Phosphonate inhibitors of protein-tyrosine and serine/threonine phosphatases

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In all, 15 aryl-containing phosphonates have been synthesized and tested for their effect on protein-tyrosine phosphatase (PTPase) activity. Two compounds, (naphth-2-yl) difluoromethylphosphonic acid (**12**) and (naphth-1-yl) difluoromethylphosphonic acid (**13**) have been found to inhibit dephosphorylation of [³²P]insulin receptors by PTP-1B, a protein tyrosine phosphatase (PTPase), with IC₅₀ values of 40–50 μM. Compound **12** competitively inhibited insulin-receptor dephosphorylation by PTP-1B. Compound **12** also inhibited PTP-1B-catalysed dephosphorylation of a synthetic tyrosine phosphorylated substrate poly(Glu⁸⁰-Tyr²⁰) at the same potency, indicating that **12** acted via interaction with the PTPase. Additionally, **12** inhibited insulin-receptor PTPase(s) and epidermal-growth-factor-receptor PTPase(s) present in solubilized membranes from CHO (Chinese-hamster ovary)/HIRc and A431 cells respectively. IC₅₀ values of 40–50 μM were obtained in all cases with compound **12**. Of note is the fact that these compounds did not have any

effect on insulin-receptor autophosphorylation. Nine out of the 15 compounds potently inhibited serine/threonine phosphatase PP-2A activity without any effect on serine/threonine phosphatase PP-1 when tested at a concentration as high as 675 μM. The most potent compounds acting toward PP-2A had IC₅₀ values of 45–50 μM. These PP-2A inhibitors could be useful tools for studying serine/threonine-phosphatase-mediated signal transduction. Two compounds, **12** and **13**, inhibited both tyrosine phosphatase PTP-1B and serine/threonine phosphatase PP-2A with similar potency; IC₅₀ values being 40–50 μM in both cases. Details of the synthesis of compounds **10**, **11** and **13** are given in Supplementary Publication SUP 50177 (6 pages), which has been deposited at the British Library Document Supply Centre, Boston Spa, Wetherby, West Yorkshire LS23 7BQ, U.K., from whom copies can be obtained on the terms indicated in Biochem. J. (1995) 305, 9.

INTRODUCTION

Protein phosphatases, including protein-tyrosine phosphatases (PTPases) are key participants in kinase-dependent signal-transduction pathways, where they can perform both positive and negative regulatory roles [1]. Maintenance of specific levels of protein-tyrosine phosphorylation and dephosphorylation is vital for normal cell proliferation, differentiation and metabolism [2–5], age [6], diabetes [7–10] and certain cancers [11–15]. A high level of expression of the PTPase PTP-1B has been reported in ovarian cancers [12] and also in insulin-resistance states [10]. Increased levels of hematopoietic PTPase expression in myeloid malignancies has been documented by Zanke et al. [11]. Furthermore, Zheng et al. [14] and Pallen [15] reported that overexpression of PTPα induced cell transformation and tumorigenesis, most likely as a consequence of the ability of PTPα to dephosphorylate and activate the *c-src* tyrosine kinase. Abnormal serine/threonine phosphatase activity has also been associated with diabetes [16, 17]. Thus the ability to inhibit disease-specific protein phosphatases may have clinical utility. Specific PTPase inhibitors would be invaluable tools for elucidating the function of individual PTPases within cells and for studying phosphatase catalytic mechanisms. Little has been reported on the design and synthesis of low-molecular-mass non-metal containing inhibitors. We have recently synthesized several arylmethyl phosphonates

and examined them for their ability to inhibit select phosphatases, including PTP-1B, and found that some of these agents can be quite effective.

A preliminary account of this work has been presented in part at the 208th National American Chemical Society Meeting held in Washington, DC, U.S.A. on 2–6 April, 1995.

EXPERIMENTAL

Materials

The Centricon 30 protein concentrator was obtained from Amicon (Beverly, MA, U.S.A.). Epidermal growth factor (EGF), monoclonal and polyclonal anti-phosphotyrosine antibodies, monoclonal anti-(insulin receptor) antibody, PTPase 1B (PTP-1B), okadaic acid and the protein serine/threonine phosphatases PP-1 and PP-2A were obtained from Upstate Biotechnology Inc. (Lake Placid, NY, U.S.A.). Phosphorylase *a* labelling kit was purchased from Life Technology Inc., Bethesda, MD, U.S.A. Wheat-germ agglutinin (WGA)-coupled to agarose was obtained from Vector Laboratories (Burlingame, CA, U.S.A.). Polyclonal anti-(EGF receptor) antibody was purchased from Oncogene Science (Uniondale, NY, U.S.A.). Peptide poly(Glu⁸⁰-Tyr²⁰) was purchased from Sigma Chemical Co., (St. Louis, MO, U.S.A.).

Abbreviations used: PTPases, protein-tyrosine phosphatases; PP-, protein-serine/threonine phosphatase; EGF, epidermal growth factor; WGA, wheat-germ agglutinin; CHO, Chinese-hamster ovary; DTT, dithiothreitol; PVDF, poly(vinylidene difluoride); pTyr, phosphotyrosine; Pmp, phosphonomethylphenylalanine.

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Figure 1 Structures of pTyr (16) and phosphonate pTyr mimetics

Inhibitors

Most of the phosphonates utilized in the present study are modelled on the phenylalanine analogues shown in Figure 1. The synthesis of phosphonates 1 [18], 2–4 [19], 5 [20], 6 [21], 7 [18], 8 and 9 [22], 12 [19], 14 and 15 [22] (Table 1) have been previously reported. Details of the synthesis of compounds 10, 11 and 13 are given in Supplementary Publication SUP 50177 (6 pages), which has been deposited at the British Library Document Supply Centre, Boston Spa, Wetherby, West Yorkshire LS23 7BQ, U.K., from whom copies can be obtained on the terms indicated in *Biochem. J.* (1995) **305**, 9.

Cell lines

The Chinese-hamster ovary (CHO) cell line transfected with an expression plasmid encoding the normal human insulin receptor [CHO/HIRc] was generously given by Dr. Morris F. White, Joslin Diabetes Center, Boston, MA, U.S.A. CHO cells overexpressing EGF receptor (CHO/EGF-R) were generously given by Dr. Roger J. Davis, University of Massachusetts Medical School, Worcester, MA, U.S.A. The cells were maintained in F-12 medium containing 10% (v/v) fetal-bovine serum and were cultured to confluence. A431 cells overexpressing EGF receptors were purchased from the American Type Culture Collection, Washington, DC, U.S.A., and were maintained in Dulbecco's modified Eagle's medium containing 10% calf serum.

Preparation of Triton X-100-solubilized membranes

Membranes from cultured cells were isolated and solubilized with Triton X-100 as previously described [23].

Receptor dephosphorylation assay in Triton X-100-solubilized membranes

This assay was essentially as described previously [23]. In brief, solubilized membrane extracts from CHO/HIRc and A431 cells were preincubated with respective ligands i.e. insulin (100 nM) or EGF (5 nM) in 100 mM Hepes (pH 7.4)/1 mM dithiothreitol (DTT)/5 mM MnCl₂ for 10 min on ice. Receptor autophosphorylation was initiated by the addition of 40 μ M [γ -³²P]ATP (10 c.p.m./fmol). After 30 min phosphorylation on ice (the phosphatase activity present in the solubilized membranes is very slow on ice), an aliquot of the reaction mixture was added to a tube containing 1 vol. of 2 $\times$ Laemmli sample buffer [24] to stop the reaction and this sample was taken as zero time for the dephosphorylation reaction. The rest of the sample was warmed to 37 °C to initiate dephosphorylation in the presence of 4 mM ATP. Dephosphorylation was monitored in the presence or absence of various concentrations of compound 12. At different

time intervals, an aliquot of the sample was transferred to a tube containing 1 vol. of 2 $\times$ Laemmli sample buffer. ³²P-labelled 95 kDa insulin-receptor β -subunit and 170 kDa EGF receptor were separated by SDS/PAGE under reducing conditions, and the loss of phosphate was quantified by Betagen counting of the fixed and dried gels (Betascop 603 blot analyser; Betagen, Weltham, MA, U.S.A.; version 2.0 of the operating software).

Assay of insulin-receptor dephosphorylation by recombinant PTP-1B

WGA-purified human insulin receptor was prepared from cultured CHO/HIRc cells. PTP-1B activity was assayed as described previously [23]. In brief, ³²P-labelled insulin receptors (20 ng of WGA eluate) were incubated with 0.05 μ g of recombinant PTP-1B at 22 °C in a 100 μ l reaction mixture containing 50 mM Hepes (pH 7.5)/BSA (0.1 mg/ml)/5 mM DTT/10 mM EDTA/0.05% Triton X-100, in the absence or the presence of various phosphonates at the indicated concentrations. Substrate over the range of 10–90 ng was used in 100 μ l of phosphatase assay mixture to determine the kinetic nature of inhibition by compound 12. The assay was terminated at various intervals by transferring an aliquot of the reaction mixture to a tube that contained 1 vol. of 2-fold-concentrated Laemmli sample buffer [24]; samples were heated at 95 °C for 5 min prior to electrophoresis in SDS/7.5%-polyacrylamide gels under reducing conditions. The loss of ³²P from the 95 kDa insulin-receptor β -subunit, present in the fixed and dried gels, was quantified with a Betascop 603 blot analyser.

Effect of compound 12 on the dephosphorylation of a synthetic peptide poly(Glu⁸⁰-Tyr²⁰) by PTP-1B

Peptide poly(Glu⁸⁰-Tyr²⁰) (300 μ g/ml) was preincubated with WGA-purified insulin receptor (250 ng/ml), and 100 nM insulin in 100 mM Hepes (pH 7.4)/1 mM DTT/5 mM MnCl₂ for 10 min on ice. Peptide phosphorylation was initiated by the addition of 40 μ M [γ -³²P]ATP (10 c.p.m./fmol). After 30 min of phosphorylation on ice, the reaction was terminated by the addition of stop solutions containing final concentrations of 5 mM DTT and 10 mM EDTA. For the dephosphorylation assay, aliquots (30 μ l) of the [³²P]poly(Glu⁸⁰-Tyr²⁰) were incubated with or without 0.05 μ g of PTP-1B at 22 °C in the absence or the presence of compound 12 (50 or 100 μ M). The concentration of PTP-1B used provided a linear initial rate of peptide dephosphorylation during a 2.5 min incubation. Additionally, no dephosphorylation was observed when incubation was performed in the absence of PTP-1B. The dephosphorylation reaction was terminated by transferring 20 μ l samples to a Whatman 3MM filter paper (2 cm $\times$ 2 cm) which was then placed into 10% (w/v) trichloroacetic acid containing 10 mM pyrophosphate and shaken for 1 h gently with four changes of the washing fluid. The filter papers were then washed once with 95% ethanol, dried, and the radioactivity was measured by Čerenkov counting.

Effect of compound 12 on insulin-receptor and EGF-receptor autophosphorylation in intact cells

Confluent CHO cells overexpressing either insulin receptors or EGF receptors in 35 mm culture dishes were incubated in F-12 medium devoid of serum for 4 h, after which the medium was replaced with 0.5 ml of serum free F-12 medium containing 100 μ M compound 12 or 1 mM vanadate. Cell monolayers were incubated for 1 h at 37 °C before the treatment with insulin (100 nM) or EGF (5 nM) of CHO/HIRc cells or CHO/EGF-R cells respectively. After 1 min the incubation was terminated by

Figure 2 Determination of inhibitory potency of (naphth-2-yl) difluoromethylphosphonic acid (**12**) against PTP-1B activity

(a) PTP-1B activity with compound **12** was measured with two different substrates. ^{32}P -labelled insulin-receptor dephosphorylation by recombinant PTP-1B was assayed in the presence of various concentrations of compound **12** as described in the legend to Table 1. The effects of four concentrations of **12** (+, 5; ▲, 25; □, 50; and ■, 100 μM) on the dephosphorylation rate of the insulin receptors by PTP-1B were compared with the rate in the absence of compound **12** (●, control). (b) shows the concentration-dependence of the inhibition of insulin-receptor dephosphorylation for 1 min by **12**. Results are expressed as means $\pm$ S.E.M. for two experiments performed in duplicate.

removing the fluid and immersing the cell dishes in liquid nitrogen. Cells were then scraped into lysing buffer (20 mM Tris/HCl, pH 7.5, containing 137 mM NaCl, 1 mM sodium vanadate, 100 mM NaF, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100, 0.2% NaN_3 , 0.2 mM phenylmethanesulphonyl fluoride, 1 mM benzamide, 8 $\mu\text{g}/\text{ml}$ aprotinin, 2 $\mu\text{g}/\text{ml}$ leupeptin) and the cell lysates incubated for 60 min on ice. After centrifugation (17000 g , 20 min) to remove cell debris, the supernatant from the cell lysate of CHO/HIRc cells was incubated at 4°C for 18 h with a monoclonal anti-(insulin receptor) antibody followed by the precipitation of the immune complexes with protein A–protein G–agarose beads. The beads were washed and the antibody–receptor complexes were eluted with Laemmli sample buffer [24] containing 5% 2-mercaptoethanol. After SDS/PAGE, proteins were electrotransferred to poly(vinylidene difluoride) (PVDF) membranes and probed with monoclonal anti-phosphotyrosine antibody. For EGF receptors, cell lysates were immunoprecipitated with polyclonal anti-(EGF receptor) antibody and immunoprecipitated proteins were then transferred to PVDF membranes and probed with monoclonal anti-phosphotyrosine antibody. Blots were developed using the ECL chemiluminescence detection system as described previously

Figure 3 Effect of (naphth-2-yl) difluoromethylphosphonic acid (**12**) on insulin-receptor dephosphorylation in solubilized membranes

Plasma membranes from CHO/HIRc cells were prepared and solubilized with Triton X-100 as described in the Experimental section. Insulin receptors present in solubilized membranes were autophosphorylated with $[\gamma\text{-}^{32}\text{P}]$ ATP/ Mn^{2+} and insulin for 30 min on ice and the dephosphorylation was initiated by transferring the reaction mixture to a water bath at 37°C after adding excess unlabelled ATP and various concentrations of compound **12** for the indicated periods. Results are expressed as percentage inhibition of insulin-receptor dephosphorylation by compound **12**. Each sample concentration was assayed in duplicate, and the results did not differ by more than 10%.

[23]. Quantification of autoradiograms was performed using ImageQuant software (version 3.3) on a Molecular dynamic densitometer (Sunnyvale, CA, U.S.A.).

Assay of protein serine/threonine phosphatase 1 and 2A

The effects of phosphonates on PP-1 and PP-2A activities were measured using a protein phosphatase assay system (GIBCO-BRL) with purified $[\text{P}^{32}]$ phosphorylase *a* as the substrate. $[\text{P}^{32}]$ phosphorylase *a* was prepared by allowing $[\gamma\text{-}^{32}\text{P}]$ ATP to react with purified phosphorylase *b* in the presence of purified phosphorylase kinase. The kinase reaction was allowed to go to completion, and the excess $[\gamma\text{-}^{32}\text{P}]$ ATP was removed by precipitating the ^{32}P -labelled phosphorylase *a* with $(\text{NH}_4)_2\text{SO}_4$, spinning down the protein and washing the pellet. The $(\text{NH}_4)_2\text{SO}_4$ was then removed by successive spins in a Centricon 30 protein concentrator. In a 60 μl assay mixture, protein serine/threonine phosphatases PP-1 (1 unit/ml) and PP-2A (1.25 unit/ml) were preincubated with and without okadaic acid or various phosphonates at the indicated concentrations for 5 min at 22°C in 20 mM Mops (pH 7.5)/60 mM 2-mercaptoethanol/0.1 M NaCl/50% glycerol/1 mg/ml BSA. The dephosphorylation reaction was initiated by the addition of 45 μg of $[\text{P}^{32}]$ phosphorylase *a*. After 10 min of dephosphorylation at 30°C, the reaction was terminated by the addition of trichloroacetic acid to a final concentration of 15%. Samples in Microfuge tubes were mixed and placed on ice for 10 min, then centrifuged at 12000 g for 5 min at 4°C. A portion of the clear supernatant (200 μl) were counted to determine the amount of radioactivity released in the assay. The assays were conducted under conditions in which the rate of dephosphorylation was linear as a function of time and enzyme dilution.

RESULTS

Effect of phosphonates on protein-tyrosine phosphatase PTP-1B

Samples at a concentration of 200 μM were tested for their inhibitory effect on the activity of a recombinant protein-tyrosine phosphatase PTP-1B. Of the 15 compounds shown in Table 1, **12**

Table 1 Effect of phosphonates on protein phosphatases

No.	Structure	Percentage inhibition*			No.	Structure	Percentage inhibition*		
		PP-1†	PP-2A†	PTB-1B‡			PP-1†	PP-2A†	PTB-1B‡
1		0	4.5±2.0	0	9		0	79.3±5.8	7.6±2.1
2		0	26.5±5.1	6±3.9	10		0	80.8±2.1	4.0±2.8
3		0	29.4±3.9	0	11		0	66.4±3.2	7.6±3.9
4		0	38.1±6.2	4±2.2	12		0	90±2.1	88±9
5		0	5±1	0	13		0	93±1	91±10
6		0	63	0	14		0	6±3.9	10±4
7		0	44±6	0	15		0	59.7±2.4	6.8±3
8		0	59±5.6	9.4±6.2					

* Results are expressed as means ± S.E.M. for two separate experiments carried out in duplicate.

† 32 P release from [32 P]-labelled phosphorylase α by PP-1 or PP-2A in the presence or absence of a 300 μ M concentration of compounds monitored at 30 °C for 5 min. Inhibition of dephosphorylation was calculated with respect to control and expressed as percentage inhibition.

‡ WGA-purified insulin receptors were phosphorylated with [32 P] ATP/Mn $^{2+}$ and used as substrate. Dephosphorylation of insulin receptors by recombinant PTB-1B was conducted in the presence or absence of the compounds (200 μ M) for 1 min at 22 °C as described in the Experimental section. Samples were resolved by SDS/PAGE under reducing conditions with quantitative analysis of the gels by Betagen counting.

and **13** potently inhibited [32 P]insulin-receptor dephosphorylation by PTP-1B, showing 88 ± 9 and 91 ± 10 % inhibition respectively. Other compounds had either significantly diminished or no effect on PTP-1B activity.

Determination of inhibitory potency of compound **12** against PTP-1B activity

[32 P]Insulin-receptor dephosphorylation by PTP-1B was measured at 22 °C in the presence or absence of the indicated concentrations of compound **12** for 1 and 2.5 min. Figure 2(a) shows that the rate of [32 P]insulin-receptor dephosphorylation

was inhibited in a dose-dependent manner at all time points tested with an apparent IC_{50} value of 35–40 μ M (Figure 2b). The potency of inhibition by compound **12** was decreased with increasing concentrations of substrate, and this inhibition was found to be competitive in nature. The maximum velocity remained constant, whereas the K_m value increased approximately 2-fold in the presence of 50 μ M inhibitor (results not shown). Compound **12** also potently inhibited dephosphorylation of another substrate, [32 P]poly(Glu 80 -Tyr 20), demonstrating ≈ 63 % and ≈ 92 % inhibition at concentrations of 50 and 100 μ M respectively (results not shown). Insulin-receptor dephosphorylation by PTP-1B was also inhibited by compound **13** in a dose-

dependent manner with an IC_{50} value of $40 \mu M$ (results not shown).

Effect of compound 12 on insulin-receptor autophosphorylation and dephosphorylation

Autophosphorylation of WGA-agarose-purified insulin receptor was conducted at $22^\circ C$ in the presence or absence of various concentrations of 12. Compound 12 did not have a direct effect on the autophosphorylation level of the insulin receptor at any concentration tested (25 – $200 \mu M$). To study the effect of compound 12 on insulin-receptor dephosphorylation we used Triton X-100-solubilized membrane preparations as the source of tyrosine phosphatase. Autophosphorylation of insulin receptor present in solubilized membranes was carried out on ice to reduce the activity of PTPase. Various amounts of compound 12 were added to prephosphorylated ^{32}P -labelled insulin receptors, and the dephosphorylation of the receptors by membrane-associated PTPases was determined. In this test, compound 12 was found to have considerable inhibitory activity against dephosphorylation of the insulin receptor. Compound 12 was active in a dose-dependent manner, with an apparent IC_{50} value of 45 – $50 \mu M$ (Figure 3). Compound 12 was also able to inhibit the dephosphorylation of the EGF receptor in solubilized membranes derived from A-431 cells with an apparent IC_{50} value of 45 – $50 \mu M$ (results not shown).

Effect of compound 12 on receptor autophosphorylation in intact cells

Intact CHO cells overexpressing either insulin receptor or EGF receptor were incubated for 1 h with compound 12 ($100 \mu M$) or vanadate ($1 mM$). The cells were then stimulated with insulin or EGF for 1 min. Vanadate significantly increased (~ 2.5 – 4 -fold) the level of hormone-stimulated receptor autophosphorylation, indicating that cellular protein tyrosine phosphatases were actively dephosphorylating both receptors during the course of the incubation. Compound 12 did not show any effect on the level of autophosphorylation of insulin receptor or EGF receptor in presence of respective ligands (results not shown).

Effect of phosphonates on serine/threonine phosphatase PP-1 and PP-2A

Phosphonates were tested for their inhibitory effects on serine/threonine phosphatases PP-1 and PP-2A. Dephosphorylation of ^{32}P phosphorylase α by PP-1 and PP-2A in the presence of $300 \mu M$ samples were monitored. Table 1 shows that several of the synthetic phosphonates inhibited PP-2A activity. Compounds 9, 10, 12 and 13 potently inhibited in the range of 80 – 90% . Compounds 6–8, 11, 14 and 15 inhibited moderately, showing approximately 40 – 65% inhibition and compounds 2–4 inhibited poorly by exhibiting inhibitory values in the range of 20 – 40% . Compounds 1 and 5 did not have any effect. All compounds when tested against the serine/threonine phosphatase PP-1 showed no inhibitory effect at concentrations of 300 and $675 \mu M$. Okadaic acid, a known serine/threonine phosphatase inhibitor, inhibited PP-2A activity with greater potency than PP-1. IC_{50} values for PP-1 and PP-2A were 18 – $20 nM$ and $\sim 0.2 nM$ respectively (results not shown).

Dose-dependent inhibition of PP-2A activity by phosphonate inhibitors

Dephosphorylation of ^{32}P phosphorylase α by PP-2A was measured in the presence of various concentrations of compounds 6,

Figure 4 Dose-dependent inhibition of PP-2A activity by phosphonates

The activity of serine/threonine phosphatase PP-2A was assayed in the presence of the indicated concentrations of various compounds for 5 min at $22^\circ C$ using ^{32}P -labelled phosphorylase α as substrate as described in the legend to Table 1. Results are expressed as means $\pm$ S.E.M. for two independent experiments performed in duplicate.

Figure 5 Activity of compound (naphth-2-yl) difluoromethylphosphonic acid (12) as both tyrosine and serine/threonine phosphatase inhibitor

Inhibition of PTP-1B-catalysed ^{32}P insulin-receptor dephosphorylation and PP-2A catalysed ^{32}P phosphorylase α dephosphorylation by compound 12 at indicated concentrations were assessed as described in the legend to Table 1.

10, 12 and 13. Figure 4 shows that all four compounds inhibited PP-2A activity in a dose-dependent manner with IC_{50} values of 200 , 60 , 50 and 45 – $50 \mu M$ respectively.

Activity of compound 12 against both tyrosine and serine/threonine phosphatase

Figure 5 shows that compound 12 inhibited both tyrosine phosphatase PTP-1B and serine/threonine phosphatase PP-2A with similar potency, having apparent IC_{50} values of 40 – $50 \mu M$.

DISCUSSION

Phosphonates were synthesized and used in various assays to determine their ability to modulate the extent of receptor dephosphorylation. Phosphonic acids are phosphate isosteres in which the ester oxygen has been replaced by a methylene unit.

One reason why such phosphate mimetics are valuable in biology [25,26] is that phosphonates are chemically and enzymically resistant to P–C bond cleavage and can act as competitive inhibitors of phosphatases. For example, low-molecular-mass acid phosphatase, which is highly specific for aryl phosphate substrates, is competitively inhibited by benzylphosphonic acid **1** [27]. One drawback of simple phosphonates is that inhibition constants are in the millimolar range [27,28]. Enhancement of inhibitory potency could theoretically be achieved by incorporating additional features into the phosphonate which promote enzymic recognition and binding. Since the natural substrates of protein phosphatases are peptidic in nature, a way to increase affinity is to include the phosphonate moiety within a peptide structure. Accordingly, 11-mer peptides in which the phosphotyrosine (pTyr) residue (compound **16**; Figure 1) has been replaced by a phosphonomethylphenylalanine (Pmp) residue (compound **17**) have been reported to inhibit competitively PTP-1B [29] and PTP 1 [30] with inhibition constants in the micromolar range (30 μ M and 10 μ M respectively).

A second means of potentially increasing binding affinity is to modify the phosphonate functionality itself. This approach has been successfully applied in the area of SH2-domain-binding peptides. We have shown that Pmp-containing peptides are less potent than native pTyr peptides in binding to SH2 domains [31]. However, modification of the Pmp residue by addition of either one (FPmp, **18**) or two (F₂Pmp, **19**) fluorine atoms at the phosphonate α -methylene (Figure 1), results in enhanced SH2 domain binding [32]. Substitution of fluorine atom or a hydroxy group (HPmp **20**) at the α -methylene of Pmp was theorized to reinstate hydrogen-bonding interactions normally afforded by the ester oxygen of the native pTyr, but lacking in the Pmp methylene bridge [33]. Additionally, phosphonate pK_a values are significantly higher than the corresponding phosphate, and fluorine substitution has been shown to lower phosphonate pK_a values to near or below native phosphate values [19]. We have recently demonstrated the applicability of this approach to peptidic PTP inhibitors by showing that a hexameric F₂Pmp-containing peptide exhibited a three orders of magnitude increase in potency relative to its Pmp homologue (IC₅₀ values of 100 nM versus 200 μ M respectively) when assayed against PTP-1B-mediated insulin-receptor dephosphorylation [34].

We therefore sought to extend these principles to the design of small phosphatase inhibitors by applying them to phenyl and naphthyl nuclei, yielding the benzylphosphonates **1–4** and naphthylmethylphosphonates **7–9**, **12** and **13** (Table 1). Additionally, three *o*-hydroxy analogues (**5**, **6** and **10**) and a chain-elongated phosphonate, **11**, were designed as bisubstrate mimetics. Finally, α -cyanocinnamamido derivatives **14** and **15** were intended as sp²-hybridized analogues of HPmp (**20**) and Pmp (**17**) respectively.

Some of the compounds inhibited serine/threonine phosphatase, while others inhibited both serine/threonine and tyrosine phosphatases. For example, compound **12** inhibited insulin-receptor dephosphorylation by membrane-associated protein tyrosine phosphatases and also by PTP-1B, a recombinant PTPase. The inhibition of insulin-receptor dephosphorylation by PTP-1B was competitive with respect to substrate. Compound **12** also inhibited phosphorylase α dephosphorylation by the serine/threonine phosphatase PP-2A. Compound **13**, which is an isomer of **12**, also inhibited PTP-1B and PP-2A. Changing substituents from the 2-position (**12**) to the 1-position (**13**) of the naphthyl nucleus did not have any effect on the potency of inhibition toward either tyrosine phosphatase PTP-1B or serine/threonine phosphatase PP-2A. Whether the mechanism(s) of inhibition for these two compounds are the same against both tyrosine phos-

phatase and serine/threonine phosphatase is not clear. Structure–activity relationships indicated that neither the side-chain structure nor the nature of the aryl ring is alone sufficient to elicit potent inhibitory effects on PP-2A. It is the combination of these which is essential, with the naphthalene ring and difluoromethyl side chain in concert providing the highest degree of inhibitory activity.

Under the conditions of the assay, nine out of 15 compounds (**6** and **8–15**) potently inhibited serine/threonine phosphatase PP-2A without any effect on the serine/threonine phosphatase PP-1. Structure–activity relationships of these compounds against PP-2A are significant. Benzylphosphonate **1** had little inhibitory activity, whereas sequential replacement of –CH₂– by –CHOH– (**2**), –CHF– (**3**) and CF₂ (**4**), increased inhibitory activity. For the phenyl series addition of an α -hydroxy group (**2**) or both an *o*-hydroxyl and a *p*-nitro group (**6**) resulted in increased inhibitory potency toward PP-2A. Alternatively, blocking the phosphonate hydroxy groups by esterification (**5**) significantly reduced inhibitory potency, indicating that an ionizable phosphonic acid is essential for inhibition. The phosphonomethylnaphthalene compounds in general showed inhibitory activity towards serine/threonine phosphatase PP-2A. In this series substitution of one H by –OH either in the –CH₂– side chain (**8**) or in the naphthyl ring (**10**) increased inhibitory potency. Also, substitution of –CH₂– by –CF₂– greatly increased inhibitory potency against PP-2A (**12** and **13**). For these analogues, substitution of H at the phosphonate α -methylene by fluorine residues increased inhibitory potency against both PTPases and PP-2A.

Several of the phosphonates showed inhibition of PP-2A without inhibiting PP-1. Although this is somewhat surprising, considering the common conserved catalytic core of PP-1 and PP-2A as recently reported by Barton et al. [35], inhibitory discrimination between PP-1 and PP-2A is not without precedent. Okadaic acid [36–39] and microcystin L [40–43] are known inhibitors of serine/threonine phosphatase which inhibit both PP-1 and PP-2A. Cohen et al. [44] have shown that okadaic acid has 150–200 fold higher inhibitory potency against PP-2A as compared with PP-1. Under the assay conditions employed in our studies, okadaic acid similarly showed higher potency against PP-2A than PP-1 (results not shown). The structural basis of the phosphonate selectivity is not known, and further studies will be required to examine this question. Additionally studies are in progress to determine inhibitory effects of these phosphonates on other known serine/threonine phosphatases, including PP-2B (calcineurin) and PP-2C. Furthermore, dual-specificity phosphatases/kinases are key to the regulation of cell-growth, differentiation and metabolism [45]. As the number of dual-specificity phosphatases increases e.g. PAC-1 [46], MKP-1 [47], VH-1 [48], CL100 [49], cdc25 [50], generation of agents which can inhibit these dual specificity phosphatases becomes obvious. Two compounds (**12** and **13**), inhibited both tyrosine phosphatase PTP-1B and serine/threonine PP-2A with equal potency *in vitro* and testing of efficacy of these compounds against known dual specificity phosphatases is needed. The variety of side-chain functionality displayed by this series could potentially make them very useful in the further characterization of the catalytic activity of phosphatases.

It is noteworthy that both naphth-1-yl (**13**)- and naphth-2-yl (**12**)-containing inhibitors gave much greater inhibition of PTP-1B than the corresponding phenyl containing compound **4** (Table 1), although the latter may appear to be closer mimic of a tyrosine residue. This evidently indicates that high-potency binding requires interactions of the inhibitor with the PTP extending beyond a single aromatic ring. This is shown in other

cases. For example, both the phosphotyrosine analogue, sulphonylphosphonic acid [23], and phenyldifluoromethylphosphonic acid (**4**) alone are not PTP inhibitors. However, when these residues are incorporated into peptides, potent inhibitors of PTPases result [23, 34], indicating that additional binding interactions were required. In order to address these questions, the binding of compound **12** with purified PTP-1B is currently being examined by X-ray crystallography (D. Barford, unpublished work).

In intact cells phosphorylation and dephosphorylation processes occur simultaneously. If these compounds can enter cells, they may be expected to inhibit PTPase activity and increase receptor autophosphorylation. CHO cells overexpressing either insulin receptor or EGF receptor were treated with compound **12** or vanadate, a known tyrosine phosphatase inhibitor, and the level of receptor autophosphorylation was measured. Autophosphorylation of both receptors was unaffected by compound **12**, whereas vanadate stimulated autophosphorylation in both cases. This indicated that compound **12** was unable to cross the plasma membrane. Because these phosphonates are highly charged, it is reasonable that they will not cross cell membranes easily. We are currently preparing modified analogues with enhanced membrane-transport properties. Such inhibitors may aid in understanding the function of protein phosphatases in the regulation of cellular processes.

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