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Identification and validation of the mode of action of the chalcone anti-myco-bacterial compounds

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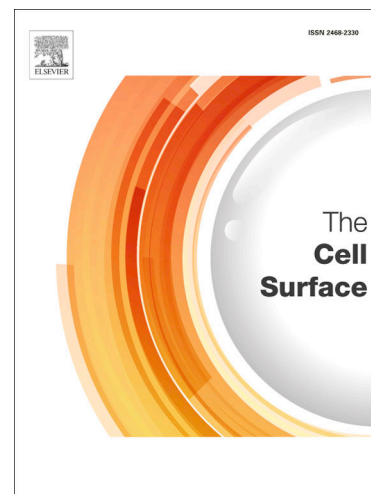
Author Credits:

Conceived and designed the experiments: Cox JAG., Anagani B., Goyal M., Benham C., Bassin JP., Besra GS. **Performed the experiments:** Cox JAG., Anagani B., Reddy TRK., Singh J. **Wrote the paper:** Goyal M., Cox JAG., Reddy TRK., Bassin JP., Anagani B. **Analyzed the data:** Cox JAG., Goyal M., Bassin JP., Singh J., Anagani B., Reddy TRK. The manuscript was reviewed by all the authors.

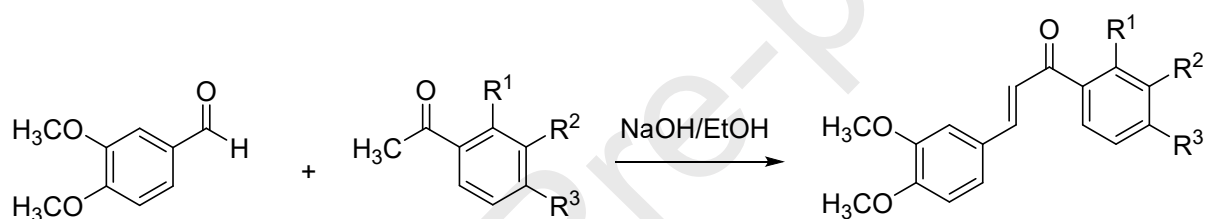**Competing Interests:** The authors declare that they have no financial or non-financial competing interests.

Figure 1: Chalcone synthesis reaction scheme **(1a-1o)**
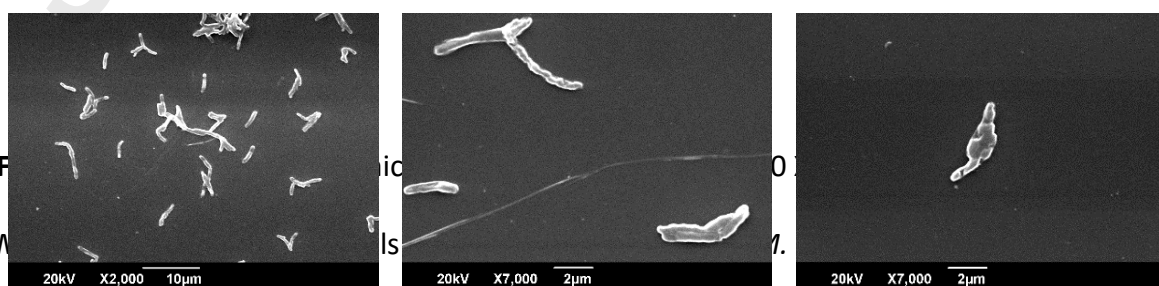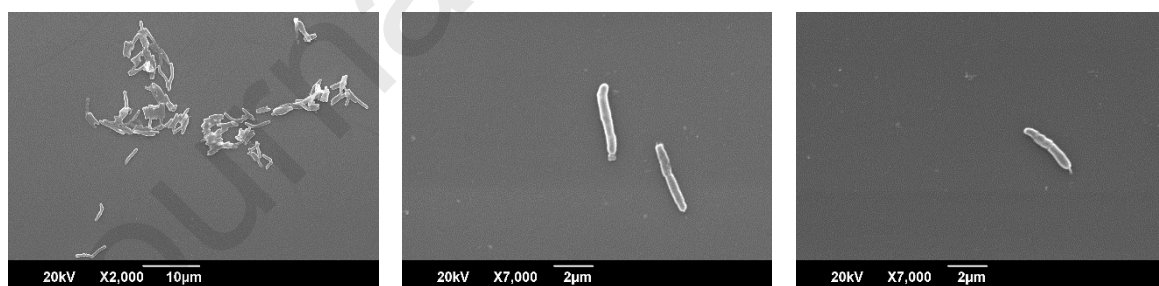
1a treated *M. bovis* BCG cells analyzed using JEOL CarryScope JCM-5700 scanning electron

microscope. There was significant proportion (76%) of cells showing morphological changes such as bulging, elongation and wrinkling of cell surface.
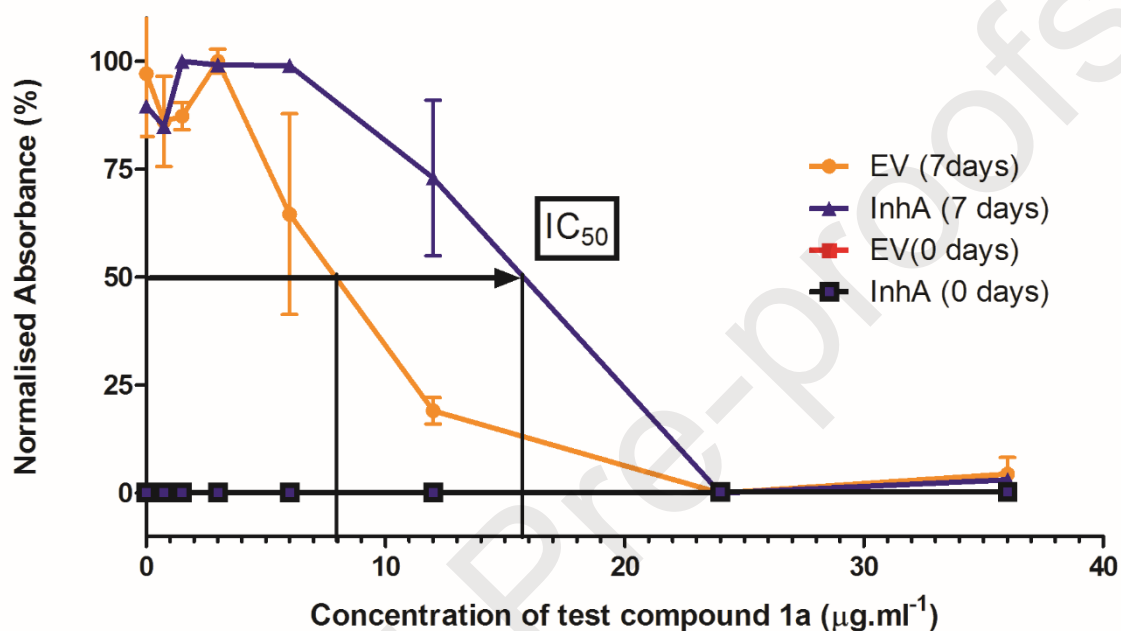
Figure 4: Investigation of the impact of InhA overexpression on sensitivity to compound **1a**. Absorbance values were read at 0 days and after 7 days for EV and InhA strain. Using *Mycobacterium bovis* BCG containing a constitutive plasmid (pMV261) expressing InhA, IC_{50} was compared to an empty vector control. The overexpressing strain demonstrated an increase in IC_{50} value 16 μg.ml^{-1} compared to 8 μg.ml^{-1} for the empty vector strain. The experiments were performed in biological replicates of *n*=3 and the error bars represent the standard error bars of mean (SEM).
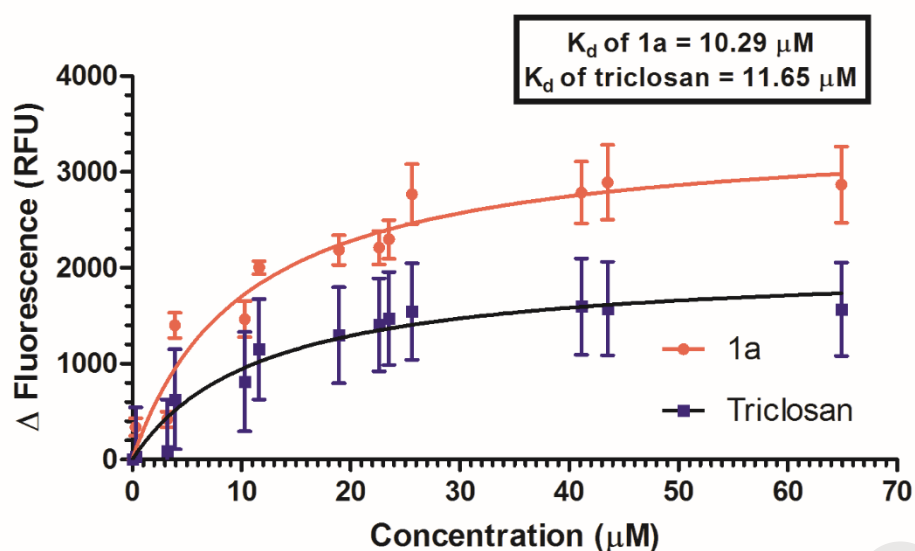
Figure 5: Saturation binding assay using intrinsic tryptophan fluorescence to quantify association of increasing concentrations of ligands **1a** and triclosan with InhA (n=3). The figure shows the K_d values (11.65 μM and 10.29 μM) for triclosan and **1a**. Data were fitted using GraphPad Prism, relative fluorescence units (RFU). The experiments were performed in biological replicates of *n*=3 and the error bars represent the standard error bars of mean (SEM).

6a

6b

Figure 6: 6a shows the predicted interaction mode of compound **1a** within the binding site of the InhA of *Mycobacterium tuberculosis*. InhA is shown as secondary structures with helices (green) and sheets (yellow). Compound **1a** is shown in the teal capped stick for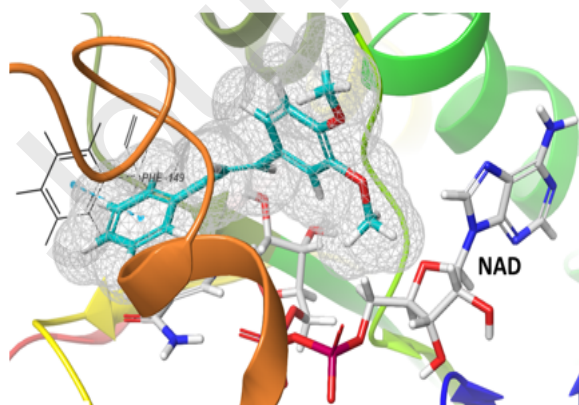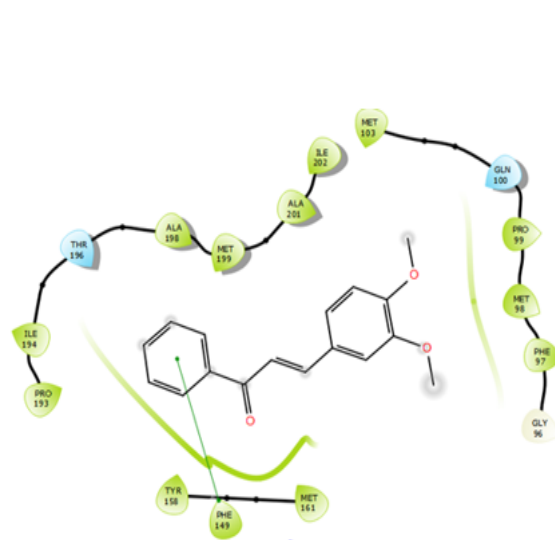mat. Van der Waals surface around compound **1a** is shown in mesh format. NAD is shown in element type format (Nitrogen in blue, Oxygen in red, Phosphorous in magenta). Pi-Pi stacking interaction is shown as teal dashed line between the phenyl rings of Phe 149 and the compound **1a.** This image has been generated by using Schrodinger drug design software. **6b** shows the ligand interaction map of the compound **1a** with the active site residues of the InhA. The proposed interaction mode of compound **1a** is shown in the stick format. Pi-Pi stacking is shown in green line.

Highlights:

· Chalcone **1a** inhibits the growth of *Mycobacterium bovis* BCG (MIC 6ug/ml), a model organism for *Mycobacterium tuberculosis*

· Chalcone **1a** directly targets inhA

· Inhibition by Chalcone **1a** results in reduction of cell wall mycolates

Table 1: Shows chalcone compounds **1a** to **1o** with various substituents at R^1, R^2 and R^3 positions with their molecular weights and predicted *Clogp* values. The MIC of each compound is shown in $\mu g.ml^{-1}$ against *M.bovis* BCG with the IC_{50} values and the selective index (SI) for J774 cell line. Lipophilicities calculated by ChemDraw Professional 15.0, part of ChemOffice (Perkin Elmer, UK).

Compound	Substituents			Mol.wt (g.mol^{-1})	*Clogp*	MIC ($\mu g.ml^{-1}$)	J-774 (IC_{50}) ($\mu g.ml^{-1}$)	SI= IC_{50}/MIC
	R^1	R^2	R^3			*M.bovis* BCG		
1a	H	H	H	270.33	3.35	6.25	50	8.0
1b	H	Br	H	349.22	4.18	-	25	-
1c	Br	H	H	349.22	4.18	25	25	1
1d	H	H	Br	349.22	4.18	12.5	25	2.0
1e	H	Cl	H	304.77	3.91	-	12.5	-

1f	Cl	H	H	304.77	3.91	12.5	12.5	1
1g	H	H	Cl	304.77	3.91	200	12.5	0.06
1h	H	H	F	288.32	3.51	25	100	4
1i	H	H	OCH_3	300.35	3.23	50	100	2
1j	H	H	CH_3	284.35	3.84	25	25	1
1k	H	H	C_6H_5	346.43	5.03	100	25	0.25
1l	H	NO_2	H	315.32	3.48	100	25	0.25
1m	CH_3	H	H	284.35	3.84	50	25	0.5
1n	H	H	NO_2	315.32	3.48	200	50	0.25
1o	Cl	H	Cl	339.21	4.47	200	6.25	0.03
RIF	-	-	-			0.1		

Identification and validation of the mode of action of the chalcone anti-mycobacterial compounds

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Highlights:

· Chalcone **1a** inhibits the growth of *Mycobacterium bovis* BCG (MIC 6.25 µg.ml^{-1}), a model organism for *Mycobacterium tuberculosis*

· Chalcone **1a** directly targets InhA

· Inhibition by Chalcone **1a** results in reduction of cell wall mycolates

Abstract

Objectives: The search for new TB drugs has become one of the great challenges for modern medicinal chemistry. An improvement in the outcomes of TB chemotherapy can be achieved by the development of new, shorter, cheap, safe and effective anti-TB regimens.

Methods: Chalcones (**1a-1o**) were synthesized and evaluated for their antimycobacterial activity against *Mycobacterium bovis* BCG using growth inhibition assays. Compound **1a** was selected as a 'hit' compound. The mode of action of compound **1a**, was identified by mycolic acid methyl esters (MAMEs) and fatty acid methyl esters (FAMEs) analysis using thin layer chromatography. Dose dependent experiments were conducted by over-expressing components of FAS-II in *M. bovis* BCG to confirm the target. Ligand binding using intrinsic tryptophan assay and molecular docking were used to further validate the target.

Results: MAMEs and FAMEs analysis showed dose-dependent reduction of MAMEs with the overall abundance of FAMEs suggesting that compound **1a** targets mycolic acid biosynthesis. Direct binding of **1a** to InhA was observed using an intrinsic tryptophan fluorescence binding assay, and a 2-fold IC$_{50}$ shift was observed with an InhA overexpressing strain confirming InhA as the cellular target.

Conclusion: The chalcone **1a** exhibits potent antimycobacterial activity, displays a good safety profile and is a direct inhibitor of InhA, a key component in mycolic acid synthesis, validating this series for further anti-TB drug development.

Key Words: Chalcones, Tuberculosis, MIC, InhA, Mycolic Acids, Docking

1 Introduction:

Antibiotic resistance is a major global healthcare crisis. It is expected that antimicrobial resistance will overtake cancer as the leading cause of death by 2050 (de Kraker et al., 2016). Tuberculosis (TB) is the most devastating infectious disease caused by *Mycobacterium tuberculosis* (MTB), claiming 1.4 million lives in 2015. It has existed for millennia and remains a global health problem (Falzon et al., 2017). The effective treatment for drug-susceptible TB is a 6-month dose regimen of four first-line drugs: isoniazid (INH), rifampicin (RIF), ethambutol (ETH) and pyrazinamide (PYZ). The most challenging problem with the current TB regimen is patient compliance attributed to the length, complexity and adverse effects commonly observed with frontline treatment. This is further complicated by the emergence of drug resistant strains. The emergence of drug-resistant TB is one of the most dangerous threats to global TB control (Brouqui et al., 2017). The treatment for RIF resistant TB (RR-TB), multidrug-resistant TB (MDR-TB), and extensively drug-resistant TB (XDR-TB) takes 18-24 months, requiring more expensive and toxic drugs (Upadhyaya et al., 2012). There is an urgent need to develop new drugs that can shorten the treatment regimen, that are able to treat MDR-TB and have less adverse effects. Phenotypic screening is emerging as an important tool in the discovery of new drugs against MTB as it allows a direct and measurable response of whole cells against a library of compounds measuring and evaluating their efficacy in bacterial killing (Kotz, 2012). Advances in high throughput screening, genome sequencing and data handling tools have further expanded its applications allowing the discovery of new anti-microbial compounds and new targets (Ferraris et al., 2018). The success of whole cell phenotypic screening is evidenced by the recent development of some new TB drugs such as bedaquiline (Matteelli et al., 2010) and others which are currently under study (Singh and Mizrahi, 2017). Whereas target-based approaches have encountered very limited success in the antibacterial field (Abrahams et al., 2012; Payne et al., 2007).

INH is a frontline anti-TB drug that inhibits the mycobacterial enoyl-reductase InhA and its activity is dependent on KatG activation, the catalase peroxidase involved in the activation of isoniazid. InhA is an essential enzyme for the biosynthesis of a major component of the mycobacterial cell wall,

mycolic acid, through fatty acid synthesis (FAS-II) system (Duan et al., 2014). InhA is also a target for second line drug ethionamide (Banerjee et al., 1994). Unfortunately, between 40 to 95% of INH-resistant MTB clinical isolates have mutations in *KatG* gene leading to decreased activation of INH (Hazbon et al., 2006; Seifert et al., 2015) and therefore this pro-drug activation step of INH mechanism of action significantly contributes to multidrug and extensively drug resistance in MTB isolates (Ramaswamy et al., 2003). It is therefore important to develop drugs that can directly inhibit InhA without requiring activation by KatG.

Chalcones are essential intermediate compounds for the synthesis of various heterocyclic compounds such as flavonoids and isoflavanoids which are abundant in edible plants (Rachmale and Patil, 2012). Many studies have shown that natural and synthetic chalcones display a wide spectrum of biological activities (Chavan et al., 2015) including anti-TB activity (Gomes et al., 2017).

In view of their therapeutic potential, a series of 15 synthetic chalcones were evaluated *in vitro* for their anti-mycobacterial activity against the MTB model organism *M. bovis* BCG using whole cell phenotypic screening. In addition, the mode of action of the hit compound was elucidated and validated using biochemical, molecular genetics and molecular docking approaches.

2 Materials and Methods:

2.1 Synthesis: Chalcones (**1a**-**1o**) were synthesized by reacting 3,4-dimethoxybenzaldehyde with various acetophenones by the Claisen-Schmidt condensation (Figure 1). The synthesized products were recrystallized from appropriate solvents and were characterized by spectral analysis, melting point, infrared spectroscopy, ^{1}H and ^{13}C NMR and mass spectrometry.

2.2 Anti-mycobacterial evaluation: Minimum inhibitory concentrations (MICs) were determined for these synthesized compounds by broth micro-dilution according to CLSI recommended procedure as described previously (Abrahams et al., 2012). To prepare stocks, *M. bovis* BCG Pasteur was grown to mid-logarithmic phase (Optical density 600 nm = 0.5-0.8) in Middlebrook 7H9 broth (Difco)

supplemented with 10 % albumin dextrose catalase supplement (ADC); 0.25% (v/v) glycerol and 0.05 % (v/v) Tween 80.

MIC of each test compound was determined against *M. bovis* BCG in a 96-well flat-bottom, microtitre plate in a final volume of 200 µl. Series of two-fold dilutions of test compounds starting from 200 µg.ml^{-1} and the standard (RIF) were prepared in Middlebrook 7H9 medium from stock solution (10 mg.ml^{-1} in DMSO). The inoculum was standardized to approximately 1×10^6 cfu.ml^{-1} and 100 µl was added to each well. All plates were sealed using seals (Sigma, UK Z380069) to prevent desiccation of the peripheral wells and incubated at 37°C without shaking for six days. A 25 µl resazurin solution (0.02% w/v in sterile distilled water) was added to each well and the plates were incubated for 24 hours. The MIC was defined as the lowest concentration where there was no colour change of resazurin (blue) to resarufin (pink). Experiments were performed with biological replicates (*n*=3).

2.3 MTT Assay for Cytotoxicity: The *in vitro* cytotoxicity of all synthesized chalcones (**1a**-**1o**) was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay against J774 cells at a concentration range of 200-0.5 µg.ml^{-1}. Exponentially growing mouse macrophages (J774) in Dulbecco's Modified Eagle's medium (DMEM) (supplemented with 10 % FBS and 1% penicillin and streptomycin) were seeded at a density of 50,000 cells.ml^{-1} into a 96-well plate and incubated overnight at 37°C (Ling et al., 2015). The medium was replaced with fresh medium containing synthesized chalcones (**1a**-**1o**) and after 24 h of incubation at 37°C, MTT was added to the cells. After 2 h, the formed crystals were dissolved in isopropanol and the absorbance was recorded using plate reader at A$_{540nm}$. The experiments were performed in biological replicates of *n*=3.

2.4 Scanning Electron Microscopy (SEM): All chemicals and reagents for electron microscopy were obtained from Sigma Aldrich, *M. bovis* BCG (Pasteur strain, ATCC 35734) cultures were grown to early log phase (optical density at 600 nm, 0.2), at which time cells were treated with **1a** (60 µg.ml^{-1}), followed by incubation at 37°C for five days. Cells were harvested by low-speed centrifugation and

washed in 0.1 M cacodylate (CACO) buffer (pH 7.3). Washed cells were then fixed on glass coverslips in 0.1 M CACO buffer (pH 7.3) containing 2.0 % glutaraldehyde–osmium tetroxide (1:1, v/v) for 45 minutes at 4°C (Parrish et al., 2001). Secondary fixation was done at 4°C overnight in a mixture of 4.0% formaldehyde and 1.0 % glutaraldehyde. Samples were post-fixed at room temperature for 1 h in 0.1 M CACO buffer containing 1% tannic acid and dehydrated through a graded ethanol series of 50, 70, 95 (twice), and 100% (three times) (Stadtländer, 2008). The samples were air dried and coated with gold and visualized using JEOL CarryScope JCM-5700 scanning electron microscope.

2.5 Extraction and Analysis of MAMES and FAMES: The inhibition of mycolic acid biosynthesis by **1a** was investigated. The qualitative and quantitative biochemical characterization of the mycolic acid pattern by thin layer chromatography (TLC) can be used to address how drugs alter mycolic acid biosynthesis (Alahari et al., 2007a, 2007b; Hartkoorn et al., 2012). To assess the effect of **1a** on both mycolic acid and fatty acid synthesis in *M. bovis* BCG, mycolic acid methyl esters (MAMES) and fatty acid methyl esters (FAMES) were prepared from cultures following labelling with [4]-acetate (Cox et al., 2016). Following the treatment with **1a** the total FAMEs and α-mycolate and keto-mycolic acid methyl esters (α- and k-MAMEs) were extracted and analyzed by autoradiography TLC using petroleum ether-diethyl ether (95:5) six times as described in Parish and Stoker (Parish and Stoker, 1998).

2.6 MIC shift assay: The mycobacterial expression vector pMV261 was cloned with *M. tuberculosis* FAS-II enzymes KasA, KasB, FabH, HadABC, MabA, MabB and InhA. Each respective plasmid was electroporated into *M. bovis* BCG and the MIC assay was performed using these transformed strains, along with an empty pMV261 control strain (Larsen et al., 2002). The data points were collected in triplicate and an IC_{50} (50% inhibition of growth) values for each strain were obtained using GraphPad Prism software. The experiments were performed in biological replicates of *n*=3.

2.7 Intrinsic tryptophan fluorescence ligand binding assay: Using intrinsic tryptophan fluorescence, a ligand binding assay was performed to measure the binding affinity of **1a** to InhA enzyme. Briefly,

InhA (10 µM) was incubated for 5 minutes in a buffer solution (25 mM HEPES, 10% glycerol and 300 mM NaCl, pH 8) at 25°C followed by addition of increasing concentrations 0.3 to 70 µM of either triclosan (positive control) or compound **1a.** DMSO concentration was maintained at less than 1% in the assay. The measurements were obtained as a change in tryptophan fluorescence by Hitachi F7000 spectrophotometer at an excitation wavelength of 350 nm and emission wavelength at 470 nm. Data were recorded using Hitachi FL Solutions 4.6 software. The experiments were performed in biological replicates of n=3.

2.8 Molecular Docking:

2.8.1 Protein preparation: The X-ray crystal structure of InhA complexed with PT506 inhibitor (5UGU) was obtained from protein data bank (www.rcsb.org). Protein preparation wizard module of Schrödinger suite was used to prepare the protein by adding hydrogens, fixing error like missing side chains, assigning correct bond orders, adjusting the ionization and tautomeric states (via epik). All the water molecules have been deleted. Optimisation of the hydrogen bonding network and the orientation of the hydroxyl/thiol groups, terminal amide groups in Asn, Gln and His states was carried out using the ProtAssign algorithm. Finally, restrained minimization was carried out on all the atoms using OPLS3 force fields, with converge heavy atoms to RMSD set to 0.3 Å (default).

2.8.2 Ligand Preparation: Compound **1a** was drawn and prepared using Ligprep module, which performed addition of hydrogens, 2D to 3D conversion, generation of ionization and tautomeric states (via Epik) at physiological pH 7.0 ± 2.0, and also generated ring conformations using default settings. Compound **1a** was energy minimized using OPLS3 force fields.

2.8.3 Docking studies: The X-ray crystal structure of InhA (PDB ID 5UGU, resolution 1.95 Å) was used for structure-based docking of compound **1a**. The Receptor Grid generation was carried out by identifying the PT506 inhibitor in the 5UGU crystal structure and excluding it from the grid generation. The triazole based inhibitor PT506 present in 5UGU crystal structure was redocked to verify our Glide docking protocol. Glide docking successfully reproduced the crystallographic binding mode of triazole based inhibitor with a high docking score of -11.00. The RMSD value between the

heavy atoms of the Glide predicted pose and the crystallographic binding pose is 0.31 Å, indicating close agreement. We have used this docking protocol to dock compound **1a** to 5UGU (InhA) using Schrödinger GLIDE, in standard precision (SP) mode. Molecular modelling studies were performed primarily using Schrödinger drug design software suite (Schrödinger, 2017).

3 Results:

The MIC values (μg.ml^{-1}) of chalcones (**1a**-**1o**) (Table 1) were obtained with RIF as the standard. The results were obtained in duplicate by testing in concentrations ranging 200-0.3 μg.ml^{-1}. Compounds that exhibited higher selectivity index (SI) values for J774 cells were considered nontoxic. Hence compound **1a** (SI=8.4) was selected for mode of action (MoA) elucidation.

Preliminary SEM examination of the **1a**-treated cells indicate definite morphological changes occurred in comparison to the control due to the action of the drug. The SEM examination revealed in general there are some degraded cells in **1a**-treated cells. The main features of the cells in control specimen (Figure 2a-2c) are smooth surface and characteristic rod like shape. Whereas the cells exposed to **1a** for five days showed elongation, wrinkles and bulging (Figure 2d-2f).

3.1 Mode of Action:

3.1.1 FAMEs and MAMEs Analysis: To identify the biological target of **1a,** inhibition of mycolic acid biosynthesis was investigated. As shown in Figure 3 there was a dose-dependent decrease in the production of α- and k-MAMEs upon treatment with **1a**. There is also an accumulation of FAMEs in a dose-dependent manner. This accumulation of FAMEs and reduction of MAMEs suggests that test compound **1a** inhibits mycolic acid biosynthesis (FAS-II inhibition) in mycobacteria which causes cessation of the essential mycolic acids. This inhibition of FAS-II is desirable as the inhibition of FAS-I is not specific since mammalian fatty acid synthases are similar to FAS-I. Original auto-radiograph of MAMEs and FAMEs is included in supplementary data (Figure S-1).

3.1.2 MIC Shift: To further corroborate that **1a** inhibits mycolic acid biosynthesis, MIC shift assay

was performed. *M. bovis* BCG containing the pMV261::*inhA* plasmid exhibited an increased IC$_{50}$ over the empty vector control (16 μg.ml^{-1} compared to 8 μg.ml^{-1} respectively). The results suggest that the ample growth of InhA over-expressor strain was observed as shown by increase in absorbance (Figure 4), indicating an increase in resistance. This further validates InhA as the target of the chalcone compound series. The data for the other overexpressing components of FAS-II are listed in supplementary data (Figure S-2).

3.1.3 Intrinsic Tryptophan Fluorescence InhA Binding Assay: Intrinsic tryptophan fluorescence was used to investigate InhA as the target enzyme for **1a**. Ligand binding assays using InhA were conducted to establish the equilibrium dissociation constant (K$_d$) values for compounds **1a** and triclosan. Binding affinity is the strength of the binding interaction between a protein and its ligand, that is measured and reported by K$_d$ values. Triclosan is well known for its direct binding affinity towards InhA. The experimental results suggest that both Triclosan and **1a** bind with similar affinity to InhA, as reflected by the K$_d$ values of 10.29 μM and 11.65 μM respectively providing the necessary evidence for target validation (Figure 5). K$_d$ values (mean$\pm$ s.d) resulting from non-linear least-squares fitting of a one-site specific binding model are listed in supplementary Table S-1.

3.1.4 Binding mode analysis of compound 1a: Predicted binding mode of the compound **1a** with the binding site of InhA (Figure 6a and 6b) suggested that it occupies the hydrophobic cavity of the substrate binding site. Substrate binding pocket is composed of hydrophobic groups from the side chains of Tyr 158, Phe 149, Met 199, Met 166 and Pro 193. Compound **1a** (SP docking score -9.14) is involved in good hydrophobic interactions with these amino acids and also involved in van der Waals interactions with the surrounding amino acids Pro193, Met 199, Phe 149. In addition, the phenyl ring of compound **1a** undergoes Pi-Pi stacking interactions with the phenyl ring of Phe 149. It appears that hydrophobic, van der Waals, Pi-Pi stacking interactions play a dominant role in the binding affinity of the compound **1a**.

4 Discussion:

Treatment of drug resistant strains of *M. tuberculosis* has become a global problem and WHO has declared the priority of new drugs to overcome antibiotic resistance. To overcome this problem many approaches including the re-purposing of broad spectrum anti-bacterials, target-based programs on antimycobacterial enzymes and phenotypic screening have been widely reported (Chetty et al., 2017). The target to drug approach for drug discovery is time consuming and costly. In comparison, a drug to target approach that involves phenotypic screening utilizes whole cells and is much quicker and establish potent inhibitors at low cost. In this study we have synthesized 15 chalcones, fully characterized them and using whole cell phenotypic screening approach showed that most of these chalcones showed antimycobacterial activity (Table 1) with MIC in the range 6.25-200 µg.ml^{-1} against *M. bovis* BCG. Compound **1a** was the most potent against *M. bovis* BCG with MIC of 6.25 µg.ml^{-1} and showed SI of 8.0. Of the fifteen compounds synthesized **1a**, where R substituents are all hydrogens, showed the best activity. The compounds that had substituents other than hydrogen did not display the same level of activity. This may reflect poor binding of the substituents in the other fourteen compounds. The compounds which also showed significant activity were those which contained a chlorine or a bromine atom. Previously reported work on *in vitro* testing on antifungal and antimycobacterial activity of a series of pyrazine analogues of chalcones showed the highest potency was exhibited by derivatives with a chlorine or a bromine atom in positions 2 and 4 of the benzoyl ring (Kucerova-Chlupacova et al., 2016). The *ortho* chloro-substituent (R^1=Cl), compound **1f**, showed the next best activity compared to compound **1a**. When the chlorine atom is positioned in the *para* position (R^3=Cl), compound **1g**, there is quite a substantial drop in activity, but when the R^3 position is occupied by a bromine atom, compound **1d**, the activity is comparable to compound **1f**. This may indicate that electronic factors do not play an important role in activity. Calculated lipophilicities of all tested compounds are provided in table 1. They do not correlate with the biological activity of the compounds. The activity of compounds **1a-1o** does not show a

correlation with electronic factors nor lipophilicities; it is therefore surmised that the conformation of particular molecules, influenced by the position of the halogen atom, may play an important role. Our results are in accordance with previous studies where it has been shown that chalcone derivatives show activity against gram-negative, gram-positive bacteria and mycobacteria (Gond et al., 2013; Lin et al., 2002; Sivakumar et al., 2007; Yadav et al., 2014). A series of 5- nitro-substituted heteroaryl chalcones were synthesised using QSAR driven approach (Gomes et al., 2017). They have shown low MICs against non-replicating *M. tuberculosis* H37Rv and RIF and INH mono-resistant strains. As the functional groups attached to the 3 carbon α,β unsaturated carbonyl structure are different from our chalcone series the results cannot be directly compared to each other. However, having a 5- nitro-heteroaryl group instead of an aromatic carbon ring B of our chalcone series seems advantageous resulting lower MICs values.

In the present study using *in vitro* biochemical and modelling experiments, we have shown that our hit compound **1a** specifically targets mycolic acid synthesis component InhA. The mycolic acids are the essential characteristic components of mycobacterial cell wall and involves two distinct fatty acid synthesis pathways- FAS-I and FAS-II. The FAS-II system enables fatty acid elongation forming meromycolic acids in the presence of condensing enzymes KasA and KasB (Kremer et al., 2002), a keto reductase MabA (Marrakchi et al., 2002), a dehydratse HadABC (Sacco et al., 2007), and an enoyl acyl carrier protein reductase InhA (Banerjee et al., 1994). The lipid analysis in our study showed dose dependent accumulation of FAMEs with depletion of MAMEs confirming that compound **1a** specifically inhibits FAS-II in mycolic acid biosynthesis (Figure 3). Furthermore, the mycobacterial strains transformed with overexpression plasmids (pMV261) containing InhA conferred an increase in resistance to compound **1a** and no resistance was observed with empty vector pMV261 (Figure 4). This further validates that mycolic acid synthesis is inhibited by compound **1a** and InhA is the possible target. InhA is one of the best validated targets in the treatment of TB. The pro drug INH is oxidatively activated by the catalase-peroxidase KatG and covalently binds to NAD to form an adduct that inhibits the activity of InhA (Rawat et al., 2003). It is anticipated that a

drug directly targeting InhA in a different location than activated INH and not requiring activation by KatG may have bactericidal and sterilizing properties superior to those of INH.

To ascertain whether compound **1a** directly targets InhA without requiring KatG activation, we conducted ligand binding tryptophan flourescence assay. Triclosan inhibits InhA directly, without requiring the activation of KatG (Gurvitz, 2010). It was observed that compound **1a** shows similar binding affinity to triclosan (Figure 5), thus suggesting that compound **1a** directly inhibits InhA. Computational studies also provided further evidence of the binding affinity of compound **1a** towards the target site InhA as shown in Figure 6 (a and b). Compound **1a** showed hydrophobic interactions with Tyr 158, Phe 149, Met 199, Met 166 and Pro 193 and is also involved in π-π interactions with Phe 149 residues of InhA. It is also involved in van der Waals interactions with surrounding amino acids. Similar to our work Yadav and co-workers by their docking studies have shown that the chalcone derivatives bind to InhA with good affinity as compared with the standard inhibitors such as triclosan and isoniazid (Yadav et al., 2014). They also showed hydrophobic interactions with Tyr and Phe residues but in addition to these residues they also showed the interaction with other amino acid residues of the InhA, including aliphatic (Gly-93 and Ile-200), sulfur containing (Met-159 and Met-206), cyclic (Pro-191), and basic (Lys-163) residues. Recently Joshi et al (2017) have used target to drug approach and shown that compounds such as pyrrolyl benzohydrazide derivatives inhibit InhA directly. Similarly Stular et al (Stular et al., 2016) using molecular docking found three novel compounds that bind InhA. Biological testing confirmed that these compounds inhibit InhA. Muradas et al (Muradás et al., 2018) have studied the inhibitory activity of quinoxaline derived chalcones against *M. tuberculosis* and showed MIC values in the range of 3.13 μg.ml^{-1} to 12.5 μg.ml^{-1} which are very similar to our results. However, in contrast to our results they showed that these chalcones did not inhibit mycolic acid synthesis as they showed low MICs against *M. tuberculosis* strains that have mutations in the *katG* or *inhA* genes. The results cannot be directly compared due to the difference in the functional groups.

5 Conclusion:

Following the identification and validation of the molecular target, target-specific optimization of compound **1a** will be pursued to improve efficacy and reduce toxicity. This will be achieved using computational and synthetic chemistry techniques to rationally design novel inhibitors based on our understanding of ligand-protein (**1a**-InhA) interaction.

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Author Contributions:

Conceived and designed the experiments: J.A.G.C., B.A., M.G., C.B., J.P.B., G.S.B. Performed the experiments: B.A., J.A.G.C., T.R.K.R., J.S. Wrote the paper: M.G., J.A.G.C., T.R.K.R., J.P.B., B.A. Analyzed the data: J.A.G.C., M.G., J.P.B., J.S., B.A., T.R.K.R. The manuscript was reviewed by all the authors.

Ethical Approval: Not Required

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