Whole cell screen of fragment library identifies gut microbiota metabolite indole propionic acid as antitubercular

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Running title: in vivo antitubercular activity of IPA

Keywords: tuberculosis, fragments, indole propionic acid, gut microbiota

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ABSTRACT Several key tuberculosis drugs including pyrazinamide, with a molecular weight of 123.1 g/mol, are smaller than the usual drug like molecules. Current drug discovery efforts focus on the screening of larger compounds with molecular weights centered around 400-500 g/mol. Fragment (molecular weight < 300 g/mol) libraries have not been systematically explored for antitubercular activity. Here we screened a collection of 1000 fragments, present in the Maybridge Ro3 library, for whole cell activity against *Mycobacterium tuberculosis*. Twenty-nine primary hits showed dose-dependent growth inhibition equal or better than pyrazinamide. The most potent hit, indole propionic acid (IPA, 3-(1H-indol-3-yl)propanoic acid), a metabolite produced by the gut microbiota, was profiled *in vivo*. The molecule was well tolerated in mice and showed adequate pharmacokinetic properties. In an acute mouse model of tuberculosis infection, IPA reduced the bacterial load in the spleen 7 fold. Our results suggest that IPA should be evaluated as an add-on to current regimens and that fragment libraries should be further explored to identify antimycobacterial lead candidates.
INTRODUCTION

Tuberculosis (TB) remains a global health threat killing 1.34 million people in 2016 (1). The high prevalence of drug resistant *Mycobacterium tuberculosis* strains is a medical urgency and calls for the development of new drugs against TB (2, 3). Some of the key tuberculosis drugs, discovered by whole cell or animal model screening such as pyrazinamide, are ‘dirty fragments’ (4). They hit multiple targets in different pathways and their molecular weights are in the range of 100–300 g/mol. This type of mechanism of action, polypharmacology, and physicochemical properties, very small and reactive, are at odds with mainstream antibacterial drug discovery. In addition, several fragment-like TB drugs are activated inside *M. tuberculosis* to generate reactive and promiscuous metabolites. For medicinal chemists however, attractive leads should inhibit a single target to facilitate lead optimization, be large enough to bind a target with high affinity, and not be reactive to minimize side effects (4).

We recently argued that the success of small dirty drugs in tuberculosis chemotherapy suggests that fragment-based whole cell screens should be ‘re’-introduced in our current antimycobacterial drug discovery efforts (4, 5). The physicochemical properties of fragments, small and moderately lipophilic, may be useful for achieving in vivo exposure and tissue penetration (6). In the case of mycobacteria, these physicochemical properties likely also have a positive impact on bacterial cell penetration: fragments might more easily penetrate the double membrane mycobacterial cell envelope since porins, the channels spanning the outer membrane, prefer small hydrophilic molecules (7, 8). Thus, fragments may have multiple advantages over larger molecules: favorable absorption and systemic pharmacokinetic properties, favorable tissue distribution and better bacterial uptake (4).
Here we carried out such a screen of a collection of fragment-sized compounds typically used for structure-based lead discovery (9) against *M. tuberculosis*. We identified a series of whole cell active hits and tested the most potent compound in the mouse.

**RESULTS**

**Whole cell screen and hit confirmation.** To screen the Maybridge library of fragment like molecules for growth inhibition, we employed a simple and robust *M. tuberculosis* screening assay in 96-well format using turbidity measurement as readout. A primary single point screen was performed with two biological replicates. Compounds that showed at least 70% growth inhibition were defined as hits (Fig. 1). From 86 primary hits, 64 could be confirmed by the same single point screen using re-ordered solids (Fig. 2). These confirmed hits were subjected to *M. tuberculosis* growth inhibition dose response assays, resulting in 29 whole cell actives with MIC$_{50}$ < 500 µM (Fig. 2, Table 1). This cutoff was chosen based on the MIC$_{50}$ of the fragment-like first line drug pyrazinamide (10).

**Bactericidal activity determination.** Bactericidal activity of the 29 most potent hits was evaluated by treatment of *M. tuberculosis* cultures with the compounds at 2× the cutoff concentration and subsequent CFU enumeration on agar. We observed a 100-fold reduction of viability for half of the whole cell actives, and three hits exhibited a 1000-fold reduction in viability (Table 1).

**Spectrum determination.** Next we assessed the spectrum of activity of the 29 *M. tuberculosis* actives against the non-tuberculous mycobacterial pathogens (NTM) *M. abscessus* and *M. avium* (11), as well as *S. aureus* and *E. coli* (Table 1). A large fraction of fragments active against *M. tuberculosis* showed activity against the two NTM species (Fig. 3). Less overlapping activity was detected for the representatives of gram-positive and gram-negative bacteria (Fig. 3).
Cytotoxicity and hemolysis determination. Cytotoxicity of the 29 hits was measured for two human cell lines, HepG2 and THP-1. Membrane toxicity was assessed by a red blood cell lysis assay. Several of the \textit{M. tuberculosis} actives displayed acceptable cytotoxicity and hemolytic activity with a selectivity index of five or above (Table 1).

\textbf{In vivo profiling of the most potent hit.} The most attractive hit based on \textit{M. tuberculosis} whole cell activity and \textit{in vitro} tolerability (F1 or 3-\((1H\)-indol-3-yl)propanoic acid, also known as indole propionic acid or IPA; MW, 189 g/mol; \textit{clogP}, 2.15; hydrogen bond acceptor, 2; hydrogen bond donor, 2; polar surface area, 53Å (18)) was selected for acute toxicity testing in mice. Three animals were dosed at 100 mg/kg on three consecutive days and monitored for seven days after receiving the last dose. None of the mice showed abnormal behavior over the course of the experiment or gross pathological changes of major organs after termination of the study. \textit{In vivo} pharmacokinetic profiling of IPA revealed adequate exposure relative to potency at 100 mg/kg delivered via the oral route (Fig. 4) with 30-33\% oral bioavailability (Table S1). Plasma levels were above the MIC\textsubscript{50} (concentration that inhibits 50\% of growth) for more than 50\% of the dosing interval. The plasma of control mice that did not receive IPA contained approximately 300 to 1,000 ng/mL of endogenous IPA (see Discussion). Average concentrations and associated standard deviation measured throughout the day in 9 naive mice were 629 +/- 233 ng/mL (Table S2). To evaluate whether \textit{in vitro} activity would translate into \textit{in vivo} efficacy, we employed the acute mouse model of tuberculosis. Mice were infected with a low dose of \textit{M. tuberculosis} by the aerosol route. At 14 days post challenge, the bacterial burden in lungs had reached \(10^5\) CFU, indicating establishment of an acute pulmonary infection (Fig. 5). At this time, disease began to disseminate from the lungs to secondary organs such as the spleen where up to 100 bacilli/animal were detected (Fig. 5). Chemotherapy was initiated 14 days post infection and drug formulations were administered at 100 mg/kg on six consecutive days per week for four
weeks. None of the animals showed signs of adverse events or abnormal behavior over the course of drug treatment, confirming that IPA is well tolerated. The first line drug isoniazid was used as a control, and reduced the bacterial load of lungs and sterilized the spleen as expected within four weeks of monotherapy (Fig. 5) (12). At the end of the experiment, mice that received IPA had a 7-fold lower bacterial load in the spleen (Fig. 5). In conclusion, we demonstrate that one fragment hit, IPA, displayed \textit{in vivo} tolerability, attractive pharmacokinetics, and activity.

\textbf{DISCUSSION}

Current tuberculosis drug discovery efforts focus on the screening of libraries of drug-like molecules, the majority of which have a molecular weight centered around 500 g/mol. Libraries of fragment-sized compounds have not been systematically interrogated for antitubercular activity (4, 5). Here we screened a collection of fragments against \textit{M. tuberculosis} and identified 29 molecules that showed MIC$_{50}$s comparable to or lower than the first-line fragment drug pyrazinamide (10). \textit{In vivo} profiling of the most potent hit, indole propionic acid (IPA, 3-(1H-indol-3-yl)propanoic acid), showed attractive tolerability, adequate pharmacokinetic properties and efficacy in a mouse model of tuberculosis. Interestingly, IPA lowered CFU only in the spleen. The reasons for the apparent organ specific effect of IPA remain to be determined. Possible explanations include unequal tissue penetration (6), differential immune response in spleen and lungs, and/or differential, organ specific, susceptibility of bacilli to IPA as a response to the specific microenvironments experienced by the pathogen (13, 14). It is interesting to note that IPA has been reported to show neuroprotective, antioxidant and anti-amyloid properties (15), and that the compound (SHP-622, VP-20629) is in early clinical development for treatment of the progressive neurodegenerative disease Friedreich’s ataxia.
Intriguingly, IPA is a metabolite produced endogenously by the gut microbiota, and can be detected in the blood of the host (16). Accordingly, we found 0.5 to 1 µg/mL of IPA in the plasma of untreated mice (Fig. 4, Table S2). Recently, Dodd et al. (17) identified the genes in the gut bacterium Clostridium sporogenes that encode production of IPA. Furthermore, the authors identified Peptostreptococcus anaerobius and three strains of Clostridium cadaveris as additional IPA producing gut bacteria. Importantly, it was shown that IPA has effects on intestinal permeability and the innate as well as the adaptive arm of the immune system (17-19). The \textit{in vitro} and \textit{in vivo} antitubercular activity of the compound demonstrated here may suggest an effect of gut microbiota on tuberculosis disease susceptibility, progression and/or severity (20, 21). To what extend the observed \textit{in vivo} activity of IPA is due to its direct antibacterial activity and its host immune modulatory function remains to be determined.

Taken together, we screened a library of 1,000 fragment-like molecules for whole cell activity against \textit{M. tuberculosis} and found that one unoptimized fragment hit, IPA, displayed \textit{in vivo} tolerability and exposure, as well as activity in a tuberculosis mouse model. Our results suggest that fragment libraries should be further explored as a source of chemical starting points. IPA should be evaluated as an add-on to current regimens and as a starting point to deliver more potent analogues. Studies are in progress to characterize the \textit{in vitro} and \textit{in vivo} antibacterial mechanism of action of this fragment compound and to determine \textit{in vivo} synergies with clinically used antituberculosis drugs.

\section*{MATERIALS AND METHODS}

\textbf{Animals and ethics assurance}. Mouse studies were carried out in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, with approval from the Institutional Animal Care and Use Committee of the New Jersey...
Medical School, Newark (CD-1 mice), National University of Singapore’s Institutional Animal Care and Use Committee (BALB/c mice). All animals were maintained under specific pathogen-free conditions and fed water and chow ad libitum, and all efforts were made to minimize suffering or discomfort. Studies in *M. tuberculosis* infected animals were performed in biosafety level 3 facilities approved for the containment of *M. tuberculosis*.

**Chemicals and drugs.** The Maybridge Ro3 library consisting of 1000 chemically diversified fragment compounds in liquid format was purchased from Maybridge USA. RIF, PZA, INH, EMB and Dimethyl sulfoxide (DMSO) were procured from Sigma-Aldrich, USA. All compounds were dissolved in 90% DMSO at 10 mM concentration and stored in aliquots at −80°C until use.

**Screening.** Primary screening was performed using exponentially growing *M. tuberculosis* H37Rv (ATCC#27294) at OD₆₀₀ ~0.4 and a final drug concentration of 1 mM in 96-well flat-bottom clear microtiter plates (Corning). The inoculum was adjusted to a final OD₆₀₀ of 0.05 in Middlebrook 7H9 (Beckton Dickinson) broth medium supplemented with 0.05% Tween 80, 0.2% glycerol, and 10% albumin-dextrose-catalase enrichment. A suspension of 200 µl of inoculum was seeded into the pre-pinned 96-well plates containing 1 µl of 200 mM fragment. RIF (10 µM) and EMB (6 µM) were included in each microtiter plate as positive controls with high and medium potency respectively. The plates were sealed with breathe-easy membrane (Diversified Biotech) and incubated for 4 days at 37°C and 80 rpm orbital shaking. Cell density was determined at 600 nm using a TECAN Infinite 200 PRO Microplate reader and percentage growth inhibition was calculated in reference to the drug-free control containing 0.5% DMSO. Compounds causing >70% growth inhibition in two biological replicates were defined as primary hits.

**Inhibitory and bactericidal activity.** The minimum inhibitory concentrations (MIC) reducing bacterial growth by 50% or 90% were determined as previously described (22).
Briefly, drug concentrations ranging from 1-1000 μM were used to inhibit growth of exponentially growing *M. tuberculosis* H37Rv in 96-well plates. Absorbance at 600 nm was measured after 10 days of incubation at 37°C and 80 rpm orbital shaking. Bactericidal activity was determined by sub-culturing bacilli treated at 1000 μM of respective compounds on Middlebrook 7H11 agar supplemented with 10% oleic acid-albumin-dextrose-catalase enrichment and 0.5% glycerol. Agar plates were incubated for 3-4 weeks at 37°C prior to counting. The absolute log CFU/ml reduction was calculated by subtracting the remaining log CFUs/ml of treated bacilli from the initial inoculum.

**Spectrum of activity.** Exponentially growing *M. bovis* BCG Pasteur (ATCC#335734), *M. smegmatis* (ATCC#700084), *M. avium* (ATCC#35717), *M. abscessus* (ATCC#19977), *Staphylococcus aureus* (ATCC#12600) and *Escherichia coli* (ATCC#25922) were used as assay strains. For *M. bovis* BCG the experimental setup described for *M. tuberculosis* was used. Cultures of *M. smegmatis*, *M. avium*, *M. abscessus* were diluted in Middlebrook 7H9 medium to a final OD of 0.005 and incubated with test compound in clear 96-well flat bottom microtiter plates for 1 day, 3 days and 4 days, respectively. Culture of *S. aureus* and *E. coli* were diluted in LB medium and incubated with test compound overnight. Ten point two-fold serial dilutions of compounds starting from 1 mM concentration were used (5, 22).

**Cytotoxicity.** The human hepatocyte cell line HepG2 (ATCC#HB-8065) and the human monocytic cell line THP-1 (ATCC#TIB-202) were maintained at 5% humidified CO₂ and 37°C in Dulbecco’s Modified Eagle Medium and Roswell Park Memorial Institute medium 1640, respectively. Media were supplemented with 10% heat inactivated fetal calf serum and 2 mM glutamine (all Gibco). THP-1 cells were differentiated using 40 ng/ml phorbol 12-myristate 13-acetate overnight (23).
The cytotoxicity assay was performed as described previously (24). Briefly, 20,000 HepG2 cells or 60,000 THP-1 cells were seeded into wells of 96-well plates and incubated overnight to allow for cell adherence. Cells were exposed to test compounds at concentrations ranging from 62.5-1000 µM for 24 hours. Cell viability was assessed by the CellTiter 96-aqueous One Solution Cell Proliferation kit following the manufacturer’s instruction (Promega). The hemolytic concentration (HC50) of compounds was determined by exposing human red blood cells (RBCs, Interstate Blood Bank, Inc. Laboratory, USA) to drugs at concentrations ranging from 62.5-1000 µM. Cytotoxicity concentration (CC50) were calculated by a non-linear logistic model equation using the GraphPad prism 6 software. Selectivity index (SI) values were defined by the ratio of CC50/MIC50 or HC50/MIC50.

**Pharmacokinetic (PK) analyses.** PK studies were performed in uninfected CD-1 mice after single doses of 3-(1H-indol-3-yl)propanoic acid (IPA) at 5 mg/kg via the intravenous (i.v.) route and 100 mg/kg via the oral route (p.o.), as described previously (25). The i.v. formulation was 5% dimethylacetamide (DMA) / 95% of a 4% Cremophor solution. The p.o. formulation was either 50% PEG400 (polyethylene glycol) / 50% D5W (dextrose 5% in sterile water) to generate a solution, or 0.5% CMC (carboxymethylcellulose) and 0.5% Tween 80 in water to generate a suspension. In the i.v. arm, blood was collected in K2EDTA coated tubes pre-dose, and at 1 min, 15 min, 1, 3, 5, 8 and 24 h post dose. In the p.o. arms, blood was collected pre-dose and at 15 min, 30 min, 1, 3, 5, 8 and 24 h post dose. Plasma was obtained by centrifugation for 10 min at 5,000 rpm and stored at -80°C until analyzed. IPA concentrations were measured as described below. The lower limit of quantification was 5 ng/ml. The PK parameters (area under the curve [AUC0-1 and AUC0-24], peak plasma concentration [Cmax], and half-life [t1/2]) were calculated from mean concentrations using Microsoft Excel (Office 2010; Microsoft Corp., Redmond, WA). AUCs were calculated...
using the linear trapezoidal rule. Half-life and elimination rate constants were calculated by linear regression using semilogarithmic concentration versus time data.

**Analytical Methods.** Neat 1mg/mL DMSO stocks of IPA were first serially diluted in 50/50 Acetonitrile water and subsequently serially diluted in drug free CD1 mouse plasma (K₂EDTA, Bioreclamation IVT, NY) to create standard curves and quality control (QC) spiking solutions. Twenty µL of standards, QC samples, control plasma, and study samples were extracted by adding 200 µL of Acetonitrile/Methanol 50/50 protein precipitation solvent containing the internal standard (10ng/mL Verapamil). Extracts were vortexed for 5 minutes and centrifuged at 4000 RPM for 5 minutes. One hundred µL of supernatant was transferred for high pressure liquid chromatography coupled to tandem mass spectrometry (LC/MS-MS) analysis and diluted with 100 µL of Milli-Q deionized water.

LC/MS-MS quantitative analysis for IPA was performed on a AB Sciex Qtrap 6500+ triple-quadrupole mass spectrometer coupled to a Shimadzu 30ACMP HPLC system, and chromatography was performed on an Agilent Zorbax SB-C8 column (2.1x30 mm; particle size, 3.5 µm) using a reverse phase gradient elution. Milli-Q deionized water with 0.1% formic acid was used for the aqueous mobile phase and 0.1% formic acid in acetonitrile for the organic mobile phase. Multiple-reaction monitoring of parent/daughter transitions in electrospray positive-ionization mode was used to quantify all molecules. MRM transitions of 190.10/130.10 and 455.40/165.20 were used for IPA and the internal standard respectively. Sample analysis was accepted if the concentrations of the quality control samples and standards were within 20% of the nominal concentration. Data processing was performed using Analyst software (version 1.6.2; Applied Biosystems Sciex).

**Animal tolerability and efficacy experiments.** Eight to ten week old female BALB/c mice were maintained in groups of 3 or 4 in individually ventilated cages under specific pathogen free conditions at the National University of Singapore biosafety level-3.
core facility. Food and water were offered *ad libitum*. Test drugs were formulated in equal volumes of polyethylene glycol 400 and 5% glucose and administered at a dose of 100 mg/kg in a volume of 200 µl by oral gavage. Acute toxicity was assessed by dosing groups of 3 mice on three consecutive days followed by a monitoring period of 7 days. Animals were subsequently euthanized by CO₂ to assess gross pathological changes. For *in vivo* efficacy determination of drug candidates, mice were infected with 100-200 CFU *M. tuberculosis* H37Rv using a full body inhalation exposure system (GlasCol). After 14 days, chemotherapy was initiated on 6 days per week for 4 weeks. INH at a dose of 25 mg/kg formulated in 0.25% methyl cellulose served as control. Mice were euthanized at designated time points by CO₂. Bacterial burden of organs was determined by plating serial dilutions of organ homogenates onto Middlebrook 7H11 agar supplemented with 20 µg/ml ampicillin and 10 µg/ml cycloheximide. Colonies were counted after 3-4 weeks of incubation at 37°C.

**ACKNOWLEDGEMENTS**

This research was supported by the Singapore Ministry of Health’s National Medical Research Council under its TCR Flagship grant NMRC/TCR/011-NUHS/2014 and the Center Grant ‘MINE’ Core #4 BSL-3 NMRC/CG/013/2013 to TD and is part of Singapore Programme of Research Investigating New Approaches to Treatment of Tuberculosis (SPRINT-TB, http://www.sprinttb.org) led by Nick Paton and managed by Pauline Yoong. DAN was supported by the Singapore International Graduate Award scholarship SING-2014-2-0626.
Figures

**FIG 1** Whole cell screening of fragments against *M. tuberculosis*. Scatter plot of relative *M. tuberculosis* growth inhibition of the primary screen performed in 96-well format is shown. Fragment compounds (1 mM, open circles) were incubated with exponentially growing *M. tuberculosis* prior to cell density measurement at 600 nm. Growth inhibition of fragments was calculated relative to drug-free control wells. Rifampicin (RIF, 10 µM, red triangles), ethambutol (EMB, 6 µM, blue diamonds) and drug-free controls (green squares). Data points shown are the average of two biological replicates. Standard deviations were < 20%.

**FIG 2** Compound progression flow-chart.

**FIG 3** Activity spectrum of 29 *M. tuberculosis* fragment hits. The Venn diagram shows overlapping activities (MIC$_{50}$ < 500 µM) of *M. tuberculosis* hits with two non-tuberculous mycobacteria (*M. avium* and *M. abscessus*), *S. aureus* and *E. coli*.

**FIG 4** Concentration-time profile of indole propionic acid (IPA) in plasma following intravenous (IV) and oral (PO) administration as indicated. The range of IPA found in control mice is indicated by the grey window. CMC: carboxymethyl cellulose based formulation (suspension); PEG: polyethylene glycol based formulation (solution). The MIC$_{50}$ and MIC$_{90}$ (concentrations that inhibit 50 and 90% of the growth in vitro, respectively) are indicated by dashed lines.

**FIG 5** Evaluation of indole propionic acid (IPA) in mice with acute TB infection. Fourteen days post infection with aerosolized *M. tuberculosis* chemotherapy for 4 weeks was initiated. IPA (100 mg/kg) and INH (25 mg/kg) were given on 6 days/week by oral gavage. Bacterial burden of lungs (A) and spleen (B) at designated time points was determined by plating organ homogenates on agar followed by incubation and colony counting. Data obtained from two independent studies were analyzed by one-way ANOVA multi comparison with Bonferroni posttest. n=4/5; *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001. Shown are data points of
individual mice, means and SD. Numbers above groups reflect mean differences as compared to the drug-free (DF) control at day 42.
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| Fragment # | IUPAC | Antibacterial activity at 1mM (log reduction) | Cytotoxicity screening (µM) |
|------------|-------|---------------------------------------------|-----------------------------|
|            |       | M. tuberculosis | M. bovis | BCG | M. smegmatis | M. avium | M. abscessus | S. aureus | E. coli | THP-1 | HepG2 | RBCs |
| F1         | 3-(1H-indol-3-yl)propanoic acid | 1.76 | 62 | 98 | 477 | 119 | >500 | >500 | >500 | >500 | >1000 | >14.7 | >1000 | >14.7 | >1000 | >14.7 |
| F2         | 1-hydroxy-4-(trifluoromethyl)-2H-chromen-2-one | 1.70 | 179 | 213 | 329 | 476 | >500 | >500 | 191 | 432 | 879.0 | 4.1 | 983 | 4.6 | >1000 | >4.7 |
| F3         | 2-methyl-1H-imidazole-4-carboxamide | 1.87 | 98 | 105 | 134 | >500 | 198 | 304 | >500 | >500 | 850.0 | 8.1 | >1000 | >9.5 | >1000 | >9.5 |
| F4         | methyl isouquinoline-3-carboxylate | 1.98 | 158 | 175 | 143 | 389 | 344 | >500 | >500 | >500 | >1000 | 5.71 | >1000 | 5.71 | >1000 | 5.71 |
| F5         | 6,7,8,9-tetrahydrobenzo[b,d][1,3]oxazines | 1.11 | 62 | 245 | 488 | >500 | >500 | >500 | >500 | >500 | >1000 | >4.1 | >1000 | >4.1 | >1000 | >4.1 |
| F6         | 6H-pyrido[3,4-d]indole | 0.43 | 113 | 97 | 205 | 121 | 302 | 254 | >500 | >500 | 230.0 | 2.4 | 476 | 4.9 | 787 | 12.5 |
| F7         | 6-methyl-4-piperazino-2-(trifluoromethyl)piperazino | 3.02 | 144 | 101 | 246 | 345 | 267 | 423 | >500 | 302 | 478.0 | 4.7 | 345 | 3.4 | >1000 | >14.9 |
| F8         | 5-(4-chlorophenyl)-N,N,N',2-trimethyl-3-furamide | 1.44 | 93 | 135 | 189 | 233 | 169 | 322 | >500 | >500 | 234.0 | 1.7 | 589 | 4.4 | 433 | 3.2 |
| F9         | methyl 3-hydroxy-1-benzo-1H-thiophene-2-carboxylate | 2.09 | 240 | 142 | >500 | >500 | >500 | >500 | 217 | 500 | 657.0 | 4.6 | 544 | 3.8 | >1000 | 7.0 |
| F10        | 5-phenylthiophene-2-carboxylic acid | 1.46 | 97 | 186 | 397 | 345 | >500 | 247 | >500 | >500 | 544.0 | 2.9 | 612 | 3.3 | >1000 | 5.4 |
| F11        | 3,4-(4,5-dimethyl-1H-pyrazole) | 3.06 | 140 | 195 | 308 | 398 | 498 | 271 | >500 | 344 | 765.0 | 3.9 | 945 | 4.8 | >1000 | 5.1 |
| F12        | 6-chloro-2(1H,4-diazepan-1-yl)-1,3-benzothiazole | 2.80 | 104 | 198 | 137 | 221 | 157 | 351 | >500 | 324 | 124.0 | 0.6 | 187 | 0.9 | >1000 | 5.1 |
| Code | Name                                                                 | Species   | Minimum | Maximum | Mean | Standard Deviation | p Value | Fold Change |
|------|----------------------------------------------------------------------|-----------|---------|---------|------|--------------------|---------|-------------|
| F13  | 2-(3-chlorophenoxy) ethanethioamide                                   | F. sp.    | 0.90    | 75      | 211  | 387                | 289     | 500         |
| F14  | isoquinoline-3-carboxylic acid                                        | F. sp.    | 1.97    | 87      | 235  | 193                | 176     | 473         |
| F15  | 4-(4-chlorophenoxy)-3,5-dimethyl-1H-pyrazole                          | F. sp.    | 2.43    | 157     | 235  | 156                | 198     | 174         |
| F16  | 2,3-dimethyl-1-(2-thiobenzyl)-1H-pyrole-3-carboxylic acid             | F. sp.    | 1.89    | 345     | 247  | 500                | >500    | 304         |
| F17  | (6-quinolin-1-yl)pyridin-2-yl)methanamine                             | F. sp.    | 3.07    | 245     | 250  | 345                | >500    | 271         |
| F18  | 2-(2,2,4,7-tetramethyl-1,2,3,4-tetrahydroquinolin-1-yl)ethan-1-ol hydrate | F. sp.    | 1.67    | 86      | 268  | 87                 | 205     | 198         |
| F19  | ethyl 2-aminos-5-methyl-4-phenothiophene-3-carboxylate                | F. sp.    | 1.98    | 117     | 270  | 267                | 491     | 214         |
| F20  | [2,2'-bithiophene]-5-carboxylic acid                                  | F. sp.    | 1.98    | 170     | 271  | 457                | 289     | >500        |
| F21  | 2-methyl-1H-indol-3-amine                                             | F. sp.    | 2.50    | 305     | 281  | 398                | 401     | >500        |
| F22  | 2-fluoro-4-hydroxybenzonitrile                                       | F. sp.    | 1.60    | 140     | 300  | 420                | 487     | 317         |
| F23  | methyl[(2-phenoxypyphenyl)methyl]amine                                | F. sp.    | 1.23    | 347     | 500  | >500               | >500    | >500        |
| F24  | 2-methyl-5-(4-methylphenyl)-3-furoic acid                             | F. sp.    | 2.02    | 120     | 316  | 193                | >500    | 458         |
| F25  | 5-chloro-1-benzothiophene-3-carboxylic acid                           | F. sp.    | 2.10    | 94      | 319  | 367                | 98      | 489         |
| F26  | 4,4'-dihydropyrazine-3-carboxylic acid                                | F. sp.    | 1.15    | 125     | 320  | >500               | >500    | >500        |
| F27  | 6-chlorobenzoylujusazon-3-ol                                           | F. sp.    | 1.05    | 210     | 395  | >500               | >500    | >500        |
| F28  | [2-(4-trifluoromethylphenyl)-1,3-diazep-4-y]methanol                   | F. sp.    | 1.21    | 215     | 434  | 398                | 235     | 325         |
| F29  | 4-phenoxypyphenol                                                      | F. sp.    | 1.09    | 118     | 439  | 387                | 250     | 411         |
aMIC$_{50}$, minimum inhibitory concentration that inhibits bacterial growth by 50%. bTHP-1, monocytic cell line; HepG2, liver cell line; RBC, red blood cells. CC$_{50}$, HC$_{50}$: cytotoxic and hemolytic concentration that kills or lyses 50% of cells relative to untreated control. SI, selectivity index calculated by CC$_{50}$/MIC$_{50}$ or HC$_{50}$/MIC$_{50}$. ‘Bactericidal activity’ shows activity against *M. tuberculosis*. **
Fragment library
1000 compounds

Primary hits, 1 mM, 70% growth inhibition
86 compounds

Confirmed hits, 1 mM, 70% growth inhibition
64 compounds

MIC<sub>50</sub> < 500 μM
29 compounds

NTM
Gram<sup>+</sup>
Gram<sup>−</sup>

In vivo profiling of most potent hit
Figure A: Lungs CFU log$_{10}$

- Day 14: 3.82
- Day 42: -0.28

Figure B: Spleen CFU log$_{10}$

- Day 14: -5.49
- Day 42: -0.82