VHR and PTP1 Protein Phosphatases Exhibit Remarkably Different Active Site Specificities toward Low Molecular Weight Nonpeptidic Substrates

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ABSTRACT: The dual-specificity protein phosphatases have recently been shown to act as key regulators of mitogenic signaling pathways as well as of the cell cycle process. The are unusual catalysts in that they can utilize protein substrates containing phosphotyrosine as well as phosphoserine/threonine. The dual-specificity phosphatases and the protein tyrosine phosphatases (PTPase) share the active site motif (H/V)**C**(X)$_5$**R**(S/T) but display little amino acid sequence identity outside of the active site. Although the dual-specificity phosphatases and the PTPases appear to bring about phosphate monoester hydrolysis through a similar mechanism, there is very limited information about the structural features that control the substrate specificity for the two groups of enzymes. As a first step in the development of selective dual-specificity phosphatase inhibitors, we have examined the active site substrate specificity of the human dual-specificity phosphatase, VHR [for VH1-Related; Ishibashi et al. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 12170−12174]. Like the tyrosine-specific PTP1, VHR also preferentially catalyzes the hydrolysis of aromatic phosphates. However, we demonstrate herein that relatively modest changes in the substitution patterns on the phosphorylated aromatic nucleus generates dramatic, and differential, swings in substrate selectivity for VHR and PTP1. For example, VHR appears to be significantly more accommodating than PTP1 toward sterically-demanding substrates. Thus, the active site specificity of these two protein phosphatases is decidedly dissimilar. In addition, we have also identified several low molecular weight compounds that are more efficient substrates than the most potent peptidic substrates ever reported for VHR. Finally, we have shown that the Michaelis constants exhibited by these substrates are accurate assessments of enzyme affinity. Consequently, it should be possible to develop phospatase-selective inhibitors based upon the distinct substrate specificities of these enzymes.

The functional role of protein tyrosine phosphatases (PTPases)[1] in cellular signaling processes is just beginning to be appreciated (Sun & Tonks, 1994; Hunter, 1995). PTPases constitute a growing family of transmembrane (receptor-like) and intracellular enzymes that rival the protein tyrosine kinases (PTKs) in terms of structural diversity and complexity. Since deregulated PTKs, such as *src*, *lck*, and *neu*, can function as dominant oncogenes, it has been assumed that, at least some PTPases, function as products of tumor suppressor genes. Indeed, mutations in SH-PTP1 lead to severe immune dysfunction and give rise to the *moth-eaten* phenotype in mice (Shultz et al., 1993). Thus, SH-PTP1 may be an important negative regulator of cytokine signaling, its loss resulting in sustained tyrosine phosphorylation and consequent enhanced proliferation (Klingmuller et al., 1995). In addition, expression of PTP1B has been shown to block transformation mediated by *neu* (Brown-Shimer et al., 1992) and to partially revert transformation by *src* (Woodord-Thomas et al., 1992). On the other hand, there is mounting evidence that some PTPases potentiate, rather than antagonize, the action of PTKs. This behavior enhances mitogenic signaling and leads to cellular transformation. Thus the receptor PTPase CD45, through its capacity to dephosphorylate and activate the *src* family of PTKs, is essential for initiating downstream signaling processes in response to stimulation of T and B cell receptors (Pingel & Thomas, 1989). SH-PTP2 and its *Drosophila* homolog *corkscrew* are positive mediators of growth factor signaling (Perkins et al., 1992; Noguchi et al., 1994). The cell cycle regulator cdc25 dephosphorylates Tyr15 of cdc2, thereby activating the cdc2/cyclin B complex which, in turn, promotes mitosis (Millar & Russell, 1992). Strikingly, ectopic expression of PTPα produces a transformed phenotype in rat embryonic fibroblasts (Zheng et al., 1992). The importance of PTPases in cellular physiology is further emphasized by the fact that they are often targets for microbial or viral intervention. For instance, the pathogenic bacteria *Yersinia* encodes a PTPase essential for its virulence (Guan & Dixon, 1990; Bliska et al., 1991), and vaccinia virus encodes a dual-specificity phosphatase VH1 (Guan et al., 1991) that is essential for viral transcription and infectivity (Liu et al., 1995).

Clearly, PTPases are participants in a diverse array of biochemical pathways. As a result, PTPase-specific inhibitors may provide further insight into the key regulatory roles played by these enzymes. In addition, such inhibitors may ultimately provide a molecular foundation upon which the design of medicinally useful compounds can be based. However, three key hurdles must be overcome before chemotherapeutic intervention through these enzyme targets can proceed. First, an intimate understanding of the active

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[1] Abbreviations: PTK, protein tyrosine kinase; PTPase, protein tyrosine phosphatase.

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site structure of these enzymes is required to assist in the design of potent inhibitory species. In this regard, the recent elucidation of the tertiary structure of several PTPases has provided a wealth of detailed information (Barford et al., 1994; Stuckey et al., 1994; Zhang, M., et al., 1994; Su et al., 1994; Jia et al., 1995). Second, it is essential that PTPases be targeted in an exquisitely selective fashion since these enzymes not only assume diverse biochemical roles, but can actually either oppose or augment signaling pathways. Third, since peptide-based inhibitors are unlikely to serve in a medicinally useful capacity, it is critical that potent non-peptidic substrates (and ultimately inhibitors) be identified. Recently, we investigated the active site substrate specificity of PTP1, a phosphatase isolated from rat brain (Zhang, Z.-Y., 1995a; Montserat et al., 1996). This enzyme utilizes a variety of structurally simplistic aromatic phosphates as substrates. Furthermore, several of these compounds are hydrolyzed nearly as efficiently as the very best peptide-based substrates identified to date. Finally, the low K_m values exhibited by several of these substrates are accurate reflections of their affinity for PTP1. In short, at least for PTP1, potent substrate (and presumably inhibitor) recognition does not require the presence of a peptide backbone. Clearly, this augurs well for the development of chemotherapeutic agents. However, we wondered whether this ability to efficiently hydrolyze low molecular weight compounds is exhibited by other members of the protein phosphatase family. We describe herein the behavior of the VHR protein phosphatase, a dual-specificity phosphatase. We have found that VHR catalyzes the hydrolysis of several low molecular weight compounds an order of magnitude more efficiently than the most potent peptide-based substrates ever reported for this enzyme. In addition, the active site specificities of VHR and PTP1 exhibit some remarkable differences.

MATERIALS AND METHODS

All phenol derivatives and common reagents were obtained from commercial suppliers and used without further purification. Solvents were distilled and dried as required. All phosphorylated phenols were prepared by one of two previously described general methods (Zhang & Van Etten, 1990; Perry & Johns, 1988), except for compounds **1**, **19**, and **21** (which were purchased from Sigma) and **25** (which was prepared from the hydrolysis of commercially available 1,2-phenylene phosphorochloridite). A more detailed description of the preparation of the aryl phosphates can be found in Montserat et al., (1996). All compounds, with the exception of **18** and **20**, were purified by recrystallization as the cyclohexylammonium salt and were characterized as such. The structures of new compounds were confirmed by ^{1}H NMR (400 Mhz), ^{13}C NMR (22.5 Mhz), ^{31}P NMR (161.9 Mhz), and fast atom bombardment mass spectral analysis (negative ion). Enzyme assay solutions were prepared with deionized/distilled water. Recombinant VHR (Zhang, Z.-Y., et al., 1995) and the catalytic domain of rat PTP1 (Hengge et al., 1995) were expressed and purified as described.

PTPase Assay. All enzyme assays were performed at 30 °C in 50 mM succinate, 1 mM EDTA, pH 6.0 buffer with a constant ionic strength of 0.15 M (adjusted with NaCl). Initial rates for the enzyme-catalyzed hydrolysis of phosphate monoesters were measured by the production of inorganic phosphate using a colorimetric method described previously (Zhang & Van Etten, 1990; Black & Jones, 1983). Michaelis−Menten kinetic parameters were determined from a direct fit of the velocity versus substrate concentration data to the Michaelis−Menten equation using the nonlinear regression program GraFit (Erithacus Software).

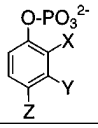
*Assay of Substrates **13**, **15**, **17**, **24**, and **25** as PTPase Inhibitors*. The inhibition constants for **13**, **15**, **17**, **24**, and **25** were determined for the VHR-catalyzed hydrolysis of **1**. At various fixed concentrations of inhibitors, the initial rates at concentrations of **1** were measured as described (Zhang, Z.-Y., 1995b). The data were fit to $v = V_{max}S/(K_m[1 + I/K_i] + S)$ using KINETASYST (IntelliKinetics, State College, PA) to obtain the inhibition constant (K_i).

RESULTS AND DISCUSSION

PTPases constitute a growing family of enzymes that can be divided into two groups, receptor-like and intracellular enzymes. The unique feature that defines the whole PTPase family is the active site sequence (H/V)**C**(X)$_5$**R**(S/T) called the PTPase signature motif in the catalytic domain (Zhang, Z.-Y., et al., 1994a). Interestingly, the PTPase signature motif can be found in the structures of dual specificity phosphatases that display little amino acid sequence identity with the classical PTPases. Unlike the PTPases which show substrate specificity strictly restricted to phosphotyrosine-containing proteins, this group of phosphatases are unusual in that they can utilize substrates containing phosphotyrosine, phosphoserine and phosphothreonine. Since the discovery of the *Vaccinia* phosphatase, VH1 (for *Vaccinia* open reading frame *H1*) (Guan et al., 1991), a number of additional dual-specific phosphatases have been identified. The mammalian dual-specific protein phosphatases have recently surfaced as key regulators of mitogenic signaling pathways as well as the cell cycle itself (Keyse, 1995). VHR (for VH1-Related; Ishibashi et al., 1992), which may be responsible for activation of cdk−cyclin complex(es) at some stage of the cell cycle (Aroca et al., 1995), has emerged as the prototype for this class of enzymes. Not only has this particular phosphatase been overexpressed and purified to homogeneity, but, in addition, it has been the subject of several detailed enzymological investigations (Zhou et al., 1994; Denu et al., 1995a,b; Zhang, Z.-Y., et al., 1995). Furthermore, PTPases and the dual-specificity phosphatases utilize a common mechanism to bring about phosphate monoester hydrolysis.

A paucity of information is available regarding the structural features that govern the substrate recognition for the dual-specificity phosphatases. A recent substrate-specificity analysis of VHR, which utilized several phosphotyrosine/phosphothreonine-containing peptides, revealed that phosphotyrosine is hydrolyzed 3 orders of magnitude more efficiently than phosphothreonine (Denu et al., 1995a). Indeed, we have found that, like the PTPases (Zhang, Z.-Y., 1995a,b), VHR is able to preferentially catalyze the hydrolysis of simple aromatic phosphates, such as phosphotyrosine, over alkyl phosphates, such as phosphoserine (Zhang, Z.-Y., et al., 1995). However, we have also found that, although simple alkyl phosphates are poor substrates for VHR, alkyl phosphates having a pendant aromatic group, such as pyridoxal 5′-phosphate, and compounds with the structure $Ar(CH_2)_nOPO_3H_2$ ($n = 1-5$) have k_{cat} and K_m values similar to those of aryl phosphates, suggesting that

Table 1: K_m and V_{max} Values for Aromatic Phosphates **1**−**9**[a]



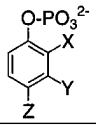
	PTP1			VHR		
	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (mM^{-1} s^{-1})	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (mM^{-1} s^{-1})
(**1**) Z = NO_2	64 ± 1	0.62 ± 0.06	103 ± 10	6.0 ± 0.2	1.7 ± 0.1	3.5 ± 0.3
(**2**) X = CH_2CH_3	0.34 ± 0.04	4.2 ± 1.1	0.08 ± 0.02	7.3 ± 0.3	0.66 ± 0.08	11 ± 1
(**3**) Y = CH_2CH_3	43 ± 3	3.1 ± 0.4	14 ± 2	3.90 ± 0.2	0.36 ± 0.07	11 ± 2
(**4**) Z = CH_2CH_3	54 ± 2	1.2 ± 0.1	45 ± 4	5.3 ± 0.5	0.74 ± 0.12	7.2 ± 1
(**5**) Y = $CH(CH_3)_2$	4.3 ± 0.4	2.1 ± 0.6	2.0 ± 0.5	3.7 ± 0.1	0.39 ± 0.02	9.5 ± 0.6
(**6**) Z = $CH(CH_3)_2$	53 ± 1	1.1 ± 0.1	48 ± 3	3.8 ± 0.2	0.25 ± 0.05	15 ± 3
(**7**) Y = C_6H_5	4.6 ± 0.6	0.32 ± 0.08	14 ± 4	2.7 ± 0.5	0.30 ± 0.10	9.0 ± 3
(**8**) Z = C_6H_5	30 ± 10	2.4 ± 0.9	13 ± 6	2.1 ± 0.4	0.24 ± 0.09	8.7 ± 3
(**9**) Z = $CH_2C_6H_5$	55 ± 3	0.30 ± 0.04	182 ± 23	3.9 ± 0.1	0.070 ± 0.005	56 ± 4

[a] Kinetic constants were determined as described under Materials and Methods. The values are given as the average ± SD.

the aromatic moieties are important for VHR binding. The fact that similar kinetic parameters are observed for substrates with 0−5 methylene units between the phosphate and the aromatic moiety also indicates that the active site of VHR is rather flexible (Zhang, Z.-Y., et al., 1995). Collectively, these results suggest the existence of a hydrophobic binding pocket at or near VHR active site which is specific for the aromatic moiety of the substrate, because simple ionic interaction between the phosphate and the active site Arg residue cannot by itself explain the distinctive recognition and binding of phosphate monoesters with the structure Ar-$(CH_2)_n OPO_3H_2$ (n = 1−5) (Zhang, Z.-Y., et. al., 1995). Interestingly, VHR hydrolyzes the peptide-based substrate DHTGFLpTEpYVATR more efficiently (by more than an order of magnitude) than simple aryl phosphates (Denu et al., 1995a). Unlike simple aromatic phosphates, peptide substrates can presumably participate in numerous noncovalent interactions with a variety of residues that are positioned in and around the active site. These interactions could promote binding and/or assist in catalysis. Indeed, there is a consensus that tyrosine-specific phosphatases, like VHR, are also unable to hydrolyze simple phosphorylated aromatic alcohols as efficiently as phosphotyrosine-containing peptides (Zhang, Z.-Y., et al., 1994b). As a consequence, it has been generally assumed that potent phosphatase inhibitors would emerge from peptide-based prototypes (Chatterjee et al., 1992; Zhang, Z.-Y., et al., 1994b; Kole et al., 1995; Chen et al., 1995). However, we have recently found that appropriately functionalized aromatic phosphates are hydrolyzed by the tyrosine-specific phosphatase PTP1 as efficiently as the very best peptide substrates reported for this enzyme (Montserat et al., 1996). In light of these surprising results, we have posed the following questions: Will other protein phosphatases likewise utilize simple aromatic phosphates as substrates in an efficient fashion? Do the K_m values exhibited by these substrates reflect their affinity for the target enzyme? Will different protein phosphatases exhibit different active site specificities with respect to unnatural substrates, even if they hydrolyze the same naturally occurring phosphorylated residue(s)? These questions have now been addressed in the context of a comparative analysis of PTP1 and VHR.

As noted above, VHR catalyzes the hydrolysis of *p*-nitrophenylphosphate (**1**), an activity displayed by many members of the tyrosine-specific family of protein phosphatases, including PTP1 (Table 1). At saturating substrate concentration, pH 6 and 30 °C, the optimal turnover number k_{cat} for PTP1 (Zhang, Z.-Y., 1995a) and VHR (Zhang, Z.-Y., et al., 1995), is 60−70 and 6 s^{-1}, respectively. Clearly, **1** is a significantly better substrate for PTP1 than for VHR, due to an order of magnitude larger k_{cat} term for the PTP1-catalyzed reaction. In marked contrast, the *ortho*-substituted aromatic phosphate **2** is a dramatically more efficient VHR substrate. This extraordinary difference in active site specificity is almost completely due to the inability of PTP1 to efficiently hydrolyze aromatic phosphates containing sterically demanding *ortho* substituents (Zhang, Z.-Y., 1995b; Montserat et al., 1996). In contrast, VHR catalyzes the hydrolysis of both **1** and **2** with near-equal efficiency. Consistent with these observations, we have previously found that α-naphthyl phosphate is a much poorer substrate than β-naphthyl phosphate for tyrosine-specific phosphatases, which is likely due to steric hindrance (Zhang, Z.-Y., et al., 1994c; Zhang, Z.-Y., 1995b). In stark contrast, the dual-specificity phosphatase VHR-catalyzed hydrolysis of α-naphthyl phosphate displays kinetic parameters (k_{cat} = 7.0 ± 0.8 s^{-1} and K_m = 0.38 ± 0.036 mM) that compare favorably to those for β-naphthyl phosphate [k_{cat} = 6.8 ± 0.4 s^{-1} and K_m = 0.50 ± 0.07 mM (Zhang et al., 1995)]. The implications of these results for inhibitor design are clear, particularly for those inhibitors (*i.e.*, suicide substrates) that require "activation" by the active site residues responsible for catalysis. In short, VHR-selectivity should be possible through the appropriate incorporation of sterically demanding substituents *ortho* to the site of phosphate hydrolysis. Decidedly different behavior is displayed with the *meta*- and *para*-substituted derivatives **3** and **4**, respectively. In terms of k_{cat}/K_m, both compounds are slightly better PTP1 substrates. In addition, the k_{cat} values associated with the PTP1-catalyzed hydrolysis of both compounds are an order of magnitude greater than those obtained with VHR. However, the Michaelis constants exhibited by these substrates are lower (particularly the *meta* derivative **3**) for the VHR-catalyzed reaction. The kinetic properties displayed by **5** and **6** reveal an additional difference in active site specificity between PTP1 and VHR. The *meta*-isopropyl substituent in **5** has a negative impact on catalytic efficacy for PTP1. Given the sensitivity of PTP1 to *ortho* substituents, it is not too surprising that large *meta* substituents might also impair hydrolytic efficacy. In contrast, VHR does not distinguish between the *meta*- and *para*-substituted derivatives **5** and **6**. *In short, the active site structure of the VHR protein phosphatase appears to be more accommodating than that of PTP1*. The phenyl-substituted derivatives, **7** and **8**, are both modest PTP1 substrates; however, the k_{cat} and K_m associated with **7** are significantly lower than those displayed

Table 2: K_m and V_{max} Values for Aromatic Phosphates **10**−**16**[a]



	PTP1			VHR		
	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (mM^{-1} s^{-1})	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (mM^{-1} s^{-1})
(**10**) Y = CO_2^-	8.3 ± 0.2	8.0 ± 0.5	1.0 ± 0.1	6.1 ± 0.2	2.1 ± 0.2	2.9 ± 0.3
(**11**) Z = CO_2^-	59 ± 5	2.1 ± 0.5	28 ± 7	3.0 ± 0.1	1.0 ± 0.2	3.0 ± 0.6
(**12**) Y = $CH_2CO_2^-$	5.8 ± 0.1	1.4 ± 0.1	4.1 ± 0.4	5.4 ± 0.3	1.9 ± 0.3	2.8 ± 0.4
(**13**) Z = $CH_2CO_2^-$	2.9 ± 0.3	0.080 ± 0.018	37 ± 9	0.99 ± 0.06	0.12 ± 0.02	8.2 ± 1
(**14**) Z = $CH_2CH_2CO_2^-$	53 ± 1	0.28 ± 0.03	188 ± 20	6.4 ± 0.4	0.43 ± 0.09	15 ± 3
(**15**) Z = *p*-$C_6H_4CO_2^-$	17 ± 1	0.090 ± 0.012	190 ± 27	2.0 ± 0.2	0.078 ± 0.020	26 ± 8
(**16**) phosphopodocarpic acid	1.1 ± 0.1	0.32 ± 0.1	4 ± 1	3.7 ± 0.2	0.082 ± 0.013	45 ± 7

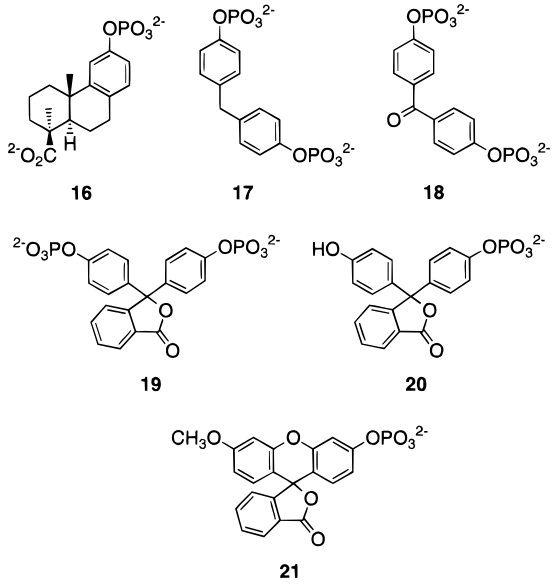
[a] Kinetic constants were determined as described under Materials and Methods. The values are given as the average ± SD.

with **8**. Both **7** and **8** are modest substrates for VHR as well. Finally, the *para*-benzyl substituent in **9** enhances the k_{cat}/K_m terms, relative to those displayed by compounds **1**−**8**, for both PTP1 and VHR. *Indeed, compound **9** is a more efficient VHR substrate than the best peptide-based substrate (DHTGFLpTEpYVATR, $k_{cat} = 4.6\ s^{-1}$, $K_m = 140\ \mu M$) ever reported for this enzyme* (Denu et al., 1995a).

We have previously shown that negatively charged substituents, appropriately positioned on the aromatic nucleus, can dramatically enhance the efficiency of PTP1-catalyzed hydrolysis of aryl phosphates (Montserat et al., 1996). Both compounds **10** and **12**, species which possess a negatively charged carboxylate positioned *meta* to the phosphate moiety (Table 2), are poor substrates for VHR and PTP1. In addition, a carboxylate directly attached to the *para* site (**11**) fails to generate potent phosphatase activity. Interestingly, the substrate efficacy of **13** is subdued due to a substantial drop in k_{cat} for both enzymes. Indeed, in terms of turnover number, **13** is the poorest VHR substrate and one of the poorest PTP1 substrates evaluated in this study. In contrast, the K_m values associated with this compound are quite respectable ($K_m = 80 \pm 18\ \mu M$ with PTP1 and 120 ± 20 μM with VHR). There are a number of possible explanations for this behavior. However, one intriguing scenario is the existence of nonproductive binding mode(s). For example, the carboxylate moiety could occupy the active site region normally reserved for the hydrolyzable phosphate group. Alternatively, the carboxlyate functionality may bind elsewhere in the active site. The latter could enhance enzyme affinity, but at the expense of the ideal active site alignment required for rapid phosphatase-catalyzed hydrolysis. Both the k_{cat} and the K_m values should decrease under either of these nonproductive binding scenarios (Fersht, 1985). In addition to **13**, it appears that both **15** and **17** may also be engaged in a combination of productive and nonproductive binding modes.

Compound **14**, which contains two methylene groups between the carboxylate acid moiety and the aromatic nucleus, displays a more robust k_{cat} relative to that exhibited by **13**. This increase is 20-fold in the case of PTP1, whereas for VHR, it amounts to only a 6-fold enhancement. In addition, for both enzymes, the K_m values associated with **14** increase slightly relative to **13**. The biphenyl derivative **15** is similar in catalytic efficiency to **14** for both PTP1 and VHR. In both instances, **15** exhibits lower K_m and k_{cat} values than those displayed by **14**. We also note that compounds **15** and **8** are structurally identical except for the carboxylate located on the *para* position of the former. In the case of PTP1, the additional carboxylate provides over an order of magnitude increase in k_{cat}/K_m due to a sharp drop in K_m. In contrast, **15** serves as only a 2.5-fold more effective VHR substrate relative to **8**. However, perhaps the most notable aspect of the PTP1- and VHR-catalyzed hydrolysis of the negatively charged substrates **10**−**15** are the similar trends observed in catalytic activity. The exception to this observation is the decreased k_{cat} values displayed by PTP1, but not by VHR, for **10** and **12**. This may be due, in part, to the apparent sensitivity of PTP1 to steric bulk positioned *meta* to the phosphate moiety.

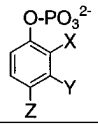
Chart 1



Podocarpic acid (**16**, see Chart 1) like compounds **10**−**15**, contains a negatively charged carboxylic moiety. However, fused to the aromatic phosphate nucleus is a sterically imposing decalin ring system. As we have noted with compounds **5** and **7**, large substituents positioned at the *meta* site curtail efficient hydrolysis by PTP1, but have little or no effect on the phosphatase activity of VHR. Indeed, podocarpic acid is hydrolyzed an order of magnitude more efficiently by VHR than by PTP1. This result, in combination with the behavior of compound **2** and α-naphthyl phosphate, demonstrates that relatively simple structural motifs are differently accommodated by the active sites of VHR and PTP1.

We also investigated the catalytic efficacy of several diphosphates (Table 3). These compounds proved to be the most efficient nonpeptidic PTPase substrates identified to data (Montserat et al., 1996). The diaryl derivative **17** is a potent PTP1 substrate, exhibiting a K_m of 16 μM. We have

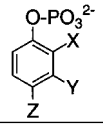
Table 3: K_m and V_{max} Values for Aromatic Phosphates **17**−**21**[a]



	PTP1			VHR		
	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (mM^{-1} s^{-1})	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (mM^{-1} s^{-1})
(**17**)	6.9 ± 0.4	0.016 ± 0.003	431 ± 85	1.2 ± 0.7	0.030 ± 0.006	40 ± 24
(**18**)	49 ± 1.1	0.13 ± 0.02	377 ± 58	2.8 ± 0.1	0.30 ± 0.03	9.3 ± 1
(**19**)	5.2 ± 0.2	0.36 ± 0.05	14 ± 2	4.3 ± 0.2	0.024 ± 0.004	180 ± 30
(**20**)	1.84 ± 0.02	2.2 ± 0.5	0.8 ± 0.2	4.6 ± 0.11	0.069 ± 0.007	67 ± 4
(**21**)	12.6 ± 1.0	0.52 ± 0.06	24 ± 3	4.9 ± 0.18	0.020 ± 0.003	245 ± 38

[a] Kinetic constants were determined as described under Materials and Methods. The values are given as the average ± SD.

Table 4: K_m and V_{max} Values for Aromatic Phosphates **22**−**26**[a]



	PTP1			VHR		
	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (mM^{-1} s^{-1})	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (mM^{-1} s^{-1})
(**22**) X = OCH_3	7.1 ± 0.6	6.6 ± 1.0	1.1 ± 0.2	5.7 ± 0.2	1.5 ± 0.8	2.8 ± 2.0
(**23**) Y = OCH_3	53 ± 1	5.6 ± 0.6	9.4 ± 0.9	5.4 ± 0.5	1.1 ± 0.3	4.9 ± 1.0
(**24**) Z = OCH_3	70 ± 4	3.2 ± 0.4	22 ± 3	5.3 ± 0.1	1.4 ± 0.1	2.8 ± 0.3
(**25**) X = OH	36 ± 1	1.3 ± 0.1	27 ± 2	3.1 ± 0.9	1.3 ± 0.1	2.4 ± 0.2
(**26**) Z = $O_2CC_6H_5$	60 ± 3	0.62 ± 0.06	97 ± 10	3.3 ± 0.1	0.15 ± 0.02	22 ± 3

[a] Kinetic constants were determined as described under Materials and Methods. The values are given as the average ± SD.

previously shown that this Michaelis constant is an accurate assessment of enzyme affinity (Montserat et al., 1996). VHR also hydrolyzes this compound with a favorable K_m (30 μM). Although **17** is the most efficient substrate that we have thus far identified for PTP1, it is a relatively modest VHR substrate. The carbonyl bridge between the aryl groups in compound **18** appears to have a dramatic impact on the K_m values obtained with both VHR and PTP1. In both cases there is an order of magnitude increase in K_m relative to the Michaelis constants exhibited by **17**. Although this is offset by an analogous increase in k_{cat} for the PTP1-catalyzed reaction, that is not true for VHR. In short, whereas **18** is efficiently hydrolyzed by PTP1, it is a significantly more modest substrate for VHR. Phenolphthalein diphosphate (**19**) and 3-*O*-methylfluorescein phosphate (**21**) are the best substrates that we have obtained for VHR. The kinetic constants associated with their hydrolysis (k_{cat} = 4.3 ± 0.2 s^{-1} and K_m = 24 ± 4 μM for **19**, k_{cat} = 4.9 ± 0.18 s^{-1} and K_m = 20 ± 3 μM for **21**) compare very favorably with those previously noted for DHTGFLpTEpYVATR (k_{cat} = 4.6 s^{-1}, K_m = 140 μM), the best peptide-based substrate reported for this enzyme. Interestingly, VHR exhibits only a 3-fold preference for the diphosphorylated derivative **19** relative to its monophosphorylated counterpart **20**. In contrast, this preference jumps to 18-fold for PTP1. The behavior of PTP1 is in keeping with a previous observation that 3,6-fluorescein diphosphate is a much better substrate than fluorescein monophosphate for a number of PTPases (Desmarais et al., 1995). This is consistent with the fact that negatively charged residues, particularly on the NH_2-terminal side of the phosphotyrosine moiety, enhance the efficacy of PTP1 peptide-based substrates (Zhang, Z.-Y., et al., 1993). The extraordinary effectiveness of both mono- and diphosphates **19**−**21** as VHR substrates stands in stark contrast to the behavior exhibited by PTP1. Indeed, compound **20** is hydrolyzed almost 2 orders of magnitude more effectively by VHR than by PTP1. These results further illustrate the striking differences in the active site specificities of VHR and PTP1.

Table 5: Comparison of K_i and K_m Values for Compounds **13**, **15**, **17**, **24**, and **25**[a]

compound	K_m (mM)	K_i (mM)
(**13**)	0.12 ± 0.2	0.15 ± 0.01
(**15**)	0.078 ± 0.020	0.16 ± 0.01
(**17**)	0.030 ± 0.006	0.059 ± 0.004
(**24**)	1.4 ± 0.1	1.4 ± 0.1
(**25**)	1.3 ± 0.1	1.2 ± 0.1

[a] Inhibition and Michaelis constants were determined as described under Materials and Methods. The values are given as the average ± SD.

Finally, we investigated the protein phosphatase substrate efficacy of several electron rich aromatic phosphates (Table 4). Compound **22**, which contains a methoxy substituent at the *ortho* position, is more efficiently hydrolyzed by VHR. Indeed, the K_m is nearly an order of magnitude lower for the VHR-catalyzed reaction. In contrast, the *meta*- and *para*-substituted derivatives **23** and **24**, respectively, are somewhat better substrates for PTP1. Compound **25**, which contains a relatively small hydroxyl group at the *ortho* position, is well tolerated by PTP1. However, this compound is a weaker substrate for VHR than the more sterically imposing derivatives **2** and **22**. Finally, the benzoate ester **26** is a reasonably effective substrate for both enzymes.

Clearly, PTP1 and VHR exhibit overlapping, bot nontheless quite distinct, active site substrate specificities. Can these differences be employed to create enzyme-selective inhibitors? In order for this question to be answered in the affirmative, some correlation must exist between active site substrate efficacy and enzyme affinity. Indeed, we have recently found such a correlation for PTP1 (Montserat et al., 1996). This appears to be the case for VHR as well. The K_i values shown for the compounds listed in Table 5 were obtained with **1** as the substrate. The hydrolysis of this compound can be easily followed by UV−visible spectroscopy *via* formation of the colored *para*-nitrophenolate product. In addition, the photophysical properties of the latter are not compromised by the products obtained from

the hydrolysis of compounds **13**, **15**, **17**, **24**, and **25**. Consequently, we were able to investigate the ability of these five compounds to serve as inhibitors of the VHR-catalyzed dephosphorylation of **1**. As is clear from Table 5, the inhibitory prowess (K_i) of these compounds nicely correlates with their respective Michaelis constants. As expected, the inhibition patterns for these compounds are competitive with respect to **1** (data not shown). Consequently, for the low molecular weight substrates evaluated herein, substrate efficacy appears to be a reliable barometer for successful inhibitor design.

In summary, we have shown that the VHR dual-specificity phosphatase will catalyze the hydrolysis of nonpeptidic aromatic phosphates. Indeed, several of the compounds evaluated herein are more efficient substrates than the most potent peptidic substrates thus far identified for VHR. In addition, we have found that the active site substrate specificities of VHR and PTP1 are not identical, in spite of the fact that both enzymes preferentially catalyze the hydrolysis of aryl phosphates. Consequently, it should be possible to develop phosphatase-selective inhibitors based upon the distinct substrate specificities of these enzymes. Finally, we have demonstrated that the Michaelis constants exhibited by these substrates are accurate assessments of enzyme affinity. Therefore, the most effective substrates in this study should serve as lead compounds for the design of even more potent substrates as well as novel inhibitors. In addition, unlike peptides, the low molecular weight compounds described herein can be rendered membrane permeant. In short, the results of this study suggest that those obstacles and complications commonly associated with peptide-based substrates and inhibitors can now be easily avoided.

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