Design of Selective Substrates and Activity-Based Probes for Hydrolase Important for Pathogenesis 1 (HIP1) from *Mycobacterium tuberculosis*

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ⓈSupporting Information

ABSTRACT: Although serine proteases are important mediators of *Mycobacterium tuberculosis* (Mtb) virulence, there are currently no tools to selectively block or visualize members of this family of enzymes. Selective reporter substrates or activity-based probes (ABPs) could provide a means to monitor infection and response to therapy using imaging methods. Here, we use a combination of substrate selectivity profiling and focused screening to identify optimized reporter substrates and ABPs for the Mtb "Hydrolase important for pathogenesis 1" (Hip1) serine protease. Hip1 is a cell-envelope-associated enzyme with minimal homology to host proteases, making it an ideal target for probe development. We identified substituted 7-amino-4-chloro-3-(2-bromoethoxy)isocoumarins as irreversible inhibitor scaffolds. Furthermore, we used specificity data to generate selective reporter substrates and to further optimize a selective chloroisocoumarin inhibitor. These new reagents are potentially useful in delineating the roles of Hip1 during pathogenesis or as diagnostic imaging tools for specifically monitoring Mtb infections.

KEYWORDS: Mycobacterium tuberculosis, hydrolase important for pathogenesis 1 (Hip1), protease, activity-based probe, fluorogenic substrate, substrate profiling
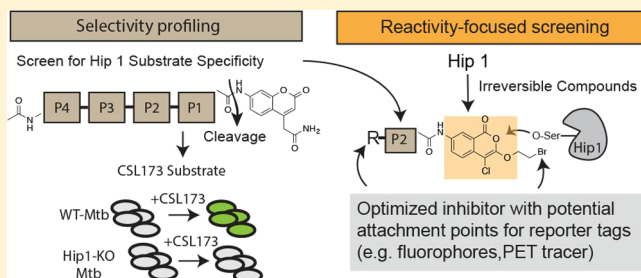
Standard pharmacological control of tuberculosis relies on combination treatment using four drugs over a period of several months.[1] Although the emergence of multidrug and extensively drug-resistant strains of *Mycobacterium tuberculosis* (Mtb) highlights the need for novel antituberculosis agents,[1,2] the lack of diagnostic methods to accurately and rapidly monitor the drug-responsiveness of a patient imposes further challenges for controlling infections in the clinic.[3] The development of targeted imaging agents that can directly bind to or be activated by enzymes expressed only by Mtb would be valuable for visualizing infection as well as monitoring the response to therapy.

Visualizing the activity of individual proteases with chemical probes has been established as a viable strategy for noninvasive in vivo imaging of conditions such as cancer[4] and inflammation.[5] Suitable chemical probes for these applications include activity-based probes (ABPs, i.e., irreversible inhibitors of a protease with a reporter tag[6]) as well as protease-activated reporters such as quenched fluorescent substrates.[7] A recent chemoproteomic study revealed that Mtb expresses more than

Table 1. Catalytic Efficiencies of Fluorogenic Substrates for *M. tuberculosis* Hip1 and Human Neutrophil Elastase[a]

	P4	P3	P2	P1	*M. tb.* Hip1			Human Neutrophil Elastase		
					k_{cat} (s^{-1})	K_M (M)	k_{cat}/K_M (s^{-1}*M^{-1})	k_{cat} (s^{-1})	K_M (M)	k_{cat}/K_M (s^{-1}*M^{-1})
WKLL-ACC	L-Trp	L-Lys	L-Leu	L-Leu	1.5E-2	7.7E-6	1.9E3	n.d.	n.d.	n.d.
CSL173	L-Igl	L-4-Cl-Phe	L-Lys	L-Leu	2.7	2.8E-7	9.6E6	3E-3	2.9E-6	1.0E3
CSL174	L-Asp	L-4-Cl-Phe	L-Lys	L-Leu	1.27	5.3E-6	2.4E5	*	*	<<1E3
CSL175	L-Igl	Gly	L-Lys	L-Leu	5.6E-1	7.3E-5	7.6E3	*	*	<<1E3
CSL176	L-Igl	L-4-Cl-Phe	L-Tyr	L-Leu	*	*	<<1E3	*	*	<<1E3

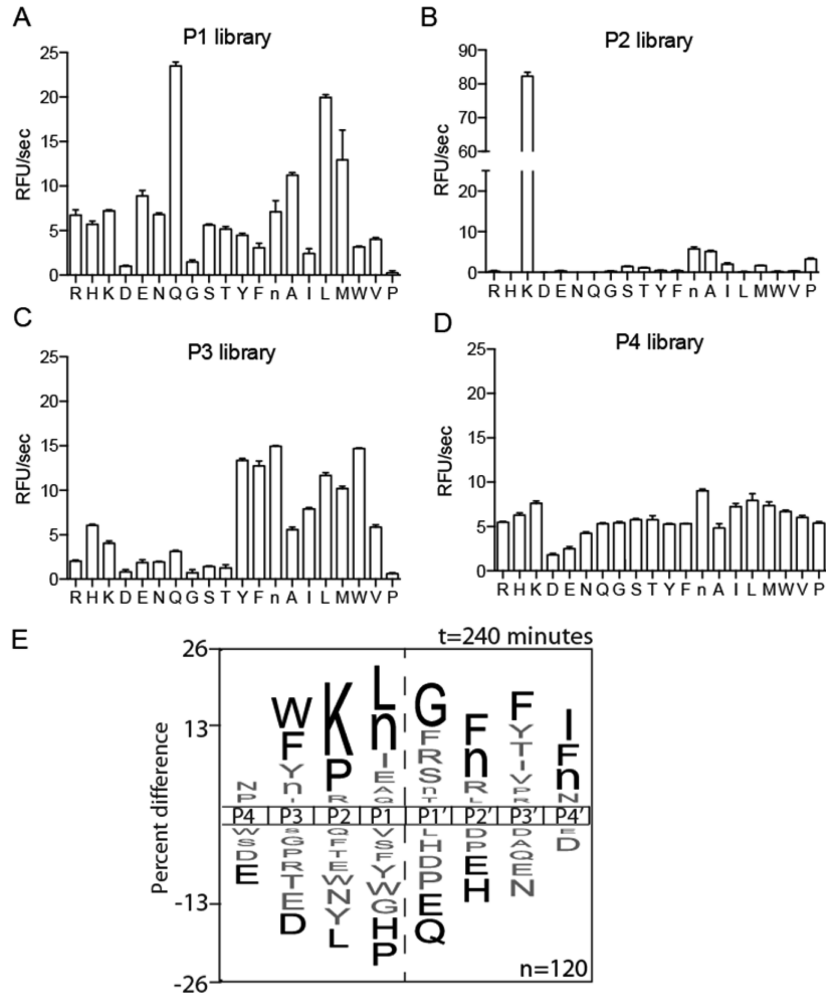[a]*No cleavage observed under 100 μM. n.d. = not determined.

Figure 1. Positional scanning-synthetic combinatorial libraries (PS-SCL)[11] were used to profile the substrate specificity of Hip1. Cleavage of the following sublibraries by Hip1 was measured in a fluorogenic substrate assay: (A) Ac-X-X-X-P1-ACC, (B) Ac-X-X-P2-X-ACC, (C) Ac-X-P3-X-X-ACC, and (D) Ac-P4-X-X-X-ACC, where X represents a mixture of all natural amino acids (excluding Cys) and norleucine, whereas single amino acids in the indicated P1–P4 positions are fixed, Ac = Acetyl. (E) Multiplex substrate profiling by mass spectrometry (MSP-MS)[12] employing a set of 228 defined 14-mer peptides to monitor cleavage by Hip1 in an MS/MS-based readout. The iceLogo plot illustrates the P4 to P4′ specificity of Hip1. Amino acids that are most frequently found at each position are shown above the horizontal line while amino acids least frequently observed are below. Lowercase "n" is norleucine and amino acid in black text have *p* value < 0.05.

70 predicted serine hydrolases (including 6 annotated serine proteases and 27 hypothetical hydrolases of unknown function)[8] that have the potential to be useful as imaging or drug targets. The prioritization of a suitable target for the design of new chemical probes for Mtb is based on a number of basic criteria. These include a lack of homology to host enzymes, expression during relevant stages of infection, potential biological accessibility to the probes, and finally ease of expression and purification to facilitate screening and optimization of lead molecules. Mtb hydrolase important for

pathogenesis 1 (Hip1, previously known as carboxylesterase A, Rv2224c, MT2282)[9] is a cell-envelope-associated serine protease whose proteolytic activity is required for the immunomodulation of host inflammatory responses.[9e] Its established role in mycobacterial virulence makes it a potential drug target.[9e] Furthermore, it shows only weak homology with other host-derived serine proteases and it can be expressed recombinantly in a proteolytically active form, therefore fullfilling all of the criteria for an optimal target for chemical probe design.

Hip1 was originally thought to be a carboxylesterase because it was unable to cleave commonly used trypsin-like protease substrates in vitro.[9a] However, a transposon mutant Hip1-KO strain was found to be deficient in proteolytic processing of the mycobacterial heat-shock protein GroEL2.[9b] This Hip1-KO strain elicited increased proinflammatory responses from both macrophages and neutrophils, indicating that the Hip1 catalytic activity is involved in a down-regulation of the host immune response.[9c,d] A later study revealed that Hip1 is indeed a serine protease that can cleave GroEL2 both in vitro and in vivo, resulting in the extracellular release of processed monomeric GroEL2 as a mediator of Hip1-dependent immunomodulatory activities.[9e] Although these studies demonstrated the physiological relevance of Hip1-dependent proteolysis using genetic methods, there are currently no chemical tools that can be used to selectively inhibit or visualize Hip1 activity in live bacteria.

In this study, we demonstrate a strategy to develop chemical probes (irreversible inhibitors, ABPs, and selective substrate probes) for Mtb Hip1 activity by combining focused compound library screening with multiple types of substrate selectivity profiling. We identified key substrate determinants that are important for recognition and turnover by Hip1 as well as a chloroisocoumarin scaffold that irreversibly inhibits Hip1. Combining the information from the specificity screen with the lead compound scaffold resulted in both selective fluorescent reporter substrates and a new class of covalent inhibitors and active-site probes for Hip1. These reagents may serve as leads for the development of clinically relevant drugs and targeted imaging probes for visualizing Mtb infections. Furthermore, the general approach and methodology presented in this study should be applicable to designing probes for virtually any serine protease of interest.

■ RESULTS AND DISCUSSION

M. tuberculosis Hip1 was produced as described previously,[8] and our initial studies revealed that it cleaved fluorogenic substrate WKLL-ACC with a k_{cat}/K_M value of 1.9×10^3 M^{-1} s^{-1} (Table 1). This substrate was designed to be cleaved by another cell envelope-associated Mtb protease, MarP,[10] and was unlikely to be optimal for Hip1. However, cleavage of the substrate confirmed that the recombinant enzyme had proteolytic activity and allowed us to determine optimal assay conditions for in-depth substrate specificity studies. We initially used positional scanning-synthetic combinatorial libraries (PS-SCL)[11] and multiplex substrate profiling by mass spectrometry (MSP-MS)[12] methods to survey the substrate specificity preferences of recombinant Hip1 (Figure 1). The PS-SCL library method allows a direct measure of the cleavage site specificity at the P1−P4 positions, and the MSP-MS method uses mass spectrometry to read out specific cleavages on 14-mer peptides and provides data for specificity-driving residues on both sides of the scissile amide bond. Our results using the PS-SCL libraries showed that, overall, Hip1 has the most pronounced

selectivity at the P2 position, showing a strong preference for lysine at this site (Figure 1B). We also observed some degree of selectivity for aliphatic residues in the P1 position along with selectivity for P1 glutamine or leucine (Figure 1A). At the P3 position, we observed specificity for aromatics and, to a lesser degree, aliphatic residues (Figure 1C). Hip1 showed overall minimal specificity at the P4 position with only a slight dislike of negatively charged residues such as aspartic and glutamic acids (Figure 1D). Importantly, the MSP-MS method produced a specificity profile that agreed well with the PS-SCL data (Figure 1E, Supporting Information Figure 1 A,B). In particular, the preference for a P2 lysine was again the most prominent specificity signature, and aliphatic P1 and aromatic P3 specificities were also confirmed. Interestingly, glutamine was neither preferred nor disfavored at the P1 position, suggesting potential differences in the way the substrates are presented in the active site for the two different methods. For the prime sites, the MSP-MS analysis indicated a preference for Gly, Phe, Arg, and Ser in the P1′ position and a variety of hydrophobic aliphatic and aromatic residues on the P2′−P4′ sides, although none of these were particularly pronounced.

The only known proteolytic substrate of Hip1, GroEL2, is a chaperone-like protein that has been reported to be cleaved at two sites in its N-terminal region (AKT-IAYDEEARR-GLERGLN, where the dashes indicate the cleavage sites).[8] The first site features a lysine in the P2 position, in agreement with the substrate preference we observed by MSP-MS and PS-SCL. The second cleavage appears to have many suboptimal amino acids in the P4−P4′ positions with the exception of glycine in the P1′ position. Therefore, it is possible that Hip1 cleaves the first site at a faster rate compared to the second site. Regardless, our Hip1 specificity data is consistent with processing events occurring in a physiological substrate. While this specificity information is valuable for the design of probes, it may be difficult to use these in vitro data to identify native substrates of Hip1 as its substrate specificity may differ for actual protein substrates in vivo.

With the exception of norleucine, the PS-SCL and MSP-MS assays utilize natural amino acids for substrate specificity analysis. This provides a starting point for designing selective substrates and for identifying putative cleavage sites on native proteins. In order to increase the specificity of substrates, it is possible to use non-natural amino acids. One method, referred to as the hybrid combinatorial substrate library (HyCoSuL), has been used to identify highly selective substrates for several important protease targets including caspases,[13] human neutrophil elastase (HNE),[14] and human neutrophil serine protease 4.[15] Therefore, we performed HyCoSuL profiling using fluorogenic substrates containing a fixed P1 alanine and 102 non-natural and D-amino acids in the P2−P4 positions (Supporting Information Figure 2). These results again confirmed the high degree of substrate specificity of Hip1 for a P2 lysine with a preference for this natural residue over all other non-natural analogs including lysine analogs such as L-ornithine, L-homoLys, and D-Lys (Supporting Information Figure 2A). In further agreement with the PS-SCL and MSP-MS results, we found that a number of non-natural aromatic amino acids were accepted in the P3 position with the most effective cleavage observed for L-4-chloro-phenylalanine (4ClPhe). In the P4 position, L-indanylglycine (Igl) and L-(benzyl)cysteine showed a 3- to 4-fold higher cleavage than for any of the natural amino acids, suggesting that this position

could be used to increase the specificity and turnover rates of selective Hip1 substrates.

Using a combination of all of the profiling data, we designed an optimized Hip1 substrate containing the L-amino acid sequence acetyl-Igl-4ClPhe-Lys-Leu-ACC (CSL173, structure in Figure 2A). With a k_{cat}/K_M value of 9.6×10^6 M^{-1} s^{-1}, this substrate was cleaved by Hip1 5000 times faster than was MarP substrate WKLL-ACC (Table 1). Furthermore, the substitution of optimal amino acids in either the P3 or P4 position with a
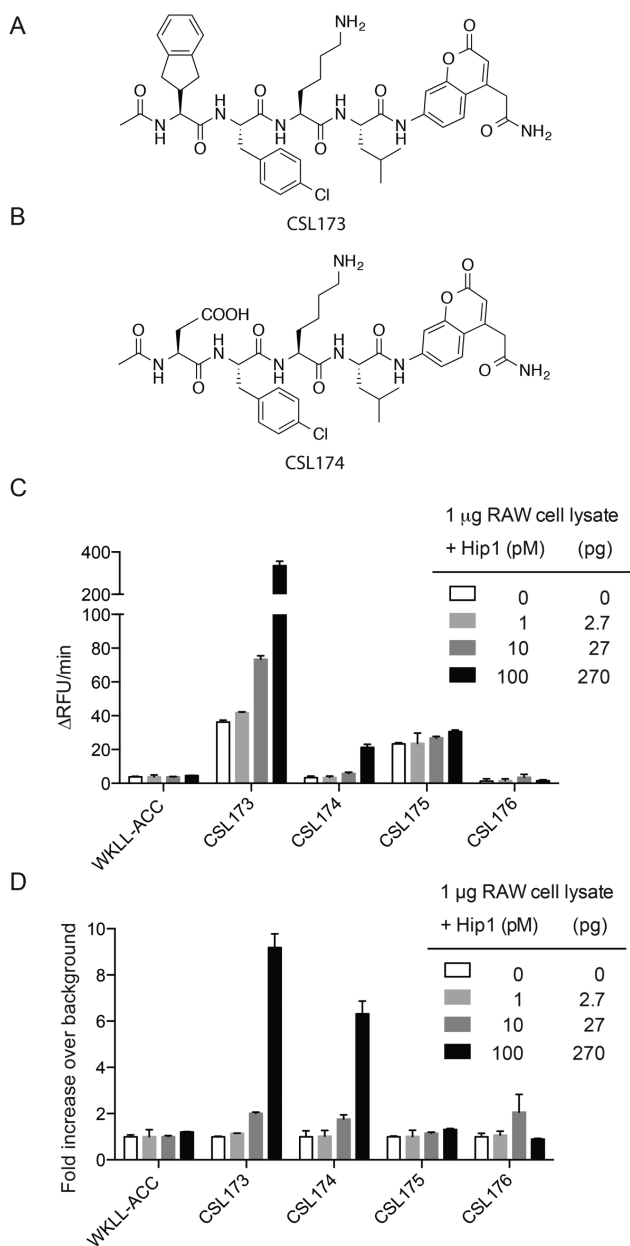

Figure 2. Selective fluorogenic substrate probe to visualize Hip1 activity. (A) Structure of optimal fluorogenic Hip1 substrate acetyl-Igl-(4Cl)Phe-Lys-Leu-ACC (CSL173) and (B) acetyl-Asp-(4Cl)Phe-Lys-Leu-ACC (CSL174). (C) Rates of cleavage of fluorogenic substrates WKLL-ACC, CSL173, CSL174, CSL175, and CSL176 (at 1 μM) in RAW cell lysates (1 μg) with increasing amounts of recombinant Hip1 (0, 2.7, 27, or 270 pg) added. Data are plotted as the rate of change in the signal over the linear portion of the progress curves. (D) Data from B plotted as a function of the signal relative to the background for each substrate.
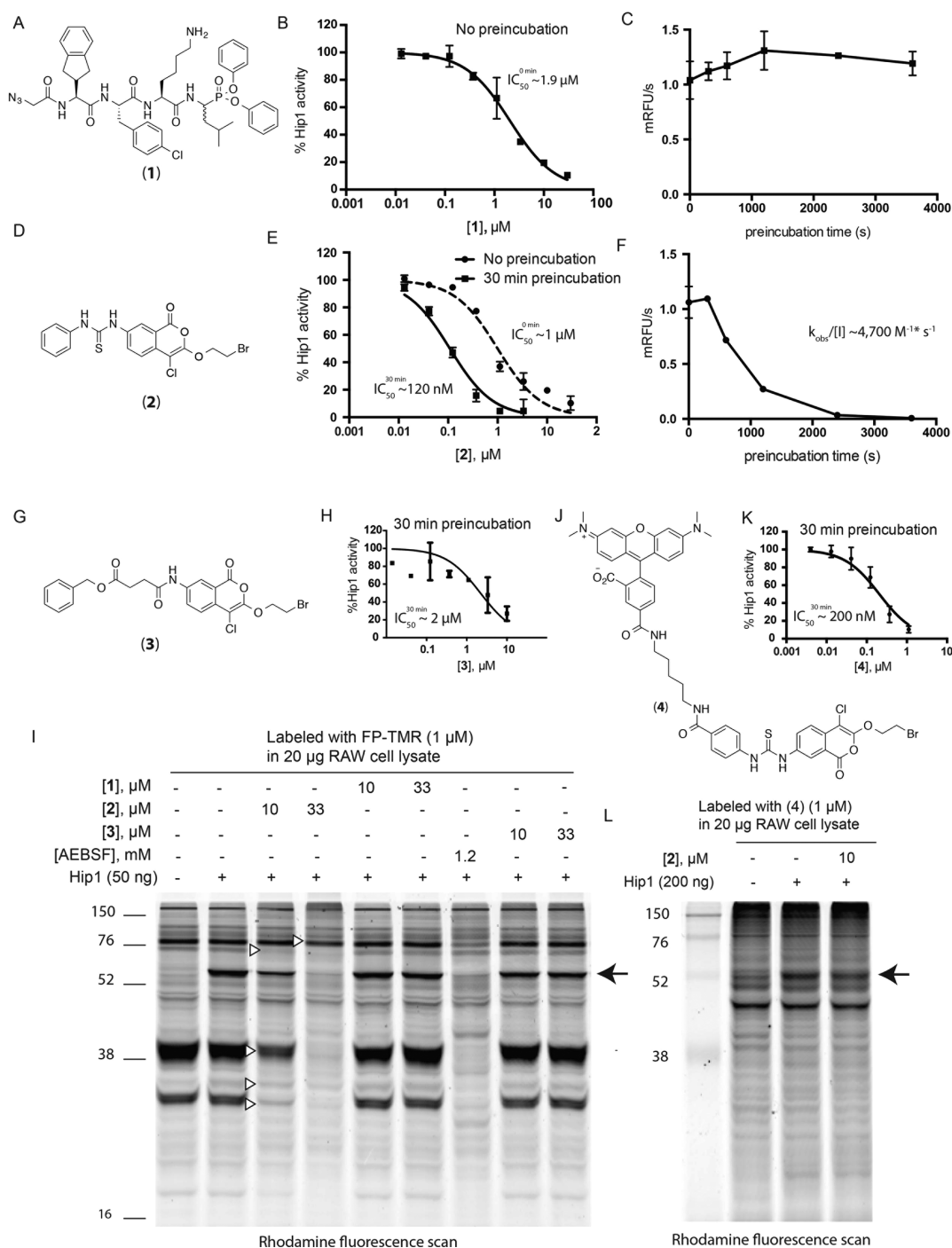
nonpreferred amino acid led to a decrease in the k_{cat}/K_M value by 1−3 orders of magnitude. In addition, when P2-Lys was replaced by Tyr, the catalytic efficiency dropped by over 10 000-fold, even though the other optimal amino acids in the P1, P3, and P4 positions were retained, again highlighting the dominant contribution of this residue to the overall substrate specificity of the protease.

Given that macrophages are important hosts for Mtb, we wanted to determine whether our newly identified selective substrates could be used to monitor Hip1 activity in the background of total extracts derived from a macrophage cell line (RAW cells). We measured the cleavage of all four substrates when added to RAW cell lysates (1 μg) that had been spiked with a range of concentrations of recombinant Hip1 (Figure 2C,D). We found that both optimal substrate CSL173 and next-best substrate CSL174 (structure in Figure 2B) showed a clear increase in signal depending on the amount of Hip1 present, with CSL173 showing a 10-fold signal over the background when only 270 pg (0.027% of total protein) of Hip1 was added. However, although the overall signals for CSL173 were higher than for CSL174, there was some cleavage in lysates that did not contain Hip1, suggesting that CSL173 is likely less selective than CSL174. However, the overall strong signal-to-noise ratio observed upon addition of small quantities of Hip1 for both probes suggests that they have the potential to be used to selectively monitor Hip1 activity in complex biological settings.

In addition to substrate probes, we wanted to develop inhibitors and ABPs that could be used to permanently label and inactivate Hip1. One of the benefits of covalent ABPs compared to substrates is that it is often easier to design highly selective ABPs for a target by making use of electrophiles that specifically react with subclasses of proteases (i.e., only serine proteases). Therefore, we used our optimal substrate as a starting point for covalent inhibitor design and attached the serine protease-specific diphenyl phosphonate (DPP) electrophile that has proven to be effective for other serine protease targets.[14,15] Conversion of the CSL173 substrate into the corresponding peptide DPP (compound **1**, Figure 3A) resulted in a compound that showed complete inhibition of the recombinant Hip1 at low micromolar concentrations (Figure 3B). However, we found that this compound did not show a time-dependent inhibition that would be expected for an irreversible inhibitor, suggesting that it acts as a reversible competitive inhibitor (Figure 3C). We reasoned that the bulky phenyl groups attached to the electrophile may not fit effectively in the Hip1 active site, preventing the phosphonate from covalently reacting with the serine active-site nucleophile.

To identify potential irreversible pharmacophores for the Hip1 ABP design, we screened Hip1 against a highly focused library of ~500 small molecules containing serine-reactive electrophiles using the fluorogenic substrate assay as a readout. We previously used this compound library to identify inhibitors of other serine protease targets.[16] The screen identified a series of 7-amino-4-chloro-3-(2-bromoethoxy)isocoumarins as hits, with the top compound **2** (JCP363, Figure 3D) inhibiting Hip1 with an IC$_{50}$ of ~120 nM (Figure 3E). Importantly, unlike the DPP inhibitor, this compound showed time-dependent inhibition, suggesting that it acts as a covalent irreversible inhibitor (Figure 3F). We also identified a structurally related 4-chloro-3-(2-bromoethoxy)isocoumarin (compound **3**, Figure 3G) that was ~17-fold less potent against Hip1 (Figure 3H)

Figure 3. Validation of Hip1 inhibitors and activity-based probes. Hip1 activity was measured using the cleavage of fluorogenic substrate WKLL-ACC. All curves show the mean ± S.D. of triplicates. (A) Chemical structure of **1**, a DPP electrophile conjugated to the Igl-(4-Cl)Phe-Lys-Leu specificity sequence identified by substrate profiling. (B) Dose–response curve for compound **1** when added to Hip1 without any preincubation. The calculated IC_{50} value is shown. (C) Time-dependent effects of compound **1** on Hip1 activity. Hip1 (3 nM) and **1** (100 nM) were preincubated for various time periods before the measurement of Hip1 activity. The values for mRFU/s are plotted for each preincubation time point. (D) Structure of chloroisocoumarin-based screening hit **2**. (E) Dose–response curves of **2** in a Hip1 activity assay. The curve for a 30 min preincubation (solid line, $IC_{50}^{30\ min}$) is compared to when **2** was added simultaneously with substrate (dashed line, $IC_{50}^{0\ min}$). The calculated IC_{50} value is indicated. (F) Time-dependent effects of compound **2** on Hip1 activity. Hip1 (3 nM) and **2** (100 nM) were preincubated for various time periods time before the measurement of Hip1 activity. The values for mRFU/s are plotted for each preincubation time point. (G) Chemical structure of **3**, an analog of **2** with ~17-fold lower inhibitory activity. (H) Dose–response curve of **3** against Hip1 in a fluorogenic substrate assay. IC_{50} is shown. (I) SDS-PAGE analysis of RAW cell lysates with or without recombinant Hip1 added were labeled with the fluorescent serine hydrolase probe FP-TMR after preincubation with chloroisocoumarin **2**, its inactive analog **3**, DPP inhibitor **1**, or general serine protease inhibitor AEBSF. The arrow indicates the position of the labeled Hip1, and the open arrowheads indicate off-target enzymes hit by the compounds. (J) Chemical structure of **4**, a fluorescent activity-based probe analog of **2**. (K) Dose–response curve of **4** in a Hip1 fluorogenic substrate assay. (L) SDS-PAGE analysis of RAW cell lysates with or without added Hip1 labeled with **4**. The arrow indicates the position of labeled Hip1.
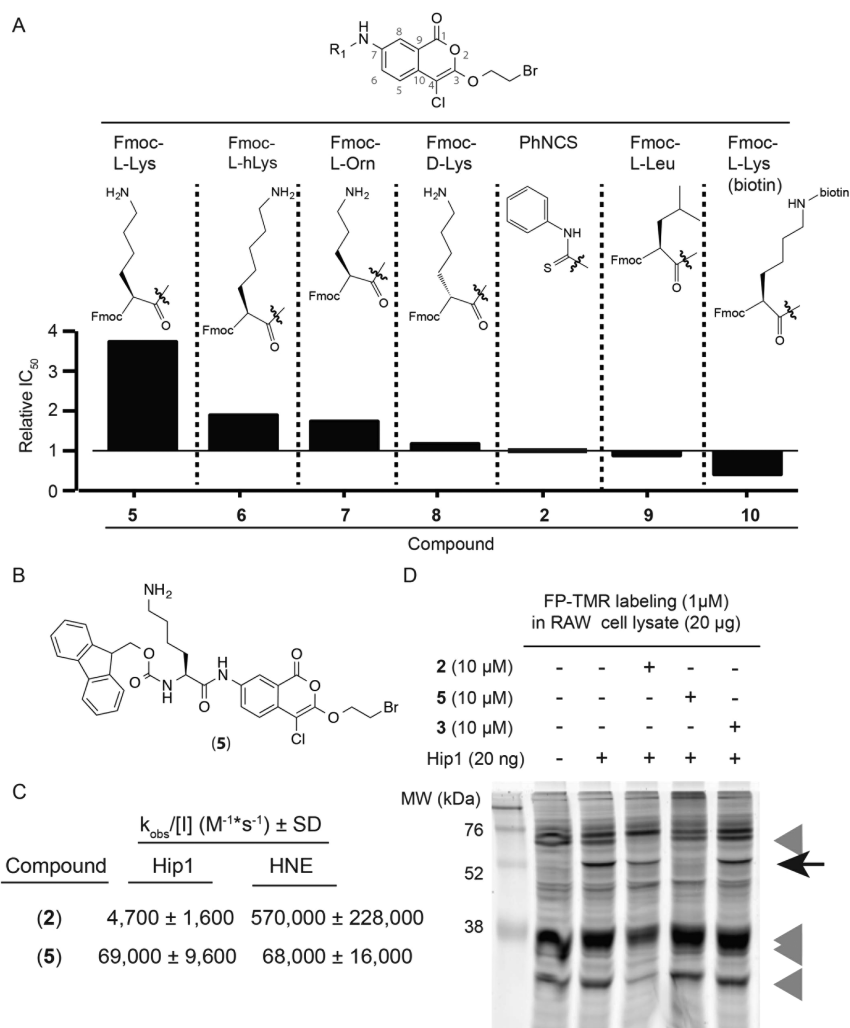
Figure 4. Optimization of chloroisocoumarin-based inhibitors using substrate selectivity information. (A) Plot of the relative IC_{50} values for a series of R_1-7-amino-4-chloro-3-(2-bromoethoxy)isocoumarins as measured using the fluorogenic substrate assay. The plotted values represent the ratio of IC_{50} for compound $2/IC_{50}$ for each indicated analog. (B) Chemical structure of optimized Hip1 inhibitor **5**. (C) Second-order rate constants $k_{obs}/[I]$ of compounds **2** and **5** calculated for Hip1 and HNE. (D) SDS-PAGE analysis of RAW cell lysates containing recombinant Hip1 that were labeled with the fluorescent serine hydrolase probe FP-TMR after preincubation with optimized Hip1 inhibitor **5**, screening hit **2**, or inactive analog **3**. The black arrowhead shows the location of labeled Hip1, and gray arrowheads show the position of enzymes hit nonspecifically by compound **2**.

and could be used as a negative control analog for future specificity studies.

To further assess the specificity of the newly identified lead chloroisocoumarin (**2**), we tested its ability to bind to recombinant Hip1 that had been added to RAW cell lysates (Figure 3I). To measure the binding and overall selectivity, we added the compound to the spiked lysate at various concentrations and then labeled active Hip1 as well as other RAW-cell-derived serine hydrolases using the general serine hydrolase probe fluorophosphonate(FP)-TMR. We also performed this competition analysis for the original DPP inhibitor **1** and the inactive control compound **3**. As expected, we found that compound **2** blocked Hip1 labeling by FP-TMR at both concentrations tested, whereas reversible compound **1** and control inactive compound **3** did not. However, we also observed that compound **2** blocked the labeling of several other prominent FP-TMR-labeled species (open arrowheads in Figure 3I), suggesting that this compound is not very selective for Hip1.

Given that our top hit was potent against Hip1 in the nanomolar concentration range, we reasoned that it could be

converted to an ABP by the addition of a fluorescent tag. The resulting fluorescent probe version of **2** (compound **4**, Figure 3J) irreversibly inhibited Hip1 with a potency similar to that of parent compound **2** (Figure 3K, ($k_{obs}/[I] \approx 1012 \pm 5$ M^{-1} s^{-1})). However, when we used probe **4** at 1 μM to label RAW lysates containing Hip1, it labeled several other proteins with only weak labeling of the desired target (Figure 3L). This result was consistent with the competition labeling studies suggesting that while compound **2** is a potent inhibitor of recombinant Hip1, it is not selective enough to be used as a probe in complex biological samples containing significant off-targets. These results are also consistent with the fact that compound **2** had previously been reported to be a potent inhibitor of the serine protease human neutrophil elastase (HNE) with $k_{obs}/[I]$ > 480 000 M^{-1} s^{-1}[15] with additional weak activity against chymotrypsin and cathepsin G.[16] Therefore, we used HNE as the most relevant off-target for testing the selectivity of substrates and inhibitors. We found that the most effective Hip1 substrate, CSL173, had a nearly 10 000-fold selectivity for Hip1 over HNE (Table 1), and the next-best substrate CSL174, which was only 40-fold less effective for Hip1 than was
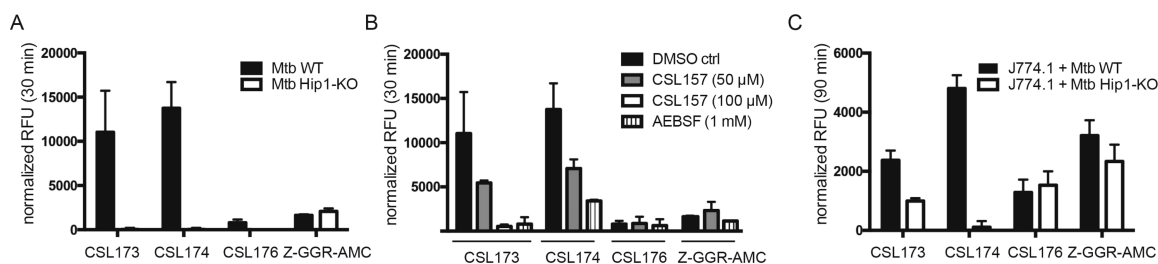
Figure 5. Validation of fluorogenic Hip1 substrates and inhibitors in live *M. tuberculosis*. (A) Proteolytic processing of CSL173, CSL174, and CSL176 and the control fluorogenic substrate Z-GGR-AMC (each at 1 μM) by live WT Mtb or Hip1-KO Mtb. Data is shown as relative fluorescence units after 30 min of incubation normalized to the background autofluorescence. (B) Proteolytic processing of the indicated fluorogenic substrates in WT Mtb after preincubation with Hip1-specific inhibitor **5** or general serine hydrolase inhibitor AEBSF at the indicated concentrations for 1 h prior to addition of substrates. (C) Fluorogenic substrate cleavage by J774.1 murine macrophages infected with either WT Mtb or Hip-1 KO Mtb (MOI = 10) for 3 h. Extracellular bacteria were washed away before substrates were added. Data is displayed as relative fluorescence units after 90 min of incubation normalized to the autofluorescence background. All graphs show mean ± SD values of triplicates.

CSL173, was not cleaved by HNE at all. This data suggests that the incorporation of binding determinants identified by Hip1 substrate profiling is likely to increase the potency and selectivity of inhibitors and covalent probes toward Hip1.

Because the major determinant for selectivity to Hip1 was the Lys in P2 (Figure 1B,E, Supporting Information Figure 2A), we generated analogs of compound **2** containing various lysine mimetics attached to the chloroisocoumarin scaffold (Figure 4A). Originally, the C_3 substituents of the chloroisocoumarins (such as the bromoethoxy group in **2**) were thought to occupy the S1 pocket of the target protease, implying that the C_7 substituent would be directed toward the S′ sites.[16] However, it was later determined that the C_3 substituent might be positioned toward the S1′ sites depending on the protease and inhibitor.[17] We therefore reasoned that it might be possible to address the S2 pocket with a substituent conjugated to the C_7 position of **2**. To verify this hypothesis, we synthesized a series of 7-R_1-CONH-4-chloro-3-bromoethoxyisocoumarins using Fmoc-protected amino acids in R_1. Compound **5**, featuring Fmoc-L-Lys at R_1 (Figure 4B), was the most potent inhibitor in this series (IC$_{50}$ of ∼34 nM, which is ∼4-fold improved over parent compound **2**, Figure 4A). R_1 amino acids with aliphatic side chains (Leu) or modified amines (L-Lys(*N*-biotin)) were less potent than **2**, whereas other substituents with primary amines of different chain lengths or stereochemistries (L-Orn, L-hLys, D-Lys) were better inhibitors than **2** but less potent than **5**. Importantly, we also observed an overall increase in the specificity of compound **5** for Hip1 over HNE compared to that for compound **2**. Specifically, compound **5** inactivated Hip1 15-fold faster than compound **2** yet was an ∼8-fold-weaker inhibitor than compound **2** for HNE. To further demonstrate this enhanced specificity of compound **5**, we again used competitive activity-based protein profiling using FP-TMR in murine macrophage lysates containing recombinant Hip1 (Figure 4E). Importantly, we found that newly designed compound **5** showed the highly selective inhibition of Hip1 labeling with markedly reduced or virtually no activity toward the many other hydrolases that were effectively targeted by compound **2**. Overall, these results confirmed that the use of our substrate specificity data to further optimize our screening hit resulted in a compound of greatly improved potency and selectivity for Hip1.

Because our long-term goal is to develop probes to visualize Hip1 activity in vivo, we proceeded to test whether the substrates can be used to monitor Hip1 activity in live bacteria. This would also allow us to determine if the peptide substrates could access the enzyme in live cells. Importantly, we observed that both of the optimized Hip1 substrates, CSL173 and CSL174, were readily processed by WT Mtb (CDC1551) but not by a previously described Hip1-KO strain[9a] (Figure 5A). In contrast, the control substrate for trypsin-like proteases (Z-GGR-AMC) was processed only weakly and at a similar rate by both strains, and control substrate CSL176 was processed by neither strain (Figure 5A). These results indicate that processing CSL173 and CSL174 by Mtb is dependent on Hip1 and that these probes readily penetrate the bacterial cell envelope where Hip1 is located. These fluorogenic substrates enabled us to determine if our optimized inhibitor **5** blocked Hip1 activity in intact bacterial cells. Using this assay, we found that compound **5** blocked Hip1 activity in a dose-dependent manner as measured by a block in the CSL174 signal (Figure 5B). These results confirmed that **5** inhibits endogenous Hip1 in its physiological environment. The relatively high concentrations required to get effective inhibition of Hip1 activity may be due to the low permeability of the inhibitor to the mycobacterial cell wall and/or may be the result of the limited incubation time (1 h) used in this assay.

As a final step of validation, we wanted to confirm that our substrate probes produce a Hip1- and Mtb-specific signal in Mtb-infected J774.1 murine macrophages. Our results confirmed that both CSL173 and CSL174 produced a specific signal in macrophages infected with WT Mtb, but not in macrophages infected with Hip1-KO Mtb. Interestingly, we observed the largest signal over background signals for CSL174 as this probe showed virtually no activity in the macrophages infected with Mtb lacking Hip1. These data are in agreement with our in vitro results using Hip1 spiked into macrophage lysates (Figure 2), where we found that CSL174 had overall lower signals in macrophage extracts than did CSL173. Thus, CSL174 is an ideal candidate for translation into an Mtb-specific quenched fluorescent substrate for noninvasive optical in vivo imaging of tuberculosis.

We believe that we have identified a valuable new reagent for use as a probe as well as a new inhibitor of Mtb protease Hip1. However, we recognize that additional medicinal chemistry efforts may be required to further enhance the selectivity and potency of the chloroisocoumarin inhibitor to avoid targeting other host-derived proteases such as HNE. For example, modifications at the C_3 position, replacing the bromoethoxy group that drives the potency for HNE,[16] might allow us to address the prime or nonprime sites of Hip1 from this position. Furthermore, both the C_3 position and the peptide moiety at

C_7 may be suitable anchor points for the attachment of tags, such as fluorophores, biotin, or a click-chemistry handle to make Hip1-selective ABPs. Whether such ABPs will readily penetrate the bacterial cell wall to reach their target will be the subject of future studies. However, the Hip1-reporter substrates presented in this work provide a target-specific readout for a cell-based assay that may be used for high-throughput screening of large compound libraries to identify new classes of cell-permeable Hip1 inhibitors. Importantly, these Hip1-selective reporter substrates may already be useful for visualizing Hip1 activity in relevant biological samples or even clinical samples. These initial probes can easily be translated into quenched fluorescent substrates for further applications such as non-invasive in vivo imaging, as has been established for the cysteine cathepsins.[7]

In conclusion, the combination of substrate profiling and focused compound screening in the design of selective chemical probes as demonstrated here for Hip1 should prove valuable for many proteases of interest. A recent chemoproteomics study identified 78 putative Mtb serine hydrolases under replicating and nonreplicating growth conditions.[8] However, with the notable exception of inhibitors for Mtb caseinolytic protease clpP1P2,[18] there are no selective inhibitors or ABPs for any of these targets. Because many of these serine hydrolases have not been functionally characterized,[8] they are currently not accessible to target-based probe design using purified recombinant enzymes. In ongoing work in our laboratory to overcome this limitation, we are establishing a competitive activity-based protein profiling approach that would allow the direct screening of focused libraries to discover candidate inhibitors and probes for unidentified serine hydrolases in complex mycobacterial lysates. The resulting lead compounds may be useful both for the study of protease function and to generate probes that can be used for target enrichment followed by mass spectrometry-based identification. Once identified, proteases can be expressed and then subjected to the target-based probe design approach presented in this study. We believe such strategies will be required to build a chemical toolbox with reagents for mycobacterial proteases that have potential value for next-generation target validation, drug development, and diagnostic monitoring efforts.

METHODS

Detailed methods can be found in the Supporting Information.

ASSOCIATED CONTENT

Ⓢ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsinfec-dis.6b00092.

> Full time-course of Hip1 cleavage profile by MSP-MS. Hybrid combinatorial substrate library (HyCoSuL) screening of Hip1. Methods, synthesis, and compound characterization. (PDF)

AUTHOR INFORMATION

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