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Competitive Activity-Based Protein Profiling Identifies Aza-β-Lactams as a Versatile Chemotype for Serine Hydrolase Inhibition

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Abstract

Serine hydrolases are one of the largest and most diverse enzyme classes in Nature. Most serine hydrolases lack selective inhibitors, which are needed for assigning functions to these enzymes. We recently discovered a set of aza-β-lactams (ABLs) that act as potent and selective inhibitors of the mammalian serine hydrolase protein-phosphatase methylesterase-1 (PME-1). The ABLs inactivate PME-1 by covalent acylation of the enzyme's serine nucleophile, suggesting that they could offer a general scaffold for serine hydrolase inhibitor discovery. Here, we have tested this hypothesis by screening ABLs more broadly against cell and tissue proteomes by competitive activity-based protein profiling (ABPP), leading to the discovery of lead inhibitors for several serine hydrolases, including the uncharacterized enzyme alpha, beta-hydrolase-10 (ABHD10). ABPP-guided medicinal chemistry yielded a compound ABL303 that potently (IC$_{50}$ value ~ 30 nM) and selectively inactivated ABHD10 in vitro and in living cells. A comparison of optimized inhibitors for PME-1 and ABHD10 indicates that modest structural changes that alter steric bulk can tailor the ABL to selectively react with distinct, sequence-unrelated serine hydrolases. Our findings, taken together, designate the ABL as a versatile reactive group for creating first-in-class serine hydrolase inhibitors.

Serine hydrolases constitute ~1% of all proteins encoded by mammalian genomes and play important roles in a wide range of (patho)physiologic processes.[1,2] Many serine hydrolases, however, remain unannotated with respect to their natural substrates and functions.[1,2] Selective chemical inhibitors have served as valuable probes for the functional annotation of serine hydrolases and led to approved drugs for treating disorders such as obesity, diabetes, microbial infections, and Alzheimer's disease.[2] Efforts to develop inhibitors for serine hydrolases have uncovered specialized chemotypes, such as lactones/lactams,[3,4] carbamates,[5–8] and ureas,[9,10] that inactivate these enzymes by covalent modification of the conserved serine nucleophile. In many cases, structural features can be introduced into the inhibitors to tailor their selectivity for individual serine hydrolases. Despite these advances, much of the serine hydrolase class still lacks selective inhibitors that are suitable for pharmacological studies in living systems.[1,2]

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To facilitate the discovery of serine hydrolase inhibitors, we have introduced a high-throughput screening-compatible variant of the activity-based protein profiling (ABPP) technology[11,12] that measures the reactivity of active site-directed fluorophosphonate (FP) probes with serine hydrolases by fluorescence polarization (fluopol).[13] We recently applied fluopol-ABPP to screen the NIH 300,000+ compound library against the serine hydrolase protein phosphatase methylesterase-1 (PME-1), resulting in the discovery of a highly potent (low nM) and selective class of aza-β-lactam (ABL) inhibitors of PME-1.[14] The lead inhibitor ABL127 (**1**) was found to irreversibly inhibit PME-1 in cells and mice through acylation of the enzyme's active-site serine nucleophile.[14]

Even though ABL127 showed high selectivity for PME-1, we wondered whether changes to its core structure could generate inhibitors for other serine hydrolases. We initially tested this premise by screening a set of ABLs and oxa-β-lactams (OBLs) (Figure 1A) against the mouse brain membrane proteome by competitive ABPP. The ABL and OBL compounds were synthesized by [2 + 2] cycloaddition chemistry using chiral 4-pyrrolidinopyridine catalysts.[15,16] Mouse brain proteome was treated with compounds **1–11**, followed by a FP-rhodamine (FP-Rh) probe,[17] and the reactions were then quenched, separated by SDS-PAGE, and FP-Rh-labeled proteins detected by in-gel fluorescence scanning (Figure 1B). At 10 uM, compounds **1–11** inhibited several serine hydrolases that, based on past ABPP studies of the mouse brain,[6–8,10] could be identified as neutral lipases (AADACL1, MAGL, ABHD6), amidases (FAAH), thioesterases (FASN), and peptidases (APEH, PREP). Interestingly, several ABLs also inhibited an as yet unidentified 27 kDa serine hydrolase that did not exhibit sensitivity to previously screened carbamate[5] or triazole urea[10] libraries. ABL117 (**7**) and ABL143 (**8**) proved to be particularly potent and selective inhibitors of this 27 kDa hydrolase, producing near-complete blockade of its FPRh labeling when tested at 1 μM (Figure 1B). Conversely, ABL113 (both enantiomers **3** and **4**) and OBL716 (**11**) were potent and selective inhibitors of APEH. Several ABLs/OBLs inhibited additional serine hydrolases found in the soluble brain proteome, including PME-1 (Figure 1C, Figure S1). Taken together, these data indicate that ABLs/OBLs are capable of inhibiting a diverse set of mammalian serine hydrolases, including enzymes that heretofore have lacked selective chemical probes.

Our attention next turned to determining the identity of the 27 kDa brain serine hydrolase that was inhibited by ABL117 and ABL143 using a competitive ABPP-MudPIT platform.[6,7] Briefly, we treated mouse brain proteome with ABL117 (2 μM) or DMSO control followed by the activity-based probe FP-biotin and then enriched FP-biotin-labeled enzymes by avidin chromatography, subjected these enzymes to an on-bead trypsin digestion, and analyzed the resulting tryptic peptide mixture by multidimensional liquid chromatography-tandem mass spectrometry (LC/LC-MS/MS). Comparison of spectral counts for serine hydrolases in DMSO- versus ABL117-treated proteomes identified two enzymes that were significantly inhibited by ABL117: PME-1 and the uncharacterized enzyme alpha, beta-hydrolase-10 (ABHD10) (Table S1).

ABHD10 is a 297 amino acid (aa) protein with a predicted MW of 33 kDa. Proteomic studies have identified ABHD10 as a mitochondrial protein[18] with a predicted leader sequence and proteolytic cleavage site at aa's 46–47. The mature ABHD10 protein would thus have a mass that closely matches the size of the ABL117-sensitive enzyme in brain proteomes. To our knowledge, inhibitors of ABHD10 have not yet been described, nor have any substrates or functions been discovered for the enzyme. These factors, combined with the very limited sequence homology (< 20%) that ABHD10 shares with other mammalian serine hydrolases, designated the enzyme as an attractive target for chemical probe development.

We first confirmed that ABHD10 was inhibited by ABL117 in a COS7 cell proteome transiently over-expressing this enzyme (Figure 1D). We next evaluated the structure-activity relationship (SAR) for ABL inhibition of ABHD10 versus other serine hydrolases by competitive ABPP of proteomes from the mouse neuronal cell line Neuro2A (Table 1 and Figure S2). Since we had already established that the ethyl substituent (R^1) on the stereogenic carbon was an important factor in promoting ABHD10 inhibition, we sought to explore other features of the core scaffold, including the carbamate substitution (R^3), the aryl ring substituent (R^2), and the configuration of the stereocenter. Increasing the steric bulk of the *O*-alkyl groups (R^3) on the carbamates from methyl (ABL117, **7**) to isopropyl (ABL243, **14**) increased potency for ABHD10 approximately four-fold, but also produced an equivalent increase in the inhibition of PREP. The PREP cross-reactivity was reduced by either substituting the *m*-methyl group on ABL243 with a fluorine atom (ABL223, **17**) or moving this methyl group to the para position (ABL303, **19**). ABL303 stood out as the most potent ABHD10 inhibitor among the tested ABLs (IC_{50} = 30 nM) with at least 40-fold selectivity over ABHD6, PREP, PME-1, and other serine hydrolases (Table 1 and Figure S2). Finally, as was observed previously for the PME-1 inhibitor ABL127, the *R*-enantiomer of ABL303 (**19**) was found to be substantially more active against ABHD10 than the *S*-enantiomer (ent-ABL303, **20**).

We next tested whether ABL303 inhibited ABHD10 with good selectivity in living cells. Gel-based ABPP confirmed excellent in situ activity for ABL303 in Neuro2A cells, where the compound inhibited ABHD10 with an IC_{50} of 21 nM (Figure 2B and D). Complete inhibition of ABHD10 was maintained for at least 6 h following a single treatment with ABL303 (250 nM, Figure S3). No detectable off-targets were observed for ABL303 until much higher concentrations ($\geq$ 1 µM) and no significant inhibition of PME-1 was observed even at 10 µM (Figure 2C, Figure S3). As previously noted,[14,19] however, gel-based ABPP lacks the resolving power to evaluate the full spectrum of serine hydrolase activities in complex biological systems. For this purpose, we assessed the cellular activity of ABL303 using the quantitative mass spectrometry-based proteomic method ABPP-SILAC.[10,14] Neuro-2A cells were cultured with isotopically light or heavy amino acids and then treated with DMSO or 100 nM ABL303 for 2 h, respectively. Proteomes were harvested, labeled with FP-biotin, and combined in a 1:1 ratio for avidin enrichment and LC/LC-MS/MS analysis on an LTQ-Velos Orbitrap instrument. Serine hydrolase activities were quantified by measuring the heavy:light ratios of the MS1 ions for their tryptic peptides. ABL303-treated cells showed a near-complete (>95%) and selective loss in ABHD10 activity among the ~40 serine hydrolase activities detected (Figure 2E). For comparison, we also assessed the activity of ABL127, which caused a complete and selective disruption of PME-1 activity in Neuro2A cells (Figure 2E). Finally, we also evaluated ABL303 at a higher concentration (250 nM) and in a second cell line, BW5147-derived mouse T-cell hybridoma cells, and, in both of these studies, we again observed complete and selective inhibition of ABHD10 (Figure S4).

In summary, we have described herein the functional proteomic analysis of a series of ABL/OBL compounds and found that they show broad reactivity with diverse branches of the serine hydrolase class, including peptidases, lipases, thioesterases, and uncharacterized enzymes. Using ABPP-guided medicinal chemistry, we developed an optimized inhibitor ABL303 that shows excellent potency and selectivity for the uncharacterized enzyme ABHD10. A comparison of the structures of ABL303 and the previously reported PME-1 inhibitor ABL127[14] indicates that only minor alterations to groups appended to the ABL are required to create compounds with excellent selectivity for individual serine hydrolases. Future studies with ABL303, in combination with metabolomic methods, may reveal the natural substrates and biochemical pathways regulated by ABHD10 in cells. More generally, our findings, combined with previous work,[14] designate the ABL as a privileged scaffold for

serine hydrolase inhibitor development that shows distinct reactivity compared to other known inhibitor classes, such as carbamates,[5–8] ureas,[9,10] and activated ketones.[20,21] That the inhibitory potential of ABLs was initially uncovered in a screen of the public NIH compound library performed against an individual serine hydrolase (PME-1) and then leveraged to create inhibitors for other enzymes from this class underscores the potential of integrated organic synthesis, high-throughput screening, and chemoproteomics to identify versatile chemotypes for small-molecule probe development.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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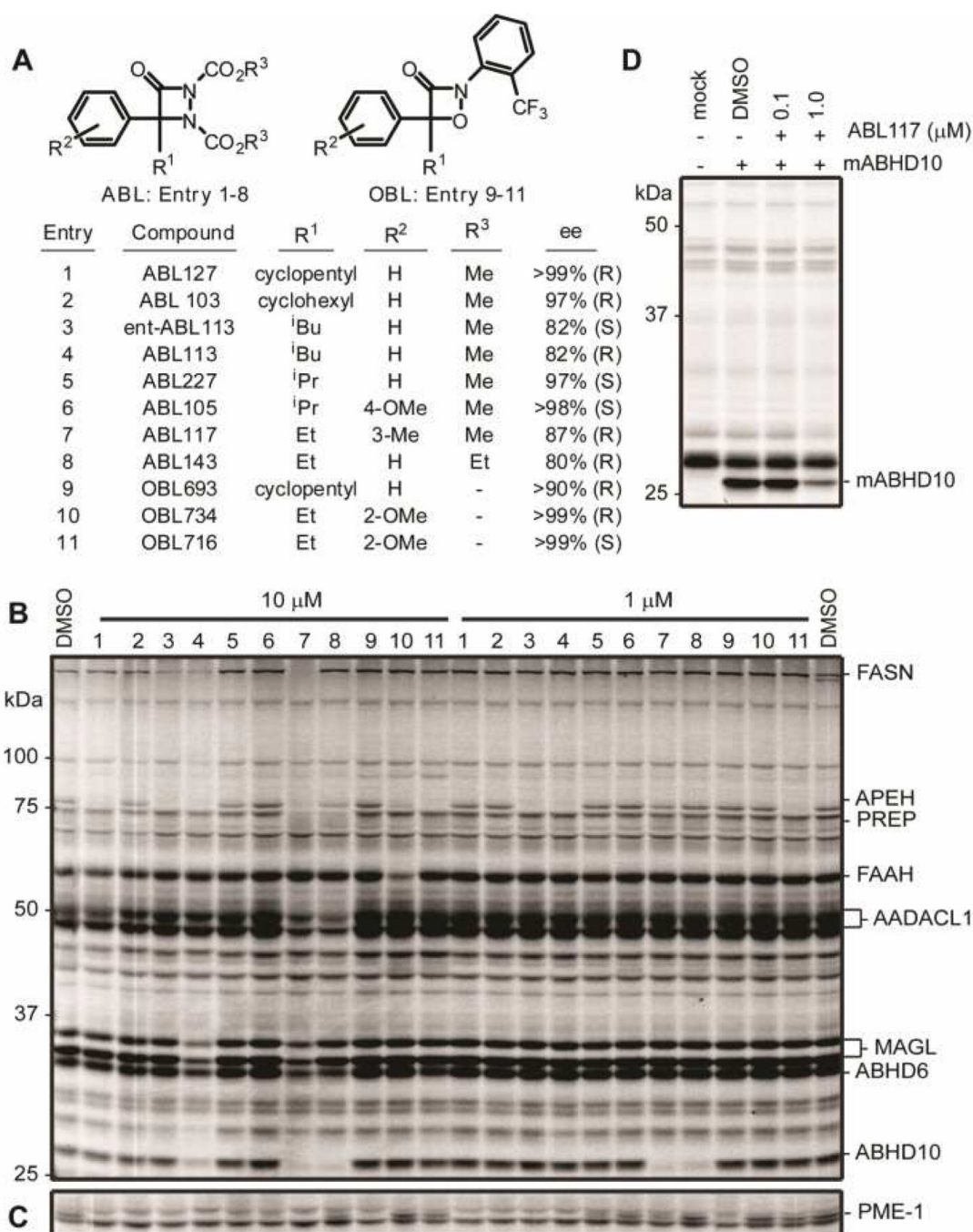
Figure 1.
Structures and competitive ABPP of ABL/OBL compounds. (A) Structures of ABL/OBL compounds. (B and C) Competitive ABPP gels of mouse brain membrane (B) and soluble (C, Figure S1) proteomes treated with ABLs/OBLs (30 min) followed by FP-Rh (1 µM, 30 min). Serine hydrolases inhibited by individual ABLs and OBLs are labeled. (D) Competitive ABPP gel of mock- versus mouse ABHD10 (mABHD10)-transfected COS-7 cell proteomes treated with ABL117, followed by FP-Rh (1 µM, 30 min). Fluorescent images shown in gray scale.
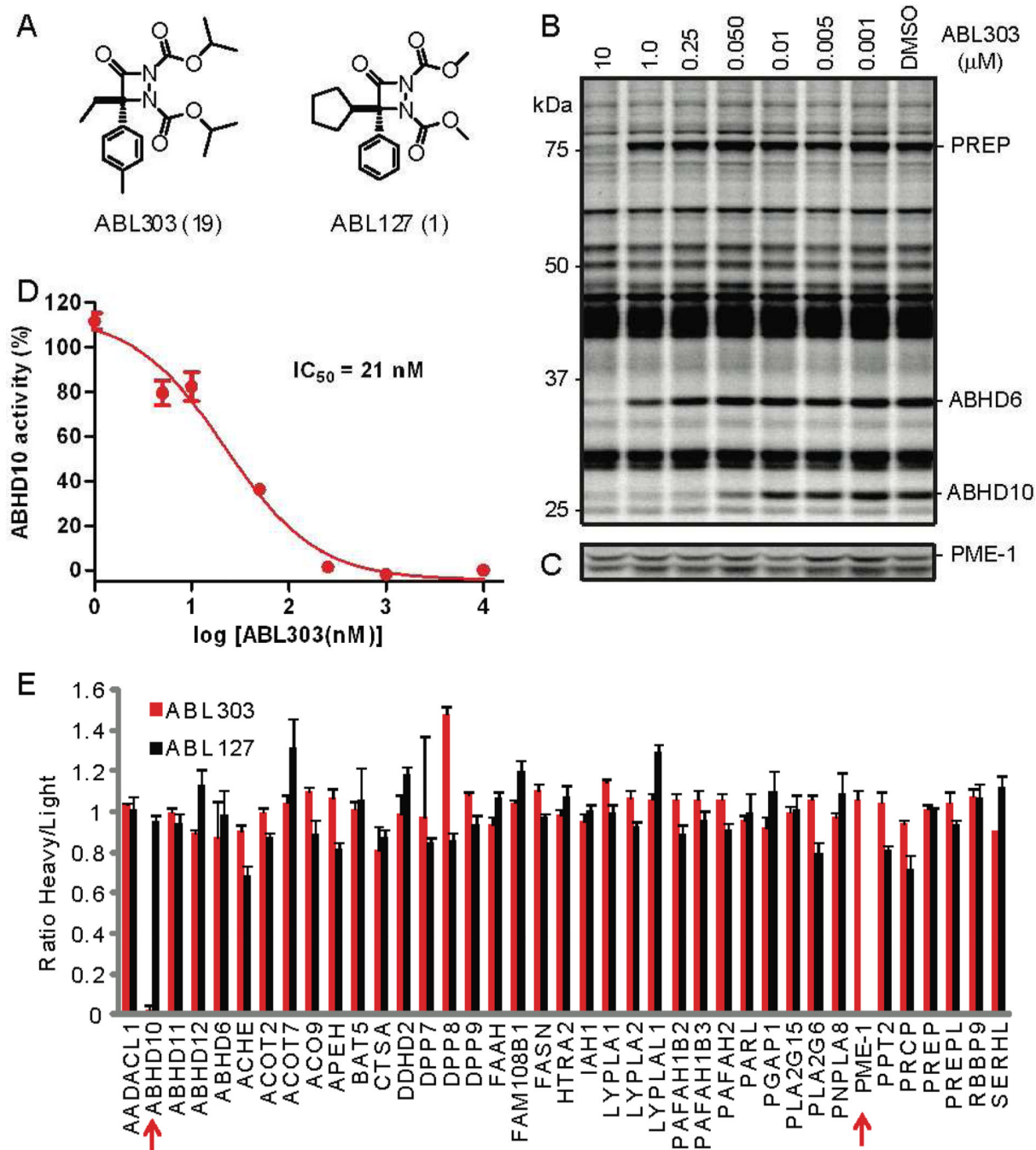
Figure 2.
Competitive ABPP of ABL303 in situ. (A) Structures of ABL probes. (B and C) Gel-based ABPP of membrane (B) and soluble (C) proteomes from Neuro-2A cells treated with ABL303 (10 - 0.001 μM, 2 h), afforded an (D) IC$_{50}$ value of 21 nM for inhibition of ABHD10 and no significant inhibition of PME-1. Data are presented as mean values ± SEM; n = 3/group. (E) ABPP-SILAC analysis of Neuro-2A cells treated with ABL303 (red) or ABL127 (black) (100 nM, 2 h; heavy samples) versus DMSO (light samples) revealed selective inhibition of ABHD10 and PME-1, respectively. Data are reported as mean values ± SEM of all peptides quantified for each serine hydrolase.

Table 1

SAR for ABL inhibitors versus ABHD10 and other representative serine hydrolases.

Entry	Compound	R^1	R^2	R^3	ee	IC$_{50}$(nM)			
						ABHD10	ABHD6	PREP	PME-1
7	ABL117	Et	3-Me	Me	86% (R)	210	1,900	2,600	250
8	ABL143	Et	H	Et	80% (R)	750	1,300	3,100	910
12	ABL91	Et	H	Me	83% (R)	1,500	10,000	>10,000	-
13	ABL123	Et	2-Me	Me	69% (R)	1,800	9,500	>10,000	-
14	ABL243	Et	3-Me	iPr	99% (R)	58	1,300	810	-
15	ABL245	Et	3-Me	Bn	86% (R)	410	180	260	220
16	ABL248	Me	3-Me	iPr	99% (R)	120	1,800	7,700	-
17	ABL223	Et	3-F	iPr	99% (R)	40	2,100	2,200	-
18	ABL179	Et	3-OMe	iPr	99% (R)	220	2,100	1,200	-
19	ABL303	Et	4-Me	iPr	99% (R)	30	3,000	4,500	1,300
20	ent-ABL303	Et	4-Me	iPr	99% (S)	>10,000	>10,000	>10,000	-