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Identification of Bidentate Salicylic Acid Inhibitors of PTP1B

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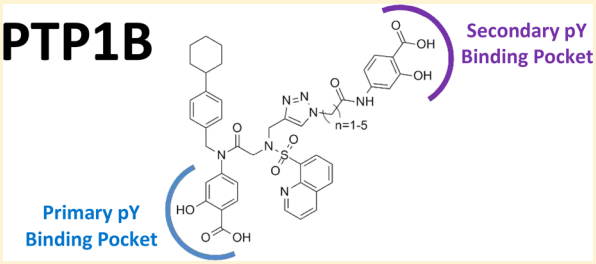
Supporting Information

ABSTRACT: PTP1B is a master regulator in the insulin and leptin metabolic pathways. Hyper-activated PTP1B results in insulin resistance and is viewed as a key factor in the onset of type II diabetes and obesity. Moreover, inhibition of PTP1B expression in cancer cells dramatically inhibits cell growth *in vitro* and *in vivo*. Herein, we report the computationally guided optimization of a salicylic acid-based PTP1B inhibitor **6**, identifying new and more potent bidentate PTP1B inhibitors, such as **20h**, which exhibited a > 4-fold improvement in activity. In CHO-IR cells, **20f**, **20h**, and **20j** suppressed PTP1B activity and restored insulin receptor phosphorylation levels. Notably, **20f**, which displayed a 5-fold selectivity for PTP1B over the closely related PTPσ protein, showed no inhibition of PTP-LAR, PRL2 A/S, MKPX, or papain. Finally, **20i** and **20j** displayed nanomolar inhibition of PTPσ, representing interesting lead compounds for further investigation.



KEYWORDS: *PTP1B, PTPσ, diabetes, insulin receptor, small molecule inhibitor, salicylic acid, bidentate inhibitors*

Phosphorylation of regulatory and signaling proteins within the cell is closely monitored by the coordinated action of protein tyrosine kinases (PTKs) and the superfamily of protein tyrosine phosphatases (PTPs). The PTKs and PTPs are responsible for the addition and removal of phosphate groups from specific tyrosine (Y) residues on proteins, respectively. This partnership is responsible for the regulation of cellular communication and homeostasis.[1] Dysregulation of phosphorylation events are directly related to malignancy states including, but not limited to, diabetes,[2] cancer,[3−5] neurological,[6] metabolic,[7−9] and autoimmune disorders.[10] Historically, drug discovery programs have predominately focused on identifying PTK inhibitors since the prevailing dogma was that phosphatases were not as attractive drug targets. PTPs were overlooked and considered housekeeping enzymes with broad specificity, as compared to their PTK counterparts. However, advancements in the past two decades in the field of molecular biology uncovered a more profound role for PTPs in cell regulation, growth, and the onset of human disease.[6,7,11] The importance of PTPs in signal transduction and cellular homeostasis has led to a flurry of discoveries, which linked their aberrant activities to numerous pathologies.[12−15] These discoveries have validated PTPs as interesting therapeutic targets.

The most studied of the PTP enzymes is PTP1B (gene code: PTPN1), which is overexpressed in muscle, liver, and adipose tissues, and implicated in the onset of type II diabetes (T2D). PTP1B overexpression leads to persistent dephosphorylation of the insulin receptor (IR), stimulating the insulin-resistant phenotype in T2D and obesity. The pivotal biological role of PTP1B has led to numerous inhibitor programmes.[8,9,16−19] Despite the identification of many potent compounds, a PTP1B-inhibiting drug has yet to reach the clinic. The most common strategy for inhibiting PTP1B, or other PTPs, has been to target the active site. However, deriving selectivity has been problematic, given that PTP active sites have, in some cases, 100% sequence homology.[9,12,20] As a result, recent inhibitor designs have focused on the simultaneous targeting of multiple subpockets proximal to the active site.[21−23] These domains are less conserved among the PTP isoforms and are seen as being a route to achieving enhanced target selectivity. In the case of PTP1B, where a proximal phosphotyrosine (pTyr)-binding site is located adjacent to the pTyr-binding active site, inhibitors have been developed that possess two pTyr groups or similar bivalent combinations of pTyr and more drug-like pTyr bioisosteres (compounds **1**−**5**, Figure 1).[24] However, bivalent pTyr-containing inhibitors that exhibit high PTP1B selectivity often exhibit poor pharmacological profiles, likely due to the charged pTyr-mimicking functionality and peptidic character.[12]

In previous efforts to target the pTyr binding site of PTP1B, scaffolds have incorporated either a salicylic or benzoic acid[25] as a cell-permeable pTyr mimetic.[26−28] Salicylic acid-based inhibitors of PTP1B were reported, with IC_{50} values of 3−8 μM, and no inhibition of PTP-LAR, PRL2 A/S, MKPX, or

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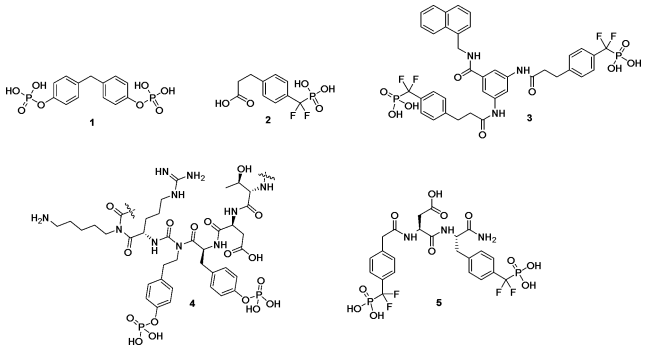

Figure 1. Chemical structures of previously described PTP1B inhibitors.

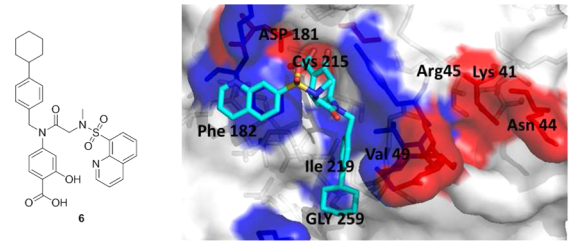

Figure 2. Docking image of compound **6** in the active site of PTP1B (PDB: 1NWL). Protein: hydrophobic residues (blue) and hydrophilic residues (red). Compound: nitrogen (blue), oxygen (red), and sulfur (gold).

papain (**6**, Figure 2).[2] However, the compounds displayed equipotent inhibition of another clinically important phosphatase, PTPσ. PTPσ is a potential target for the development of novel therapies for spinal cord injury and multiple neurodegenerative diseases.[29−31] Moreover, PTPσ inhibition has recently been identified as a target for improving memory loss.[32] In this study, it was hypothesized that incorporation of a second carboxylate group to compound **6** might provide a second pTyr mimetic for more potent and selective targeting of PTP1B. It was reasoned that such a scaffold might improve cell penetrating properties more than current pTyr mimetics utilized in bidentate PTP1B inhibitors.[22]

To identify the site of carboxylate binding on **6**, *in silico* docking studies were performed with PTP1B using Genetic Optimization for Ligand Docking (GOLD) software (Figure 2). Results indicated that the salicylic acid was binding within the pTyr-recognition site (Asp181 and Cys215, Figure 2) and the cyclohexylbenzyl group was docked within the large hydrophobic pocket (Ile219, Gly259, and Val49, Figure 2). The quinoline group was found to form $\pi-\pi$ interactions with Phe182, as well as to H-bond with the peptide backbone via the quinoline nitrogen. Since the *N*-methyl group was not involved in binding and was oriented toward the second pTyr binding site, it was selected for attaching the second salicylic acid substituent.

Copper-catalyzed click chemistry was selected to couple the two components via a triazole linker of varying alkyl chain length.[33,34] As opposed to a flexible and rather hydrophobic hydrocarbon linker, the flat triazole provides additional polarity and rigidity and has been deemed the ideal linker for development of bridged inhibitors in recent literature.[33,34] To study the binding mode of the proposed disalicylic acids, comparative *in silico* docking analysis was conducted with PTP1B and a proposed bidentate inhibitor **20f**, superimposed

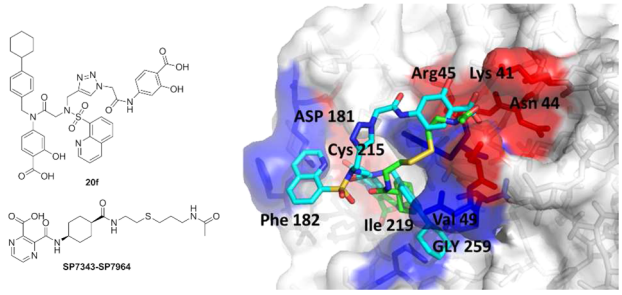

Figure 3. Comparative docking studies of **20f** (cyan) and inhibitor **SP7343-SP7964** (green) bound to PTP1B (PDB: 1NWL). Protein: hydrophobic residues (blue) and hydrophilic residues (red). Compounds: nitrogen (blue), oxygen (red), and sulfur (gold).

with known bidentate PTP1B inhibitor, **SP7343-SP7964** (K_i = 4.1 mM, Figure 3). Docking revealed that **20f** bound analogously to **SP7343-SP7964**, with both salicylic acids interacting with the respective pTyr-binding sites. The interactions between the cyclohexylbenzyl group of **6** and the hydrophobic pocket of the active site were retained, despite the addition of the second salicylate group. Next, the linker length between both salicylic acid groups was explored *in silico* with analysis suggesting to incorporate linkers ranging from one to five carbons (Supporting Information). The *N*-sulfonamide aryl group, R, was either a quinoline, as previously used in **6**, or a tolyl group, as previous investigations with this substituent had indicated activity against PTP1B.[2]

The focused library was screened for activity at 10 μM using a previously described DiFMUP assay against the non-receptor tyrosine phosphatases PTP1B and TC-PTP, receptor-like tyrosine phosphatases PTPσ and PTP-LAR, the dual specificity phosphatases PRL2 A/S (active mutant) and MKPX, and a human cysteine-based protease papain (used here as a control for oxidation) (Figure 4). Compounds **20f−j** inhibited TC-PTP, PTP1B, and PTPσ enzymatic activity by over 90% compared to the DMSO control. Compounds **20f−j** showed modest increases in activity as compared to **6** (Supporting Information). Negligible effects were observed against PRL2 A/S, PTP-LAR (D1D2), MKPX, or CD45D1D2. MKPX and PRL2 A/S are dual specificity phosphatases that recognize phosphorylated Ser and Thr residues, as well as pTyr. The selectivity may be the result of unfavorable binding interactions with the catalytic pockets of PTP-LAR (D1D2), MKPX, CD45D1D2, and PRL2 A/S. Tolyl inhibitors **20a−e** were significantly less active and less selective compared to the quinoline analogues. This was likely due to poor water solubility under the assay conditions and/or less favorable binding interactions of the tolyl with the PTP1B active site as compared to the quinoline derivatives (Comparative docking studies provided in the Supporting Information). Quinoline compounds **20f−j** inhibited the receptor-like PTPs, such as PTPσ, more potently than the non-receptor PTPs like PTP1B. Thus, **20f−j** were further evaluated to quantify relative levels of PTP1B and PTPσ inhibition.

IC_{50} values were determined for each compound (Table 1, with representative curves shown in Figure 5). IC_{50} values for **20f-j** ranged from 0.6−6.1 μM. Two inhibitors, **20f** and **20h**, possessed > 2-fold improved potency for PTP1B over **6** (**20f**, IC_{50} = 3.6 μM; **20h**, IC_{50} = 1.7 μM; **6**, IC_{50} = 6.2 μM), with **20h** also displaying a 4-fold selectivity for PTP1B over PTPσ. Notably, **20i** was identified as the first nanomolar PTPσ

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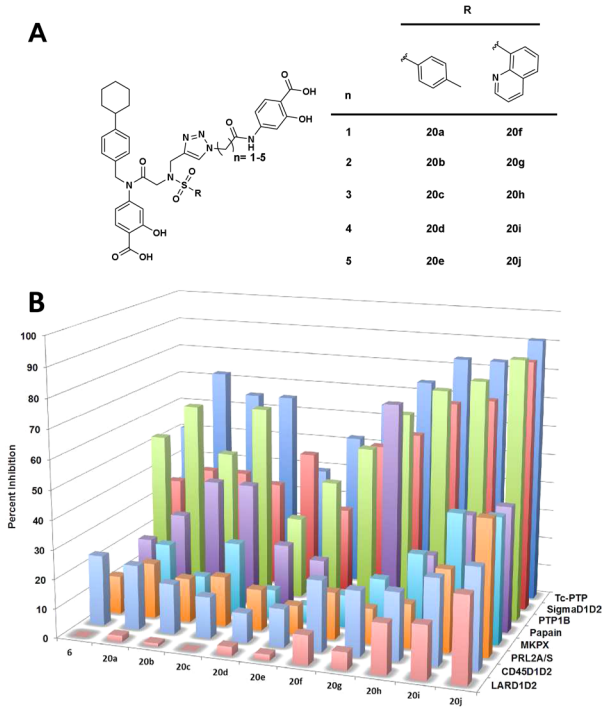

Figure 4. (A) Structures of inhibitors **20a–j**; (B) preliminary high-throughput kinetic enzyme activity screen for compounds **6** and **20a–j** at 10 μM against phosphatases PTP1B, TC-PTP, PTPσ (D1D2), PTP-LAR (D1D2), PRL2 A/S, and MKPX, with the protease papain present as a negative control. Experiments were performed in duplicate. Graph bars represent percent inhibition (100 = complete inhibition; 0 = no inhibition).

Table 1. IC_{50} (μM) Values for 6 and 20f–j against PTP1B and PTPσ in Dose-Dependent Enzymatic Assays[a]

compd	PTP1B	PTPσ
6	6.2 ± 1.1	4.3 ± 1.2
20f	3.6 ± 0.9	15.6 ± 1.1
20g	6.1 ± 0.4	11.8 ± 0.1
20h	1.7 ± 0.4	7.9 ± 1.3
20i	2.3 ± 0.2	0.6 ± 0.9
20j	2.4 ± 1.3	0.9 ± 0.1

[a]Based on initial enzyme screen.

inhibitor, also with approximately 4-fold selectivity over PTP1B (PTP1B IC_{50} = 2.3 μM; PTPσ IC_{50} = 0.6 μM).

Compounds **20i** and **20j** were screened against a panel of PTPs employing the DiFMUP assay. The dose-dependent enzyme activity rate curves of **20i** and **20j** showed significantly greater activity against PTP1B and PTPσ, compared to PTP-LAR (D1D2), MKPX, and CD45D1D2 (Figure 5). The limited discrimination of the compounds between PTP1B and TC-PTP was anticipated, given > 74% homology in their catalytic domains and adjacent pockets. This demonstrated for the first time a scaffold that displayed potency and selectivity for PTP1B over PTPσ. Moreover, this study identified potent PTPσ inhibitors, **20i** and **20j**. However, given there is no cell-based assay for assessing the enzymatic activity of PTPσ, the inhibition profiles of **20i** and **20j** against PTPσ could not be explored further at this time.

The cytotoxicities of PTP1B inhibitors **20f**, **20h**, and **20j** were determined in Chinese hamster ovary (CHO) cells, which express the human insulin receptor (CHO-IR). A Titer-Blue

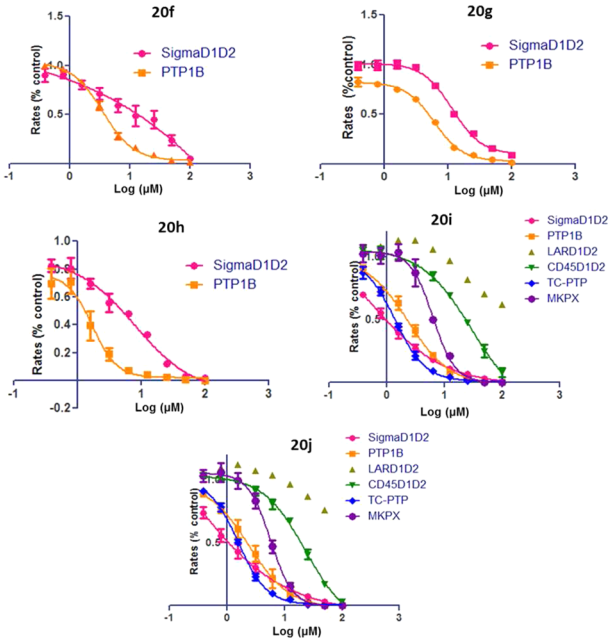

Figure 5. Dose-dependent curves for inhibitors **20f–j**. Inhibitors were tested against an array of phosphatases. Individual plots overlay inhibitor-specific data for the various phosphatases.

Cell viability assay (Promega) showed no toxicity at 0.78 to 50 μM concentrations (Supporting Information).[2] Having established that the compounds were nontoxic, **20f**, **20h**, and **20j** were subjected to Western blot analysis against PTP1B in CHO cells.[2] Briefly, the insulin-signaling cascade depends on the phosphorylation state of the insulin receptor (IR). In this signal transduction pathway, insulin binds to the IR on the extracellular plasma membrane and promotes autophosphorylation of the IR (pIR). Since pIR is a direct substrate for PTP1B, the IR phosphorylation state is largely dependent on the activity levels of PTP1B within the cytoplasm. Thus, monitoring pIR levels is a useful reporter for PTP1B activity. CHO cells were incubated with increasing concentrations of inhibitor in serum-free media, then stimulated with insulin for 15 min. Western blot analysis determined the relative levels of pIR (Figure 6). Compounds **20f**, **20h**, and **20j** dose-dependently increased the pIR signal, relative to the DMSO control (Figure 6A). Although vanadate induced a strong response (50 μM), it suffers as a therapeutic due to poor metabolic stability and low bioavailability.[35] In addition, the compounds displayed improved activity in cells relative to **6** (Supporting Information). The densitometry graph showed **20f**, **20h**, and **20j** had a significant effect on the pIR/IR levels compared to the DMSO control (Figure 6B).[2]

Finally, comparative Michaelis–Menten kinetic studies were performed for **6** and **20f** against PTP1B (Figure 7). Compound **20f** showed an approximate 3-fold greater competitive inhibition (K_i = 1.83 ± 0.39 μM) compared to **6** (K_i = 5.66 ± 2.11 μM). In comparison to **6**, the bidentate approach yielded a more direct, competitive inhibitor of the active site. In general, in the field of PTP inhibition, mixed inhibitors tend to display a higher degree of toxicity, attributed to their promiscuous mode of action.[12,36,37] Thus, we have optimized a mixed inhibitor, **6**, to produce **20f**, a competitive and presumed active site binder of PTP1B.

In summary, identifying selective and potent small molecule inhibitors of the PTP family of proteins (> 200 members)

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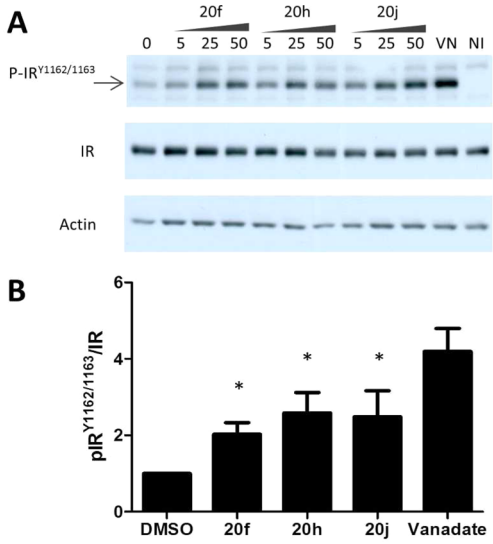

Figure 6. (A) Dose-dependent response of compounds **20f**, **20h**, and **20j** on the phosphorylated insulin receptor was determined by Western blot. Cells were treated with compounds (5−50 μM), DMSO as a solvent control, 50 μM vanadate (VN), or left unstimulated (NI). (B) Densitometry graph showing the response of **20f**, **20h**, and **20j** at 25 μM on the levels of pIR and IR vs a DMSO control, $^*P < 0.05$. Values correspond to mean ± standard error of the mean ($n = 4$). Statistical analysis was performed with two-tailed, unpaired, Student's t test.

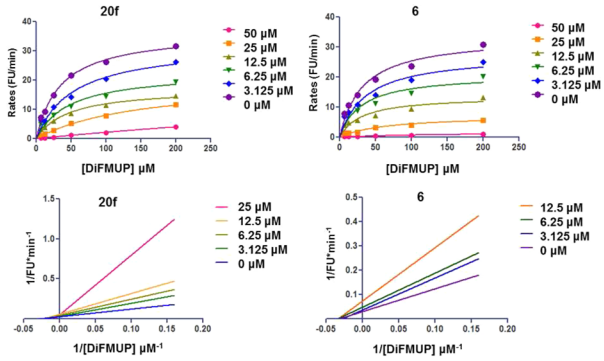

Figure 7. Michaelis−Menten rate data as a function of substrate concentration for **20f** and **6**. Curves represent the hydrolysis of DiFMUP and subsequent fluorescent detection at 450 nm (excitation at 358 nm). The bottom curves show the Lineweaver−Burke transformation detailing competitive inhibition for **20f** and **6**.

remains a daunting task due to the high degree of sequence homology. Inspired by current approaches utilizing bidentate scaffolds, a family of disalicylic acid derivatives, based on PTP1B inhibitor **6**, were developed to achieve more potent and selective inhibition of PTP1B. A number of compounds were identified that displayed improved potency (> 5-fold) and selectivity for PTP1B over PTPσ (4-fold) relative to **6**. Compounds **20f−j** did not inhibit other PTP family members such as PTP-LAR (D1D2). In particular, **20h** represents an interesting lead for further investigation against PTP1B, as it demonstrated whole-cell PTP1B inhibition and > 4-fold selectivity over PTPσ. Finally, **20i** and **20j** displayed nanomolar inhibition for PTPσ and thus represent interesting leads for further investigation against this novel target.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmedchemlett.5b00171.

Information on compound synthesis, characterization, detailed results, and experimental procedures (PDF)

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Notes

The authors declare no competing financial interest.

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