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Article

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

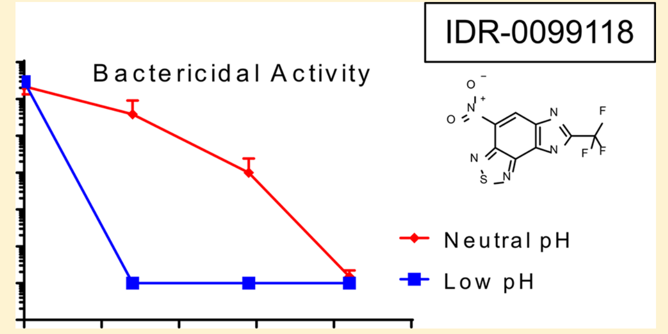
Julie Early,[†] Juliane Ollinger,[†] Crystal Darby,[‡] Torey Alling,[†] Steven Mullen,[†] Allen Casey,[†] Ben Gold,[‡] Jason Ochoada,[§,∥] Todd Wiernicki,[§] Thierry Masquelin,[§] Carl Nathan,[‡] Philip A. Hipskind,[§,⊥] and Tanya Parish*[,†]

[†]TB Discovery Research, Infectious Disease Research Institute, 1616 Eastlake Avenue E, Suite 400, Seattle, Washington 98102, United States

[‡]Department of Microbiology and Immunology, Weill Cornell Medical College, 1300 York Avenue, Box 62, New York, New York 10065, United States

[§]Lilly Research Laboratories, Eli Lilly and Company, 307 E Merrill Street, Indianapolis, Indiana 46285, United States

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*

Tuberculosis (TB), caused by *Mycobacterium tuberculosis*, is the leading cause of death among infectious diseases.[1] Treatment for tuberculosis is 6−9 months long for drug sensitive strains and even longer for resistant strains.[1] 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.[2] *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.[3,4] *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.[5] 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.[6−11] The acidic microenvironment itself may or may not be bactericidal to *M. tuberculosis* but can make *M. tuberculosis* more vulnerable to other stresses.[12−15] 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),[16,17] are unable to survive acid stress *in vitro* and are attenuated in mouse models of infection.[18−20] Survival in low pH is partly dependent on cell density, a phenomenon attributed to cell to cell signaling in other bacteria.[15,20,21] OmpA, an outer membrane protein,[22] and Ppm1 and PonA, both involved in cell wall synthesis,[15] are also involved in *M. tuberculosis* survival under low pH. Genes that are induced when the external pH in culture drops rapidly[23] are also up-regulated in human and

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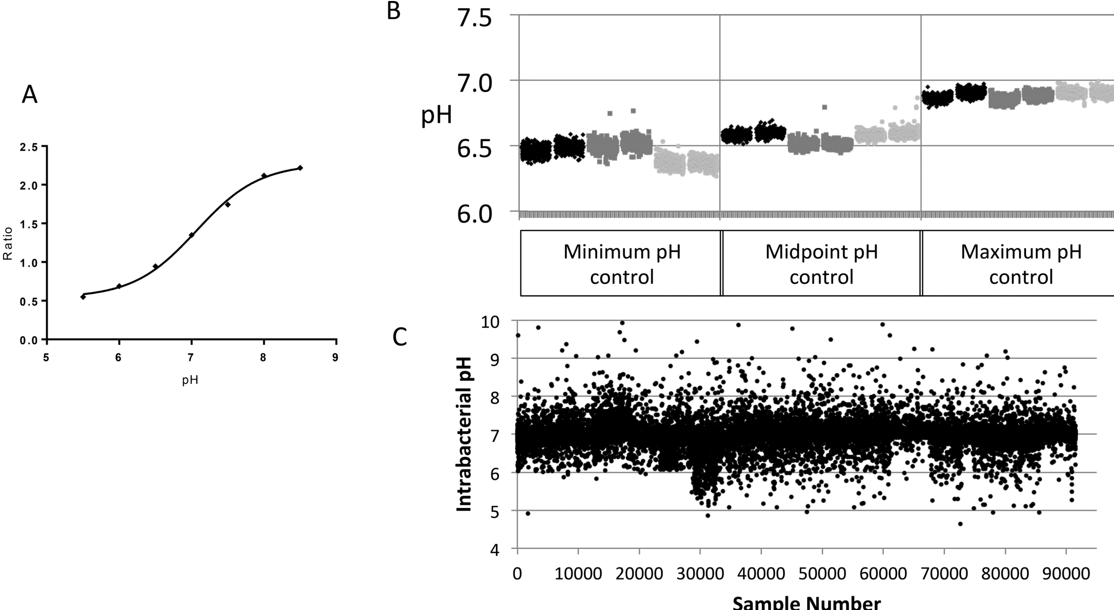

Figure 1. High throughput screen. (A) Lysates were generated from *M. tuberculosis* expressing pH-sensitive green fluorescent protein in buffered solution. Fluorescence was measured at excitation/emission 400/516 nm and 485/516 nm, and the ratio was calculated. Data are average and standard deviation from 2 independent experiments. The line of best fit using the least-squares method was derived to generate a standard curve. (B) Duplicate plates for minimum signal (5 μM monensin), midpoint signal (0.5 μM monensin), and maximum signal (DMSO) were tested on 3 different days using *M. tuberculosis* incubated in phosphocitrate buffer at pH 4.5 for 48 h. Day 1, black; day 2, dark gray; day 3, light gray. The Z' was >0.5 for each plate. (C) For each compound, the intrabacterial pH was calculated based on the standard curve in panel A; compounds that dropped the pH < 6.5 were considered active.

mouse infected lungs and in intracellular bacteria.[24−27] Thus, pH sensing and homeostasis are important processes for *M. tuberculosis* survival in its host. Regulatory mechanisms such as PhoPR[28,29] and the transcription factor WhiB3 are involved in the response to low pH,[30] since they control the pH-mediated expression of genes including *lipF* and the *apr* locus.[31] In addition adenylyl cyclases may sense external pH changes.[32,33] Further evidence suggests that pH homeostasis is a viable drug target, as both nitazoxanide and the imidazopyridine series disrupt intrabacterial pH (pH_{IB}),[34,35] as do the benzoxaninones targeting MarP,[18]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.[36]

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).[37] This approach was previously used to identify compounds that disrupt pH homeostasis from a small library of 1980 natural products.[37] 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.[37] 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 (pH_{IB}) 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.[38] 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 OD_{590} 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 (5 μM monensin), and midpoint signal (0.5 μM monensin) on three separate days[39] (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/

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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 ($MAC_{6.5}$) as the minimum concentration required to decrease *M. tuberculosis* $pH_{IB} < 6.5$. CCCP was most effective, with a $MAC_{6.5}$ of 0.5 μM. PZA had activity but only at higher concentrations, with a $MAC_{6.5}$ of 69 μM.

Table 1. Reference Compounds[a]

compound	$MAC_{6.5}$ (μM)
carbonyl cyanide 3-chlorophenylhydrazone	0.5 ± 0.0
metronidazole	>100
pyrazinamide	69 ± 26
ethambutol	>100
rifampicin	>100
ofloxacin	>100
D-cycloserine	>100
kanamycin	>100
isoniazid	>100
moxifloxacin	>100

[a]*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. $MAC_{6.5}$ 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.[37] 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 of >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 $pH_{IB} < 6.5$, 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 $MAC_{6.5}$ 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 multistress assay.[40] 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[a]

Structure	Compound ID	MAC6.5	Non-replicating IC_{90} (μM)	Replicating IC_{90} (μM)	MIC at pH 5.5 (μM)	MIC at pH 6.7 (μM)	Cytotoxicity HepG2 IC_{50} (μM)
	IDR-0020850	2	93 ± 10	>100	35 ± 4	>200	19 ± 2.9
	IDR-0054790	1	98 ± 3.5	>100	96 ± 4	>200	46 ± 6.8
	IDR-0099118	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
	RIF	See Table 1	N/D	0.0030 ± 0.001	0.0040 ± 0.001	0.0030 ± 0.001	>100
	INH	See Table 1	N/D	0.35 ± 0.09	0.55 ± 0.20	0.35 ± 0.09	>100
	PZA	See Table 1	N/D	>200	>200	>200	>100

[a]Compound activity was measured against *M. tuberculosis* under different conditions. Data are the average ± standard deviation for at least two independent experiments, except $MAC_{6.5}$ was determined from a single experiment.

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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 benzothiadiazole compound. Compound IDR-0040669 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 pH. 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 $MAC_{6.5}$ 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 nonreplicating state.[41] In order to determine if pH was a determining factor for compound activity, we determined the MIC against replicating bacteria at pH 5.5 and pH 6.7. We selected pH 5.5, since it is the lowest pH at which we were able to measure reproducible growth. All five compounds showed activity at pH 5.5 (Table 2). Two of the compounds had activity at pH 6.7, but to a lesser degree; three of the compounds had no activity at pH 6.7, confirming that they have pH-dependent activity against *M. tuberculosis*. The reference compounds RIF and INH were roughly equipotent against replicating *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 $MAC_{6.5}$ 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 disrupters 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).

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 five 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. Compound IDR-0081053 was effective at sterilizing cultures in a concentration-dependent fashion but only at the highest concentrations (7 days for 100 μM, 14 days for 50 μM, and 21 days for 25 μM).

Table 3. Ionophore and Membrane Potential Testing[a]

compound	calcium ionophore EC_{50} (μM)	membrane potential (μM)
IDR-0020850	>100	>100
IDR-0054790	>100	>100
IDR-0099118	>100	>100
IDR-0040669	>100	>100
IDR-0081053	>100	>100
A23187	0.15 ± 0.008	>100
amphotericin	>100	0.62 ± 0.1311

[a]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. EC_{50} is the concentration required to effect 50% of the maximal response. Results are the average ± standard deviation from 2 independent runs.

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).

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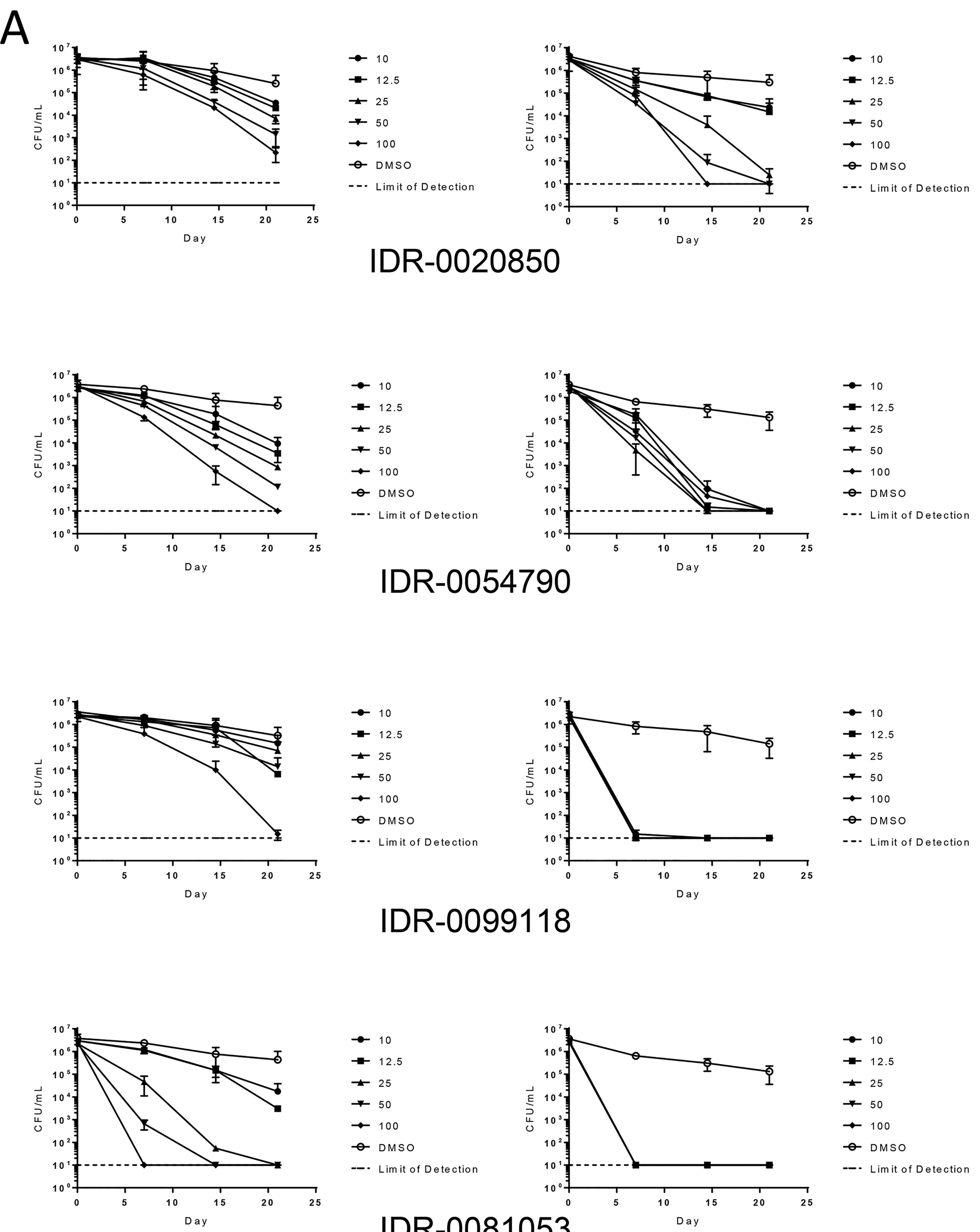

Figure 2. continued

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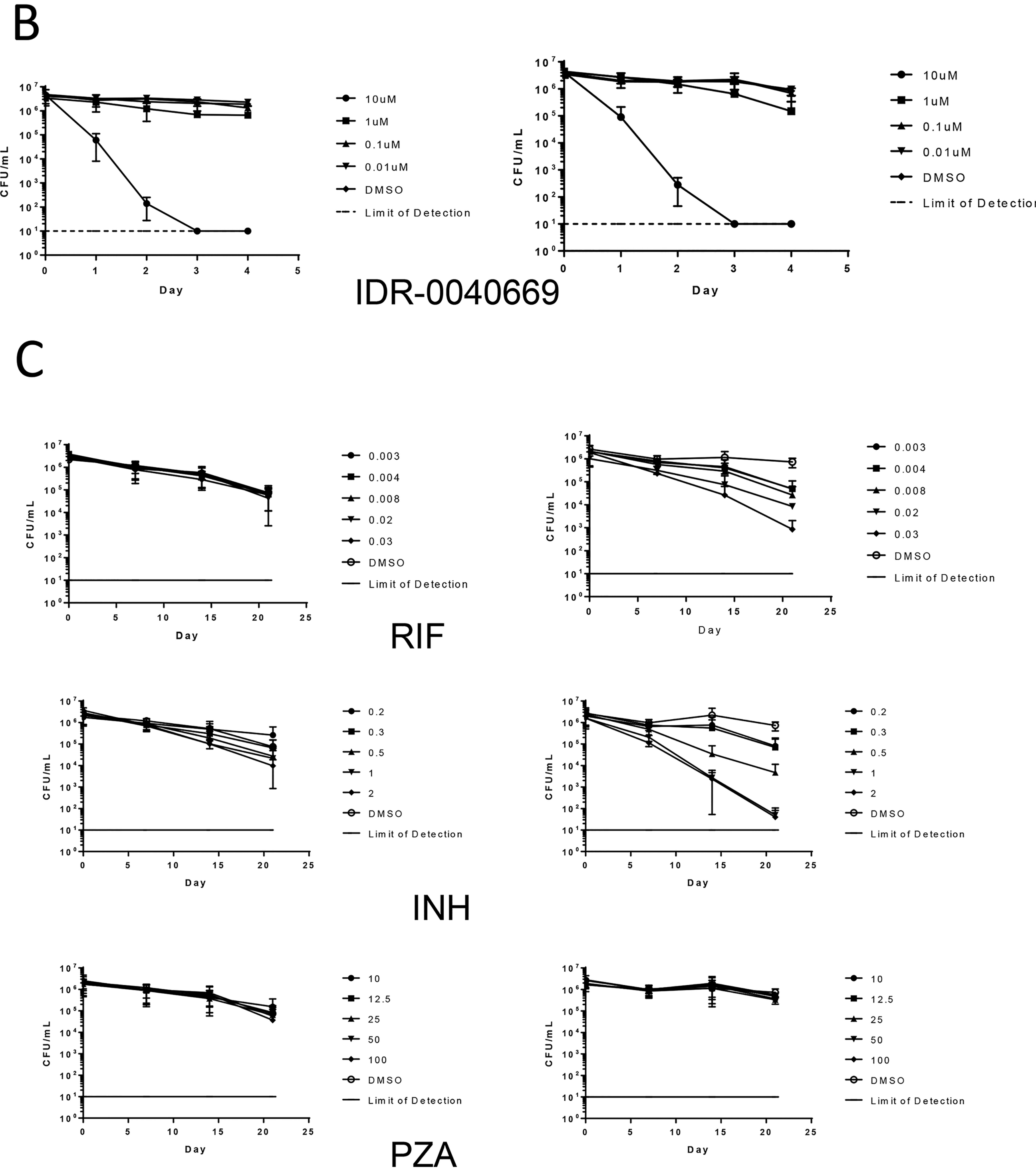

Figure 2. Kill kinetics. Compounds were tested for their ability to kill *M. tuberculosis* in phosphate citrate buffer at pH 6.8 (left panels) and pH 4.5 buffer (right panels): (A, B) test compounds; (C) control compounds. Data are the average and standard deviation from two independent experiments.

■ DISCUSSION

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,[37] although it is not known how low the pH_{IB} 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.[42]

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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 cultured in Middlebrook 7H9 (Difco) medium supplemented with 0.05% w/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 ($OD_{590} \approx 1$), 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 fit line was generated.

Screen. *M. tuberculosis* was cultured in 7H9-Tw-OADC-Hyg to late logarithmic phase, washed, and resuspended in phosphate citrate buffer, pH 4.5 (0.0896 M Na_2HPO_4, 0.0552 M citric acid) plus 0.05% v/v tyloxapol to an OD of 0.6. To prepare assay plates, 24 μL of buffer was dispensed into sterile black, clear bottom 384-well plates (Greiner). Controls or compounds were added as follows: 1 μL of 250 μM monensin (Sigma-Aldrich) in column 1; 1 μL of 25 μM monensin in column 23; 1 μL of DMSO in columns; and 1 μL of 40 μM test compound in columns 3−22. An additional 25 μL of buffer was dispensed in column 24. Plates were inoculated with 25 μL of culture in columns 1−23 using a Multidrop Combi (Thermo Scientific). Plates were incubated for 2 days in a humidified incubator at 37 °C. The following QC criteria had to be satisfied for each plate and each run: % CV of maximum acidification control <20, % CV of minimum acidification control <20, % CV of background <20, and Z' of controls >0.5.

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 ($MAC_{6.5}$).

Ionophore and Membrane Potential Assays. HEK-293 cells (ATCC CRL-1573) were seeded at 15000 cells/well onto poly(D-lysine) 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% CO_2. 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. The membrane potential assay was carried out as described above for the calcium ionophore assay except membrane potential red dye R8126 (Molecular Devices) was used and detected at excitation 510 nm and emission 565−625 nm to indicate voltage depolarization changes across the cell. Amphotericin B (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 mc^{2}6220 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% O_2 and 5% CO_2 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% O_2 and 5% CO_2 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 OD_{590} was 0.04 and plates were incubated for 6 days, while the starting OD_{590} was 0.02 and plates were incubated for 5 days for samples at pH 6.8. MICs were determined by measuring growth by OD_{590} and three parameter nonlinear fit.

Kill Kinetics. Late logarithmic phase H37Rv (OD_{590} 0.6−1.0) was harvested and resuspended in phosphate citrate buffer plus 0.05% tyloxapol. Cultures were inoculated to ~10^6 CFU/mL, compounds were added (final concentration 2% DMSO), and cultures were incubated standing at 37 °C. Aliquots were plated for CFU every 7 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

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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% CO_2. 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, CellTiter-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 curves were fitted using the Levenberg–Marquardt algorithm, and the concentration that produced 50% of the growth inhibitory response was reported as the IC_{50}.

AUTHOR INFORMATION

Corresponding Author

*E-mail: Tanya.Parish@idri.org.

ORCID

Ben Gold: 0000-0002-9562-8241

Tanya Parish: 0000-0001-7507-0423

Present Addresses

‖J.O.: St. Jude Children's Research Hospital, 262 Danny Thomas Pl., Memphis, Tennessee 38105, United States.

⊥P.A.H.: Lgenia, 412 S Maple St., Suite 104, Fortville, Indiana 46040, United States.

Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

rGFP, ratiometric green fluorescent reporter protein; Ex, excitation; Em, emission; pH_{IB}, intrabacterial pH; NCGC, NCATS Chemical Genomics Center; DMSO, dimethyl sulfoxide; CV, coefficient of variation; CCCP, 3-chlorophenylhydrazone; $MAC_{6.5}$, minimum concentration required to decrease *M. tuberculosis* pH_{IB} < 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

REFERENCES

(1) WHO (2017) *Global Tuberculosis Report*; ISBN 978 92 4 156551 6.

(2) Moreira, A. L., Wang, J., Tsenova-Berkova, L., Hellmann, W., Freedman, V. H., and Kaplan, G. (1997) Sequestration of Mycobacterium tuberculosis in tight vacuoles in vivo in lung macrophages of mice infected by the respiratory route. *Infect. Immun. 65* (1), 305−8.

(3) Hestvik, A. L., Hmama, Z., and Av-Gay, Y. (2005) Mycobacterial manipulation of the host cell. *FEMS Microbiol Rev. 29* (5), 1041−50.

(4) Houben, E. N., Nguyen, L., and Pieters, J. (2006) Interaction of pathogenic mycobacteria with the host immune system. *Curr. Opin. Microbiol. 9* (1), 76−85.

(5) Yates, R. M., Hermetter, A., Taylor, G. A., and Russell, D. G. (2007) Macrophage activation downregulates the degradative capacity of the phagosome. *Traffic 8* (3), 241−50.

(6) MacMicking, J. D., Taylor, G. A., and McKinney, J. D. (2003) Immune control of tuberculosis by IFN-gamma-inducible LRG-47. *Science 302* (5645), 654−9.

(7) Schaible, U. E., Sturgill-Koszycki, S., Schlesinger, P. H., and Russell, D. G. (1998) Cytokine activation leads to acidification and increases maturation of Mycobacterium avium-containing phagosomes in murine macrophages. *J. Immunol 160* (3), 1290−6.

(8) Via, L. E., Fratti, R. A., McFalone, M., Pagan-Ramos, E., Deretic, D., and Deretic, V. (1998) Effects of cytokines on mycobacterial phagosome maturation. *J. Cell Sci. 111* (Pt 7), 897−905.

(9) Sprick, M. G. (1956) Phagocytosis of M. tuberculosis and M. smegmatis stained with indicator dyes. *Am. Rev. Tuberc 74* (4), 552−65.

(10) Sibley, L. D., Franzblau, S. G., and Krahenbuhl, J. L. (1987) Intracellular fate of Mycobacterium leprae in normal and activated mouse macrophages. *Infect. Immun. 55* (3), 680−5.

(11) Vandal, O. H., Nathan, C. F., and Ehrt, S. (2009) Acid resistance in Mycobacterium tuberculosis. *J. Bacteriol. 191* (15), 4714−21.

(12) Jackett, P. S., Aber, V. R., and Lowrie, D. B. (1978) Virulence and resistance to superoxide, low pH and hydrogen peroxide among strains of Mycobacterium tuberculosis. *J. Gen. Microbiol. 104* (1), 37−45.

(13) Stuehr, D. J., and Nathan, C. F. (1989) Nitric oxide. A macrophage product responsible for cytostasis and respiratory inhibition in tumor target cells. *J. Exp. Med. 169* (5), 1543−55.

(14) Turk, V., Turk, B., and Turk, D. (2001) Lysosomal cysteine proteases: facts and opportunities. *EMBO J. 20* (17), 4629−33.

(15) Vandal, O. H., Roberts, J. A., Odaira, T., Schnappinger, D., Nathan, C. F., and Ehrt, S. (2009) Acid-susceptible mutants of Mycobacterium tuberculosis share hypersusceptibility to cell wall and oxidative stress and to the host environment. *J. Bacteriol. 191* (2), 625−31.

(16) Vandal, O. H., Pierini, L. M., Schnappinger, D., Nathan, C. F., and Ehrt, S. (2008) A membrane protein preserves intrabacterial pH in intraphagosomal *Mycobacterium tuberculosis*. *Nat. Med. 14* (8), 849−854.

(17) Small, J. L., O'Donoghue, A. J., Boritsch, E. C., Tsodikov, O. V., Knudsen, G. M., Vandal, O., Craik, C. S., and Ehrt, S. (2013) Substrate Specificity of MarP, a Periplasmic Protease Required for Resistance to Acid and Oxidative Stress in Mycobacterium tuberculosis. *J. Biol. Chem. 288* (18), 12489−12499.

(18) Zhao, N., Darby, C. M., Small, J., Bachovchin, D. A., Jiang, X., Burns-Huang, K. E., Botella, H., Ehrt, S., Boger, D. L., Anderson, E. D., Cravatt, B. F., Speers, A. E., Fernandez-Vega, V., Hodder, P. S., Eberhart, C., Rosen, H., Spicer, T. P., and Nathan, C. F. (2015) Target-Based Screen Against a Periplasmic Serine Protease That Regulates Intrabacterial pH Homeostasis in Mycobacterium tuberculosis. *ACS Chem. Biol. 10*, 364.

(19) Biswas, T., Small, J., Vandal, O., Odaira, T., Deng, H., Ehrt, S., and Tsodikov, O. V. (2010) Structural Insight into Serine Protease Rv3671c that Protects M. tuberculosis from Oxidative and Acidic Stress. *Structure 18* (10), 1353−1363.

(20) Mates, A. K., Sayed, A. K., and Foster, J. W. (2007) Products of the Escherichia coli acid fitness island attenuate metabolite stress at

DOI: 10.1021/acsinfecdis.8b00256
ACS Infect. Dis. 2019, 5, 272−280

extremely low pH and mediate a cell density-dependent acid resistance. *J. Bacteriol. 189* (7), 2759−68.

(21) Li, Y. H., Hanna, M. N., Svensater, G., Ellen, R. P., and Cvitkovitch, D. G. (2001) Cell density modulates acid adaptation in Streptococcus mutans: implications for survival in biofilms. *J. Bacteriol. 183* (23), 6875−84.

(22) Raynaud, C., Papavinasasundaram, K. G., Speight, R. A., Springer, B., Sander, P., Bottger, E. C., Colston, M. J., and Draper, P. (2002) The functions of OmpATb, a pore-forming protein of Mycobacterium tuberculosis. *Mol. Microbiol. 46* (1), 191−201.

(23) Fisher, M. A., Plikaytis, B. B., and Shinnick, T. M. (2002) Microarray analysis of the Mycobacterium tuberculosis transcriptional response to the acidic conditions found in phagosomes. *J. Bacteriol. 184* (14), 4025−32.

(24) Honer Zu Bentrup, K., Miczak, A., Swenson, D. L., and Russell, D. G. (1999) Characterization of activity and expression of isocitrate lyase in Mycobacterium avium and Mycobacterium tuberculosis. *J. Bacteriol. 181* (23), 7161−7.

(25) Graham, J. E., and Clark-Curtiss, J. E. (1999) Identification of Mycobacterium tuberculosis RNAs synthesized in response to phagocytosis by human macrophages by selective capture of transcribed sequences (SCOTS). *Proc. Natl. Acad. Sci. U. S. A. 96* (20), 11554−9.

(26) McKinney, J. D., zu Bentrup, K. H., Munoz-Elias, E. J., Miczak, A., Chen, B., Chan, W. T., Swenson, D., Sacchettini, J. C., Jacobs, W. R., Jr., and Russell, D. G. (2000) Persistence of Mycobacterium tuberculosis in macrophages and mice requires the glyoxylate shunt enzyme isocitrate lyase. *Nature 406* (6797), 735−8.

(27) Bacon, J., and Marsh, P. D. (2007) Transcriptional responses of Mycobacterium tuberculosis exposed to adverse conditions in vitro. *Curr. Mol. Med. 7* (3), 277−86.

(28) Ryndak, M., Wang, S., and Smith, I. (2008) PhoP, a key player in Mycobacterium tuberculosis virulence. *Trends Microbiol. 16* (11), 528−34.

(29) Parish, T. (2014) Two-Component Regulatory Systems of Mycobacteria. *Microbiol. Spectrum 2* (1), No. Mgm2-0010-2013.

(30) Mehta, M., Rajmani, R. S., and Singh, A. (2016) Mycobacterium tuberculosis WhiB3 Responds to Vacuolar pH-induced Changes in Mycothiol Redox Potential to Modulate Phagosomal Maturation and Virulence. *J. Biol. Chem. 291* (6), 2888−903.

(31) Tan, S., Sukumar, N., Abramovitch, R. B., Parish, T., and Russell, D. G. (2013) Mycobacterium tuberculosis responds to chloride and pH as synergistic cues to the immune status of its host cell. *PLoS Pathog. 9* (4), No. e1003282.

(32) Cook, G. M., Berney, M., Gebhard, S., Heinemann, M., Cox, R. A., Danilchanka, O., and Niederweis, M. (2009) Physiology of mycobacteria. *Adv. Microb. Physiol. 55*, 81−182, () 318−319.

(33) Tews, I., Findeisen, F., Sinning, I., Schultz, A., Schultz, J. E., and Linder, J. U. (2005) The structure of a pH-sensing mycobacterial adenylyl cyclase holoenzyme. *Science 308* (5724), 1020−3.

(34) de Carvalho, L. P., Darby, C. M., Rhee, K. Y., and Nathan, C. (2011) Nitazoxanide disrupts membrane potential and intrabacterial pH Homeostasis of *Mycobacterium tuberculosis*. *ACS Med. Chem. Lett. 2* (11), 849−854.

(35) Moraski, G. C., Markley, L. D., Cramer, J., Hipskind, P. A., Boshoff, H., Bailey, M., Alling, T., Ollinger, J., Parish, T., and Miller, M. J. (2013) Advancement of Imidazo[1,2-a]pyridines with Improved Pharmacokinetics and Nanomolar Activity Against Mycobacterium tuberculosis. *ACS Med. Chem. Lett. 4* (7), 675−679.

(36) McDermott, W., and Tompsett, R. (1954) Activation of pyrazinamide and nicotinamide in acidic environments in vitro. *Am. Rev. Tuberc 70* (4), 748−54.

(37) Darby, C. M., Ingolfsson, H. I., Jiang, X., Shen, C., Sun, M., Zhao, N., Burns, K., Liu, G., Ehrt, S., Warren, J. D., Anderson, O. S., Brickner, S. J., and Nathan, C. (2013) Whole cell screen for inhibitors of pH homeostasis in Mycobacterium tuberculosis. *PLoS One 8* (7), No. e68942.

(38) Huczynski, A., Janczak, J., Lowicki, D., and Brzezinski, B. (2012) Monensin A acid complexes as a model of electrogenic transport of sodium cation. *Biochim. Biophys. Acta, Biomembr. 1818* (9), 2108−19.

(39) Eli Lilly & Company (2004) *Assay Guidance Manual*, Eli Lilly & Company and the National Center for Advancing Translational Sciences.

(40) Gold, B., Warrier, T., and Nathan, C. (2015) A multi-stress model for high throughput screening against non-replicating *Mycobacterium tuberculosis*. in *Mycobacteria Protocols* (Parish, T., and Roberts, D. M., Eds.), pp 293−315, Methods in Molecular Biology, Vol. *1285*, Springer.

(41) Gold, B., Pingle, M., Brickner, S. J., Shah, N., Roberts, J., Rundell, M., Bracken, W. C., Warrier, T., Somersan, S., Venugopal, A., Darby, C., Jiang, X., Warren, J. D., Fernandez, J., Ouerfelli, O., Nuermberger, E. L., Cunningham-Bussel, A., Rath, P., Chidawanyika, T., Deng, H., Realubit, R., Glickman, J. F., and Nathan, C. F. (2012) Nonsteroidal anti-inflammatory drug sensitizes Mycobacterium tuberculosis to endogenous and exogenous antimicrobials. *Proc. Natl. Acad. Sci. U. S. A. 109* (40), 16004−11.

(42) Rao, M., Streur, T. L., Aldwell, F. E., and Cook, G. M. (2001) Intracellular pH regulation by Mycobacterium smegmatis and Mycobacterium bovis BCG. *Microbiology 147* (4), 1017−24.

DOI: 10.1021/acsinfecdis.8b00256
ACS Infect. Dis. 2019, 5, 272−280