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ACS Infectious Diseases

Letter

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Identification of Novel Anti-mycobacterial Compounds by Screening a Pharmaceutical Small-Molecule Library against Nonreplicating *Mycobacterium tuberculosis*

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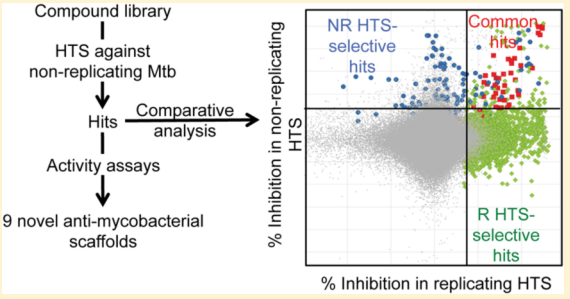
Supporting Information

ABSTRACT: Identification of compounds that target metabolically diverse subpopulations of *Mycobacterium tuberculosis* (Mtb) may contribute to shortening the course of treatment for tuberculosis. This study screened 270,000 compounds from GlaxoSmithKline's collection against Mtb in a nonreplicating (NR) state imposed in vitro by a combination of four host-relevant stresses. Evaluation of 166 confirmed hits led to detailed characterization of 19 compounds for potency, specificity, cytotoxicity, and stability. Compounds representing five scaffolds depended on reactive nitrogen species for selective activity against NR Mtb, and two were stable in the assay conditions. Four novel scaffolds with activity against replicating (R) Mtb were also identified. However, none of the 19 compounds was active against Mtb in both NR and R states. There was minimal overlap between compounds found active against NR Mtb and those previously identified as active against R Mtb, supporting the hypothesis that NR Mtb depends on distinct metabolic pathways for survival.



KEYWORDS: *nonreplicating M. tuberculosis, high-throughput screen, reactive nitrogen species*

INTRODUCTION

Tuberculosis (TB) causes more deaths than any other bacterial infection. Standard treatment of drug-sensitive TB involves four drugs for 2 months and two of them for an additinoal 4 months, a far longer treatment than for most bacterial infections, whereas treatment of drug-resistant TB often requires >24 months.[1] Although a 95% cure rate is attainable for drug-sensitive TB, patient noncompliance is frequent, especially in resource-poor settings, contributing to an epidemic of drug-resistant TB.

Prolonged treatment may be necessary because subpopulations of *Mycobacterium tuberculosis* (Mtb) enter a nonreplicating (NR), phenotypically drug-tolerant state in response to stringent host microenvironments, such as hypoxia in granulomatous lesions, reduced nutrient availability in macrophages, low pH in mature phagolysosomes or inflammatory sites, and reactive nitrogen species and reactive oxygen species generated by host and mycobacterial enzymes.[2−4] None of the first- and second-line TB drugs that are active against replicating (R) Mtb in vitro are comparably active against NR Mtb.[5,6]

These considerations have prompted two types of efforts to find leads for drugs that can kill NR Mtb. A target-based approach seeks inhibitors of enzymes used by Mtb to survive adverse environments in the host. The first compounds found to be selectively bactericidal toward NR Mtb were inhibitors of Mtb's dihydrolipoamide transferase (DlaT).[6] Other examples include inhibitors of the Mtb proteasome and spectinamides that inhibit the Mtb ribosome.[7,8]

A second approach, taken here, is to use whole cells to identify compounds active against NR Mtb. We screened virulent Mtb H37Rv rendered NR by simultaneous exposure to four host-relevant stresses, hypoxia (1% O_2), mild acid (pH 5.0), reactive nitrogen species generated from 0.5 mM nitrite

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under acidic conditions, and restriction to butyrate (0.05%) as the carbon source,[9] using the newest and most drug-like compounds of the GlaxoSmithKline (GSK) collection. Systematic evaluation and progression of hits from the NR screen led to the identification of nine novel anti-mycobacterial scaffolds, of which five were selectively active against NR Mtb, whereas four were selectively active against R Mtb. Primary hits from the NR screen were also compared with those from a previous GSK screen against R Mtb with the same compound set, revealing remarkably little overlap.

RESULTS AND DISCUSSION

We operationally defined NR conditions as those that resulted in neither a net gain nor a loss in colony-forming units (CFU) over the period of exposure, recognizing that this does not exclude some degree of balanced replication and death.[9] These criteria were met when applied to Mtb H37Rv (Figure S1A in the Supporting Information (SI)). For high-throughput screening (HTS), we judged the ability of a compound to kill Mtb during 3 days of exposure under NR conditions by recording the impairment of Mtb's outgrowth as reflected by optical density in a second stage of the assay conducted under R conditions. For the R stage, the culture was diluted 5-fold in nutrient-rich medium at pH 6.6 without nitrite and incubated in 20% O_2. Because the screen was conducted at 10 μM, compounds that caused significant growth inhibition only against R Mtb below 2 μM could score as positive in the outgrowth phase following their carry-over at a 5-fold dilution from the NR phase. Nonetheless, limiting the dilution factor to 5 made it possible to conduct both the NR stage and the R stage of the assay in the same wells of 384-well plates. Positives were retested in a two-plate assay (see Methods), wherein we increased the dilution factor between the NR and R stages to 21-fold and confirmed in CFU assays. Thus, the screen under NR conditions could potentially identify three classes of actives based on the metabolic state of the bacteria they targeted: NR-specific, R-specific, and dually NR- and R-active.

To conduct the primary HTS in a 384-well, one-plate format, we determined the following optimal conditions: an initial $OD_{580\ nm}$ of 0.1 for the inoculum, 3 days in the NR stage, and 7 days in the R stage. With rifampin (1 μg/mL) as positive control and DMSO (1%) as vehicle control, we consistently achieved Z' values of >0.6 (Figure S1B in the SI). To maximize the number of candidate actives, despite the inevitability of accruing a high proportion of false positives, we used statistically derived cutoff values to define candidate hits. The primary screen identified ~2700 hits (hit rate of 1.01%) based on an average cutoff of 27% growth inhibition (Figure 1A).

Approximately 500 hits confirmed when retested at the screening concentration of 10 μM (Figure 1A). Known inhibitors of dividing bacteria such as rifamycins and bacterial topoisomerase/mycobacterial gyrase inhibitors were identified at this step, validating the ability of the assay to detect potent R-specific or dual actives. All but 2 of the 142 hits that were bacterial topoisomerase/mycobacterial gyrase inhibitors were excluded from further analysis in an effort to focus on novel compound classes. We chose to include 47 that had percent inhibition values >80 in the primary screen but did not confirm in the single-shot assay (% inhibition < 30), yielding a final list of 363 hits (Figure 1A). A cutoff of 50% growth inhibition at 100 μM (IC_{50} < 100 μM) in concentration response curve (CRC) assays under NR conditions reduced the compounds of interest to 166; 8 of these were from the 47 that did not

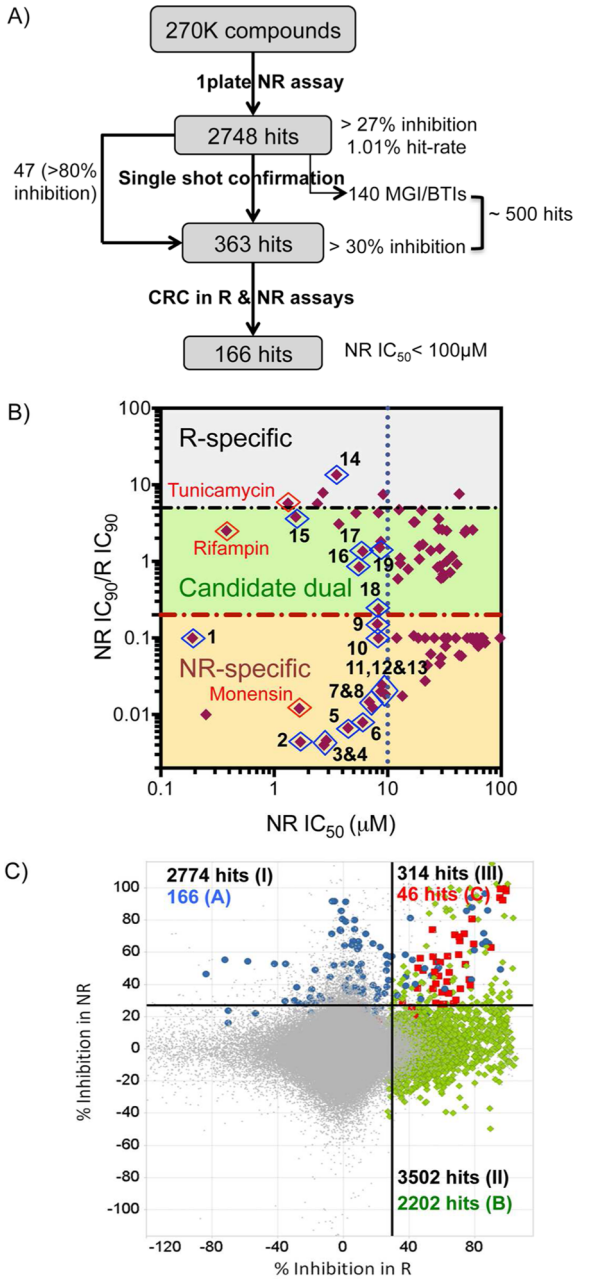

Figure 1. HTS against NR Mtb. (A) Hit progression. MGI, mycobacterial gyrase inhibitor; BTI, bacterial topoisomerase inhibitor. (B) Activity profiles of 90 nontoxic hits with NR IC_{50} < 100 μM. Ratios were calculated from IC_{90} values from NR and R CRCs. Hits were tentatively classified as R-specific (ratio > 5), dual (5 > ratio > 0.2), or NR-specific (ratio < 0.2) actives. Hits with no detected activity in the R assay were assigned an arbitrary R IC_{90} value of 1000 to calculate the ratio. Blue diamonds highlight novel structures selected for characterization (NR IC_{50} < 10 μM; Table S3). Red diamonds highlight known anti-mycobacterials. (C) Distribution of hits from NR and ATP R HTS against Mtb H37Rv. Percent inhibition of a compound in primary screening assay is plotted on the two axes. A total of 2774 hits inhibited NR Mtb (class I; 27% mean inhibition), whereas 3502 inhibited R Mtb (class II; 35% mean inhibition). Three hundred and fourteen hits (class III) were common to both primary screens. Among the confirmed hits, 46 noncytotoxic hits (red squares; class C) were common to both screens, whereas 166 (blue circles; class A) were confirmed in NR HTS and 2022 (green diamonds; class B) were confirmed in ATP R HTS.

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confirm in the single-shot assay. Sixty were excluded because of toxicity (TOX_{50} < 100 μM) toward a human hepatocellular carcinoma cell line, HepG2 (Table 1 in the SI). The decision to exclude bacterial topoisomerase/mycobacterial gyrase inhibitors precluded calculation of the confirmation rate.

Clustering of nontoxic confirmed hits from NR HTS identified 60 clusters of which 21 clusters have at least 2 members, whereas 39 were singletons. Table 2 in the SI summarizes cluster properties such as cluster size, lowest NR IC_{50} per cluster, and several physicochemical characteristics. We have also included a chemical name that describes the type of scaffold for those clusters with at least three members (before exclusion of cytotoxic compounds). This type of analysis showed that confirmed hits from the NR HTS are not restricted to a specific chemotype; instead, they fall into diverse structural groups.

To classify the 106 nontoxic confirmed hits as being more likely NR-specific, R-specific, or dual active, we compared results from two kinds of CRC assays: the two-stage, one-plate, NR assay and a single-stage, one-plate, R assay (see Methods), performed head-to-head on the same compounds. The ratio of IC_{90} values in NR versus R assay was used to distinguish the classes (Figure 1B and Table 1 in the SI). A ratio >5 (the dilution factor in the NR assay going from NR stage to R stage) suggested that drug carry-over to the outgrowth stage could account for the observed activity and thus implied R-selectivity, whereas a ratio ranging from 0.2 to 5 suggested dual activity. Very high IC_{90} values or inability to achieve 90% inhibition at any tested concentration in the R assay indicated NR-selectivity. Sixteen noncytotoxic hits were excluded at this stage due to IC_{90} > 100 μM in NR or R and NR IC_{50} > 50 μM. By these criteria, 45 compounds appeared to be NR-specific, whereas 39 appeared to be dual actives and 6 were candidate R-specific actives. A detailed schematic of the pipeline used for characterization of confirmed hits is included in Figure S2 in the SI.

The same compound library was previously screened against R Mtb H37Rv (unpublished data). That screen will be referred to as "ATP R HTS" because ATP levels were used as a reflection of bacterial biomass and the term helps distinguish the two screens in the following discussion. Of the ~2700 and ~3500 primary (that is, not yet confirmed) hits identified by the NR and ATP R HTS, respectively, only 314 were identified in common (Figure 1C). Among the 166 NR HTS hits confirmed by CRC assay (NR IC_{50} < 100 μM; highlighted as blue circles in Figure 1C) and the 2202 ATP R HTS hits confirmed by CRC assay (highlighted as green diamonds in Figure 1C), there were 46 compounds in common (highlighted as red squares in Figure 1C). Of these, 19 were cytotoxic to HepG2 cell line. Among the remaining 27 common confirmed hits, 18 were candidate dual actives based on CRC analyses performed in conjunction with the NR screen. Surprisingly, ATP R HTS identified none of the confirmed R-specific actives identified by the NR HTS. This could be due to differences in read-outs used by both screens: ATP levels versus optical density. Nevertheless, the observation that ATP R HTS missed 53% of the candidate dual and 95.4% of the NR-specific actives demonstrates that the two HTS were almost completely orthogonal. This suggests that distinct physiological states make Mtb dependent on different targets, or differentially affect Mtb's uptake, catabolism, or retention of certain compounds. A third possibility is suggested by the observation of Gold et al. that the NR conditions may modify some of the compounds tested, either abrogating or conferring activity.[9]

One of the NR-selective compounds was monensin, an ionophore, which was reported to disrupt Mtb's intrabacterial pH homeostasis in a screen of natural products against Mtb, and thus served as an internal positive control for NR assay conditions.[10] Among novel classes of NR-specific actives, 13 (highlighted in Figure 1B and Table 3 in the SI) were selected for further study, on the basis of their favorable physicochemical properties, potency (IC_{50} < 10 μM in the NR assay), and availability as pure powders. Three singletons, **2**, **6**, and **12**, had IC_{90} values of 6−25 μM in two-plate NR assays (Figure 2A,B). Five actives with IC_{90} values of 6−12.5 μM belonged to two chemical families, pyridazinones (**4**, **5**, and **7**) and chromanones (**3** and **8**). A representative structure from each family is shown in Figure 2A. Five compounds were excluded due to inactivity of resupplied powders (**1**, **10**, and **13**), cytotoxicity (**9**), or poor solubility (**11**) (Table 3 in the SI), bringing the number of novel NR-specific scaffolds to five (**2**, **6**, **12**, pyridazinones, and chromanones).

One or more of the four individual stresses in the NR assay may be necessary for Mtb's susceptibility to these compounds. Hence, we tested the effect of excluding each stress separately. All eight compounds tested proved to be dependent on reactive nitrogen species for their activity (Figure 2B). Next, we analyzed the stability of these actives in NR assay conditions, because the combination of mild acid and nitrite can cause some compounds to undergo structural modifications.[9] LC-MS of samples after 3 days of exposure to cell-free NR medium showed that chromatogram peaks of **6** and **12** were 100% preserved relative to time 0, whereas only 20% of **2** remained (Figure 2B). All of the other hits, **3**, **8**, **4**, **5**, and **7**, were unstable in NR medium, with <50% of original sample preserved. Next, we tested if exposure of compounds to NR conditions altered their NR-selectivity by conferring activity against R Mtb. After pre-exposure to NR conditions, among the unstable compounds, **2** had negligible activity against R Mtb, whereas the other unstable compounds acquired activity against R Mtb. This gain of activity against R Mtb could be due to the formation of toxic products; hence, we deprioritized all unstable compounds except **2**. The stable compounds **6** and **12**, as expected, did not acquire activity against R Mtb (Figure S3A in the SI).

12 and **2** were selected for potential use as tool compounds in mechanism of action studies because of their structural diversity, specificity toward NR Mtb, and distinct stability profiles in assay conditions. To determine if **2** itself or the conversion product of **2** mediates the NR activity associated with **2**, we pre-exposed **2** to NR conditions for 3 days before adding it to Mtb under NR conditions. **2** significantly lost activity toward NR Mtb after pre-exposure, with a 32-fold increase in IC_{90} (Figure S3B in the SI). In comparison, IC_{90} of **12** increased only 4-fold after pre-exposure (Figure S3B in the SI). Both compounds were bactericidal as shown by reduction in CFU to limit of detection (5-$\log_{10}$ decrease) at their IC_{90} after 3 and 7 days of exposure (Figure 2C). Thus, **2** itself was active, and its effects were exerted quickly enough to be manifest before it was modified. We were unable to isolate the conversion product(s) of **2**.

12 and **2** were dependent on acidified nitrite, suggesting that they may disrupt Mtb's adaptation to reactive nitrogen species. Generation of reactive nitrogen species by host inducible nitric oxide synthase (iNOS) is vital for efficient control of TB in

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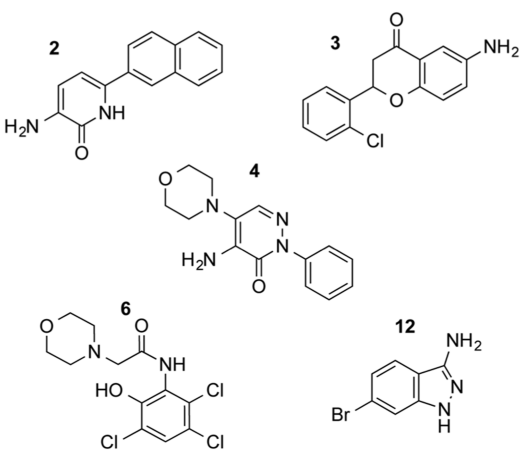
A)



B)

Cpd No:	NR IC_{90} (μM)				$Stability_{NR}$ (% remaining after 72h)
	Complete- at pH 5.0, with 1% O_2 & 0.5 mM $NaNO_2$	w/o $NaNO_2$	at 20% O_2	at pH 7.0	
2	6.25	>100	3.12	>100	20
3	6.25	>100	6.25	>100	0
4	6.25	>100	6.25	>100	30
5	12.5	>100	12.5	>100	0
6	6.25	>100	3.12	>100	100
7	6.25	>100	6.25	>100	0
8	6.25	>100	12.5	>100	0
12	12.5	>100	6.25	>100	100

C)

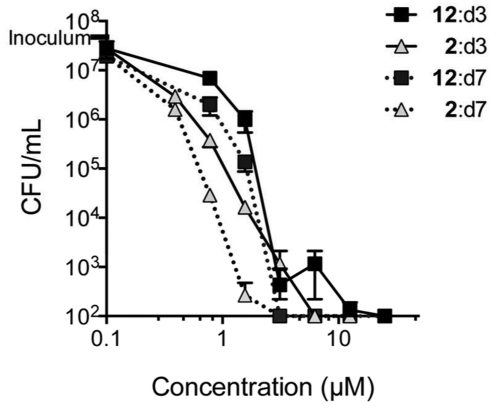


Figure 2. Characterization of NR-specific actives. (A) Representative structures of five novel scaffolds active against NR Mtb. (B) IC_{90} against Mtb after exclusion of nitrite, hypoxia, or acid stress from the two-plate assay. "Complete" refers to standard four-stress NR conditions. Stability in NR medium after 3 days is expressed relative to amount at time 0. (C) CFU for NR Mtb after 3 and 7 days of exposure to **12** or **2**.

mice.[11] However, Mtb withstands micromolar concentrations of reactive nitrogen species, which trigger a dormancy-associated transcriptional response.[12] To determine if dependence on reactive nitrogen species for activity reflected codependence on the low pH used to foster reactive nitrogen species generation from nitrite, we added diethylenetriamine NO adduct (DETA-NO), a slow-release NO donor, to Mtb under R conditions in the presence and absence of **12** and **2**. Both compounds reduced Mtb CFU to an extent dependent on the concentration of NO (Figure S4A in the SI). We also performed detailed CFU analysis of **12**'s dependence on NO for activity and observed cidal activity at 100, 30, and 10 μM **12** in the presence of 100 μM DETA-NO. A slight reduction in CFU was seen at 100 μM **12** in the presence of 30 μM DETA-NO, but it was static at lower concentrations of DETA-NO (Figure S4B in the SI). Recent studies have shown that Mtb's transcriptional adaptation to NO overlaps with but is distinct from its transcriptional response to the nitrite it generates from respiration of nitrate.[13] We therefore measured the activity of **12** and **2** in the presence of 1 mM nitrite and nitrate under R conditions and observed no inhibition (Figure S4C in the SI). We conclude that the activity of these two compounds against Mtb in NR conditions requires the presence of reactive nitrogen species other than nitrite, such as NO or others of its products. In the presence of an NO donor, the two compounds were inactive against *Staphylococcus aureus* (Figure S5 in the SI) or *Escherichia coli* (data not shown). Nor did an NO donor render **12** cytotoxic against the J774 mouse macrophage cell line (data not shown).

Among 45 nontoxic hits that had activity against both R and NR Mtb in CRC assays were 39 candidate dual and 6 candidate R-specific actives (Figure 1B and Figure S2 in the SI). Five novel scaffolds with NR IC_{50} < 10 μM were selected for further characterization (highlighted in Figure 1B and listed in Table 3 in the SI). **18** and **16** were excluded due to cytotoxicity and unavailability of powder, respectively. An authentic dual active is expected to reduce CFUs of both NR and R Mtb. Although all of the remaining compounds tested were bactericidal against R Mtb, with up to 6-log_{10} decrease in CFUs, CFUs of NR Mtb were not substantially reduced (Figure 3A,B), except that 25 μM RIF and 50 μM MOXI displayed up to 3 and 1.5 log_{10} killing of NR Mtb, respectively. Thus, no compounds with potent bactericidal activity against both R and NR Mtb were identified, although exclusion of the novel BTI/MGIs may have biased results against them. In other screening campaigns against NR Mtb conducted with 113,000 compounds provided to the Weill Cornell group by academic institutions, there were very few authentic dual actives and most of them were cytotoxic[9] (and unpublished data). However, rare compounds were identified that were potently mycobactericidal under both R and NR conditions, were not cytotoxic to mammalian cells, and had a narrow microbicidal spectrum,[14,15] suggesting that it is appropriate to continue to search for dual active compounds.

The only structurally distinct R-specific scaffold we identified, **14** (Figure 3C), was a pyrido-benzimidazole with submicromolar IC_{90} values and bactericidal activity, with up to 4 log_{10} reduction of CFUs after 8 days of exposure at 6.25 and 12.5 μM in R conditions; there was no effect on NR Mtb (Figure 3D). **14** had no activity toward *Staphylococcus aureus*, *Escherichia coli*, or *Pseudomonas aeruginosa* (Figure S6 in the SI), although an analogue of **14** was reported to be active against R Mtb.[16] Thus, we identified four novel scaffolds (**14**, **15**, **17**, and **19**) with activity against dividing Mtb.

In conclusion, we identified novel, nontoxic scaffolds that are bactericidal toward Mtb in either but not both of two metabolic states: when it stops replicating while coping with reactive nitrogen species and when it is dividing. These novel scaffolds could prove to be useful tool compounds to identify mechanisms used by Mtb to adapt to these distinct states. Of the five NR-specific scaffolds, three were found to be unstable in the NR assay conditions. The observation that one of the

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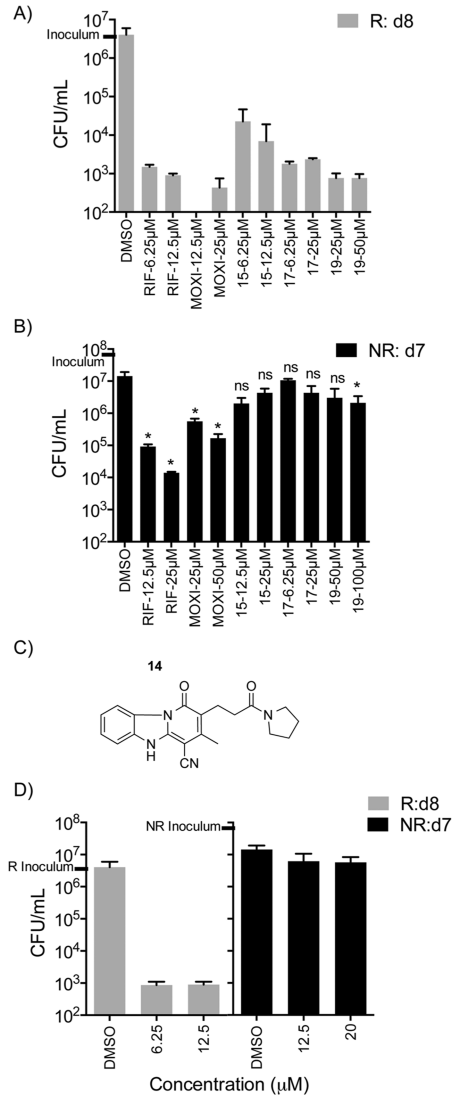

Figure 3. Characterization of dual and R-specific actives. CFU assay in (A) R conditions (8 days exposure) and (B) NR conditions (7 days exposure) with RIF, MOXI, **15**, **17**, or **19**. (C) Structure of the R-specific active, **14**. (D) CFU after exposure to **14** for 8 days under R conditions or for 7 days under NR conditions. Limit of detection = 100 CFU/mL. Data are representative of two independent experiments. (∗) *P* value <0.05 with two-tailed paired *t* test analysis.

unstable compounds, **2**, is a fast-acting cidal compound despite its conversion product being inactive against NR Mtb (Figure S3B) suggests that instability in NR assay conditions that mimic Mtb's host environment might not always be a liability. Structure−activity relationship studies are ongoing with the NR active, **12**, and the R active, **14**, to increase potency and improve pharmacokinetics in mice for efficacy determination in murine model of tuberculosis. Recently, others have performed phenotypic screens against Mtb subjected to individual stresses among the four that we combined here, such as hypoxia, starvation, and acid, and have identified scores of new inhibitors.[10,17,18] These and the compounds reported here may provide starting points for drug development aimed toward shortening TB therapy.

METHODS

Bacterial Strains and Culture Conditions. Mtb H37Rv was grown at 37 °C in Difco Middlebrook 7H9 (BD) medium with 0.2% glycerol, 10% ADC (BD) or ADN (0.5% bovine serum albumin (BSA; Roche), 0.2% dextrose, 0.085% NaCl, and 0.02% tyloxapol. Sauton-based minimal medium for NR assays contained 0.05% KH_2PO_4, 0.05% $MgSO_4$, 0.005% ferric ammonium citrate, 0.0001% $ZnSO_4$, 0.1% NH_4Cl, 0.05% butyrate, 0.5% BSA, 0.085% NaCl, and 0.02% tyloxapol. pH was set to 5 with 2 N NaOH and 0.5 mM $NaNO_2$ was added from freshly prepared 1 M stock. Reagents were from Sigma-Aldrich, unless specified.

HTS and CRC Assays (One-Plate Assay). One hundred and fifty nanoliters of 100× (1 mM) test compound was dispensed acoustically (Echo555) to tissue culture-treated, clear-bottom, black 384-well plates (Greiner-BioOne 781091). Columns 6 and 13 carried 150 nL of 100% DMSO (100× of vehicle control) and 150 nL of 100 μg/mL RIF (100× of positive control), respectively. Log-phase Mtb was washed twice with phosphate buffer saline with 0.02% tyloxapol and suspended at OD_{580} of 0.1 in NR medium. After addition of $NaNO_2$, 15 μL was dispensed into each well using a MultidropCombinL dispenser (Thermo Scientific). Plates were incubated for 3 days at 37 °C in oxygen-controlled incubators (Binder GmbH) at 1% O_2 and 5% CO_2. Sixty microliters of complete 7H9 medium was then added to each well to allow outgrowth at 37 °C with 20% O_2 and 5% CO_2 for 7 days. OD_{580} was recorded. These assay conditions were also used to test CRCs of compounds from 100 to 0.097 μM against NR Mtb. CRCs in R conditions were performed with log phase Mtb at an OD of 0.02 (one-fifth of 0.1). Percent inhibition data were analyzed with ACTIVITYBASE (idbs) using nonlinear regression analysis to plot the best-fit curves.

Statistical Analysis. Hits were defined as compounds producing inhibition that exceeded the mean ±3 SD calculated as described[19] for all plates tested on a given day, after correction using an in-house pattern recognition algorithm.[20]

Ninety-six-Well Plate Assays (Two-Plate Assay). NR and R Mtb suspensions were prepared with OD of 0.1 and 0.005, respectively. Compounds were added to 200 μL/well of Mtb in 96-well plates (Costar). The two-plate NR assay included incubation with 1% O_2 and 5% CO_2 for 3 days followed by transfer of 10 μL into 200 μL of 7H9 medium in a separate 96-well plate for 10−14 days of outgrowth with 20% O_2 and 5% CO_2. R assay allowed bacterial division for 7−10 days with 20% O_2 and 5% CO_2. OD_{580} was the read out. IC_{90} was defined as the concentration that led to growth inhibition ≥90%.

Molecular Clustering. Molecules were clustered by applying an in-house complete linkage hierarchical algorithm using Chemaxon (www.chemaxon.com) topological fingerprints with Tanimoto similarities.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsinfecdis.5b00025.

Supplementary methods, figures, and tables (PDF)

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Notes

The authors declare no competing financial interest.

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