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A Substrate-Free Activity-Based Protein Profiling Screen for the Discovery of Selective PREPL Inhibitors

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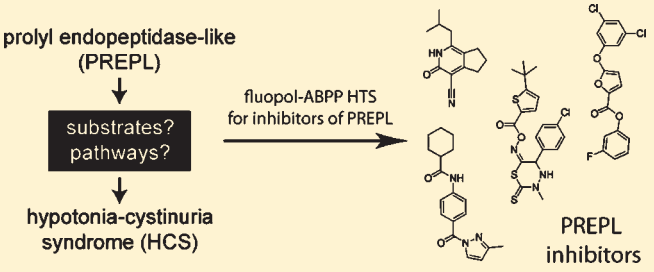
Supporting Information

ABSTRACT: Peptidases play vital roles in physiology through the biosynthesis, degradation, and regulation of peptides. Prolyl endopeptidase-like (PREPL) is a newly described member of the prolyl peptidase family, with significant homology to mammalian prolyl endopeptidase and the bacterial peptidase oligopeptidase B. The biochemistry and biology of PREPL are of fundamental interest due to this enzyme's homology to the biomedically important prolyl peptidases and its localization in the central nervous system. Furthermore, genetic studies of patients suffering from hypotonia-cystinuria syndrome (HCS) have revealed a deletion of a portion of the genome that includes the *PREPL* gene. HCS symptoms thought to be caused by lack of PREPL include neuromuscular and mild cognitive deficits. A number of complementary approaches, ranging from biochemistry to genetics, will be required to understand the biochemical, cellular, physiological, and pathological mechanisms regulated by PREPL. We are particularly interested in investigating physiological substrates and pathways controlled by PREPL. Here, we use a fluorescence polarization activity-based protein profiling (fluopol-ABPP) assay to discover selective small-molecule inhibitors of PREPL. Fluopol-ABPP is a substrate-free approach that is ideally suited for studying serine hydrolases for which no substrates are known, such as PREPL. After screening over 300 000 compounds using fluopol-ABPP, we employed a number of secondary assays to confirm assay hits and characterize a group of 3-oxo-1-phenyl-2,3,5,6,7,8-hexahydroisoquinoline-4-carbonitrile and 1-alkyl-3-oxo-3,5,6,7-tetrahydro-2*H*-cyclopenta[*c*]pyridine-4-carbonitrile PREPL inhibitors that are able to block PREPL activity in cells. Moreover, when administered to mice, 1-isobutyl-3-oxo-3,5,6,7-tetrahydro-2*H*-cyclopenta[*c*]pyridine-4-carbonitrile distributes to the brain, indicating that it may be useful for *in vivo* studies. The application of fluopol-ABPP has led to the first reported PREPL inhibitors, and these inhibitors will be of great value in studying the biochemistry of PREPL and in eventually understanding the link between PREPL and HCS.



INTRODUCTION

The prolyl peptidases are a family of biomedically relevant enzymes (Figure 1A) that cleave peptides on the C-terminal side of proline residues.[1−3] These enzymes participate in a variety of biological processes including peptide biosynthesis, catabolism, and bioactive peptide regulation. For example, prolyl endopeptidase (PEP) regulates the production of the anti-fibrotic peptide Ac-SDKP.[4−6] The prolyl peptidases have also been targeted in drug development. As an example, dipeptidyl peptidase 4 (DPP4) inhibitors form a mechanistically distinct class of anti-diabetic drugs.[2,7−9] These inhibitors slow the DPP4-mediated inactivation of the insulinotropic peptide glucagon-like peptide 1 (GLP-1),[10−12] resulting in increased insulin levels and improved physiological glucose tolerance.[2,3,13] These findings have fueled

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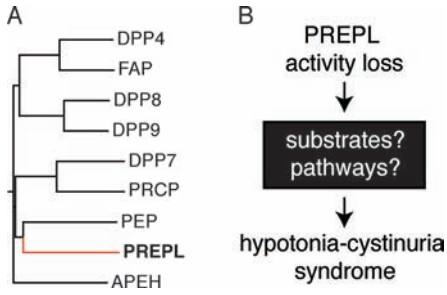

Figure 1. PREPL, a mammalian disease-associated peptidase. (A) Dendrogram of *Mus musculus* PREPL, PEP, DPP4, FAP, DPP7, DPP8, DPP9, prolyl carboxypeptidase (PRCP), and aminopeptide hydrolase (APEH). (B) Genetic studies of patients with hypotonia−cystinuria syndrome (HCS) revealed a deletion in the genome that includes PREPL. PREPL loss is thought to lead to hypotonia, but the substrates and biochemical pathways regulated by PREPL remain enigmatic.

interest in the biochemistry and physiological functions of the prolyl peptidases.

The most recently discovered member of this family is prolyl endopeptidase-like (PREPL).[14−19] Deletions in *PREPL* and the neighboring gene *SLC3A1* have been identified in patients suffering from hypotonia−cystinuria syndrome (HCS).[14,17,19] Since prior work has linked *SLC3A1* deletion to cystinuria,[20,21] the data suggest that the loss of PREPL is associated with the low muscle tone (hypotonia) observed in these patients (Figure 1B).[17] PREPL is primarily found within the nervous system,[22] specifically neurons,[23] which together with the hypotonia phenotype suggests that this peptidase might be involved in neuromuscular function. To date, however, no substrate for PREPL has been identified,[16,18] and PREPL has not been shown to cleave any PEP substrates. Our current lack of knowledge about the substrates and pathways regulated by PREPL prevents any insight into the mechanistic connection between PREPL and HCS, despite the strong genetic association.

With no specific PREPL inhibitors and no PREPL knockout mice available, we decided to screen for small-molecule PREPL inhibitors, which would provide a valuable tool for investigating the catalytic functions of this enzyme. The first step in discovering a small-molecule inhibitor for PREPL was the development of a high-quality assay of enzyme activity.[24] This can be particularly challenging for targets like PREPL that do not have any known substrates. As a member of the serine hydrolase superfamily, however, PREPL has a catalytic serine nucleophile that can be labeled in an activity-dependent manner by fluorophosphonate activity-based probes.[16,25] Recently, a platform has been introduced that uses this labeling reaction for high-throughput screening (HTS). This platform, referred to as fluorescence polarization activity-based protein profiling (fluopol-ABPP),[26,27] has already been used to identify novel inhibitors for several enzymes from multiple mechanistic classes, including RBBP9,[26] PME-1,[27] GSTO1,[26] and PAD4.[28] Here, we use fluopol-ABPP to discover selective PREPL inhibitors.

EXPERIMENTAL SECTION

Materials. Fluorophosphonate-rhodamine (FP-Rh)[29] and fluorophosphonate-polyethyleneglycol-rhodamine (FP-PEG-Rh)[30] were synthesized following previously described protocols. Polyclonal antibodies were generated by Open Biosystems in rabbits using a peptide epitope (EELGLDSTDAFEALKKYLKF) derived from murine PREPL.

Cloning, Expression, and Purification of PREPL. The *Mus musculus Prepl* (mPrepl) gene was PCR amplified from an Open Biosystems clone containing the full-length open reading frame (pCMV_mPrepl, Open Biosystems clone ID 3585402) using forward primer AAA AGG ATC CCA TGG ATG CAT TTG AAA AAG TGA G and reverse primer AAA AGG TAC CTC AGA ACT TTA GGT ATT TCT TCA GC. The resulting insert was then ligated into the pTrcHisB expression vector (Invitrogen) using the *Bam*HI and *Kpn*I restriction sites. The resulting vector, pTrcHisB_mPrepl, was amplified in Top10 cells, purified, and sequenced to confirm the correct coding sequence.

Expression was carried out in Rosetta 2(DE3)pLysS competent cells (EMD Biosciences), by growing a starter culture overnight, diluting 1:100 into fresh media the next morning, and inducing this culture with 1 mM IPTG at OD 0.5. After 12−15 h at 37 °C, cells were harvested and frozen. The pellets were resuspended in 20 mM Na_2HPO_4, 0.75 M NaCl, pH 7.4 (lysis buffer), with 1% Triton X-100 and lysed by sonication at 4 °C. The lysate was centrifuged at 5000*g* for 10 min, whereupon the supernatant was applied to a Ni^{2+}-charged IMAC Sepharose 6 Fast Flow resin (GE Healthcare). The resin was then washed with 20 mM Na_2HPO_4, 0.75 M NaCl, 30 mM imidazole, pH 7.4 (wash buffer), containing 1% Triton X-100. This was followed by a wash with wash buffer to which no Triton X-100 had been added. After these wash steps, PREPL was eluted from the solid support with 20 mM Na_2HPO_4, 0.75 M NaCl, 200 mM imidazole, pH 7.4 (elution buffer). The protein was concentrated and exchanged into PBS using Amicon Ultra-15 10 kDa molecular weight cutoff filters (Millipore). The PREPL concentration was determined by Bradford Assay and brought to 2 mg/mL in PBS and 10% glycerol. The protein was then frozen at −80 °C. The activity of each enzyme batch was assessed through a labeling reaction with FP-Rh activity-based probe (ABP). The labeled enzyme was run out on an SDS-PAGE gel and analyzed using a Typhoon flatbed scanner (GE Healthcare Life Sciences). The presence of a fluorescent band at approximately 75 kDa, which corresponds to the molecular weight of PREPL, demonstrated that the enzyme batch was active. The gels were then Coomassie stained to determine the overall purity of the sample (see Supporting Information Figure S1 for representative examples of ABPP and Coomassie gels).

To obtain a negative control for the screen, catalytically inactive PREPL(S470A) was prepared by site-directed mutagenesis from the pTrcHisB_mPrepl vector using a QuickChange site-directed mutagenesis kit (Stratagene). The following primers were used to introduce the mutation: CGC TGA GCG CTT TCG CTG CTG GAG GTG TGC TCG and CGA GCA CAC CTC CAG CAG CGA AAG CGC TCA GCG. The vector was sequenced to confirm the mutation. This construct, pTrcHisB_mPrepl(S470A), was used to express the inactive enzyme. PREPL(S470A) was purified in the same way as the wild-type (WT) enzyme and showed no activity by ABPP.

PREPL Fluopol-ABPP Assay and Screening. Using the fluopol-ABPP assay,[26] an initial screen was carried out against 18 000 compounds from the Maybridge Hitfinder Collection and a validation fraction from the National Institutes of Health (NIH) Molecular Libraries Small Molecule Repository. Prior to the start of the assay, 10 μL of assay buffer [0.01% pluronic acid (Invitrogen), 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM DTT (Invitrogen)] containing PREPL (33 nM, 30 nM final concentration) was dispensed into 384-well microtiter plates. Test compound (50 nL) in DMSO or DMSO alone (0.59% final concentration DMSO; 10 μM final compound concentration for the Maybridge library and 5 μM compound concentration for the NIH validation set) was added to the appropriate wells and incubated for 30 min at 25 °C. The assay was initiated by dispensing 1.0 μL of FP-PEG-Rh probe (750 nM, 75 nM final concentration) in assay buffer to all wells. The reactions were incubated for 15 min at room temperature and read on the Perkin-Elmer Envision with the optimized

BODIPY TMR FP dual emission label 2100-8070 consisting of the following filters and mirror modules: BODIPY TMR FP dial mirror module (2100-4080), BODIPY TMR FP excitation filter (2100-5050), BODIPY TMR FP emission filter S-pol (2100-5160), and BODIPY TMR FP emission filter P-pol (2100-5170). The percent inhibition for each compound was calculated as follows: percent inhibition = (test compound mP − median negative control mP)/(median positive control mP − median negative control mP) × 100. Test compound was defined as wells containing PREPL in the presence of test compound, negative controls were defined as wells containing PREPL and DMSO, and positive controls were defined as wells containing no PREPL protein. As an initial filter to remove nonselective serine hydrolase inhibitors, we selected compounds that inhibited PREPL >50% relative to control reactions and simultaneously showed <20% inhibition in previous fluopol-ABPP screens against serine hydrolases PME-1 and Cgi67.

MLPCN Screen. This initial screen was followed up by screening the 324 751-compound Molecular Libraries Probe Productions Centers Network (MLPCN) library in collaboration with the MLPCN at the Scripps Research Institute. Prior to the start of the assay, 4.0 μL of assay buffer [0.01% pluronic acid (Invitrogen), 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM DTT (Invitrogen)] containing 37.5 nM PREPL was dispensed into 1536-well microtiter plates. Next, 30 nL of test compound in DMSO or DMSO alone (0.59% final concentration DMSO; 5.9 μM compound) was added to the appropriate wells and incubated for 30 min at 25 °C. The assay was started by dispensing 1.0 μL of 375 nM FP-PEG-Rh probe in assay buffer to all wells. Plates were centrifuged, and after 15 min of incubation at 25 °C, fluorescence polarization was read on a Viewlux microplate reader (Perkin-Elmer) using a BODIPY TMR FP filter set and a BODIPY dichroic mirror (excitation = 525 nm, emission = 598 nm). Fluorescence polarization was read for 15 s for each polarization plane (parallel and perpendicular). The well fluorescence polarization value (mP) was obtained via the Perkin-Elmer Viewlux software. The percent inhibition for each compound was calculated as described above. A mathematical algorithm was used to determine nominally inhibiting compounds in the primary screen. Two values were calculated: (i) the average percent inhibition of all compounds tested and (ii) 3 times their standard deviation. The sum of these two values was used as a cutoff parameter; i.e., any compound that exhibited greater inhibition than the cutoff parameter was declared active. The reported PubChem Activity Score (http://pubchem.ncbi.nlm.nih.gov/) has been normalized to 100% observed primary inhibition. Negative percent inhibition values are reported as activity score zero. The activity score range for active compounds is 100−11, and that for inactive is 11−0.

Active compounds in the primary screen (AID 2751, PubChem's BioAssay identifier) were followed up in a confirmation screen performed in triplicate (AID 2803). Active compounds from this confirmation screen and from the initial 18 000-compound screen were carried forward through a series of secondary assays to establish their ability to inhibit PREPL and their feasibility for use in cells and tissues.

Single-Concentration ABPP Assays as a Secondary Screen To Confirm PREPL Inhibition. PREPL (17 μL, 117 nM, final conentration 100 nM) in assay buffer [PBS with 0.012% Pluronic (Invitrogen)] was preincubated with inhibitor (1 μL, 400 μM, final concentration 20 μM) for 15 min at room temperature. FP-Rh (2 μL, 1 μM, final concentration 100 nM) was then added and allowed to react for 7 min, whereupon the reaction was quenched by the addition of 10 μL SDS-PAGE loading buffer. The samples were separated by SDS-PAGE and visualized by in-gel fluorescence scanning. The percentage activity remaining was determined by measuring the integrated optical intensity of the bands using ImageQuant software. A reduction of the band intensity by more than 20% was taken to indicate that the compound was capable of inhibiting PREPL, confirming the result from the high-throughput screen.

Competitive ABPP Assays in Complex Proteomes. For *in vitro* experiments, brain proteomes were diluted to 1 mg/mL in PBS (pH 7.4) and spiked with 40 nM final concentration of recombinant PREPL. Pluronic was added to a final concentration of 0.01%. These proteomes were incubated with DMSO or 50 μM inhibitor (final concentration) for 30 min at room temperature. FP-Rh was then added at a final concentration of 0.5 μM. After 15 min, the reactions were quenched with SDS-PAGE loading buffer, separated by SDS-PAGE (10% acrylamide), and visualized in-gel with a Typhoon flatbed fluorescence scanner (GE Healthcare Life Sciences). Bands were quantified to assess the extent of labeling and determine whether or not a compound was able to inhibit PREPL in a complex proteome.

Determination of IC_{50} Values. For determination of *in vitro* IC_{50} values, compounds were preincubated with PREPL (100 nM final concentration in PBS, pH 7.4, with 0.01% final concentration of Pluronic) at the indicated concentrations for 15 min at room temperature. The reactions were performed in triplicate. The samples were then labeled with FP-Rh (100 nM final concentration) for 7 min, quenched, separated by SDS-PAGE, and visualized by in-gel fluorescence scanning. The percentage activity remaining was determined by measuring the integrated optical intensity of the bands using ImageQuant software. IC_{50} values were determined from a dose−response curve generated with Prism (GraphPad Software) using the following equation: $Y = \text{bottom} + (\text{top} - \text{bottom})/(1 + 10^{(X - \log IC_{50})})$.

PEP Selectivity Assay. PEP (0.70 μg/mL) was preincubated with 20 μM inhibitor for 15 min in PEP assay buffer (25 mM Na_2HPO_4, 0.5 mM EDTA, 2 mM DTT). Z-Gly-Pro-AMC (Bachem) in PEP assay buffer was then added to a final concentration of 133 μM. The cleavage of this fluorogenic substrate was monitored on a Spectramax Gemini XS fluorescence plate reader (Molecular Devices), with excitation at 360 nm and emission at 460 nm.

***In Situ* ABPP Assays To Determine Cellular Activity of PREPL Inhibitors.** Neuro2A cells were plated at 1.5×10^6 cells per well in six-well plates. The next day, cells were transfected with pCMV_mPrepL (Open Biosystems Clone ID 3585402) using Lipofectamine 2000 transfection reagent (Invitrogen). Two days post-transfection, inhibition and labeling were carried out in the intact cells before harvesting. First, the medium covering the cells was removed and replaced with medium containing 50 μM inhibitors. Cells were incubated in this medium for 1.5 h. A cell-permeable FP-alkyne probe was then added to the medium to a final concentration of 10 μM and let incubate for 1 h. Cells were washed three times with PBS and harvested with a cell scraper. The cell pellet was washed three times (by suspending in 1 mL of PBS, centrifuging at 1400*g* for 3 min, and then aspirating off PBS). After the final wash, the cell pellet was resuspended in 80 μL of PBS. Cells were lysed by seven freeze−thaw cycles and centrifuged at 4 °C for 30 min (20000*g*). The supernatant was transferred to a new tube and the protein concentration obtained by Bradford assay. The samples were diluted to 4 mg/mL with PBS. To 43 μL of 4 mg/mL protein sample were added 2 μL of 1.25 mM rhodamine-azide, TCEP (1 μL, 50 mM), ligand (3 μL, 1.67 mM), and $CuSO_4$ (1 μL, 50 mM).[31,32] This reaction was allowed to proceed for 1 h at room temperature and then quenched with SDS-PAGE loading buffer. Samples were separated by SDS-PAGE and visualized by in-gel fluorescence scanning.

Animal Studies. The C57BL/6J mice used in this study were either purchased (Jackson Labs, Bar Harbor, ME) or taken from a breeding colony. Animals were kept on a 12-h light, 12-h dark schedule and fed *ad libitum*. For brain collection, animals were first euthanized with CO_2, followed by rapid isolation of the brain. The tissue was flash frozen in liquid N_2 and stored at −80 °C. All animal care and use procedures were in strict accordance with the standing committee on the use of animals in research and teaching at Harvard University and the National

Institutes of Health guidelines for the humane treatment of laboratory animals.

***In Vivo* Distribution of PREPL inhibitors by LC-MS/MS.** 1-Isobutyl-3-oxo-2,3,5,6,7,8-hexahydroisoquinoline-4-carbonitrile (**8**, 20 μg) was delivered to a mouse (20 g) via intraperitoneal injection (200 μL of 95:5 PBS/DMSO) for a final dose of 1 mg/kg. After 30 min, the mouse was sacrificed and the brain was rapidly isolated and flash frozen in liquid nitrogen. The sample was subsequently Dounce homogenized in chloroform/methanol/water (2:1:1), and this homogenate was centrifuged to separate organic and aqueous layers.[33] The organic layer was isolated and concentrated under a stream of nitrogen. The residue was then dissolved in chloroform for liquid chromatography−tandem mass spectrometry (LC-MS/MS) analysis. Detection of inhibitor **8** was performed using an Agilent 6410 Triple Quad instrument with an electrospray ionization source operating in positive ionization mode. MS analysis was performed in multiple reaction monitoring (MRM) mode, which could specifically detect inhibitor **8** in biological matrices. The capillary voltage was set to 4 kV, and the fragmentor voltage was 161 V. A drying gas temperature of 350 °C was used, with a flow rate of 8 L/min and a nebulizer pressure of 35 psi. Data were collected for two MRM transitions from the m/z 217.1 precursor ion, m/z 173.1 and m/z 155.1, with collision energies set to 36 and 45 V, respectively. Liquid chromatography was performed using a Gemini C18 5-μm, 110-Å, 50 × 4.60 mm column (Phenomenex). Mobile phases A and B were composed of GC-grade water and methanol (Burdick & Jackson), respectively, and both phases contained 0.1% formic acid and 5 mM ammonium formate. The mobile phase composition was held at 5% B with a flow rate of 0.1 mL/min for 5 min, increased linearly from 5% to 100% B over 40 min, during which the flow rate was 0.4 mL/min, held at 100% B for 8 min at 0.5 mL/min, and then held at 5% B for 7 min with a flow rate of 0.5 mL/min.

RESULTS AND DISCUSSION

Expression and Purification of PREPL for High-Throughput Screening. Murine *Prepl* (amino acids 1−638) was cloned into the pTrcHisB expression vector and expressed in Rosetta 2(DE3) pLysS competent cells. We found that Rosetta cells,[34] which are codon optimized for mammalian proteins, were required for adequate protein expression. Recombinant PREPL was purified using standard immobilized metal affinity chromatography methods and analyzed by SDS-PAGE, followed by Coomassie staining, to assess the purity of the protein, which was typically >90% (Supporting Information Figure S1). FP-Rh labeling of recombinant PREPL was used to test the activity of the enzyme (Supporting Information Figure S1). Together, these data enabled us to conclude that the PREPL was folded and active, permitting the optimization of the fluopol-ABPP assay necessary for screening.

To optimize the fluopol-ABPP assay properly, it was necessary to generate a catalytically inactive mutant of PREPL. To accomplish this, we replaced the PREPL active-site serine at position 470[14,16] with an alanine by site-directed mutagenesis. PREPL(S470A) was expressed and purified in the same manner as the WT protein. This protein did not, however, react with FP-Rh,

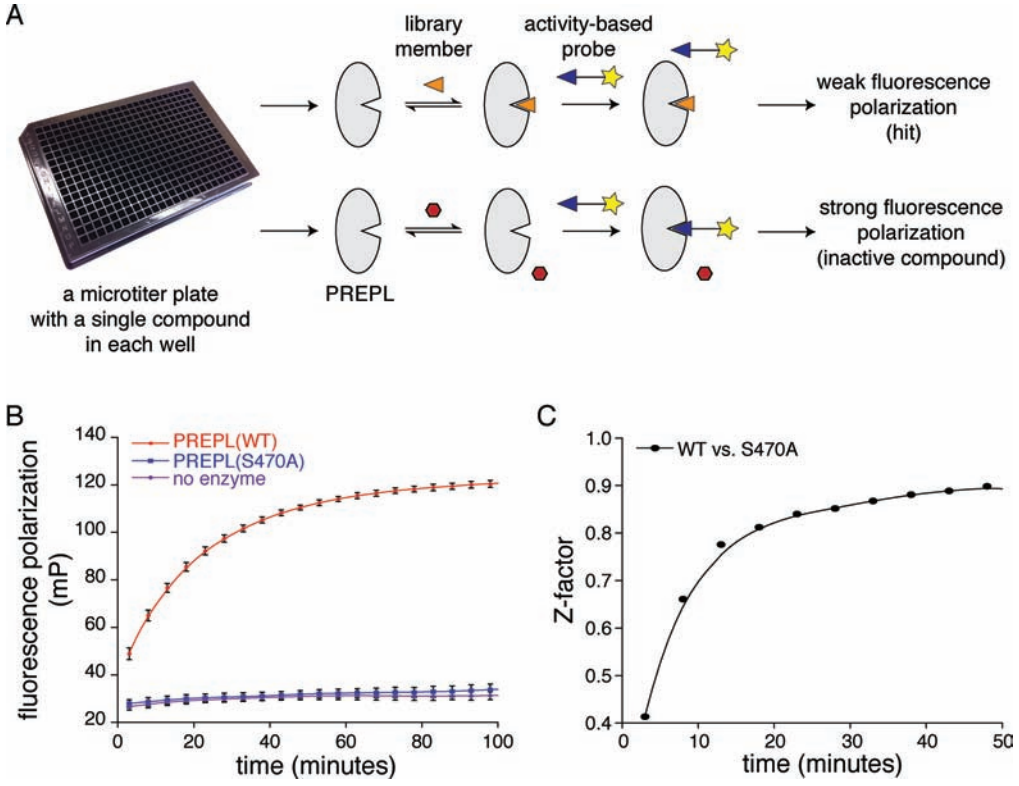

Figure 2. Fluopol-ABPP assay for the discovery of selective PREPL inhibitors. (A) Recombinantly expressed and purified PREPL is added, along with a single compound, to individual wells in a microtiter plate. The FP-PEG-Rh ABP, which covalently labels serine hydrolases like PREPL, is then added to each sample. The presence of an inhibitor reduces PREPL labeling by FP-PEG-Rh, leading to a weaker fluorescence polarization signal (top). Inactive compounds will not reduce enzyme labeling with FP-PEG-Rh, which results in a much stronger fluorescence polarization signal. The assay can be used to identify active compounds during a high-throughput screen. (B) Fluorescence polarization signal (mP) of PREPL(WT), PREPL(S470A), and no enzyme control with respect to time. (C) Calculation of the *Z*-factor by comparing PREPL(WT) and PREPL(S470A) fluorescence polarization signals at different time points.

indicating that it was no longer catalytically active. Together, PREPL and PREPL(S470A) provided us with the necessary reagents for assay development and screening.

Optimization of the Fluopol-ABPP Assay. The fluopol-ABPP assay[26,27] takes advantage of the fluorophosphonate labeling of serine hydrolases, in this case a candidate serine peptidase, to enable the substrate-free identification of enzyme inhibitors. In this assay, a purified enzyme and a compound are dispensed into the wells of a 384- or 1536-well microtiter plate. The assay is then initiated by the introduction of a fluorescent ABP, typically FP-Rh,[29] into each well. In the presence of inactive compounds, the enzyme is labeled with FP-Rh, resulting in a large increase in the fluorescence polarization signal, since the tumbling rate of a protein-bound fluorescent ABP is lower than that of an unbound ABP. An inhibitor, however, will block the interaction between the ABP and the enzyme, leading to a lower fluorescence polarization signal and the identification of a "hit" compound (Figure 2A). In this way, the fluopol-ABPP assay can be used to screen for inhibitors of probe-sensitive targets in the absence of a known natural substrate, which was essential in the case of PREPL.

In order to detect reversible as well as irreversible inhibitors of PREPL in the fluopol-ABPP screen, it was necessary to identify a kinetically relevant time point for this particular enzyme. In a competitive labeling reaction, an irreversible inhibitor such as FP-Rh will always outcompete a reversible inhibitor given enough time. Therefore, it is crucial to pick conditions (time point, enzyme concentration, labeling reagent) where the enzyme is incompletely labeled and the effect of a reversible inhibitor can be detected.[26,35] In addition, the *Z*-factor at the selected time point should be suitable for HTS (typically >0.5).[36]

The conditions for the PREPL fluopol-ABPP assay were established by testing various parameters. For example, several different concentrations of enzyme (5, 1, and 0.5 μM) were incubated with 75 nM FP-Rh, and labeling was assessed at different time points. In all cases, however, complete labeling was observed already by 5 min (Supporting Information Figure S2). To slow down the reaction, we used a different probe, FP-PEG-Rh, where a PEG linker connects the fluorophosphonate reactive group with the rhodamine dye (Supporting Information Figure S3). This probe modification often leads to slower reactions with serine hydrolases.[30] With the FP-PEG-Rh probe, a time-dependent increase in fluorescence polarization signal was observed as the probe reacted with PREPL. Importantly, wells containing catalytically inactive PREPL(S470A) mutant, where

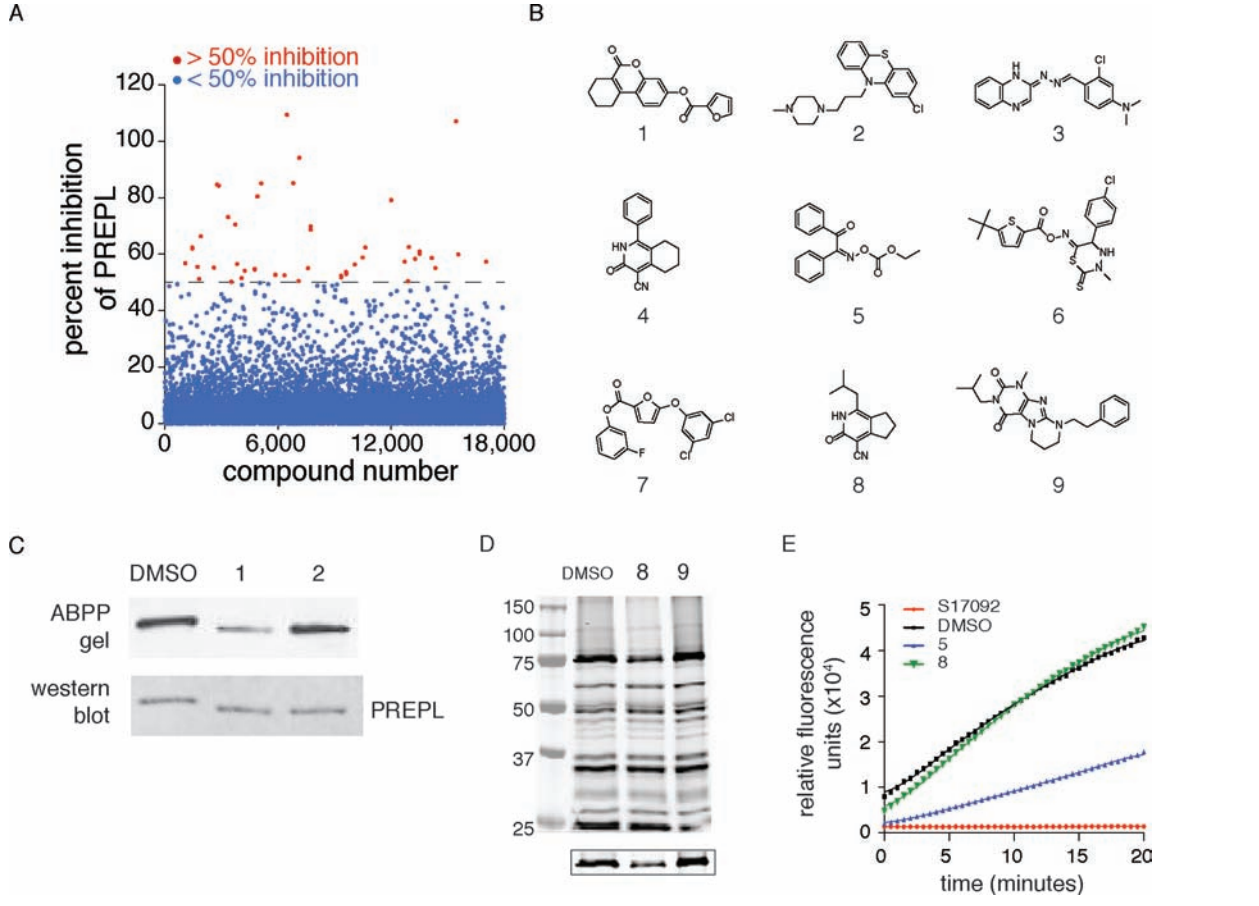

Figure 3. Identification of selective PREPL inhibitors. (A) Screening 18 000 compounds using the fluopol-ABPP assay identified 39 candidate inhibitors with >50% PREPL inhibition and <20% inhibition in two other serine hydrolase fluopol-ABPP screens (CGI67 and PME-1). In the MLPCN screen, 725 such inhibitors were identified (this number was narrowed down to 556 by removing ester-containing compounds). (B) Some representative hits from the fluopol-ABPP screen. (C) Gel-based ABPP assays were then used to confirm PREPL inhibition by some of these compounds. (D) A gel-based competitive ABPP with PREPL-spiked mouse brain lysates. Inhibitors are assessed for their ability to selectively inhibit PREPL in a complex proteome. The PREPL band is located at 75 kDa and shown in more detail below the main gel. (E) Candidate inhibitors were also screened for cross-reactivity against the highly related peptidase PEP. Examples of cross-reactive (**5**) and non-cross-reactive (**8**) compounds are shown. The control for 100% PEP inhibition, S17092, is a specific PEP inhibitor.

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the active-site serine had been replaced with an alanine, or wells without enzyme showed no increase in fluorescence polarization upon addition of the FP-PEG-Rh probe (Figure 2B). For HTS analysis, we selected the kinetically relevant time point at 15 min (Figure 2B), where PREPL generated a high *Z*-factor (0.83) relative to control reactions (Figure 2C).

Screening for Inhibitors of PREPL. Prior to embarking on an HTS screen of a large (>100 000) compound library, we first wanted to demonstrate that our screening strategy could discover *bona fide* PREPL inhibitors. Therefore, we performed an initial screen against a pilot library containing 18 000 compounds. A total of 39 compounds were found that inhibited fluorescence polarization in the PREPL screen by at least 50% and simultaneously did not inhibit fluorescence polarization by more than 20% in two previously performed screens against the Maybridge Library, FAM108B (CGI67) and protein phosphatase methylesterase 1 (PME-1)[27] (Figure 3A and Supporting Information Table S1; http://pubchem.ncbi.nlm.nih.gov/). Comparison of candidate PREPL inhibitors to hits in these other screens is intended to filter out promiscuous serine hydrolase inhibitors that are active against several enzymes.

In order to identify additional scaffolds that could serve as selective PREPL inhibitors, we proceeded to screen PREPL against the MLPCN library, which contained 324 751 compounds at the time of the screen. The screen was performed essentially as described for the initial 18 000-compound library screen, with some minor modifications (see Experimental Section). This fluopol-ABPP screen (AID 2751) initially identified 2221 compounds that were active against PREPL. Retesting these compounds in triplicate against PREPL (AID 2803) removed a number of false positives and resulted in a total of 1333 hits.

To filter out compounds that were clearly not selective, the list was then narrowed down to compounds which inhibited PREPL by greater than 50% and simultaneously did not show greater than 20% inhibition in previous fluopol-ABPP screens against three other serine hydrolases (RBBP9, AID 1515; PME-1, AID 2130; LYPLA2, AID 2177) and a thiol-reactive glutathione S-transferase (GSTO1, AID 1974). This step removed an additional 608 molecules to afford 725 compounds. Finally, the list was narrowed down to 556 inhibitors by removing compounds containing esters, which we suspected would be unstable in cells or tissues (Supporting Information Table S2).

Secondary Assays To Validate the Substrate Confirmation Workflow. Of the 595 compounds identified in the 18 000- and 324 751-compound screens, 590 were commercially available and purchased. A number of hits were subsequently confirmed using a competitive gel-based ABPP approach against purified, recombinant PREPL (Figure 3C).[35] In this assay, recombinant PREPL was incubated with inhibitors at 20 μM or vehicle for 15 min and then treated with FP-Rh for 7 min prior to quenching with SDS-PAGE loading buffer. The samples were analyzed by SDS-PAGE, and the activity of each compound was determined on the basis of the intensity of the PREPL band. A few inhibitors were also tested against the poorer PREPL substrate 4-methylumbelliferyl-*p*-guanidinobenzoate (MUGB) and were active in this assay, demonstrating assay independence. In particular, compound **8** inhibited breakdown of MUGB by PREPL, indicating that this inhibitor binds to and specifically inhibits PREPL (Supporting Information Figure S4).

Identification of Selective PREPL Inhibitors. These candidate inhibitors were then assayed for ability to inhibit PREPL in a complex proteome using gel-based ABPP with FP-Rh (Figure 3D).

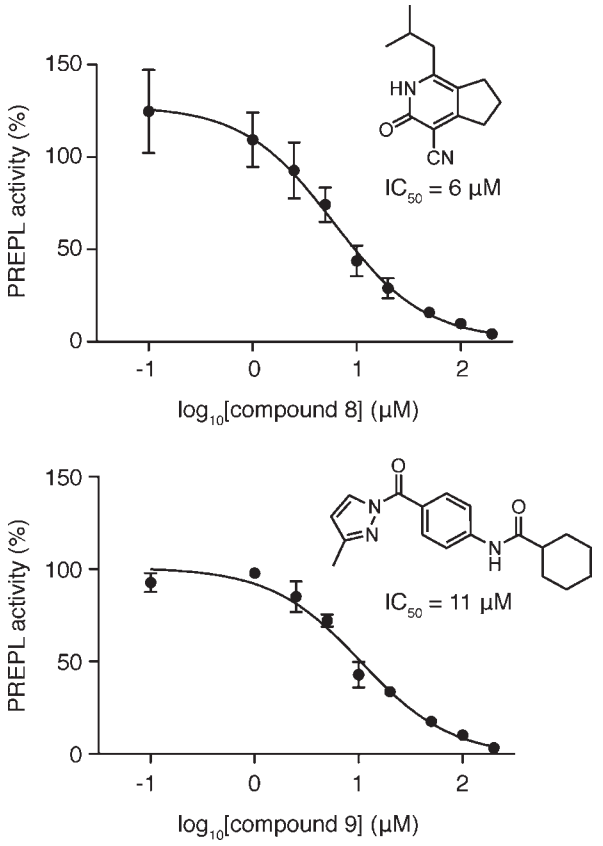

Figure 4. IC_{50} data for two of the representative inhibitors identified from the MLPCN screen that were selective for PREPL (over PEP) and were able to inhibit PREPL in a complex proteome (brain). The IC_{50} values of these compounds were determined using a gel-based ABPP approach and calculated using the Prism software. The 95% confidence interval for compound **8** is 3−12 μM, and for compound **9** is 7−16 μM.

By visualizing the labeling of all brain serine hydrolases simultaneously, these assays also enabled us to assess the selectivity of candidate inhibitors for PREPL over many mechanistically related enzymes. Selective inhibitors should specifically reduce the intensity of the PREPL band with no effect on the signal intensity of any of the other labeling events in the proteome. There were no promiscuous inhibitors identified in this assay; however, several of the candidate inhibitors did not inhibit PREPL in the whole proteome and were excluded from future studies.

As further confirmation of selectivity, candidate inhibitors were also directly tested against the PREPL homologue PEP (Figure 1A) using a well-established fluorogenic PEP substrate assay with Z-Gly-Pro-AMC (Figure 3E).[37] We considered inhibitors exhibiting >20% reduction in PEP activity as cross-reactive and discarded these compounds from future studies. The most promising inhibitors remaining after these selectivity assays were then tested at different concentrations against FP-Rh to determine their IC_{50} values, which were typically in the low micromolar range (Figure 4). Together, these assays identified 12 selective PREPL inhibitors (compounds **4**, **6**−**8**, **10**−**18**) (Figure 3B and Supporting Information Table S3).

Cell-Based ABPP Assays. Having found inhibitors selective for PREPL *in vitro*, we wanted to explore the activity of these inhibitors in cells. To be able to detect reversible as well as

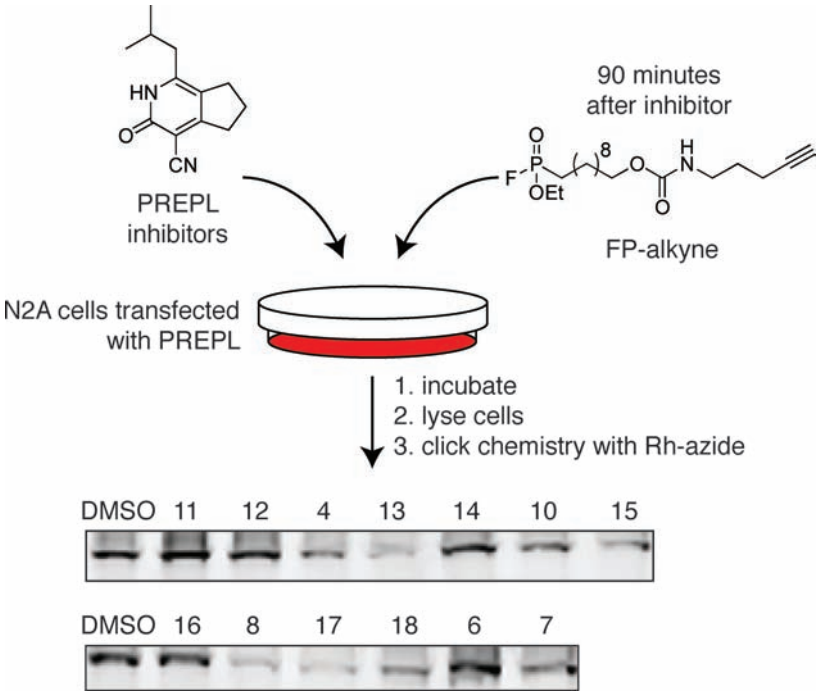

Figure 5. Inhibition of PREPL in cells was determined with a competitive ABPP assay using the cell-permeable FP-alkyne ABP. Inhibitors were added to the media of PREPL-transfected Neuro2A cells at 50 μM final concentration, followed by the addition of the FP-alkyne probe. Inhibitors that are active *in situ* will reduce labeling by the activity-based probe, resulting in a lighter band on the gel, as seen for compounds **8** and **17**, for example.

irreversible inhibitors in this assay, we used a click chemistry-based approach where PREPL-transfected Neuro2A cells were first incubated with inhibitor, followed by addition of a cell-permeable FP-alkyne probe to the media.[31,32,38] After the cells were washed and harvested, the labeled proteome was reacted with rhodamine−azide (Rh−N_3) and separated by SDS-PAGE gel. The extent of inhibition was determined from the reduction in fluorescence of the PREPL band in samples containing inhibitor relative to vehicle-treated samples (Figure 5 and Supporting Information Figure S5). All of the carbonitrile compounds (**4**, **8**, **13**, **17**, and **18**) were able to cross the cell membrane and inhibit PREPL intracellularly, while the other inhibitors did not display significant inhibition of PREPL in situ. Compounds that were inactive in this assay either were unable to enter cells or may have been unstable (i.e., prone to hydrolysis) in intact cells (Supporting Information Figure S5). Together with the prior selectivity assays, these experiments revealed that inhibitors **4**, **8**, **13**, **17**, and **18** are all excellent tools for studying PREPL activity in cells.

Central Nervous System Delivery of 1-Isobutyl-3-oxo-3,5,6,7-tetrahydro-2*H*-cyclopenta[*c*]pyridine-4-carbonitrile (8). We selected 1-isobutyl-3-oxo-3,5,6,7-tetrahydro-2*H*-cyclopenta[*c*]pyridine-4-carbonitrile (**8**), the most potent carbonitrile inhibitor in the brain proteome ABPP assay, and tested whether this compound could enter the brains of mice.[39] Thirty minutes after an intraperitoneal injection of inhibitor **8**, the mouse was sacrificed and the brain collected and frozen. The sample was subsequently extracted and analyzed to determine whether compound **8** was present in the brain. The analysis was performed using the highly selective MRM detection method on a triple-quadrupole LC-MS system,[40] which relied on characteristic transitions that occurred during the fragmentation of inhibitor **8** (Supporting Information Figure S6). We saw a strong

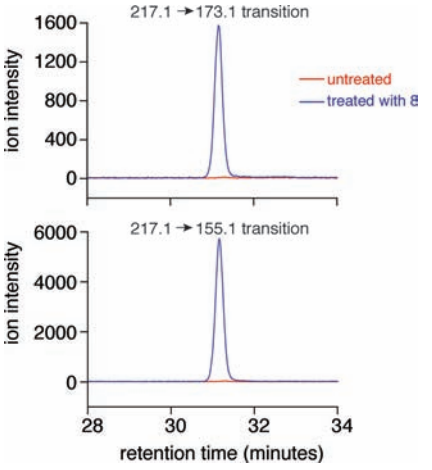

Figure 6. Compound **8** is able to enter the brain. Brain extracts from mice treated with **8** and untreated mice were analyzed on a triple-quadrupole mass spectrometer in the multiple reaction monitoring mode. Two characteristic transitions for compound **8** (m/z 217.1-to- 173.1 and m/z 217.1-to-155.1) were monitored, and chromatograms are shown for these transitions for mice treated with **8** and untreated mice.

signal for compound **8** in the inhibitor-treated mice, while there was no signal in the untreated control, as expected (Figure 6 and Supporting Information Figure S7). Since inhibitor **8** had already been shown to selectively inhibit PREPL in brain proteomes, these data suggest that we may be able to use this inhibitor to study PREPL *in vivo*. Physiological experiments will require the optimization of the dosing, but the discovery of a selective and bioavailable inhibitor is a promising first step.

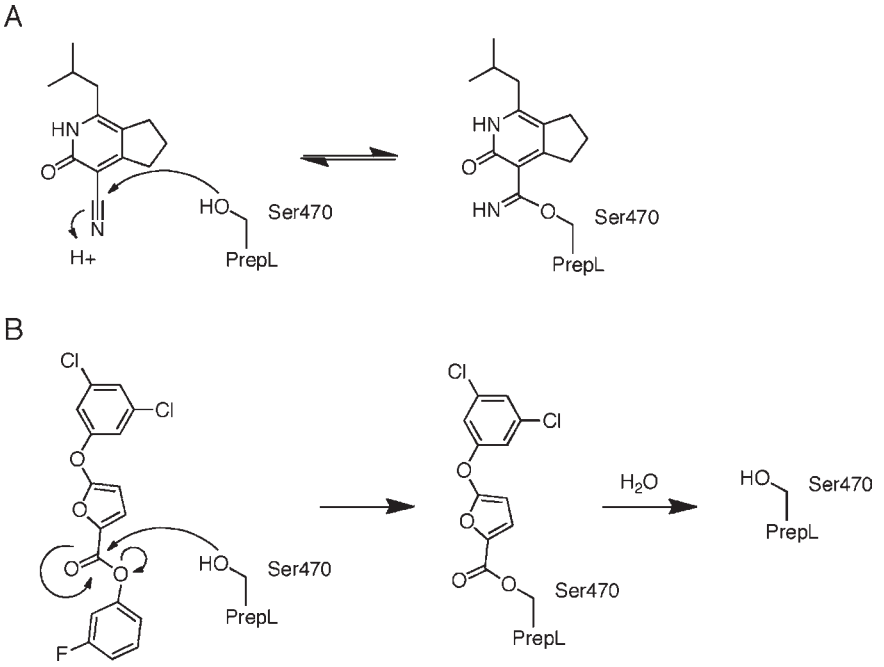

Figure 7. Potential mechanisms of PREPL inhibition by the different inhibitor chemotypes. (A) We suspect that, in analogy to known DPP4 inhibitors,[9,41] the carbonitrile inhibitors are functioning through the reversible attack of Ser470 of PREPL to form an imidate adduct. (B) Similarly, the carbonyl groups (ester and oxime esters) are electrophilic and can react with PREPL to form an acyl-enzyme intermediate; they are then likely removed by hydrolysis of the acyl-enzyme intermediate to regenerate PREPL.

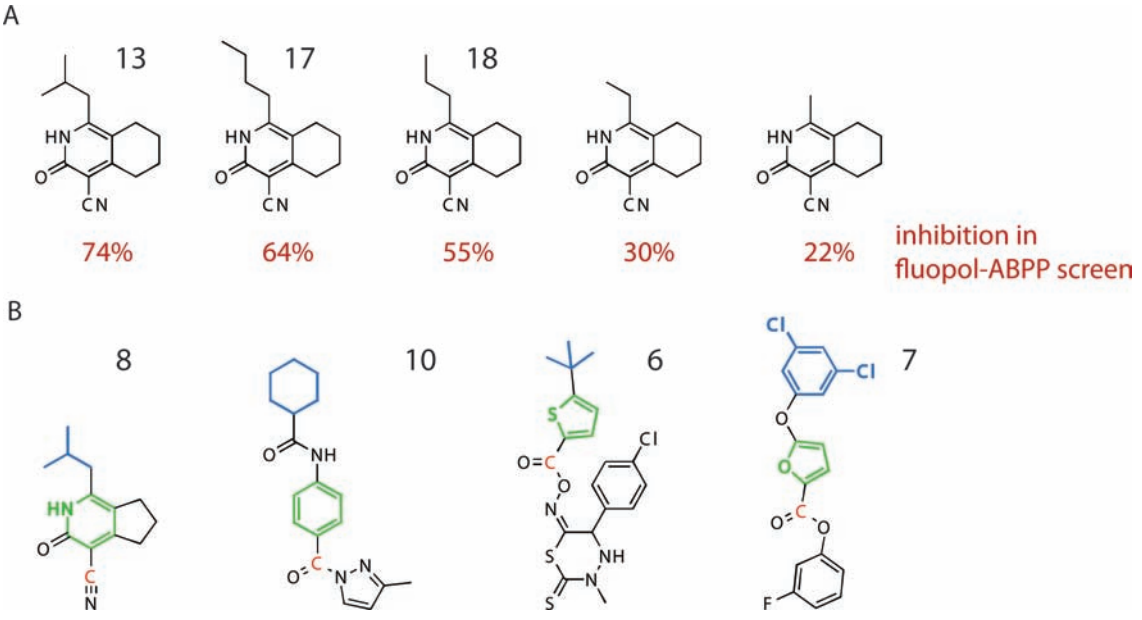

Figure 8. Analysis of the common structural features found among different inhibitor classes. (A) Comparison of the percent inhibition of different carbonitrile derivatives in the original fluopol-ABPP screen demonstrates that increased hydrophobicity opposite from the carbonitrile group on the aromatic ring leads to improved inhibition. (B) Comparison of the different inhibitor classes indicates that the three key elements in PREPL inhibitors identified in this study are an electrophilic carbonyl or carbonitrile (red), aromatic ring (green), and a hydrophobic group (blue).

Proposed Mechanism of Inhibition for Identified PREPL Inhibitors. The fluopol-ABPP screen and subsequent selectivity experiments resulted in a set of selective PREPL inhibitors (Supporting Information Tables S3 and S4). Mechanistically, some of these compounds may be inhibiting PREPL by reacting with or binding to the nucleophilic serine (Ser470) in the PREPL active site (Figure 7). Carbonitriles have emerged as a potent chemotype in the design of DPP4 inhibitors.[41−43] For example, saxagliptin (BMS-477118), a FDA-approved DPP4 inhibitor, contains a carbonitrile.[41] Structural studies of DPP4 with

carbonitrile-containing compounds demonstrate that the carbonitrile is electrophilic and reacts with the serine nucleophile to form a covalent imidate adduct.[43] We propose that the carbonitrile compounds identified in our screen as PREPL inhibitors likely operate through an analogous mechanism. In this model, Ser470 attacks the carbonitrile to form a covalent imidate adduct (Figure 7A). Importantly, crystallographic studies have shown that such covalent imidate adducts can be removed from the active site by soaking in a noncovalent binder, which demonstrates that these carbonitriles are covalent-reversible inhibitors.[43]

In the second group of inhibitors we identified, the compounds possess electrophilic acyl groups that could react with Ser470 to form acyl-enzyme intermediates. Two of the compounds from the initial screen are activated esters, a 3-fluorophenol ester (**7**) and an oxime ester (**6**). The oxime ester functional group has recently been shown to be a covalent inhibitor of serine hydrolases, and the acyl-enzyme intermediate has been confirmed by mass spectrometry.[44] In our case, compounds **6** and **7** likely interact with PREPL through the nucleophilic addition of Ser470 to the electrophilic carbonyl of these inhibitors to form an acyl-enzyme intermediate (Figure 7B). This group can then be removed through subsequent hydrolysis of the acyl-enzyme intermediate with water (Figure 7B).[45] It is also likely that the acyl pyrazole group in compound **9** is electrophilic enough to react with an activated serine nucleophile. Studies with organic compounds have demonstrated that alcohols and amines are able to hydrolyze acyl pyrazoles,[46−48] but no studies with macromolecules, such as serine hydrolases, have to our knowledge been reported. While the available evidence supports the inhibition mechanisms proposed above, these will need to be verified through additional mechanistic and structural studies.

Comparison of Inhibitors To Identify Structural Elements Necessary for PREPL Inhibition. At first glance, the structures of the PREPL inhibitors seemed remarkably different (Figures 3B and 8). To gain insight into features that may favor PREPL inhibition, we analyzed the relative activities of the carbonitrile compounds in the MLPCN screen. We compared the percent inhibition of carbonitriles that differed by a single R group in the 1 position (Figure 8A). The data for methyl (22%), ethyl (30%), propyl (55%), and isopropyl (74%) groups suggest that PREPL inhibition is correlated with increased hydrophobicity (and branching) at the 1 position. On the basis of these data, we propose a model for the key elements necessary for PREPL inhibition (Figure 8B). In our model, the structural features shared by PREPL inhibitors include an electrophilic carbonyl or carbonitrile (red), an aromatic ring (green), and a bulky hydrophobic group (blue). These hypotheses will need to be confirmed by targeted modification of PREPL at the critical sites in future structure−activity relationship studies.

CONCLUSIONS

This study identified the first set of PREPL inhibitors, and these compounds will find immediate use as chemical probes to investigate the biochemistry and biology of this enzyme. In addition, this study highlights the distinct advantage of the fluopol-ABPP methodology, which enabled the identification of chemical inhibitors of PREPL using a substrate-free format. The ability to screen for inhibitors without needing substrates is of paramount importance when studying novel and uncharacterized enzymes, such as in the case of the mammalian peptidase PREPL. This is the first reported use of the fluopol-ABPP assay to identify peptidase inhibitors. We anticipate that these compounds will be useful in structural studies of PREPL by helping to define key elements of the PREPL active site. Furthermore, we specifically plan to use these inhibitors to study the biochemical function of PREPL by coupling inhibition of this enzyme to mass spectrometry approaches for substrate discovery that we have developed.[6,33,49,50] In doing so, these inhibitors will prove to be useful chemical probes in further exploring the biochemistry of this uncharacterized peptidase and might eventually help reveal the connection between PREPL and HCS.

ASSOCIATED CONTENT

Supporting Information. Experimental results for PREPL expression and purification; comparison of labeling kinetics for FP-Rh and FP-PEG-Rh; structures of these probes; full gels for the *in situ* inhibition experiment; mass spectrometry data for characterization and quantitation of **8** and inhibition with **8** using an alternative assay; full lists of hits from the two screens; further data on the activities of the carbonitrile compounds; complete list of inhibitors described in this study; complete refs 7, 8, 41, and 43. This information is available free of charge via the Internet at http://pubs.acs.org.

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