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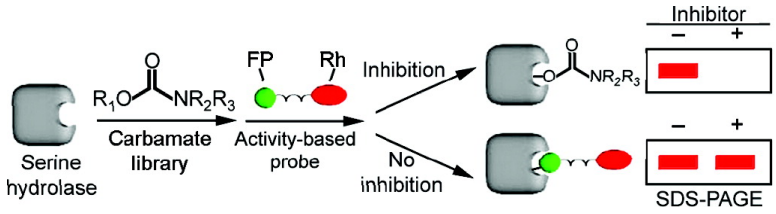

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A Functional Proteomic Strategy to Discover Inhibitors for Uncharacterized Hydrolases

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Hydrolytic enzymes constitute one of the largest and most diverse protein classes in Nature. Of the four major classes of hydrolytic enzymes (aspartyl, cysteine, metallo, serine), serine hydrolases (SHs) are a particularly expansive family in mammals, representing ~1% of the predicted protein products of the human genome.[1] SHs play key roles in nearly all physiological and pathological processes and are targeted by drugs that treat diseases such as diabetes, Alzheimer's disease, obesity, and blood clotting disorders.[2] The mammalian SH superfamily contains a remarkable number of uncharacterized members, with at least 40−50% of these enzymes lacking experimentally verified endogenous substrates and products. Assignment of metabolic and cellular functions to these enzymes requires the development of pharmacological tools to selectively perturb their activity. We describe herein a functional proteomic strategy to systematically develop potent and selective inhibitors for uncharacterized SHs.

SHs are nearly universally susceptible to inactivation by fluorophosph(on)ates (FPs), which covalently modify the catalytic serine nucleophile in these enzymes.[3] Reporter-tagged versions of FPs (Figure 1) have been shown to serve as versatile activity-based protein profiling (ABPP) probes for SHs,[4] leading to the discovery of enzymes dysregulated in aggressive cancer cells, activated chondrocytes, and fatty livers.[5] Beyond their application for SH discovery in disease models, reporter-tagged FPs have formed the basis of competitive assays to evaluate the specificity of SH inhibitors in native proteomes.[6] In select cases, inhibitors for uncharacterized SHs have emerged from competitive ABPP experiments and used, in combination with metabolomic methods,[7] to determine the endogenous metabolic function of the enzymes.[8] Nonetheless, it remains unclear whether ABPP methods can be systematically applied to engender potent and selective inhibitors for uncharacterized SHs. We set out to test this premise by performing competitive ABPP with a representative unannotated SH, α/β-hydrolase domain 6 (ABHD6) and a library of SH-directed inhibitors based on the carbamate reactive group (Figure 1). Carbamates have proven to be a versatile class of SH inhibitors, with multiple agents in active clinical use or development, including those that target the SHs acetylcholinesterase (AChE) and FAAH,[9] which regulate acetylcholine and endocannabinoid signaling in the nervous system, respectively. ABHD6 represented an attractive initial target for competitive ABPP, as this enzyme, like AChE and FAAH, is highly expressed in mammalian brain (Supporting Figure 1).

COS-7 cells were transiently transfected with the human ABHD6 cDNA, resulting in high expression levels of active, recombinant enzyme in the membrane proteome (Figure 2A).[10] This proteome was treated with individual members of an initial library of 55 carbamates (50 μM; see Supporting Information for compound structures) for 1 h, followed by the addition of the ABPP probe FP-rhodamine (FP-Rh;[4c] 1 μM) for 1 h. Reactions were then

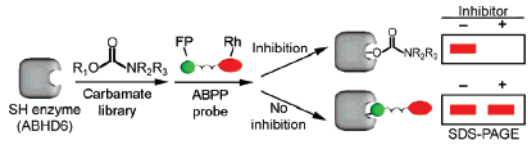

Figure 1. Functional proteomic strategy to discover carbamate inhibitors for uncharacterized SH enzymes. Rh, rhodamine.

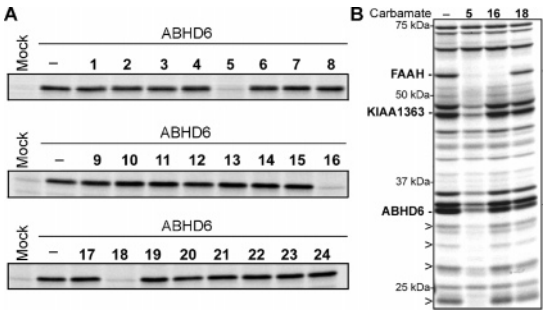

Figure 2. Discovery of lead inhibitors for ABHD6. (A) Screening of representative carbamates for inhibition of FP-Rh labeling of ABHD6. Active compounds shown: **5**, **16**, and **18** (see Supporting Figure 2 for complete carbamate library screen). (B) Selectivity of lead inhibitors (10 μM) was assessed by performing competitive ABPP with the mouse brain membrane proteome. Representative SH targets identified in previous studies[6] are shown in bold. Arrowheads designate additional SHs sensitive to carbamate **5**. Fluorescent gels shown in grayscale.

analyzed by SDS-PAGE and in-gel fluorescence scanning. Multiple carbamates blocked FP-Rh labeling of ABHD6, including compounds **5**, **16**, and **18** (Figure 2A). These inhibitors were tested over a concentration range of 0.01−50 μM to generate IC_{50} values for ABHD6 inhibition of 2.3, 1.0, and 0.35 μM, respectively (see Supporting Figure 2 for IC_{50} curves). Carbamates **5**, **16**, and **18** were next assessed for their selectivity in competitive ABPP assays with the mouse brain membrane proteome. Marked differences in selectivity were observed (Figure 2B). Carbamate **5** inhibited several brain SHs, including FAAH, KIAA1363, and multiple enzymes with masses between 20 and 40 kDa (arrowheads, Figure 2B). Carbamate **16** also cross-reacted with FAAH. In contrast, carbamate **18** selectively inhibited, albeit incompletely, a single 35 kDa FP-Rh-reactive band provisionally assigned as ABHD6.

The superior selectivity exhibited by carbamate **18** suggested that this agent might serve as a useful lead for generating higher affinity ABHD6 inhibitors. Approximately 20 derivatives of **18** were synthesized with various modifications to the *O*-aryl group and screened for activity against ABHD6 (Supporting Figure 3). Modification of the terminal phenyl ring of **18** with a *p*-carboxamide group improved potency ~5-fold, generating an agent **70** that inhibited ABHD6 with an IC_{50} value of 70 nM (Figure 3A,B). This boost in potency was achieved with no discernible loss in selectivity as judged by competitive ABPP with brain membrane proteome (Supporting Figure 4). Curiously, however, even at concentrations

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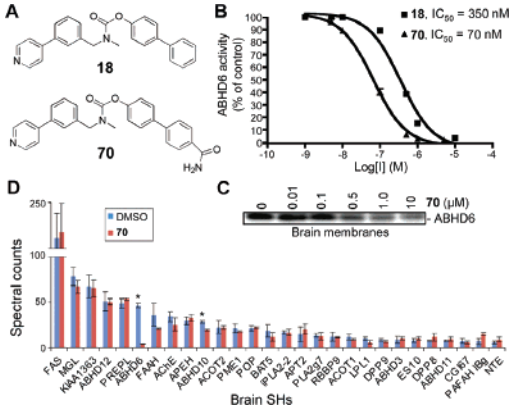

Figure 3. Development of carbamate **70**, a potent and selective inhibitor of ABHD6. (A) Structures of carbamates **18** and **70**. (B) IC_{50} curves for inhibition of FP labeling of recombinant ABHD6 by **18** and **70**. Data represent the average fluorescent signals ± standard error (SE) for three independent competitive ABPP experiments. See Supporting Figure 5 for gel images. (C) Blockade of FP labeling of endogenous ABHD6 in brain membrane proteome by **70** (see Supporting Figure 4 for quantification of signals). (D) ABPP-MudPIT analysis of the impact of **70** (10 μM, 1 h preincubation) on the labeling of brain membrane SH activities by FP-biotin (5 μM, 1 h). See Supporting Table 1 for full names of brain SHs. Data represent the average spectral counts ± standard error (SE) for three independent experiments. ∗, $p < 0.01$ for **70**- versus DMSO-treated proteomes.

up to 10 μM, carbamate **70** failed to completely block FP labeling of the 35 kDa brain protein band assigned as ABHD6 (Figure 3C and Supporting Figure 4). We speculated that the residual 35 kDa FP signal observed in competitive ABPP reactions with **70** (and previously with **18**) might represent an additional brain SH that co-migrates with ABHD6. Consistent with this premise, analysis of the brain soluble proteome identified a 35 kDa SH activity that was insensitive to **70** (Supporting Figure 4).

To more thoroughly characterize the target profile of carbamate **70**, we performed competitive ABPP experiments with mouse brain membrane proteome and FP-biotin[4a] and analyzed the reactions using a shotgun LC-MS method termed ABPP-MudPIT.[11] The relative levels of SH activities in **70**-treated versus DMSO-treated (control) proteomes were quantified by spectral counting.[11] For these studies, we selected a concentration of **70** (10 μM) that was well above the calculated IC_{50} value (70 nM), so as to discern the degree of selectivity exhibited by this agent for ABHD6 relative to other brain SHs. Under these conditions, carbamate **70** was found to block greater than 90% of the activity of ABHD6 (Figure 3D). In contrast, none of the other 27 SH activities identified in the brain membrane proteome were significantly altered by **70**, with the exception of ABHD10, which showed a modest (30%) but significant reduction in activity in **70**-treated proteomes.[12] Follow-up studies, however, failed to reveal any inhibition of recombinant ABHD10 by **70** at concentrations up to 100 μM, indicating that this enzyme is unlikely to be a true target of **70**. Collectively, these data indicate that **70** is a highly selective inhibitor of ABHD6 compared to other brain SHs. From a technical perspective, the results also highlight the superior resolution afforded by LC-MS compared to gel-based methods for broadly assessing the protein targets of chemical probes.

In summary, we have described herein a functional proteomic strategy to develop inhibitors for uncharacterized SHs, and its application to create a potent and selective inhibitor of ABHD6. The enriched expression of ABHD6 in brain tissue suggests a role for this enzyme in nervous system metabolism and/or signaling. ABHD6 is also highly elevated in Epstein−Barr virus-transformed B cells,[13] indicating that it may contribute to cancer pathogenesis. These expression patterns thus designate cell and organ targets where the future application of **70**, in conjunction with metabolomic methods,[7,8] may reveal endogenous biochemical and (patho)-physiological functions for ABHD6. Projecting beyond ABHD6, it is noteworthy to emphasize that the development of **70** required screening fewer than 75 compounds. This achievement reinforces the idea that carbamates may offer a privileged molecular scaffold for the streamlined development of SH inhibitors that display an excellent combination of potency and selectivity. Of course, our competitive ABPP studies only speak to the specificity of carbamates *within* the SH family; potential protein targets outside of this enzyme class are not discriminated. Conversion of carbamates into probes using bio-orthogonal reactions such as the Cu(I)-catalyzed Huisgen's azide−alkyne cycloaddition (click chemistry)[14] provides a complementary route to directly visualize protein targets of carbamates.[15] We anticipate that continued efforts to screen structurally diverse libraries of carbamates against the daunting number of uncharacterized SHs that populate eukaryotic and prokaryotic proteomes will engender a suite of valuable pharmacological tools for annotating new biochemical pathways.

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Supporting Information Available: Synthesis and experimental protocols; figures showing tissue distribution of ABHD6, IC_{50} curves for **5** and **16**, and SDS-PAGE analysis of competitive ABPP experiments; table of brain membrane SH enzymes. This material is available free of charge via the Internet at http://pubs.acs.org.

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