Academic cross-fertilization by public screening yields a remarkable class of protein phosphatase methylesterase-1 inhibitors

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National Institutes of Health (NIH)-sponsored screening centers provide academic researchers with a special opportunity to pursue small-molecule probes for protein targets that are outside the current interest of, or beyond the standard technologies employed by, the pharmaceutical industry. Here, we describe the outcome of an inhibitor screen for one such target, the enzyme protein phosphatase methylesterase-1 (PME-1), which regulates the methylesterification state of protein phosphatase 2A (PP2A) and is implicated in cancer and neurodegeneration. Inhibitors of PME-1 have not yet been described, which we attribute, at least in part, to a dearth of substrate assays compatible with high-throughput screening. We show that PME-1 is assayable by fluorescence polarization-activity-based protein profiling (fluoplop-ABPP) and use this platform to screen the 300,000+ member NIH small-molecule library. This screen identified an unusual class of compounds, the azah-lactams (ABLs), as potent (IC50 values of approximately 10 nM) covalent PME-1 inhibitors. Interestingly, ABLs did not derive from a commercial vendor but rather an academic contribution to the public library. We show using competitive-ABPP that ABLs are exclusively selective for PME-1 in living cells and mice, where enzyme inactivation leads to substantial reductions in demethylated PP2A. In summary, we have combined advanced synthetic and chemoproteomic methods to discover a class of ABL inhibitors that can be used to selectively perturb PME-1 activity in diverse biological systems. More generally, these results illustrate how public screening centers can serve as hubs to create spontaneous collaborative opportunities between synthetic chemistry and chemical biology labs interested in creating first-in-class pharmacological probes for challenging protein targets.

Protein phosphorylation is a pervasive and dynamic posttranslational protein modification in eukaryotic cells. In mammals, more than 500 protein kinases catalyze the phosphorylation of serine, threonine, and tyrosine residues on proteins (1). A much more limited number of phosphatases are responsible for reversing these phosphorylation events (2). For instance, protein phosphatase 2A (PP2A) and PP1 are thought to be responsible together for >90% of the total serine/threonine phosphatase activity in mammalian cells (3). Specificity is imparted on PP2A activity by multiple mechanisms, including dynamic interactions between the catalytic subunit (C) and different protein-binding partners (B subunits), as well as a variety of posttranslational chemical modifications (2, 4). Within the latter category is an unusual methylesterification event found at the C terminus of the catalytic subunit of PP2A that is introduced and removed by a specific methyltransferase (leucine carboxymethyltransferase-1 or LCMT1) (5, 6) and methylesterase (protein phosphatase methylesterase-1 or PME-1) (7), respectively (Fig. 1A). PP2A carboxymethylation (hereafter referred to as “methylation”) has been proposed to regulate PP2A activity, at least in part, by modulating the binding interaction of the C subunit with various regulatory B subunits (8–10). A predicted outcome of these shifts in subunit association is the targeting of PP2A to different protein substrates in cells. PME-1 has also been hypothesized to stabilize inactive forms of nuclear PP2A (11), and recent structural studies have shed light on the physical interactions between PME-1 and the PP2A holoenzyme (12).

Notwithstanding the aforementioned models and findings, the actual functional consequences of perturbing PP2A methylation remain largely unexplored. In yeast, LCMT1 deletion caused severe growth defects under stress conditions, while PME-1 deletion did not result in an observable cellular phenotype (9). Disruption of the PME-1 gene in mice, on the other hand, caused early postnatal lethality (13), which has limited the experimental opportunities to explore methylation of PP2A in animals. Recent studies have found that RNA-interference knockdown of PME-1 in cancer cells leads to activation of PP2A and corresponding suppression of protumorigenic phosphorylation cascades (14), indicating that PME-1 could be an attractive drug target in oncology. Changes in PP2A methylation have also been implicated in Alzheimer’s disease, where this modification may stimulate PP2A’s ability to promote neural differentiation (15).

Despite the critical role that PME-1 plays in regulating PP2A structure and function, PME-1 inhibitors have not yet been described. This deficiency may be due to a lack of PME-1 activity assays that are compatible with high-throughput screening (HTS). Assessment of PME-1 activity typically involves either Western blotting with antibodies that recognize specific methylation states of PP2A (7, 13) or monitoring the release of 3H-methanol from radiolabeled-C subunits (16), but neither assay is easily adapted for HTS. PME-1 is, however, a serine hydrolase and therefore susceptible to labeling by active-site-directed fluorophosphonate (FP) probes (17). We have recently shown that FP probes can form the basis for a fluorescence polarization-activity-based protein profiling (fluoplop-ABPP) assay suitable for HTS (18). Here, we apply fluoplop-ABPP to screen the 300,000+ National Institutes of Health (NIH) compound library for PME-1 inhibitors. From this screen, we identified a set of azah-lactam (ABL) compounds that act as remarkably potent and selective pharmacological probes for evaluating the binding interaction of the C subunit with various regulatory B subunits (8–10). A predicted outcome of these shifts in subunit association is the targeting of PP2A to different protein substrates in cells. PME-1 has also been hypothesized to stabilize inactive forms of nuclear PP2A (11), and recent structural studies have shed light on the physical interactions between PME-1 and the PP2A holoenzyme (12).
PME-1 inhibitors. We show that these ABLs covalently inactivate PME-1 with high specificity in living cells and animals, where disruption of this enzyme leads to substantial decreases in demethylated PP2A.

**Results**

**PME-1 Inhibitor Screening by Fluopol-ABPP.** Because PME-1 is a serine hydrolase that is known to interact with reporter-tagged FP probes (17, 19), we reasoned that this enzyme would be assayable by competitive ABPP methods. However, lower-throughput, gel-based competitive ABPP screens have not succeeded in identifying lead PME-1 inhibitors (20), indicating the need to survey larger compound libraries. We therefore asked whether PME-1 could be assayed using the recently introduced, HTS-compatible fluopol-ABPP platform (18). This technique, where compounds are tested for their ability to block the increase in fluopol signal generated by reaction of a fluorescent activity-based probe with a much larger protein target, has enabled inhibitor screening for a wide range of probe-reactive enzymes (http://pubchem.ncbi.nlm.nih.gov/). We confirmed that purified, recombinant wild-type PME-1, but not a mutant PME-1 in which the serine nucleophile was replaced with alanine (S156A), labels with a fluorophosphonate rhodamine (FP-Rh) (21) probe (Fig. 1B). This reaction generates a strong, time-dependent increase in fluopol signal that is not observed in the absence of enzyme or with the S156A mutant PME-1 enzyme (Fig. 1C). In collaboration with the Molecular Libraries Probe Production Centers Network (MLPCN), we screened 315,002 compounds for PME-1 inhibition using fluopol-ABPP (see Fig. 1D for a representative subset of the primary screening data). Following a confirmation screen on initial hits, we identified 1,068 compounds as potential PME-1 inhibitors. After a confirmation screen on initial hits, the filter yielded approximately 300 candidate PME-1 inhibitors.

**Discovery of aza-β-lactam (ABL) Inhibitors of PME-1.** The approximately 300 hit compounds were next analyzed by gel-based competitive ABPP (18, 22) in soluble lysates from HEK 293T cells overexpressing PME-1. This convenient selectivity screen was performed on other enzymes (http://pubchem.ncbi.nlm.nih.gov/), and compounds with isopropyl substituents, ABL105 and ABL107, exhibited lower IC₅₀ values (92 and 24 nM, respectively) but were still good inhibitors of PME-1. Interestingly, a strong preference for the R enantiomer of ABLs was observed, as the S enantiomer of ABL127 (ent-ABL127) was at least two orders of magnitude less potent at inhibiting PME-1 (Fig. 2). Indeed, some of the apparent activity of the S enantiomer may be due to the small amount of residual R enantiomer in the >99% ee sample.

Before proceeding further, we wanted to confirm that ABLs could inhibit the ability of PME-1 to demethylate PP2A. We therefore treated HEK 293T soluble lysates with ABL127 (500 nM, 30 min) or DMSO before adding purified recombinant PME-1 for an additional hour. In DMSO-treated lysates, we observed the expected time-dependent increase in demethylated PP2A and concomitant decrease in methylated PP2A, as determined by immunoblotting with antibodies that specifically recognize either form of the C terminus of PP2A (Fig. 2E). In lysates containing ABL127, however, we observed little or no change in the methylation state of PP2A (Fig. 2E), indicating that ABL127 blocks PME-1’s activity on its physiological substrate. As ABL127 and ABL103 are highly similar structures and exhibit almost identical activity against PME-1, to assess their potency (Fig. 2C). The two compounds bearing cycloalkyl substituents at R, ABL127 and ABL103, were extraordinarily potent inhibitors of PME-1, with IC₅₀ values of 4.2 and 2.1 nM, respectively (Fig. 2C and Fig. S2). The two compounds with isopropyl substituents, ABL105 and ABL107, were designated as hits for competitive ABPP methods. However, lower-throughput, gel-based competitive ABPP screens have not succeeded in identifying lead PME-1 inhibitors (20), indicating the need to survey larger compound libraries. We therefore asked whether PME-1 could be assayed using the recently introduced, HTS-compatible fluopol-ABPP platform (18). This technique, where compounds are tested for their ability to block the increase in fluopol signal generated by reaction of a fluorescent activity-based probe with a much larger protein target, has enabled inhibitor screening for a wide range of probe-reactive enzymes (http://pubchem.ncbi.nlm.nih.gov/). We confirmed that purified, recombinant wild-type PME-1, but not a mutant PME-1 in which the serine nucleophile was replaced with alanine (S156A), labels with a fluorophosphonate rhodamine (FP-Rh) (21) probe (Fig. 1B). This reaction generates a strong, time-dependent increase in fluopol signal that is not observed in the absence of enzyme or with the S156A mutant PME-1 enzyme (Fig. 1C). In collaboration with the Molecular Libraries Probe Production Centers Network (MLPCN), we screened 315,002 compounds for PME-1 inhibition using fluopol-ABPP (see Fig. 1D for a representative subset of the primary screening data). Following a confirmation screen on initial hits, we identified 1,068 compounds as potential PME-1 inhibitors. As an initial filter, we selected compounds for follow-up studies that had <5% hit rates in all other bioassays reported in the PubChem database, <30% inhibition in three fluopol-ABPP screens performed on other enzymes (http://pubchem.ncbi.nlm.nih.gov/), and >40% inhibition of PME-1 in the confirmation screen. This filter yielded approximately 300 candidate PME-1 inhibitors.

**ABLs Covalently Inhibit PME-1.** Based on scientific precedent showing that other serine hydrolases can react with and open β-lactam
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In both cell lines, we observed highly potent and selective inhibitory activity of ABL127 for 1 h, harvested the soluble proteomes, and then reacted these lysates with the FP-Rh probe (Fig. 4A and Fig. S3). In both cell lines, we observed highly potent and selective inhibition of PME-1 with IC₅₀ values of 11.1 nM and 6.4 nM, respectively (Fig. 4B and Fig. S3). Although these gel-based competitive ABPP studies did not reveal any additional serine hydrolase targets of ABL127 at concentrations under 10 μM, we wanted to verify this selectivity profile by higher-resolution LC-MS/MS methods. To accomplish this, we employed an advanced version of our competitive ABPP-MudPIT technology (20, 25) that utilizes stable-isotope labeling of amino acids in cell culture (SILAC) (26). SILAC involves differential labeling of proteins with stable isotopes to generate isotopically “light” and “heavy” samples, which, when pooled and analyzed by MS, yield accurate quantification by comparing intensities of light and heavy peptide peaks. SILAC has previously been used to identify enzymes targets of activity-based probes (27) and small-molecule-binding proteins in cell lysates (28). In our competitive ABPP-SILAC experiments, cells grown in light and heavy media were treated with DMSO or ABL127, respectively, for 1 h. Proteomes were then harvested, combined at a 1:1 total protein ratio, and treated with the activity-based probe FP-biotin (29). FP-biotin-labeled proteins were then enriched with streptavidin-conjugated beads, digested on-bead with trypsin, and the resulting peptides analyzed by liquid chromatography-high-resolution tandem MS using an LTQ-Orbitrap mass spectrometer. Specifically enriched proteins were identified and quantified based on analysis of MS2 spectra and MS1 profiles, respectively. This analysis revealed complete and selective in situ inhibition of PME-1 by ABL127 with no activity against >50 other serine hydrolases detected in MDA-MB-231 and HEK 293T cells (Fig. 4C and Fig. S4).

We next investigated the impact of ABL127 incubation on the methylation state of PP2A in cells. As expected, in both MDA-MB-231 and HEK 293T cells (Fig. 4D), PP2A demethylation values were obtained from tryptic digests of purified, recombinant PME-1 (10 μM) treated with ABL127 (50 μM, red trace) or DMSO (black trace).
in the levels of demethylated PP2A (35% and 80%, respectively; Fig. 4D and E). A trend toward increases in methylated PP2A was also observed in ABL127-treated HEK 293T cells, but this change did not reach statistical significance (p = 0.12) (Fig. 4D and E). No difference in methylated PP2A was observed in MDA-MB-231 cells treated with ABL127 (Fig. 4D and E). These outcomes might be expected if the vast majority of PP2A is constitutively methylated under standard cell culture conditions. To investigate this hypothesis, we stably overexpressed PME-1 in HEK 293T cells (Fig. 4F), which resulted in a dramatic increase in demethylated PP2A and a significant decrease in methylated PP2A relative to a control cell line stably expressing GFP (Fig. 4G and H). Importantly, treatment of PME-1-transfected cells with ABL127 for only 1 h reduced the amount of demethylated PP2A back to the level observed in GFP-overexpressing control cells (Fig. 4G and H). These ABL127-treated cells also showed a significant increase in methylated PP2A (Fig. 4G and H). Time course studies revealed that a single treatment of ABL127 resulted in sustained inactivation of PME-1 and reductions in demethylated PP2A for at least 24 h (Fig. 4I). These data, taken together, indicate that ABL127 selectively inactivates PME-1 in living cells, which in turn causes significant changes in the methylation state of PP2A.

**A Clickable ABL Confirms Proteome-Wide Selectivity for PME-1.** Our competitive ABPP results showed that ABL127 is highly selective for PME-1 among members of the serine hydrolase family, but they did not address the possibility that ABL127 might react with other proteins in the proteome. To investigate this possibility, we synthesized ABL112, an analog of ABL127 that contains alkyne groups to serve as latent affinity handles amenable to modification by reporter tags using the copper(I)-catalyzed azide–alkyne cycloaddition reaction (“click chemistry”) (30) (Fig. 5A). We confirmed that ABL112 retains inhibitory activity for PME-1 by gel-based competitive ABPP in MDA-MB-231 lysates, where it showed only a threefold reduction in potency (IC50 = 13.8 nM; Fig. 5B) compared to the parent inhibitor ABL127 (Fig. S5). Next, we treated MDA-MB-231 cells with ABL127 (100 nM)
ABL12 Inactivates PME-1 in Mice. As mentioned earlier, PME-1 (−/−) mice are not viable (13), which has hindered experimental efforts to characterize this enzyme’s function (and the functional significance of PP2A methylation) in animals. Pharmacological inhibition of PME-1 would thus offer a potentially powerful means to study this enzyme in vivo. With this goal in mind, we asked whether ABL127 could inhibit PME-1 in mice. C57Bl/6 mice were treated with ABL127 (50 mg/kg, i.p., 2 h) or vehicle, sacrificed, and their brain proteomes assayed for PME-1 activity by competitive ABPP. Gel-based profiles indicated that brain PME-1 was inactivated by ABL127 (Fig. 6A), but overlapping serine hydrolase activities precluded a confident assessment of the extent of inactivation. For enhanced resolution of the activity state of PME-1 and other brain serine hydrolases, we performed competitive ABPP-MudPIT studies using FP-biotin. These LC-MS profiles confirmed complete inactivation of PME-1 (Fig. 6B) and no substantial reductions in any of the other approximately 40 brain serine hydrolases detected in this experiment. We also observed an approximately 35% reduction in the amount of demethylated PP2A in brain tissue from mice treated with ABL127 (Fig. 6C and D). These results confirm that ABL127 can selectively inhibit PME-1 in mice and this inhibition alters the methylation state of brain PP2A.

Discussion

We report herein a class of ABL inhibitors that show remarkable selectivity for PME-1 and equipotent activity in both cell-free and living cell assays. The lead ABL, ABL127 (designated as NIH Probe ML174), is capable of inactivating PME-1 in both human cancer cells and mice, suggesting that it should serve as a versatile pharmacological probe for evaluating PME-1 function in a multitude of living systems. We found that PME-1 inhibition causes a significant reduction in demethylated PP2A and, in cells with high levels of PME-1 activity, also a concomitant increase in methylated PP2A. Future studies with ABL127 should facilitate a more detailed understanding of the role that methylation plays in regulating PP2A function. Will, for instance, alterations in methylation state impact the composition and/or stability of specific PP2A complexes? Structural studies have confirmed that

These results demonstrate that the remarkable selectivity displayed by ABL127 for PME-1 extends not only across the serine hydrolase family but also the greater mammalian proteome.

**Fig. 5.** A clickable ABL reveals proteome-wide selectivity for PME-1. (A) Structure of the ABL alkyne probe ABL112. (B) ABL112 inactivates PME-1 (IC50 = 13.8 nM, 95% confidence limits 6.5–29 nM) in the MDA-MB-231 soluble proteome (1 mg/mL protein) as determined by gel-based competitive ABPP (see Fig. S5). (C) MDA-MB-231 cells were incubated with DMSO or ABL127 (100 nM, 30 min) followed by ABL112 (10–200 nM, 2 h). Soluble cell proteomes (0.5 mg/mL) were then subjected to a standard “click” reaction using RhN3 (30) and ABL112-labeled proteins were visualized by in-gel fluorescence scanning. PME-1 was the only protein labeled by ABL112 and competed by ABL127. ABL112 labeled an additional 80 kDa protein that was not competed by ABL127, suggesting it may be a target for ABL112, but not ABL127 (see Fig. S5). (D) Mouse brain soluble lysates (1 mg/mL) were incubated (30 min) with DMSO or ABL127 (100 nM). ABL112 (10 nM) was then added for an additional 30 minutes before analysis by click chemistry-based ABPP.

or DMSO for 30 min before adding ABL112 at a range of concentrations and incubating for another 2 h. Cells were then lysed and the soluble proteomes were subjected to a “click” reaction with an azide-Rh tag (RhN3), separated by SDS/PAGE, and ABL112-labeled proteins were visualized by in-gel fluorescence scanning (Fig. 5C). This analysis identified PME-1 as the only ABL112-labeled protein that could be competed by pretreatment with ABL127. One additional ABL112-reactive protein at approximately 80 kDa was also detected, but the labeling of this protein was not competed by ABL127 or ent-ABL127 (Fig. S5), indicating that it is exclusively a target of the clickable probe ABL112 but not ABL127. Consistent with this premise, competitive ABPP analysis identified an 80 kDa FP-Rh-labeled protein in MDA-MB-231 lysates that was sensitive to inhibition by ABL127, but not ABL112-labeled proteins were visualized by in-gel fluorescence scanning. PME-1 was the only protein labeled by ABL112 and competed by ABL127, suggesting it may be a target for ABL112, but not ABL127 (see Fig. S5). (D) Mouse brain soluble lysates (1 mg/mL) were incubated (30 min) with DMSO or ABL127 (100 nM). ABL112 (10 nM) was then added for an additional 30 minutes before analysis by click chemistry-based ABPP.
PME-1 is a component of this complex, where its interactions with the catalytic subunit of PP2A appear to be mediated, at least in part, by the PME-1 active site (12). It is thus possible that inhibition of PME-1 will affect PP2A complexes not only through altering methylation but also through disrupting physical interactions between PME-1 and the catalytic subunit of PP2A. Determining how such changes in PP2A complexes affect substrate interactions and the broader phosphoproteome should represent an exciting area of research. On this note, recent studies point to an important role for PME-1 in negatively regulating the tumor-suppressive function of PP2A in cancer cells (14). PME-1 inhibitors may thus have utility as anticancer agents. Finally, we speculate that the net effect of PME-1 inhibition on PP2A methylation state will be dictated by the expression levels of not only PME-1 (see Fig. 3g) but also LCMT1. Imbalances in the relative expression of these two enzymes may thus mark specific cellular states where PME-1 inhibitors will produce their most dramatic pharmacological effects.

There were several keys to the success of our probe development effort. First, screening for inhibitors of PME-1 benefited from the fluopel-ABPP technology, which circumvented the limited throughput of previously described substrate assays for this enzyme. Second, we were fortunate that the NIH compound library contained several members of the ABL class of small molecules. These chiral compounds, which represent an academic contribution to the NIH library, occupy an unusual portion of structural space that is poorly accessed by commercial compound collections. Although at the time of their original synthesis (23) it may not have been possible to predict whether these ABLs would show specific biological activity, their incorporation into the NIH library provided a forum for screening against many proteins and cellular targets, culminating in their identification as PME-1 inhibitors. We then used advanced chemoproteomic assays to confirm the remarkable selectivity displayed by ABLs for PME-1 across (and beyond) the serine hydrolase superfamily.

That the mechanism for PME-1 inhibition involves acylation of the enzyme’s conserved serine nucleophile (Fig. 3) suggests that exploration of a more structurally diverse set of ABLs might uncover inhibitors for other serine hydrolases. In this way, the chemical information gained from a single high-throughput screen may be leveraged to initiate probe development programs for additional enzyme targets.

Projecting forward, this research provides an example of how public small-molecule screening centers can serve as a portal for spawing academic collaborations between chemical biology and synthetic chemistry labs. By continuing to develop versatile high-throughput screens and combining them with a small-molecule library of expanding structural diversity conferred by advanced synthetic methodologies, academic biologists and chemists are well-positioned to collaboratively deliver pharmacological probes for a wide range of proteins and pathways in cell biology.

Materials and Methods

PME-1 Protein Expression and Purification. Human recombinant PME-1 was expressed in BL21(DE3) Escherichia coli and purified at approximately 5 mg/L as detailed in SI Materials and Methods.

PME-1 Fluopel-ABPP Assay. See SI Materials and Methods for details.

Competitive ABPP Assays in Proteomes. See SI Materials and Methods for details.

Synthesis of ABLs. See SI Materials and Methods for details.

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