Identification of Compounds with pH-Dependent Bactericidal Activity against *Mycobacterium tuberculosis*

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ABSTRACT: To find new inhibitors of *Mycobacterium tuberculosis* that have novel mechanisms of action, we miniaturized a high throughput screen to identify compounds that disrupt pH homeostasis. We adapted and validated a 384-well format assay to determine intrabacterial pH using a ratiometric green fluorescent protein. We screened 89000 small molecules under nonreplicating conditions and confirmed 556 hits that reduced intrabacterial pH (below pH 6.5). We selected five compounds that disrupt intrabacterial pH homeostasis and also showed some activity against nonreplicating bacteria in a 4-stress model, but with no (or greatly reduced) activity against replicating bacteria. The compounds selected were two benzamide sulfonamides, a benzothiadiazole, a bissulfone, and a thiadiazole, none of which are known antibacterial agents. All of these five compounds demonstrated bactericidal activity against nonreplicating bacteria in buffer. Four of the five compounds demonstrated increased activity under low pH conditions. None of the five compounds acted as ionophores or as general disrupters of membrane potential. These compounds are useful starting points for work to elucidate their mechanism of action and their utility for drug discovery.

KEYWORDS: *Mycobacterium tuberculosis*, antibacterial, bactericidal, drug discovery, pH homeostasis, phenotypic screen

**T**uberculosis (TB), caused by *Mycobacterium tuberculosis*, is the leading cause of death among infectious diseases. Treatment for tuberculosis is 6–9 months long for drug sensitive strains and even longer for resistant strains. Given the long treatment time and prevalence of the disease, there is urgent need for new drugs.

*M. tuberculosis* is an intracellular pathogen that can replicate in macrophages and persist within the lung for long periods. *M. tuberculosis* has numerous mechanisms by which it can evade the immune system, including its ability to inhibit fusion of phagosomes and lysosomes, and avoid antimicrobial activities such as acidification of the compartment it occupies. *M. tuberculosis* can survive and replicate within resting macrophages. However, activated macrophages can mount an effective response to *M. tuberculosis* infection, and if acidification of the compartment occupied by *M. tuberculosis* occurs, bacterial killing is observed. The pH of phagosomes occupied by *M. tuberculosis* ranges from 4.5 to 6.2, which is dependent on the state of activation of the macrophage. The acidic microenvironment itself may or may not be bactericidal to *M. tuberculosis* but can make *M. tuberculosis* more vulnerable to other stresses.

As a result, acidification of the microenvironment is a mechanism for inducing bacterial death. Thus, the ability of *M. tuberculosis* to maintain its internal pH within narrow parameters is critical for survival in macrophages.

In the host environment, *M. tuberculosis* must be able to sense external pH and to regulate its internal pH to maintain metabolic activity and viability. *M. tuberculosis* mutants defective in blocking acidification, for instance, by deletion of the periplasmic serine protease Rv3671c (MarP), are unable to survive acid stress in vitro and are attenuated in mouse models of infection. Survival in low pH is partly dependent on cell density, a phenomenon attributed to cell to cell signaling in other bacteria, and are attenuated in mouse models of infection. Survival in low pH is partly dependent on cell density, a phenomenon attributed to cell to cell signaling in other bacteria, and are attenuated in mouse models of infection. In cell wall synthesis, *M. tuberculosis* survival under low pH, Genes that are induced when the external pH in culture drops rapidly are up-regulated in human and...
mouse infected lungs and in intracellular bacteria. Thus, pH sensing and homeostasis are important processes for M. tuberculosis survival in its host. Regulatory mechanisms such as PhoPR and the transcription factor WhiB3 are involved in the response to low pH, since they control the pH-mediated expression of genes including lipF and the apr locus. In addition adenyl cyclases may sense external pH changes. Further evidence suggests that pH homeostasis is a viable drug target, as both nitazoxanide and the imidazopyridine series disrupt intrabacterial pH (pHIB), as do the benzoxaninones targeting MarP, and pyrazinamide, a compound that is active against M. tuberculosis under acidic conditions through an unclear mechanism of action, is a critical part of the TB treatment regimen.

We are interested in developing antitubercular agents with novel mechanisms and targets. We focused on an approach to find compounds that work at low pH and might not be otherwise found in a standard screening campaign. Our screening approach was to identify compounds that disrupt the ability of M. tuberculosis to maintain its internal pH in an acidic environment using a fluorescent reporter protein that can monitor pH. We reasoned compounds disrupting pH homeostasis would have bactericidal properties under acidic conditions.

**RESULTS**

**Assay Development and Validation.** We adapted a medium throughput screen (96-well format) that uses a pH-sensitive fluorescent reporter or pHLUOR (rGFP). This approach was previously used to identify compounds that disrupt pH homeostasis from a small library of 1980 natural products. We wanted to increase the throughput of the screen to enable us to screen tens of thousands of compounds, so we adapted and miniaturized the assay to 384-well format.

We generated a recombinant strain of M. tuberculosis H37Rv expressing a ratiometric GFP (rGFP) using the same vector as previously described. The pHLUOR demonstrates a pH-dependent excitation wavelength; we confirmed that we could generate a similar calibration curve to determine pH from the ratio of the fluorescence of Ex395/Em510 and Ex475/Em510 using M. tuberculosis lysates in buffered solutions (Figure 1A). These data confirmed that the rGFP was working as expected and the standard curve was used to derive intrabacterial pH (pHIB) for all future work. The standard curve and line of best fit were confirmed with a second set of lysates at the end of the screen.

We selected similar conditions to the published screen using 0.15 M phosphate citrate buffer, pH 4.5, plus tyloxapol; we used the ionophore monensin as a control. After optimization for higher throughput, our final assay parameters were as follows: 25 μL of late logarithmic phase M. tuberculosis in phosphate citrate buffer at an OD590 of 0.6; 24 μL of phosphate citrate buffer, pH 4.5; 1 μL of compound; Greiner black 384-well plates with a clear bottom; 48 h incubation with compound at 37 °C. We validated the robustness of the assay according to NCGC guidelines by performing a reproducibility test. We ran duplicate plates for maximum signal (DMSO only), minimum signal (0.5 μM monensin), and midpoint signal (0.5 μM monensin) on three separate days (Figure 1B). We used the standard curve to convert the ratio of the two fluorescence readings into pH. The average pH was 6.45 ± 0.07 for 5 μM monensin, 6.56 ± 0.05 for 0.5 μM monensin, and 6.88 ± 0.03 for DMSO. The assay passed reproducibility testing for both intraexperiment and interexperiment variability measures; % CV was <20%, signal/background and signal/...
noise were >5, and the Z’ of controls for plates was >0.5 (Figure 1B).

Once we had validated the screen for reproducibility, we tested several reference compounds (Table 1). None of the compounds we tested were active, with the exception of carbonyl cyanide 3-chlorophenylhydrazone (CCCP), a known proton ionophore and pyrazinamide (PZA). We defined the minimum active concentration 6.5 (MAC₆₅) as the minimum concentration required to decrease M. tuberculosis pH < 6.5. CCCP was most effective, with a MAC₆₅ of 0.5 μM. PZA had activity but only at higher concentrations, with a MAC₆₅ of 69 μM.

Table 1. Reference Compounds

| compound                                      | MAC₆₅ (μM) |
|----------------------------------------------|-----------|
| carbonyl cyanide 3-chlorophenylhydrazone      | 0.5 ± 0.0 |
| metronidazole                                | >100      |
| pyrazinamidide                               | 69 ± 26   |
| ethambutol                                   | >100      |
| rifampicin                                   | >100      |
| ofloxacin                                    | >100      |
| n-cycloserine                                | >100      |
| kanamycin                                    | >100      |
| isoniazid                                    | >100      |
| moxifloxacin                                 | >100      |

“M. tuberculosis expressing the pH-sensitive green fluorescent protein was incubated with compounds in phosphocitrate buffer at pH 4.5 for 48 h. Fluorescence was measured at excitation/emission 395/510 and 475/510, and the ratio was converted into pH using the standard curve from Figure 1. MAC₆₅ is the concentration required to reduce the intrabacterial pH < 6.5. The assay was repeated at least twice; results are average ± standard deviation.

High Throughput Screen. Once we had validated the assay, we selected a diverse compound set to screen. We selected the screening concentration using three plates from the library selected at random. We tested each plate at 5 μM, 10 μM, and 20 μM. We defined compounds as active if they reduced the pH below 6.5. Out of 949 compounds tested at 5 μM, only 1 compound was active, while 2 compounds were active at 10 μM, and 6 compounds were active at 20 μM (data not shown). We selected 20 μM as the test concentration for the screen, since it had the highest hit rate. We screened a set of 89,273 compounds from the Eli Lilly corporate collection, which were selected to represent the chemical diversity in the entire library >800,000 compounds (using methods proprietary to Lilly). We screened the compounds at a fixed concentration of 20 μM (Figure 1C). The Z’ for each plate was determined, which ranged from 0.53 to 0.8 (average 0.77). We identified 605 hits that reduced pH < 6.5, making a hit rate of 0.7%.

Confirmation of Hit Activity. We confirmed the activity of our hit compounds. Compounds were resupplied from Eli Lilly and tested as a 10-point serial dilution series. We calculated the MAC₆₅ for each compound using a starting concentration of 200 μM. We confirmed 556 of the 605 hits, a confirmation rate of 92%.

Activity of Compounds against Replicating and Nonreplicating M. tuberculosis. In order to select the best compounds for further work, we determined which compounds had activity in other antitubercular assays. We determined the minimum inhibitory concentration (MIC) against replicating bacteria grown under standard aerobic conditions, as well as against nonreplicating bacteria generated using a multitarget assay. We were particularly interested in finding molecules with activity against nonreplicating bacteria, which can be hard to target. Forty-eight compounds were

Table 2. Key Hit Compounds

| Structure | Compound ID | MAC₆₅ | Non-replicating IC₅₀ | Replicating IC₅₀ | MIC at pH 5.5 | MIC at pH 6.7 | Cytotoxicity HepG2 IC₅₀ |
|-----------|-------------|-------|-----------------------|------------------|---------------|---------------|------------------------|
| IDR-020850| 2           | 93 ± 10 | >100                  | 35 ± 4           | >200          | 19 ± 2.9      |                         |
| IDR-0054790| 1           | 98 ± 3.5 | >100                  | 98 ± 4           | >200          | 46 ± 6.8      |                         |
| IDR-009118| 8           | 59 ± 1.4 | >100                  | 115 ± 15         | >200          | 82 ± 22.5     |                         |
| IDR-0040669| 2           | 3.0 ± 2.6 | 100                  | 13 ± 2           | 50 ± 4        | 9.1 ± 1.5     |                         |
| IDR-0081053| 2           | 34 ± 13 | >100                  | 28 ± 3           | 50 ± 8        | 44 ± 3.4      |                         |

“Compound activity was measured against M. tuberculosis under different conditions. Data are the average ± standard deviation for at least two independent experiments, except MAC₆₅ was determined from a single experiment.
active in nonreplicating conditions. From this set, compounds
were identified and grouped into structural classes for further
study, and five compounds with a range of activity from 1.4 to
100 μM against nonreplicating bacteria were selected for
further study (Table 2). Compounds IDR-0020850 and IDR-
0054790 are benzamide sulfonamides, where IDR-0020850 has
a benzothiazole substitution on the amide. Compound IDR-
0099118 is a similar benzothiazidine compound. Compound
IDR-0040699 is a bissulfone. Compound IDR-0081053 is a
thiadiazole. None of the described actives are known
antibacterial agents. Other compounds with similar in vitro
profiles showed diverse structural features different from
compounds discussed here and are currently under evaluation
separately.

Activity of compounds against M. tuberculosis at low
dH. We selected five compounds for further study based on
their properties in vitro (Table 2); in particular, these five
confirmed activity from the screen by exhibiting a MAC6.5 less
than 10 μM, showed activity against M. tuberculosis in the
multistress model, and were less effective against aerobically
grown, actively replicating bacteria. Four of the compounds
had no activity against replicating bacilli, and one compound
had activity, but was 50-fold less active (Table 2). The
multistress model uses several conditions relevant to the
infection setting, namely, an acidic pH of 5.0, low oxygen, low
nutrient, and nitric oxide, all of which induce the non-
replicating state.41 In order to determine if pH was a
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infection setting, namely, an acidic pH of 5.0, low oxygen, low
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nutrient, and nitric oxide, all of which induce the non-
replicating state.41 In order to determine if pH was a

| compound | calcium ionophore EC50 (μM) | membrane potential (μM) |
|----------|--------------------------|------------------------|
| IDR-0020850 | 100                      | 100                    |
| IDR-0054790 | 100                      | 100                    |
| IDR-0099118 | 100                      | 100                    |
| IDR-0040699 | 100                      | 100                    |
| IDR-0081053 | 100                      | 100                    |
| A23187  | 0.15 ± 0.008              | 100                    |
| amphotericin | 100              | 0.62 ± 0.1311          |

HEK-293 cells were seeded in 384-well plate with compounds
prepared as serial dilutions. Calcium ionophore activity was measured
using calcium dye and FLIPR reader; A23187 was the positive
control. Disruption of membrane potential was measured using the
membrane potential red dye; amphotericin B was the positive control.
EC50 is the concentration required to effect 50% of the maximal
response. Results are the average ± standard deviation from 2
independent runs.

Bactericidal activity against M. tuberculosis. We
demonstrated that compounds disrupted pH homeostasis and were
active in vitro in short-term assays (up to 7 days)
dependent on bacterial growth. Our next step was to determine
if this activity translated into effective bactericidal activity over
the course of 21 days. We determined the kill kinetics of the
compounds against nonreplicating M. tuberculosis under the 2 conditions. PZA
was not active in either condition, up to 200 μM. We also
determined if these compounds were cytotoxic to mammalian
cells, and all of them were more potent in the assay that
determined the MAC6.5 than they were in the cytotoxicity
assay.

Lack of Nonspecific Activity. One potential mechanism
for increased activity at low pH would be if the compounds
exhibited ionophore/protonophore activity or were general
membrane disruptors or pore formers, similar to monensin. We
tested the five compounds in a number of assays to exclude this
possibility. We tested for ionophore activity, using HEK-293
cells and calcium, as well as for disruption of membrane
potential in HEK-293 cells (Table 3). None of the compounds
had activity, suggesting they are not calcium ionophores and
their mode of action does not involve disrupting membrane
potential in mammalian cells (Table 3).

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dependent on bacterial growth. Our next step was to determine
if this activity translated into effective bactericidal activity over
the course of 21 days. We determined the kill kinetics of the
compounds against nonreplicating M. tuberculosis at near-neutral
(pH 6.8) and acidic (pH 4.5) conditions (Figure 2).
Surprisingly, all the compounds showed activity against bacilli in
buffer pH 6.8, despite having no activity against replicating
bacteria, suggesting their bactericidal activity may be specific
for nonreplicating organisms. In some cases this activity was
pronounced; for example, compound IDR-0040669 was rapidly
bactericidal, resulting in sterilization after 3 days at a
concentration as low as 10 μM (Figure 2B). In contrast, 3
compounds, IDR-0020850, IDR-0054790, and IDR-0099118,
had a slower kill that appeared to be concentration dependent.
Composé IDR-0081053 was effective at sterilizing cultures in a
concentration-dependent fashion but only at the highest
consentration (7 days for 100 μM, 14 days for 50 μM, and 21
days for 25 μM).

All compounds were more effective at killing under low pH,
except IDR-0040669 (Figure 2). Compounds IDR-0020850
and IDR-0054790 were slightly better at low pH, whereas
compounds IDR-0099118 and IDR-0081053 were markedly
more efficient at killing M. tuberculosis under acidic conditions.
Thus, we confirmed that 4 of the 5 compounds had pH-
dependent bactericidal activity. In these experiments, we also
saw that RIF and INH were slightly more active in the acidic
conditions (Figure 2C). This has not been noted before, but we
see this reproducibly, suggesting that mechanisms other
than disrupting pH homeostasis could also lead to the
identification of compounds with condition-dependent, or
condition-enhanced activity. Since INH inhibits mycolic acid
production and therefore cell wall integrity, and inhibition of
transcription can prevent an adaptive transcriptional response,
these factors may contribute to enhanced activity at low pH.
PZA was inactive at the concentrations we tested in both
conditions. This is consistent with previous reports, which
suggest that even at low pH, the effective concentration of PZA
is very high (>500 μM). To rule out any strain-dependent
effects, we confirmed that compounds 1–5 were similarly
active against the Erdman strain under low pH (data not
shown).

Compound Stability at Low pH. We confirmed the
purity of the five compounds by LCMS as >95%. We
determined the stability of compounds in buffer at pH 4.5 or
pH 6.8 over 5 days. Four of the compounds were stable in
buffers at both pH values (50% remaining after 5 days), while
compound IDR-0054790 appeared to degrade to an unknown
species in the low pH buffer (<20% at pH 6.8 and no
detectable parental compound at pH 4.5 at day 5).
Figure 2. continued
We performed a high throughput screen using a pH-sensing strain of *M. tuberculosis* to identify compounds that interrupt the pH homeostasis. From our hits, we focused on compounds that were active against nonreplicating *M. tuberculosis* and might therefore be able to contribute to shortening the duration of therapy. Although this screen was tested against live cells of *M. tuberculosis*, one major limitation was that activity from the screen might not translate into the ability to kill bacteria. We were able to find compounds with bactericidal activity, which in 4/5 cases was pH-dependent, confirming the validity of the approach. We used a cutoff for hits in our screen of pH 6.5 as previously described, although it is not known how low the pH needs to drop, or for how long, to result in death. In *Mycobacterium bovis* BCG or *Mycobacterium smegmatis*, a reduction to pH 6 by CCCP had a lethal effect.

Figure 2. Kill kinetics. Compounds were tested for their ability to kill *M. tuberculosis* in phosphate citrate buffer at pH 6.8 (left panels) and pH 4.5 buffer (right panels): (A, B) test compounds; (C) control compounds. Data are the average and standard deviation from two independent experiments.
We demonstrate here that a reduction in *M. tuberculosis* to pH 6.5 is sufficient, since compounds had bactericidal activity.

The five compounds we selected were more active against *M. tuberculosis* at low pH. Four of the compounds were more active against replicating *M. tuberculosis* in the acidic condition compared to neutral pH. Two compounds were more active against nonreplicating *M. tuberculosis* in the acidic condition compared to the neutral condition. This increased activity at low pH could be partially due to *M. tuberculosis* being more generally vulnerable in the low pH condition. However, the increase in susceptibility was more marked for these compounds than for reference compounds. Further study regarding mechanism of action of these five compounds would shed light on why some compounds are pH-dependent under replicating conditions and others are pH-dependent under nonreplicating conditions.

In summary, we screened a set of ~89,000 compounds, identified compounds that interrupt pH homeostasis, and confirmed 556 hits. Five compounds were selected and demonstrated pH-dependent bactericidal activity, confirming the utility of the screen. Further work to elucidate their mechanism of action and their utility as starting points for drug discovery is warranted.

### MATERIALS AND METHODS

**Growth of *M. tuberculosis***. *M. tuberculosis H37Rv* was cultivated in Middlebrook 7H9 (Difco) medium supplemented with 0.05% v/v Tween 80 and 10% v/v oleic acid, albumin, dextrose, and catalase (OADC; Becton Dickinson) (7H9-Tw-OADC). Recombinant *M. tuberculosis* expressing a codon-optimized pH-sensitive green fluorescent protein (GFP) under the control of a constitutively expressed promoter was constructed in H37Rv and cultured with hygromycin B (Hyg) at 50 μg/mL.11,16

**Generation of rGFP Standard Curve.** *M. tuberculosis* expressing a codon-optimized pH-sensitive green fluorescent protein (rGFP) was grown to late logarithmic phase (OD₅₉₀ ≈ 1), washed, resuspended in phosphate buffered saline (PBS), and lysed using a Fastprep instrument (MP Biomedicals). Cell-free extracts were recovered and diluted to 10 μg/μL total protein in phosphate citrate buffers adjusted to pH 5.5–8.5 in increments of 0.5 units. Fluorescence was measured at excitation/emission 395/510 and 475/510, and the ratio was calculated. The ratio was plotted versus the pH to generate a standard curve, and a three parameter nonlinear regression was calculated. The ratio was plotted versus the pH to generate a standard curve, and the concentration at which the pH response curves were plotted using four-parameter logistic nonlinear regression, and the concentration at which the pH reached 6.5 was calculated and recorded as the minimum active concentration 6.5 (MAC₆₅₅).

**Ionophore and Membrane Potential Assays.** HEK-293 cells (ATCC CRL-1573) were seeded at 15,000 cells/well onto poly(ethylene) coated black/clear bottom 384-well plates in 25 μL of minimum essential medium, 10% FBS, 100 IU/mL penicillin, 100 μg/mL streptomycin, and 2 mM Glutamax. Compounds were diluted in DMSO and tested as 10-point, 3-fold serially diluted samples starting at 100 μM. For the calcium ionophore assay, 25 μL of calcium 5 dye (Molecular Devices) was suspended in Hank's Balanced Salt Solution (HBSS) with 20 mM HEPES, pH 7.4, and added to 384-well cell plates containing HEK-293 cells, then incubated for 60 min at 37 °C and 5% CO₂. Compounds were then added to the dye-loaded cell plates. Using a FLIPR Tetra plate reader (Molecular Devices), fluorescent signal (excitation 470–490 nm; emission 515–575 nm) was detected at 1 s intervals for 300 s. Emission maximum results, a measure of intracellular calcium, were plotted using XLfit software (IDBS, Inc.). Calcium ionophore A23187 (Sigma) was used as a positive control.

**Replicating and Nonreplicating Minimum Inhibitory Concentration (MIC) Determination.** Compounds were tested in nonreplicating and replicating conditions against the mc²6220 strain as previously described.40,41 Briefly, logarithmically replicating *M. tuberculosis* was exposed to compound in a modified Sauton's medium at pH 5.5 in 1% O₂ and 5% CO₂ with 0.05% μM butyrate in the presence of 0.5 mM nitrite for 6–7 days, then diluted 5-fold and outgrown in 7H9 with glucose and glycerol at pH 6.6 in 21% O₂ and 5% CO₂ for 7–10 days. For replicating data, compounds were tested as described in the outgrowth step.

**MIC Determination at pH 5.5 & pH 6.8.** Middlebrook 7H9-OADC medium with 0.05% tyloxapol was adjusted to pH 5.5 using hydrochloric acid and filter sterilized. To allow sufficient growth at pH 5.5, the starting OD₅₉₀ was 0.04 and plates were incubated for 6 days, while the starting OD₅₉₀ was 0.02 and plates were incubated for 5 days for samples at pH 6.8. MICs were determined by measuring growth by OD₅₉₀ and three parameter nonlinear fit.

**Kill Kinetics.** Late logarithmic phase H37Rv (OD₅₀₀ 0.6–1.0) was harvested and resuspended in phosphate citrate buffer plus 0.05% tyloxapol. Cultures were inoculated to ~10⁶ CFU/mL, compounds were added (final concentration 2% DMSO), and cultures were incubated standing at 37 °C. Aliquots were plated for CFU every 3 days. Plates were incubated at 37 °C for 4 weeks before counting.

**Compound Stability.** Compounds were diluted to 20 μM in phosphate citrate buffer with tyloxapol at pH 4.5 or pH 6.8 and incubated at 37 °C. Samples were taken and injected into an Agilent 1100 HPLC system using the following Phenomenex Gemini C18 column, 3 mm × 50 mm; flow rate 0.45 mL/min; gradient 5–95% acetonitrile in water over 8 min with all solvents containing 0.05% formic acid. UV

**Dose Response.** Compounds were tested as 10-point, 3-fold serial dilutions, typically starting at 200 μM. Compound response curves were plotted using four-parameter logistic nonlinear regression, and the concentration at which the pH reached 6.5 was calculated and recorded as the minimum active concentration 6.5 (MAC₆₅₅).
detection was monitored at 214 and 254 nm using an Agilent diode array detector (G1315B). Mass spectroscopy data was generated via electrospray ionization in positive mode using an Agilent LC/MSD (G1956B). The area under the UV peaks was determined using Agilent ChemStation software (B.01.03-SR2) to estimate % parent remaining.

**Cytotoxicity.** HepG2 human liver cells (ATCC) were seeded in DMEM, 10% FBS, 1 mM sodium pyruvate, 2 mM Glutagro (Corning), 100 IU/mL penicillin, and 100 μg/mL streptomycin containing 25 mM glucose and incubated in 37 °C, 5% CO₂. Three-fold serially diluted compounds were added to the cells 24 h post cell seeding to 1% DMSO final concentration, and cells were incubated for 72 h. Next, CellTitler-Glo was added, and RLU were measured using a Synergy4 plate reader. Raw data were normalized by the average RLU value from 1% DMSO treated wells and expressed as % growth. Growth inhibition was correlated and fitted using the Levenberg–Marquardt algorithm, and the concentration that produced 50% of the growth inhibitory response was reported as the IC₅₀.

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**Notes**

The authors declare no competing financial interest.

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### ABBREVIATIONS

rGFP, ratioometric green fluorescent reporter protein; Ex, excitation; Em, emission; pHᵢ₅₀, intrabacterial pH; NCGC, NCATS Chemical Genomics Center; DMSO, dimethyl sulfoxide; CV, coefficient of variation; CCCP, 3-chlorophenylhydrazone; MAC₆.₅, minimum concentration required to decrease M. tuberculosis pHᵢ₅₀ < 6.5; PZA, pyrazinamide; MIC, minimum inhibitory concentration; Rif, rifampicin; INH, isoniazid; LCMS, liquid chromatography–mass spectrometry; OADC, oleic acid, albumin, dextrose, and catalase; Hyg, hygromycin B; PBS, phosphate buffered saline; FBS, fetal bovine serum; HBSS, Hank’s Balanced Salt Solution; CFU, colony forming units; UV, ultraviolet; RLU, relative light units.

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