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Target-Based Screen Against a Periplasmic Serine Protease That Regulates Intrabacterial pH Homeostasis in *Mycobacterium tuberculosis*

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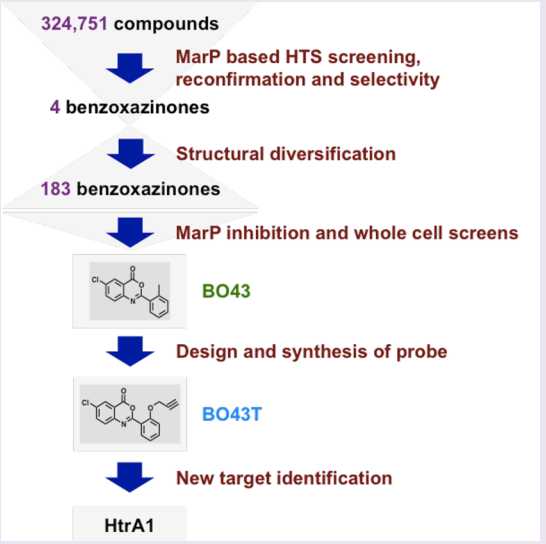
Supporting Information

ABSTRACT: *Mycobacterium tuberculosis* (*Mtb*) maintains its intrabacterial pH (pH_{IB}) near neutrality in the acidic environment of phagosomes within activated macrophages. A previously reported genetic screen revealed that *Mtb* loses this ability when the mycobacterial acid resistance protease (*marP*) gene is disrupted. In the present study, a high throughput screen (HTS) of compounds against the protease domain of MarP identified benzoxazinones as inhibitors of MarP. A potent benzoxazinone, BO43 (6-chloro-2-(2′-methylphenyl)-4H-1,3-benzoxazin-4-one), acylated MarP and lowered *Mtb*'s pH_{IB} and survival during incubation at pH 4.5. BO43 had similar effects on MarP-deficient *Mtb*, suggesting the existence of additional target(s). Reaction of an alkynylbenzoxazinone, BO43T, with *Mycobacterium bovis* variant *bacille Calmette-Guérin* (*BCG*) followed by click chemistry with azido-biotin identified both the MarP homologue and the high temperature requirement A1 (HtrA1) homologue, an essential protein. Thus, the chemical probe identified through a target-based screen not only reacted with its intended target in the intact cells but also implicated an additional enzyme that had eluded a genetic screen biased against essential genes.



Tuberculosis (TB) is the second leading cause of death among infectious diseases worldwide, surpassed only by HIV/AIDS, and is the leading cause of death in people whose mortality is attributed to HIV/AIDS.[1] Moreover, TB is a major cause of antibiotic-resistant infections. The urgent search for new drugs for TB includes efforts to identify inhibitors of pathways that *Mycobacterium tuberculosis* (*Mtb*) uses to persist in growth-restricting environments in the host, alongside the traditional approach of targeting pathways that *Mtb* uses to proliferate in bacteriologic media *in vitro*.

One stress that *Mtb* is thought to encounter in the host, but not in growth-supporting culture media, is mild acidity. In pathologic sites such as granulomas, where infiltrating host cells outstrip a meager microvasculature, hypoxia is presumed to increase host cell glycolysis, and the limited perfusion that contributes to hypoxia is expected to allow acid products of glycolysis to accumulate. Further, *Mtb* in the phagosomes of activated macrophages encounters a pH near 4.5.[2] When *Mtb* is exposed *in vitro* to pH's of 4.5−5.5, it stops replicating but can survive for days.[3−5] A screen of transposon mutants of *Mtb* for those that succumbed at pH 4.5 identified a gene whose disruption prevented *Mtb* from maintaining intrabacterial neutrality in the face of extrabacterial acidity.[4] The gene product was named MarP for mycobacterial acid resistance protease (*marP*) because homology modeling, mutation analysis, crystallography, and substrate profiling identified MarP as a transmembrane serine peptidase with its protease domain in the periplasm.[4,6,7] When incubated *in vitro* at pH 4.5 and when phagocytized by activated macrophages, the *Mtb* MarP transposon mutant (*marP::Tn*) dropped its intrabacterial pH (pH_{IB}) below 5.5 (the limit of

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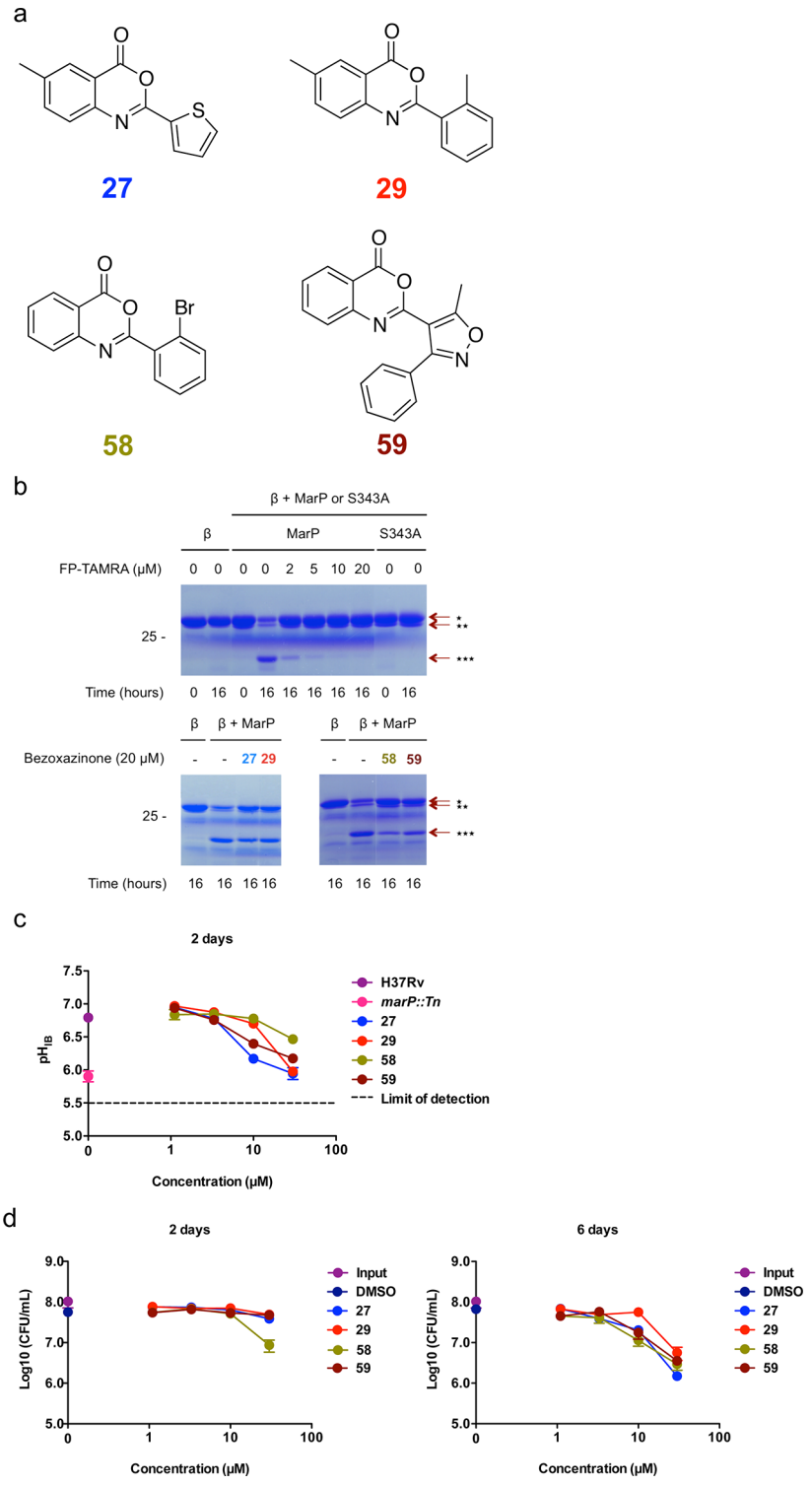

Figure 1. Biochemical and biological activity of 4 benzoxazinones identified in the MLSC screen. (a) Structures of benzoxazinones 27, 29, 58, 59 and BO43. (b) Proteolytic activity of recombinant MarP and its S343A active site mutant on *β* casein (*β*) (top) and inhibition of this proteolysis by 27, 29, 58, 59 (bottom), ★ indicates *β* casein, ★★ indicates recombinant MarP or its S343A active site mutant, ★★★ indicates main proteolytic product. (c) pH_{IB}-disrupting activity of 30 mM of 27, 29, 58, and 59 on *Mtb* at pH 4.5 after 2 days. (d) CFU activity of 27, 29, 58, and 59 on *Mtb* at pH 4.5 at 2 days (left) and 6 days (right). Means ± S. E. M. of triplicate samples shown in d and e represent three independent experiments. Some error bars are smaller than the symbols.

detection of the assay), and MarP-deficient *Mtb* was markedly attenuated in mice.[4]

An earlier study used a whole cell screen of a natural product library to identify inhibitors of *Mtb*'s pH_{IB} homeostasis.[8] None of the inhibitors identified in that study acted on MarP.[8] Accordingly, we undertook a high throughput screen (HTS) of 324 751 synthetic organic compounds against MarP itself in an effort to identify additional potential starting points for development of drugs that render *Mtb* vulnerable to conditions encountered in the host.

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In combination, the three screens, the functional screen of a transposon mutant library at low pH, the chemical screen of whole *Mtb* at low pH, and the chemical screen of the protein identified in the genetic screen, reported here, illustrate the complementarity of genetic and chemical approaches.[4,8] The present findings reveal additional complexity in *Mtb*'s mechanisms of maintaining pH_{IB} homeostasis.

RESULTS AND DISCUSSION

Identification of Benzoxazinone BO43 as an Inhibitor of MarP. We screened an NIH-supported Molecular Libraries Screening Center (MLSC) collection of 324 751 small, synthetic organic compounds at 6.0 μM against 3.8 μM purified, recombinant extracellular domain (ECD) of MarP by competition with an activity-based probe, a fluorophosphonate-rhodamine (FP-Rh, Supplementary Figure S1), recording the decrease in fluorescence polarization afforded by compounds that interfered with binding of the probe to the active site serine hydroxyl.[7,9−12] In an assay with $Z' = 0.69 \pm 0.05$ ($N = 271$ plates), 157 compounds inhibited polarization by >37% (data are deposited in PubChem with the identifier AID 2761). Of the 142 that could be resupplied, 60 were confirmed in the same assay. These were tested at 5.9 μM (first 3 enzymes) or 7.9 μM (fourth enzymes) against four mammalian serine hydrolases, for which assay details with FP-Rh are available in Pubchem: phosphatase methylesterase-1 (PME-1; AID 2130), lysophopholipase 2 (LYPLA2; AID 2177), prolyl oligopeptidase-like (PREPL; AID 2751), and retinoblastoma binding protein 9 (RBBP9; AID 1515). Nine compounds met four criteria: they did not inhibit the mammalian serine hydrolases; they did not autofluoresce; at 20 μM, they inhibited MarP's cleavage of β casein, used as a surrogate substrate in an assay monitored by SDS-PAGE, given that the natural substrate of MarP is unknown; at 30 μM, they reduced *Mtb*'s pH_{IB} to <6.5 within 48 h of incubation of *Mtb* in phosphate-citrate buffer at pH 4.5 (data not shown).[6]

Among the nine compounds were four 4H-1,3-benzoxazin-4-ones (benzoxazinones 27, 29, 58, and 59 in Figure 1a), three coumarins (6, 52, and 55 in Supporting Information Figure S2), and two singletons (22 and 41 in Supporting Information Figure S2). Benzoxazinones are synthetically tractable and can reversibly acylate the active site serine hydroxyl in serine hydrolases.[13] Thus, we focused on benzoxazinones with the expectation that their predicted covalent reaction with MarP, though reversible, might help us use a pull-down technique to test whether the inhibitor actually bound MarP within *Mtb* itself. Inactivity of these four benzoxazinones against four mammalian serine hydrolases and their inhibition of MarP cleavage of β casein are shown in Table 1 and Figure 1b. Fluorophosphonate-tetramethylrhodamine (FP-TAMRA, Supporting Information Figure S1) was used as an alternative activity-based probe against MarP in this assay (Table 1).[6,12]

Table 1. Inhibition (%) of 27, 29, 58, and 59 against MarP and 4 Mammalian Serine Proteases: RBBP9, PME-1, LYPLA2, and PREPL

Benzoxazinones	MarP	RBBP9	PME-1	LYPLA2	PREPL
27	63	-3	-6	-1	-10
29	75	-	-3	0	13
58	66	-	9	-2	10
59	62	-4	-7	-10	20

First, we asked if the functional impact of the benzoxazinones on intact *Mtb* was consistent with inhibition of MarP. When the bacteria were incubated in buffer at pH 4.5, 27, and 29 (each at 30 μM), it brought pH_{IB} to the same level as seen in the MarP-deficient *Mtb* in the same experiments (Figure 1c). Within 6 days, the benzoxazinones killed between 90% and 99% of *Mtb* incubated at pH 4.5 (Figure 1d), consistent with the impact of *marP* disruption in earlier studies.[4] In contrast, there was no detectable loss of viable *Mtb* treated with DMSO alone at pH 4.5 (Figure 1d), nor did the benzoxazinones affect *Mtb*'s survival at pH 6.6 (data not shown).

We then examined 183 additional benzoxazinone analogs belonging to 14 structural classes (A−N) based on the nature, number, and distribution of heteroatoms in the two fused rings. This included replacement of the fused benzene ring with other heterocycles including thienyl, the presence of a third fused ring, and additional candidate acylating replacements for the oxazinone with systematic replacements of each of the heteroatoms. Each class contained a range of added substituents, including the incorporation of substituted C2 aryl groups (e.g., phenyl, thienyl, furanyl; Supporting Information Figure S3). Each compound was studied in three assays: inhibition of β casein hydrolysis by ECD of recombinant MarP, disruption of pH_{IB} in *Mtb*, and loss of viability of *Mtb* during incubation at pH 4.5. The benzoxazinone that was effective in all three assays at the lowest concentration was BO43 (Figure 2a−d), a member of the B series (Supporting Information Figure S3). At 3.13 μM, BO43 was able to drop the pH_{IB} of *Mtb* to the limit of detection, pH 5.5, and drop colony forming unites (CFU) by 1 $\log_{10}$ when *Mtb* was treated at pH 4.5. By comparison, 29 only exhibited a moderate effect on pH_{IB} at 30 μM after 2 days and dropped CFU by 1 $\log_{10}$ only after 6 days under the same conditions (Figure 1c and d).

Resynthesized BO43 (Supporting Information Figure S4) demonstrated the same activities described above. To confirm that BO43 reacted with MarP, we used LC-MS to compare the mass of MarP with and without exposure to BO43. The observed mass shift of 271.04142 Da of 25% of the MarP in the incubation was consistent with covalent acylation in a 1:1 stoichiometry by the expected mechanism (Figure 2e).[13]

To determine the IC_{50} of BO43 against MarP, we took advantage of the identification, through positional scanning, of Tyr-Lys-Leu-Leu-7-amino-4-methylcoumarin (YKLL-AMC) as a preferred MarP oligopeptide substrate.[7] BO43 inhibited MarP-dependent peptidolysis with a K_i of 185 nM (Figure 2f). In contrast, the chemical congener 2DT (Figure 2a) did not inhibit MarP at any concentration tested (Figure 2f).

To confirm the expected reversibility of the effect of BO43 on whole mycobacteria, we used *Mycobacterium bovis* variant *bacille Calmette-Guérin* (*BCG*) transformed with pH-sensitive GFP (*BCG*-pHGFP), after determining that the impact of benzoxazinones on *BCG*'s pH_{IB} and viability at low extrabacterial pH's were comparable to benzoxazinones' effects on *Mtb* (data not shown). The pH_{IB}-lowering effect of BO43 on *BCG* reverted within 4 h of removal of the compound and was reimposed by reintroduction of BO43 (Figure 2g).

A high degree of selectivity of BO43 for MarP among serine hydrolases was indicated by the observation that BO43 (20 μM) competed for binding of FP-Rh (1 μM) to none of >50 polypeptides that were detected to bind the probe in cytosol or membrane fractions of a human melanoma cell line (Supporting Information Figure S5). In contrast, 5D (Figure 2a), a benzoxazinone from the D series that was not able to disrupt pH_{IB} homeostasis of *Mtb*, competed with binding to four polypeptides (Supporting Information Figure S5). Another human cell line, the HepG2 hepatocarcinoma, maintained its ATP levels during

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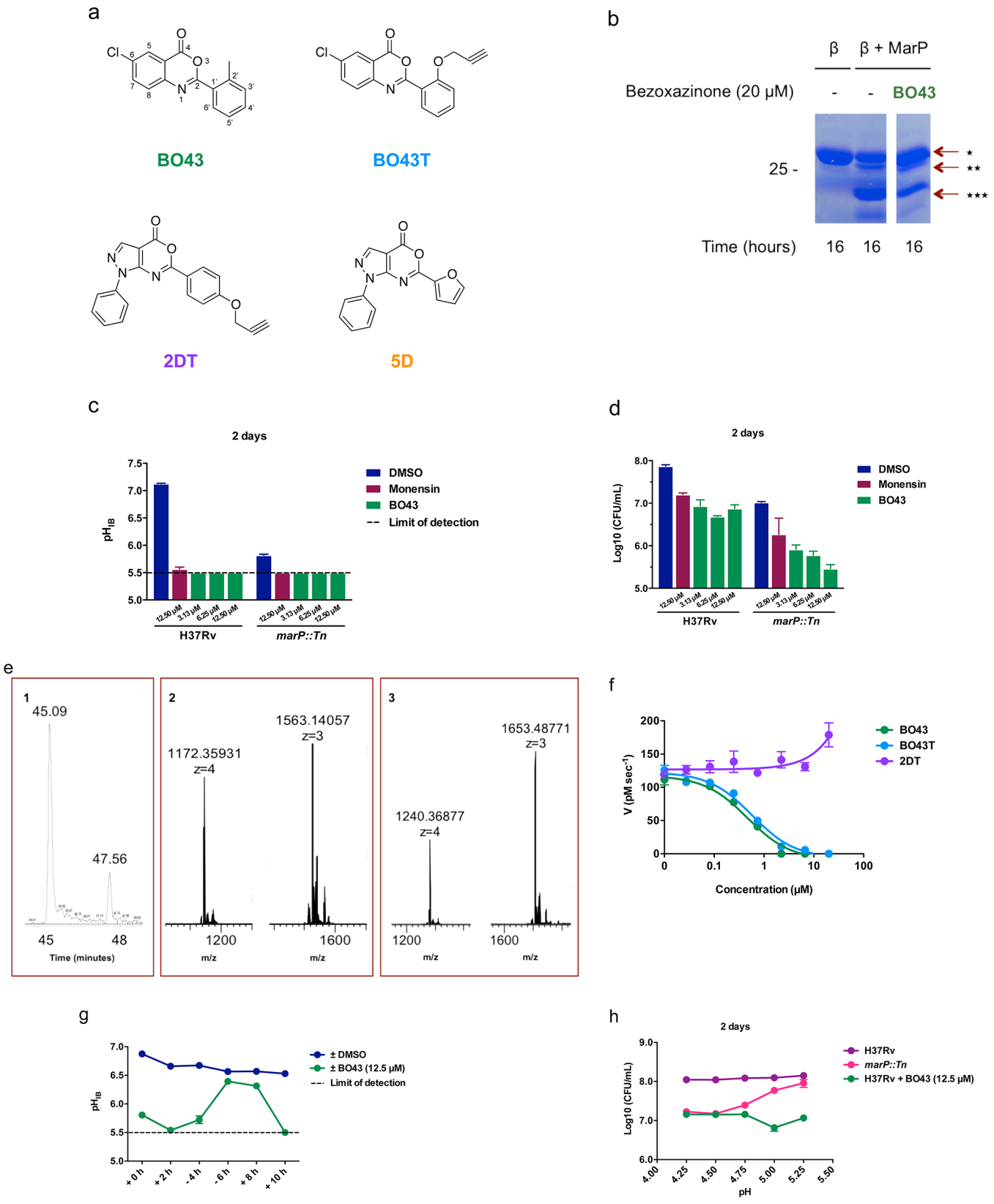

Figure 2. Biochemical and biological activities of BO43, BO43T, 2DT, and 5D on *Mtb* or *BCG*. (a) Structures of BO43, BO43T, 2DT, and 5D. (b) Proteolytic activity of recombinant MarP on *β* casein (*β*) and inhibition by BO43; ★ indicates *β* casein, ★★ indicates recombinant MarP, ★★★ indicates main proteolytic product. (c) Activity of BO43 on pH_{IB} of *Mtb* H37Rv and *Mtb marP::Tn* at pH 4.5. (d) CFU activity of BO43 on *Mtb* H37Rv and *Mtb marP::Tn* at pH 4.5. (e) HPLC-MS analysis of MarP labeling by BO43 (1, HPLC trace of MarP following incubation with BO43; 2, MS of peak at 45.09 min; 3, MS of peak at 47.56 min). (f) Inhibition of recombinant MarP mediated proteolysis by BO43, BO43T, and 2DT on YKLL-AMC. (g) Reversible activity of BO43 on pH_{IB} of *BCG* at pH 4.5. "+" indicates treatment of compounds, "−" indicates no treatment with compounds. (h) Survival of *Mtb* H37Rv treated with BO43 and of *Mtb marP::Tn* at different pH's. Means ± S. E. M. of triplicate samples shown in c, d, f, g, and h represent three independent experiments. Some error bars are smaller than the symbols.

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48 h of incubation with BO43 up to the highest concentration tested, 100 μM (data not shown).

Impact of BO43 on Wild Type and MarP-Deficient *Mtb*. As noted, incubation of *Mtb* at pH 4.5 with as little as 3.13 μM BO43 lowered pH_{IB} of *Mtb* to <5.5 (the limit of detection for the pH-sensitive GFP). This was as extensive an effect as seen with the ionophore monensin, used as a postive control, but was a greater reduction than afforded by genetic disruption of MarP (Figure 2c), suggesting that BO43 might have another target(s) besides MarP.[8] The existence of an additional target was further supported by the observation that when the *Mtb* were incubated at pH 4.5, BO43 reduced the viability of wild type *Mtb* to the level seen with untreated MarP-deficient *Mtb* but decreased the viability of MarP-deficient *Mtb* even further (Figure 2d). Moreover, BO43 could kill wild type *Mtb* equally well at extrabacterial pH's ranging from 4.25 to 5.25, but MarP deficiency only rendered *Mtb* vulnerable to pH's of 5.0 or lower (Figure 2h).

Given that BO43 had another target(s) besides MarP (for simplicity, possibilities are discussed below as if there is one other target), two new questions arose: Was MarP a target in intact mycobacteria? What was the other target? To approach these questions, we designed a pull-down probe based on the benzoxazinone structure−activity relationships (SAR).

SAR of BO43-Based Benzoxazinone Analogs. The following SAR guided us in the design of a probe for affinity purification of the benzoxazinone targets (Supporting Information Table S1). Chloride at position 6 (BO43, BO45, BO63, BO68, BO69, BO71) contributed greatly to activity. However, a methyl (BO21, BO28), methoxy (BO17, BO18), or acetoxy group (BO31, BO51, BO54, BO55, BO56, BO59, BO65) at the same position was not beneficial. Likewise, t-butyl (BO30) or amino (BO76) substitution was inferior to chloride at position 6. Furan or thiophene at position 2 provided bioactive isosteres (BO39 and BO45). In contrast, other heterocycles at position 2, such as pyridine (BO5, BO25, BO27, BO28, BO30), 1,2,3-thiadiazole (BO6), or isoxazole (BO8), offered no improvement. Among analogs with benzene at position 2 without substitutions at positions 5, 6, 7, and 8, halogen (BO57, BO72) and methyl (BO62) substitution at position 3′ gave better activity than amide (BO35) or alkoxy (BO46) substitution. Favored substitutions at position 4′ were alkyl (BO41, BO52), alkoxy (BO50, BO60), halogen (BO40), or an aromatic (BO37), but not carbonylamino (BO49, BO70) or acetoxy (BO36). The potency of BO43 depended on both chloride at position 6 and methyl at position 2′ (compare to BO11, BO71, BO10).

Pull-Down of Benzoxazinone Targets in *BCG*. To identify targets of BO43 in whole mycobacteria, we synthesized an alkyne version, BO43T (Figure 2a and Supporting Information Figure S6), using a congener, 2DT (Figure 2a and Supporting Information Figure S7), as a negative control. As predicted from the SAR, BO43T conserved key biochemical and biological activities of BO43, inhibiting peptidolysis by MarP with K_i 262 nM (Figures 2f and 3a and b). We treated intact *BCG* with BO43T at pH 4.5, washed the cells, prepared lysates, and incubated them with azido-biotin under click chemistry conditions to promote the 1,3-cycloaddition reaction. BO43T-binding proteins on streptavidin beads were then separated by SDS-PAGE.[14] Among these, the MarP homologue encoded by *Mb3695c* was identified in the membrane fraction by immunoblotting with an antibody raised against the purified protein (Figure 3c, left). Detection with fluorescently tagged streptavidin revealed several bands (Figure 3c, right), mostly in the membrane fraction. Peptide mass fingerprinting of the proteins pulled down from the membrane fraction identified the high temperature requirement A1 (HtrA1) homologue Mb1255 as the one protein that met all three of the following criteria: it was selectively identified in the BO43T-treated sample but not the DMSO treated sample, was identified from the most peptides with the highest ion counts, and met the first two criteria in each of the 2 independent experiments performed (Supporting Information Tables S2 and S3).

Two kinds of screens are often used to initiate discovery of antibacterial compounds: biochemical screens against bacterial targets or phenotypic screens against whole cells. Target-based screens have the advantage that the target is presumed to be already known, and the disadvantage that inhibitors identified through biochemical assays frequently fail to act against intact bacteria. The present results illustrate several ways in which the foregoing characterization of target-based screens may oversimplify both their pitfalls and advantages.

In this study, a target-based screen yielded an inhibitor that did act on the target in the intact bacterium but also acted on another target, most likely HtrA1. In doing so, the chemical probe biochemically implicated an essential enzyme as a key target. The earlier genetic screen did not identify HtrA1, presumably because essentiality of HtraA1 precluded representation of HtraA1 loss-of-function mutants in the transposon mutant library. This experience illustrates the importance of using multiple approaches for target identification, even when the target is presumed to be known based on the search strategy. It also adds to growing evidence that antibacterials with whole cell activity frequently have more than one target or mechanism of action and shows that this can be true even when the agent in question was selected through a target-based screen.[15,16] The benzoxazinone was thus useful as a tool compound, but its rapid metabolism by liver microsomes (data not shown) suggests its prospects are not favorable for drug development.

The essentiality of HtrA1 means that a knockout strain cannot be developed and tested for its capacity to maintain pH_{IB} homeostasis when the bacteria are faced with an acidic extrabacterial environment. We are continuing extensive efforts to construct a strain in which HtrA1 can be conditionally regulated so that we can test the predicted vulnerability of HtrA1-deficient *Mtb* to pathophysiologically relevant concentrations of acid and to other stresses that *Mtb* may encounter in the host.

Interest in HtrA1 as a potential target for an antimycobacterial agent is supported by roles of its homologues in other bacteria, where the nonessentiality of the homologues has made it feasible to phenotype knockout strains.[17] In *Listeria monocytogenes* and *Streptococcus mutans*, HtrA family proteins control protein turnover and folding in response to low pH stress.[18−20] In *Escherichia coli*, HtrA (DegS) functions as a site-1 protease (S1P), which can activate stress responses by performing the first proteolytic cleavage of membrane bound antisigma factors.[21] A second proteolytic cleavage by a membrane embedded site-2 protease (S2P) then releases the antisigma factor into the cytosol, where it is degraded, allowing the σ factor to modulate transcription. Rv2869c encodes a S2P in *Mtb*.[22] It is also possible that the chaperone and proteolytic functions of HtrA1 are regulated by other proteins in the periplasmic space, including proteases such as MarP.[23,24]

Future studies will focus on the possible inter-relation of *Mtb's* MarP and HtrA1, neither of whose natural substrates is known. The possibility exists that they serve some roles singularly and others redundantly, depending on the degree of extrabacterial

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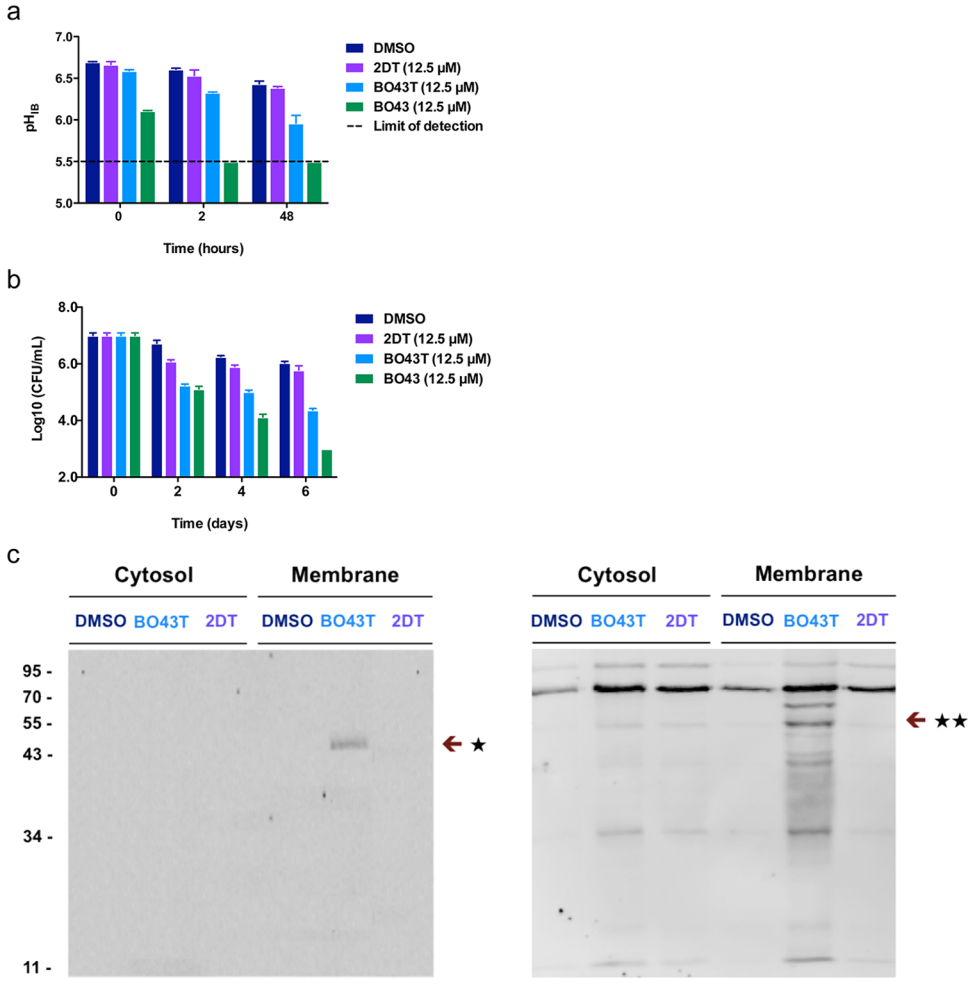

Figure 3. BO43T probe to identify additional targets of BO43. (a) pH_{IB} activity of BO43, BO43T, and 2DT on *BCG* at pH 4.5. (b) CFU activity of BO43, BO43T, and 2DT on *BCG* at pH 4.5. (c) MarP immunoblot (left) and streptavidin-fluorescence (right) of BO43T treated *BCG* following *in situ* labeling and pull-down; ★ indicates MarP homologue (Mb3695c), ★★ indicates HtrA1 homologue (Mb1255). Means ± S. E. M. of triplicate samples shown in a and b represent three independent experiments. Some error bars are smaller than the symbols.

acidity that the mycobacteria encounter. Because BO43 did not kill *Mtb* at neutrality, where HtrA1 is essential, it is likely that BO43 does not inhibit HtrA1 completely and/or that its ability to inhibit HtrA1 is pH-dependent. Moreover, the CFU data in Figure 2d raise the possibility that MarP, when present, may increase the ability of HtrA1 to remain functional in the presence of BO43, perhaps through a proteolytic event exerted on an HtrA1-binding or HtrA1-regulating protein.

Deeper understanding of pH_{IB} homeostasis in *Mtb* may help reveal the mechanisms of action of other compounds that have been found to disrupt it, such as the FDA-approved anti-infective nitazoxanide and the natural product agrimophol.[8,25]

METHODS

Compounds. A library of 324 754 compounds was from NIH. The structures of the FP-Rh and FP-TAMRA have been reported (Supporting Information Figure S1).[11,12] The 60 compounds for the ABPP assay and the 183 benzoxazinone congeners for SAR study were supplied by Dr. Benjamin F. Cravatt and Dr. Dale L. Boger, respectively. BO43, BO43T, and 2DT were synthesized according to procedures described in the Supporting Information.

Strains and Media. *Mtb* H37Rv, *Mtb marP::Tn*, and *BCG* (ATCC) were transformed with a pH-sensitive ratiometric GFP. The strains were cultivated in Middlebrook 7H9 at pH 6.6 with 0.2% v/v glycerol, 0.5% v/v bovine serum albumin (BSA), 0.2% v/v dextrose, 0.085% v/v NaCl, and 0.05% v/v Tween 80 (7H9) or on Middlebrook 7H11 plates containing 0.5% v/v glycerol and 10% v/v OADC (oleic acid, albumin, dextrose and catalase supplement). A total of 50 μg mL^{-1} hygromycin (VWR) was contained in the 7H9 and 7H11 when needed. Acidic buffer was 200 mM sodium phosphate and 100 mM citrate at pH 4.5 with 0.02% v/v Tyloxapol (Pcit-Tyl-4.5). Neutral buffer was 20 mM Tris buffer adjusted to pH 7.4 with HCl (Tris-HCl-7.4). HepG2 cells were grown in Eagle's Minimum Essential Medium with L-glutamine and Earle's salts, 10% v/v FBS and 1% v/v nonessential amino acids.

Target-Based HTS. The methods associated with the HTS can be found on the Pubchem Web site under the identifier AID 2606 (https://pubchem.ncbi.nlm.nih.gov/assay/assay.cgi?aid=2606) as summarized in the Supporting Information.

pH_{IB} Measurement, CFU Assay, and Toxicity Assessment. Effects of compounds on pH_{IB} and CFU were determined as reported.[8] In the pH_{IB} reversibility assay, 200 μL *BCG*-pHGFP was washed in Pcit-Tyl-4.5, resuspended in Pcit-Tyl-4.5 at OD_{580} 0.4, treated with DMSO or 12.5 μM BO43 and incubated at 37 °C for 2 h. The culture was then washed in Pcit-Tyl-4.5 and incubated in 200 μL of fresh Pcit-Tyl-4.5 without BO43.

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Four hours later, BO43 was added again at the same concentration as before, and pH_{IB} was recorded over another 4 h. A total of 25 μL of HepG2 cell suspension was incubated with BO43 with concentrations of 0−100 μM at 37 °C for 48 h. Promega CellTiter-Glo Luminescent Cell Viability Assay kit was then applied to determine ATP levels.

Proteolysis and Competitive ABPP Assays. Recombinant ECD of MarP was overexpressed and purified as described.[6] In the proteolysis assay, 500 nM of ECD of MarP was incubated with 0−20 μM BO43, BO43T, or 2DT for 35 min at RT in 50 mM Tris-HCl-7.4, 500 mM NaCl, and 0.01% v/v Triton X-100. The reaction was initiated by the addition of MarP and inhibitor mixture to 50 μM YKLL-AMC in a final reaction volume of 200 μL. Change in fluorescence was recorded at excitation at 354 nM and emission at 435 nM for 1.5 h at 37 °C. The IC_{50} was calculated by nonlinear curve fitting of log-transformed inhibitor concentration data using Prism Software.[7] In the competitive ABPP assay, soluble and membrane proteomes of human melanoma line C8161 were treated with 20 μM BO43T, 5D, or DMSO as a solvent control for 30 min followed by competition with 1 μM FP-Rh for 10 min at RT. Reactions were quenched with SDS-PAGE loading buffer. Samples were subjected to SDS-PAGE and visualized in-gel using a flatbed fluorescence scanner (MiraBio).[6,12]

***In situ* Labeling of BO43T in *BCG*.** Middle log phase cultures of *BCG* were washed with Pcit-Tyl-4.5 twice and adjusted to OD_{580} 0.2. A total of 200 mL of culture was incubated with 100 μL of DMSO, BO43T (10 mM, DMSO stock), and 2DT (10 mM, DMSO stock), respectively, in Pcit-Tyl-4.5 at 37 °C for 2 h. Subsequently, *BCG* was washed with PBS twice, collected by centrifugation, and suspended in 1 mL of PBS for beating four times with glass beads. The cell lysate was fractionated by ultracentrifugation at 414 630*g* at 4 °C for 1 h to separate cytosolic (supernatant) and membrane fractions (pellet). The pellet was washed three times with PBS, dissolved in PBS containing 1% v/v Triton-X100 during rotation at 4 °C for 1 h, and then centrifuged at 414 630*g* to provide soluble membrane fraction (supernatant). Cytosolic and membrane fractions were depleted of endogenously biotinylated and agarose-binding proteins by rotating with one-fifth volume of prewashed streptavidin agarose (Sigma) at 4 °C for 1 h. Concentrations of both fractions were finally adjusted to 1 mg mL^{-1}.

Click Chemistry-Based Conjugation of BO43T and Biotin and Identification of BO43T-Binding Proteins. To 500 μL of each cytosolic and membrane fraction the following were added in this order: 11.3 μL of 5 mM azido-biotin in DMSO, 11.3 μL of 50 mM Tris(2-carboxyethyl)phosphine (TCEP) in water, 34.0 μL of 1.7 mM Tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine (TBTA) in mixed solvent of DMSO and *t*-butanol (1:4), and 11.3 μL of 50 mM $CuSO_4{\cdot}5H_2O$ in water. Samples were mixed and allowed to react at RT for 1 h, during which the majority of proteins precipitated. The precipitate was collected by centrifugation at 13 000*g* for 5 min, boiled in 4X SDS loading buffer at 95 °C for 10 min, run on SDS-PAGE, and electrobotted to nitrocellulose membranes. The nitrocellulose membranes were treated with blocking buffer (Odyssey) at RT for 1 h, exposed to a IRDye 800CW Streptavidin (Li-COR) at RT for 1 h, washed with Tris-buffered saline with 0.05% v/v Tween 20 (TBST) buffer three times (10 min each time), and visualized by using an infrared imaging system (Odyssey). Other aliquots of the precipitate formed in the process of click chemistry were washed in cold methanol three times and dissolved in 500 μL of 1% v/v SDS/PBS. One-fifth volume of prewashed streptavidin agarose (Sigma) was added, and the mixture rotated at 4 °C for 1 h. The beads were washed three times in PBS by centrifugation at 13 000*g* for 5 min, boiled in 4X SDS loading buffer at 95 °C for 10 min, and centrifuged again. The supernatant was run on SDS/PAGE. Excised lanes were treated with trypsin and the resulting peptides analyzed by MALDI-TOF MS.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Methods, Tables S1−S3, and Figures S1−S7. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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