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Conformational and SAR Analysis of NAALADase and PSMA Inhibitors

A. Jayne Oliver,[a] Olaf Wiest,[a,*] Paul Helquist,[a] Marvin J. Miller[a] and Martin Tenniswood[b]

[a]*Walther Cancer Research Center, Department of Chemistry and Biochemistry, University of Notre Dame, Notre Dame IN 46556-5670, USA*

[b]*Department of Biological Sciences, University of Notre Dame, Notre Dame IN 46556-5670, USA*

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Abstract—Prostate specific membrane antigen (PSMA) is a 110 kDa type II transmembrane protein that is expressed exclusively by prostate tumor cells and as such is a clear cellular target in the development of a new method for fast and reliable diagnosis of prostate cancer. PSMA is highly homologous to the neuropeptidase NAALADase, and it has been shown that inhibitors of NAALADase also strongly bind to PSMA. In an effort to better understand the structural basis of the inhibitory activity of more than 60 NAALADase inhibitors synthesized and tested by our group, we used Monte Carlo calculations employing the Merck Molecular Force Field to explore the conformational space available to a set of PSMA inhibitors. Conformational analysis indicated that the lower the number of unique conformations accessible by an inhibitor, the greater the biological activity displayed by the compound against LnCAP cells. This suggests that the difference in activity is largely entropy based. The key conformations associated with high activity are used to develop a simple pharmacophore model that led to the design of new, conformationally restricted analogues with potentially high activity in rational drug design.

Introduction

On the order of 180,000 men per annum are diagnosed with prostate cancer in the USA, of which 37,000 will die primarily due to two factors. The first is the nature of the disease itself, whereby the androgen independent prostate tumor cells generally only proliferate by 5% daily, hence rendering traditional chemotherapies ineffective as these therapies typically depend on targeting proliferating cells. Secondly, there are inherent difficulties in correct and early diagnosis of the condition. Although the current diagnostic method of serum prostate specific antigen (PSA) measurement is valuable, the test has serious limitations. Of patients diagnosed with prostate cancer as indicated by an abnormal PSA level, only 30% actually had the disease whilst a further 30% of the patients correctly diagnosed display a condition where the disease has extended beyond the prostate, and a cure is unlikely.[1] There is therefore an essential need to develop a new method for fast and reliable diagnosis of prostate cancer.

An alternative cellular target for the diagnosis and treatment of prostate cancer is the less well-known prostate specific membrane antigen, PSMA. This protein is highly homologous to the neuropeptidase, NAALADase (*N*-acetylated-alpha-linked acidic dipeptidase) that releases the neuro-transmitter, glutamate, from both the neuronal peptide *N*-acetylaspartylglutamate (NAAG) **1** and folate polyglutamate.[2–6]

NAALADase and other neuronal enzymes have been associated with disorders that involve improper glutamatergic neurotransmission such as in schizophrenia, seizures and other neuro-degenerative conditions such as Alzheimer's and Huntington's disease.[7] As such, efforts have been made to design and synthesize inhibitors of NAALADase. Among the most effective inhibitors are hydrolysis transition state analogues of NAAG that contain a phosphinate or phosphonate isosteric mimic **4** of the transition state leading to the tetrahedral

*Corresponding author. Tel.: +574-631-5876; fax: +574-631-6652; e-mail: owiest@nd.edu

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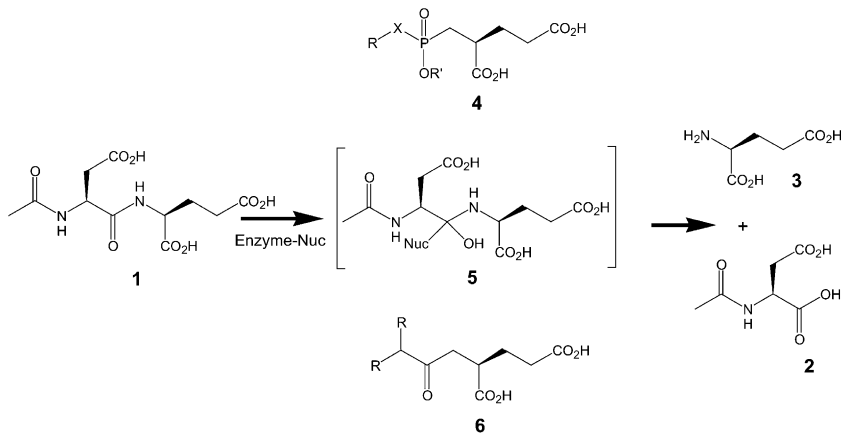


Scheme 1. Hydrolytic cleavage of the neuro-peptide NAAG by NAALADase via a tetrahedral intermediate **5**.

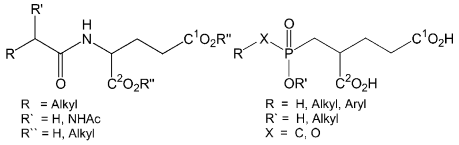


Figure 1. Substrate (left) and transition state (right) analogue inhibitor of NAALADase.

intermediate **5** (Scheme 1) generated during cleavage of NAAG 1.[8] We have found that various analogues of **1** are also potent inhibitors of PSMA.[9]

PSMA is also known as membrane glutamate carboxypeptidase mGCP, which is homologous to the transferrin receptor.[10] Since the amino acid sequence of PSMA has been established, the domain structure of PSMA can be predicted and the catalytic domain assigned to the M28 family of metallopeptidases. A recent crystal structure of *Aeromonas proteolitica* aminopeptidase (AAP), another member of this family, provides us with valuable information on the structure of the active site of such enzymes. X-ray crystal structures of AAP bound to transition state analogue inhibitor D-iodophenylalanine hydroxamate shows that a single oxygen of the inhibitor bridges between both Zn^{2+} ions with the concomitant loss of the native bridging water molecule.[11,12] This structure is similar to that seen for the AAP L-leucine phosphonic acid complexed structures, which again is an example of a transition state analogue inhibitor. Therefore it seems likely that the phosphorus substituted glutamic acid analogues **4**, will interact with the active site of PSMA in a similar way.[13,14]

In our initial earlier work, we therefore synthesized a diverse range of PSMA inhibitors based on two fundamental structures, the natural substrate and the phosphinate or phosphonate containing transition state analogues (Fig. 1).

These inhibitors were designed based on three key concepts. First, these compounds have the ability to inhibit catalytic activity, due to the presence of a tetrahedral intermediate considered to ligate to the zinc ions of the active site. Second, molecules are designed that inhibit PSMA due to the ability to utilize the specificity pocket. Third, the ability to suitably derivatize the inhibitor at position R by linking a suitably labeled head group would allow detection of prostate derived cells in circulation by florescence or MRI imaging whilst not impairing the ability of the inhibitor to access the active site. The head group of the inhibitors were also derivatized to investigate the effects of polar, nonpolar and ion chelating head groups such as hydroxamates and carboxylates on the overall activity of the compounds and transport properties of such molecules.

The activity of these inhibitors as determined by their IC_{50} in the NAALADase assay described earlier[9] ranged from 12.5 nMol to 12 mMol.[9] However, prior to our present studies, there was no clear rationalization for the structural basis of the different activities, since closely related compounds showed quite different activities. For this contribution, we have used Monte Carlo techniques and cluster analysis to develop a structure–activity relationship for the NAALADase inhibitors. We will show how a qualitative model can be refined using these methods to yield a simple pharmacophore model that accounts for the observed IC_{50} values and is in agreement with the information from the three dimensional structure of AAP. The understanding of the active conformations and the simple pharmacophore model derived from them can then be used to screen for the ability of other compounds to adopt the conformation associated with high activity and design new, potentially highly active PSMA inhibitors. Finally, we will show how the results of the conformational analysis can be used for the design of new class of conformationally restricted NAALADase and PSMA inhibitors.

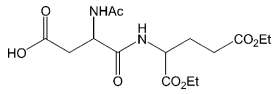
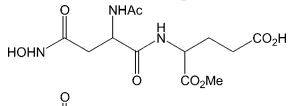
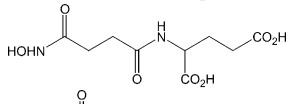
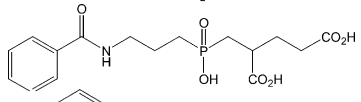
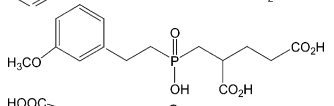
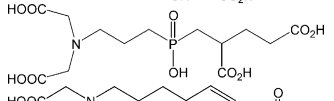
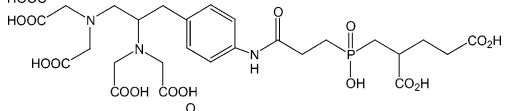
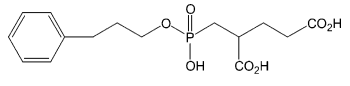
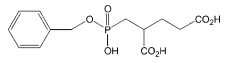
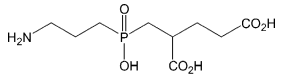
Computational methodology

Monte Carlo calculations using the Merck Molecular Force Field[15–19] (MMFF) as implemented in MACROMODEL,[20] were employed to investigate the conformational space available to each of a set of sixty NAALADase inhibitors. Solvation effects in water were included via the implicit Generalized Born Model

(GBS). The Merck Molecular Force Field was selected as the most suitable force field for these calculations as it seeks to achieve accuracy for small molecules in a combined organic/protein force field that is equally applicable to proteins and other systems of biological significance. The core portion of MMFF has primarily been derived from high-quality computational data where some 500 molecular structures used in the parameterization were optimized at the HF/6-31G* level. Parameterization was also based on a wide variety of chemical systems of interest in organic and medicinal chemistry, including many combinations of functional groups for which little experimental data are available. The methodology accurately describes intermolecular interactions in hydrogen-bonded systems and was tested extensively on a variety of small organic molecules.[15–19]

Although we studied more than 60 compounds tested earlier,[9] we will only discuss a representative subset of ten high, medium and low activity compounds, displayed in Table 1, where high activity is defined as an IC_{50} of less than 1 μM, medium activity as an IC_{50} of 1–10 μM and low activity as an IC_{50} greater than 10 μM.[9] Since the exact protonation state of a given compound in a physiological system is not known and might vary between different types of transition state analogues, we decided to consider only the fully protonated species for reasons of consistency. It was found that 20,000 Monte Carlo steps were sufficient to converge the conformational search for all the inhibitors in the data set. Typically, the calculations were found to converge on the global minima early in the first half of the search, whilst increasing the number of steps to 40,000 did not increase the number of unique conformations produced.

Table 1. Representative high, medium and low activity compounds[9]

		IC_{50} [μM]
7		1113.4
8		18.7
9		15.8
10		2.8
11		2.6
12		1.7
13		0.3
14		0.02
15		0.012
16		0.98

The conformations within 3 kcal/mol of the global minimum of each inhibitor generated by the Monte Carlo search were subjected to cluster analysis.[21] Although the use of a 3 kcal/mol threshold for the thermally accessible conformations is somewhat arbitrary, the results of the analysis presented here do not change significantly if other threshold values of less than 5 kcal/mol are used. Cluster analysis is performed by the XCluster program embedded within Macromodel. Monte Carlo calculations produce a large number of unique conformations when the potential energy surface available to a molecule is searched. It is sometimes of interest not only to find the global minimum structure but also how many of the conformations found are related to each other, whether they form conformationally related clusters or a random distribution. XCluster analyzes the output file, which contains the unique conformations generated by the Monte Carlo calculation, and searches for structurally related clusters by analyzing the matrix pairwise similarities. Conformations are said to form clusters when the similarities within the group are greater than the similarity between the groups. Significant clusters are thus distinguished by high values of separation ratio defined as the ratio of the smallest intercluster distance to the threshold distance that defines the cluster. All significant clusters were therefore identified and representative conformations generated by the XCluster routine. To generate the RMS deviations, the average structure found by the cluster analysis was superimposed onto the structure of the substrate and transition state analogues.

Results and Discussion

Conformational analysis

We started our investigation by calculating the conformational space accessible to each inhibitor, to identify any conformational trends that correlate with high biological activity. First, we tested the hypothesis that PSMA inhibitors would adopt a conformation similar to that of the natural substrate in the active site, in part by either mimicking the tetrahedral transition state generated during peptide hydrolysis, or by adopting a suitable configuration of the glutamate tail group. High activity compounds could then be pre-oriented to place the C1 and C2 carboxylates in a suitable orientation to interact with the basic amino acids of the specificity pocket and allow a suitable orientation to bind to the Zn^{2+} ions in the active site.

The importance of the conformational similarity of the backbone region was assessed by rigid body superimposition of the atoms 1–3 of the peptide bonds to be cleaved onto the corresponding atoms of the transition state mimic and the substrate analogue as shown in

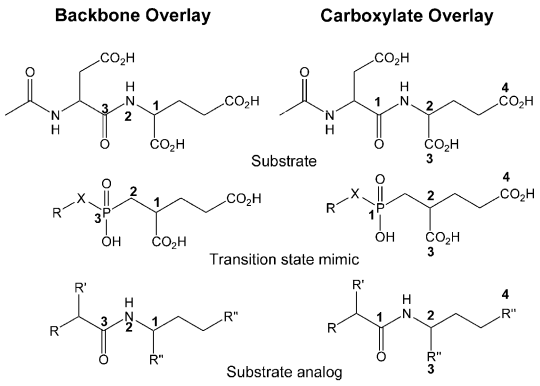


Figure 2. Rigid body superimposition of peptide backbone (left) and carboxylic acid tail groups (right) of substrate and inhibitors.

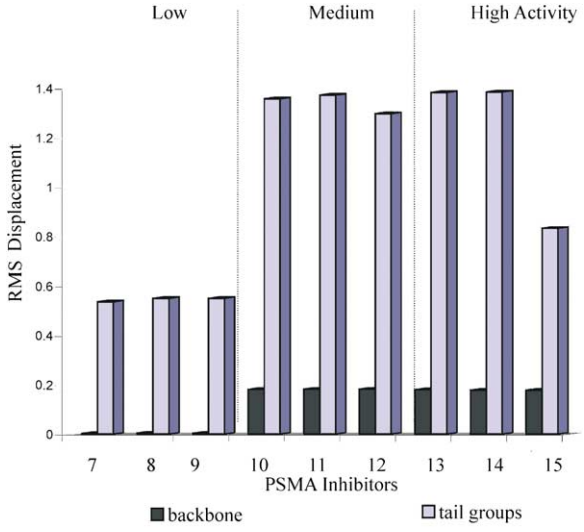


Figure 3. RMS deviations (Å) for substrate inhibitor superimpositions.

Figure 2 left. In analogy, the importance of the positioning of the carboxylate side groups was evaluated by overlaying the atoms as indicated in Figure 2 right. The root mean square deviation of the atoms of the representative conformation generated by Xcluster from a perfect superimposition onto the relative substrate atoms was then calculated and correlated with the experimentally determined PSMA activities found by Tenniswood and coworkers.[9]

Figure 3 shows the RMS deviations for the rigid body superposition of the peptide backbone of each inhibitor shown in Table 1 (**7**–**15**) onto the substrate and the RMS deviations for the rigid body superposition of the carboxylic acid tails of each inhibitor onto the corresponding atoms of the substrate. It can be seen that as we move across the series from the low to the high activity species, there is no correlation of the biological activity of the inhibitors with the RMS deviation. Instead, the RMS deviations appear as great for the high activity compounds as for the low activity compounds. This is consistent with the idea that the conformation of the free substrate used as a template is quite different from the one responsible for the activity

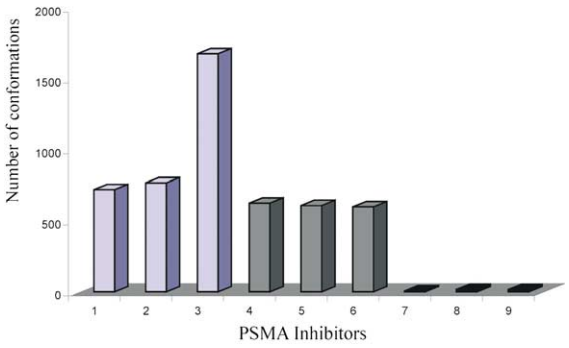


Figure 4. Number of unique conformations within 3kcal/mol of global minima.

of the inhibitors. A large number of conformations for some of the calculated compounds are within 3 kcal/mol of the global minimum and are thus easily accessible energetically. It can be postulated that different tail groups will alter the conformation of the inhibitor, therefore aiding or impairing their ability to adopt suitable zinc binding conformations or to access the specificity pocket.

Upon further analysis of the data, it can be seen that the *absolute* number of thermally accessible conformations correlates better with the experimentally observed activity. Figure 4 shows the plot of the number of unique conformations within 3 kcal/mol of the global minimum for representative members of the low, medium, and high activity groups (**7**–**15**). It can be seen that the most active compounds with IC_{50} values of less than 0.5 μM distinguish themselves by a very small number of thermally accessible conformations.

The origin of this effect can be illustrated as shown in Figure 5, where the overlay of the conformations within 3 kcal/mol of the global minimum for the low activity compound **7** are plotted on the left, while on the right, the analogous plot for the highly active compound **13** is shown. It is clear from this representation that **7** will have a substantial entropic penalty when binding to the active site of the enzyme. On the other hand, **13** is already pre-organized with five accessible conformations falling into two well defined clusters. The phosphinate group and carboxylic acid tail groups that are essential for binding overlay in each case very closely. This trend is typical of the high activity inhibitors in the data set. On further analysis, it is found that when the relative dihedral angles of C2 and C1 carbons to P-C′-C″ (defined as shown in Fig. 6) are compared for the high activity compounds, the C2 carbonyl is always found at ±60°, whilst C1 adopts a wide variety of positions ranging either between 70° and 120°, or between −120° and −160°. This is in line with the experimental evidence that suggests that the C2 group is more critical for activity as discussed below.

These results suggest that a major reason for the low activity of compounds such as **7** is the entropic penalty of constricting the large conformational space accessible to the molecule. Therefore, more active compounds could be obtained if the conformational space is

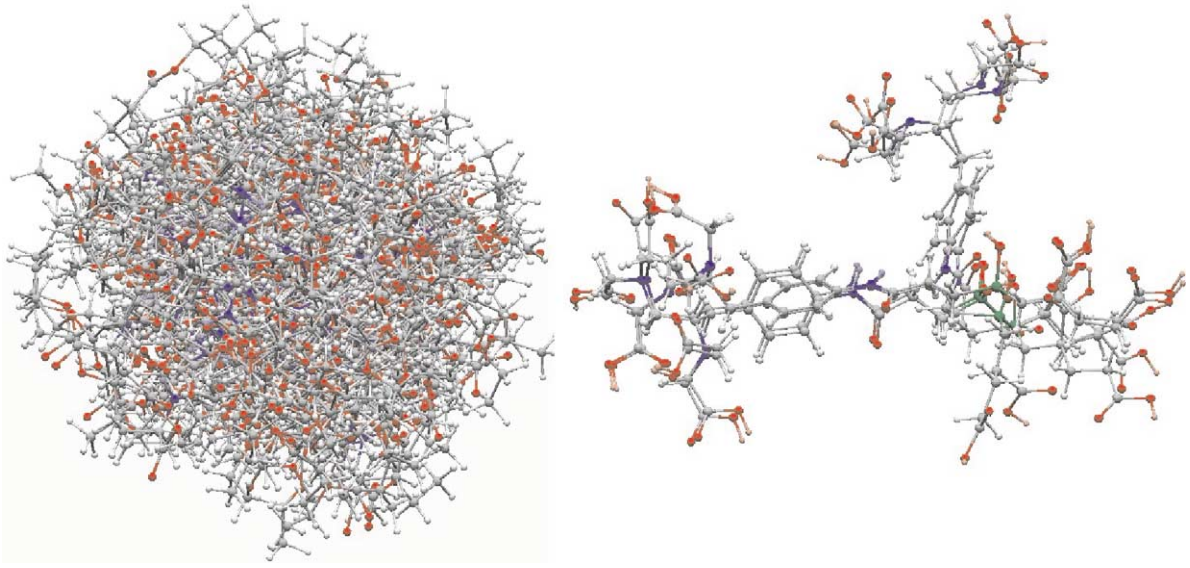


Figure 5. Overlay of all conformations within 3kcal/mol of global minima of compound **7** (left) and **13** (right).

restricted to the appropriate arrangement, for example through incorporation of the relevant groups into cyclic systems, as shown in Figure 7. The restricted conformations obtained for the high activity inhibitors such as **15** provide a basic pharmacophore identifying the spatial arrangement of the pharmacophoric elements and therefore lend themselves to providing a method of primary screening for biological activity of other compounds prior to synthesis. This simple model has allowed the assessment of the suitability of a number of conformationally restricted glutamate analogues **17** and **18**.

By superimposing the representative conformations of the glutamate analogues onto the simple pharmacophore, we can assess what ring sizes and diastereoisomers adopt conformations that are most like the conformations displayed by the high activity compounds whereby the analogues that generate conformations which superimpose the required key pharmacophoric elements (phosphinate or phosphonate center, C1 and C2 carboxylic acid tail groups) onto the corresponding atoms of the pharmacophore are predicted to have a greater activity. In this case we used the lowest energy conformation of the most active compound (compound **15**, Table 1) to represent the spatial arrangement of the pharmacophoric elements. The diastereomers of both the five and six membered ring restricted glutamate analogues **17** and **18** were then superimposed onto **15** as shown in Figure 8. It was found that in each case, the trans arrangement of the carboxylic acid groups afforded the best overlay with the pharmacophore, whereby the carboxylic acid groups are forced equatorial to the ring and result in a lower rms displacement than the corresponding *cis* isomers. Hence the trans isomers in each case were selected for synthesis, which will be reported separately.[22]

Structure–activity relationships. While the accessible conformational space is an important factor in determining the activity of the inhibitors, an examination of

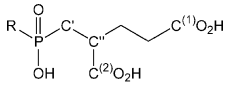


Figure 6. Illustration of measured dihedrals.

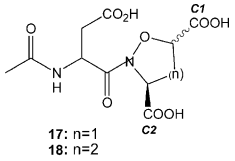


Figure 7. Conformationally restricted glutamate analogues.

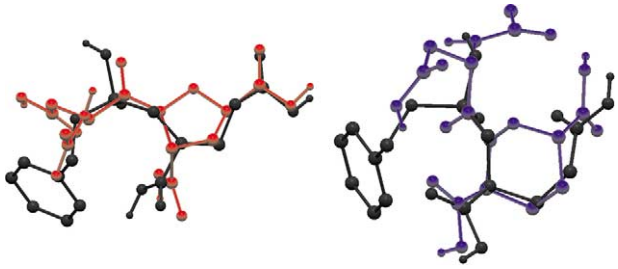


Figure 8. Overlay of **15** (black) and *trans*-**17** (red) and *trans*-**18** (blue).

the 60 compounds that were synthesized and biologically tested by Tenniswood and coworkers[9] for their ability to inhibit PSMA provides further information on other features of the structure–activity relationships (SAR).

First, it can be noted that the most active species in the data set had both C1 and C2 carboxylic acid tail groups in free, unblocked form analogous to the glutamate moiety of the NAAG substrate. As demonstrated in compounds **19**–**21**, shown in Figure 9, there is a greater than 40-fold activity penalty for blocking the C2 carboxylate, and a 50-fold decrease in activity is experienced when the C1 carboxylate is esterified. These effects appear to be additive, resulting in a complete inactivation when both carboxylic acids are esterified, as

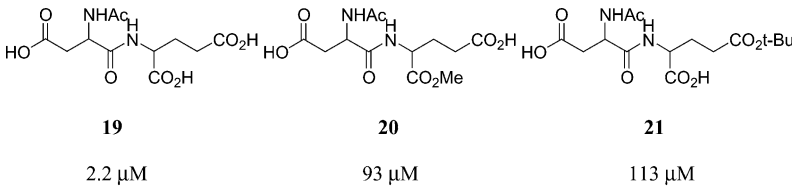


Figure 9.

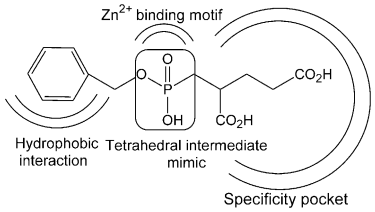


Figure 10. Qualitative representation of PSMA inhibitor/active site interactions.

can be seen for **7**. These observations are also true of the transition state analogues. Second, it is observed that the phosphonic and phosphinic acid derivatives **10**–**16** are significantly more active when compared to the substrate analogue inhibitors **7**–**9** or **19**–**21**, supporting the fact that these compounds in part are active due to their ability to mimic the tetrahedral transition state observed during enzymatic substrate hydrolysis. Therefore, the presence of a mimic such as a phosphinate and phosphonate center is essential in the design of PSMA inhibitors. Thirdly, intramolecular hydrogen bonding in some inhibitors results in a decrease in the number of conformations displayed, hence digressing from the relationship of the low number of conformations to high activity. Compound **10** in Table 1 is such an example, where each of the conformations within 3 kcal/mol of the global minimum is found to place the amide head group within hydrogen bonding distance of the phosphinate hydroxyl, hence reducing the number of accessible conformations. It is difficult to assess whether this effect is desired or not, whether the hydrogen bonding will aid in increasing activity by holding the C2 group in a position that is required for favorable interaction with the PSMA active site, or whether the hydrogen bonding reduces activity by impairing the ability of the inhibitor to develop favorable interactions with its environment, or by restricting its ability to form other low energy conformations. Fourth, the presence of an electron donating oxygen adjacent to the phosphorus as in the most active compounds **14** and **15** also appears to be desirable for increased activity. This is consistent with the idea that the phosphonic acid derivatives are more active than the phosphinic acid derivatives based on the former compounds ability to donate electrons to the phosphorus center, which will aid in the generation of a greater negative charge on the anion and subsequently greater interaction with the Zn^{2+} of the active site. As such, phosphonic acid derivatives were included in a subset of inhibitors, which were designed, subjected to conformational analysis, synthesized, and will be subsequently tested for their biological activity.[22] Finally, compounds which have an aromatic ring within three to five bonds of the phosphorus center also seem to display an increased high activity, implying that this region of the inhibitor may be placed in a hydrophobic pocket or interact with aromatic species involved in π stacking. Conversely, it can be noted that the presence of any functional group with the potential to generate a formal negative charge within the 3 to 5-bond linker region (apart from the position directly adjacent to the phosphorus) appears to result in a decrease in the activity of the inhibitor. These observations give some indication of what parameters are considered important in the design of new inhibitors and also provide additional information about the environment within the active site of the enzyme as summarized in Figure 10, which gives a qualitative representation of the interactions between the key elements of the pharmacophor represented by the most active compound **15**, and the PSMA active site.

Conclusions

The activity of PSMA and NAALADase inhibitors that are based on either substrate or transition state analogues can be understood in terms of the number of energetically accessible conformations, indicating the importance of the entropic penalty upon binding of very flexible derivatives. Based on this conformational analysis, it was predicted that conformationally restricted glutamates that place the carboxylates in 1,4-*trans* positions of a six-membered ring are promising candidates for new PSMA inhibitors. In addition, several other factors were found to increase the activity of the inhibitors. These results indicate that a number of criteria must be met to induce inhibition of PSMA. It is evident that the presence of the C1 and C2 carboxylic acids is essential to activity. If the entire dataset is considered,[9] it becomes apparent that the influence of the carboxylic acid at C1 has a slightly smaller influence than the one at C2. The presence of a tetrahedral transition state mimic such as a phosphinate or phosphonate is also essential. It can be concluded that the phosphonic acid derivatives are particularly active whilst simple structure activity relationships imply that the presence of an aromatic ring 3- to 5-bond lengths from the phosphate center provides a favorable interaction within the enzyme active site. It is also proposed that any negatively charged group found within the 3- to 5-bond region decreases activity. Some compounds produce large numbers of conformations and also display high activity such as those containing an iodide substituent.[9] Also, intramolecular hydrogen bonding results in the generation of low numbers of conformations associated with some compounds with low activity. Ongoing stud-

ies in our laboratories will assess how these findings correlate with the recently proposed homology model of NAALADase.[23]

Supporting information

Full details of the conformational searches of all compounds discussed are available from the authors upon request.

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References and Notes

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