptides in

chemical biology and in drug discovery, different approaches to overcome their limitations are needed.

There are several strategies available that can be utilized for the development of drugs that modulates the physiological events that are triggered by peptides. One common approach is high-throughput screening (HTS) of libraries of small molecules. This approach only considers the macromolecular target and can be utilized if the target is known and a suitable biochemical assay is available. Peptide-based therapeutics is another, currently growing, area that instead focuses on the biologically active peptide and aims to circumvent the limitation of peptides by introducing various modifications (e.g. PEG and phosphoesters linkages) or by employing different ways of administration (e.g. parenteral, mucosal, oral and transdermal routes). As a complement to HTS and peptide modifications, rational design based on step-wise transformation of peptides into low-molecular-weight and bioavailable drug-like molecules that mimic the action of peptides, i.e. peptidomimetics, is a viable way to overcome the problems associated with peptides (Hruby, 2002; Olson, 1993; Ripka, 1998; Vagner, 2008). Peptidomimetics are molecules with significantly reduced peptide character that mimic the bioactive conformation of peptides, and thus retain the ability to interact with the biological target and cause the same biological effect (Grauer, 2009). As these compounds are non-peptides which often possess desired improved pharmacokinetic properties, such as better absorption, metabolic stability and/or bioavailability. There are several successful examples where a rational design approach has resulted in approved drugs, e.g. the development of protease inhibitors such as angiotensin-converting enzyme (ACE) inhibitors, HIV protease inhibitors, and hepatitis C virus (HCV) inhibitors.

3.3.1.1 Strategy for the Development of Peptidomimetics

To transform biologically active peptides into small drug-like pseudopeptides or peptidomimetics in a rational way, a stepwise procedure, similar to the one outlined in Fig. 3.3.2, can be employed. Biological evaluation (both *in vitro* and *in vivo*), evaluation of

physicochemical and pharmacokinetic properties, and computational modeling guide the stepwise structural modifications and should be run in parallel with the different steps. The strategy will be described in the following section, and further exemplified in a case study related to a metabolite to the neuropeptide Substance P (Fransson, 2008; 2010; 2013).

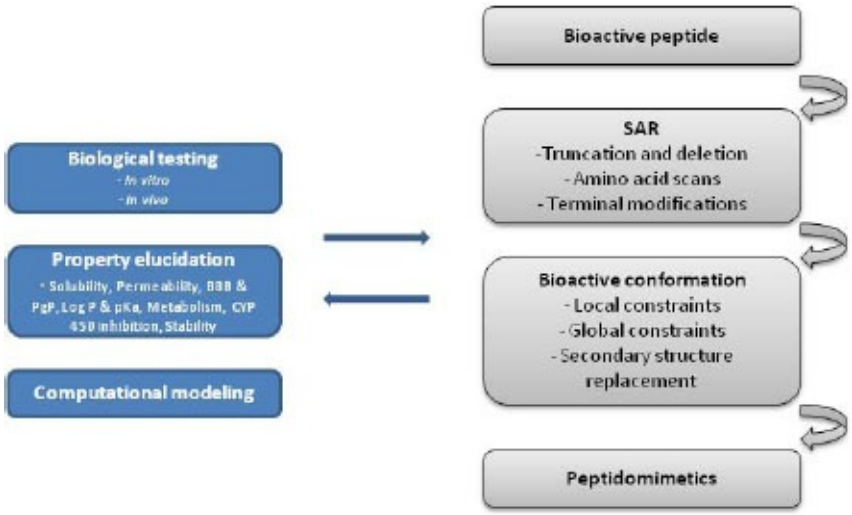


Figure 3.3.2: A step-wise strategy for development of drug-like peptidomimetics.

3.3.1.1.1 Property Elucidation

The activity at the target and the exposure (e.g. concentration and duration) determine the efficacy of a drug. In the body, several barriers to drug exposure can be found, for example cell membranes, metabolic enzymes, efflux transporters, and binding proteins. How a compound performs at a specific barrier is connected to its drug properties. In the GI tract, compounds can cross the cellular membrane barrier by three major mechanisms (van de Waterbeemd, 2001). The two most common are transcellular absorption, i.e. passive transfer by diffusion across the lipid membranes, and paracellular absorption, which proceeds through aqueous pores at the tight junctions between the cells. The third mechanism is active uptake by transport proteins that usually transport nutrients across the membrane (Kerns, 2008; van de Waterbeemd, 2001). The most important mechanism for drug absorption is passive diffusion, and about 95% of all commercial drugs are absorbed by this route (Kerns, 2008). Metabolizing enzymes in the GI tract, e.g. the cytochrome P450 (CYP) enzymes and proteolytic enzymes as well as efflux

transporters, e.g. P-glycoprotein (PgP), are expressed, which limit the oral absorption of compounds (Fig. 3.3.3) (van de Waterbeemd, 2001). In addition to avoiding the efflux of PgP and metabolism by gut wall enzymes, good permeability is important to maximize the oral absorption of a compound. In general, therapeutic peptides suffer from short half-lives due to rapid degradation by proteolytic enzymes of the GI tract and blood plasma, rapid clearance from the circulation by liver and kidneys, as well as limited permeability across physiological barriers because of their hydrophilic structure (Vlieghe, 2010). Especially CYP3A4 and PgP have been shown to have a significant impact on the bioavailability of peptidic and peptidomimetic drugs (Wacher, 1998). One means of enhancing oral bioavailability is to increase passive diffusion by changing the physiochemical properties (i.e. decreasing the hydrophilicity) of the compound (Wang, 1999). It should be noted that increasing the lipophilicity, in order to improve membrane permeability, can also lead to increased efflux and metabolism. Two other strategies that can be used to improve permeability are reducing hydrogen bonding and decreasing the polarity (Kerns, 2008).

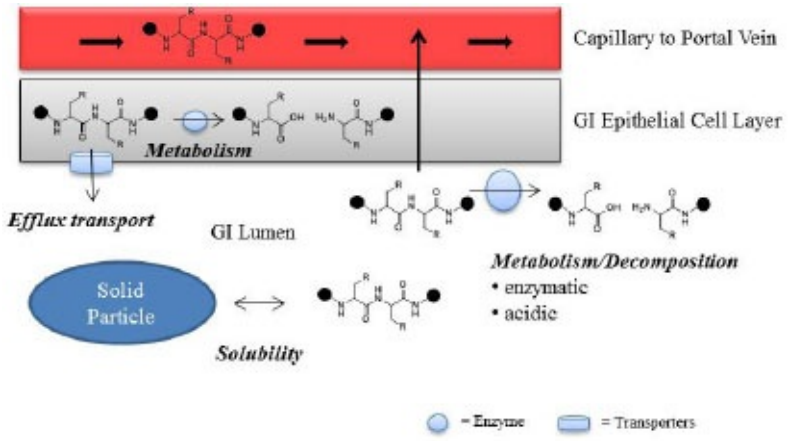


Figure 3.3.3: Illustration of the barriers to drug absorption in the GI tract.

In the bloodstream, enzymatic hydrolysis by proteolytic enzymes and plasma protein binding (PPB) constitute barriers preventing drugs from penetrating into the tissues. The affinity of a compound to plasma proteins determines the ratio of bound to unbound ("free") drug in solution, and only the unbound drug can enter the tissues. If a compound has a high binding affinity it can be difficult to achieve concentrations in the tissue sufficient to produce the desired

pharmacological effect. High PPB also reduces the clearance of a compound and thus increases the PK half-life, since it prevents the drug from permeating into the liver and kidneys (Kerns, 2008).

A further dimension that must be considered when working with central nervous system (CNS) active agents is the uptake in the brain. Here, the blood–brain barrier (BBB) constitutes an additional barrier to absorption. It has been shown that the penetration of a compound into the brain decreases with increasing polar surface area (Van de Waterbeemd, 1998). However, it is not only the physiochemical properties that influence brain uptake. Transporters, especially PgP, and metabolizing enzymes are also limiting factors. Several structure modification strategies to reduce PgP efflux have been described (Kerns, 2008). These include reducing the number of hydrogen bond donors, decreasing the hydrogen bond acceptor potential of a compound, and decreasing the overall lipophilicity of the structure.

During rational peptide lead optimization various *in vitro* property assays (e.g. solubility, permeability, chemical stability, metabolism, protein binding and transport) are used to assess the drug-like properties of the generated peptide analogues (Kerns, 2008). Extensive *in vitro* profiling regarding the PK data of lead compounds in the early stages of development can thus provide the medicinal chemist with information on the structure-property relationship important for the further development of orally active compounds.

3.3.1.1.2 Structure–Activity Relationship

In order to develop peptidomimetics with retained affinity to the biological target it is crucial to investigate what parts of the peptide are directly involved in target recognition. Thus, attainment of the structure–activity relationships (SARs) and identification of the minimal active sequence of the peptide required for biological activity are important first steps in the process. This is normally achieved by the evaluation of binding affinities of peptide analogs to the target protein. In practice, such information is normally gathered through amino acid scans, truncations and N- and C-terminal modifications (Grauer, 2009; Hruby, 2002; Wiley, 1993; Vlieghe, 2010). Amino acid scans determine

the importance of amino acid side chains by systematically replacing each residue within the peptide with alanine, glycine or the corresponding d-amino acid. Alanine and glycine are the smallest amino acids available, having a methyl and a hydrogen side chain, respectively. Thus, they should have only a small impact on the overall binding affinity, unless they substitute a crucial amino acid in the original peptide. N- or C-terminal truncation removing one amino acid at a time provides information about the minimal sequence needed for biological activity. The importance of a basic N-terminal or an acidic C-terminal is determined by the introduction of capping groups. These initial investigations will lead to the identification of the essential residues in the peptide and potential pharmacophoric groups, i.e. structural features that are required for the biological activity. Based on the information gained from these studies, further structural modifications are undertaken to improve stability, potency and selectivity.

3.3.1.1.3 Bioactive Conformation

One of the central steps in peptidomimetic design is to elucidate the bioactive conformation of the peptide, i.e. the conformation adopted when it is bound to the macromolecular target. Conformational restrictions are frequently used to explore the bioactive conformation and to enhance bioavailability by improving enzymatic stability, (Grauer, 2009; Veber, 1985). A constraint that leads to a reduction in the loss of conformational entropy upon interaction with the target can also increase the binding affinity (Rizo, 1992). Global and local constraints can be achieved by cyclization (Hruby, 2002), *N*-methylation (Rizo, 1992), isosteric substitution (Rizo, 1992; Sewald, 2002) or by secondary structure replacement (which will be discussed in more detail below) (Kim, 2000) as exemplified in Fig. 3.3.4.

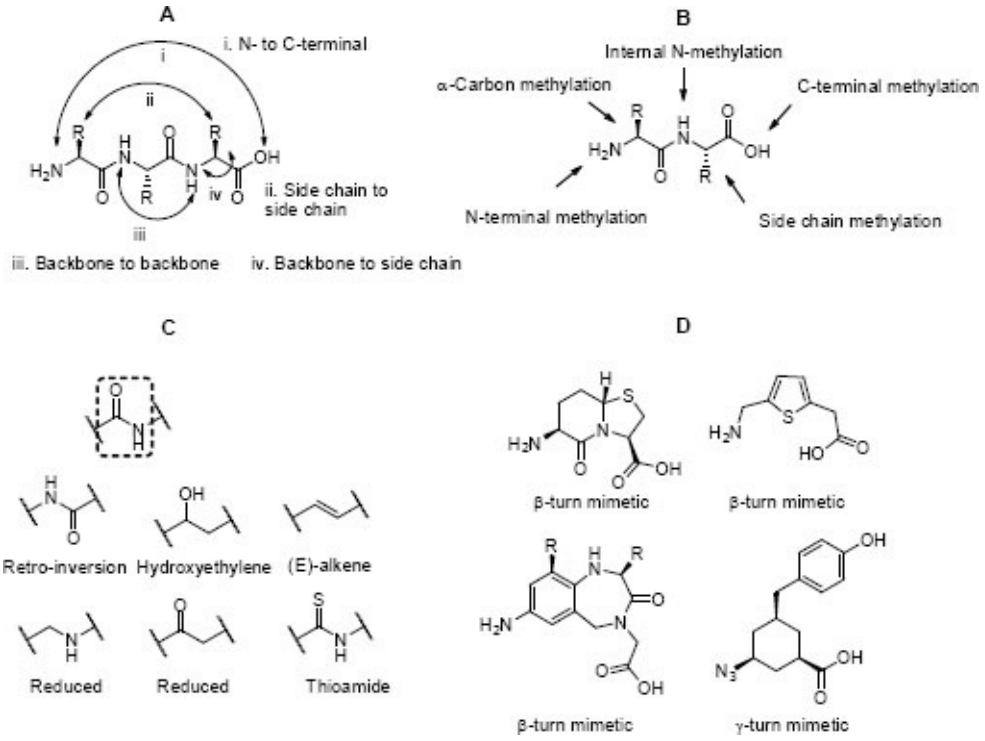


Figure 3.3.4: A) Cyclization strategies that can be performed in a peptide sequence to introduce global constraints (Rizo & Gierasch, 1992). **B)** Positions in a peptide that can be methylated. Methylation of both the backbone atoms and the side chains can introduce local constraints (Rizo, 1992). **C)** Isosteric replacement of the peptide bond can introduce local constraints. It should be noted that such replacements are not always real constraints, but alter the overall conformational behavior of the peptide backbone to varying degrees. In some cases the flexibility is increased (Rizo, 1992; Sewald, 2002). **D)** Secondary structure mimetics can induce a desired conformation when introduced into the peptide backbone (Rosenström, 2006; Schmidt, 1998; Sewald, 2002).

Many high-quality 3D structures of ligands, when bound to their macromolecular target, have been obtained using X-ray crystallography. However, this technique is not readily applicable to all types of systems. For example, enzymes have been easier to crystalize as compared to membrane bound G-protein coupled receptors (GPCRs), although the number of crystalized receptors are increasing. To model the receptor-bound conformation when the 3D structure of the target is unknown, ligand-based experimental or theoretical studies have to be performed. Conformations adopted by flexible linear peptides are numerous, and are strongly influenced by interactions with the environment (Rose, 1985). Therefore, to limit the conformational flexibility, conformational constraints can be introduced into the peptide to provide information about the bioactive conformation. In many cases the constrained part of

the peptide is not completely rigid (e.g., using side chain cyclizations as constraints) and therefore several differently constrained analogues of the peptide may be required in order to derive a putative receptor-bound conformation. When peptides bind to their receptors they become an integral part of the protein structure and thus can be predicted to adopt secondary structure motifs as part of the bioactive conformation (Hruby, 2002). Therefore, conformational constraints or organic scaffolds that induce or mimic secondary structures can give valuable information when searching for the bioactive conformation and may provide a valuable starting point for the development of peptidomimetic drugs.

3.3.1.1.3.1 Secondary Structure Mimetics

The main secondary structures found in proteins and peptides are the α-helix, β-sheet and turns. For peptide ligands, secondary structures correspond to local rigidified structure motifs with a specific arrangement of the residue side chains, which could provide important recognition elements during receptor binding and activation (Rose, 1985). Thus, mimicking such structural elements of the peptide with organic scaffolds is a rational approach in the development of peptidomimetic compounds. In addition, such an approach may also give compounds with increased metabolic stability and higher receptor specificity, and provide a rational basis for exploring the conformational effect on activity (Hruby, 1982). There are indications that turn structures are present in several peptide ligands when bound to their receptors, and this makes it appealing to mimic turns (Rose, 1985). The major types of turns present in proteins and peptides are the β-turn and the γ-turn, depicted in Fig. 3.3.5, where turn mimetic examples also can be found.

Venkatachalam first suggested the presence of β-turns in proteins based on modeling studies in 1968 (Venkatachalam, 1968). The β-turn causes reversal of the peptide backbone and is defined as a sequence of four amino acid residues in a non-helical segment with a distance of less than 7 Å between C $\alpha_{,i}$ and C $\alpha_{,i+3}$ (Lewis, 1973). The β-turns are often, but not necessarily, stabilized by a CO_i–NH_{i+3} hydrogen bond. The β-turn has been divided into several types, depending on the values of the

backbone torsion angles of residues *i*+1 and *i*+2. The most common β-turns found in proteins are types I, I′, II, II′, and VIII (Table 3.3.1) (Hutchinson, 1994; Richardson, 1981; Venkatachalam, 1968; Wilmot, 1988). In addition to these, other classes such as III, III′, VIa, VIb and VII have been proposed (Lewis, 1973; Venkatachalam, 1968). The miscellaneous category IV has been introduced to accommodate β-turns that do not fit any specific type.

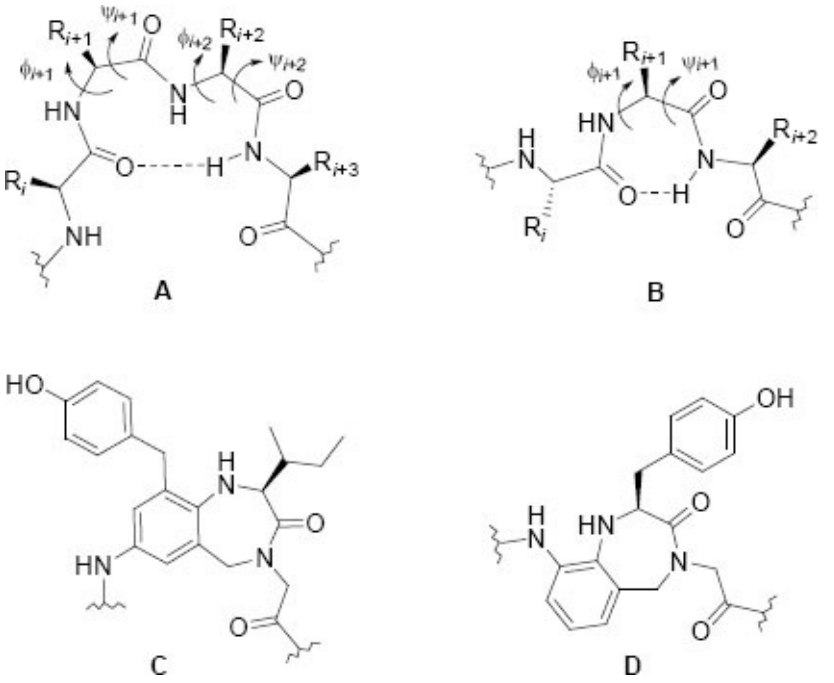


Figure 3.3.5: A β-turn (A) and a γ-turn (B) and examples of a β-turn mimetic (C) (Rosenström, 2006) and a γ-turn mimetic (D) (Rosenström, 2005). The backbone torsion angles phi (φ) and psi (ψ) are indicated for the relevant residues in the peptide turns.

Table 3.3.1 Idealized backbone torsion angles for the most common β-turn types (Venkatachalam, 1968; Wilmot, 1988) and the backbone torsion angles for the classic and inverse γ-turn (Matthews, 1972; Nemethy, 1972).

	Residue i+1		Residue i+2	
β-Turn	Phi (φ)	Psi (ψ)	Phi (φ)	Psi (ψ)
I	-60	-30	-90	0
I′	60	30	90	0
II	-60	120	80	0
II′	60	-120	-80	0
VIII	-60	-30	-120	120
γ-Turn (classic)	70 to 85	-60 to -70		
γ-Turn (inverse)	-70 to -85	60 to 70		

The γ-turn is not as common as the β-turn but has been found to be

adopted by peptides as short as tripeptides (Motta, 2005). A γ-turn spans over three amino acid residues with a hydrogen bond between CO_i and NH_{i+2}, resulting in the shape of a seven-membered ring. There are two types of γ-turns: the classic γ-turn with the *i*+1 residue side chain in an axial geometry and the inverse γ-turn with the side chain in an equatorial geometry (Table 3.3.1) (Matthews, 1972; Nemethy, 1972). Although the classic γ-turn was proposed first, it has been shown to be very rare and the inverse γ-turn is the significantly more common of the two (Milnerwhite, 1988). The classic γ-turn conformation causes reversal of the backbone direction and is mainly found in β-hairpin structures, as a tight turn, forming an antiparallel β-sheet structure. The inverse γ-turn can be found as backbone kinks and rarely causes reversal of the backbone direction (Milnerwhite, 1988).

The information gained from the investigation of the bioactive conformation can provide indications regarding the presence of a turn structure in the bioactive conformation. Attempts to replace this part of the peptide with a turn mimetic would then be a rational step towards a peptide mimicking compound. However, how does one know if a particular organic scaffold indeed does mimic a turn, and in that case, which specific class of turns? One way of characterizing a potential turn mimicking moiety is to compare the geometry with experimental turn structures found in proteins (Claerhout, 2012; Rosenström, 2006; Whitby, 2011). The great abundance of protein 3D structures determined by X-ray crystallography in the Protein Data Bank (Berman, 2000) provides a good source of experimental protein secondary structures that can be used for this purpose. Using this method, both β- and γ-turn mimicking scaffolds have been characterized, which are exemplified below.

β-Turn Mimicking Scaffold C

Since turn mimicking moieties usually have reduced peptidic character and thus do not have a straightforward atom-to-atom match to the peptide, the phi and psi dihedral angles used to classify β-turns can be difficult to use for classifying β-turn mimetics. Instead, another rational classification approach is to compare the geometries of the mimicking

moiety and the different β-turn types by superimposing important structural features. The main features in a protein turn are the incoming and outgoing directions of the protein backbone and the side chains. If the corresponding directions can be identified in the peptidomimetic moiety, the characterization can be done by superimposing the relevant atoms to the corresponding protein turn structures. The quality of the match can be quantified by the root-mean-square atom pair distance between the two structures. In the case of scaffold **C** (Fig. 3.3.6) low energy conformations of the scaffold were superimposed to more than 13,000 β-turn structures collected from protein 3D structures in PDB. Out of the well-defined β-turn types, scaffold **C** had best match to type II β-turns (Fig. 3.3.6) and thus may be suited as a mimetic of this class of β-turns. In angiotensin II (Ang II, Table 3.3.2, entry 1) there have been indications of a turn structure present in the central part of the peptide and scaffold **C** has been used to replace amino acids in the center of Ang II to yield high affinity Ang II type 1 (AT_1) and Ang II type 2 (AT_2) receptor ligands (Table 3.3.2, entries 2 and 3).

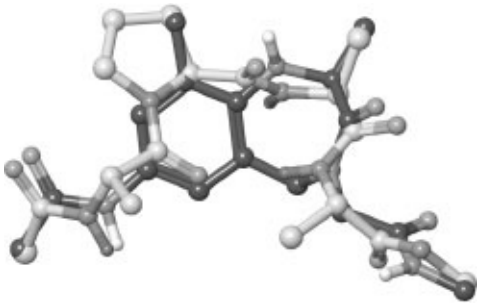
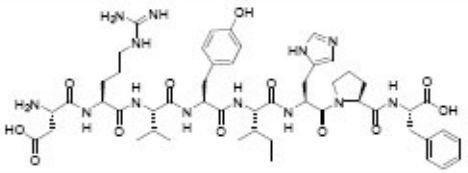
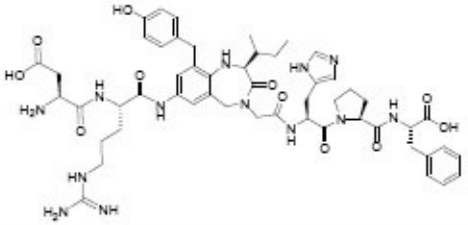
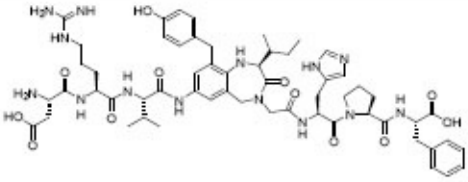
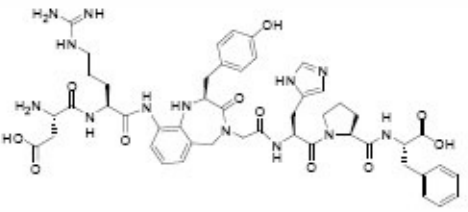
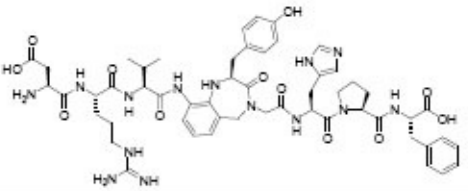


Figure 3.3.6: Low energy conformation of β-turn mimetic scaffold **C** (dark grey carbons) superimposed to a type II β-turn (light grey carbons; PDB ID 1H2C, chain A, sequence Ile^{142}-Pro^{143}-Asp^{144}-His^{145}). For clarity, only C_β is shown for the side chains and non-polar hydrogen atoms are omitted.

Table 3.3.2: AT_1- and AT_2 receptor affinity of Ang II and Ang II mimicking pseudopeptides.

Entry		AT_1[a] K_i (nM) ± SEM	AT_2[b] K_i (nM) ± SEM	Reference
1		0.24 ± 0.07	0.23 ± 0.03	Rosenström, 2005
2		1,668 ± 20	4.7 ± 0.3	Rosenström, 2006
3		14.9 ± 0.4	1.8 ± 0.04	Rosenström, 2006
4		> 10,000	2.8 ± 0.2	Rosenström, 2005
5		> 10,000	0.8 ± 0.1	Rosenström, 2005

[a] Rat liver membranes

[b] Pig uterus myometrium

γ-Turn Mimicking Scaffold D

In the case of the γ-turn mimicking scaffold **D** (Fig. 3.3.7) an idealized γ-turn was first used to perform geometry comparison with respect to distances and angles in the turn structure. In scaffold **D** two amino acid residues corresponding to *i*+1 and *i*+2 can be directly mapped onto a peptide as seen in Fig. 3.3.7. The third atom building up the C_α atom triangle is part of a benzene ring, but is easily mapped to a peptide backbone via atom count either from the N-terminal or from residue *i*+1. As seen in Fig. 3.3.7 the C_α –C_α distances and angles match very well

between scaffold **D** and an idealized peptide γ-turn, which indicate that this scaffold is a promising γ-turn mimetic. Scaffold **D** has been experimentally evaluated and has successfully been used to replace two or three amino acids in Ang II, which furnished selective, high affinity pseudopeptides (Table 3.3.2, entries 4 and 5) that act as agonists at the AT_2 receptor.

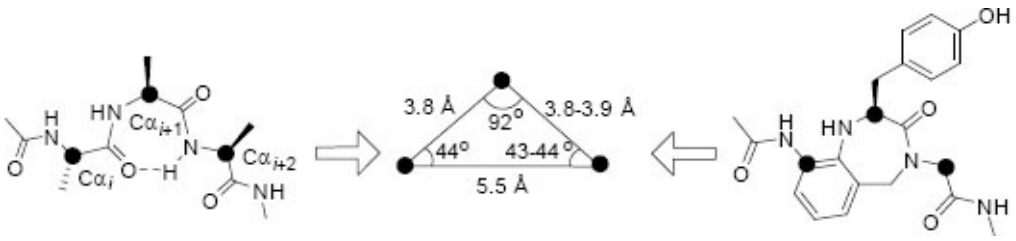


Figure 3.3.7: Comparison of Cα distances and angles in an ideal γ-turn model peptide (left) and corresponding atoms in the γ-turn mimicking scaffold **D** (right).

Although the distances and angles correspond well between an idealized γ-turn and scaffold **D**, such an analysis does not include the incoming and outgoing directions of the peptide backbone or the direction of the side chain of residue *i*+1. As in the case of the β-turn mimetic classification, one way of characterizing the γ-turn mimetic is to compare relevant parts to a determined 3D structure of a γ-turn. In Fig. 3.3.8, an energy-minimized conformation of scaffold **D** can be seen superimposed on a γ-turn found in a protein 3D structure, which shows the good match between the structures.

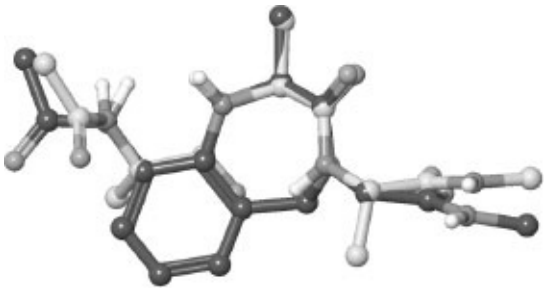


Figure 3.3.8: Low energy conformation of scaffold **D** superimposed on an inverse γ-turn (light grey carbons, PDB ID 1NNF, chain A, sequence Thr^{82}-Ala^{83}-Gln^{84}). For clarity, only C_β is shown for the side chains and non-polar hydrogen atoms are omitted.

3.3.2 A Case study of Rational Peptide Lead Optimization: Development of Small and Constrained Peptides Targeting the

Substance P 1-7 Binding site

Substance P (SP) was the first neuropeptide to be identified (Von Euler, 1931). In the beginning of the 1970s the peptide sequence, H-Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH2, was disclosed (Chang, 1971) and a decade later its belonging to the tachykinin family was affirmed (Erspamer, 1981). Together with neurokinin A (NKA) and neurokinin B (NKB), SP is the most well-known member of this family (Harrison, 2001). Three mammalian tachykinin receptors are known today: the neurokinin (NK) receptors NK1, NK2 and NK3 (Otsuka, 1993). SP is the preferred endogenous ligand for the NK1 receptor, where it acts as a neurotransmitter and a neuromodulator in both the central and peripheral nervous system. In the brain, SP and its corresponding receptor are expressed in areas related to depression (Kramer, 1998), anxiety (De Araujo, 2001) and stress (Culman, 1995; Ebner, 2008) as well as in areas involved in motivation and reward (Hasenohrl, 1991; Huston, 1993). In the spinal cord, SP is expressed in pain processing pathways (Zubrzycka, 2000).

3.3.2.1 SP_{1-7} and its Binding Site

Several of the degradation products of SP have been found to be bioactive, and especially the C-terminal fragments can mimic the effects of the mother peptide. For example, infusion of the C-terminal metabolite SP_{5-11} into the spinal cord induces nociceptive reactions (Skilling, 1990) and when injected into the dorsal periaqueductal gray matter in rats the C-terminal fragment SP_{6-11} was shown to produce anxiogenic effects mediated via the NK1 receptor (De Araujo, 2001). The N-terminal fragment SP_{1-7} is one of the major metabolites of SP. In contrast to the C-terminal metabolites of SP, SP_{1-7} has been shown to oppose several effects of SP, e.g. the nociceptive effect (Sakurada, 2004), the inflammatory effect (Wiktelius, 2006) and the potentiating effect on opioid withdrawal symptoms (Kreeger, 1993; Zhou, 2003). Interestingly, these effects were not found to be mediated through the NK1 receptor and although the actions of this heptapeptide are well-known, no explicit receptor has yet been identified. However, the potential

existence of a specific receptor for the N-terminal partial sequences of SP in mouse spinal cord was discussed at the beginning of the 1980s (Piercey, 1982) and in 1990 Igwe et al. characterized the specific binding site of SP_{1-7} in mouse brain and spinal cord (Igwe, 1990). Binding was found to be specific, saturable and reversible, which strongly supported the existence of an N-terminal-directed SP receptor. In 2006, Nyberg and coworkers demonstrated the presence of specific binding sites for SP_{1-7} in rat spinal cord in accordance with previously reported results from the mouse spinal cord and brain (Botros, 2006; Igwe, 1990). A binding assay was developed using spinal cord tissue homogenate and measurements of the binding affinity for various compounds by displacement of tritiated SP_{1-7} ($[^3H]$-SP_{1-7}), which was a prerequisite for the medicinal chemistry program which will be described below.

Initially, ligands for opioid and neurokinin receptors, as well as various N-terminal SP fragments, were screened against the SP_{1-7} binding site. In short, none of these ligands did bind efficiently to this binding site. However, interestingly, the high-affinity μ-opioid receptor agonists endomorphin-2 (EM-2) and endomorphin-1 (EM-1) were shown to interact differentially with the binding site of SP_{1-7}. Thus, EM-2 had only a 10-fold lower affinity than SP_{1-7}, whereas EM-1 had a 1,400-fold lower affinity. To rule out involvement of the μ-opioid receptor, the binding affinity of SP_{1-7} to the μ-opioid receptor and the ability of the heptapeptide to activate it were further investigated (Botros, 2006). However, no specific binding or any activation of the μ-opioid receptor was observed, which is indicative of a specific target protein for SP_{1-7}, identical to neither the tachykinin receptors nor the μ-opioid receptor.

3.3.2.2 SAR and Truncation Studies of SP_{1-7} and EM-2

3.3.2.2.1 Strategy

A SAR study of the two peptide leads, SP_{1-7} and EM-2, was initiated, with the intention of identifying the pharmacophoric groups. This design strategy included Ala scans, truncations and C- and N-terminal modifications of the two target peptides (Fig. 3.3.9). Thus, a series of

peptide analogs, in which each amino acid residue of the two target peptides was replaced sequentially with an alanine, was synthesized. In the truncation studies, one amino acid at a time was removed from the N-terminal. Both C-terminal carboxylic acids and carboxamides were included.

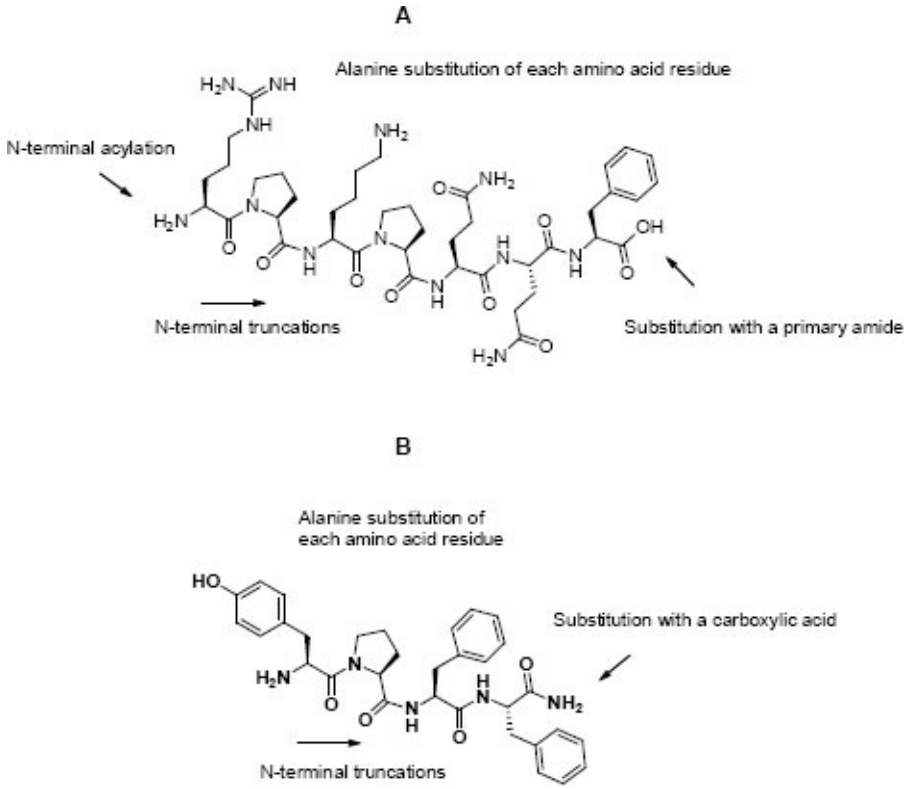
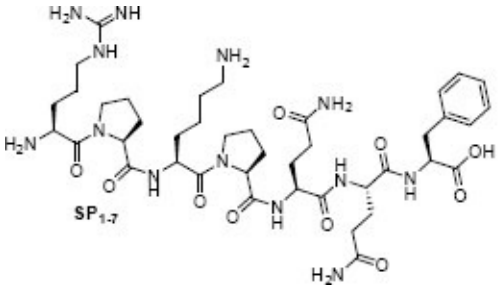


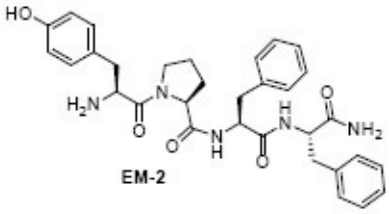
Figure 3.3.9: Illustration of the modifications of **A**) SP_{1-7} and **B**) EM-2, used in the SAR study.

3.3.2.2.2 Structure–activity Relationship

The two lead peptides, SP_{1-7} (**1**) and EM-2 (**2**), the Ala-substituted peptides **3**–**13**, the N- and C-terminally modified analogs **14**–**16** and **28**–**29**, the truncated analogs **17**– **27**, and the (d) and (l) variants **30**–**32** were prepared and biochemically evaluated (Tables 3.3.3 and 3.3.4).

Table 3.3.3: K_i values of SP_{1-7} and EM-2 analogs for inhibition of [^{3}H]-SP_{1-7} binding to rat spinal cord membrane.





Compound	Sequence	K_i ± SEM (nM)
1 ($SP_{1–7}$)	H-Arg-Pro-Lys-Pro-Gln-Gln-Phe-OH	1.6 ± 0.1
2 (EM-2)	H-Tyr-Pro-Phe-Phe-NH_2	8.7 ± 0.1
	Alanine-substituted $SP_{1–7}$	
3	H-**Ala**-Pro-Lys-Pro-Gln-Gln-Phe-OH	12.3 ± 0.4
4	H-Arg-**Ala**-Lys-Pro-Gln-Gln-Phe-OH	1.7 ± 0.1
5	H-Arg-Pro-**Ala**-Pro-Gln-Gln-Phe-OH	2.8 ± 0.1
6	H-Arg-Pro-Lys-**Ala**-Gln-Gln-Phe-OH	2.8 ± 0.1
7	H-Arg-Pro-Lys-Pro-**Ala**-Gln-Phe-OH	78.6 ± 5.1
8	H-Arg-Pro-Lys-Pro-Gln-**Ala**-Phe-OH	365 ± 3
9	H-Arg-Pro-Lys-Pro-Gln-Gln-**Ala**-OH	> 10 000
	Alanine-substituted EM-2	
10	H-**Ala**-Pro-Phe-Phe-NH_2	11.5 ± 0.1
11	H-Tyr-**Ala**-Phe-Phe-NH_2	10.2 ± 0.3
12	H-Tyr-Pro-**Ala**-Phe-NH_2	9.4 ± 0.1
13	H-Tyr-Pro-Phe-**Ala**-NH_2	1460 ± 15
	Terminally Modified $SP_{1–7}$ and EM-2	
14	**Ac**-Arg-Pro-Lys-Pro-Gln-Gln-Phe-OH	7.1 ± 0.0

15	H-Arg-Pro-Lys-Pro-Gln-Gln-Phe-**NH_2**	0.3 ± 0.0
16	H-Tyr-Pro-Phe-Phe-**OH**	30.2 ± 1.7
	Truncated $SP_{1–7}$ Peptides	
17	H-Pro-Lys-Pro-Gln-Gln-Phe-OH	29.6 ± 0.8
18	H-Pro-Lys-Pro-Gln-Gln-Phe-NH_2	2.8 ± 0.25
19	H-Lys-Pro-Gln-Gln-Phe-OH	30.9 ± 0.4
20	H-Lys-Pro-Gln-Gln-Phe-NH_2	4.4 ± 0.1
21	H-Pro-Gln-Gln-Phe-OH	26.2 ± 0.7
22	H-Pro-Gln-Gln-Phe-NH_2	4.5 ± 0.3
23	H-Gln-Gln-Phe-OH	20.4 ± 0.8
24	H-Gln-Gln-Phe-NH_2	1.9 ± 0.05
	Truncated EM-2 Peptides	
25	H-Pro-Phe-Phe-NH_2	10.9 ± 0.7
26	H-Phe-Phe-NH_2	1.5 ± 0.1
27	H-Phe-NH_2	5028 ± 31

The results were remarkably consistent for $SP_{1–7}$ and EM-2 peptides (Table 3.3.3). Substitution with alanine in the N-terminal part of $SP_{1–7}$ was well tolerated without affecting the binding affinity significantly (cf. **1** with **4**, **5** and **6**), although replacement of the basic amino acid arginine rendered a 10-fold lower affinity (cf. **1** and **3**). Likewise, the three N-terminal amino acid residues in EM-2 (tyrosine, proline and the internal phenylalanine) could be substituted with an alanine and still retain binding affinity (cf. **2** with **10**, **11** and **12**). However, removal of the

two primary amide functions of the side chains of the glutamine in the C-terminal of SP_{1-7} resulted in a considerable decrease in affinity (**7** and **8**). The C-terminal phenylalanine was absolutely crucial for strong affinity in both SP_{1-7} and EM-2. Replacement of the C-terminal phenylalanine in SP_{1-7} gave analog **9**, which was devoid of affinity. Making the same substitution in EM-2 resulted in compound **13** with a K_i value of 1460 nM. The finding that the C-terminal phenylalanine plays such an important role in binding affinity is in line with the peptide scan discussed above, where the C-terminal fragment SP_{1-6} possessed very weak binding affinity to the SP_{1-7} binding site (Botros, 2006; Igwe, 1990). Moreover, the potency of SP_{1-7} showed a five-fold increase upon amidation of the terminal carboxyl group (**15**). Similarly, the binding affinity of EM-2 was reduced by a factor of four upon removal of the amide function (**16**). Since SP_{1-7} is a proteolytic product of SP resulting in a C-terminal carboxylic acid, it was surprising that the amidated analog **15** led to improved binding affinity.

As deduced from the two Ala scans, the N-terminal parts of SP_{1-7} and EM-2 does not seem to be engaged in molecular recognition or binding to the target protein, a fact that prompted synthesis of the truncated analogs **17**–**24** and **25**–**27**. For SP_{1-7}, removal of the N-terminal arginine rendered the hexapeptide **17** with a 20-fold reduction in affinity compared to SP_{1-7} and a little more than 2-times lower affinity than the alanine derivative **3**. The affinity could, however, be recovered by amidation of **17**, resulting in peptide **18**, which was 10 times more potent. Further truncation down to the tripeptide level was possible without loss of affinity, and C-terminal amidation of all the truncated SP_{1-7} analogs improved the affinity 5–10-fold. Hence, the tripeptide H-Gln-Gln-Phe-NH_2 (**24**) exhibited a K_i of 1.9 nM.

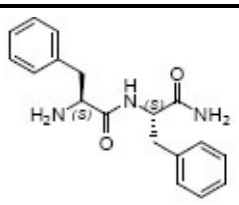
For EM-2, simultaneous removal of the two N-terminal amino acids tyrosine and proline improved the binding affinity six times, resulting in the notable discovery of the dipeptide H-Phe-Phe-NH_2 (**26**) with a K_i value similar to that of SP_{1-7} itself. It should be emphasized that the Tyr-Pro sequence is the critical fragment for binding to the μ-receptor, whereas the two C-terminal phenylalanines are not, facts that highlight

the double nature of EM-2 (Fichna, 2007; Kruszynski, 2005; Okada, 2003). In the Ala scan of EM-2, substitution of the internal phenylalanine was accepted (**12**), but truncation down to a single phenylalanine resulted in **27** with no binding affinity (K_i of 5028 nM).

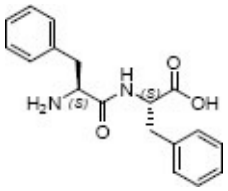
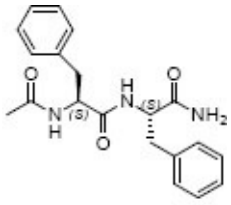
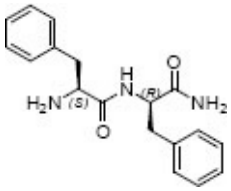
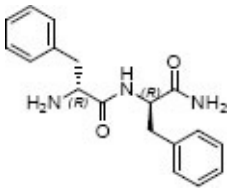
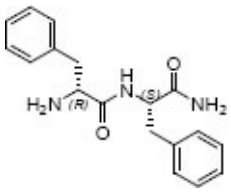
The binding features of the new dipeptide lead compound H-Phe-Phe-NH_2 were further explored via the synthesis of peptides **28**–**32** (Table 3.3.4). As observed for SP_{1-7} and EM-2, the C-terminal function should be a primary amide. The corresponding carboxylic acid was devoid of activity (cf. **26** with **28**). All four stereoisomers of H-Phe-Phe-NH_2 (**26**, **30**, **31** and **32**) were synthesized and evaluated. The natural l-Phe-l-Phe isomer (**26**) was found to be preferred, followed by the d,d compound (**31**), although with a 40-fold lower affinity. As mentioned above, incorporation of d-amino acids into neuropeptides can change their biological function from an agonist to an antagonist, e.g. d-SP_{1-7}, which makes it interesting to evaluate the analogs **30**, **31** and **32** in animal studies concerning their functional activities.

Due to the smaller size of **26** in comparison to the heptapeptide SP_{1-7}, lower selectivity can be expected. Moreover, **26** resembles ligands for the NK3 receptor (Boden, 1995; 1994). Hence, the possible binding affinity of **26** to the human neurokinin receptors NK1 and NK3 was studied. Binding was evaluated in agonist radioligand binding assays relying on the displacement of [Sar^9, $Met(O_2)^{11}$]-SP from NK-1 receptors, and [$MePhe^7$]-NKB from NK-3 receptors (Anthes, 2002; Heuillet, 1993), **26** was tested at a concentration of 10 µM, but showed no affinity for any of the receptors.

Table 3.3.4: K_i values of Phe-Phe analogs for inhibition of [^{3}H]-SP_{1-7} binding to rat spinal cord membrane.

Compound	Sequence[a]	K_i ± SEM (nM)
26		1.5 ± 0.1

Terminally Modified Phe-Phe Peptides

28		> 10 000
29		18.5 ± 1.7
	Phe-Phe Analogs	
30		540 ± 20
31		64 ± 2
32		175 ± 13

[a] S configuration = l configuration and R configuration = d configuration.

3.3.2.2.3 Effects of SP_{1-7} and its Analogs

As mentioned in the introduction, SP_{1-7} has been shown to influence opioid withdrawal symptoms and possess antinociceptive properties. Consequently, the synthesized compounds **15** and **26** were evaluated in different *in vivo* models. The amidated C-terminal analog SP_{1-7}-NH_2 (**15**) was demonstrated to attenuate the expression of naloxone-precipitated withdrawal in morphine-dependent rats when administered intracerebroventricularly (Zhou, 2009; Zhou, 2003). In agreement with the binding affinities obtained in the SAR study, the C-terminal amide analog is more efficient in reducing opioid withdrawal symptoms than SP_{1-7}.

SP_{1-7}-NH_2 (**15**) and also **26** have been further tested regarding their potential antinociceptive effect in both non-diabetic and diabetic mice after intrathecal administration (Fig. 3.3.10) (Carlsson, 2010; Fransson, 2010b; Nyberg, 2010; Ohsawa, 2010; 2011). The use of diabetic mice for evaluation is due to their reduced pain threshold compared to non-diabetic mice, a reduction thought to arise from hyperalgesia caused by neuropathy, which makes them a good model for studying neuropathic pain. Interestingly, morphine was unable to induce any antinociceptive effect in the diabetic mice, whereas SP_{1-7} showed a dose-dependent antinociceptive effect in both diabetic and non-diabetic mice (Carlsson, 2010). The effect was higher in diabetic mice, which suggests that the compound is more effective on neuropathic pain and that SP_{1-7} ameliorates signs of hyperalgesia (Fig. 3.3.10). In agreement with the results obtained from the opioid withdrawal test, SP_{1-7}-NH_2 proved to be more efficient in reducing pain as compared to the native heptapeptide. It was also demonstrated that the dipeptide **26**, which possessed the same binding affinity as SP_{1-7} (**1**), showed greater antinociceptive potency in diabetic mice than SP_{1-7} (Fransson, 2010b; Nyberg, 2010; Ohsawa, 2010).

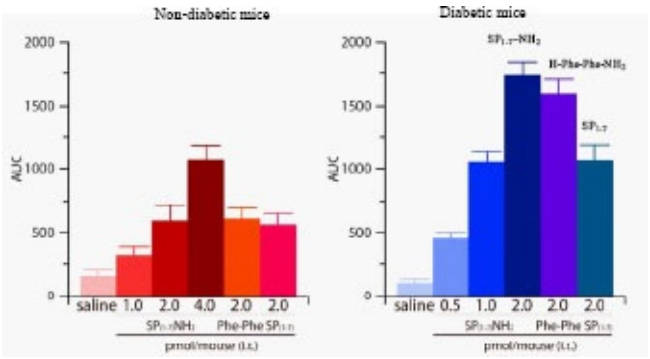


Figure 3.3.10: The antinociceptive effect of SP_{1-7} (**1**), SP_{1-7}-NH_2 (**15**) and H-Phe-Phe-NH_2 (**26**) in non-diabetic (left) and diabetic (right) mice. The antinociceptive effect was evaluated by the AUC calculated from the time-response curve of tail-flick latency. Each column represents the mean with S.E.M. (n = 6).

3.3.2.3 Design and Synthesis of Small Constrained H-Phe-Phe-NH_2 Analogs

3.3.2.3.1 Strategy

The potent dipeptide lead H-Phe-Phe-NH_2 (**26**), discussed above, was chosen for further optimization studies with the overall aim of developing metabolically stable and selective SP_{1-7} analogs. The introduction of local constraints can enhance stability, selectivity and bioavailability. The intestinal permeability is an important factor in the development of orally bioavailable drugs. In the intestine, the di/tri-peptide transporter PepT1 enables the absorption of small peptides from the digestion of dietary proteins. This transport system has also been shown to transport a variety of peptidomimetic drugs, such as β-lactam antibiotics and ACE inhibitors and might be exploited in order to increase the absorption of our small compounds (Brandsch, 2009; Brodin, 2002; Rubio-Aliaga, 2002). A known problem with peptides is their susceptibility to efflux. For peptides targeting functions in the CNS, uptake in the brain, i.e. crossing the BBB, is a crucial factor. As a defense mechanism preventing harmful substances from entering the brain, the BBB is equipped with efflux transporters (Witt, 2001). PgP is one of the most important, and can actively transport substances out of the brain (Giacomini, 2010). Such transporters can be an obstacle to entering the CNS.

A series of H-Phe-Phe-NH_2 analogs (**33**–**43**, Tables 3.3.5 and 3.3.6) incorporating different types of constraints were designed, synthesized and evaluated regarding their binding affinity, stability, uptake and permeability (Fig. 3.3.11). *N*-methyl and α-methyl amino acids were incorporated, substituting one residue at a time. Furthermore, β-methylation of the phenylalanine side chain was used to reduce the conformational flexibility, which can be advantageous upon binding. This approach has been successful in other projects in obtaining neuropeptide analogs resistant to metabolism while still retaining their biological activity (Veber, 1985). Both N- and C-terminal rigidifications were accomplished by the introduction of a 3-phenylproline derivative (Sewald, 2002).

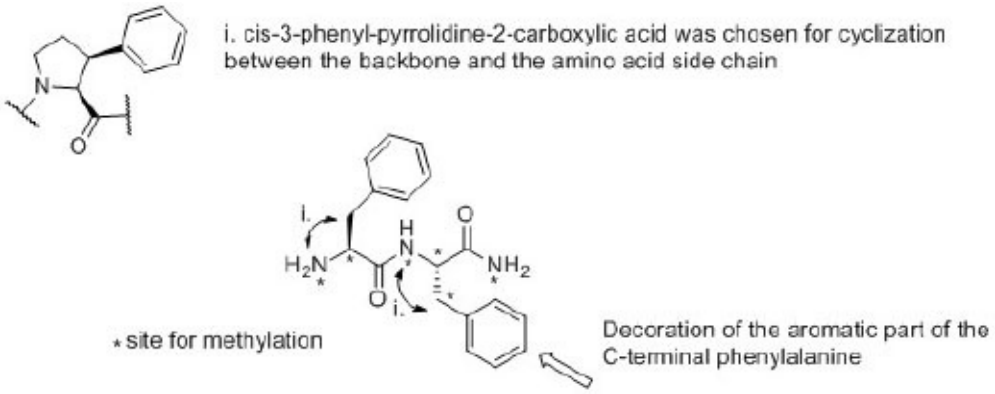
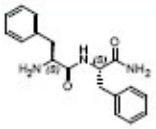
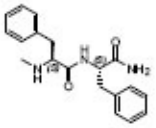
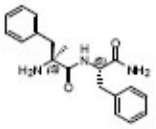
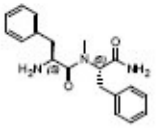
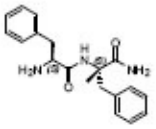
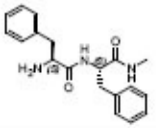


Figure 3.3.11: Overview of different modification strategies.

3.3.2.3.2 Structure–activity relationship and ADME properties

The binding affinities of the dipeptides were evaluated as described previously. The dipeptide lead H-Phe-Phe-NH_2 was re-tested with the new peptides for a more accurate comparison of the K_i values. The metabolic stability was evaluated by incubating the peptides with pooled human liver microsomes. *In vitro* half-life ($t_{1/2}$) and *in vitro* intrinsic clearance (Cl_{int}) were calculated using previously reported models (Houston, 1994; Obach, 1999). The K_i values and metabolic stabilities ($t_{1/2}$ and Cl_{int}) of the methylated analogs **33**–**37** are presented in Table 3.3.5, while the results of the rigidified and C-terminal-phenylalanine-modified analogs **38**–**43** are presented in Table 3.3.6.

Table 3.3.5: Binding affinity (K_i values) and metabolic stability (Cl_{int} and $t_{1/2}$) of the methylated H-Phe-Phe-NH_2 analogs

Compound	Structure	Binding affinity K_i ± SEM (nM)	Clearance[c] Cl_{int}[d] (µL min^{-1} mg^{-1})	Half-life[c] $t_{1/2}$[e] (min)
26		8.4 ± 0.4[a] (1.5 ± 0.1)[b]	121 ± 39	12 ± 4
33		189 ± 3	2.7 ± 1.5	597 ± 40
34		70 ± 3	64 ± 11	22 ± 4
35		9.4 ± 0.1	92 ± 0	15 ± 1
36		26 ± 1	38 ± 15	40 ± 16
37		136 ± 2	175 ± 5	7.9 ± 0.2

[a] K_i value determined on the same occasion as for **33–46.** [b] Previously determined K_i value [c] The metabolic stability data are expressed as mean ± SD.

[d] Cl_{int} = *in vitro* intrinsic clearance. [e] $t_{1/2}$ = *in vitro* half-life.

In the methylated series, only compound **35** with internal *N*-methylation, retained binding affinity comparable to that of H-Phe-Phe-NH_2 (**26**); unfortunately, no significant improvement in the metabolic stability was achieved. Methylation of the N-terminal resulted in 22-fold lower affinity, but was found to have a pronounced impact on the stability, which increased the half-life 50 times (cf. **26** and **33**). C-terminal methylation (**37**) was accompanied by a roughly 20-fold decrease in binding affinity, but without improvement of the stability. A potential reason for the reduced binding affinity of **37** is that the secondary amide loses a hydrogen bond donor feature compared to **26**, which may be important for the affinity.

Incorporation of an α-methyl amino acid (**34** and **36**) also reduced the binding affinity, but less than for the *N*-methyl amino acids in the terminal parts of H-Phe-Phe-NH_2. A 2- to 3-fold increase in the half-life

was thus observed for the α-carbon methylated analogs.

When the *cis* 3-phenylproline derivative was incorporated into the N-terminal part of H-Phe-Phe-NH_2 replacing phenylalanine (**38** and **39**), the binding affinity decreased 4 and 6 times, respectively. A possible reason for this loss in affinity is that these compounds may have problems adopting an optimal binding conformation because of the introduced rigidification provided by the proline analogue. Replacement of the C-terminal phenylalanine by the *cis* 3-phenylproline moiety in the *S*,*S*,*S* configuration (**40**) resulted in a more potent ligand than H-Phe-Phe-NH_2. This rigidification also increased the half-life by 7-fold (cf. **26** and **40**).

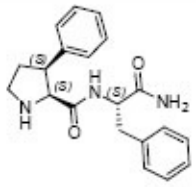
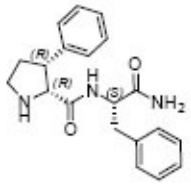
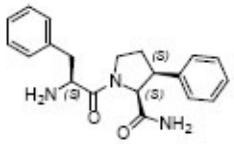
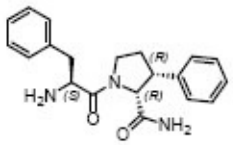
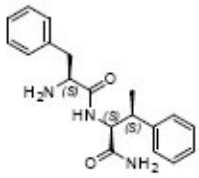
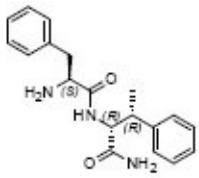
β-Methylation of the C-terminal phenylalanine gave compounds **42** and **43**, with lower binding affinity than H-Phe-Phe-NH_2. When comparing the metabolic stability in all the diastereomeric pairs (**38** vs. **39**, **40** vs. **41**, and **42** vs. **43**), the natural *S*,*S*,*S* configuration was more easily metabolized. Incorporation of d-amino acids is a known strategy to improve metabolic stabilization (Humphrey, 1986; Veber, 1985).

The physiochemical properties of all the synthesized compounds were further explored by evaluation of their intestinal epithelial permeability. This was determined from transport rates across a Caco-2 cell monolayer, and is expressed as the apparent permeability coefficient (P_{app}) (Hubatsch, 2007). A good relationship between the permeability across the Caco-2 monolayer and the extent of absorption *in vivo* has been reported (Stewart, 1995). Each compound was investigated in the apical to basolateral (a–b) and basolateral to apical (b–a) direction. One of several efflux transporters present in the Caco-2 cells is PgP. Measurement of the efflux (the ba/ab ratio) can thus indicate whether or not the compounds are substrates for PgP.

Since uptake transporters can enhance absorption, the possibility of the peptides being actively transported was studied in Chinese hamster ovary (CHO) cells stably transfected with the PepT1 transporter (CHO)-PepT1, using CHO-K1 cells as the control. The results are expressed as pmol mg^{-1} of protein/min. For peptides being actively transported the PeptT1/K1 ratio should be greater than one. Uptake and permeability are

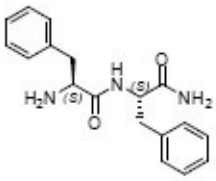
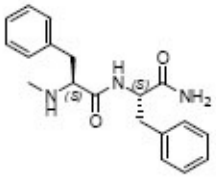
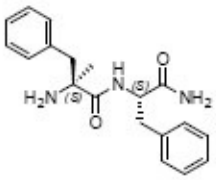
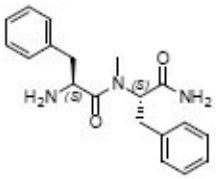
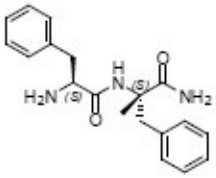
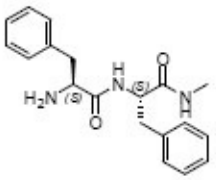
reported in Tables 3.3.7 and 3.3.8.

Table 3.3.6: Binding affinity (K_i values) and metabolic stability (Cl_{int} and $t_{1/2}$) of the rigidified and C-terminal-modified H-Phe-Phe-NH_2 analogs

Compound	Structure	Binding affinity K_i ± SEM (nM)	Clearance[c] Cl_{int}[d] (µL min^{-1} mg^{-1})	Half-life[c] $t_{1/2}$[e] (min)
38[a]		34 ± 3	28 ± 3	50 ± 6
39[a]		51 ± 2	16 ± 9	103 ± 6
40[a]		2.4 ± 0.6[b]	16 ± 3	88 ± 15
41[a]		93 ± 0	7.6 ± 1.9	187 ± 5
42[a]		18 ± 1	39 ± 4	36 ± 3
43[a]		68 ± 1	16 ± 7	98 ± 4

[a] The stereochemistry of each diastereomer pair was estimated from the pharmacophore model. [b] IC_{50} value. Analog **40** was tested at a 2:1 ratio with analog **41**. It was tested once, at six different concentrations, in triplicate. [c] The metabolic stability data are expressed as mean ± SD. [d] Cl_{int} = *in vitro* intrinsic clearance. [e] $t_{1/2}$ = *in vitro* half-life.

Table 3.3.7: Active uptake and permeability data for the methylated H-Phe-Phe-NH_2 analogs.

Compd.	Structure	Uptake[a] (pmol mg^{-1} protein min^{-1}) CHO-PepT1	CHO-K1	Ratio PepT1/K1	Caco-2 permeability[a] P_{app}[b] (10^{-6} cm s^{-1}) a–b[c]	b–a[d]	Ratio ab/ba	Ratio ba/ab
26		0.3 ± 0.0	0.3 ± 0.0	1.0	0.02 ± 0.00	0.2 ± 0.0	0.1	11
33		2.2 ± 0.7	2.4 ± 0.5	0.9	14 ± 1	124 ± 21	0.1	9
34		1.1 ± 0.2	1.5 ± 0.2	0.7	18 ± 0	87 ± 0	0.2	5
35		19 ± 0	23 ± 3	0.8	9.0 ± 2.2	124 ± 19	0.1	14
36		33 ± 2	32 ± 9	1.0	20 ± 2	224 ± 5	0.1	11
37		0.3 ± 0.1	0.4 ± 0.1	0.7	0.3 ± 0.1	0.9 ± 0.2	0.3	3

[a] The results are expressed as mean ± SD. [b] P_{app} = apparent permeability coefficient. [c] a–b = apical to basolateral. [d] b–a = basolateral to apical.

A P_{app} value below 0.2 × 10^{-6} cm s^{-1} indicates low permeability, a P_{app} value ranging from 0.2 × 10^{-6} cm s^{-1} to 1.6 × 10^{-6} cm s^{-1} indicates moderate permeability and a P_{app} value above 1.6 × 10^{-6} cm s^{-1} indicates high permeability, in this particular setting (Bergström, 2003). The permeability in the a–b direction increased substantially for all the methylated analogs (ranging from 0.3 × 10^{-6} cm s^{-1} to 20 × 10^{-6} cm s^{-1}) compared to H-Phe-Phe-NH_2 (**26**, 0.02 × 10^{-6} cm s^{-1}). All the compounds,

except **37**, were classified as having high permeability, with the highest permeability observed for the α-methylated analogs **34** and **36**, 18×10^{-6} cm s^{-1} and 20×10^{-6} cm s^{-1}, respectively. However, the methylated compounds were not actively transported, as can be seen from the PepT1/K1 ratio. Furthermore, the peptides also displayed efflux (the ba/ab ratio ranging from 3 to 14), where compounds **35** and **36** showed the highest tendency towards efflux and were equal to **26**.

Compared to the methylated analogs, the rigidified and the C-terminal-phenylalanine-modified analogs possessed much lower permeability (ranging from 0.02×10^{-6} cm s^{-1} to 4.4×10^{-6} cm s^{-1}). Satisfyingly, the high affinity analog **40** turned out to have high permeability (3.6×10^{-6} cm s^{-1}). In this series, the efflux was much higher; 8–95 times, in the b–a direction than in the a–b direction. The stereochemistry of the compounds also seemed to influence the predisposition for efflux. Thus, the compounds **38**, **40** and **42** all showed lower efflux ratios than their diastereomeric counterparts **39**, **41** and **43**. The replacement of the phenylalanine in the N- or C-terminal by the 3-phenylproline moiety seemed advantageous in improving the active uptake of these compounds, since a slight increase in the PepT1/K1 ratio was observed (cf. **26** vs. **38**, **39**, **40** and **41**).

3.3.3 Conclusion

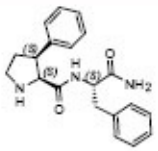
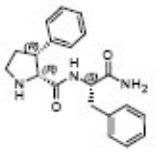
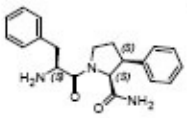
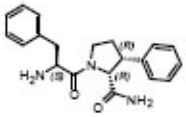
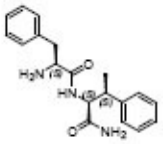
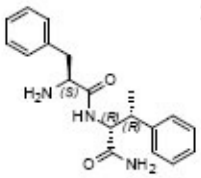
The case study presented herein is an illustrative example of rational design of drug-like molecules mimicking the actions of a bioactive peptide. It highlights the importance working in an iterative manner, where structural modifications of compounds are evaluated both from a pharmacodynamic and pharmacokinetic perspective.

The optimization process, starting with the bioactive heptapeptide SP_{1-7} and the tetrapeptide EM-2, resulted in the remarkable discovery of the dipeptide H-Phe-Phe-NH_2, which was equipotent to endogenous SP_{1-7} and had a higher binding affinity than EM-2. However, in the ADME assessment, the dipeptide showed poor metabolic stability and permeability, and suffered from high efflux rates. By introducing local constraints in the C-terminal of H-Phe-Phe-NH_2, via the introduction of

the *cis* 3-phenyl-pyrrolidine moiety, the pharmacokinetic properties could be substantially improved and the binding affinity retained.

It should be emphasized that although numerous strategies and attempts for transforming a bioactive peptide into small peptidomimetics have been reported, it is not straightforward, it is case dependent, and there is no guarantee of success. The decreasing number of approved drugs during recent years has put enormous pressure on the pharmaceutical industry, resulting in a revival of interest in peptides as potential drug candidates (Vlieghe, 2010). By using synthetic strategies to limit metabolism and exploring alternative routes of administration, a number of peptidic drugs have been brought to the market (Vlieghe, 2010), showing that it is not necessary to remove the peptide character completely, and that small pseudopeptides can be useful as drugs.

Table 3.3.8: Active uptake and permeability data for the rigidified and C-terminal-modified H-Phe-Phe-NH_2 analogs.

Compd.	Structure	Uptake[a] (pmol mg^{-1} / protein min^{-1}) CHO-PepT1	CHO-K1	Ratio PepT1/ K1	Caco-2 permeability[a] P_{app}[b] (10^{-6} cm s^{-1}) a–b[c]	b–a[d]	Ratio ab/ba	Ratio ba/ab
38		31 ± 4	22 ± 2	1.4	0.5 ± 0.0	26 ± 1	0.0	53
39		15 ± 2	11 ± 1	1.4	0.8 ± 0.1	76 ± 1	0.0	95
40		13 ± 2	11 ± 1	1.2	3.6 ± 0.4	75 ± 2	0.1	21
41		11 ± 1	8.7 ± 0.8	1.3	0.6 ± 0.0	51 ± 2	0.0	86
42		0.6 ± 0.1	0.7 ± 0.0	0.9	0.7 ± 0.0	5.4 ± 0.4	0.1	8
43		25 ± 4	22 ± 3	1.1	4.4 ± 0.1	171 ± 6	0.0	39

[a] The results are expressed as mean ± SD. [b] P_{app} = apparent permeability coefficient. [c] a–b = apical to basolateral. [d] b–a = basolateral to apical.

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3.4 Synthetic Vs. Natural Bioactive Compounds Against Tropical Disease

3.4.1 Introduction

Tropical diseases, although largely neglected by the commercial pharmaceutical industry, are a major problem for people in poor and underdeveloped nations. Perhaps the most significant of these threats comes from malaria. Roughly half of the world's population live in malaria endemic regions, and the WHO estimates that it caused 627,000 deaths in 2012 (CDC, August 18, 2014). Nearly 500 million cases of malaria are confirmed worldwide annually, with roughly 80% of those caused by *Plasmodium vivax*, a persistent strain of the parasite (Goncalves, 2014). Due to the constant emergence of resistance, there is an ongoing need for the development of new drugs for malaria (Rosenthal, 2003). For this reason, and in the interest of outlining a generic drug development paradigm in limited space, we will focus the topic of the current discussion on the historical successes and failures of natural products in treating malaria and the efforts in the modern era to combat drug resistance and poor drug tolerance with synthetic drugs.

3.4.2 Early History of Malaria Treatment; Quinine and Artemisinin

"The angel of disease and death, ascending from his oozy bed, along the marshy margins of the bottom grounds [...] floats in his aerial chariot, and in seasons favorable to his progress, spreads mortal desolation as he flies", so it was written in an Ohio newspaper article from 1820 (Findley, 1968). From this quote one can see how parasitic disease plagued the early frontiersmen of the 19th century. However, the unhappy coexistence of humans and parasitic disease goes back much further into the past. In fact, the earliest recorded descriptions of a

disease with symptoms consistent with those of malaria date back to 2700 BC from imperial China. During this period traditional Chinese medical practitioners discovered the use of sweet wormwood (*Artemesia annua*) to treat this mysterious fever. Meanwhile, on the other side of the planet Quechuas from South America used the bark of the cinchona tree in the treatment of similar fevers. Jesuit monks brought this bark from the new world and introduced its powerful medicinal properties to Europe during the 17th century, however, the active component, quinine, was not isolated until 1820 (Faurant, 2011). Quinine is known for several unpleasant and potentially severe adverse reactions including cinchonism, hearing impairment, increased risk of hemolysis in G6PD deficient patients, and is linked to a severe syndrome in some patients, dubbed blackwater fever. Modern efforts suggest the latter may be the result of redox active metabolites (Marcsisin, 2013). As we will explore, many of the ongoing efforts in anti-malarial therapy revolve around not only circumventing resistance, but in mitigating many of the safety risks associated with historically used drugs. These reasons and the outbreak of war in malaria endemic regions of the world led to a surge of interest in the development of safe and effective drugs to treat malaria that we will explore further.

3.4.3 Post World War II and the Development of Synthetic Anti-malarials

"Doctor [...] this shall be a long war if for every division I have facing the enemy I must count on a second division in the hospital with malaria and a third division convalescing from this debilitating disease!" Thus opined Gen. Douglas MacArthur concerning the ravages of malaria during the second world war (Coates, 1963). This constant struggle with non-combat related injury from disease was a tremendous burden on medical logistical chains, morale, and overall readiness on fronts across Africa, Asia, and even in parts of Europe. This reality was a major shaping force for a massive effort to prevent and treat malaria.

During WWI, Germany had no access to quinine. As a result, a large-scale effort ensued in the intervening years between WWI and WWII in

which the Germans synthesized and screened thousands of compounds for anti-malarial activity. Among these were several 8-aminoquinolines, atabrine (quinacrine), and a drug they discarded for toxicity called Resochin (chloroquine) (Coates, 1963; Vale, 2009). After access to quinine from Indonesia was cut to Allied forces by the entry of Japan into WWII, US and British chemists began a similar push resulting in the adoption of atabrine as the drug of choice for malaria treatment and prophylaxis during the later years of the war. Post-war, powerful new anti-malarial drugs began to emerge as the culmination of follow-on efforts by US and British chemists to exploit the original efforts by Germany. Many of these drugs are still in use today, with robust analog efforts being employed around them to overcome developing resistance.

3.4.4 Modern Efforts in Antimalarial Drug Development

Modern efforts in anti-malarial drug discovery in general follow six separate approaches including optimization of therapy with existing agents, development of analogs of existing agents, natural products, repurposing of drugs from other therapeutic areas, reversal of resistance, and discovery of compounds active against novel targets (Rosenthal, 2003). Table 3.4.1 illustrates examples from current literature from each approach. It is interesting to note that many of these approaches revolve around existing agents and their analogs, many of which were known since the 1920s, 30s, or 40s. For the purposes of this discussion, we will focus on ongoing work in existing classes and development of novel classes of anti-malarials, as discussions of combinations of existing drugs can become lengthy and are better suited in a clinical pharmacology text.

3.4.4.1 Quinine, 4-Aminoquinolines, and Quinoline Methanols

For natural products, quinine is a success story that has endured for centuries. Quinine is an aryl amino alchohol derived from the bark of the cinchona tree (Fig. 3.4.1). Despite its discovery over 400 years ago, it remains an enormously important drug in the treatment of malaria in the developing world. Quinine is rapidly absorbed, both orally and

parenterally, broadly distributed throughout the body, and has proven highly effective for the treatment of uncomplicated or severe malaria (Achan, 2011). In the age of resistance, quinine has made a resurgence in usage. Although quinine resistance has been reported, it is generally low-grade, and is largely found in Asia and South America (Noedl, 2006; Parola, 2001).

Perhaps a larger issue still in play for quinine is tolerance. Quinine has several notable and potential severe adverse events associated with usage. Most common side-effects include tinnitus and hearing impairment, but more severe effects can include vertigo, vomiting, abdominal pain, or hypotension (Achan, 2011). The most severe and potentially least understood is a syndrome called blackwater fever characterized by hemolysis and hemoglobinuria (George, 2009). Recent studies suggest a potential link between CYP mediated oxidative metabolites of quinine and this potentially fatal reaction (Marcsisin, 2013). It should also be noted that while clinically different from the hemolytic events observed with the 8-aminoquinolines (which we will discuss in a later section), as with the 8-aminoquinolines a link may exist between glucose-6-phosphate (G6PD) deficiency and hemolysis (Hue, 2009). This is significant as it suggests common metabolic pathways for some quinoline drugs that should be avoided or at least considered when developing new drugs in these classes. For quinine, the formation of redox active quinones may lead to increased oxidative stress under conditions of high parasitemia or in G6PD deficient individuals that ultimately results in the destruction of red-cells through mechanisms which are not fully understood (Fig. 3.4.2) (Marcsisin, 2013).

Arguably one of the most successful classes of drugs to arise from WWII efforts is the 4-aminoquinolines. Fig. 3.4.3 illustrates two members of the class, chloroquine and amodiaquine, and can serve as a generic paradigm for its structure. Chloroquine was originally discovered by Hans Andersag in 1934 while working for Bayer, however, the drug was discarded for perceived toxicity. After its re-discovery by British and American chemists, it quickly won favor as a safe and effective anti-malarial (CDC, August 18, 2014). In fact, chloroquine was used extensively in post-war eradication efforts. While the mechanism of

action for all 4-aminoquinolines is not exactly known, they are known to be effective in treating only erythrocytic stages of infection. Poor compliance or poor management of care with such drugs can rapidly lead to resistance. This was exactly the case with chloroquine. By the early 1950's, chloroquine resistance was identified along the Thai-Cambodian border and Colombia. Within two decades it had spread to every malaria endemic region of the world (Farooq & Mahajan, 2004).

While the mechanism of action for the 4-aminoquinolines is not completely understood, the most widely cited hypothesis is that accumulation in the digestive vacuole of the parasite interferes with hemoglobin digestion. Resistance is believed to be conferred by the presence of a chloroquine specific P-glycoprotein pump (Foley & Tilley, 1998). Several loci in the *P. falciparum* genome have been implicated in this resistance (Farooq & Mahajan, 2004). This mechanism, or a variant thereof may well be important in quinine, mefloquine, or other quinoline resistance mechanisms, however, it should be noted that more lipophilic quinolines do not concentrate in the food vacuole to the same extent as chloroquine, and therefore other mechanisms need to be explored (including potential alternative targets of efficacy) when considering these drugs.

A considerable effort is still ongoing to circumvent resistance through analog campaigns or co-administration with other drugs (Hanboonkunupakarn, 2014; Le Garlantezec, 2014; I. Opsenica, 2011; I. M. Opsenica, 2013; Thriemer, 2014). While it is tempting to synthesize analogs of a compound with emerging or established resistance, in the author's opinion, such efforts should be entered into with full knowledge that selection pressures in malaria endemic areas quickly erode the efficacy of new drugs from old classes. It is advisable to pursue a combination approach wherein fast acting short half-life drugs (e.g. artemesinins) are combined with long half-life (longer exposure of parasite to drug) quinolines prone to emergence of resistance. Further, future efforts in this class should pay careful attention to early signs of cross resistance in chloroquine resistant strains. While small jumps in IC50 in a resistant strain may not raise alarms if those IC50s are still significantly less than the anticipated maximum concentrations achieved in plasma post-exposure, they could be indicative of shared

mechanisms of resistance that might worsen over time with large exposures in environments with poorly controlled administration or where monotherapy is used.

Mefloquine, a quinoline methanol, was developed by the US Army in the 1970s, and was considered very desirable for both treatment and prophylaxis due to its long half-life (Croft, 2007). In recent years mefloquine usage has decreased due to emerging resistance, and more significantly, poor tolerance (Farooq & Mahajan, 2004; Milner, 2010). Mefloquine has been linked to neurological side-effects including vertigo, loss of balance, and polyneuropathy, which have recently been labeled as potentially irreversible by the FDA (Nevin, 2014). Extensive efforts have shown promise in improving the therapeutic index of mefloquine by opening or removing the piperadine side-chain (Dow, 2006; 2011; Milner, 2011). With the notoriously poor predictive power of models of CNS toxicity and the stigma attached to the quinoline methanol class, these efforts have largely been abandoned. Further, recent trials with enantiomerically pure mefloquine (currently marketed as a racemic mixture) have shown little promise in improving tolerability (Nevin, 2014).

3.4.4.2 8-Aminoquinolines

Another powerful class of anti-malarial drugs to emerge from war efforts is the 8-aminoquinolines. Although still quinoline based, this class has several key features which make it unique. One primary feature of this class is its ability to act as a causal prophylactic, or to prevent the initial infection of parasites in the liver. Further, the class has powerful anti-hypnozoite activity. Hypnozoites being the dormant liver stage of *P. vivax* and *P. ovale*, this imparts a separate prophylactic use, namely presumptive anti-relapse therapy or PART. Combined with this unique exoerythrocytic activity is the gametocytocidal activity, whereby 8-aminoquinolines kill the sexual stages of the parasite blocking further transmission of infection. This combination of activities makes this class highly attractive for prophylaxis, treatment of relapsing strains of malaria, and/or elimination efforts. The class has also shown *in vitro* anti-leishmanicidal activity and has clinical utility in the treatment of

pneumocystic pneumonia, and trypanosomiasis (Vale, 2009). However, as with other classes that we have reviewed here tolerability is a substantial problem for the 8-aminoquinolines. From this class, currently only primaquine is clinically available for these uses.

Administration of 8-aminoquinolines is known to cause methemoglobinemia and hemolysis in G6PD deficient individuals. Both of these effects are linked to oxidative metabolites rather than the parent drug itself (Ganesan, 2009; Ganesan, 2012). Interestingly, these same metabolites were recently shown to play a crucial role in activity (Bennett, 2013; Marcsisin, 2014; Pybus, 2013). For both primaquine and tafenoquine (8-aminoquinoline, currently in clinical development), activity is mediated by CYP 2D6 (Fig. 3.4.2). The mechanism of activity for the 8-aminoquinolines is not fully understood, however, contemporary thought is that redox active metabolites produce peroxides and superoxides (oxidative stress), which ultimately interferes with electron transport in the parasite. Not surprisingly, what is also unknown at present is whether the toxicity can be mitigated while preserving efficacy. Work in this area is of paramount importance, as new 8-aminoquinolines with similar profiles to primaquine are of little clinical utility.

The WHO currently calls for a 30 mg dose of primaquine as a chemoprophylaxis in areas where *P. vivax* is the predominant risk, however significant fractions of the population (10% in Europeans and as much as 50% in some Asians) possess 2D6 alleles with reduced function (Deye & Magill, 2014). It is therefore likely that primaquine prophylaxis and even anti-relapse therapy will fail in many of these individuals. All things considered, the importance of this class cannot be overemphasized and it cannot be abandoned without more study. As stated previously, primaquine is currently the only clinically available drug for the treatment of relapsing malaria. As such, the challenges posed for future 8-aminoquinoline development include re-routing metabolism through a non-CYP 2D6 pathway, circumventing hemolytic toxicity, or both. The primaquine enamine bulaquine showed some promise in recent animal studies at improving the therapeutic index (Lal, 2003; Mehrotra, 2007). This appears to be due to differential pharmacokinetic exposure patterns as compared to parent primaquine.

The addition of the enamine group on the side-chain creates an almost time-release effect as it is hydrolyzed to release primaquine. This leads to an interesting hypothesis that perhaps while the same metabolites may well be responsible for efficacy and toxicity, efficacy could be exposure driven while toxicity is concentration dependent, providing an in-road to improvements in therapeutic index. Although in the author's opinion, circumstantial evidence overwhelmingly points to an inextricable link between efficacy and toxicity in this class that may make it difficult, if not impossible, to support the large-scale development of a new candidate.

3.4.4.3 Artemisinins and other Endoperoxides

Although sweet wormwood was used for over 2000 years in China to treat malaria, the active ingredient, artemisinin, was not identified until the 1970's (Fig. 3.4.5) (Faurant, 2011). At present, several artemisinin compounds are clinically available for use including artemisinin, artesunate, dihydroartemisinin (DHA), and artemether. Other experimental drugs exist but are not clinically available. Due to poor tolerability and hence compliance with quinine, artemisinin compounds have drastically risen in popularity for the treatment of falciparum malaria (Shanks, 2006). Artemisinin resistance was first reported in Southeast Asia in 2008, and evidence suggests that it is spreading (Ashley, 2014; Dondorp, 2009; Thriemer, 2014). Widespread resistance to the most powerful new weapon in the fight against malaria could de-rail many ongoing efforts to eradicate the disease in the endemic world. As such, it is widely acknowledged that combination therapy should be the standard of care. The WHO currently recommends artemisinin combination therapy (ACT) consisting of DHApiperaquine, followed with a 0.75 mg kg^{-1} single dose of primaquine for the treatment of uncomplicated malaria (WHO, 2010).

At present, research is still ongoing as to the exact killing mechanism for the functional endoperoxide bridge contained in the artemisinins, however, it is thought to be a result of membrane depolarization and subsequent interference with electron transport (Antoine, 2014). Further development of synthetic and semi-synthetic artemisinins is still

ongoing as well as the development of novel endoperoxide containing compounds which have proven effective as anti-malarial agents in pre-clinical studies (Lanteri, 2014; Oliveira, 2014).

3.4.4.4 Repurposed Drugs

For the treatment of malaria and many other tropical diseases, limitations in funding have led to discoveries in the area of repurposing of drugs commonly used for other indications. Broad spectrum antibiotics have proven highly successful in the treatment of malaria. Doxycycline, a synthetic tetracycline, was shown to have partial prophylactic efficacy against malaria in the 1970's (Andrews, 2014). In fact, despite its own set of tolerance issues, doxycycline is now the prophylactic drug of choice for the US Army due to more serious concerns with mefloquine (Kime, 2012). It is also effective for use in treatment when partnered with a fast acting anti-malarial like quinine or quinidine (Tan & Centers for Disease Control and Prevention, 2011). Other antibiotics including clindamycin and sulfonamide antibiotics like sulphadiazine and sulphadoxine have also proven effective in the treatment of malaria (Andrews, 2014). Many of these target folate synthesis, which is a common pathway for other classical anti-malarials. Antifolate drugs block the synthesis of tetrahydrofolate, which in turn shuts down nucleic acid synthesis (Shanks, 2006). Among these is dapsone. Dapsone, a sulfone, was first used to treat leprosy but was introduced by GSK as Lapdap (dapsone/chloroproguanil) in the late 1990s. This drug was removed from the market in 2008 due to hemolytic anemia similar to that of the 8-aminoquinolines. Exploration of this strategy still continues with significant effort. Several examples can be found in the literature with protease inhibitors, antibiotics, and quinolones found to act against various targets in the parasite (Rosenthal, 2003). With pre-existing and well established safety profiles, re-purposing of old drugs is an attractive strategy if for no other reason than bypassing several regulatory hurdles and saving money in development. Another potential benefit for the developing world is often the cost of these drugs once marketed. Doxycycline, for example, is pennies per dose compared to Malarone (atovaquone/proguanil) and

inexpensive drugs are far more likely in that regard to make a meaningful impact on the fight against parasitic disease worldwide.

3.4.5 Natural Products in the Treatment of Malaria

As we have noted many of the progenitive compounds (quinine, artemisinin) for the treatment of malaria are naturally derived. Efforts continue to isolate natural compounds with anti-protozoal activity (Mohammed, 2014; Singh, 2014; Traore, 2014). Natural sources including plants, microbes, and animals provide libraries with rich chemical diversity, however, isolation and purification of active components presents a significant challenge in natural product development. The complexity of isolation is highly dependent on structure, which can often be quite complex with compounds isolated from natural sources. This of course leads to a second challenge for the development of natural products, namely synthesis. Once a hit is identified from a mixture, large quantities are often needed for further pre-clinical or clinical testing. The likelihood of identifying a clinic-ready compound from a natural source in today's regulatory environment is small. The more likely scenario involves the identification of a hit compound, which can be potentially modified to improve drug-like characteristics or decrease potential liabilities from toxicity. As we will discuss in the next section, the source of starting material for a drug development project is largely irrelevant once a hit is identified, as all compounds should be gated through the same testing scheme to ensure the end product matches the target product profile.

3.4.6 Considerations for Anti-parasitic Drug Development

In any drug candidate's journey down a development pipeline there are many pitfalls. Sadly, activity against a biological target or even *in vivo* efficacy does not make a successful drug. Careful consideration should be given early to liabilities that might lead to late stage failure, which could be costly and drain resources vital to the discovery of a potential candidate. In 1991, the leading cause of attrition for drug candidates was

poor pharmacokinetics and/or bioavailability (40%), yet by 2000 this had decreased to less than 10% (Kola & Landis, 2004). This is largely attributable to the advent of *in vitro* screens for physiochemical properties likely to create downstream liabilities. The placement of these screens early in the pipeline forces compounds with poor solubility, bioavailability, and metabolic stability to fail during far cheaper stages of development and spares the opportunity cost otherwise missed in development of these compounds. Fig. 3.4.6 outlines a generic paradigm for screening compounds. In this paradigm early hits from *in vitro* activity screens are passed through an absorption, distribution, metabolism, and excretion (ADME) gate before passing into more expensive animal models. The source of compounds could be from commercially available large libraries, libraries of natural product extracts, small scale synthesis, etc. The process remains unchanged. However, feedback from all stages should be iteratively incorporated into the next round of screening. Acceptable physiochemical properties for a drug ultimately depend on the target product profile for intended use; however, it is reasonable to assume that compounds with low solubility or poor permeability may not fit a profile for oral prophylaxis, for example. It should also be noted that many of these properties go hand-in-hand, and that it is often necessary to strike a balance. For example, lipophilicity, permeability, and metabolic instability tend to correlate since CYPs tend to favor lipophilic drugs. From this example, if one were trying to develop a long half-life lipophilic drug to concentrate in the food vacuole of a parasite, it might be necessary to trade off some lipophilicity (and with it potentially some biological activity) to enhance half-life. The subtleties of this interplay of course depend again on the intended use of the drug. Feedback from all stages of screening should be used to inform later rounds. In such a manner, successive rounds of screening should get closer and closer to the desired chemical space with optimum physiochemical properties and biological activity for intended use.

From the example of the 8-aminoquinolines, a very modern consideration for drug development arises. Pro-drugs should be fully metabolically characterized, with all pertinent pathways identified. Consideration should be given to the pharmacogenomic make-up of the

target population. Pro-drugs activated by CYPs 3A4, 2D6, or 2C19 may lack efficacy in some populations due to high polymorphism. If at all possible, non-CYP mediated conversion is desirable to avoid such liabilities in later development. Likewise, toxicity can be affected in a similar manner if, for example, the formation of a reactive metabolite is mediated by a highly polymorphic CYP.

Table 3.4.1: Approaches to antimalarial drug discovery and development. Adapted from Philip J. Rosenthal's review on Antimalarial drug discovery: old and new approaches (Rosenthal, 2003).

Approach	**Examples**
Optimize therapy with existing drugs (combinations)	Amodiaquine/sulfadoxine/pyrimethamine Amodiaquine/artesunate Artesunate/sulfadoxine/pyrimethamine Artesunate/mefloquine Artemether/lumefantrine Chlorproguanil/dapsone Chlorproguanil/dapsone/artesunate Atovaquone/proguanil
Develop analogs of existing drugs	New aminoquinolines New endoperoxides New folate antagonists
Natural Products	New natural products
Repurposing of drugs from other therapeutic areas	Folate antagonists Antibiotics Atovaquone Iron chelators
Reversing drug resistance	Verapamil, desipramine, trifluoperazine, chlorpheniramine
Discovery of compounds active against novel targets	Antibiotics, Quinolones, etc.

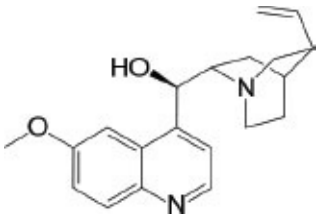

Figure 3.4.1: The structure of quinine.

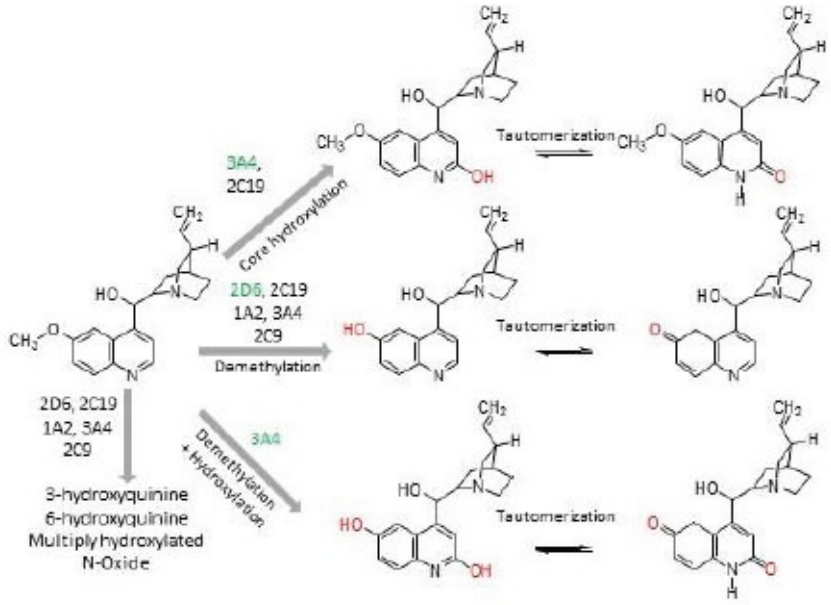

Figure 3.4.2: Metabolism of quinine leads to the formation of redox active quinones. Adapted from Marcsisin 2013 (Marcsisin, 2013).

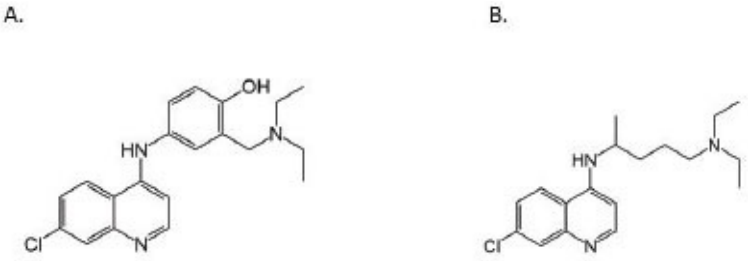

Figure 3.4.3: Two important members of the 4-aminoquinoline class: A. Amodiaquine B. Chloroquine

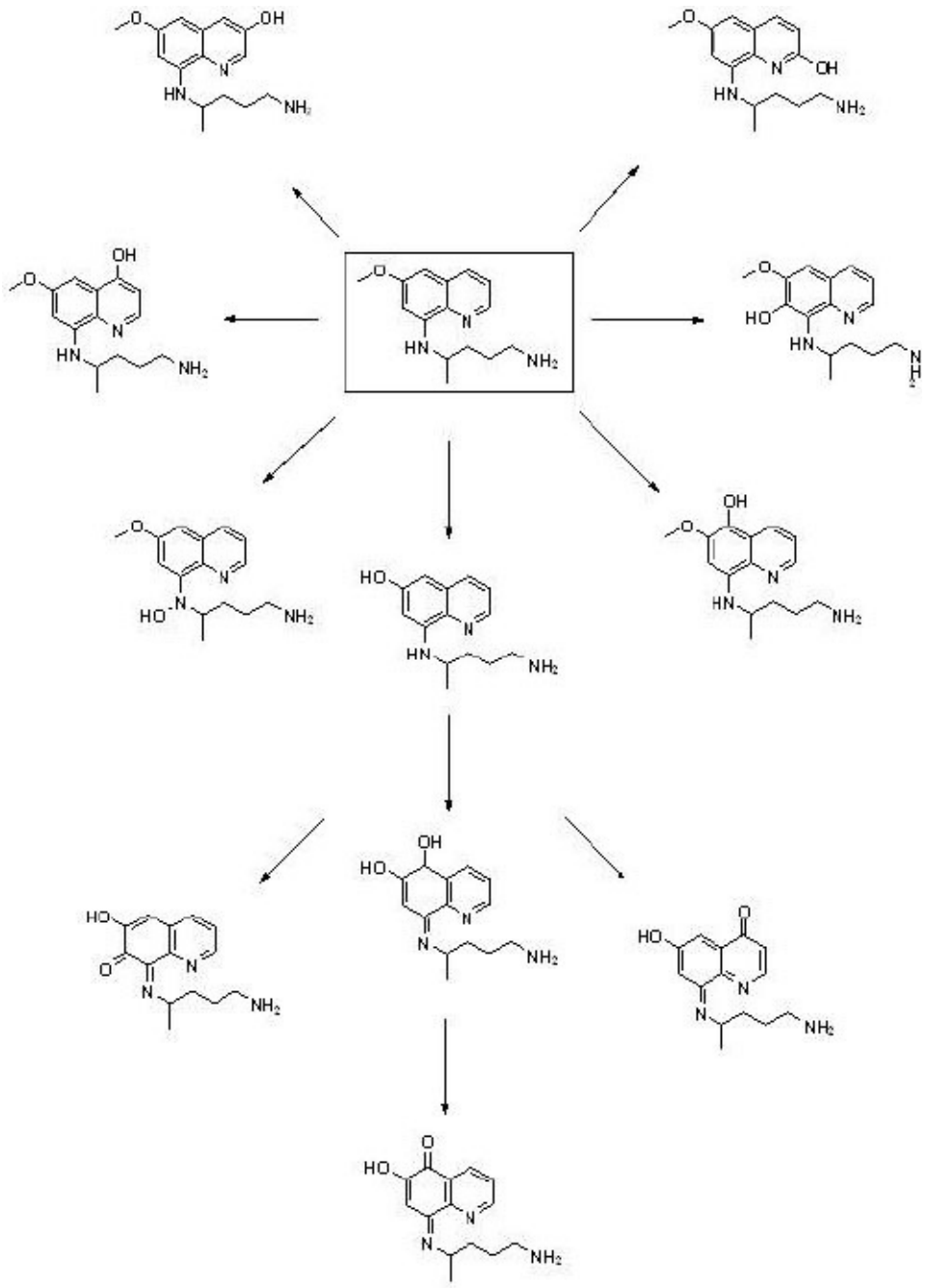

Figure 3.4.4: CYP 2D6 mediated metabolism of primaquine (Pybus, 2013). Several potentially oxidative metabolites formed by CYP 2D6 may be key in primaquine activity. Carboxyprimaquine is the primary metabolite found is plasma. Its production is likely MAO mediated and the pathway is not shown.

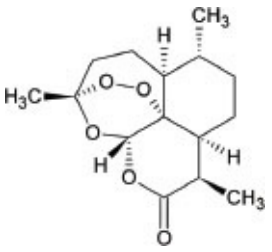

Figure 3.4.5: Artemisinin was identified as the active ingredient in sweet wormwood in the 1970s. Several synthetic artemisinins and other endoperoxides now exist for the treatment of malaria.

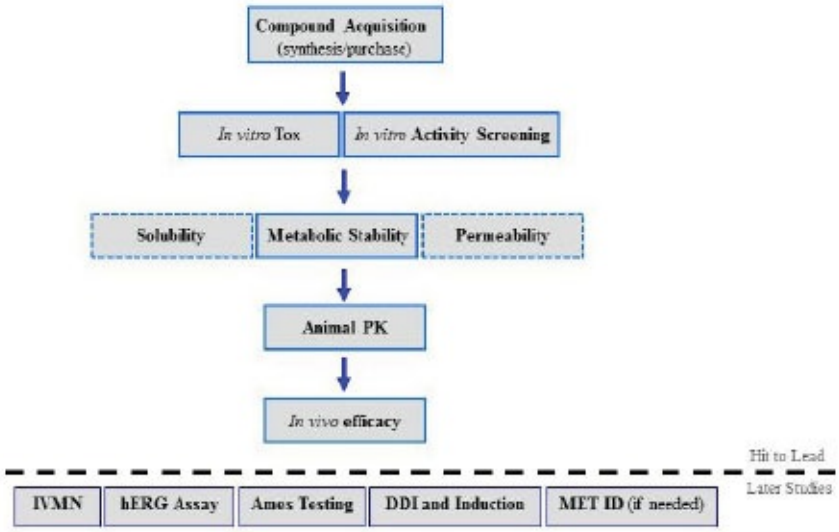

Figure 3.4.6: Generic paradigm for drug discovery that uses ADME properties to down-select potential candidates prior to expensive and labor intensive animal modeling. Dashed lines indicate a screen that is not necessarily a mandatory gate, but which can aid in the interpretation of potential later stage failure.

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3.5 Current Trends and Developments for Nanotechnology in Cancer

Abstract: In spite of the incessant development in medicine and technology, cancer continues to be one of the leading causes of death worldwide. Conventional systems for cancer therapy that are available in the market have limited and unspecific access to tumor sites. Thus, in the recent years, nanotechnology has been applied to the field of medicine, opening new avenues to the treatment, diagnosis, and monitoring of cancer diseases. This horizon has become closer with a considerable number of nano-formulations being recently approved for commercialization or reaching preclinical and clinical stages. In this context, remarkable advances in nanotechnology led to the emergence of nanodelivery systems that can specifically target an extensive variety of malignant tissues, control precisely the release of the cargos, as well as to improve the biological effects of the immunostimulatory molecules via different mechanisms for cancer immunotherapy. In addition, imaging techniques combined with nanotechnology render extraordinary sensitive and powerful diagnostic and imaging tools. Multifunctional systems encompassing both therapeutic and diagnostic functions provide great advantage for tracking, in real time, the drug payloads delivered to the tumour site. The bench-to-bedside translation of the nanomedicines and technologies have introduced a new era in the design and development of innovative, but simultaneously complex targeting nanoparticles for delivery of both therapeutic and diagnostic agents to tumors. In this chapter, we focus on the current trends and developments of nanotechnology for cancer, highlighting some of the most advanced drug delivery nanosystems, targeting strategies, the nanotools used for cancer imaging and diagnostics, as well as the recent nanotechnological approaches used for cancer immunotherapy.

Keywords: Nanotechnology, nanomedicine, drug delivery, targeting, cancer immunotherapy, diagnostics, imaging

3.5.1 Introduction

Cancer is one of the leading causes of death in the present days, killing millions of people every year (Sutradhar, 2014). Cancer is a pathological problem derived from genetic instability and multiple molecular alterations, caused by uncontrollable cell division which invade the surrounding tissue and destroy it (Mody, 2011). More than 8.2 million people died from cancer in 2012, and around 60% of new cases occurred in Africa and Asia, but 30% of cancers were prevented (WHO, 2014). In 2014, more than 1.6 million new cancer cases and more than half million cancer deaths are estimated to occur in the United States (ACS, 2014). During the most recent 5 years of existing data (2006–2010), delay-adjusted cancer incidence rates declined slightly in men (by 0.6% per year) and were stable in women, while cancer death rates decreased by 1.8% per year in men and by 1.4% per year in women. The combined cancer death rates (deaths per 100,000 inhabitants) have been continuously declining for 2 decades, from 215.1 in 1991 to 171.8 in 2010. This 20% decline translates to the avoidance of more than 1.3 million cancer deaths (almost 1 million among men and more than 0.3 million among women) during this time period (Siegel, 2014). In Europe, around one quarter of the total population is affected by new cancer cases, with about 3.2 million new patients every year (WHO, 2014). Breast, colorectal, lung, liver and stomach cancers are some of the most common cases of cancers.

While there have been many advances in cancer treatment, such as chemotherapy and radiotherapy for cancer, they are still far from being ideal. The problematic issue is to achieve the desired concentration of therapeutic agents at the tumor site, thereby destroying cancer cells, while minimizing the damage to normal cells. In order to overcome some of the problems of conventional anticancer therapeutics, cancer nanotechnology has been implemented, which indicates a major breakthrough in cancer detection, diagnosis and treatment of cancer (Misra, 2010).

Nanotechnology is one of the fastest areas of growth and development in the 21st century, where several material types (e.g., organic, inorganic, and polymeric based), medicines and devices are

used to manipulate matter with size in the range of 1–100 nm (Reddy, 2011) (Fig. 3.5.1). Some of the major challenges in nanotechnology are a proper identification of neoplasic early biomarkers, as well as the understanding of their evolution, screening and early detection, development of technology to provide different selective therapeutic nanoplatforms and the ability to pass through biological and physical barriers (Pinheiro AV1, 2011). Some nanosystems are developed to overcome drug resistance, since they control the efflux of P-glycoprotein, the major mechanism of drug resistance (Dong, 2010). Therefore, nanotechnology has brought new hope for cancer detection due to the development of nanozised probes (Mody, 2011).

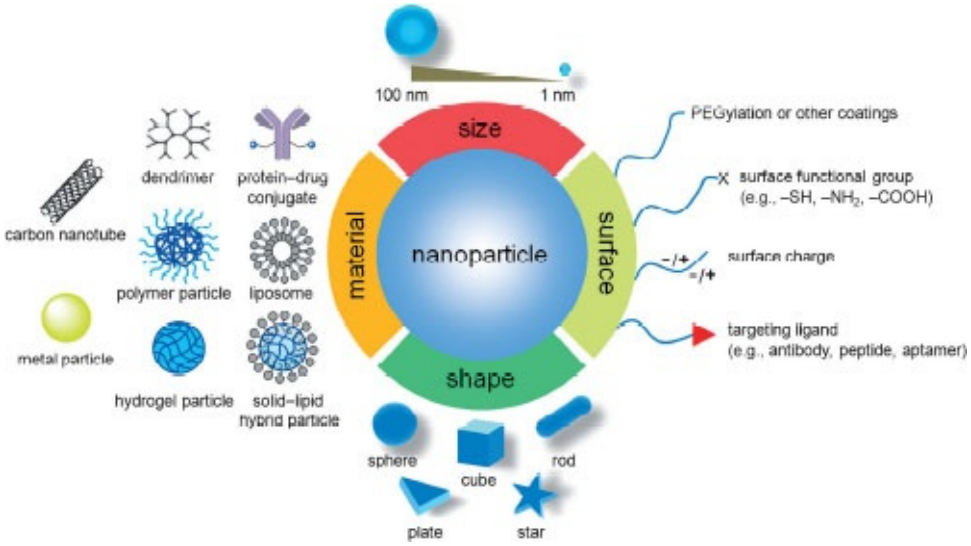

Figure 3.5.1: A summary of nanoparticles that have been explored as carriers for drug delivery in cancer therapy, together with illustrations of their biophysicochemical properties. Reprinted with permission from (Sun, 2014).

Nanotechnology applied to the field of medicine or “nanomedicine”, entails strong advantages in the treatment, diagnosis, monitoring, and control of biological systems, providing several innovative tools, some of them taking the form of nanoparticles, polymeric nanoconstructs, nanofibers, nanoscale microfabrication-based devices and sensors. (Moghimi, 2005). These nanotools aid in the design of imaging agents and diagnostics, allowing easy treatment and early detection of cancer (Table 3.5.1).

Nanomedicines are being used as vehicles to deliver therapeutic agents to a specific location in the body (Markman, 2013). The selective

nanomedicines for cancer treatment have revolutionized the research field of cancer and technology due to the possibility to precisely synthetize nanoparticulate systems with variable size, shape and physicochemical properties (Paulo, 2011). Nanomedicine can be applied to actively or passively target the tumor tissue (Krishnan, 2014). In passive targeting, the physicochemical properties of the drug nanocarrier are molded so that it escapes from the immune system and accumulates in the target tissue. Active targeting involves the attachment of an antibody, carrier protein or other ligand to the nanovector, so that it has affinity to a cell receptor or biomarker (Danhier, 2010). Targeted nanoparticulate imaging agents offer new opportunities for accurate cancer diagnosis. Due to the highly engineerable nanomaterials, nanoparticles offer great advantages such as increased sensitivity to contrast and the avidity of binding and cleavage specificity (Gautier, 2013). Considering the nanoparticles and their ability to reach a specific location and generate detectable signals, these nanoparticles can also be used to study the biophysical interactions (Sumer, 2008). Drug development and imaging nanomedicines are thus interconnected, due to the advantages of the application and non-invasiveness in high yield imaging studies. All imaging modalities can be used in drug studies to provide anatomical, pharmacokinetic and pharmacodynamic information (Wang, 2010).

Table 3.5.1: Nanomedicines approved by one or more regulatory bodies. Reprinted from (Wang, 2013).

Product	Nanoplatform/agent	Indication	Status	Company
Doxil	PEGylated liposome/ doxorubicin hydrochloride	Ovarian cancer	Approved 11/17/1995 FDA50718	Ortho Biotech (acquired by JNJ)
Myocet	Non-PEGylated liposomal doxorubicin nanomedicine	Metastatic breast cancer	Approved in Europe and Canada, in combination with cyclophosphamide	Sopherion Therapeutics, LLC in North America and Cephalon, Inc. in Europe
DaunoXome	Lipid encapsulation of daunorubicin	First-line treatment for patients with advanced HIV-associated Kaposi's sarcoma	Approved in the USA	Galen Ltd.
ThermoDox	Heat-activated liposomal encapsulation of doxorubicin	Breast cancer, primary liver cancer	Received Fast Track Designation, approval expected by 2013	Celsion
Abraxane	Nanoparticulate albumin/paclitaxel	Various cancers	Approved 1/7/2005 FDA21660	Celgene
Rexin-G	Targeting protein tagged phospholipid/ microRNA-122	Sarcoma, osteosarcoma, pancreatic cancer, and other solid tumor	Fully approved in Philippine Phase II/III (Fast Track Designation, Orphan Drug Status Acquired) in USA	Epeius Biotechnologies Corp.
Oncaspar	PEGylated asparaginase	Acute lymphoblastic leukemia	Approved 24/06/2006	Enzon Pharmaceuticals, Inc.
Resovist	Iron oxide nanoparticles coated with carboxydextran	Liver/spleen lesion imaging	In 2001, approved for the European market	Bayer Schering Pharma AG
Feridex	Iron oxide nanoparticles coated with dextran	Liver/spleen lesion imaging	Approved by US-FDA in 1996	Berlex Laboratories
Endorem	Iron oxide nanoparticles coated with dextran	Liver/spleen lesion imaging	Approved in Europe	Guerbet

The current nanoplatforms used for the purpose of targeting drug delivery to cancer tissues can also be addressed to selectively deliver imaging agents (Chen, 2014). For example, the first evaluation of the effectiveness of a particular formulation to a patient can be performed with imaging agents, in order to verify that the delivery system will primarily target the cancerous tissues, even before any drug regimen test is initiated (Toy, 2014). However, imaging is not only used for detection, it is also important to determine the stage and the precise locations of cancer. Distribution of multistage systems have shown potential to meet the challenges of drug targeting and overcome biological barriers (Miele, 2012). Image-based nanomedicines have also a crucial importance in cancer diagnostics due to their highly sensitive detection probes (Pan,

2010). Imaging techniques, which are methods of producing images of the body, are an important element of early detection of cancer diseases (Shukla-Dave, 2014).

Transformation procedures leading to malignancy can be detected by a matter of routine screening, non-invasive means, such as standard proteomic analysis of blood samples or *in vivo* imaging of molecular profiles (Bellisola, 2012). Some of the main challenges for nanomedicines are the development for the detection and monitoring of cancer markers *in vivo*, creation of technological platforms for early detection of cancer biomarkers *ex vivo*, and engineering of nanoparticles to prevent biophysical and biological barriers (Ferrari, 2005).

In this chapter we focus on the current trends and developments of nanotechnology for cancer, highlighting some of the most advanced drug delivery nanosystems, targeting strategies, nanotools used for cancer imaging and diagnostics, as well as recent nanotechnological approaches used for cancer immunotherapy.

3.5.2 Drug Delivery Nanosystems in Cancer Therapy

Cancer drug delivery is a demanding subject that requires deep and comprehensive research. Thus, this area of research has been under intensive investigation during the last few years. For example, problems associated with selective targeting, improved penetration through biological barriers and a controlled release of therapeutic cargos over time are details that influence the design of nanocarriers for drug delivery. Therefore, recently, sophisticated nanosystems have been developed in order to provide delivery of one or more therapeutic molecules in a controlled, selective and “smart” way to the desired cancer cells/tissues.

3.5.2.1 Controlled Drug Delivery

Conventional systems for cancer therapy that are available in the market have limited and unspecific access to tumor sites, and thus, it is well-known that patients experience serious side effects when treated with

anticancer drugs, frequently associated to low therapeutic efficacy (Siegel, 2011). Another factor that needs to be considered for drug delivery is the physicochemical properties of the drugs themselves. Many of the anticancer drugs used in the clinic at the present are rather hydrophobic, and thus, their poor solubility may lead to local toxicity associated with the fact that they may not be soluble enough to go through the aqueous environment surrounding the tumor cells and cross the cell membrane to ultimately reach the intracellular targets (Owen, 2012). On the other hand, successful utilization of hydrophilic drugs (e.g., macromolecules such as proteins or nucleic acids) has been stalled by a number of obstacles, such as poor cell internalization, because of their inability to cross the lipid bilayer of the cell membrane, as well as short half-life in the bloodstream, due to poor stability against proteolytic and hydrolytic degradation (Fattal, 2009; Ishihara, 2010; Sun, 2014).

Therefore, by applying nanotechnology, many scientists have focused on investigating new ways to develop novel drug delivery systems with the aim to maintain high therapeutic drug levels at the malignant cell sites and as low as possible in healthy cells, hence overcoming and improving the poor physicochemical properties of the drug (Grinberg, 2014; Sun, 2014). For this purpose, several strategies to both target the tumor site and release the drug(s) in a controlled fashion have been developed in order to selectively deliver therapeutic cargos over time. In this section, we will briefly focus on the most recent controlled release strategies and give some examples of the nanosystems used to achieve the abovementioned aims.

It is possible to fine-tune and control the release of payloads from the nanocarrier through diverse mechanisms. For example, for porous materials, controlling the pore size and the surface chemistry of the pore walls can lead to different diffusion release profiles, either improving or sustaining the release of the loaded cargos. Porous hollow Fe_3O_4 nanoparticles were able to provide the delivery of cisplatin via a slow diffusion-controlled process, exhibiting different kinetic profiles of cisplatin depending of the pore gap sizes (Cheng, 2009). Porous silicon (PSi) materials are another good example of a biomaterial widely used for biomedical applications (Santos, 2014). The small and tunable size of

the pores, high surface area and large pore volume enables high-drug-loading capacity. The confinement of large amounts of drug molecules, together with their interaction with the nanocarriers, and depending on the physicochemical properties of the PSi materials, leads to a tunable and controllable release of cargos (Mäkilä, 2014; Salonen, 2008). Controlled release of drug molecules can be also achieved through the uniform incorporation of the drug into matrix-based systems (water-insoluble polymer carriers). These systems enable sustained drug release because the diffusion of drug molecules from the inner matrix of the polymers to the surface takes time and may depend on the physicochemical properties of the drug, on the interactions between the drug and the polymeric matrix, and on the density of the matrix itself (Sun, 2014). Ma have synthesized a PCL-Tween 80 copolymer nanoparticulate matrix from ε-caprolactone and *Tween 80* to maintain the release of cargos through diffusion for as long as 28 days, with a better in vitro cytotoxicity towards C6 glioma cells compared to the commercial docetaxel (Taxotere) at the same drug concentration (Ma, 2011). Sustained release can similarly be achieved by using nanocarriers made of erodible or degradable materials. The release can be tailored by tuning the erosion kinetics of nanoparticles through the selection of biodegradable polymers, such as poly(lactic acid) (PLA), poly(lactic-co-glycolic acid) (PLGA), polyethylene glycol (PEG), polycaprolactone (PCL) among others (Sun, 2014), and through different encapsulation methods (Zilberman, 2008). Sun have successfully constructed a biodegradable nanoparticle based on a triblock copolymer PEG-b-PCLb-poly (2-aminoethylethylene phosphate) (Fig. 3.5.2) with a proven fruitful delivery of small interfering RNA (siRNA) and paclitaxel for synergistic tumor suppression (Sun, 2011).

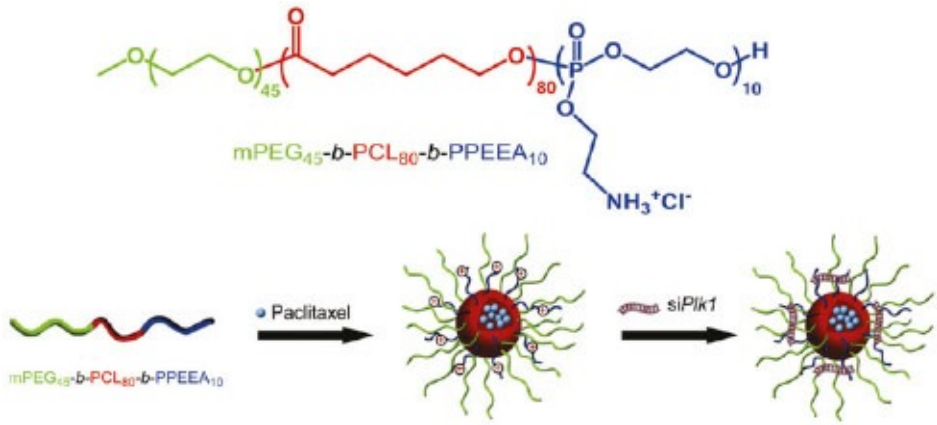

Figure 3.5.2: Chemical structure of the triblock copolymer PEG-b-PCL-b-poly (2-aminoethylethylene phosphate) and schematic illustration of the micellar nanoparticle formation and loading of paclitaxel and siRNA. Reprinted with permission from (Sun, 2011).

Nowadays, the most advanced systems are not so simple and combine a series of different functionalities that render tunable release of cargos, triggered release properties, selectivity, and the possibility of tracking the nanocarriers by imaging and even combined therapy of several active substances. Such systems are further described in the sections below.

3.5.2.2 Stimuli-responsive Controlled Drug Delivery Systems

Nanoparticulate systems have been widely used for drug delivery when combined to molecules that have the particularity to react to certain physiological or pathological changes in the surrounding environment (Jhaveri, 2014). This is an approach that offers additional opportunities to enhance tumor accumulation of drugs that are administered systemically, increasing the chances of a more successful therapeutic outcome when treating solid tumors and avoiding unnecessary drug distribution in healthy tissues, thus minimizing the toxic side effects of the drugs (Nakayama, 2014). Different strategies have been investigated and nowadays there are different nanosized platforms capable of responding to different stimuli (Fig. 3.5.3) that can be tumor extrinsic, such as application of light, magnetic field, heat or ultrasounds; or intrinsic to the tumor-like pH, reductive potential changes or up-regulation of proteins or enzyme expression (MacEwan, 2010; Nakayama, 2014; Yang, 2013; Zhu, 2013). In this section we will explain different tumor-intrinsic stimuli-responsive strategies recently applied in the research field.

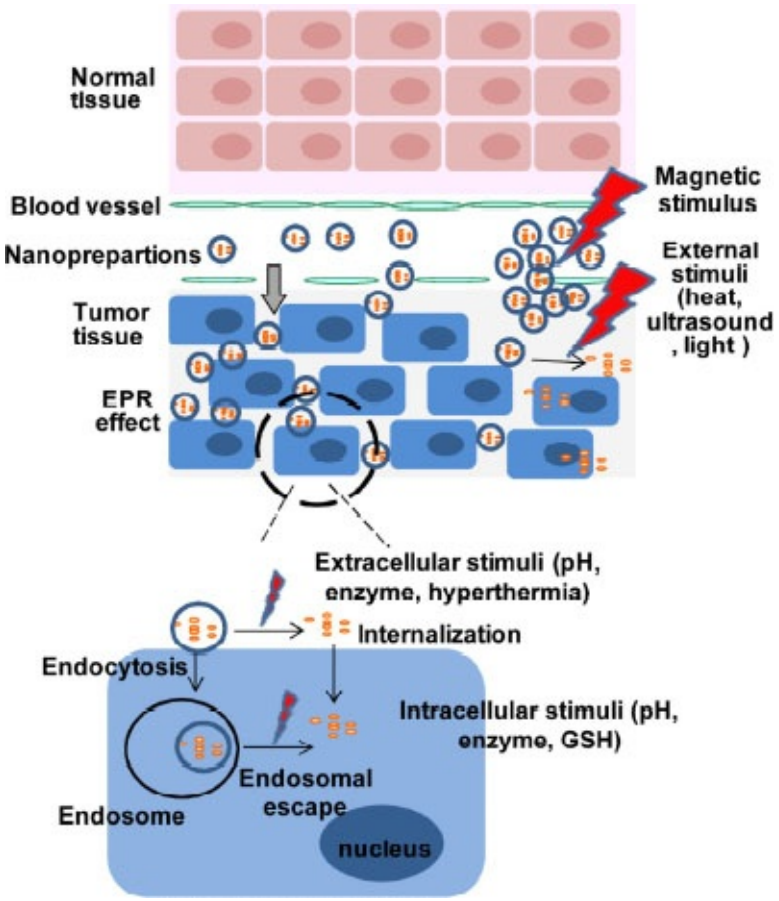

Figure 3.5.3: Stimulus-responsive delivery strategies for tumor targeting. Abbreviations: EPR, enhanced permeability and retention effect; GSH, glutathione. Reprinted with permission from (Zhu, 2013).

Among the nanosystems generated to trigger the release of cargos in a pH-dependent manner, many systems have been developed taking into account the difference in pH values existing between the healthy tissues (pH = 7.4) and the extracellular environment of solid tumors (pH = 6.5–6.8) (Helmlinger, 2002). Other systems exploit the pH values of the internal cellular organelles, such as lysosomes and endosomes (Sorkin, 2002). It is well-known that, environmentally, these cellular compartments have characteristic features, such as lower pH and reductive potential, which are divergent from the extracellular surroundings, offering a chance to exploit these special properties to release actively the cargos intracellularly, inside endosomal vesicles and/or lysosomal organelles (Elzoghby, 2012; MacEwan, 2010). For example, mesoporous silica nanoparticles (MSN) have been widely used for stimuli-responsive drug delivery in cancer therapeutics and were proved to be a versatile, easily functionalized platform, enabling an easy

manipulation of the nanopore openings, with noncytotoxic solid supports for nanovalve-controlled drug delivery (Fig. 3.5.4) (Coti, 2009; Tarn, 2013; Xia, 2009).

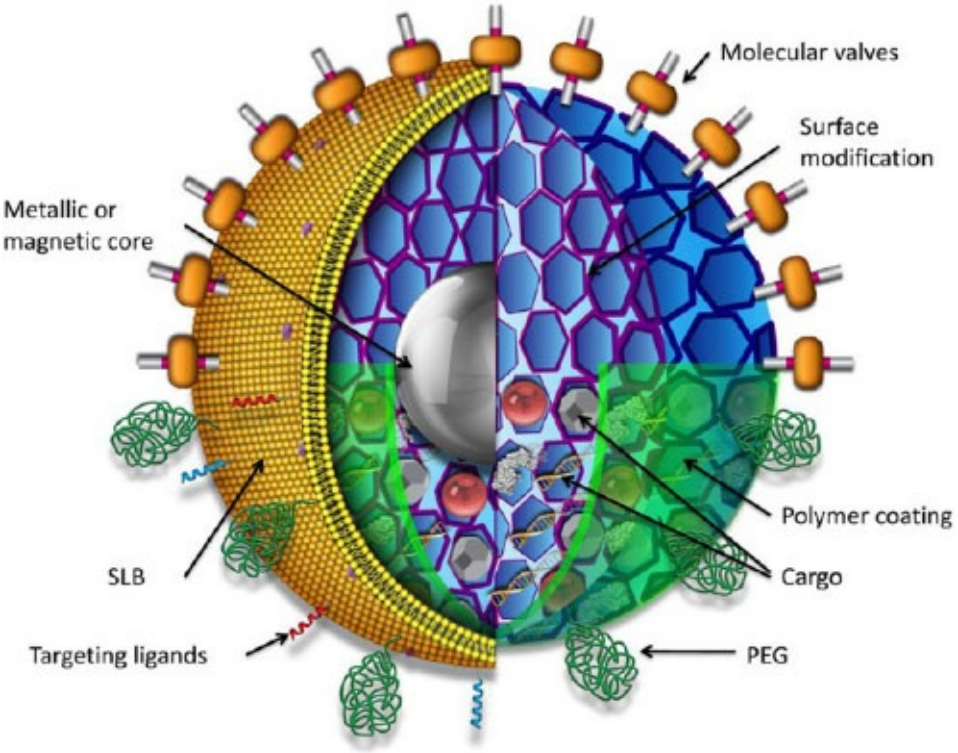

Figure 3.5.4: Schematic of a multifunctional mesoporous silica nanoparticle showing a core/shell design, surface modifications, and multiple types of cargos. Reprinted with permission from (Tarn, 2013).

It is also known that MSNs are endocytosed by cells in an energy-dependent manner and colocalize to the lysosomes (Yanes, 2012). Thus, many researchers have reported pH-responsive MSNs modified with different kinds of gatekeepers. The activated release of anti-cancer drugs from mesoporous materials due to a pH change in the environment has mainly been achieved by using polyelectrolytes, supramolecular nanovalves, pH-sensitive linkers, and acid-decomposable inorganic materials (Yang, 2014; Zhang, 2014b). In a study reported by Meng, (Meng, 2010), a nanovalve-based delivery system was designed to meet the pH features of the acidic compartments of lysosomal organelles. The nanosystem had the ability to have the nanovalves closed at physiologic pH by non-covalent interactions, but open in response to changes in pH acidic conditions inside endosomal compartments by dissociation at pH 6 or lower after cellular uptake. The principle used here was based on

the construction of stalks that were covalently attached to the nanopore openings, and the same stalks were bonded to a capping agent, in this case β-cyclodextrin (β-CD), that responded to the changes in the pH environment by opening or closing the nanopore accordingly in a reversible manner. In addition, the authors were able to demonstrate that this nanosystem was taken-up into the acidic endosomal compartments in THP-1 and KB-31 cells, showing also an efficient entrapment of dye molecules and anticancer drug doxorubicin inside the nanopores at physiological pH. In contrast, when the pH was decreased to values below 6, it caused dissociation of the β-CD caps and released the entrapped cargos, leading to apoptotic events of the cells. The opposite systems was also designed, having the β-CD rings immobilized and the stalks movable, making also possible the storage and delivery of different payloads (Zhao, 2010).

Other stimuli-responsive approaches are related to the use of pH-sensitive linkers that connect the drug molecule with the nanoparticle itself. Lee created a delivery system that linked doxorubicin to the MSN surface by hydrazone bonds, which were sensitive to the pH environmental changes, cleaving at low pH values from 4–6 (compatible with pH in the lysosome) and being intact at physiological pH, thus maintaining the drug molecules safely connected to the carrier during circulation in the bloodstream (Lee, 2010). Polymer coatings have also been used as coating agents to prevent the fast release of drug payloads. For example, Liu used poly(4-vinyl pyridine) (PVP) as a pH-responsive capping nanoshell to coat MSNs, where the releasing kinetics of the molecules was dependent on the protonation degree of PVP at different pH values. As the pH decreased, the drug release rate increased, since the protonated polymer became swollen and permeable to the trapped molecule. This approach may be useful to deliver drug cargos into microenvironments with lower pH, such as the tumor region and/or even the endosomal organelles (Liu, 2011).

Redox-responsive drug delivery approaches have also been recently exploited for cancer therapy applications (Remant, 2014; Shi, 2014), taking advantage of the fact that tumor cells have greater reducing features compared to healthy tissues, thus showing a much higher concentration of glutathione in the cytosol of tumor cells (Kuppusamy,

2002). Chuan have developed a novel nanosystem consisting of a self-assembling poly(ethyleneglycol)-disulfide-paclitaxel (PEG-SS-PTX/PTX) delivery vector containing free paclitaxel (PTX) and conjugated PTX in the same nanoparticulate system with a redox-triggered drug-release mechanism (Fig. 3.5.5). The principle is based on the instability of disulfide bonds in a reductive environment. The authors were able to improve the drug loading method, obtain programmed drug release, reduce the drug toxicity, and enhance the antitumor efficacy (Chuan, 2014).

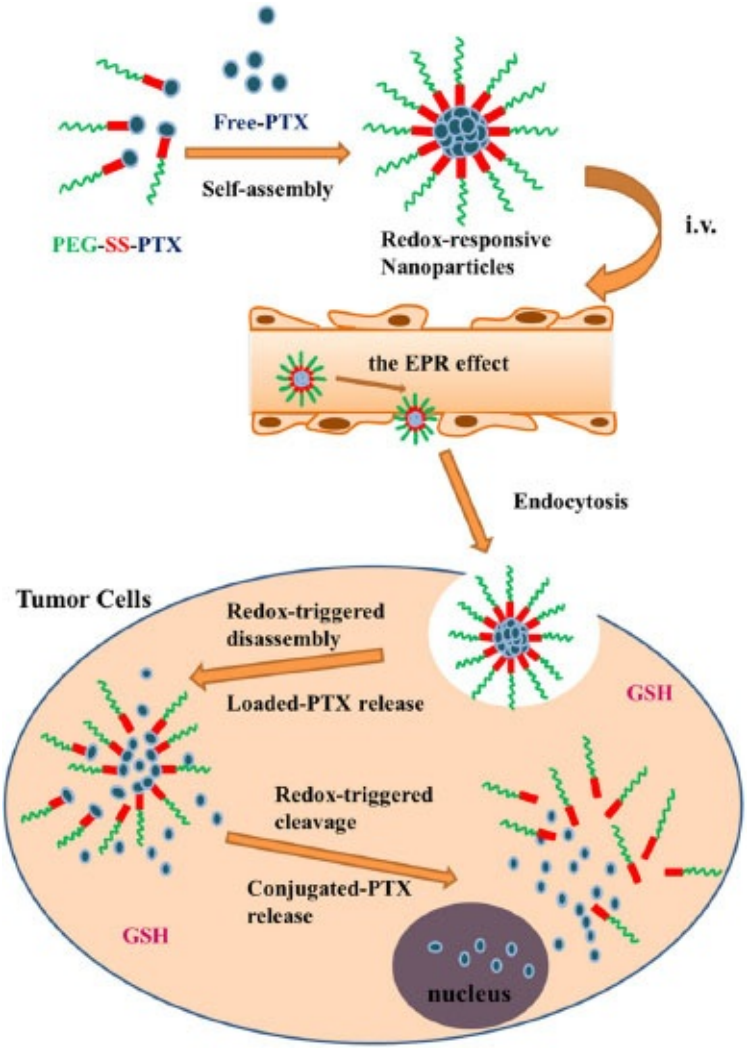

Figure 3.5.5: Schematic representation of the self-assembly, accumulation at the tumor site, uptake by tumor cells, and triggered intracellular drug release of redox-responsive PEG-SS-PTX/PTX nanoparticles. The programmed drug release undergoes two phases: release of loaded PTX and conjugated PTX. Abbreviations: PTX, paclitaxel, GSH, glutathione, EPR, enhanced permeability and retention effect. Reprinted with permission from (Chuan, 2014).

Variations in the composition and expression of local enzymes are other

potential factors that may provide an opportunity to selectively deliver drugs efficiently in the tumor sites via an enzyme-triggered mechanism. One of the most popular approaches is based on the altered expression of matrix metalloproteinases (MMPs), since in the MMP family, the up-regulation of MMP2 and MMP9 is generally recognized as involved in the invasion, progression and metastasis of most human tumors (Mansour, 2003). Zhu developed a multifunctional complex drug delivery system that has prolonged blood circulation time, targets specifically to tumor cells, responds to the up-regulated matrix metalloprotease 2 (MMP2) in the tumor microenvironment and has enhanced intracellular delivery by coupling specific moieties to the surface of a liposomal nanovector (Zhu, 2012). They were able to show a significant increase in the tumor targetability, as well as tumor cell internalization of the nanocarrier, and thus, this approach may be useful for selective drug delivery of cargos in the tumor sites. Other up-regulated enzymes, namely cancer-associated enzymes, have been utilized for the same purpose in liposomal formulations, yielding positive results of fast release kinetics in the specific tumor site (Basel, 2011).

3.5.2.3 Combination Therapy

Cancer is a multifactorial disease and often the clinical treatment requires the administration of more than one drug molecule in order to achieve successful therapeutic outcomes. In addition, cancer cells frequently become multidrug resistant, by developing defense mechanisms against the therapeutic approach, like expression of different receptors/target proteins, self-repairing mechanisms or/and alterations in their own metabolism, evolving towards a weaker response to the treatment (Holohan, 2013). The combination of different therapeutics can lead to reduced drug resistance and synergize the therapeutic effect of different active substances, and thus, a dual positive outcome on the improvement of therapeutic effectiveness and decrease of deleterious effects (Hu, 2010). Despite the positive advantages of the association of multidrugs in a delivery system, it is rather challenging to efficiently co-load different drug molecules, often with distinct physicochemical properties, in the same ordinary

nanocarrier (Hu, 2010; Kratz, 2012; Zhang, 2011), and even more challenging to deliver such cargos *in vivo*, where it is absolutely necessary to supply the proper drug ratio at the tumor level (Mayer, 2006). Several nanoparticulate systems such as lipid-based, inorganic and polymeric nanoparticles, among other types, have been used to co-load two or more different payloads adopting different strategies (Hu, 2010). Some include the physical adsorption of drugs to the nanovector, with the disadvantage that only drugs with similar physicochemical properties can be loaded simultaneously (Herranz-Blanco, 2014; Liu, 2012; 2014a). It is also possible to co-load molecules with different physicochemical properties via a sequential adsorption procedure (Liu, 2013a). Others have followed the incorporation of therapeutics into the particle matrix during particle preparation (Doane, 2013), and covalently bonded the drug molecules to the polymeric backbone after particle preparation (Aryal, 2011).

Despite the obstacles encountered in cancer therapy, extensive progress has been made so far and nowadays there are several nanoformulations that combine two or more different anticancer drugs (Liao, 2014; Tardi, 2009) or even two different classes of therapeutics (Deng, 2013; Liu, 2014b; Saad, 2008). For example, a nanoparticulate system was developed by Deng using a layer-by-layer deposition approach (Deng, 2013). Liposomes were chosen to be encapsulated by polycationic PLA through the layer-by-layer deposition, and finally coated by a layer of hyaluronic acid that enhances the *in vivo* stability of the nanosystem (Poon, 2011). This system co-delivered siRNA molecules and doxorubicin loaded in the core of the liposomes, which were able to knockdown a drug resistance pathway in tumor cells (Whitehead, 2009). This means they can be applied in the treatment of triple-negative breast cancer form, which is an estrogen, progesterone and human epidermal growth factor receptor 2 (HER2)-negative cancer type with a more aggressive effect than other breast cancers (Kassam, 2009; Livasy, 2006). The successful co-delivery of both siRNA and doxorubicin was achieved, as well as significantly enhanced DXR efficacy by 4-fold *in vitro* (Fig. 3.5.6). The *in vivo* results showed an 8-fold decrease in tumor volume compared to the control treatments, with no observed toxicity to the animals. An ultimate outer layer of hyaluronic acid (HA) was

deposited to increase the stability of the system (Deng, 2013).

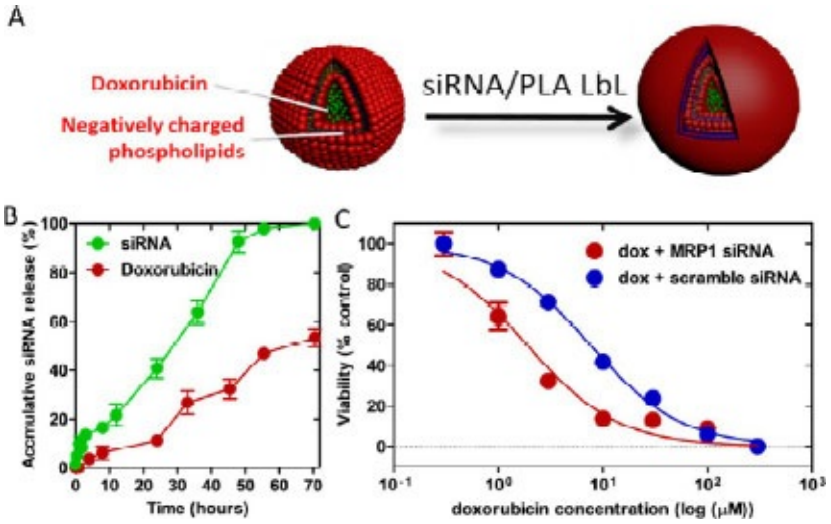

Figure 3.5.6: The co-delivery of siRNA and doxorubicin using the modular doxorubicin-liposome/PLA/siRNA/PLA/HA LbL nanoparticle platform. (A) Schematics of the siRNA-doxorubicin LbL liposomes. (B) Release profile of the two therapeutics components: siRNA and doxorubicin from the LbL liposomal nanoparticles in tissue culture medium over 72 h ($n = 3$). (C) Evaluation of the siRNA-enhanced cytotoxicity of the combo therapy in MDA-MB-468 cells. The results represent mean ± standard deviation ($n = 3$; $P < 0.05$). Reprinted with permission from (Deng, 2013).

Another study that combines siRNA and the co-delivery of paclitaxel in a two-in-one micelleplex system showed that it is possible to reduce by hundreds of times the dose of paclitaxel given by associating it with siRNA, and equally achieve a synergistic therapeutic effect (Sun, 2011). This and other systems are able to provide the co-delivery of different drugs and therapeutic classes to the tumor sites *in vivo*, giving hope to the decrease of harmful side effects of the anticancer drugs.

As described before, there are a variety of strategies that can be applied to combine different molecules for treatment of tumor diseases. In addition, there is the possibility that the nanocarrier itself is modified in such a way that it also has some therapeutic activity. Interestingly, the following study combined doxorubicin to the pro-apoptotic effect of the nanocarrier itself by means of incorporation of a sphingolipid C6-ceramide into the liposomal formulation, targeting at the same time nucleolin, a protein that is greatly expressed in cancer and endothelial cells of tumor angiogenic blood vessels (Shi, 2007). In this work, the sinergistic combination of DXR:C6-Cer in a 1:2 molar ratio was identified

and tested, which increased the cell toxicity potential, and thus, enabled at least a 4–6-fold dose reduction of doxorubicin, independently of the cell line tested. Hence, this approach might be valid to overcome drug resistance and, at the same time, decrease the deleterious effects of doxorubicin (Fonseca, 2014).

3.5.3 Cancer Targeting

Recent developments in nanotechnology are expected to revolutionize the current scenario of cancer therapy and diagnosis. This horizon has become closer with a considerable number of nano-formulations being recently approved for commercialization or reaching preclinical and clinical stages. The unique features of engineered nanotherapeutics can be translated into potential and attractive advantages over the conventionally practiced chemotherapeutic modalities, including: the reversion of unfavorable physicochemical properties of cytostatic drug molecules, resulting in improved pharmacokinetic and pharmacodynamics profiles (Alexis, 2008; Bertrand, 2012; Bertrand, 2014; Kipp, 2004); improvement of drug stability by confining the therapeutic agents inside the nanocarriers, preventing their degradation (Whitehead, 2009); delivery of the bioactive payload to the tumor site through passive and/or active targeting strategies, consequently improving its therapeutic efficacy and reducing adverse side effects by hindering the drug release during systemic circulation; and designing multifunctional nanoplatforms for combined therapy and/or theranostic applications, encompassing both therapeutic and diagnostic functions (Fernandez-Fernandez, 2011; Janib, 2010; Sanna, 2014).

Since the concept of the “magic bullet” was introduced, nearly one hundred years ago (Strebhardt, 2008), significant efforts have been focused on a deeper understanding of the tumor biology and microenvironment, as well as on the screening of receptors overexpressed by the cancer cells, with the ultimate goal of defining strategies for specifically targeting the tumor tissues. In this context, remarkable advances in nanotechnology led to the emergence of targeting nanodelivery systems as innovative as complex, which exploit

both passive and active mechanisms for targeting an extensive variety of malignant tissues (Fig. 3.5.7) (Davis, 2008; Farokhzad, 2009; Ferrari, 2005; Nicolas, 2013). This section briefly describes the fundamentals behind tumor targeting, scrutinizes different strategies that have been employed for targeting cancer tissues, and highlights the most recent breakthroughs on designing and developing targeting drug delivery nanoplatforms for application in cancer therapy.

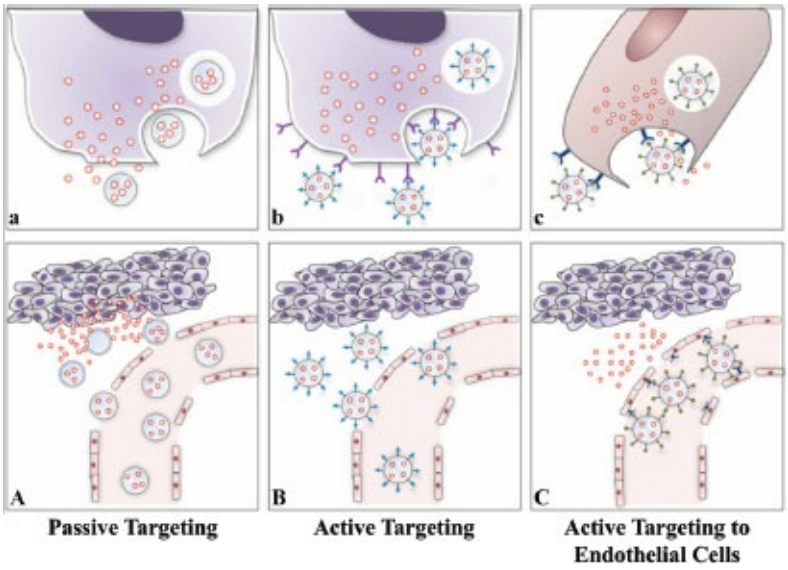

Figure 3.5.7: Strategies adopted for drug targeting and localization of nanosystems to tumor cells and tissues. (**A**) Passive targeting. Circulating nanoparticles passively extravasate in solid tumor tissue via the enhanced permeability of blood vessels, i.e., through the disorganized and leaky vasculature surrounding the solid tumor coupled with the absence of lymphatic drainage, and preferentially accumulate in tumor cells (the EPR effect). (**a**) The drug is released into the extracellular matrix and diffuses through the cells and tissue. (**B**) Active targeting. Once nanoparticles passively extravasate and concentrate in the target tissue via the EPR effect, the presence of ligands grafted onto the nanoparticle surface enable active targeting of xthe nanoparticles to receptors that are overexpressed on tumor cells or tissue, resulting in enhanced uptake and internalization via receptor-mediated endocytosis. (**b**) Tumor-specific ligands on the nanoparticles bind to cell surface receptors, triggering internalization of the nanoparticles into the cell through endosomes on which, due to an internal acidic pH, the drug is released from the nanoparticles and diffuses into the cytoplasm. (**C**) Active targeting to endothelial cells. Nanoparticles can be targeted to bind to angiogenic endothelial cell surface receptors with the aims of enhancing drug accumulation in the tumor endothelium, thereby inhibiting growth of blood vessels supplying the tumor rather than inhibiting tumor cells per se (**c**); and improving delivery of chemotherapeutic agents to tumor cells via the EPR effect with the potential to act synergistically in targeting both the vascular tissue and tumor cells. Adapted

and reproduced with permission from (Lammers, 2012). Abbreviation: EPR, enhanced permeability and retention.

3.5.3.1 Passive Targeting

3.5.3.1.1 The Fundamentals of Passive Targeting and the EPR Effect

The accumulation of drug molecules and nanosystems in certain target tissues, relying exclusively on their pathophysiological features (i.e., not involving any ligand-driven mechanism), can be referred as "passive targeting" (Arias, 2011; Jhaveri, 2014). The majority of nanoparticulate systems with the size ranging between ~10–500 nm present the capability of exploiting the unique intrinsic characteristics of the tumor microenvironment, and eventually accumulating at the tumor (Torchilin, 2011). The rationale behind the preferential distribution of nanoparticles to malignant tissues, after intravenous administration, has been intensively investigated and, nowadays, it is well recognized that such behavior relies on the EPR effect (Fang, 2011; Maeda, 2001; Maeda, 2000; Matsumura, 1986; Torchilin, 2011).

Contrarily to what happens in normal tissues, the microvasculature of solid tumors is characterized by a low degree of differentiation, a discontinuous highly-fenestrated endothelium and a disrupted basal membrane, essentially resulting from an active angiogenesis stimulated by a frenetic and metabolically demanding tumor growth (Carmeliet, 2000; Jain, 1998; 2010). The interendothelial fenestrations of this imperfect vascular architecture result in an increased permeability and consequent lower resistance to the extravasation selective accumulation of nanoparticles and active macromolecules from the blood vessels lumen to the tumor extravascular space (Fig. 3.5.7A) (Danquah, 2011). Additionally, the tumor tissues exhibit a compromised lymphatic drainage function, hindering the renewal of the interstitial fluid and, consequently, an effective clearance of the extravased nanoparticles leading to their accumulation in the tumor interstitium (Padera, 2004). The aforementioned pathophysiological phenomena occurring in the tumor milieu represent the fundamentals of the EPR effect, and consequently, of the EPR effect-driven passive targeting of nanosystems

to tumors.

3.5.3.1.2 Physicochemical Properties of Nanoparticles Affecting the Passive Targeting

The physicochemical properties of the nanocarriers, namely the size, surface charge, shape and hydrophobicity, play a major role on exploiting the EPR effect and, consequently, the passive mechanism as a strategy to target tumors, not only for influencing the extravasation capacity of the nanoparticles from the tumor surrounding capillaries and their accumulation in the tumor site, but also, and equally relevant, for influencing their stability in blood circulation, which significantly impacts the clearance by the reticuloendothelial system (RES), and ultimately the blood circulation kinetic profiles (Alexis, 2008; Bertrand, 2012; Dreher, 2006; Maeda, 2001). The meticulous and rational design of the nanoparticles intended for cancer therapeutic and diagnostic applications is, therefore, of utmost importance when taking a passive targeting strategy into consideration.

The size of the nanocarriers represents a crucial parameter that significantly influences the extent and kinetics of accumulation in the tumor tissues. In order for the nanoplatforms to extravasate from the tumor microvasculature to the tumor interstitial fluid, their hydrodynamic radius needs to be lower than the threshold of the capillary interendothelial gaps. In contrary, nanoparticles sized over the referred limit are more likely to be engulfed by the RES (Arias, 2011). Recent works have been shown the impact of the nanocarriers size on the efficiency of tumor passive targeting (Cabral, 2011; Huo, 2013; Lee, 2013b; Perrault, 2009; Wong, 2011). Chan evaluated the influence of the nanoparticles' size and surface chemistry on the *in vivo* passive targeting of tumors, and it was observed that nanoparticles of reduced size are capable of extravasate to the tumor interstitium, contrarily to their larger counterpartes, which were confined inside the neovasculature (Perrault, 2009). The histological data (Fig.3.5.8) revealed that, although no difference was observed on the extension of tumor penetration by nanoparticles with variable sizes (20, 60 and 100 nm) after 1 h, the smaller particles diffused inside the tumor mass throughout time, while

the larger ones were mostly restrained inside the blood vessels.

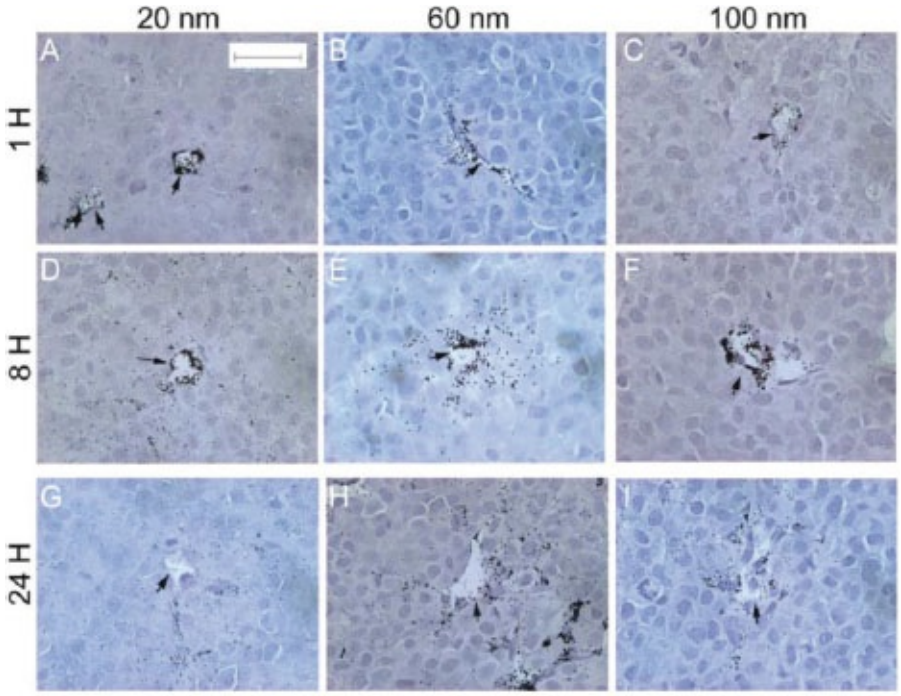

Figure 3.5.8: Histological data of the nanoparticle uptake in tumors when different sized nanoparticles were used *in vivo*. Reproduced with permission from (Perrault, 2009).

In a study conducted by Tseng, magnetothermally-responsive doxorubicin loaded supramolecular magnetic nanoparticles (Dox-SMNPs) were synthesized with the sizes of 70, 100 and 160 nm, and subsequently injected intravenously into DLD-1 colorectal adenocarcinoma tumor bearing mice, aiming to examine the influence of the nanoparticles' size on their retention at the tumor site (Jain, 1987). The two-dimension (2D) micro-positron emission tomography (PET) cross sectional images displayed a considerably higher accumulation of the 70 nm nanoparticles at the malignant tissue, comparatively to their larger counterparts (Fig. 3.5.9).

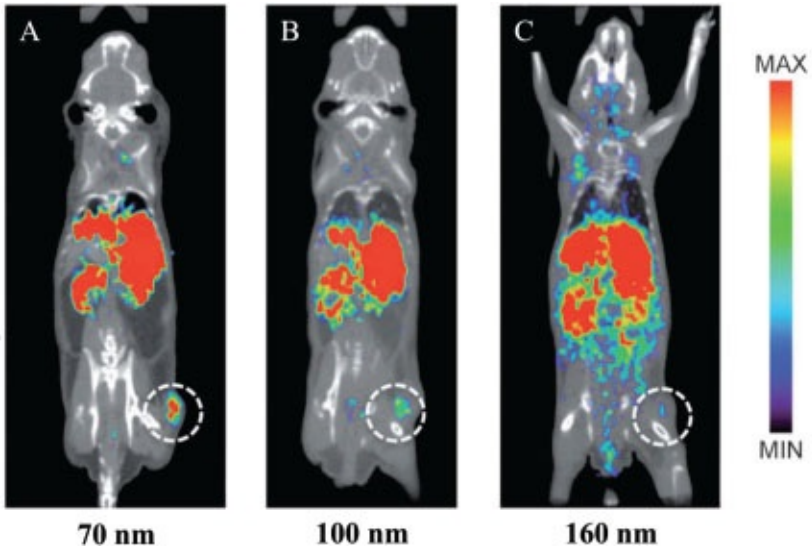

Figure 3.5.9: 2D micro-PET cross-sections images, created using a filtered back-projection, of mice bearing a DLD-1 tumor (n = 3) at 36 h post-injection of 70 (A), 100 (B), and 160 nm (C) ^{64}Cu-labeled Dox-SMNPs. Each image was scaled to its own maximum. The 70 nm Dox-SMNPs show the highest tumor-specific uptake among the three studies. Abbreviations: Dox-SMNPs, doxorubicin-loaded supramolecular magnetic nanoparticles. Adapted and reproduced with permission from (Lee, 2013b).

Similarly to the size, the surface charge of nanoparticles exert an effect on their behavior in the systemic circulation, including the circulation time, interaction with elements of the RES and predisposition for interacting with the tumor cells, affecting cellular association and internalization (Alexis, 2008; He, 2010). In this regard, positively charged nanocarriers are recognized as owing more propensity for interacting and, consequently, accumulating at the tumor tissues, as suggested by studies performed in animal models (He, 2010; Hu-Lieskovan, 2005). An enhanced interaction between positively charged nanoparticles and the endothelial cells of the tumor microvasculature is suspected to be one of the mechanisms involved on the referred predisposition, and the application of this phenomenon on the targeting of the endothelium of the tumor vasculature for delivery of antiangiogenic agents has already been explored (Fasol, 2012; Lohr, 2012; Schmitt-Sody, 2003; Strieth, 2008).

The coating of nanodelivery systems with hydrophilic polymers, such as PEG, has been widely used for improving biocompatibility, as well as for sterically stabilizing the nanocarriers (Alexis, 2008; Otsuka, 2003; Torchilin, 2006). The so-called PEGylation of the nanoparticles'

surface renders the nanocarriers capable of evading the recognition and opsonization by the RES, consequently reducing their clearance from the systemic circulation, prolonging the circulation time, and ultimately promoting the accumulation in the tumor site through the EPR effect (Arias, 2011; Danhier, 2010; Maruyama, 2011; Sawant, 2012; Sultana, 2013). The aforementioned formulations strategies have been explored on the engineering of nanoparticulate drug delivery platforms that exclusively exploit a passive mechanism for targeting cancer and, in fact, a bench-to-bedside translation can already be considered as a reality with many of those being approved for commercialization or undergoing clinical trials for treating a wide range of cancers (Table 3.5.2).

3.5.3.1.3 Challenges and Future Prospects of Passive Targeting

Despite the potential of the passive targeting that has been discussed above, this mechanism presents some limitations and faces some challenges, being now considered as a more complex and heterogeneous phenomenon than previously assumed (Prabhakar, 2013).The inter- and intratumoral variability of the tumors result in significant differences on the physiology of the tumor microvasculature and microenvironment and, consequently, on the therapeutic response to passive targeted nanomedicines (Jain, 2010).

In addition, the capability of the loaded therapeutic agents to succesfully reach their pharmacological target, thus exerting their therapeutic effect, does not solely depend on the biodistribution of the nanocarrier and its accumulation in the tumor site. An effective drug delivery might be hindered by an increased interstitial fluid pressure which induces the eflux of therapeutics back in the systemic circulation, as well as by the inability of the drug molecules to homogeneously diffuse inside the tumor mass (Dreher, 2006; Sawant, 2012).

Therefore, the screening of individual tumor genetic profiles and their particular predisposition for the EPR effect, paralellely to the specific fine-tuning of the drug release profiles based on the bahavior of the drugs in the tumor microenvironment, may become crucial when designing and engineering personalized and optimized

nanotherapeutics exploiting cancer passive targeting.

Table 3.5.2: Examples of non-targeted nanosystems in clinical use for anticancer therapy. Adapted with permission from (Sanna, 2014).

Name	Formulation	Bioactive compound	Indication	Status
Liposomes				
DaunoXome®	Non-PEGylated liposomes	Daunorubicin	Kaposi's sarcoma	Approved
Myocet®	Non-PEGylated liposomes	Doxorubicin	Breast cancer	Approved
Onco TCS®	Non-PEGylated liposomes	Vincristine	Non-Hodgkin's lymphoma	Approved
Depocyt®	Non-PEGylated liposomes	Cytarabine	Leukemia Glioblastoma	Phase III Phase I/II
Doxil®/Caelyx®	PEGylated liposomes	Doxorubicin	Breast cancer, ovarian cancer, multiple myeloma,Kaposi's sarcoma	Approved
Thermodox®	PEGylated liposomes	Doxorubicin	Liver cancer, breast cancer	Phase III
SPI-77	PEGylated liposomes	Cisplatin	Ovarian cancer	Phase II
NL CPT	PEGylated liposomes	Irinotecan	Glioma	Phase I
Polymeric nanoparticles				
Genexol-PM®	PEG-PLA	Paclitaxel	Breast cancer, lung cancer, ovarian cancer	Phase II
NK105	PEG-poly(aspartic acid)	Paclitaxel	Gastric cancer Breast cancer	Phase I Phase III
NK911	PEG-poly(aspartic acid)	Doxorubicin	Various solid tumors	Phase II
Opaxio™	PGA-paclitaxel	Paclitaxel	Lung cancer, ovarian cancer	Phase III
CRLX101	PEG-cyclodextrin	Camptothecin	Non-small-cell lung cancer	Phase II
NC-6004	PEG-poly(glutamic acid)	Cisplatin	Pancreatic cancer	Phase II
ProLindac™	HPMA	DACH-Pt	Ovarian cancer	Phase II
Others				
Abraxane®	Albumin-based	Paclitaxel	Breast cancer	Approved
Paclical®	Micellar retinoid-derived	Paclitaxel	Ovarian cancer	Phase III
NC-4016	Micellar PEG/polyamino acid	Oxaliplatin	Various solid tumors	Phase I/II
Oncaspar®	PEG-L-asparaginase	Asparagine specific enzyme	Acute lymphoblastic leukemia	Approved

Note: DaunoXome® (Galen US Inc., Souderton, PA, USA); Myocet® (Sopherion Therapeutics Inc., Princeton, NJ, USA); Onco TCS® (Inex Pharmaceuticals Corp., Burnay, BC, Canada, and Enzon Pharmaceuticals Inc., Bridgewater, NJ, USA); Depocyt® (Pacira Pharmaceuticals Inc., San Diego, CA, USA); Doxil®/Caelyx® (Janssen Biotech Inc., Horsham, PA, USA / Janssen-Cilag Pty Ltd, Macquarie Park, NSW, Australia); Thermodox® (Celsion Corporation, Lawrenceville, NJ, USA); Genexol-PM® (Samyang Biopharmaceuticals Corporation, Jongno-gu, Seoul, Korea); Opaxio™ (Cell Therapeutics, Inc., Seattle, WA, USA); ProLindac™ (Access Pharmaceuticals Inc., Dallas, TX, USA); Abraxane® (Celgene Corporation, Inc., Berkeley Heights, NJ, USA); Paclical® (Oasmia Pharmaceutical AB, Uppsala, Sweden); Oncaspar® (Enzon Pharmaceuticals Inc., Bridgewater, NJ, USA).

Abbreviations: PEG, poly(ethylene glycol); HPMA, hydroxypropylmethacrylamide; DACH-Pt,

diaminocyclohexane-platinum.

3.5.3.2 Active Targeting

3.5.3.2.1 The Fundamentals of Active Targeting

The active targeting of nanoparticles, also denominated ligand-mediated targeting, involves the surface-functionalization of the nanoparticles with active moieties possessing intrinsic affinity to specific receptors/antigens overexpressed in the diseased tissues or cells, functioning not only as driven-force for the nanocarries' accumulation at the target site, but also as a mechanism for enhanced cellular association and internalization by receptor-mediated endocytosis (Fig. 3.5.7B) (ACS, 2014; Allen, 2002; Arias, 2011; Cho, 2008; Couvreur, 2006; Danhier, 2010; Kamaly, 2012; Lammers, 2012; Peer, 2007; Shi, 2011; Torchilin, 2006). Considering the specific cellular recognition and consequent enhancement of cellular uptake as the main achievement of the active targeting, this strategy is assumed to ultimately result in an improved therapeutic efficacy of the targeted nanoparticulate systems, comparatively to their non-targeted counterparts (Kamaly, 2012). The active targeting mechanism requires the proximity of the ligand-anchored nanoparticles to the targeted antigen, enabling their recognition and the interaction between the two components. Therefore, the intrinsic characteristics of the nanocarriers influencing the EPR effect and the blood circulation kinetics also play an important role on the delivery and therapeutic and efficiency of active-targeted nanoparticles, since those primarily need to extravasate from the tumor microvasculature in order to interact with their specific targets located in the extravascular tissue (Lammers, 2012; Taurin, 2012). Hence, the active and passive strategies for targeting of nanoparticles to tumors are currently considered as complementary to ultimately improve the therapeutic efficiency of anticancer nanodelivery systems.

3.5.3.2.2 Factors Affecting Tumor Active Targeting

The meticulous and rational design of actively targeted nanocarriers is

of utmost importance for the efficient targeting and recognition of the targeted receptor, both *in vitro* and *in vivo*. This optimal engineering of the nanoparticles' architecture should encompass not only the physicochemical properties of the nanoplatform, including its size, shape and surface properties (i.e., charge and hydrophobicity), but also parameters associated with the targeting ligand, such as the affinity of the ligand to the targeting receptor, the ligand density and the conjugation chemistry (Berretta, 2011; Chen, 2012; Humrich, 2010; Lesterhuis, 2004; Signori, 2010). The combination of these factors can synergistically impact the systemic circulation kinetics, biodistribution profiles and targeting efficiency of actively-targeted nanodelivery systems, therefore dictating their performance *in vivo* (Fig. 3.5.10).

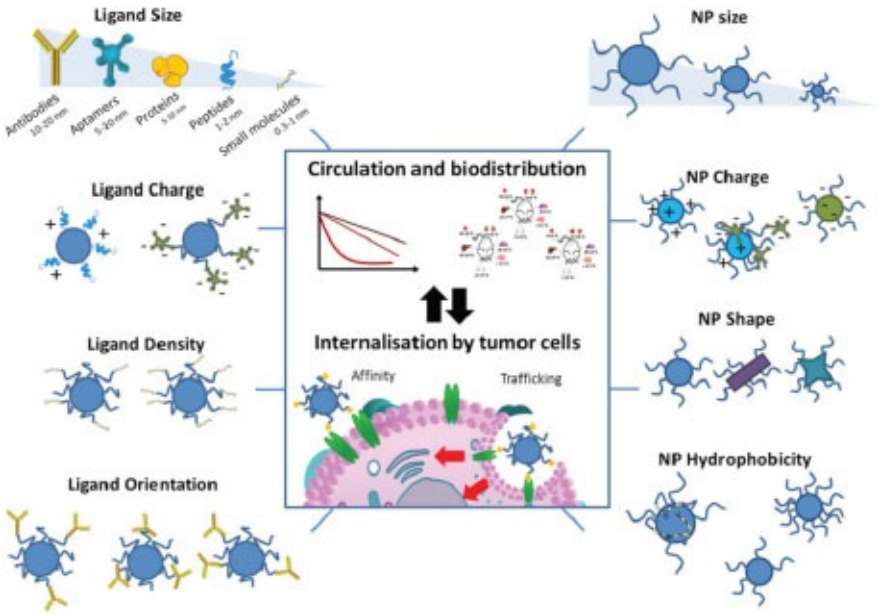

Figure 3.5.10: The physicochemical properties of the ligand and the nanoparticles affect their blood circulation profiles, their biodistribution and their ability to be internalized by cancer cells. Reproduced with permission from (Bertrand, 2014).

The optimization of the targeted nanoparticles' size is therefore crucial for their extravasation from the tumor vasculature and accumulation at the tumor size. Rationally, when a ligand functionalization is intended, the increase on the hydrodynamic radius after conjugation of the targeting moiety must be taken into consideration, particularly in the case of large ligands, such as antibodies, proteins or aptamers, under penalty of negatively influencing the targeting efficiency by hindering the accumulation of the nanocarrier in the tumor (Humrich, 2010). The

size of targeted nanoparticles was also revealed to impact their intracellular trafficking, with smaller particles being increasingly found deposited in the cytoplasm and nucleus of cancer cells, in a study conducted by Lee (Kapsenberg, 2003). In parallel, the influence of the shape of actively targeted nanoparticles on their cell internalization by cancer cells has also been recently investigated (Coosemans, 2014; Hanke, 2013; Subbotin, 2014).

The surface of the actively targeted nanoparticles also impact on the interaction between those and the targeted cells' surface, therefore contributing for the efficacy of the targeting. Cationic nanoparticulate systems are acknowledged for more extensively interacting with negatively-charged cell membranes, resulting in a non-specific increased cellular association and internalization (Offringa, 2009). However, an excess in the positive charge of the nanoparticles might increase toxicity and stimulate immunologic response (Moghimi, 2005). When the nanoparticles' surface is functionalized with a targeting moiety, the surface charge is not only influenced by the intrinsic charge of the ligand and the nanoplatform, but also by the ligand density.

Besides the surface charge, the hydrophobicity of the nanocarriers can also interfere with the way they interact with targeted cells, since the adsorption of plasma proteins onto the surface of non-sterically stabilized nanoparticles may decrease the capacity of those to bind to the targeted receptor (Homma, 2005). Contrarily, the nanoparticles' surface functionalization with high molecular weight polymers, such as PEG, may hinder the targeting moieties to efficiently bind to their antigen (Lowin, 1995; Peters, 1991). The development of a PEG-shielded actively-targeted nanoplatform with prolonged circulation time and capable of undergoing cleavage of the steric protection in the tumor interstitium, consequently exposing the targeting moiety to the cancer cells, might constitute an interesting approach demanding further investigation (Arroyo, 2002).

Finally, the density of the targeting molecules functionalizing the nanoparticles' surface significantly influences the avidity of targeting nanotherapeutics to the complementary receptor. Theoretically, a higher density of the targeting moieties generates a favorable binding enthalpy

in the system, consequently inducing the interaction between the ligand and the antigen (Callard, 1977; Liu, 2007), and also stimulates the overexpression of the receptor at the cells' surface, therefore resulting in an enhanced internalization of the actively targeted nanoparticles by the tumor cells. Nevertheless, various aspects, including the inappropriate orientation of the targeting ligand, the steric hindrance of subjacent molecules and a competitive behavior towards the receptor binding may revert this relation between the ligand density and the efficiency of cell association (James, 1981; Valdez, 2000).

3.5.3.2.3 Ligands for Tumor Active Targeting

The adequate selection of the most suitable targeting moiety to functionalize the surface of nanoparticles is absolutely imperative for the efficiency of their targeting to specific receptors overexpressed by the tumor tissues, therefore representing one of the major aspects to be considered during the design of actively-targeted nanomedicines. A variety of targeting moieties are available to functionalize the surface of carriers through different approaches, and those encompass monoclonal antibodies and their fragments, proteins and peptides, aptamers and other nucleic acids, as well as small molecules (Yu, 2010).

3.5.3.2.3.1 Monoclonal Antibodies

In the past few years, monoclonal antibodies (mAb) have remained the preferred and most extensively used and investigated type of targeting moieties, due to the high affinity and specificity they render to the nanoparticles. This potential of the application of mAb in targeted cancer therapy led to the approval of a large number of these macromolecules for clinical use, including rituximab, cetuximab and trastuzumab, as well as to the recent development of chimeric and humanized derivatives with modulated immunogenicity (Kamaly, 2012; Ndungu, 2012; Yoshida, 2010). However, despite the aforementioned attractive features of mAb for targeting anti-tumor nanotherapy, the performance of these molecules *in vivo* still present some limitations and challenges, including their large size and molecular weight, their immunogenicity resulting in the prompt recognition by the RES and

rapid nanoparticle clearance from the systemic circulation (Taniguchi, 2012; Vivek, 2014), as well as their liability to environmental alterations and organic solvents, which challenges the processes of scale-up and manufacturing, impacts the cost/effectiveness of the formulations and compromises their stability. In this regard, there is an emerging interest on engineering antibody fragments, including Fab fragments, single chain variable fragments (scFv) minibodies, diabodies and nanobodies, for functionalizing the surface of nanodelivery systems, maintaining their targeting specificity and considerably reducing their size and immunogenicity (Peer, 2007; Scott, 2012; Weiner, 2000). Antibody fragment-targeted nanomedicines have already reached clinical trials, as for example MCC-465 and SGT-53 (Table 3.5.3). MCC-465 exhibited an adequate biodistribution and highly efficient delivery of doxorubicin for the treatment of stomach cancer in preclinical studies (Baxevanis, 2008), and SGT-53 sustained the tumor growth in different cancers, including head and neck, breast and prostate, by delivering the p53 supressor protein after targeting the transferrin-receptor (Tf-R) overexpressed by the tumor cells (Bonsignori, 2012; Le Garff-Tavernier, 2014).

3.5.3.2.3.2 Proteins and Peptides

Recent advances in bioinformatics and cancer proteomics enabled the high-throughput screening of protein antigens overexpressed in tumors. These biomarkers can be selectively recognized by targeting proteins and peptides that functionalize the nanoparticles' surface, resulting in their receptor-mediated endocytosis (Mody, 2011). Transferrin (Tf), a serum glycoprotein involved in the iron homeostasis and regulation of cell growth has focused attention for specifically binding to the internalizing transferrin-receptor (Tf-R), approximately 100-fold overexpressed in various tumors comparatively to healthy cells (Chandrasekhar, 2013). Therefore, transferrin has been exploited as a potential ligand for decorating nanoparticulate drug delivery systems intended to target Tf-R and some nano-formulations are presently under clinical evaluation (Prang, 2005). However, the feasibility of using proteins for targeting purposes has been limited by the immunogenicity of these molecules, resulting in their increased predisposition for

opsonization by the RES. In addition, the expression of the targeted receptors by nonmalignant tissues may originate unwanted off-target effects, compromising one of the main advantages on using targeting therapy.

In order to circumvent the aforementioned bottlenecks, attention has been focused on the synthesis of peptide-based ligands exhibiting smaller molecular sizes and simpler 3D conformation, consequently resulting in higher stability and relatively lower immunogenicity when compared with the majority of the proteins. The identification of new ligand-receptor combinations normally derive from the application of recently developed high-throughput screening techniques and phage/plasmid/bacterial peptide display libraries (Paulo, 2011). The RGD sequence (i.e., arginine-glycine-aspartic acid) has been extensively included in an immense variety of peptides sharing the capability to specifically target the $\alpha_v\beta_3$ integrin receptors, an endothelial cell surface receptor overexpressed in neovascular endothelial cells (Fass, 2008; Kamaly, 2012; Miele, 2012). Despite the potential and promising application of RGD peptides on the targeting of endothelium of the tumor microvasculature, the expression of the $\alpha_v\beta_3$ integrin receptor by normal and inflamed tissues limits its clinical translation. In that sense, investigations have been undergoing with the purpose of generating new RGD analogs with improved targeting specificity (Kamaly, 2012; Nembrini, 2011).

3.5.3.2.3.3 Aptamers

Nucleic acid-based aptamers are small, single-stranded RNA or DNA oligonucleotides, where the conformational structure can be designed, rendering them the capability of binding antigens with high affinity and specificity (Bellisola, 2012; Pan, 2010). Candidates for targeting a specific receptor have been screened with oligonucleotide libraries using high throughput screening methodologies. The Promising aptamers are subsequently selected and amplified in detriment of the remaining ones. Recently, an *in vitro* chemical technique denominated "systemic evolution of ligands by exponential enrichment" (SELEX) has been explored for identification of the referred candidates (Wang, 2013). This

technique has been used by Langer and Farokhzad for developing customized controlled-release nanoplatforms capable of specifically targeting the prostate-specific membrane antigen (PMSA) (Ferris, 2010; Ma, 2013; Monjanel, 2014; Nembrini, 2011; Tao, 2012). Aptamers present attractive intrinsic features to be used as targeting ligands, such as smaller size and reduced immugenicity, in comparison with mAb or other macromolecules, resulting in improved stability and biodistribution profiles (Oki, 2012; Pawelec, 1999). Similarly to the other classes of targeting moieties, the use of aptamers presents some limitations, which hinder their clinical success. First of all, the susceptibility of nucleic acids to the action of nucleases, the stability of the aptamer conjugation onto the nanoparticles' surface becomes a major concern. Second of all, the negative charge of the aptamers attributed to the phosphodiester backbone is suspected to impact the systemic circulation kinetics of the actively-targeted nanosystem. Finally, although presenting a relatively small molecular size, the stiffness of the nucleic acid conformation may be translated into an increased hydrodynamic radius of the targeting nanosystem after ligand conjugation, hence influencing its accumulation at the targeted site, as previously discussed in this section.

3.5.3.2.3.4 Small Molecules

Small molecules, usually defined as possessing a molecular weight < 500 Da, are currently considered as a potential group of targeting moieties, due to the attractiveness of their properties. These targeting molecules present a small size, improved stability, and almost no immunogenicity. In addition, the availability of simple coupling chemistries for their conjugation onto the nanoparticles' surface enable the control of the functionalization process and, consequently, the fine-tuning of the ligand density and charge on the nanosystems' surface, which play a significant role in the stability, blood circulation kinetics and targeting efficiency, as previously discussed. Furthermore, a wide range of ligands with different physiochemical properties and chemical functionalities are available and the low production costs, reproducible scale-up and simple manufacturing render these small molecular weight compounds very promising targeting ligands for tumor targeted therapy

(ACS, 2014; Kamaly, 2012; Moghimi, 2005).

Folic acid has been one of the most employed and studied molecules among the numerous targeting ligands used in cancer targeted therapy, fundamentally due to its high affinity to the folate receptor (FR), which was found of being frequently overexpressed on many tumors, including ovarian, breast, brain, renal, colon and lung cancers (ACS, 2014; Annabi, 2014; Bachmann, 2010; Lee, 2011). Unfortunately, the FR expression seems to rely on inter-tumor variability and, therefore, the tumor predisposition for accumulating folate-targeted nanomedicines needs to be individually evaluated (Bachmann, 2010; Kamaly, 2012). Recently, folate-targeted imaging strategies have emerged for identifying FR-positive patients, permitting the selection of those that would in fact benefit from the administration of the folate-targeted nanoparticles (Bimbo, 2013). Interestingly, these images would possibly enable the quantification of the receptor displayed in the cellular surface and readily available to interact with the targeting ligand. Additionally to the tumor inter-variability of the FR overexpression, this receptor is also expressed in healthy tissues, a fact that might constitute a major downside on the administration of folate-targeted nanosystems for specific drug delivery to cancer.

Definitively, a successful case on the field of tumor targeted therapy, particularly when a small molecule is used as a targeting moiety, is the one of BIND-014 (Table 3.5.3). This nanomedicine relies on the targeting affinity of a urea-based small molecule denominated S,S-2-[3-[5-amino-1-carboxypentyl]-ureido]-pentanedioic acid (ACUPA) to PSMA (Binjawadagi, 2014; Nembrini, 2011). Docetaxel-loaded nanoparticles with a ligand density of approximately 200 ACUPA molecules per particle exhibited an optimized targeting of PSMA-positive prostate tumors, with no visible change in the systemic circulation kinetics (Binjawadagi, 2014; Chen, 2012).

Table 3.5.3: Tumor-targeted nanomedicines in clinical development. Adapted and reprinted with permission from (Bertrand, 2014).

Name	Formulation	Targeting ligand	Bioactive compound	Indication	Status
Liposomes					
CALAA-01	Cyclodextrin-based NP containing anti-RRM2	Transferrin	siRNA	Various solid tumors	Phase I
MBP-426	Liposomes	Transferrin	Oxaliplatin	Gastro-esophageal adenocarcinoma	Phase II
MCC-465	PEGylated liposomes	F(ab') 2 fragment of human Ab GAH	Doxorubicin	Metastatic stomach cancer	Phase I
SGT53	Liposomes	Anti-transferrin receptor single-chain Ab fragment (TfRscFv)	p53 gene	Solid tumors	Phase I
C225-ILS-DOX	PEGylated liposomes	Antigen-binding fragments (Fab) of cetuximab	Doxorubicin	Advanced solid tumors	Phase I
Polymeric nanoparticles					
BIND-014 (Accurins™)	PEGylated PL(G)A	PSMA (Small molecule)	Docetaxel	Solid tumors	Phase II
Atu027	Liposomes	Protein kinase N3	siRNA	Solid tumors	Phase I
C-VISA-BikDD	Liposomes	Proapoptotic gene	BikDD plasmid DNA	Pancreatic cancer	Phase I

Note: Accurins™ (BIND Therapeutics, Inc., Cambridge, MA, USA).

Abbreviations: NP, nanoparticles; PEG, poly(ethylene glycol).

3.5.4 Nanotechnology and Immunotherapy

Cancer immunotherapy is a therapeutic strategy in which the body's own defense mechanisms can become activated to fight against the abnormally fast growing cells by reinforcing the immune system. Nowadays, this method has attracted a lot of attention because of the discovery and application of different synthetic and natural immunogenic materials (Egilmez, 2012; Gajewski, 2013; King, 2008). The three most common methods for cancer immunotherapy include the application of immunoadjuvant materials for non-specific treatment, vaccination using antigens and the application of monoclonal antibodies (Chandramohan, 2013; Gedeon, 2014; Monjazeb, 2012; Nembrini, 2011). Non-specific immunotherapies can be given as a single therapy or at the same time with another treatment, such as chemo- or radiotherapy for improving the therapeutic responses. Interferons and

interleukins (ILs) are the most common non-specific anticancer immunotherapies loaded inside nanoparticles for activating different immunological pathways (Rosenberg, 1988). Cancer vaccination is another approach to help the body fighting against cancer (Nembrini, 2011). Vaccines contain an agent that resembles a disease-causing environment and are able to improve the immunity against a particular disease. While traditional vaccines are often made from dead or weakened forms of the microbe or their toxins, nowadays the investigation is more focused on the development of new vaccines made from surface proteins and DNA of the immunogenic cells (Kalkanidis, 2006; Silva, 2013; Singh, 2007). The immune system can recognize the immunogenic molecule, keep a record of it, and more easily recognize and destroy any of the cells containing the immunogenic molecule (Signori, 2010).

Monoclonal antibodies (mAb) can be used for immunotherapy through a number of mechanisms, such as blocking the function of the targeted molecules, antibody dependent cellular cytotoxicity effect, inducing apoptosis in the cells expressing the target antigen, and increasing the phagocytosis of the target cells by macrophages or by modulating the signaling pathways of the target cells (Chandramohan, 2013; Ferris, 2010; Gedeon, 2014). In addition to immunotherapy, mAb can be applied for cancer targeting and diagnosis (Ma, 2013; Tao, 2012).

3.5.4.1 Nano-based Cancer Immunotherapy

One of the main shortcomings of the current immunostimulatory materials is the lack of dendritic cell (DC) targeting and short time immunostimulative responses, as the concentration of the immunogenic molecules declines in the body over a short period of time. In addition, existing immunoactivating compounds mostly elicit Immunoglobulin G (IgG) isotype Ab and are not able to induce the secretion of various Ab isotypes (Mallapragada, 2008). Thereby, induced immunotherapeutic protections are not long-lasting. Accordingly, new strategies are essential to be developed for more efficient activation of the immune system against cancer.

In general, the ideal properties of a good anticancer

immunotherapeutic formulation are high safety, the capability of eliciting the desired immune responses after a single dose, absence of a booster dose, absence of the premature drug release, simple and affordable preparation process, easy administration and scaling-up process, and high physicochemical stability of the immunogenic agents and excipients throughout the process, storage and administration (Oyewumi, 2010). To combine all these properties in one formulation, nanoparticles have been suggested as versatile systems capable of improving the biological effects of the immunostimulatory molecules via different mechanisms. One of the benefits of nanovaccines is that the morphology, size distribution, entrapment efficiency, release kinetics and other physicochemical properties, which affect the obtained immune responses can be controlled, leading to the successful development of promising vaccines (Kendall, 2006). In addition, the systemic severe side effects of high dose administration of the immunostimulants, such as toll-like receptor ligands (Heikenwalder, 2004), can be minimized using nanoparticles. Nanoparticles can also reduce the needed dose and limit the non-specific immune responses (Diwan, 2004).

Nanomaterial-based immunotherapy is a relatively new interdisciplinary field holding great promise by combining materials science, chemistry and immunology. The immunostimulative biomolecules can be either captured within or conjugated on the surface of the nanoparticles (Nembrini, 2011; Xiang, 2013). The former method offers distinct advantages, such as reduced dose of antigen, efficient uptake and processing by antigen presenting cells (APCs), increased stability during storage and long-term immune response to the therapy (Foster, 2010; Katare, 2003). Although the entrapment of immunogenic biomolecules within the particles is offered as the best possible protective strategy, the main drawback of this method is the unavailability of the loaded antigens upon administration because of the slow drug release profiles (Kazzaz, 2000). In addition, the loaded bio-immunogenics can physically or chemically degrade during the loading process (Jung, 2001). This method also causes a lower extent of immunity compared to the nanoparticles that have immunostimulative molecules chemically conjugated or physically adsorbed on their

surface with the aim to induce rapid and short immune responses. However, this method also suffers from low stability and rapid degradation (Kalkanidis, 2006; Sloat, 2010).

It is currently known that the immunostimulatory products with large hydrophobic structures are more immunostimulative than hydrophilic compounds. Therefore, it is proposed that microbially derived adjuvants can be replaced with hydrophobic nanomaterials. These nanosystems can also simultaneously serve as delivery vehicles for the immunostimulative molecules (Kipper, 2002), with the aim of facilitating single-dose vaccination and eliminate the need for booster shots through sustaining the release of immunotherapeutic payloads and potentiating their effect via the intrinsic adjuvanticity of the nanoparticles. In addition, most of the nanoparticles applied for cancer immunotherapy possess high safety, and controllable rate of degradation for the antigen release (Kersten, 2004). Owing to these benefits, it can be possible to avoid the need for surgical removal of cancer tissues and circumvent the disadvantages of conventional anticancer formulations by combining chemo- and immunotherapeutic approaches using such nanostructures.

Below we discuss the adjuvanticity of nanoparticles as well as the current progresses in the development of nanovaccines, particularly those with high potential to be used for cancer therapy.

3.5.4.2 Nanoparticulate Adjuvants for Cancer Immunotherapy

Adjuvants are immunogenic compounds capable of accelerating and extending the immunostimulative response of biomolecules. Currently, alum salts are the most widely used immune adjuvants (Correia-Pinto, 2013), owing to their potential in triggering the so-called “inflammasome” mechanism in the cells, that leads to the release of danger signals and subsequent secretion of pro-inflammatory biomolecules, resulting in the activation of the immune system (Marrack, 2009). Despite the popularity of immunogenic alum salts and other conventional adjuvants over the last few decades, they suffer from major limitations, such as adverse local reactions, degradation during freeze-drying, lack of inducing cellular immune responses and necessity

of multi-dosing to reach long lasting protection (Correia-Pinto, 2013). Hence, for cancer immunotherapy, the scientific community has been developing nanoparticulate adjuvants that are able to show intrinsic immuno-adjuvanticity and also to act as vehicles for the delivery of antigens and immunotherapeutic biomolecules (Park, 2013). This strategy can provide an opportunity for simultaneous humoral and cell-mediated immunity induction, which can lead to improved therapeutic effects (Rappuoli, 2011; Wu, 2006). Nanoparticles may also assist the interaction of the delivered antigens with APCs, enhancing the antigen-based immune responses (Hamdy, 2011; Park, 2013). Moreover, co-encapsulation of anticancer drug molecules with immunostimulative biomolecules can be obtained for synergised multifunctional purposes (Roy, 2013). Accordingly, cancer nanovaccines have recently attracted a lot of interest owing to their unique properties to overcome the limitations of immuno-therapeutics, including low interaction with APCs, inherent instability of biomacromolecules and lack of cross-presentation to T lymphocytes (Hamdy, 2011; Park, 2013).

The impact of nanoparticles on the maturation of DCs for cancer immunotherapy is usually evaluated by studying the expression of co-stimulatory molecules and the major histocompatibility complex (MHC) classes I and II bioreceptor molecules, the production of cytokines, and the activation of signalling pathways (Almeida, 2014; Klippstein, 2010). For example, the effects of poly(lactic-co-glycolic acid) (PLGA) nanoparticles on the maturation of DCs were studied in mouse spleens (Reischl, 2009). The results showed a dose-dependent expression of co-stimulatory molecules, such as CD80, CD86, CD40, and MHC class I, as well as enhanced secretion of inflammatory cytokines and chemokines, such as tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6). In addition, increased activity of mitogen-activated protein kinase and nuclear factor–κB (NF-κB) signalling pathways was observed. Joshi (Joshi, 2013) have also demonstrated that the delivery of vaccine antigens with an appropriate nanoparticulate adjuvant can stimulate the immune responses against cancer, reducing tumor growth and improving the survival rate in mice. Accordingly, the authors developed an amphiphilic polyanhydride copolymer based on 1,8-bis(pcarboxyphenoxy)-3,6-dioxaoctane (CPTEG) and 1,6-bis(p-

carboxyphenoxy) hexane (CPH) with inherent adjuvant properties for immunogenicity, antigen-loading properties and antitumor activity. It was shown that mice treated with ovalbumin-encapsulated CPTEG:CPH particles elicited very high $CD8^+$ T cell responses on days 14 and 20, leading to a delay of the tumor progression and extended survival time. These results suggest that the immunoadjuvant properties of the nanoparticles can enhance antigen-specific immuno-cellular responses, and thus, they can be potentially applied for the promotion of antitumor responses in cancer patients.

3.5.4.3 Nanoparticle Based DC Targeting for Cancer Immunotherapy

Nanoparticles have attracted more attention than microparticles for DC targeting because of the inverse relationship between the efficiency of the uptake by DCs and the particle size (Hamdy, 2011). Moreover, positively charged nanoparticles are more preferred since they can highly interact with the immune cells via binding to the negatively charged surface of the cells (Dobrovolskaia, 2012; Gjetting, 2014). After the internalization of nanoparticles into the DCs, loaded cancer cell antigens can be released in endosomes, degrade, and eventually bind to the MHC II molecules on the surface of the DCs to present the antigenic molecules to the $CD4^+$ T cells (Trombetta, 2005). If the nanoparticles escape from the endosome and release their immunostimulative antigens in the cytoplasm, antigens degrade to small peptides by the proteosomes and, finally, form a complex with the MHC I in order to be recognized by the $CD8^+$ T cells (Davis, 2004; Trombetta, 2005). Finally, activated T cells can recognize cancer cells expressing antigens on their surface and destroy them. These explanations show the substantial role of the nanoparticle uptake by DCs for the subsequent immune cell activation. For example, Ma (Ma, 2012) have shown that tumor antigenic peptides loaded in PLGA nanoparticles can be highly colocalized in the DCs in 30 min (Fig. 3.5.11) and induce significantly stronger antitumor cytotoxicity of T lymphocytes than the free antigen peptide.

DC targeting can be achieved via both passive and active processes. The efficiency of the anticancer nanovaccines to passively target DCs is strongly dependent on the size, surface charge,

hydrophobicity/hydrophilicity, and the interaction of nanoparticle with the plasma proteins and cell-surface receptors (Bachmann, 2010). It has been reported that nanoparticles with a size smaller than 500 nm are taken-up more efficiently by the DCs (Foged, 2005). In addition, a pre-clinical study performed in mice suggested that the nanoparticle size of 40–50 nm was optimal for nanovaccines (Fifis, 2004). However, nanoparticles up to 300 nm have already demonstrated effective induction of the $CD4^+$ and $CD8^+$ T cell responses (Fifis, 2004; Kasturi, 2011; Reichert, 2011; Shima, 2013; Tomic, 2014). Positively charged particles are taken-up more efficiently than those with a negative or neutral charge by the DCs *in vitro* (Foged, 2005). However, some *in vivo* studies have revealed no significant difference in the immunostimulatory effect between positively and negatively charged particles (Yotsumoto, 2004). Some studies have also shown that the particles with positive surface charge can immobilize the nanovaccines in negatively charged components presented in the extracellular matrix of the cells through electrostatic interactions (van den Berg, 2010), resulting in the inhibition of the immunostimulative responses, owing to the reduced tissue penetration.

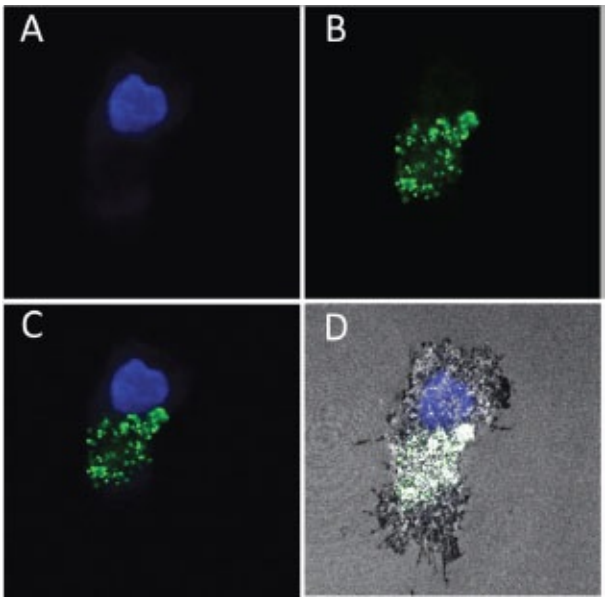

Figure 3.5.11: Confocal microscopy imaging of the PLGA nanoparticles in human DCs. (**A**) 4′,6-Diamidino-2-phenylindole channel. (**B**) Fluorescein isothiocyanate channel. (**C**) 4′,6-Diamidino-2-phenylindole and fluorescein isothiocyanate channels overlaid. (**D**) 4′,6-Diamidino-2-phenylindole, fluorescein isothiocyanate, and reflection channels overlaid. Reprinted with permission from (Ma, 2012).

Since DCs have the intrinsic ability of phagocyting foreign material, passive DC targeting for cancer immunotherapy is possible by directing the nanoparticles to sites rich in DCs. For this purpose, the route of administration of the nanoparticles is very important. For example, while subcutaneously administered small nanoparticles (< 100 nm) can be quickly transported into the lymphatic system to interact with the DC localized in the lymph node (Cubas, 2009; Manolova, 2008; Reddy, 2007), larger nanoparticles (> 500 nm) stay for a longer time in the injection site of the skin and predominantly interact with the DCs or monocytes available in the skin (Manolova, 2008; Moon, 2012). To avoid the size dependency of passive DC targeting to the lymph node, direct injection into the blood is suggested as an efficient way to reach both blood and splenic DCs and induce strong immune responses by a variety of particles (Perche, 2011; Tacken, 2011). However, the macrophage uptake is still one of the main drawbacks of the cancer immunotherapy using this strategy.

DCs also express many cell surface receptors, such as mannose (Chenevier, 2002; Ramakrishna, 2004), CD11c (Cruz, 2014), DEC-205 (Cruz, 2014), Langerin (Flacher, 2010), DC-SIGN (Cruz, 2010; Varga, 2013), clec9A (Caminschi, 2008), and DCIR2 (Dudziak, 2007), which can facilitate actively targeted cellular endocytosis of the nanoparticles via appropriate surface modification with the ligands that target these receptors on the surface of the DCs (Foged, 2002). It has already been reported that the conjugation of such targeting moieties on the surface of the nanoparticles increases their uptake by the DCs and enhances the DC maturation, as evidenced by the high secretion of cytokine and up-regulation of CD83 and CD86 surface maturation markers (Kempf, 2003). The type of the target receptor can have a substantial effect on the immunological response of nano-immunotherapeutics. A study by Dudziak showed that the targeting antigens to DEC-205 results mainly in cross-presentation of antigens to the CD8 T cells, whereas antigens targeted to DCIR2 are preferentially presented via the MHC class II molecules to the CD4 T cells (Dudziak, 2007). In addition, Idoyaga (Idoyaga, 2011) have shown that, while antigens can be targeted to the splenic $CD8\alpha^+$ DC subset by antigen conjugation to antibodies against Langerin and DEC205, DCIR2 antibodies can specifically target $CD8\alpha^-$

DCs. In addition, it has been proved that effective active DC targeting can be achieved by reducing the nonspecific interaction of the nanoparticles with plasma constituents and other cells in the bloodstream, by introducing a hydrophilic biomaterial consisting of PEG onto the surface of the particles (Hillaireau, 2009).

Polysaccharides (Weber, 2010), PLA (Primard, 2010), PLGA (Binjawadagi, 2014), polyanhydrides (Camacho, 2011), and polyphosphazene (Garlapati, 2012) are among the most widely studied nanomaterials prepared for immunostimulative purposes. Different techniques have been employed to prepare the nano-immunotherapeutics using the abovementioned polymers, including spray drying, emulsification/solvent evaporation, and coacervation (Kalkanidis, 2006; Oyewumi, 2010). All of these methods can produce polymeric nanoparticles with a large surface area that improve the interaction between immune cells and the immunostimulative payload (Bachmann, 2010), leading to enhanced uptake of antigens by DCs and improved immune responses (Silva, 2013). The main reason for the great interest in immunostimulatory potential of the abovementioned nanoparticles is the superior biocompatibility, versatile chemistry, high protection of the loaded biomolecules, high loading efficiency, efficient endocytosis and high presentation of the immunogenic molecules (Binjawadagi, 2014; Camacho, 2011; Garlapati, 2012; Jiang, 2005; Primard, 2010; Weber, 2010). For example, some of these nanocarriers have shown endosomal escape properties, potentiating the proteosome-dependent processing of the immunogenic payload and cross-presentation through MHC class I (Jia, 2013; Silva, 2013). Another benefit of these nanoparticles is the ability to control the release pattern of the loaded immunostimulative compound and to act as an adjuvant and increase the therapeutic effect of the formulation (Cohen, 1994; Nandakumar, 2012; Oyewumi, 2010). For example, Gómez (Gomez, 2006) have reported the ability of poly(methyl vinyl ether-alt-maleic anhydrate) (PMVE-MAh) nanocarriers for vaccination using OVA as a model antigen. The results showed higher humoral immunity (IgG titers) in response to the OVA-loaded nanoparticles compared to the alum, indicating the potential of PMVE-MAh for antigen delivery and improving immune responses (Fig. 3.5.12). Despite all the advantages

nanomaterials render to immunostimulative formulations, several hurdles, such as reproducibility, stability during production, and appropriate methods for non-thermal sterilization, need to be taken into account for developing proper immunotherapeutic approaches (Nandedkar, 2009; Sharma, 2009). For example, the reproducibility of the formulation can be affected by the variation in the size of the nanoparticles, which in turn can modify the immunogenic property of the final product (Fifis, 2004).

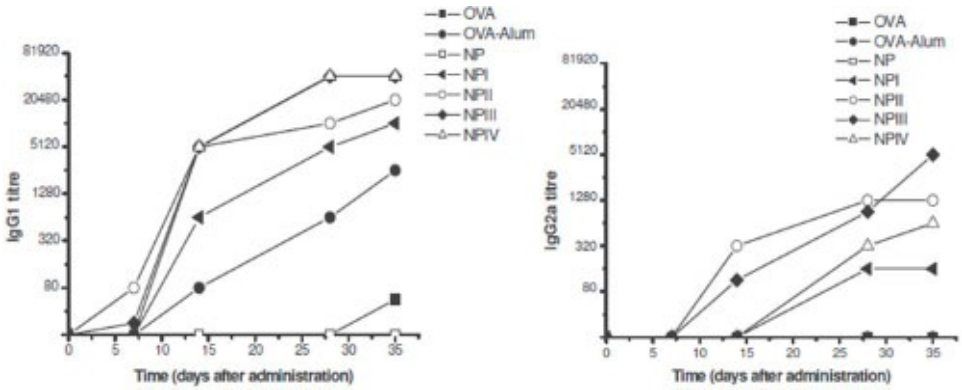

Figure 3.5.12: Anti-OVA IgG1 and IgG2a titers in sera of female BALB/c mice after intradermal immunisation with 10 μg of ovalbumin as follows: ovalbumin solution (OVA), ovalbumin adsorbed to Alum (OVA-Alum), blank nanoparticles (NP), ovalbumin coated nanoparticles (NP I), ovalbumin encapsulated nanoparticles (NP II) and 1,3-diaminopropane cross-linked ovalbumin encapsulated nanoparticles (NP III and NP IV). The antibody titer is defined as the reciprocal dilution giving an optical density. Reprinted with permission from (Gomez, 2006).

All the above-mentioned examples demonstrate the high potential of nanoparticles to synergistically improve the immunostimulative effect of antigens and other immunogenic molecules. However, using nanoparticles as anticancer immunoadjuvants and the delivery of immunostimulative biomolecules still suffers from several important drawbacks, including the problems associated with the scaling-up of the system, expensive preparation processes, and limitations for producing sterile products (Sangha, 2007). Accordingly, despite the great versatility and promising features observed for the potential of such anticancer therapeutic systems, intensive research studies are still needed in order to develop nano-formulations that can be produced in large scale and applied in the clinic.

3.5.5 Cancer Imaging, Diagnostics and Multifuctional Nanosystems

The early diagnosis of tumors through screening based on imaging might contribute to the reduction in the mortality for certain types of cancers. Real-time imaging is a most valuable technique that enables visualization of the tissue morphology and cell function, thus allowing the identification of pathological situations that generally cannot be aquired in *in vitro* analysis (Fass, 2008). Imaging techniques are being combined with nanotechnology, rendering, in many cases, extraordinarily sensitive and powerful diagnostic and imaging tools that, by other means, were not available with small or microscale tools, thus enhancing the tissue contrast or allowing the identification of specific biological changes (Toy, 2014). As it is necessary to specifically target the tumor sites in order to achieve a more successful outcome out of the treatment, it is also of utmost importance that the diagnostic and imaging tools, such as those extensively used in the medical practice: Magnetic Resonance Imaging (MRI), Computed Tomography (CT) and Positron Emission Topography (PET), are reliable enough to facilitate the right medical decisions, which can be improved by alliyng them to nanotechnology (Lee, 2013a; Perez-Medina, 2014).

Contrast agents are frequently employed to assist and complement the visualization of abnormalities in a diagnostic image, interacting with the incident radiation to yield noticeable alterations in the final aquired images. They increase the power of the imaging techniques since there is an increased sensitivity, allowing the diagnosis of many diseases that are not possible to detect using conventional methods (Power, 2011). However, nanotechnology is impacting this area of diagnostic imaging by opening new avenues of novel imaging techniques, and by allowing the enhancement of the sensitivity, pharmacokinetics, pharmacodinamics and biocompatibility features of several contrast agents, as well as providing a high signal-to-noise ratio (Rosen JE, 2011). It is becoming inevitable to not associate imaging and therapeutic properties in a single nanoplatform. Multifunctional nanocarriers are becoming more and more fashionable and many studies have been focusing on the production of multistage nanoparticulate systems.

The employment of nanoparticulate systems to image and diagnose cancer has been extensively applied and new approaches have been developed (Choi, 2012). Some advantages of using nanoparticulate-based probes for imaging purposes is attained to their low cytotoxicity profiles and physicochemical properties (Santos, 2013a). In general, a surface area-to-volume ratio and effective surface functionality, are particularities used as tools to tune the nanoparticle properties for tissue or cell targeting *in vivo* through high affinity to certain cell biomarkers (Davis, 2008). Biochemical changes *in vivo*, such as enhanced receptor density in a certain tumor type could be imaged and measured by probing with specific nanoparticulate systems avid to bind to it (Jin, 2014; Satpathy, 2015). These probes shall attain certain features that allow them to be detected and promote their accumulation at the site of interest, be safe and biocompatible, as well as to have limited side effects and to avoid the hostile environments encountered *in vivo*, such as avoidance of interactions with plasma proteins and the recognition by the phagocytic cells and consequent removal from the systemic circulation by the mononuclear phagocyte system (Santos, 2013a). For example, superparamagnetic iron oxide nanoparticles (SPIONs), along with many other clinical applications, are some of the nanoparticulate systems that are most used as highly effective contrast agents for MRI diagnosis of solid tumors (Ittrich, 2013; Rosen, 2012). Compared to other traditional contrast agents, SPIONs exhibit several advantageous properties, among them the greater magnetic signal strength and longer lasting contrast enhancement, low cytotoxic effects, biodegradability and improved delineation of tumor margins (Corot, 2006; Varallyay, 2002; Wang, 2001). Such nanoparticulate systems have been used to target solid tumors, either in a passive way, (Zolata, 2014), taking advantage of the leaky and damaged tumor vasculature through the enhanced permeability and retention effect (Brannon-Peppas, 2004; Rosen, 2012), by targeting actively the tumor sites by attaching a targeting ligand to the surface of the SPIONs, or by taking into account the tumor pathological features, such its lower pH microenvironment. The internalization of SPIONs enables a longer and effective imaging time by improvement of the contrast-enhancing effect of the particles through the accumulation of a high number of SPIONs in the tumor tissue, therefore being a more promissing and sensitive molecular

diagnostics approach than passive targeting imaging. To make it possible to biofunctionalize the SPIONs, it is necessary to provide them with a surface coating system in order to create a desirable platform for conjugation of targeting ligands or to make the SPIONs sensitive to tumor characteristic microenvironment (Fig. 3.5.13) (Jin, 2014; Xie, 2010).

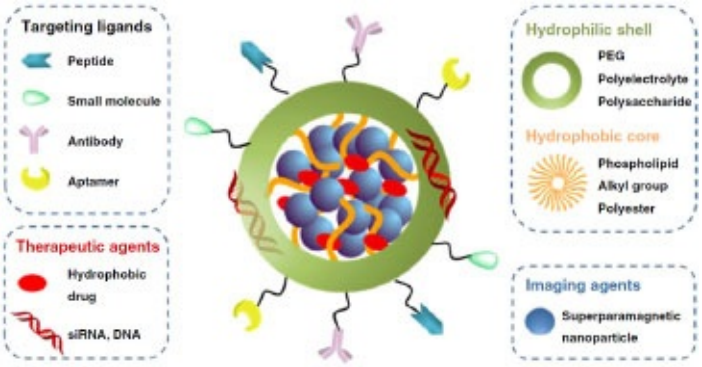

Figure 3.5.13: Schematic representation illustration of the polymer micelle composite system of surface-engineered SPION for imaging and therapy applications. Adapted from (Jin, 2014).

Recently, several studies utilizing SPIONs for the active targeting of several types of cancer were conducted and showed promising results (Kievit, 2012; Ling, 2014; Melancon, 2011; Zhang, 2014a; Zhang, 2014c). "CLIO" nanoparticles, which are SPIONs cross-linked with a dextran coating, have appeared as a powerful multistage nanovector for targeted imaging and diagnostic applications, allowing a wide and versatile conjugation of targeting moieties and compatible with diagnostic imaging MRI, optical and PET techniques (Tassa, 2011). Imaging and diagnosis can be achieved with this nanoscaled platform, as well as the delivery of therapeutic cargos to the tumor sites, making multifunctional systems or "theranostic" (Choi, 2012). For example, in a recent study, CLIO nanoparticles were developed with the purpose of producing a multifunctional platform. Taking advantage of the eminent levels of MMPs in the tumor microenvironment, in particular MMP-14, which has an important role in the tumor invasion features and provides a direct cellular target for prodrug activation (Devy, 2009; Landry, 2005), it was found that functionalization of the CLIO nanoparticulate system with the MMP-14 cleavable peptide-conjugate of azademethylcolchicine leads to vascular collapse of the tumor (Reyes-Aldasoro, 2008). This theranostic nanovector provided an opportunity to image selectively the

tumor site and, at the same time, track the drug payloads delivered to the tumour site. In addition, the conjugation of a drug molecule to nanoparticles allowed for a higher amount of drug cargo to be delivered, leading to a more effective therapeutic outcome and, again, decreasing the associated toxicity of the anticancer drug to healthy tissues (Ansari, 2014).

PSi nanomaterials are other example of materials that have been proven to be biocompatible and possess remarkable features for biomedical applications, including imaging and drug delivery (Liu, 2013b; Santos, 2013b; Shahbazi, 2012). Besides the vast medical applications (Park, 2009; Santos, 2014; Shahbazi, 2012), and taking into account a special property of this material, its photoluminescence (Anglin, 2008; Hua, 2005), PSi has been explored for clinical diagnosis and imaging applications (Godin, 2011; Santos, 2013a), such as acting as a self-reporting drug delivery systems using optical imaging in living animals (Cheng, 2008), multicolor near infrared imaging in live mice (Erogbogbo, 2010), incorporation of gadolidium (Ananta, 2010) and SPIONs inside the nanoporous structure for MRI applications (Kinsella, 2011), as well as radiolabeling of PSi with radioisotopes for imaging purposes (Bimbo, 2010; Sarparanta, 2012).

Liposomal formulations have also been used as theranostic nanoplatforms for both cancer therapeutics and imaging, considering its safety and wide clinical use. Recent studies included the encapsulation of Magnevist, a contrast agent for MRI with *in vivo* contrast-enhancing effects into liposomes, which showed, together with doxorubicin, an improved biocompatibility when coated with hyaluronic acid ceramide polymer, aimed for targeted imaging and drug delivery (Park, 2014). SPIONs have been successfully encapsulated inside immuno-liposomes targeting endothelial cells in tumor vasculature and applied in MRI imaging (Zhang, 2014a). Multimodal fluorescent labeled liposomes with thermo-responsive release properties of the contrast agents are some of the more advanced imaging platforms fabricated so far (Kokuryo, 2014), and together with near infrared (NIR) fluorescent dyes, have been similarly loaded inside theranostic liposomes with improved label efficiency (Xie, 2014).

The abovementioned nanoparticulate systems are revolutionary for the diagnostic and imaging fields, but other materials such as gold nanomaterials, carbon nanotubes and silica nanoparticles have also shown light absorbing properties, that when scattered or emited, yield specific types of diagnostic/therapeutic signals, e.g. under ultrasound, heat, Raman or fluorescence signals. These types of nanoparticles may function as theranostic nanoparticles on their own and/or can be associated with other molecules for increases in targeting and selectivity (Choi, 2012).

3.5.6 Conclusions and Future Prospects

Nanotechnology has provided the opportunity to explore new clues for the diagnosis and treatment of various diseases, in particular, cancer. With the advances of nanomedicine, the direct use of nanoparticles in prevention, diagnostic and treatment of cancer, has become a reality. In the past decades, the use of nanotechnology for simultaneous drug delivery, imaging and diagnostics has grown exponentially. There is a great evolution of the biological and physical resources to improve the use of nanoparticles for finding the best treatment for cancer. The controlled size and multifunctionality are the main reasons for the growing applications of nanomedicines as anticancer agents. Nanoparticles can synergistically improve the immunostimulative effect of antigens and other immunogenic molecules. However, using nanoparticles as anticancer immune adjuvants and delivery of immunostimulative biomolecules still suffers from several important drawbacks, including the problems associated with the scaling-up of the system, expensive preparation processes, and limitations for producing sterile products

Tremendous and fast advancements are being achieved to produce better and improved nanosystems able to provide precise information for a better understanding of the tumor diseases, as well as for facilitating medical decisions in the clinic, adding therapeutic functions to the novel nanoplatforms that can, in the near future, change the practical course of the treatment and diagnosis of cancer. The bench-to-

bedside translation of the nanomedicines and technologies have introduced a new era in the design and development of innovative but simultaneously complex targeting nanoparticles for delivery of therapeutic and diagnostic agents to tumors. Accordingly, despite the great versatility and promising features observed for such anticancer therapeutic systems, intensive research studies are still needed at the pre-clinical level in order to develop nano-formulations that can be produced in large scale and applied in the clinic.

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Biomedical Chemistry provides readers with an understanding of how fundamental chemical concepts are used to combat some diseases. The authors explain the interdisciplinary relationship of chemistry with biology, physics, pharmacy and medicine. The results of chemical research can be applied to understand chemical processes in cells and in the body, and new methods for drug transportation. Also, basic chemical ideas and determination of disease etiology are approached by developing techniques to ensure optimum interaction between drugs and human cells. This book is an excellent resource for students and researchers in health-related fields with frontier topics in medicinal and pharmaceutical chemistry, organic chemistry and biochemistry.

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