Arylstibonic acids are potent and isoform-selective inhibitors of Cdc25a and Cdc25b phosphatases

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Keywords: Arylstibonic acids, protein tyrosine phosphatase, Cdc25 inhibitor

Abstract

In this study, a library of arylstibonic acids were screened as potential protein tyrosine phosphatase inhibitors. Two compounds, namely 13778 and P6949 were identified as potent Cdc25 phosphatase inhibitors, an important key regulator of the eukaryotic cell cycle. These arylstibonic acids were also tested against other members of the protein tyrosine phosphatase family and were found to display good specificity and potency for Cdc25 phosphatases.
Introduction

Phosphatases are important regulators of many cellular functions, which in turn impact disease and human health. In particular, protein tyrosine phosphatases (PTPs), which counteract the many protein tyrosine kinases involved in cellular signaling, and have a significant role in the development of cancer, diabetes and obesity (1, 2). The PTP family is defined by its homology in the phosphatase domain, which is characterized by a cysteine separated by five amino acids from an arginine (CX6R motif). Within this family of CX6R phosphatases are the cell division cycle 25 (Cdc25) phosphatases, which play a key role in cell cycle regulation by controlling cyclin-dependent kinase dephosphorylation and activation (3). There are three isoforms of Cdc25 phosphatases encoded by the human genome, namely Cdc25a, Cdc25b and Cdc25c. Cdc25a appears to regulate the G1/S transition, whereas Cdc25b and Cdc25c both are regulators of the S/G2 transition (4). It has been reported that Cdc25 phosphatases, in particular Cdc25a and Cdc25b, are over-expressed in various primary human cancers (5). Therefore, Cdc25 phosphatases are potential targets for anticancer therapeutics.

Results and Discussion

Recently, arylstibonic acids have been characterized as potent inhibitors of the DNA repair endonuclease APE1 and type 1B topoisomerases (6, 7). Interestingly, APE1 shares some homology in its catalytic site with the type II phosphoinositide 5-phosphatase (5’-P) active site of the synaptojanins, which is distinct from the active sites of the CX6R family of phosphatases (8). From this homology we surmised that the arylstibonates might selectively target the 5’-P active site of synaptojanin. Instead, we surprisingly found that a subset of these arylstibonates were potent and isoform selective Cdc25 phosphatase inhibitors.

We began by performing a preliminary screen of the arylstibonic acid library against the activities of representative phosphatases from the 5’-P and CX6R families. Interestingly, in addition to its 5’-P activity, synaptojanin also contains an independent phosphatase active site, referred to as SAC1, which is related to the CX6R family of the PTPs. Thus, we had the opportunity to assess the selectivity of these arylstibonates for either active site in a single bifunctional enzyme. In addition,
we also screened PTP-β, another CX₃R family member. We used screening conditions where the substrate concentration was fixed near its $K_{M}$, employing just 5 µM of each arylstibonate. This screen revealed that some compounds provided 40-50% inhibition of the 5'-P activity of synaptojanin under these conditions, consistent with the expectation based on APE1 homology (6). However, the structurally unrelated CX₃R phosphatases SAC1 and PTP-β were found to be much better targets for several compounds (Figure 1).
Figure 1. Inhibition of synaptojanin, SAC1 and PTP-β activities by arylstibonic acids.

(Top panel) A library of arylstibonic acids were screened in activity assays against synaptojanin, SAC1 and PTP-β. Shown are the percentages of inhibition in the presence of 5 μM of arylstibonic acids. The most potent compounds for synaptojanin (≥ 40% inhibition), SAC1 (≥ 40% inhibition) and PTP-β (≥ 90% inhibition) are shown. The activity of synaptojanin was measured in the presence of 50 μM PI(4)P presented in octylglucoside mixed micelles; SAC activity was assessed using 50 μM I(1,4,5)P₃ as substrate and PTP-β activity was assessed using 1 mM p-NPP substrate. Error bars represent the standard error of triplicate repeats. (Bottom panel) Chemical structures of arylstibonic acids inhibiting synaptojanin, SAC domain and PTP-β. For the full list of tested arylstibonates refer to (7). All other compounds showed an inhibition of less than 40% against the tested enzymes (data not shown).

The most potent inhibition was observed against PTP-β, with several compounds (13755, P6953, 13778, P6968, P6981, P6949) nearly wiping out phosphatase activity at 5 μM inhibitor concentration. We then performed a full dose response study over the range 50 nM to 5 μM using the four most selective and effective PTP-β phosphatase inhibitors and employing p-NPP as substrate (Fig. 2). We found that compounds 13778 and P6949 were the most potent inhibitors (IC₅₀ ~100 nanomolar range).

Figure 2. Potency of selected arylstibonates on tyrosine phosphatase activity.

PTP-β inhibitors identified in the screen presented in Fig. 1 were tested for their potency in the presence of enzyme with increasing concentrations of the arylstibonates 13778 and P6949. Error bars represent the standard error of triplicate repeats.

The initial screen of the arystibonic acid library of 40 compounds, which have discrete structural differences, allows us the assessment of structure-function relationships. The screen using 5 μM of arystibonates reveal that substitution in the aromatic ring is vital for
compounds inhibitory effect, as arylstibonate 13744 does not show any inhibitory effect towards PTP-β (see Table 1). Interestingly, the positioning of aromatic substitution does not have a significant effect on PTP-β inhibition, as demonstrated by comparing compound 13759 and 13760. However, carboxylic acid rather than nitro functionality seems to be more important for PTP-β inhibition. Removal of the nitro group lessened inhibition (13760) but the decrease in inhibition observed when the carboxylic acid is removed is more severe (13743). Twelve out of the thirteen carboxylic acid functional compounds in the library inhibited PTP-β by more than 60% again highlighting the dependence of PTP inhibition on carboxylic acid functionality. The interaction between the stibonates and PTP-β compound is clearly controlled by electrostatic interaction. If the carboxylic acid COOH group is replaced the less electronegative hydroxyl group, (comparing 13745 with 13760), then inhibition is abolished. Replacing the COOH with ester functionality also abolishes the inhibition (13771 and P6983). Extending the length of the carboxylated side chain greatly improves the potency of compound 13759, making 13778 and P6949 highly potent inhibitors.

<table>
<thead>
<tr>
<th>Compound (5 μM)</th>
<th>X</th>
<th>Y</th>
<th>Inhibition of PTP-β</th>
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<tbody>
<tr>
<td>13744</td>
<td>H</td>
<td>H</td>
<td>No inhibition</td>
</tr>
<tr>
<td>13759</td>
<td>CO₂H</td>
<td>H</td>
<td>78%</td>
</tr>
<tr>
<td>13760</td>
<td>H</td>
<td>CO₂H</td>
<td>72%</td>
</tr>
<tr>
<td>P6966</td>
<td>CO₂H</td>
<td>CO₂H</td>
<td>51%</td>
</tr>
<tr>
<td>13771</td>
<td>CO₂CH₃</td>
<td>H</td>
<td>No inhibition</td>
</tr>
<tr>
<td>P6983</td>
<td>CH₂CH(CO₂Et)₂</td>
<td>H</td>
<td>No inhibition</td>
</tr>
</tbody>
</table>
Table 1. Structure activity relationships of arylstibonic acids as PTP-β inhibitors.

<table>
<thead>
<tr>
<th></th>
<th>Structure</th>
<th>Inhibition</th>
</tr>
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<tbody>
<tr>
<td>13778</td>
<td>C$_2$H$_2$CO$_2$H</td>
<td>H</td>
</tr>
<tr>
<td>P6949</td>
<td>C$_2$H$_4$CO$_2$H</td>
<td>H</td>
</tr>
<tr>
<td>13743</td>
<td>NO$_2$</td>
<td>H</td>
</tr>
<tr>
<td>P6981</td>
<td>CH$_2$CH(CO$_2$H)$_2$</td>
<td>H</td>
</tr>
<tr>
<td>P6982</td>
<td>H</td>
<td>CH$_2$CH(CO$_2$H)$_2$</td>
</tr>
<tr>
<td>13755</td>
<td>NO$_2$</td>
<td>CO$_2$H</td>
</tr>
<tr>
<td>13745</td>
<td>H</td>
<td>OH</td>
</tr>
</tbody>
</table>

The finding that arylstibonates were potent inhibitors of PTP-β prompted the testing of 13778 and P6949 against a panel of nine CX$_3$R family members using the artificial substrate O-methylfluorescein phosphate (OMFP) (see Table 2). Included in this panel were several enzymes (Cdc25s, PTEN, MKP-3, VHR, and PTPMT1) that are members of the dual-specificity phosphatase (DSP) subfamily which are characterized by a broader substrate specificity. Although they share the same catalytic mechanism as the classical PTPs, the DSP active site allows these enzymes to accommodate phosphoserine, phosphothreonine, phosphotyrosine and even non-protein substrates. After determining the $K_m$ and $V_{max}$ values of each enzyme for OMFP (not shown), dose response curves for arylstibonates 13778 and P6949 were then constructed using OMFP concentrations near the $K_m$ value for each enzyme (Fig. 3). Arylstibonate 13778 and P6949 were found to inhibit some of the tested PTPs with even higher potency than PTP-β. The best potency was observed for arylstibonate 13778 against Cdc25a phosphatase with an IC$_{50} = 0.11 \pm 0.01$ μM, followed by Cdc25b (IC$_{50} = 0.60 \pm 0.07$ μM). Interestingly, the activity of Cdc25c was not sufficiently affected by even 10 μM of compound 13778, which indicates that 13778 has an isoform-selectivity against Cdc25 phosphatases. Another inhibited phosphatase was PTPMT1 (IC$_{50} = 1.62$ μM ± 0.19 μM), which was recently identified as a DSP anchored at the inner membrane of the mitochondria.
(9). PTP1B is inhibited by 13778 with an IC50 of ~ 8 µM which is over 10-fold higher than PTP-β, once again showing the potential for significant selectivity of these compounds, even when the active site sequences of the enzymes are closely matched (see below).

Arylstibonate P6949 showed similar selectivity as 13778 (Fig. 3b). Cdc25a, 25b as well as PTP-β were the best targets, while Cdc25c was not affected at the concentrations tested, once again underlining the isoform-selectivity of these inhibitors within the Cdc25 family. Similar selectivity by compounds 13778 and P6949 is expected based on their similar structures, which only differ by the presence of the double bond in the carboxylate side chain of 13778. However, despite their similar selectivities, the potency of compound P6949 is significantly lower than that of 13778 (~30-fold lower for Cdc25a and about 10-fold lower for Cdc25b), indicating a significant beneficial contribution from the double bond-containing side chain of 13778. We determined that the inhibition was reversible for both compounds using a standard dilution approach, where the enzyme was pre-incubated with four times the IC50 concentration of the arylstibonate inhibitor, followed by a 10-fold dilution with reaction buffer (Fig. 4)(10).
Figure 3. Potency of arylstibonate 13778 and P6949 on members of the PTP family.

(a) IC$_{50}$ curves of PTPs in the presence of arylstibonate 13778. (b) IC$_{50}$ curves of PTPs in the presence of arylstibonate P6949. Data shown are the average of 3 independent experiments performed in triplicates.

(c) Alignment of amino acid sequences of the catalytic motif of PTPs assayed.

Table 2. IC$_{50}$ values of PTPs assayed in the presence of 13778 and P6949 using OMFP as substrate.
In order to investigate the mode of inhibition, we studied the steady-state kinetic parameters of Cdc25a and 25B in the presence of varying inhibitor concentrations. As shown in Fig. 4, the apparent $K_m$ values for both enzymes increase as the concentrations of 13778 and P6949 increase, and that $V_{max}$ drops, but not to zero. These trends indicate a partial mixed-type mode of inhibition, and that the inhibitors bind to a site different from that of the substrate. This behavior might explain why the arylstibonate inhibitors have different potency towards the three Cdc25 isoforms, because alignment of the amino sequences of all three isoforms within the catalytic CX5R motif are identical (Fig. 3c). Contrary to our initial
expectations, these findings indicate that the arylstibonic acid moiety is not a mimic of the aryl phosphate substrate. Secondary plots of the enzymological data (not shown) reveal that 13778 has $K_i$ values of 72 nM and 369 nM for Cdc25a and 25B, while P6949 has $K_i$ values of 1.7 µM for Cdc25a and 4.7 µM for Cdc25b.

In conclusion, a focused 40 member arylstibonic acid library was tested against the synaptojanin I-5-P and various members of the PTP family. Compounds 13778 and P6949 were identified as inhibitors of Cdc25a and Cdc25b phosphatases, with 13778 showing nanomolar potency, and favorable selectivity. Within the Cdc25 family, 13778 and P6949 show 100-fold isoform selectivity for Cdc25a and Cdc25b as compared to Cdc25c, which makes both compounds a valuable enrichment to the currently existing portfolio of lead structures in the search for isoform-selective Cdc25 inhibitors (11). A fascinating aspect of these inhibitors is their partial mixed type mode of inhibition, which suggests an allosteric effect on the active site structure. Future improvements would be to identify and characterize this site and use this information to design inhibitors with greater potency and/or selectivity. As over-expression of Cdc25a and/or Cdc25b, but not Cdc25c, has been detected in numerous cancers, these compounds comprise potentially valuable leads for selective inhibition of Cdc25a and Cdc25b without affecting the activity of Cdc25c, a feature that could be exploited in future drug development (5).

Material and Methods

Compounds

All arylstibonic acids were obtained from the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, National Cancer Institute, Bethesda, MD. Compounds were originally screened from DMSO solutions and activities of P6949 and 13778 containing the antimony element
were confirmed from dry powders. Because antimony is paramagnet, the fine features of the 400 MHz proton NMR spectra were broadened extensively, and carbon spectra were not obtainable due to due unfavorable relaxation effects. The purity of each compound was determined as greater than 97% based on proton NMR spectra. Microanalysis was performed by A P Dickerson, University of Cambridge.

**P6949** 3-(3-stibonophenyl)propanoic acid, $^1$H NMR (D$_2$O): $\delta$ 2.0-3.0 (m, 4H), 7.0-8.0 (m, 4H); ESI-MS: m/z 320.9, 322.9 [MH$^+$].

**13778** (E)-3-(3-stibonophenyl)acrylic acid, $^1$H NMR (DMSO-d$_6$): $\delta$ 6.0-6.5 (m, 1H), 7.0-8.2 (m, 5H), 12.0-12.8 (br, 1H). ESI-MS: no signal obtained.

Microanalysis calculated for C$_9$H$_9$O$_5$Sb: C, 33.89; H, 2.84; N, 0.00. Found: C, 33.90; H, 2.70; N, 0.00.

**Protein Expression and purification**

Human PTEN and rat synaptojanin (amino acids 1-1042), the latter containing the SAC1 and 5-phosphatase activities were expressed as Glutathione S-transferase (GST)-fusion proteins and subsequently purified as described before (12). Protein expression was induced in the *E. coli* strain XL-1 blue for 24 h using 1 mM isopropyl β-D-1-thiogalactopyranoside at 18°C for synaptojanin and at 23°C for PTEN. Cells were harvested and stored at -20°C. The harvested cells were re-suspended in lysis buffer containing 50 mM Tris (pH 7.4), 10 mM benzamidine hydrochloride, 100 µg/mL soybean trypsin inhibitor, 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride, 2 mM dithiothreitol (DTT) and 1% Triton X-100. Lysis was performed by adding lysozyme to the cell suspension at a concentration of 2 mg/mL and sonication. Cell debris was removed by centrifugation at 18000 g for 1 h at 4°C. The supernatant was loaded onto a glutathione sepharose column, pre-equilibrated with 50 mM Tris (pH 7.4), 140 mM NaCl and 2.7 mM KCl. After loading, the column was washed twice with 50 mM Tris (pH 7.4), 140 mM NaCl, 2.7 mM KCl and 2 mM DTT. Another two washes
were performed using the same buffer with 500 mM NaCl. The GST-tagged PTEN was eluted using 20 mM glutathione in 50 mM Tris (pH 7.4), 250 mM NaCl, 20% glycerol and 2 mM DTT. Protein concentration was determined using Bradford assay. Protein integrity was confirmed by Western blot using GST antibody (Novagen). The catalytic domains of Cdc25a, b and c with N-terminal His tags were expressed from pET21a in E.coli strain BL21 (DE3) and purified by ion exchange (Sepharose SP Fast Flow) and gel filtration (Sephadex G-50) chromatography. Recombinant human His-VHR was obtained from Enzo Life Sciences (Exeter, UK) and PTP-β was purchased from Sigma-Aldrich (Dorset, UK).

**Phosphatases Assays**

All enzyme preparations were tested for linearity to ensure that suitable amounts of enzyme were employed in all kinetic studies.

*Colorimetric quantification of phosphate release using malachite green assay*

Phosphatase activities were determined by measuring the amount of released phosphates for the 5-inositol phosphatase (IP₃ as a substrate) and SAC1 phosphatases activities of synpatojanin1 (12). In the case of lipid substrates the phosphatase reaction was started by adding the corresponding substrates presented in octylglucoside mixed micelles as described before (13). Reactions were carried out in a 96-well plate with buffer containing 4 mM MgCl₂, 100 mM Tris (pH 7.4) in a total volume of 80 µL at 37°C. Appropriate amounts of denatured enzyme (active enzyme was heated at 95°C for 10 minutes) were employed in place of active enzyme for comparison (control). In order to stop the reaction, same volume of color reagent (5 mM malachite green, 17 mM ammonium heptamolybdate, 77 mM bismuth citrate and 1.7 M HCl) was added to the assay. The mixture was allowed to develop for 10 min and the absorbance was read at 625 nm.

*Colorimetric PTP-β assay using para-nitrophenol phosphate as substrate (p-NPP)*
Phosphatase activity of PTP-β was measured using p-NPP as substrate (14). As above, reactions were carried out in a 96-well plate in the presence of 25 mM Hepes (pH 7.4), 50 mM NaCl and 5 mM DTT. The reaction was initialized by added p-NPP dissolved in H₂O. The enzymatic release of p-nitrophenol was measured at 405 nm.

3-O-Methylfluorescein Phosphate (OMFP) assay

Enzyme kinetic studies using OMFP were performed as previously described (10). OMFP was dissolved in DMSO to a concentration of 20 mM and then further diluted with 1% DMSO to the tested concentrations. Assays were performed in 100 mM Tris (pH 7.4) containing 2 mM DTT at room temperature (20 °C).

For inhibition studies, enzymes were pre-incubated with arylstibonates at RT for 10 min. Reactions were then initialized by adding OMFP. The hydrolysis of OMFP to OMF was monitored by measuring the change of fluorescence units (FU) in a 96-well microtiter plate (excitation at 485 nm and emission at 525 nm) using a Varian fluorescence spectrophotometer.

Acknowledgments

We thank Bob Shoemaker and Rhone Akee and the Developmental Therapeutics Program at the National Cancer Institute, Frederick Maryland for providing the arylstibonic acid small molecule library. We would also like to thank Jack E. Dixon (University of California at San Diego) for the generous gift of the expression plasmid of PTPMT1. This work was supported by an EPSRC Centre for Doctoral Training Studentship from the Institute of Chemical Biology (Imperial College London) awarded to J.K., Cancer research UK (D.M.) and a NIH grant GM068626 to J.T.S.

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