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Glutathione-Responsive Selenosulfide Prodrugs as a Platform Strategy for Potent and Selective Mechanism-Based Inhibition of Protein Tyrosine Phosphatases

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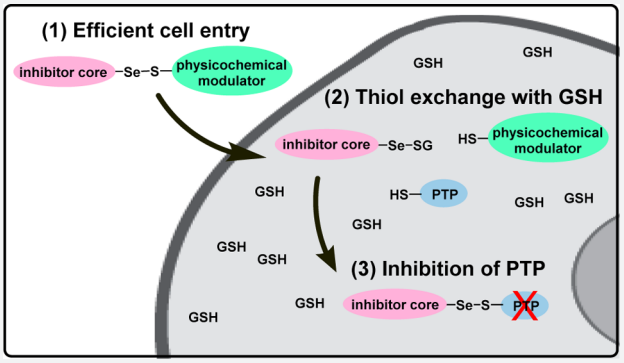
Supporting Information

ABSTRACT: Dysregulation of protein tyrosine phosphorylation has been implicated in a number of human diseases, including cancer, diabetes, and neurodegenerative diseases. As a result of their essential role in regulating protein tyrosine phosphorylation levels, protein tyrosine phosphatases (PTPs) have emerged as important yet challenging therapeutic targets. Here we report on the development and application of a glutathione-responsive motif to facilitate the efficient intracellular delivery of a novel class of selenosulfide phosphatase inhibitors for the selective active site directed inhibition of the targeted PTP by selenosulfide exchange with the active site cysteine. The strategy leverages the large difference in extracellular and intracellular glutathione levels to deliver selenosulfide phosphatase inhibitors to cells. As an initial exploration of the prodrug platform and the corresponding selenosulfide covalent inhibitor class, potent and selective inhibitors were developed for two therapeutically relevant PTP targets: the *Mycobacterium tuberculosis* virulence factor *m*PTPA and the CNS-specific tyrosine phosphatase, striatal-enriched protein tyrosine phosphatase (STEP). The lead selenosulfide inhibitors enable potent and selective inhibition of their respective targets over a panel of human PTPs and a representative cysteine protease. Kinetic parameters of the inhibitors were characterized, including reversibility of inhibition and rapid rate of GSH exchange at intracellular GSH concentrations. Additionally, active site covalent inhibitor-labeling with an *m*PTPA inhibitor was rigorously confirmed by mass spectrometry, and cellular activity was demonstrated with a STEP prodrug inhibitor in cortical neurons.



INTRODUCTION

Protein tyrosine phosphorylation is a prominent post-translational modification that is essential for nearly all cellular functions. Protein tyrosine kinases and phosphatases catalyze phosphorylation and dephosphorylation reactions, respectively, to carefully regulate tyrosine phosphorylation levels.[1,2] A number of human diseases, including cancer, diabetes, and neurodegenerative diseases, have been linked to dysregulation of protein tyrosine phosphorylation.[3,4] Additionally, PTPs have been identified as virulence factors in infectious diseases, including tuberculosis.[3,5,6] Because of the importance of careful regulation of tyrosine phosphorylation levels in maintaining proper cellular function, dozens of protein tyrosine kinase inhibitors have been approved as drugs that have greatly benefited the treatment of human diseases.[3,7] In contrast, despite their complementary role in regulating protein phosphorylation levels, PTPs remain challenging targets, and, to date, no PTP inhibitors have progressed into the clinic.[7−9]

PTPs catalyze tyrosine dephosphorylation through attack of an active site cysteine to form a covalent phosphocysteine intermediate that rapidly hydrolyzes to regenerate the active enzyme.[10] Nature regulates PTP activity through oxidation of the catalytic cysteine, rendering the PTP inactive by the formation of covalent adducts such as sulfinic acids, disulfides with other cysteine residues, and sulfenamide bonds with backbone amides.[11,12] For some modifications, subsequent reaction of the covalently modified enzyme with low molecular weight thiols can serve to reactivate PTPs.[13−16] The role of cysteine adducts in the context of PTP regulation is an incredibly active area of research.[17,18]

Herein, a mechanism-based platform for phosphatase inhibition is described whereby a selenosulfide pharmacophore reacts with the active site cysteine of the PTP to form a covalent selenosulfide adduct that inhibits the PTP (Figure

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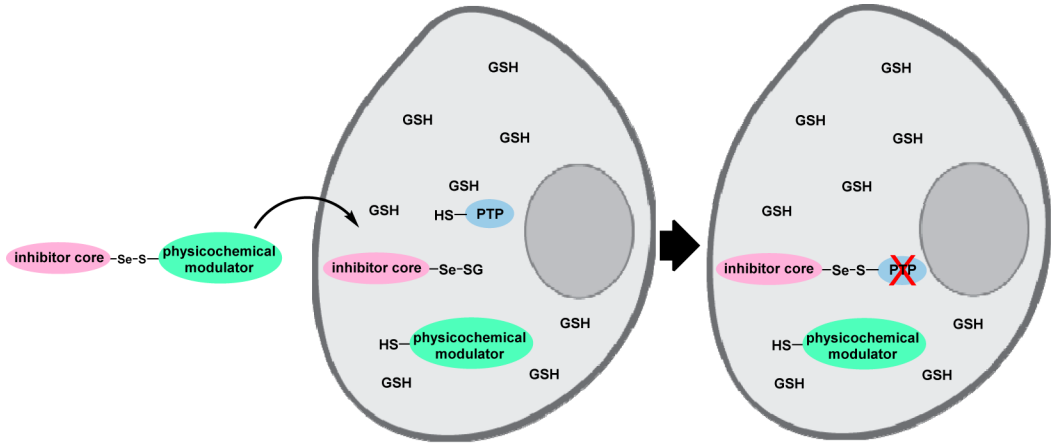

Figure 1. Selenosulfide prodrug strategy for PTP inhibition.

1).[19,20] Significantly, a modest seleninic acid inhibitor of PTP1B has previously been shown to generate a selenosulfide adduct with the active site cysteine of PTP1B as determined by X-ray crystallography.[21] Selenium is an essential element[22] and many examples of the incorporation of selenium into biologically active structures have been reported in the literature.[23−28] Potency and selectivity for a specific PTP can be achieved by introducing functionality on the seleno portion of the inhibitor to provide additional noncovalent binding interactions with the PTP of interest (vide infra).

In addition to potent inhibition, the selenosulfide PTP pharmacophore class provides a unique opportunity to tune the physicochemical properties of the inhibitors. Selenium−sulfur bonds are known to undergo rapid exchange with external thiols through nucleophilic attack at selenium.[29−31] Leveraging this rapid selenosulfide-thiol exchange allows for the construction of a general prodrug platform for enhancing the physicochemical properties of the inhibitor by intracellular glutathione (GSH)-activation. Indeed, numerous disulfide-based protein and small molecule prodrug strategies have been developed that rely on high intracellular GSH levels.[32−34] Moreover, active transport of activated disulfides into cells is increasingly recognized as an important mechanism for cell delivery and possibly might also be applicable to selenosulfides.[35,36] Here we demonstrate our selenosulfide PTP prodrug approach by the development of potent and selective inhibitors to the *Mycobacterium tuberculosis* virulence factor *m*PTPA and the CNS-specific tyrosine phosphatase, striatal-enriched protein tyrosine phosphatase (STEP).

RESULTS AND DISCUSSION

We first applied the selenosulfide prodrug strategy to the development of a potent and selective inhibitor of the *Mycobacterium tuberculosis* (*Mtb*) virulence factor *m*PTPA. Tuberculosis is caused by *Mtb*, which infects a third of the world's population and results in over 1 million deaths annually.[37] Limited treatment options exist for the over 50 million people who develop multidrug resistant tuberculosis, and this has spurred a search for new treatment strategies.[38] Recent work focused on pathogen virulence factors has resulted in the identification of two secreted PTPs, *m*PTPA and *m*PTPB, as targets for the treatment of tuberculosis.[5,6,39] In particular, a genetic deletion of *ptpA* attenuated mycobacterial survival within human macrophages and resulted in increased phagosome−lysosome fusion and activity. Notably, because *m*PTPA is secreted into the macrophage cytosol where it acts on host proteins, targeting *m*PTPA bypasses intrinsic resistance factors such as the highly impermeable *Mtb* cell wall and drug efflux mechanisms.

Our lab previously identified active site directed reversible inhibitor **1** with single-digit micromolar inhibition of *m*PTPA and greater than 10-fold selectivity across multiple PTPs (Figure 2a).[40] We envisioned combining the selenosulfide PTP pharmacophore with the *m*PTPA-specific aryl structure of **1** to create a *m*PTPA selective selenosulfide inhibitor. Excitingly, GSH selenosulfide inhibitor **2** is a potent irreversible inhibitor with a k_{inact}/K_i of 360 000 ± 17 000 s^{-1} M^{-1} (Figure 2a). Irreversible inhibition was rigorously established by dialysis to remove the inhibitor even over extended times with no regain of *m*PTPA activity observed (Figure 2b and Table S1). However, when the fully inhibited enzyme was incubated with 1 mM GSH or DTT, partial or full regain of *m*PTPA activity occurred, respectively, as would be expected due to selenosulfide thiol interchange of the inhibited enzyme (Figure 2c). Given that inhibition of *m*PTPA with **2** is partially reversible in the presence of intracellular concentrations of GSH (1 mM), its inhibitory activity was also characterized at 1 mM GSH and found to be sub-micromolar under these more challenging conditions with an IC_{50} of 0.53 ± 0.10 μM (Figure 2a).

Truncated inhibitors were next prepared and evaluated in order to examine the importance of the aryl scaffold for the inhibition of *m*PTPA. As expected, the fully elaborated aryl core of the inhibitor is essential for achieving potent inhibition of *m*PTPA (Figure 2d).

To demonstrate that selenosulfide **2** inhibits *m*PTPA through a covalent modification, we analyzed selenosulfide-treated and untreated *m*PTPA by mass spectrometry (Figure 2e). The mass of untreated *m*PTPA obtained by mass spectrometry was 18.172 kDa. This mass coincides well with the expected mass of 18.173 kDa predicted based on the protein sequence. As expected, upon treatment of *m*PTPA with selenosulfide **2**, even

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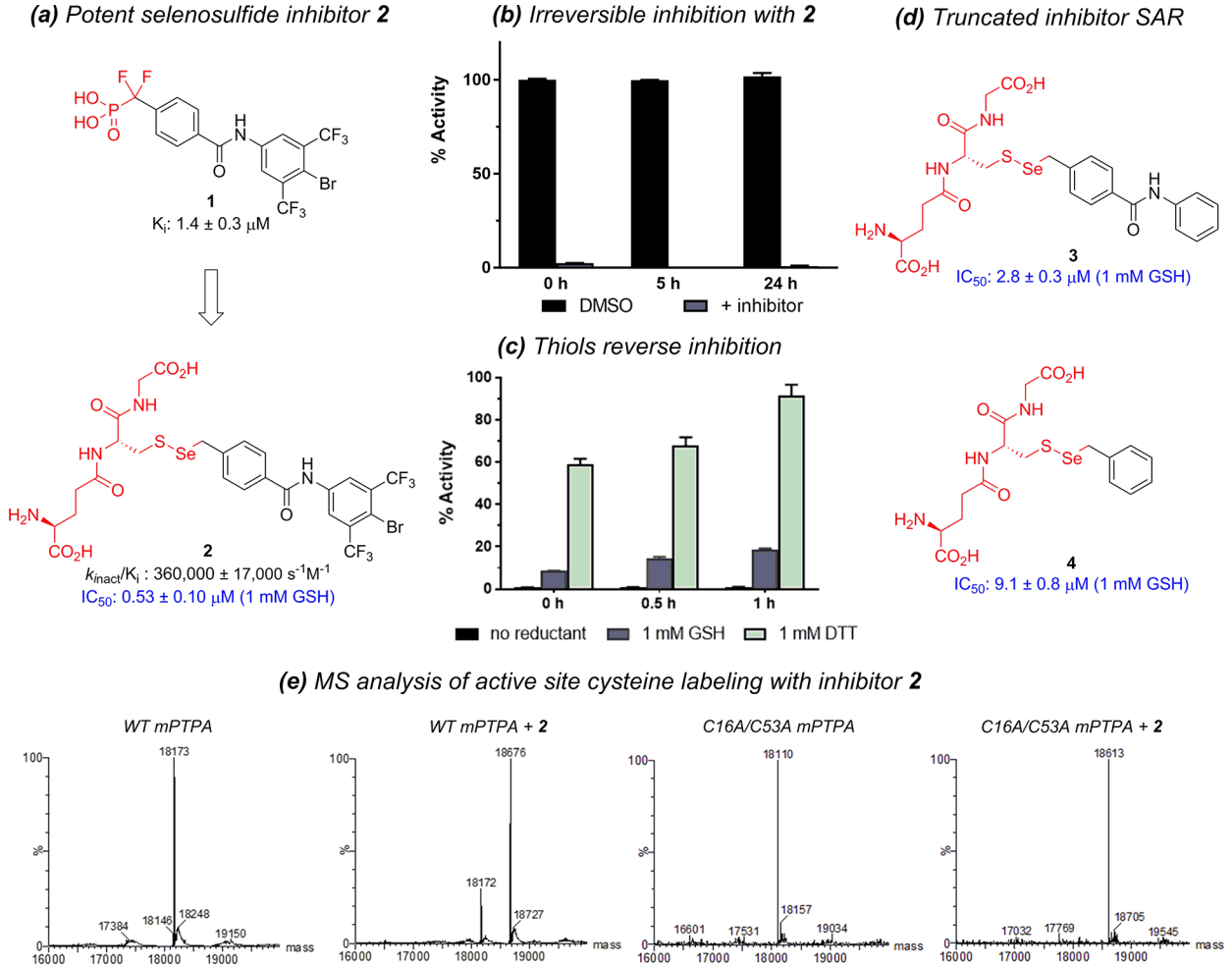

Figure 2. Selenosulfide inhibitors of *m*PTPA. (a) Combining the *m*PTPA-specific aryl scaffold of previously developed active site directed reversible inhibitor **1** with the selenosulfide PTP pharmacophore to generate potent *m*PTPA selenosulfide inhibitor **2** assayed with or without intracellular concentrations of GSH (1 mM). (b) Irreversible inhibition with **2**. *m*PTPA (200 nM) and inhibitor **2** (1 μM) (or DMSO control) were incubated for 60 min in the absence of GSH to inhibit enzymatic activity prior to dialysis. The enzymatic activity of *m*PTPA was measured using the pNPP assay at 0, 5, and 24 h after dialysis. (c) Reactivation of inhibited *m*PTPA by the addition of thiols. *m*PTPA (200 nM) and **2** (5 μM) were incubated for 10 min, and following addition of GSH or DTT (1 mM each) or water (no reductant), for another 0, 0.5, and 1 h after which enzymatic activity was measured. (d) Structure–activity relationship (SAR) of *m*PTPA selenosulfide inhibitors. (e) Deconvoluted ESI mass spectrum of WT *m*PTPA, WT *m*PTPA inhibited with selenosulfide **2** in the presence of 1 mM GSH, C16A/C53A *m*PTPA, and C16A/C53A *m*PTPA inhibited with selenosulfide **2** in the presence of 1 mM GSH.

in the presence of 1 mM GSH, a modified mass of 18.676 kDa was observed. This mass is consistent with the formation of a covalent selenosulfide bond between the *m*PTPA catalytic cysteine and seleno portion of the inhibitor, which should result in a mass increase of 0.505 kDa.

While the MS data showed a clear mass ion peak corresponding to covalent modification of *m*PTPA, in addition to the active site cysteine (C11), the wild type enzyme also contains another two cysteines (C16 and C53). Sequence analysis after trypsin-digestion of a covalently inhibited protein typically has been employed to identify the location of covalent modification.[41,42] This approach, however, is not suitable for selenosulfide inhibitor **2** because the enzyme is inhibited reversibly via rapid thiol interchange. To rigorously evaluate whether or not inhibitor labeling occurs on the active site cysteine, we performed mass spectrometry analysis with the double mutant C16A/C53A *m*PTPA that now only contains the active site cysteine (C11). The mutant enzyme is catalytically active, though with 10-fold and 2-fold reduction in k_{cat} and K_M relative to wild type enzyme, respectively. Treatment of the double mutant C16A/C53A *m*PTPA with selenosulfide **2** in the presence of 1 mM GSH resulted in a modified mass of 18.613 kDa, a 0.505 kDa increase corresponding to inhibitor labeling, in comparison to its untreated form (18.110 kDa) (Figure 2e). This result clearly establishes that covalent modification by selenosulfide inhibitor **2** occurs at the active site cysteine of *m*PTPA.

The high structural homology among PTPs often leads to difficulty in achieving high selectivity.[9] For this reason, we next evaluated the selectivity of lead *m*PTPA inhibitor **2** against *m*PTPB, a panel of human PTPs, and a generic cysteine protease (Table 1). Selenosulfide **2** exhibited good to outstanding selectivity against all human PTPs screened. Notably, we observed >50-fold selectivity against the highly homologous human LMW-PTP. Additionally, **2** showed >100-fold selectivity for *m*PTPA over *m*PTPB, which could serve to aid in biochemical and pharmacological studies designed to decipher the biological role of *m*PTPA independent of *m*PTPB.

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Table 1. Selectivity Profile of *m*PTPA Inhibitor 2 against a Panel of Human PTPs, *m*PTPB, and a Cysteine Protease

enzyme class	enzyme	IC_{50} (μM)[a]	selectivity
phosphatases	*m*PTPA	0.53 ± 0.10	
	*m*PTPB	>100	>100
	STEP	6.2 ± 2.9	12
	LMW-PTP	43 ± 11	81
	PTP1B	50 ± 20	94
	CD45	>100	>100
	LAR	>100	>100
	TC-PTP	>100	>100
cysteine protease	cathepsin B	>100	>100

[a]IC_{50} values were determined in the presence of a physiological intracellular concentration of GSH (1 mM).

Moreover, the inhibition of cathepsin B was evaluated as a representative example of the cysteine proteases, another important class of active site cysteine enzymes. Inhibition of cathepsin B was not observed.

Application of the selenosulfide inhibitor strategy resulted in the development of a potent and selective *m*PTPA inhibitor. The next step would be evaluation of a prodrug of **2** in a cellular assay. As *m*PTPA is a secreted virulence factor, a simple bacterial cytotoxicity assay is not relevant. Moreover, in prior animal models, inhibitors to both *m*PTPA and *m*PTPB were required to achieve significant antibacterial activity.[43] For this reason we chose to develop selenosulfide prodrug inhibitors to another PTP target to provide for more straightforward analysis of activity in a cellular context.

The CNS-specific tyrosine phosphatase STEP has been implicated as an important therapeutic target for neurological diseases, including Alzheimer's disease.[44,45] In addition, we have reported a benzopentathiepin-based inhibitor that covalently labels the enzyme in a reversible fashion in the presence of thiols like GSH. This inhibitor was active in both cell-based assays and animal models, but its poor solubility and instability remain significant challenges to further development.[46] We also previously identified active site directed reversible inhibitors **5** and **6** as single-digit micromolar inhibitors of STEP. These inhibitors have greater than 20-fold selectivity across multiple phosphatases and inhibit STEP in cortical neurons (Figure 3a).[47] We combined the selenosulfide PTP pharmacophore with the STEP-specific aryl scaffold of **5** and **6** to obtain STEP inhibitor **7**. Potent irreversible inhibition of STEP with inhibitor **7** was observed with a k_{inact}/K_i = 1 210 000 ± 140 000 s^{-1} M^{-1} (Figure 3a). Irreversible inhibition was demonstrated by dialysis to remove the inhibitor without any regain in enzyme activity (Figure 3b and Table S1). However, when the inhibited enzyme was incubated with 1 mM GSH or DTT recovery of STEP activity occurred due to the selenosulfide thiol interchange of the inhibited enzyme (Figure 3c). Under cellular concentrations of GSH (1 mM) where inhibition is reversible, inhibitor **7** retains good activity with an IC_{50} of 1.5 ± 0.7 μM (Figure 3a). Truncated inhibitors were next prepared and evaluated in order to examine the importance of the aryl scaffold for the inhibition of STEP. As expected, the fully elaborated aryl core of the inhibitor is essential for achieving potent inhibition of STEP (Figure 3d).

STEP inhibitor **7** was evaluated against a panel of human PTPs and a generic cysteine protease (Table 2). Inhibitor **7** exhibited good selectivity against most phosphatases screened, with >20-fold selectivity against TC-PTP, PTP1B, LAR, CD45, and MKP5. We observed 8-fold selectivity against human LMW-PTP and only very modest selectivity against the highly

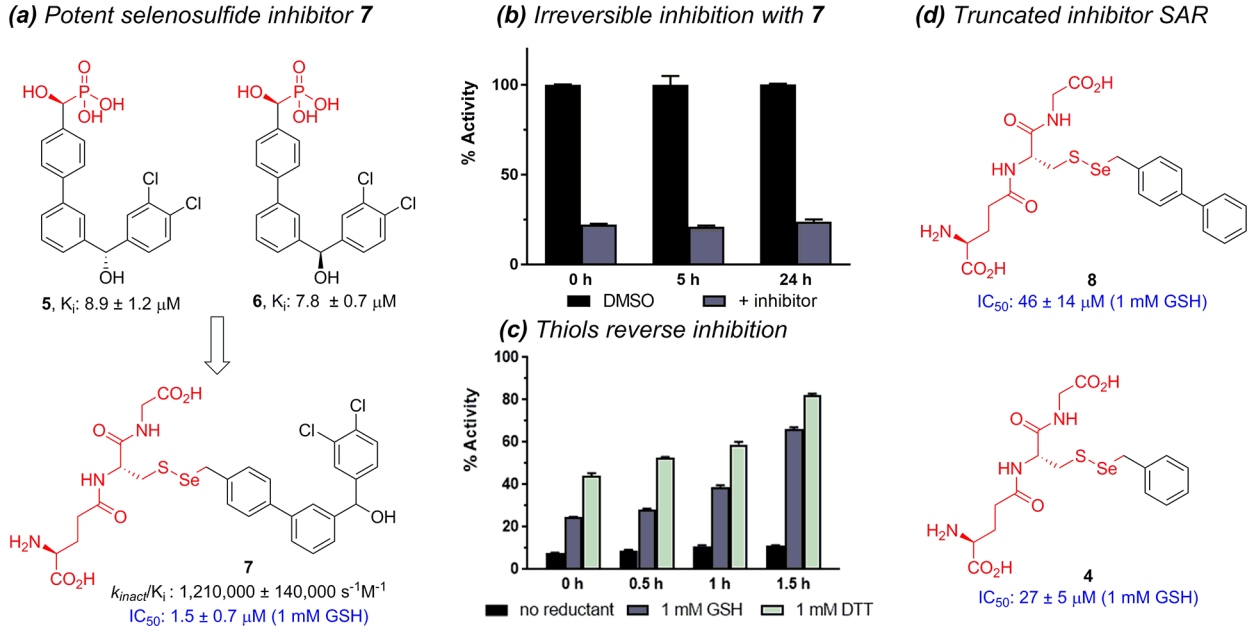

Figure 3. Selenosulfide inhibitors of STEP. (a) Combining the STEP-specific aryl scaffold of previously developed active site directed reversible inhibitors **5** and **6** with the selenosulfide PTP pharmacophore to generate potent STEP selenosulfide inhibitor **7** assayed with or without physiological intracellular concentrations of GSH (1 mM). (b) Irreversible inhibition with **7**. STEP (200 nM) and inhibitor **7** (1 μM) (or DMSO control) were incubated for 60 min in the absence of GSH to inhibit enzymatic activity prior to dialysis. The enzymatic activity of STEP was measured using the pNPP assay at 0, 5, and 24 h after dialysis. (c) Reactivation of inhibited STEP by the addition of thiols. STEP (200 nM) and **7** (5 μM) were incubated for 10 min, and following addition of GSH or DTT (1 mM each) or water (no reductant), for another 0, 0.5, 1, and 1.5 h after which enzymatic activity was measured. (d) Structure–activity relationship of STEP selenosulfide inhibitors.

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Table 2. Selectivity Profile of STEP Inhibitor 7 against a Panel of Human PTPs and a Cysteine Protease

enzyme class	enzyme	IC_{50} (μM)[a]	selectivity
phosphatases	STEP	1.5 ± 0.7	
	HePTP	2.5 ± 0.1	1.7
	LMW-PTP	12 ± 3	8.0
	TC-PTP	40 ± 3	27
	PTP1B	71 ± 7	27
	LAR	68 ± 19	45
	CD45	>200	>100
	MKP5	>200	>100
cysteine protease	cathepsin B	>200	>100

[a]IC_{50} values were determined in the presence of a physiological intracellular concentration of GSH (1 mM).

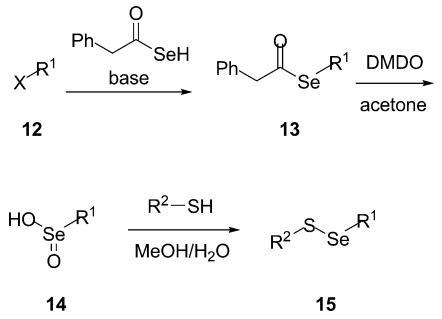
homologous tyrosine phosphatase HePTP.[48,49] No inhibition of cathepsin B was observed.

Following the evaluation of potency and selectivity of lead GSH selenosulfide inhibitor **7**, we prepared and evaluated selenosulfide prodrugs for inhibition of STEP in cells (Figure 4). To promote cell-permeability of the inhibitor scaffold, two functionalized selenosulfide inhibitors **9** and **10** were prepared with the previously established inhibitor core. In the presence of a cellular concentration of GSH, very rapid thiol exchange to give the GSH selenosulfide inhibitor **7** is expected to occur. Indeed, the very similar STEP inhibitory potency of **9** and **10** with GSH selenosulfide **7** with 1 mM GSH is consistent with expected rapid thiol exchange (Figure 4a). The rapid rate of thiol exchange in the presence of a cellular concentration of GSH was also rigorously established by treatment of selenosulfide **11** with 1 mM GSH resulting in an equilibrium mixture of **11** and the GSH-adduct **4** within 2 min as determined by LCMS analysis (Figure 4b).

Following evaluation of GSH-exchange and inhibitor potency against purified enzyme, prodrug inhibitors **9** and **10** were evaluated for their ability to inhibit STEP in rat cortical neurons. Inhibition was evaluated by monitoring phosphorylation levels of known STEP substrates pNR2B, pPyk2, and pERK.[50−54] Statistically significant increases in the phosphorylation levels of all three STEP substrates were observed upon treatment with **9** (Figure 4c), while inhibitor **10** showed more modest increases in phosphorylation levels (data not shown). Finally, in preliminary studies for future in vivo evaluation, **9** was determined to have a reasonable 73% stability to rat plasma over 1 h as well as good stability to rat liver microsomes (77% and 82% remaining after 1 h with and without NADPH).[55]

Inhibitor Synthesis. As a general approach toward the preparation of the selenosulfide PTP inhibitors described in this article, appropriately functionalized alkyl halide precursors **12** can be transformed to the corresponding selenate esters **13** (Scheme 1). Using a procedure developed by Zhang, Knapp, and co-workers, oxidation of the selenate esters **13** with dimethyl dioxirane (DMDO) results in the seleninic acids **14**.[21] Treatment of seleninic acids **14** with an excess of the desired thiol then provides the selenosulfide prodrug inhibitors **15**. An advantageous aspect of this approach is that the physicochemical-modulating thiol component of an inhibitor is installed in the final step of the sequence. This enables rapid preparation of inhibitors with a range of thiol physicochemical modulators appended to a given inhibitor core.

Scheme 1. General Approach Towards the Preparation of Selenosulfide Inhibitors



CONCLUSIONS

A GSH-responsive prodrug strategy has been developed to facilitate the efficient intracellular delivery of a novel class of selenosulfide PTP inhibitors. As an initial exploration into this

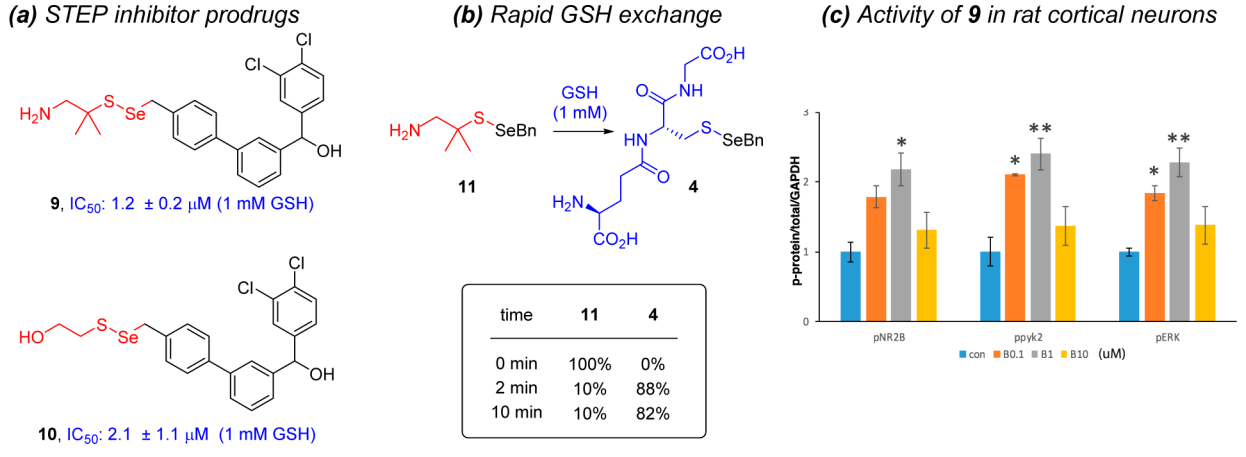


Figure 4. Selenosulfide STEP inhibitor prodrugs. (a) Evaluation of STEP selenosulfide prodrug inhibitors **9** and **10** in the presence of a physiological intracellular concentration of GSH (1 mM). (b) Monitoring selenosulfide thiol exchange with GSH, amounts of **11** and **4** present determined by LCMS-UV. (c) Inhibition of STEP in rat cortical neurons treated with **9**. Concentrations of 0.1, 1.0, or 10 μM of **9** for 1 h in HBS buffer and analyzed by Western blotting (*p < 0.05; **p < 0.01 one-way ANOVA, Dunnett's *post hoc*). Data represent the phosphosignal normalized to the total protein signal and GAPDH + s.e.m. (n = 3−5 in each group).

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prodrug strategy, we have developed inhibitors for two PTPs: the *Mtb* virulence factor *m*PTPA and the brain-specific tyrosine phosphatase STEP. The lead molecules described in this work enable potent and selective inhibition of *m*PTPA and STEP, respectively. We have further characterized the expected covalently modification resulting from selenosulfide inhibitor treatment by mass spectrometry and have demonstrated cellular activity. These studies demonstrate the feasibility of the selenosulfide prodrug approach, which potentially could be applied to many other PTPs.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acscentsci.7b00486.

All synthetic procedures, characterization, analytical data, reversibility of inhibition by dialysis and thiols, details on generation of mutant *m*PTPA, mass spectrometry experiments, rate of GSH-exchange of selenosulfide **11**, cell culture and Western blotting. (PDF)

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Notes

The authors declare no competing financial interest.

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