Identification of the anti-mycobacterial functional properties of piperidinol derivatives

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Supporting information is included.

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Abstract

Background and purpose: Tuberculosis (TB) remains a major global health threat and is now the leading cause of death from a single infectious agent worldwide. The current TB drug regimen is inadequate and new anti-tubercular agents are urgently required to be able to successfully combat the increasing prevalence of drug-resistant TB. The purpose of this study was to investigate a piperidinol compound derivative that is highly active against the *Mycobacterium tuberculosis* bacillus.

Experimental approach: The antibacterial properties of the piperidinol compound and its corresponding bis-Mannich base analogue were evaluated against *Mycobacterium smegmatis* and Gram-negative organisms. Cytotoxicity studies were undertaken in order to determine the selectivity index for these compounds. Spontaneous resistant mutants of *Mycobacterium smegmatis* were generated against the piperidinol and corresponding bis-Mannich base lead derivatives and whole genome sequencing employed to determine genetic modifications as result to selection pressure in the presence of these compounds.

Key results: The piperidinol and the bis-Mannich base analogue were found to be selective for mycobacteria and rapidly kill this organism with a cytotoxicity selectivity index for mycobacteria of >30 fold. Whole genome sequencing of *M. smegmatis* strains resistant to the lead compounds led to the identification of a number of single nucleotide polymorphisms (SNPs) indicating multiple-targets.

Conclusion and implications: Our results indicate that the piperidinol moiety represents an attractive compound class in the pursuit of novel anti-tubercular agents.

Abbreviations:

CFU: colony forming units; C_{lint}: intrinsic clearance; *E. coli*: *Eschericia coli*; Fractional inhibitory concentration (FIC); HIV: human immunodeficiency virus; INH: isoniazid; INDEL: insertion/deletion of bases; Luria-Bertani (LB); MIC: minimum inhibitory concentration; MBC: minimum bactericidal concentration; *M. bovis* BCG: *Mycobacterium bovis* bacillus Calmette-Guérin; *M. smegmatis*: *Mycobacterium smegmatis*; *M. tuberculosis*: *Mycobacterium tuberculosis*; NAT: Arylamine *N*-acetyltransferase; *P. putida*: *Pseudomonas putida*; REMA: resazurin microtiter assay; RIF: rifampicin; SET solution: sucrose-EDTA-Tris-HCl; SNP: single nucleotide polymorphism; TB: Tuberculosis; TE buffer: Tris-EDTA; WT: wild-type

Tables of Links

TARGETS
Enzymes[a]
Arylamine *N*-acetyltransferase

LIGANDS
Piperidinol (compound **1**) SMILE: CN1CCC(C2=CC=CC=C2)(O)C(C(C3=CC=CC=C3)=O)C1 PUBCHEM CID:20784
Bis-Mannich base (compound **2**) SMILE: O=C(CCN(C)CCC(C1=CC=CC=C1)=O)C2=CC=CC=C2 PUBCHEM CID: 408365

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Southan *et al.*, 2016) and are permanently archived in the Concise Guide to PHARMACOLOGY 2015/16 (Alexander *et al.*, 2015)

Introduction

Tuberculosis (TB), caused by the bacillus *Mycobacterium tuberculosis*, is a major global pathogen and has now surpassed human immunodeficiency virus (HIV) to be the leading cause of death from a single infectious agent (WHO, 2015). In 2014, 9 million new cases of active TB were reported and 1.5 million deaths resulted from TB infection, with a significant number of deaths occurring in individuals who are co-infected with HIV and TB (WHO, 2015, Dye and Williams, 2010). The current drug-regimen to treat TB is over 40 years old and is hindered by its long duration, which is typically 6-9 months in the case of drug-sensitive strains and frequently accompanied by toxic side-effects (Zumla *et al.*, 2015). A variety of reasons, including patient non-compliance, have led to the emergence of drug resistant TB strains that are highly resistant to most, if not, all – in the case of totally drug resistant strains of TB – of the current antibiotics that are available (Udwadia *et al.*, 2012, Klopper *et al.*, 2013). Together this highlights the urgent need for the discovery of new antibiotics that can be used to shorten treatment time, tackle the increasing problem of clinical drug resistance and be used in combination with HIV treatments. However, there has been relatively little interest in the pursuit of new therapeutic agents for TB by the pharmaceutical industry, which may be due in part to the greatest burden of disease being present in developing countries (Cole, 2014). Despite this, over the last 10 years there has been an increase in the discovery and development of new TB drugs either through high-throughput phenotypic screens or target-based approaches (Reynolds *et al.*, 2012, Stanley *et al.*, 2012, Lechartier *et al.*, 2014, Christophe *et al.*, 2009). This has resulted in a promising TB drug pipeline (Wallis *et al.*, 2016, Zumla *et al.*, 2013), comprising new antibiotic agents including the accelerated approval of bedaquiline (Andries *et al.*, 2005), which is the first FDA-approved TB drug in 40 years, and delamanid for use in multi-drug resistant TB (Gler *et al.*, 2012), along with new therapeutic regimens (Zumla *et al.*, 2015, Zumla *et al.*, 2013).

The mycobacterial *N*-acetyltransferase (NAT) enzyme has been identified as a potential target for new TB agents (Bhakta *et al.*, 2004). Deletion of the *nat* gene in *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) results in a significant decrease in the mycolates found in the mycobacterial cell wall, with increased antibiotic susceptibility and killing within macrophages (Bhakta *et al.*, 2004, Anderton *et al.*, 2006). In previous work, a high-throughput target-based screen of 5,000 drug-like compounds was undertaken to find specific inhibitors of NAT enzymes (Westwood *et al.*, 2010, Abuhammad *et al.*, 2012, Fullam *et al.*,

2011, Fullam *et al.*, 2013). From this screen the piperidinol derivative: 3-benzoyl-4-phenyl-1-methylpiperidinol (Figure 1), compound **1** was identified as an inhibitor of mycobacterial NAT enzymes and a potent inhibitor of *M. tuberculosis* (Abuhammad *et al.*, 2014, Abuhammad *et al.*, 2012, Westwood *et al.*, 2010, Fullam *et al.*, 2009, Fullam *et al.*, 2008). The unique *in vitro* mechanism of inhibition of compound **1** with the NAT enzyme from *Mycobacterium marinum* has been elucidated and involves a specific covalent modification of a cysteine residue located within the active site of the NAT enzyme (Abuhammad *et al.*, 2012, Abuhammad *et al.*, 2010, Fullam *et al.*, 2009, Fullam *et al.*, 2008).

The aim of this study was to further evaluate the antimicrobial properties of the lead cyclic piperidinol derivative **1** along with its analogous bis-Mannich base derivative **2** (Figure 1) which depending on the *in vivo* physiological conditions encountered may be produced *via* a retro-Aldol mechanism from compound **1**. In order to achieve this, a combination of approaches involving anti-microbial susceptibility testing, cytotoxicity studies, resistance mapping by whole genome sequencing and genetic validation using the non-pathogenic organism *M. smegmatis* have been undertaken. The combined results demonstrate that the piperidinol scaffold **1** and its derivative **2** are specific for the *Mycobacterium genus*, resulting in rapid killing, have an encouraging cytotoxicity selectivity index with a multiple and diverse mechanism of action.

Materials and Methods

Chemical synthesis of 4-hydroxy-1-methyl-4-phenylpiperidin-3-yl)(phenyl)methanone: compound 1 and 3,3'-(methylazanediyl)bis(1-phenylpropan-1-one): compound 2

The synthesis of 4-hydroxy-1-methyl-4-phenylpiperidin-3-yl)(phenyl)methanone (compound **1**) and 3,3'-(methylazanediyl)bis(1-phenylpropan-1-one) (compound **2**) were carried out as described previously (Abuhammad *et al.*, 2012, Wang *et al.*, 2000, Gul *et al.*, 2002, Blicke and Burckhalter, 1942). Full experimental details and characterisation data of the compounds used in this study are in Supplementary Information (Supplementary Figures 1 and 2).

Bacterial strains, cell lines, culture conditions and chemicals

M. smegmatis MC^2155 was routinely grown at 37 ^oC in 7H9 broth (BD Difco) supplemented with 0.2 % glycerol and 0.05 % Tween 80 or Luria-Bertani (LB) medium supplemented with 0.05 % Tween 80. *Escherichia coli (E. coli)* and *Pseudomonas putida (P. putida)* were routinely cultured in LB-medium at 37 ^oC and 30 ^oC respectively. Human alveolar basal epithelial A549 cells (Public Health England, ECACC 86012804) were cultured at 37 ^oC with 5 % CO_2 atmosphere in Ham's F-12K (Kaighn's) Medium (Gibco, UK) supplemented with 10 % fetal-bovine serum, 100 Units/mL penicillin, 100 µg/mL streptomycin, and 250 ng/mL amphotericin B (HyClone). Ovine red blood cells were purchased from TCS Biosciences. All chemicals were purchased from Sigma-Aldrich unless otherwise stated.

Determination of minimum inhibitory concentration

The measurement of the minimum inhibitory concentration (MIC) of compounds **1** and **2** were determined using the resazurin reduction microplate assay (REMA) as described previously (Palomino *et al.*, 2002). *M. smegmatis*, *E. coli* and *P. putida* were grown to mid-log phase and the inoculum standardised to 1 x 10^6 colony forming units (CFU)/mL before addition to the prepared 96-well flat-bottom microtiter plate with 2-fold serial dilutions of each drug in media. An antibiotic control was also added to each plate (rifampicin for *M. smegmatis*, ampicillin for *E. coli* and tetracycline for *P. putida*) and the last column of the plate was used as a control without the addition of compound. In the case of *M. smegmatis* the plates were incubated for 24 h at 37 ^oC without shaking before addition of 25 µL resazurin (one tablet of resazurin (VWR) dissolved in 30 mL of sterile PBS). Following a further incubation at 37 ^oC the plates were assessed for colour development. The MIC values were determined as the lowest concentration of drug that prevented the colour change of

resazurin (blue – no bacterial growth) to resofurin (pink – bacterial growth). The MIC values were determined with 5 independent experimental repeats and data are presented as mean ± SEM.

Determination of the minimum bactericidal activity (MBC)

The MBC was determined by setting up a microtiter plate as performed for the MIC determination. Instead of adding resazurin at 24 h, each well from the microtiter plate was plated on to solid-LB medium and the CFUs determined after a 3-4 day incubation time at 37 ^{o}C. The lowest concentration at which no CFUs were counted was determined to be the MBC. The MBCs were carried out with 5 independent experimental repeats.

Time kill studies

Cultures of *M. smegmatis* (10^5 CFU) were incubated with 2x MBC of compound **1** (625 μg/mL), 2x MBC compound **2** (312.5 μg/mL) and 2x MBC rifampicin (25 μg/mL), along with a no-drug control. Cultures were collected at defined time intervals (0, 30, 60, 90, 120, 180, 240, 300, 360 min and 24 h) and plated on solid LB-media and incubated at 37 ^{o}C for 3 days. Cell viability was assessed by determining the CFUs. The time kill studies were carried out with 5 independent experimental repeats and data are presented as mean ± SEM.

Determination of compound interactions using a REMA checkerboard assay

In order to determine whether the compounds interacted with isoniazid a checkerboard assay was employed. Fractional inhibitory concentrations (FICs) were calculated by use of the following formula: FIC (X + Y) = [MIC of compound X in combination with Y]/[MIC of X alone]. To evaluate interaction profiles the fractional inhibitory index (ΣFIC) was calculated as FIC of compound X + FIC of compound Y where ΣFICs of ≤ 0.5 designate synergistic activity, ΣFICs of ≥ 4.0 indicate antagonism, and values in between correspond to additivity (Rand *et al.*, 1993, Lechartier *et al.*, 2012).

Cytotoxicity assay

The cytotoxicity of the compounds was measured against a human lung epithelial cell line (A549). Briefly, cells were incubated (10^6 cells/well) with 2-fold serial dilutions of compounds (compound **1**: 31.25-500 μg/mL; compound **2**: 62.5-1000 μg/mL) in a 12 well plate (Thermo Fisher #150628), including a cell only control. Following incubation for 24 h

at 37 ^{o}C, in the presence of 5 % CO_2 cell viability was determined by adding 100 μL resazurin for 1 h at 37 ^{o}C and measuring the absorbance of the resofurin metabolite at 570 nm using a BioTek Synergy HT microplate reader. All assays were undertaken with 5 independent experimental repeats and data are presented as mean ± SEM.

Haemolysis assay

The haemolysis activity of compounds **1** and **2** were tested against ovine red blood cells. Stock concentrations of compounds **1** (500 μg/mL) and **2** (1 mg/mL) were prepared in PBS containing 5 % DMSO. The compounds were serially diluted giving final concentrations of compound **1** of 15.6-250 μg/mL and compound **2** of 31.25-500 μg/mL. A positive control comprised lysis buffer (10 mM Tris pH 7.8, 0.32 M sucrose, 5 mM $MgCl_2$, 10 % Triton X-100) and the negative control comprised PBS containing 5 % DMSO. 150 μL of each compound was added to 150 μL of ovine blood and incubated at 22 ^{o}C for 1 h, after which the samples were centrifuged for 5 min (2,000 rpm, 22 ^{o}C). 10 μL of the supernatant was added to 90 μL of PBS in a 96 well flat bottom microtiter plate (Thermo Fisher, #167008) and the absorbance read at 450 nm (BioTek HT Synergy). All assays were undertaken with 5 independent experimental repeats and data are presented as mean ± SEM.

Agglutination assay

The agglutination activity of compounds **1** and **2** were tested against ovine red blood cells. Briefly, 150 μL of ovine blood was incubated with 150 μL compound **1** or **2** (2-fold dilutions: final concentration of compound **1** 15.6-250 μg/mL, final concentration of compound **2** 31.25-500 μg/mL). Separately, 150 μL of 25 % polyethylenimine was added as positive control or 150 μL PBS as a negative control. Following addition of the compounds the ovine blood was incubated at room temperature for 1 h, after which 50 μL of the sample was added to a round bottom 96 well microtiter plate (Corning, #3790), which was then incubated at room temperature for a further 30 min. The plate was then assessed for signs of agglutination. All assays were undertaken with 5 independent experimental repeats and data are presented as mean ± SEM.

Metabolic stability *in vitro*

The intrinsic clearance (CL_{int}) of compounds **1** and **2** were determined using mouse (CD-1) liver microsomes (Gibco). Briefly, microsomes (100 μg final protein concentration) and test

compound in 0.1 M phosphate buffer pH 7.4 were prepared. In parallel an NADPH-regenerating system (Promega) was prepared in 0.1 M phosphate buffer (pH 7.4). The solutions were pre-incubated at 37 °C for 10 min before assessment of the intrinsic clearance was initiated by mixing the two solutions (50 μL of each; final compound concentration 1 μg/ml) at 37 °C. After 0, 5, 10, 15, 20 and 30 min the reactions were terminated by the addition of 100 μL of acetonitrile containing 1 μg/mL verapamil and placed on ice for 30 min. The samples were then centrifuged at 12,000 × *g* for 10 min and analysed by LC-MS to determine the quantity of the parent compound remaining over time. Carbamazepine (1 μg/mL) was used as a control for low intrinsic clearance.

Generation of spontaneous resistant mutants of *M. smegmatis* to compounds 1 and 2.

M. smegmatis resistant mutants were generated by plating 10^8 mid-log cells (OD_{600} 0.6) on LB-agar containing 2.5x MIC of compound **1** and 5x MIC of compound **2**. In the case of compound **1**, following the initial identification of mutants from the initial 2.5x MIC plate the selected colonies were inoculated into liquid LB media containing 2x MIC compound **1** twice before selecting on solid medium containing 2.5x MIC compound **1** and then proceeding as for compound **2**. In the case of compound **2**, following identification, resistant mutants were subsequently inoculated into liquid media in the absence of the compound to mid-log phase and selected on solid LB-agar containing 5x MIC of compound **2**. The colonies were subsequently inoculated into LB-medium in the absence of compound before selecting on LB-agar plate containing either 2.5x MIC of compound **1** or 5x MIC of compound **2** to confirm resistance to the compound. Following this validation step for the generation and identification of spontaneous mutants, the mutant strains and wild-type *M. smegmatis* were inoculated in parallel into 50 mL liquid media containing 2x MIC of the respective compound (no compound was added in the case of the wild-type *M. smegmatis* strain) and grown to mid-log phase. Genomic DNA was prepared (total of 9 samples (7 resistant mutant strains and 2 wild-type strains)) by centrifugation of the culture and resuspension in 25 % sucrose, 50 mM EDTA, 50 mM Tris HCl, pH 8.0 (SET solution) and 50 μL of 20 mg/mL lysozyme and incubated at 37 ^{o}C for 16 h. After this incubation step, 5 μL of 10 mg/mL RNAse A was added and the resuspension incubated at 37 ^{o}C for 30 min, after which 250 μL of Proteinase K solution (400 mg/mL Proteinase K, 100 mM Tris HCl pH 8.0, 0.5 % SDS) was added and incubated at 55 ^{o}C for 2 h. DNA was extracted using phenol-chloroform-isoamyl alcohol (24:24:1), chloroform-isoamyl alcohol (24:1), precipitated with ethanol, dried by air and

resuspended in TE buffer (10 mM Tris, 1 mM EDTA pH 8.0). The amount and purity of the DNA was checked with a NanoDrop (Thermo Scientific).

Genomic DNA from each *M. smegmatis* mutant and wild-type strain was converted into Nextera XT libraries for sequencing according to the manufacturer's instructions with a few modifications. Libraries were prepared using the dual indexing principal and amplified libraries were purified using 25 μL AMPure XP beads and 45 μL of purified library, retained and stored at -20 °C until denaturing for sequencing. Libraries were pooled in equimolar concentrations (determined by analysis on Agilent Bioanalyser 2100 and HS dsDNA qubit assay) and 12 pM sequenced on an Illumina MiSeq platform V2 2x 250 bp paired end protocol. Reads were aligned to the *M. smegmatis* str. MC^2155 (accession number NC_008596.1) using the Burrows-Wheeler Aligner (BWA) MEM algorithm v0.7.5a-r405 (Li and Durbin, 2009). Resulting BAM files were manipulated with SAMtools (Li *et al.*, 2009) to produce mpileup files, with the following parameters 'mpileup -B -f ' .VarScan v2.3 (Koboldt *et al.*, 2012) was used for single nucleotide polymorphism (SNP) and insertion/deletion (INDEL) calling, with the following parameters ' --min-var-freq .80 --p-value 0.05 --min-avg-qual 30'. SNPs that were common to both the wild-type control and the mutant strain were not considered in further analysis. Read data has been submitted to the EBI under the accession number PRJEB15140.

Genetic validation

Genes identified as putative targets from the whole-genome sequencing data were cloned into the replicative vector pMV261 for over-expression in mycobacteria under control of the *hsp60* promoter. The gene of interest was amplified by PCR (Q5 High-Fidelity Polymerase (New England Biolabs) from *M. smegmatis* genomic DNA. The primers used are as shown in Supplementary Table 1. The resulting PCR product was digested with the appropriate restriction enzymes and ligated (T4 DNA ligase, NEB) into the pMV261 vector. The resulting construct was transformed into *E. coli* TOP10 (Invitrogen) and verified by sequencing (GATC Biotech). The resulting constructs were electroporated into *M. smegmatis* along with an empty pMV261 vector control.

Data and analysis

Blinding and randomisation were not applied in these studies. All data are represented by mean ± SEM of at least five individual experiments and analysed using GraphPad Prism

version 7.0a software program (GraphPad Software Inc., San Diego, CA, USA). Statistical significance between different groups was determined by unpaired *t*-test for two groups or one-way ANOVA with Bonferroni's *post hoc* test to compare all pairs of columns for more than two groups. A value of $P < 0.01$ was considered statistically significant. The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology (Curtis *et al.*, 2015).

Results

Antimicrobial properties of compound 1 and 2

Compound **1** has previously been shown to be highly active against *M. tuberculosis* with a MIC of < 5 μg/mL (Abuhammad *et al.*, 2012, Jeney and Zsolnai, 1956). We used the resazurin microplate assay (REMA) to determine the MIC for compound **1** and its bis-Mannich base derivative **2** (Figure 1) against *M. smegmatis*. In addition, in order to determine the specificity of compounds **1** and **2** for the *Mycobacterium genus* these compounds were also tested for their potential to inhibit the growth of the Gram-negative organisms *E. coli* and *P. putida*. The MIC values for compounds **1** and **2** are shown in Table 1 and indicate that the piperidinol class of compounds and its corresponding bis-Mannich base are able to inhibit the growth of *M. smegmatis* with MIC values of 62.5 μg/mL and 125 μg/mL respectively. Whilst the MIC for compounds **1** and **2** are higher than that for *M. tuberculosis* it is clear that these compounds do not have any effect on the growth of *E. coli* or *P. putida* at concentrations as high as 250 μg/mL.

To further understand the inhibitory properties of compounds **1** and **2** the MBCs were determined against *M. smegmatis*. The MBCs were found to be 312.5 μg/mL for compound **1** and ranged between 78-156 μg/mL for compound **2** indicating that both compounds are bactericidal against actively growing *M. smegmatis*. There is good correlation between the MIC and MBC values for compound **2**, whereas compound **1** displayed an MBC 5x greater than its MIC value.

To determine whether compounds **1** and **2** are able to kill *M. smegmatis* completely and the rate at which the antimicrobial activity is exerted, time-kill experiments were conducted in which the reduction of CFUs was quantified as a function of exposure time of *M. smegmatis* to 2x MBC of compounds **1** and **2** over a 24-hour time period. The kill curve is shown in Figure 2. A monophasic kill curve was observed for both compounds **1** and **2** with a 2-log reduction in CFUs after 90 minutes for compound **1** and 3 hours for compound **2** with apparent complete eradication of viable *M. smegmatis* within a 3 hour time period for compound **1** and a 5 hour time period for compound **2**. In comparison, rifampicin was added at 2x MBC and no reduction in CFUs were observed during this time period (Figure 2) (One-way ANOVA: $P < 0.001$).

Compound interaction assessed by a checkerboard assay

To evaluate the impact of potential compound combinations and interactions on *M. smegmatis* a checkerboard assay was used. Isoniazid was serially-diluted two-fold horizontally across the plate (0 μg/mL - 100 μg/mL) and either compound **1** or **2** (0 μg/mL - 1000 μg/mL) was serially diluted two-fold vertically down the plate. In addition, control wells in which either isoniazid, compound **1** or compound **2** alone were tested (Supplementary Figure 3). The sum of the fractional inhibitory index (ΣFIC) for each combination was calculated whereby $\Sigma FIC \leq 0.5$ denotes synergy and $\Sigma FIC \geq 4$ denotes antagonism. Compound **1** has a ΣFIC of 1.5 and compound **2** has a ΣFIC of 3. Importantly, no antagonistic interaction is observed between either compound **1** or compound **2** and the front-line anti-tubercular agent isoniazid.

Cytotoxicity of compounds 1 and 2

To determine the selectivity index of compounds **1** and **2** we measured the cytotoxicity of both compounds against the A549 human lung epithelium cell line using the resazurin reduction viability assay and the haemolytic activity of compounds **1** and **2** against ovine red blood cells using both a haemoglobin release assay and an agglutination assay (Figure 3 and Supplementary Figure 4). The maximum tolerated dose of compounds **1** and **2** against A549 cells was found to be between 125 μg/mL-250 μg/mL (*t*-test compound **1**: $P < 0.001$; *t*-test compound **2**: $P < 0.001$) (Figure 3A). No lysis or agglutination of ovine blood red cells was observed at any of the concentrations tested up to 250 μg/mL for compound **1** (*t*-test: $P < 0.001$) and up to 500 μg/mL for compound **2** (*t*-test: $P < 0.001$) (Figure 3B and Supplementary Figure 4). These results therefore indicate that compounds **1** and **2** have a higher selectivity for the clinically relevant pathogenic organism *M. tuberculosis* compared to the human and ovine cells tested with a selectivity index of > 30-fold. These results are in agreement with previous studies that also found low toxicity to compound **1** for the mouse macrophage cell line RAW 264.7 and human cell line U937 (Abuhammad *et al.*, 2012).

Metabolic stability of compounds 1 and 2

We subsequently evaluated the *in vitro* metabolic stabilities of compound **1** and **2** using mouse liver microsomes and LC-MS and found that both compounds had high intrinsic clearance rates $[CL_{int}] > 48$ μL/min/mg protein. A slow $[CL_{int}]$ control using carbamazepine was used for comparison (Supplementary Figure 5).

Target identification of compounds 1 and 2

Compound **1** has shown encouraging inhibitory potency against *M. tuberculosis* and biochemical

studies have shown that compound **1** inhibits mycobacterial NAT enzymes (Abuhammad *et al.*, 2014, Abuhammad *et al.*, 2012, Jeney and Zsolnai, 1956). In order to identify any additional intracellular targets for the piperidinol class of compounds spontaneous resistant mutants in *M. smegmatis* were generated. Using the determined MIC values of compounds **1** and **2** (Table 1) *M. smegmatis* resistant mutants were generated on solid LB-agar containing 2.5x MIC of compound **1** (due to solubility issues) and 5x MIC compound **2**. Three colonies for compound **1** and four colonies for compound **2** were selected for subsequent inoculation in liquid medium in the absence of any compound and re-selection on solid medium in the presence of either compound **1** at 2.5 x MIC and compound **2** at 5x MIC. All colonies selected from the original plate maintained their ability to grow on solid medium with the addition of compound **1** and **2** following this procedure. Two of the resistant mutants to compound **1** and two of the resistant mutants to compound **2** were re-tested in the REMA assay whereby it was shown that the MIC of the mutants to compound **1** increased 5 fold (*t*-test: $P < 0.001$) and the mutants to compound **2** increased by 2-3-fold (*t*-test: $P < 0.01$) when compared to the wild-type *M. smegmatis* strain (Table 2). Interestingly isolate 3 to compound **1** also had increased resistance to compound **2** by 2-3 fold (*t*-test: $P < 0.01$) and similarly isolate 2 to compound **2** also increased its resistance to compound **1** between 2-3 fold (*t*-test: $P < 0.01$) indicating cross-resistance and a potential common resistance mechanism. Each mutant remained fully susceptible to isoniazid and rifampicin (Table 2).

Given that resistance to compounds **1** and **2** was maintained in the absence of any selection pressure on the mutant strains generated, it was postulated that a genetic resistance mechanism is involved in the observed resistant phenotype. To identify the genetic mutation causing the observed phenotype the genomes of the mutant strains were subjected to whole-genome sequencing using Illumina technology (Table 3).

Bioinformatics analysis identified nineteen putative SNPs compared to the laboratory *M. smegmatis* wild-type control strains. Three SNPs were predicted to occur in pseudogenes, six SNPs were predicted to occur in intergenic regions and two SNPS were predicted as synonymous mutations. Alleles with non-synonymous SNPs were found to occur in six *M. smegmatis* genes (Table 3) and included two genes with unknown function (MSMEG_2746 and MSMEG_3244), a gene predicted to be involved with homing (HNH) endonuclease function (MSMEG_2148), a putative 3-dehydroquinate synthase gene (MSMEG_3033, *aroB*), a putative monooxygenase gene (MSMEG_4083) and a putative gene involved in amino acid transport (MSMEG_5807). It is interesting to note that more alleles with non-synonymous SNPs were identified for the resistant mutants isolated from compound **2** which were generated at 5x MIC compound **2** compared to 2.5x MIC compound **1** due to solubility issues encountered. It was particularly interesting to find that four of the predicted SNPs occurred in the *aroB* gene resulting in an amino acid change of G278R and C352G for all four resistant isolates to compound **2** and two additional SNPs in *aroB* resulting in the additional amino acid change of G280D and L359P in three out of four of these resistant isolates (Table 3). These results led us to investigate the role of this potential *aroB* target further.

The *aroB* gene from *M. smegmatis* was expressed using the *hsp60* promoter from the replicative pMV261 vector and the susceptibility of this overexpressing strain to compounds **1** and **2** was undertaken using the REMA assay and the MICs determined. The results demonstrate that over-expression of *aroB* leads to partial resistance to both compound **1** and **2** when compared to the wild-type strain (*t*-test compound **1**: $P < 0.01$; *t*-test compound **2**: $P < 0.01$) although there is little difference between overexpression of *aroB* compared to empty vector alone (Table 4).

Discussion and Conclusions

Piperidinol compound **1** and its corresponding bis-Mannich base **2** have previously been demonstrated to have potent anti-tubercular activity against both *M. tuberculosis* and *M. bovis* BCG (Abuhammad *et al.*, 2014, Abuhammad *et al.*, 2012, Jeney and Zsolnai, 1956) and we have shown these compounds also have specificity for the *Mycobacterium genus* and good cytotoxicity selectivity. Despite no evident signs of cytotoxicity, the compounds appear to have a high intrinsic clearance rate in mouse microsomes and hence further chemical optimisation of these compounds will be required in order to improve their metabolic degradation profile whilst retaining anti-tubercular activity.

Treatment of TB is increasingly hindered by the emergence of drug-resistant strains and this has led to increased efforts in the identification of new potent inhibitors of *M. tuberculosis* and pharmacologically validated targets. In order to tackle this problem, there has been a recent increase in the utilisation of chemical genomics approaches to determine the cellular targets of these newly identified anti-tubercular agents. The generation of spontaneous resistant mutants followed by whole genome sequencing has been successfully used to determine a number of intracellular targets including, for example, SQ109, an FDA approved drug to be used only for drug-resistant strains of TB (Tahlan *et al.*, 2012), dinitrobenzamide and benzothiazole derivatives which are in the late stages of pre-clinical development (Christophe *et al.*, 2009, Neres *et al.*, 2012) and the natural product pyridomycin (Hartkoorn *et al.*, 2012). From this approach a range of new and previously pharmacologically validated targets have been identified, including the MmpL3 membrane transporter (Tahlan *et al.*, 2012) that is involved in the export of trehalose monomycolate and cell wall biosynthesis, and InhA, an enoyl (ACP)-reductase that is a key component in the type II fatty acid synthase system (FasII) and is the cellular target for isoniazid (Banerjee *et al.*, 1994).

The piperidinol compound **1** has been shown to inhibit the NAT enzyme from *M. marinum* through the generation of a polyvinyl moiety that forms a covalent adduct with the cysteine residue located in the NAT active-site (Abuhammad *et al.*, 2014, Abuhammad *et al.*, 2012, Abuhammad *et al.*, 2010). However, the potential link between the anti-tubercular activity observed of **1** and the potential endogenous role of NAT in the bactericidal activity of this piperidinol derivative has yet to be evaluated and other potential targets have not been precluded (Abuhammad *et al.*, 2014, Abuhammad *et al.*, 2012). Therefore, in this study, we

utilised a chemical genetic approach as a route to determine other potential cellular targets of compounds **1** and **2**. Given that *M. smegmatis* has been successfully used in high-throughput screening campaigns to identify new TB drugs, including the clinically approved anti-tubercular agent bedaquiline (Andries *et al.*, 2005), we chose to use this organism to generate spontaneous resistant mutants due to the ease and speed of manipulation despite the slight increase in the MIC of compounds **1** and **2** compared to *M. tuberculosis*. Interrogation of the resultant resistant isolates' genomes has led to the identification of a multiple number of high quality SNPs for each mutant. The high number of SNPs observed for this piperidinol scaffold is higher than that for similar chemical bioinformatics studies (Hartkoorn *et al.*, 2012, Hartkoorn *et al.*, 2014, Tahlan *et al.*, 2012) suggesting a potential diverse activity mechanism profile.

Mycobacteria have a highly unique cell wall structure, comprising a mycolic acid-arabinogalactan-peptidoglycan (mAGP) core (Brennan and Nikaido, 1995). Investigation into the endogenous role of the NAT enzyme in mycobacteria by deletion of the *nat* gene in *M. bovis* BCG has determined that NAT has a key role in the biosynthesis of mycobacterial mycolic acids and has been postulated to have a potential role in maintaining the homeostasis of the acetyl CoA pool (Bhakta *et al.*, 2004). NAT enzymes utilize the cofactor acetyl CoA as an acetyl donor for arylamine substrates (Sim *et al.*, 2007). Whilst, under these experimental conditions, our studies did not identify any SNPs in the *nat* gene, it was of particular interest to note the identification of a number of SNPs in the *aroB* enzyme in all resistant isolates generated with compound **2**. The *aroB* gene putatively encodes for a 3-dehydroquinate synthase enzyme involved in the second step of the shikimate pathway with AroB catalysing the cyclisation of 3-deoxy-D-arabino-heptulosonate-7-phosphate in 3-dehydroquinate. The 3-dehydroquinate is subsequently metabolised in the shikimate pathway to chorismate which is required for the biosynthesis of all aromatic amino acids and other key metabolites. In *M. tuberculosis* the *aroB* gene (Rv2538) is predicted to be essential for the survival of *M. tuberculosis* (Sassetti *et al.*, 2003, Parish and Stoker, 2002). Enzymes within the shikimate pathway represent excellent targets for the development of new antibiotics as they are essential for bacterial survival with no human counterpart (Bentley, 1990, Ducati *et al.*, 2007). Given the role of the AroB enzyme in the essential biosynthesis of aromatic amino acids, naphthoquinones, menaquinones, and mycobactin in *M. tuberculosis* we selected this prospective genetic target for further validation. Overexpression of *aroB* using the replicative pMV261 vector, with a relative copy number of 2-3 fold (Stover *et al.*, 1991), resulted in

partial resistance of these strains to compounds **1** and **2**, which could in part be explained by the presence of the chromosomal wild-type *aroB* gene still being expressed. Partial resistance has been found to occur in a similar chemical bioinformatics study to investigate the mechanism of resistance to clofazimine, a drug that is used in the multidrug therapy of leprosy (Hartkoorn *et al.*, 2014). Although, it is possible that these studies have also identified off-target compensatory pathways in response to these compounds. However, it is probable that given the high number of SNPs identified for this piperidinol derivative for *M. smegmatis* that these compounds are active against a diverse set of intracellular targets which subsequently results in difficulties in restoring the observed chemical-genetic phenotype through overexpression of a single gene.

In summary, these studies indicate that the cyclic piperidinol class of compound along with its bis-Mannich base derivate are specific for the *Mycobacterium genus* and rapidly kill *M. smegmatis* with encouraging cytotoxicity profiles. Genetic interrogation of spontaneous resistant mutants of *M. smegmatis* generated under the selective pressure of compounds **1** and **2** has led to the identification of potential genetic targets and pathways that are involved in the multiple and diverse mode of action for these piperidinol chemical moieties. Previous *in vitro* biochemical studies demonstrated the formation of a polyvinyl ketone moiety which is likely to be highly reactive at the proteomic level. Given the increase in TB drug-resistant strains that often results from drugs acting on a single biological target, chemical moieties with anti-tubercular inhibitory activities that act upon multiple cellular targets are highly attractive for the development of new TB agents from a clinical standpoint and are worthy of further investigation.

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Author contributions

Conceived and designed the experiments: CG ET GK MIG MJP EF. Performed the experiments: CG ET GK DP CF TB JH EF. Analysed the data: CG ET GK CG DP AM MIG EF. Analysed bioinformatics data: ET, GK, ADM. Contributed reagents/materials/analysis tools: CG GK AM DP MIG MJP EF. Wrote the paper: CG ET ADM EF

Conflicts of interest:

The authors declare no conflicts of interest.

Supporting Information

Supporting information is provided for the synthetic protocols for compounds **1** and **2** along with 5 Supplementary Figures and 1 Supplementary table.

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Table 1. Minimum inhibitory concentrations of compounds 1 and 2

Bacterium	Compound **1** MIC (μg/mL)	Compound **2** MIC (μg/mL)	Reference
M. tuberculosis	5	nd	(Abuhammad *et al.*, 2012, Jeney and Zsolnai, 1956)
M. bovis BCG	6.25-12.5	nd	(Abuhammad *et al.*, 2014)
M. smegmatis	62.5±0	125±0	This study
E. coli	250±0	250±0	This study
P. putida	250±0	>500±0	This study

nd – not determined. All assays in this study were undertaken with n=5 and data are presented as mean ± SEM.

Table 2. Activity of compounds 1 and 2 against selected resistant mutants

	Isolate[a]	MIC compound **1** (μg/mL)	MIC compound **2** (μg/mL)	MIC INH (μg/mL)	MIC RIF (μg/mL)
Compound 1	1	375.0±62.5	156±0	25±0	5±0
Compound 1	3	375.0±62.5	375.0±62.5	12.5±0	10±0
Compound 2	1	171.6±38.2	375.0±62.5	12.5±0	10±0
Compound 2	2	265.6±46.9	281.3±31.3	12.5±0	3.2±0.5
n/a	*M. smegmatis* WT	56.2±6.3	112.5±12.5	12.5±0	3.2±0.5

[a]Isolate refers to mutant numbers listed in Table 3
INH: isoniazid; RIF: rifampicin; WT: wild-type strain: n/a: not applicable
The averages of five independent experiments are shown and the data are presented as mean ± SEM.

Table 3: Single nucleotide polymorphisms detected in *Mycobacterium smegmatis* mutants resistant to compounds 1 and 2

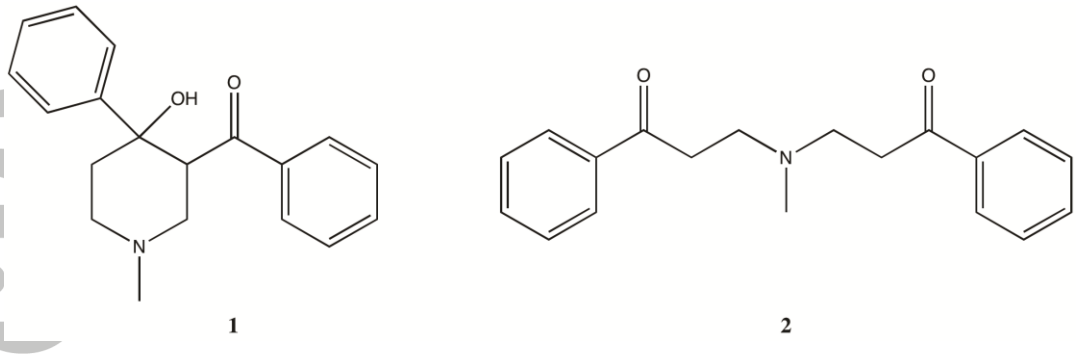
SNP locus *M. smegmatis chromosome position*	Codon Change	Amino acid change	Gene	Annotation	Compound 1				Compound 2		
2223880	cCc→cGc	P380R	MSMEG_2148	HNH	–	–	–	–	R	R	R
2223882	Aaa→Caa	K381Q		endonuclease	–	–	–	–	K	K	K
2816638	gGa→gAa	G332E	MSMEG_2746	Unknown	E	E	–	–	E	–	–
3105561	Ggg→Agg	G278R	MSMEG_3033	aroB	–	–	–	R	R	R	R
3105568	gGc→gAc	G280D			–	–	–	–	D	D	D
3105783	Tgc→Ggc	C352G			–	–	–	G	G	G	G
3105805	cTa→cCa	L359P			–	–	–	–	P	P	P
3328246*	ccT→ccC	P384P	MSMEG_3244	Hypothetical	–	–	–	–	P*	P*	P*
4158542*	ttT→ttC	F227F	MSMEG_4083	Monooxygenase	–	–	–	F*	F*	F*	F*
5874050	gCc→gGc	A306G	MSMEG_5807	Amino acid transport	–	G	–	–	–	–	–

Genomic positions are relevant to *M. smegmatis* str. MC^{2}155 (accession number NC_008596.1)
WT alleles are represented by '– '; * represents non-synonymous SNP

Table 4: Susceptibility profiles of *M. smegmatis* strains to compounds 1 and 2

	MIC (μg/mL)		
Strain	Compound 1	Compound 2	Rifampicin
M. smegmatis mc²155 – Wild type	56.2±6.3	112.5±12.5	7.5±1.1
M. smegmatis:*aroB*	112.5±12.5	300.0±49.9	7.5±1.1
M. smegmatis:pMV261	112.5±12.5	275.0±61.2	7.5±1.1

The averages of five independent experiments are shown and the data are presented as mean ± SEM.



1 **2**

Figure 1. Compounds identified as inhibitors of the NAT enzyme. Structures of piperidinol compound **1** and the bis-Mannich base compound **2**

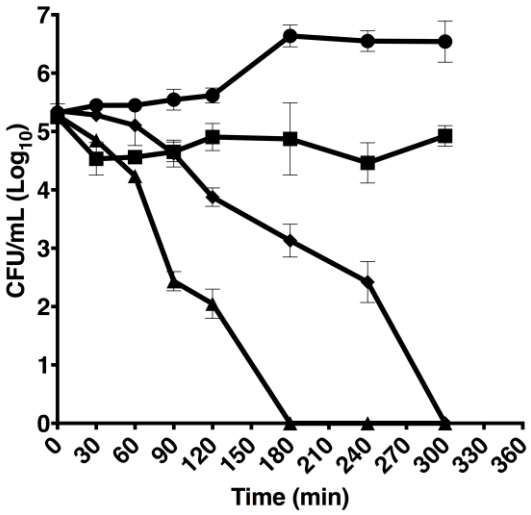


Figure 2. Time kill curve of compounds **1** and **2**. Exponentially growing cultures of *M. smegmatis* were treated with 2x MBC of compound **1** (▲), 2x MBC compound **2** (◆), 2x MBC rifampicin (■) and a no drug control (③). CFUs were counted at the time points indicated. The averages of five independent experiments are shown and the data are presented as mean ± SEM.

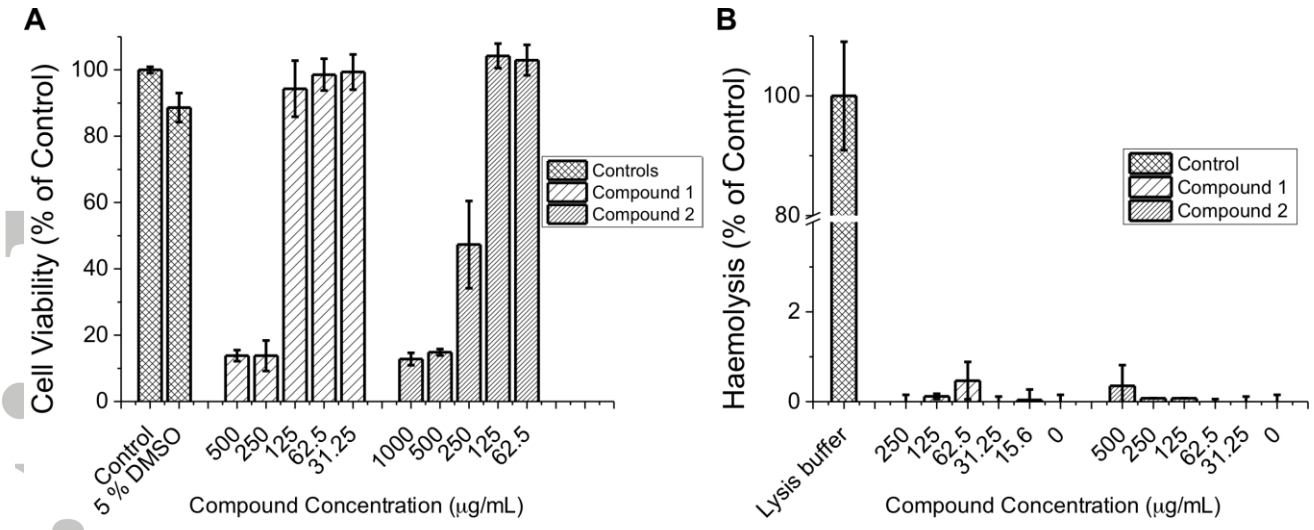


Figure 3. Cytotoxicity testing of compounds **1** and **2** against human A549 human lung epithelium cell line and ovine blood. A) Effect on the cell viability of the human A549 cell line in the presence of compound **1** or **2**. A549 cells were exposed to compound **1** or **2** at the final concentrations indicated for 24 h and the cell viability determined after this time by the addition of resazurin. Percentage cell viability is compared to an A549 cell only control. B) Effect on haemolysis of ovine red blood cells in the presence of compounds **1** or **2**. Ovine red blood cells were exposed to compound **1** or **2** at the final concentrations indicated for 1 h after which the percentage haemolysis was determined by measuring the absorbance at 450nm Percentage lysis is compared to the 100 % lysis reading upon addition of lysis buffer (10 mM Tris pH 7.8, 0.32 M sucrose, 5 mM $MgCl_2$, 10 % Triton X-100). In both cases the averages of five independent experiments are shown and the data are presented as mean ± SEM.