[Difluro(phosphono)methyl]phenylalanine-containing peptide inhibitors of protein tyrosine phosphatases

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Peptides containing the non-hydrolysable phosphotyrosine analogue 4-[difluro(phosphono)methyl]phenylalanine [Phe(CF_2P)] were synthesized and tested as inhibitors of the protein tyrosine phosphatases (PTPs) PTP1B, CD45, PTPβ, LAR and SHP-1. We have identified peptides containing two adjacent Phe(CF_2P) residues as potent inhibitors of PTPs. The tripeptide having the sequence Glu-Phe(CF_2P)-Phe(CF_2P) is a potent and selective inhibitor of PTP1B. This peptide inhibits PTP1B with an IC_{50} of 40 nM, which is at least 100-fold lower than with other PTPs. A second tripeptide, Pro-Phe(CF_2P)-Phe(CF_2P), is most potent against PTPβ, with an IC_{50} of 200 nM, and inhibits PTP1B with an IC_{50} of 300 nM. These data suggest that it is possible to develop selective, active-site-directed, reversible, potent inhibitors of PTPs.

Key words: tyrosine phosphatases PTP1B, CD45, LAR, PTPβ and SHP-1.

INTRODUCTION

Phosphorylation of proteins on tyrosine residues plays an essential role in regulating a wide variety of cell functions. It is controlled by the competing activities of protein tyrosine kinases and protein tyrosine phosphatases (PTPs). Over 50 PTPs have been identified so far and the functions of some of these enzymes are beginning to be elucidated [1–8]. Several avenues have been used to delineate the functions of these enzymes. Some of the successful approaches with PTPs include overexpression of either wild-type or mutated proteins (e.g. dominant negatives) in various cell lines, and generation of cell lines and animals in which the expression of the protein has been disrupted (e.g. see [9–14]). Another approach is to use selective inhibitors of PTPs to delineate the function of these enzymes.

All PTPs have a catalytic Cys residue at the active site [1–6]. Thus oxidizing and alkylating agents irreversibly inhibit PTPs. For example, pervanadate is an oxidizing agent that potently and irreversibly inactivates PTPs [15]. Vanadate is a phosphate analogue and inhibits PTPs by binding reversibly to the enzyme at the active site [15]. However, vanadate is a non-specific PTP inhibitor. In addition to these inhibitors, peptides containing non-hydrolysable analogues of phosphotyrosine have been reported as inhibitors. These include peptides that contain sulphotyrosine, thiophosphotyrosine, *O*-(dicarboxymethyl)tyrosine and phosphonomethylphenylalanine [Phe(CH_2P)] residues [16–20, 24]. In the case of Phe(CH_2P)-containing peptides, replacement of the hydrogen of the phosphonomethyl with fluorines, as in [4-difluro(phosphono)methyl]phenylalanine [Phe(CF_2P)], has been shown to dramatically increase the inhibitory potency of some of the peptides towards PTP1B [19]. The binding affinity of some of these Phe(CF_2P) peptides to PTP appears to be unaffected by changes in the pH [20]. This suggests that the enhanced potency of these peptides is not due to pK_a effects [20–22], but may be due to an enhanced binding interaction between the fluorine atoms and the enzyme [20–23].

In the present study we have synthesized a series of Phe(CF_2P)-containing peptides and have evaluated the ability of these peptides to inhibit PTP1B, CD45, LAR, PTPβ and SHP-1. We show that some of these peptides are potent inhibitors of PTPs, and demonstrate that it is possible to identify very potent, selective, active-site-directed inhibitors for these enzymes.

EXPERIMENTAL

Materials

N-Fluoren-9-ylmethoxycarbonyl-4-[(*O*,*O*′-diethylphosphono) difluoromethyl]-L-phenylalanine [Fmoc-Phe(CF_2-PO_3Et_2)] was prepared by the procedure of Smyth and Burke [25]. The 5-[4-(9-fluorenylmethyloxycarbonyl)aminomethyl-3,5,-dimethoxyphenoxy]valeric acid (PAL) handle used for solid-phase peptide synthesis was prepared using the procedures of Albericio et al. [26]. Unless stated otherwise, all reactions were carried out at ambient temperature. 3,6-Fluorescein diphosphate (FDP) was synthesized and purified as in Scheigetz et al. [27]. GT-Sepharose was from Pharmacia.

Peptide synthesis

The tripeptides were prepared using Fmoc chemistry on PAL resin [26], with 1,3-di-isopropylcarbodi-imide (DIPCDI)/1-hydroxybenzotriazole (HOBt) as the coupling reagents, and 50% piperidine/dimethylformamide (DMF) for Fmoc deprotection. The fully protected resin-bound peptides were cleaved from the resin using trifluoroacetic acid (TFA)/water (9:1, v/v) to yield the ethyl-protected phosphonate tripeptide amides. The ethyl protective groups were removed from the Phe(CF_2P) side chain with a mixture of trimethylsilyltriflate/TFA/dimethyl sulphide [28] to yield the crude, fully deprotected tripeptide amide. The tripeptides were purified to homogeneity by reverse-phase HPLC. The fractions containing pure tripeptide were

Abbreviations used: DIPCDI, 1,3-di-isopropylcarbodi-imide; DMF, dimethylformamide; FDP, 3,6-fluorescein diphosphate; Fmoc, *N*-fluoren-9-ylmethoxycarbonyl; GST, glutathione S-transferase; HOBt, 1-hydroxybenzotriazole; PAL, 5-[4-(9-fluorenylmethyloxycarbonyl)aminomethyl-3,5,-dimethoxyphenoxy]valeric acid; Phe(CF_2P), [difluro(phosphono)methyl]phenylalanine (denoted as F* in the one-letter code); Phe(CF_2-PO_3Et_2), 4-[(*O*,*O*′-diethylphosphono)difluoromethyl]-L-phenylalanine; Phe(CH_2P), phosphonomethylphenylalanine; PTP, protein tyrosine phosphatase; TFA, trifluoroacetic acid.

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collected and lyophilized to dryness. Fast-atom bombardment MS of each peptide exhibited a molecular ion (MH^+ or MNa^+) consistent with the assigned structure. The synthesis of the tripeptide EF*F*-amide [where F* = Phe(CF_2P)] described below is representative.

A reaction chamber containing PAL resin (~ 0.6 mmol/g; 350 mg) was charged successively with Fmoc-Phe(CF_2-PO_3Et_2) (0.78 mmol; 450 mg in 0.8 ml of DMF), HOBt (0.8 ml of a 1 M solution in DMF) and CH_2Cl_2 (1.6 ml). After 30 s, DIPCDI (0.15 ml; 0.96 mmol) was added and the mixture was agitated for 15 h. The resin was washed successively with DMF (3 × 10 ml) and CH_2Cl_2 (3 × 10 ml) and air dried. The resin was treated with piperidine (3 ml) and DMF (3 ml) for 1 h. The resin was washed successively with DMF (3 × 10 ml) and CH_2Cl_2 (3 × 10 ml) and air dried. The sequence was repeated as described above, except using Fmoc-Phe(CF_2-PO_3Et_2) (423 mg), HOBt (0.74 ml of a 1 M solution in DMF) and DIPCDI (0.14 ml). The resin was divided into five equal portions (~ 0.042 mmol/reactor), and one portion was treated as in the above sequence but using Fmoc-L-glutamic acid γ-t-butyl ester (0.3 ml of a 1 M solution in DMF), HOBt (0.3 ml of a 1 M solution in DMF), CH_2Cl_2 (0.6 ml) and DIPCDI (56 ml). After the final piperidine cleavage and washing, the resin was treated with 2.5 ml of TFA/water (9:1, v/v) and tri-isopropylsilane (0.1 ml) for 1 h. The resin was filtered off, washed with TFA/water (9:1, v/v; 3 × 2 ml), and the combined filtrate and washings were concentrated *in vacuo* at 35 °C. The residue was taken up in water (5 ml), washed with ether (2 × 2 ml) and lyophilized to dryness. The residual material was treated with trimethylsilyltriflate/TFA/dimethyl sulphide (1:5:3, by vol.; 2.5 ml) and tri-isopropylsilane (0.1 ml). After stirring for 15 h, the mixture was concentrated *in vacuo* at 25 °C, taken up in water (5 ml) and washed with ether (2 × 2 ml). The aqueous solution containing the crude tripeptide amide was lyophilized to dryness and purified by reverse-phase HPLC (Nova-Pak C18 column; 25 mm × 100 mm) using a mobile-phase gradient from 100% water (containing 0.2% TFA) to 50% acetonitrile in water (containing 0.2% TFA) over 40 min (20 ml/min) and monitoring at 230 nm. The fraction eluting at approx. 14.3 min was collected, concentrated and lyophilized to dryness to yield the tripeptide EF*F*-amide as a white foam (10.3 mg).

The peptides DADEF*L, DEF*L and EF*L used in the experiment shown in Figure 1 were synthesized on a 9650 Plus PepSynthesizer (PerSeptive Biosystems) using the resin Fmoc-PAL- poly(ethylene glycol)-polystyrene. Peptides were synthesized in the fast cycle using *O*-(7-azabenzotriazol-1-yl)-1,1,3,3,tetramethyluronium hexafluorophosphate ('HATU') as the coupling reagent [29].

Purification of various PTPs

The intracellular domains of PTPβ and CD45 and the catalytic domain of PTP1B were expressed as glutathione S-transferase (GST) fusion proteins in *Escherichia coli* and purified on GT-Sepharose beads. PTPβ and CD45 were cleaved with thrombin from GT-Sepharose beads, as described previously for SHP-2 by Huyer et al. [30], and were used without further purification. These proteins were greater than 80% pure. GST–PTP1B was eluted from GT-Sepharose beads with 10 mM glutathione and was greater than 90% pure. The SHP-1 catalytic domain was purified as described by Townley et al. [31]. The intracellular domain of rat LAR was purified as described by Pot et al. [32].

Assay of PTP activity

The reaction mixture in 170 μl contained 50 mM Bis-Tris (pH 6.3), 2 mM EDTA, 5 mM dithiothreitol, either 5 μM FDP (PTP1B and SHP-1) or 20 μM FDP (CD45, PTPβ and LAR) and 10 μl of the Phe(CF_2P)-containing peptide (0–2 mM in DMSO; 10 concentrations used). The reaction was initiated by adding 20 μl of the enzyme diluted in 50 mM Bis-Tris (pH 6.3) containing 2 mM EDTA, 5 mM dithiothreitol, 20% glycerol and 0.1 mg/ml BSA. The amount of enzyme used per well was 20 ng for CD45 and PTPβ, 100 ng for PTP1B and LAR and 50 ng for SHP-1 The assay was carried out at room temperature in 96-well plates. The phosphatase activity was followed by monitoring the appearance of the fluorescent product fluorescein monophosphate ([15,29]; Z. Huang, Q. Wang, H. D. Ly, A. Govindarajan, J. Scheigetz, R. Zamboni and C. Ramachandran, unpublished work) continuously for 15–30 min using a Cytofluor II plate reader (PerSeptive Biosystems), with excitation at 440 nm (slit width 20 nm) and emission at 530 nm (slit width 25 nm). The formation of the product, fluorescein monophosphate, increased linearly with time. All the assays were carried out at least in duplicate, and the average results are presented. Variations between experiments were less than 10%.

RESULTS

Inhibition of PTPs by DADEF*L

Initial experiments were performed to confirm the observations of Burke et al. [17]. The Phe(CF_2P)-containing hexapeptide DADEF*L, corresponding to the epidermal growth factor receptor autophosphorylation site 992, was synthesized and its ability to inhibit various purified PTPs was tested. We used the continuous fluorescence assay with the fluorogenic substrate FDP ([15,29]; Z. Huang, Q. Wang, H. D. Ly, A. Giovindarajan, J. Scheigetz, R. Zamboni and C. Ramachandran, unpublished work). The initial rate of dephosphorylation was determined at various concentrations of inhibitor, as shown for PTP1B in Figure 1. In confirmation of the observation of Burke et al. [17], this peptide inhibited PTP1B with an IC_{50} of 30 nM. This peptide also inhibited PTPβ with an IC_{50} of 9 μM (results not shown). It did not inhibit CD45 or LAR up to 100 μM (results not shown). Deletion of the two amino acids at the N-terminus (Asp and Ala) decreased the ability of the peptide to inhibit PTP1B by over 60-fold. Removal of the next amino acid (Asp) decreased the ability of the peptide to inhibit PTP1B by a further 20-fold. The importance of the N-terminal acidic residues for inhibition of PTP1B is consistent with the substrate selectivity data reported

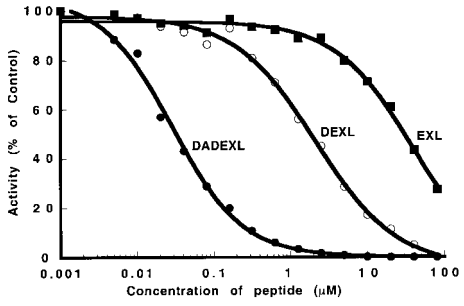

Figure 1 Inhibition of PTP1B by DADEF*L and effects of N-terminal deletions

Peptides of the sequences indicated were tested at 10 different concentrations against PT1B using FDP as a substrate. The initial rate of fluorescein monophosphate formation (0–500 s) is plotted against the concentration of the peptide, and the data are fitted to a four-parameter equation using the KaleidaGraph program (version 3.05). Results are expressed as means of two experiments.

Table 1 Sequences of the $X_1F^*X_2$-NH_2 peptides

Peptides of the sequence $X_1F^*X_2$ were synthesized and tested at 100 μM as inhibitors of PTPs using FDP as substrate, as described in the Experimental section. None of the peptides inhibited CD45, PTPβ or LAR. The peptides that inhibited PTP1B and their IC_{50} values are shown in Table 2.

X_1	X_2
Pro	Pro, Tyr, Gln, Asp
Glu	Pro, Tyr, Gln, Asp
Ile	Pro, Tyr, Gln, Asp
Ser	Pro, Tyr, Gln, Asp
Lys	Pro, Tyr, Gln, Asp
Gln	Gln

Table 2 IC_{50} values for the inhibition of PTPs by $X_1F^*X_2$-NH_2 peptides

The IC_{50} values for the tripeptide amides against various PTPs were determined using FDP as substrate, as illustrated in Figure 1.

		IC_{50} (μM)				
X_1	X_2	PTP1B	CD45	PTPβ	LAR	SHP-1
Glu	Tyr	16	> 100	> 100	> 100	> 10
Glu	Gln	10	> 100	> 100	> 100	> 10
Ile	Gln	13	> 100	> 100	> 100	> 10
Gln	Gln	10	> 100	> 100	> 100	> 10

Table 3 IC_{50} values for the inhibition of PTPs by XF^*F^*-NH_2 peptides

Tripeptide amides of the sequence XF*F* were synthesized, and the IC_{50} values against various PTPs were determined using FDP as substrate, as illustrated in Figure 1

	IC_{50} (μM)				
X	PTP1B	CD45	PTPβ	LAR	SHP-1
Glu	0.04	7.5	4.2	45	6
Ile	0.2	100	3	170	> 10
Pro	0.3	> 100	0.2	100	> 10
Ser	0.3	100	9	> 100	> 10
Lys	0.7	> 100	35	> 100	> 10

Table 4 IC_{50} values for the inhibition of PTPs by F^*XF^*-NH_2 peptides

Tripeptide amides of the sequence F*XF* were synthesized, and the IC_{50} values against various PTPs were determined using FDP as substrate, as illustrated in Figure 1

	IC_{50} (μM)				
X	PTP1B	CD45	PTPβ	LAR	SHP-1
Glu	0.4	> 50	50	> 50	22
Ile	0.4	> 50	20	> 50	26
Ser	0.6	> 50	15	> 50	40
Lys	2.1	> 50	30	> 50	37
Pro	11.7	> 50	20	> 50	25

by Zhang et al. [16] using peptides containing phosphotyrosine. The inhibition of PTP activity by these Phe(CF_2P)-containing peptides is reversible on dilution and is competitive with the substrate FDP (results not shown).

Tripeptide inhibitors of tyrosine phosphatases

In an attempt to evaluate the contribution of amino acids around Phe(CF_2P) in mediating the inhibition of PTPs, 21 tripeptide amides of the sequence $X_1F^*X_2$ were synthesized, wherein X_1 and X_2 are an acidic (Asp), basic (Lys), hydrophobic (Ile), uncharged polar (Gln) or aromatic hydroxy (Tyr) amino acid, or the imino acid (Pro), as indicated in Table 1. These peptides were tested at 100 μM against PTP1B, CD45, PTPβ and LAR. None of the peptides inhibited CD45, PTPβ or LAR at this concentration. Only four peptides inhibited PTP1B significantly. The sequences of these peptides and their IC_{50} values are shown in Table 2. Peptides containing Glu, Ile or Gln at the N-terminus and Gln at the C-terminus inhibited PTP1B with an IC_{50} of 10–13 μM. The only other peptide that inhibited PTP1B had the sequence EF*Y and its IC_{50} was 16 μM. These tripeptides are much poorer inhibitors than the hexapeptide shown in Figure 1.

Peptides containing two Phe(CF_2P) residues

The deletion studies using the DADEF*L peptide shown in Figure 1 suggest that acidic amino acids N-terminal to Phe(CF_2P) are essential for high-affinity binding to PTP1B. Large numbers of tyrosine kinases have several, closely spaced, phosphorylated tyrosine residues, and in some cases these are on adjacent residues. In the case of the Src homology 2 (SH2) domain, it has been shown that having two adjacent phosphotyrosine residues in an appropriate peptide sequence enhances the affinity of these peptides for this domain [33]. Hence we synthesized a series of tripeptide amides having the sequence XF*F*, where X is an acidic (Glu), basic (Lys), hydrophobic (Ile) or aliphatic hydroxy (Ser) amino acid, or the imino acid (Pro), to examine their affinity for various PTPs.

These peptides were initially tested at concentrations of 10, 50 and 100 μM. From these data, those enzymes that were inhibited substantially by these peptides were examined further to determine their IC_{50} values. Data in Table 3 show that the peptide of the sequence EF*F* was very potent against PTP1B, with an IC_{50} of 40 nM. This peptide also inhibited PTPβ, SHP-1, CD45 and LAR, with IC_{50} values of 4.2, 6, 7.5 and 45 μM respectively. Thus this tripeptide is highly selective for inhibition of PTP1B, binding it about 100-fold more tightly than other PTPs tested, and is approximately equipotent with the hexapeptide DADEF*L described by Burke et al. [17]. The peptide of sequence IF*F* was also found to be potent against PTP1B, with an IC_{50} of 0.2 μM. It also inhibited PTPβ, CD45 and LAR, with IC_{50} values of 3, 100 and 170 μM respectively. The peptide of sequence PF*F* was more potent against PTPβ, with an IC_{50} of 0.2 μM. It also inhibited PTP1B and LAR, with IC_{50} values of 0.3 and 100 μM respectively. The Ser-containing peptide SF*F* also inhibited PTP1B, CD45 and PTPβ, with IC_{50} values of 0.3, 100 and 9 μM respectively. The tripeptide with a basic amino acid at the N-terminus (KF*F*) was the poorest inhibitor of the PTPs tested. The data in Table 3 clearly demonstrate that it is possible to identify selective high-affinity peptide inhibitors for PTPs.

The requirement of having two adjacent Phe(CF_2P) residues was investigated next by synthesizing peptides of the same composition as shown in Table 3, but with the Phe(CF_2P) residues flanking the natural amino acid (F*XF*). The IC_{50} values for this series of peptides were determined and are shown in Table 4. In general, these tripeptides were relatively less potent than the peptides shown in Table 3, and were also less selective.

Again, the peptides in this series were most potent against PTP1B, with IC_{50} values ranging from 0.4 to 11.7 μM, with the best peptides in this series being those containing Glu and Ile. The peptide containing Lys had the lowest affinity. These peptides also inhibited PTPβ, with IC_{50} values ranging from 15 to 50 μM, and SHP-1, with IC_{50} values of 22–40 μM. The IC_{50} values of these peptides against CD45 and LAR were all greater than 50 μM.

DISCUSSION

The activity of a PTP towards various phosphotyrosine-containing proteins in the cell is determined by the inherent activity of its catalytic domain, its localization in particular compartments of the cell, and the regulation of its catalytic activity by diverse structural elements present in these enzymes [1–7]. The selectivity of the catalytic domains has been investigated for some enzymes using phosphotyrosine-containing peptide substrates, as well as by various arylphosphates [34–39]. In addition, peptide inhibitors containing phosphotyrosine mimics such as sulphotyrosine, thiophosphotyrosine, *O*-(dicarboxymethyl)tyrosine, Phe(CH_2P) and Phe(CF_2P) have been synthesized [16–20,23,24]. It has been suggested that, in the case of Phe(CF_2P), the fluorine may be mimicking the oxygen of the phosphotyrosine by hydrogen-bonding to the enzyme, thereby accounting for the enhanced affinity of Phe(CF_2P)-containing peptides for PTP1B [20–23]. With this in mind, we set out to identify selective and potent inhibitors of PTPs that contain one or more residues of the phosphotyrosine mimic Phe(CF_2P) in order to understand further the effect of Phe(CF_2P) on different PTPs.

Initial experiments confirmed the observations of Burke et al. [17], in that the Phe(CF_2P)-containing hexapeptide DADEF*L was a potent inhibitor of PTP1B (Figure 1). This peptide binds to PTP1B 300-fold more effectively than to PTPβ. It also binds to PTP1B greater than 3000-fold more effectively than to either CD45 or LAR. The two Asp residues at the N-terminus of this peptide are essential for potency. These observations are consistent with the findings of Zhang et al. [16], who found that acidic residues N-terminal to phosphotyrosine are essential for the efficient turnover of phosphotyrosine-containing peptides.

Having confirmed the observations of Burke et al. [17], we examined the role of amino acid residues on either side of Phe(CF_2P) in the binding of peptides to a variety of PTPs. Tripeptides containing a single Phe(CF_2P) group in the middle and a variety of different amino acids at the N- and C-termini were essentially inactive up to 100 μM against CD45, LAR and PTPβ (Table 1). A few peptides containing Glu, Gln or Ile at the N-terminus and Tyr or Gln at the C-terminus inhibited PTP1B, with IC_{50} values of 10–16 μM.

The poor potency of these tripeptides containing a single Phe(CF_2P) residue and the requirement for acidic residues N-terminal to Phe(CF_2P), for mediating binding to PTP1B, prompted us to investigate the effect of a second Phe(CF_2P) residue on binding to PTP. Tripeptides having two Phe(CF_2P) residues at the C-terminal end (Table 3) or two Phe(CF_2P) residues separated by a natural amino acid in the middle (Table 4) were prepared. Analysis of these tripeptides demonstrated that the acidic peptide EF*F* was the most potent tripeptide inhibitor of PTP1B, with an IC_{50} value of 40 nM. This tripeptide also inhibited CD45, LAR, PTPβ and SHP-1, but with potencies that were 100–1000-fold lower. A peptide of the same composition, but with the sequence F*EF*, was 10-fold less potent against PTP1B than was EF*F*. This observation demonstrates that it is not the overall charge of the peptide, but its sequence, that is important for high-affinity binding. It will be of interest to determine the reason for the enhanced potency and selectivity imposed by the second Phe(CF_2P) residue. Studies are in progress to determine the structure of the tripeptide inhibitors bound to PTP1B. After the present work was completed, a second non-catalytic arylphosphate binding site on PTP1B was identified [40], which is most likely to be the binding site for one of the Phe(CF_2P) residues.

Replacement of the N-terminal Glu with Pro in the peptide EF*F* decreased the inhibitory potency of the peptide against PTP1B about 8-fold (Table 3). Interestingly, the inhibitory potency of this peptide against PTPβ was increased from 4.2 μM to 0.2 μM. Thus a single amino acid substitution altered the selectivity of the tripeptide for inhibition of PTP1B over PTPβ from 100 to 0.5. This observation provides additional evidence that it is possible to identify selective active-site-directed inhibitors of PTPs. Systematic analysis with more exhaustive libraries of peptides containing Phe(CF_2P) and other natural and synthetic amino acids will aid in the identification of inhibitors that are selective towards various PTPs. These reagents will be of great use in deciphering the signalling pathways in which various PTPs participate, thereby providing novel targets for therapeutic intervention.

In conclusion, we have identified tripeptide inhibitors that are potent and selective against PTP1B, as well as a potent tripeptide inhibitor for PTPβ. To our knowledge these are the most potent, most selective and smallest peptide inhibitors of PTPs reported thus far.

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