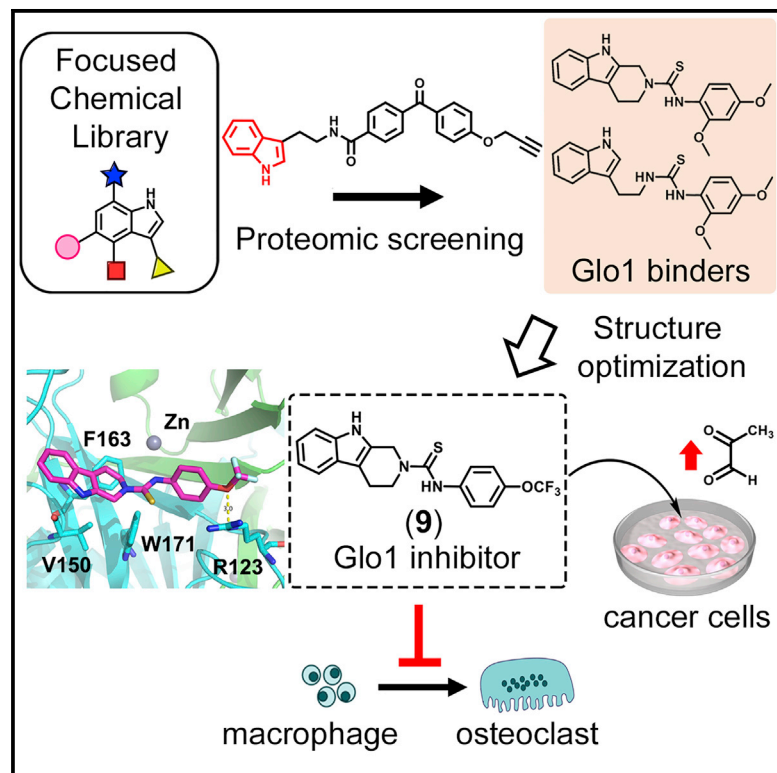


Cell Chemical Biology

Chemoproteomic Profiling of a Pharmacophore-Focused Chemical Library

Graphical Abstract



Authors

Louvy Lynn Punzalan, Lulu Jiang, Di Mao, ..., Makiya Nishikawa, Lu Zhou, Motonari Uesugi

Correspondence

zhoulu@fudan.edu.cn (L.Z.),
uesugi@scl.kyoto-u.ac.jp (M.U.)

In Brief

Punzalan et al. applied a chemical proteomic approach to screen a focused chemical library for new biologically active molecules. The study resulted in the identification of a Glo1 inhibitor with a unique binding mode of inhibition.

Highlights

- A competition protein profiling was applied to screen a focused chemical library
- Occurrence of probe-labeling enhancement of the target protein was observed
- Molecule 9 revealed an unknown small-molecule binding mode for Glo1 inhibition
- Molecule 9 increased cellular MG level and suppressed osteoclast formation

Article

Chemoproteomic Profiling of a Pharmacophore-Focused Chemical Library

Louvy Lynn Punzalan,¹ Lulu Jiang,² Di Mao,¹ Amarjyoti Das Mahapatra,¹ Shinichi Sato,¹ Yasushi Takemoto,¹ Mari Tsujimura,³ Kosuke Kusamori,³ Makiya Nishikawa,³ Lu Zhou,^{2,*} and Motonari Uesugi^{1,2,4,5,*}

¹Institute for Chemical Research, Kyoto University, Uji, Kyoto 611-0011, Japan

²School of Pharmacy, Fudan University, Shanghai 201203, China

³Faculty of Pharmaceutical Sciences, Tokyo University of Science, 2641 Yamazaki, Noda, Chiba 278-8510, Japan

⁴Institute for Integrated Cell-Material Sciences (WPI-iCeMS), Kyoto University, Uji, Kyoto 611-0011, Japan

⁵Lead Contact

*Correspondence: zhoulu@fudan.edu.cn (L.Z.), uesugi@sci.kyoto-u.ac.jp (M.U.)

<https://doi.org/10.1016/j.chembiol.2020.04.007>

SUMMARY

Pharmacophore-focused chemical libraries are continuously being created in drug discovery programs, yet screening assays to maximize the usage of such libraries are not fully explored. Here, we report a chemical proteomics approach to reutilizing a focused chemical library of 1,800 indole-containing molecules for discovering uncharacterized ligand-protein pairs. Gel-based protein profiling of the library using a photo-affinity indole probe 1 enabled us to find new ligands for glyoxalase 1 (Glo1), an enzyme involved in the detoxification of methylglyoxal. Structure optimization of the ligands yielded an inhibitor for Glo1 (9). Molecule 9 increased the cellular methylglyoxal levels in human cells and suppressed the osteoclast formation of mouse bone marrow-derived macrophages. X-ray structure analyses revealed that the molecule lies at a site abutting the substrate binding site, which is consistent with the enzyme kinetic profile of 9. Overall, this study exemplifies how chemical proteomics can be used to exploit existing focused chemical libraries.

INTRODUCTION

Focused chemical libraries are designed based on biologically active pharmacophores to target a specific protein or family of proteins (Harris et al., 2011). These chemical libraries are used by pharmaceutical companies and researchers to discover or optimize seed molecules. Despite the high cost and time-consuming synthesis, up-to-date chemical libraries are continuously being generated. Meanwhile, existing chemical libraries are subjected to a range of target-based and phenotypic screening to maximize their usage. In our case, an in-house indole-based chemical library (1,800 compounds) was exploited to screen for small molecules that disrupt protein-protein interactions via tryptophan. The screening eventually led to the discovery of wrenchnolol, a small molecule that impairs the expression of Her2 oncogene (Asada et al., 2003), and JI130, a chemical modulator of transcription factor Hes1 (Perron et al., 2018). Although both studies were successful in discovering small-molecule probes, a large fraction of the library remains unexplored.

The indole ring is considered as a “privileged structure” that is capable of binding to various receptors and enzymes (Welsch et al., 2010; de Sa Alves et al., 2009). This could be attributed to indole’s large hydrophobic surface, which could interact with hydrophobic pockets and surfaces of proteins, and to its N-H group, which could form key hydrogen bond in-

teractions. In fact, a number of biologically active natural products and currently used drugs have indole structures (Kaushik et al., 2013; Zhang et al., 2015a, 2015b). Because of the established biological importance of the indole group, we are keen to reuse our indole-focused library for finding new biologically active molecules. Instead of performing another target-based screening in which tryptophan or indole structures are known to play an important role, we embarked on a chemical proteomic approach to discovering unexpected targets of the indole structures.

Chemical proteomics has enabled the screening for a number of covalent ligands (Bachovchin et al., 2010; Zuhl et al., 2012; Cognetta et al., 2015; Suciú et al., 2018) and recently Cravatt and coworkers described how chemical proteomic analysis could assist the discovery of reversible ligands and their protein targets (Parker et al., 2017). In brief, they prepared several photo-affinity probes containing small-molecule fragments found in drugs, which are appended with diazirine and alkyne groups. Competition profiling with small molecules bearing the drug structures provided seed inhibitors for proteins involved in metabolism (Parker et al., 2017). In this study, we extend the chemoproteomic technique to screen our focused chemical library of 1,800 indole-containing molecules for uncharacterized bioactive ligands and to identify their protein targets in cells. The screening strategy unintentionally led to a Glo1 inhibitor with a unique inhibition mechanism.

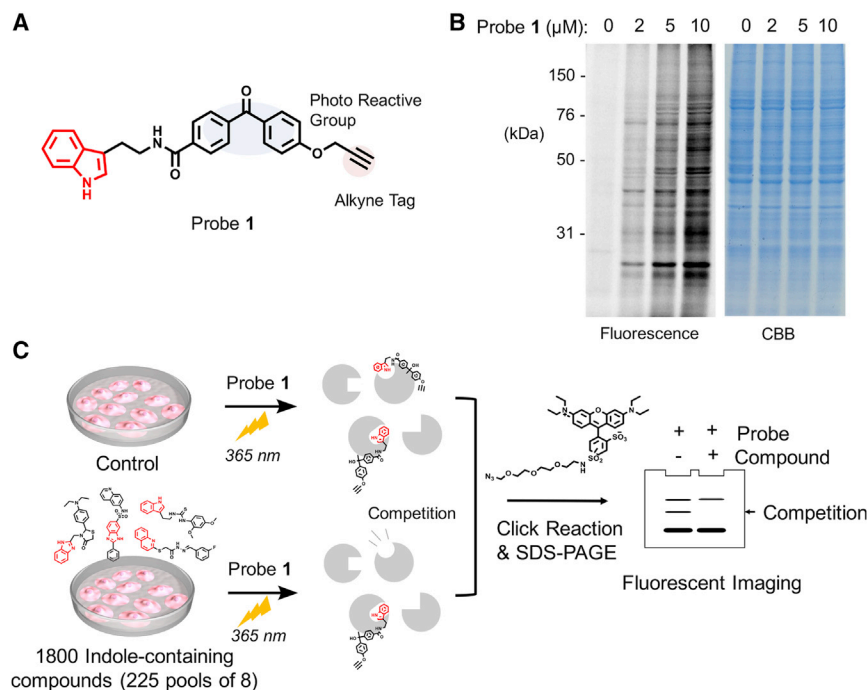


Figure 1. Proteomic Profiling to Screen for Indole-Based Ligands

(A) Structure of the photo-affinity indole probe **1**. (B) Fluorescent gel images showing the concentration-dependent protein labeling of probe **1**-interacting proteins. CBB, Coomassie blue. (C) Experimental workflow of the screening method.

RESULTS

Chemoproteomic Screening Identified Glo1-Binding Compounds **7oa3** and **7qc8**

We first prepared an indole-containing photo-affinity probe **1** (Figure 1A), in which an indole scaffold is equipped with a benzo-phenone for photo-crosslinking and an alkyne group for conjugation to an azide reporter tag. We then profiled the proteins that photo-react with probe **1** in HEK293 cells. The cells were treated with probe **1** for 30 min, irradiated with ultraviolet light, and lysed. The resulting cell lysate was reacted with sulforhodamine B-PEG3-azide via copper-catalyzed azide-alkyne cycloaddition (CuAAC) and was characterized by SDS-PAGE. Fluorescent images of the gel showed the concentration-dependent labeling of the various proteins that interact with probe **1** in HEK293 cells (Figure 1B). These probe **1**-binding proteins were subsequently identified by biotin pulldown experiment: the proteins labeled with probe **1** were reacted with biotin- N_3 , enriched using streptavidin-agarose beads, and submitted for shotgun liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis (Figure S1A). The proteins enriched by probe **1**, but not detected in DMSO-treated samples, vary from enzyme, receptors, and scaffolding proteins (Figure S1B). These proteins are the potential target proteins of the indole compounds in the chemoproteomic screening. We next introduced the library molecules as competitors of the probe during the protein profiling to search for small molecule-protein pairs (Figure 1C). For the screening purpose, a gel-based protein profiling was chosen because it is more accessible and cost-effective to profile a library of thousands of molecules. In this context, a compound that has stronger affinity to a probe **1**-binding protein will compete with the probe, resulting in the loss or decrease of fluorescence labeling of the protein in the gel profile. To speed up the screening process, 8 compounds (30 μ M each) from the library were randomly

combined giving a total of 225 test pools. HEK293 cells were co-treated with probe **1** (5 μ M) and the test pools ($\sim$ 240 μ M) for 30 min and subjected to the same treatments as mentioned above. Of the 225 test pools screened, only 2 pools, **7oa3** and **7qc8**, abolished the probe labeling of a protein whose approximate molecular weight is 25 kDa (Figure 2A). Deconvolution of the screening hit pools by examining the competition profile of the individual compounds identified **7oa3** and **7qc8** to be responsible for the observed competition (Figure 2B). Both compounds exhibited a dose-dependent

reduction in the probe labeling of the $\sim$ 25-kDa protein (Figure S1C), and these two compounds share an indole-thiourea structure (Figure 2C).

Encouraged by this result, we pursued the identification of the $\sim$ 25-kDa protein. The probe **1**-labeled proteins were separated by SDS-PAGE followed by silver staining. The protein band with the desired molecular weight was successfully enriched and was submitted for LC-MS/MS analysis (Figure 2D). The major protein identified was glyoxalase 1 or Glo1, a cytosolic enzyme involved in the detoxification of methylglyoxal (MG). Western blot analysis using a Glo1 antibody confirmed the $\sim$ 25-kDa band as Glo1 (Figure 2E). The enzyme was successfully enriched by probe **1** (5 μ M), and the co-treatment of the probe with the screening hits (50 μ M) resulted in the loss of Glo1 enrichment (Figure 2E), which is consistent with the results of the fluorescence gel profile. Noticeably, a band shift from 22 to $\sim$ 25 kDa was observed for Glo1, suggesting that the probe is covalently bound to the enzyme. The formation of a higher-molecular-weight protein upon covalent binding of a probe to a protein was similarly observed by other groups (Zhang et al., 2019).

To predict where the probe and the screening hits bind to Glo1, we performed competition profiling with reported Glo1 inhibitors. In our previous study, Glo1 was isolated as another target protein of the widely used anti-inflammatory drug, indometacin (Sato et al., 2007). This drug also contains an indole moiety in its structure (Figure S2A). We initially thought that the Glo1 binders may share the same binding site with indometacin but the competition profiling suggests otherwise (Figure S2B and S2C). The drug does not compete with probe **1**, proposing that the identified Glo1 binders, **7oa3** and **7qc8**, occupy a different binding site from indometacin. We also tested BBGC (Figure S2D), a cell-permeable competitive Glo1 inhibitor. Co-treatment of the probe and BBGC abolished the labeling of the $\sim$ 25-kDa band (Figure S2E) and eliminated the enrichment of Glo1 in

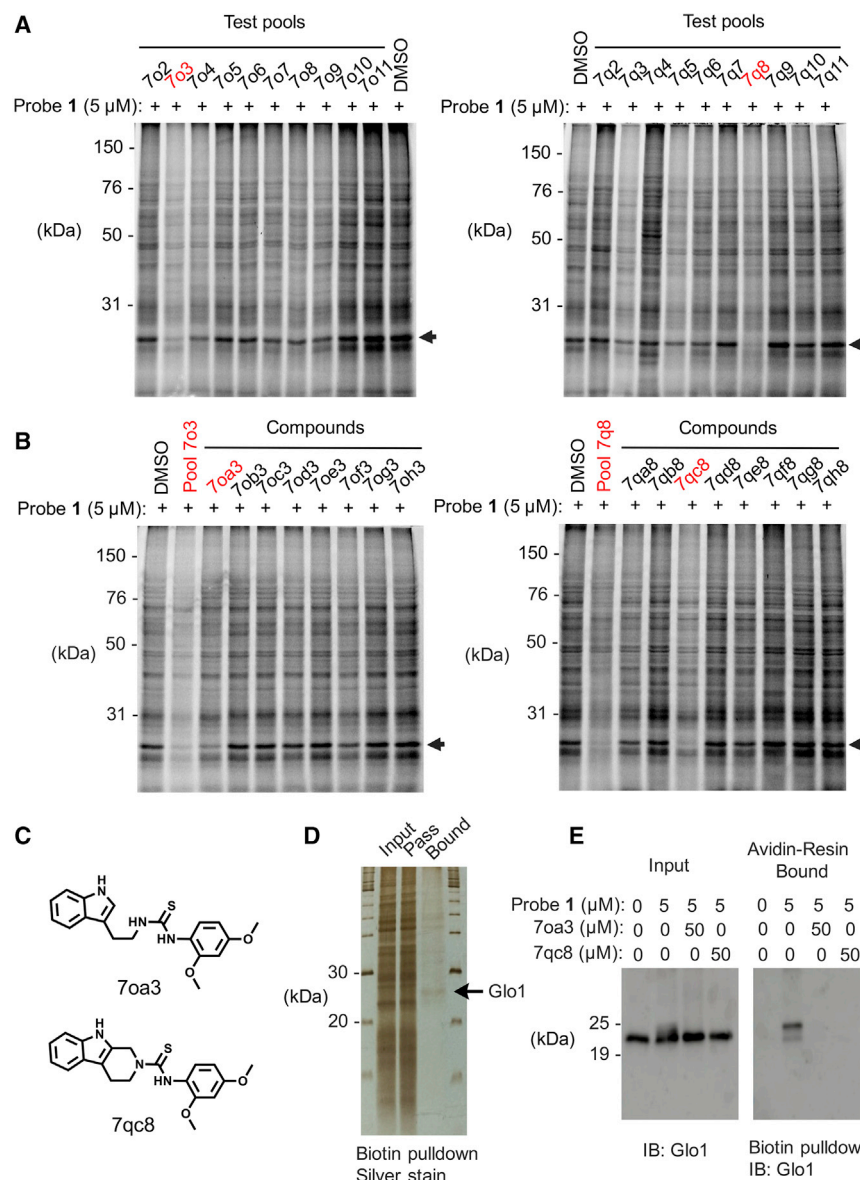


Figure 2. Discovery of Glo1-Binding Compounds 7oa3 and 7qc8

(A) Fluorescence gel images of HEK293 cells co-treated with probe 1 (5 μM) and various test pools (eight compounds each, ~240 μM): 7o2-7o11 test pools (left gel), 7q2-7q11 test pools (right gel). The loss of the ~25-kDa band (black arrow) was observed in the presence of test pools, 7o3 and 7q8.

(B) Fluorescence gel images of HEK293 cells co-treated with probe 1 (5 μM) and the individual compounds (30 μM) of 7o3 (left gel) and 7q8 (right gel).

(C) Chemical structures of the screening hits, 7oa3 and 7qc8.

(D) Silver-stained gel image of proteins enriched by biotin pull-down of probe 1. The ~25-kDa band (black arrow) was identified as Glo1.

(E) Western blot analysis showed the enrichment of Glo1 by probe 1 (5 μM), which was abolished in the presence of 7oa3 or 7qc8 (50 μM).

methacin, probe 1 and the screening hits showed marginal hGlo1 inhibition (20%–30%) at 5 μM (Figure 3B).

Since the binding ligands exhibited low functional activity, several indole-thiourea compounds were synthesized with the goal of finding a more effective hGlo1 inhibitor (Figures 3C and 3D). The indole and thiourea groups were maintained while various phenyl substituents were evaluated (2–9). Most of the derivatives prepared have the same inhibitory activity as the screening hits, especially the 7oa3 series (2–5). For 7qc8, replacement of the 2,4-dimethoxy groups with 2,4-dichloride (7) resulted in substantial improvement in hGlo1 inhibition (half maximal inhibitory concentration [IC₅₀] = 6.8 μM), while introduction of *p*-trifluoromethoxy (9) achieved the most significant hGlo1 inhibition (IC₅₀ = 2.7 μM). The rigid structure of the

tetrahydropyridindole appears to enhance the hGlo1 inhibition, as the *p*-trifluoromethoxy counterpart of 7oa3 (5) displayed a weaker inhibition.

Structure Optimization of 7oa3 and 7qc8 Yielded Glo1 Inhibitor 9

We proceeded to evaluate if probe 1 and the screening hits could modulate the activity of human Glo1 (hGlo1). Glo1 is part of the glyoxalase system that carries out the detoxification of MG, a reactive dicarbonyl that is formed during glycolysis (Figure 3A) (Thornalley, 1993, 1996). Glo1 catalyzes the isomerization of the hemithioacetal formed from the non-enzymatic reaction of MG and glutathione (GSH). The resulting thioester, S-D-lactoylglutathione is then hydrolyzed to D-lactate by glyoxalase 2, Glo2. Glo1's activity was measured *in vitro* by monitoring the initial formation of S-D-lactoylglutathione spectrophotometrically. Indometacin, which has a Glo1 inhibitory activity (K_i = 18.1 μM), was used as a positive control. Similar to indo-

A second set of derivatives was prepared based on the structure of 9 (10–12). Removing the indole ring (10) lost the hGlo1 inhibitory activity and adding a methoxy group at C5 of the indole ring (11) reduced the hGlo1 inhibition. This implies the importance of the indole ring for the hGlo1 inhibitory activity of these compounds. Separating the indole group from the pyrido ring (12) showed less inhibitory activity, reinforcing the favorable effect of the rigid structure of the tetrahydropyridindole on hGlo1 inhibition. Other types of substituents on the phenyl ring were further explored (13–16). Introduction of *p*-methoxy (13) showed a weaker hGlo1 inhibitory activity, while placing *p*-trifluoromethyl (14) *p*-nitro (15), and 3,5-bis(trifluoromethyl) (16) exhibited similar inhibitory activity as 9 (IC₅₀ of 14 = 4.7 μM, IC₅₀ of 15 = 2.3 μM, IC₅₀ of 16 = 2.9 μM). These results highlight the

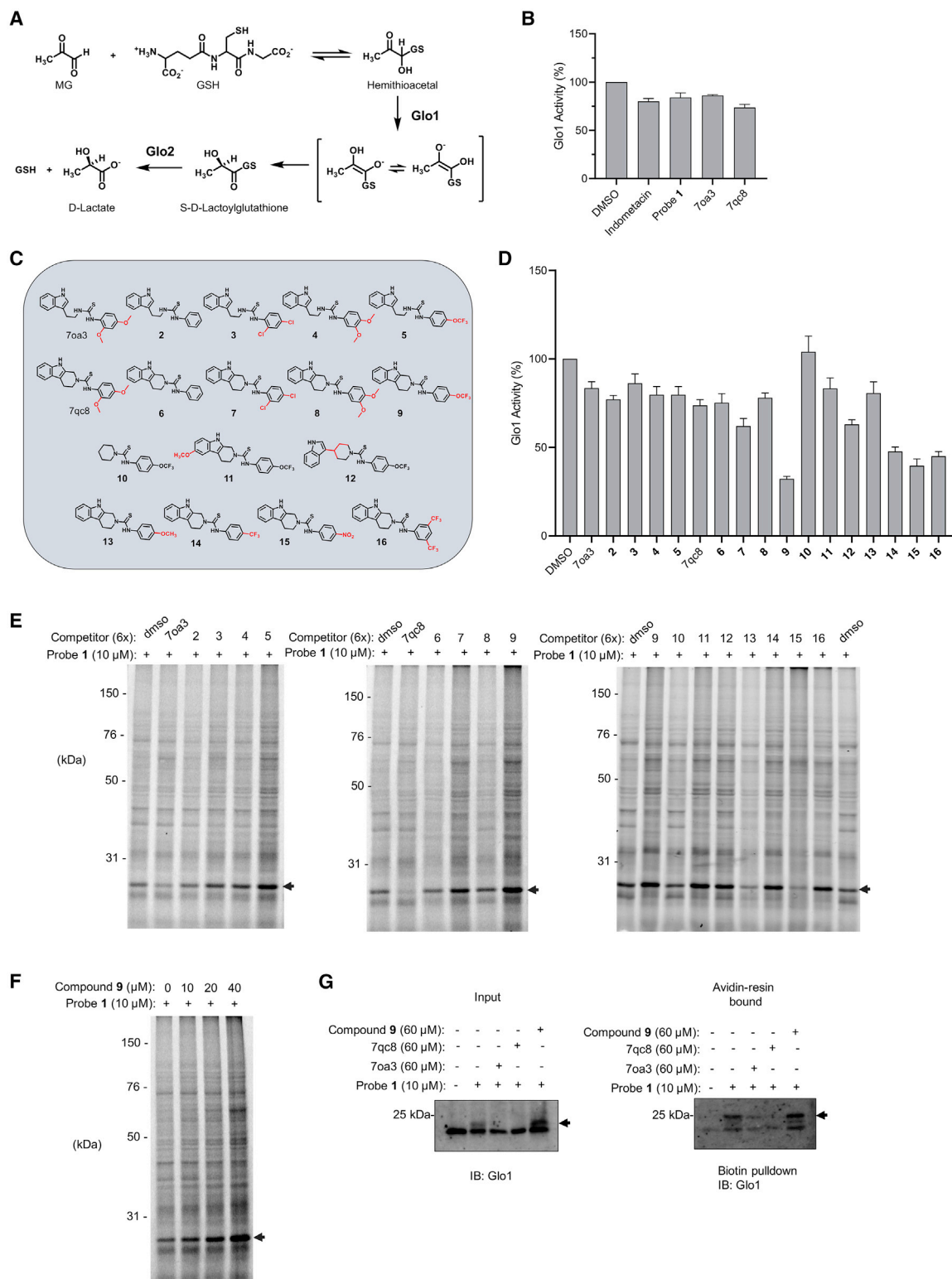


Figure 3. Glo1 Activity, Structure Optimization, and Protein Profiling of 7oa3 and 7qc8 Derivatives

(A) Detoxification of methylglyoxal (MG) by the glyoxalase system.

(B) The activity of recombinant human Glo1 in the presence of indomethacin, probe 1, 7oa3, and 7qc8 (5 μ M each). Data represent average values $\pm$ SD (n = 3).

(C) The structure of compounds 2–16. The modified groups are indicated in red.

(D) The activity of recombinant human Glo1 in the presence of compounds 2–16 (5 μ M). Data represent average values $\pm$ SD (n = 3).

(legend continued on next page)

advantage of placing high electron withdrawing groups on the phenyl ring for the hGlo1 inhibitory activity of the tetrahydropyridindole-thiourea series.

We analyzed the gel profile of the derivatives to determine if they interact with Glo1 in HEK293 cells. To our surprise, the compounds exhibited a different competition profile than the screening hits, 7oa3 and 7qc8. An unusual fluorescence enhancement was observed for the ~25-kDa protein upon the co-treatment of the probe and some of the derivatives (**5**, **9**, **11**, **12**, **14**, and **16**) (Figure 3E). We further evaluated molecule **9**, the compound which is the most potent *in vitro*, and observed a concentration-dependent enhancement of probe labeling of the ~25-kDa band (Figure 3F). Biotin pulldown in the presence of molecule **9** confirmed that the enhanced band is Glo1, wherein a higher amount of the enzyme was enriched by the probe when co-treated with molecule **9** (Figure 3G). We can infer from these results that molecule **9** and some of the other derivatives predominantly interact with Glo1 in HEK293 cells but the binding site is different from that of the probe and the screening hits, 7oa3 and 7qc8. Modifications in the structure of the screening hits could have shifted the binding site of the derivatives.

Glo1 Inhibitor **9** Exhibits Tight Binding and Mixed Type of Inhibition

To understand the binding and enzyme inhibition mechanism of the tetrahydropyridindole-thiourea scaffold, compound **9** was subjected to further biochemical assays. Isothermal titration calorimetry (ITC) experiments revealed that **9** has a strong binding affinity to hGlo1 with an apparent K_d of 81 nM (Figure S3). Kinetic studies of **9** showed a concentration-dependent decrease of V_{max} and increase of K_m , which is a property of a mixed-type inhibition (Figure 4A). Mixed-type inhibition is perceived as a “mixture” of competitive inhibition and uncompetitive inhibition (Figure 4B), wherein the inhibitor could bind both to the free enzyme (competitive) and the enzyme-substrate complex (uncompetitive) but has greater affinity toward one state over the other (Ramsay and Tip-ton, 2017). We estimated the inhibitory constant K_i (E) and K'_i (ES) using a graphical method and found the values to be ~1.32 and ~4.02 μ M, respectively (Figures 4C and 4D). Most Glo1 inhibitors, such as the glutathione derivatives (More and Vince, 2006, 2009; Jin et al., 2017), curcumins (Liu et al., 2010; Yuan et al., 2011), flavonoids (Takasawa et al., 2008, 2011), methyl-gerfelin (Kawatani et al., 2008), 18- β -glycyrrhetic acid (Zhang et al., 2015a, 2015b), and small molecules with metal-binding pharmacophores (Chiba et al., 2012; Al-Balas et al., 2016; Perez et al., 2019), behave as competitive inhibitors. Molecule **9** represents the first reported mixed-type hGlo1 inhibitor.

Molecule **9** Lies at a Site Abutting the Substrate Binding Site

Glo1 forms a swapping dimer that creates two active sites situated at the dimer interface (Cameron et al., 1997). To verify the binding mode of **9**, we obtained the crystal structure of mouse

Glo1 (mGlo1) complexed with **9** at 1.95 Å resolution. We observed an electron density near one of the active site pockets of the Glo1 homodimer and could assign it to molecule **9** (Figures 5A and 5B). The final structural model contains one protein homodimer, two zinc ions, and one molecule of **9**. Most of the Glo1-inhibitor complexes in literature depict one inhibitor molecule bound to each active site (PDB: 2ZA0, 1QIN, 1QIP). However, Hu and coworkers have described a similar case as ours where only one active site of the Glo1 dimer is occupied by a small molecule (Zhai et al., 2013; Zhang et al., 2015a, 2015b). This could be due to the preparation of the enzyme-inhibitor complex and the solubility property of our molecule. In the model of the mGlo1-**9** complex (Figure 5B), molecule **9** is found lying at the border of one of the active sites. The tetrahydropyridindole scaffold sits on the hydrophobic region formed by Phe₁₆₃, Val₁₅₀, and Trp₁₇₁, outside the substrate binding site, while the oxygen atom of the trifluoromethoxy phenyl group is located within the H-bond distance (3.0 Å) of the guanidinium side chain of Arg₁₂₃. Superimposition of the mGlo1-**9** complex with the hGLO1-HIPC-GSH complex, which mimics the interaction of the enediolate intermediate and Glo1 (Cameron et al., 1999), shows that molecule **9** is positioned adjacent to the substrate (Figure 5C).

We performed another kinetic study of molecule **9** with mGlo1 to determine if the inhibition mechanism is the same as that we observed in hGlo1. The kinetic profile suggests that molecule **9** behaves as an uncompetitive inhibitor of mGlo1 (Figures 5D and 5E), and thereby the inhibition by the molecule is enhanced in the presence of the substrate. The kinetics studies are in accord with the obtained crystal structure of mGlo1-**9**. The binding mode of **9** depicts that the enzyme can accommodate both the substrate and the inhibitor (Figure 5C). To support the proposed binding mode, we performed point mutation of the two key residues, Phe₁₆₃ and Arg₁₂₃, which are found to interact with molecule **9**, and of Lys₁₅₆, an amino acid in the substrate binding site that interacts with HIPC-GSH (Cameron et al., 1999) (Figure S4A). Although the F163G mutation resulted in a decrease in Glo1's enzymatic activity, all the mutants maintained the enzymatic activity (Figure S4B). We examined the ability of molecule **9** to inhibit the three mGlo1 mutants *in vitro*, and confirmed that Phe₁₆₃ is important for the inhibition mode of **9** as the molecule consistently displayed less inhibition against F163G mGlo1 (Figure 5F). Neither K156G or R123G, on the other hand, have a significant effect on the inhibitory activity of **9** because the molecule has retained its inhibition against the two mutants. From these results, Arg₁₂₃ seems dispensable in the inhibition mode of the molecule.

Molecule **9** Disrupts the Function of Glo1 in Cells

Finally, compound **9** was evaluated for its ability to inhibit Glo1's function in human A549 cells. As the major enzyme involved in the detoxification of MG, silencing or inhibition of Glo1 by shRNA or small-molecule inhibitors have been shown to increase cellular MG (Distler et al., 2012; Nokin et al., 2017; Stratmann et al., 2017;

(E) Fluorescence gel images of HEK293 cells co-treated with probe **1** (10 μ M) and derivatives **2–16** (60 μ M).

(F) Fluorescence gel image of HEK293 cells co-treated with probe **1** (10 μ M) and increasing concentration of molecule **9** (0, 10, 20, and 40 μ M). A concentration-dependent fluorescence enhancement was observed for the ~25-kDa protein.

(G) Western blot analysis showing the enrichment of the Glo1 in the presence of the probe **1** (10 μ M), which was further enhanced in the presence of molecule **9** (60 μ M).

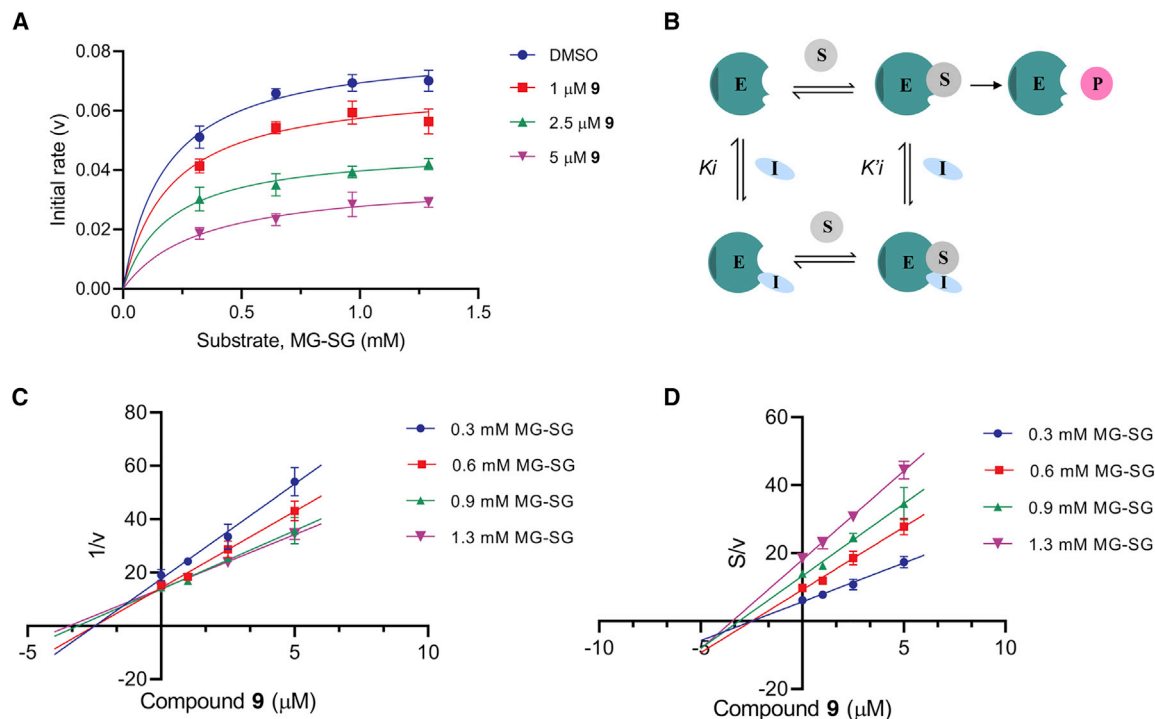


Figure 4. Molecule 9 Exhibits a Mixed Type of Inhibition

(A) Michaelis-Menten plot showing a concentration-dependent decrease of V_{max} and increase in K_m . Data represent mean $\pm$ SD (n = 3).

(B) Illustration of the mixed-type inhibition exhibited by 9.

(C) Graphical estimation of the inhibitory constant K_i (E) and K'_i (ES) by Dixon plot of initial velocity versus varying 9 concentrations (left graph) and the plot of substrate concentration/initial velocity (S/v) versus varying 9 concentrations (right graph), respectively. Data represent mean $\pm$ SD (n = 3).

Galligan et al., 2018). The cellular MG levels were evaluated through derivatization with *o*-phenylenediamine (*o*-pd) and subsequent high-performance liquid chromatography quantification of the quinoxaline products (Figure S5). Higher levels of MG were detected in cells treated with 9 (25 μM) and BBGC (25 μM), a cell-permeable Glo1 inhibitor (Sakamoto et al., 2001), but not with compound 10 (25 μM) or DMSO (Figure 6A).

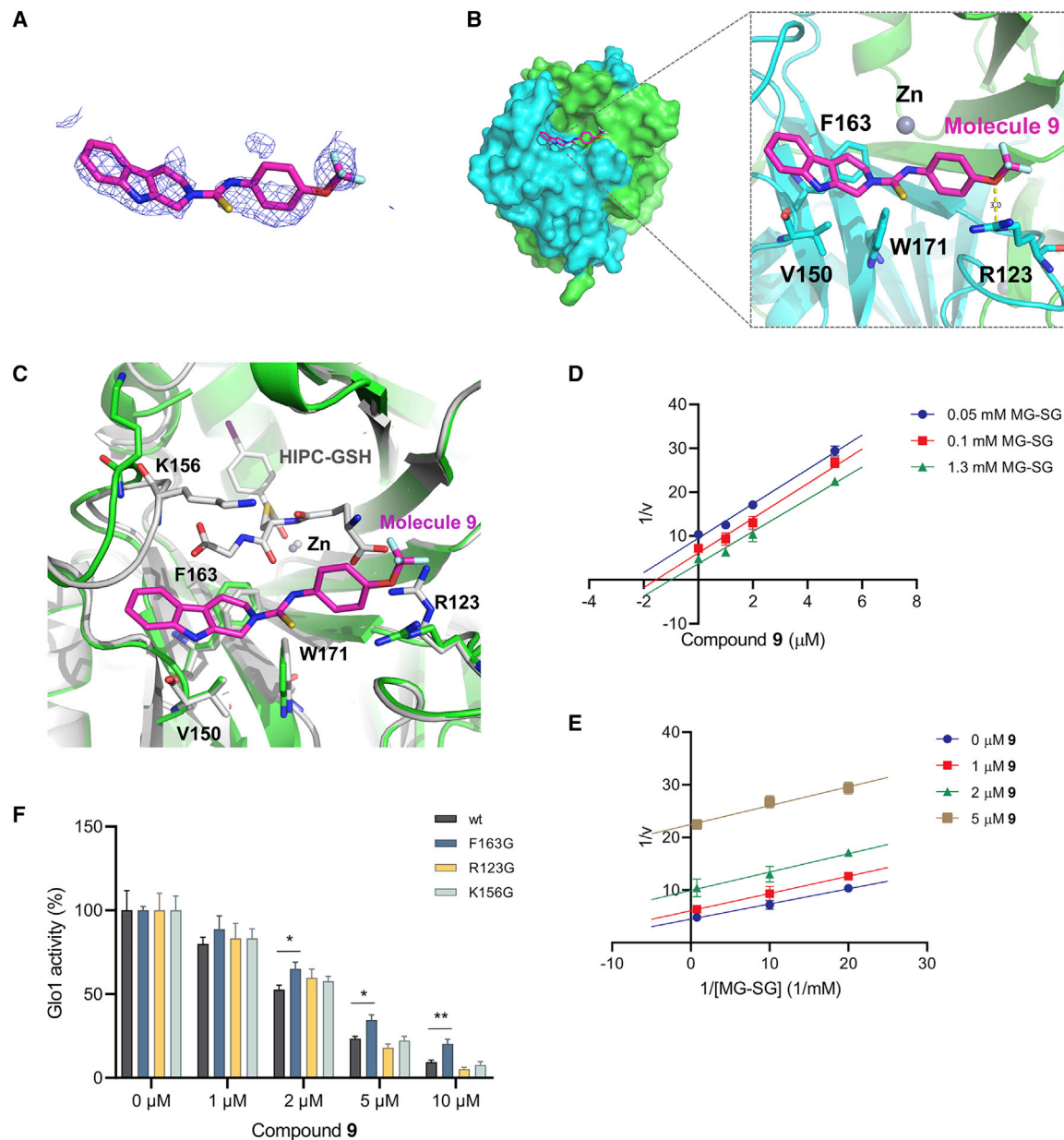
Glo1's activity was reported to be important for osteoclastogenesis, a process required in bone remodeling (Kawatani et al., 2008). The inhibition of Glo1 by a small molecule or exogenous addition of MG was described to suppress osteoclast formation (Kawatani et al., 2008). Molecule 9 was examined as to whether it gives the same phenotype. Mouse bone marrow-derived macrophages were added with M-CSF and sRANKL in the presence of the compounds to induce osteoclast formation. After 96 h, the cells treated with DMSO or 10 (10 μM) displayed pink multinucleated stained cells in TRAP+ staining denoting successful osteoclast formation (Figures 6B and 6C). The cells treated with 9 (10 μM) and MG (500 μM), on the other hand, showed no pink multinucleated stained cells signifying the absence of osteoclast. These results, taken together, are consistent with the notion that molecule 9 inhibits the enzymatic activity of Glo1 in cells.

DISCUSSION

Target-based screening and phenotypic screening are the traditional ways to discover new bioactive ligands from a collection of

molecules. Here, we demonstrate how chemical proteomics could be an alternative strategy to screen a chemical library for biologically active ligands. Using a pharmacophore-based photo-affinity probe, we performed a small-molecule competition profiling of the library molecules in cellular context. This approach of screening provides the advantage of revealing unexpected small molecule-protein interactions in cells. Indeed, introduction of our indole-oriented chemical library as competitors of probe 1 resulted in the unbiased discovery of indole-thiourea Glo1 binders. Assessment of the biological activity of the identified ligands showed that binding of a small molecule may not necessarily modulate the function of a protein. To attain functional relevance, a small molecule must bind to at least one of the following sites of the protein; the active site, protein-protein interface, or at an allosteric site that alters the conformation of the protein (Hardy and Wells, 2004; Xu et al., 2016; Zerbe et al., 2012; Suplatov and Švedas, 2015). Such binding preferences, however, cannot be controlled by a simple photo-affinity-based probe. For binding ligands that fail to modulate the activity of its target protein, structure-activity relationship studies and protein profiling done in parallel will help generate cell permeable functioning ligands.

On a positive note, the indefinite binding of the probe to a particular protein could reveal unknown small-molecule binding modes and unpredicted allosteric sites of proteins. In the study, we found a shallow hydrophobic pocket outside Glo1's active site formed by Phe₁₆₃, Val₁₅₀, and Trp₁₇₁ that can be occupied by small hydrophobic molecules. In the mGlo-9 complex,



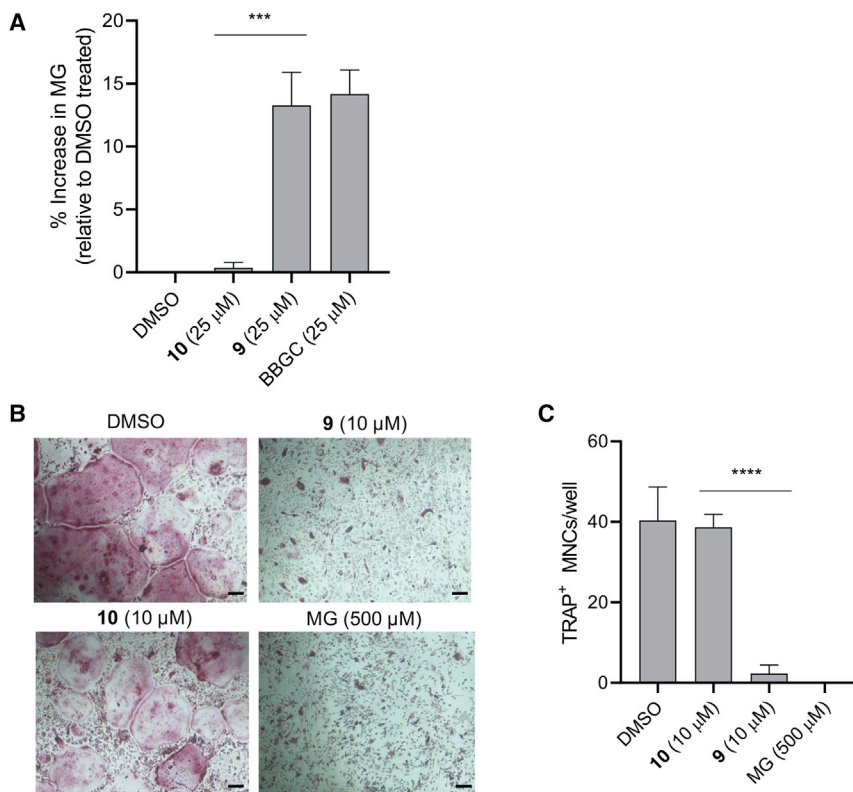


Figure 6. Molecule 9 Disrupts Glo1's Function in Cells

(A) The percentage increase in cellular MG levels of A549 cells after 24 h treatment of **9** (25 μ M), **10** (25 μ M), and BBGC (25 μ M) was detected by *o*-pd derivatization followed by high-performance liquid chromatography measurement of the quinoxaline adduct. Data represent average values $\pm$ SD ($n = 3$), *** $p = 0.0004$.

(B) Mouse bone marrow-derived macrophage cells were treated with M-CSF and sRANKL in the presence of **9** (10 μ M), **10** (10 μ M), and MG (500 μ M) for 96 h and was stained for TRAP (pink stain). Scale bars, 200 μ m.

(C) Cell count of TRAP⁺ multinucleated cells (MNCs). Data represent average values $\pm$ SD ($n = 3$), **** $p < 0.0001$.

molecule **9** binds at the border of the active site leaving the active site unoccupied. It is unlikely that binding of **9** to this site is sufficient to inhibit the enzyme's activity. The enzyme kinetics profile of **9** explained the inhibition mechanism for the observed binding mode of the molecule. With mGlo1, **9** exhibits an uncompetitive inhibition, which denotes that, in the presence of the substrate, the molecule binds to the shallow hydrophobic pocket next to the substrate, which consequently inhibits the product formation. Obtaining a ternary complex of mGlo1-**9**-substrate in the future would be meaningful to validate the proposed inhibition mode of **9**. Meanwhile, for hGlo1, the molecule displays a mixed type of inhibition (competitive and uncompetitive). The Glo1 gene is conserved in human and mouse, wherein the hGlo1 and mGlo1 gene sequence has 89.7% identities and 93.5% similarities (Zhai et al., 2013). We believe that the observed binding mode of **9** in mGlo1 is one of the binding modes exhibited by the molecule in hGlo1. The presented binding mode could also explain the difference in the K_d value (binding affinity) and K_i value (inhibitory activity) of the molecule toward hGlo1. Molecule **9** is highly hydrophobic in nature, which explains its strong affinity to Glo1. However, the binding of **9** to the shallow pocket requires the presence of the substrate to inhibit the enzyme's activity (uncompetitive inhibition). At higher concentrations of the inhibitor, the substrate has probably been displaced by two or more molecules of **9** to fully inhibit the enzyme's activity (competitive inhibition). Collectively, these findings provide a fresh perspective in designing Glo1 inhibitors.

Another valuable observation in the study was the occurrence of probe binding enhancement in the presence of another ligand. An increase in the fluorescence labeling of the ~25-kDa band

was observed in the competition profile of probe **1** and molecule **9**, which suggests that binding of **9** did not weaken the probe's interaction to Glo1 but instead augmented the probe's affinity to the enzyme. This incident is reasonable as the experiments suggest that probe **1** and molecule **9** share different Glo1 binding sites and are possibly positioned adjacent to each other. Probe **1** is believed to bind at the active site, since BBGC, a

known competitive inhibitor of Glo1, competed with the binding of the probe, while molecule **9** is described by the crystal structure to bind at the border of the active site, adjacent to the substrate. Based on our results, an increase in fluorescence labeling in gel profiles is likely to happen, particularly for affinity-based probes, when the binding of a small molecule enhances the affinity of the probe to the protein. Small molecules exhibiting this behavior may bind to a site adjacent to the probe or at an allosteric site triggering a conformational change in the protein that consequently result in a stronger affinity of the probe toward the protein. If the probe is designed to bind to a particular site of the protein, the occurrence of probe binding enhancement in the presence of another ligand could uncover new ways of modulating the protein.

For future prospective, screening a pharmacophore-focused library with the intention to find novel targets of the pharmacophore would benefit from a photo-affinity probe with less promiscuous photo-reactive groups, such as diazirine (Parker et al., 2017) and thienyl-substituted α -ketoamide (Ota et al., 2018). This will narrow the target proteins to only those that specifically bind to the pharmacophore. In our case, the benzophenone group has an influence on the set of partner proteins of probe **1** (Figures S6 and S6B). We also learn that our control probe containing only benzophenone has an affinity to Glo1 (Figure S6C). Nonetheless, the competition profile of the control probe with the indole-thiourea compounds is different from that of probe **1**: the control probe was less sensitive to the inhibition of 7oa3 and 7qc8 and did not enhance the enriched Glo1 in the presence of molecule **9**. Of note, while a gel-based platform is ideal for profiling a library of thousands of molecules and has proven to

be successful for screening (Li et al., 2007; Bachovchin et al., 2010; Zhou et al., 2019; Baggelaar et al., 2019) and selectivity studies (Janssen et al., 2018), this method is not sensitive enough to analyze the whole proteome-wide selectivity of the molecule. Thus, quantitative chemical proteomic analysis of the final seed molecule is valuable to help advance the study of the ligand-protein pair. Overall, we believe a chemical proteomic approach of screening will benefit focused chemical libraries that have been fully exhausted in target and phenotypic-based screening. We intend that this study serve as a guide for those who would like to expand the reuses of their existing chemical libraries through chemical proteomics.

SIGNIFICANCE

This study demonstrates how chemical proteomics can be used as an alternative screening strategy to exploit focused chemical libraries that have been exhausted in target-based and phenotypic screening. The chemoproteomic way of screening is an unbiased approach to discovering ligand-protein pairs in cells. Our structure optimization, activity assay, and protein profiling show that cell-permeable functioning ligands can be generated from the screening hits. Of note, the study also disclosed the occurrence of probe-labeling enhancement that has not been described in other competition profiling of small molecules. We explained that such a pattern also denotes the small-molecule interaction to the protein and could be referred to as an identified “hit” in future screening studies. Moreover, the ligand-protein pair (9-Glo1) described here, uncovers a unique binding mode for Glo1 inhibition. Albeit the inhibition mechanism is distinct from other reported Glo1 inhibitors, molecule **9** is effective in inhibiting the enzyme function *in vitro* and in cells, and thus serves as a useful tool in Glo1 studies. Taken together, we show that chemical proteomics could be a valuable method for identifying biologically active ligands from focused chemical libraries and may reveal unknown small-molecule binding modes for inhibition/activation.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- RESOURCE AVAILABILITY
 - Lead Contact
 - Materials Availability
 - Data and Code Availability
- EXPERIMENTAL MODEL AND SUBJECT DETAILS
 - Cell Line
 - Bacterial Strain
- METHOD DETAILS
 - Ligand and Protein Pair Screening
 - Competition Profiling of 7oa3 and 7qc8 Derivatives, 2-16
 - Identification of Probe 1-Binding Proteins
 - Identification of the ~25 kDa Protein by Biotin-Pull Down
 - Western Blot Analysis of Glo1 Enrichment

- Preparation of Recombinant 6-His-tagged mGlo1 (Wild-type and Mutants)
- In Vitro Glo1 Assay
- Isothermal Titration Microcalorimetry (ITC) Measurement
- Cellular Methylglyoxal Quantification
- Osteoclast Formation
- Crystal Structure Analysis
- Chemistry Materials
- Chemical Synthesis and Characterization
- QUANTIFICATION AND STATISTICAL ANALYSIS

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at <https://doi.org/10.1016/j.chembiol.2020.04.007>.

ACKNOWLEDGMENTS

The authors thank Dr. M. Kawatani for the protocol of osteoclast formation; Dr. T. Morii and Dr. S. Futaki for providing access to their ITC machine and fluorescence gel imager; Mr. H. Hakariya for helping in the DNA sequencing; Dr. R. Nakagawa and Dr. S. Kuraku for the LC-MS/MS analysis; Dr. F. Quiocho for editing the manuscript. This work was supported by JSPS (17H06408 and 19H00922 to M.U.) and the International Collaborative Research Program of Institute for Chemical Research, Kyoto University (grant no. 2019-61). This work was inspired by the international and interdisciplinary environments of WPI-iCeMS, JSPS A3 Foresight Program “Asian Chemical Probe Research Hub,” and JSPS CORE-to-CORE Program “Asian Chemical Biology Initiative.”

AUTHOR CONTRIBUTIONS

Conceptualization, L.L.P. and M.U.; Methodology, L.L.P., S.S., and Y.T.; Investigation, L.L.P., L.J., D.M., A.D.M., and M.T.; Visualization, L.L.P. and L.J.; Writing – Original Draft, L.L.P.; Writing – Review & Editing, M.U.; Resources, S.S., K.K., and M.N.; Funding Acquisition, M.U.; Supervision, L.Z. and M.U.

DECLARATION OF INTERESTS

The authors declare no competing interests.

Received: October 6, 2019

Revised: March 28, 2020

Accepted: April 15, 2020

Published: May 12, 2020

REFERENCES

- Adams, P., Grosse-Kunstleve, R., Hung, L.-W., Ioerger, T., McCoy, A., Moriarty, N., Read, R., Sacchettini, J., Sauter, N., and Terwilliger, T. (2002). PHENIX: building new software for automated crystallographic structure determination. *Acta Crystallogr. D Biol. Crystallogr.* 58, 1948–1954.
- Al-Balas, Q., Hassan, M., Al-Shar'i, N., Mhaidat, N., Almaaytah, A., Al-Mahasneh, F., and Isawi, I. (2016). Novel glyoxalase-I inhibitors possessing a “zinc-binding feature” as potential anticancer agents. *Drug Des. Devel. Ther.* 10, 2623–2629.
- Asada, S., Choi, Y., and Uesugi, M. (2003). A gene-expression inhibitor that targets an alpha-helix-mediated protein interaction. *J. Am. Chem. Soc.* 125, 4992–4993.
- Bachovchin, D., Ji, T., Li, W., Simon, G., Blankman, J., Adibekian, A., Hoover, H., Niessen, S., and Cravatt, B. (2010). Superfamily-wide portrait of serine hydrolase inhibition achieved by library-versus-library screening. *Proc. Natl. Acad. Sci. U S A* 107, 20941–20946.
- Baggelaar, M., Dulk, H., Florea, B., Fazio, D., Bernabò, N., Raspa, M., Janssen, A., Scavizzi, F., Barboni, B., Overkleeft, H., et al. (2019). ABHD2 inhibitor

identified by activity-based protein profiling reduces acrosome reaction. *ACS Chem. Biol.* **14**, 2295–2304.

Cameron, A., Olin, B., Ridderström, M., Mannervick, B., and Jones, T.A. (1997). Crystal structure of human glyoxalase I-evidence for gene duplication and 3D domain swapping. *EMBO J.* **16**, 3386–3395.

Cameron, A., Ridderström, M., Olin, B., Kavarana, M., Creighton, D., and Mannervik, B. (1999). Reaction mechanism of glyoxalase I explored by an X-ray crystallographic analysis of the human enzyme in complex with a transition state analogue. *Biochemistry* **38**, 13480–13490.

Chiba, T., Ohwada, J., Sakamoto, H., Kobayashi, T., Fukami, T., Irie, M., Miura, T., Ohara, K., and Koyano, H. (2012). Design and evaluation of azaindole-substituted N-hydroxypyridones as glyoxalase I inhibitors. *Bioorg. Med. Chem. Lett.* **22**, 7846–7849.

Cognetta, A., 3rd., Niphakis, M., Lee, H.-C., Martini, M., Hulce, J., and Cravatt, B. (2015). Selective N-hydroxyhydantoin carbamate inhibitors of mammalian serine hydrolases. *Chem. Biol.* **22**, 928–937.

Cornish-Bowden, A. (1974). A simple graphical method for determining the inhibition constants of mixed, uncompetitive, non-competitive inhibitors. *Biochem. J.* **137**, 143–144.

de Sa Alves, F., Barreiro, E., and Fraga, C.A.M. (2009). From nature to drug discovery: the indole scaffold as a 'privileged structure'. *Mini. Rev. Med. Chem.* **9**, 782–793.

Dhar, A., Desai, K., Liu, J., and Wu, L. (2009). Methylglyoxal, protein binding and biological samples: are we getting the true measure? *J. Chromatogr. B* **877**, 1093–1100.

Distler, M., Plant, L., Sokoloff, G., Hawk, A., Aneas, I., Wuenschell, G., Termini, J., Meredith, S., Nobrega, M., and Palmer, A. (2012). Glyoxalase 1 increases anxiety by reducing GABA_A receptor agonist methylglyoxal. *J. Clin. Invest.* **122**, 2306–2315.

Emsley, P., and Cowtan, K. (2004). Coot: model-building tools for molecular graphics. *Acta Crystallogr. D. Biol. Crystallogr.* **60**, 2126–2132.

Frisco-Cabanas, H., Watanabe, M., Okumura, N., Kusamori, K., Takemoto, N., Takaya, J., Sato, S., Yamazoe, S., Takakura, Y., Kinoshita, S., et al. (2014). Synthetic molecules that protect cells from anoikis and their use in cell transplantation. *Angew. Chem. Int. Ed.* **53**, 11208–11213.

Galligan, J., Weply, J., Streeter, M., Kingsley, P., Mitchener, M., Wauchope, O., Beavers, W., Rose, K., Wang, T., Spiegel, D., and Marnett, L. (2018). Methylglyoxal-derived posttranslational arginine modifications are abundant histone marks. *Proc. Natl. Acad. Sci. U S A* **115**, 9228–9233.

Hardy, J., and Wells, J. (2004). Searching for new allosteric sites in enzymes. *Curr. Opin. Struct. Biol.* **14**, 706–715.

Harris, C.J., Hill, R., Sheppard, D., Slater, M., and Stouten, P. (2011). The design and application of target-focused compound libraries. *Comb. Chem. High Throughput Screen* **14**, 521–531.

Janssen, A., Vliet, D., Bakker, A., Jiang, M., Grimm, S., Campiani, G., Butini, S., and Stelt, M. (2018). Development of multiplexed activity-based protein profiling assay to evaluate activity of endocannabinoid hydrolase inhibitors. *ACS Chem. Biol.* **13**, 2406–2413.

Jin, T., Zhai, J., Liu, X., Yue, Y., Huang, M., Li, Z., Ni, C., Deng, Q., Sang, Y., Yao, Z., et al. (2017). Design, synthesis and biological evaluation of potent human glyoxalase I inhibitors. *Chem. Pharm. Bull.* **65**, 455–460.

Kaushik, N.K., Kaushik, N., Attri, P., Kumar, N., Kim, C.H., Verma, A.K., and Choi, E.H. (2013). Biomedical importance of indoles. *Molecules* **18**, 6620–6662.

Kawatani, M., Okumura, H., Honda, K., Kanoh, N., Muroi, M., Dohmae, N., Takami, M., Kitagawa, M., Futamura, Y., Imoto, M., and Osada, H. (2008). The identification of an osteoclastogenesis inhibitor through the inhibition of glyoxalase I. *Proc. Natl. Acad. Sci. U S A* **105**, 11691–11696.

Li, W., Blankman, J., and Cravatt, B. (2007). A functional proteomic strategy to discover inhibitors for uncharacterized hydrolases. *J. Am. Chem. Soc.* **129**, 9594–9595.

Liu, M., Yuan, M., Luo, M., Bu, X., Luo, H.-B., and Hu, X. (2010). Binding of curcumin with glyoxalase I: molecular docking, molecular dynamics simulations, and kinetics analysis. *Biophys. Chem.* **147**, 28–34.

More, S., and Vince, R. (2006). A metabolically stable tight-binding transition-state inhibitor of glyoxalase-I. *Bioorg. Med. Chem. Lett.* **16**, 6039–6042.

More, S., and Vince, R. (2009). Inhibition of glyoxalase I: the first low-nanomolar tight-binding inhibitors. *J. Med. Chem.* **52**, 4650–4656.

Nokin, M.-J., Durieux, F., Bellier, J., Peulen, O., Uchida, K., Spiegel, D., Cochrane, J., Hutton, C., Castronovo, V., and Bellahcène, A. (2017). Hormetic potential of methylglyoxal, a side-product of glycolysis, in switching tumours from growth to death. *Sci. Rep.* **7**, 11722.

Ota, E., Usui, K., Oonuma, K., Koshino, H., Nishiyama, S., Hirai, G., and Sodeoka, M. (2018). Thienyl-substituted α -ketoamide: a less hydrophobic reactive group for photo-affinity labeling. *ACS Chem. Biol.* **13**, 876–880.

Otwinowski, Z., and Minor, W. (1997). Processing of X-ray diffraction data collected in oscillation mode. *Methods Enzymol.* **276**, 307–326.

Parker, C., Galmozzi, A., Wang, Y., Correia, B., Sasaki, K., Joslyn, C., Kim, A., Cavallaro, C., Lawrence, M., Johnson, S., et al. (2017). Ligand and target discovery by fragment-based screening in human cells. *Cell* **168**, 527–541.

Perez, C., Barkley-Levenson, A., Dick, B., Glatt, P., Martinez, Y., Siegel, D., Momper, J., Palmer, A., and Cohen, S. (2019). Metal-binding pharmacophore library yields the discovery of a glyoxalase 1 inhibitor. *J. Med. Chem.* **62**, 1609–1625.

Perron, A., Nishikawa, Y., Iwata, J., Shimojo, H., Takaya, J., Kobayashi, K., Imayoshi, I., Mbenza, N.M., Takenoya, M., Kageyama, R., et al. (2018). Small-molecule screening yields a compound that inhibits the cancer-associated transcription factor Hes1 via the PHB2 chaperone. *J. Biol. Chem.* **293**, 8285–8294.

Ramsay, R., and Tipton, K. (2017). Assessment of enzyme inhibition: a review with examples from the development of monoamine oxidase and cholinesterase inhibitory drugs. *Molecules* **22**, 1192.

Sakamoto, H., Mashima, T., Sato, S., Hashimoto, Y., Yamori, T., and Tsuruo, T. (2001). Selective activation of apoptosis program by S-p-bromobenzylglutathione cyclopentyl diester in glyoxalase I-overexpressing human lung cancer cells. *Clin. Cancer Res.* **7**, 2513–2518.

Sato, S., Kwon, Y., Kamisuki, S., Srivastava, N., Mao, Q., Kawazoe, Y., and Uesugi, M. (2007). Polyproline-rod approach to isolating protein targets of bioactive small molecules: isolation of a new target of indomethacin. *J. Am. Chem. Soc.* **129**, 873–880.

Stratmann, B., Goldstein, B., Thornalley, P., Rabbani, N., and Tschoepe, D. (2017). Intracellular accumulation of methylglyoxal by glyoxalase 1 knock down alters collagen homeostasis in L6 myoblasts. *Int. J. Mol. Sci.* **18**, 480.

Suciu, R., Cognetta, A., 3rd., Potter, Z., and Cravatt, B. (2018). Selective irreversible inhibitors of the wnt-deacetylating enzyme NOTUM developed by activity-based protein profiling. *ACS Med. Chem. Lett.* **9**, 563–568.

Suplatov, D., and Švedas, V. (2015). Study of functional and allosteric sites in protein superfamilies. *Acta Nat.* **7**, 34–45.

Takasawa, R., Takahashi, S., Saeki, K., Sunaga, S., Yoshimori, A., and Tanuma, S. (2008). Structure-activity relationship of human GLO I inhibitory natural flavonoids and their growth inhibitory effects. *Bioorg. Med. Chem. Lett.* **16**, 3969–3975.

Takasawa, R., Tao, A., Saeki, K., Shionozaki, N., Tanaka, R., Uchiro, H., Takahashi, S., Yoshimori, A., and Tanuma, S. (2011). Discovery of a new type inhibitor of human glyoxalase I by myricetin-based 4-point pharmacophore. *Bioorg. Med. Chem. Lett.* **21**, 4337–4342.

Thornalley, P. (1993). The glyoxalase system in health and disease. *Mol. Aspects Med.* **14**, 287–371.

Thornalley, P. (1996). Pharmacology of methylglyoxal: formation, modification of proteins and nucleic acids, and enzymatic detoxification—a role in pathogenesis and antiproliferative chemotherapy. *Gen. Pharmacol.* **27**, 565–573.

Welsch, M., Snyder, S., and Stockwell, B. (2010). Privileged scaffolds for library design and drug discovery. *Curr. Opin. Chem. Biol.* **14**, 347–361.

Winn, M., Ballard, C., Cowtan, K., Dodson, E., Emsley, P., Evans, P., Keegan, R., Krissinel, E., Leslie, A., McCoy, A., et al. (2011). Overview of the CCP4 suite and current developments. *Acta Crystallogr. D. Biol. Crystallogr.* **67**, 235–242.

Cell Chemical Biology

Article



Xu, D., Jalal, S., Sledge, G., Jr., and Meroueh, S. (2016). Small-molecule binding sites to explore protein–protein interactions in the cancer proteome. *Mol. Biosyst.* **12**, 3067–3087.

Yuan, M., Luo, M., Song, Y., Xu, Q., Wang, X., Cao, Y., Bu, X., Ren, Y., and Hu, X. (2011). Identification of curcumin derivatives as human glyoxalase I inhibitors: a combination of biological evaluation, molecular docking, 3D-QSAR and molecular dynamics simulation studies. *Bioorg. Med. Chem. Lett.* **19**, 1189–1196.

Zhai, J., Zhang, H., Zhang, L., Zhao, Y., Chen, S., Chen, Y., Peng, X., Li, Q., Yuan, M., and Hu, X. (2013). Zopolrestat as a human glyoxalase I inhibitor and its structural basis. *ChemMedChem* **8**, 1462–1464.

Zerbe, B., Hall, D., Vajda, S., Whitty, A., and Kozakov, D. (2012). Relationship between hot spot residues and ligand binding hot spots in protein–protein interfaces. *J. Chem. Info. Model.* **52**, 2236–2244.

Zhang, H., Huang, Q., Zhai, J., Zhao, Y.-N., Zhang, L.-P., Chen, Y.-Y., Zhang, R.-W., Li, Q., and Hu, X.-P. (2015a). Structural basis for 18- β -glycyrrhetic

acid as a novel non-GSH analog glyoxalase I inhibitor. *Acta Pharmacol. Sin.* **36**, 1145–1150.

Zhang, M.Z., Chen, Q., and Yang, G.F. (2015b). A review on recent developments of indole-containing antiviral agents. *Eur. J. Med. Chem.* **89**, 421–441.

Zhang, X., Crowley, V., Wucherpfennig, T., Dix, M., and Cravatt, B. (2019). Electrophilic PROTACS that degrade nuclear proteins by engaging DCAF16. *Nat. Chem. Biol.* **15**, 737–746.

Zhou, J., Mock, E., Martella, A., Kantae, V., Di, X., Burggraaff, L., Baggelaar, M., Al-Ayed, K., Bakker, A., Florea, B., et al. (2019). Activity-based protein profiling identifies α -ketoamides as inhibitors for phospholipase A2 group XVI. *ACS Chem. Biol.* **14**, 164–169.

Zuhl, A., Mohr, J., Bachovchin, D., Niessen, S., Hsu, K.-L., Berlin, J., Dochnahl, M., Lopez-Alberca, M., Fu, G., and Cravatt, B. (2012). Competitive activity-based protein profiling identifies aza- β -lactams as a versatile chemotype for serine hydrolase inhibition. *J. Am. Chem. Soc.* **134**, 5068–5071.

STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Glo1 antibody, anti-mouse	Novus Biological	Cat# NBP1-19015; RRID: AB_1625385
Mouse secondary antibody	GE Healthcare Life Sciences	Cat# NA931; RRID: AB_772210
Chemicals, Peptides, and Recombinant Proteins		
Sulforhodamine B-PEG3-azide	Click Chemistry Tools	AZ106
Tris(3-hydroxypropyltriazolylmethyl) amine, THPTA	Sigma	762342
Azide-PEG3-biotin conjugate	Sigma	762024
Sodium L-Ascorbate	TCI	A0539
Copper (II) Sulfate	Wako	030-04442
Softlink™ Soft Release Avidin Resin	Promega	V201B
D-Biotin	Nacalai Tesque	04822-91
Recombinant human Glo1	R&D Systems	4959-GL
Recombinant mouse Glo1	Sato et al., 2007 ; and this study	N/A
Recombinant mouse Glo1 _{R123G} mutant	This study	N/A
Recombinant mouse Glo1 _{K156G} mutant	This study	N/A
Recombinant mouse Glo1 _{F163G} mutant	This study	N/A
Indometacin	Sigma	I7378
Methylglyoxal solution (40% in water)	Sigma	M0252
Glutathione	Wako	073-02013
o-phenylenediamine	TCI	P1805
2-methylquinoxaline	TCI	M0580
5-methylquinoxaline	TCI	M1371
M-CSF	R&D Systems	216-MC
TGF-β1	R&D Systems	240-B
sRANKL	Peprtech	310-01
L-Glutamine	Nacalai Tesque	169-19
TRAP stain	Cosmo Bio	PMC-AK04F-COS
Ni Sepharose 6 Fast Flow	GE Healthcare	17531801
Kanamycin sulphate	Fujifilm Wako	119-00703
Isopropyl-β-D-galactopyranoside, IPTG	Wako	099-02534
Critical Commercial Assay		
In-Fusion HD Cloning Kit	Takara Biolab	639648
Deposited Data		
Crystal structure (Molecule 9/Glo1 complex)	This study	PDB ID: 6L0U
Crystal structure (HIPC-GSH/Glo1 complex)	Cameron et al., 1999	PDB ID: 1QIN
Crystal structure (NBC-GSH/Glo1 complex)	Cameron et al., 1999	PDB ID: 1QIP
Crystal structure (M-GFN/Glo1 complex)	Kawatani et al., 2008	PDB ID: 2ZA0
Experimental Models: Cell Lines		
HEK293 (female)	RIKEN Cell Bank	RCB1637
A549 (male)	RIKEN Cell Bank	RCB0098

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Experimental Models: Organisms/Strains		
Five-week-old ddY mice (male)	Sankyo Labo Service Co., Inc. (Tokyo, Japan)	N/A
<i>Escherichia coli</i> BL21-CodonPlus (DE3)-RIPL cells	Agilent Technologies	230280
Oligonucleotides		
(Glo1-Nco infusion) 5'-AGGAGATATACCATGGC AGAGCCACAGCCGGCGTC-3'	Life Technologies Japan	Custom Primers
(Glo1-Xho infusion) 5'-GGTGGTGGTGCTCGAGA ATAATCGTTGCTATTTGTTAGG	Life Technologies Japan	Custom Primers
R123G mGlo1 mutant (forward) 5'-CAACTCGGACCCTGGTGA TTTGGTCAC-3'	Life Technologies Japan	Custom Primers
R123G mGlo1 mutant (reverse) 5'-GTGACCAAATCCACCAGGGT CCGAGTTG-3'	Life Technologies Japan	Custom Primers
K156G mGlo1 mutant (forward) 5'-GTCCTTTCATTCCCCGTCATC AGG-3'	Life Technologies Japan	Custom Primers
K156G mGlo1 mutant (reverse) 5'-GTCCTTTCATTCCCCGTCATC AGG-3'	Life Technologies Japan	Custom Primers
F163G mGlo1 mutant (forward) 5'-CTGGCGGGCATTCAAGACCC-3'	Life Technologies Japan	Custom Primers
F163G mGlo1 mutant (reverse) 5'-GGGTCTTGAATGCCCGCCAG-3'	Life Technologies Japan	Custom Primers
Recombinant DNA		
pET28b expression vector	Novagen	69865-3
Plasmid: 6-His-tagged mGlo1	Sato et al., 2007	N/A
Plasmid: 6-His-tagged mGlo1 _{R123G} mutant	This study	N/A
Plasmid: 6-His-tagged mGlo1 _{K156G} mutant	This study	N/A
Plasmid: 6-His-tagged mGlo1 _{F163G} mutant	This study	N/A
Software and Algorithms		
GraphPad Prism Version 8.0	GraphPad Software Inc.	https://www.graphpad.com/scientific-software/prism/
ChemDraw Professional 18.0	Perkin Elmer	www.perkinelmer.com
ImageJ	ImageJ developers	https://imagej.net
Phenix	Adams et al., 2002	https://www.phenix-online.org/
HKL3000	Otwinowski and Minor, 1997	http://www.hkl-xray.com/hkl-3000
CCP4	Winn et al., 2011	http://www.ccp4.ac.uk/
COOT	Emsley and Cowtan, 2004	https://www2.mrc-lmb.cam.ac.uk/personal/pemsley/coot/
PyMOL	Schrödinger, Inc.	https://pymol.org/2/

RESOURCE AVAILABILITY

Lead Contact

Further information and requests for resources and reagents should be directed to the Lead Contact, Motonari Uesugi (uesugi@scl.kyoto-u.ac.jp).

Materials Availability

All unique/stable reagents generated in this study are available from the Lead Contact without restrictions.

Data and Code Availability

The PDB file that supports the findings of this study has been deposited in Protein Data Bank under accession code 6L0U.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Cell Line

HEK293 cells (female) were cultured in Dulbecco's modified Eagles' medium (DMEM, GIBCO) supplemented with 10% (v/v) fetal bovine serum (FBS, Biosera), and penicillin-streptomycin mixed solution (Nacalai Tesque, 100 units/mL each). A549 cells (male) were cultured in RPMI 1640 medium (GIBCO) supplemented with 10% (v/v) fetal bovine serum (FBS, Biosera) and penicillin-streptomycin mixed solution (Nacalai Tesque, 100 units/mL each). All cells were maintained at 37°C under 5% CO₂ atmosphere.

The bone marrow cells used for the osteoclast formation were derived from a five-week-old ddY male mice purchased from Sankyo Labo Service Co., Inc. (Tokyo, Japan). The bone marrow cells were cultured in alpha-mem medium (Sigma Aldrich) supplemented with 10% calf serum (Biosera), L-glutamine (0.292 g/L, Nacalai Tesque), M-CSF (50 ng/mL, R&D systems), and TGF-β1 (1 ng/mL, R&D systems) at 37°C under 5% CO₂ atmosphere.

Bacterial Strain

The *Escherichia coli* BL21-CodonPlus(DE3)-RIPL competent cells (Agilent Technologies) were derived from Agilent's high performance BL21-Gold competent cell line. The *Escherichia coli* BL21-CodonPlus(DE3)-RIPL competent cells were stored at -80°C upon receipt, and were thawed on ice just before the transformation procedure. The bacterial cells were grown in LB (Lennox, Nacalai Tesque) growth media at 37°C.

METHOD DETAILS

Ligand and Protein Pair Screening

HEK293 cells were grown in a 12-well plate until it reached 90% confluency. The medium was replaced with a new free-serum medium (1 mL) containing probe **1** (5 μM) and 8 small molecules (30 μM each) from the indole-focused chemical library (1,800 molecules, Tripos Receptor Research). After 30 mins incubation at 37°C, the plate was placed on ice and exposed to 365 nm light for 20 min. Treated cells were collected into 1.5-mL Eppendorf tube by scraping, washed with 1x PBS and incubated with 100 μL lysis buffer (1x PBS with 1% NP-40) on ice for 30 min. After centrifugation (14,000 rpm, 15 min, 4°C), 50 μL aliquot of the soluble fractions was transferred into a 96-well plate. Sulforhodamine B-PEG3-azide (Click Chemistry Tools, 1 μL of 2 mM) was added followed by the catalyst mixture (7.25 μL of 50 mM CuSO₄; 5 μL of 50 mM tris(3-hydroxypropyltriazolylmethyl)amine, THPTA; 1.25 μL of 200 mM sodium ascorbate). The plate was shaken for 1 h. The reaction was stopped by adding 6x SDS-PAGE reducing loading buffer (Nacalai Tesque). The protein was resolved in a 10% SDS-PAGE acrylamide gel and the fluorescence image was obtained using TyphoonTM FLA 900.

Competition Profiling of 7oa3 and 7qc8 Derivatives, 2-16

The same protocol as mentioned above was followed for the competition profile of probe **1** (10 μM) and the derivatives **2-16** (6x, 60 μM).

Identification of Probe 1-Binding Proteins

Twenty-four (24) 10-cm dishes of HEK293 cells were prepared. Twelve (12) dishes of cells were treated with probe **1** (5 μM), while the other twelve (12) dishes were treated with DMSO in a free-serum media (10 mL) for 30 mins at 37°C. The cells were exposed to 365 nm light for 20 min. The cells from two 10-cm dishes were collected into a 1.5-mL Eppendorf tube, centrifuge (3,000 rpm, 4 min, 4°C) and washed with 1x PBS. The cells were added with cold 1x PBS with 1% NP-40 and 1x protease inhibitor (1 mL), and were lysed by freeze-thaw method using liquid N₂. The soluble fraction was collected after centrifugation (14000 rpm, 15 min, 4°C). Aliquots (500 μL) of the soluble fraction were placed into a 2-mL Eppendorf tubes and were added with Azide-PEG3-biotin conjugate (Sigma, 10 μL of 10 mM stock) and catalyst mixture (72.5 μL of 50 mM CuSO₄, 50 μL of 50 mM THPTA, 12.5 μL of 200 mM sodium ascorbate). The tubes were rotated for 1 h at room temperature and were added with cold acetone (1.5 mL, -20°C). The samples were placed at -20°C overnight to precipitate the proteins. The next day, the pellets were centrifuged (10,000 g, 10 min, 4°C), washed with cold acetone three times and air dried for 10 min. The proteins were solubilized in 1x PBS with 1% SDS (100 μL) by sonication.

The samples were added with 1x RIPA buffer (900 μ L) and were placed on ice for 30 min before centrifugation (14,000 rpm, 15 min, 4°C). All the resulting supernatant was collected into a 15-mL corning tube. Softlink Soft Release Avidin Resin (Promega, 60 μ L) was added and the sample was rotated at 4°C overnight. The supernatant was aspirated out the resin and the resin was washed five times with 1x RIPA buffer containing 0.1% SDS (1 mL). The bound proteins were eluted by adding 2x SDS-PAGE loading buffer (Nalacai Tesque) (100 μ L) with subsequent heating at 95°C for 3 min. The detergent was removed from the protein sample by chloroform: methanol (4:1) precipitation. The precipitated proteins are then submitted to the Laboratory for Phyloinformatics in RIKEN Center for Biosystems Dynamic Research (BDR) for shotgun LC-MS/MS analysis. The proteins that were enriched in probe **1**-treated cells but not in DMSO-treated cells were listed in [Figure S1](#).

Identification of the ~25 kDa Protein by Biotin-Pull Down

Twelve (12) 10-cm dishes of HEK293 cells were prepared. Each 10-cm dish of HEK293 cells were treated with probe **1** (5 μ M) in a free-serum medium (10 mL) for 30 min at 37°C. The protocol of the initial LC-MS/MS sample preparation was followed, expect that the eluted probe **1**-binding proteins were resolved on a 12% SDS-PAGE acrylamide gel. The resulting gel was stained using Silver stain MS kit (Wako). The band observed near 25 kDa was cut and submitted for analysis to the Laboratory for Phyloinformatics in RIKEN Center for Biosystems Dynamic Research (BDR).

Western Blot Analysis of Glo1 Enrichment

HEK293 cells in 10-cm dishes were prepared. At 90% confluency, the cells were treated with the vehicle (0.1% DMSO), the probe (**1** or control probe; 5 μ M- 10 μ M), and the probe (5 μ M-10 μ M) with competitor (7oa3, 7qc8, **9**, indometacin, BBGC; 50 μ M- 60 μ M) in a free-serum medium. The same treatment and photo-crosslinking condition as the protein identification was followed. Cells were collected and lysed in 1x PBS with 1% NP-40 and 1x protease inhibitor (600 μ L) by freeze-thaw method. After centrifugation, an aliquot (500 μ L) of the soluble fraction was subjected to copper-catalyzed click reaction as indicated above. The proteins were precipitated overnight using cold acetone (1.5 mL, -20°C). The pellets were centrifuged, washed three times with cold acetone and air dried. The pellets were suspended in 1x PBS with 1% SDS (100 μ L), sonicated and added with 1x RIPA buffer (900 μ L). The resulting supernatant was incubated with Softlink Soft Release Avidin Resin (Progema, 20 μ L) and was rotated overnight at 4°C. The resin was washed three times with 1x RIPA buffer containing 0.1% SDS. The resin was added with 2x SDS-PAGE loading buffer (Nalacai Tesque) (40 μ L) and heated at 95°C for 3 min to elute the bound proteins. The proteins were resolved on a 12% SDS-PAGE acrylamide gel and transferred onto a nitrocellulose blotting membrane (GE Healthcare). The membrane was blocked for 1 h with 5% skim milk and incubated overnight with mouse anti-glo1 antibody (Novus Biologicals, 1:200) in blocking buffer. The membrane was washed five times with PBS-T followed by 1 h incubation at room temperature with ECL-peroxidase-labeled anti-mouse (GE Healthcare). The membrane was washed five times before adding Amersham Biosciences ECL Prime Western blotting detection reagents (GE Healthcare). The bands were observed using ImageQuant LAS 500 imaging system (GE Healthcare).

Preparation of Recombinant 6-His-tagged mGlo1 (Wild-type and Mutants)

Using the pET28b-6-His-mGlo1 ([Sato et al., 2007](#)), the point mutations were introduced using specific primers (see [Key Resources Table](#)) and PCR. The resulting Glo1 cDNA was inserted into pET28b expression vector (Novagen) by using In-Fusion HD Cloning Kit (Takara Biolab). The plasmid constructs (pET28b-6-His-mGlo1 and the mGlo1 mutants: pET28b-6-His-mGlo1_{F163G}, pET28b-6-His-mGlo1_{R123G}, pET28b-6-His-mxGlo1_{K156G}) were verified by DNA sequencing. The mGlo1 DNA plasmids were introduced into *Escherichia coli* BL21-CodonPlus(DE3)-RIPL cells (Agilent). In brief, the plasmid construct (2 μ L) was added to BL21 competent cells (20 μ L) on ice. The resulting sample was incubated on ice for 30 min, heat-shocked at 42°C for 45 s, and placed back on ice for 2 min. To the BL21 cell mixture, LB media (200 μ L) was added, and the sample was incubated at 37°C for 1 hour. The bacterial mixture was spread on a LB agar plate with kanamycin (30 μ g/mL, Wako), and the bacterial plate was incubated at 37°C overnight. A single colony was picked and placed in LB media with kanamycin (30 μ g/mL). The expression of the recombinant proteins was induced with 0.4 mM isopropyl- β -D-galactopyranoside (IPTG, Wako) at 37°C for 4 h. The bacterial culture was collected by centrifugation (10,000 rpm, 5 min, 4°C) and was lysed by sonication in lysis buffer (20 mM Tris-HCl pH 8.0, 100 mM NaCl, 10% glycerol, 0.1% NP-40, 1x protease inhibitor). The soluble fraction was collected by centrifugation (16,000 rpm, 30 min, 4°C) and the 6-His-tagged mGlo1 proteins were purified by using Ni Sepharose 6 Fast Flow (GE Healthcare).

In Vitro Glo1 Assay

In vitro Glo1 assay was performed as indicated in the protocol of R&D systems. The assay buffer used was 0.1 M sodium phosphate pH 7.0 (Nalacai Tesque). Glutathione, GSH (Wako) in distilled water (100 mM) and methylglyoxal, MG (Sigma) in assay buffer (100 mM) were prepared fresh. The substrate (MG-SG) mixture was generated by pre-incubating GSH and MG in the assay buffer at 25°C. Recombinant human glyoxalase 1, rhGlo1 (R&D Systems) or recombinant 6-His-mGlo1 in assay buffer was incubated either with DMSO or the compound. The substrate mixture was added to the enzyme and the change in absorbance at 240 nm was recorded for 3 min using Hitachi U-3010 Spectrophotometer. The final concentration of rhGlo1 and 6-His-mGlo1 were 9.5 nM and 19 nM, respectively. For the kinetic studies, various concentrations of the substrate (MG-SG) were prepared by varying the concentration of MG while maintaining the concentration of GSH. The substrate concentration was calculated using the equation: $3\text{ mM} = Kd = \frac{[MG][GSH]}{[MGSG]}$. The inhibitory constants, K_i (E) and K_i' (ES) values were graphically estimated as previously described ([Cornish-Bowden, 1974](#)).

Isothermal Titration Microcalorimetry (ITC) Measurement

The binding of Glo1 to compound **9** was investigated using a MicroCal iTC200 microcalorimeter. A solution of recombinant human Glo1 (R&D Systems, 22.7 nM) was titrated with a solution of compound **9** (3.5 μ M) in 1x PBS containing 1% DMSO at 25°C. The concentration of compound **9** was determined using the λ_{max} absorbance in DMSO ($\epsilon_{280} = 34,720 \text{ M}^{-1} \text{ cm}^{-1}$).

Cellular Methylglyoxal Quantification

The cellular methylglyoxal levels were measured as previously described (Dhar et al., 2009). A549 cells were treated with the compounds in fresh medium. After 24 h, the cells were collected and washed twice with 1x PBS. The cells were suspended in 1x PBS (450 μ L), sonicated and centrifuged (14,000 rpm, 15 min, 4°C). An aliquot (400 μ L) of the soluble fraction was added with perchloric acid, 60% (Wako) to give a final concentration of 0.45 N. An internal standard, 5-methylquinoxaline, 5-MQ (TCI, 2 μ M) and *o*-phenylenediamine, *o*-pd (TCI, 1 mM) were added and the samples were incubated at room temperature for 21–24 h. The samples were centrifuged (14,000 rpm, 10 min, room temperature) to remove the precipitates and were filtered before injection. HPLC measurement: The 2-methylquinoxaline derivative, 2-MQ and the internal standard 5-MQ were measured using a Shimadzu HPLC with LC-20AD liquid chromatograph and SPD-M20A prominence diode array detector. The column used was a C18 reversed phase column (Interstil, ODS-3, 5 μ M, 4.6 $\times$ 150 mm). HPLC-grade acetonitrile (Nacalai Tesque, Solvent B) and HPLC-grade water (Nacalai Tesque, Solvent A) with 0.1% TFA were used as solvents. The mobile phase is 20% acetonitrile with a flow rate of 1 mL/min. The sample injection volume is 150 μ L and the detection wavelength is 315 nm. Samples were calibrated by comparison with 2-MQ standard.

Osteoclast Formation

Mouse bone marrow cells were isolated as previously reported with some modification (Frisco-Cabanos et al., 2014). Five-week-old ddY male mice were euthanized with isoflurane under anesthesia. Femurs and tibiae were excised from the mice and cleaned by removing all connective tissue. After disinfection with 70% ethanol, the cells in the bone were isolated by flushing with RPMI containing 10% heat-inactivated fetal bovine serum (Biosera, UK) using a 26-gauge needle and a syringe. The cell mixtures were filtered through 70- μ m and 40- μ m cell strainers (BD Falcon, Franklin Lakes, NJ, USA). The cells were centrifuged at 450 $\times$ g for 10 min at 4°C, and contaminating red blood cells in the pellets were lysed by incubation in a hypo-osmotic solution of Tris-ammonium chloride (0.15 M ammonium chloride: 0.28 M Tris, pH 7.2, 9:1) for 5 min at room temperature. The remaining cells were centrifuged at 450 $\times$ g for 5 min at 4°C, and the pellets were used as bone marrow cells. The bone marrow cells were cultured in α -mem medium (Sigma Aldrich) supplemented with 10% calf serum (Biosera) and L-glutamine (0.292 g/L, Nacalai Tesque) with M-CSF (50 ng/mL, R&D systems) and TGF- β 1 (1 ng/mL, R&D systems). After 72 h of culture, floating cells were removed and attached cells were used as BMMs. The BMMs cells were seeded in 96-well type-1 collagen coated plates (Iwaki) and cultured with RANK (50 ng/mL, Peprotech), M-CSF (50 ng/mL, R&D systems) and the compounds. After 96 h, the cells were fixed with 4% paraformaldehyde and stained for TRAP (Cosmo Bio) as indicated in the protocol of the kit. The animal experiments were conducted in accordance with the principles and procedures outlined in the National Institute of Health Guide for the Care and Use of Laboratory Animals. The protocols for animal experiments were approved by the Animals Experimentation Committee of Tokyo University of Science.

Crystal Structure Analysis

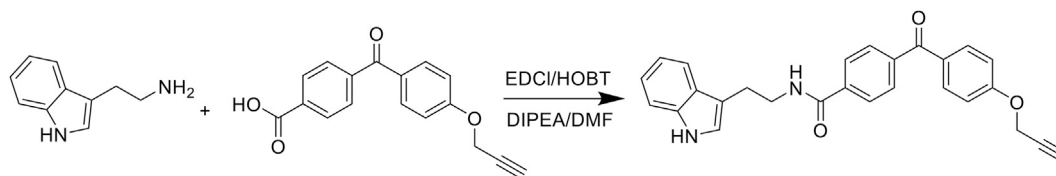
The pET28b-6-His-mGlo1 (Sato et al., 2007) was introduced into *E. coli* BL21(DE3) competent cells as described above. The expression of recombinant mGlo1 was induced with isopropyl- β -D-galactopyranoside (IPTG) at 37°C until the OD₆₀₀ value was 0.8. The 6-His tagged mGlo1 were purified by using Ni-NTA column followed by size exclusion chromatography. The purified mGlo1 were stored at a concentration of 15 mg/mL in a buffer containing 20 mM Tris-HCl pH 8.0, 100 mM NaCl and 1 mM DTT. The crystals of Glo1 were obtained using the hanging drop vapor-diffusion method at 16°C in a crystallization buffer containing 100 mM MES monohydrate pH 6.0, 12% (w/v) PEG2000 and 16% (v/v) 2-propanol. To obtain the crystal of Glo1 in complexed with compound **9**, the Glo1 crystals were soaked in the crystallization buffer containing compound **9** (0.5 mM, 20% DMSO) and then quickly frozen in liquid nitrogen using 20% glycerol as cryo-buffer. Diffraction data were collected at beamline BL18U1 and BL19U1 in Shanghai Synchrotron Radiation Facility (SSRF). The data set was handled with HKL3000 (Otwinowski and Minor, 1997) and the structure was determined using molecular replacement in CCP4 (Winn et al., 2011) using the X-ray structure model of Glo1 derived from PDB entry 2ZA0. The model was refined to 1.95 Å resolution using Phenix (Adams et al., 2002) and the ligand was built using eLBOW. Manual rebuilding of the model was completed using the molecular graphics program COOT (Emsley and Cowtan, 2004). Water molecules were incorporated into the model during structure refinement. All the graphs were plotted by PyMOL. Data collection and refinement statistics are summarized in Table S1. The atomic coordinate has been deposited in the Protein Data Bank with entry code 6LOU.

Chemistry Materials

Chemical reagents were purchased from Sigma-Aldrich Japan, Wako Pure Chemical Industries, Ltd. (Japan) and Tokyo Chemical Industry (TCI). Reaction progress was monitored by thin-layer chromatography, TLC (Merck silica gel 60 F-254 plates) using UV illumination and phosphomolybdic acid stain for detection. Some compounds were purified using a flash column system (Silica gel 60, Nacalai Tesque). Mass spectra were collected using Shimadzu LCMS-2010 in ESI mode. ¹H-NMR were collected using JEOL JNM-ECP 300 MHz.

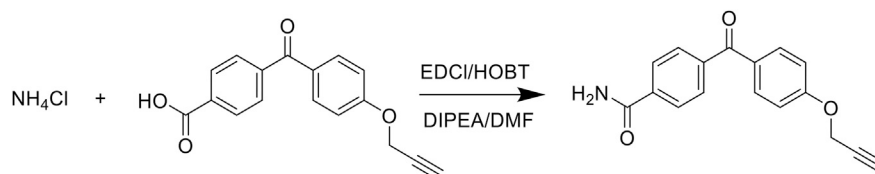
Chemical Synthesis and Characterization

Synthesis of Probe 1:



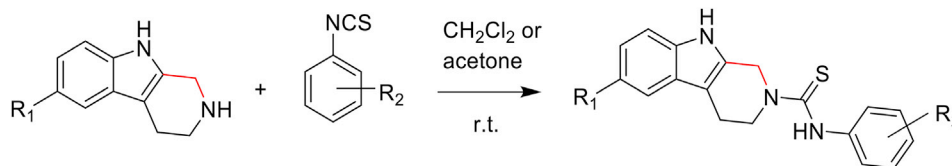
To a solution of tryptamine (8 mg, 50 μ mol) in DMF (500 μ L) was added 4-(4-(prop-2-yn-1-yloxy)benzoyl)benzoic acid (**2**) (16.8 mg, 60 μ mol), EDCI (14.4 mg, 75 μ mol), HOBT (11.5 mg, 75 μ mol) and DIPEA (13 mg, 100 μ mol). The reaction mixture was stirred overnight at room temperature and then pulled into 10 mL H₂O. The mixture was then extracted with ethyl acetate. The organic layer was next washed with NaHCO₃ and brine, dried with Na₂SO₄ and concentrated by vacuum. The residue was finally purified with flash chromatography to give probe **1** as a white solid (12 mg, 57%). ¹H-NMR (300 MHz, CDCl₃): δ 8.08 (br, 1H), 7.81 (d, J = 6.9 Hz, 2H), 7.75-7.65 (m 5H), 7.55-7.52 (m, 1H), 7.41 (d, J = 8.1 Hz, 1H), 7.24-7.10 (m, 2H), 7.49 (d, J = 8.7 Hz, 2H), 6.35 (br, 1H), 4.78 (s, 2H), 3.83 (t, J = 6.3, 2H), 3.14 (t, J = 6.6 Hz, 2H), 2.57 (s, 1H); MS (ESI-TOF) calculated for C₂₇H₂₂N₂O₃ 423.1703 (M+H⁺), found 423.1704.

Synthesis of Control Probe

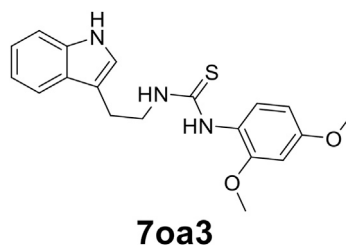


To a solution of NH₄Cl (3 mg, 56 μ mol) in DMF (500 μ L) was added 4-(4-(prop-2-yn-1-yloxy)benzoyl)benzoic acid (**2**) (16.8 mg, 60 μ mol), EDCI (14.4 mg, 75 μ mol), HOBT (11.5 mg, 75 μ mol) and DIPEA (19.2 mg, 150 μ mol). The reaction mixture was stirred overnight at room temperature and then pulled into 10 mL H₂O. The mixture was then extracted with ethyl acetate. The organic layer was next washed with NaHCO₃ and brine, dried with Na₂SO₄ and concentrated by vacuum. The residue was purified with flash chromatography to give control probe as a white solid (16 mg, quantitative). ¹H-NMR (300 MHz, CDCl₃): δ 7.93 (m, 2H), 7.82 (m, 4H), 7.67 (d, J = 9 Hz, 2H), 6.14 (br, 1H), 5.73 (br, 1H), 4.79 (s, 2H), 2.57 (s, 1H); MS (ESI-TOF) calculated for C₁₇H₁₃NO₃ 280.0968 (M+H⁺), found 280.0969.

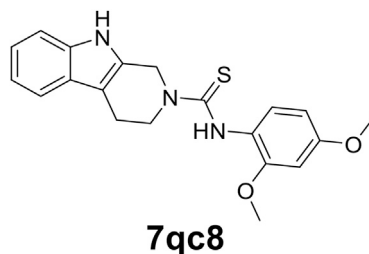
Synthesis of Screening Hits 7oa3 and 7qc8 and Derivatives 2-18



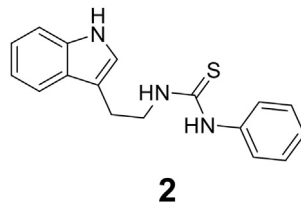
General Procedure. To a solution of substituted phenyl thioisocyanate in dry dichloromethane or dry acetone (1 eq), the amine in dry dichloromethane or dry acetone (1 eq) was added dropwise and the reaction mixture was stirred at room temperature overnight. The reaction was quenched with water, and was washed with NH₄Cl and NaHCO₃. The organic solvent was dried over MgSO₄, filtered and concentrated under vacuum. The crude compound was purified either by flash chromatography using hexane/ethyl acetate solvent mixture or by recrystallization in dichloromethane.



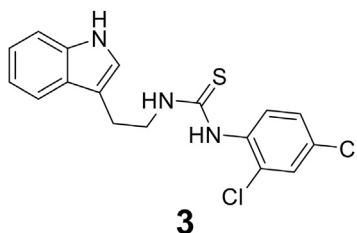
7oa3. White precipitate (90 mg, 80% yield). $^1\text{H-NMR}$ (300 MHz, CDCl_3): δ 8.00 (bs, 1H), 7.58 (d, J = 8.1 Hz, 1H), 7.36 (d, J = 8.1 Hz, 1H), 7.22-7.17 (m, 2H), 7.11-7.09 (m, 1H), 6.95 (s, 1H), 6.79 (bs, 1H), 6.38 (s, 1H), 6.24-6.21 (m, 1H), 5.94 (bs, 1H), 3.95 (q, J = 6.6 Hz, 2H), 3.78 (s, 3H), 3.64 (s, 3H), 3.05 (t, J = 6.6 Hz, 2H); MS (ESI-TOF) calculated for $\text{C}_{19}\text{H}_{21}\text{N}_3\text{O}_2\text{S}$ 356.1427 ($\text{M}+\text{H}^+$), found 356.1427.



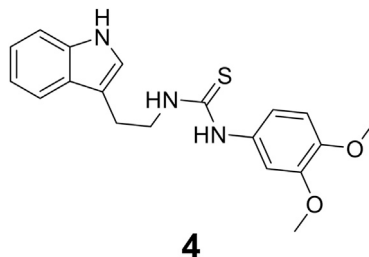
7qc8. Off white precipitate (56 mg, 50% yield). $^1\text{H-NMR}$ (300 MHz, CDCl_3): δ 7.82 (bs, 1H), 7.62 (d, J = 9.3 Hz, 1H), 7.49 (d, J = 7.8 Hz, 1H), 7.34 (d, J = 8.4 Hz, 1H), 7.21-7.1 (m, 2H), 6.5-6.48 (m, 2H), 5.16 (s, 2H), 4.17-4.11 (m, 2H), 3.84 (s, 3H), 3.81 (s, 3H), 2.95-2.93 (m, 2H); MS (ESI-TOF) calculated for $\text{C}_{20}\text{H}_{21}\text{N}_3\text{O}_2\text{S}$ 368.1427 ($\text{M}+\text{H}^+$), found 368.1428.



Compound 2. White precipitate (88 mg, 80% yield). $^1\text{H-NMR}$ (300 MHz, MeOD-D_4): δ 7.62 (d, J = 7.8 Hz, 1H), 7.32 (d, J = 8.1 Hz, 1H), 7.27-7.22 (m, 2H), 7.15-6.96 (m, 6H), 3.86 (t, J = 6.9 Hz, 2H), 3.06 (t, J = 6.9 Hz, 2H); MS (ESI-TOF) calculated for $\text{C}_{17}\text{H}_{17}\text{N}_3\text{S}$ 296.1216 ($\text{M}+\text{H}^+$), found 296.1216.

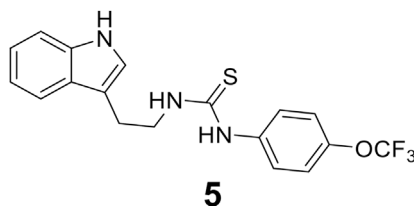


Compound 3. White precipitate (88 mg, 79% yield). $^1\text{H-NMR}$ (300 MHz, CDCl_3): δ 8.04 (bs, 1H), 7.56 (d, J = 7.5 Hz, 1H), 7.41-7.37 (m, 2H), 7.29-7.21 (m, 2H), 7.11 (t, J = 7.8 Hz, 1H), 7.01 (s, 1H), 6.9 (m, 1H), 5.9 (bs, 1H), 3.98-3.97 (m, 2H), 3.11 (t, J = 6.6 Hz, 2H); MS (ESI-TOF) calculated for $\text{C}_{17}\text{H}_{15}\text{Cl}_2\text{N}_3\text{S}$ 364.0437 ($\text{M}+\text{H}^+$), found 364.0434.

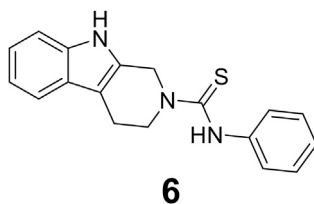


Compound 4. White precipitate (24 mg, 42% yield). $^1\text{H-NMR}$ (300 MHz, Acetone-D_6): δ 10.0 (bs, 1H), δ 8.54 (bs, 1H), 7.68 (d, J = 8.1 Hz, 1H), 7.36 (d, J = 8.1 Hz, 1H), 7.16-6.97 (m, 3H), 6.84-6.82 (m, 2H), 6.68-6.65 (m, 1H), 3.94-3.88 (m, 2H), 3.77 (s, 3H), 3.67 (s, 3H),

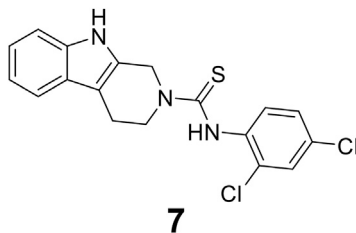
3.11–3.07 (m, 2H); MS (ESI-TOF) calculated for $C_{19}H_{21}N_3O_2S$ 356.1427 ($M+H^+$), found 356.1423.



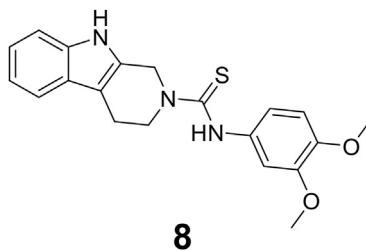
Compound 5. White precipitate (76 mg, 64% yield). 1H -NMR (300 MHz, MeOD- D_4): δ 7.63 (d, J = 7.8 Hz, 1H), 7.33 (d, J = 6.6 Hz, 1H), 7.24–7.21 (m, 2H), 7.14–6.97 (m, 5H), 3.87 (m, 2H), 3.1–3.06 (m, 2H); MS (ESI-TOF) calculated for $C_{18}H_{16}F_3N_3OS$ 380.1039 ($M+H^+$), found 380.1038.



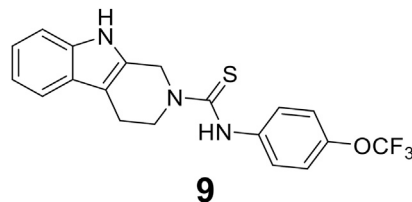
Compound 6. Off white precipitate (36 mg, 42% yield). 1H -NMR (300 MHz, Acetone- D_6): δ 9.99 (bs, 1H), 8.79 (bs, 1H), 7.45 (d, J = 7.8 Hz, 1H), 7.37–7.26 (m, 5H), 7.13–6.98 (m, 3H), 5.19 (s, 2H), 4.32–4.28 (m, 2H), 2.92–2.94 (m, 2H); MS (ESI-TOF) calculated for $C_{18}H_{17}N_3S$ 308.1216 ($M+H^+$), found 308.1217.



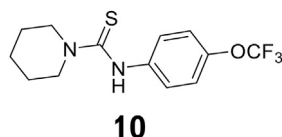
Compound 7. White precipitate (59 mg, 51% yield). 1H -NMR (300 MHz, Acetone- D_6): δ 10.1 (bs, 1H, NH), 8.62 (bs, 1H, NH), 7.52–7.44 (m, 3H), 7.36–7.34 (m, 2H), 7.08–7.01 (m, 2H), 5.3 (s, 2H), 4.35–4.34 (m, 2H), 2.97 (m, 2H); MS (ESI-TOF) calculated for $C_{18}H_{15}Cl_2N_3S$ 376.0437 ($M+H^+$), found 376.0438.



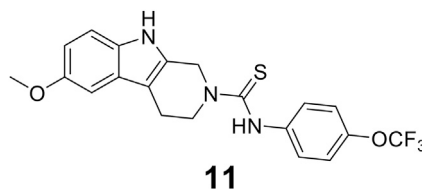
Compound 8. White precipitate (70 mg, 73% yield). 1H -NMR (300 MHz, Acetone- D_6): δ 9.99 (bs, 1H, NH), 8.68 (bs, 1H, NH), 7.44 (d, J = 8.1 Hz, 1H), 7.35 (d, J = 8.1 Hz, 1H), 7.10–6.98 (m, 3H), 6.87–6.79 (m, 2H), 5.19 (s, 2H), 4.31–4.28 (m, 2H), 3.78 (s, 3H), 3.74 (s, 3H), 2.91 (m, 2H); MS (ESI-TOF) calculated for $C_{20}H_{21}N_3O_2S$ 368.1427 ($M+H^+$), found 368.1424.



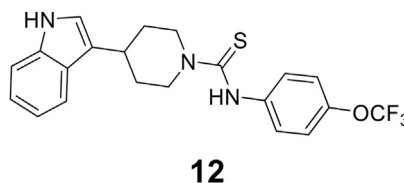
Compound 9. Off white precipitate (79 mg, 72% yield). ^1H -NMR (300 MHz, Acetone- D_6): δ 9.99 (bs, 1H), 8.89 (bs, 1H), 7.48 (d, J = 6.9 Hz, 2H), 7.42 (d, J = 7.5 Hz, 1H), 7.32 (d, J =7.8 Hz, 1H), 7.23 (d, J = 7.8 Hz, 2H), 7.07–6.96 (m, 2H), 5.2 (s, 2H), 4.29–4.28 (m, 2H), 2.91 (m, 2H); ^{13}C -NMR (150 MHz, Acetone- D_6): δ 181.74, 144.55, 140.19, 135.98, 130.92, 126.78, 126.26, 122.56, 120.87, 120.77, 120.66, 119.18, 118.48, 117.50, 110.97, 106.91, 47.12, 46.54, 20.77; MS (ESI-TOF) calculated for $\text{C}_{19}\text{H}_{16}\text{F}_3\text{N}_3\text{OS}$ 392.1039 ($\text{M}+\text{H}^+$), found 392.1038.



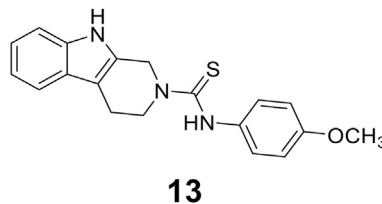
Compound 10. White precipitate (81 mg, 91% yield). ^1H -NMR (300 MHz, Acetone- D_6): δ 8.69 (bs, 1H), 7.49–7.46 (m, 2H), 7.25–7.22 (m, 2H), 3.95 (m, 4H), 1.63 (m, 6H); MS (ESI-TOF) calculated for $\text{C}_{13}\text{H}_{15}\text{F}_3\text{N}_2\text{OS}$ 305.0930 ($\text{M}+\text{H}^+$), found 305.0930.



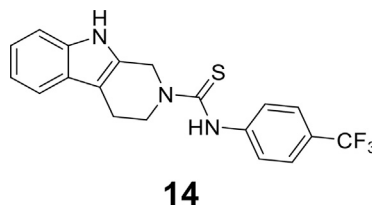
Compound 11. Light cream precipitate (120 mg, 57% yield). ^1H NMR (300 MHz, $\text{DMSO}-d_6$): δ 10.744 (s, 1H, NH), 9.595 (s, 1H, NH), 7.446–7.392 (m, 2H), 7.317–7.167 (m, 3H), 6.961–6.931 (m, 1H), 6.734–6.675 (m, 1H), 5.132 (s, 2H), 4.218 (s, 2H), 3.786–3.731 (m, 3H), 2.828 (s, 2H); MS (ESI-TOF) calculated for $\text{C}_{20}\text{H}_{18}\text{F}_3\text{N}_3\text{O}_2\text{S}$ 422.1145 ($\text{M}+\text{H}^+$), found 422.1147.



Compound 12. White precipitate (155 mg, 74% yield). ^1H NMR (300 MHz, $\text{DMSO}-d_6$): δ 10.81 (s, 1H, NH), 9.39 (s, 1H, NH), 7.59 (t, 1H, J =6.9 Hz), 7.44–7.39 (m, 2H), 7.36–7.26 (m, 3H), 7.14–7.13 (m, 1H), 7.10–7.01 (m, 1H), 6.98–6.94 (m, 1H), 4.86 (d, 2H, J = 12.3), 3.29–3.15 (m, 2H), 2.04 (d, 2H, J = 12.6 Hz), 1.70–1.62 (m, 2H); MS (ESI-TOF) calculated for $\text{C}_{21}\text{H}_{20}\text{F}_3\text{N}_3\text{OS}$ 420.1352 ($\text{M}+\text{H}^+$), found 420.1354.

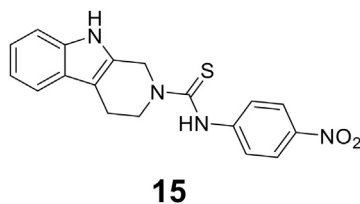


Compound 13. White precipitate (106 mg, 63% yield). ^1H NMR (300 MHz, $\text{DMSO}-d_6$): δ 10.90 (s, 1H, NH), 9.38 (s, 1H, NH), 7.41 (d, 1H, J =7.8Hz), 7.32–7.28 (m, 1H), 7.18–7.14 (m, 2H), 7.07–6.94 (m, 2H), 6.89–6.85 (m, 2H), 5.15 (s, 2H), 4.19 (s, 2H), 3.74–3.73 (m, 3H), 2.82 (s, 2H); MS (ESI-TOF) calculated for $\text{C}_{19}\text{H}_{19}\text{N}_3\text{OS}$ 338.1322 ($\text{M}+\text{H}^+$), found 338.1319.

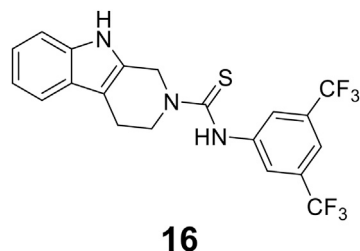


Compound 14. White precipitate (141 mg, 75% yield). ^1H NMR (300 MHz, $\text{DMSO}-d_6$): δ 10.92 (s, 1H, NH), 9.78 (s, 1H, NH), 7.64–7.55 (m, 4H), 7.44 (d, 1H, J = 6.9 Hz), 7.32 (d, 1H, J = 7.5 Hz), 7.06–6.99 (m, 2H), 5.15 (s, 2H), 4.25 (s, 2H), 2.88 (s, 2H); MS (ESI-TOF)

calculated for $C_{19}H_{16}F_3N_3S$ 376.1090 ($M+H^+$), found 376.1088.



Compound 15. Yellow precipitate (130 mg, 74% yield). 1H NMR (300 MHz, DMSO- d_6): δ 10.92 (s, 1H, NH), 9.856 (s, 1H, NH), 8.18 (d, 2H, J = 8.1 Hz), 7.62 (d, 2H, J = 8.4 Hz), 7.44 (d, 1H, J = 7.8 Hz), 7.32 (d, 1H, J = 7.5 Hz), 7.09 – 6.96 (m, 2H), 5.15 (s, 2H), 4.26 (s, 2H), 2.90 (s, 2H); MS (ESI-TOF) calculated for $C_{18}H_{16}N_4O_2S$ 353.1067 ($M+H^+$), found 353.1066.



Compound 16. White precipitate (129 mg, 58% yield). 1H NMR (300 MHz, DMSO- d_6): δ 10.94 (s, 1H, NH), 9.96 (s, 1H, NH), 8.11 (s, 2H), 7.78 (s, 1H), 7.44 (d, 1H, J = 7.5 Hz), 7.32 (t, 1H, J = 6.9 Hz), 7.08 – 6.96 (m, 2H), 5.21 (s, 2H), 4.25 (d, 2H, J = 4.8), 2.90 (s, 2H); MS (ESI-TOF) calculated for $C_{20}H_{15}F_6N_3S$ 444.0964 ($M+H^+$), found 444.0963.

QUANTIFICATION AND STATISTICAL ANALYSIS

All data fitting and statistical analysis were performed using GraphPad Prism version 8.0. Reported values, exact n values, and statistical significance are indicated in the Figure Legends. Statistical significance was defined as $p < 0.05$ and determined by 2-tailed Student's t tests.