Leucine Aminopeptidase as a Target for Inhibitor Design

J. Grembecka and P. Kafarski*

Institute of Organic Chemistry, Biochemistry and Biotechnology, Wroclaw University of Technology, Wybrzeze Wyspianskiego 27, 50-370 WROCLAW, POLAND

Abstract: In this review we focus on the most effective and the most promising inhibitors of leucine aminopeptidase. Their binding modes to the enzyme, the attempt to explain the origin of the inhibitory activity, as well as the structure – activity relationship for some of these compounds are discussed. Besides, the structural and electronic requirements of the enzyme active site and the binding pockets, together with the specificity towards the ligands, based on the structural and kinetic data, are presented.

1. INTRODUCTION

Aminoexopeptidases catalyse the hydrolysis of the N-terminal peptide bond in proteins and peptides. They have a broad substrate specificity and are wildly distributed in many tissues and cells in plants, animals, bacteria and viruses, which indicate their significant role in various biological processes [1]. Aminopeptidases are essential for protein maturation, activation and the determination of their stability as well as in the degradation and regulation of hormonal and nonhormonal peptides. Altered aminopeptidase activity is associated with certain pathological disorders, such as: ageing, cancers, cataracts, cystic fibrosis and leukemias [1,2]. Since aminopeptidases appear to be involved in many important biological processes, the compounds that inhibit these enzymes are of medical and therapeutic significance.

One of the first discovered and most widely studied aminopeptidase with respect to sequence, composition, structure and mechanism of action is cytosolic leucine aminopeptidase (LAP, α-aminoacyl-peptide hydrolase (cytosol), E.C. 3.4.11.1). This enzyme is of significant biological and medical importance due to its altered activity, observed in some diseases, such as cancer [3], eye lens ageing and cataract [4]. Human leucine aminopeptidase may be important in the processing of antigenic peptides and in the determination of the immunodominance of various peptides [5]. Moreover, LAP may play an important role in early events of HIV infection and thus serum activity of this enzyme may be useful as a surrogate marker for HIV infection and a response to chemotherapy [6].

2. LEUCINE AMINOPEPTIDASE STRUCTURE

The structure of leucine aminopeptidase, its mechanism of action and the homology with other aminopeptidases were recently reviewed [1,2,7]. Thus, in this paper, we reduced the description of LAP structure to the size, which allows to explain the binding mode and the activity of the enzyme inhibitors.

2.1. Overall LAP Structure

Leucine aminopeptidase is a species specific but not organ specific [8,9]. The primary structure of the enzyme from beef and hog appear 91.5 % sequence homology, while 81% homology is observed for bovine and human enzymes. As the enzymes are also kinetically very similar, it is likely, that they share the same active site design [2]. The enzyme from porcine kidney (pkLAP) is the most extensively studied as a target for inhibitors, because of its availability (porcine kidney enzyme is commercially available). However, the enzyme from bovine eye lens (blLAP) is the only one for which X-ray structures of the native form [10,11] as well as those complexed with the inhibitors, namely: amastatin [12], bestatin [13,14], leucinal [11] and phosphonic analogue of leucine (Leu^P) [15] were reported. This revealed the binding modes and the nature of the interactions of these inhibitors with the enzyme. Based on very close similarity with respect to kinetic properties and apparent amino acid sequence of LAPs isolated from different sources [8], it seems that the model of leucine aminopeptidase proposed for bovine lens enzyme can be applicable to design the inhibitors for LAP from porcine and human tissues.

blLAP is a hexamer build of six identical protomers. Each of the subunits consists of 487 amino acid residues, forming single side chain and contains three Zn^{2+} ions [11,15]. Two zinc ions (Zn488 and Zn489) are placed in the active site of the enzyme and participate in the catalysis and in the substrate and inhibitor binding. The third zinc ion (Zn490) was found about 12Å far away the active site and probably serves a structural role.

The six blLAP protomers are assembled in a way, that they form 15Å in radius and 10Å high solvent cavity in the centre of the hexamer [16]. This causes that the access to blLAP active site is limited to the molecules with the maximal diameter of 7Å [17].

*Address correspondence to this author at the Institute of Organic Chemistry, Biochemistry and Biotechnology, Wroclaw University of Technology, Wybrzeze Wyspianskiego 27, 50-370 WROCLAW, POLAND, tel. (+48)(+71)3203682, fax (+48)(+71)3284064, e-mail kafarski@kchf.ch.pwr.wroc.pl

1389-5575/01 $20.00+.00

The binding the inhibitors to blLAP do not result in structural changes of the enzyme and the native structure of blLAP is very similar to the structures of the enzyme complexes with all mentioned above inhibitors [11,12,14,15,18].

2.2. LAP Active Site Structure

Two zinc ions: Zn488 and Zn489, which are present in the active site of leucine aminopeptidase are essential for the catalytic activity. They may by replaced by other divalent positively charged ions with different exchange kinetics. The readily exchangeable site1 (Zn488) can stoichiometically bind Zn^{2+}, Mn^{2+}, Mg^{2+} or Co^{2+}, while tightly bound Zn489 (site2) can only be replaced by Co^{2+} in the case when both sites are unoccupied [7,19-22]. The substitution of zinc by other ions decrease blLAP activity affecting both K_m and k_{cat}.

pkLAP contains one zinc ion in the native structure (corresponding to Zn489, site2) and unoccupied binding site1 per protomer [23]. The incubation of pkLAP with Mg^{2+} or Mn^{2+} results in the occupancy of the site1 by one of these ions and causes the significant increase of the enzymatic activity, with the most active MnZn form [24]. The substitution of the site1 affects both k_{cat} and K_m significantly [25]. Some activity exhibited by non-activated native pkLAP indicate that Zn489 is more important for catalysis than the metal ion in the site 1. This may arise from the catalytic role of Zn489 which binds the N-terminal α-amino group of the substrate of L-configuration, for which LAP is specific [26].

The three dimensional structure of the native blLAP revealed that Zn488 is coordinated by the carboxylate oxygens of Asp255, Glu334 and by the carbonyl and carboxyl oxygens of Asp332, while Zn489 is coordinated by the carboxylate oxygen atoms of Asp273, Glu334, Asp255 and the side chain amino group of Lys250, which is probably unprotonated [10,11], Fig. (**1a**). These two zinc ions are separated by about 3Å in the native structure of the enzyme and bridged by one water molecule (or hydroxide ion) [11]. The position of this water molecule is very close to the position of the oxygen atoms O1 of LAP inhibitors, (L-leucinal and L-Leu^P, Fig. (**1b** and **1c**)) [11,15]. Another LAP active site residues, which are important for the substrate and inhibitor binding, include: Lys262, which side chain amino group is involved in the hydrogen bond with the carbonyl oxygen of the intermediate or the oxygen atoms

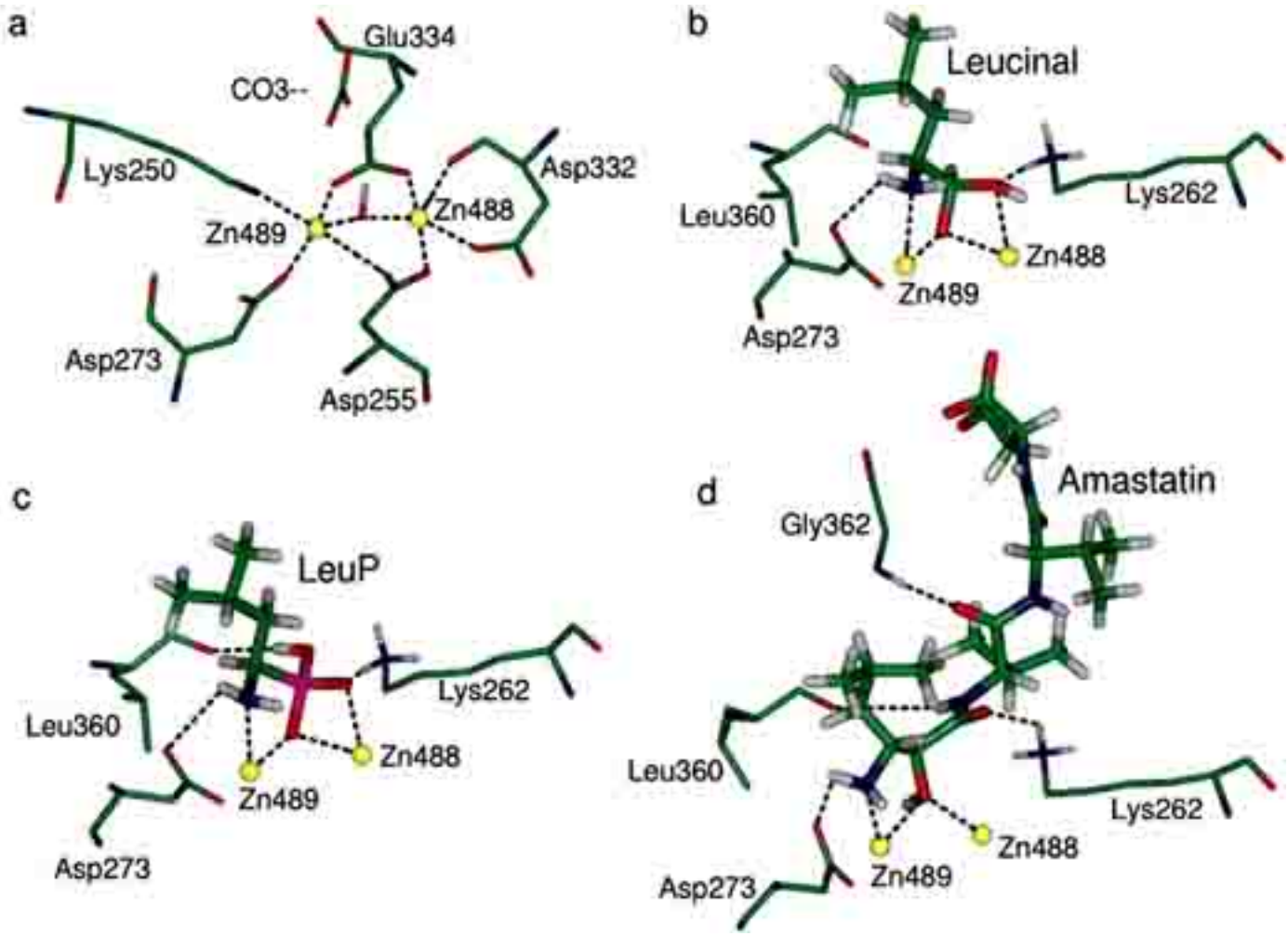

Fig. (1). The important amino acid residues of leucine aminopeptidase active site and the binding mode of LAP inhibitors: a) the active site of the native blLAP (pdb code – 1lam), b) blLAP-leucinal complex (pdb code – 1lan), c) blLAP-Leu^P complex (pdb code - 1lcp), d) blLAP amastatin complex (pdb code – 1bll). The hydrogen atoms were added in Insight_97.0/Builder program.

of the inhibitors, Fig. (**1b**-**1d**); Leu360, which carbonyl oxygen is involved in the hydrogen bond with the amide group of the scissle peptide bond of substrate or peptide analogues as well as hydroxyl group of amino acid analogues, Fig. (**1c** and **1d**); Asp273, which carboxyl oxygen forms the hydrogen bond with the N-terminal amine group of the substrate and inhibitors, and the amide group of Gly362, which interact with the P1' carboxyl group of the dipeptide substrate or its analogues.

Apart from the zinc ions, two positively charged moieties are also present in the enzyme active site. These are: amino group of Lys262, which participates in the substrate binding and the transition state stabilization during the hydrolysis of the peptide bond and guanidinyl moiety of Arg336, which adopt two distinct conformations (one of which is close to zinc ions) [10]. In the native structure of LAP, the guanidine and amide groups of Arg336 as well as the amide group of Gly335 and the carbonyl group of Leu360 are involved in the net of the hydrogen bonds with the active site bicarbonate ion, Fig. (**2**) [11]. According to the recently proposed mechanism of the peptide hydrolysis by LAP [27], this bicarbonate ion functions as a general base that accepts a proton from the zinc-bridging water nucleophile and shuttles it to the leaving amine group. The two zinc ions are involved in the deprotonation of the nucleophile and together with Lys262 cause the polarization of the substrate carbonyl group and the transition state stabilization. In the cocrystal LAP structures with the inhibitors (leucinal, Leu^P), this bicarbonate ion is replaced by two water molecules which probably form a bihydroxie ion ($H_3O_2^-$).

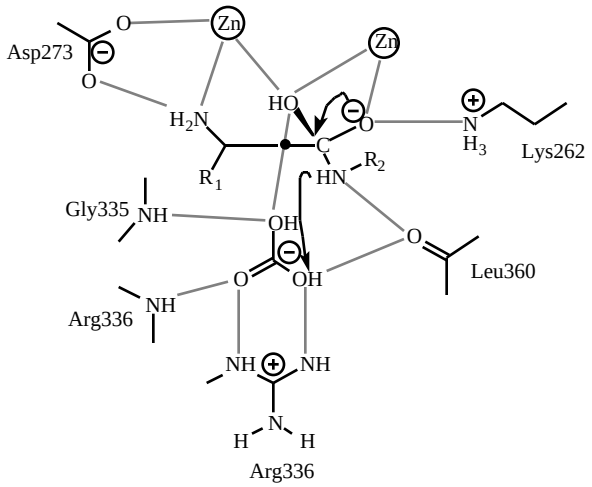

Fig. (2). Schematic representation of the mechanism of peptide hydrolysis by leucine aminopeptidase, proposed by Sträter at all [27].

3. LEUCINE AMINOPEPTIDASE INHIBITORS

The inhibitors of leucine aminopeptidase, can be subdivided into two categories: amino acid and peptide analogues. The analogues of amino acids contain the metal-binding moiety, which interact with LAP active site and the N-terminal hydrophobic group, bound in the S1 enzyme binding pocket, while peptide analogues contain the additional (P1', P2' etc.) residues, which enhance their complexation. Leucine aminopeptidase was extensively studied as a target for many classes of inhibitors. However, due to space restrictions, we focus in this review on the most potent and the most interesting ones, which resemble the substrate or the intermediate, and thus their binding modes may be deduced based on the crystal structures of the enzyme with the transition state analogues, as well as on some computer-aided modelling.

3.1. Analogues of Amino Acids

Due to the presence of two zinc ions in the LAP active site, the enzyme is inhibited by chelating agents, such as amino acid hydroxamates and amino thiols as well as boronic and phosphonic acid derivatives and aminoaldehydes, which are able to mimic the tetrahedral transition state after their binding by the enzyme.

3.1.1. Aminoaldehydes

α-Aminoaldehydes are low molecular, strong and competitive inhibitors of leucine aminopeptidase [28]. Among them L-leucinal, Fig. (**3**), is the most potent one, with K_i value 60nM towards pkblLAP. This inhibitor binds to LAP as a hydrated, *gem-diol* form, Fig. (**1b**), resembling the intermediate formed after the attack of a water molecule on the scissle peptide bond of the substrate. This explains very strong potency of this inhibitor. The nitrogen atom of the terminal α-amino group is coordinated to Zn489 and is probably unprotonated [29], which was seen from the significant decrease of the K_i values for L-leucinal with the increase in pH above 7.5-8.0. The gem-diolate oxygen O1, may be unprotonated upon binding, similarly to the O1 atom of Leu^P [15] and interacts with Zn489 and Zn488, replacing the water molecule bridging both zinc ions in the native blLAP structure. This oxygen atom is also involved in the hydrogen bonding with a water molecule present in the LAP active site [11]. Second oxygen atom of L-leucinal, O2, is Zn488 ligand and forms the hydrogen bond with the side chain amino group of Lys262. α-Amino group of the inhibitor is additionally involved in the hydrogen bond with the carboxyl oxygen of Asp273. Two active site metal ions in blLAP-leucinal complex are at the distance of 3.2 Å and are coordinated by six ligands in a distorted octahedral coordination geometry [11].

Hydrates of 3-amino-2-hydroxy-propionaldehyde are moderate, competitive LAP inhibitors, with K_i=85μM for the most active $HCl \times H_2NCH(i\text{-}Bu)CH(OH)CH(OH)_2$. Their binding mode is expected to be similar to α-aminoaldehydes [30].

3.1.1. α-Aminoboronic Acid Analogues

α-Aminoboronic acid derivatives form an another group of competitive, low molecular LAP inhibitors, with K_i values 0.13, 0.05 and 90 μM found for pinacol esters of H-boroLeu (Leu^B), H-boroPhe and H-boroAla [31]. It was reported that α-amino boronic acids derivatives might be bound to LAP active site as pinacol or propanediol esters. However, our attempts to dock these esters into LAP active

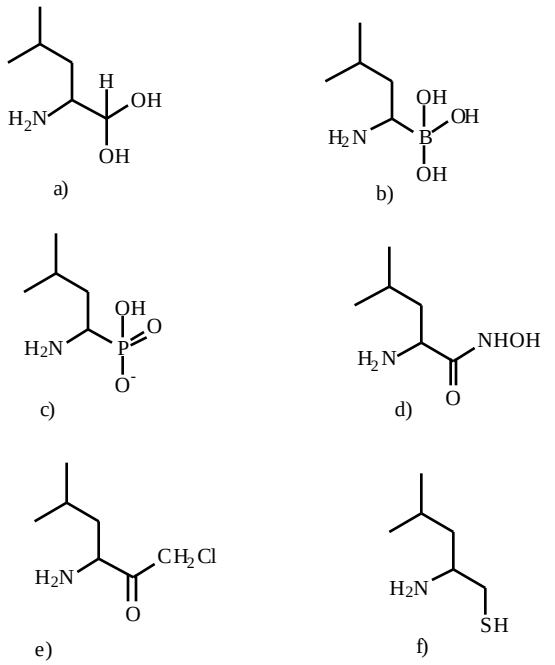

Fig. (3). The structures of amino acids analogues: a) leucinal, b) Leu^B, c) Leu^P, d) leucine hydroxamic acid, e) $LeuCH_2Cl$, f) leucinethiol.

site showed, that there are neither space nor favourable interactions for these hydrophobic groups (particularly for the pinacol moiety) [Grembecka, unpublished results]. The significant steric hindrances between the pinacol group and zinc ions as well as Asp332, which carbonyl and carboxyl oxygens are Zn488 ligands, were observed. Thus, the binding of the pinacol esters of boronic acids would demand the large conformational changes of the enzyme active site, which are strongly unfavourable, because of the strong interactions between zinc ions and their ligands, which keep the active site structure rigid. Consequently, we suggest that these compounds are bound to LAP as free acids, in a similar manner, as it is for the inhibition of serine proteases by peptide boronic acids [32]. Our assumption is supported by the fact, that the inhibitory measurements were carried out in conditions, which may promote the hydrolysis of these esters.

The α-amino boronic acids are potent leucine aminopeptidase inhibitors and exhibit the slow-binding kinetics, which is revealed by the slow decrease in the reaction rate, and which varies with the inhibitor concentration. The preincubation of the enzyme with the inhibitor for an appropriate period of time gave the linear reaction velocities, which are defined as the final steady state velocities. The slow-binding process involves rapid formation of the initial collision complex, its slow transformation to the tight complex and even slower deformation of this complex [2].

The boron atom of the aminoboronates has trigonal geometry and may form a negatively charged tetrahedral boronate ion in the active site of enzymes. Such structures are expected to act as the transition state analogues for proteases, Fig. (**3**) and such a tetrahedral complex of the phenylethane boronic acid with subtilisin (serine protease) was already observed by means of X-ray [33]. It seems that the slow binding kinetics exhibited by α-aminoboronic acid derivatives may involve the transformation of the trigonal boronic acid into the tetrahedral boronic ion. It is possible that the water molecule, present in the native LAP structure as di-zinc ligand [11] may be involved in the formation of the tetrahedral boronic moiety.

3.1.3. Phosphonic Acid Analogues of Amino Acids

Phosphonic acid analogues of L-amino acids are effective inhibitors of leucine aminopeptidase [34-36]. They are interesting inhibitors of the enzyme because oppositely to boronates and aldehydes, which are potent inhibitors of the serine proteases and form the covalent bond with the serine in the active site of these enzymes [37], the phosphonate analogues are not able to form such adducts and are rather weak inhibitors of serine proteases. However, as a result of the tetrahedral geometry of the phosphorus atom, these compounds are able to serve as the transition state analogues of the enzymatic peptide hydrolysis. The phosphonic and phosphinic acid analogues are the most successful inhibitors of other zinc proteases [38], which provide the interaction of the negatively charged phosphonate with a positively charged zinc ion in the enzyme active site.

The phosphonic acid analogues of L-amino acids were shown to be competitive inhibitors in relation to the substrate with the most active ones (similarly to Leu^B) exhibiting the slow-binding kinetics towards pkLAP. The origin of slow-binding process for these compounds is not clear. The most potent among them are the phosphonic analogues of the natural amino acids: L-leucine (Leu^P, K_i=0.23μM), Fig. (**3**), L-phenylalanine (Phe^P, K_i=0.42μM) [34] and L-valine (Val^P, K_i=0.15μM) [35]. L-isomers of these compounds are bound considerably more tightly than D-isomers, (with K_i values for L and D Leu^P being 0.23μM and 220μM, respectively) [34].

Phosphonic and boronic acids analogues of leucine are weaker inhibitors of the enzyme than L-leucinal. The similar binding mode of L-leucinal and L-Leu^P, revealed by the X-ray studies Fig. (**1b** and **1c**) [11,15], demonstrates that weaker binding of Leu^P than leucinal is mainly due to the different electronic and steric properties of these two moieties. This was confirmed by the calculations of the interaction energy of these two inhibitors with LAP active site [39] [Grembecka, unpublished results]. The P-C bond is 0.3Å longer than C-C bond of the putative intermediate, and a P-O bond is 0.1Å longer than a C-O bond. Moreover, the phosphonic amino acid analogues contain one sp^2 hybridized oxygen (O2) at the tetrahedral phosphorus, whereas in the *gem*-diolate transition state all atoms bound to the former carbonyl carbon atom are sp^3 hybridized [15], which is reproduced in L-leucinal after binding [11]. Thus the latter compound better resembles the transition state than phosphonic acid analogue. Another difference in the interactions of Leu^P and leucinal with LAP active site is the presence of the hydrogen bond between the O3H group of Leu^P and the carbonyl oxygen of Leu360, which is absent for leucinal. However, stronger binding of leucinal suggest that

this hydrogen bound, which is also present in the interactions of the transition state with the enzyme, do not influence significantly the inhibitor binding.

The modification of the phosphonic moiety in Leu^P structure, i.e. the replacement of one of its oxygen atoms by hydrogen, metoxyl, methyl or chloromethyl groups, resulted in significant lowering of the inhibitory activity [34,35]. The inhibition constants found for these derivatives correlate reasonably with their calculated total interaction energy with the enzyme active site [39]. These studies confirmed that the interactions of the unsubstituted phosphonic group with LAP active site are the strongest, and showed that the interactions with the enzyme Zn488 and Lys262, are very important for inhibitory activity.

The lack of the amino group in L-Leu^P structure resulted in 3300 times lowering of the activity [34], while its replacement by the hydroxyl moiety ($Leu^{P(OH)}$), lowered the activity about two orders of magnitude (K_i =28.5μM) [35]. This difference arises from both steric and electrostatic differences of Leu^P and $Leu^{P(OH)}$, which in turn is reflected in their interactions with Zn489 and Asp273.

3.1.4. Hydroxamate Amino Acid Analogues

The hydroxamate derivatives of amino and aliphatic acids are effective, competitive inhibitors of cytosolic leucine aminopeptidase and do not exhibit the slow-binding kinetics [40]. The enzyme is inhibited about 10 times more effectively by D-leucine and D-valine hydroxamic acids, Fig. (**3**) than by the appropriate L-isomers (K_i values for D and L-leucine hydroxamic acid are 1.3 $*10^{-6}$ M and 4.7 $*10^{-5}$ M, respectively). The activity differences between these D and L isomers are not such large as for the phosphonic acid analogues, where L-isomer was about 3 orders of magnitude more active than D-enantiomer [34]. Moreover, the absence of the α-amino group in the D-leucine hydroxamic acid do not result in the significant activity decrease (K_i=4.3$*10^{-6}$ M). These finding suggest that a particularly favourable geometry for binding existing between D-isomer and the enzyme, results from the formation of the bidentate complex between the hydroxamate fragment of the molecule and zinc ions, similarly as it was observed in X-ray structure of thermolysin with hydroxamate: $ClCH_2CO$-DL-(NOH)$LeuOCH_3$ [41].

The activity of L and D leucine hydroxamic acids increase about 200 fold after the replacement of Zn^{2+} by Mn^{2+} or Mg^{2+} in metal site1 pkLAP [40].

The studies of N-aminoacyl-O-4 nitrobenzoyl hydroxamates and the N-terminal free derivatives of these compounds exhibited that the protection of amino group do not result in any inhibition of the enzyme [42]. In opposition, the deprotected derivatives are competitive LAP inhibitors, with the lowest K_i value (39.5μM) for H-Phe-NHO-Nbz.

The hydroxymethylketones are weak LAP inhibitors, with K_i=160μM for HCl, H_2NCH(i-Bu)$COCH_2OH$) (DL). This compound may be compared with L-leucine hydroxamate and differs only with the replacement of the -NH- group by the $-CH_2-$ moiety, which results in several times lower activity. Hydroxymethylketones probably resemble structurally the hydroxamic acid, although the hydrate form, $-C(OH)_2-CH_2-OH$, which would make them similar to the transition state structure, may also be considered.

3.1.5. Chloromethyl Ketone Amino Acid Analogues

Chloromethyl ketones are moderate, competitive inhibitors of blLAP (K_i =0.226mM for $LeuCH_2Cl$, Fig. (**3**) and 0.337mM for $PheCH_2Cl$) [43]. Their binding mode in LAP active site is unknown, although the susceptibility of these compounds to the reversible addition of the nucleophiles is well known [44]. These strong electrophilic carbonyl compounds being the simple structural analogues of good substrates, might react reversibly with nucleophiles at the active site of aminopeptidase to form adducts resembling the "tetrahedral" intermediates forming upon hydrolysis of substrates.

There are some discrepancies in the literature concerning leucine aminopeptidase properties and its inhibitors. This is a result of the fact that the investigators and suppliers were not precise in the specifying the identity and source of the studied enzymes, which may lead to some difficulties in the distinguishing between cytosolic leucine aminopeptidase (E.C. 3.4.11.1) and membrane alanyl aminopeptidase (mAPP) (E.C. 3.4.11.2) [45]. The latter was referred in its earlier days as aminopeptidase M (microsomal or membrane aminopeptidase), reflecting its tight association with a microsomal membrane fraction in pig kidney from which it was purified. This enzyme was frequently confused with the cytosolic leucine aminopeptidase, because of their overlapping substrate specificity and similar tissue distributions. In some papers it is only mentioned that the studies concern pig kidney leucine aminopeptidase (without the description of the enzyme origin – cytosolic or microsomal). For example in the paper of Birch [46] chloromethyl ketones are studied as 'pig kidney leucine aminopeptidase inhibitors' and the results of these studies are in some other papers cited as those concerning cytosolic leucine aminopeptidase. However, the description of the experimental conditions (e.i. the lack of activation by metal ions, the absence of these ions in the assay mixture as well as low pH value (7.2) and potassium phosphate buffer used in these studies [46]) suggest that the membrane alanyl leucine aminopeptidase was studied. This may be also supported by the significant weaker activity of Leu and Phe chloromethyl ketones towards Mn^{2+} activated LAP reported by Fittkau [43] comparing with the data presented by Birch [46], where also no metal activation of porcine kidney enzyme was performed.

Moreover, as it was pointed out by Wilkes and Prescott [40] in the paper of Chan at all [47] 'microsomal leucine aminopeptidase' is the description given for the enzyme used in the experiments, while in the review of the literature and in the discussion of the results, the references relate entirely to the cytosolic leucine aminopeptidase. Thus, to avoid the mistake and in cases where there is no clear indication in the text, which enzyme was studied, it is worth to analyse the description of the enzyme preparation and the assay

conditions, which are different for cytosolic and microsomal leucine aminopeptidases.

3.1.5. Thiol Analogues of Amino Acids

The thiol compounds, which are known as strong metal chelators, are surprisingly poor LAP inhibitors (for L and D-leucinethiol, Fig. (**3**) K_i values are above 500μM with respect to pkLAP) [48]. The poor LAP inhibition by L-leucinethiol was explained as a result of the use of the particular $Mg^{2+}Zn^{2+}$ metallohybrid of LAP [49] [7] in the studies. Mg^{2+} (a hard acid) does not have as great affinity for thiol compounds (soft bases) as does Zn^{2+} (a soft acid). The marked difference in the relative hardness of Zn^{2+} and Mg^{2+} may particularly explain the unexpectedly weak binding of thiol compounds to pkLAP. The marked difference in the activity of bestatin, the strongest dipeptide LAP inhibitor, with respect to two $Mg^{2+}Zn^{2+}$ and $Mn^{2+}Zn^{2+}$ pkLAP isozyms, where K_i values are $2*10^{-8}$M and $5.8*10^{-10}$M respectively [49], may suggest that the thiol compounds would also be strongly bound to $Mn^{2+}Zn^{2+}$ pkLAP. The studies with Mn^{2+} activated enzyme would probably clarify this problem.

3.2. Analogues of Peptides

Based on the substrate binding and interactions, it may be assumed that the analogues of peptides should be able to interact with two metal ions and other active site residues of leucine aminopeptidase as well as with the enzyme S1, S1', S2' etc. binding pockets. Such pattern of binding may enhance their affinity.

3.2.1. Bestatin and Amastatin Analogues

The most potent, natural leucine aminopeptidase inhibitors: bestatin, a dipeptide analogue ([(2S,3R)–3-amino-2-hydroxy-4-phenylbutanoyl]-(S)-leucine), and amastatin, a tetrapeptide analogue ([(2S,3R)-3-amino-2-hydroxy-5-methylhexanoyl-S-valyl-S-valyl-S-aspartic acid]), Fig. (**4**), are slow and tight binding inhibitors of the enzyme, assumed as the transition state analogues [50,51]. Bestatin is the strongest known LAP inhibitor with K_i values being $5.8*10^{-10}$M, $2.8*10^{-8}$M and $2.8*10^{-8}$M for pkLAP $Mn^{2+}Zn^{2+}$, $Mg^{2+}Zn^{2+}$and $Zn^{2+}Zn^{2+}$ isozymes respectively, and $2.8*10^{-9}$M with respect to $Zn^{2+}Zn^{2+}$ blLAP [49]. For amastatin these values are $2*10^{-7}$M towards $Mg^{2+}Zn^{2+}$ pkLAP [52] and $3*10^{-8}$M with respect to $Mn^{2+}Zn^{2+}$ pkLAP [53].

The X-ray structures of these inhibitors with blLAP revealed their similar binding mode to the enzyme, Fig. (**1d**) [10,12-14,54] and they were used as the models for the determination of the binding of substrate transition state. The P1 residues of bestatin and amastatin have the additional carbon atom (C2) of the S configuration, with attached hydroxyl group and these P1 side chains are configured like that of a D-amino acid Fig. (**4**). This is in the contrast with LAP substrate specificity, because N-terminal D-amino acids appear to be poorly bound to LAP and their amides and peptides are not cleaved [26].

Bestatin and amastatin coordinate to the active site Zn489 in the bidentate mode by the nitrogen atom of the N-terminal α-amino group and P1 hydroxyl oxygen, Fig. (**1d**). This oxygen atom, which position is similar to the position of a water molecule in the native blLAP structure, interact also with Zn488. The P1 carbonyl oxygens of these inhibitors do not interact with Zn488 but are involved in hydrogen bonding with the side chain amino group of Lys262. Moreover, the P1' amide nitrogen forms the hydrogen bond with the carbonyl oxygen of Leu360. Finally, the C-terminal carboxyl oxygen of bestatin and the P1' carbonyl oxygen of amastatin are involved in the hydrogen bonding with the amide nitrogen of Gly362. The studies with bestatin isostere in which the P1 carbonyl group was replaced by the methylene group unable to form the hydrogen bond with Lys262, showed its 50000 times lower activity with respect to pkLAP (IC_{50} > 1mM) [55].

Bestatin contains three chiral carbon atoms and the studies of its stereoisomers revealed that the isomers with 2S configuration in the 3-amino-2-hydroxy-4-phenylbutanoyl (AHPBA) moiety, Fig. (**4**), inhibit pkLAP markedly [56]. Epibestatin (2R,3R)-AHPBA-(S)-Leu, which differs in the configuration of C2 atom, is about 3 orders of magnitude weaker inhibitor, while this difference is about 100-fold for amastatin and epiamastatin [52,56]. Other stereoisomers with 2R configuration show only slight inhibition of the enzyme (I_{50}>800μM). This is not surprising because for 2R isomers the interactions of the C2 hydroxyl oxygen with Zn288 and Zn489 are lost, if assuming their similar binding mode to this of bestatin.

The change of the configuration on the $C_{3\alpha}$ of the P1 residue of bestatin to S resulted in lowering of the activity about 10 times, while R configuration of the C-terminal Leu residue resulted in lowering the activity more than 300 times [56].

The replacement of the C-2 hydrogen of bestatin with a methyl group, reduces the inhibition 200-fold [52]. Our attempts to dock this inhibitor in LAP active site exhibited that such a poor activity may result from the existence of unfavourable steric hindrances between the attached methyl group and the enzyme Leu360 and Arg336 residues.

The deletion of the 2S hydroxyl group in amastatin, results in the loose of interactions with two zinc ions and in the lowering the activity by more that 30000 times (K_i=$68000*10^{-8}$M with respect to pkLAP) [52]. This inhibitor, however, still exhibits the slow-binding kinetics. Desaminoamastatin, which lacks the interactions of the 3-amino group with Zn489 and Asp273, is also very weak inhibitor of the enzyme (K_i=$74000*10^{-8}$M), but not slow binding one.

The isopropyl side chain of P1' valine residue of amastatin makes very little contact with the enzyme S1' pocket, which is formed by Asn330, Ala333 and Ile421 side chains [10,12]. The closest enzyme residues to valine side chain are Asp332 and Arg336 (3.6Å and 3.8Å away, respectively). The P2' and P3' residues of amastatin are very close to the solvent cavity at the centre of the blLAP hexamer and there are only few enzyme-inhibitor

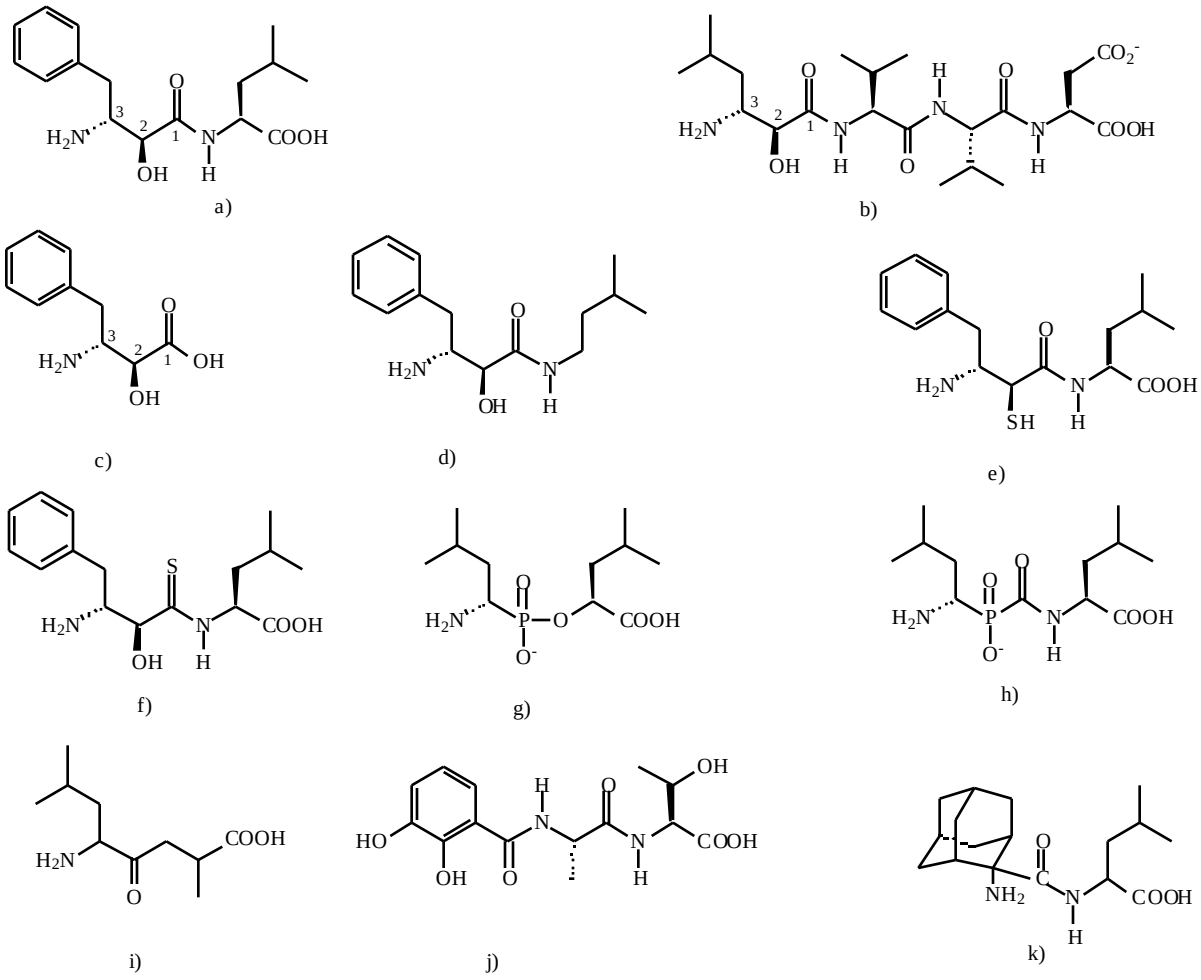

Fig. (4). The structures of peptide analogues: a) bestatin, b) amastatin, c) AHPBA, d) (2S,3R)-AHPBA-Iaa e) thiolbestatin, f) bestatin thioamide, g) Leu^{P}Leu, h) phosphonic acid analogue of bestatin, i) Leu^{K}Ala, j) (2,3-dihydroxybenzoyl)-L-alanyl-L-threonine, k) leucyladamantanine.

interactions. This may explain the fact that bestatin-dipeptide analogue, which has Leu in the P1' position, interact strongly with the S1' binding pocket and is more active inhibitor of the enzyme than amastatin.

The tripeptide analogue of amastatin, which lacks one Val residue is about 6-times weaker inhibitor, while the amastatin derivative containing the isoamyl amide moiety in the P3' position is only 4 times weaker pkLAP inhibitor (K_i=89*10^{-8}M) This supports the finding that exposed to the solvent C-terminal carboxyl group of amastatin is not required for activity. However, the substitution of the C-terminal, P1' residue in bestatin by Iaa (isoamylamine), yielding (2S,3R)-AHPHA-Iaa, Fig. (**4**), lowered the activity by a factor of 25, which means that this C-terminal COOH group, which is now involved in the hydrogen bonding with Gly362, ensure tighter binding.

The slow binding character of inhibition by amastatin and bestatin may be a result of the di-metal nature of the LAP active site. The binding modes of amastatin and bestatin by LAP determined by X-ray studies, indicate that these inhibitors most likely enter the active site via their N-terminus [12]. Thus, they encounter first the Zn488 and it is likely that they coordinate to this site before achievement of the site 2, which is placed deeper. This could involve the formation of the "initial" collision complex followed be the "final"- tightened complex. The K_i values measured for the "initial " and "final" blLAP-bestatin complexes are 1.1 10^{-7}M and 1.3*10^{-9}M, respectively [49], while for amastatin these values are K_i=2*10^{-5}M and K_i=2*10^{-8}M [52]. These assumptions were supported by the detection of two pre-steady-state intermediates in the hydrolysis of a C-terminal dansyl-modified dipeptide by di-metal forms of pkLAP under low temperature conditions [25,57] and one intermediate observed in the hydrolysis by single-metal native pkLAP. The ligand would bypass the unoccupied metal binding site 1 and bind directly to the site 2, which resulted in one intermediate [7]. The similar effects would also result in the slow-binding kinetics of some phosphonic and boronic amino acid analogues.

Zn^{2+} environment in blLAP structure changes slightly upon binding the inhibitors (Zn^{2+}-Zn^{2+} distances are: 2.9Å, 3.1Å, 3.3Å and 3.4Å in native, blLAP-bestatin, blLAP-amastatin and blLAP-Leu^{P} complexes, respectively [12]). The increased separation may be due to the coordination of both active site Zn^{2+} by the P1 hydroxyl oxygen, which may also result in the slow-binding of these inhibitors to leucine aminopeptidase. However, this is not supported by

the absence of slow binding kinetics for leucinal, which O1 atom also interacts with two zinc ions and for which the separation between these ions increase to 3.2Å.

The slow-binding inhibition of LAP cannot be explained rather as the molecular conformational change of the enzyme residues, because such changes are not observed when comparing the native blLAP and these complexed with the inhibitors: bestatin, amastatin, Leu^P and leucinal [12]. The r.m.s. deviation for the backbone atoms between the native blLAP and the blLAP-inhibitor complexes are lower than 0.5Å. Among the side chain atoms, only Arg336 was found in two different conformations in the blLAP-amastatin complex, one of which is closer to the di-zinc centre. However, for this conformational change, the energy barrier is not high (the adjustment in the diherdral angles about freely rotating single bonds of Arg336) and does not result probably in the slow binding kinetics.

Moreover, the structures of bestatin in the single crystal structure and in the blLAP-bestatin complex differ only by torsion angle changes, involving both the backbone and side chain atoms and these changes could be accomplished without crossing any large energy barriers [10]. Thus, the structural changes of the inhibitors are probably not responsible for their slow-binding kinetics.

3.2.2. Sulfur Containing Analogues of Bestatin

Sulfur containing analogues of bestatin were designed with the assumption that the replacement of the hydroxyl group, which interact strongly with two zinc ions in LAP active site by sufhydryl group should lead to the tighter binding of the inhibitors. However, these analogues of bestatin are significantly weaker inhibitors than bestatin itself [58]. The activity of α-thiolbestatin (K_i=0.55μM), Fig. (**4**), is about 30 times lower than that of bestatin, while α-thiolepibestatin (K_i=1.0μM) is 17 times more active than epibestatin. The decrease in the activity observed after the incorporation of the α-thiol group instead of the hydroxyl moiety to the P1 residue may result from the use of $Mg^{2+}Zn^{2+}$ pkLAP isozyme in the study, and is similar in nature to this observed for the thiol amino acid analogues.

The replacement of the P1' Leu residue of bestatin by the methoxy group, yielded the inhibitor (2S,3R)-AHPBA-OMe, which is 1650 times weaker (K_i=33μM) than bestatin, while the replacement of α-hydroxyl group in this analogue with α-thiol group, yielded (2S,3R)-α-thiol of AHPBA-OMe, an inhibitor, which activity is about 10 times lower (K_i=300μM) than the model compound. The lack of the carboxyl group in α-thiolbestatin (K_i=16.7μM) resulted in the lowering of the activity by about 30 times, comparing both with the model compound and with the α-hydroxy derivative (2S,3R)-AHPBA-Iaa. The substitution of the -OMe- moiety in (2S,3R)-α-thiol of AHPBA-OMe by the amide functionality and Iaa residue, favoured the binding to the enzyme by a factor of about 20, which may be caused by the additional stabilizing interactions of either the additional amide proton or the introduced isoamyl side chain. It is interesting, that oppositely to bestatin, the change of the configuration of C2 atom of this compound, did not change the activity significantly (K_i=26μM for (2R,3R)-α-thiol of AHPBA-Iaa) [58].

The replacement of the oxygen by the sulfur atom at the scissle bond of peptide substrates represent a minimal change in the structure [59,60] that may enhance the binding to the metal ion of aminopeptidases. Except for the length, the geometrical and the conformational properties of the thioamide bond closely resemble those of the amide. However, the thioamides are poorer pkLAP inhibitors than the amides itself [58]. Bestatin thioamide, for example (Fig. (**4**)), is about 10 times less active than the model compound. The similar difference of the activity was observed for the (2S,3R)-AHPBA-Iaa (K_i=0.5μM) and its thioamide analogue (K_i=4.4μM). Sulfur atom of the thioamide is larger and has the reduced hydrogen bonding capabilities, if compared with the oxygen of a peptide bond of bestatin, (involved in the hydrogen bond with Lys262) and this probably results in their poorer binding.

3.2.3. Phosphonic Analogues of Peptides

The phosphonic analogues of the presumed tetrahedral intermediate, the depsipeptides of leucylleucine (Leu^PLeu), Fig. (**4**), and phenylalanylleucine (Phe^PLeu) are only modest, competitive and apparently not slow-binding inhibitors of pkLAP, with K_i values of 58μM and 340μM respectively [34]. They are weaker LAP inhibitors than sole amino acid analogues (e.i. Leu^P and Phe^P). Although the binding modes of these compounds to LAP were not determined by X-ray studies, it seems that being the competitive inhibitors of the enzyme, they are placed in a similar manner to other transition state analogues. The oxygen atom of the dipeptide phosphonic analogues laying in a place of -NH- group of the substrate intermediate, cannot be involved in the hydrogen bonding with the carbonyl oxygen of Leu360, which is characteristic for the transition state structure. The electrostatic repulsion between these two oxygens may result in lower activity of this analogue. Moreover, this oxygen may have an influence on the polarization of the phosphonic group, weakening its interactions with two zinc ions and Lys262 in the active site of the enzyme. The bestatin phosphonic analogue, in which the carbinyl moiety was replaced by the tetrahedral phosphorus Fig. (**4**) is also quite moderate inhibitor of pkLAP (K_i=56μM). As it was revealed from our attempts to dock this inhibitor into blLAP active site, it is not as active as bestatin probably because of the slightly different geometrical arrangement of the phosphonic group in the active site of the enzyme and the presence of the additional oxygen atom, which respond to the position of C2 hydrogen atom in bestatin [Grembecka, unpublished results]. The close contact and unfavourable interactions of this oxygen with the carbonyl group of Leu360 may reflect the modest activity of this analogue.

3.2.4. Ketomethylene Peptide Analogues

The incorporation of the ketomethylene moiety instead of the amide bond provide the moderate or rather poor leucine aminopeptidase inhibitors, which are several thousand weaker than bestatin [61] and do not exhibit slow binding kinetics. In the case of the ketomethylene inhibitors, the

inhibitor affinity decrease with the increase of the peptide chain length was not observed and the analogues: Leu^K(R)-Ala, Fig. (**4**) and Leu^K(R)-Ala-Val-Iaa (K_i=0.35mM) are approximately equipotent, similarly to Leu^K(RS)-Phe, which activity is close to Leu^K(RS)-Phe-Val-Iaa (K_i=25μM). The replacement of Ala in the P1' position by Phe residue, enhance the activity more than 14 times in dipeptide and tetrapeptide analogues. Poor LAP inhibition by the ketomethylene isosters may be explained by the hydrolytic stability of these substrate analogues which do not resemble the tetrahedral transition state.

3.2.5. Peptide LAP inhibitors

Bacillus circulans was found to produce a compound, Bu-2743E ((2,3-dihydroxybenzoyl)-L-alanyl-L-threonine) Fig. (**4**), which showed potent inhibitory activity towards pkLAP (IC_{50}=12.5μM), but is still significantly weaker than bestatin. The C-terminal methyl ester of this inhibitor showed no inhibition towards the enzyme [62, 63]. The shift in the position of the hydroxyl groups in the catechoyl moiety to positions 3 and 4 lowered the activity more than 10 times (IC_{50}>200μM), an activity similar to this observed in the case of derivative, which contains only one hydroxyl group at the position of 2.

The tripeptide analogue, Z-Thr(Bu^t)-Phe-Pro-OH is quite strong, competitive and reversible inhibitor of leucine aminopeptidase (K_i=10^{-5}M) and the removal of its *tert*-butyl group, results in the tripeptide, which is quite good LAP substrate (K_m=5*10^{-5}M) [64]. This behaviour may be particularly explained by the observation of the NMR spectra of the compound in D_2O, where the exchange of the amide proton of the Phe residue was observed for the *tert*-butyl derivative, whereas no exchange was observed for the free tripeptide. It was suggested that in the latter one, the amide proton may be engaged in the intermolecular hydrogen bonds, being stabilized against the exchange with the solvent, while in the *tert*-butyl derivative this proton is exposed to the solvent. However, the connection between such observations and the functions of these compounds as LAP inhibitors or substrates is not clear. It is possible that the peptides containing the *tert*-butyl groups are protected against LAP enzymatic attack. This would be additionally supported by the fact that dipeptide derivatives in which adamantanine is placed at the P1 position Fig. (**4**), were not hydrolysed by leucine aminopeptidase and were weak inhibitors with the most active L- leucyladamantanine (K_i=1.19mM), which is a competitive pkLAP inhibitor [65].

4. THE S1 BINDING POCKET OF THE ENZYME

The S1 pocket of leucine aminopeptidase, which binds the N-terminal side chain of the intermediate is very important for the specificity of LAP in the binding of inhibitors and substrates. This pocket has a hydrophobic character and is formed by the residues of Met270, Ala451, Thr359, Gly362 and Met454. Consequently, the analogues of the natural amino acids with the hydrophobic side chains (e.i. Leu or Phe analogues) are the most effective LAP inhibitors. This may also explain why the inhibitors with short side chains (Gly or Ala analogues), or containing polar groups (Ser or Lys analogues) appear to be weak inhibitors of the enzyme [28,34,35,66]. For example, glycinal and alaninal exhibit about 4 and 3 orders of magnitude lower activity towards LAP than leucinal [28,66]. It is important to note, that this is in the agreement with the substrate specificity of the enzyme [24].

The importance of hydrophobic site was demonstrated when studying aminophosphonate inhibitors of LAP [34,35]. When comparing experimentally measured activities for Ala^P (K_i=240μM) and its higher homologue – 1-aminopropanephosphonic acid (K_i=3.6μM), the strong difference in their affinities were found, despite the small differences in their structures. Thus, the presence of only one additional methyl group increased the activity almost 67 times. Such a large difference in the activity, resulting from the presence of the additional methyl or ethyl group, were not observed for other analogues bound in the same binding pocket. This suggest that there is a minimal hydrophobic contact required for proper binding and further extensions of the hydrophobic side chain does not influence the affinity in such a drastic manner. The presence of the considered methyl group in the enzyme binding site could be needed not only to increase the contact area between the inhibitor and the enzyme, but also to "anchor" the inhibitor in the S1 binding pocket. The side chain of Ala^P interacts mainly with Met270 residue, while the analogue with additional methyl group is involved also in the interactions with Ala451 and maybe these contacts ensure the "anchoring".

The studies indicated that there is considerable space in the hydrophobic binding site of blLAP [67,68]. Based on the search performed using the LUDI program [69], it was found that the inhibitors with larger side chains than those present in Leu or Phe structures can be bound quite effectively [70]. Among the designed inhibitors there are the compounds with long hydrophobic side chains as well as the compounds containing polar groups, which can form hydrogen bonds with the additional enzyme residues, e.i. with carbonyl group of Ala451, carboxyl group of Asp365, amide group of Ala265 and amide and carbonyl groups of Ala263. These residues are placed far from the active site of LAP and the inhibitors interacting with them should contain a long hydrophobic chain with a terminal polar group. Obviously, they are not analogues of the natural amino acids and based only on the substrate analogy it would be rather impossible to design these potential LAP inhibitors. This finally shows the utility of the computer-aided methods for the design of enzyme inhibitors.

5. CONCLUDING REMARKS

The analysis of the leucine aminopeptidase inhibitors exhibit, that bestatin like compounds are the most tightly bound by the enzyme, while leucinal is the most potent among simple amino acid analogues. This comes from the fact that these inhibitors both sterically and electrostatically most closely resemble the structure of the presumed transition state during peptide bond hydrolysis. The X-ray studies of the native blLAP structure and the structures of the enzyme with its inhibitors allowed to understand the origin of the inhibitory potency, being also very useful in the

design of new enzyme inhibitors. The interactions with two zinc ions, as well as with positively charged amino group of Lys262 in the enzyme active site, which are electrostatic in their nature, appear to be the most important for tight binding of LAP inhibitors. The presence in the inhibitor structure the oxygen atom, which position resemble that of the water molecule bridging two zinc ions in the native blLAP is required for strong binding. Its absence results in lowering the activity by a factor of 100. The P1 carbonyl oxygen in the peptide analogues and O2 oxygen atom in the amino acid analogues, involved in the hydrogen bonding with Lys262 (important for transition state stabilization) ensure potent inhibitory activity. The same phenomena may be to some degree responsible for very weak activity of the thiol amino acid and peptide analogues as well as ketomethylene peptide analogues, which lack the possibility to form such a bond .

The lack of the α-amino group in the structures of LAP inhibitors makes these compounds only slightly active or totally inactive, while N-substituted derivatives are at least two fold less tightly bound than the appropriate unsubstituted derivatives. This confirms the necessity of the presence of a free α-amino group, able to interact with Zn489 and Asp273.

The structure-inhibition relationship for leucine aminopeptidase closely parallel to the substrate specificity of the enzyme. Leucine aminopeptidase is strongly specific for the N-terminal hydrophobic amino acids, which was exhibited by the 3-4 orders of magnitude decrease in the activity of the inhibitors which lacks or have short hydrocarbon side chain in the P1 position. The structure-based design of LAP inhibitors showed the possibility of binding in the S1 binding pocket the compounds, which contain long hydrophobic side chain bearing terminal polar group. The latter should be placed at a position, which ensures its involvement in the hydrogen bonds with some enzyme residues placed deep in this binding pocket.

Similarly to the S1 pocket, the S1' enzyme pocket is hydrophobic in its nature. However, the increase of the side chain in the P1' position does not result in such a drastic enhance of the activity (several times) as it is for the P1 residues. The presence of the free -COO^- group in the P1' position, which is involved in the formation of the hydrogen bond with Gly362, enhance the activity more than 20 times and confirms the importance of this bond for tighter binding.

The P2' and P3' residues, which are exposed to the solvent, are not important for binding and their presence usually lower the activity. Thus the inhibitory potencies of tri or tetrapeptides are lower than these of dipeptide analogues (e.i. bestatin and amastatin). This is also in agreement with the substrate specificity of cytosolic leucine aminopeptidase, which hydrolyse preferably di- or tripeptides.

The crystal structures of blLAP both as a free enzyme and in the complexes with inhibitors, revealed the nature of their interactions with the enzyme as well as explained the substrate specificity. This knowledge, together with plenty of kinetic data available for LAP inhibition by compounds of varying structures, may be very useful in the design of new potent enzyme inhibitors, as well as in explaining the origin of their inhibitory potency and evaluation of the mechanism of peptide hydrolysis by the enzyme.

ACKNOWLEDGEMENTS

This work has been supported by KBN grant 6 PO4B 001 15.

ABBREVIATIONS

AHPBA	=	3-Amino-2-hydroxy-4-phenylbutanoic acid
Ala^P	=	Phosphonic acid analogue of alanine
blLAP	=	Bovine lens leucine aminopeptidase
Bu^t	=	*tert*-Butyl group
Bu-2743E	=	2,3- Dihydroxybenzoyl)-L-alanyl-L-threonine
Iaa	=	Isoamylamine
LAP	=	Cytosolic leucine aminopeptidase
Leu^B	=	Boronic acid analogue of leucine
$LeuCH_2Cl$	=	Chloromethyl ketone analogue of leucine
Leu^KAla	=	Ketomethyl analogue of leucylalanine
Leu^P	=	Phosphonic acid analogue of leucine
Leu^PLeu	=	Phosphonic analogue of leucylleucine
$Leu^{P(OH)}$	=	Phosphonic acid analogue of α-hydroxyleucine
mAPP	=	Membrane alanyl aminopeptidase
$PheCH_2Cl$	=	Chloromethyl ketone analogue of phenylalanine
Phe^P	=	Phosphonic acid analogue of phenylalanine
Phe^PLeu	=	Phosphonic analogue of phenylalanylleucine
pkLAP	=	Porcine kidney leucine aminopeptidase
Val^P	=	Phosphonic acid analogue of valine

REFERENCES

[1] Taylor, A. *Trends Biochem. Sci.*, **1993**, *18*, 167.

[2] Taylor, A. *FASEB J.*, **1993**, *7*, 290.

[3] Umezawa, H. *Recent Results Cancer Res.*, **1980**, *75*, 115.

[4] Taylor, A., Daims, M.A., Lee, J., Surgenor, T. *Curr. Eye Res.*, **1982**, *2*, 47.

[5] Beninga, J., Rock, K.L., Goldberg, A.L. *J. Biol. Chem.*, **1998**, *273*, 18734.

[6] Pulido-Cejudo, G., Conway, B., Proulx, P., Brown, R., Izaguirre, C.A. *Antiviral Res.*, **1997**, *36*, 167.

[7] Kim, H., Lipscomb, W.N. *Adv. Enzymol. Relat. Areas Mol. Biol.*, **1994**, *68*, 153.

[8] Taylor, A., Volz, K.W., Lipscomb, W.N., Takemoto, L.J. *J. Biol. Chem.*, **1984**, *259*, 14757.

[9] Taylor, A., Surgenor, T., Thomson, D.K.R., Graham, R.J., Oettgen, H.C. *Exp. Eye Res.*, **1984**, *38*, 217.

[10] Burley, S.K., David, P.R., Lipscomb, W.N. *Proc. Natl. Acad. Sci. U. S. A.*, **1991**, *88*, 6916.

[11] Sträter, N., Lipscomb, W.N. *Biochemistry*, **1995**, *34*, 14792.

[12] Kim, H., Lipscomb, W.N. *Biochemistry*, **1993**, *32*, 8465.

[13] Burley, S.K., David, P.R., Sweet, R.M., Taylor, A., Lipscomb, W.N. *J. Mol. Biol.*, **1992**, *224*, 113.

[14] Kim, H., Burley, S.K., Lipscomb, W.N. *J. Mol. Biol.*, **1993**, *230*, 722.

[15] Sträter, N., Lipscomb, W.N. *Biochemistry*, **1995**, *34*, 9200.

[16] Burley, S.K., David, P.R., Taylor, A., Lipscomb, W.N. *Proc. Nat. Acad. Sci. U. S. A.*, **1990**, *87*, 6878.

[17] Fittkau, S., Kammerer, G., Damerau, W. *Ophthalmic Res.*, **1979**, *11*, 381.

[18] Sträter, N., Lipscomb, W.N., Klabunde, T., Krebs, B. *Angew. Chem. Int. Ed. Engl.*, **1996**, *35*, 2024.

[19] Allen, M.P., Yamada, A.H., Carpenter, F.H. *Biochemistry*, **1983**, *22*, 3778.

[20] Thompson, G.A., Carpenter, F.H. *J. Biol. Chem.*, **1976**, *251*, 1618.

[21] Thompson, G.A., Carpenter, F.H. *J. Biol. Chem.*, **1976**, *251*, 53.

[22] Carpenter, F.H., Vahl, J.M. *J. Biol. Chem.*, **1973**, *248*, 294.

[23] Van Wart, H.E., Lin, S.H. *Biochemistry*, **1981**, *20*, 5682.

[24] Smith, E.L., Spackman, D.H. *J. Biol. Chem.*, **1955**, *212*, 271.

[25] Lin, W.-Y., Lin, S.H., Morris, R.J., Van Wart, H.E. *Biochemistry*, **1988**, *27*, 5068.

[26] Smith, E.L., Hill, R.L. *The enzymes*; Boyer, P. D., Lardy, H. and Myrback, K., Ed., Academic Press: New York, 4, part A pp. 37-62.

[27] Sträter, N., Sun, L., Kantrowitz, E.R., Lipscomb, W.N. *Proc. Natl. Acad. Sci. U. S. A.*, **1999**, *96*, 11151.

[28] Andersson, L., Isley, T.C., Wolfenden, R. *Biochemistry*, **1982**, *21*, 4177.

[29] Andersson, L., MacNeela, J., Wolfenden, R. *Biochemistry*, **1985**, *24*, 330.

[30] Tarnus, C., Remy, J.M., d'Orchymont, H. *Bioorg. Med. Chem.*, **1996**, *4*, 1287.

[31] Shenvi, A.B. *Biochemistry*, **1986**, *25*, 1286.

[32] Kettner, C.A., Shenvi, A.B. *J. Biol. Chem.*, **1984**, *259*, 15106.

[33] Matthews, D.A., Alden, R.A., Birktoft, J.J., Freer, S.T., Kraut, J. *J. Biol. Chem.*, **1975**, *250*, 7120.

[34] Giannousis, P.P., Bartlett, P.A. *J. Med. Chem.*, **1987**, *30*, 1603.

[35] Lejczak, B., Kafarski, P., Zygmunt, J. *Biochemistry*, **1989**, *28*, 3549.

[36] Lejczak, B., Popiel de Choszczak, M., Kafarski, P. *J. Enzym. Inhib.*, **1993**, *7*, 97.

[37] Tschesche, H.J. *Medicinal Chemistry Advances*; De Las Herras, F. G. and Vega, S., Ed., Pergamon Press: Oxford, U.K., **1981;** pp. 411-422.

[38] Matthews, B.W. *Acc. Chem. Res.*, **1988**, *21*, 333.

[39] Grembecka, J., Kedzierski, P., Sokalski, W.A. *Chem. Phys. Lett.*, **1999**, *313*, 385.

[40] Wilkes, S.H., Prescott, J.M. *J. Biol. Chem.*, **1983**, *258*, 13517.

[41] Holmes, M.A., Tronrud, D.E., Matthews, B.W. *Biochemistry*, **1983**, *22*, 7955.

[42] Demuth, H.-U., Stockel, A., Schierhorn, A., Fittkau, S., Kirschke, H., Bromme, D. *Biochem. Biophys. Acta*, **1993**, *1202*, 265.

[43] Fittkau, S., Forster, U., Pascual, C., Schunck, W.H. *Eur. J. Biochem.*, **1974**, *44*, 523.

[44] Lewis, C.A., Wolfenden, R. *Biochemistry*, **1977**, *16*, 4886.

[45] Strater, N., Lipscomb, W.N. *Handbook of Proteolytic Enzymes*; Barret, A. J., Rawlings, N. D. and Woessner, J. F., Ed., Academic Press: New York, **1998;** pp. 1384-1389.

[46] Birch, P.L., el-Obeid, H.A., Akhtar, M. *Arch. Biochem. Biophys.*, **1972**, *148*, 447.

[47] Chan, W.W., Dennis, P., Demmer, W., Brand, K. *J. Biol. Chem.*, **1982**, *257*, 7955.

[48] Ocain, T.D., Rich, D.H. *Biochem. Biophys. Res. Commun.*, **1987**, *145*, 1038.

[49] Taylor, A., Peltier, C.Z., Torre, F.J., Hakamian, N. *Biochemistry*, **1993**, *32*, 784.

[50] Umezawa, H., Aoyagi, T., Suda, H., Hamada, M., Takeuchi, T. *J. Antibiot.*, **1976**, *29*, 97.

[51] Aoyagi, T., Tobe, H., Kojima, F., Hamada, M., Takeuchi, T., Umezawa, H. *J. Antibiot.*, **1978**, *31*, 636.

[52] Rich, D.H., Moon, B.J., Harbeson, S. *J. Med. Chem.*, **1984**, *27*, 417.

[53] Wilkes, S.H., Prescott, J.M. *J. Biol. Chem.,* **1985**, *260*, 13154.

[54] Ricci, J.S., Bousvaros, A., Taylor, A. *J. Org. Chem.,* **1982**, *47*, 3063.

[55] Harbeson, S.L., Rich, D.H. *Biochemistry,* **1988**, *27*, 7301.

[56] Suda, H., Aoyagi, T., Takeuchi, T., Umezawa, H. *Arch. Biochem. Biophys.*, **1976**, *177*, 196.

[57] Van Wart, H.E., Lin, S.H. *Proc. Natl. Acad. Sci. U. S. A.*, **1983**, *80*, 7506.

[58] Ocain, T.D., Rich, D.H. *J. Med. Chem.,* **1988**, *31*, 2193.

[59] La Cour, T.F.M., Hansen, H.A.S., Clausen, K., Lawesson, S.-O. *Int. J. Pept. Protein Res.*, **1983**, *22*, 309.

[60] Guthrie, D.J.S., Williams, C.H., Elmore, D.T. *Int. J. Pept. Protein Res.*, **1986**, *28*, 208.

[61] Harbeson, S.L., Rich, D.H. *J. Med. Chem.,* **1989**, *32*, 1378.

[62] Nakonieczna, L., Pastuszak, J.J., Chmiak, A. *Z. Naturforsch.*, **1989**, *44b*, 811.

[63] Kobaru, S., Tsunakawa, M., Hanada, M., Konishi, M., Tomita, K., Kawaguchi, H. *J. Antibiot. (Tokyo)*, **1983**, *36*, 1396.

[64] Jost, R., Masson, A., Zuber, H. *FEBS Lett.,* **1972**, *23*, 211.

[65] Nagasawa, H.T., Elberling, J.A., Shirota, F.N. *J. Med. Chem.*, **1975**, *18*, 826.

[66] Frick, L., Wolfenden, R. *Biochim. Biophys. Acta*, **1985**, *829*, 311.

[67] Taylor, A., Tisdell, F.E., Carpenter, F.H. *Arch. Biochem. Biophys.*, **1981**, *210*, 90.

[68] Taylor, A., Sawan, S., James, T.L. *J. Biol. Chem.,* **1982**, *257*, 11571.

[69] Ligand_Design 97.0 Molecular Modelling Program Package; Molecular Simulations Inc.: San Diego, **1997**.

[70] Grembecka, J., Sokalski, W.A., Kafarski, P. *J. Comp. Aided. Mol. Design*, **2000**, *19*, 531.