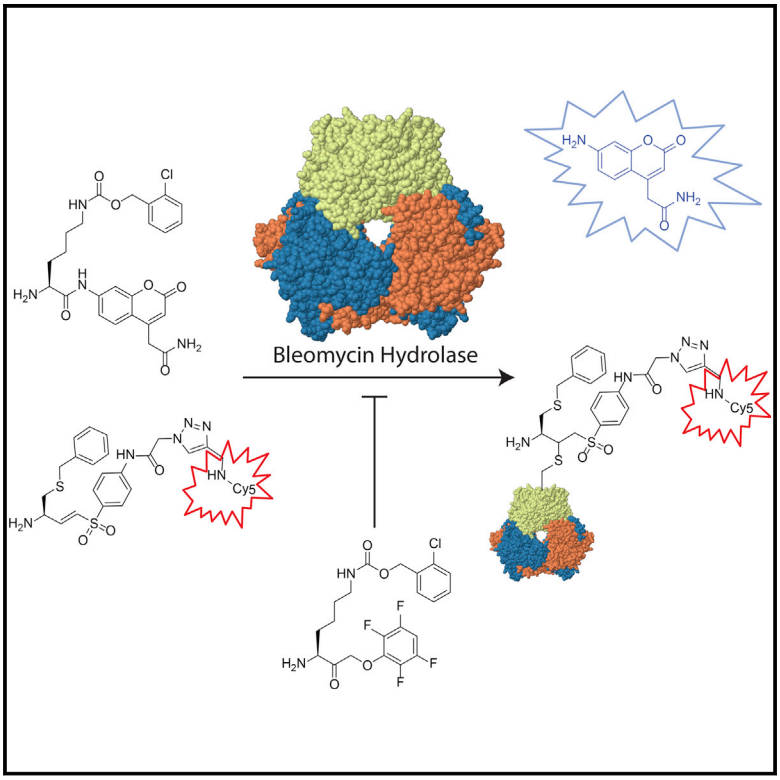
Brief Communication

Chemistry & Biology

Design and Synthesis of Activity-Based Probes and Inhibitors for Bleomycin Hydrolase

Graphical Abstract



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In Brief

Bleomycin hydrolase is a neutral cysteine aminopeptidase that has been ascribed roles in many physiological and pathological processes, but its primary biological function remains enigmatic. van der Linden et al. describe the synthesis and evaluation of activity-based probes, irreversible inhibitors, and fluorogenic substrates for bleomycin hydrolase.

Highlights

- Application of substrate screening to identify bleomycin hydrolase-specific scaffolds
- Identification of cell-permeable irreversible inhibitors for bleomycin hydrolase
- Identification of cell-permeable activity-based probes for bleomycin hydrolase

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Design and Synthesis of Activity-Based Probes and Inhibitors for Bleomycin Hydrolase

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SUMMARY

Bleomycin hydrolase (BLMH) is a neutral cysteine aminopeptidase that has been ascribed roles in many physiological and pathological processes, yet its primary biological function remains enigmatic. In this work, we describe the results of screening of a library of fluorogenic substrates to identify non-natural amino acids that are optimally recognized by BLMH. This screen identified several substrates with k_{cat}/K_M values that are substantially improved over the previously reported fluorogenic substrates for this enzyme. The substrate sequences were used to design activity-based probes that showed potent labeling of recombinant BLMH as well as endogenously expressed BLMH in cell extracts, and in intact cells. Importantly, we identify potent BLMH inhibitors that are able to fully inhibit endogenous BLMH activity in intact cells. These probes and inhibitors will be valuable new reagents to study BLMH function in cellular and animal models of human diseases where BLMH is likely to be involved.

INTRODUCTION

Bleomycin hydrolase (BLMH) is a cysteine aminopeptidase that is ubiquitously expressed in mammalian tissue (Brömme et al., 1996). BLMH was initially discovered for its ability to inactivate bleomycin (Umezawa et al., 1972; Schwartz et al., 1999), a drug used extensively to treat cancer. BLMH is a cytosolic neutral protease with a barrel-like structure composed of six monomers of 50 kDa each (Brömme et al., 1996; O'Farrell et al., 1999). The active sites of BLMH are located within the barrel (Hibino et al., 2013). After expression, the C terminus of the protein undergoes self-cleavage yielding an enzyme with broad-specificity aminopeptidase activity (Joshua-Tor et al., 1995; Zheng et al., 1998). While the physiological roles of BLMH remain obscure, it has been suggested to be important in several physiological and pathological processes. BLMH null mice have reduced neonatal survival, brain pathologies (Montoya et al., 2007), and a dermatitis phenotype. BLMH is involved in the production of free amino acids as moisturizing agents in the skin (Kamata et al., 2009), and therefore plays an important role in maintaining epidermal integrity (Kamata et al., 2011). BLMH has also been shown to play a role in peptide trimming downstream of the proteasome, and thus has a role in the production of peptides for antigen presentation (Stoltze et al., 2000; Kim et al., 2009); however, this role seems to be redundant (Towne et al., 2007).

In addition to its aminopeptidase activity, BLMH has the ability to hydrolyze homocysteine lactone, a reactive metabolite produced from methionine, which causes protein damage and hyperhomocysteinemia and is implicated in multiple human diseases, including Alzheimer's disease. BLMH is implicated in protection against homocysteine toxicity (Zimny et al., 2006; Borowczyk et al., 2012). However, recent data showing that another enzyme exists with higher homocysteine lactonase activity has called into question the role for BLMH in homocysteine detoxification (Marsillach et al., 2014). BLMH polymorphisms are also associated with sporadic Alzheimer's disease (Montoya et al., 1998; Papassotiropoulos et al., 2000). Ectopic expression of BLMH increases processing of amyloid precursor, suggesting a regulatory role for BLMH in the secretion of amyloid precursor protein and β-amyloid, which are major components of Alzheimer's disease-associated plaques (Lefterov et al., 2000, 2001). However, other studies have shown reduced homocysteine lactonase activity in brains of Alzheimer patients that correlated with a reduction in BLMH levels, thus suggesting a protective role for BLMH (Suszynska et al., 2010).

At present, BLMH aminopeptidase activity has only been measured using fluorogenic substrates (Brömme et al., 1996; Zimny et al., 2006). While these substrates provide a relatively rapid and simple readout of enzyme activity, the resulting data are often difficult to interpret because other aminopeptidases are likely to be active toward the reported substrates (Rut et al., 2015). Activity-based probes circumvent this problem by covalently attaching to target enzymes, allowing direct identification and quantification of enzyme activity (Sanman and Bogyo, 2014). Furthermore, by screening substrate libraries of increased diversity, it should be possible to identify sequences that are optimized for BLMH and not cleaved by other aminopeptidases.

In this article, we present a screen of a diverse substrate library made up of both natural and non-natural amino acids to identify optimal binding elements for BLMH. Using this approach we were able to design selective substrates, activity-based probes, and inhibitors for BLMH. These reagents can be used for biochemical studies of the purified enzyme as well as to monitor

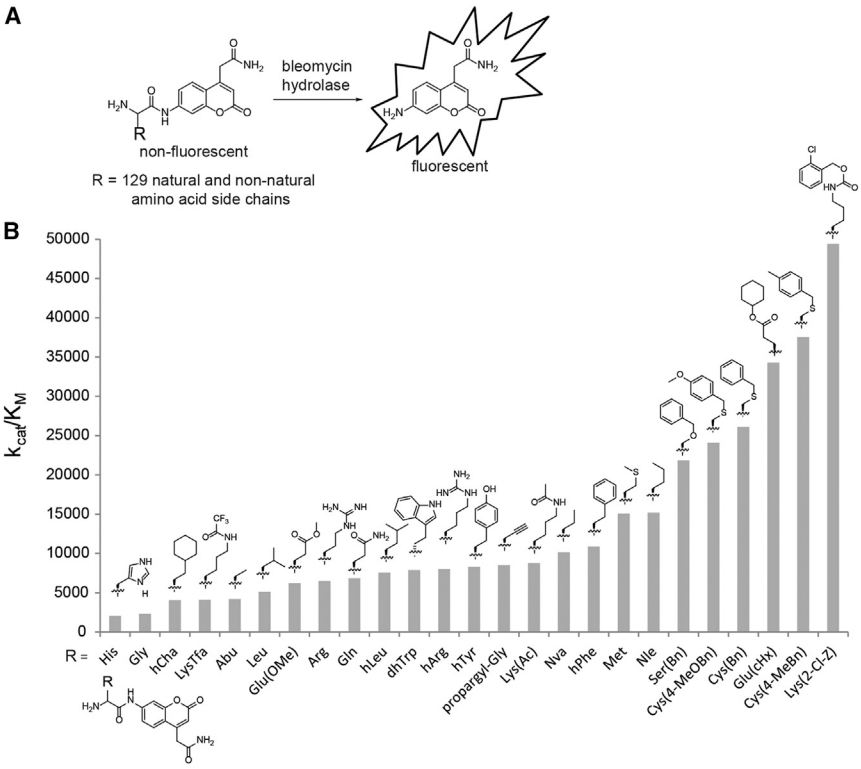


Figure 1. Screening of a Diverse Fluorogenic Substrate Library

(A) Diagram of the fluorogenic substrate library screening approach.

(B) Ranking of the top BLMH substrates ordered by k_{cat}/K_M values. The substrates containing Lys(2-Cl-Cbz) and Cys(Bn) are effective, with activities that are substantially higher than the best natural amino acid.

and inhibit the endogenous protease target in cellular extracts, intact cells, and potentially whole organisms.

RESULTS

We screened a hybrid tailored amino acid substrate library that was recently described and used to find highly efficient substrates of a number of aminopeptidases (Drag et al., 2010; Rut et al., 2015). This library is made up of a diverse set of natural and non-natural amino acids linked to a fluorogenic reporter that provides a signal when the substrate is cleaved by a protease. Because bleomycin hydrolase is an aminopeptidase, we screened the library of single amino acid-ACC (7-amino-4-carbamoylmethylcoumarin) substrates against the recombinant protease (Figure 1A). Interestingly, this screen identified non-natural amino acid-containing substrates that had k_{cat}/K_M values greater than the best natural amino acid, methionine (Figure 1B). The top two substrates that we chose for further development into inhibitors and active site probes were Lys(2-Cl-Cbz)-ACC and S-benzylated cysteine.

To design activity-based probes, we choose electrophiles that would covalently label the active site nucleophile, but that also allowed incorporation of a tag that would not interfere with the free amino group required for aminopeptidase recognition. Therefore, we initially used the vinyl sulfone, since this electrophile has been extensively applied to probes of cathepsins and also the proteasome (Verdoes et al., 2006; Yuan et al., 2006). We synthesized a reagent that allows introduction of a vinyl sulfone equipped with an azide via the Horner-Wadsworth-Emmons (HWE) reaction (Figure 2A). Diethyl (iodo)methylphosphonate was reacted with 4-aminothiophenol sodium salt to yield aminothioether **1**. We then chloroacetylated the amine group in **1** and performed a subsequent substitution reaction to generate azide **2**. Oxidation of the thioether yielded sulfone **3**, which can be used as a general reagent to make vinyl sulfone probes with a Click handle. We converted commercially available Boc-Lys(2-Cl-Cbz)-OH (**4a**) and Boc-Cys(Bn)-OH (**5a**) to the corresponding Weinreb amides and reduced them to their respective aldehydes (**4c** and **5c**), and reacted in the HWE olefination reaction to yield the azide-labeled phenyl vinyl sulfone inhibitors **4d** and **5d**. We obtained the final Cy5 modified activity-based probes WL1256 and WL1259 using Click chemistry (Figure 2A).

To test the newly synthesized probes we incubated recombinant BLMH (rBLMH) with increasing concentrations of each probe, then measured labeling by SDS-PAGE analysis followed by scanning of the gel for fluorescent-labeled protein (Figure 2B). The labeling confirmed that both probes efficiently labeled the recombinant protein, as indicated by the presence of a doublet of 52 kDa corresponding to the expected size of rBLMH. The appearance of multiple labeled species is likely due to autoprocessing of BLMH, as has been previously described (Zheng et al., 1998). We next tested the limit of sensitivity of the probes by labeling with a set probe concentration (1 μM) and decreasing the amount of the rBLMH in the labeling reaction (Figure 2C). Ultimately, the probe WL1259 showed the most potent labeling of the target, and was therefore used for validation studies targeting the endogenously expressed enzyme.

To confirm that our optimal probe WL1259 was a viable tool for the study of BLMH function, we performed probe labeling studies in lysates from fibroblasts derived from wild-type (WT) and BLMH knockout (KO) mice (Figure 2D). Importantly, these results confirmed that the probe labeled a protein of the expected size of 52 kDa in WT lysate that was confirmed to be native BLMH due to its absence in the KO cell lysate. We observed similar results when the probe was used to label intact fibroblast cells derived from WT and BLMH KO mice (Figure 2E). These data confirmed that the probe was able to enter cells and label the native BLMH.

Given the success of the activity-based probes in both lysates and intact cells, we used the same general scaffolds to generate inhibitors of BLMH that could be used to block its function in vivo. We initially synthesized phenyl vinyl sulfone derivatives of Lys(2-Cl-Cbz) and Cys(Bn), as these most closely matched the

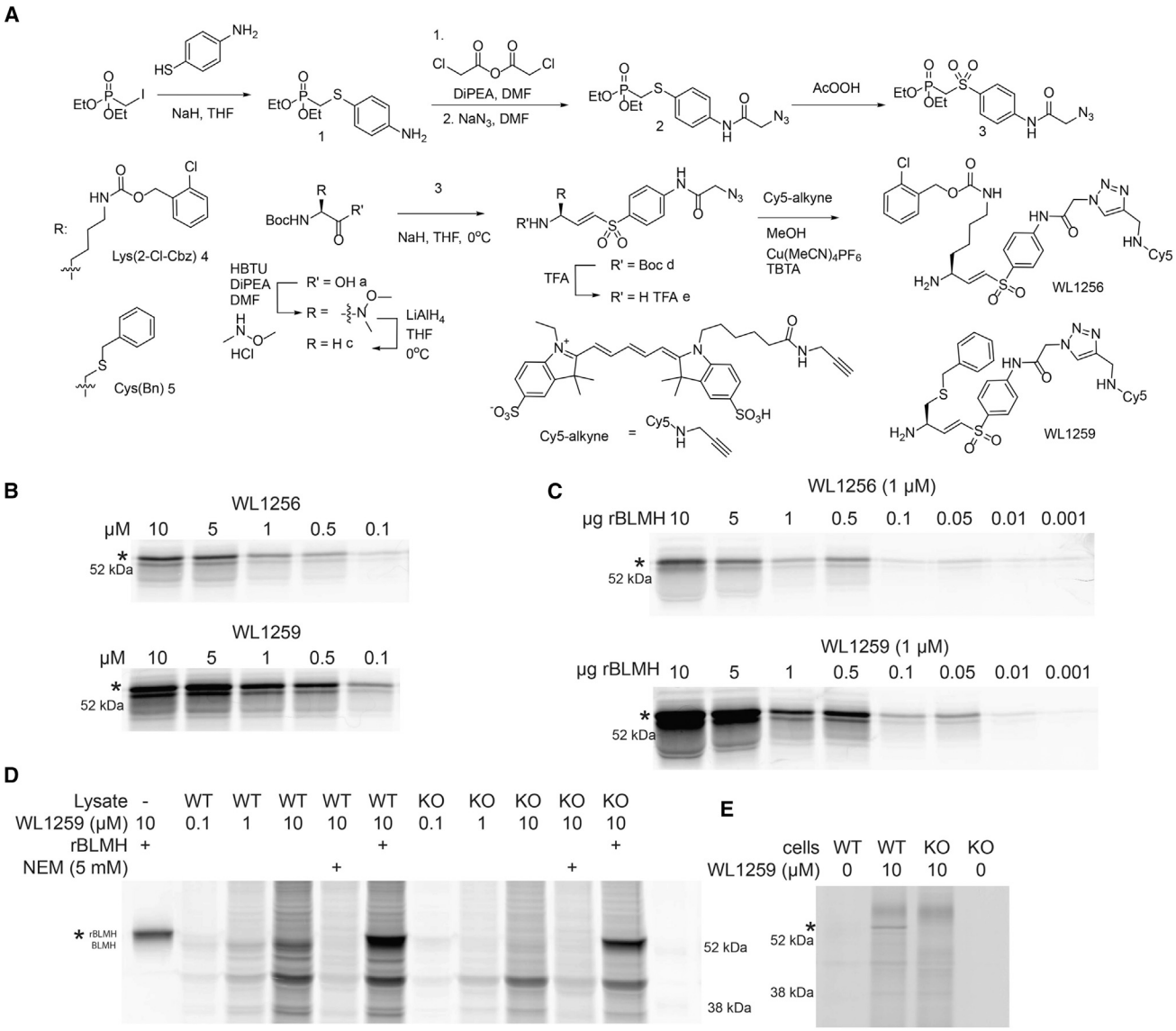


Figure 2. Synthesis and Evaluation of BLMH Probes Based on the Library Screening Result

(A) Synthesis scheme for the azide-intermediate and final Cy5-labeled probes.

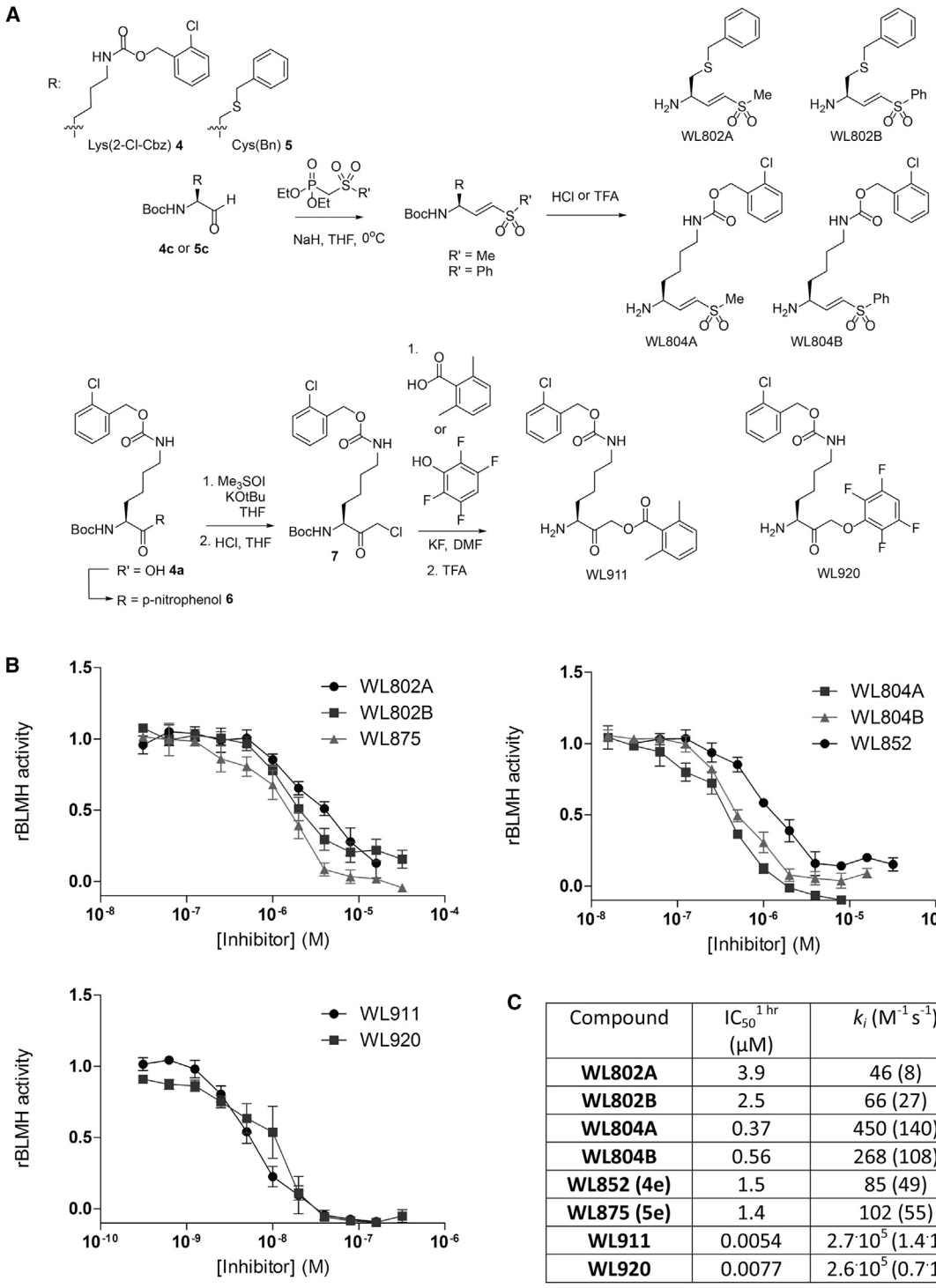
(B) Labeling of 1 μg rBLMH over a range of probe concentrations. Samples were analyzed by SDS-PAGE followed by scanning of the gel using a flatbed laser scanner to detect the Cy5 signal.

(C) Labeling of decreasing amounts of rBLMH using 1 μM of each of the two primary Cy5 probes.

(D) Assessment of BLMH labeling by the selected probe, WL1259 in wild-type (WT) and BLMH knockout (KO) fibroblast lysates. rBLMH is shown in the first lane as a standard, but appears as a slightly higher molecular weight than the native enzyme due to the presence of HIS_6 tag.

(E) Intact WT and BLMH KO cells were incubated with WL1259 (10 μM) for 3 hr at 37°C, then cells were lysed and analyzed by SDS-PAGE as in (D). The location of BLMH is indicated by a star and is only visible in the WT cells treated with the probe.

activity-based probes WL1256 and WL1259. We also synthesized the methyl sulfone versions of the compounds to see whether the smaller methyl group would reduce steric hindrance in the active site and result in greater potency. Both classes of vinyl sulfone compounds were generated using the same chemistry as described for the probe synthesis (Bogyo et al., 1997). We also synthesized the acyloxymethyl ketone (AOMK) and phenoxymethyl ketone (PMK) version of the lead compounds, as these two electrophiles have been extensively used to target cysteine proteases (Powers et al., 2002; Kato et al., 2005; Deu et al., 2010). The synthesis of all the potential BLMH inhibitors is shown in Figure 3A (Wang et al., 2004). To measure the inhibitory potencies of the inhibitors, we used a fluorogenic substrate assay with the reported BLMH substrate Met-AMC and rBLMH (Figure 3B). We found that while the original vinyl sulfone compounds had overall good potencies, the AOMK and PMK were more potent inhibitors of BLMH by several orders of magnitude (Figure 3C). These data confirm that our chosen scaffold can



C

Compound	$IC_{50}^{1\ hr}$ (μM)	k_i ($M^{-1}\ s^{-1}$)
WL802A	3.9	46 (8)
WL802B	2.5	66 (27)
WL804A	0.37	450 (140)
WL804B	0.56	268 (108)
WL852 (4e)	1.5	85 (49)
WL875 (5e)	1.4	102 (55)
WL911	0.0054	$2.7 \cdot 10^5$ ($1.4 \cdot 10^5$)
WL920	0.0077	$2.6 \cdot 10^5$ ($0.7 \cdot 10^5$)

Figure 3. Synthesis and Evaluation of BLMH Inhibitors

(A) Synthesis of six inhibitors for BLMH containing multiple different cysteine-reactive electrophiles.

(B) Fluorogenic substrate assay using rBLMH to determine the IC_{50} of inhibitors. Fluorogenic substrate experiments were performed in triplicate for each point and were normalized. Error bars represent SEM.

(C) Calculated IC_{50} and k_i values for the BLMH inhibitors. k_i values were derived from curve fitting of the normalized data and are presented as the average ± SD.

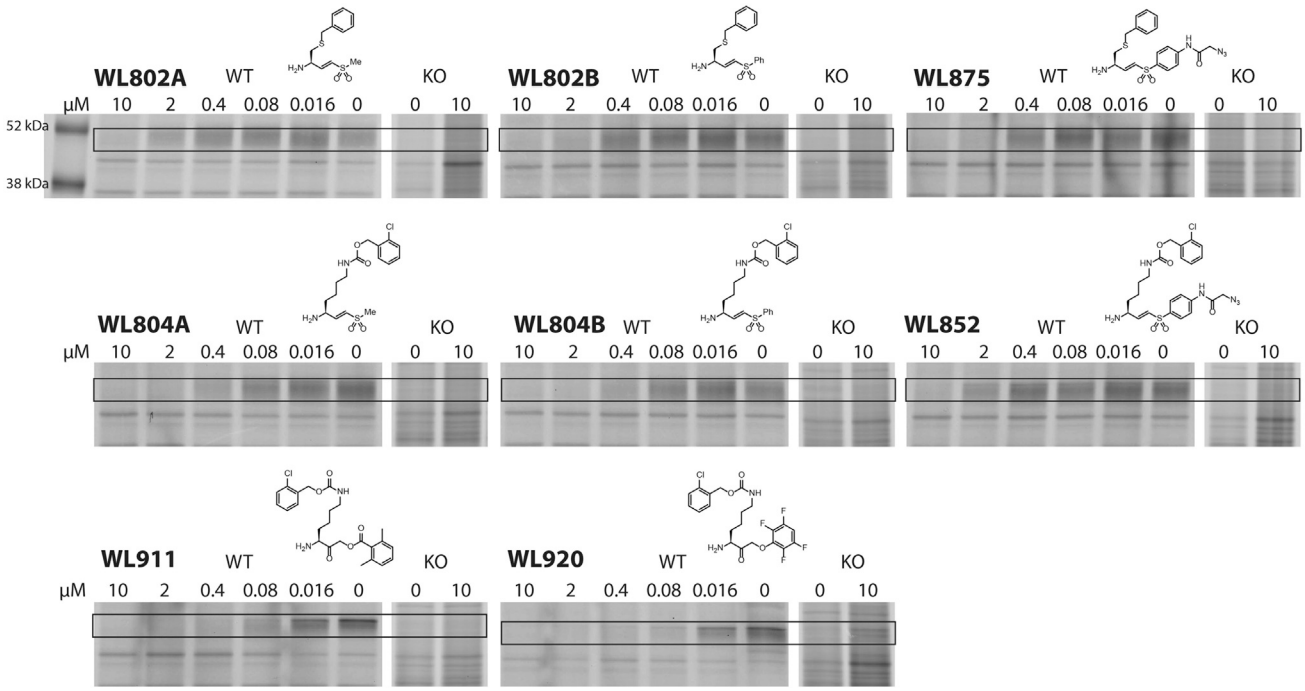


Figure 4. Inhibition of Endogenous BLMH in Intact Cells Using the Optimized Inhibitors

Embryonic fibroblast cells derived from wild-type (WT) and BLMH knockout (KO) mice were incubated with inhibitors at the indicated concentrations for 3 hr at 37°C. Cells were washed and lysed, and the lysate incubated with 1 μM WL1259 for 1 hr at 37°C. Samples were analyzed by SDS-PAGE followed by scanning of the gel by a flatbed laser scanner to observe fluorescence. The box indicates the location of native BLMH that is absent in the knockout (KO) cells.

be used to yield highly potent inhibitors that are effective in the low nanomolar concentration range.

As a final test of potency and cell permeability, we treated intact mouse embryonic fibroblasts (MEF) or BLMH KO mouse fibroblasts with a range of doses of each inhibitor. We then lysed cells and labeled them with WL1259, and measured residual activity of the native BLMH enzyme by SDS-PAGE analysis (Figure 4). We found that all of the vinyl sulfones were able to penetrate cells and completely inhibit BLMH, but only at micromolar concentrations, consistent with the measured potencies of the compounds against the recombinant enzyme. The AOMK (WL911) and PMK (WL920) derivatives, on the other hand, were able to completely block activity of the native BLMH at mid to high nanomolar concentrations. Thus, we have identified a class of highly potent inhibitors of this enigmatic protease that can be used on intact cells to block enzyme activity and allow studies of protease function.

SIGNIFICANCE

Although BLMH has been studied for many years, chemical tools to study its function have not been reported. Here, we describe activity-based probes and potent cell-permeable inhibitors of BLMH. Given that this enzyme has been postulated to be involved in many physiological processes important in human diseases, such as antigen processing, homocysteine lactone detoxification, and Alzheimer's disease, the inhibitors and probes presented here will be highly valuable reagents for further study of BLMH, and can be used to shed light on its still enigmatic primary biological functions.

EXPERIMENTAL PROCEDURES

Synthesis of Inhibitors and Probes

Detailed methods and compound characterization for all inhibitors and activity-based probes can be found in the Supplemental Materials and Methods section.

Cloning, Expression, and Purification of rBLMH

Details regarding cloning, expression, and purification of rBMLH can be found in the Supplemental Information.

Screening of Fluorescent Substrates

BLMH was assayed in 100 mM Tris-HCl (pH 7.5), 1 mM EDTA, and 1 mM DTT. Assays were performed at 37°C and enzyme was incubated at 37°C for 30 min before adding substrate. Screening of the library was carried out at 2 μM substrate concentration, with 20 nM enzyme. Release of fluorophore was monitored continuously with excitation at 355 nm and emission at 460 nm for 30–45 min, and the linear portion of the progress curve was used to calculate velocity. All experiments were repeated at least three times. Analysis of the results was based on total relative fluorescence units for each substrate, setting the highest value to 100% and adjusting the other results accordingly.

Determination of Kinetic Parameters k_{cat}, K_M, and k_{cat}/K_M

Enzyme assay conditions were as follows: 100-μl reaction with eight different substrate concentrations. Release of ACC fluorophore was monitored as above. Absolute ACC concentrations were calculated by the hydrolysis of three independent ACC-coupled substrates at known concentration, and average value was determined. Concentration of DMSO in the assay was less than 1%.

Fluorogenic Substrate Assay

BLMH activity was measured in black 96-well plates (n = 3). rBLMH (1 nM) was incubated with inhibitors (100× DMSO stock) in 100 mM Tris (pH 7.5), 1 mM EDTA, and 1 mM DTT for 1 hr at 37°C. Substrate (100 μM Met-AMC) was added, and 7-amino-4-methylcoumarin (AMC) fluorescence was monitored every minute for 45 min at 37°C using a Biotek plate reader. Half-maximal inhibitory concentration (IC_{50}) values were calculated using Graphpad Prism. k_i values were calculated using the formula $v/v_0 = \exp(-k_i[I]t)$.

BLMH KO Mouse Generation

KO mice were bred from frozen heterozygous embryos obtained from Jackson. The deletion of the BLMH gene was confirmed by genotyping, and loss of protein expression was confirmed by western blot. All animal care and experimentation was conducted in accordance with current NIH and Stanford University Institutional Animal Care and Use Committee guidelines.

Labeling Experiments in Cell Lysate

Mouse immortalized fibroblasts and mouse BLMH KO immortalized fibroblasts were cultured on DMEM (Gibco) supplemented with 10% fetal calf serum (Gibco), 100 units/ml penicillin, and 100 μg/ml streptomycin (Gibco) in a 5% CO_2 humidified incubator at 37°C. Cells were harvested, washed twice with PBS, and permeated in lysis buffer (50 mM Tris [pH 7.4], 150 mM NaCl, 20 mM $MgCl_2$, 0.5% NP-40, 2 mM DTT) for 20 min on ice and centrifuged at 16,100 relative centrifugal force for 20 min at 4°C. The supernatant was collected and the protein content determined by bicinchoninic acid (BCA) assay (Pierce). Total lysate (15 or 10 μg total protein) was incubated with the inhibitors (10× solution in DMSO) for 1 hr at 37°C. Reaction mixtures were boiled in Laemmli buffer containing β-mercaptoethanol for 3 min before being resolved by 15% SDS-PAGE. In-gel detection of fluorescently labeled proteins was performed directly in the wet gel slabs on the Typhoon Variable Mode Imager (Amersham Biosciences) using the Cy3/Tamra settings (λ_{ex} 532 nm, λ_{em} 560 nm) for WL1189 and WL1192, or Cy5 settings (λ_{ex} 650 nm, λ_{em} 670 nm) for WL1256 and WL1259.

Labeling/Inhibition Experiments in Living Cells

Cells (50,000) were seeded and grown overnight. Stock solutions of inhibitors or probes (100×) were added to 0.5 ml of medium and the cells were incubated for 3 hr at 37°C. Cells were harvested and washed twice with PBS, and lysate was prepared as described above. The protein content was determined by BCA assay (Pierce). For cells labeled with probe, the lysate was immediately boiled in Laemmli sample buffer and resolved as described above. Lysates (20 μg total protein, diluted with lysis buffer) from the inhibitor-treated cells were labeled with 1 μM probe D for 1 hr at 37°C, and boiled in Laemmli sample buffer and resolved as described above. Staining of the gel with Coomassie brilliant blue was used to confirm equal protein loading.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Materials and Methods and can be found with this article online at http://dx.doi.org/10.1016/j.chembiol.2015.07.010.

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