α-Haloacetophenone Derivatives As Photoreversible Covalent Inhibitors of Protein Tyrosine Phosphatases

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Phosphorylation of proteins on tyrosine residues is one of the most important posttranslational modifications, playing central roles both in physiological processes such as transmembrane signaling and in pathological processes such as cancer and immune dysfunction.[1]

The levels of tyrosine phosphorylation are regulated by the opposing actions of protein tyrosine kinases (PTKs), which catalyze the formation of phosphotyrosine (pY) in proteins, and protein tyrosine phosphatases (PTPs), which hydrolyze pY residues to give back tyrosine and inorganic phosphate. More than 100 PTPs have been identified to date, and it is estimated that the human genome contains as many as 500 PTP genes.[2] The precise functions of these PTPs in physiological and pathological states have remained largely unknown. Specific PTP inhibitors would provide valuable tools in studying the functions of these enzymes as well as potential therapeutic agents. It is with this premise that there has been a recently intensified interest in developing PTP inhibitors.[3] Here we report that α-halogenated acetophenones act as a novel class of potent, covalent PTP inhibitors, whose inhibitory effects can be conveniently reversed by photolysis at 350 nm.

PTPs of all origins share a common catalytic domain of ∼250 amino acids, containing the unique "signature motif", (I/V)-HC×AG××R(S/T).[2] PTP-catalyzed pY hydrolysis proceeds through a nucleophilic attack on the phosphate group by the side-chain thiol of the conserved cysteine in the signature motif, forming a covalent phosphocysteinyl enzyme intermediate, which is subsequently hydrolyzed by a water molecule (Scheme 1).[4] We envisioned that α-haloacetophenone **1** could bind to the PTP active site as a pY mimetic; its phenyl ring could engage in hydrophobic interactions with the protein as the phenyl ring of a substrate does, and the electron-rich halogen atom could mimic the negatively charged phosphate oxyanions. Binding of **1** to the PTP active site in such a manner would place the α-carbon, which is highly susceptible to nucleophilic attack, next to the catalytic cysteine. An S_N2 reaction between **1** and the cysteine thiol would result in the formation of a covalent enzyme−inhibitor adduct through a stable thioether linkage and loss of phosphatase activity.

α-Bromo- and α-chloroacetophenone derivatives (**1a**−**d**) were prepared[5] and assayed against the prototypical phosphatase PTP1B,[6] a Src homology 2 (SH2) domain-containing phosphatase SHP-1,[7] and the catalytic domain of SHP-1, SHP-1(ΔSH2).[8] All four compounds resulted in time-dependent inhibition of the PTPs, and their inhibition kinetics can be described by equation

$$E + I \overset{K_I}{\rightleftharpoons} E{\bullet}I \xrightarrow{k_{inact}} E{-}I$$

where K_I is the dissociation constant of the noncovalent complex, E•I, and k_{inact} is the first-order rate constant for conversion of the E•I complex into a covalent complex, E−I. Inhibitor **1a** binds to PTPs with the highest affinity, having K_I values of 43 and 42 μM and k_{inact} values of 0.40 and 0.57 min^{-1} for SHP-1(ΔSH2) and PTP1B, respectively (Table 1). Inhibitors **1b** and **1c** bind to SHP-1(ΔSH2) with 3−5-fold lower affinity but have 4−6-fold higher k_{inact}, therefore having similar overall potency as **1a**. The α-chloro derivative (**1d**) is 13-fold less potent than its α-bromo counterpart (**1c**), mainly because of lower binding affinity (higher K_I). This may be due to the smaller size of the chlorine atom, rendering α-chloroacetyl group a less effective mimetic of the phosphate group than the α-bromoacetyl group. Note that **1a** has lower affinity to wild-type SHP-1 than its catalytic domain (K_I = 530 vs 43 μM), likely due to the fact that the SH2 domains can directly bind to the PTP active site and interfere with substrate/inhibitor binding.[9] As controls, **1a** was assayed against alkaline phosphatases, acid phosphatases, and a dual-specificity phosphatase VHR.[10] Incubation with 10 mM **1a** for 10 min resulted in no significant inhibition of any of the alkaline or acid

Scheme 1. Mechanism of Catalysis and Inactivation by **1**

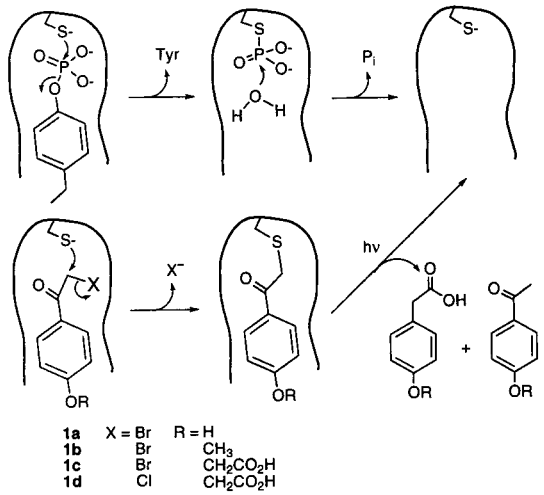

Table 1. Kinetic Constant of PTP Inhibition by **1a**−**d**[a]

enzyme	inhibitor	K_I (μM)	k_{inact} (min^{-1})
SHP-1(ΔSH2)	**1a**	43 ± 10	0.40 ± 0.10
	1b	128 ± 10	2.4 ± 0.2
	1c	193 ± 38	1.8 ± 0.3
	1d	2540 ± 610	1.8 ± 0.4
	$BrCH_2CO_2H$	77000 ± 14000	1.4 ± 0.2
SHP-1	**1a**	530 ± 120	2.6 ± 0.2
PTP1B	**1a**	42 ± 5	0.57 ± 0.05
VHR	**1a**	8900 ± 4500	3.4 ± 1.6

[a] Data reported are the mean ±SD from three or more independent experiments carried out at pH 7.4 and at room temperature.

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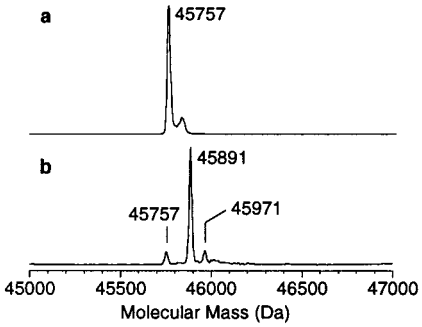

Figure 1. Reconstructed ESI/MS spectra for (a) native SHP-1(ΔSH2); and (b) **1a** inactivated SHP-1(ΔSH2).

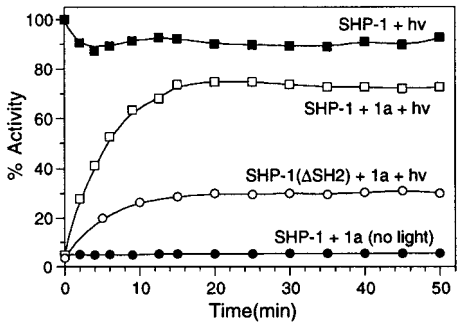

Figure 2. Photolytic reactivation of **1a** inactivated SHP-1 and SHP-1(ΔSH2). All activities are relative to those of the untreated enzymes.

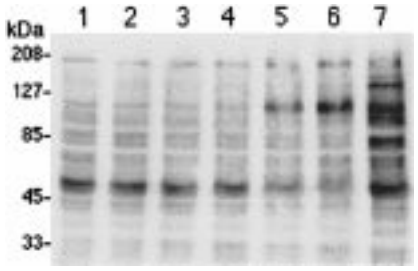

Figure 3. Western blot analysis of B cell phosphoproteins. 5×10^6 Ramos human B cells were incubated at 37 °C for 3 min with nothing (lane 1), 0.1% DMSO (lane 2), or with 30 μM (lane 3), 100 μM (lane 4), 300 μM (lane 5), or 1000 μM (lane 6) **1a** in 0.1% DMSO. Lane 7 is a positive control with 100 μM phenylarsine oxide. The resulting cell lysates were separated by SDS-PAGE, transferred to nitrocellulose filters, and probed with an anti-pY antibody.

phosphatases tested. Although VHR was inhibited by **1a**, its binding affinity (K_I = 8.9 mM) is 200-fold lower than that of PTP1B or SHP-1(ΔSH2).[11] In another control, α-bromoacetate was found to inhibit SHP-1(ΔSH2) with ~2000-fold lower affinity than **1a** (Table 1). Finally, the reaction rate of **1c** with 2-mercaptoethanol at pH 7.4 was determined to be 0.21 $M^{-1}min^{-1}$, 45000-fold lower than that with SHP-1(ΔSH2) (k_{inact}/K_I = 9300 $M^{-1}min^{-1}$).[12] Taken together, these results demonstrate that **1a**−**d** are general PTP-specific inhibitors.

The mechanism of PTP inhibition by **1a**−**d** was investigated by mass spectrometry. Electrospray ionization mass spectrometric (ESI MS) analysis of native SHP-1(ΔSH2) gave a single peak at a molecular mass of 45 757 ± 5 Da (calculated molecular mass is 45 744 Da). After incubation with **1a** (200 μM) for 7 min, the inactivated protein (57 μM) gave a major peak at 45891 ± 5 Da (Figure 1). The increase of 133.9 ± 0.5 Da in mass can be accounted for by the attachment of a *p*-hydroxyphenacyl group to the protein (Scheme 1). The absence of any M + 268 or M + 402 peak indicates a 1:1 enzyme/inhibitor stoichiometry, despite the presence of four cysteines in the enzyme and excess **1a** in the reaction. Matrix assisted laser desorption ionization mass spectrometric analysis of the trypsin digest of **1a** inactivated SHP-1(ΔSH2) showed disappearance of the peak at m/z = 2044 (corresponding to the active-site fragment Q_{440}ESLPHAGPII-VHCSAGIGR$_{459}$) and appearance of a new peak at m/z = 2178 (data not shown). This suggests that the inhibitor is attached to this active-site fragment, presumably at the catalytic Cys-453. Consistent with this notion, mutation of Cys-453 to serine completely abolished the modification by **1a** as judged by ESI MS analysis (not shown).

Givens et al. used *p*-hydroxyphenacyl and *p*-methoxyphenacyl groups as photolabile protecting groups to generate caged molecules.[13] We thus irradiated the inactivated PTPs at 350 nm to determine whether they could be reactivated. Indeed, after irradiation of SHP-1 (4.7 μM) that had been completely inactivated by treatment with 100 μM **1a** at 4 °C, ~80% of original activity was recovered after ~15 min (Figure 2). SHP-1(ΔSH2) and PTP1B that had been inactivated with excess **1a**−**d** were also reactivated by the same procedure, with activity recovery at ~30% of the untreated enzyme. Irradiation at 350 nm has no effect on the native enzymes. The mechanism of the photolytic reaction has not yet been investigated, but the photolysis products are likely to be *p*-hydroxy- (for **1a**) or *p*-alkoxyphenylacetate (for **1b**−**d**) and the corresponding acetophenones.[13]

The potential of **1a** as a tool for cellular studies was tested with human B cells. After incubation with varying concentrations of **1a** for 3 min, the cells were lysed, and the cellular proteins were separated on an SDS-PAGE gel, followed by western blot analysis using an anti-pY antibody (Figure 3). Treatment with ≥300 μM **1a** resulted in the hyperphosphorylation of a protein at 110 kDa, consistent with inhibition of a PTP(s) which normally dephosphorylates this protein. Interestingly, **1a** also decreased the phosphorylation level of a 50 kDa protein at concentrations as low as 30 μM. Presumably, inhibition of a PTP(s) by **1a** led to the inactivation of a downstream kinase, as some kinases require the removal of inhibitory pY residues by PTPs for activation.[14] Therefore, while other scenarios are formally possible, the simplest explanation is that **1a** penetrated into the cells and resulted in inhibition of PTPs.

In summary, α-haloacetophenone derivatives are shown to be potent, photoreversible, and membrane permeable inhibitors of PTPs. Work is already in progress to further improve their binding affinity and specificity to individual PTPs by attaching specific ligands to the para position. These inhibitors will provide powerful tools for controlling cellular events by switching on and off PTPs. The use of neutral halogenated groups as phosphate mimetic may find general utility in designing membrane permeable ligands for other phosphate-binding modules such as SH2 domains.

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Supporting Information Available: Experimental details for synthesis, in vitro and in vivo inhibition assays, and photolytic reactivation (PDF). This information is available free of charge via the Internet at http://pubs.acs.org.

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