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Article

Phosphonic analogues of phenylglycine as inhibitors of aminopeptidases: Comparison of porcine aminopeptidase N, bovine leucine aminopeptidase and aminopeptidase from barley seeds

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Abstract: Inhibitory activity of 14 phosphonic analogues of phenylglycine, substituted in aromatic ring by fluorine and chlorine, was determined towards porcine aminopeptidase N. The obtained data served as a basis for studying their interaction with the enzyme as modelled by the use of Schrödinger Release 2018 program. The observed linearity between modelled Gibbs free energy differences and inhibitory constants indicated the usefulness of this program. The obtained binding mode was compared with this modelled for bovine lens leucine aminopeptidase. Although both enzymes differ in the number of zinc ions present in the active site, they are considered to exhibit similar activity towards substrates and inhibitors. Our studies seem to support that supposition since the modes of binding of the studied inhibitors are quite similar. Additionally, inhibitory activity of the phosphonic analogues of phenylglycine towards barley aminopetpidase was determined showing that this enzyme could be considered as neutral aminopeptidase.

Keywords: aminopeptidases, inhibitors, aminophosphonate, phenylglycine analogues, fluorine substituted, molecular modeling

1. Introduction

Aminopeptidases constitute a widespread group of exopeptidases with capacity to cleave peptide bond from the N-terminus of protein substrates [1,2]. They play a central role in the protein turnover, protein maturation, and generation or catabolism of bioactive peptides that are important in a variety of physiological processes [1,3]. Most important, human aminopeptidases are considered as important targets for search for potential anti-cancer agents [4-6] and the search for their inhibitors is an extensively developing field of research [6-11].

It is well known that libraries of structurally related, low-molecular enzyme inhibitors are a good starting point for the design of more potent ones following analysis of their interactions with enzymes at molecular level [13]. Quite frequently molecular modeling is of use as a tool in such studies. Having in hand a series of phosphonic analogues of phenylglycine [12], variably substituted in aromatic ring we decided to compare their activity towards commonly studied aminopeptidase N (alanine aminopeptidase, pAPN) from porcine kidney [11,14,15], a model enzyme for human one. Human enzyme is overexpressed in various types of cancer and on the surface of vasculature undergoing angiogenesis and thus being considered as a promising target for anticancer therapy [4,10-16].

In order to understand better the binding of these inhibitors molecular modeling was performed for porcine alanyl aminopeptidase (pAPN) bearing one zinc ion in the active site. For comparison also the mode of their binding to leucine aminopeptidase from bovine lens (blLAP) containing two zinc ions in the active site was determined by modeling.

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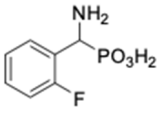
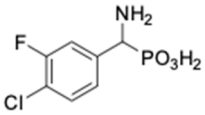
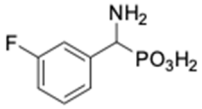
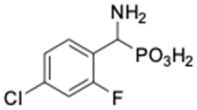
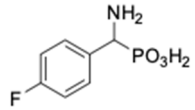
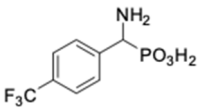
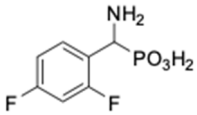
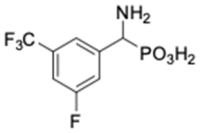
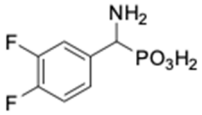
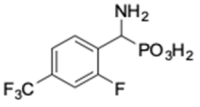
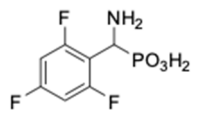
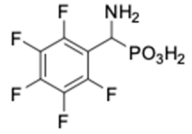
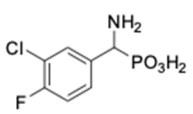
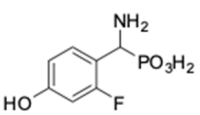
Finally inhibitory activity of the analogues of phenylglycines was determined for newly isolated and only preliminarily characterized, metalloaminopeptidase from barley seeds [17,18]. These studies have been done in order to determine the similarity between plant and mammalian enzymes.

2. Results and Discussion

The analogues of phenylglycine studied in this work contain mostly aromatic rings variably substituted with fluorine and chlorine atoms. Fluorine is considered as a good mimic of hydrogen in medicinal chemistry since it has been shown as being well tolerated by a variety of proteins. This results from the fact that carbon-to-fluorine bond is only slightly (about 20%) longer than carbon-to-hydrogen bond and thus do not introduce much steric perturbation. On the other the hand, electron-withdrawing nature of fluorine significantly affects electrostatic interactions. Chlorine is a spacious and strongly electronegative substituent.

Table 1. Inhibitory potencies of phosphonic analogues of phenylglycine towards porcine APN and aminopeptidase from barley seeds.

Compound	Structure	pAPN Ki [μM]	barley AP Ki [μM]	Compound	Structure	pAPN Ki [μM]	barley AP Ki [μM]
1		232±22	691±68	**8**		69.1±6.5	103±10
2		122±10	311±27	**9**		299±11	818±43
3		123±2.7	1105±57	**10**		56.3±2.2	686±70
4		258±23	1425±83	**11**		164±7.7	1033±73
5		101±3.8	549±54	**12**		un	un
6		un[1]	un	**13**		un	un[2]
7		45.4±4.5	62.1±2.3	**14**		116±22	un[3]

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[1] unactive at concentration of 1mM
[2] IC_{50}= 2.43 mM
[3] IC_{50}= 1.03 mM

As seen from Table 1 all of the evaluated compounds exhibited moderate, micromolar inhibitory activity towards pAPN and appeared to be competitive inhibitors. This is typical for low-molecular weight inhibitors.

The obtained results were additionally analysed by means of molecular modeling. As seen from Figure 1 modelled affinities of the aminophosphonates **7**, **8**, **9**, **10**, **11** (given as Gibbs free energy differences) are in linear agreement with observed K_i values, thus indicating correctness of the used approach (Figure 1).

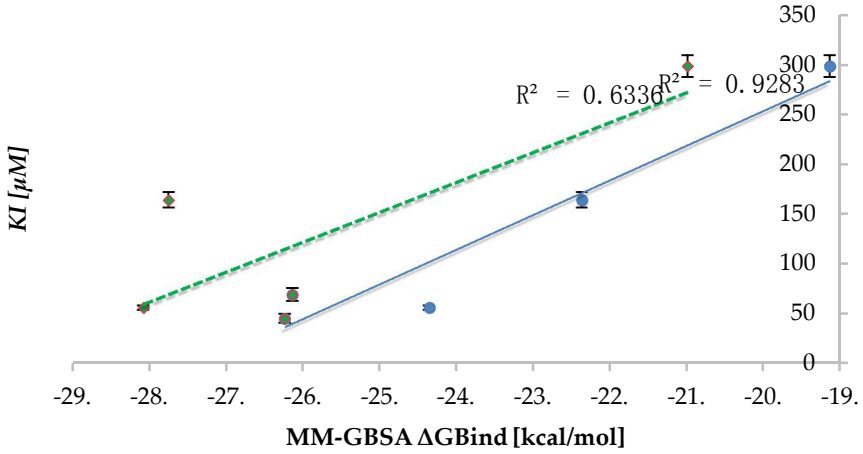

Figure 1. Relationship between experimentally determined values of K_i and calculated Gibbs free energy differences determined for pAPN. Blue line express the dependence for aminophosphonates of *S* configuration green dotted line for *R* configuration.

Quite interestingly this relationship is much better for analogues of *S*-configuration (corresponding to *D*-configuration of amino acids), with R^2 of 0.9283 versus 0.63357 for *R* isomers. This is commonly seen that inhibitory potency of low-molecular inhibitors to aminopeptidases is only slightly dependent on their configuration and that the expansion of their structure promotes stronger dependence [19].

For each pair of enantiomers the nature of binding of phosphonic analogues of phenylglycine to porcine aminopeptidase is quite complicated and depended on the absolute configuration of the α-carbon. This is seen from significant differences in energy binding values determined for both enantiomeric forms. The general tendency in binding mode is well exemplified by comparison of binding of both enantiomers of compounds **7** and **10** (Figure 2). As seen from Figure both enantiomers presented slightly different positioning in the active site.

The dominant component of the total interaction energy of the studied inhibitors with enzyme is the electrostatic one between phosphonate anion and zinc cation. The phosphonate group is involved in bidental zinc ion complexation and additionally forms hydrogen bonds with Tyr472, Glu406, Glu350 and Gln208. Especially, interaction with the hydroxyl group of Tyr472 indicates its important role for the hydrolytic mechanism, namely stabilization of the transition state.

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The hydrogen bonds are formed commonly between amino moiety and active site residues Glu350, Glu384 and Ala348 for isomers *S*.

Architecture of S1 pocket consists of hydrophobic walls with an open end resulting from the presence of Met349, Phe467 and Tyr472. The presence of Phe467 interacting with aromatic ring of R-enantiomer of **7** (most likely via formation of charge-transfer complex) affects the change of the relative positioning of aromatic portion of this isomer versus Tyr472 from face-to-face (observed for *S*-isomer) to face-to-edge presumably additionally increasing stabilization of *S*-aminophosphonate. Formation of charge-transfer interaction is, most likely, responsible for completely different binding of compound S-**7** than all the other analogues (Figure 3).

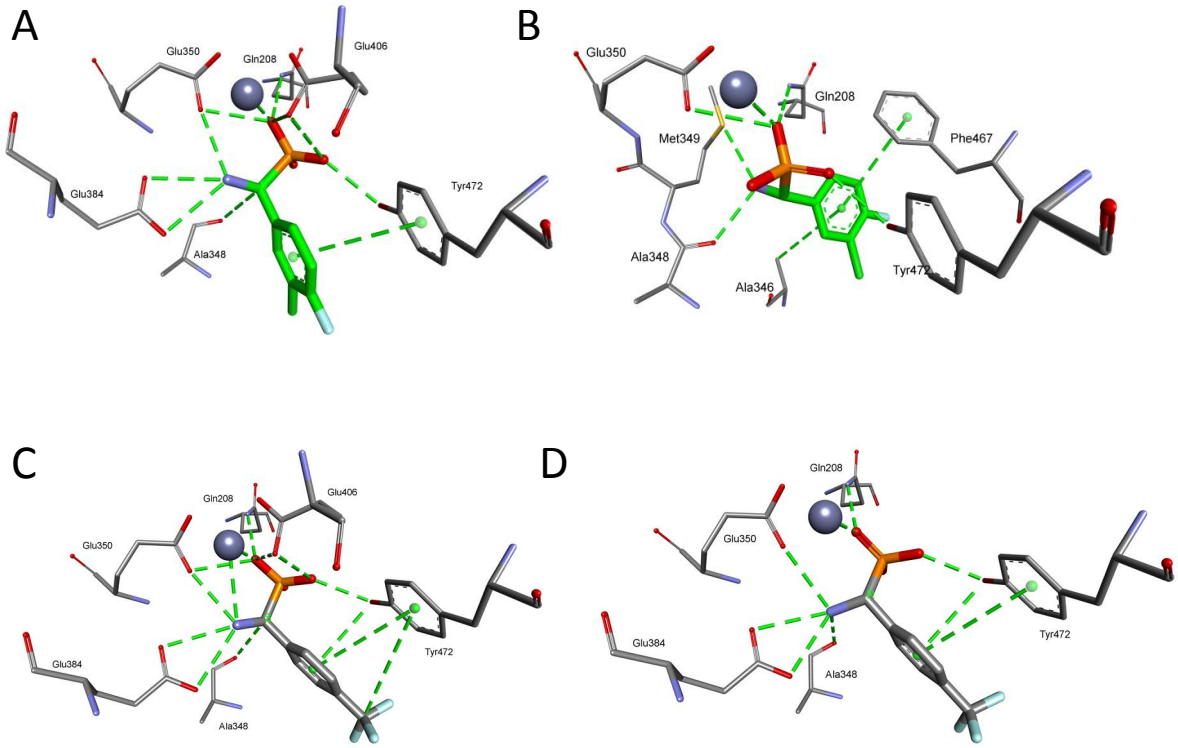


Figure 2. Mode of binding of both enantiomers of representative examples of phosphonic analogues of phenylglycine to porcine alanyl aminopeptidase A: *S*-isomer; B: *R*-isomer of compound **7**, C: *S*-isomer; D: *R*-isomer of compound **10**

All that subtle effects are responsible for the unexpected higher affinity predicted for phosphonic analogues of *S*-phenylglycines over their *R* isomers.

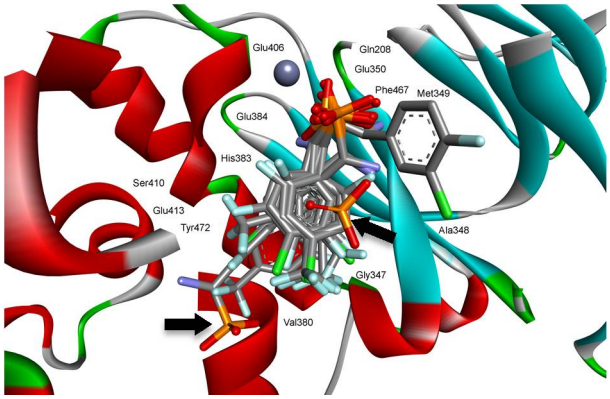
Generally, aromatic fragments of phenylglycine analogs fill the spacious, hydrophobic pocket of APN in a slightly different manner for each inhibitor in dependence on its structure (Figure 3). Quite

 doi:10.20944/preprints201905.0046.v1

interestingly, modeling of binding mode of compound **14** provided different results – its phosphonic moiety (despite on the enantiomeric form) does not interact with zinc ion and it is located in hydrophobic niche of the enzyme; that is differently than all of the phosphonic analogues of phenylglycine (Figure 3, phosphonic moieties indicated by black arrows). This, most likely results from competitive interaction of phenolic hydroxyl with His383 and Glu384 and phosphoric moiety benefices from ionic interaction with amine with Glu413.

Figure 3. Mode of binding of all the studied analogs of glycine (both enantiomers shown) in the hydrophobic cleft of porcine aminopeptidase (APN). Phosphonic groups of enanatiomers of compound **14** are shown by black arrows.

In the MEROPS peptidase information database [20] 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. The compounds, which have been reported as APN (belonging to M1 family) are known to exert also the inhibitory activity versus other zinc-dependent metallopeptidases. The most commonly studied one is bovine lens leucine aminopeptidase (blLAP, M17 enzyme), containing two zinc ions in its active site [21]. These two enzymes are reported to display similar activity-relationship towards both, synthetic substrates as well as inhibitors. In order to compare pAPN and blLAP, we have also modelled affinity of the studied compounds to blLAP. The obtained pattern of affinities of the aminophosphonates **7**, **8**, **9**, **10**,



and **11** (given as relation of Gibbs free energy differences) towards both enzymes are not linear indicating that pattern of activity of phosphonic analogues of phenylglycines towards these enzymes might be quite different, and thus indicates the differences between the two enzymes.

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Analysis of the binding of amino(3-fluoro-4-trifluoromethylphenyl)methylphosphonic acid (compound **11**) and of amino(2-fluoro-4-hydroxyphenyl)methylphosphonic acid (compound **14**) to bovine lens leucine aminopeptidase (Figure 4) showed that each of their two enantiomers is bound in a slightly different manner. This is also reflected in calculated Gibbs free energy difference, which is equal -36.06 kcal/mol for *S*-**11**, being -37.51 kcal/mol for its *R*-isomer and -33.30 kcal/mol for *S*-**14** and -37.62 kcal/mol for its *R* izomer. Thus, the inhibitory potency of phosphonic analogues of phenylglycine depended only slightly on their absolute configuration.

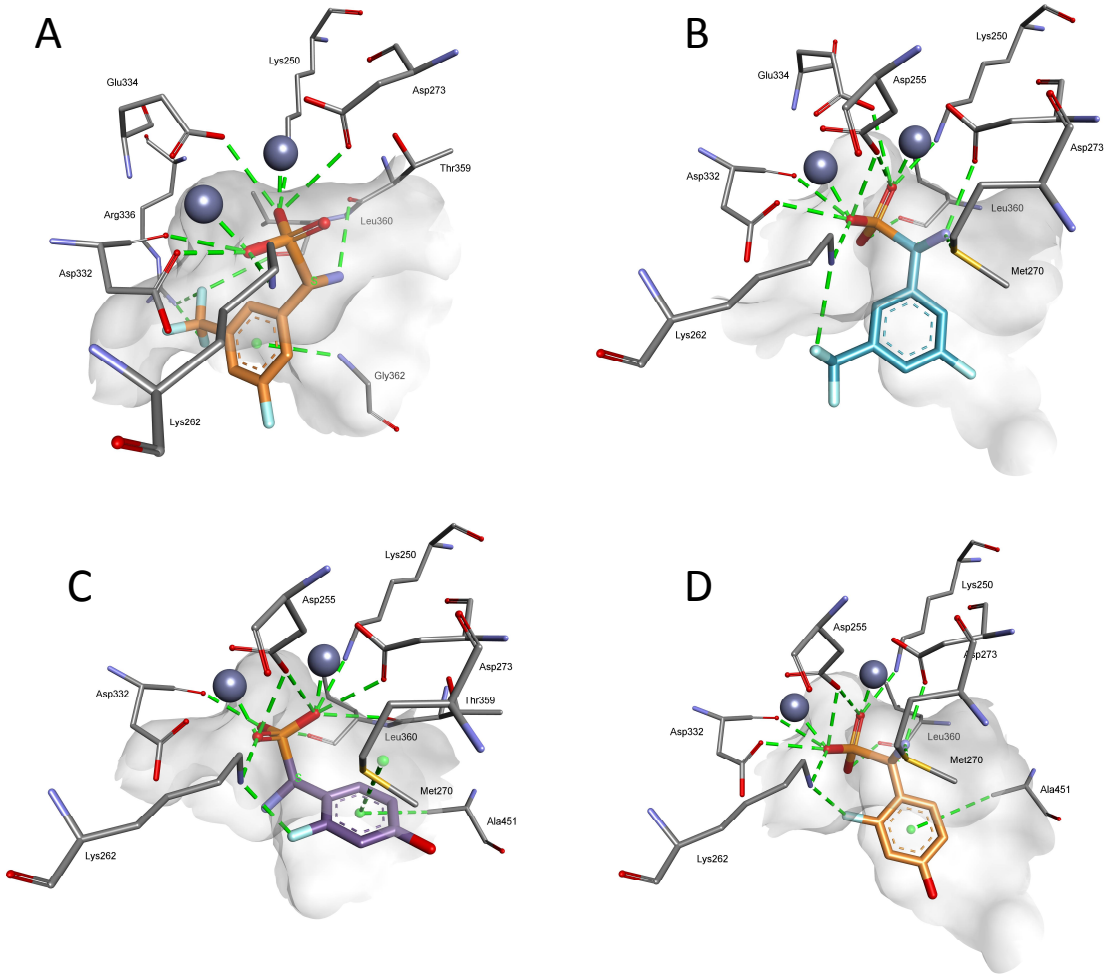

Figure 4. Mode of binding of both enantiomers of representative analogues of phenylglycine by bovine lens leucine aminopeptidase. A: *S*-isomer; B: *R*-isomer of compound **11**, C: *S*-isomer; D: *R*-isomer of compound **14**

There are not observed any of the characteristics hydrophobic contacts between aromatic rings and the surrounding residues. Lack of these interactions can partially explain lower differences in the free energies of binding. Also in this case the dominant component of the total interaction energy inhibitor and the enzyme is electrostatic one, occurring between phosphonate anion and zinc cations. As shown earlier phosphonate anion is bound stronger to one of the zinc ions. The electrostatic binding is strongly enforcement by interactions with Lys262, Asp332, Asp273 and Asp255, with the latter one being more pronounced in the case of *R*-isomers. Furthermore, an extra binding between one Asp273 and amino group of both enantiomers of compounds **11** and **14** is visible. In comparison with pAPN, amino group of inhibitors is not bound in a specific manner; there are various enzyme

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side chains involved in that binding of different analogues of phenylglycine and this effect is dependent on their structure.

Generally, architecture of binding the hydrophobic parts of the phosphonic analogues of phenylglycine is also dependent on the structure of their side chains and the aromatic fragments of these molecules are located in a spacious hydrophobic cleft of blLAP in a manner similar to that observed for pAPN. Exception is that hydrophobic portion of compound **10** is located in a significantly different manner. It is worth to mention that in the case of blLAP both isomers of compounds **14** are bound standardly - with phosphonic moiety interacting with zinc ions (Figure 5).

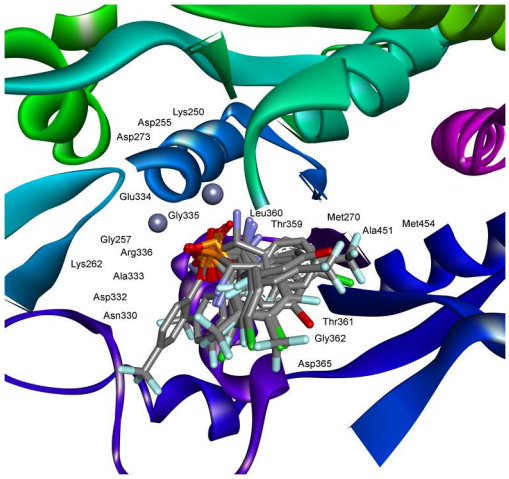

Figure 5. Mode of binding of all the studied analogs of glycine (both enantiomers shown) in the hydrophobic cleft of bovine lens leucine aminopeptidase (blLAP).

Genome analysis has indicated that plants, like animals, possess a variety of aminopeptidase genes, however, they have not been characterized suitably at the enzyme level so far [22,23]. Since the three-dimensional structures of plant enzymes are not known, it is only postulated that most plant enzymes belong to the class of leucine aminopeptidases (LAPs). Thus, in plants, there are basically two classes of LAPs [24]: the neutral ones (APN and its orthologs), which are constitutively expressed and detected in all plants, whereas the stress-induced acidic ones (LAPA) are expressed only in a subset of the *Solanaceae*.

Comparison of inhibitory potency against mammalian and plant aminopeptidases indicated that barley enzyme is less susceptible to the action of these aminophosphonates. Differences in inhibitory activities are well visible when comparing the effects of individual compounds toward both aminopeptidases. However, general trend for both enzymes is quite similar. Thus, compounds **7** and **8** were the most potent inhibitors and possess similar inhibitory activity towards both enzymes, whereas practically no inhibitory action was observed in the case of compounds **12** and **13**. The biggest differences were obtained for compounds **3**, **4** and **10**. These differences most likely reflect the differences in the architecture of these two enzymes in a part, which is interacting with *para* substituent of inhibitor. Our studies, although very peliminary, seem to indicate that barley seed enzyme falls into the family of leucine aminopeptidases.

Relations of of calculated Gibbs free energy differences obtained for pAPN and blLAP versus inhibitory activity of phosphonic analogues of phenylglycines **7**, **8**, **9**, **10**, and **11** towards aminopeptidase from barley seeds are almost linear and quite similar to each other (Figure 6). Thus, it is difficult to judge if plant enzyme should be considered as a member of M1 or of M17 family.

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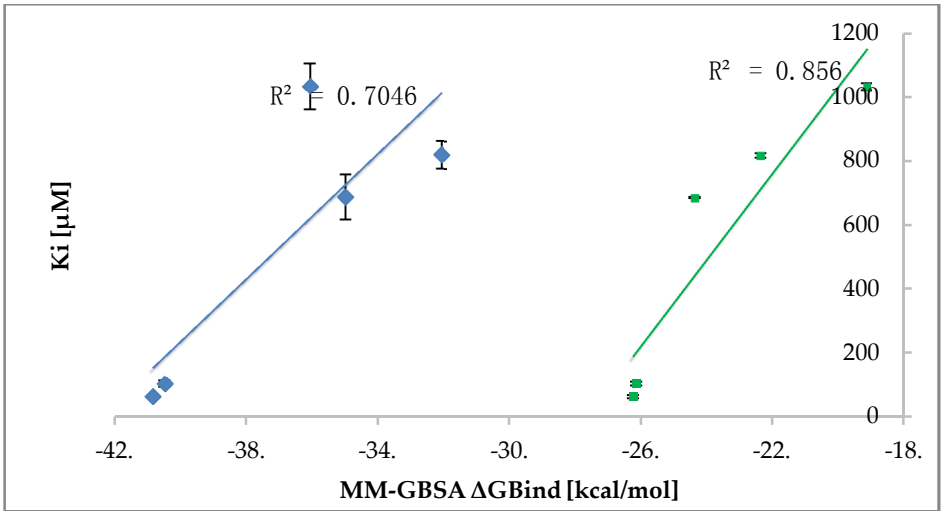

Figure 6. Plot of K_i vakues determined for compounds **7**, **8**, **9**, **10**, and **11** towards barley seed aminopeptidase versus Gibbs energy difference found for binding of *S*-isomers of these compounds to pAPN (green line) and biLAp (blue line)

3. Conclusions

Low-molecular enzyme inhibitors are commonly considered as a first step of designing more complex, more effective and more specific inhibitors of chosen enzyme. Being only roughly specific towards the enzyme they usually exhibit the effect at micromolar range. The studies presented in this work, although being in good agreement with general scheme of inhibitor design, show that this concept might be somewhat misleading. Our results indicate the different mode of interaction of single phosphonic analogues of phenylglycine with the enzyme spacious hydrophobic site, which binds aromatic portions of these molecules. Such a dispersion of binding modes of relatively simple and structurally similar compounds cause that it is not possible to choose one, specific lead compounds for further inhibitor design, even taking into consideration the most active ones because the differences in activity are small.

4. Materials and Methods

4.1. Compounds

Aminobenzylphosphonic acid were available from previous studies [25] and were synthesized based on the procedure decribed by Oleksyszyn and Soroka [26,27].

4.2. *Enzyme Preparations*

Microsomal leucine aminopeptidase (APN, EC 3.4.11.2) from porcine kidney was purchased from Sigma Aldrich. It was applied directly in kinetic measurements after dissolving in 50 mM potassium phosphate (pH 7.2). Such a solution might be stored in 4^0C not longer than 2 days**.**
Aminopeptidase from barley seeds (*Hordeum vulgare* L.) was isolated and purified basing on the procedure described previously [17]. The following steps were used: (i) ammonium sulfate precipitation; (ii) gel chromatography (Sephadex G-25, Sephacryl HR 300), followed by (iii) ion chromatography (DEAE – Sepharose). The molecular mass of the extracted enzyme was ~ 58kDa. Due to the low stability of the concentrated enzyme solution, it was prepared directly before each kinetic study.

4.3. *Kinetic characterization of aminopeptidases*

 doi:10.20944/preprints201905.0046.v1

Activity of pAPN (K_M = 0.52mM) was determined as described in literature [28], in 50mM potassium phosphate buffer, pH 7.2, using commercially available substrate *L*-Leucine-*p*-nitroanilide (*L*-Leu-*p*Na) dissolved in DMSO (used in 0.4-0.1 mM range) as substrate. The assay mixture, totally 1.05 ml, contained: the assay buffer, 0.025 ml of the substrate dissolved in DMSO, 0.05 ml of the potential inhibitor solution with appropriate concentrations and 0.01 ml of the enzyme solution (4μg/ml final concentration). The progress of enzymatic reaction was monitored spectrophotometrically (UV-VIS Spectrophotometer JASCO V-730) by following change in absorbance at 405 nm (formation of *p*-nitroanilide). The extinction coefficient for *L*-Leu-pNa was 9620 $M^{-1}cm^{-1}$.
Activity of aminopeptidase from barley seeds was determined in 50mM Tris-HCl, pH 8.0, containing 50mM NaCl and 10mM β-mercaptoethanol using *L*-Leu-pNa dissolved in DMSO. Kinetics parameters of the reaction were measured as described above. K_M value of barley seeds aminopeptidase was found to be 0.21 mM ± 0.014 and was determined by using the Lineweaver – Burk weighted regression method.

4.4. *Inhibitory studies*

The assay mixture, totally 1.095 ml, contained: 50mM Tris-HCl buffer (pH 8.0) with 50mM NaCl and 10mM β-mercaptoethanol, 0.025 ml of the substrate *L*-Leu-pNa dissolved in DMSO (used in 0.4-0.1 mM range), 0.05 ml of varying concentrations of the potential inhibitor (dependent on the potency of the compound) and 0.02 ml enzyme (0.030 mg of protein). The progress of the enzymatic reaction was measured at 37^0C for 10 min. Inhibition constants were determined considering four substrate concentrations and five concentrations of each inhibitor, with each measurement being repeated twice.
The rate of enzymatic reaction was studied following the reaction progress (changes in absorbance of *L*-Leu-pNa over time). Kinetics constants, namely V_{max}, K_i, and IC_{50} and type of the inhibition were determined using Lineweaver-Burk, Dixon and Hanes Wolf procedures. For each parameter relative errors were calculated. In the case of K_i and IC_{50} the range of errors did not exceed 10% for each of the tested inhibitors.

4.5. *Molecular modeling*

Crystal structures of enzyme were obtained from the RCSB Protein Data Bank (PDB). Bovine M17 aminopeptidase 2J9A [29] and porcine M1 aminopeptidase 6VB0 [30] were prepared using the Schrödinger's Protein Preparation Wizard module [31]. Water and ligands were deleted followed by addition of hydrogen atoms, and het states generated using the Epik with consideration of experimental pH. The next step was optimization of structure with the use of PROPKA in the experimental pH as well and final minimization with OPLS3 force field.
All ligands were prepared and optimized with the OPLS3 force field in LigPrep Schrödinger's module [32] taking into consideration stereochemistry of alpha carbon atoms. Inhibitors were docked with the Induced Fit Docking Protocule [33]. The zinc atoms were selected as cetroid of box with size of 20 Å. In addition the metals atoms were selected as H-bond and metal constraint atoms in the protocol for both enzymes. Glide docking and prime retirement were followed as the recommended procedures when the XP precision was selected in the Glide re-docking. All of the docked ligands were primed again with Prime MM-GBSA protocol 34] and the compounds with the lower ΔG_{Bind} for each inhibitor were selected as final results.

Author Contributions: Individual contributions: conceptualization, P.K. and W.W.; methodology, M.P. and M.T.; investigation, W.W and M.T.; writing—original draft preparation, P.K. and W.W.; writing—review and editing, P.K.; visualization, M.T.; funding acquisition, P.K.

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Conflicts of Interest: The authors declare no conflict of interest.

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