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Identification of Novel Inhibitors of *M. tuberculosis* Growth Using Whole Cell Based High-Throughput Screening

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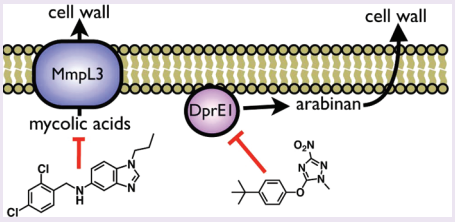
Supporting Information

ABSTRACT: Despite the urgent need for new antitubercular drugs, few are on the horizon. To combat the problem of emerging drug resistance, structurally unique chemical entities that inhibit new targets will be required. Here we describe our investigations using whole cell screening of a diverse collection of small molecules as a methodology for identifying novel inhibitors that target new pathways for *Mycobacterium tuberculosis* drug discovery. We find that conducting primary screens using model mycobacterial species may limit the potential for identifying new inhibitors with efficacy against *M. tuberculosis*. In addition, we confirm the importance of developing *in vitro* assay conditions that are reflective of *in vivo* biology for maximizing the proportion of hits from whole cell screening that are likely to have activity *in vivo*. Finally, we describe the identification and characterization of two novel inhibitors that target steps in *M. tuberculosis* cell wall biosynthesis. The first is a novel benzimidazole that targets mycobacterial membrane protein large 3 (MmpL3), a proposed transporter for cell wall mycolic acids. The second is a nitrotriazole that inhibits decaprenylphosphoryl-β-D-ribose 2′-epimerase (DprE1), an epimerase required for cell wall biosynthesis. These proteins are both among the small number of new targets that have been identified by forward chemical genetics using resistance generation coupled with genome sequencing. This suggests that methodologies currently employed for screening and target identification may lead to a bias in target discovery and that alternative methods should be explored.



The alarming rise in resistance to drugs currently used to treat tuberculosis has spurred a significant increase in efforts geared toward identifying new drugs effective against *Mycobacterium tuberculosis*. Currently there are only six new compounds in the clinical development pipeline that were specifically developed for treating tuberculosis.[1] Of these potential drugs, only two function by inhibiting a completely novel target.[2] Two of the compounds are inhibitors of ribosomal function, and two have complex mechanisms with multiple targets.[2] In order to meet future therapeutic needs, the discovery of new chemotypes and the chemical and biological validation of new targets are required. Although several promising inhibitors have been identified that encompass both novel chemical scaffolds and novel targets, more work is needed to ensure that a sufficient number of new drugs will be available to combat the continual emergence of drug resistance.

There is as yet no clear consensus on the best methods for identifying novel inhibitors of *M. tuberculosis* that will be effective *in vivo*. Approaches range from target-based discovery to whole cell screening efforts against *M. tuberculosis* or related mycobacteria.[3] Target-based approaches rely on knowledge of protein essentiality and function, either through direct studies in mycobacterial species or through homology to proteins in other species. One challenge with target-based approaches is that knowledge of protein essentiality is not sufficient to guarantee "druggability".[4] In addition, compounds identified in target-based screens often lack activity against whole cells. Whole cell screening approaches circumvent these issues but also have inherent challenges. Screening directly against *M. tuberculosis* is extremely challenging as a BSL3 level containment facility equipped with high-throughput screening equipment is required, and procedures required for handling this organism are laborious. Many groups have therefore relied upon the use of model mycobacterial species such as *Mycobacterium smegmatis*. However, it is unclear to what degree these organisms can be relied upon as adequate surrogates for

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M. tuberculosis. In addition, despite the fact that it is becoming clear that the conditions used for culturing *M. tuberculosis* during whole cell screening are critical to identifying inhibitors that will be effective *in vivo*,[5] there is no clear consensus on the conditions that best reflect *in vivo* biology. Finally, the elucidation of the target of a promising small molecule identified in a whole cell screen can be extremely challenging.

Herein we report our experience in whole cell screening using a library of ~20,000 diverse small molecules from the Broad Institute collection against *M. tuberculosis* and the related mycobacterial species *M. smegmatis* and *Mycobacterium bovis* BCG. We found a surprisingly small overlap in the small molecules that are effective against these three species, highlighting the importance for directly screening against *M. tuberculosis*. In addition, we describe the characterization of 91 hits derived from this screen. We also describe testing of 1113 possible leads obtained from a 341,808 compound screen conducted by the NIAID and Southern Research Institute.[6−8] From these combined screening efforts we identified novel inhibitors that target decaprenylphosphoryl-β-D-ribose 2′-epimerase (DprE1), an enzyme required for cell wall biosynthesis, and mycobacterial membrane protein large 3 (MmpL3), a proposed exporter of newly synthesized cell wall mycolic acids. Interestingly, these two proteins now appear to be the targets of several chemically unrelated small molecules. Notably, these targets were all identified using a similar method of resistance generation and whole genome sequencing of mutants.[9−11] This repeated identification of the same targets by the same methodology, albeit using multiple structurally unrelated inhibitors, may imply either that these targets are highly "druggable" and/or the prioritization of molecules whose targets are identified by resistance generation leads to a bias in identified targets.

RESULTS AND DISCUSSION

Screening To Identify *M. tuberculosis* Growth Inhibitors. To identify compounds with activity against *M. tuberculosis* strain H37Rv, we employed a high-throughput screen using constitutive GFP fluorescence as a reporter of bacterial number[12] to screen a total of 20,000 compounds selected from the larger screening collection of the Broad Institute for maximal chemical diversity and consisting of commercially available compounds, natural products, compounds generated by Diversity Oriented Synthesis,[13] and other synthetic compounds. The complete data from the screen has been deposited in the publicly accessible database Chembank (http://chembank.broadinstitute.org/). Cells were seeded into 384-well plates at a low density and were incubated in the presence of ~25 μM small molecules for a total of 3 days at which time GFP fluorescence was used as a readout of bacterial growth (*Z*′-factor = 0.8 for rifampicin and *Z*′-factor = 0.7 for clofazimine, coefficient of variation = 0.05). Dose responses of the known antitubercular drugs clofazimine and rifampicin were used as positive controls for selection of a *z*-score cutoff to define hits from the screen (see Methods). Although the absolute magnitude of the *z*-scores obtained from day to day using this assay varied, the relative performance of a given compound from day to day was highly reproducible. Independent screening of the same set of 4608 compounds on different days showed extremely highly correlated *z*-scores with R^2 = 0.95 (Figure 1a). Using a *z*-score cutoff of −4, we identified 91 hits that were cherry-picked for further analysis.

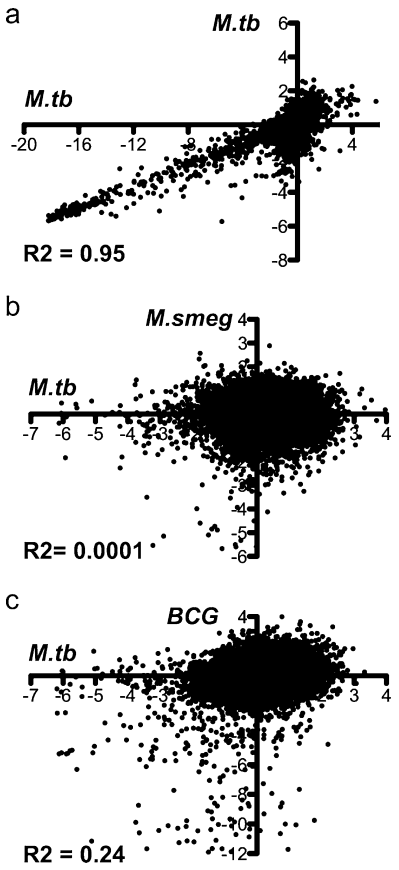

Figure 1. Screening validation and cross-species comparison of hits identified in primary screens against *M. tuberculosis*, *M. smegmatis*, and *M. bovis* BCG. (a) Reproducibility of a GFP reporter based screen for identifying inhibitors of *M. tuberculosis* replication. To determine the reproducibility of the GFP-based assay, the same set of 4308 unique compounds was screened against *M. tuberculosis* on two different days. A high degree of correlation is observed for *z*-scores obtained using the same assay against *M. tuberculosis* on different screening days. (b) Correlation of hits identified in primary screens against *M. tuberculosis* and *M. smegmatis*. 11,147 unique compounds were screened against *M. tuberculosis* using the GFP assay and *M. smegmatis* using an OD_{600} based assay. Plotted are composite *z*-scores calculated for each compound in both the *M. tuberculosis* (*x*-axis) and *M. smegmatis* (*y*-axis) screens. (c) Correlation of hits identified in primary screens against *M. tuberculosis* and *M. bovis* BCG. 13,715 unique compounds were screened against *M. tuberculosis* and *M. bovis* BCG using GFP-based assays. Plotted are composite *z*-scores calculated for each compound in both the *M. tuberculosis* (*x*-axis) and *M. bovis* BCG (*y*-axis) screens. All screens were conducted using duplicate wells for each compound, and composite *z*-scores for the duplicates are plotted. R^2 values were calculated using the two-tailed Pearson product moment correlation test.

The 91 hits obtained from the primary screen were cherry picked and retested in a dose−response assay. Seventy-nine of the 91 compounds (87%) retested at concentrations at or below the screening concentration (Supplementary Tables S1 and S2). Forty-five compounds retested with an IC_{90} of 10 μM or less, and 6 compounds retested with an IC_{90} of less than 1 μM. The active hits were subjected to a number of secondary screens to assess mammalian cell toxicity and activity against *M. tuberculosis* in infected macrophages (Supplementary Tables S1 and S2). The majority of the hit compounds are unique structures that have not been reported to have antimicrobial activity. We thus sought to further characterize these small

molecules to assess their potential for development as tool compounds for the identification and validation of novel targets for drug development.

Cross Species Comparison of Growth Inhibitors. To determine whether the fast-growing mycobacterial species *M. smegmatis* could be used in subsequent follow-up studies, the active hits identified against *M. tuberculosis* were tested for activity against *M. smegmatis*. Approximately 20% of these hits exhibited activity against *M. smegmatis* at the highest concentration tested against *M. tuberculosis* using a low stringency cutoff of a *z*-score < −1.5 as criteria for activity against *M. smegmatis*. That relatively few of the hits from our *M. tuberculosis* primary screen had activity against *M. smegmatis* suggests that the spectrum of compounds that kill *M. tuberculosis* and *M. smegmatis* are potentially quite different, either because of differences in essential targets, in target affinity for an inhibitor, or in cell entry. This is surprising, as *M. smegmatis* whole cell screening was recently used to identify a novel inhibitor of mycobacterial ATP synthase with activity against both *M. smegmatis* and *M. tuberculosis*.[2] However, a recent study comparing a set of 5000 compounds screened across *M. smegmatis, M. bovis* BCG, and *M. tuberculosis* found that only 50% of the compounds that kill *M. tuberculosis* were detected using *M. smegmatis*.[14] A significant proportion of the compounds screened in this study were known pharmacologically active compounds, which could create a potential bias. In our study comparing compounds that were rigorously selected for good activity against *M. tuberculosis,* we found an even smaller degree of overlap in the compounds that are effective against both organisms. This finding has potential implications for drug discovery efforts using *M. smegmatis* as a surrogate organism for whole cell screening. To extend this analysis to a larger number of compounds with less likelihood of inherent structural bias, 11,147 compounds from the primary screen against *M. tuberculosis* were tested in a large-scale primary screen against *M. smegmatis* (*Z*′-factor = 0.8 using hygromycin). These compounds were randomly selected from the full set used against *M. tuberculosis* in the primary screen. Consistent with the previous observation that few of the 91 primary *M. tuberculosis* hits had *M. smegmatis* activity, this unbiased screen revealed little overlap in the compounds with activity against *M. tuberculosis* and those with activity against *M. smegmatis* (Figure 1b) using composite *z*-scores from the primary screens as the basis for comparison (R^2 = 0.0001). The majority of active compounds have species-specific activity.

The surprising lack of concordance in activity of small molecules observed against *M. tuberculosis* and *M. smegmatis* led us to test the degree of overlapping hits when *M. bovis* BCG is used as the screening organism. *M. bovis* BCG is the *M. tuberculosis* vaccine strain that is often used as a surrogate organism for biological studies due to its high degree of relatedness but relative lack of pathogenicity. To this end, 13,715 compounds used in the primary screen against *M. tuberculosis* were randomly selected and were screened in a GFP-based screen using *M. bovis* BCG (*Z*′-factor = 0.8 using hygromycin). Comparison of the hits from the primary screen against *M. tuberculosis* and *M. bovis* BCG by composite *z*-score (Figure 1c) revealed a higher degree of correlation (R^2 = 0.24) than observed in the comparison with *M. smegmatis*. However, the majority of the hits from the individual primary screens still appear to be specific for either *M. tuberculosis* or *M. bovis* BCG. These results are surprising as most effective antitubercular drugs are effective against all three mycobacterial species, although MICs range for these organisms. It is noteworthy that our studies revealed less overlap between mycobacterial species than that identified in the previous study, perhaps due to the larger and less biased composition of the compound collection that we used for cross-species comparison. While in the previous study of ∼5000 compounds a significant proportion of compounds were known bioactives with the inherent potential for structural bias, our larger compound collection consists of uncharacterized structurally diverse and unbiased small molecules. Finally, while it is possible that performing the primary screens with a higher average concentration of inhibitor would result in enhanced correlation of hits, these results suggest a note of caution in the use of model mycobacterial species for conducting primary screens to identify novel drug-like molecules that are effective against *M. tuberculosis*.

Characterization of Gliotoxin Analogues. One of the most potent inhibitors identified in our screen against *M. tuberculosis* was an acetylated analogue of the known fungal toxin gliotoxin (Table S1, Table S2, compound 3). Gliotoxin is an epidithiodiketopiperazine toxin characterized by a bridged disulfide ring (Figure 2a).[15,16] Gliotoxin is known to have

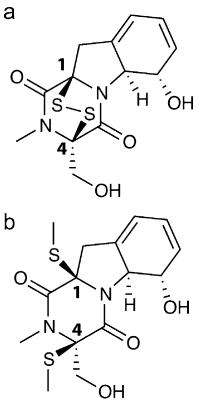

Figure 2. Structures of gliotoxin analogues. (a) Gliotoxin. (b) Dimethylthioether of reduced gliotoxin.

toxicity against a variety of cell types from a wide range of species, including mycobacteria.[17] The toxic effects of gliotoxin on mammalian cells are dependent on the presence of the strained disulfide bond that reacts with cysteine residues on proteins, resulting in protein inactivation.[18] We determined that gliotoxin has an IC_{90} of 0.14 μM against *M. tuberculosis*. Gliotoxin is therefore extremely active against *M. tuberculosis*, with an IC_{90} that is comparable to those of drugs currently used for treating tuberculosis. However, as expected, we found that gliotoxin was toxic to mammalian cells, with an IC_{90} of 8.2 μM. Interestingly, among bacterial species, *M. tuberculosis* appears particularly sensitive to gliotoxin, as we found that the IC_{90} of gliotoxin against other bacterial species including *M. smegmatis, Pseudomonas aeruginosa, Staphylococcus aureus, and Enterococcus faecalis* were >25 μM (data not shown). We were unable to generate mutants of *M. tuberculosis* that were resistant to gliotoxin, indirectly supporting the hypothesis that gliotoxin may have a nonspecific mechanism of action. To determine whether the disulfide bond is required for gliotoxin activity against *M. tuberculosis*, we tested an analogue of gliotoxin where formation of the disulfide bond is prevented by methylation, resulting in dimethyl thioethers at the 1 and 4 positions (Figure 2b). This gliotoxin analogue was significantly less potent than

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gliotoxin itself, with an IC_{90} of >50 μM. While the disulfide bond may serve as a structural scaffolding requirement for specific target inhibition, the reducing potential of the disulfide bond in formation of mixed disulfides with *M. tuberculosis* proteins may also account for gliotoxin activity against *M. tuberculosis*.

Strategies for Target Identification. One of the major hurdles of drug development is the identification of the target of small molecules identified in whole cell screens. Recent successes in the field of antitubercular therapeutics[2,9,11] utilize resistance generation coupled with next generation sequencing for target identification. Using this approach to focus on compounds with a high likelihood of a specific and novel target in *M. tuberculosis*, we eliminated compounds that had significant toxicity against mammalian cells, that had structures suggestive of detergents or ionophores, or that were structurally related to known bioactive agents. We selected a set of 25 compounds to generate resistant mutants and were successful for three structural classes of compounds described below.

Identification of Glycerol-Dependent Growth Inhibitors. Compound A039 (1-(4-ethoxyphenyl)-2-(4-methoxyphenyl)-6,7,8,9-tetrahydro-5*H*-imidazo[1,2-*a*]azepin-1-ium chloride) (Figure 3a) is a highly active bactericidal substituted imidazole with an IC_{90} of 1.5 μM and limited toxicity against mammalian cells (Supplementary Tables S1 and S2, compound 7). High-level resistance (>25 times the MIC) was observed in mutants generated to compound A039 (Figure 3b). Four mutants derived from resistance generated in parallel using four independent parent clones were subjected to whole genome sequencing using Illumina based technology. In each of the four mutants analyzed, we identified a frame shift-mutation in the *glpK* gene resulting from a single base-pair insertion at codon 191 (^{191}GGT > ^{191}GGGT) in each of the four mutants. Frame shift mutations in *glpK* have been previously associated with resistance to inhibitors of *M. tuberculosis* replication identified in whole cell screens, and it has been demonstrated that the activity of these compounds is linked to metabolism of glycerol contained in standard 7H9 mycobacterial media.[5] To confirm that the observed activity of compound A039 against *M. tuberculosis* is dependent upon growth in glycerol-containing media, we compared the activity of A039 against *M. tuberculosis* grown on glycerol or acetate as the sole carbon source. Activity of A039 was dependent on the presence of glycerol (Figure 3c). Compounds with glycerol-specific activity have been demonstrated to induce "self-poisoning" of *M. tuberculosis* by promoting accumulation of glycerol phosphate and rapid ATP depletion and have no efficacy in animal models of tuberculosis.[5] These hits are therefore artifacts of the standard laboratory growth conditions used to culture *M. tuberculosis*.

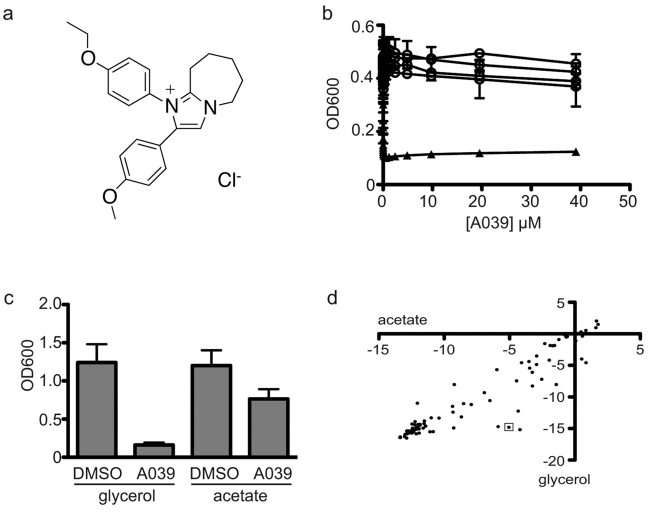

Figure 3. Identification and characterization of compounds with glycerol-dependent killing mechanisms. (a) Structure of compound A039. (b) Dose–response curves for mutants with high-level resistance to compound A039. Black triangles represent wild-type *M. tuberculosis* and open circles represent four independently generated resistant mutants. Points represent the average of duplicates. The experiment was repeated three times. (c) Compound A039 killing of *M. tuberculosis* requires glycerol. Bacteria were cultured on media containing either glycerol or acetate as the sole carbon source for a period of 7 days prior to dilution into media containing acetate or glycerol as the sole carbon source with the addition of 2 μM of compound A039 or DMSO as a control. After 14 days of growth the OD_{600} was measured. Columns represent the average of duplicates with error bars representing standard deviation. The experiment was repeated three times. (d) Comparison of *z*-scores obtained from retesting hits using acetate (*x*-axis) or glycerol (*y*-axis) as the sole carbon source. Plotted is the composite *z*-score from each screen calculated by screening in duplicate.

While the prior study reported the identification of a compound series with glycerol-dependent activity from screening of *M. tuberculosis*, we sought to determine the relative frequency with which this phenomenon of identifying glycerol-dependent inhibitors occurs. To determine the number of hits from our primary screen with a glycerol-dependent mechanism of action, we subjected the hits from the *M. tuberculosis* primary screen to secondary screens using either glycerol or acetate as a sole carbon source. Approximately ~10% of the hits were shifted toward greater activity in glycerol-containing media compared with acetate-containing media, including compound A039 (Figure 3d). These results indicate that the artifact resulting from growth on glycerol can result in large numbers of spurious hits from screens conducted on glycerol-containing media and reinforce the need for conducting primary screens on alternate carbon sources or performing an early secondary screen to rapidly identify hits with a mechanism of action (MOA) that is glycerol-independent.

Identification of a Novel Inhibitor of MmpL3. C215 (*N*-(2,4-dichlorobenzyl)-1-propyl-1*H*-benzo[*d*]imidazol-5-amine) is an inhibitor (Figure 4a) that we identified in HTS with glycerol-independent activity against *M. tuberculosis*, limited nonspecific toxicity against mammalian cells, an IC_{90} of 16 μM against *M. tuberculosis*, and efficacy against *M. tuberculosis* growing in macrophages (Supplementary Tables S1 and S2, compound 72). Four independent resistant mutants were generated to C215. These mutants had IC_{90} values that were shifted only ~2-fold relative to the wild-type parents used for resistance generation (Figure 4b). Sequencing of the genomes of the four resistant mutants identified *Rv0206c* as the only gene with mutations identified in all four resistant mutants, suggesting that mutation of *Rv0206c* confers resistance to C215. *Rv0206c* MmpL3 is a member of the resistance, nodulation, and cell division (RND) family of proteins that are thought to encode lipid transporters in *M. tuberculosis*.[19] AU12345, a recently identified adamantyl urea compound with activity against *M. tuberculosis*, targets MmpL3, resulting in inhibition of the transport of mycolic acids across the inner membrane.[11] In addition, SQ109, a 1,2-diamine related to ethambutol, was also shown to target MmpL3.[20] These compounds were used to demonstrate that MmpL3 encodes

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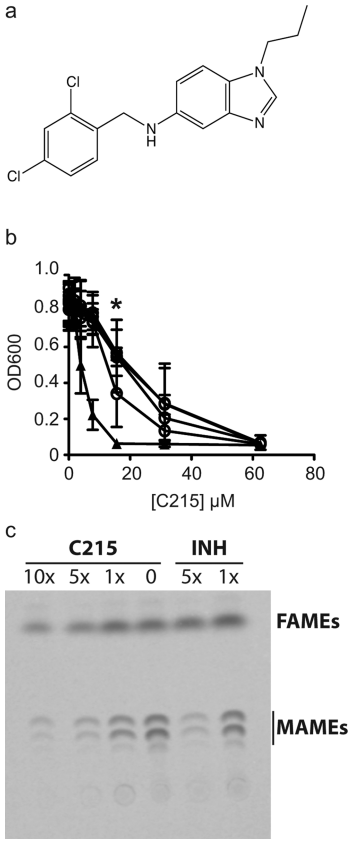

Figure 4. Benzimidazole C215 kills *M. tuberculosis* by targeting MmpL3. (a) Structure of C215. (b) Dose–response curves of resistant mutants generated to inhibitor C215. Black triangles represent wild-type *M. tuberculosis*, and open circles represent four independently generated resistant mutants. Bacterial numbers are determined by OD_{600}, and points represent the average 12 replicates from three independent experiments. * $p < 0.0001$ by Mann–Whitney U. (c) Treatment with C215 inhibits mycolic acid transport. Fatty acid methyl esters (FAMEs) and mycolic acid methyl esters (MAMEs) are labeled. The experiment was repeated twice.

a transporter of trehalose-monomycolate (TDM), an essential precursor to myolic acid containing lipid species in the outer mycolate membrane. The target of AU12345 was identified using the method of resistance generation coupled to genome sequencing, and resistant mutants were found to harbor a single mutation in *mmpL3* (G758A). In contrast, in our analysis of C215 resistant mutants, a different mutation in *mmpL3* was identified in each of the resistant mutants we isolated (L320P, T667A, V684A, V51A). To demonstrate that C215 kills *M. tuberculosis* by inhibiting MmpL3 and transport of mycolic acids across the inner membrane, we treated cells with 1x, 5x, and 10x the MIC of C215 and examined cell wall mycolic acids. We observed a dose-dependent decrease in cell wall bound mycolic acids (α, methoxy, and keto) upon treatment with C215, similar to the effect previously reported for AU12345, confirming that MmpL3 is the likely target of C215. This represents the fourth identification of a small molecule that kills *M. tuberculosis* by inhibiting MmpL3.[11,20,21]

Identification of a Novel DprE1 Inhibitor. Recent screens of 341,808 compounds conducted at the Southern Research Institute under the auspices of the National Institutes of Health resulted in a selected set of 1113 hits that were made available from NIAID.[6−8] This screen was conducted using 7H12 medium that utilizes palmitate as the major carbon source. We obtained these compounds and screened them in two independent screens using the GFP-based assay with either glycerol or acetate as the primary carbon source. The compounds were screened at an average concentration of 20 $\mu g\ mL^{-1}$, slightly higher than the 15 $\mu g\ mL^{-1}$ used in the original NIAID/SRI screen. We observed a high degree of correlation between the composite *z*-scores obtained from the glycerol- and acetate-based screens (Supplementary Figure S1). Using a *z*-score cutoff of −4 for this screen yielded 751 (67%) hits on glycerol and 698 (63%) hits on acetate. While this set of compounds was selected for their activity against *M. tuberculosis*, the relatively low proportion of active compounds again suggests that activity can be dependent on growth conditions and screening methodologies. The GFP assay we used depends upon bacterial replication and compounds that score as hits in this assay necessarily inhibit growth. For this reason, our assay utilizes culture conditions that support robust growth of the bacteria. In contrast, the resazurin (alamar blue)-based assay used by the NIAID/SRI for their *M. tuberculosis* screening measures the reductive potential of living cells and does not require active growth conditions.[22] Thus, their screen using resazurin as a reporter of bacterial viability utilized a long chain fatty acid as the primary carbon source, conditions that do not support robust growth of the bacteria. The differences in the conditions employed for screening thus likely account for the variation in activity observed.

From the set of compounds obtained from the NIAID we confirmed that compound 377790 (1-(4-(*tert*-butyl)benzyl)-3-nitro-1*H*-1,2,4-triazole), a novel nitro-substituted triazole (Figure 5a, Supplementary Table S3), has good activity against *M. tuberculosis* with an IC_{90} of 0.5 μM. Of note, several classes of antibiotics that are active against *M. tuberculosis* are characterized by the presence of a nitro-substituted heterocycle, including the nitroimidazole metronidazole,[23] various nitrofurans,[24] and the promising new agent PA-824, a bicyclic nitroimidazole.[25,26] These compounds all have activity against nonreplicating, dormant *M. tuberculosis*, suggesting the exciting possibility that they may be more effective in treating latent *M. tuberculosis* infection than currently used first-line drugs. Although the MOA of these compounds is still under investigation, the reduction of the nitro-group and subsequent formation of reactive intermediates is critical for activity against nonreplicating populations of cells. Resistant mutants generated against such compounds often have acquired mutations in activating enzymes. For example, FGD1, a specific glucose-6-phosphate dehydrogenase, and its deazaflavin cofactor F_{420} provide electrons for reductive activation of PA-824, and mutation in either of these enzymes confers resistance to this drug.[25]

SAR analysis conducted around 377790 suggests that the nitro-group is essential for activity, as analogues lacking the nitro-group or with various substitutions in place of the nitro group are significantly less active than the original nitro-containing hit (Supplementary Table S3). The requirement for the nitro group could indicate that the mechanism of action of these compounds requires reduction of the nitro group to a reactive species, similar to PA-824, or that the nitro group is required for binding of the inhibitors to its target.

To elucidate the mechanism of action of these compounds, we generated resistance to compound 377790. Mutants generated from selections were resistant at >60 times the IC_{90} (Figure 5b). The genomes of two independently generated

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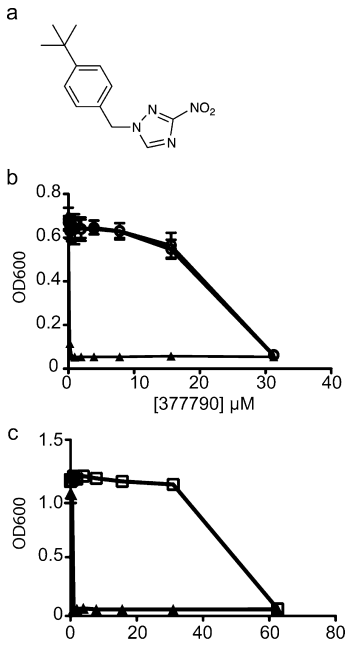

Figure 5. Nitro-triazole inhibitors kill *M. tuberculosis* by targeting DprE1. (a) Structure of nitrotriazole inbibitor 377790. (b) Dose–response curves for two high-level resistant mutants independently generated to inhibitor 377790. Black triangles represent wild type *M. tuberculosis*, and open circles represent resistant mutants. (c) Dose–response curves for DprE1 overexpression and wild-type *M. tuberculosis* tested against inhibitor 377790. Black triangles represent wild type *M. tuberculosis*, and open squares represent overexpression of DprE1. Points represent the average of quadruplicates with error bars representing standard deviation. The experiments were repeated three times.

resistant mutants to 377790 were sequenced. All four resistant mutants contained a single common mutation in *dprE1*, a gene that encodes the epimerase DprE1, resulting in the amino acid change C387S. Recently, a novel class of benzothiazinones (BTZs) that have activity against *M. tuberculosis via* inhibition of DprE1 were identified.[9] These compounds, though structurally dissimilar to the triazole compounds described here, also possess a nitro group functionality and are thought to inhibit DprE1 by formation of a covalent bond *via* reduction of the nitro group to a nitroso-derivative that reacts specifically with Cys387.[27,28] Mutation of Cys387 also confers a high degree of resistance to BTZs. The requirement for the nitro-group in the triazoles is consistent with a mechanism in which this functionality is required for covalent modification of the DprE1 enzyme. As observed with BTZs, overexpression of DprE1 confers resistance to compound 377790 and other nitro-triazole analogues (Figure 5c, Supplementary Table S3), further confirming that these compounds likely target DprE1. This is the third reported discovery of a small molecule that targets this enzyme.[10,27]

In this report we describe the identification of two novel series of inhibitors with clearly defined molecular targets. To identify the targets of these inhibitors we employed the method of resistance generation coupled with whole genome resequencing. Using this method, we identified the target of the novel benzimidazole inhibitor C215 as the cell wall mycolic acid transporter MmpL3 and the target of the novel nitro-triazole inhibitor 377790 as the epimerase DprE1. In recent years, three new targets have been identified from inhibitors discovered in whole cell screens. These targets are DprE1, MmpL3, and ATP synthase. It is interesting to note that several structurally unrelated inhibitors have now been reported for MmpL3. Prior to our study, the two reported DprE1 inhibitors were structurally related, suggesting that the apparent promiscuity of MmpL3 could be an isolated case. However, our identification of a completely structurally distinct inhibitor of DprE1 argues that both MmpL3 and DprE1 are highly druggable and can be inhibited by a wide variety of chemical structures. In addition, these findings suggest that whole cell screens, coupled with whole genome sequencing of resistant mutants, appear to be revealing the same targets repeatedly despite the fact that there are likely many essential and druggable proteins in *M. tuberculosis* that have yet to be uncovered. Alternatively, the repeated identification of the same targets may suggest that the use of resistance generation for target identification may bias the nature of targets identified to those that easily accommodate mutations in their active site while preserving target function. Thus, selecting compounds for prioritization on the basis of the ability to identify a target using resistance generation may result in lead compounds with biased mechanisms of action.

Most target identification efforts in the field of *M. tuberculosis* anti-infectives currently rely on resistance generation, resulting in a possible quandary. By focusing on small molecules with an identified target based on this method, such as inhibitors of DprE1 or MmpL3, candidates may be prioritized to which clinical resistance may be easily and rapidly engendered. This bias highlights the need for multiple approaches to identify targets of promising inhibitors of *M. tuberculosis.* In fact, one might argue that prioritization should be given to those candidates for which we are unable to generate resistance, as this may be a desirable property of a new antibiotic. Thus, the development and use of parallel strategies for target identification are of vital importance to the discovery efforts geared toward the identification of the next generation *M. tuberculosis* drugs. Nonetheless, the identification of novel inhibitors with clearly defined targets is still relatively rare in the field of *M. tuberculosis* drug discovery, and the two novel inhibitors described in this report will be useful in future investigations related to drug discovery and *M. tuberculosis* cell wall biosynthesis.

All currently used antibiotics were discovered using whole cell screening, highlighting the importance of this approach. In this report we describe our experiences with whole cell high-throughput screening to identify novel inhibitors of *M. tuberculosis* replication using both an unbiased collection of >20 K compounds from the Broad Institute and a set of 1113 hit compounds made available to the *M. tuberculosis* screening community by the NIAID. This latter data set provides independent verification and testing of the NIAID compounds to help define the importance of this collection to *M. tuberculosis* drug discovery efforts. In addition, by analyzing the primary and secondary screening data from the Broad collection, we were able to expand upon and quantify observations related to whole cell screening. This study using a large compound collection with no predicted potential for structural bias found an even smaller degree of overlap in compounds then previously reported[14] that are active against three mycobacterial species, clearly demonstrating the potential pitfalls of using surrogate mycobacterial species for identifying inhibitors of *M. tuberculosis* growth. In addition, this work describes the surprisingly high rate at which such hits are

identified from whole cell screening that bear the recognized characteristic of dependence on growth on standard glycerol containing *M. tuberculosis* media. This is particularly important, as inhibitors of this nature have no potential for *in vivo* activity, suggesting that a major shift away from the standard growth media used for the study of *M. tuberculosis* may be warranted, particularly with respect to chemical biological investigations. Finally, we note the interesting phenomenon of repeated identification of the same targets DprE1 and MmpL3 by the methods utilized, which may suggest that they are highly targetable but also raise the caveat that they may be targets that are easily mutated to confer resistance. In summary, this work provides insights and perspectives that should help guide future *M. tuberculosis* screening efforts and antibiotic discovery in general.

METHODS

Bacterial Strains and Growth Conditions. Bacterial strains used were *M. tuberculosis* H37Rv, *M. bovis* BCG strain Pasteur, and *M. smegmatis* MC^2155. GFP was expressed using a pUV15tetORm derivative[29] with *tetR* deleted for constitutive expression. Mycobacterial strains were cultured in Middlebrook 7H9 medium with 0.05% (v/v) Tween-80 and 10% (v/v) OADC with either 0.25% (v/v) glycerol or 10 μM acetate.

Compound Library. The 20,502 compounds screened against *M. tuberculosis* were obtained from the Broad Institute small molecule library. The categories of compounds were as follows: 30% commercially available compounds purchased predominantly from ChemDiv, Maybridge (Thermo Fisher Scientific) and TimTec; 40% compounds synthesized by Broad chemists using Diversity Oriented Synthesis (DOS) or other strategies for organic synthesis; 30% natural products consisting of natural product extracts and purified natural products.

Screening, Hit Selection, and IC_{90} determinations. For *M. tuberculosis* and BCG screening assays, bacteria expressing GFP was grown to midlog phase (OD_{600} = 0.6–0.8), diluted, and plated into 384-well plates into which compounds had previously been pinned for a final OD_{600} of 0.025 and compound concentration of 25 μM (Broad Library) or 20 $\mu g\ mL^{-1}$ (NIH inhibitors). Plates were incubated for a period of 72 h, at which time GFP fluorescence was read. Each compound was screened in duplicate, and composite *z*-scores were calculated using DMSO controls as reference. Hits from the *M. tuberculosis* screen were defined as compounds with a composite *z*-score of less than −4. This *z*-score cutoff was selected using the average of the *z*-scores of the concentrations of the control antibiotics clofazimine and rifampicin that gave a Z′-factor of 0, meaning that the distance separating the positive and negative controls is 3x the sum of the standard deviations of the two populations. For dose–response curves and IC_{90} determination of hits from the screens, bacteria were grown to midlog phase and plated in 96-well plates at OD_{600}= 0.05 in the presence of small molecule inhibitors for 7 days unless otherwise indicated, and growth was assessed by reading OD_{600}. The IC_{90} was defined as the minimum concentration that inhibited growth by 90% relative to the DMSO control. For the *M. smegmatis* screen, OD_{600} was used as the readout, and the plates were read after 2 days of incubation.

Secondary Screening Assays. Hits from the primary screen against *M. tuberculosis* were cherry picked from the library and arrayed in 384-well plates as a dose–response curve using 2-fold serial dilutions from the primary screening concentration. Preliminary cherry pick plate MIC values were determined using the GFP-based assay and are reported as the minimum concentration that gives the maximum inhibition observed using rifampicin controls. Macrophage activity was assessed by infecting J774 macrophages plated into 96-well plates with a strain of *M. tuberculosis* constitutively expressing firefly luciferase. After 3 days of infection in the presence of inhibitors, the infected monolayers were washed and lysed, and luminescence was used as a reporter of bacterial viability. To determine macrophage toxicity, J774 macrophages were incubated with small molecules for a period of 3 days upon which time CellTiter-Glo (Promega Corporation) was used as a readout for macrophage viability.

Generation of Resistant Mutants. The MIC of each compound on solid media was identified by plating 10^7 bacteria on agar containing a dose response in 96-well plate format. The MIC was defined as the lowest concentration resulting in inhibition of bacterial growth. Resistant mutants were generated by plating *M. tuberculosis* cells onto agar pads containing 2x and 10x the agar MIC of each compound using four independently derived wild-type clones. Colonies that arose on inhibitor-containing plates were inoculated into liquid media containing 1x the liquid MIC of the inhibitor. These cultures were grown to midlog, and samples were retested in a liquid MIC assay to confirm that a shift relative to the wild-type MIC was observed.

Metabolic Labeling of Mycolic Acids and Compound Treatment. Cultures (10 mL) of *M. tuberculosis* were grown to OD_{600} = 0.6 in inkwell bottles, and then 1x, 5x, or 10x the MIC of C215 was added to the cultures simultaneously with 10 μCi of ^{14}C acetate. After 20 h of labeling, the cultures were washed once with water and saponified with tetrabutylammonium hydroxide, and methyl esters of fatty acids and mycolic acids were created by addition of methyl iodide. Fatty acid methyl esters (FAMEs) and mycolic acid methyl esters (MAMEs) were extracted from the aqueous layer using methylene chloride and run out on TLC plates using 95:5 hexanes/ethyl acetate. TLC plates loading was normalized according to the OD_{600} of the cells after the 20 h treatment period. FAMEs and MAMEs were visualized by phosphorimaging.

ASSOCIATED CONTENT

Supporting Information

Supplementary figure and tables and materials and methods describing the synthesis of triazole compounds NT19–NT27. 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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