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LETTER

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Nitazoxanide Disrupts Membrane Potential and Intrabacterial pH Homeostasis of *Mycobacterium tuberculosis*

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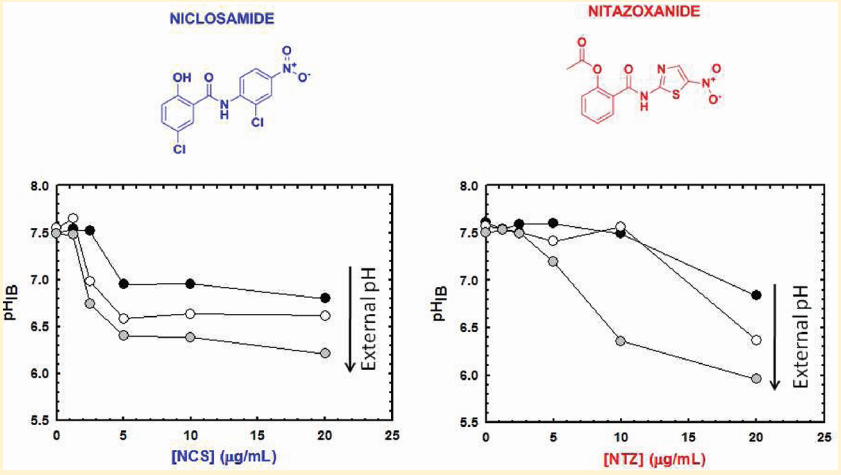
Supporting Information

ABSTRACT:



Nitazoxanide (Alinia), a nitro-thiazolyl antiparasitic drug, kills diverse microorganisms by unknown mechanisms. Here we identified two actions of nitazoxanide against *Mycobacterium tuberculosis* (Mtb): disruption of Mtb's membrane potential and pH homeostasis. Both actions were shared by a structurally related antimycobacterial compound, niclosamide. Reactive nitrogen intermediates were reported to synergize with nitazoxanide and its deacetylated derivative tizoxanide in killing Mtb. Herein, however, we could not attribute this to increased uptake of nitazoxanide or tizoxanide as monitored by targeted metabolomics, nor to increased impact of nitazoxanide on Mtb's membrane potential or intrabacterial pH. Thus, further mechanisms of action of nitazoxanide or tizoxanide may await discovery. The multiple mechanisms of action may contribute to Mtb's ultralow frequency of resistance against nitazoxanide.

KEYWORDS: Nitazoxanide, tizoxanide, *Mycobacterium tuberculosis*, niclosamide, pH homeostasis, membrane potential, metabolomics

The worldwide dissemination of multidrug (MDR) and extensively drug resistant (XDR) *Mycobacterium tuberculosis* (Mtb) strains poses a serious threat to human welfare that, in part, reflects the inadequacy of contemporary tuberculosis (TB) chemotherapy. New chemical entities with novel mechanisms of action that can be used against drug-susceptible and drug-resistant strains while shortening the course of treatment could contribute to saving nearly 2 million lives yearly.

Among the ideal characteristics of a new TB drug would be mycobactericidal activity against both replicating and nonreplicating populations of Mtb.[1−3] Targeting both populations is central to sterilization of Mtb and a clinical cure. Drug candidates active against both populations are thus being intensely sought.[4−9] Elimination of replicating and nonreplicating Mtb subpopulations in patients is currently achieved only with strains of Mtb that are sensitive to a combination of multiple drugs consisting of isoniazid and ethambutol (active primarily against replicating Mtb) with rifampicin (active against replicating Mtb and, to a lesser but significant extent, against nonreplicating Mtb) and pyrazinamide (active only against Mtb residing in acidic compartments, whether or not replicating).

In an effort to find novel chemical entities active against both replicating and nonreplicating Mtb subpopulations, we identified nitazoxanide (NTZ) as a promising candidate.[5] Nitazoxanide (Alinia, Romark Laboratories), shown in Scheme 1, is a nitro-thiazolyl antiparasitic drug approved by the FDA in 2002 for the treatment of infections caused by *Giardia* and *Cryptosporidium*.[10,11] NTZ is a pro-drug that is deacetylated in the

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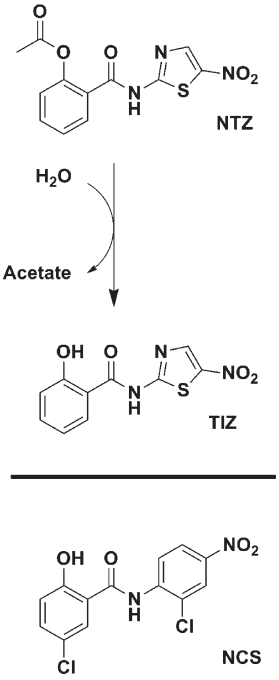
Scheme 1. Structures of NTZ, TIZ, and NCS



gastrointestinal tract to tizoxanide (TIZ), which is believed to be the active form (Scheme 1). NTZ is also active against several anaerobic and microaerophilic bacteria, such as *Clostridium difficile* and *Helicobacter pylori*,[12−15] and, surprisingly, against hepatitis B and hepatitis C viruses.[16,17] These activities are accompanied by an outstanding safety profile.[18,19]

That NTZ exhibited activity against Mtb came as a surprise,[5] as Mtb does not possess a homologue for the putative bacterial target, pyruvate ferredoxin oxidoreductase (PFOR),[20] nor does Mtb require a microaerophilic or anaerobic atmosphere to manifest susceptibility to NTZ. NTZ's activity against both replicating and nonreplicating Mtb and its ultralow frequency of resistance thus led us to hypothesize that NTZ has a novel mechanism of action (MOA) against Mtb that may involve more than one target.[5]

NTZ and TIZ display considerable structural similarity with niclosamide (NCS). On the basis of this similarity, we wondered if these compounds share similar pharmacodynamic properties. NCS (Scheme 1) is a nitroaromatic compound with anticestode activity and has been shown to function as an uncoupler of oxidative phosphorylation.[21] NCS was also found to be active against Mtb.[22] Interference with oxidative phosphorylation could affect maintenance of membrane potential and pH homeostasis, which are essential for mycobacterial survival.[23]

On the basis of these observations, we characterized the ability of NTZ to accumulate in Mtb using targeted metabolomics and examined its effect on membrane potential (MP) and intrabacterial pH (pH_{IB}).

To determine if only NTZ, only TIZ, or both compounds enter Mtb, we used liquid chromatography and accurate mass time-of-flight mass spectrometry. Cell lysates were prepared from Mtb treated with NTZ at 0-, 1-, 4-, and 10-fold the minimum inhibitory concentration (MIC) of 1 μg/mL (3.25 μM). Identification of NTZ and TIZ was based on their accurate masses, retention times, and isotopic envelopes, using pure chemicals as standards. Targeted analysis of the metabolome of Mtb indicated that there are no endogenous metabolites with m/z values that matched those calculated for NTZ and TIZ ($(M + H)^+$ = 308.0336 and 266.0230, respectively), allowing identification and quantification of NTZ and TIZ in Mtb. Exposure of Mtb to increased concentrations of NTZ led to the appearance in the lysates of ions with m/z values of 308.0346 and 266.0240 (Figure 1a and b), within 4 ppm of the m/z of the standards. The newly appearing ions displayed isotopic envelopes very similar to those of the standards and of the calculated isotopic envelopes and displayed identical retention times to the standards (Supporting Information). Control experiments using $U^{13}C$-acetate indicated that the NTZ observed in the lysate was not a product of intracellular acetylation of TIZ (data not shown), as there was no ^{13}C labeling of NTZ.

Acidified nitrite (ASN) forms nitrous acid, which slowly dismutates to produce nitric oxide and nitrogen dioxide. In a previous study,[5] ASN was shown to synergize with NTZ in killing Mtb. We therefore tested whether the synergy might be explained by an ASN-dependent increase in the intrabacterial accumulation of NTZ and TIZ. However, incubation of Mtb in ASN led to only a slight increase in the pool size of NTZ and no increase in TIZ (Figure 1c and d).

These results indicate that both NTZ and TIZ are able to penetrate and accumulate inside Mtb. The concentration of TIZ in Mtb was roughly 1000-fold greater than the concentration of NTZ (Figure 1c and d). To our knowledge, this is the first observation of accumulation of NTZ in a bacterium. Intrabacterial accumulation of NTZ raises the possibility that NTZ itself might be an active species against some organisms. This information may aid the design of more potent, antimycobacterial-specific analogues of NTZ with improved bioavailability. For instance, the acetyl group linked to TIZ's phenolic hydroxyl could be replaced with ester, amide, or ether functionalities, to generate a collection of analogues for further studies.

NTZ's antimycobacterial mechanism of action is undefined. Structural similarities between TIZ and NCS suggest that these two drugs might act in a similar fashion. NCS was found to uncouple oxidative phosphorylation in some cells, by affecting the mitochondrial enzymes carrying out proton transfer across compartments,[21] and to kill Mtb.[22] Thus, we probed the effect of NTZ and NCS on Mtb's MP, using the fluorescent, membrane-permeable dye 3,3′-diethyloxicarbocianide chloride ($DiOC_2$). As shown in the top panels of Figure 2, both NTZ and NCS caused a concentration-dependent collapse of Mtb's MP at pH 7.4 that was as marked as that induced by CCCP, a strong uncoupler used as a positive control. Under identical conditions, rifampicin, despite its marked mycobactericidal activity, did not alter the MP. Although the baseline MP was slightly affected at pH 5.5, NTZ and NCS also collapsed Mtb's MP at this pH (Figure 2). However, this effect was nearly abrogated at pH 4.5 (Supporting Information) due to the collapse in MP caused by the lower pH itself. In contrast, addition of 0.5 mM $NaNO_2$ at pH 5.5 caused no significant changes in the observed effects (Figure 2e and f). Thus, both NTZ and NCS altered Mtb's MP, but reactive nitrogen intermediates (RNI) did not do so during the period examined.

pH_{IB} and MP are intrinsically coupled to one another for bacteria to maintain a specific proton motive force (reviewed in refs 24 and 25). We therefore sought to probe whether NTZ and NCS also disturbed Mtb's pH_{IB}. To do so, we used a strain of H37Rv carrying a plasmid containing a pH-sensitive ratiometric GFP[26,27] and monitored the concentration-dependent effects of NTZ and NCS on Mtb's pH_{IB} at pH 7.5, 6.5, and 5.5 in the

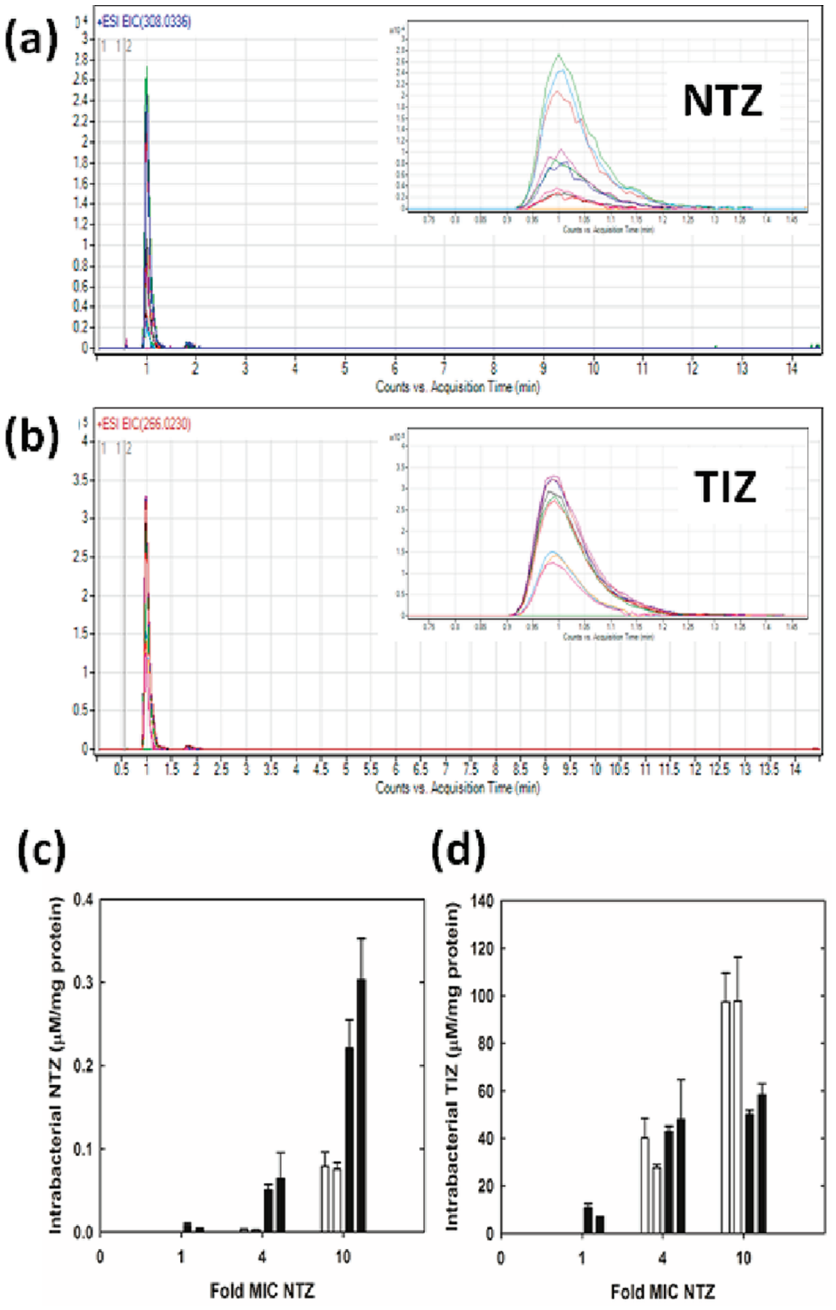


Figure 1. Targeted metabolomic analysis of NTZ and TIZ in Mtb: (a) Extracted ion chromatogram for NTZ-treated Mtb. The inset shows the blow up of the NTZ-peaks obtained from Mtb treated with 10-, 4-, 1-fold the MIC and untreated cells, at pH 5.5 + $NaNO_2$. Triplicates are shown. (b) Extracted ion chromatogram for NTZ-treated Mtb. The inset shows the blow up of the TIZ-peaks obtained from Mtb treated with 10-, 4-, 1-fold the MIC and untreated cells, at pH 5.5 + $NaNO_2$. Triplicates are shown. (c) Quantification of NTZ in Mtb as a function of the concentration of NTZ, cultured at pH 6.6 and pH 5.5 + 0.5 mM $NaNO_2$. Data shown are the average of three biological replicates, and the error is the standard deviation. (d) Quantification of TIZ in Mtb as a function of the concentration of NTZ, cultured at pH 6.6 and pH 5.5 + 0.5 mM $NaNO_2$. Two bars in each condition indicate average data from two independent experiments. Open and closed bars indicate data obtained at pH 6.6 and at pH 5.5 + $NaNO_2$, respectively.

presence or absence of $NaNO_2$. Figure 3 shows representative data after a 16-h exposure to NTZ, NCS, or vehicle alone (DMSO). The pH_{IB} of untreated Mtb remained constant for 3 days (data not shown). NTZ reduced Mtb's pH_{IB} in a concentration-dependent manner (Figure 3a). This effect increased as the external pH decreased. Addition of $NaNO_2$ (Figure 3b) caused a slight increase in potency of NTZ with respect to its impact on pH_{IB}. NCS was far more effective than NTZ at disrupting Mtb's pH_{IB} homeostasis (Figure 3c), and this effect was unperturbed by addition of $NaNO_2$ (Figure 3d).

The lack of influence of RNI on uptake of NTZ and TIZ, the lack of synergism of RNI with NTZ on Mtb's MP, and the minimal impact of RNI on NTZ's disruption of Mtb's pH_{IB} homeostasis stand in stark contrast to the ability of RNI to synergize with NTZ for killing Mtb. Thus, the mechanism of synergy between RNI and NTZ remains to be defined, and,

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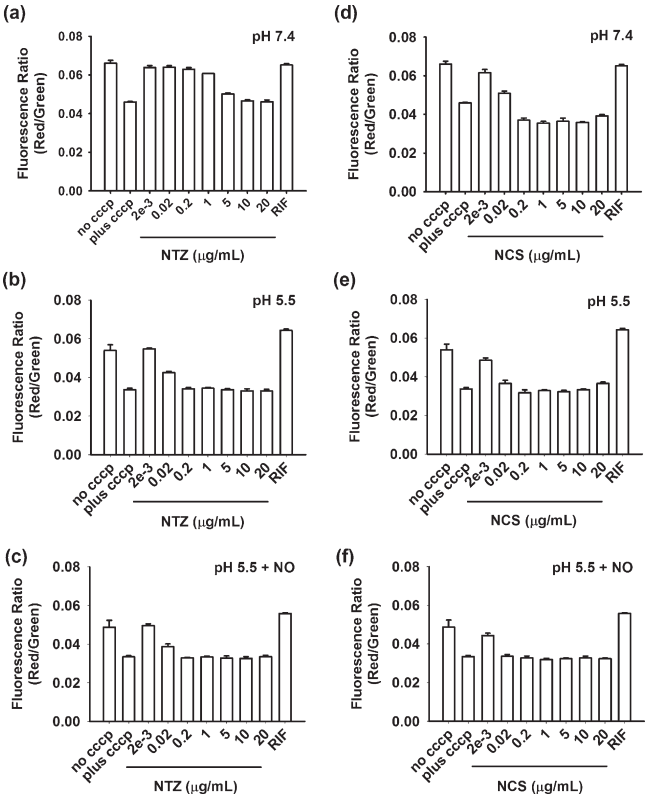

Figure 2. NTZ and NCS disrupt Mtb's membrane potential: (a and d) effect of NTZ, NCS, and control on the membrane potential at pH 7.4; (b and e) effect of NTZ, NCS, and control on the membrane potential at pH 5.5; (c and f) effect of NTZ, NCS, and control on the membrane potential at pH 5.5 + 0.5 mM $NaNO_2$. Data are the average of triplicates and are representative of two independent experiments. Error bars indicate standard deviation.

along with it, the possibility that NTZ targets yet another pathway in Mtb besides membrane potential and intrabacterial pH homeostasis. In summary, the results presented here indicate that NTZ/TIZ are membrane active compounds, as recently suggested by Hurdle et al.,[28] although this activity is not responsible for RNI-potentiated killing. Furthermore, NTZ and TIZ share similar mechanistic properties with NCS, although the magnitude and onset of the effects are distinct. Disruption of membrane potential was also observed when NTZ and TIZ were tested against several nematodes, trematodes, and cestodes, such as *Caenorhabditis elegans*, *Ascaris suum*, *Schistosoma mansoni*, and *Hymenolepsis diminuta*,[29] but other mechanisms were also evident. These observations strongly suggest that NTZ might have multiple targets in several organisms.

EXPERIMENTAL PROCEDURES

LC-MS solvents were Optima LC-MS grade from Fischer Scientific. Nitazoxanide from Atlantic SciTech Group was dissolved fresh for each experiment in dimethylsulfoxide (DMSO). TIZ was prepared as described.[5] Other chemicals were from Sigma-Aldrich and were the highest purity available. All data are representative of at least two independent experiments.

M. tuberculosis H37Rv (Mtb) and *M. tuberculosis* H37Rv [p-UV15-RM-GFP] (Mtb pHRMGFP) were cultured in a biosafety level 3 facility at 37 °C in Middlebrook 7H9 broth or on 7H10 agar (Difco)

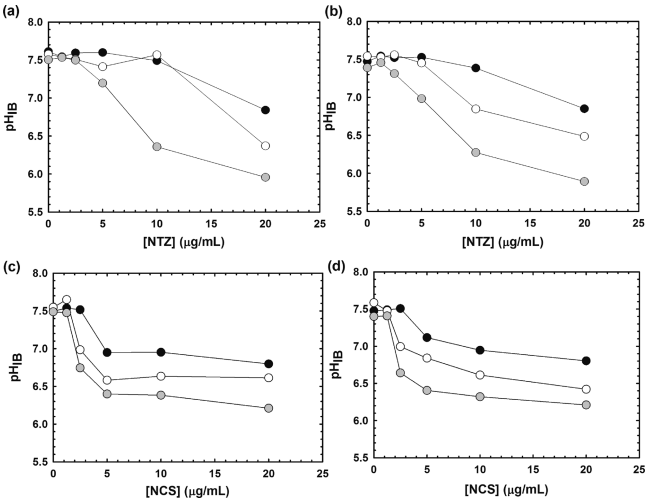

Figure 3. NTZ and NCS disrupt Mtb's intrabacterial pH. (a) NTZ effect on pHIB in 7H9 media at different pH values; (b) NTZ effect on pHIB in 7H9 media containing 0.5 mM $NaNO_2$ at different pH values; (c) NCS effect on pHIB at different pH values; (d) NCS effect on pHIB in 7H9 media containing 0.5 mM $NaNO_2$ at different pH values. These results were obtained after 4 h of exposure to drug or vehicle alone. Black circles indicate pH 7.5, white circles indicate pH 6.5, and gray circles indicate pH 5.5.

supplemented with 0.2% glycerol, 0.2% dextrose, 0.02% Tyloxapol (broth only), and 0.085% NaCl. Tyloxapol was used as a dispersal agent instead of Tween 80 due to the instability of Tween 80 at lower pH values.

Targeted metabolomic profiling was used to detect the presence of NTZ and TIZ inside Mtb as described.[30,31] In short, 0.5 mL of a culture at OD_{580} = 1.0 was transferred to a nitrocellulose filter, which was placed on 7H10 agar at 37 °C for 6 days. On the sixth day, the filter was moved to a 7H10 plate containing drug or vehicle plus dextrose and glycerol and incubated at 37 °C for 16 h. The filter was then plunged into 1 mL of acetonitrile/methanol/water (2:2:1, v/v/v), at *ca.* −40 °C. The bacteria were lysed by bead-beating. The insoluble fraction was pelleted by centrifugation at 4 °C for 10 min and the soluble fraction frozen at −80 °C until analysis. For LC-MS, samples were thawed and diluted with an equal volume of 0.2% acetic acid (v/v) in acetonitrile. Samples (2 μL) were injected into a Cogent Diamond Silica Hydride Type-C column (100 Å, 2.1 mm × 150 mm, 4 μm), using an Agilent LC 1200 liquid chromatography platform connected to an Agilent Accurate Mass-TOF mass spectrometer (6220). Chromatographic separation was accomplished using gradient C as described.[32] The concentration of NTZ and TIZ in the samples was obtained by using standard curves with chemical standards and by using the residual peptide content.

Mtb cultures were grown to midlog phase (OD_{580} ~ 0.6) and concentrated by centrifugation and resuspension to an OD_{580} ~ 1.0 in Middlebrook broth 7H9 with 0.2% glycerol, 0.2% dextrose, 0.085% NaCl, and 0.02% Tyloxypol at indicated pH values. NTZ or NCS was added to achieve a final concentration of 0.002, 0.02, 0.2, 1, 5, 10, or 20 μg/mL. Cultures were immediately exposed to 15 μM $DiOC_2^3$ for 20 min at room temperature. Cultures were subsequently washed to remove extracellular dye and resuspended in fresh 7H9 broth. As a positive control for membrane depolarization, 5 μM of the protonophore carbonyl-cyanide 3-chlorophenylhydrazone (CCCP) (Invitrogen) was included. DMSO and rifampicin (0.4 μg/mL) were used as negative controls. The assay was performed in black with clear-bottom 96-well plates (Costar), and a SpectraMax M5 spectrofluorimeter (Molecular Devices) was used to measure green fluorescence (488 nm/530 nm) and shifts to red fluorescence (488 nm/610 nm), as a result of aggregation of dye molecules due to the

presence of a large membrane potential. MP was measured as a ratio of red fluorescence (which depended on cell size and membrane potential) to green fluorescence (which depended on cell size alone). Each condition was measured in triplicate, and each experiment was performed twice.

We used *M. tuberculosis* expressing a pH-sensitive ratiometric green fluorescent protein (Mtb-pHRMGFP).[26,27] Mtb-pHRMGFP cultures were grown to midlog phase ($OD_{580} \sim 0.6$), resuspended, and diluted to an $OD_{580} \sim 0.3$ in 7H9 medium at indicated pH values. Fluorescence was measured using a SpectraMax M5 spectrofluorimeter at excitation 395 nm, emission 510 nm (reading 1) and at excitation 475 nm, emission 510 nm (reading 2). pH_{IB} was inferred from the ratio of reading 1 to reading 2 (excitation 475 nm) by reference to a calibration curve performed on lysates of Mtb-pHRMGFP.

■ ASSOCIATED CONTENT

Supporting Information. Retention time information for NTZ and TIZ. Additional data on collapse of membrane potential by NTZ and NCS, at pH 4.5. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

L.P.S.C. performed pH_{IB} measurements and metabolomics, designed experiments, and analyzed and interpreted data. C.M.D. performed pH_{IB} and membrane potential measurements, designed experiments, and analyzed and interpreted data. K.Y.R designed and performed metabolomic experiments and analyzed and interpreted the data. C.N. designed experiments and interpreted data. All authors prepared the manuscript.

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■ ABBREVIATIONS

Mtb, *Mycobacterium tuberculosis*; MDR, multidrug resistant; XDR, extensively drug resistant; NTZ, nitazoxanide; TIZ, tizoxanide; NCS, niclosamide; pH_{IB}, intrabacterial pH; MP, membrane potential; pHRMGFP, pH-sensitive ratiometric green fluorescent protein; DMSO, demethylsulfoxide

■ REFERENCES

(1) Mitchison, D. A. The search for new sterilizing anti-tuberculosis drugs. *Front. Biosci.* **2004**, *9*, 1059–1072.

(2) Nathan, C.; Gold, B.; Lin, G.; Stegman, M.; de Carvalho, L. P.; Vandal, O.; Venugopal, A.; Bryk, R. A philosophy of anti-infectives as a guide in the search for new drugs for tuberculosis. *Tuberculosis* **2008**, *88* (Suppl 1), S25–33.

(3) Koul, A.; Arnoult, E.; Lounis, N.; Guillemont, J.; Andries, K. The challenge of new drug discovery for tuberculosis. *Nature* **2011**, *469*, 483–490.

(4) Makarov, V.; Manina, G.; Mikusova, K.; Mollmann, U.; Ryabova, O.; Saint-Joanis, B.; Dhar, N.; Pasca, M. R.; Buroni, S.; Lucarelli, A. P.; Milano, A.; De Rossi, E.; Belanova, M.; Bobovska, A.; Dianiskova, P.; Kordulakova, J.; Sala, C.; Fullam, E.; Schneider, P.; McKinney, J. D.; Brodin, P.; Christophe, T.; Waddell, S.; Butcher, P.; Albrethsen, J.; Rosenkrands, I.; Brosch, R.; Nandi, V.; Bharath, S.; Gaonkar, S.; Shandil, R. K.; Balasubramanian, V.; Balganesh, T.; Tyagi, S.; Grosset, J.; Riccardi, G.; Cole, S. T. Benzothiazinones kill *Mycobacterium tuberculosis* by blocking arabinan synthesis. *Science* **2009**, *324*, 801–804.

(5) de Carvalho, L. P.; Lin, G.; Jiang, X.; Nathan, C. Nitazoxanide kills replicating and nonreplicating *Mycobacterium tuberculosis* and evades resistance. *J. Med. Chem.* **2009**, *52*, 5789–5792.

(6) Stover, C. K.; Warrener, P.; VanDevanter, D. R.; Sherman, D. R.; Arain, T. M.; Langhorne, M. H.; Anderson, S. W.; Towell, J. A.; Yuan, Y.; McMurray, D. N.; Kreiswirth, B. N.; Barry, C. E.; Baker, W. R. A small-molecule nitroimidazopyran drug candidate for the treatment of tuberculosis. *Nature* **2000**, *405*, 962–966.

(7) Hugonnet, J. E.; Tremblay, L. W.; Boshoff, H. I.; Barry, C. E., 3rd; Blanchard, J. S. Meropenem-clavulanate is effective against extensively drug-resistant *Mycobacterium tuberculosis*. *Science* **2009**, *323*, 1215–1218.

(8) Andries, K.; Verhasselt, P.; Guillemont, J.; Gohlmann, H. W.; Neefs, J. M.; Winkler, H.; Van Gestel, J.; Timmerman, P.; Zhu, M.; Lee, E.; Williams, P.; de Chaffoy, D.; Huitric, E.; Hoffner, S.; Cambau, E.; Truffot-Pernot, C.; Lounis, N.; Jarlier, V. A diarylquinoline drug active on the ATP synthase of *Mycobacterium tuberculosis*. *Science* **2005**, *307*, 223–227.

(9) Lilienkampf, A.; Mao, J.; Wan, B.; Wang, Y.; Franzblau, S. G.; Kozikowski, A. P. Structure-activity relationships for a series of quinoline-based compounds active against replicating and nonreplicating *Mycobacterium tuberculosis*. *J. Med. Chem.* **2009**, *52*, 2109–2118.

(10) Adagu, I. S.; Nolder, D.; Warhurst, D. C.; Rossignol, J. F. In vitro activity of nitazoxanide and related compounds against isolates of *Giardia intestinalis*, *Entamoeba histolytica* and *Trichomonas vaginalis*. *J. Antimicrob. Chemother.* **2002**, *49*, 103–111.

(11) Theodos, C. M.; Griffiths, J. K.; D'Onfro, J.; Fairfield, A.; Tzipori, S. Efficacy of nitazoxanide against *Cryptosporidium parvum* in cell culture and in animal models. *Antimicrob. Agents Chemother.* **1998**, *42*, 1959–1965.

(12) Dubreuil, L.; Houcke, I.; Mouton, Y.; Rossignol, J. F. In vitro evaluation of activities of nitazoxanide and tizoxanide against anaerobes and aerobic organisms. *Antimicrob. Agents Chemother.* **1996**, *40*, 2266–2270.

(13) Megraud, F.; Occhialini, A.; Rossignol, J. F. Nitazoxanide, a potential drug for eradication of *Helicobacter pylori* with no cross-resistance to metronidazole. *Antimicrob. Agents Chemother.* **1998**, *42*, 2836–2840.

(14) McVay, C. S.; Rolfe, R. D. In vitro and in vivo activities of nitazoxanide against *Clostridium difficile*. *Antimicrob. Agents Chemother.* **2000**, *44*, 2254–2258.

(15) Pankuch, G. A.; Appelbaum, P. C. Activities of tizoxanide and nitazoxanide compared to those of five other thiazolides and three other agents against anaerobic species. *Antimicrob. Agents Chemother.* **2006**, *50*, 1112–1117.

(16) Korba, B. E.; Elazar, M.; Lui, P.; Rossignol, J. F.; Glenn, J. S. Potential for hepatitis C virus resistance to nitazoxanide or tizoxanide. *Antimicrob. Agents Chemother.* **2008**, *52*, 4069–4071.

(17) Korba, B. E.; Montero, A. B.; Farrar, K.; Gaye, K.; Mukerjee, S.; Ayers, M. S.; Rossignol, J. F. Nitazoxanide, tizoxanide and other

thiazolides are potent inhibitors of hepatitis B virus and hepatitis C virus replication. *Antiviral Res.* **2008**, *77*, 56–63.

(18) Rossignol, J. F. Nitazoxanide in the treatment of acquired immune deficiency syndrome-related cryptosporidiosis: results of the United States compassionate use program in 365 patients. *Aliment. Pharmacol. Ther.* **2006**, *24*, 887–894.

(19) Rossignol, J. F.; Elfert, A.; El-Gohary, Y.; Keeffe, E. B. Improved virologic response in chronic hepatitis C genotype 4 treated with nitazoxanide, peginterferon, and ribavirin. *Gastroenterology* **2009**, *136*, 856–862.

(20) Hoffman, P. S.; Sisson, G.; Croxen, M. A.; Welch, K.; Harman, W. D.; Cremades, N.; Morash, M. G. Antiparasitic drug nitazoxanide inhibits the pyruvate oxidoreductases of *Helicobacter pylori*, selected anaerobic bacteria and parasites, and *Campylobacter jejuni*. *Antimicrob. Agents Chemother.* **2007**, *51*, 868–876.

(21) Weinbach, E. C.; Garbus, J. Mechanism of action of reagents that uncouple oxidative phosphorylation. *Nature* **1969**, *221*, 1016–1018.

(22) Sun, Z.; Zhang, Y. Antituberculosis activity of certain antifungal and antihelmintic drugs. *Tuberc. Lung Disease* **1999**, *79*, 319–320.

(23) Vandal, O. H.; Nathan, C. F.; Ehrt, S. Acid resistance in *Mycobacterium tuberculosis*. *J. Bacteriol.* **2009**, *191*, 4714–4721.

(24) Slonczewski, J. L.; Fujisawa, M.; Dopson, M.; Krulwich, T. A. Cytoplasmic pH measurement and homeostasis in bacteria and archaea. *Adv. Microb. Physiol.* **2009**, *55* (1−79), 317.

(25) Krulwich, T. A.; Sachs, G.; Padan, E. Molecular aspects of bacterial pH sensing and homeostasis. *Nat. Rev. Microbiol.* **2011**, *9*, 330–343.

(26) Miesenbock, G.; De Angelis, D. A.; Rothman, J. E. Visualizing secretion and synaptic transmission with pH-sensitive green fluorescent proteins. *Nature* **1998**, *394*, 192–195.

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

(28) Hurdle, J. G.; O'Neill, A. J.; Chopra, I.; Lee, R. E. Targeting bacterial membrane function: an underexploited mechanism for treating persistent infections. *Nat. Rev. Microbiol.* **2011**, *9*, 62–75.

(29) Atherton, R. R. D. Ph.D. Thesis: Mechanism of Action of Nitazoxanide and Related Drugs Against Helminths. Department of Infectious and Tropical Diseases, London School of Hygiene and Tropical Medicine: London, 2003.

(30) de Carvalho, L. P.; Fischer, S. M.; Marrero, J.; Nathan, C.; Ehrt, S.; Rhee, K. Y. Metabolomics of *Mycobacterium tuberculosis* reveals compartmentalized co-catabolism of carbon substrates. *Chem. Biol.* **2010**, *17*, 1122–1131.

(31) de Carvalho, L. P.; Zhao, H.; Dickinson, C. E.; Arango, N. M.; Lima, C. D.; Fischer, S. M.; Ouerfelli, O.; Nathan, C.; Rhee, K. Y. Activity-based metabolomic profiling of enzymatic function: identification of *Rv1248c* as a mycobacterial 2-hydroxy-3-oxoadipate synthase. *Chem. Biol.* **2010**, *17*, 323–332.

(32) Pesek, J. J.; Matyska, M. T.; Fischer, S. M.; Sana, T. R. Analysis of hydrophilic metabolites by high-performance liquid chromatography-mass spectrometry using a silica hydride-based stationary phase. *J. Chromatogr., A* **2008**, *1204*, 48–55.