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Metallo-aminopeptidase inhibitors

Artur Mucha\textsuperscript{a,**}, Marcin Drag\textsuperscript{b}, John P. Dalton\textsuperscript{c,*}, Paweł Kafarski\textsuperscript{a}

\textsuperscript{a} Department of Bioorganic Chemistry, Faculty of Chemistry, Wrocław University of Technology, Wybrzeże Wyspianskiego 27, 50-370 Wrocław, Poland

\textsuperscript{b} Department of Medicinal Chemistry and Microbiology, Faculty of Chemistry, Wrocław University of Technology, Wybrzeże Wyspianskiego 27, 50-370 Wrocław, Poland

\textsuperscript{c} Institute of Parasitology, McGill University, 2111 Lakeshore Road, Sainte Anne de Bellevue, QC H9X 3V9, Canada

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\begin{abstract}
Aminopeptidases are enzymes that selectively hydrolyze an amino acid residue from the N-terminus of proteins and peptides. They are important for the proper functioning of prokaryotic and eukaryotic cells, but very often are central players in the devastating human diseases like cancer, malaria and diabetes. The largest aminopeptidase group include enzymes containing metal ion(s) in their active centers, which often determines the type of inhibitors that are the most suitable for them. Effective ligands mostly bind in a non-covalent mode by forming complexes with the metal ion(s). Here, we present several approaches for the design of inhibitors for metallo-aminopeptidases. The optimized structures should be considered as potential leads in the drug discovery process against endogenous and infectious diseases.
\end{abstract}

\begin{keywords}
Aminopeptidase
Inhibitor
Non-covalent ligand
Drug design
Cancer
Malaria
Drugs
Diseases
Enzymes
\end{keywords}

1. Introduction

Amino-terminal modifications of nascent polypeptides are the most common processing events, occurring on nearly all proteins. Aminopeptidases are a class of enzymes that play a pivotal role in this processing. Aminopeptidases (EC 3.4.11 – hydrolases/peptidases/aminopeptidases, according to the classification of the International Union of Biochemistry and Molecular Biology) are proteolytic enzymes that hydrolyze peptide bonds from the amino termini of polypeptide chains. They may hydrolyze the first peptide bond in a polypeptide chain with the release of a single amino acid residue (aminopeptidases in a strict sense) or may remove dipeptides or tripeptides (dipeptidyl- and tripeptidyl-peptidases) from polypeptide substrates. Regarding catalytic mechanism, most of the aminopeptidases are metallo-enzymes but cysteine and serine peptidases are also included in this group. This review focuses on the strict metallo-aminopeptidases because they constitute the largest and the most homogenous class of these enzymes and use one or two metal ions in their active sites to specifically release the N-terminal amino acid residues of polypeptides and proteins. Since 1990 over 5000 papers dealing with aminopeptidases have been published (Medline database).

Aminopeptidases are ubiquitous enzymes widely distributed throughout the biological kingdoms and are found in many subcellular organelles, in cytoplasm, and as membrane components where they perform essential cellular functions. Aminopeptidases act in concert with other peptidases to complete diverse proteolytic pathways. They play a vital role in a range of biological processes and disease situations. Processes as distinct as angiogenesis, antigen presentation, neuropetide and hormone processing, pregnancy and reproduction, protein turnover, memory, inflammation, tumor growth, cancer and metastasis, blood pressure and hypertension all involve one or more critical aminopeptidases. These enzymes can efficiently retrieve amino acids from dietary proteins and endogenous proteins degraded during protein turnover, thereby also covering a nutritional role.

In addition to the book of Hooper and Lendeckel [1], which describes role of aminopeptidases in biology and medicine, several excellent reviews on various aspects of their biology and the application of their inhibitors have been published [2–4]. We therefore limit our review to selected recent achievements in this field, although we present this in a certain historical context.

*Corresponding author. Tel./fax: + 1 514 398 8668.
**Corresponding author. Tel.: +48 71 320 3446; fax: +48 71 328 4064.
E-mail addresses: artur.mucha@pwr.wroc.pl (A. Mucha), john.dalton@mcgill.ca (J.P. Dalton).

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doi:10.1016/j.biochi.2010.04.026
2. Classification of metallo-aminopeptidases

The classification of aminopeptidases has often been based on mechanism of catalysis, the structure of the active site, substrate specificity (broad or narrow) and molecular properties. The nomenclature of many peptidases has been determined by their preferences or requirements for a particular N-terminal amino acid. The rapidly accumulating data covering new representatives of proteolytic enzymes required the development of an integrated source of information [5]. Such a role was fulfilled by the MEROPS database (http://merops.sanger.ac.uk/), which uses hierarchical, structure-based classification of these enzymes. This database relies on the fact that enzymes performing the same (or similar) chemical functions in different organisms generally turn out to have similar overall three-dimensional structures, and also show significant conservation of their amino acid sequences, polypeptide chain lengths and domain organization. In particular, in the regions of their active sites, a high degree of conservation of residue identities and structural positions is observed. In the MEROPS peptidase information database each protease is assigned to a certain family on the basis of statistically significant similarities in amino acid sequence, and families that are thought to be homologous are grouped together into clans. Clans consist of families of peptidases that are believed to share a single evolutionary origin, evidenced by similarities in their tertiary structures and/or their active site architectures.

Fifteen clans of metalloproteases have been identified, with metallo-aminopeptidases found in six which are designated as, MA (the largest one, containing over 35 families), MF, MG, MH, MN and MQ. The families in clan MA are united by the presence of an HEXXH motif in which the two His residues are zinc ligands and the Glu has a catalytic function. Clans MF (two zinc ions in the active site), MG (with the pita-bread fold and containing two cobalt or two manganese ions in their active centers) and MQ (typically with two zinc ions) consists of only one family of peptidases each (M17, M24 and M29, respectively). The MH clan forms the most heterogeneous group and contains a variety of zinc-dependent exopeptidases. Their structures show similar protein folds and are co-catalytic zinc peptidases containing two atoms of zinc per molecule, which have five amino acid ligands. ClanMN contains only one enzyme — D-amino acid–specific aminopeptidase from Bacillus subtilis.

Although metallo-aminopeptidases occur in all types of organisms, the mammalian enzymes were amongst the first proteases discovered in tissues and therefore they have been most intensively studied. Human enzymes are particularly increasing in interest since the alterations in their function and regulation underline many human diseases. For example, leucine aminopeptidases (LAPs, EC 3.4.11.1), belonging to M17 family, have been the most extensively studied because they play a key role in the metabolism of proteins and biologically active peptides. These enzymes, perhaps the first that were isolated, are cytosolic exopeptidases of broad substrate specificity that are ubiquitous in nature being present not only in animals, but also in plants and bacteria [6]. They have medical and biological importance because of their functions in the metabolism of hormones and neurotransmission, cell maturation, and turnover of proteins, including utilization of exogenous proteins as nutrient substances and elimination of nonfunctional proteins. Human LAP is important in processing of antigenic peptides and in determination of immunodominance of various peptides [7–9] as well as in development of human eye lens cataract [10]. The protease plays a vital role in progression of cancers [11]. It may also have an important function in early events of HIV infection and thus serum activity of this enzyme may be useful as a surrogate marker for HIV infection and response to chemotherapy [12].

Another example of medically important enzyme is microsomal aminopeptidase (belonging to M1 family), known also as aminopeptidase N, CD13 or alanyl aminopeptidase (EC 3.4.11.2) [13]. In biological systems, their primary peptide substrates include a wide variety of neuropeptides and hormones [14,15]. It has been established as a myelomonocytic marker in leukemia typing [16], as a receptor for human coronavirus 229E and cytomegalovirus [17,18], as a mediator of both inflammation and cell invasion [16], as a regulator of blood pressure and the pathogenesis of hypertension [19], and as a regulator of analgesia via metabolism of endorphins and enkephalins [20]. Furthermore, it also regulates IL-8 bioavailability in the endometrium and therefore may contribute to the process of angiogenesis [21]. It also plays key roles in physiological and pathological processes, such as embryogenesis, immune responses, angiogenesis, tumor cell invasion, and metastasis [22].

Methionine aminopeptidases (aminopeptidase M, MetAPs, EC 3.4.11.18), belonging to M24 family, are an example of peptidases that exhibit narrow specificity [23]. Generally they are responsible for the removal of methionine from the amino-terminus of newly synthesized proteins. They maintain stringent specificity for the N-terminal methionine and accept no other natural amino acid residues. They also have a strong preference for small and uncharged second residues in peptide chains. Since the mammalian enzymes play a critical role in the regulation of post-translational processing and protein synthesis they play an important role in the development and malignancy of different types of cancer [24–28]. Human aminopeptidase M is also involved in neurofibromatosis, one of the most common tumor predisposition syndromes [29].

Although scarce, there are also reports on aminopeptidase isolation and characterization from other vertebrate species, as exemplified by recent findings in fishes (carp and red sea bream) [30,31] and birds (chicken) [32]. Far more information is known about insect aminopeptidase N, which is one of the membrane proteins identified as a receptor to Cry proteins in various species [33–36]. Cry proteins produced by Bacillus thuringiensis are toxic to insects and thus this strain is exploited commercially as a bioinsecticide. Aminopeptidases involved in the degradation of insect neuropeptides have been also studied in some respects [37].

The other groups of metallo-aminopeptidases explored intensively are of bacterial origin. The first studies on these enzymes were carried out over 40 years ago, and since then a large number of aminopeptidases of microbial origin have been characterized. They may be localized in cytoplasm, on membranes associated with the cell envelope or secreted into the extracellular media [4]. The interest in these enzymes stems from their potential to act as targets to combat bacterial diseases. In this respect, a wide variety of structurally diverse aminopeptidases have been recently isolated and characterized from a range of bacterial species. These include: aminopeptidase P isolated from common strain of Escherichia coli [38], aminopeptidase M from pathogenic Mycobacterium tuberculosis [39] and leucine aminopeptidase from Helicobacter pylori [40], cold-active aminopeptidase from psychrotrophic Colwellia psychrerythraea [41], a thermonphilic enzyme from Geobacillus thermodenaturans [42], an extracellular zinc metalloprotease and putative virulence factor involved in pathogenicity of the fish pathogen Vibrio anguillarum [43], and a lysine–specific enzyme from unusually resistant, hyperthermophilic archaeon Pyrococcus furiosus [44]. This clearly indicates that bacterial aminopeptidases are widely distributed and are of vital importance.

In recent years there has been a considerable interest in aminopeptidases of parasitic protozoans, which cause important diseases in humans, animals and birds. The alanine and leucine aminopeptidases from Plasmodium falciparum, a causative agent of malaria, the most significant parasitic disease of humans, are the most comprehensively studied [45,46]. These aminopeptidases are
promising targets for designing anti-malarial drugs (for a recent review see [47]). Another important enzyme of *Plasmodium* — aspartyl aminopeptidase is being considered as an additional target for drug design [48,49]. Intensive studies on the role and biochemistry of aminopeptidases isolated from other parasitic organisms, including *Legionella pneumophila* (causative agent of Legionnaires’ disease) [50], *Eimeria tenella* (causes hemorrhagic cecal coccidiosis in young poultry) [51], *Babesia gibsoni* (parasite found in red blood cells and transmitted by ticks) [52] and *Microsporidia* (causing diseases in immunosuppressed patients) [53] are ongoing.

Diseases caused also by trematodes, commonly known as blood-flukes, affect hundreds of million people in impoverished areas of Africa, Central and South America and East Asia, with those caused by *Schistosoma* spp. are considered by the World Health Organization as second in importance only to malaria. Leucine aminopeptidase is thought to play a central role in hatching of the miracidium from the schistosome egg and therefore is intensively studied as candidate for drug design [54,55]. Similar motivation has driven studies on the leucine aminopeptidase from *Paragonimus westermani*, a tissue-invading trematode that causes inflammatory lung disease as well as systemic infections including cerebral invasion in carnivorous mammals [56]. Specific parasite aminopeptidases might also be considered as targets for vaccine design, as shown for *Fasciola hepatica* [57], a vector of an important freshwater snail-borne helminthiasis that produces a chronic liver infection of cattle and sheep.

3. The catalytic mechanism of aminopeptidases

Understanding the mechanism of action for each family of metallo-aminopeptidases is of a key importance to rational design of more potent and more specific inhibitors of these enzymes and, consequently, to obtain drugs of improved properties. Therefore, a substantial effort has been made in studying the mode of binding of their substrates and transition state inhibitors in enzymatic binding sites, as well as in the elucidation of the three-dimensional structure of active sites and the detailed mechanisms of catalyzed reactions.

A feature common to all metallo-aminopeptidase active sites is that the metal ion (in most cases zinc) is surrounded by a shell of hydrophilic groups that is embedded within a larger environment of hydrophobic groups. In addition, amino acid side chains serving as ligands usually form hydrogen bond contacts with neighboring residues, perhaps to preorder the metal ion binding site and to decrease its entropic cost of binding. The structures of active sites suggest that a number of reaction paths are possible. Two catalytic roles of metal ions might be considered. First, they might stabilize a highly reactive hydroxide ion, thereby ensuring that an activated nucleophile is available for catalysis at physiological pH (mechanism A in Fig. 1). Second, the positively charged metal ion may serve as an electrophilic catalyst complexing an oxygen atom of the scissile peptide bond to facilitate the nucleophilic attack of a water molecule (mechanism B).

There is also a possibility that the active site glutamate (especially in the case of M17 family of peptidases) acts as a nucleophile resulting in formation of a covalent enzyme—inhibitor complex followed by its fast hydrolysis by water (mechanism C in Fig. 2). The glutamate assisted process is being considered as a less probable mechanism than A and B. In the case of the enzymes containing binuclear metal centers the substrate carbonyl oxygen is coordinated to one of them and a hydroxide ion bridging two metal ions acts as the nucleophilic agent (mechanism D in Fig. 2). This may explain the fact that several dinuclear metallopeptidases retain some catalytic activity when converted into mononuclear proteins.
mechanism.

Finally, in all cases the additional role of a metal ion is to stabilize ones but typically exhibit faster rates with dinuclear active sites. Finally, in all cases the additional role of a metal ion is to stabilize developing negative charge(s) in the transition state of the catalytic mechanism.

There is no single method for the elucidation of the mechanism of certain enzymatic reaction. Thus, a combination of several methods is required. Enzyme kinetics cannot prove which modes of catalysis are used by an enzyme. However, some kinetic data can suggest possibilities to be examined by other techniques. The evaluation of the role of metal ion is usually studied by metal exchange technique. In particular, the role of each metal ion in the dinuclear sites of aminopeptidases from the M17 family towards peptide hydrolysis was studied by kinetic and spectroscopic methods after replacement of one of active site zinc ions by Mn(II), Co(II), Ni(II), Zn(II), and Cd(II) [58–60].

Important data about detailed mechanisms of action of aminopeptidases are also gained by production of altered enzymes by means of site-directed mutagenesis [61–66]. Good examples are the studies on the role of active site glutamic acid in various aminopeptidases of M17 family [66,67]. This was achieved by replacing glutamic acid either by structurally related aspartic acid (possessing an acidic side group), glutamine (lacking an acidic side group), or structurally different alanine, and studies of kinetic properties of the mutated enzyme.

The method that provides the most important insights into domains organization and architecture of active sites of aminopeptidases, and thus into mechanisms of their action, is crystallography. In that respect crystal structures of native enzymes and those complexed with small ligands are extremely useful (for representative recent examples see [68–73]), especially those determined for enzymes bound with inhibitors being considered as transition state analogues [70,72,74–76].

There is also an emerging power in the application of computer-aided methods as a mean to study mechanisms of enzymatic reactions. The calculations enable the researcher to choose one of the several possible reaction pathways (and thus to determine reaction mechanism) and to establish the structures of transition states. These methods are based on the knowledge of enzyme three-dimensional structure available either by crystallographic methods or obtained by computations using homology approach techniques (for representative examples considering metallo-aminopeptidases see [77–80]). Besides the elucidation of the functional roles of active-site residues, an estimation of the environment effects are also possible. In the case of metallo-aminopeptidases such popular techniques such as studies employing modified substrates [81], studies on isotopic effects [67] or construction of the chemical models of enzyme active sites [82] are quite scarce.

4. Inhibitors

Due to their association in several medical disorders, metallo-aminopeptidases are considered important targets for the design of inhibitors, which could potentially enter clinical trials as candidates for drugs. The presence of the metal ion(s) in the active center has determined a general strategy applied for the development of such synthetic ligands. These possess two fundamental structural features, a specific war-head portion dedicated to recognize the active site of the enzymes and specific functional groups that create appropriate complexes with the metal ions. A metal ligand fragment can be incorporated into a backbone containing optimized side chain(s) that are able to interact with the enzyme binding pocket(s). As the result, non-covalent inhibitors of the amino acid/peptide structure (natural substrate, transition state or product analogues) have appeared the most suitable for this purpose. Indeed, the majority of compounds designed to date have such characteristics. Bidentate tetrahedral phosphonates and aldehydes (hydrated in the gem-diolate form), bidentate planar carboxylates and hydroxamates, as well as monodentate thiols, are classical examples.

Recently, a variety of promising heteroaromatic or miscellaneous (heteroaromatic based sulfonamides/carboxylates/amides) inhibitors of metallo-aminopeptidases have been identified from random screening of compound libraries. Their structure usually involve a bidentate N,N, N,O or O,O donor system incorporated into a rigid hydrophobic environment, and thus such compounds also act in a non-covalent mode.

Finally, natural products are a also a source of the appropriate effectors of this group of peptidases, with bestatin being a prototypical representative. Its peptide-like framework offers a choice of heteroatom groups that get involved in an active site metal ion complexation. Other natural non-covalent heteroatom-rich systems are based on terpene or polyphenol scaffolds. Fumagillin and ovalicin are quite unique examples of very specific inhibitors that possess an electrophilic moiety (an epoxide) capable of reacting with the nucleophilic His side chain in the active site.

The proven medicinal importance of aminopeptidase N (APN, CD13), leucine aminopeptidase (LAP) and methionine aminopeptidases (MetAPs) has resulted in the focus on design of inhibitors primarily for these three enzymes and extensive thematic reviews have been recently published [6,83–86]. Here, we present a selection of several recent and historic approaches for design and optimization of the inhibitors of metallo-aminopeptidases, which could serve as lead compounds in the future studies of this group of proteases.

5. Phosphorus containing amino acid and peptide analogues

Among de novo constructed targeted molecules, organophosphorus compounds, namely $\alpha$-aminokane phosphonates (general formula 1, Fig. 3) and phosphorus containing pseudodipeptides (predominantly phosphinic, 2), have probably contributed the most to the inhibition studies on the neutral aminopeptidases: APN (alanyl M1) and LAP (leucine M17). Although the phosphonate/phosphinate group is a rather weak zinc complexing moiety, it offers other advantageous structural and electronic features. Similar to other amino acid and peptide mimetics used as protease inhibitors, this is the effect of the incorporation of a covalent or non-covalent binding group (here involved in coordination of a catalytic metal ion(s) in the enzyme active site) into a substrate structure. For the phosphorus modified compounds it is also uniquely combined with its tetrahedral shape that is considered to mimic the high energy transition state of the peptide bond hydrolysis. Additionally, the P1 side chain of the aminophosphonic acid analogues (or more effectively, both P1 and P1’ residues of the pseudopeptides phosphoryl moiety) gives further possibility of structural optimization of substituents interacting with the S1 and S1’ binding pockets of the enzyme (Fig. 3). Accordingly, $\alpha$-aminophosphonates and their derivatives act as competitive inhibitors of numerous metallo-aminopeptidases, frequently

![Fig. 3. The general structure of $\alpha$-aminokane phosphonate acids (1) and phosphinic pseudodipeptides (2) applied for inhibition of neutral aminopeptidases.](image-url)
displaying slow-binding kinetics. Among them, various amino acid analogues have been developed and tested for inhibition of mammalian (porcine kidney) LAP [87–91] and APN [89–92].

Isosteres of natural hydrophobic aliphatic amino acids, such as valine and leucine (3 and 4, Fig. 4), appeared to be efficient inhibitors of LAP with a $K_i = 0.15$ [90] and 0.23 $\mu$M [87] for the $R$ ($L$) enantiomers. The $D$ ($S$) enantiopes were strongly discriminated, by 2–3 orders in magnitude for the given examples. Non-coded arylalkyl derivatives, exemplified by phosphonic homophenylalanine (5) and homotyroline (6), were bound preferentially to an even slightly greater extent ($K_i = 0.14$ and 0.12 $\mu$M, respectively, for the racemic mixture, Fig. 4) [91]. Promising affinity ($K_i < 1.0$ $\mu$M) was also found for extended linear homologues, namely 1-aminooctanephosphonic acid [87] and 1-aminooctanephosphonic acid [91]. These results indicate that the $S_1$ binding pocket of the leucine aminopeptidase can accommodate hydrophobic ligands even bulkier than indicated by its substrate preferences (the hydrolase cleaves substrates with the broad band specificity, however, those of the hydrophobic character at the $N$-termini, like leucine, methionine, isoleucine, valine, etc., are processed noticeably faster [79,84]).

Somehow similar situations are observed for the aminyl aminopeptidase APN, both the mammalian one [91] and the orthologue from a lower organism, the protozoan $P.$ falciparum [93]. For example, phosphonic amino acid analogues of strong hydrophobic character, such as phenylalkyl or (cycloalkyl)alkyl (compounds 7 and 8, Fig. 4), inhibited the porcine kidney enzyme with the $K_i$ values at low micromolar range [91]. An interesting attempt of mapping of the APN $S_1$ binding pocket and rationalisation of these data in the context of the substrate-inhibitor structural relationship has been recently undertaken [94].

The specificity of APN was determined using an extensive collection of fluorogenic substrates bearing both natural and non-natural $P_1$ residues. Then, the obtained kinetic parameters were correlated with the activity of the corresponding $\alpha$-aminophosphonic inhibitors. Surprisingly, not the turnover velocity (expressed by the $k_{\text{cat}}/K_m$) but only the strength of substrate binding (described by the $K_m$ value) predicted the most reliably structural features responsible for the inhibitory potency. Thus, the appropriate $P_1$ residue incorporated into the $\alpha$-aminophosphonate core was proved to be indispensable for the tight binding of a ligand to APN.

Because of the resemblance in the substrate specificity, the two aminopeptidases (LAP and APN) are frequently studied together to refer the selectivity of newly developed ligands [89–91]. In general, $\alpha$-aminoalane phosphonic acids are much more potent inhibitors of the leucine aminopeptidase. For example, hydrophobic aliphatic compounds, such as 3 and 4 (Fig. 4), expressed an affinity of more than two orders of magnitude higher for LAP compared to APN as indicated by the appropriate $K_i$ values [90]. The reason for this observation seems to be the presence of the two zinc ions in the binding site of the cytosolic aminopeptidase (LAP contain two zinc ions while APN contain one). Involvement of the $N-C-P$ portion in interactions with both metal ions definitely results with a tighter binding. Contrarily, the leucine aminopeptidase does not readily accept $P_1$ substituents containing a nitrogen atom. Thus, to achieve a high differentiating factor in favor of APN, bulky hydrophobic residues should be appropriately modified with a heteroatom. Compounds 9 and 10 (Fig. 4), bearing an additional amino moiety, was obtained by aziridinephosphonate ring opening with an amine and are low micromolar inhibitors of APN but do not effect LAP [90].

It is worth noting that among the phosphorus analogues of amino acids studied so far, $N$-cyclohexyl-1,2-diaminoethanephosphonic acid (9) appeared the most active towards APN.

The availability of a broad collection of the applied $\alpha$-aminophosphonates allowed a systematic structure–activity relationship in this context of the $S_1$ binding pocket preferences and the specificity of LAP and APN. However, to achieve more significant inhibition these compounds needed to be extended to interact with the $S_1$ pocket as well. Three kinds of phosphonate elongation/modification were envisaged to provide such dipeptide transition state mimetics. Formally, their structure is the result of the replacement of the scissile amide bond by the phosphonodepsipeptidamide or phosphonate moiety. The potential of all three variations for aminopeptidase inhibition was evaluated in detail for the Leu-Leu mimetics and LAP as the target [87,95]. The synthesis of the phosphonate analogue (compound 11, X = O, Fig. 5) was described in the early work of Bartlett, however, the compound showed only moderate potency towards the enzyme studied [87]. Due to this, a systematic computer-aided approach was undertaken by Grembecka et al. to design new generations of more active inhibitors. The methodology was preliminary validated to rationalize the structure–activity relationship obtained for various phosphorus containing amino acid analogues [96,97].

Then, when applied for pseudopeptides, it positively confirmed the idea of addition of the $P_1$’ residue, albeit only via $P-N$ or $P-C$ bonding [95]. Pseudodepsidipeptide bond ($P-O$) was found to be unfavourable because of entropic effects upon inhibitor binding and oxygen–oxygen electrostatic repulsions with the carbonyl of Ala113. The two other types of analogues (12, $X = NH$ and 13, $X = CH_2$, Fig. 5) were synthesized and evaluated [95]. Disappointingly, the fully unprotected phosphonamidate 12 appeared to be unstable at pH below 11. The hydrolysis of the $P-N$ bond that occurred, which effected two component amino acids, was clearly

**Fig. 4.** Selected phosphonic amino acids analogues and their inhibition of the mammalian leucine and alanine aminopeptidases.
correlated with the presence of free neighboring amino group (from the other side, crucial for the effective binding) [98]. Thus, despite being the most promising compounds according to the computed predictions (compound 12 was theoretically calculated to bind with the affinity of \( K_i = 5 \) nM), phosphonamidates were excluded for practical reasons. In turn, phosphinic pseudopeptides exhibited perfect hydrolytic stability and inhibition constants in nanomolar range, and were ranked among the most effective ligands of LAP reported to date. The mixture of two diastereoisomers of the Leu-Leu analogue 13 showed \( K_i = 65 \) nM, similarly to the phosphinate hPhe-Phe and hPhe-Tyr mimetics, both containing arylalkyl P1 and P1’ residues (compounds 14 and 15, respectively, Fig. 5, tested as the mixture of four diastereoisomers) [95]. Chiral chromatography performed for compound 14 allowed separation and assignment of the activity of its individual stereoisomers. The final \( K_i \) value for \( \text{RS-S} \)-counterpart of the \( \text{LL} \) natural peptide configuration, was determined as 45 nM [99].

Interestingly, compounds 14 and 15, particularly the latter one, appeared also effective inhibitors of APN (\( K_i = 276 \) nM and 36 nM, respectively, for the mixture of four diastereoisomers) [95]. Clear preference for the Tyr residue at the P1’ position indicated the significance of the terminal phenolic OH group. This observation was explained by formation of a very specific hydrogen bond (to the carboxylate of Glu 413 of APN) with the use of a model homologous to the leukotriene A4 hydrolase structure. Further exploration of the P1’ structure and its termini, in the context of LAP versus APN selectivity, was undertaken via a parallel N-alkylation strategy of appropriate amino acid building blocks. Unfortunately, the final products appeared only moderate and poorly selective inhibitors (\( K_i \) for both enzymes varied at 0.5–20 \( \mu \)M range) [100].

Importantly, phosphinic pseudopeptides were found to be excellent inhibitors of parasite counterparts of LAP and APN and useful tools for their validation as potential targets in a novel treatment of malaria [47]. \( P. \ falciparum \) M1 and M17 aminopeptidases are responsible for the cleavage of the neutral residues in the terminal stages of the host haemoglobin degradation. Thus, being a limiting stage in generating amino acids essential to parasite growth and development, they represent an attractive target for the development of novel anti-malarial drugs. Compounds 14 and 15 inhibited recombinant M17 enzyme with the potency superior to that observed for the mammalian peptidase (\( K_i = 13.2 \) and 10.4 nM, respectively) [101]. The affinity of 14 measured for the \( \beta M1 \) was also elevated (\( K_i = 78.4 \) nM) when compared to porcine APN (\( K_i = 276 \) nM). In addition, the phosphinates efficiently controlled the growth of \( P. \ falciparum \) in culture, including malaria cells lines that were resistant to the well-known anti-malarial chloroquine. Finally, in vivo studies using a non-lethal \( P. \ chabaudi \) murine malaria model demonstrated that treatment of mice with compound 14 reduced infection by 92% compared with controls [101]. Recently, this compound was co-crystallized with \( \beta M1 \) [75] and \( \beta M17 \) [76] to give an insight into the active site architecture and the mechanism of action of both aminopeptidases which will greatly facilitate the design of new optimized ligands with potential as anti-parasite agents.

Appropriate phosphinic pseudopeptide building blocks can be further elongated by means of solution or solid-phase peptide synthesis to produce tripeptide analogues that possess an additional P2’ substituent. Such optimized compounds were reported to be the most potent organophosphorus inhibitors of the alanyl (APN) and glutamyl (aminopeptidase A, APA, EC 3.4.11.7) metallo-aminopeptidases reported to date. Chen et al. described a series of nanomolar ligands of mammalian APN, exemplified by the Ala-Phe-Phe analogue (16, Fig. 6) [102]. They exhibited promising

\( 11, \ X = O \quad K_i = 58 \ \mu\text{M (RS,RS)} \)
\( 12, \ X = \text{NH} \quad \text{unstable at pH < 11} \)
\( 13, \ X = \text{CH}_2 \quad K_i = 65 \ \text{nM (RRS)} \)

\( 14, \ X = \text{H} \quad K_i = 66 \ \text{nM} \)
\( K_i = 45 \ \text{nM (R,S )} \)
\( K_i = 13.2 \ \text{nM (rec.} \ P. \ falciparum \ M17) \)

Fig. 5. Phosphorus containing pseudopeptides as inhibitors of the M17 leucine aminopeptidase.

\( 15, \ X = \text{OH} \quad K_i = 67 \ \text{nM} \)
\( K_i = 36 \ \text{nM (APN)} \)

\( 16, \ K_i = 2.2 \ \text{nM (mammalian APN)} \)
\( K_i = 1.5 \ \text{nM (bacterial APN)} \)

\( 17, \ K_i = 0.8 \ \text{nM (APA)} \)

Fig. 6. Phosphinic pseudotripeptides as highly effective inhibitors of the alanyl and glutamyl metallo-aminopeptidases.

\( 18 \quad 19 \)
\( 20 \quad 21 \)

Fig. 7. The general structures of phosphonic compounds \( \sigma \)-functionalized with an additional heteroatom group to enhance the coordination of the catalytic zinc ion in the active site of the aminopeptidase.
anti-nociceptive activity thanks to the dual action against APN and nephrilysin which caused an analgesic response after administration in mice [103]. Compound 16 inhibited equipotently both the mammalian alanyl aminopeptidase as well as its bacterial orthologue. It also served as a ligand to resolve the three-dimensional structure of the latter enzyme [70]. Related phosphinate tripeptidic analogues were used for co-crystallization with the leukotriene A₄ hydrolase/aminopeptidase, a prototypic M1 family member. As the result, substrate and transition state binding details together with a presumed catalytic mechanism, scarce data for the M1 enzymes, were reported [104].

A phosphinic pseudotripeptide, the Glu-Leu-Ala analogue (17, Fig. 6, tested as mixture of four diastereoisomers), showed high affinity towards the zinc glutamyl aminopeptidase (mice recombinant) expressed by its $K_i$ equal to 0.8 nM [105]. Within a series of compounds, N-terminal pseudoglutamyl residue was found to be crucial for the high potency and selectivity. For example, the differentiating factor between APA and APN (crucial for the high potency and selectivity. For example, the compounds, N-terminal pseudoglutamyl residue was found to be for LAP but the inhibition achieved for the Leu-Leu analogue (P' (R = Ph, (CH₂)₃Ph or CH₂(p-OMe-C₆H₄)) allowed for regulation of the enzyme activity with the IC₅₀ value to 0.25 μM (this result corresponded to $K_i = 143$ nM) [106,107]. Interestingly, all four structural variations 18–21 produced comparable, low micromolar or sub-micromolar IC₅₀ values of inhibition.

6. Bestatin and derivatives

Originally isolated from Streptomyces olivoreticuli (MD976-C7) more than 30 years ago by Umezawa and co-workers, bestatin ((2S,3R)-3-amino-2-hydroxy-4-phenylbutanoyl-L-leucine = Ubenimex, Fig. 8) was one of the first potent inhibitors of metalloaminopeptidases with broad spectrum of action [108]. Bestatin has been extensively investigated in biological systems both in vitro and in vivo, which resulted in discovery of several interesting properties of this compound such as ability to induce apoptosis in cancer cells, anti-angiogenic, anti-malarial or immunomodulatory effects [109]. Presently, bestatin is on the market in Japan where it is applied for treatment of cancer and bacterial infections. Examples of successful inhibition of aminopeptidases by bestatin include aminopeptidase N (CD13), leucine aminopeptidase (LAP), aminopeptidase B (EC 3.4.11.6) or LTA₄ hydrolase [110–112]. Bestatin can act as slow (LAP) or fast (APN) binding, competitive inhibitor of aminopeptidases [113]. It resembles a Phe-Leu dipeptide substrate, however its Phe residue is β-amino-α-hydroxy amino acid [114]. This α-hydroxy group together with the neighboring carbonyl group coordinate a zinc ion, which results in a competitive active site-directed inhibition. Bestatin is weaker inhibitor of aminopeptidases containing one metal ion in the active center (APN, APB) and much stronger of enzymes with two metal ions (LAP). This feature is explained by larger amount of interactions made by inhibitor with both enzyme metal ions in the active center along with additional contacts made by side chains of the inhibitor in S1 and S1’ pockets of the enzyme.

To date several stereoselective, synthetic methods leading to desired bestatin diastereomers have been described [115–119] (see recent reviews presenting available synthetic methods for bestatin and some modifications [120,121]). Absolute configuration is a key issue responsible for good inhibitory effect and discrimination of the appropriate binding partners (substrates and inhibitors) for most of the proteases. Diastereomer of bestatin with inverse conformation at carbon atom with hydroxy group ((2R,3R)-3-amino-2-hydroxy-4-phenylbutanoyl-L-leucine) is known as epibestatin and is frequently used as negative control in biological experiments [122]. The structure of bestatin has also been a subject of several modifications in hope to improve its inhibitory and pharmacological properties. Examples of such derivatives are bestatin thioamide (compound 23, Fig. 9) [123], para-hydroxybestatin (24) [124] or 2-thiolbestatin (25) [125]. The scaffold of bestatin was also used for the design of Activity

![Fig. 8. The structure of bestatin and its inhibition of selected mammalian aminopeptidases.](image)

![Fig. 9. Structure of bestatin derivatives and their inhibition of selected aminopeptidases.](image)

| Compound | IC₅₀ (μM) | Kᵢ (μM) | Activity |
|----------|----------|---------|----------|
| 23       | 0.02     | 0.033   | APN      |
| 24       | 0.03     | 0.007   | APB      |
| 25       | 0.55     | 4.4     | APN      |
Based Probes (ABPs) for detection of mammalian and malaria M1 aminopeptidase N [126]. These were obtained by solid-phase synthesis compounds (for example 26, Fig. 10) and effectively labeled recombinant APN as well as metallo-aminopeptidases in sophisticated proteome system originated from *P. falciparum* cells. Success with bestatin resulted in more intense research of this group of compounds and isolation and characterization of several other compounds bearing a similar scaffold structure. Examples of such compounds of natural origin are phebestin ([25R]-3-amino-2-hydroxy-4-phenylbutanoyl-L-valyl-L-phenylanil ne, compound 27, Fig. 11) [127], probestin ([25R]-3-amino-2-hydroxy-4-phenylbutanoyl-L-valyl-L-prolyl-L-proline, 28) [128] and amastatin ([25R]-3-amino-2-hydroxy-5-methylhexanoyl-L-valine-L-valine-L-aspartic acid, 29) [113,129]. These compounds are tri- and tetrapeptides and are better inhibitors of aminopeptidase N when compared to bestatin. This is due to an increased amount of contacts made with S1, S1', S2' and S3' pockets of the enzyme and side chains of the inhibitors, which in most cases have very hydrophobic character (Phe, Leu, Val).

### 7. Hydroxamic acids

Hydroxamic acids (N-hydroxyamides) can be considered as analogues of carboxylic acids and amides that uniquely combine the features of both of these groups. Simultaneously, the hydroxamate moiety is an effective planar bidentate O,O metal chelating system. This close structural similarity to the products/substrates of the enzyme and side chains of the inhibitors, which in most cases have very hydrophobic character (Phe, Leu, Val).

The most promising applications have been associated with inhibition of matrix metalloproteinases as perspective targets for anti-cancer therapy. Matrix metalloproteinases, involved in normal and abnormal tissue remodeling, angiogenesis and tumor metastasis, have been potently regulated by hydroxamates (e.g. Batimastat, Marimastat) that reached advanced phases of the clinical trials. These finally failed because of side effects associated with cross interactions with other metal containing proteins [130,131].

Fundamental work on the metallo-aminopeptidases inhibition by α-amino hydroxamates was performed by Chan et al. and concerned the leucine aminopeptidase [132]. The derivatives of hydrophobic amino acids (exemplified by L-Leu-NHOH, compound 30, Fig. 12) regulated the enzyme activity with the *K*$_i$ values of micromolar range. Other C-terminally modified compounds, such as the L-Leu amide, alcohol, or hydrazide, together with the amino acid alone, were much less potent, typically at least by a 100-fold ratio. These observations were consistent with a subsequent study by a series of phenylalanine derivatives targeted towards APN. Compound 31 (Fig. 12) appeared a moderate inhibitor of the enzyme [133]. Both L-Leu-NHOH and L-Phe-NHOH were overpowered by the corresponding thioles which indicated a much tighter individual sulfur–zinc interaction than the bidentate oxygen–zinc binding. Since then, a choice of hydroxamic acid inhibitors (not necessarily based on the amino acid skeleton) of different metallo-aminopeptidases have been reported in the literature. A representative selection from recent examples is presented in Fig. 13. Compound 32, bearing a hydrophobic substituent, was identified by screening of a 3000 natural and synthetic compound library and appeared equipotent to bestatin for APN [134]. Compound 32 controlled the basic fibroblast growth-factor-induced invasion of endothelial cells at low micromolar range.

![Fig. 10. Structure of Activity Based Probe based on structure of bestatin for labeling of aminopeptidases.](image)

![Fig. 11. The structures of phebestin, probestin and amastatin and their activity towards aminopeptidase N.](image)

![Fig. 12. Amino acid derived hydroxamate inhibitors of neutral aminopeptidases.](image)
The hydroxamate was highly selective as it exerted no action towards members of the matrix metalloprotease family.

Interesting hydroxamate based inhibitors were also developed for the methionine aminopeptidases from different organisms. Hu et al. described the synthesis of compounds possessing an additional substitution at the hydroxamate nitrogen atom [135]. These N-hydroxydipeptides, derivatives of Met-Gly (compound 33, Fig. 13), allowed for structural optimization of both sides of the molecule. The products inhibited the bacterial as well as both forms of human MetAP, with a slight preference to E. coli enzyme. A cooperative action of the N-terminal amino group and two hydroxamate oxygen atoms towards two catalytic metal ions in the active center was suggested as the binding pattern.

The bacterial form of MetAP1 served also as a model for comparison of the activity of 5-aryl-furane-2-carboxylic acids with other variants of the C-termini [136]. Such heteroaromatic hydroxamic acids (exemplified by 34, Fig. 13) were found to be superior inhibitors to corresponding acids, esters, amides, hydrazides, alcohols and nitriles towards distinct metalloforms of the EcMetAP1.

Certain metallo-aminopeptidases are promising targets in anticancer therapy in humans, but they can also be exploited in the development of antibacterial, antifungal and antiparasitic agents. The zinc P. falciparum M1 aminopeptidase is being actively explored in this context (as described in the section devoted to organophosphorus compounds). The dual function amide-hydroxamate template was also used to target the malaria enzyme [137]. The compounds developed consisted of three portions: hydroxamic acid termini dedicated for Zn(II) complexation, a hydrophobic α-substituent and the amide function that served for extensive structural diversified. The use of bulky hydrophobic amines for the amide formation (as exemplified by 35, Fig. 14) yielded potent inhibitors of the parasite enzyme, with the IC₅₀ in low nanomolar range [138]. Importantly, consecutive iterative optimization gave derivatives that were characterized by spectacular selectivity versus the mammalian orthologue. Compounds were active in parasite growth inhibition and displayed good pharmacokinetic properties [138].

Tosedostat (CHR-2797, 36, Fig. 15) [139] seems to be the most attractive novel pharmacologically active product among hydroxamic acid metallopeptidase inhibitors. This orally available cyclopentyl ester prodrug is converted in vivo into the intracellularly active acid metabolite. The latter is a potent inhibitor of a number of aminopeptidases, including leucine aminopeptidase, aminopeptidase N, puromycin sensitive aminopeptidase and leukotriene A₄ hydrolase/aminopeptidase (with the IC₅₀ in nanomolar range) [140]. CHR-2797 exerted anti-proliferative effects against tumor cell lines in vitro and in vivo, and exhibited 300 times more potency than bestatin. The proposed mechanism of tumor cell killing involves depletion of amino acids by blocking protein processing/recycling [140]. CHR-2797 is well tolerated and can be safely administered at doses that result in the metabolite activity as evidenced in preclinical models [141]. Recently, Tosedostat demonstrated promising efficacy in patients with acute myeloid leukemia and myelodysplastic syndrome in the phase II of clinical trials [142].

8. 2-Amino-thiols

2-Amino-thiols are synthetic analogues of amino acids in which the carboxylate function has been replaced by a thiomethyl residue. The chemical transformations that are usually applied for their synthesis, involve a reduction of the ester group of an N-protected amino ester to the hydroxymethyl function, followed by an...
activation of the latter and its subsequent substitution with a sulfur containing nucleophile [143–146]. Alternatively, the Mitsunobu reaction is utilized as the OH replacement procedure [145,146]. As the optically active substrates are easily available and there is no risk of racemization, final products are obtained in the enantio-merically pure form. When the mode of binding to metallo-aminopeptidases is considered, 2-aminothiols are typical analogues of the N-terminal portion of the peptide substrates that act in reversible competitive manner. The thiol group is a termini designed to interact non-covalently with the catalytic metal ion(s). Except for the functional groups essential for the polar contacts, 2-aminothiols contain a side chain that occupies the S1 pocket. Despite a structural simplicity and low molecular weight, the potency of these compounds against metallo-aminopeptidases is very high. The reason for this is a strong affinity of the sulfur atom for divalent soft acid metal ions, such as Zn(II). Consequently, 2-aminothiols exhibit much higher activity than amino acid analogues of the corresponding complexity, but containing other metal chelating moieties, such as phosphonate or hydroxamate ones. Frequently, these compounds are discriminated with an affinity more than 1000-fold ratio lower in comparison to the analogous thiol compounds. In general, 2-aminothiols are one-handed inhibitors directed specifically towards the S1 subsite part. The availability of extended ligands that possess an additional Pn portion is limited because of a complex synthesis and diastereomeric purity. Nevertheless, 2-aminothiols have found some fundamental and practical applications connected with metallo-aminopeptidase inhibition. The most spectacular achievements are associated with the regulation of action of aminopeptidases A and N, enzymes involved in the brain renin–angiotensin cascade that represent perspective targets in a hypertension therapy.

A series of representative 2-aminothiol compounds, derived from the natural amino acids, is given in Fig. 16. l-Lysine thiol (the absolute configuration S, compound 37) was shown to be an extremely potent inhibitor of arginyl aminopeptidase (aminopeptidase B, APB) with a subnanomolar Ki value [143]. l-Leucine thiol (compound 38) binds to the same target but with a lower activity. More interestingly, it exhibited a 700-fold ratio stereochemical preference towards the enzyme for the natural configuration (Ki = 980 µM for the D (R) enantiomer). Unexpectedly, compound 38 poorly inhibited leucine aminopeptidase (LAP), what was explained by the use of the zinc–magnesium hybrid enzyme in these experiments [143].

According to an earlier study, l-leucine thiol (38) was shown to be a potent competitive inhibitor of the microsomal aminopeptidase from porcine kidney (APN, Ki = 22 nM) [144]. This compound was then used as a lead structure to optimize the size of a neutral hydrophobic P1 residue, in the context of construction of potentially analgesic dual inhibitors of neprilysin (NEP) and APN [133]. A broad series of inhibitors was synthesized that showed high equipotent efficacy for the aminopeptidase N (Ki = 11–50 nM). Among them, the methionine thiol (compound 39, Fig. 16) appeared the most active in vitro. 2-Aminothiols were at least four orders in magnitude more effective than carboxylates, phosphonates and hydroxamates of the corresponding structure [145]. Despite such potency, the use of selected compounds in intravenous administration did not induce anti-nociceptive responses in mice on the hot plate test, evidently because of difficulties to cross the blood–brain barrier. Indeed, the oxidation to the disulfide form increases the lipophilicity made them efficient prodrugs. Much more significant response was stimulated by application of a mixed prodrug which structure was disulfide based combination of two thiol inhibitors, each directed towards a certain enzyme, either APN or NEP [147].

Elucidation of an intrinsic function in the brain and, therefore, the medical potential of selected aminopeptidase in the renin–angiotensin system stimulated continuation of studies on 2-aminothiols. These two metallo-aminopeptidases, namely APN and glutamyl aminopeptidase (aminopeptidase A, APA), are supposed to participate in metabolism of brain angiotensin II and III (AngII and AngIII). Glu-thiol (compound 40, Fig. 17) [148] was described as the first efficient inhibitor of APA, however it was equipotent to APN (Ki = 140 versus 120 nM, respectively). To study the physiological importance of these enzymes, specific agents, discriminating between the two targets to a high degree are indispensable. In a quest for a selective inhibition, a number of 2-aminothiol derivatives were synthesized and evaluated [145,146]. It appeared to be a relatively simple task to identify the P1 residue responsible for high nanomolar potency of an APN ligand. An extended P1 hydrophobic portion, favorably terminated with a heteroatom hydrophobic portion, favorably terminated with a heteroatom (as exemplified by compounds 41 and 42, Fig. 17), represented such an option. The affinity of these compounds towards APA was of 100-fold ratio lower. Anyway, according to recent data l-methionine thiol developed earlier [149] gave even more

![Fig. 16](image1)

Potent 2-aminothiol inhibitors of the aminopeptidases B and N, the analogues of the natural amino acids.

![Fig. 17](image2)

Optimization of the lead Glu-thiol structure (40) to achieve selective inhibition of aminopeptidase N versus glutamyl aminopeptidase (compounds 40, 41 and 43 were tested as the S enantiomers, whereas compound 42 as the racemic mixture).
impressive differentiation ($K_i = 8 \text{ nM}$ for APN versus $K_i = 17.2 \text{ \mu M}$ for APA, respectively [149]).

Such a convenient modification was not found for the opposite case. A compromise was achieved for short side chains with an acid function other than carbonyl at the terminus (such as 43, Fig. 17) [145,146]. The affinity of the sulfonic acid 43 for APA was not elevated in comparison to the lead, but a drop in potency measured for APN was more significant and finally resulted in a 100-fold discrimination ratio. These parameters were furthermore improved by exploration of the $S_1'$ subsite of the aminopeptidase A. The synthesis of tripeptidomimetics that contained an optimal $P_1$ substituent, attached as the 3-amino-2-mercaptopropan-1-ol to the N-termini of dipeptide libraries, allowed determination of the advantageous $P_1'$ and $P_2'$ side chains [150]. The $S_1'$ binding pocket accommodated hydrophobic residues whereas the $S_2'$ pocket preferred negatively charged residues. As a result, an exceptionally potent compound (44, Fig. 17) characterized by a striking 20,000-fold selectivity ratio, was identified. Interestingly, the most active diasteroisomer exhibited non-natural configuration $R$ at the $P_1$ portion which was explained by sterical constrains of the whole molecule [150]. Despite a remarkable activity (a first subnanomolar thiol inhibitor of APA), compound 44 was poorly bioavailable. A limited $P_1'$ fragment optimization starting from the lead 43 gave a much simpler molecule: (3S,4S)-3-amino-4-mercapto-6-phenylhexane-1-sulfonic acid [151]. The substitution of the $C_3$ atom with a hydrophobic arylalkyl fragment resulted with a satisfactory potent APA inhibition ($K_i = 30 \text{ nM}$) (however, the kinetic data for APN were not reported).

Over-activity of the renin–angiotensin system in the brain has been implicated in the development and maintenance of hypertension. Using specific agents described above, it was demonstrated that APA and APN are involved in the metabolism of angiotensin II and III, respectively, within this system. AngII (2–8) is generated from AngII by APA assisted cleavage of the N-terminal Asp-Arg bond, whereas APN functions in subsequent inactivation of AngIII. APA specific sulfonate 43 increased the half-life of AngII in mice, blocked the AngII to AngI conversion which in turn controlled vasopressin release [152,153]. The dose-dependent decrease of blood pressure was achieved only by intra-cerebroventricular injection. Contrarily, selective inhibitors of APN (39 and 41), administered by the same route, caused elevated blood pressure. Thus, the level of angiotensin II and III in the brain, in particular that controlled by the aminopeptidases, was suggested to be a potential target of hypertension treatment [153,154]. To this end, the disulfide dimer of the lead 43 was reported as a first orally available candidate for the therapy based on aminopeptidase A inhibition [154,155].

9. Epoxide (covalent) inhibitors

The majority of inhibitors discovered for metallo-aminopeptidases are compounds which interact with enzyme in a non-covalent way. However, for some enzyme covalent inhibitors have also been found. Fumagillin ((2Z,4E,6E,8E)-10-[(5-methoxy-4-[2-methyl-3-(3-methylbut-2-eny)oxy]iran-2-yl]-1-oxaazepiro[2.5][octan-6-yl][oxy]-10-oxodeca-2,4,6,8-tetraenoic acid, 45, Fig. 18) and ovalicin ((1S,2R,3S,4R,5S)-7-hydroxy-8-methoxy-7-[2-methyl-3-(3-methylbut-2-eny)oxy]iran-2-yl]-4-oxaazepiro[2.5][octan-2-one, 46], which are fungal metabolites inhibit methionine aminopeptidase type 2 (MetAP2) by the formation of a covalent, irreversible bond between a reactive epoxide and His-79 present in the active center of the enzyme [156,157]. These compounds are not effective towards other aminopeptidases. Selectivity towards MetAP2 and complete lack of selectivity towards MetAP1 is explained by difference in only one amino acid (Ala-202 in MetAP2 and threonine in MetAP1) around the active center of these enzymes as determined by comparison of crystal structures [158,159]. This approach revealed also that tight binding of fumagillin and derivatives to MetAP2 is also a combination of several other interactions made by the inhibitor components with the surface around the active center of the enzyme. In biological studies fumagillin and ovalicin have been found as potent inhibitors of angiogenesis due to their inhibition of endothelial cell proliferation. This resulted in extensive SAR studies and synthesis of several derivatives of these compounds. Compound TNP-470 (47, Fig. 18) has entered clinical trials and was evaluated for application in anti-angiogenic therapy [156,160].

10. Heteroatom scaffolds

Numerous competitive inhibitors of metallo-aminopeptidases that have been recently developed, frequently starting from leads identified by random screening (e.g. high throughput screening utilizing fluorogenic [161,162] and chromogenic assays [163,164] or virtual screening [165,166]), are based on a heteroatom-rich fragment. Such an appropriate heteroaromatic (or rarely heterocyclic) scaffold contains a set of the nitrogen, oxygen and/or sulfur atoms involved in coordination of the catalytic metal ion(s). Structural rigidity and constraint of this portion of a ligand is often privileged for formation of hydrophobic interactions, specifically $-\pi-\pi$ stacking, with the neighboring residues of the active site. The synthetic procedures used for obtaining such compounds should allow the combination with reliable methods of the substituent(s) diversification. This can be performed prior to a ring formation/aromatization or by parallel decoration of the target scaffold with the use of a simple chemistry, such as the amide bond formation. An extensive structural optimization of certain scaffolds has led to very potent inhibition of selected aminopeptidases by surprisingly low molecular weight compounds (even below 200). Methionine aminopeptidases (particularly human MetAP2), are representative examples of successful application of this strategy.

1,2,4-Triazoles were originally described in the patent literature as potent reversible non-peptidic inhibitors of the methionine aminopeptidase type 2 [167,168]. Various substituted compounds bound to the cobalt form of MetAP2 with the affinity expressed by
the IC₅₀ values in the range of 0.1 nM–100 µM. The optimized synthesis of an array of 3-anilino-5-benzylthio-1,2,4-triazoles (compounds 48–50, Fig. 19) and their biological relevance was also subsequently presented [169]. Iterative refinement of the inhibitor lead structure allowed the identification of compounds with the potency against MetAP2 in the picomolar range. Systematic modifications of the key structural fragments revealed certain tendencies responsible for elevation or decrease in their activity. The 3° and 4° substitution of the aniline phenyl ring with small residues was the most favorable (compounds 49 and 50, Fig. 19).

Contrarily, any changes in the optimal benzylthio S1 residue structure were found deleterious, similarly to methylation of any of the triazole nitrogen atoms (to a relatively smaller extent for the N4). Selected derivatives inhibited human endothelial cell proliferation and the growth of new blood vessels in a model of angiogenesis.

Low molecular weight 4-aryl substituted 1,2,3-triazoles were found to act as almost equipotent reversible inhibitors of the human MetAP2 [170] as described for 1,2,4 derivatives. Diversification of the position 4 was performed by the use of various aromatic aldehydes that were transformed into the appropriate alkyne substrates for the cycloaddition. Several effective ligands of the cobalt-activated MetAP2 were identified, showing the Kₚ values down to 1 nM. For example, Kₚ = 15 nM for compound 51 (Fig. 19) corresponded to Kₛ = 4.2 nM and IC₅₀ = 10 nM in full kinetic analysis [170]. SAR studies performed for the phenyl ring excluded bulky 3° and 4° substitution showed that only small alkyl chains were tolerated whereas the halogens were favorable in these positions (see for example compound 52 in Fig. 19). Contrarily, a 2° addition of a bulky residue, even a heteroaromatic one, was well accepted and indicated some additional room in the active site. Replacement of the whole phenyl ring by a substituted pyridyl was also profitable. The use of the pyrazole based scaffolds instead of the triazole one, revealed the key role of N1 and N2 atoms in high affinity. Regarding their biological activity, the compounds inhibited human and mouse endothelial cell growth.

Enzyme-ligand crystal structures accompanied studies on the triazoles [169,170]. They revealed, among others, the significance of the P1 aromatic ring of a certain size (substituted or not, present in potent MetAP2 inhibitors) that should occupy the hydrophobic pocket formed by Phe219, His331, Tyr444 and His231, and form the appropriate π–π stacking. The size of the pocket (narrower for

![Fig. 19](https://example.com/fig19.png)

**Selected potent inhibitors of the human MetAP2 based on the 1,2,3 and 1,2,4-triazole scaffolds. Compounds 53 inhibited the bacterial MetAP1 and allowed the elucidation of the triazoles binding mode to the enzyme catalytic site.**

![Fig. 20](https://example.com/fig20.png)

**Inhibition of the cobalt dependent *Escherichia coli* methionine aminopeptidase type 1 by thiazole based compounds.**
MetAP1) was postulated to be a discrimination factor between the two forms of the methionine aminopeptidase. The difference in the binding affinity is usually 3–4 orders in magnitude for MetAP2 for the triazoles studied (for example, $K_{\text{IC}50} = 0.5 \text{nM}$ versus 3900 nM for 48, $IC_{50} = 10 \text{nM}$ versus 7180 nM for 51, Fig. 19). The structural data provided a rationale for SAR discussion, but first of all confirmed the significance of the key interactions between N1 and N2 nitrogen atoms of the inhibitors and the cobalt ions. The molecular mechanism of triazole action was elucidated originally using the crystal structures of the Staphylococcus aureus orthologue of the MetAP1 enzyme complexed with 3,5-disubstituted-1,2,4-triazoles (such as compound 53, Fig. 19) [171]. According to these results, each of the triazole N1 and N2 nitrogen atoms interacts with one of the two cobalt ions such that the N–N bond is nearly co-linear with the Co to Co. The distances between the nitrogen and the coordinated cobalt are very similar to each other and tight (close to 2 Å) which indicates strong interactions [171]. The triazole ring forms a stacking contact with the hydrophobic face of the imidazole side chain of H76, a residue that is believed to be involved in the catalytic process. Somewhat surprisingly, the substituent of the substrate-like methionine structure of 53 docks to the S1’ subsite, and not to the S1 site. In turn, its natural position is occupied by the thiobenzyl residue as described above.

MetAP is believed to be a dinuclear cobalt dependent enzyme as Co(II) activates all its known forms, however, the identity of the metal cofactor is still ambiguous. Triazoles that inhibited exclusively the cobalt-activated human MetAP2 even with high nanomolar potency, although failed in cell proliferation assays, were used to shed light on this matter [172,173]. Such metal dependent action indicates that other divalent ions must be considered as the relevant cofactors, particularly in respect to the fact that the architecture of the enzyme active site does not depend on the identity of the bound metal. In this context, manganese was postulated as the physiologically relevant metal [172]. Indeed, the triazoles investigated were not effective towards such activated MetAP form.

Contrary to triazoles, 1,3-thiazoles appeared selective inhibitors of the methionine aminopeptidase type 1 of both bacterial and human origin. The essential function of MetAP1 in bacteria suggested that the enzyme could be a promising target for the development of novel broad spectrum antibiotic agents. Accordingly, thiazole based active compounds were identified by screening of libraries towards the E. coli methionine aminopeptidase. It is worth noting that selected leads did not represent typical heteroaromatic scaffolds. These were not the central core of the molecule, but rather a proximity group linked with another hydrophobic portion (frequently heteroaromatic as well) by a carbon—carbon, amide or carbonyl based bonding. Thus, the complexing mode of the enzyme catalytic ion(s) was not as characteristic as for triazoles and usually involved participation of two neighboring heteroatom groups.

The random screening of 4500 compounds by Nan and co-workers led to the amide of pyridine-2-carboxylic acid and thiazol-2-ylamine (54, Fig. 20) that inhibited the E. coli MetAP1 with the $IC_{50} \approx 5 \mu M$ and served as a perspective lead compound [174]. All primary chemical mutation in the basic structure, such as change in position of pyridine substitution or heteroatom replacement by an isosteric group, appeared deleterious for the potency [174,175]. Most probably, the combination of three electronegative groups that are involved in the metal ions coordination, namely the aromatic nitrogen of the pyridine ring, the carbonyl moiety in position 2 and a heteroatom of the thiazole, forms a system essential for the tight binding. Nevertheless, substitution of the position 3 in pyridine with an amide or ester group appeared profitable in terms of optimization of interactions with the binding pocket. The affinity was elevated to nanomolar range, as for example for compound 55, Fig. 20 [174]. Importantly, introduction of a bulky cyclic or aromatic residue at the proximity of the amide allowed for efficient discrimination between the bacterial enzymes from different sources. For $X = \text{benzoyloamino or cyclohexancarbamino}$, by substituting the structure 54 the selectivity factor between E. coli and the Saccharomyces cerevisiae exceeded 100-fold ratio ($IC_{50} \approx 0.89$ and $0.87 \mu M$ for EcMetAP1, respectively and $IC_{50} > 100 \mu M$ for ScMetAP1 for both compounds). Wide optimization of the X moiety demonstrated that introduction of an additional heteroatom to the extended amide structure was also well tolerated (56, Fig. 20) [175,176]. Finally, replacement of the whole pyridine ring by another thiazole system gave compound 58 (Fig. 20) with low nanomolar potency [177].

Interestingly, 3-substituted pyridine-2-carboxylic acid thiazol-2-ylamides selectively inhibited human MetAP1 versus MetAP2, as exemplified by a 200-fold differentiating factor for compound 57 (Fig. 20) [178]. Despite their moderate potency in vitro, the treatment of tumor cell lines with such specific agents suggested a role of MetAP1 in G2/M phase transition. Furthermore, this study
confirmed the previously reported results on variations in the metal binding in the active site of the Co(II)-EcMetAP in the presence of the thiazole based ligands [165]. According to the appropriate crystal structures, these compounds seemed to be responsible for driving a third metal ion into the enzyme active site. The auxiliary cobalt atom was not present in the native enzyme, even when crystallized in the presence of high concentration of the metal salt. The additional cobalt interacts with a single histidine residue of the enzyme, three water molecules at the entrance of the active center and two nitrogen atoms of the ligand (those of the pyridine and the amide). Inhibitor binding is stabilized by several contacts, mostly of the hydrophobic character. Obviously, such an unusual situation must be taken into consideration when SAR of the active isoforms of the enzyme (for example see 63, Fig. 21). Similarly as before, they complexed to the auxiliary Co(II) that is normally not involved in the catalytic process [164]. Nevertheless, they appeared to be novel potential anti-malarial agents. Three active isoforms of PfMetAP1 were obtained after cloning, expression and purification, and tested in vitro. The inhibitors were remarkably selective towards the 1b type, with the highest obtained potency with $IC_{50} = 112$ nM. The most active compound 63 inhibited also the P. falciparum proliferation of both chloroquine sensitive and dependent erythrocyte cultures with $IC_{50} = 0.9$ and 3.1 μM, respectively [180]. Consequently, this compound was used in murine malaria models and positively confirmed PfMetAP1b as a promising target for development of new anti-malarials.

### 11. Boronic acids

Boronic acids as inhibitors of aminopeptidases were described first by Baker et al. for Aeromonas aminopeptidase (EC 3.4.11.10) [181,182]. In this report simple aliphatic derivatives were used as competitive, transition state analogues that bound to the active site of a ten times larger library [161]. The main goal of the study was to find inhibitors. These compounds bind the driven active site auxiliary cobalt ion by employing nitrogen atoms from each of the rings that are placed in co-planar manner. Thiabendazoles (benzimidazoles substituted in position 2 with the thiazole ring) and their derivatives (compounds 60–62, Fig. 21) regulated the Co(II)-EcMetAP activity with $K_i$ value in sub-micromolar range, although they appeared ineffective in vivo [166]. This was explained with the use of the MetAP-ligand crystal structures that revealed variations in the metal binding in the active site [165,166] as described above for pyridine-2-carboxylic acid thiazol-2-ylamides.

A family of related 2-(2-pyridinyl)pyrimidines was identified upon screening of a 175,000 member library for inhibitors of human and P. falciparum methionine aminopeptidases [164,180]. Interestingly, the compounds were virtually equipotent towards both mammalian types of the enzyme (for example see 63, Fig. 21). Similarly as before, they complexed to the auxiliary Co(II) that is normally not involved in the catalytic process [164]. Nevertheless, they appeared to be novel potential anti-malarial agents. Three active isoforms of PfMetAP1 were obtained after cloning, expression and purification, and tested in vitro. The inhibitors were remarkably selective towards the 1b type, with the highest obtained potency with $IC_{50} = 112$ nM. The most active compound 63 inhibited also the P. falciparum proliferation of both chloroquine sensitive and dependent erythrocyte cultures with $IC_{50} = 0.9$ and 3.1 μM, respectively [180]. Consequently, this compound was used in murine malaria models and positively confirmed PfMetAP1b as a promising target for development of new anti-malarials.

### Additional Resources

- [Fig. 23](#): Structures and activity of $\alpha$-aminoboronic acids.
- [Fig. 24](#): Structures of $\alpha$-aminoaldehydes.
- [Fig. 25](#): Structure and activity of 3-amino-2-hydroxy-propionaldehyde and 3-amino-1-hydroxypropan-2-one phenylalanine derivatives.
center of enzyme with good efficiency. Among five tested derivatives 1-butaneboronic acid (64, Fig. 22) was the best inhibitor of the enzyme. In another approach, Shenvi described a series of α-amino- boronic acids as effective inhibitors of human enkephalin degrading aminopeptidase (HEDA), microsomal leucine aminopeptidase and cytosolic leucine aminopeptidase [183]. The advantage of these inhibitors over simple aliphatic derivatives was the presence of the free amine group at carbon α, a feature that is known to improve binding of the ligand to aminopeptidases. Detailed analysis of kinetic data for cytosolic leucine aminopeptidase revealed biphasic slow-binding inhibition mechanism of α-amino- boronic acids. This suggested that slow-binding step is responsible for formation of tetrahedral boronate molecule from trigonal boronic acid. The inhibitory activity of the tested derivatives (65–68) also strongly correlated with the side chain type used in the study (Fig. 23).

12. Aldehydes

Aldehyde derivatives of amino acids have been also described as inhibitors of aminopeptidases. Andersson et al. first reported this group of compounds as very effective transition states analogue inhibitors (upon hydration they formed a gem-diolate involved in zinc complexation) of porcine cytosolic and microsomal leucine aminopeptidases [184]. The most effective inhibitor described in this report was L-leucinal (compound 70, Fig. 24, $K_i = 6$ nM for LAP and $K_i = 76$ nM for APN). Importance of the aldehyde war-head was demonstrated in this report by comparison of the value of L-leucinal inhibitory constant with those found for simple amino acid L-leucine as well as its hydroxy derivative L-leucinol. These compounds were around 4–5 orders of magnitude less effective towards both aminopeptidases tested. Additionally, glycine aldehyde derivative (glycinyl, compound 69, Fig. 24) investigated in this study was also much less effective ($K_i = 0.68$ nM for LAP). This result confirmed the importance of the side chain in binding efficiency of the designed inhibitors, but also proved that information from the substrate activity screening can be used for the design of the inhibitors. L-Leucine p-nitroanilide substrate is much more efficiently processed by LAP than analogous glycine derivative.

Another group of inhibitors for aminopeptidases with aldehyde scaffold were proposed by Tarnus et al., 3-amino-2-hydroxy-propionaldehyde and 3-amino-1-hydroxypropan-2-one derivatives (71 and 72, respectively, Fig. 25) [185]. These compounds designed as general inhibitors of metallo-aminopeptidases were micromolar competitive inhibitors of LAP and APN. For some derivatives selective inhibition of APN over LAP was observed. Unfortunately, due to their susceptibility to oligomerization as well as very high reactivity aldehyde derivatives are not the best candidates for in vivo studies. However, they are an interesting alternative for design of inhibitors, which can be used for investigation of aminopeptidases in vitro.

13. Sulfonamides

Sulfonamides belong to a group of the most recognized compounds with biomedical relevance. Easily obtained in the reaction of appropriate sulfonyl chlorides with amines, they offer a great potential of structural variations of both substrates. Indeed, since the discovery of the antimicrobial properties, sulfonamides have found a vast number of other biological applications connected with regulation of enzymatic activity. In the context of proteases inhibition, a rationale for the sulfonamide moiety application is the isosteric and isoenergetic resemblance to the high energy tetrahedral transition state that is present in the amide bond hydrolysis (similarly to the phosphorus containing peptide analogues). Surprisingly, there are not many recent examples of the use of this strategy towards metallo-aminopeptidase targets. What is more significant, the mode of sulfonamides action in those rare cases is miscellaneous and does not follow the theoretically considered pattern of transition state interactions. The title functional group plays more of a role as a linker between hydrophobic portions of the inhibitor, with another metal binding entity incorporated into one of them. Alternatively, a cooperative action of two heteroatom-rich moieties (including the sulfonamide one) is observed.

The most advanced studies on this topic seemed to be performed in Abbott Laboratories and concerned anthranilic acid sulfonamides as inhibitors of the human recombinant methionine aminopeptidase type 2 (as a target for orally available drugs in an anti-cancer therapy) [186–189]. A series of sulfonamide compounds, such as 73 (Fig. 26), was identified using affinity selection by a mass spectroscopy screening method [186]. They exhibited micromolar activity for the manganese form of the human MetAP2 and were moderately potent in a cell proliferation assay. Thanks to promising pharmacokinetics and synthetic viability they were pointed out as novel attractive leads. Consecutive X-ray studies allowed for the rational design of a new generation of ligands and tracking of the efficiency of structural optimizations. First iteration
enlargement of the aromatic portion of the anthranilic acid to a naphthalene or tetrahydronaphthalene system (compound [186]). As revealed by the crystal structure, tetrahydronaphthyl ring fitted tightly into a hydrophobic pocket of the active site. The carboxylate was an actual metal chelating group, whereas the sulfonamide moiety simply ensured the proper twist of the molecule to point the aromatic rings into a lipophilic environment. Unfortunately, these inhibitors exhibited a limited cellular activity and extensive binding to human serum albumin. A positively charged ortho substitution in the arylsulfonamide ring was predicted to obey these drawbacks, in particular to disrupt interactions with albumin [187]. A set of substituents to the molecule [186]. As revealed by the crystal structure, tetrahydronaphthyl ring fitted tightly into a hydrophobic pocket of the active site. The carboxylate was an actual metal chelating group, whereas the sulfonamide moiety simply ensured the proper twist of the molecule to point the aromatic rings into a lipophilic environment. Unfortunately, these inhibitors exhibited a limited cellular activity and extensive binding to human serum albumin. A positively charged ortho substitution in the arylsulfonamide ring was predicted to obey these drawbacks, in particular to disrupt interactions with albumin [187]. A set of substituents to the structure based on complex amines and diamines was introduced at this position and positively validated the approach. The modified products (exemplified by 75, Fig. 26) showed potent activity for MetAP2, associated with high selectivity versus related aminopeptidases (3000-fold ratio less active for MetAP1, for example) [187]. Importantly, their efficiency in cell proliferation assays was improved by a greater than 100-fold gain in potency and ranked in low nanomolar range. Similar parameters were achieved for 5,6 disubstituted anthranilic acids containing and additional heteroatom group that was presumed to tighten interactions with manganese ions [188]. Since they exhibit strong anti-cancer activity, enhanced accessibility and oral available such sulfonamides could be considered as optimized for therapeutic use [189].

A cooperative binding mode was observed for quinoline-based sulfonamides potent towards the E. coli MetAP1. These inhibitors were discovered by screening of a 100,000 member small organic compounds library [190]. Selected hits were screened for different metalloforms of the enzyme, with the highest affinity towards the cobaltated form displayed by compound 76 (Fig. 27). The sulfonamides behaved as typical competitive inhibitors; however, as disclosed by the X-ray structural studies, their mode of interactions was not typical. Consistent with data described for the heteroaromatic ligands, the enzyme active site was loaded with three metal ions. The inhibitor bound as a bidentate sulfonamide/quinoline N,N donor to the auxiliary manganese or cobalt atom [190]. Although the methanesulfonyl fragment was deeply buried, the molecule had no direct interactions with the catalytic metal ions.

14. Tetralone derivatives

First described in 1994 by Schalk et al. derivatives of 3-amino-2-tetralone were found to be nanomolar inhibitors of porcine kidney aminopeptidase N [191]. These compounds (77 and 78, Fig. 28) have non-peptidic character and are competitive inhibitors of the enzyme. The possible mechanism of their coordination with enzyme zinc ion is via carboxyl and amine groups located on the neighboring carbons in the cyclohexyl scaffold. Interestingly, these compounds were almost completely inactive towards aspartate and arginine aminopeptidases and only slightly active towards LAP.

In another approach Albrecht et al. synthesized various new derivatives of 3-amino-2-tetralone [192]. Several methyl ketone, substituted oximes or hydroxamic acids, phosphinic acids and hydrazides derivatives (exemplified by compounds 79–81, Fig. 29) were obtained and tested towards leucine aminopeptidase, aminopeptidase N, Aeromonas proteolytica aminopeptidase, and leukotriene A4 hydrolase. Even if theoretically equipped with better zinc chelating groups, these compounds were rather weak (up to low micromolar Ki values) inhibitors of aminopeptidases with one active site zinc and very weak inhibitors of aminopeptidases with two zinc ions.

15. Gallic acid derivatives

Inhibitors bearing gallic acid in the structure were designed based on the previously known biological properties of this compound as well as by assumption that methoxy (natural gallic acid has three free hydroxy groups) substituted hydrophobic ring of this compound will tightly bind in the S1 pocket of aminopeptidase N. Several amino acids derivatives of this compound like 4-amino-L-proline, L-iso-glutamine and cyclo-L-iso-glutamine have been obtained [193–195]. Among them galloylalamide derivatives based on proline scaffold (compounds 82 and 83, Fig. 30) were extremely good inhibitors of aminopeptidase N. The best compounds had IC_{50} values in low nanomolar range [194].

In another study L-iso-glutamine and cyclo-L-iso-glutamine derivatives (compounds 84 and 85, Fig. 31) have been synthesized. These compounds were not as good inhibitors as proline derivatives and had IC_{50} values in middle micromolar range. Among them...
Curcumin invasion of APN-negative cells, what further strengthened growth factor [201]. Interestingly, curcumin did not in [199,200]. This compound interacted with CD13 in irreversible and of action as well as biological targets are extensively investigated is known as potent antitumor agent and currently its mechanism has been also described as inhibitor of aminopeptidase N. Curcumin cell invasion as well as induced angiogenesis by basic non-competitive mode and strongly inhibited APN-positive tumor IC

a couple of compounds were potent inhibitors of aminopeptidase N with IC50 values similar to bestatin (IC50 = 2.4 μM) [195].

16. Betulinic acid

Betulinic acid (86, Fig. 32) is a triterpene isolated from bark of several different plants (birch bark is rich source of this compound) [196]. This compound has been found to be potent inducer of apoptosis in cancer cells and is currently in clinical trials [197]. It is proposed that betulinic acid acts by increasing mitochondrial membrane permeability, which subsequently facilitates release of apoptosis stimulating proteins (cytochrome C). However, several others biological targets for this compound have been proposed and one of them is membrane aminopeptidase N (CD13). Melzig et al. determined the IC50 for betulinic acid against aminopeptidase to be 7.3 μM [198].

17. Curcumin

Naturally occurring in plants polyphenol—curcumin (87, Fig. 33) has been also described as inhibitor of aminopeptidase N. Curcumin is known as potent antitumor agent and currently its mechanism of action as well as biological targets are extensively investigated [199,200]. This compound interacted with CD13 in irreversible and non-competitive mode and strongly inhibited APN-positive tumor cell invasion as well as induced angiogenesis by basic fibroblast growth factor [201]. Interestingly, curcumin did not influence invasion of APN-negative cells, what further strengthened hypothesis that anti-invasive activity of this compound is a result of CD13 inhibition.

18. Concluding remarks and perspectives

Aminopeptidases play pivotal roles in the turnover of proteins and the regulation of intracellular amino acids pools. They are essential to the metabolism, growth and development of all cells and tissues, and are part of the processes that regulate our immune and neurological systems. They also perform a broad spectrum of functions outside the cell, on the surface or even in the surrounding milieu (receptors, hormone processing and regulation etc). Their involvement in the cause or maintenance of certain pathological diseases, particularly cancer, has focused our attention on their structure and function in the hope of developing new treatments. More recently, we have learned that aminopeptidases are also central to the cellular physiology of many parasites, including malaria, which has opened new avenues for development of anti-infectious disease drugs. Crucial to the development of new drugs, however, is our detailed understanding of the mechanism of binding and interaction of inhibitory compounds to the active site of their targets. The present review highlights how this has been progressing well for several aminopeptidases, including leucine, alanine and methionine aminopeptidases, but also exposes our lack of information on a large number of aminopeptidase families. Clearly, these gaps will be filled in time due to the improvements in inhibitor discovery and design (e.g. screening of chemical libraries followed by medicinal chemistry) and in methods for three-dimensional structure determination. The challenge will be to discover inhibitors with selectivity not only for specific enzyme types so as to avoid off-target effects on other systems, but can be delivered to block specific functions or physiological roles of a particular aminopeptidase since each enzyme often performs a variety of defined and ancillary roles.

Acknowledgements

Marcin Drag is supported by Foundation for Polish Science. Artur Mucha is supported by the Polish Ministry of Science and Higher Education (grant number N N302 159937). John P. Dalton is recipient of a Canada Research Chair (Tier 1) from the Canadian Institutes of Health Research (CIHR).

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