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Rapid Development of a Potent Photo-Triggered Inhibitor of the Serine Hydrolase RBBP9

Xiaodan Liu^a, Melissa Dix^b, Anna E. Speers^b, Daniel A. Bachovchin^b, Andrea M. Zuhl^b, Benjamin F. Cravatt^b, and Thomas Kodadek^a

Thomas Kodadek: Kodadek@scripps.edu

^aDepartment of Chemistry, The Scripps Research Institute, 130 Scripps Way, Jupiter, FL 33458

^bDepartment of Chemical Physiology and the Skaggs Institute for Chemical Biology, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, California 92037

Abstract

The serine hydrolases constitute a large class of enzymes that play important roles in physiology. There is great interest in the development of potent and selective pharmacological inhibitors to these proteins. Traditional active site inhibitors often have limited selectivity within this superfamily and are tedious and expensive to discover. Using the serine hydrolase RBBP9 as a model target, we report here a rapid and relatively inexpensive route to highly selective peptoid-based inhibitors that can be activated with visible light. This technology provides rapid access to photo-activated tool compounds capable of selectively blocking the function of particular serine hydrolases.

Keywords

peptoid library; one-bead-one-compound; serine hydrolase; ABPP; CALI; RBBP9

Introduction

Pharmacological inhibitors with high affinity and selectivity are useful tools to probe the function of the target protein and may serve as drug leads. Ideally, one would like to have such reagents for the entire proteome, though this is unlikely to be achieved in the near future. A more realistic, though still daunting, goal would be to develop highly selective inhibitors of an entire class of proteins. For example, the serine hydrolases (SHs) constitute about 1% of the human proteome and play diverse roles in physiology.^[1] Selective inhibitors exist for only a small fraction of these enzymes. Unfortunately, the most commonly used type of high-throughput screening technology is not capable of addressing even this more circumscribed goal. Robotic screening of hundreds of thousands of small molecules via a functional assay, such as monitoring enzyme inhibition in the wells of microtiter plates, is highly effective for single targets or a small number of them, but is expensive and often requires purified protein and tailored substrate assays. This makes it difficult to apply this technology to large numbers of enzymes. Moreover, it is almost always the case that initial screening hits must be optimized via relatively tedious medicinal chemistry efforts in order to be truly useful probe molecules, another major barrier to the development of probes against dozens or hundreds of proteins in a timely fashion.

Correspondence to: Benjamin F. Cravatt; Thomas Kodadek, Kodadek@scripps.edu.

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An alternative to functional screens is an assay that scores binding of compounds in a library to the target protein. These assays, employing one bead one compound (OBOC)^[2] or DNA-encoded^[3] libraries, can be conducted under conditions that encourage the isolation of only highly selective “hits”, for example by making the labeled target protein a minor component of a complex protein mixture. This methodology also allows very large numbers of compounds to be assayed rapidly and inexpensively. Binding assays have their own limitations however. They do not necessarily result in the isolation of inhibitors. Inhibitors or even silent ligands that do not affect the function of the target protein substantially can be identified in such screens. A labeled protein is required and this requires either modification of purified protein with a tag such as biotin or the creation of a functional epitope-tagged species. Unless one has a good antibody, it is difficult to screen against a native protein in this format. Finally, binding screens are also unlikely to yield primary hits of the desired potency and so optimization is still an issue.

Here we report a melding of several technologies that address some of the major problems inherent in developing a strategy to tackle the isolation of potent, selective inhibitors for the SH family. Using the important SH RBBP9 and OBOC peptoid libraries as a model system, we demonstrate that protein labeled by an activity-based probe (a biotin-tethered fluorophosphonate) can be successfully used as a target in a binding screen to rapidly and inexpensively discover modest affinity ligands to that protein (Scheme 1). Moreover, we demonstrate that reasonably potent inhibitors of RBBP9 can be created by tethering chromophores to these primary hit molecules that generate singlet oxygen when photolyzed with visible light. Finally, we demonstrate that the photo-triggered compounds are highly selective RBBP9 inhibitors by employing activity-based protein profiling (ABPP) assays to evaluate their effect on a number of SHs in a crude cellular extract.

Results

Establishment and validation of general magnetic screening method

We selected the putative serine hydrolase RBBP9 as an initial target for peptoid library screening since this enzyme is implicated in pancreatic cancer,^[4] but still lacks known substrates and potent and selective inhibitors, even though it has been subject to a high throughput screen based on blocking its reaction with the serine hydrolase-directed activity-based probe fluorophosphonate-rhodamine (FP-Rh).^[5] FP is a specific irreversible inhibitor of SHs that, when coupled to a fluorophore or biotin tag, facilitates the detection and enrichment of active enzymes from complex biological systems (Figure 1A.). Our plan was to identify selective RBBP9-binding peptoids by first labeling RBBP9 with FP-biotin to generate the covalent, biotin-displaying RBBP9 adduct, followed by incubation of this target with a one-bead-one-compound (OBOC) peptoid library in the presence of an excess of unlabeled competitor proteins. Tentagel beads that display peptoids that retain significant amounts of the enzyme would then be removed from the population by subsequent incubation with streptavidin-coated magnetic beads (SA-MB) followed by exposure to a strong magnet (Scheme 1).

Before applying this strategy to a screen, it was important to verify the efficiency of labeling and recognition of biotinylated RBBP9 by SA-MB. The labeling efficiency was evaluated by two methods: whole protein mass spectrometry (Figure 1B) and SDS/PAGE followed by blotting with streptavidin-HRP (Figure 1C.). The mass spectrometry data show that, after incubation with three equivalents of FP-biotin, there is no mass corresponding to the unmodified protein observed and the mass peak representing RBBP9 shifted completely to a higher mass that equals the mass of RBBP9 plus one molecule of FP-biotin, suggesting essentially complete conversion of RBBP9 to the modified form. Furthermore, only the FP-

biotinylated RBBP9 (not its unmodified form) was detected by streptavidin-HRP after SDS-PAGE, also indicating the successful FP-biotinylation of RBBP9.

Next we investigated whether the FP-biotinylated RBBP9 in its native form can be recognized by SA-MB. This is essential if we are to employ this interaction to isolate peptoid-displaying Tentagel beads that retain the labeled protein. To address this, after incubation with either unlabeled or FP-biotin labeled RBBP9, SA-MB were subject to thorough washing followed by on-bead trypsin digestion (Figure 2.). In the mass spectrum of the tryptic digest, mainly the tryptic peptide fragments from streptavidin were detected from the incubation of SA-MB with unlabeled RBBP9 (Figure 2A.). In contrast the mass spectrum obtained following incubation with labeled RBBP9 showed all the major tryptic peptide fragments from RBBP9 (Figure 2B.). The overall spectrum is comparable with a standard RBBP9 trypsin digestion performed in solution (Figure 2C). This demonstrates binding of the magnetic bead-bound streptavidin molecules to the biotinylated protein.

Library screening and validation of “hit” peptoids

A library of tetrameric peptoids with a theoretical diversity of 160,000 compounds was constructed by split and pool sub-monomer synthesis^[6] (Scheme 2.) on TentaGel HL NH₂ beads with a diameter of ~75 μm (4×10⁶ beads/g, ~100 pmol peptoids/bead), which we have shown to be a superior support for on-bead magnetic screening.^[7] Two different constant dipeptoid sequences (Nmpa-Nmpa or Nlys-Nlys) were introduced as a spacer prior to library synthesis. Twenty amines were employed in the library synthesis. One of the primary amines in 1,4-diaminobutane was protected with t-Boc, an acid-labile protecting group that was removed without disturbing the linkage of the peptoids to the beads after completion of library synthesis.

After deprotection in 95% TFA solution, the library beads were washed thoroughly with DMF followed by DCM, and then lyophilized to completely remove residual DCM. To assess the quality of the library, forty beads were picked randomly and subjected to MALDI mass spectrometry analysis. In each mass spectrum, a single predominant peak was observed. Tandem MALDI-TOF-TOF mass spectrometry allowed each of the peptoids to be sequenced and all twenty amines employed in the library construction were represented in at least one of the sequences (data not shown). We concluded that the library was of high quality and suitable for screening.

Approximately 500,000 beads (about three-fold coverage of the theoretical diversity) were incubated with *E. coli* extract to block the potential non-specific binding sites on the beads. After washing with TBST once, the beads were then mixed with SA-MB, and then the conical tube was placed on a magnet separator. The library beads with surface-bound magnetic beads were attracted to the wall, while the nonbinding beads settled to the bottom of the tube. Around 500 beads were removed during this pre-screening process to remove streptavidin and magnetic bead ligands from the library. The pre-screening procedure was repeated and 50 beads were removed during the second round at which point the library was deemed ready for screening.

The library was then incubated with FP-biotinylated RBBP9 in *E. coli* extract. After washing, the beads were further incubated with SA-MB. After removing the non-binding beads from the bottom of the tube, the positive beads (9 beads out of the library with the Nlys spacer, and 23 beads out of the library with the Nmpa spacer, 0.05% and 0.14 % hit rate respectively) were retrieved by removing the magnet separator. These beads were completely stripped to remove bound proteins.

The primary “hit” beads were then subjected to extensive washing (DMF-DCM-PBS-TBST) and then equilibrated in TBST overnight before re-probing. The beads were re-blocked in *E. coli* extract followed by incubation with a higher concentration of FP-biotinylated RBBP9 (5 μ M) in a reduced volume of *E. coli* extract (1 mL). After washing, the beads were allowed to incubate with streptavidin-coated red quantum dots (Qdot). The beads were visualized under a fluorescence microscope by irradiation of the beads through a DAPI filter (390–410 nm band pass filter), and 17 beads that displayed a red halo on the surface were removed using a micropipette. The beads were stripped before being subjected to CNBr cleavage and MALDI sequencing. Initial validation of these “hit” compounds was performed by resynthesizing them on the same TentaGel beads that we used to construct the library, then repeating the Qdot re-probing process. Based on these criteria, six of the 17 peptoids were confirmed to bind to FP-biotinylated RBBP9.

Determination of binding affinity

To validate the binding of the “hit” compounds in a solution experiment, we turned to fluorescence polarization spectroscopy. Fluorescein conjugates of the “hit” peptoids were synthesized through the reaction of fluorescein-5-maleimide with a cysteine residue incorporated at the C-terminal end of the peptoid. The fluorescein-peptoid conjugate was incubated with the indicated concentrations of RBBP9 in the presence of either BSA or Tween-20 in PBS containing 2 mM DTT. Fluorescence polarization measurement indicated that fluorescein-labeled hit **1** and hit **6** bound to RBBP9 protein with equilibrium dissociation constant (K_d), of approximately 50 and 2 μ M, respectively (Figure 3). For the other 4 hits, they were either not observed to bind to RBBP9 or their binding didn't reach the saturation plateau at 500 μ M of RBBP9 (Figure S1). These data suggest that the conditions employed in the screen allowed even very weak ligands to score as hits.

Potentiation of “hit” peptoids by chromophore-assisted light inactivation (CALI)

The inhibition potency of the “hit” compounds **1** and **6** were first evaluated against purified RBBP9 by gel based ABPP. Up to a concentration of 500 μ M (Figure S2), neither of them inhibited RBBP9 reaction with FP-Rh. This is not surprising since the product of this reaction was employed as a screening target, a protocol that will almost certainly select ligands that bind outside of the enzyme active site and which are unlikely to inhibit the labeling of this site. We conclude that **1** and **6** are ligands for RBBP9, but not intrinsic inhibitors. Of course, this means that a standard ABPP experiment cannot be employed to assess the selectivity of compounds **1** and **6** for RBBP9 relative to the other SHs in a cellular extract. Therefore, we proceeded to employ these peptoids as “directing agents” for the delivery of a singlet oxygen-generation moiety.

Two chromophores were investigated. First, (Ru(II)(bpy)₃)²⁺, an exceptionally efficient photocatalyst for singlet oxygen generation^[8] was appended to Hit **6** via an alkyne-azide cycloaddition reaction to yield the conjugate that we will call Ru(II)-**6** (Scheme 3A). The ruthenium reagent was synthesized in two steps. First, 3-azidopropylamine was prepared from the corresponding bromide via reaction with sodium azide^[9] Secondly, the commercially available bis(2,2'-bipyridine)-4'-methyl-4-carboxybipyridine-ruthenium N-succinimidyl ester was allowed to react with the 3-azidopropylamine. Peptoid **6** was synthesized on Knorr Amide resin with a C-terminal propargylamine. The “click” reaction was catalyzed with tetrakis(acetonitrile)copper hexafluorophosphate using a microwave-assisted protocol (see Experimental). In addition to the ruthenium conjugate, we also tethered hit **1** to eosin (2',4',5',7'-tetrabromofluorescein), which was recently reported to be an efficient CALI “warhead”.^[10] Eosin-**1** was constructed via the reaction of Eosin-5-maleimide and a cysteine thiol appended to the Hit **1** (Scheme 3B).

The potency of these compounds was then tested in an ABPP assay in which the purified RBBP9 was incubated with the indicated concentration of the compounds, after which the solution was split into equal portions. One tube was subjected to irradiation with visible light, while the other was not. In the absence of irradiation, Ru(II)-**6** and Eosin-**1** did not inhibit RBBP9 labeling by FP-Rh even at the highest concentration examined (Figure 4). However, with visible light irradiation, RBBP9 labeling by FP-Rh was inhibited around 70% by 20 nM of Ru(II)-**6** or Eosin-**1**. Thus incorporating a chromophore into the hit compound converted the modest affinity ligand, which did not inhibit the enzyme, into a potent light-triggered inhibitor.

Evaluation of the selectivity in cell-line soluble proteome

The selectivity of the photo-activated inhibitors was evaluated by competitive ABPP assay in a complex proteome comprised of the soluble fraction of a HeLa cell extract doped with purified RBBP9. Doping was necessary because native RBBP9 could not be detected by gel-based ABPP in the crude extract (Figure 6C). These gel-based ABPP assays allowed us to determine the selectivity of the “hit” compounds for RBBP9 compared to a number of different serine hydrolases. Upon light irradiation, Ru(II)-**6** selectively abolished FP-Rh labeling of RBBP9 at a concentration of 450 nM, but did not block the labeling of most of other serine hydrolases detected (Figure 5A.). Free Ru(II)(bpy)₃²⁺ and a scrambled version of Ru(II)-**6** were used as controls. At the indicated concentration, both compounds blocked the labeling of most serine hydrolases, suggesting non-specific interactions with those proteins. Similarly, Eosin-**1** (250 nM) also showed selective inhibition of RBBP9 (Figure 5B.). In contrast, eosin exhibited largely non-specific effects. Eosin-**1** consists of three aromatic amine monomers adjacent to the linker region and 1, 4-butanediamine at the N-terminus. The scrambled version of Eosin-**1** was generated by switching the last two amine monomers at N-terminus of Eosin-**1** (Figure S3). Scrambled Eosin-**1** (250 nM) with this slight structural change inhibited the FP-Rh labeling of RBBP9 and several other serine hydrolases as indicated by arrows in Figure 5B.

Compound **1** showed a lower binding affinity (50 μ M) than that of compound **6** (2 μ M) in the fluorescence polarization study (Figure 3). However, Eosin-**1** was shown to be a more potent inhibitor than Ru(II)-**6** towards recombinant RBBP9 (Figure 4) or RBBP9 doped in a complex cellular proteome (Figure 5). This discrepancy may possibly be caused by the different chromophores attaching to the hit compounds. Eosin was reported to generate 13 times as much singlet oxygen as the ruthenium complex.^[10]

Covalent modification of RBBP9 during photoinactivation

The Ru(II)- and eosin-peptoid conjugates, when irradiated, are anticipated to inactivate RBBP9 through various covalent modifications brought about by reaction with singlet oxygen, which are likely to be heterogeneous. Therefore, there is a concern that disappearance of the sharp activity-labeled RBBP9 band in a gel-based analysis could be due to “smearing” of the protein in the gel due to differences in the electrophoretic mobility of the different oxidized forms of the protein, rather than the loss of activity of the protein. To probe this potential artifact more carefully, an ABPP experiment with RBBP9 was repeated in the presence or absence of Ru(II)-**6** and the presence or absence of light and the gel was stained with Coomassie Blue in addition to being probed with streptavidin. As shown in Figure 6A, photolysis of the solution containing RBBP9 and Ru(II)-**6**, followed by addition of FP-biotin did not result in the production of a streptavidin-reactive band, as expected, but also resulted in the loss of the Coomassie Blue-stained band. This renewed our concern regarding the potential artifact discussed above. However, staining with silver did allow the visualization of a distinct band with slightly higher mobility than the unmodified RBBP9 that was not streptavidin-reactive (Figure 6A). These data suggest that the

irradiation of RBBP in the presence of Ru(II)-6 did indeed ablate the catalytic activity of RBBP9 via oxidative protein modification, but that these same modifications interfere with protein staining by the Coomassie dye. In order to confirm this view, whole protein MALDI mass spectrometry analysis was conducted. As discussed previously, and shown again in Figure 6B, in the absence of the photo-triggered compounds, treatment of RBBP9 with FP-biotin results in the formation of a product whose mass is consistent with a covalent RBBP9-FP-biotin adduct. A similar result is observed in the presence of Ru(II)-6, but without irradiation. In stark contrast, no evidence for labeling of RBBP9 with FP-biotin was observed after photolysis of the enzyme in the presence of Ru(II)-6. Instead, the peak observed has a mass that was identical to that observed when RBBP9 and Ru(II)-6 were photolyzed in the absence of the FP-biotin reagent. This peak reflects the population of covalently modified and catalytically inactive protein.

The identity and mobility of RBBP9 on gel were also confirmed by probing the gel with western blotting with anti-RBBP9 antibody (Figure 6C). In the absence of light, Ru(II)-6 treatment did not impact the mobility of RBBP9 on gel. However, with increasing Ru(II)-6, in response to light irradiation, a ladder of bands with higher molecular weight was observed, suggesting the photo-damaged RBBP9 may form oligomers or crosslink with other proteins. In addition to this, there are some extra higher molecular weight bands observed on the ABPP gel in the presence of light activated hit compounds (Figure 5). Higher order crosslinks between proteins induced by Ru(II)(bpy)₃²⁺ and light were also reported in a previous study from our laboratory.^[11]

Discussion

The development of pharmacological tool compounds for entire families of proteins is a challenging endeavor. In this pilot study, a single hydrolase, RBBP9 was used as a model to address whether combining three different technologies, activity-based protein labeling,^[12] on-bead screening of OBOC libraries and CALI, could potentially accelerate efforts in this regard. To eliminate the need to develop specialized functional assays for individual SH, we demonstrated that RBBP9 labeled with a FP-biotin chimera could be employed successfully as a screening target in the context of a complex protein mixture using an OBOC library of tetrameric peptoids (Scheme 1). A rapid and convenient magnetic method to “fish out” hits (bead-displayed peptoids binding the RBBP9-FP-biotin conjugate) from the large collection of beads was employed. This approach to hit identification eliminates the need to develop a specific functional assay for each SH one wishes to target and should be applicable to any soluble SH that can be expressed in reasonable quantities.

Post-screening analysis revealed that the best of these hits evince a low- to mid-micromolar affinity for the target protein, though none of them proved to be inhibitor of RBBP9. The latter observation is not surprising, since the screen was conducted against an adduct of the enzyme and a mechanism-based inhibitor. These peptoids are almost certainly ‘exosite’ ligands that bind to one or more surfaces of the protein distinct from the active site, though this was not addressed experimentally in this study. This could be considered a weakness of this strategy relative to more traditional functional screening in which one can ask for an inhibitor directly.

To circumvent this limitation and, more importantly, to attempt to avoid the time-consuming optimization of primary screening hits through structure-activity relationship studies of derivatives, we turned to the use of CALI.^[13] In this technology, a photo-activated singlet oxygen-generating compound is added to the protein ligand of interest. Local production of singlet oxygen in the context of the ligand-protein complex can result in protein inactivation. Of course, for modest affinity ligands, a good deal of singlet oxygen will be generated by

unbound ligand, but the short diffusion radius of singlet oxygen limits the amount of non-specific protein damage caused by these events.

CALI allows protein ligands that are not intrinsic inhibitors to be employed as photo-triggered inhibitors. Modest potency inhibitors generally evince higher apparent potencies because of the different mechanism of protein inhibition, which no longer requires constant association of the compound with the protein (i.e., the compound can employ a “hit and run” mechanism). CALI technology has not been used widely for protein inhibition due to difficulties caused by inefficient, singlet-oxygen-sensitive chromophores. Recently however, two new CALI “warheads”, eosin^[10] and a Ru(II)(bpy)₃²⁺ complex^[8, 14], have been reported to provide superior results and these were employed in this study. We hoped that equipping the modest affinity RBBP9-binding peptoids with CALI warheads would transform them into reasonably potent photo-triggered inhibitors that would be useful tool compounds without the requirement for tedious optimization.

Indeed, using labeling of RBBP9 by an FP-Rh or FP-biotin conjugate as an activity assay, we demonstrated that moderately potent inhibition of the purified protein could be observed in the presence of one of the peptoid-chromophore conjugates and visible light (Figure 4). The data show that under the conditions employed, the Ru(II) photo-peptoid exhibited nearly complete inhibition of RBBP9 reaction with the activity reagent at a concentration of approximately 500 nM (Figure 4), whereas the fluorescence polarization data indicate that almost 10 μ M of the peptoid is required to saturate the protein. This 20-fold differential between the apparent potency of the photo-inhibitor relative to the equilibrium dissociation constant was attributed to the fact that the enzyme is deactivated through covalent modification rather than simple binding of the compound to the enzyme. Actually, this 20-fold effect was more modest than has been observed in similar experiments previously using different peptoids targeted to different proteins, where effects of several hundred-fold were observed.^[14] This could be due to RBBP9 being less sensitive to singlet oxygen or to the fact that peptoid **1** carries an imidazole side chain, which is likely to react with singlet oxygen, possibly leading to reduced affinity for RBBP9. These issues, which were tangential to the central question addressed in this paper, will be addressed in a future study. The important point is that a sub-micromolar inhibitor of RBBP9 was obtained without the requirement for tedious optimization of the primary screening hit.

To address the selectivity of Ru(II)-**6** for photo-triggered inactivation of RBBP9 relative to other serine hydrolases, an ABPP experiment was conducted in a HeLa cell extract doped with recombinant RBBP9. As shown in Figure 5, nearly complete inhibition of the reaction of RBBP9 with the FP-Rh reagent was observed in the presence of 450 nM Ru(II)-**6** when the extract was irradiated with visible light. This effect was dose-dependent, as little inhibition of activity was observed at 10-fold or 100-fold lower Ru(II)-**6** concentrations. As hoped, little effect was seen on the labeling of other serine hydrolases in the extract at 450 nM Ru(II)-**6**, suggesting that selective binding of the peptoid to RBBP9 delivers the photo-triggered warhead to this enzyme selectively, greatly limiting damage to other proteins in the extract. In contrast, irradiation of a “naked” Ru(II)(bpy)₃²⁺ complex, or a conjugate of the warhead with a scrambled peptoid that does not bind RBBP9 resulted in significant non-selective inhibition of many serine hydrolases (Figure 5, compare lanes 4 and 6 with lane 12). This was somewhat surprising as we had expected that the Ru(II)(bpy)₃²⁺ complex or the scrambled peptoid would have little effect on any of the hydrolases at these concentrations. Yet the data show that the peptoids **1** and **6** appear to suppress non-selective reactions. These data underscore the importance of isolating highly selective protein ligands in the screening step.

The data shown in this paper support the idea that Ru(II)(bpy)₃²⁺ conjugates of peptoids identified in a simple binding screen using an activity-labeled serine hydrolase target are reasonably potent and selective inhibitors. The relative ease and low expense of this protocol suggests that it is indeed a viable option for the high-throughput and massively parallel development of selective photo-triggered inhibitors of large numbers of proteins in this class.

This study thus establishes proof of concept for several facets of a comprehensive strategy to develop tool compounds against large numbers of serine hydrolases. However, there remain some important questions to be addressed in future work. Labeling of the native RBBP9 could not be detected in HeLa cells (Figure 5B, lane 1) and MDA-435 cells (Figure 6C), presumably due to its low level in the extract. We also failed to detect peptides derived from native RBBP9 when a mass spectrometry-based ABPP analysis was conducted with this extract (data not shown). Thus, we have been unable to validate the ability of Ru(II)-6 to inactivate native RBBP9 in living cells when irradiated. This important question could be addressed in future studies targeting more abundant hydrolases. However, it is worthwhile mentioning that the Ru(II)(bpy)₃²⁺ moiety is cell permeable,^[14] as are peptoids in general.^[8] Therefore, we anticipate that these conjugates will likely be valuable in studies of the role of intracellular serine hydrolases in cultured cells, but this point remains to be demonstrated experimentally. Of course, even assuming that this is true, the utility of these compounds will be limited to studies of the proteins in cultured cells, not whole organisms such as mice, since the wavelengths of light required to trigger singlet oxygen production via the Ru(II)(bpy)₃²⁺ chromophore do not penetrate significantly into mammalian tissue. However, for studies in some model organisms, such as the optically transparent zebrafish embryo, peptoid-Ru(II)(bpy)₃²⁺ conjugates would be expected to be of significant utility.

Another question mark is the ability to carry out massively parallel screens by exposing a bead library to multiple serine hydrolases labeled with an activity-based agent such as FP-biotin. The issue here will be whether the level of native serine hydrolases will be high enough to allow for visualization of the beads that display ligands that bind them. Another challenge will be to assign hits to a particular SH if there are many such proteins tagged in the mixture. It may be possible to do this by mass spectrometric analysis of the protein bound to the bead or via an ABPP-based profiling experiment for each strong hit identified in the screen.

Finally, while this study employed peptoid libraries as a source of SH ligands, we have begun to report the development of second generation libraries of peptoid-inspired oligomers^[15] that retain many of the favourable features of these compounds, including ease of library synthesis, but contain much greater chemodiversity in the backbone and incorporate significant conformational constraints. These libraries may provide higher affinity initial hits than do the “floppy” peptoids. Others have reported the creation of complex cyclic peptide libraries^[16] and encoded small molecule libraries^[17] on TentaGel beads and these are potential sources of higher affinity ligands as well.

Experimental Section

Syntheses of peptoid libraries

A tetrameric “one bead one compound” peptoid library^[6] was synthesized using the split-and-pool method on TentaGel HL NH₂ beads (0.6 g, ~ 0.3mmol) by a microwave-assisted protocol.^[18] A linker sequence including a methionine for cyanogen bromide cleavage and two constant Nlys or Nmpa residues preceded the peptoid library. Twenty amines with diverse functional groups were used as building blocks. The synthesized library has a theoretical diversity of 20⁴ = 160, 000 compounds. Briefly, TentaGel beads swelled in N,N-

Dimethylformamide (DMF) were treated with Fmoc-Met-OH, HBTU, HOBT, and NMM overnight followed by piperidine deprotection. The beads were then treated with 1 M bromoacetic acid and 1 M diisopropylcarbodiimide (DIC) in anhydrous DMF. The reaction was performed in a microwave oven set to 10% power (2×15 sec). After washing with DMF, the beads were treated with primary amine (2 M) in the microwave oven as described above. The beads were washed with DMF and distributed equally into ten peptide synthesis reaction vessels after adding two spacers (Nlys-Nlys or Nmpa-Nmpa). The coupling procedure was repeated until the desired length was achieved. The completed library was incubated with a cocktail of 95% trifluoroacetic acid (TFA), 2.5% triisopropylsilane and 2.5% water for 2 hours to remove side chain protecting groups, and then the library beads were washed thoroughly with DMF and dichloromethane (DCM). The solvent was drained and the library beads were dried completely on a lyophilizer and stored at 4 °C.

Preparation of FP-biotinylated RBBP9

Recombinant RBBP9 was expressed and purified following the published protocol.^[5] RBBP9 (20 μ M) was incubated with FP-biotin (60 μ M) in PBS buffer (2.5 mL) for 1 h at 25 °C. The reaction solution was then passed through a PD-10 column to remove unreacted FP-biotin. The concentration of final collected FP-biotinylated RBBP9 was 14 μ M in PBS buffer (3.5 mL).

The labeling efficiency was evaluated with MALDI mass spectrometry. Briefly, final FP-biotinylated RBBP9 solution (2 μ L) was mixed with 0.1% TFA (10 μ L). Millipore ZipTip C₄ pipette tips were used to desalt and concentrate the protein. Sinapic acid (20 mg/mL) prepared in ACN : 0.1% TFA (7:3, v/v) was used as the MALDI matrix to detect RBBP9. The labeling was also confirmed using Western blotting. Briefly, 2 μ L of the final FP-biotinylated RBBP9 solution was diluted with PBS buffer to 20 μ L and then mixed with 7 μ L of SDS-PAGE gel loading buffer (Invitrogen). On one gel, 20 μ L of samples was loaded, electrophoresed and then subjected to Coomassie blue staining; on the other gel, 2 μ L of sample was loaded, electrophoresed and then detected by blotting with streptavidin-HRP as described previously.^[12]

The FP-biotinylated RBBP9 (20 μ L) prepared as described above was diluted with 5% BSA in TE buffer to a final volume of 600 μ L in a 1.5 mL centrifuge tube, followed by the addition of SA-MB (50 μ L Pierce). After incubation for 1 hour at 25 °C, the magnetic beads were collected with a magnetic separation stand for 1.5 mL centrifuge tubes (Promega). The beads were pooled on the side of the centrifuge tube when the centrifuge tube was positioned on the magnetic stand. The supernatant was removed with a pipette tip, and TBST buffer was added to wash the beads. After thoroughly washing five times with TBST buffer, 500 μ L NH₄HCO₃ (50 mM, pH 8.5) containing 0.3 μ g of trypsin was added to the beads. The trypsin digestion was allowed to proceed for 12 hr on a rotator at 37 °C. Supernatant was collected on the magnetic stand, and dried in a Speedvac concentrator. Samples were reconstituted in 20 μ L of 1% TFA and desalted with ZipTip C₁₈ (Millipore) according to the manufacturer's protocol.

The same concentration of RBBP9 without FP-biotin labeling was used as control in the experiments described above.

Library screening and identification of hits

The library beads were further washed with PBS buffer (20×5 ml) and TBST buffer (20×5 ml). Before screening, the library beads were transferred to a 15 mL conical centrifuge tube and equilibrated in TBST buffer overnight. The beads were blocked with E. coli lysate (1 mg/mL) at room temperature for 1 hour. The lysate was removed, and the beads were

washed with TBST twice. For pre-screening, 200 μ L of SA-MB were incubated with the library beads in 10 mL of TBST buffer for 1 hour at 25 °C. The conical centrifuge tube was placed in a magnetic bead separator (DynaMag-15 magnet, Invitrogen).^[7] Beads that retained the SA-MB were held on the side of the tube. These bead-displayed peptoids exhibited interactions with magnetic beads that would interfere with subsequent screening against the target protein. These were discarded. The beads that remained at the bottom of the conical tube were collected. The pre-screening process was repeated twice to completely remove the undesired binders.

After pre-screening, the library was washed with TBST five times, and equilibrated in TBST overnight. The beads were blocked with *E. coli* lysate (1 mg/mL) at room temperature for 1 hour. Then *E. coli* lysate was removed by centrifugation (100 g, 5 min). The library beads were incubated with 10 mL of *E. coli* lysate (1 mg/mL) containing FP-biotinylated RBBP9 (1.5 μ M, 0.03 mg/mL) for 2 hrs at 25 °C, following by TBST washing five times. 200 μ L of SA-MB were incubated with the library beads in 10 mL of TBST buffer in for 1 hour at 25 °C. Again, beads that retained the SA-MB were held on the side of the tube, which were the “hit” beads. The beads that remained at the bottom of the conical tube were transferred to another tube, and another 200 μ L of SA-MB were used to probe the library again in case the first round missed some hits.

The “hit” beads (~500) were washed with 70% acetonitrile (ACN) + 0.1% TFA, and then heated at 95 °C for 10 min to remove proteins and any other bead-bound species. The beads were then washed with TBST thoroughly and equilibrated with TBST overnight. The smaller amount of beads were then blocked with *E. coli* lysate (1 mg/mL) for 1 hour, followed by incubation with 2 mL of *E. coli* lysate (1 mg/mL) containing FP-biotinylated RBBP9 (5 μ M) for 1 hour at 25 °C. After washing with TBST buffer five times, the beads was incubated with Streptavidin Qdot 655 (20 nM, Invitrogen) in TBST buffer (1 mL) for 1 h at 25 °C. The peptoids that bind to the FP-biotinylated RBBP9/Streptavidin Qdots were visualized under a fluorescence microscope by irradiation of the beads through a DAPI filter (390–410 nm band pass filter). The beads appearing as red ringed discs were picked up using a micropipette. Beads were then transferred, one by one, into the wells of a 96-well plate, followed by rinsing with ACN + 0.1% TFA. 100 μ L of cleavage solution (0.6 M CNBr, ACN : acetic acid : H₂O 5 : 4 : 1, v/v/v) was then added to each well. The plates were covered and gently shaken overnight at 25 °C. The cleavage solution was evaporated in a Speedvac concentrator. The dried residuals were reconstituted in 10 μ L of water, and 0.3 μ L was taken and mixed with 0.3 μ L of matrix (-Cyano-4-hydroxycinnamic acid, 25 mM in ACN : 0.1% TFA, 1:1, v/v). The mixture was spotted on a MALDI plate and sequenced by MALDI-TOF-TOF mass spectrometry.

Synthesis of fluorescein-Hits and fluorescence polarization

After removing the Fmoc protecting group on Knorr Amide MBHA resin (capacity: 0.78 mmol/g), Fmoc-Cys (tert)-OH was conjugated to the beads, followed by the peptoid residues involved in the Hits with the microwave-assisted protocol in a scale of 5 μ mol, respectively. The resulting compounds were cleaved from the resin with cleavage cocktail (95% TFA, 2.5% water and 2.5% triisopropylsilane). TFA was evaporated and the crude products were reconstituted in PBS buffer (10 mL) and the pH was adjusted to pH 7.2 with NaOH (1M). Then, 500 μ L of Fluorescein-5-maleimide (10 mM, prepared in DMSO) was added to the peptoids and the mixture was incubated at 25 °C on a rotator overnight in the dark. The Fluorescein-labeled peptoid was purified by RP-HPLC and characterized by MALDI mass spectrometry. The fluoresceinated hits (10 nM) was incubated with indicated concentrations of RBBP9 in the presence of either BSA (1 mg/mL) or tween-20 (0.1%) in PBS buffer (pH 7.4) in a final volume of 120 μ L at 25 °C for 2h in the dark. The fluorescence polarization was measured on a Panvera Beacon 2000 instrument (Invitrogen).

Gel-based ABPP experiments with recombinant enzyme

Recombinant RBBP9 (400 nM) in assay buffer was incubated with DMSO or the indicated compound for 1 hr at 25 °C before the addition of FP-Rh at a final concentration of 1 μ M in 20 μ L total reaction volume. The reaction was incubated for 20 min at 25 °C, quenched with 4 $\times$ SDS-PAGE loading buffer (Invitrogen), separated by SDS-PAGE and visualized in-gel using a flatbed fluorescence scanner (Typhoon 9410, Amersham Biosciences). The percentage activity remaining was determined by measuring the integrated optical density of the bands with software ImageJ 1.44p.

Competitive ABPP assays in proteomes

The soluble HeLa cell proteome was diluted to 1 mg/ml in PBS. Recombinant RBBP9 (400 nM) was added for comparison. The proteome was pre-incubated with either DMSO or candidate inhibitor at the indicated concentration in a 50 μ L reaction volume for 1 hr at 25 °C. FP-rhodamine was then added at a final concentration of 1 μ M. After 20 min, the reactions were quenched and analyzed as described above.

Western blotting of RBBP9 in MDA-435 cells

Cell lysates with or without doped-in RBBP9 were treated as described in the section of competitive ABPP assay in proteomes above. As control, recombinant RBBP9 was also treated as described in the section of gel-based ABPP experiments with recombinant enzyme. Then samples (15 μ L) were separated by 4–20% SDS-PAGE and transferred onto a PVDF membrane. The membrane was blotted with primary anti-RBBP9 monoclonal antibody (Novus Biologicals) followed by secondary conjugated antibody (goat anti-mouse IgG horseradish peroxidase, Bio-Rad). Bands were detected using ECL Western Blotting Substrate analysis system (Pierce, Rockford, IL).

Preparation of ruthenium-conjugated peptoids

Ruthenium-azide. To a stirred solution of Bis-(2,2'-bipyridine)-4'-methyl-4-carboxybipyridin-ruthenium-N-succinimidylester-bis-(hexafluorophosphate) (5.0 mg, 0.005 mmol, in 3 mL DMF) was added 4-azidopropylamine (0.025 mmol),^[9] and DIPEA (0.025 mmol). After stirring for 6 hr at 25 °C, the reaction mixture was diluted with dH₂O (0.1 % TFA, 3 mL) and directly purified by preparative HPLC. Fractions containing product were lyophilized to afford the desired compound (0.004 mmol) as a red solid. IonTrap: [M]²⁺ calculated, 355.1; observed, 355.1.

Ru (II)-6

To a stirred solution of ruthenium-azide (0.004 mmol) in DMSO (300 μ L) was added 30 μ L of DIEA, alkyne-hit6 (0.008 mmol) and Tetrakis(acetonitrile)copper hexafluorophosphate (0.0004 mmole, 10 mole %). The reaction mixture was microwaved (100 % power) for 20 sec, and allowed to stir at 25 °C for 1 h. This sequence was repeated four times before quenching the reaction with 0.1 % TFA (3 mL). The mixture was applied directly to the preparative HPLC to give 0.0022 mmol of Ru-Hit6. MALDI / TOF: [M]⁺ calculated 1681.80, observed 1681.51. Further purification on analytical HPLC was performed before biological evaluation. After HPLC purification, the pH of the fraction containing the desired compound was adjusted to 7.2 by addition of NaOH and then dried on Speedvac concentrator. The dried compound was redissolved in DMSO, and the concentration was determined by measuring the absorbance at 456 nm, which is the specific absorbance peak for ruthenium. A standard curve was generated by plotting the UV absorbance at 456 nm of Tris (2,2'-bipyridyl) dichlororuthenium (II) hexahydrate at the indicated concentrations on a Nanodrop 2000C spectrophotometer (Thermo).

Eosin-1

Hit peptoid with a C-terminal cysteine residue was synthesized as described above. After cleavage, TFA was evaporated and the crude products were redissolved in PBS buffer (10 mL) and the pH was adjusted to pH 7.2 with NaOH (1M). Then, 500 μ L of Eosin-5-maleimide (10 mM, prepared in DMSO) was added to the peptoids and the mixture was incubated at 25 °C on a rotator overnight in the dark. The Eosin-1 was purified by RP-HPLC and characterized by MALDI mass spectrometry. MALDI / TOF: $[M]^+$ calculated 1851.19, observed 1851.00. Further purification on analytical HPLC was performed before biological evaluation. After HPLC purification, the fraction was neutralized with NaOH to pH 7.2 and dried on Speedvac concentrator. The dried compound was redissolved in DMSO, and the concentration was determined by measuring the absorbance at 540 nm, which is the specific absorbance peak for eosin. A standard curve was generated by plotting the UV absorbance at 540 nm of eosin at indicated concentration on a Nanodrop 2000C spectrophotometer (Thermo)

Visible light irradiation

Irradiation was done using a 240-W xenon arc lamp (Oriel). Light was filtered first through distilled water (10 cm) and then through a 380 to 2,500 nm cut-on filter (Oriel). Samples were positioned at a 20 cm distance from the light source and irradiated for 10 min.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Activity based labeling of RBBP9 with biotinylated fluorophosphonate probe. A) General mechanism for covalent modification of serine hydrolases active site by fluorophosphonate-biotin. B) Whole protein mass spectra for unmodified and FP-biotin modified RBBP9. C) Gel analysis for the recognition of FP-biotinylated RBBP9 by Streptavidin.

Figure 2.

Mass spectra of on-bead tryptic digestion from Streptavidin-coated magnetic beads after incubation with RBBP9. A) Streptavidin-coated magnetic beads were incubated with unmodified RBBP9. B) Streptavidin-coated magnetic beads were incubated with FP-biotinylated RBBP9. C) Mass spectra of a standard in-solution RBBP9 digestion.

Figure 3. Structure of the hit compounds identified from the screening and the binding affinity evaluation by fluorescence polarization assay. A) Fluorescein-1. B) Fluorescein-6.

Figure 5. Selective inhibition of RBBP9 in cellular proteome. Evaluation of A) Ru(II)-6, B) Eosin-1 by competitive ABPP in soluble proteome of HeLa cells (1 mg/mL). Recombinant human RBBP9 (400 nM) was doped into this proteome for comparison.

Figure 6.

Photo-inactivated RBBP9 by Ru(II)-6 does not react with ABPP probe. A) The photo-inactivated RBBP9 does not response to Coomassie blue staining. Silver staining and Streptavidin-HRP blotting suggest that photo-damaged RBBP9 does not react with FP-biotin. B) Mass Spectra of RBBP9 labeling with FP-Biotin after incubation with Ru(II)-6 in the absence of presence of light activation. C) No endogenous level of RBBP9 was detected by western-blotting with anti-RBBP9 antibody. For the recombinant RBBP9 and RBBP9 doped in cell proteome complex, a ladder of higher molecular weight bands observed with increased Ru(II)-6 upon light activation.

Scheme 1.

Screening Strategy of ABPP Probe facilitated Peptoid Library Screening against Serine Hydrolases. RBBP9 was allowed to react with FP-biotin to yield the active site biotinylated protein. The FP-biotinylated RBBP9 was incubated with OBOC peptoid library. The hit compounds that bound to RBBP9 can be pull out with Streptavidin-coated magnetic beads by Streptavidin-biotin interaction.

Scheme 2.

Synthesis of a 4-mer peptoid library with 20 different amines (diversity: 160,000) for screening.

Scheme 3.

Synthesis of photo-activated inhibitors. A) Synthesis of Ru(II)-6 via alkyne-azide click reaction. B) Synthesis of Eosin-1 through the reaction of maleimide and thiol.