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Indoleamides are active against drug-resistant *Mycobacterium tuberculosis*

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Abstract

Responsible for nearly two million deaths each year, the infectious disease tuberculosis remains a serious global health challenge. The emergence of multidrug- and extensively drug-resistant strains of *Mycobacterium tuberculosis* confounds control efforts, and new drugs with novel molecular targets are desperately needed. Here we describe lead compounds, the indoleamides, with potent activity against both drug-susceptible and drug-resistant strains of *M. tuberculosis* by targeting the mycolic acid transporter MmpL3. We identify a single mutation in *mmpL3* which confers high resistance to the indoleamide class while remaining susceptible to currently used first- and second-line tuberculosis drugs, indicating a lack of cross-resistance. Importantly, an indoleamide derivative exhibits dose-dependent anti-mycobacterial activity when orally administered to *M. tuberculosis*-infected mice. The bioavailability of the indoleamides, combined

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Author contributions

W.R.B.; A.P.K.; and S.L. conceived the original idea. S.L. and W.R.B. designed and discussed the biological work. S.L. and H.G. performed the biological experiments. A.P.K.; O.K.O.; M.P.; H.G.; G.C.; and S.K.T. designed, discussed and synthesized the compounds. S.L. and N.C.A. analyzed the data and prepared the tables and figures. N.C.A.; S.L.; and O.K.O. wrote the manuscript. All authors edited the manuscript.

Competing financial interests

The authors declare no competing financial interests.

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with their ability to kill tubercle bacilli, indicates great potential for translational developments of this structure class for the treatment of drug-resistant tuberculosis.

Introduction

Tuberculosis (TB) is a human infectious disease responsible for significant worldwide morbidity and mortality, accountable for an estimated 8.7 million incident cases and 1.4 million deaths in 2011[1]. Although effective therapy exists for TB caused by drug-susceptible *Mycobacterium tuberculosis*, this therapy requires daily administration of multiple drugs for a minimum of 6 months. Strict adherence to treatment is necessary for successful outcome. However, the intensity and duration of effective therapy challenge patient compliance and thus contribute to treatment failures, leading to increased disease, continued *M. tuberculosis* transmission and ultimately selection of drug-resistant organisms. The development of drug resistance is especially alarming, as transmission of drug-resistant bacilli can lead to primary infections refractory to standard TB therapy. In 2011, the World Health Organization (WHO) reported that 3.7% of new TB cases were due to infection with multidrug-resistant (MDR) *M. tuberculosis*[1]. The tragic development of MDR- and extensively drug-resistant- (XDR-) TB has kindled a worldwide push for the development of new therapy options for this disease, and new drugs are desperately needed to enable effective worldwide TB control.

The current WHO-endorsed standard regimen for the treatment of drug-susceptible TB consists of daily rifampin, isoniazid, pyrazinamide and ethambutol for two months, followed by four months of daily isoniazid and rifampin. This first-line regimen, referred to as the “short course” (as previous treatment regimens ranged from 18–24 months in duration), utilizes some of the oldest antibiotics in modern medicine, with isoniazid and pyrazinamide developed in the 1950s and ethambutol and rifampin developed in the 1960s. That the most recent first-line anti-TB drugs are over 50 years old illustrates the paucity of drug development advances in this field. In December 2012, the United States Food and Drug Administration (FDA) granted accelerated approval of bedaquiline, a diarylquinoline antimycobacterial drug, for the treatment of MDR-TB (infection with *M. tuberculosis* resistant to rifampin and isoniazid), including XDR-TB (resistance to rifampin, isoniazid, a quinolone and one of the injectable drugs: kanamycin, amikacin or capreomycin), when no other treatment options exist[2]. The FDA approval of bedaquiline is a landmark event in TB chemotherapy, representing the introduction of a new drug class and being the first new TB drug approved in half a century. However, the nature of the approval, being only permitted for use when other treatment options are exhausted, indicates that bedaquiline will be added to otherwise failing drug regimens, and as such it can be anticipated that microbial resistance to this new compound will eventually emerge. Thus, it is imperative that TB drug development efforts continue to push forward.

Whole-cell phenotypic high-throughput screening is a powerful tool for evaluation of the antimicrobial activity of compounds in large chemical libraries. Indeed, such high-throughput compound screening with the proxy nonpathogenic organism *M. smegmatis* identified the diarylquinoline precursor to bedaquiline, which was subsequently optimized

for activity against *M. tuberculosis*[3]. This method has been adapted for direct utility with *M. tuberculosis* and has led to the identification of a number of promising lead compounds[4]. A recent phenotypic screening of a library of 6,800 compounds identified several chemotypes with anti-*M. tuberculosis* activity[5–8]. We synthesized and preliminarily characterized one molecular class, indoleamides, which was active against both drug-susceptible and drug-resistant *M. tuberculosis*[9].

Here we further characterize three lead compounds from this class both *in vitro* and *in vivo*. Our work indicates that these compounds target the mycobacterial membrane protein, large-3 (MmpL3), a mycolic acid transporter, and that the indoleamides are orally bioavailable and effective *in vivo* in a mouse model of TB, indicating promising translational potential.

Results

Indoleamides are active against *M. tuberculosis*

A high-throughput screen of compounds[8] identified a structurally simple indole-2-carboxamide, compound **1**, with activity against *M. tuberculosis* (Fig. 1a). We used the indoleamide scaffold as a basis for the development of structural analogues, which yielded compounds **2** and **3** (Fig. 1b). The minimum inhibitory concentration (MIC) values of each of these compounds were determined against different *M. tuberculosis* strains, including a fully drug-susceptible laboratory reference strain, H37Rv, and five clinical isolates originally obtained from pulmonary TB patients in KwaZulu-Natal, South Africa[7, 10]. The patient isolates included a drug-susceptible strain (V4207), two confirmed MDR strains (V2475 and KZN494) and two XDR strains (TF274 and R506). As expected, the control strains H37Rv and V4207 were susceptible to the first-line and second-line drugs tested; the MIC values for compounds **1**, **2** and **3** were 0.125-0.25, 0.0156–0.0313 and 0.0039 μg/mL, respectively[9], concentrations that are within a feasible range for translational utility. The MDR strains were resistant to isoniazid and rifampin but susceptible to the second-line drugs tested, and the XDR strains were resistant to all tested drugs[7]. However, the indoleamide compounds exhibited MIC values of ≤1 μg/mL for all strains tested, suggesting that this structure class inhibits *M. tuberculosis* via a novel molecular interaction, and, importantly, that these compounds may be effective against MDR and XDR strains.

To further investigate the *in vitro* anti-mycobacterial activity of these indoleamide compounds, we determined their minimum bactericidal concentration (MBC) values against the H37Rv strain. For compounds **1**, **2** and **3**, the MBC values were 0.25, 0.0313 and 0.0078 μg/mL, respectively. Since compounds **2** and **3** exhibited lower MIC values for all *M. tuberculosis* strains tested than the original hit molecule, we assessed the kill kinetics of these two indoleamide derivatives at concentrations of 4X and 16X the MIC with the H37Rv reference strain. The 4X MIC of both compounds killed at least 4 $\log_{10}$ colony forming units (CFUs) within 3 or 5 days for compounds **2** and **3**, respectively (Fig. 1c), suggesting aggressive bactericidal activity towards *M. tuberculosis*.

Indoleamide physicochemical properties

In addition to their promising *in vitro* bactericidal activity against *M. tuberculosis*, the indoleamides have physicochemical properties that indicate great potential for absorption and permeation as orally available compounds. Namely, they comply with at least three of the four physicochemical parameters defined by the Lipinski "rule-of-five" which predict aqueous solubility and intestinal permeability[11]. All three indoleamide compounds had less than 5 hydrogen bond donors, less than 10 hydrogen bond acceptors, and molecular weights less than 500 g/mole (Fig. 1a,b). In terms of lipophilicity, compound **1** also had a CLogP value of less than 5, while compounds **2** and **3** had CLogP values just above 5. The ease of synthesis coupled with the promising physicochemical properties render these compounds attractive for further development as novel anti-tuberculosis drugs.

Furthermore, we assessed the potential cytotoxicity of our indoleamide compounds on mammalian cells using the Vero cell line. The half maximal inhibitory concentration (IC_{50}) value for Vero cell viability was high for all three tested compounds (>64 μg/mL for compounds **1** and **2**, and 16 μg/mL for compound **3**), indicating that they were non-toxic in this model system. Their low MIC values and toxicity profiles resulted in very high selectivity index values, ranging from >256 for compound **1** to >2048 for compound **2** and 4000 for compound **3**.

mmpL3 mutation confers resistance to indoleamides

Initial *in vitro* experiments and structural analyses indicated that the indoleamides may represent a promising new anti-*M. tuberculosis* structure class for drug development; however, their bacterial target was unknown. Thus, we selected *M. tuberculosis* colonies with phenotypic resistance to compound **2** by growing the H37Rv reference strain on 7H10 agar plates containing a range of compound concentrations. We obtained one single CFU on a plate containing compound **2** at 8X the MIC. This isolate, referred to as IAR2 (indoleamide-resistant, compound **2**) was able to multiply when inoculated into 7H9 liquid media with the same concentration of compound **2**, indicating IAR2 was a true resistant mutant selected at a frequency of one in 3×10^7 CFUs.

To identify mutations associated with resistance, whole genome sequencing was performed on both the IAR2 and parental H37Rv strains of *M. tuberculosis* using the Ion Torrent Personal Genome Machine platform. We obtained sequences for greater than 95% of each genome with approximately 30X coverage (Table 1), with the average read lengths of 98 and 118 bases for IAR2 and H37Rv, respectively. Relative to the H37Rv parental strain, the IAR2 genome contained a T to A single nucleotide polymorphism (SNP) at position 862 within the *Rv0206c* gene, encoding for MmpL3, a mycolic acid transporter. This SNP, which was further validated by Sanger sequencing, resulted in a serine to threonine missense mutation at position 288 of the cognate protein (Fig. 2a). This exact SNP was identified in 14/14 reads at this allele in the IAR2 genome (Table 2).

We then re-evaluated the MIC values of each of our indoleamide compounds for the IAR2 mutant and found the MIC to be much higher than the parental H37Rv strain (Table 3). The MIC upshift of this structure class ranged from 32 to 64-fold for compounds **2** and **3** to

1024-fold or greater for compound **1**, suggesting that MmpL3, a mycolic acid transporter, is the target of the indoleamide compounds. Interestingly, in the last year, three different compounds have been reported to also target MmpL3: the urea derivative AU1235[12], the pyrrole derivative BM212[13,14], and the diamine SQ109[15] (Fig. 2b). We therefore determined the MIC values of these three compounds for the IAR2 mutant and found that the MIC for each compound was higher for IAR2 than for the parental H37Rv strain (Table 3).

The IAR2 mutant is not cross-resistant to TB drugs

To assess the novelty of the microbial target of the indoleamide scaffold and the possible translational utility of this class of compounds for the treatment of both drug-susceptible and drug-resistant TB, we determined the MIC values of commonly used first-line (isoniazid, rifampin and ethambutol) and second-line (levofloxacin, moxifloxacin, kanamycin, capreomycin and amikacin) TB drugs on the IAR2 mutant and its H37Rv parental strain. All of the tested drugs exhibited the same MIC values for IAR2 as for H37Rv (except for rifampin, which actually had a lower MIC value for the mutant strain, Table 3). These results demonstrate that MmpL3 may be a validated molecular target in *M. tuberculosis* and that the S288T mutation in this target does not result in any cross-resistance to drugs currently used for TB treatment.

An indoleamide inhibits *M. tuberculosis* growth *in vivo*

All of the *in vitro* experiments indicated that our indoleamide compounds may represent a new structure class active against a membrane transporter in *M. tuberculosis* (MmpL3) that is not targeted by existing TB drugs, prompting evaluation of the activity during *in vivo* infection. As compound **3** exhibited a dose-dependent mycobactericidal effect *in vitro*, we analyzed the effect of administration of this most potent compound to *M. tuberculosis*-infected mice. Female BALB/c mice were infected by aerosol with *M. tuberculosis* H37Rv (day 1 implantation of 3.0 $\log_{10}$ CFU/lung), and two weeks after infection, when the bacterial burden was 6.5 $\log_{10}$ CFU/lung, compound **3** was administered daily to the mice by oral gavage at doses of 33, 100 and 300 mg/kg. After four weeks of treatment, the lung CFU counts were significantly lower in mice receiving any dose of compound **3** compared to untreated mice, and the bacterial burden in the lungs declined in a dose-dependent manner (Fig. 3, Table 4). Pharmacokinetic studies indicate that the 100 mg/kg dose results in a maximum concentration of 0.49 μg/mL in plasma and 2.47 μg/g in the lungs (Table 5), well above the *in vitro* MIC value of 0.0039 μg/mL. Furthermore, in both plasma and lung, the concentration of compound **3** remained above the MIC for nearly 24 hours (Fig. 4). These data indicate that compound **3** is orally bioavailable in the mice and active against *M. tuberculosis in vivo*.

Discussion

New drugs for the treatment of TB, including those that are effective against MDR- and XDR-TB, are greatly needed in the global effort to control this deadly disease. Whole-cell phenotypic screening has been demonstrated to be an effective method for the identification of novel structural classes of antimicrobial compounds, and in fact has proven more likely to

generate lead compounds than rationale drug-design approaches[11]. However, appreciable limitations of this method include the lack of information regarding the target(s) of compounds, *in vivo* availability and tolerability. While the former limitation does not necessarily preclude the forward development of hit compounds, knowledge of the target(s) allows for effective lead optimization, providing a molecular basis for structure-activity relationship analyses and also indicating potential pathways for toxic activity within eukaryotic cells. The latter limitation is critical, and the demonstration of safe *in vivo* activity of a compound is absolutely essential for its continued development. Here, we describe a new structural class, the indoleamides, with promising activity against *M. tuberculosis*. Importantly, we have both identified the mycobacterial target and demonstrated *in vivo* availability and efficacy of this chemotype, overcoming two of the major hurdles in preclinical drug development.

Using the original hit compound **1** (Fig. 1a) identified from high-throughput screening, as well as two additional derivatives of this molecule (compounds **2** and **3**, Fig. 1b), we demonstrated that these indoleamides were highly active against drug-susceptible, MDR and XDR *M. tuberculosis* strains[9], suggesting that these molecular entities may interact with a novel mycobacterial target. Indeed, the whole genome sequencing of an *in vitro*-selected mutant resistant to compound **2** revealed a mutation in the gene encoding for the mycolic acid transporter MmpL3 (Fig. 2a). Although currently not the known target of any licensed drug, MmpL3 has recently been identified as the target of several anti-mycobacterial compounds, strongly indicating that this transporter represents a *bona fide* target for anti-tuberculosis drug development. Our indoleamide-resistant mutant, IAR2, exhibited full sensitivity to currently used first- and second-line TB drugs (Table 3), indicating a lack of cross-resistance. Importantly, we also demonstrated that an indoleamide derivative (compound **3**) was orally bioavailable and active against *M. tuberculosis* in a mouse model of TB (Fig. 3). These studies suggest that the indoleamide structural class represents a valuable source of possible agents effective against both drug-susceptible and drug-resistant TB. Interestingly, the indoleamide structural class was also identified to be active on *M. tuberculosis* by an independent group[16], verifying the antitubercular property of this class.

The mycobacterial MmpL proteins belong to the resistance, nodulation and [cell] division (RND) family of membrane transporters[17]. RND family proteins are known to mediate the transport of a wide variety of substrates, including antimicrobial compounds, across cell membranes, and are also established as virulence factors for several bacterial pathogens[18]. *M. tuberculosis* strains encode up to 14 known MmpL family proteins, of which MmpL3 has been the least characterized due to difficulties in deleting its cognate gene, suggesting essentiality for the microorganism[17, 19, 20]. Interestingly, MmpL3 has recently been identified as the target for a number of structurally distinct compounds: the pyrrole derivative BM212[13, 14], the urea derivatives AU1235[12] and 1-adamantyl-3-heteroaryl ureas[21], the diamine SQ109[15] (Fig. 2b) and tetrahydropyrazolo[1,5-*a*]pyrimidine-3-carboxamide and *N*-benzyl-6',7'-dihydrospiro[piperidine-4,4'-thieno[3,2-*c*]pyran] analogues[22]; these studies have also revealed a role for MmpL3 in the transport of mycolic acids across the *M. tuberculosis* cell membrane. The molecular mechanisms involved in mycolic acid synthesis and assembly of the cell wall are well-appreciated molecular targets

for both growth inhibition and killing of mycobacteria, being affected by key TB drugs including isoniazid and ethambutol[23]. Thus, our finding that the indoleamide scaffold targets MmpL3 further corroborates the accumulating evidence that compound-based interactions with this protein interfere with *M. tuberculosis* growth. That we were able to target MmpL3 with an orally bioavailable compound suggests real translational possibility for the indoleamide structural class.

Our indoleamide-resistant *M. tuberculosis* strain, IAR2, was derived *in vitro* in the presence of compound **2**, and we found that this strain contained a SNP in the gene encoding for MmpL3 resulting in an S288T amino acid change, which is predicted to occur in the fourth trans-membrane domain of the transporter (Fig. 2a). This alteration in MmpL3 was associated with decreased susceptibility to all of the indoleamides (compounds **1**, **2** and **3**), and interestingly also resulted in decreased susceptibility to the other known MmpL3-targeting compounds SQ109 and AU1235, and possibly BM212, as the increase in MIC value was only 2-fold (Table 3). *In vitro*-selected *M. tuberculosis* mutants resistant to these compounds were found to have different MmpL3-associated mutations, as illustrated in Fig. 2a. Thus, it is intriguing that the S288T mutations conferred resistance to these compounds. However, it is possible that this amino acid substitution in the trans-membrane domain of MmpL3 alters the transporter structure in such a way that SQ109, BM212 and AU1235 cannot adequately access their targets within the protein. It would be of great interest to determine if the *M. tuberculosis* strains resistant to these compounds are also resistant to the indoleamides.

Certainly, our work provides further validation that MmpL3 is a viable target for anti-TB drug development. Furthermore, we demonstrated that the IAR2 mutant was fully susceptible to the commonly used first- and second-line TB drugs (Table 3). Considering that the AU1235-resistant mutant described by Grzegorzewicz and colleagues was also susceptible to the currently approved TB drugs[12], our data strongly suggest that targeting MmpL3 is a valid strategy for the treatment of drug-resistant TB.

A key finding in our work is that the indoleamide structure class exhibited oral bioavailability and effectiveness *in vivo* in a mouse model of TB, thus demonstrating that these two large obstacles of high-throughput screening-based drug development can likely be overcome with members of this structure class. Moreover, lead optimization could result in increased *in vivo* activity of this group. The compound SQ109, which was identified from a phenotypic compound screen of a directed combinatorial library, has been shown to also be a very promising agent that also targets MmpL3, that was proven to be safe and well-tolerated in Phase I and early Phase II clinical trials[24, 25]. Our identification of an additional MmpL3-targeting class of compounds considerably bolsters the SQ109 work and could be developed in a complementary context, providing another effective, orally available option for TB treatment. Furthermore, it would be incredibly beneficial to examine whether combination of these two compounds could provide a synergistic effect for the complete inhibition of this essential target.

In summary, we have identified a novel structural class, the indoleamides, which interact with a validated target in *M. tuberculosis*, the MmpL3 transporter, and show vigorous

activity against both drug-susceptible and drug-resistant (including MDR and XDR) *M. tuberculosis* strains. Our studies build upon and complement new and exciting findings in this field and strongly suggest that the indoleamides have serious translational potential for development into a real tool for TB treatment and control.

Methods

Chemistry

Starting materials, reagents, and solvents were purchased from commercial suppliers and used without further purification unless otherwise stated. Anhydrous dichloromethane (CH_2Cl_2) was obtained by distillation over calcium hydride. Thin layer chromatography (TLC) was performed with Merck 60 F254 silica gel plates. Flash chromatography was performed using CombiFlash Rf system with RediSep columns or alternatively using Merck silica gel (40–60 mesh). Final compounds were purified by preparative high performance liquid chromatography (HPLC) using an ACE 5-AQ (21.2 mm × 150 mm) column, with detection at 254 and 280 nm on a Shimadzu SCL-10A VP detector, flow rate = 17.0 mL/min. Method 1: 50–100% CH_3OH/H_2O in 30 min; 100% CH_3OH in 5 min; 100–50% CH_3OH/H_2O in 4 min. Method 2: 25–100% CH_3OH/H_2O in 30 min; 100% CH_3OH in 5 min; 100–25% CH_3OH/H_2O in 4 min. Method 3: 15–100% CH_3OH/ H_2O in 30 min; 100% CH_3OH in 5 min; 100–15% CH_3OH/H_2O in 4 min. Both solvents contained 0.05 vol % of trifluoroacetic acid (TFA). The purities of all final compounds were determined to be >95% using the Agilent 1100 HPLC system with a Synergi 4 μm Hydro-RP 80A column, on a variable wavelength detector G1314A. Sequence: Flow rate = 1.4 mL/min; gradient elution over 20 minutes, from 30% CH_3OH-H_2O to 100% CH_3OH (both solvents contained 0.05 vol % of TFA). ^1H nuclear magnetic resonance (NMR) and ^{13}C NMR spectra were recorded on a Bruker spectrometer at 400 MHz and 100 MHz, respectively, with tetramethylsilane (TMS) as an internal standard. ^1H and ^{13}C chemical shifts are reported in δ (parts per million). Standard abbreviations indicating multiplicity were used as follows: s = singlet, d = doublet, dd = doublet of doublets, t = triplet, q = quadruplet, m = multiplet and br = broad. High-resolution mass spectroscopy (HRMS) experiments were performed on Q-TOF-2TM instrument (Micromass).

Synthesis of compounds

The target compounds were synthesized by employing an efficient amide coupling protocol. To a solution of 4,6-dimethyl-1*H*-indole-2-carboxylic acid (1 equiv) in anhydrous CH_2Cl_2 (4 mL/mmol) at room temperature were added anhydrous hydroxybenzotriazole (HOBt, 1 equiv) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC•HCl, 1 equiv) under an argon atmosphere. After stirring for 10 min, the appropriate substituted amine (1 equiv) and triethylamine (1.5 equiv) were added, and the reaction mixture was stirred at room temperature until disappearance of the starting material (usually 12 to 16 h). After this time water (2 mL) was added, and the mixture was extracted with ethyl acetate (EtOAc) (3 × 10 mL), the combined organic layers were separated, washed with brine, dried over anhydrous sodium sulfate, filtered, and concentrated under reduced pressure. The residue was subjected to flash chromatography (EtOAc–hexane 1:4) to obtain the indole-2-

carboxamides in good yields prior to further HPLC purification. Structure-activity relationships of these compounds are reported in Onajole *et al*.[9]

Bacterial strains

Wild type *M. tuberculosis* H37Rv lab strain was obtained from the Johns Hopkins Center for Tuberculosis Research laboratory stocks. The KwaZulu-Natal clinical isolates used in this study were a kind gift from Dr. William R. Jacobs, Jr., at the Albert Einstein College of Medicine.

MIC and MBC assays

MIC was determined using microplate alamar blue assay[7, 8]. Plates were then read using a fluorescence microplate reader at 544 ex/590 em. Percentage inhibition was calculated based on the relative fluorescence units and the minimum concentration that resulted in at least 90% inhibition was identified as MIC. For this assay, 7H9 broth without Tween-80 was used as the assay media.

For MIC and MBC determination using tube-broth dilution methods, compounds **1**, **2** and **3** were 2-fold serially diluted at a volume of 2.5 mL in 7H9 without Tween-80. Mid-log phase H37Rv culture was diluted, and 0.1 mL of the diluted culture containing 10^5 CFUs was added to each of the assay tubes. Media control, positive control (isoniazid) and growth control (no compound) were included. Tubes were incubated at 37 °C. At day 7 and day 14, pellet formation was observed and recorded and the minimum concentration that prevented pellet formation was identified as MIC. The end point CFUs per tube for the treatment was determined on the tubes that did not show pellet on Day 14. The minimum concentration that killed 99% of the inoculum was identified as the MBC.

Kill kinetic assay

M. tuberculosis H37Rv culture was diluted to an OD_{600} of 0.001 and then divided to five of 10 mL aliquots and supplemented with a final concentration of 0.016 μg/mL (4X MIC) or 0.064 μg/mL (16X MIC) of compound **3**, or 0.125 μg/mL (4X MIC) or 0.5 μg/mL (16X MIC) of compound **2**. At day 0, 1, 3, and 5, cultures were diluted and plated. CFUs per mL were enumerated after 4 weeks of incubation.

Cytotoxicity assay

Vero cell linage (ATCC CCL-81) was grown in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS). Flat-bottomed 96-well plate was seeded with 4×10^4 cells. The plate was incubated at 37 °C with 5% CO_2 for 16 h. For compound preparation, 2-fold serial dilution was made using a deep-well block using DMEM containing 5% FBS with a volume of 200 μL. Culture media was replaced with 160 μL of the compound-containing media, with 100% DMSO as positive (100% kill) control and media only as blank (100% viability) control. The plate was incubated for 72 h and then washed twice with PBS before adding 100 μL of DMEM with 5% FBS medium freshly mixed with 10% alamar blue. The plate was incubated for 2 h and then immediately read with a fluorescence microplate reader at 544Ex/590Em. The minimum concentration that killed at least 50% of the cells was identified as IC_{50}.

Selection of indoleamide-resistant mutant

To select for resistance, 7H10 agar plates containing 2X, 4X, 8X and 16X MIC of compound **2** were prepared. Late log phase *M. tuberculosis* H37Rv culture (OD_{600} approximately 1.0) was spread on these plates and incubated at 37 °C for 4 weeks. Colonies were recovered and propagated in 7H9 broth containing correspondent level of the compound.

Deep sequencing and target identification

Genomic DNA was isolated from both the parental wild type (H37Rv) and the resistant mutant (IAR2) strain by using the lysozyme and cetyltrimethylammonium bromide in glucose-tris-EDTA buffer methods. 5 μg DNA was subjected to Covaris S2 DNA shearing system to prepare DNA fragments. The library was prepared and enriched by using the Ion OneTouch and Ion OneTouch Template Kit systems. Enriched template-positive Ion Sphere Particles was sequenced using the Ion Torrent Personal Genome Machine following the Ion 316 Chip protocol and the Ion Sequencing Kit User Guide v2.0 (Life Technologies). After on-machine filtering, all reads were tempted to be aligned to the published *M. tuberculosis* H37Rv sequence[26] by using the Burrows-Wheeler Aligner algorithms[27]. SNPs were analyzed and called by the GATK package.

Mouse aerosol infection and monotherapy model

Four-to-six-week-old female BALB/c mice were aerosol-infected with *M. tuberculosis* H37Rv. From 14 days after infection, group of five mice were treated with 33.3, 100 and 300 mg/kg of compound **3** by oral gavage, daily (5 days per week). Isoniazid at 10 mg/kg was administered as positive control. Infected but untreated mice were negative control. At day −13, 0, 7, 14, and 28 from treatment start, 5 mice from each treatment were sacrificed and the lungs removed. The lungs were bead-beaten to homogenate, diluted and plated on 7H11 selective agar plates. All animal procedures were approved by the Institutional Animal Care and Use Committee of the Johns Hopkins University School of Medicine.

In vivo pharmacokinetic evaluation

Female BALB/c mice (20 g each, Charles River Laboratories) were given a single dose of compound **3** at 100 mg/kg by oral gavage in a volume of 0.2 mL. At 0.125, 0.25, 0.5, 1, 2, 4, 8 and 24 h after compound administration, animals (n=3 per time point) were euthanized and cardiac blood (~0.7 mL) was collected. Mouse lungs were removed, weighed and stored at −80 °C. Plasma was separated by centrifugation at 12,000 × *g* for 20 min at 4 °C and stored at −80 °C. Mouse lungs were homogenized by bead-beating in 0.5 mL of liquid chromatography/mass spectrometry (LC/MS) water and supernatants were recovered by centrifugation at 4 °C for 20 min. Concentrations of compound **3** in plasma and lung homogenate supernatants were analyzed with LC-tandem MS (LC-MS/MS, AB SCIEX QTRAP 5500 system) with compound **2** as internal standard. MS detection of mass transitions 299.01/146.1 and 299.01/131.1 was carried out. Concentration calculation was done with MultiQuant Software (Version 2.1, AB SCIEX). The pharmacokinetic profile of the test compound was analyzed from plasma and lung concentration-time data after oral administration. The peak concentration (C_{max}), the time of peak (T_{max}), and the area under

the concentration curve from time 0 to 24 h ($AUC_{0–24}$) were calculated by using GraphPad Prism 4.

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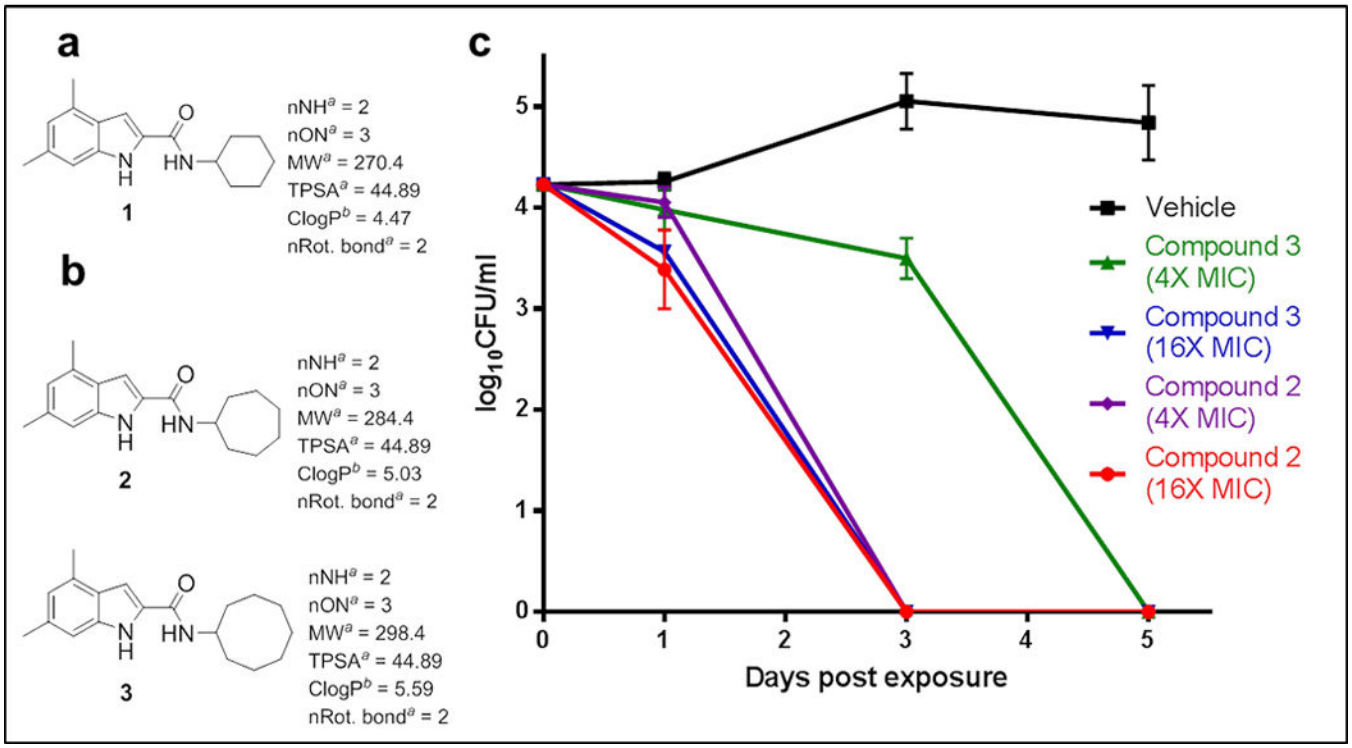

Figure 1. Indoleamide compounds are active *in vitro* against *Mycobacterium tuberculosis*

(**a**) Structure of compound **1**, the initial hit indoleamide. (**b**) Structures of compounds **2** and **3**, derivatives of compound **1**. (**c**) *In vitro* kill curve of *M. tuberculosis* exposed to 4X and 16X MIC of the indoleamide derivative compounds **2** and **3**. Data are presented as mean ±S.E.M. (n=3). nNH, number of hydrogen bond donors; nON, number of hydrogen bond acceptors; MW, molecular weight; TPSA, topological polar surface area; nRot. bond, number of rotatable bonds; MIC, minimum inhibitory concentration.[a]Calculated using molinspiration online service;[b]Calculated using ChemDraw Ultra 13.0, CambridgeSoft.

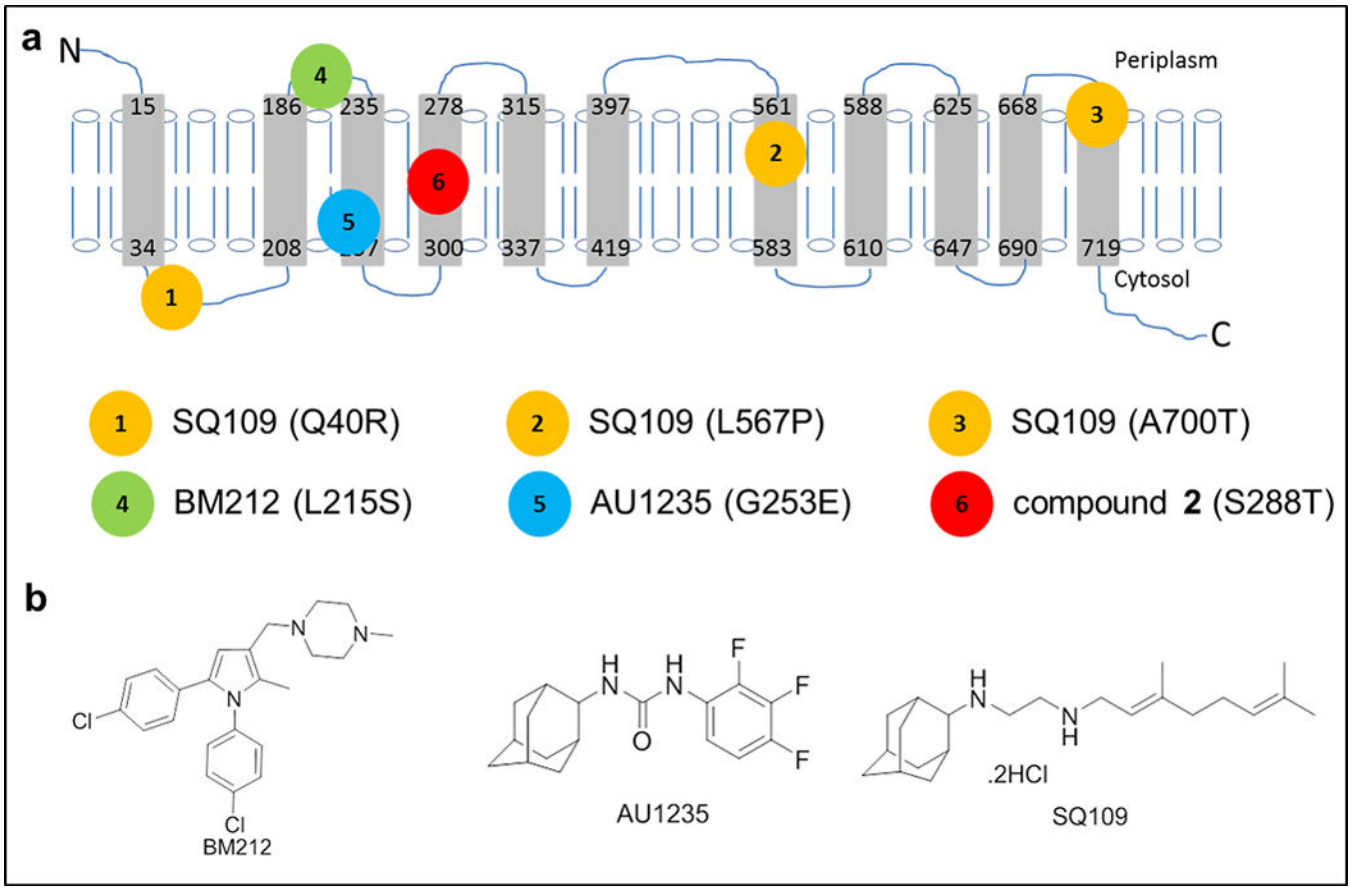

Figure 2. MmpL3 is a validated target in *Mycobacterium tuberculosis*

(**a**) Illustration of the topology of the MmpL3 mycolic acid transporter protein in the *M. tuberculosis* inner membrane. Colored circles represent the locations of amino acid changes associated with resistance to compounds known to target this protein: the diamide SQ109[15], the pyrrole derivative BM212[13], and the urea derivative AU1235[12]. (**b**) Structures of BM212, AU1235 and SQ109.

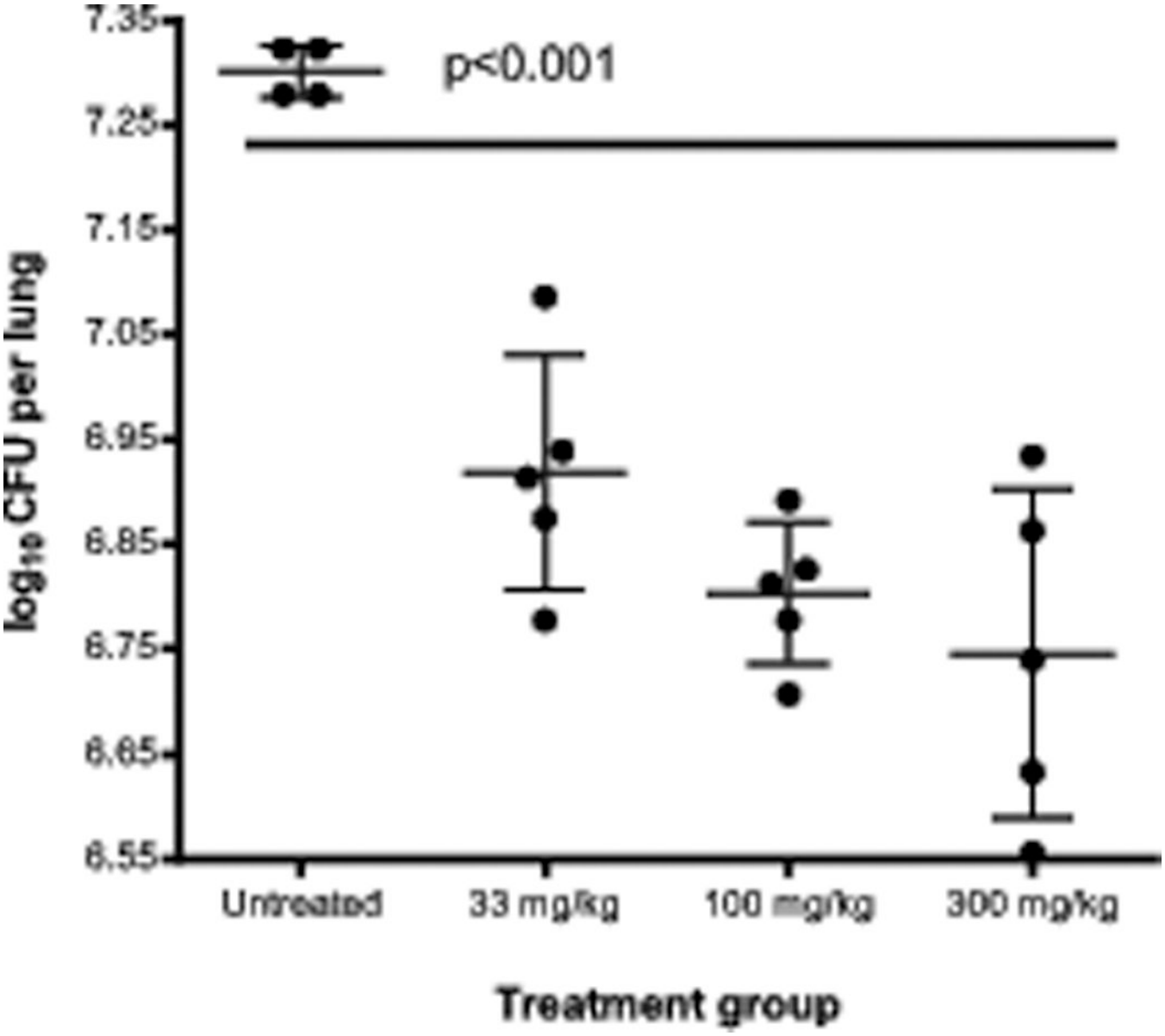

Figure 3. The indoleamide compound 3 is active against *Mycobacterium tuberculosis* in a dose-dependent manner during *in vivo* infection of BALB/c mice

Lung CFU counts were assessed 4 weeks after starting daily oral administration of compound **3**. Each dot represents CFUs from the lungs of an individual mouse, and the bars indicate mean±S.D. CFU counts in each group (n=5 for treated groups and n=4 for untreated control because of one accidental death prematurely). Statistical significance was assessed using the one-way ANOVA with Tukey's multiple comparison test. CFU, colony forming unit.

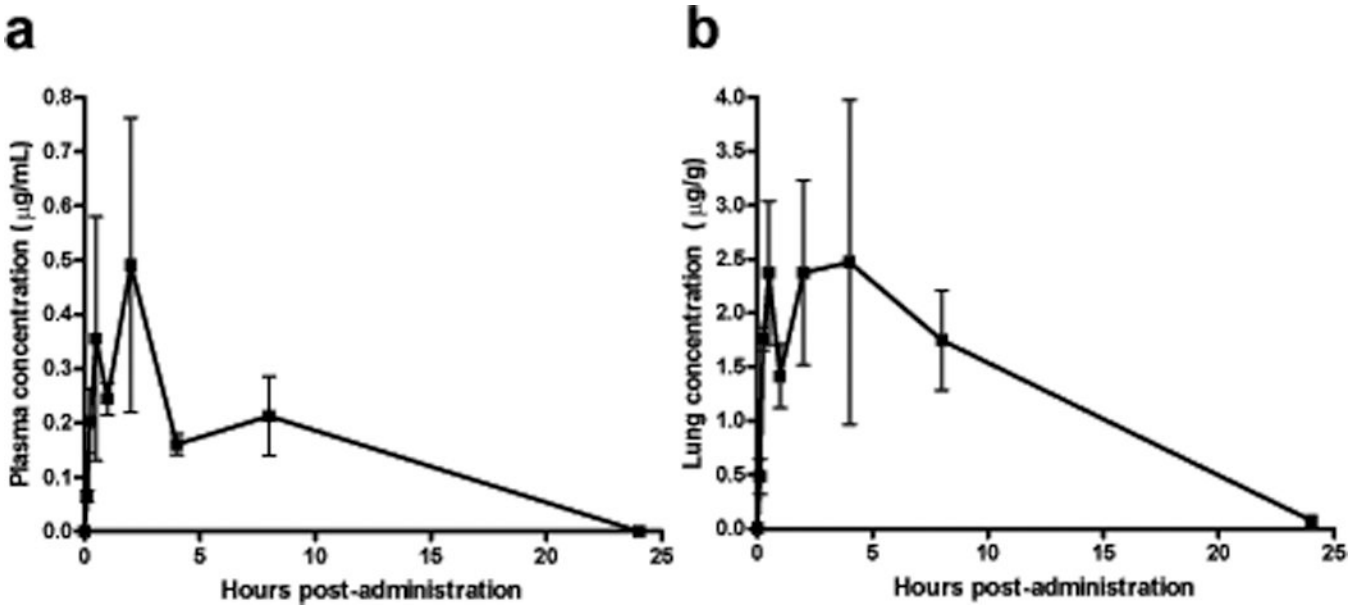

Figure 4. Pharmacokinetic analysis of compound 3 in female BALB/c mice

(**a**) Concentration in plasma and (**b**) concentration in lung following a single 100 mg/kg dose administered by oral gavage. Data are presented as mean±S.E.M. (n=3).

Table 1

Summary statistics of whole genome sequencing.

Sample	Chip	Total Bases	AQ17	AQ20	Perfect	Percent Coverage	Average Coverage Depth	SNPs	Indels	Gaps
H37Rv	314	50.41	40.73	36.97	32.48	98%	11.43X	81	41	687
	316	131.43	105.54	94.00	81.71	96%	29.80X	79	10	1831
IAR2	314	38.52	33.81	31.30	28.73	99%	8.73X	82	26	559
	316	154.07	126.29	113.06	103.64	98%	34.94X	89	14	1236

Sequencing was performed using the Ion Torrent Personal Genome Machine platform. Each genome was sequenced twice. The reference sequence for the annotation of both strains is the published *M. tuberculosis* H37Rv genome, NCBI Reference Sequence NC_000962[26].

Chip, Ion Torrent semiconductor chip type; Total Bases, total mega bases of DNA sequenced; AQ17, mega bases of DNA with one mismatch in the first 50 bases relative to the reference strain; AQ20, mega bases of DNA with one mismatch in the first 100 bases relative to the reference strain; Perfect, mega bases of DNA with perfect alignment relative to the reference strain; SNPs, Single nucleotide polymorphisms relative to the published reference genome Indels, Insertions/deletions relative to the published reference genome; Gaps, Gaps in the complete sequence relative to the published reference genome.

Table 2

Single nucleotide polymorphisms identified in the *Mycobacterium tuberculosis* IAR2 isolate

SNP Description	SNP/Coverage	Locus Tag	Gene Name	SNP Class	AA Change
A 246,457 T	14/14	Rv0206c	*mmpL3*	Missense	S 288 T
A 340,613 G	2/2	Rv0280	*PPE3*	Missense	D 417 G
C 1,655,844 T	2/2	Rv1468c	*PE_PGRS29*	Missense	S 293 N

The reference sequence for the annotation of both strains is the published *M. tuberculosis* H37Rv genome, NCBI Reference Sequence NC_000962[26]. In addition to the SNP in *mmpL3*, two other SNPs were identified, but only with 2 sequence reads each.

SNP Description, the position of the SNP relative to the reference genome with the reference base to the left of the position and the observed base to the right; SNP/Coverage, the number of times the described SNP was observed over the total number of transcripts covering that allele; AA amino acid.

Table 3

MIC of indoleamides and three additional compounds reported to target the MmpL3 mycolic acid transporter.

Compound	MIC (μg/mL) for H37Rv	MIC (μg/mL) for IAR2	Fold change in MIC for IAR2
compound **1**	0.125-0.25	≥128	≥(512–1024)
compound **2**	0.0156–0.0313	1	32–64
compound **3**	0.0039	0.25	64
AU1235	0.0313–0.0625	>64	>(1024–2048)
SQ109	0.25	4	16
BM212	2	4	2
Isoniazid	0.04	0.04	0
Rifampin	0.125	0.03125	0.25
Ethambutol	1	1	0
Levofloxacin	0.25	0.25	0
Moxifloxacin	0.0625-0.125	0.0625-0.125	0
Kanamycin	2	2	0
Capreomycin	1	1	0
Amikacin	1	1	0

MIC, minimum inhibitory concentration

Table 4

Bacterial burden in mouse lungs.

	Mean lung CFU counts (standard deviation) at the following time points:				
Treatment	**Day -14**	**Day 0**	**Day 7**	**Day 14**	**Day 28**
Untreated	2.971 (0.039)	6.545 (0.046)	7.136 (0.285)	6.936 (0.366)	7.300 (0.025)
Isoniazid (10 mg/kg)	---	---	5.508 (0.124)	5.266 (0.089)	4.561 (0.088)
Compound **3** (33.3 mg/kg)	---	---	7.184 (0.244)	7.001 (0.206)	6.919 (0.112)
Compound **3 (**100 mg/kg)	---	---	6.902 (0.243)	7.122 (0.148)	6.803 (0.068)
Compound **3** (300 mg/kg)	---	---	6.768 (0.329)	6.981 (0.305)	6.746 (0.157)

Mean colony forming unit (CFU) counts from the lungs of *M. tuberculosis*-infected mice before and during treatment with compound **3**. Five mice per group were sacrificed at each time point, except for untreated control at Day 28, which was four mice because of an accidental death prematurely. Day -14 represents the day after infection, and day 0 represents the day of treatment initiation. Drugs were administered daily (5 days per week) by oral gavage.

Table 5

In vivo pharmacokinetic parameters of compound 3 in female BALB/c mice.

	C_{max}(SEM)	T_{max}	$AUC_{0–24}$
Plasma	0.49 (0.271) μg/mL	2.00 h	3.71 mg·h/L
Lung	2.47 (1.507) μg/g	4.00 h	31.40 mg·h/kg

A single 100 mg/kg dose of compound **3** was administered to 24 mice (3 per time point). Plasma and lung concentration of compound **3** was determined by liquid chromatography-tandem mass spectrometry.

C_{max}, maximum concentration; T_{max}, time to maximum concentration, $AUC_{0–24}$, area under the concentration curve during the first 24 hours post-administration; SEM, standard error of the mean.