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Oxime esters as selective, covalent inhibitors of the serine hydrolase retinoblastoma-binding protein 9 (RBBP9)

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ABSTRACT

We recently described a fluorescence polarization platform for competitive activity-based protein profiling (fluopol-ABPP) that enables high-throughput inhibitor screening for enzymes with poorly characterized biochemical activity. Here, we report the discovery of a class of oxime ester inhibitors for the unannotated serine hydrolase RBBP9 from a full-deck (200,000+ compound) fluopol-ABPP screen conducted in collaboration with the Molecular Libraries Screening Center Network (MLSCN). We show that these compounds covalently inhibit RBBP9 by modifying enzyme's active site serine nucleophile and, based on competitive ABPP in cell and tissue proteomes, are selective for RBBP9 relative to other mammalian serine hydrolases.

In both academia and industry, high-throughput screening (HTS) of large compound libraries has emerged as a powerful means to identify lead scaffolds for chemical probes and drugs.[1,2] A wide array of HTS-compatible assays have been introduced, ranging from classical in vitro substrate assays for enzyme inhibitors to in situ screens that profile cellular phenotypes. Still, the development of an accurate and reproducible assay, the critical first step in an HTS campaign, can be difficult, even for well-characterized biological systems. For example, a target-based HTS program that seeks to identify modulators of a specific protein must optimize the readout of biochemical activity so that it is consistent between wells and plates, has enough sensitivity to detect compounds with weak activity, and is economically feasible. As a consequence, the estimated 30–50% of the human proteome with poorly characterized biochemical activities has largely remained outside the general scope of HTS.

To address this problem, our laboratory has recently adapted the chemical proteomic technology activity-based protein profiling (ABPP)[3,4] for HTS.[5] ABPP employs reactive chemical probes to covalently label the active sites of mechanistically related enzymes, regardless of their degree of biochemical annotation, enabling the direct evaluation of the functional state of either purified enzymes or enzymes in complex biological systems. ABPP can be performed in a competitive format to discover lead inhibitors, where compounds are assayed for their ability to impede probe labeling of enzymes.[6,7] Importantly, this strategy, when employed in complex proteomes, enables the simultaneous optimization of both the potency and selectivity of inhibitors against many enzymes in parallel. Competitive ABPP has traditionally required a gel-based readout, limiting the throughput to hundreds of compounds, but has nevertheless led to the identification of many selective inhibitors,[6–10] including several for uncharacterized enzymes.[8,10]

To create an HTS-amenable version of competitive ABPP, we modified this platform such that probe labeling of purified enzymes could be monitored by fluorescence polarization (fluopol-ABBP).[5] We initially applied fluopol-ABPP to the retinoblastoma-binding protein-9 (RBBP9), a poorly characterized serine hydrolase that reacts with reporter-tagged fluorophosphonate probes [FP–biotin and FP–rhodamine (FP–Rh)].[11,12] RBBP9, originally discovered as a protein that confers resistance to the growth-inhibitory effects of TGF-β1, has been shown to bind the retinoblastoma (Rb) protein and transform rat epithelial cell lines.[13] Most recently, RBBP9 has been found to promote anchorage-independent growth and pancreatic carcinogenesis through overriding TGF-β-mediated antiproliferative signaling.[14] Although these data suggest that RBBP9 plays an important role in cancer, the biochemical function of this enzyme and identity of its endogenous substrate remain unknown.

From an initial ~20,000 compound fluopol-ABPP screen, we identified the natural product emetine as a reversible inhibitor of

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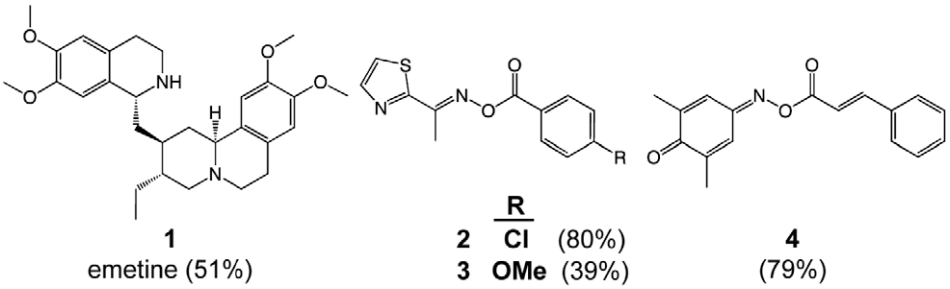

Figure 1. Structures of HTS lead RBBP9 inhibitors. Normalized percent inhibition of RBBP9 (compounds at 7.9 μM) from fluopol-ABPP PubChem Bioassay AID 1537 is shown in parentheses.

RBBP9 that selectively blocked FP–Rh labeling of this enzyme compared to other members of the serine hydrolase family[5] (Fig. 1). Although emetine does not interact with other serine hydrolases, it is a cytotoxic natural product and has been shown to inhibit translation in vitro, possibly through direct interactions with the ribosome.[15] Emetine has also been shown to antagonize α2-adrenergic receptors.[16] As these additional targets could complicate the use of emetine in biological studies of RBBP9, we sought to identify additional classes of inhibitors for this enzyme. Toward this end, we report herein the discovery of an oxime ester set of inhibitors of RBBP9 from a 200,000+ compound fluopol-ABPP screen. We show that these oxime ester compounds covalently modify the active site serine of RBBP9 and are selective for RBBP9 relative to other mammalian serine hydrolases in proteomes.

In collaboration with the Molecular Libraries Screening Center Network at The Scripps Research Institute, we screened 217,969 individual compounds (7.9 μM) for RBBP9 inhibition by fluopol-ABPP (PubChem AID: 1515). A robust average *Z′* factor (0.75) enabled the identification of 445 potential inhibitors, defined as those that reduced the RBBP9-induced fluorescence polarization signal by greater than 14.2%. A confirmation screen, performed identically to the primary screen and assaying hit compounds in triplicate, verified 137 as active (PubChem AID: 1537). Notably, this group included the natural product emetine, confirming the reproducibility of our small- and large-scale screens. The confirmed hit compounds were next analyzed by gel-based competitive ABPP in the membrane fraction of mouse brain doped with exogenous RBBP9 and in the soluble fraction of RBBP9-transfected HEK 293T cells (Fig. 2A and B). Briefly, the proteomes were incubated with compounds (20 μM) for 30 min, followed by addition of FP–Rh (1 μM). The reactions were quenched after 10 min, separated by SDS-PAGE, and quantified by in-gel fluorescence scanning. These assays enabled us to discard dozens of weak and/or promiscuous inhibitors and aggregation-based inhibitors (which showed a characteristic profile of blocking probe labeling of most serine hydrolases in the soluble proteome, while exhibiting negligible activity in the membrane proteome[5]) in favor of compounds that selectively inhibited RBBP9, including three with oxime esters (inhibitors **2**–**4**, Fig. 1).

Compounds **2** and **3**, differing only in the *para* substituent of the phenyl ring were highly selective for RBBP9, inhibiting only a single additional hydrolase target (75 kDa) at high concentrations (Fig. 2B). Compound **4**, a differently substituted oxime ester possessing two potentially cysteine-reactive chemical moieties (see below), was less selective for RBBP9, inhibiting the same 75 kDa hydrolase described above, as well as 30 and 40 kDa hydrolases in the HEK 293T soluble proteome (Fig. 2A).

The IC_{50} values of compounds **1**–**4** against purified RBBP9 as determined by gel-based ABPP correlated closely with the percent inhibition at 7.9 μM observed in the fluopol-ABPP confirmation screen (Fig. 1 and Tables 1 and 2). Emetine (**1**) inhibited RBBP9 in the initial fluopol-ABPP assay by 51% and, remarkably, inhibited this enzyme in the gel-based assay with an IC_{50} of 7.8 μM. Compounds **2** and **4** both inhibited RBBP9 in the HTS confirmation screen by ~80% and gave more potent IC_{50} values (1.9 μM and 1.2 μM, respectively) in the gel-based assay. Compound **3** inhibited

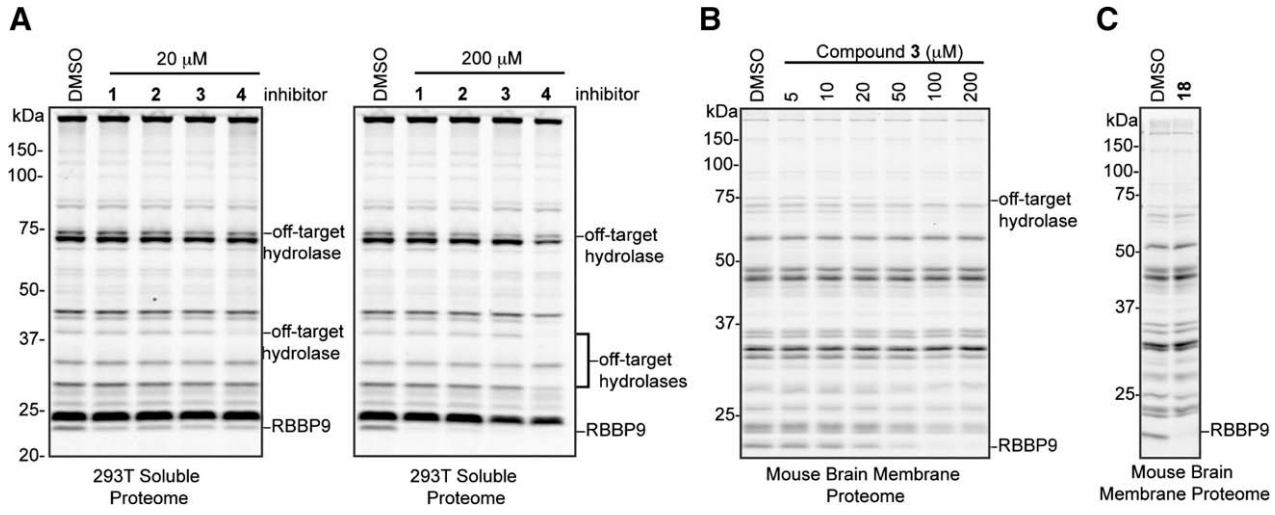

Figure 2. Selectivity profiling of oxime ester RBBP9 inhibitors. (A) Evaluation of RBBP9 inhibitors by competitive ABPP with the FP–Rh probe in the RBBP9-transfected HEK 293T soluble proteome at 20 μM (left panel) and at 200 μM (right panel). Fluorescent gels are shown in gray scale. (B) Evaluation of the selectivity of oxime ester **3** at indicated concentrations and (C) oxime ester **18** at 200 μM by competitive ABPP in the mouse brain membrane proteome doped with recombinant RBBP9.