



Identification and validation of the mode of action of the chalcone antimycobacterial compounds



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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 IC₅₀ 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.

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

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(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 and 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 (Fig. 1). The synthesized products were recrystallized from appropriate solvents and were characterized by spectral analysis, melting point, infrared spectroscopy, ^1H 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 $\mu\text{g}\cdot\text{ml}^{-1}$ and the standard (RIF) were prepared in Middlebrook 7H9 medium from stock solution (10 $\text{mg}\cdot\text{ml}^{-1}$ 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 h. 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 $\mu\text{g}\cdot\text{ml}^{-1}$. 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 $A_{540\text{nm}}$. 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 $\mu\text{g}\cdot\text{ml}^{-1}$), 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 min 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,b; 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 κ -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).

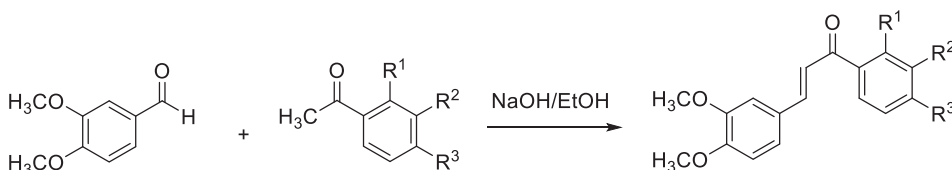


Fig. 1. Chalcone synthesis reaction scheme (**1a-1o**).

Table 1

Shows chalcone compounds **1a** to **1o** with various substituents at R¹, R² and R³ positions with their molecular weights and predicted *Clogp* values. The MIC of each compound is shown in $\mu\text{g.ml}^{-1}$ against *M. bovis* BCG with the IC₅₀ 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)	<i>Clogp</i>	MIC ($\mu\text{g.ml}^{-1}$) <i>M. bovis</i> BCG	J-774 (IC ₅₀) ($\mu\text{g.ml}^{-1}$)	SI = IC ₅₀ /MIC
	R ¹	R ²	R ³					
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 ₆ H 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	–	–

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 IC₅₀ (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 min in a buffer solution (25 mM HEPES, 10% glycerol and 300 mM NaCl, pH 8) at 25 °C followed by addition of increasing concentrations 0.3–70 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 ($\mu\text{g.ml}^{-1}$) 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 $\mu\text{g.ml}^{-1}$. 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 (Fig. 2a-c) are smooth surface and characteristic rod like shape. Whereas the cells exposed to **1a** for five days showed elongation, wrinkles and bulging (Fig. 2d-f).

3.1. Mode of action

3.1.1. FAMES and MAMES analysis

To identify the biological target of **1a**, inhibition of mycolic acid

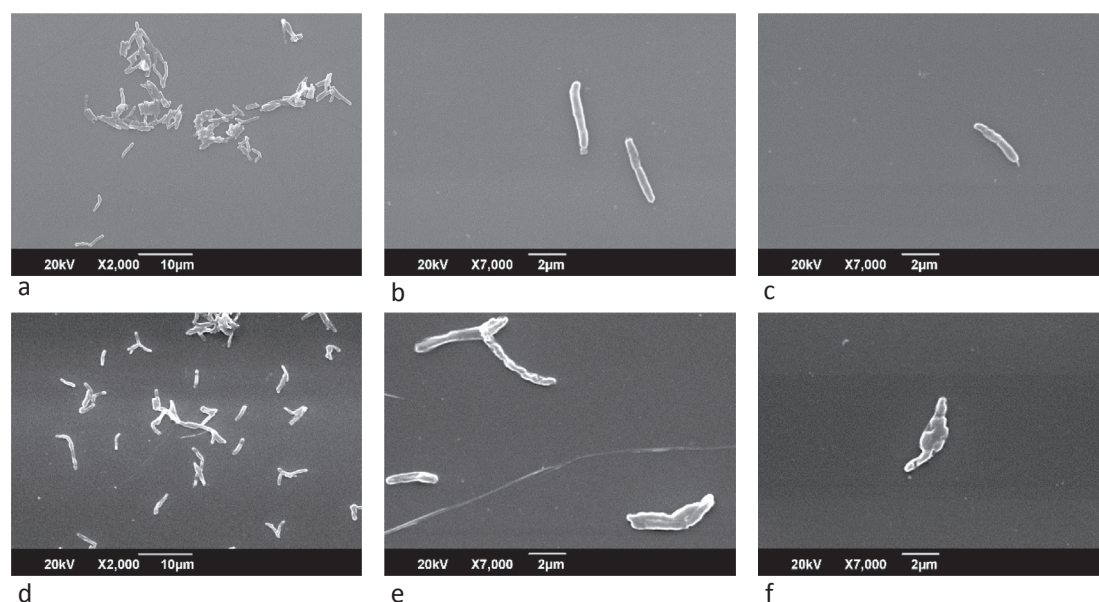


Fig. 2. Scanning electron micrographs of untreated and 10 X MIC (60 µg/ml) **1a** treated *Mycobacterium bovis* BCG cells for 5 days. (a-c) untreated *M. bovis* BCG cells; (d-f) 60 µg/ml **1a** treated *M. bovis* BCG cells analyzed using JEOL CarryScope JCM-5700 scanning electron microscope. There was significant proportion (76%) of cells showing morphological changes such as bulging, elongation and wrinkling of cell surface.

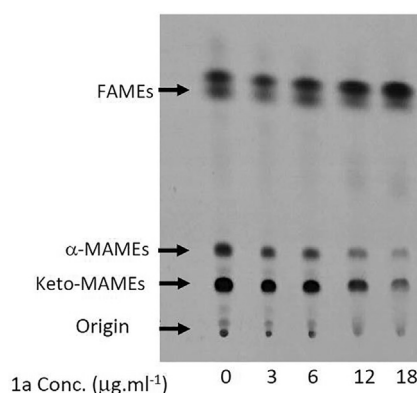


Fig. 3. Inhibition of mycolic acid biosynthesis by Chalcone **1a**. *Mycobacterium bovis* BCG cultures, labelled with [14 C]-acetate were exposed to increasing concentrations of **1a**. The α - and k-MAMES and FAMES were extracted and analysed by autoradiography-TLC. From left to right: Untreated, treated with 3 µg.ml $^{-1}$ of **1a**, treated with 6 µg.ml $^{-1}$ of **1a**, treated with 12 µg.ml $^{-1}$ of **1a** and treated with 18 µg.ml $^{-1}$ of **1a**.

biosynthesis was investigated. As shown in Fig. 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 (Fig. 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 (Fig. 4), indicating an

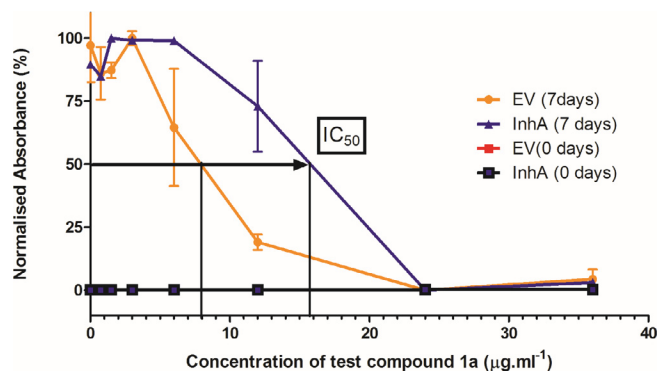


Fig. 4. Investigation of the impact of *InhA* overexpression on sensitivity to compound **1a**. Absorbance values were read at 0 days and after 7 days for EV and *InhA* strain. Using *Mycobacterium bovis* BCG containing a constitutive plasmid (pMV261) expressing *InhA*, IC $_{50}$ was compared to an 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).

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 (Fig. 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 (Fig. 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.

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 dehydratase 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 (Fig. 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 (Fig. 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 fluorescence 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 (Fig. 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 Fig. 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 $\pi-\pi$ 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. (2016) using molecular docking found three novel compounds that bind InhA. Biological testing confirmed that these compounds inhibit InhA. Muradás et al. (2018) have studied the inhibitory activity of quinoxaline derived chalcones against *M. tuberculosis* and showed MIC values in the range of 3.13–12.5 $\mu\text{g}\cdot\text{ml}^{-1}$ 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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Declarations

None.

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Competing interests

The authors declare that they have no financial or non-financial competing interests.

Author contributions

Conceptualization: J.A.G.C., B.A., M.G., C.B., J.P.B., G.S.B. Investigation and Methodology: B.A., J.A.G.C., T.R.K.R., J.S. Writing - original draft, Writing - review & editing: M.G., J.A.G.C., T.R.K.R., J.P.B., B.A. Formal Analysis: J.A.G.C., M.G., J.P.B., J.S., B.A., T.R.K.R. Supervision: M.G; J.P.B; J.A.G.C. Project Administration: M.G; J.P.B; J.A.G.C. Software: T.R.K.R. The manuscript was reviewed by all the authors.

Ethical approval

Not Required.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.tcs.2020.100041>.

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