International Journal of Antimicrobial Agents 42 (2013) 27–35

Contents lists available at SciVerse ScienceDirect

International Journal of Antimicrobial Agents

journal homepage: http://www.elsevier.com/locate/ijantimicag

Identification and characterisation of small-molecule inhibitors of Rv3097c-encoded lipase (LipY) of *Mycobacterium tuberculosis* that selectively inhibit growth of bacilli in hypoxia☆

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ARTICLE INFO

Article history:
Received 15 October 2012
Accepted 17 March 2013

Keywords:
Mycobacterium
Rv3097c
Lipase
Inhibitor
Hypoxia
Latent TB

ABSTRACT

The mycobacterial Rv3097c-encoded lipase LipY is considered as a true lipase involved in the hydrolysis of triacylglycerol stored in lipid inclusion bodies for the survival of dormant mycobacteria. To date, orlistat is the only known LipY inhibitor. In view of the important emerging role of this enzyme, a search for small-molecule inhibitors of LipY was made, leading to the identification of some new compounds (**8a–8d**, **8f**, **8h** and **8i**) with potent inhibitory activities against recombinant LipY, with no cytotoxicity [50% inhibitory concentration (CC_{50}) ≥500 μg/mL]. The compounds **6a**, **8c** and **8f** potently inhibited (>90%) the growth of *Mycobacterium tuberculosis* H_{37}Rv grown under hypoxia (oxygen-depleted condition) but had no effect on aerobically grown bacilli, suggesting that these new small molecules are highly selective towards the growth inhibition of hypoxic cultures of *M. tuberculosis* and hence provide new leads for combating latent tuberculosis.

1. Introduction

Tuberculosis (TB) is a globally challenging disease primarily caused by *Mycobacterium tuberculosis*. Despite the availability of highly efficacious treatment(s) for decades, TB remains a major global health problem with an estimated 9 million new cases and 2 million deaths every year [1]. In addition, over 2 billion people harbour latent TB infection, thus representing an enormous reservoir of *M. tuberculosis* that can subsequently progress to active TB. Effective treatment of TB can be achieved by combination of three to four drugs [2]. However, the spread of TB remains unchecked for a number of reasons, including the emergence of multidrug-resistant TB (MDR-TB) and extensively drug-resistant TB (XDR-TB), latent TB infection, and its synergy with HIV/AIDS [1]. This highlights the need for new therapies that act via a novel mechanism of action to shorten the treatment duration of active drug-sensitive TB, of MDR-TB and XDR-TB, and of persistent and latent bacilli [1].

Determination of the complete genome sequence of the most widely known H_{37}Rv strain of *M. tuberculosis* has opened new perspectives for developing novel drugs to treat TB [3]. Comparative sequence analysis of the *M. tuberculosis* genome has revealed the presence of nearly 250 genes encoding lipolytic enzymes involved in lipid metabolism, a major pathway in *Mycobacterium* spp. [4]. The complex cell wall of mycobacteria contains unusual lipids that are essential for survival and virulence [5,6]. In addition, the cytosol of mycobacteria contains organites (lipid inclusion bodies) that store important lipids [triacylglycerol (TAG)] for long-term survival during the dormancy phase [7]. Utilisation of the stored TAG requires its hydrolysis and the release of fatty acids for catabolism by β-oxidation.

In recent years, mycobacterial lipases have received considerable attention [8]. Deb et al. [9] cloned the putative lipase genes of *M. tuberculosis* and expressed them in *Escherichia coli*. They compared the enzyme activity of recombinant enzymes in vitro and suggested that the newly identified Rv3097c-encoded enzyme LipY was active as a true lipase [10]. Upregulation of LipY was observed ex vivo in infected murine macrophages and in *M. tuberculosis* growing in the lungs of infected mice [11,12]. Under conditions of nutrient starvation and oxygen-depleted hypoxic conditions, extensive LipY-induced breakdown of TAG was observed [9,13], emphasising the role of LipY in survival of mycobacteria in dormant

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http://dx.doi.org/10.1016/j.ijantimicag.2013.03.007

conditions. A strong humoral immune response against LipY observed in clinical TB suggested the importance of the Rv3097c antigen in immunopathogenicity of *M. tuberculosis* [10]. Interestingly, extensive LipY-induced hydrolysis of TAG in *Mycobacterium bovis* bacillus Calmette-Guerin (BCG) reduced the efficacy of the BCG vaccine to protect against infection with *M. tuberculosis*, and it was found that loss of efficacy of the vaccine correlated with suppression of host immune responses [14]. A striking observation was made that synthesis of a unique phenolic glycolipid in some strains of *M. tuberculosis* conferred a hypervirulent phenotype by inhibiting the innate immune response of the host [15].

Cumulative results suggested that LipY is most likely the major lipase involved in the hydrolysis of stored TAG. The *N*-terminal domain of LipY is homologous to proteins belonging to the PE-PGRS family, whereas the *C*-terminal resembles the conserved hormone-sensitive lipase family. Pair-wise alignment of the amino acid sequence of LipY with 35 representative lipases from all of the eight reported families of bacterial lipases indicated that LipY shared only 12–23% global amino acid identity with known bacterial lipases [9]. To date, there is no knowledge of any compound that can potently inhibit the mycobacterial active lipase encoded by Rv3097c.

Orlistat, a US Food and Drug Administration (FDA)-approved anti-obesity drug [16–18], has recently been shown to inhibit and disrupt cell wall formation in several mycobacterial species through inhibition of the essential *M. tuberculosis* lipase Rv3802c. The three-dimensional (3D) structure of *M. tuberculosis* lipase Rv3802c is yet to be elucidated, although a moderate-resolution crystal structure of MSMEG_6394 [Protein Data Bank (PDB) ID: 3aja] reported by Crellin et al. [19] is considered as a close homologue of Rv3802c. West et al. [20] recently reported several lead compounds exhibiting nanomolar inhibitory activity against the mycobacterial lipase Rv3802c.

In view of the emerging role of Rv3097c-encoded lipase from *M. tuberculosis* in latent TB, here we report identification of a new class of Rv3097c-encoded lipase inhibitors. These new LipY inhibitors selectively inhibited the growth of *M. tuberculosis* growing in hypoxia but not of aerobically growing bacilli and thus represent an interesting scaffold for identification of candidate molecules for the treatment of dormant bacilli.

2. Materials and methods

2.1. Chemistry

The synthesis of title compounds (Table 1) is outlined in Fig. 1. Reaction of compound **1** with diethyl ethoxymethylenemalonate (**2**) under reflux produced compound **3**, which was then refluxed to produce compound **4** (Scheme 1). Treatment of **4** with methanolic ammonia under reflux yielded amide **5**, which upon reaction with 3-phenylpropanal produced compound **6a**, whereas reaction of compound **4** with hydrazine hydrate produced hydrazide **7**, which upon reaction with benzaldehyde(s) produced compounds **8a–k** (Scheme 2).

2.2. Bacterial and plasmid strains

Mycobacterium tuberculosis $H_{37}Rv$ (TMC-702) was used as the template for PCR amplification of the *lipY* gene. *Mycobacterium smegmatis* mc^2155 strain was received from Central JALMA Institute for Leprosy and other Mycobacterial Diseases (India). Vector pSD26 was the expression vector for LipY in the *M. smegmatis* host strain.

2.3. Cloning and expression of the lipY gene

The *lipY* gene was amplified by PCR using *M. tuberculosis* $H_{37}Rv$ genomic DNA and the following primers: upstream primer *pSDLipY*-up 5′-AAA GGA TCC GTG TCT TAT GTT GTT GCG TTG-3′; and downstream primer *pSDLipY* 5′-AAG GAT CCG GCG GCG ATA CCG AGT TGC TG-3′ [primers contain *Bam*HI sites (underlined)]. The PCR-amplified gene was cloned at the *Bam*HI site into pSD26. The vector pSD26 is a *M. smegmatis* expression vector containing the mycobacterial inducible acetamidase promoter that allows translational fusion with the 6× His tag [21]. The recombinant plasmid was electroporated into *M. smegmatis* mc^2155 strain and then hygromycin-resistant colonies were selected. The presence of the insert (1314 bp) was confirmed in transformants by restriction digestion and PCR amplification.

2.4. Purification of recombinant protein

Mycobacterium smegmatis [pSD26::rv3097c] was grown in 100 mL of Middlebrook 7H9 (MB7H9) complete medium (Difco) with hygromycin (Sigma, St Louis, MO) (50 μg/mL) at 37 °C to an optical density at 600 nm of 0.6. The culture was divided into two aliquots. One aliquot was induced with 0.2% acetamide (Sigma) for 7 h at 37 °C, whilst the other formed an uninduced control and received an equal volume of sterilised Milli-Q water. Cells were harvested by centrifugation at 6000 rpm for 10 min at 4 °C, washed with 20 mM Tris–HCl (pH 7.5) and re-suspended in 15 mL of 20 mM Tris–HCl (pH 7.5) containing phenylmethanesulfonyl fluoride (PMSF). Cells were disrupted by sonication in ice for 10–15 min and the lysate was centrifuged at 20 000 × *g* at 4 °C for 20 min to separate the cytoplasmic fraction from the cell wall. It is known that LipY is partitioned in the pellet [10]. Recombinant LipY (rLipY) was purified using Ni-nitrilotriacetic (NTA) beads under denaturing conditions by solubilising the inclusion body in 8 M urea, which did not inhibit the activity of LipY. Briefly, the pellet was re-suspended in 10 mL of binding buffer [20 mM Tris–HCl (pH 7.5), 200 mM NaCl, 0.1 mM PMSF and 8 M urea] and was incubated at 37 °C for 1 h followed by centrifugation at 12 000 rpm for 20 min at 4 °C. The supernatant was loaded on the Ni-NTA agarose column (3 mL bed volume), pre-equilibrated with 15 mL of the binding buffer. The flow through was passed five times through the Ni-NTA agarose column. Unbound proteins were washed out by passing 15 mL of binding buffer. The column with bound proteins was washed with washing buffer (the binding buffer with 2, 5, 10 and 30 mM imidazole, 10 mL each). rLipY was eluted in the presence of 400 mM imidazole and its molecular weight was determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).

2.5. Mycobacterial LipY inhibition assay

Lipase activity was assayed according to the method reported by Mishra et al. [10], with some modifications, using *p*-nitrophenyl stearate as substrate. Orlistat was used as a positive control. The assay was performed in 96-well plates (in 200 μL of reaction mixture) containing 50 μL (0.5 μg) of protein in elution buffer, 150 μL of sodium phosphate buffer saline [100 mM sodium phosphate buffer (pH 8.0), 200 mM NaCl] and a final concentration of 0.5 mM *p*-nitrophenyl stearate (20 mM *p*-nitrophenyl stearate stock solution prepared in acetonitrile). Test compounds were dissolved in 10% dimethyl sulphoxide (DMSO). The mixture was incubated for 1 h with intermittent shaking at 37 °C and the release of *p*-nitrophenol was measured spectrophotometrically at 405 nm. The blank reaction was set with protein sample substituted by elution buffer. Assays were performed in triplicate and protein activity was expressed as the average 'nM of *p*-nitrophenol released/mg of protein/min'. The Michaelis–Menten constant (K_m)

Table 1
Structures and in vitro inhibitory activities (IC_{50} values) against *Mycobacterium tuberculosis* LipY of the evaluated compounds (**6a**, **8a–k**).

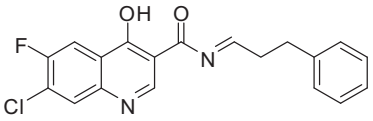
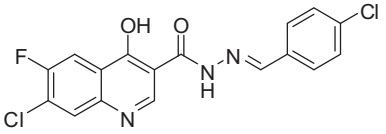
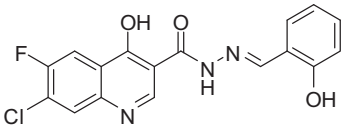
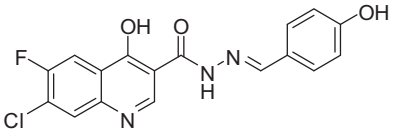
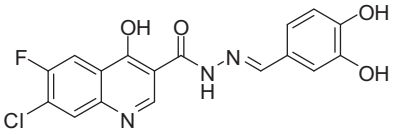
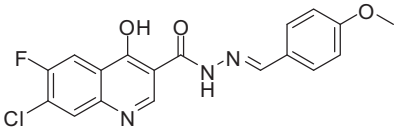
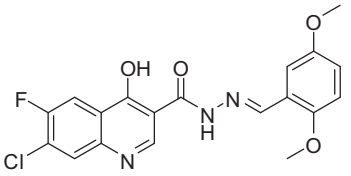
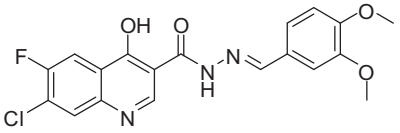
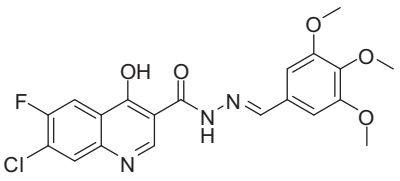
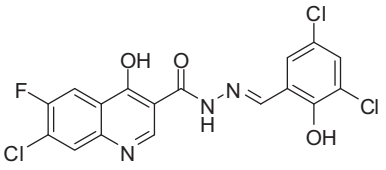
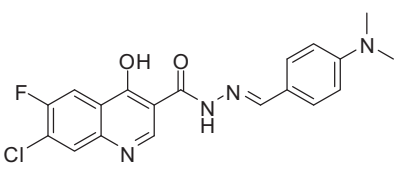
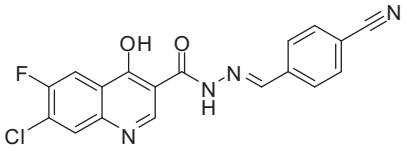
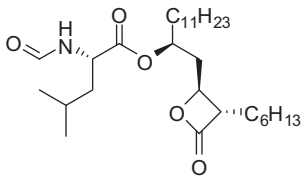
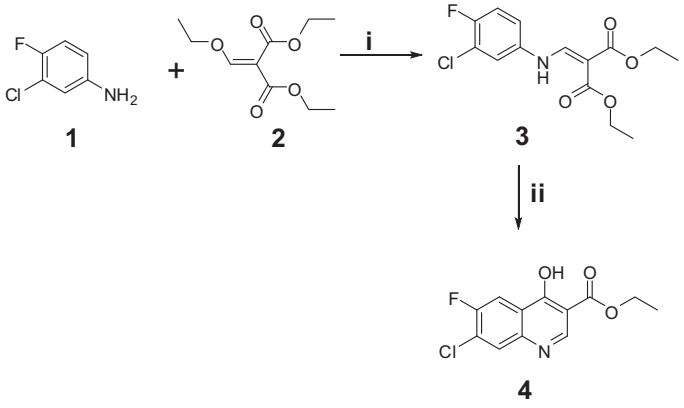
Compound	Chemical structure	XP GlideScore	LipY IC_{50} (mean ± S.E.M.) (μM)	Vero cell CC_{50} (μg/mL)
6a		−5.30	25.00 ± 1.31	>500
8a		−8.68	7.75 ± 0.21	>500
8b		−6.64	12.50 ± 0.32	137.89
8c		−5.64	12.50 ± 0.17	>500
8d		−6.63	9.25 ± 0.17	>500
8e		−5.42	>100	N/D
8f		−8.73	8.25 ± 0.43	>500
8g		−5.67	18.00 ± 0.11	N/D
8h		−5.58	9.25 ± 0.21	N/D
8i		−9.26	5.13 ± 0.20	>500
8j		−4.99	>100	N/D

Table 1 (*Continued*)

Compound	Chemical structure	XP GlideScore	LipY IC_{50} (mean ± S.E.M.) (μM)	Vero cell CC_{50} (μg/mL)
8k		−5.37	>50	N/D
Orlistat			<1.50	N/D

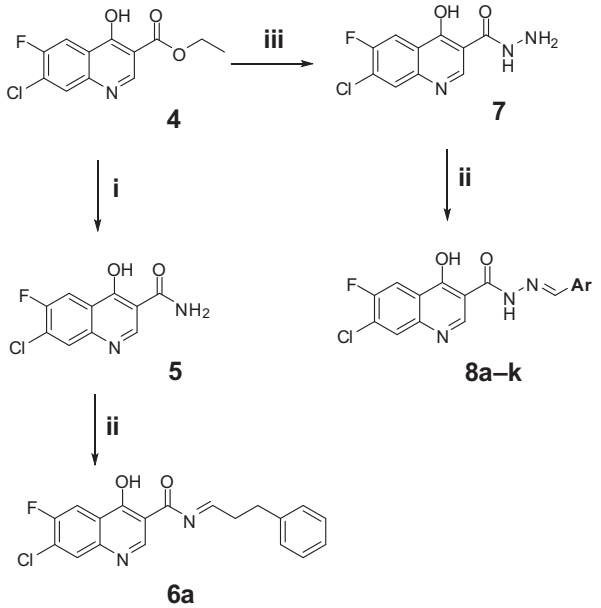
IC_{50}, concentration of inhibitor that caused 50% inhibition of the activity at 500 μM substrate concentration; CC_{50}, % inhibitory concentration; S.E.M., standard error of the mean; N/D, not determined.

Scheme 1: Synthesis of the intermediate compound 4[a]



[a] (**i**) Reflux, 3–5 h; (**ii**) Dowtherm, reflux at 250 °C.

Scheme 2: Synthesis of the title compounds (6a, 8a-k)[a]



[a] (**i**) Methanolic ammonia, reflux; (**ii**) aldehyde, acetic acid, ethanol, reflux; (**iii**) hydrazine hydrate, reflux.

Fig. 1. Outline for synthesis of title compounds.

and maximum enzyme reaction rate (V_{max}) of the purified rLipY lipase enzyme were 317 μM and 514.54 nM/mg/min, respectively. rLipY inhibitory activity was assayed at varying concentrations (100–1.56 μg/mL) to determine the IC_{50} value, which was defined as the concentration of inhibitor that caused 50% inhibition of the activity at 500 μM substrate concentration.

2.6. Cytotoxicity assay

The compounds were tested for cytotoxicity in an in vitro model with Vero monkey kidney cells using the resazurin assay [22]. Vero cells (ATCC CCL-81) were seeded overnight at 3×10^4 cells/well in 96-well plates at 37 °C in RPMI supplemented with 10% heat-inactivated fetal bovine serum and 5% CO_2. Cells were exposed to dilutions of experimental and control drugs in triplicate for 2 h with each compound at a range of concentrations from 100 μg/mL to 1.56 μg/mL. Rifampicin was used as a control at the same concentration. Each well had 100 μL of the test material in serially descending concentrations. After 72 h of incubation, 10 μL of resazurin indicator solution (0.1%) was added and incubation was continued for 4–5 h. Any colour change from purple to pink or colourless was recorded as positive. Fluorescence of each sample was measured with an excitation wavelength at 530 nm and an emission wavelength at 590 nm using a BMG POLARStar Galaxy microplate reader (BMG Labtech, Cary, NC). CC_{50} values (50% inhibitory concentrations) were calculated by plotting fluorescence values using Microsoft Excel (Microsoft Corp., Redmond, WA).

2.7. Resazurin microtitre assay

Drug susceptibility testing using resazurin was performed under aerobic and hypoxic conditions as described [23] for *M. tuberculosis*. Viable cells convert non-fluorescent resazurin dye to red fluorescent resorufin dye in response to chemical reduction resulting from cell growth. Continued cell growth maintains a reduced environment, whilst inhibition of growth maintains an oxidised environment. Reduction related to growth causes the redox indicator to change from the oxidised form (non-fluorescent, purple colour) to the reduced form (fluorescent, red colour).

2.7.1. Assay under aerobic conditions

For aerobic cultures, the assay was performed in 96-well microplates. Initial drug dilutions were prepared in either DMSO or sterile deionised water, and subsequent two-fold serial dilutions were performed in 0.1 mL of MB7H9-S/Dubos-S medium (Difco) supplemented with 0.05% glycerol (Sigma) (without Tween 80) in the microplates. Approximately 5×10^5 CFU of *M. tuberculosis* H_{37}Rv was added per well in a volume of 0.1 mL. Control wells contained bacteria only (B), medium only (M) or drug only (to detect autofluorescence of the drug) and were used to calculate percentage inhibition of viability. Plates were incubated at 37 °C for 6 days. Thereafter, 20 μL of 0.02% resazurin was added. The wells were observed after 48 h for colour change from blue to pink. The fluorescent signal was monitored using 530–560 nm excitation wavelength and 590 nm emission wavelength in bottom-reading mode.

2.7.2. Assay under hypoxia

Several 3 mL culture aliquots of *M. tuberculosis* containing 1.5×10^6 CFU/mL ($A_{595} \sim 0.003$) were injected into 5 mL uncoated Vacutainer® tubes and kept static at 37 °C to allow self-generation of hypoxia. The medium contained the oxygen indicator methylene blue (1.5 mg/L). Decolourisation of the medium suggested depletion of dissolved oxygen as an indicator of hypoxia/anoxia. As reported previously [24,25], a viable non-replicating persistent culture could be obtained after 22 days with less than 1% dissolved oxygen in the medium and the cells were sensitive to metronidazole (MTZ) and resistant to isoniazid (INH). Compounds to be tested were injected (100 μL/tube) at different concentrations and incubated for 6 days at 37 °C under static conditions. Then, 350 μL of 0.02% resazurin was added to each tube and was incubated for 48 h at 37 °C. After this incubation, 200 μL aliquots from each tube were transferred into a 96-well microplate and fluorescence was determined.

During these measurements, three controls were performed: (i) without any drug; (ii) with MTZ; and (iii) with INH at different concentrations [25]. Background fluorescence from medium (M) and drug wells was subtracted. Percentage reduction in viability was determined as: 1 − (test well fluorescence/mean fluorescence of triplicate B wells) × 100 as described previously by Collins and Franzblau [26].

2.8. Computational studies

The homology model of *M. tuberculosis* LipY protein was developed using the Prime [27,28] module considering its closest structural homologue triglyceride lipase from *Staphylococcus aureus* (PDB ID: 3d7r) [29], sharing 22.6% sequence identity and 41.6% sequence similarity. The 3D structures of ligands were sketched and prepared in Maestro using the optimised potentials for liquid simulations–all atom (OPLS-AA)-2005 force field. Molecular docking studies were carried out using Glide within a grid generated using the O^{γ} atom of the Ser309 residue specifying an active site radius of 10 Å. The predicted binding poses were ranked using the XP GlideScore and the top-ranked poses were used for further analysis.

3. Results

3.1. Cloning, expression and purification of Rv3097c-encoded LipY

Expression of recombinant Rv3097c-encoded LipY protein was achieved in the acetamide-inducible vector pSD26 in a *M. smegmatis* host strain. rLipY protein was purified using Ni-NTA beads under denaturing conditions by solubilising the inclusion body in 8 M urea, which did not inhibit the activity of LipY. SDS-PAGE revealed a single protein band of 47 kDa (Fig. 2). The purified protein was used for characterisation of its enzymatic properties and for inhibitor screening.

3.2. Target-based biological screening and structure-based drug design (SBDD)

Target-based in vitro screening of an in-house proprietary collection of compounds against purified rLipY enzyme led to the identification of an acyl-imine class of new molecule (**6a**) exhibiting moderate inhibition of mycobacterial Rv3097c-encoded rLipY, with an IC_{50} of 25 μM (Fig. 3). In the follow-up stage, design of a new series of compounds considering the hit **6a** was done using the SBDD technique. The first theoretical model of Rv3097c-encoded LipY was modelled based on the triglyceride lipase from *S. aureus* (PDB ID: 3d7r). The local and global Cα-root mean square deviations (RMSD) of the developed model and the template were 1.02 and 5.53, respectively. The developed LipY model contains a canonical α/β hydrolase domain with an eight-stranded parallel β-sheet (β1–β8) surrounded by five α-helices (H3–H5, H7 and H8) and is comprised of a catalytic triad formed by Ser309, Asp383 and His413 residues similar to the other serine esterases (Fig. 4). The Ser309 residue was located at the bend of the tight turn connecting the β5 strand and H5 helix, a feature known as the 'nucleophilic elbow' that is conserved among all members of

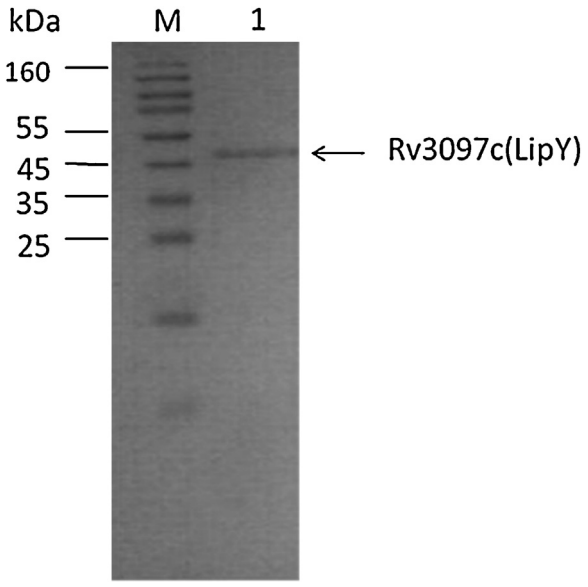


Fig. 2. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (12.5%) analysis of recombinant Rv3097c (LipY) protein. Lane M, pre-stained protein molecular weight ladder (MBI Fermentas, Amherst NY); lane 1, purified recombinant LipY protein.

the α/β hydrolase superfamily, and is the nucleophile that attacks the substrate to form a covalent acyl–enzyme intermediate. The active site of LipY was comprised of four subsites, namely the catalytic triad, an exposed wide pocket, a short deep pocket and an extended hydrophobic channel (Fig. 5). Molecular docking of **6a** into the active site of the LipY model using Glide [30] revealed a quinoline moiety being accommodated in the extended hydrophobic channel, and a 4-hydroxyl group formed two bonds with Trp339 and Asp414 residues, whereas the terminal phenyl ring linked to amidic NH was accommodated in the short deep pocket (Fig. 5). The acyl-imine linker was located in a highly polar region, where the hydrazine linker (Fig. 3) was recognised to be a better substitute through docking studies. Therefore, we embarked on the synthesis and biological evaluation of the series of hydrazides **8a–8k**, making major variations in the benzylidene fragment. The in vitro rLipY inhibitory activities and the XP GlideScore of these hydrazides are summarised in Table 1. The majority of these hydrazides exhibited improved rLipY inhibitory activity compared with **6a**, thus validating better suitability of the hydrazine than the acyl-imine for binding with LipY. Compound **8i** (IC_{50} = 5.13 μM) exhibited the highest rLipY inhibitory activity, whilst another four compounds (**8a**, **8d**, **8f** and **8h**) also exhibited promising inhibition of rLipY (IC_{50} = 7.75–9.25 μM). The nature of groups present on the phenyl ring of the benzylidene in terms of hydrophobic and electronic interactions with the residues present on the rim of the interface cavity (Fig. 5) explained well the observed inhibitory activity of the compounds against mycobacterial rLipY (Table 1). This was also supported by the quantitative structure–activity relationship (QSAR) studies between the observed rLipY inhibitory activity (pIC_{50}) and the computed physicochemical parameters for hydrophobicity ($\Sigma\pi$), sterics (ΣMR) and electronic ($\Sigma\sigma$, ΣF and ΣR), which revealed that the electronic parameter followed by hydrophobicity influence the rLipY inhibitory activity variations (Eq. (1)).

$$pIC_{50} = 0.517(\pm 0.148)\Sigma F + 4.709 \quad (1)$$

$N = 9$, $R = 0.796$, $R^2 = 0.634$, SEE = 0.133

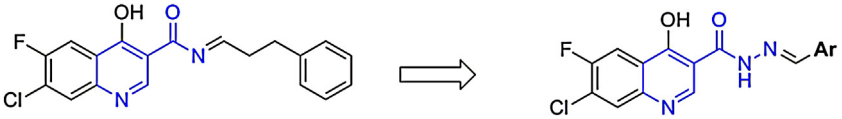
3.3. In vitro cytotoxicity

Vero monkey kidney cells were used for evaluation of the in vitro cytotoxicity of synthesised title compounds (Table 1) using the resazurin assay, where the maximum concentration for the test compounds was 500 μg/mL. The CC_{50} value, which is defined as the concentration causing 50% reduction in the cell number compared with that for the untreated controls, was determined for the tested compounds. The observed CC_{50} values for all the tested compounds are summarised in Table 1.

3.4. Inhibition of mycobacterial viability in aerobic and anaerobic conditions

Selected compounds (**6a, 8a–d, 8f–i)** with rLipY IC_{50} values ≤25 μM were evaluated for their potential to inhibit the viability of aerobically grown and anaerobic hypoxic cultures of *M. tuberculosis* H_{37}Rv at two concentrations (50 μg/mL and 100 μg/mL) using the resazurin reduction assay. Compounds **6a**, **8c** and **8f** caused >90% viability inhibition of hypoxic cultures of bacilli, whereas compounds **8a**, **8b** and **8i** caused 28–53% inhibition at 50 μg/mL. These compounds did not show any growth inhibition of aerobic cultures up to 100 μg/mL (Table 2). In this study, the observed results with the two controls, namely INH (a known inhibitor of aerobic culture) and MTZ (a known inhibitor of anaerobic culture), were similar to the reported results [20,25]. Importantly, compounds **6a**, **8c** and **8f** exhibited promising growth-inhibitory activity of the dormant bacilli grown under hypoxia but not of the bacilli grown under aerobic condition, hence these new molecules can be considered as selective inhibitors of the growth/replication of dormant bacilli under hypoxia targeting LipY.

As the comparison of the relative enzyme inhibitory activities in relation to inhibition of whole-cell mycobacterial viability did not correlate, and the least active compound **6a** in the enzyme assay was almost equally effective as compound **8f,** which was also not the most active compound (**8i**) in the enzyme assay (Table 2), it appeared that the rate-limiting factor for whole-cell activity may be transport of the compound/drug. Hence, log *P* values of six



Compound 6a
Log P: 4.17
tPSA: 62.02
MW: 357.78
M. tuberculosis LipY IC_{50} = 25 μM

Designed Prototype

Ar = substituted aryl

Fig. 3. Chemical structure and LipY inhibitory activity of the preliminary hit **6a**, and the structural formula of the designed new prototype. tPSA, total polar surface area; MW, molecular weight; IC_{50}, concentration of inhibitor that caused 50% inhibition of the activity at 500 μM substrate concentration.

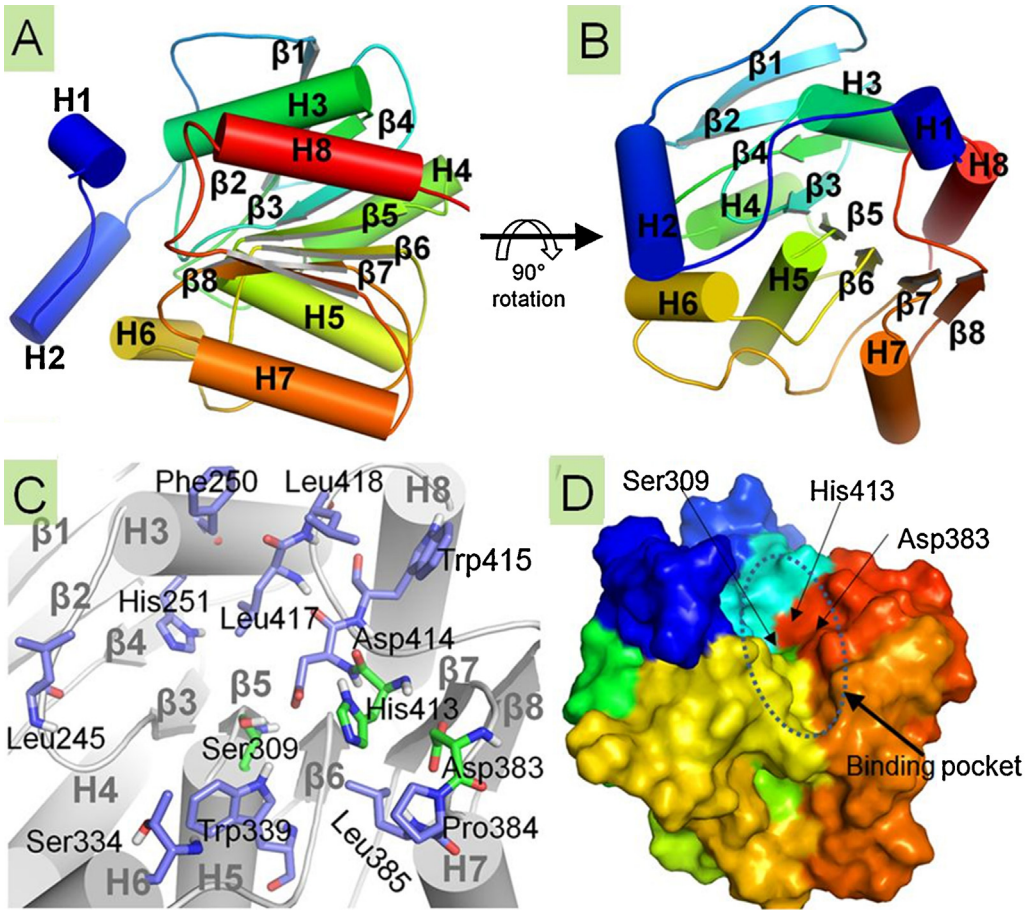


Fig. 4. (A) Schematic representation and annotation of the *Mycobacterium tuberculosis* LipY protein model. The secondary structural elements are coloured based on the position with respect to the *N*- (blue) and *C*- (red) terminals. (B) Schematic representation of the LipY protein after rotation by 90°. (C) Top view of the active site of *M. tuberculosis* LipY, depicting the orientations of the active site amino acids. The secondary structural elements are shown in schematic representation, coloured grey, and labelled. Residues forming the catalytic triad are shown as green-coloured carbon, and the other conserved residues are shown as magenta-coloured carbon. (D) Surface representation of the *M. tuberculosis* LipY protein, depicting the active site residues and the depth of the binding pocket. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
Inhibition of viability of *Mycobacterium tuberculosis* $H_{37}Rv$ by selected compounds in aerobic and anaerobic (hypoxia) conditions.

Compound	Concentration (μg/mL)	% Survival (mean ± S.D.)		log *P*[a]	$[\log P - \log P)_0]^{2}$[b]
		Aerobic	Hypoxia		
6a	100	95.83 ± 1.87	5.23 ± 1.44	4.17	0.10
	50	96.23 ± 1.11	6.19 ± 1.24		
	25	N/D	62.09 ± 1.40		
8a	100	94.57 ± 0.89	41.37 ± 1.61	4.33	0.23
	50	95.06 ± 0.66	47.06 ± 1.37		
8b	100	96.67 ± 0.23	46.72 ± 1.39	3.39	0.21
	50	98.12 ± 0.39	54.09 ± 1.89		
8c	100	97.45 ± 2.83	5.02 ± 3.51	3.39	0.21
	50	97.67 ± 2.54	7.65 ± 3.87		
	25	N/D	60.4 ± 1.34		
8f	100	97.07 ± 1.52	4.06 ± 0.85	3.52	0.11
	50	97.51 ± 0.43	5.52 ± 4.06		
	25	N/D	56.6 ± 1.15		
8i	100	97.07 ± 1.32	64.72 ± 1.62	4.50	0.42
	50	97.92 ± 0.50	72.31 ± 3.48		
INH	1.6	4.11 ± 2.35	88.21 ± 1.36		
	0.8	4.59 ± 2.42	93.84 ± 1.24		
MTZ	128	92.5 ± 1.25	7.54 ± 2.17		
	64	91.77 ± 1.16	14.09 ± 0.71		

S.D., standard deviation; N/D, not determined. INH, isoniazid; MTZ, metronidazole.
[a] Log *P* values were calculated using ChemDraw software (http://www.cambridgesoft.com).
[b] $(\log P)_0 = 3.85$ calculated from Eq. (1).

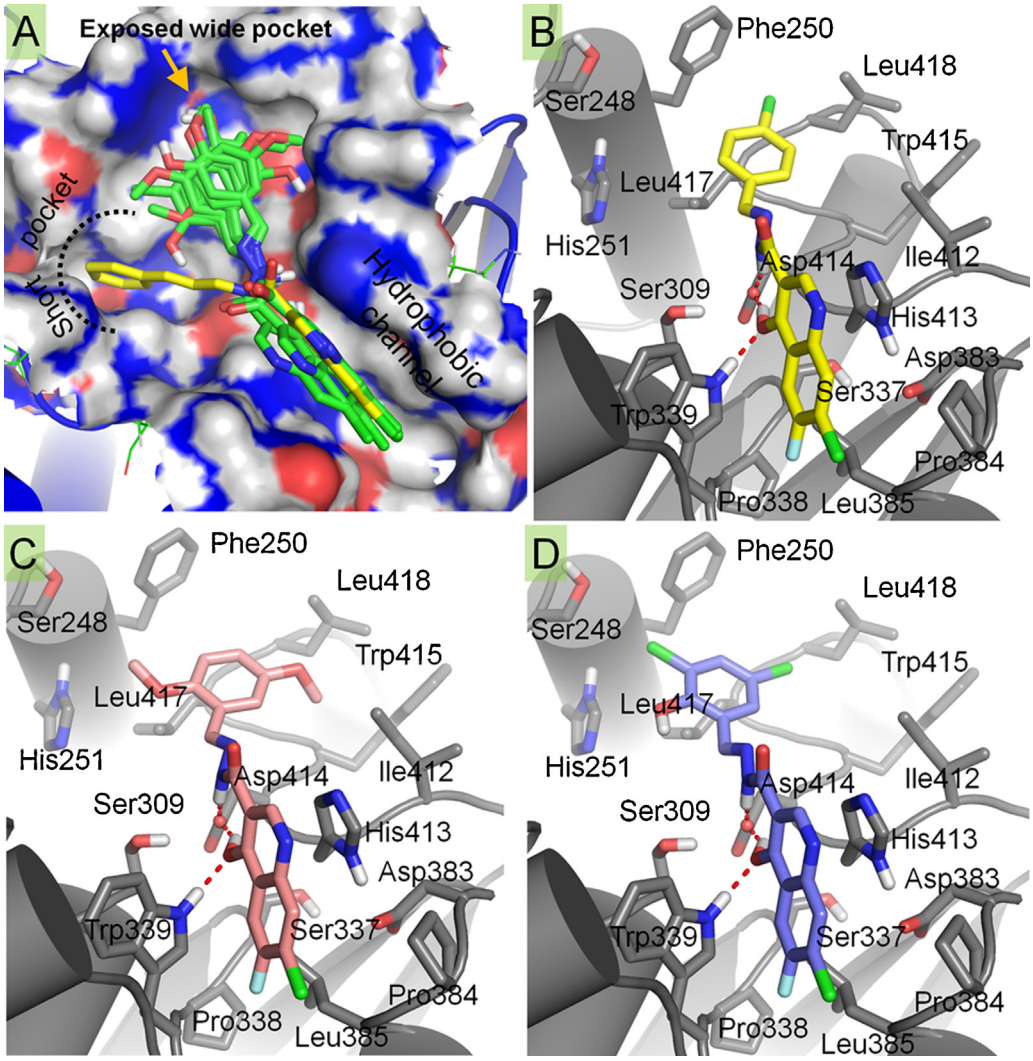


Fig. 5. (A) Perspective view of the docked orientations of 12 characterised new compounds in the binding site of modelled LipY protein. (B–D) Binding pose of compounds **8a** (B) **8f** (C) and **8i** (D), depicting important direct or indirect interactions of active compounds into the active site of the LipY model.

compounds (**6a**, **8a–c**, **8f**, **8i**) were calculated using ChemDraw software (http://www.cambridgesoft.com) and were correlated with the whole-cell activity log [$p/(100-p)$], where p denotes the percentage inhibition/killing (i.e. 100 – %survival).

$$\log[p/(100-p)] = -38.768(\pm 18.006)\log P + 5.042(\pm 2.304)(\log P)^2 + 72.919 \quad (2)$$

$N = 6$, $R = 0.830$, $R^2 = 0.689$, SEE = 0.520

The obtained parabolic correlation (Eq. (2)) with optimum $(\log P)_0$ value of 3.85 well explained the observed whole-cell activity data and further supported the hypothesis that transport/penetration of the compound is the rate-limiting step for whole-cell activity. Since the most active compound **8i** with a log P value of 4.50 has the highest deviation [$\log P - (\log P)_0 = 0.65$] from the optimum $(\log P)_0$, it may not be available within the cell for binding with the lipase enzyme owing to lack of or poor penetration. On the other hand, compounds **6a** ($\log P = 4.17$) and **8f** ($\log P = 3.52$) with minimum deviations of 0.32 and 0.33, respectively, are effective against dormant bacilli in the whole-cell assay.

4. Discussion

The mycobacterial Rv3097c-encoded lipase LipY is considered as a true lipase, essential for the survival of dormant mycobacterium, and hence is an attractive target for the development of drugs against latent TB. To date, the anti-obesity drug orlistat is the only known inhibitor of this attractive target LipY.

Biological screening of in-house compounds against purified rLipY afforded an interesting acyl-imine class of compound **6a** (IC_{50} = 25 μM). Further synthesis and screening of 11 hydrazides (**8a–k**) against this enzyme suggested some promising compounds with better inhibitory activities than **6a**. Five compounds (**8a**, **8d**, **8f**, **8h** and **8i**) have shown potent inhibition of rLipY, with IC_{50} values in the range of 5.13–9.25 μM, where **8i** (IC_{50} = 5.13 μM) showed the highest rLipY inhibitory activity and approximately five-fold better activity than **6a** (IC_{50} = 25 μM) (Table 1). This suggested the better suitability of the hydrazide linker compared with the acyl-imine. Most of these compounds, except **8b** (CC_{50} = 137.89 μg/mL), lacked any cytotoxicity up to 500 μg/mL, suggesting that the observed antimycobacterial activities are not due to any general cytotoxicity.

Molecular docking in combination with QSAR studies on these compounds has revealed the particular importance of groups

present on the benzylidene group in terms of hydrophobic and electronic interactions with the residues present on the rim of the interface cavity (Fig. 5). The electronic parameter ΣF contributes positively and describes ca. 64% of the variance in enzyme activity.

Furthermore, compounds **6a, 8c** and **8f** exhibited >90% inhibition of viability of the dormant bacilli even at a concentration of 50 μg/mL, whilst **8a**, **8b** and **8i** caused 28–53% inhibition of hypoxic cultures. Interestingly, these compounds did not show any growth-inhibitory effect on aerobic cultures up to 100 μg/mL and hence these compounds may be considered as selective inhibitors of the growth/replication of dormant bacilli under hypoxia targeting Rv3097c-encoded lipase LipY. The observed parabolic relationship between the whole-cell activity and log *P* with an optimum $(\log P)_0$ value of 3.85 (Eq. (2)) indicates that the rate-limiting factor for the whole-cell activity is cell wall transport/penetration.

One of the major impediments to TB control is the ability of *M. tuberculosis* to enclose in lung granuloma where the bacilli live in a low-oxygen environment with slow metabolism and enter an almost non-replicating state. The energy requirement is met by hydrolysing TAG. Hence, the role of Rv3097c lipase appears very important. Bacilli in granuloma become refractory to frontline anti-TB drugs [2,9,10,13]. The lead compounds and data presented here become significant because the compounds appear to inhibit LipY lipase. However, it would have been interesting if the level of TAG in the cell in the presence of the inhibitors was measured.

In conclusion, the present study reports the discovery of some new small molecules as potent mycobacterial lipase inhibitors exhibiting low micromolar inhibitory activities against mycobacterial rLipY. Some compounds selectively inhibited mycobacterial growth in hypoxia condition and are better than MTZ. Therefore, these compounds represent interesting scaffolds to medicinal chemists for identification of promising candidate molecules for the treatment of latent TB.

Funding: No funding sources.

Competing interests: None declared.

Ethical approval: Not required.

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