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Transition State Analogues of Enzymatic Reaction as Potential Drugs

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1. Introduction

All chemical transformations pass through an unstable structure called the transition state, which is poised between the chemical structures of the substrates and products. The transition states for chemical reactions are proposed to have lifetimes near 10^{-13} sec, the time for a single bond vibration. Thus, the transition state is the critical configuration of a reaction system situated at the highest point of the most favorable reaction path on the potential-energy surface, with its characteristics governing the dynamic behavior of reacting systems decisively. It is used primarily to understand qualitatively how chemical reactions take place.

Yet transition state structure is crucial to understanding enzymatic catalysis, because enzymes function by lowering activation energy. Linus Pauling coiled an accepted view, that incredible catalytic rate enhancements caused by enzyme is governed by tight binding to the unstable transition state structure in 1948. Because reaction rate is proportional to the fraction of the reactant in the transition state complex, the enzyme was proposed to increase the concentration of these reactive species. This proposal was further formalized by Wolfenden (1972) and coworkers, who hypothesized that the rate increase imposed by enzymes is proportional to the affinity of the enzyme to the transition state structure relative to the Michaelis complex.

Transition state structures of enzymatic targets for cancer, autoimmunity, malaria and bacterial antibiotics have been explored by the systematic application of kinetic isotope effects and computational chemistry. Today the combination of experimental and computational access to transition-state information permits the design of transition-state analogs as powerful enzymatic inhibitors and exploration of protein features linked to transition-state structure.

Molecular electrostatic potential maps of transition states serve as blueprints to guide synthesis of transition state analogue inhibitors of chosen enzymes. Substances, that ideally

mimic geometric and electrostatic features of a transition state (or other intermediates of high energy) are considered as excellent enzyme inhibitors (Fig.1). They bind up to 10^8 times tighter than substrate. Thus, the goal of transition-state analogs design is to create stable chemical structures with van der Waals geometry and molecular electrostatic potential surfaces as close as possible to those of the transition state.

Although some reviews on the subject have been published, this concept has not been reviewed in detail [Wolfenden, 1999; Robertson, 2005; Schramm, 2005; Schramm, 2007; Dyba-ła-Defratyka et al. 2008; Schramm, 2011]. In this review the current trends, alongside with appropriate case studies in designing of such inhibitors will be presented.

2. Choice of the target enzyme

The sequencing of the human genome has promised a revolution in medicine. The genome encodes 20,000- 25,000 human genes, and thousands more proteins as a result of alternative gene splicing. Many of these hold the keys to treating disease, especially numerous enzymes of undefined so far physiologic functions [Gonzaga-Jagureui et al., 2012]. Out of 1200 registered drugs over 300 act as enzyme inhibitors. Most of them are simple analogs of substrates of certain enzymatic reaction. Analogy to transition state as a mean to obtain effective inhibitors emerged in 1970s [Lienhard, 1973]. Through the 1970s and 1980s, most of the known examples were natural products [Wolfenden, 1976]. The situation has changed in 1990s when synthetic inhibitors became the predominate examples of transition-state inhibitors. In 1995, there were transition-state analogues for at least 130 enzymes [Radzicka & Wolfenden, 1995].
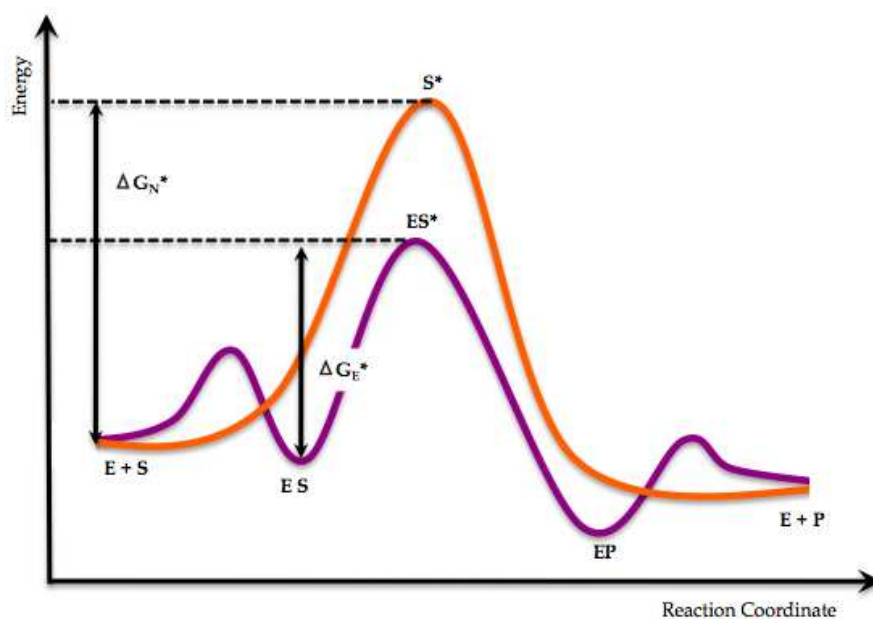

Figure 1. Progress of the enzymatic reaction versus uncatalyzed one.

The design of transition-state inhibitors is likely to become more frequent in the future, alongside with the development of theory and technology for understanding enzyme transition states. Today the sequence of information required to obtain transition state analog of enzymatic reaction considers: choice of the suitable enzyme (most likely suited to kinetic isotope effect measurement), selection of presumable mechanism(s) of catalyzed reaction, measurement of kinetic isotope effects (KIE), computer-aided calculations matching the intrinsic KIEs, construction of steric and electronic map of transition state and synthesis of stable compound(s) matching this map [Schramm, 2007]. This procedure has been developed gradually in parallel with the advances in KIE enzymology, computational chemistry, and synthetic organic chemistry.

3. Determination of transition state architecture

At present, the most reliable method to determine three-dimensional architecture of transition state is through the use of computational methods in conjunction with experimentally measured kinetic isotope effect (KIE).

Isotopic substitution is a useful technique for probing reaction mechanisms. The change of an isotope may affect the reaction rate in a number of ways, providing clues to the pathway of the reaction. The advantage of isotopic substitution is that this is the least disturbing structural change that can be effected in a molecule. Replacement of one isotope of the substrate by another at vicinity where bonds are being or re-hybridizing typically leads to a change in the rate of the reaction. Thus, kinetic isotope effects measurements compare k_{cat}/K_M values between isotope-labeled and natural abundance reactants. This provides information about which bonds are broken or formed, and identifies changes in hybridization that occur during the rate limiting step of a reaction. It is reached by conversion of atom-by-atom KIE values to a specific static model with fixed bond angles and lengths by computational matching to a quantum chemical model of the reaction of interest. Substrate, intermediate and product geometries are located as the global minima. Transition-state structures are located with a single imaginary frequency, characteristic of true potential energy saddle points.

Such an analysis was performed recently for human thymidine phosphorylase, an enzyme responsible for thymidine homeostasis, action of which promotes angiogenesis. Thus, inhibitors of this enzyme might be considered as promising anticancer agents. Its transition state was characterized using multiple kinetic isotope effect measurements applying isotopically (^{3}H, ^{14}C and ^{15}N) enriched thymidines, which were synthesized enzymatically [Schwartz et al, 2010]. A transition state constrained to match the intrinsic KIEs was found using density functional theory. In the proposed mechanism (Fig.2), departure of the thymine results in a discrete ribocation intermediate. Thymine likely leaves deprotonated at N1 and undergoes enzyme-catalyzed protonation before the next step. In the following step, the intermediate undergoes nucleophilic attack from an activated water molecule to form the products. The latter step is a reaction rate limiting step as determined by energetics of its transition state.

The transition state model predicts that deoxyribose adopts a mild 3′-*endo* conformation during nucleophilic capture (Fig. 2).

Such studies, although cumbersome and difficult, are being recently more and more popular, as demonstrated by representative studies on *Escherichia coli t*-RNA-specific adenosine deaminase [Luo & Schramm, 2008], glucoside hydrolases [Lee et al, 2004], human purine nucleoside phosphorylase [Murkin et al., 2007], *Trypasnosoma cruzi trans*-sialidase [Pierdominici-Sottile et al., 2011], *L*-dopa decarboxylase [Lin & Gao, 2011] or *cis*-prenyltransferase [Hu et al., 2010].
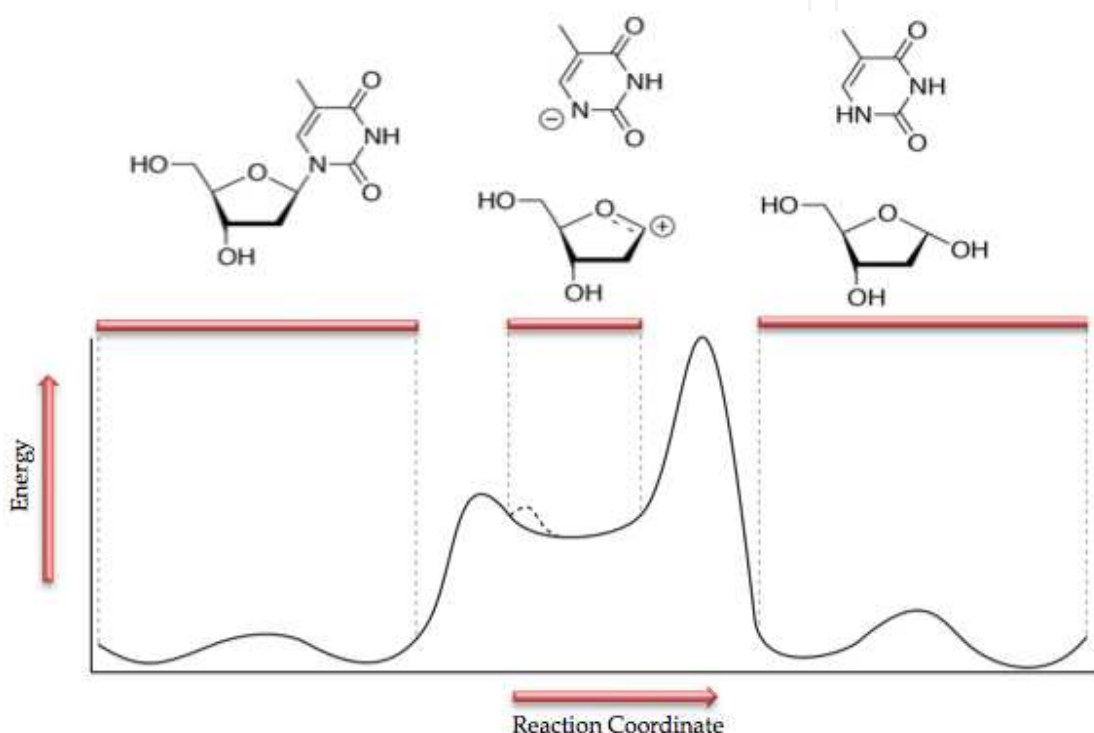

Figure 2. Mechanism of human thymidine phosphorylase catalyzed depirymidation of thymidine. The dash line represents protonation step.

Computational chemistry provides techniques for the generation and exploration of the multi-dimensional energy surfaces that govern chemical reactivity. Therefore, energy minima and saddle points can be located and characterized. The pathways that interconnect them can be determined. Thus, computational methods are increasingly at the forefront of elucidating mechanisms of enzyme-catalyzed reactions, and shedding light on the determinants of specificity and efficiency of catalysis [Kollman et al., 2002; Parks et al., 2010; Williams, 2010; Lonsdake et al., 2012].

At the beginning of a molecular modeling study choice upon the specific catalytic process to model has to be undertaken. This decision may sound simple, but it includes the nontrivial task of exhaustively searching the literature to determine what is already known about the selected enzymatic system, either from experiments or from previous computational stud-

ies. Reaction mechanisms may have already been proposed in the literature, and thus provide a logical starting point for modeling studies. The three-dimensional structure of the enzyme, preferably with a bound substrate analog, reaction product or inhibitor, is among the most critical sources of information. In practice, this usually means that a high-resolution X-ray crystal structure of a reacting enzyme complex is required.

Molecular mechanics methods are important in simulations of enzymes, even though these methods cannot model chemical reactions. For that molecular dynamics simulations, or combination of molecular mechanics with quantum mechanical methods are commonly used [Senn & Thiel, 2007; Hou & Cui, 2011; Kosugi & Hayashi, 2012; Londsdale et al., 2012]. Enzymes are large molecules consisting of thousands of atoms whereas the active site may comprise only around 100 atoms. Since quantum chemical calculations are nowadays affordable only for up to a few hundred atoms (depending on the level of accuracy) the system is split into two regions: a small region encapsulating the reaction at the active site is modeled with a quantum mechanical methods, while the rest of the enzyme alongside with surrounding water is modeled using molecular mechanics (Fig. 3.)
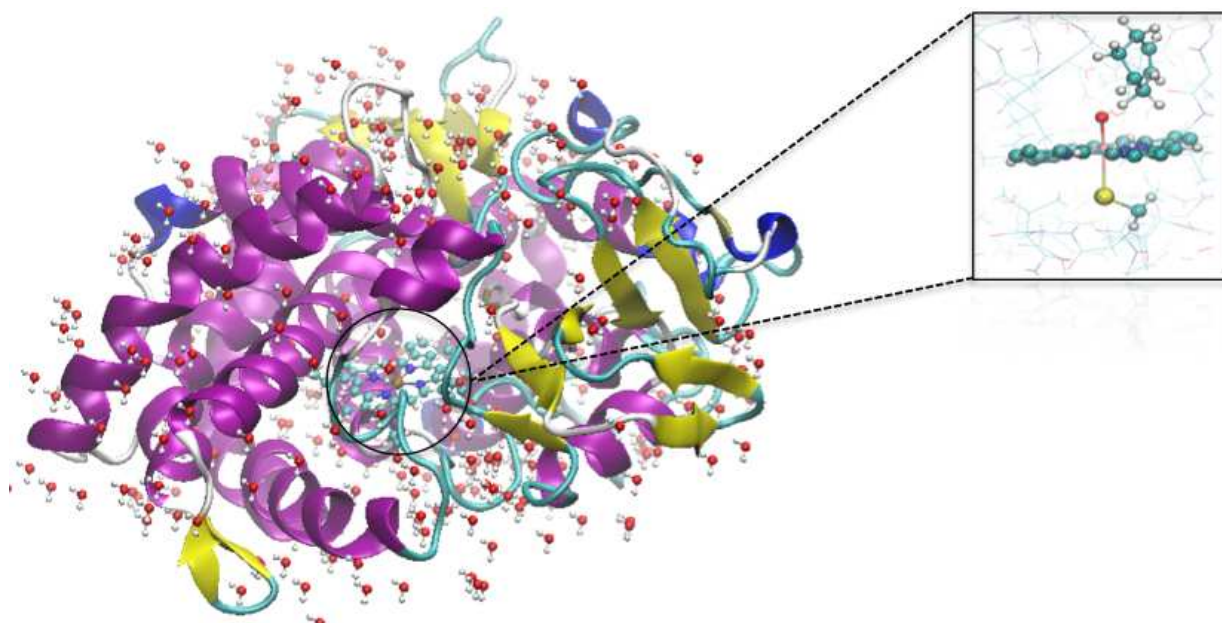

Figure 3. Quantum mechanics/molecular mechanics calculation of an enzymatic reaction illustrated by cytochrome P450 with bound cyclohexene [Lonsdake et al., 2010].

These calculations do not take in the consideration such an important factor as protein dynamic. There is an agreement that fast (at nano- or picosecond scale) protein motions couple directly to transition state formation in enzymatically catalyzed reactions and are an integral part of the reaction coordinate. Slower protein dynamic motions also influence the heights of barriers in enzymatic reactions, however detailed description of these effects require elaboration of new computational methods [Saen-oon et al., 2008].

4. Inhibitors of proteinases

First inhibitors being transition state analogs were designed for proteolytic enzymes. The design was based on the resemblance of transition state of phosphonamidates and phosphonic, phosphinic (Fig. 4) acids to the sp^3 intermediate of the hydrolysis of peptide bond. Because the lengths of oxygen-to-phosphorus and carbon-to-phosphorus bonds are significantly longer than the corresponding carbon-to-carbon and carbon-to-oxygen bonds, organophosphorus fragment of the molecule might be considered as "swollen" tetrahedral intermediate and thus can be treated similar to the transition state of this reaction.
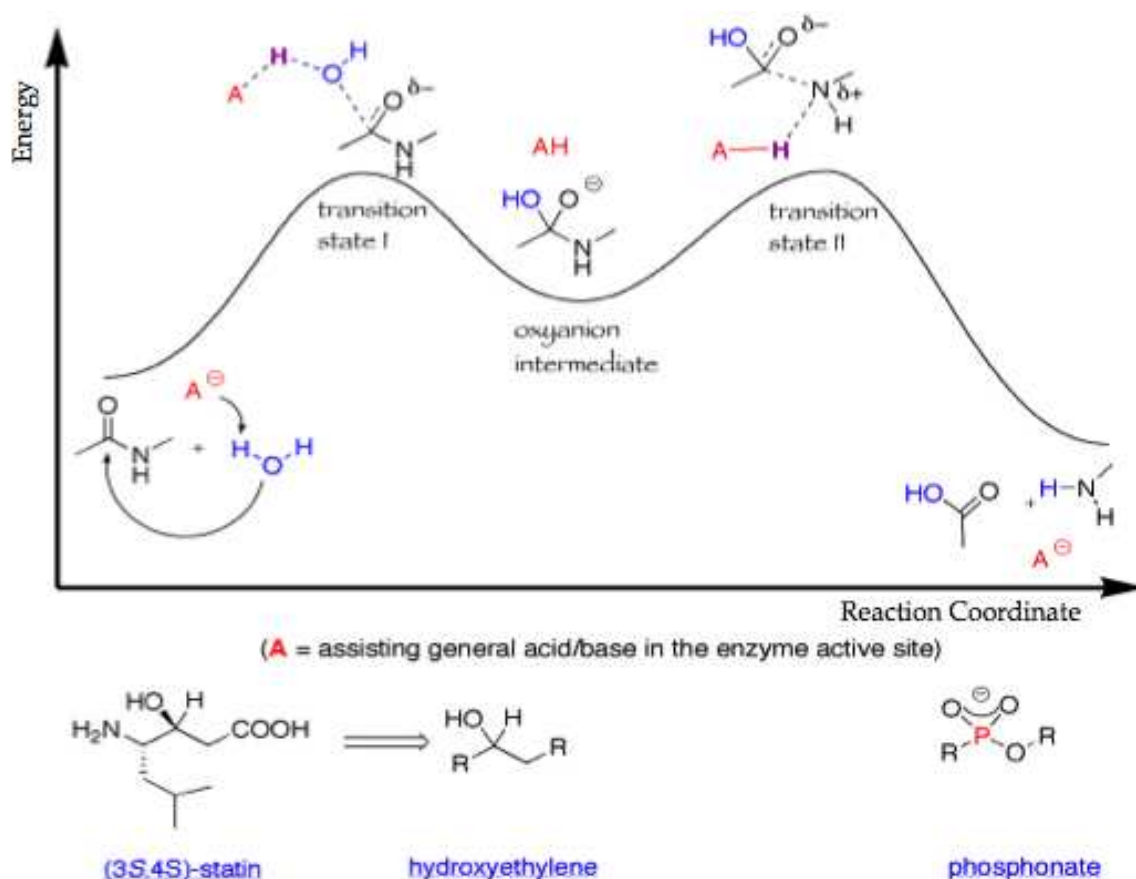

Figure 4. Organophosphoprus compounds as transition state analog inhibitors of hydrolysis of peptide bonds.

Crystallography of enzyme-inhibitor complexes and molecular modeling studies had shown that their potent inhibitory activities result from both: resemblance to the transition state and strong electrostatic interactions between positively charged active-site metal ions (predominantly zinc ions) and negatively charged phosphonic acid (or related) group [Mucha et al., 2010; Mucha et al., 2011]. Although the phosphonate/phosphinate group is a rather weak zinc complexing moiety, it offers other advantageous structural and electronic features [Collinsova & Jiráček, 2000].
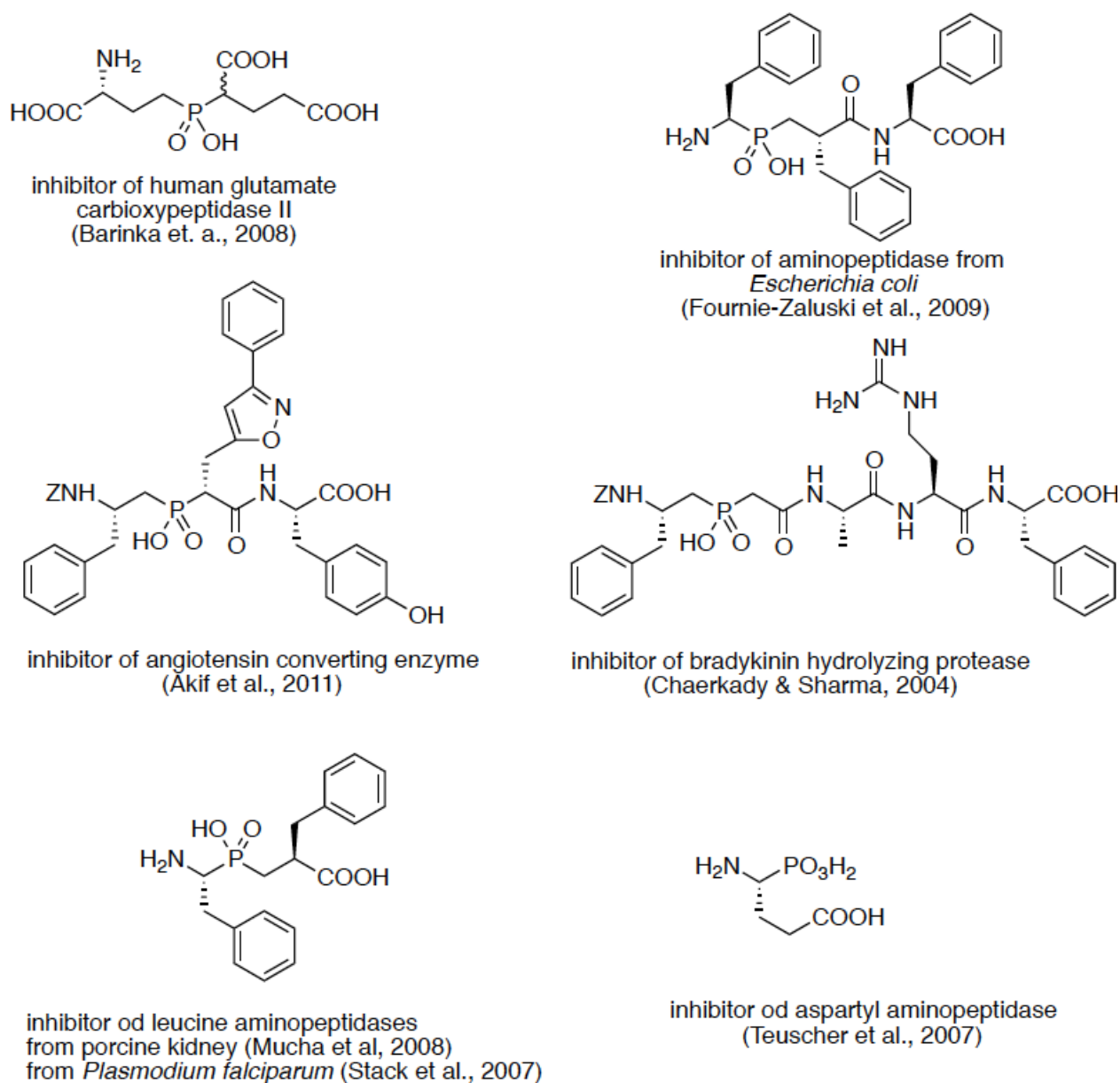
inhibitor of human glutamate
carbioxypeptidase II
(Barinka et. a., 2008)

inhibitor of aminopeptidase from
Escherichia coli
(Fournie-Zaluski et al., 2009)

inhibitor of angiotensin converting enzyme
(Akif et al., 2011)

inhibitor of bradykinin hydrolyzing protease
(Chaerkady & Sharma, 2004)

inhibitor od leucine aminopeptidases
from porcine kidney (Mucha et al, 2008)
from *Plasmodium falciparum* (Stack et al., 2007)

inhibitor od aspartyl aminopeptidase
(Teuscher et al., 2007)

Figure 5. Representative organophosphorus inhibitors of metalloproteinases.

Simple phosphonic acid analogs of amino acids and pseudopeptides, containing phosphi-
nate moiety replacing scissile peptide bond, are acting via this mechanism and rank
amongst most potent inhibitors of metalloproteinases (Fig. 5). Inhibitors of neutral alanyl
(M1) and leucine (M17) aminopeptidases are among the most recognized and most inten-
sively studied representatives of metal-containing exopeptidases of biomedical significance
[Lowther & Matthews, 2002; Grembecka et al., 2003; Vassiliou et. al. 2007]. Functions related
to tumorigenesis and invasion makes these enzymes molecular targets for the development
of potential anticancer drugs [Grembecka & Kafarski, 2001; Zhang & Xu, 2008; Fournié-Za-
luski et al., 2009; Grzywa et al., 2010]. The recognized role of neutral aminopeptidase in the
pathogenesis of hypertension provides also an opportunity for regulating arterial blood

pressure by their inhibitors [Banegas et al., 2006; Bodineau et al., 2008]. Additionally, two of these pseudodipeptides appear to be excellent inhibitors when applied to *Plasmodium falciparum* M1 and M17 aminopeptidases (Fig. 5), the protozoan counterparts of neutral and leucine aminopeptidases [Stack et al., 2007; Cunningham et al, 2008; McGowan et al., 2009; McGowan et al., 2010]. They efficiently controlled the growth of *P. falciparium* in cultures, including those of malaria cells lines resistant to chloroquine, and significantly reduced malaria infections in murine model (*Plasmodium chabaudi*) [Skinner-Adams et al., 2007]. These findings positively validated *P. falciparum* M1 and M17 aminopeptidases as promising targets for a novel treatment of malaria and identify new leads with anti-parasite potential [Skinner-Adams et al., 2010; Thivierge et al., 2012].

The design and development of pseudopeptidic inhibitors of aminopeptidases are greatly facilitated by two factors. First, the results of extensive structure-activity relationship studies, available for a wide collection of fluorogenic substrates, have defined the requirements of the S1 binding pockets of these enzymes [Drag & Salvesen, 2009; Drag et al., 2010; Gajda et al., 2012; Poręba et al., 2011; Poręba 2012]. Second, computer-aided analysis of numerous crystal structures available for leucine aminopeptidase has pointed to this enzyme as a primary molecular target for extending and optimizing interactions within the S1' pocket [Grembecka et al., 2001; Jørgensen et al., 2002; Evdokimov et al., 2007; Khandelwal et al., 2005; Khaliullin et al. 2010; Li et al., 2010].

Phosphinic pseudopeptides have also clearly revealed their potential for the regulation of matrix metalloproteinases (MMPs, matrixins), zinc-dependent endopeptidases implicated in the breakdown of the extracellular matrix [Yiotakis at al., 2004; Fisher & Mobashery, 2006]. Cleavage of the matrix component (collagen, lamanin, elastin, gelatin, etc.) is physiologically essential for tissue remodeling processes such as morphogenesis, embryogenesis and reproduction [Overall & Kleifield, 2006; Sang et. al., 2006]. Overexpression or inadequate level of matrix metalloproteinases leads to pathological states such as osteoarthritis, rheumatoid arthritis and inflammation, but it is most associated with tumor growth, invasion, and metastasis. Angiogenetic process favored by these enzymes is essential for vascoularization and growth of tumors. Thus, they were the first proteinase targets seriously considered for combating cancer. Despite that preliminary clinical/preclinical studies on MMP inhibition in tumor models brought positive results the outcome in the drug market has been so far unsatisfactory. The spectacular failure of the last-step clinical trials is mainly due to a lack of selectivity and serious side effects [Fisher & Mobashery, 2006]. The field is now resurging with careful reinvestigation of the precise roles of each particular MMP member and a focus on the development of selective inhibitors that fully discriminate between different members of the MMP family [Reiter et al., 2003; Matziari et al., 2007; Zucker & Cao, 2009; Devel et al., 2010; Johnson et al., 2011]. Such selectivity had been reached by variation of peptide scaffold by means of combinatorial pseudopeptide synthesis [Buchardt et al, 2000; Dive et al., 2004] or by application of molecular modeling based on crystallographic studies of these enzymes [Rao, 2005; Pirard, 2007; Verma & Hansch, 2007; Anzellotti & Farrell, 2008; Kalva et al., 2012]. Representative selective inhibitors of this class are shown in Figure 6.
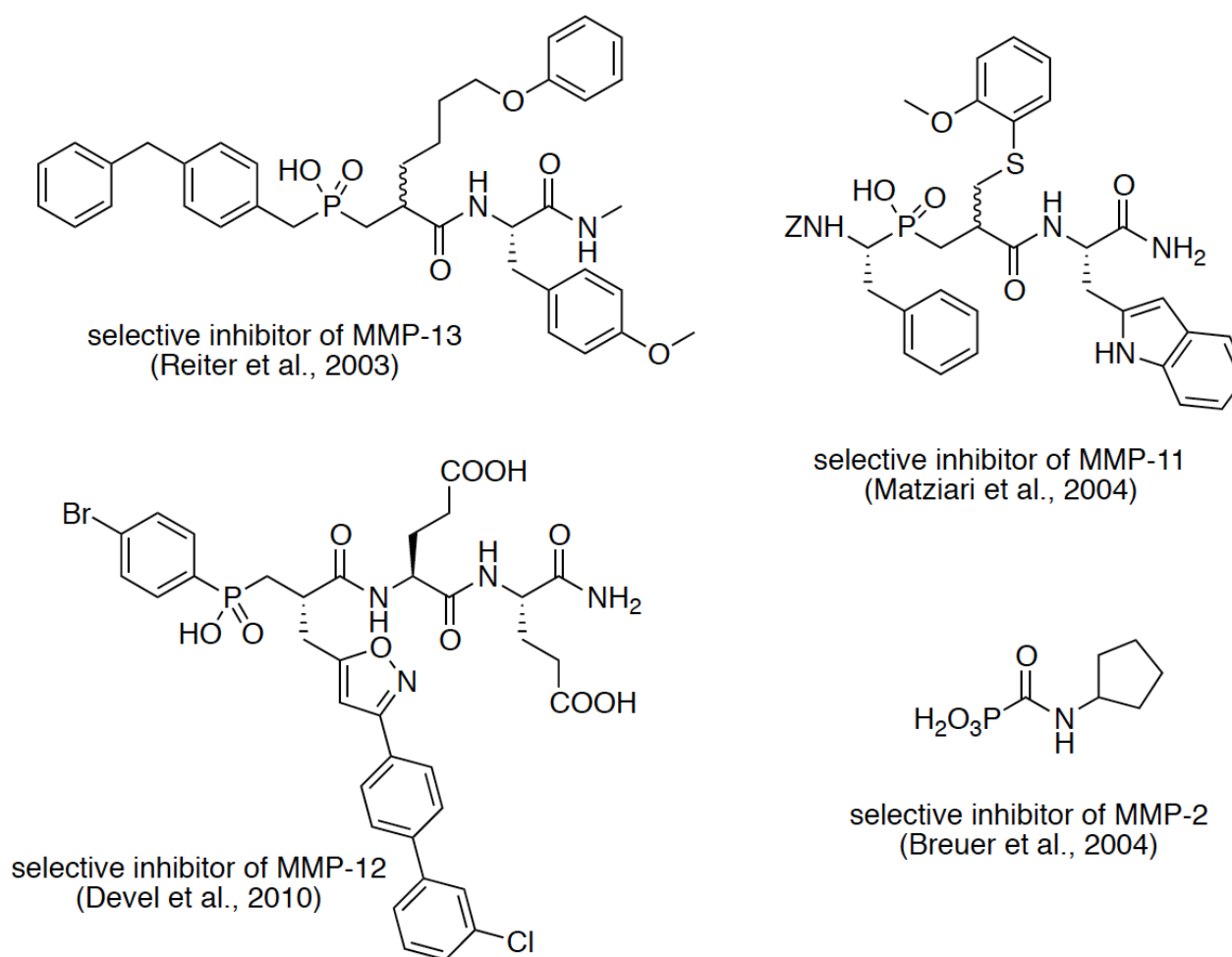
Figure 6. Representative inhibitors selective against chosen matrix metalloproteinases.

Quite interesting approach is preparation of hybrid systems as this composed of a phosphinate transition state analogue that has been incorporated within a triple-helical peptide template. The template sequence was based on the $\alpha 1(V)436$-450 collagen region, which is hydrolyzed at the Gly_{439}-Val_{440} bond selectively by MMP-2 and MMP-9. In that manner highly selective inhibitor towards these two gelatinases was found [Lauer-Fields et al., 2007; Lauer-Fields et al., 2008].

Phosphinic transition state analog approach has been also recently applied for the design and synthesis of novel potent inhibitors of other proteinases of medicinal importance. Thus, inhibitors of angiotensin converting enzyme [Mores et al., 2008; Julien et al. 2010; Akif et al., 2011] are potential drugs against hypertension, aspartyl aminopeptidase as antimalarial agent [Teuscher et al., 2007], inhibitors of cathepsin C and renal dipeptidase may be considered as potential anticancer agents [Gurulingappa et al., 2003: Mucha et al., 2004], inhibitors of sortase, which is bacterial virulence protein [Kruger et al., 2004], whereas inhibition of pyroglutamyl peptidase II enhances the analeptic effect of thyrotropin [Matziari et al., 2008; Lazcano et al. 2012].

It is worth mentioning that *Monopril®*, the sodium salt of fosinopril [Fig. 7], the ester prodrug of an angiotensin-converting enzyme (ACE) inhibitor fosinoprilat, is perhaps one of the most effective implementation of transition state analogy in medicine [Powell et al., 1984].
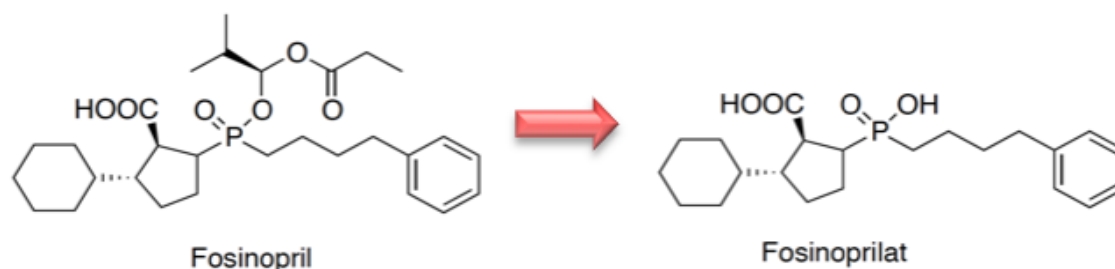

Figure 7. Fosinopril and fosinoprilat.

Sulfonamides also mimic both shape and electronic environment of the transition state of peptide bond hydrolysis. This approach was used for introduction of transition state inhibitors of HIV-protease, thermolysin and thrombine as well as haptens for generation of catalytic antibodies (Fig. 8) [Moree, et al., 1993; Moree, et al., 1995; Löwik et al., 2000; Liskamp & Kruijzer, 2004; Turcotte et al., 2012]. Unfortunately most of them appeared to be ineffective. This might be explained by non-typical bonding of potent inhibitor of this class with HIV protease (Fig. 9). It appeared that sulfonamide moiety displaces water molecule from active site and forms hydrogen bonds with two isoleucines, not as expected with catalytic aspartic acids [Meanwell, 2011]. Thus, sulfonamide group does not act as transition state analogue.
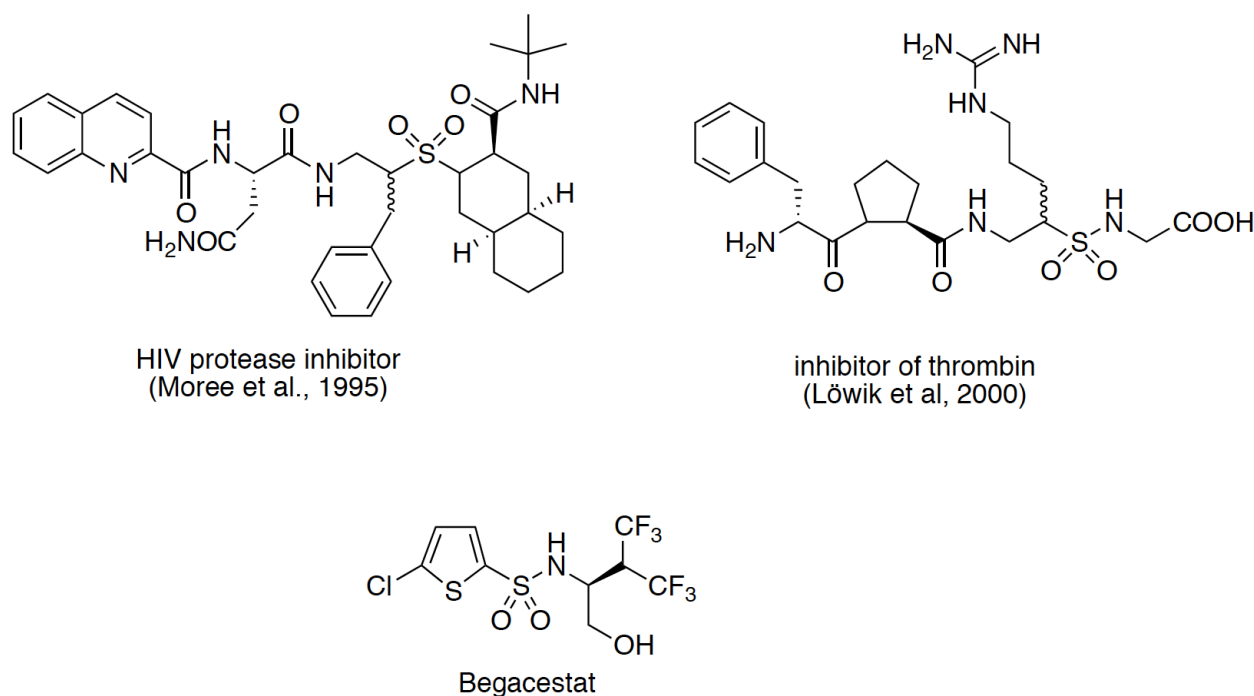

Figure 8. Sufonamides as inhibitors of proteases.

Begacestat (Fig. 8), an effective and potent inhibitor of γ-secretase is an exception here [Mayer et al., 2008; Martone et al., 2009]. γ-Secretase catalyzes the final step in the generation of amyloid β peptides from amyloid precursor protein. Amyloid β-peptides aggregate to form neurotoxic oligomers, senile plaques, and congophilic angiopathy, some of the cardinal pathologies associated with Alzheimer's disease. Begacestat appeared to be well tolerated in mouse and dog toxicity studies and has been advanced to human clinical trials for the treatment of this neurological disease.
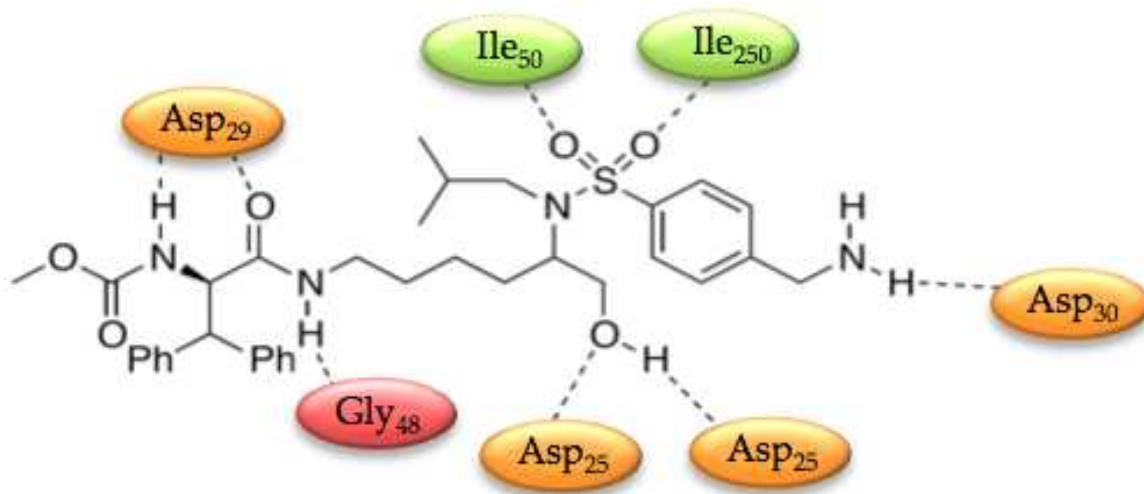

Figure 9. Mode of binding of sulfonamide HIV protease inhibitor.

inhibitor of angiotensin converting enzyme
(Mutahi et al., 2002)

HIV protease inhibitors
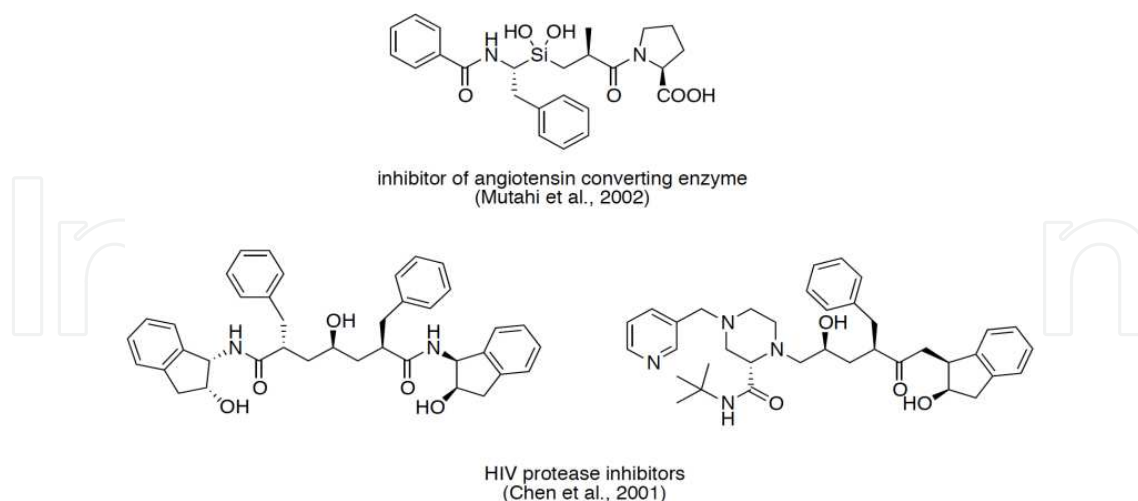(Chen et al., 2001)

Figure 10. Silenediols as proteinase inhibitors.

Dialkylsilanediols are tetrahedral functional groups that can mimic hydrated carbonyls and thus might be also considered as „swollen" intermediates of peptide bond hydrolysis. When silanediols are embedded in a peptide-like structure, they are recognized by proteinases and

act as hydrolytically stable entities. Thus, dialkylsilanol is an effective functional group for the design of active site-directed protease inhibitors. This concept has been successfully tested by replacing the presumed tetrahedral carbon of thermolysin, HIV-protease and angiotensin converting enzyme substrates with silanediol groups (Fig. 10), which resulted in potent inhibitors of these enzymes [Juers et al., 2005; Sieburth & Chen, 2006; Bo et al., 2011; Meanwell, 2011].

5. Hydroxyethylene intermediate analogs as inhibitors of proteases

Aspartic proteases generally bind 6-10 amino acid regions of their polypeptide substrates, which are typically processed with the aid of two catalytic aspartic acid residues in the active site. Thus, there is usually considerable scope for building inhibitor specificity for a particular aspartic protease by taking advantage of the collective interactions between a putative inhibitor on both sides of its scissile amide bond, and a substantial portion of the substrate-binding groove of the enzyme [Eder et al., 2007]. A very effective group of their inhibitors are simple hydroxyethylene analogs of tetrahedral oxyanion intermediates of the hydrolysis of peptide bonds. This approach is based on the structure of bestatin, a general inhibitor of aminopeptidases and aspartyl proteinases isolated in 1976 from *Streptomyces olivoreticuli* [Umezawa et al., 1976].

Today, HIV protease inhibitors constitute around 40% of available drugs against HIV. Nearly all of them contain hydroxyethylene unit as transition state analog mimic, to mention only Darunavir, Atazanavir, Fosamprenavir, Lopinavir (or Ritonavir) or the oldest one Saquinavir (Fig. 11) [Brik & Wong, 2003; Pokorna et. al, 2009].

Availability of anti-HIV drugs enabled to introduce highly active antiretroviral therapy (HAART), which resulted in dramatic decrease of the mortality and morbidity for a wide variety of opportunistic viral, bacterial, fungal and parasitic infections among HIV-infected individuals in economically developed countries [Andreoni & Perno, 2012]. Thus, the design, development and clinical success of HIV protease inhibitors represent one of the most remarkable achievements of molecular medicine. However, both the academias, as well as, the industry need to continue in their effort to develop novel, more potent compounds. This is mainly connected with HIV drug resistance, which in turn results from the high mutation rate, caused by the lack of proofreading activity of the viral reverse transcriptase. The pattern of mutations associated with the viral resistance is extremely complex as shown in Figure 12 [Pokorna et al., 2009]. Taken together, in spite of the indisputable success of the HAART and benefit to patients, new approaches to the antiviral treatment are highly desirable [Clarke, 2007; Adachi et al., 2009; Pokorna et al., 2009; Alfonso & Mozote, 2011].

Quite interestingly, small modifications of core structure of the inhibitor results in minute changes in inhibitor affinity to HIV protease as demonstrated in Figure 13 [Wu et al., 2008; Mahalingham et al., 2010].

Studies conducted in order to evaluate the influence of these antiviral drugs on the development of parasites, which are known to co-infect HIV-positive individuals, surprisingly
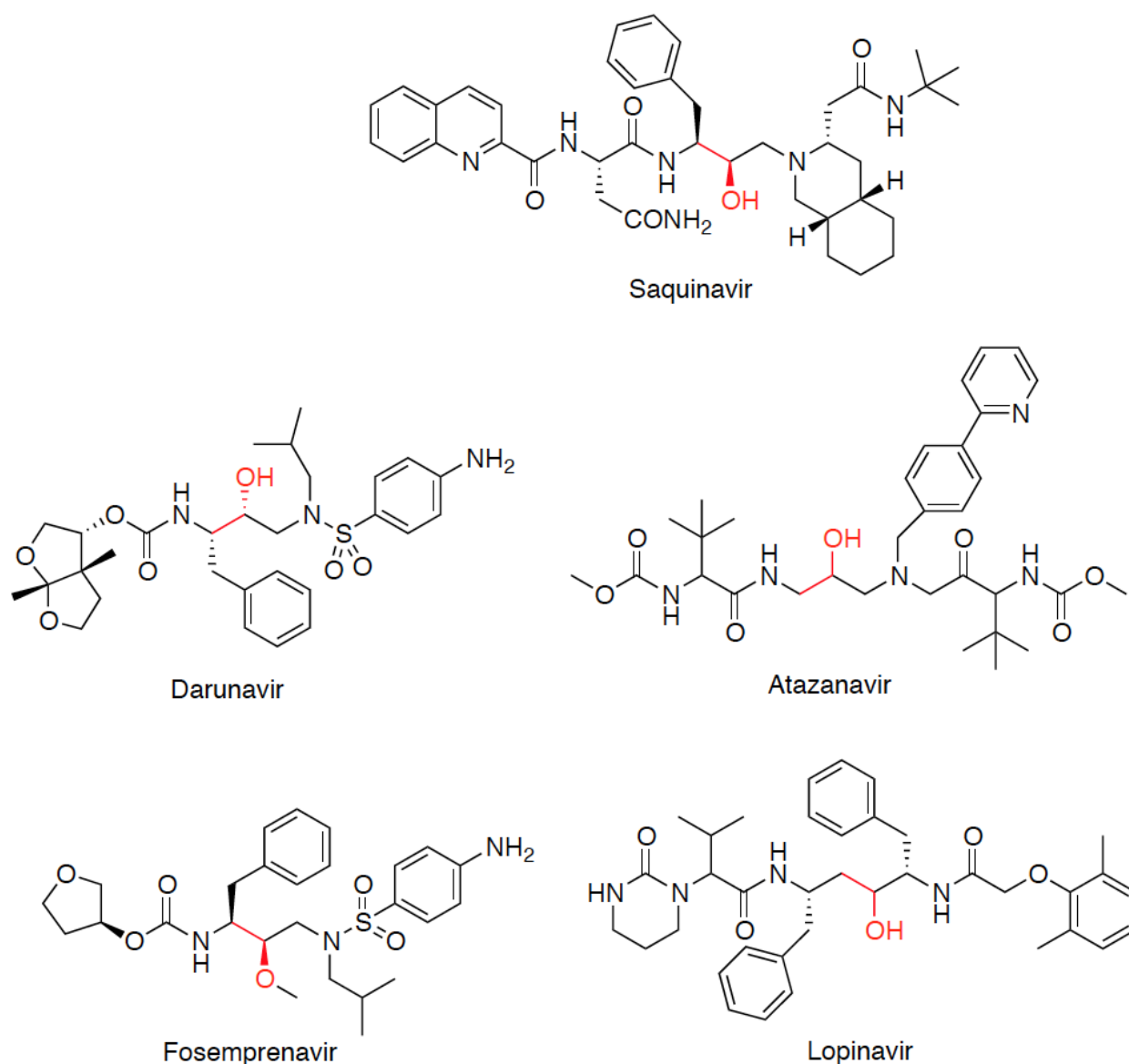
Figure 11. Representative examples of clinical HIV protease drugs.

shown, that these drugs exhibit marked antiprotozoal activity. For example Saquinavir, Lopinavir, Indinavir directly inhibited the grown of *Plasmodium falciparum* in vitro at clinically relevant concentrations. This findings suggest that some inhibitors of HIV protease are active against the most virulent human malaria parasite *P. falciparum* that is known to express number of aspartic proteases (plasmepsins) [Skinner-Adams, et al., 2004; Alfonso & Mozote, 2011].

More than 25 million people are suffering from dementia, and the annual socioeconomic worldwide costs have been estimated to exceed U.S. $200 billion. γ-Secretase, along with β -secretase produces the amyloid β-protein of Alzheimer's disease. Because of its key role in the pathogenesis γ-secretase has been a prime target for drug discovery, and many inhibitors of this protease have been developed. These enzymes are also effectively inhibited by
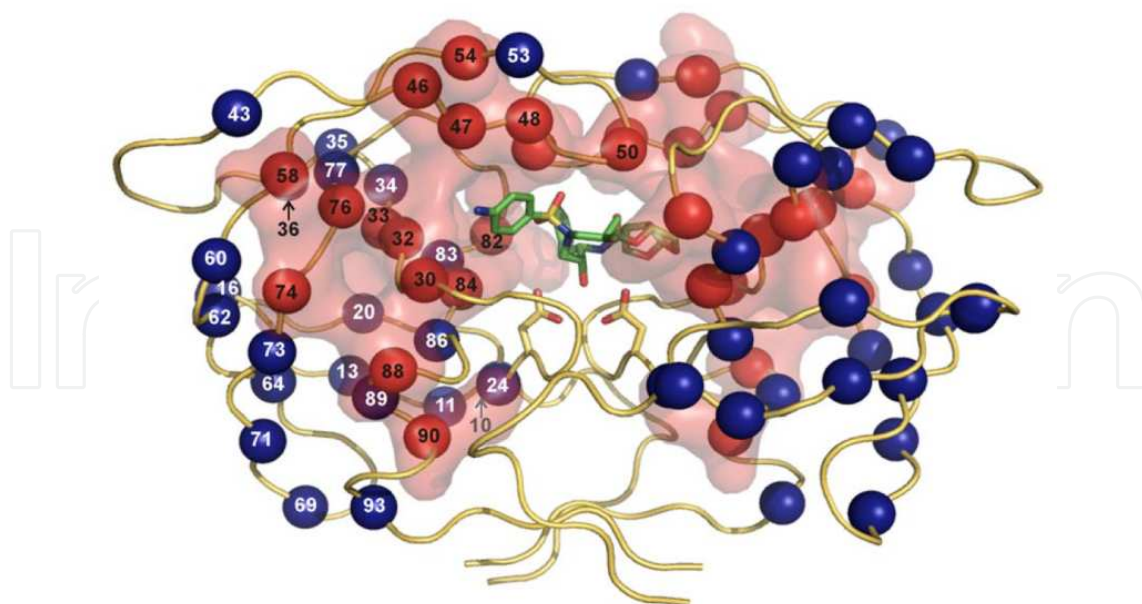
Figure 12. Three-dimensional structure of HIV protease complexed with Darunavir. Mutations associated with resistance to clinically used inhibitors are depicted as balls.
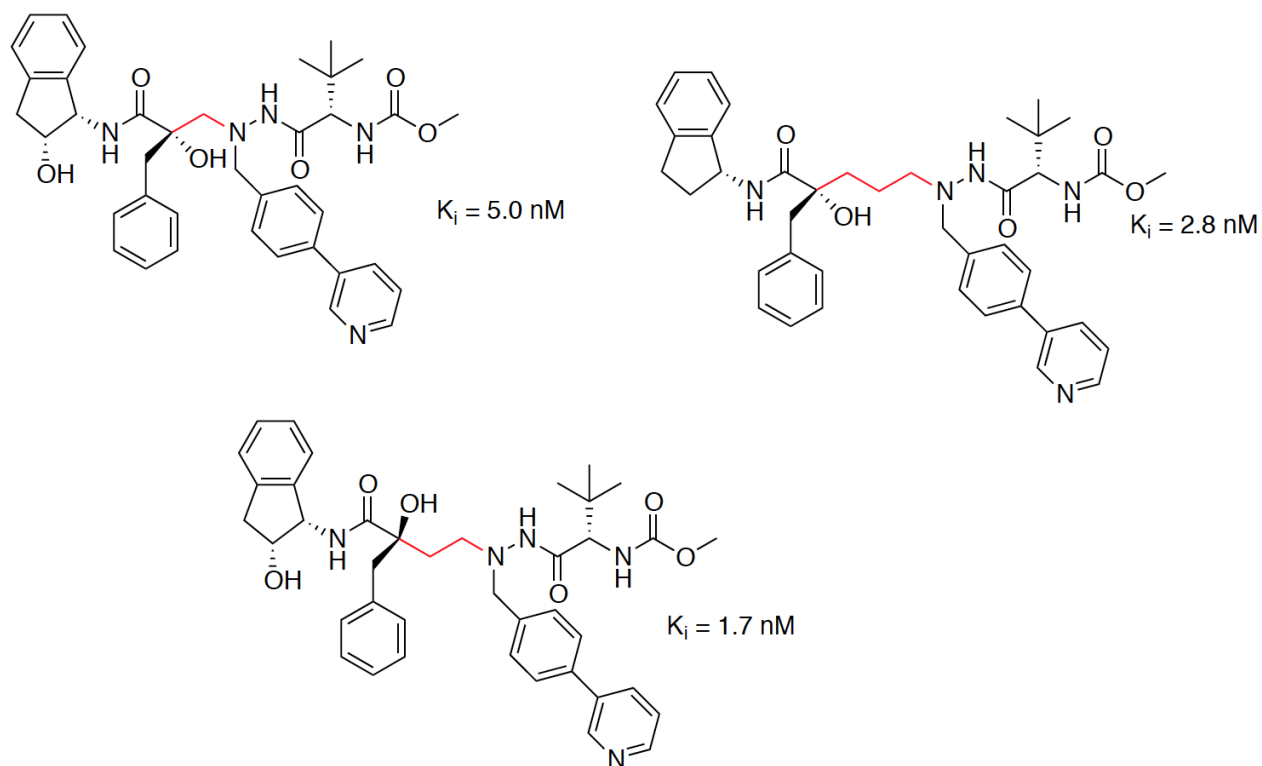

$K_i = 5.0$ nM

$K_i = 2.8$ nM

$K_i = 1.7$ nM

Figure 13. Influence of linker length on activity of HIV protease inhibitors.

peptidomimetics containing hydroxyethylene fragment replacing hydrolyzed peptide bond. Only one drug (Semagacestat, Fig. 14) reached phase III clinical studies so far, however, uncovered evidence of cognitive worsening in treated patients compared with placebo led to suspension of the trials in 2010. Anyway, design, synthesis and evaluation of new low-molecular, nanomolar inhibitors of secretases, structure of which significantly drifted away from peptidic transition state analogs (Fig. 14), is still challenging and brought new promising results [Osterman et al., 2006; Wolfe, 2012]. Due to rapid technological progress in chemistry, bioinformatics, structural biology and computer technology, computer-aided drug design plays more and more important role in this respect [Avram et al., 2006; Fujimoto et al., 2008; Xu et al., 2009; Al-Tel et al. 2011; Hamada et al., 2012].
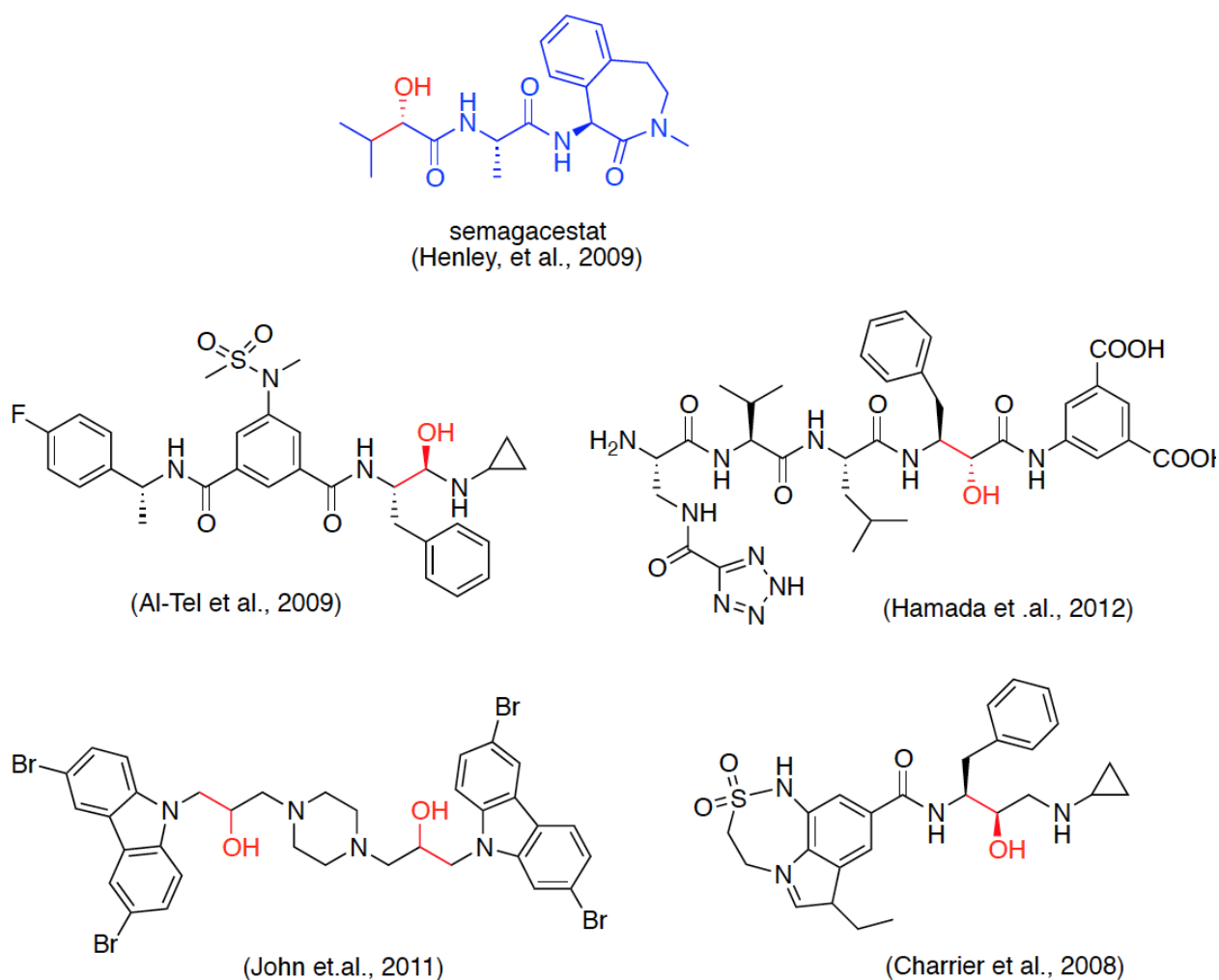

Figure 14. Inhibitors of secretases.

Hypertension is a major risk factor for cardiovascular diseases such as stroke, myocardial infarction, and heart failure, the leading causes of death in the Western world. Inhibitors of the renin–angiotensin system have proven to be successful treatments for hypertension. As renin specifically catalyses the rate limiting step of this system, it represents the optimal tar-

get for antihypertensive drugs. Aliskiren (Fig. 15), a promising drug lowering blood pressure in sodium-depleted marmosets and hypertensive human patients, was developed using a combination of crystal structure analysis of renin–inhibitor complexes and computational methods [Wood et al., 2003]. The therapy was introduced under the names Takturna and Rasilez.
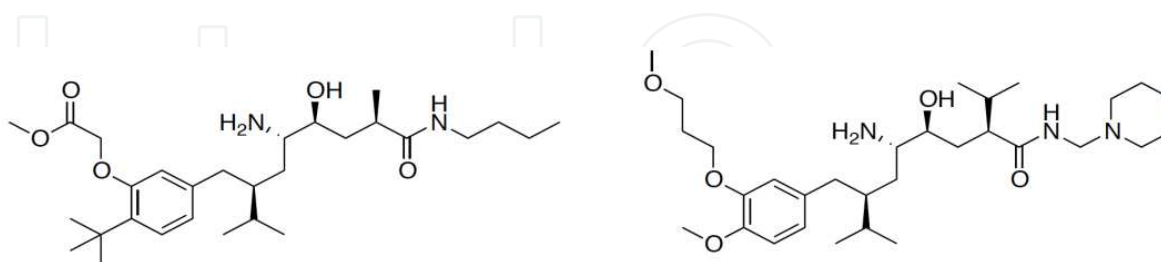
Figure 15. Aliskiren (left hand side) and its more potent analog.

Another possibility arose from use of fluoroketone derivatives. α-monofluoroketones are approximately 50% hydrated, whereas the α,α-difluoroketones are 100% hydrated in aqueous solutions (Fig. 16). The latter ones are obviously of choice because of their striking similarity to phosphinic inhibitors (two hydroxyls placed at terahedral atom). This approach is applied very rarely but gave good inhibitors of fungal endothiapepsin [Tuan et al., 2007] and matrix metalloprotease [Reiter et al., 2000] (Fig. 16).
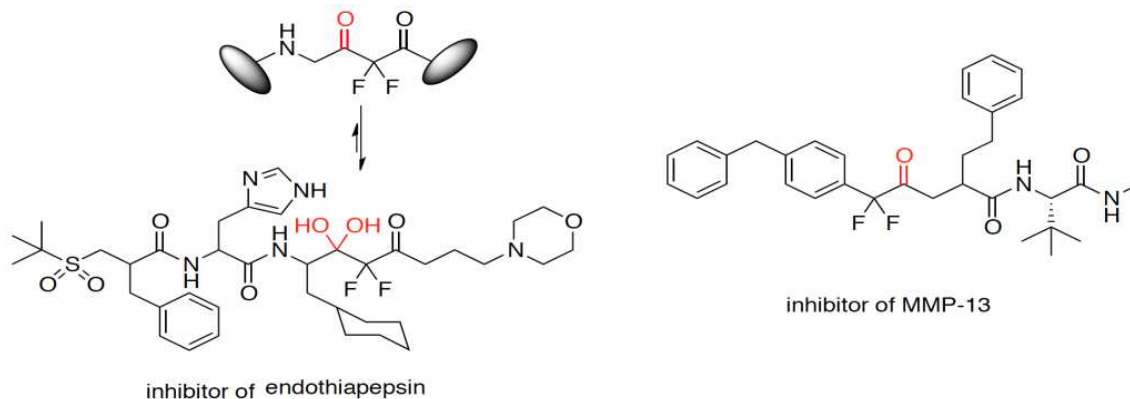
inhibitor of endothiapepsin

inhibitor of MMP-13

Figure 16. Difluoroketones as transition state inhibitor.

6. Suicide substrates yielding transition state analogues

Peptide aldehydes and boronic acids are inhibitors of serine and threonine enzymes forming both, hydrogen and covalent bonds in the enzyme active site. Tetrahedral adduct generated from these compounds upon their action on enzymes bear a closer relationship to the structure of the true intermediate and they may be considered as suicidal substrates. There is,

however, an important difference between these two types of inhibitors. The boronic acid derivative possesses a negative charge, whereas the hemiacetal adduct is neutral (Fig 17).
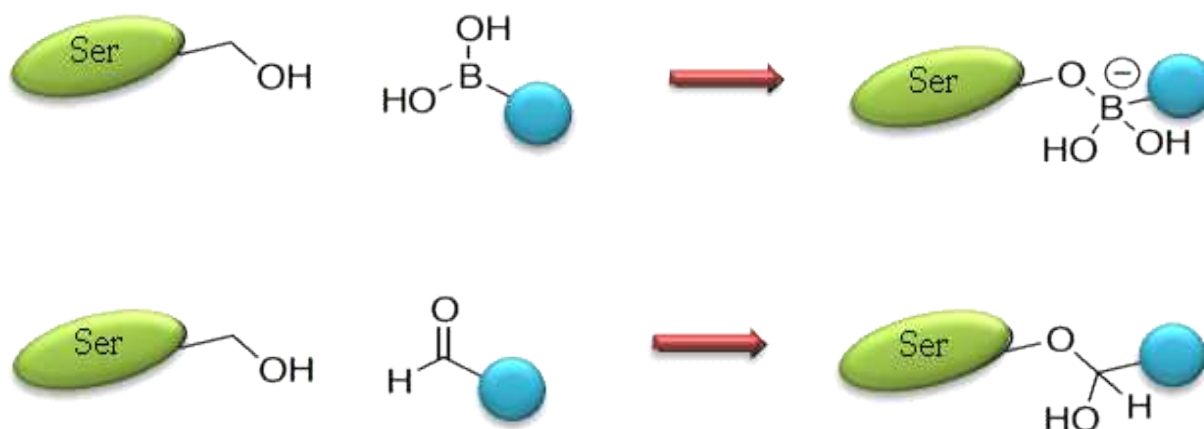

Figure 17. Suicidal substrates yielding transition state analogs

Hence, the peptidyl boronic acid adduct is a better transition state analog than the hemiacetal formed from peptidic aldehyde [Polgár, 2005]. Aldehydes typically have a low prevalence in drugs and drug candidates because of their potential chemical reactivity and susceptibility to be engaged in a reduction/oxidation pathways in vivo. Therefore peptidyl boronic acids are considered as far better drug candidates. Additionally slight changes in pH can result in release of the inhibitor from the active site, which is profitous.

In 2003, bortezomib (Fig. 18), a first-in-class therapeutic, gained approval from the US Federal Drug Administration for the treatment of relapsed multiple myeloma and mantle cell lymphoma. Approval in the UK, for multiple myeloma, followed in 2006. It possesses a unique mode of action. Bortezomib acts as inhibitor of the 26S proteasome, the key regulator of intracellular protein degradation, found in the nucleus and cytosol of all eukaryotic cells, and forming part of the critical ubiquitin-proteasome system. This inhibition results in disruption of homeostatic mechanisms within the cell that can lead to cell death.
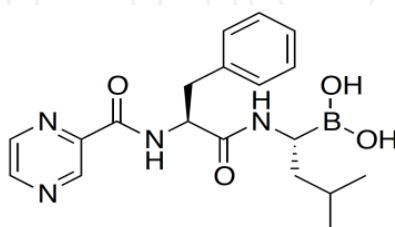

Figure 18. Structure of bortezomib.

This finding initiated intensive researches on boronic inhibitors of serine and threonine proteases [Trippier & McGuigan, 2010]. For, example recently inhibitors of Lon proteases (bacterial ATP-dependent protease conferring bacterial virulence) afforded interesting

antibacterial agents [Frase and Lee, 2007], inhibitors of prostate-specific antigen for prostate cancer imaging and therapy [LeBeau et al., 2008], antifungal inhibitors of kexin (regulatory proteins from *Candida*) [Holyoak et al., 2004; Wheatley & Holyoak, 2007], inhibitor of HCV NS3 protease as potential drug against hepatitis [Zhang et al., 2003; Venkatraman et al., 2009], and anticancer and antibacterial inhibitors of proteasome [Hu et al., 2006]. Representative examples of these inhibitors are shown in Figure 19.
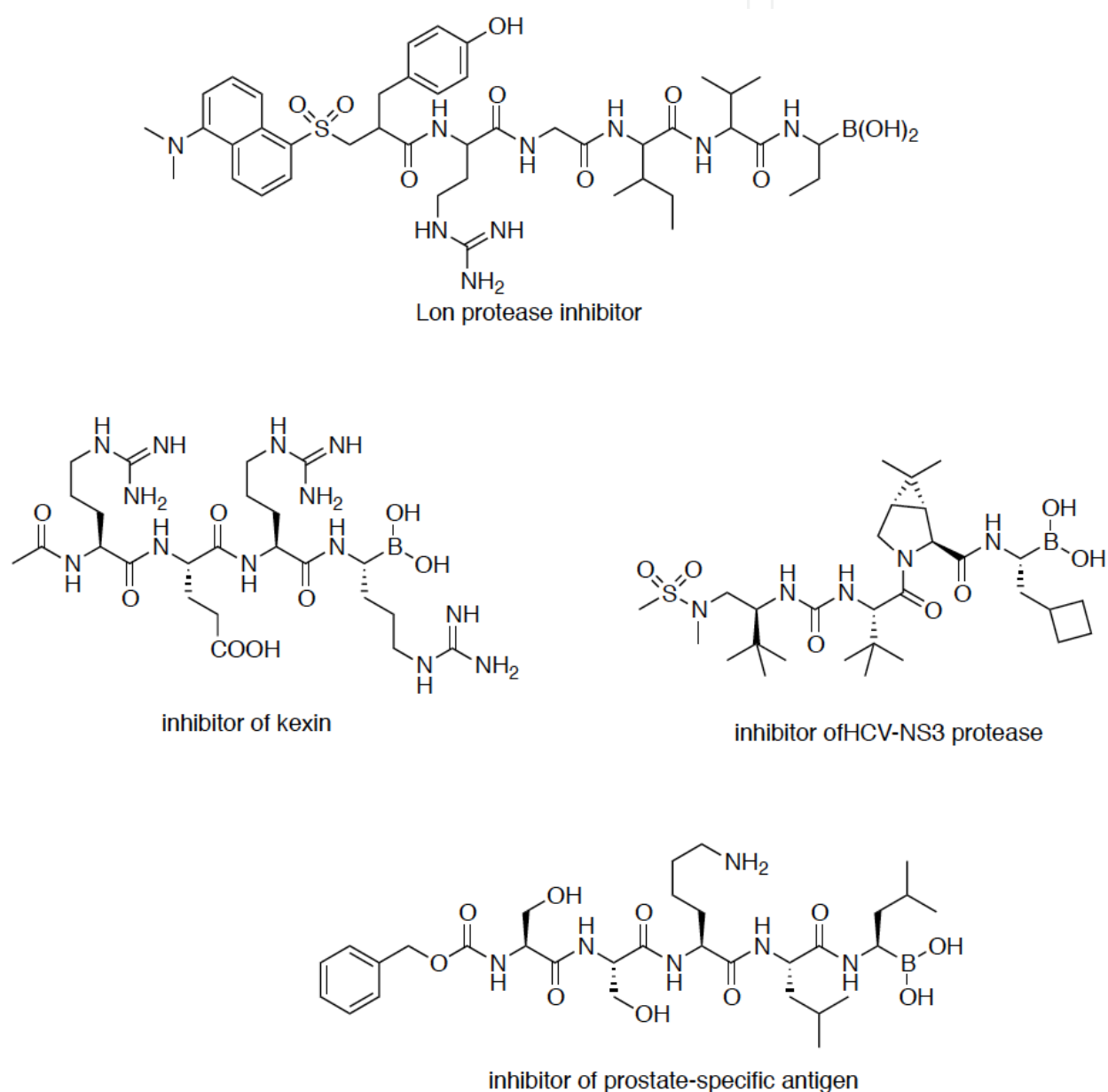

Figure 19. Boronic acid based inhibitors.

7. Other hydrolases

The data considering transition state analogue inhibitors of other hydrolases are practically limited to inhibitors of β–lactamases, arginase and urease.

Antibiotic resistance, especially to widely prescribed β-lactam antibiotics, is a serious threat to public health and is responsible for the increase in morbidity, mortality, and health care costs related to the treatment of bacterial infections. In most cases emergence of antibiotic-resistant bacteria is primarily driven by overuse of β-lactam antibiotics in food and agricultural products. The most prominent resistance mechanism is related to the expression of β-lactamases, which hydrolyze β–lactam fragment of the drug molecule. In nature, four classes of these enzymes exist. Three of them are serine-based, whereas fourth is zinc-dependent-hydrolase. To counteract β-lactamases, mechanism-based inhibitors were developed to be administered in concert with β-lactam antibiotics. Presently, there are three commercially available β-lactamase inhibitors (clavulanate, sulbactam, and tazobactam). The new approach to obtain such inhibitors is combination of structure of potent β-lactam antibiotics with a boronic [Thomson, et al., 2007; Eidam et al., 2010: Ke, et al., 2011; Chan, et al., 2012] or phosphonic [Nukaga, et al., 2004] acid moieties with the goal of mimicking the transition state and creating a high-affinity, reversible inhibitor that cannot be inactivated by β-lactamases since they do not bear hydrolyzable β-lactam ring.

analog of Ceftazidime
(Ke et al., 2011)

analog of Cefoperazone
(Ke et al., 2011)

(Eidam et al., 2010)
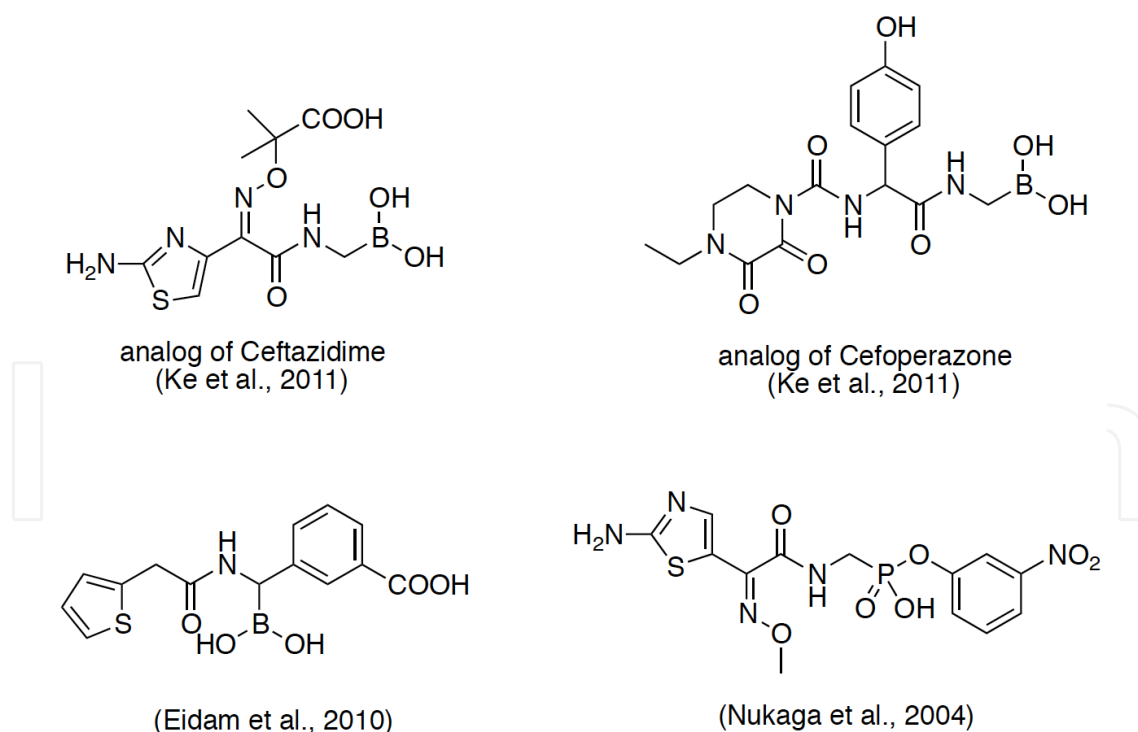
(Nukaga et al., 2004)

Figure 20. Transition state inhibitors of lactamases.

Arginase is a binuclear manganese metalloenzyme that serves as a therapeutic target for the treatment of asthma, erectile dysfunction, and atherosclerosis. The hydrolysis of *L*-arginine

to *L*-ornithine and urea (Fig. 21) is also the final cytosolic step of the urea cycle in mammalian liver. *S*-(2-Boronoethyl)-*L*-cysteine is one of the most effective inhibitor of the enzyme (Fig 21). The specificity determinants of amino acid recognition by arginase were identified by X-Ray structure of human arginase I enzyme complexed with this inhibitor. These studies undoubtedly shown that boronate adopts tetrahedral configuration [Cama et al., 2003 & 2003a; Shishova, et al., 2009]. Also aldehydes and sulfonamides similar to boronic acids appeared to be promising inhibitors of arginases [Shin et al., 2004].
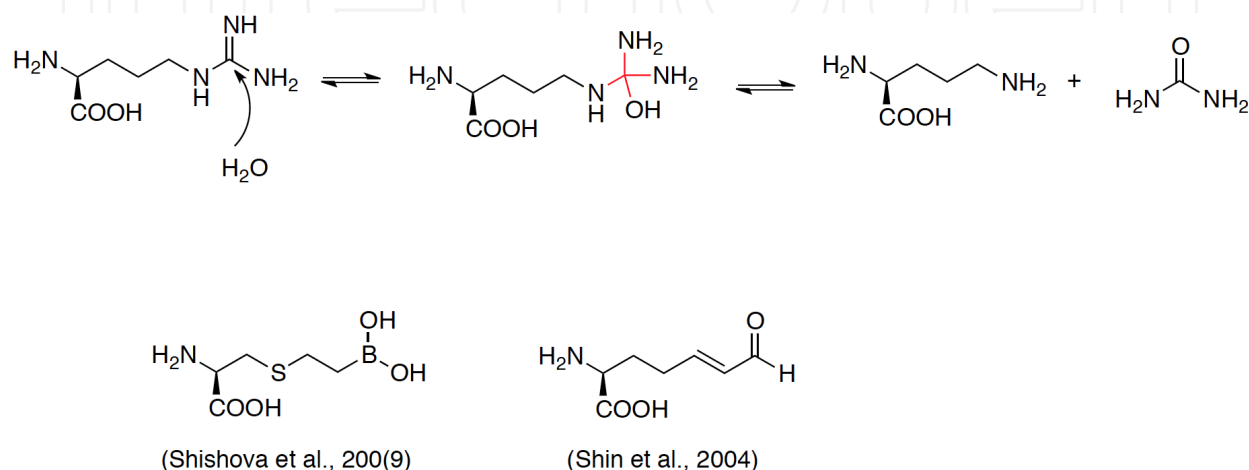

(Shishova et al., 200(9))   (Shin et al., 2004)

Figure 21. Arginase catalyzed reaction and representative inhibitors of the enzyme.

Urease catalyzes hydrolysis of urea in the last step of organic nitrogen mineralization to give ammonia and carbamate, which decomposes to give a second molecule of ammonia and bicarbonate (Fig. 22). The hydrolysis of the reaction products induces an overall pH increase that has negative implications both in human and animal health as well as in the ecosphere. Urease is a virulence factor in infections of urinary (*Proteus mirabilis, Ureaplasma urealyticum*) and gastrointestinal tracts (*Helicobacter pylori*), causing severe diseases such as peptic ulcers, stomach cancer, and formation of urinary stones. The efficiency of soil nitrogen fertilization with urea (the most used fertilizer worldwide) decreases due to ammonia volatilization and root damage caused by soil pH increase. Thus, control of the activity of urease through the use of inhibitors could counteract these negative effects [Kosikowska & Berlicki, 2011; Zambelli et al., 2011]. Di- and triamides of phosphoric acid represent a group of urease inhibitors with the highest activity. It is the direct consequence of their similarity to the tetrahedral transition state of the enzymatic reaction of urea hydrolysis. Takeda Chemicals have patented a large group of N-acyltriamido phosphates and found over 90 examples with nanomolar activity against *H. pylori* urease, with flurofamide being the most effective (Fig. 22) [Kosikowska & Berlicki, 2011].

Recently design, synthesis, and evaluation of novel ogranophosphonate inhibitors of bacterial urease have been described as an attractive alternative to known phosphoramidates. On the basis of the crystal structure of *Bacillus pasteurii* urease, several phosphinic acids and their short peptides have been designed by using the computer-aided techniques. The step-

wise scheme of inhibitor design, shown in Figure 21, led to the synthesis of compounds with low structural complexity, high hydrolytic stability and satisfactory biological activity against various ureases, including cytoplasmic urease from pathogenic *Proteus* species [Vassiliou et al., 2008; Berlicki et al., 2012; Vassiliou et al., 2012].

8. Peptide bond formation by ribosome

Ribosomes are molecular machines that synthesize proteins in the cell. Recent biochemical analyses and high-resolution crystal structures of the bacterial ribosome have shown that the active site for the formation of peptide bonds – the peptidyl-transferase center – is composed solely of rRNA. Thus, the ribosome is the largest known RNA catalyst and the only natural ribozyme that has a synthetic activity. Peptide bond formation during ribosomal protein synthesis involves an aminolysis reaction between the aminoacyl α-amino group and the carbonyl ester of the growing peptide via a transition state with a developing negative charge - the oxyanion. Therefore the observed intermediates and transition states are similar to those observed in proteinases (Fig. 23). Structural and molecular dynamic studies have suggested that the ribosome may stabilize the oxyanion in the transition state of peptide bond formation via a highly ordered water molecule [Rodnina et al., 2006; Carrasco et al., 2011].
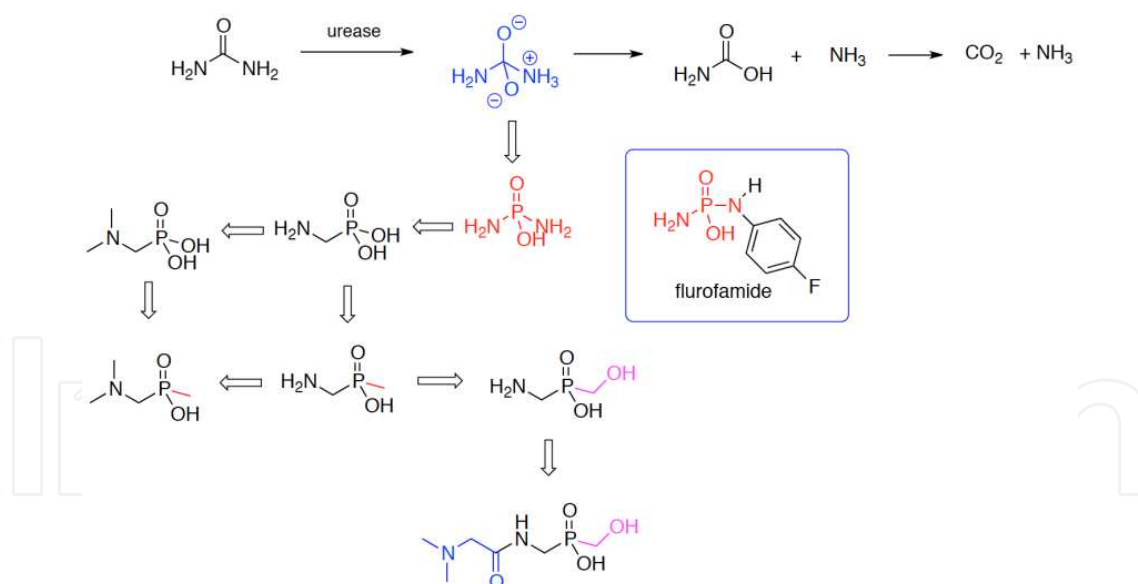
Figure 22. Urease hydrolysed reaction and evolution of the structure of its inhibitors.

To biochemically elucidate how the ribosome stabilizes the developing negative charge in the transition state of peptide bond formation, a series of tetrahedral transition state mimics were synthesized. Their relative binding affinities for the ribosomes also were measured (Fig. 23). The obtained results confirmed high affinity of predicted mechanism of ribosome action [Green & Lorsch, 2002; Weinger et al., 2004; Carrasco, et al., 2011].

Figure 23. Transition state analog inhibitor of peptidyl transferase.
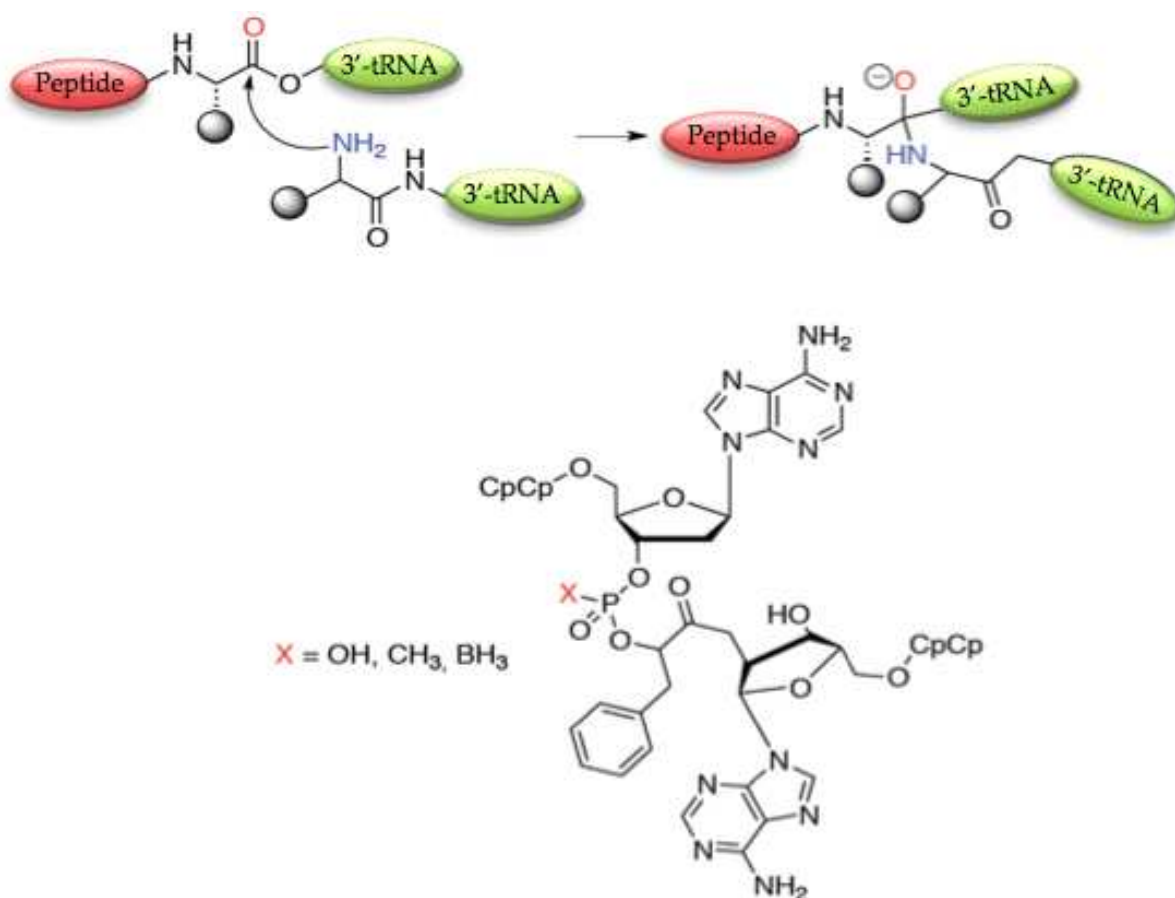
9. Enzymes forming amide and ester bonds via carboxylic-phosphate anhydride

Activation of carboxylic group of amino acid by ATP-phosphorylation yielding mixed carboxylic-phosphate anhydride is quite popular mechanism of synthesis of amide and ester bonds.

D-Alanyl-*D*-alanine ligase is one of the key enzymes in peptidoglycan biosynthesis and is an important target for antibacterial drugs. The enzyme catalyzes the condensation of two alanine molecules using ATP to produce *D*-Ala-*D*-Ala (Fig. 24), which is the terminal peptide of a peptidoglycan monomer. Analogs of *D*-Ala-*D*-Ala, in which phosphonate or phosphinate moiety replaces peptide bond appear to be potent inhibitors. As determined by kinetic [Ellsworth et al., 1996], X-Ray [Wu et al., 2008], and molecular modeling studies the inhibitor behaves as substrate and reacts with ATP to produce ADP and a tight-binding phosphorylated transition state analogue, which exerts inhibitory action against the enzyme (Fig. 24). Thus, these compounds might be rather considered as suicide substrates.
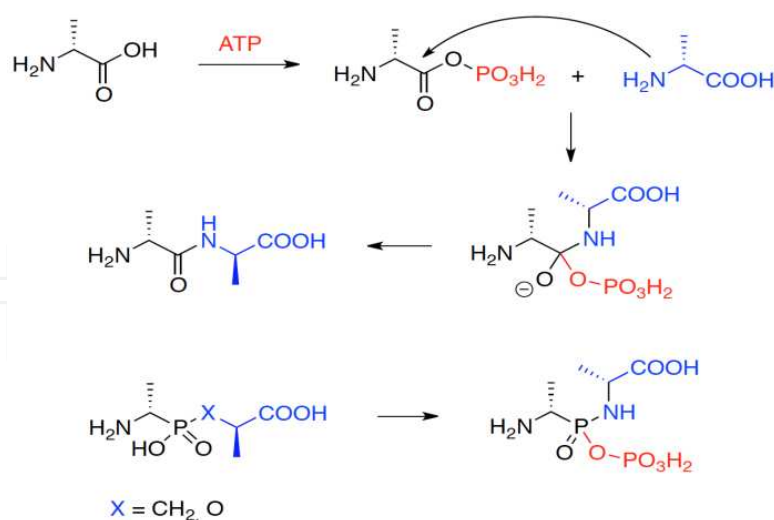
Figure 24. Mechanism of condensation of two molecules of D-Ala catalyzed by *D*-alanine-*D*-alanine ligase.

Similarly acting inhibitors have been found for glutamine synthetase (phosphinothricin and methionine sulfoximine and their analogs) [Berlicki et al., 2005; Berlicki & Kafarski 2006; Berlicki, 2008], γ-glutamylcysteine synthetase [Hibi et al., 2004], or penicillin binding proteins [Dzekieva et al., 2010; Dzekieva et al., 2012] (Fig. 25).

phosphinothricin

methionine sulfoximine

inhibitor of penicillin binding protein

inhibitor of glutamylcysteine syntetase
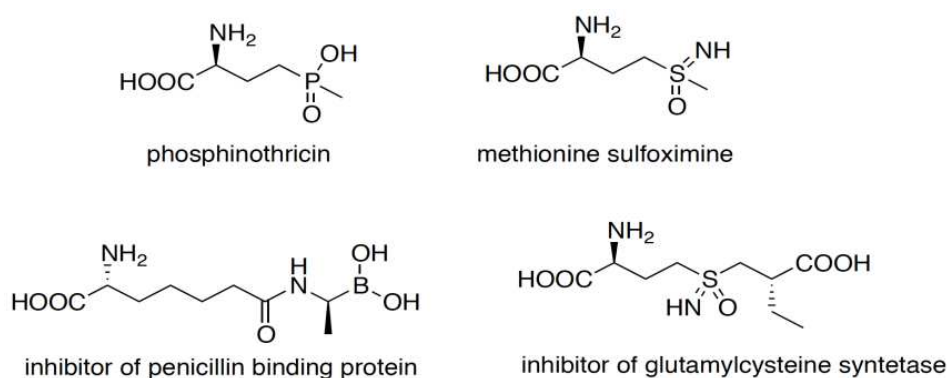
Figure 25. Inhibitors activated by ATP.

10. Nucleotide deaminases

Enzymes of the deaminase superfamily catalyze deamination of bases in nucleotides and nucleic acids across in diverse biological contexts. Representatives that act on free nucleotides or bases are primarily involved in the salvage of pyrimidines and purines, or in their catabolism in bacteria, eukaryotes and phages. Other members of the deaminase superfamily catalyze the *in situ* deamination of bases in both RNA and DNA. Such modifications play a central role in RNA editing, which is critical for generating the appropriate anti-codon sequences for decoding the genetic code, modification of the sequences of microRNA and oth-

er transcripts and alteration of the reading frames in mRNAs, defense against viruses via hypermutation-based inactivation, and somatic hypermutation or class switching of antigen receptor genes in vertebrates [Iyer et al., 2011].

Adenosine deaminase (ADA) is an enzyme present in all organisms and catalyzes the irreversible deamination of adenosine and deoxyadenosine to inosine and deoxyinosine (Fig. 26). Both adenosine and deoxyadenosine are biologically active purines that can have a deep impact on cellular physiology. For example it plays a vital role in regulating T-cell coactivation. Deficiency of this enzyme in humans causes severe combined immunodeficiency. Increased serum activity of this enzyme have been found in many infectious diseases caused by microorganisms infecting the macrophages, in leprosy, brucellosis, HIV infections, viral hepatitis, infectious mononucleosis, liver cirrhosis and tuberculosis. Its extended transition state inhibitor – conformycin was isolated from *Nocardia interforma* and *Streptomyces kaniharaensis*. Analogs of conformycin (Fig. 26) are proposed as an antineoplastic synergists and immunosuppressants [Wolfenden, 2003].

The wide potential of these inhibitors may be illustrated by the fact that deaminoformycin was recently applied to evaluate mechanisms responsible for lethality caused by genetic and herbicide-based activity of adenosine deaminase [Sabina et al., 2007], as well as identification of highly selective inhibitor of purine salvage pathway in malaria parasites [Tyler et al., 2007]. This is because of a unique feature of *Plasmodium falciparum* enzyme that catalyzes the deamination of both adenosine and 5'-methylthioadenosine.
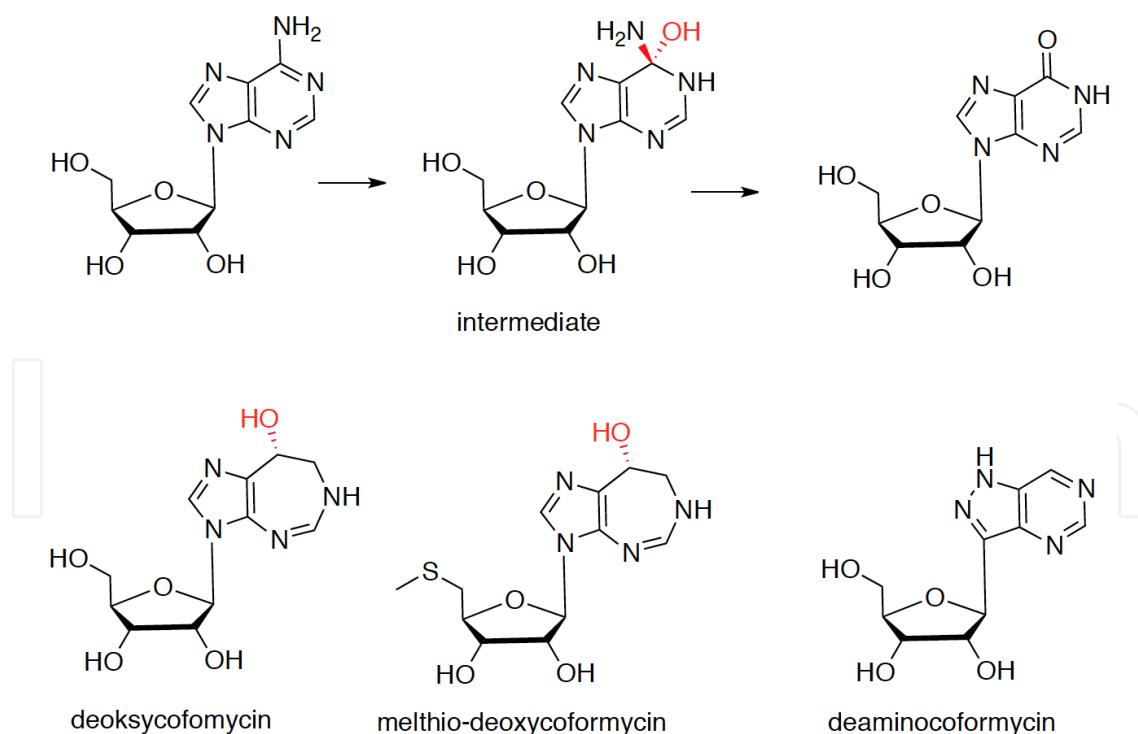

Figure 26. Inhibitors of adenosine deaminase.

Guanine deaminase is an enzyme that hydrolyzes guanine to form xanthine that is unsuitable for DNA/RNA buildup. This enzyme has been found in normal or transformed human

organs and sera. One of the approaches for antiviral/anticancer therapy is to design structural mimics of natural guanine as nucleic acid building blocks, with an anticipation that such analogs would be incorporated into DNA/RNA of virus for cancer cells, interrupting their normal replicative processes. Unfortunately these potent anticancer mimics are believed to be substrates for the enzyme guanine deaminase, which converts them into their respective inactive forms. A potent inhibitor would restore the original potency of these anticancer compounds. Such an activity was determined for azepinomycin [Isshiki et al., 1987] and its analog designed as transition state of this reaction (Fig. 27) [Chakraborty et al., 2011].
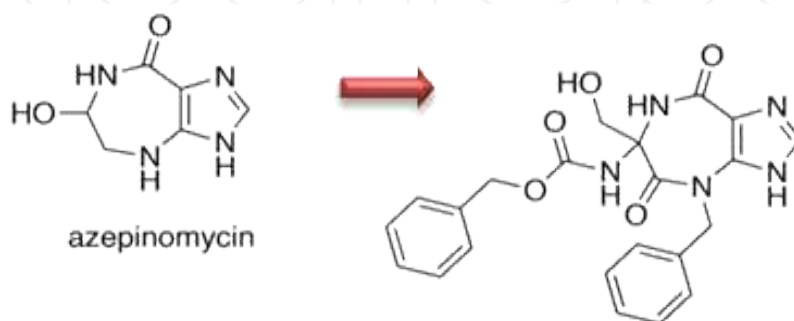

Figure 27. Apizenomycin as a template for guanine deaminase inhibitor.

11. Glycosidases and related enzymes

Glycoside hydrolases, the enzymes catalyzing hydrolysis of the glycosidic bond in di-, oligo- and polysaccharides, and glycoconjugates, are ubiquitous in Nature and fundamental to existence. The extreme stability of the glycosidic bond caused that they have evolved into highly proficient catalysts, with an estimated 10^{17} fold rate enhancement over the uncatalysed reaction. Such rate enhancements mean that enzymes bind the substrate at the transition state with extraordinary affinity [Gloster & Davies, 2010].

In the most cases of glycoside hydrolysis, the short-lived transition state possesses substantial oxocarbenium character (Fig. 27) resembling classical S_N1 reaction intermediate. Under these conditions the anomeric carbon possesses trigonal character, which causes sp^2 hybridisation predominantly along the bond between the anomeric carbon and endocyclic oxygen and significant relative positive charge accumulation on the pyranose ring [Lee et al., 2004; Biarnés et al., 2011; Davies at al., 2012].

The quest for potent and selective inhibitors of glycosidases is extremely active at present. This results from the involvement of glycosidases in lysosomal storage disorders, cancer, viral infections, diabetes and many others. Consequently a plethora of glycosidase inhibitors have been already synthesized and evaluated. The number of them is continually growing. It is outside the scope of this chapter to mention all of them in detail. One of the most appealing ways to design a transition state analog would be to incorporate both the features of

geometry and charge present at the transition state. Distortion of the ring to generate compounds which may resemble the geometry of the transition state can be done by introducing a double bond in the pseudo-glycoside ring itself, whereas introduction of the charge might be done by application of sulfonium or ammonium ions [Rempel & Withers, 2008; Gloster & Davies, 2010; Sumida et al., 2012].
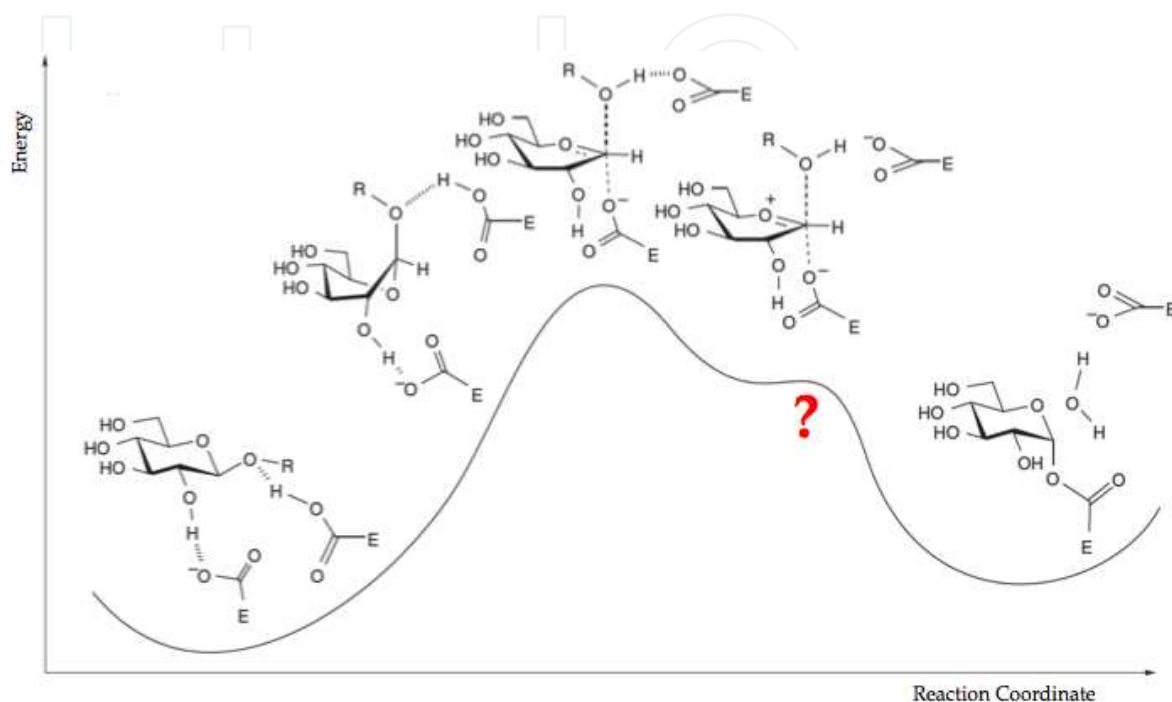
Figure 28. Mechanism of β-glucosidase action [after Vasella et al., 2002].

Some representatives, which fulfills these requirements are: salicinol, one of the active principles in the aqueous extracts of *Salacia reticulata* that is traditionally used in Sri Lanka and India for the treatment of diabetes [Ghavami et al., 2001] and its structurally variable analogues [Liu et al., 2006; Bhat et al., 2007; Mohan & Pinto, 2008].

Figure 29. Salicinol and its analogs.

Sialic acids play an important role in a variety of biological processes. They are usually attached to the terminal positions of glycoproteins, glycolipids and oligosaccharides. From more than 100 different sialic acids, *N*-acetylneuraminic acid (NeuAc) is the most abundant one. Sialidases or neuraminidases are a family of exo-glycoside hydrolases that catalyze the

cleavage of terminal sialic acid residues from sialylated oligosaccharides, glycoproteins, and glycolipids. Aberrant expression of different human sialidases was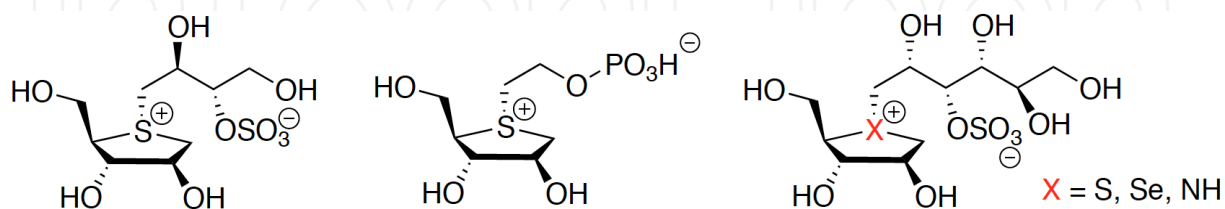 found to associate with various pathological conditions, including lysosomal storage diseases such as sialidosis and galactosialidosis. Non-specific transition-state analog of sialidase, 2-deoxy-2,3-dehydro-N -acetylneuraminic acid (DANA, Fig. 30) is a good starting point for the synthesis of specific inhibitors of human enzymes [Streicher & Busse, 2006; Li et al., 2011].
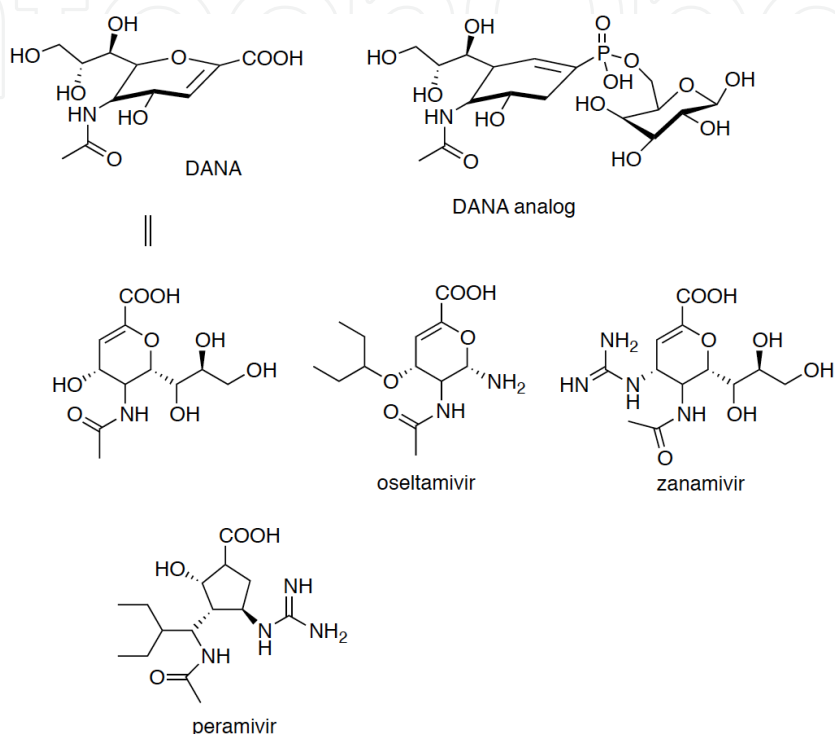

Figure 30. Chemical structures of neuraminidaze inhibitors.

Influenza viruses, in particular those of type A that can infect animals and humans, continue to represent a major threat to public health and animal health worldwide. The social and economic burden associated with a pandemic is substantial. Two viral surface glycoproteins, the sialoside-hydrolysing neuraminidase and the sialic acid-binding hemagglutinin, have become important targets for such approach. Most likely, the function of flu virus neuraminidase is to remove sialic acid receptors for the virus from the host cells, and also, perhaps more importantly, from the newly formed virus particles themselves [Nelson & Holmes, 2007; Medina & Garcia-Sastre, 2011]. Three inhibitors of neuraminidase have been successfully introduced as anti-influenza drugs, all of them being transition state analog inhibitors. They were designed by systematic reduction of DANA structure using crystallographic data and computer-aided methods [Wei, et al., 2006]. Relenza (zanamivir) was the first inhibitor to be synthesized which specifically inhibited neuraminidases of both Type A and Type B influenza viruses and is effective in controlling influenza infections. In people is given as a powder by oral inhalation [Palese et al., 1974]. Interestingly, it is weaker inhibitor of neuramidase than DANA, however, DANA inhibited influenza virus replication in tissue culture

but failed to prevent disease in flu-infected animals. In order to produce a neuraminidase inhibitor, which was orally bioavailable and which was taken orally in capsules or as a suspension, Tamiflu (oseltavimir) was developed in 1997 [Kim et al.]. Third drug, which has been authorized for the emergency use of treatment of certain hospitalized patients with known or suspected 2009 H1N1 influenza, is permavir [Chand, et al., 2005]. Structures of these drugs are presented in Figure 30.

All three drugs soon became lead structures for the design and preparation of new, presumably more effective ones. Syntheses and eveluation of phosphinic analogs and significantly simplified analogs of permavir (Fig. 31) have been recently described [Kati et al., 2001; Bianco et al., 2005; Shie et al., 2007; Udommaneethanakit et al., 2009].
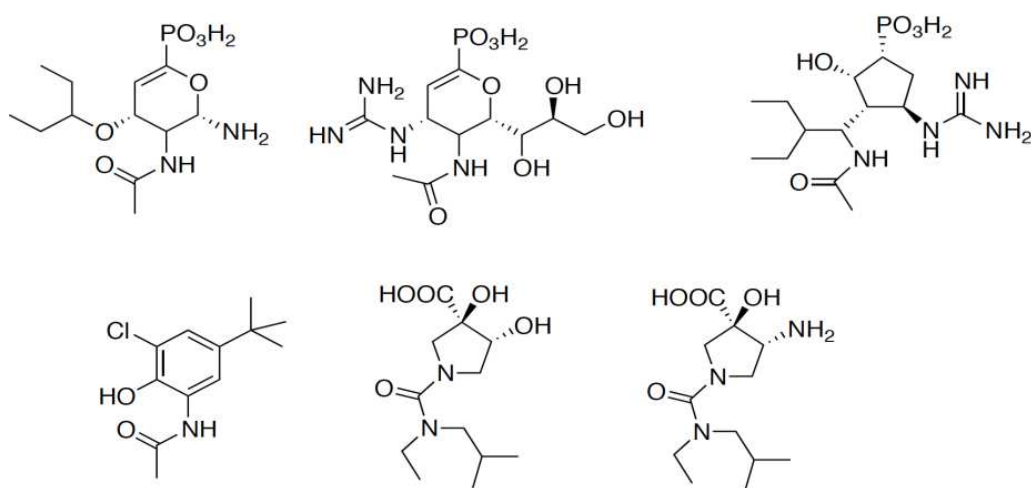
Figure 31. Second generation of influenza neuraminidase inhibitors.

Modified phosphonic analogs of oseltamivir were used to functionalize gold nanoparticles and were found to bind strongly and selectively to all seasonal and pandemic influenza virus strains, and thus could serve as prototypes for novel virus sensors. This may be helpul in fast influenza diagnosis [Stanley et al., 2012].

12. Transition state analogues of nucleic acids metabolism

N-Ribosyltransferases are a general class of enzymes that catalyze nucleophilic displacement reactions by migration of the cationic ribooxacarbenium carbon from the fixed purine to phosphate and water nucleophiles, respectively. Two major classes of these enzymes are hydrolases and phosphorylases. Hydrolases, which release the heterocycle to generate a free sugar ribosyl unit, include enzymes for DNA repair, RNA depurinations by plant toxins, and purine and pyrimidine nucleoside and nucleotide metabolism. Phosphorylases, which transfer ribosyl groups to phosphate, are also involved in the pathway for nucleoside salvage. Genetic defects in this pathway prevent normal purine catabolism in humans.

The focus on transition states for a family of *N*-ribosyltransferases roots from physiologic importance of these enzymes. Similarly as in the case of glycosidases, most sugar transferases form transition states with cationic charge at the anomeric carbon. The geometry is altered at this center from sp^3 (tetrahedral geometry) in the reactant sugar to sp^2 (trigonal planargeometry) at the transition state (Fig. 32) [Schramm, 2002; Murkin et al., 2007; Silva et al., 2011].
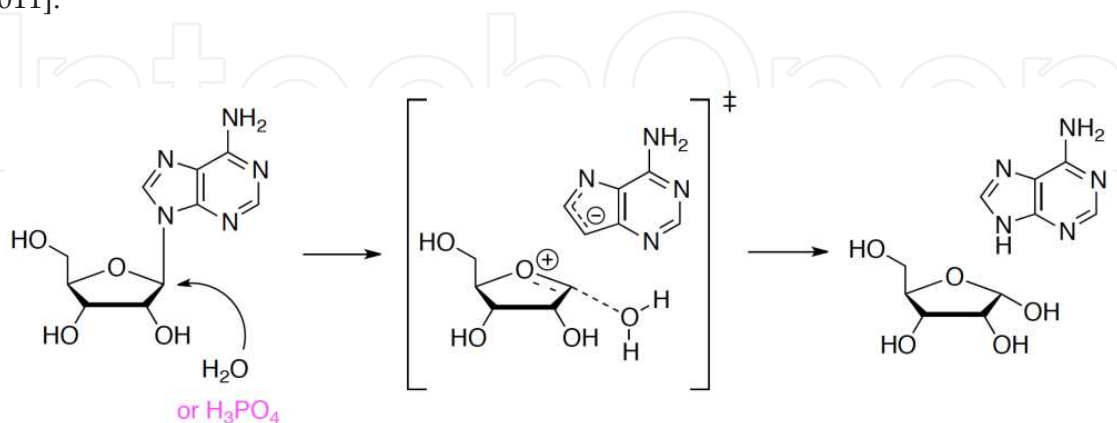

Figure 32. Course of reaction catalyzed by *N*-ribosyltransferases.

Newborns with a genetic deficiency of purine nucleoside phosphorylase are normal, but exhibit a specific T-cell immunodeficiency during the first years of development. All other cell and organ systems remain functional. Human purine nucleoside phosphorylase degrades deoxyguanosine, and apoptosis of T-cells occurs as a consequence of the accumulation of deoxyguanosine in the circulation. Thus, control of T-cell proliferation is desirable in T-cell cancers, autoimmune diseases, and tissue transplant rejection. The search for powerful inhibitors of these enzymes as anti-T-cell agents has culminated in the discovery of immucillins. The atomic replacements between inosine and immunocilin H make an insignificant change in atomic size, but a dramatic change in the molecular electrostatic potential surface (Fig. 33). Thus, analysis of the molecular electrostatic potential surface similarity between transition state and immucilin confirmed utility of this simple approach in helping to design effective inhibitor [Schramm, 2002; Schramm, 2007].

Evolution of immucilin structure, performed using standard structural analogy techniques, enabled to obtain new inhibitors of purine nucleotide phosphorylase of nano- to picomolar affinities to the enzyme (Fig. 34) [Evans et al., 2008; Edwards et al., 2009; Ho et al. 2010; Rejman et al., 2012].

Plasmodium parasites (causative agents of malaria) are purine auxotrophs and require preformed purine bases for synthesis of nucleotides, cofactors, and nucleic acids. The purine phosphoribosyltransferases catalyze transfer the 5-phosphoribosyl group from 5-phospho-α-D-ribofuranosyl-1-pyrophosphate to salvage hypoxanthine, guanine, or xanthine to form intracellular nucleosides. Purine salvage in *Plasmodium falciparum* uses hypoxanthine formed in erythrocytes or in parasites by the sequential actions of adenosine deaminase and purine nucleoside phosphorylase
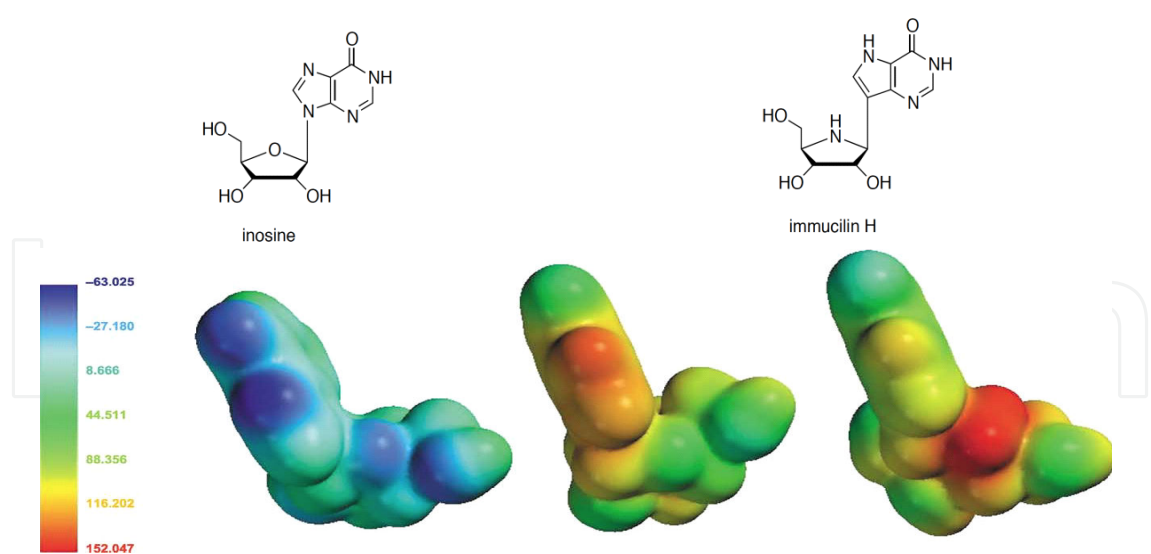
Figure 33. Molecular electrostatic potential surfaces for inosine, the transition state of purine nucleoside phosphorylase and immucilin H.
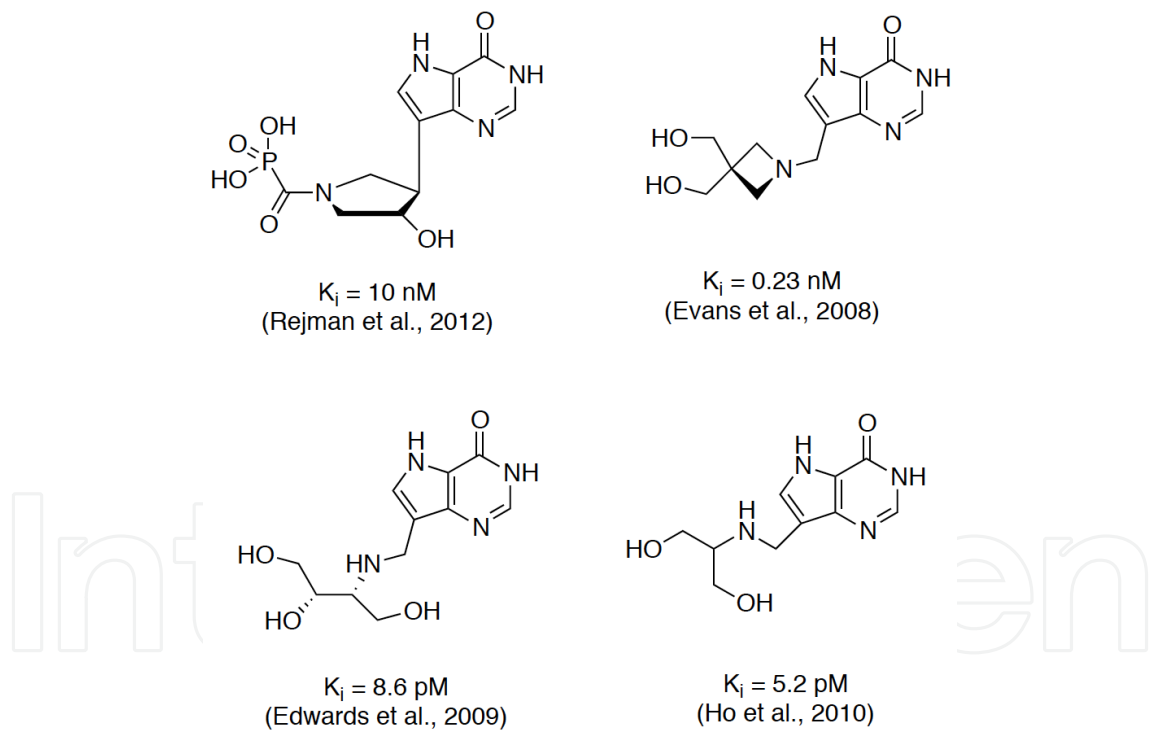
Figure 34. Effective inhibitors of purine nucleotide phosphorylase.

Therefore, effective inhibitors of both enzymes influence the life cycle of P. falciparum and these pathways have been targets for antimalarials since the discovery that *Plasmodium* parasites are purine auxotrophs.

Immunocillins HP and GP [Shi et al., 1999] and BCX4945 [Cassera et al., 2011] (Fig. 35) appear to be effective inhibitors of phosphoribosyltransferases and are also able to influence

purine nucleoside phosphorylase, being dual inhibitor of the process. Especially the efficacy, oral availability, chemical stability, unique mechanism of action and low toxicity of BCX4945 demonstrate potential for combination therapies with this novel antimalarial agent. Similar studies have been also carried out for acyclic nucleoside phosphonates [Keough et al., 2009].
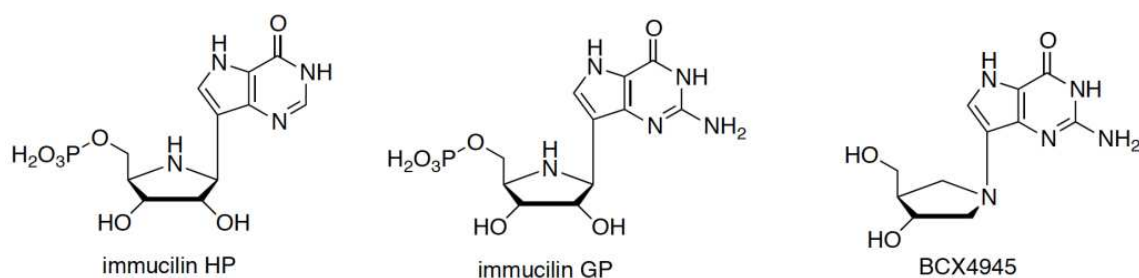
Figure 35. Antimalarial agents of dual inhibitory action.

Acknowledgements

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