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Phospho-Azatyrosine, a Less Effective Protein-Tyrosine Phosphatase Substrate Than Phosphotyrosine

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Abstract—Azatyrosine (AzaTyr, **4**) is a natural product isolated from *Streptomyces chibanesis*, whose structure is characterized by a nitrogen atom in the aryl ring of a tyrosyl residue. This seemingly minor modification to the tyrosyl residue results in profound physiological effects, as AzaTyr has been shown to promote permanent reversion of *ras*-dependent transformed cells to the normal phenotype in culture and to inhibit chemical induction of carcinogenesis in transgenic mice bearing oncogenic human *ras*. The mechanisms underlying these effects are not known, however *ras*-pathways involve an intricate balance between both protein-tyrosine kinases (PTKs) and protein-tyrosine phosphatases (PTPs). The present study was undertaken to examine the general utility of AzaTyr as a structural motif for PTP inhibitor design by examining the phospho-azatyrosine (pAzaTyr)-containing peptide Ac-Asp-Ala-Asp-Glu-*pAzaTyr*-Leu-amide (**8**) in a PTP1 enzyme system. Kinetic analysis indicated that **8** binds with a K_m value of 210 μM and a catalytic turnover rate, k_{cat} of 52 s^{-1}. This represents a greater than 50-fold reduction in binding affinity relative to the parent phosphotyrosine-containing peptide, indicating that the aryl nitrogen adversely affects binding affinity. The much lower PTP affinity of the pAzaTyr-containing peptide reduces the potential utility of the AzaTyr pharmacophore for PTP inhibitor design. These results are discussed from the point of view that incorporation of AzaTyr residues into proteins could result in perturbation of protein-tyrosine phosphorylation/dephosphorylation cascades that control signal transduction processes, including *ras*-dependent pathways. Published by Elsevier Science Ltd.

Protein-tyrosine kinase (PTK)-dependent signal transduction is critical for the maintenance of normal cellular growth and differentiation. Subversion or inappropriate activation of PTK-dependent signalling can contribute to a variety of diseases, and agents which attenuate these pathogenic alterations may potentially afford new therapeutic approaches to immune disorders and cancers. Since signalling is dependent upon an interplay of phosphorylation by PTKs and dephosphorylation by protein-tyrosine phosphatases (PTPs), inhibitors of either PTKs or PTPs may be therapeutically useful. Because the phosphotyrosyl residue (pTyr, **1**) provides the defining pharmacophore around which all PTK-dependent signalling is centered, the structure of pTyr itself can serve as a motif upon which both PTK and PTP inhibitors can be derived.[1] This paradigm has proven particularly useful in the structure-based design of PTP inhibitors, where peptides bearing nonhydrolyzable or poorly hydrolyzable pTyr mimetics have demonstrated very high PTP inhibitory potency. Exemplary of this are peptides containing difluorophosphonomethyl phenylalanine (F_2Pmp, **2**),[2] sulfonyltyrosine[3] and fluoro-*O*-(α-malonyl)tyrosine (FOMT, **3**).[4] Inhibitory potency of these peptides is dependent on the recognition and binding of the pTyr mimetics within the catalytic pocket in manners that are catalytically unproductive. There is therefore significant interest in the developing new Tyr analogues that can function as PTP-inhibitory pTyr mimetics.[1]

Azatyrosine (AzaTyr, **4**) is a natural product isolated from *Streptomyces chibanesis*,[5] whose structure is characterized by a nitrogen atom in the aryl ring of a tyrosyl residue. This seemingly minor modification to the Tyr residue results in profound physiological effects, as AzaTyr has been shown to promote permanent reversion of

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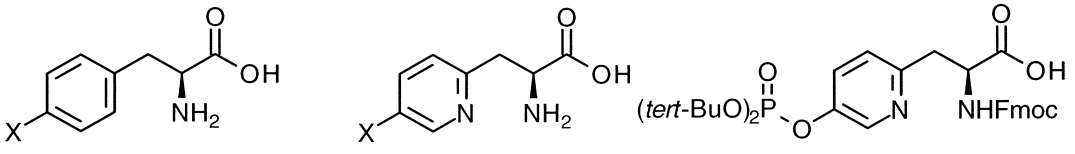

pTyr (**1**) X = O-PO_3H_2
F_2Pmp (**2**) X = CF_2-PO_3H_2
FOMT (**3**) X = O-$CF(CO_2H)_2$

AzaTyr (**4**) X = OH
pAzaTyr (**5**) X = O-PO_3H_2

(**6**)

ras-dependent transformed cells to the normal phenotype in culture[6] and to inhibit chemical induction of carcinogenesis in transgenic mice bearing oncogenic human *ras*.[7] The mechanisms underlying these effects are not known, however *ras*-pathways involve an intricate balance in the interactions of both PTKs and PTPs and represent major signal transduction routes for many growth factors and cytokines. Because PTPs have been shown to have both positive and negative signalling effects,[8] we were intrigued whether AzaTyr could potentially exert at least part of its physiological effects through the inhibition of PTPs. This was particularly pertinent in light of the ability of other pTyr mimetics to induce potent PTP inhibition when incorporated into appropriate high affinity peptide sequences.[4,9] We therefore undertook the present study to examine the general utility of AzaTyr as a structural motif for PTP inhibitor design.

Materials and Methods

Preparation of pAzaTyr-containing peptide 8

Synthesis of Ac-Asp-Ala-Asp-Glu-*pAzaTyr*-Leu-amide (**8**) was achieved by solid-phase techniques using Fmoc-based protocols and the recently reported pAzaTyr reagent **6**.[10]

Enzyme assay and data analysis

The recombinant catalytic domain of mammalian protein-tyrosine phosphatase 1 (PTP1) was created by inserting a stop codon at residue 323, yielding PTP1U323, in order to eliminate the hydrophobic membrane 'targeting' domain of the molecule. Homogeneous recombinant catalytic domain PTP1U323, from here on referred to as PTP1, was purified as described.[11] The PTP activity was assayed at 30 °C in a reaction mixture (200 μL) containing appropriate concentrations of the pAzaTyr-containing peptide (**8**). The buffer used was pH 7.0, 50 mM 3,3-dimethylglutarate, 1 mM EDTA. The ionic strength of the solution was kept at 0.15 M using NaCl. The reaction was initiated by addition of PTP1 and followed by the production of inorganic phosphate. The reaction was quenched after 2–3 min by addition of 100 μL of 10% trichloroacetic acid, followed by addition of 200 μL of a mixture composed of 80 μL of 2% ammonium molybdate and 120 μL of 14% ascorbic acid in 50% trichloroacetic acid. The nonenzymatic hydrolysis of the substrate was corrected by measuring the control without the addition of enzyme. As previously described,[12] Michaelis–Menten kinetic parameters k_{cat} and K_m were determined by analyzing the experimental data through a nonlinear least squares fit algorithm using the equation: $v = k_{cat}[E][S]/(K_m + [S])$.

Results and Discussion

As part of our larger effort to derive synthetic pTyr mimetics which inhibit PTPs by binding with high affinity within the catalytic site,[1,2,4,9,13–15] we considered the effects of placing nitrogens within the aromatic ring of pTyr itself. Such modifications could potentially change pTyr-containing substrates into inhibitors either by interfering with the hydrolysis of the phosphate moiety, or by slowing the release of product once hydrolysis had occurred. For this reason, AzaTyr was of considerable interest as a tyrosine analogue, since in its phosphorylated state, it could potentially serve as such an inhibitor. We were also attracted to the AzaTyr structure because of its ability to revert *ras*-transformed cells and the possibility that inhibition of pTyr-dependent enzymes within the *ras*-pathway might contribute to this effect. We therefore undertook the preparation of an appropriate phospho-AzaTyr (pAzaTyr, **5**)-containing peptide to examine whether introduction of a nitrogen into the pTyr ring would result in switching from a substrate to an inhibitor. This study was intended to provide important structure–activity data on inhibitor design, and potential insight into at least one possible mechanism for the anti-oncogenic activity of AzaTyr.

Our previous work with PTP inhibitors utilized the PTP1B enzyme as well as its closely related rat homologue, PTP1. These have proven to be valuable systems, since the X-ray structure of PTP1B has been solved in both ligated and unligated forms[15–17] allowing computer-assisted molecular modeling analysis of the interaction of potential inhibitor molecules.[4,15,18] Additionally, due to homology among PTPs within the pTyr binding domain,[8] results obtained with PTP1B also potentially provide structural information relevant to other PTPs. For these reasons, we chose to utilize PTP1 as a general tyrosine phosphatase model in the present study. Consistent with our choice of PTP1 was the utilization of the epidermal growth factor receptor-derived peptide sequence Asp-Ala-Asp-Glu-Xxx-Leu-amide for incorporation of the pAzaTyr residue. This sequence has been reported to bind PTP1 with a high affinity when

Xxx = pTyr (peptide **7**).[19] Synthesis of the target peptide, Ac-Asp-Ala-Asp-Glu-*pAzaTyr*-Leu-amide (**8**) was achieved by solid-phase techniques employing our recently reported protected pAzaTyr derivative (**6**).[10]

Examination of pAzaTyr-containing peptide **8** in a PTP1 system showed that it bound with a K_m value of 210 μM and exhibited a catalytic turnover rate, k_{cat} of 52 s^{-1} (Table 1). This is in comparison to the corresponding pTyr-containing parent peptide **7**, which has been reported to bind with a K_m value of 3.6 μM, having a turnover of 68 s^{-1}.[19] These results indicate that the pAzaTyr-containing peptide **8** undergoes a greater than 50-fold decrease in binding affinity relative to the parent peptide **7**, yet once bound, catalytic phosphate monoester hydrolysis and release of products are only moderately impaired. This may be interpreted to mean that introduction of a nitrogen into the aryl ring of pTyr in the 3-position relative to the phosphate ester introduces unfavorable binding interactions between the aryl ring and residues within the catalytic pocket, yet does not dramatically interfere with the actual process of catalysis itself.

The fact that k_{cat} for **8** is minimally perturbed compared to the parent peptide **7** is in accord with known chemical mechanisms for PTPs. PTP catalysis proceeds through a covalent cysteinyl phosphate intermediate which is subsequently hydrolyzed by H_2O.[8] Under most conditions, the rate-limiting step is the hydrolysis of this intermediate. The similar k_{cat} value observed for the pAzaTyr-containing peptide suggests that this modification does not perturb the rate-limiting step. However, the incorporation of a nitrogen into the 3-position of the aromatic ring has a profound effect on the catalytic efficiency of PTP1, reducing the k_{cat}/K_m term, which is a measure of substrate specificity, by 76-fold. Thus, although the pAzaTyr peptide can still be processed by PTP1, it is a much poorer substrate than the normal pTyr peptide.

In vivo, the level of protein-tyrosine phosphorylation, and, thus, the strength and duration of the signal transmitted, are balanced by the opposing actions of PTKs and PTPs. The introduction of pAzaTyr into proteins would greatly diminish the ability of PTPs to remove the phosphoryl moiety. It is conceivable that a reduction of the affinity of normal PTP substrates could lead to a perturbation of cellular homeostasis. It remains to be seen whether AzaTyr can serve as an efficient substrate for PTKs. Indeed nitration of tyrosine residues has been shown to block kinase activity.[20]

It was our intent in this study to examine the effect on PTP interaction of introducing a nitrogen into the pTyr aryl ring. Our interest was particularly directed toward the potential use of this or similar ring-modified pTyr mimetics for PTP inhibitor design. The results of our present study indicate that nitrogen placement in the 3-position relative to phopsho-group, significantly reduce binding affinity. This renders the concept of limited use for PTP inhibitor design. We were also interested in the potential ability of pAzaTyr to inhibit PTPs as one potential mechanism contributing to the ability of AzaTyr to revert the *ras*-transformed phenotype. The much reduced PTP affinity of pAzaTyr-containing substrate tends to support its role in impairing PTP action. It has been reported that radioactive azatyrosine becomes incorporated into cellular protein, and that incorporation can be competed with by tyrosine in a manner concomitant with reduction in azatyrosine's ability to revert transformed to normal phenotype.[21] It remains to be seen, however, whether pAzaTyr-containing proteins once formed, could alter PTK-dependent cell signalling by other mechanisms, including effects on PTKs themselves, or on SH2 domain and protein-tyrosine binding domains.

Table 1. Kinetic constants measured against PTP1 preparations

	k_{cat} (s^{-1})	K_m (μM)
Ac-D-A-D-E-pTyr-L-NH_2 (**7**)	68	3.6
Ac-D-A-D-E-pAzaTyr-L-NH_2 (**8**)	52	210

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