Platensimycin Activity against Mycobacterial \(\beta\)-Ketoacyl-ACP Synthases

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Abstract

Background: There is an urgent need for the discovery and development of new drugs against Mycobacterium tuberculosis, the causative agent of tuberculosis, especially due to the recent emergence of multi-drug and extensively-drug resistant strains. Within, we have examined the susceptibility of mycobacteria to the natural product platensimycin.

Methods and Findings: We have demonstrated that platensimycin has bacteriostatic activity against the fast growing Mycobacterium smegmatis (MIC = 14 \(\mu\)g/ml) and against Mycobacterium tuberculosis (MIC = 12 \(\mu\)g/ml). Growth in the presence of platensimycin specifically inhibited the biosynthesis of mycolic acids suggesting that the antibiotic targeted the components of the mycolate biosynthesis complex. Given the inhibitory activity of platensimycin against \(\beta\)-ketoacyl-ACP synthases from Staphylococcus aureus, M. tuberculosis KasA, KasB or FabH were overexpressed in M. smegmatis to establish whether these mycobacterial KAS enzymes were targets of platensimycin. In M. smegmatis overexpression of kasA or kasB increased the MIC of the strains from 14 \(\mu\)g/ml to 30 and 124 \(\mu\)g/ml respectively. However, overexpression of fabH on did not affect the MIC. Additionally, consistent with the overexpression data, in vitro assays using purified proteins demonstrated that platensimycin inhibited Mt-KasA and Mt-KasB, but not Mt-FabH.

Significance: Our results have shown that platensimycin is active against mycobacterial KasA and KasB and is thus an exciting lead compound against M. tuberculosis and the development of new synthetic analogues.

Introduction

Platensimycin (Figure 1A) is a secondary metabolite from Streptomyces platensis [1,2,3] which has been shown to possess potent anti-microbial activity against Gram-positive bacteria including methicillin-resistant Staphylococcus aureus (MRSA) and vancomycin-resistant Enterococcus (VRE). The low mammalian cell toxicity and the lack of antifungal activity indicates that platensimycin acts selectively [3]. As a result platensimycin represents a promising new chemical class of antibiotics with \(in vivo\) activities of approximately 1 \(\mu\)g/ml towards S. aureus, Enterococcus faecalis and Streptococcus pneumoniae [3]. Platensimycin targets fatty acid biosynthesis in these species by inhibiting FabF and FabH, two \(\beta\)-ketoacyl-ACP synthases (KAS) of the bacterial multi-enzyme fatty acid synthase complex FAS-II [2,3]. Mycobacterium tuberculosis, the causative agent of tuberculosis contains three distinct \(\beta\)-ketoacyl-ACP synthases, KasA, KasB and FabH [4,5]. Of these, FabH acts as a pivotal link between a mammalian-like Fatty Acid Synthase I (FAS-I), a multifunctional enzyme that conducts \(de novo\) synthesis of C\(_{16}\) and C\(_{26}\) fatty acids, and Fatty Acid Synthase-II (FAS-II) a bacterial-type multi-enzyme complex that extends FAS-I products to long chain C\(_{48}\)–C\(_{56}\) fatty acids termed meromycolic acids. FAS-I derived C\(_{26}\) and meromycolic acids then undergo a Claisen-type condensation to form mycolic acids [6,7], \(\alpha\)-alkyl \(\beta\)-hydroxy fatty acids which are important and essential constituents of the mycobacterial cell wall (Figure 2). KasA and KasB are two distinct ketosynthases that are part of a core FAS-II complex which also includes a keto-reductase (FabG1, MabA), a multicomponent dehydratase (Rv0636\(+\) Rv0635 or Rv0637) and an enoyl reductase (InhA) [8,9,10,11,12,13,14,15,16]. This core complex is involved in a reductive cycle that elongates an acyl carrier protein (ACP)-bound acyl chain by iterative addition of two carbons using malonyl-ACP as a substrate, finally resulting in the formation of a meromycolate chain.

While kasA is an essential gene in mycobacteria [11], deletion of Mycobacterium marinum kasB [17] and M. tuberculosis kasB [10] resulted in viable strains that produced shorter meromycolate chains and were attenuated in macrophages and mice. In this study we have examined the whole cell susceptibility of M. smegmatis and M. tuberculosis to platensimycin. In addition, using discrete enzymes assays using purified Mt-KasA, Mt-KasB and Mt-FabH, we have established platensimycin as a promising lead compound for drug development.
Results

Whole cell activity of platensimycin against *Mycobacterium smegmatis*

Platensimycin has been previously shown to be an effective inhibitor of Gram-positive bacteria with MIC values as low as 1 μg/ml for *S. aureus*, *E. faecalis* and *S. pneumoniae* [3]. Platensimycin was initially tested for inhibitory properties against the non-pathogenic, fast growing *M. smegmatis mc²155* which has been used in a number of studies as a surrogate for *M. tuberculosis*. The MIC<sub>99</sub> of *M. smegmatis* in liquid medium was found to be 14 μg/ml (Table 1). We then monitored the growth of *M. smegmatis* in LB broth in the presence or absence of 14 μg/ml platensimycin for a period of 72 hours. While *M. smegmatis* grew normally in medium devoid of platensimycin, the culture in the medium containing platensimycin showed a decrease in OD<sub>600</sub> values with time (data not shown) resulting in clumping after 24 hours of incubation (Figure 3A). Monitoring of viable colony forming units (CFU) demonstrated that the culture grown in the presence of platensimycin possessed a 2 log decrease in CFU (Figure 3B). The plateau shape observed with the treated cells, rather than a killing curve, would suggest that platensimycin is bacteristatic in nature. Further experimentation utilising cells exposed to platensimycin for 72 hours showed that after washing and re-inoculation into fresh media, treated cultures could be revived confirming that the antibiotic is bacteristatic against *M. smegmatis*.

Activity of platensimycin against slow growing mycobacteria

To test the antimycobacterial potency of platensimycin against slow growing mycobacteria we first tested the activity of the antibiotic against *M. tuberculosis* CDC1551 and H37Rv. The MIC of platensimycin required to inhibit the growth of 99% of both *M. tuberculosis* strains on solid medium was 12 μg/ml (Table 1) indicating a comparable potency for this drug against this slow growing pathogen. Surprisingly, growth of the vaccine strain *M. bovis* BCG in the presence of platensimycin was different to that of *M. tuberculosis* and the strain grew normally in medium containing up to 128 μg/ml of platensimycin. In an effort to investigate the

![Figure 1. Structure of platensimycin (A) and platencin (B).](https://doi.org/10.1371/journal.pone.0006306.g001)

![Figure 2. Structures of the major mycolic acids of *M. tuberculosis* and *M. smegmatis*.](https://doi.org/10.1371/journal.pone.0006306.g002)
apparent resistance of BCG to platensimycin we sought to test the effects of increased membrane permeability by generating a M. bovis BCG ΔkasB mutant (Figure 4). It had been previously shown that a ΔkasB null mutant in M. tuberculosis synthesised shorter mycolic acids with almost a complete loss of trans-cyclopropanation of oxygenated mycolic acids that resulted in increased susceptibility to lipophilic antibiotics [10]. Interestingly, the M. bovis BCG ΔkasB (Table 1) mutant was sensitive to platensimycin (MIC 61 μg/ml) suggesting that the increased permeability in comparison to the parental M. bovis BCG strain has indeed increased the sensitivity of M. bovis BCG to platensimycin. However the high MIC of the mutant BCG strain in comparison to that in M. tuberculosis indicates that it is still unclear whether the resistance of BCG to platensimycin was solely due to decreased permeability to the drug.

Platensimycin inhibits biosynthesis of fatty acids and mycolic acids

To study the biochemical effects of platensimycin treatment, cultures of M. smegmatis mc²155 were metabolically labelled with [14C] acetate following exposure to platensimycin. Fatty acids and mycolic acids were extracted from [14C] labelled cells and methylated using phase-transfer catalysis and iodomethane. Extracts of total fatty acid methyl esters (FAMEs) and mycolic acid methyl esters (MAMEs) from untreated and platensimycin treated cultures (5–60 μg/ml) were analysed by TLC-autoradiography. Biosynthesis of fatty acids and α- and epoxy-mycolic acids (Figure 2) was significantly inhibited upon platensimycin treatment (20–40 μg/ml) (Figure 5A). Interestingly, an accumulation of α'-MAMEs was observed at lower concentrations (10–20 μg/ml) of platensimycin, similar to studies observed upon treatment of M. smegmatis with thiolactomycin (TLM), a known inhibitor of KasA and KasB (Figure 5A) [18]. The inhibition of fatty acids is in contrast to studies involving the FAS-II inhibitor isoniazid (INH) where inhibition of mycolic acid biosynthesis leads to an accumulation of fatty acids [19]. These results suggest that platensimycin also inhibits fatty acid biosynthesis via inhibition of mycobacterial FAS-I. Further analysis of the same samples by 2D-Ag²⁺TLC reinforced these findings and revealed more clearly that synthesis of α (α₁ and α₂) and epoxy mycolic acids (Figure 6A) was abolished at lower concentrations, in comparison with the initial accumulation and then cessation α'-mycolic acid biosynthesis (Figure 6A). Furthermore, extracts of cell wall bound mycolic acids, afforded similar profiles upon platensimycin treatment (Figure 6B).

Table 1. Influence of Mt-KasA, Mt-KasB and Mt-FabH overexpression on platensimycin in whole cell inhibition of M. tuberculosis, M. smegmatis and M. bovis BCG.

| Strain                  | MIC₉₀ (μg/ml) |
|-------------------------|--------------|
| M. tuberculosis CDC1551 | 12           |
| M. tuberculosis H37Rv   | 12           |
| M. smegmatis pVV16      | 14           |
| M. smegmatis pVV16-KasA | 30           |
| M. smegmatis pVV16-KasB | 124          |
| M. smegmatis pVV16-KasAB| 126          |
| M. smegmatis pVV16-FabH | 16           |
| M. bovis BCG pVV16      | >128         |
| M. bovis BCG pVV16-KasA | >128         |
| M. bovis BCG pVV16-KasB | >128         |
| M. bovis BCG pVV16-KasB | >128         |

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Figure 3. In vivo effect of platensimycin against M. smegmatis. (A) Clarification of cultures due to clumping and cellular lysis at time point 72 h. (B) Cultures were grown to an OD₆₀₀ of 0.4 upon which 14 μg/ml of platensimycin was added, samples were taken over a 72 h period. Viable counts were calculated as per the methods where the mean CFU per millilitre from three independent experiments was calculated. ●, M. smegmatis; ○, M. smegmatis + platensimycin.

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In addition, analysis of $^{14}$C labelled lipids extracted from platensimycin-treated cultures also revealed that the synthesis of mycolate containing lipids glucose monomycolate (GMM) and trehalose dimycolate (TDM) were reduced (Figure 6C; based on co-migration with authentic standards). The corresponding extracts did not show any platensimycin-derived effects on diacyltrehalose and glycopeptidolipid biosynthesis indicating that the inhibitory effect of platensimycin was specific to mycolate-containing glycolipids. These results demonstrated that platensimycin targeted fatty acid and mycolic acid biosynthesis in M. smegmatis.

### Platensimycin resistance of M. smegmatis strains overexpressing Mt-KasA, Mt-KasB or Mt-FabH

The use of gene overexpression to identify cellular targets of anti-mycobacterial drugs has been highly successful [20,21,22]. Given that the targets of platensimycin in other bacteria were β-ketoacyl synthases, we tested the effects of overexpression of Mt-KasA, Mt-KasB or Mt-FabH on platensimycin resistance in M. smegmatis. The three β-ketoacyl-ACP synthases, cloned into the E. coli-Mycobacterium shuttle vector pVV16 were introduced into M. smegmatis by electroporation. Multiple copies and constitutive expression driven by the hsp60 promoter ensures overexpression of the cloned genes in the host Mycobacterium. First, levels of each recombinant protein was assessed by western blot to confirm that any observed change in resistance could be attributed due to increased levels of the target protein (data not shown). Overexpression of Mt-KasA conferred a modest 2-fold increase in resistance to platensimycin, increasing the MIC from 14 to 30 μg/ml (Table 1). On the other hand, Mt-KasB overexpression resulted in a substantial 9-fold increase in the MIC to 124 μg/ml, respectively (Table 1). A combination of Mt-KasA/B overexpression failed to substantially enhance resistance to platensimycin further and possessed a MIC of 126 μg/ml. Interestingly, though platensimycin had a minimal effect on FabH of other bacteria [2], overexpression of Mt-FabH in theory should confer a small degree of resistance to platensimycin but no significant change was observed (Table 1). These observations were similar to resistance studies conducted with TLM and strains overexpressing Mt-FabH [23]. The nine-fold increase in resistance to platensimycin by M. smegmatis overexpressing Mt-kasB suggests that platensimycin preferentially targets KasB.

To further confirm the observed effects of overexpression on MICs, the ability of the recombinant M. smegmatis strains to incorporate $^{14}$C-acetate into fatty acids and mycolic acids was examined. TLC analysis of FAMEs and MAMEs extracted from different strains treated with platensimycin revealed that whilst mycolic acid biosynthesis was only partially restored in the Mt-KasA overproducing strain, overexpression of either Mt-KasB or Mt-KasAB fully restored fatty acid and mycolic acid biosynthesis (Figure 5 B–D).

### Activity of platensimycin against M. tuberculosis β-ketoacyl-ACP synthases and FAS-I

To evaluate the effect of platensimycin on in vitro enzymatic activity, the impact upon $^{14}$C malonate incorporation into fatty acids in cell free extracts of M. smegmatis enriched with FAS-I, and either purified Mt-KasA, Mt-KasB or Mt-FabH was assessed in discrete assays as described earlier [13,21,24]. In these assays $^{14}$C-malonyl-CoA is transacylated to AcpM via mtFabD prior to the addition of the relevant substrates (C<sub>10</sub>-AcpM and C<sub>16</sub>-CoA) and the enzyme of interest. Upon completion and termination of the experiment the radiolabelled acyl derivates are extracted using organic solvents. The assay was performed with a titre of platensimycin present in triplicate. The results were formulated into a graph where the 50% activity was calculated and noted as the IC<sub>50</sub>. Platensimycin was active against both Mt-KasA and Mt-KasB possessing IC<sub>50</sub> values of 2 μg/ml (4.53 μM) and 4.2 μg/ml (9.51 μM), respectively (Table 2). These results are consistent with the in vitro inhibition of S. aureus FabF and E. coli FabF by platensimycin.
Figure 5. TLC-autoradiography of FAMEs and MAMEs from *M. smegmatis* strains overexpressing Mt-KasA, Mt-KasB and Mt-FabH following platensimycin treatment. Platensimycin (0–60 μg/ml) was titrated into *M. smegmatis* cultures at an OD$_{600}$ nm of 0.4 prior to labelling with 1 μCi/ml [1,2-$^{14}$C]acetate for 12 h. [14C]-FAMEs and MAMEs were extracted and resolved by TLC. An equivalent aliquot of the resulting solution of FAMEs and MAMEs was subjected to TLC using silica gel plates developed twice in petroleum ether-acetone (95:5). Autoradiograms were produced by overnight exposure to Kodak X-Omat film to reveal [14C]labeled FAMEs and MAMEs. (A) *M. smegmatis* pVV16, (B) *M. smegmatis* pVV16-Mt-KasA, (C) *M. smegmatis* pVV16-Mt-KasB, and (D) *M. smegmatis* pVV16-Mt-KasAB.

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Interestingly, the IC\textsubscript{50} values obtained with platensimycin are significantly lower than those obtained with another mycobacterial KAS inhibitor TLM (KasA = 20 \textmu M, KasB 90 \textmu M) \cite{25}. While studies by Wang \textit{et al.} \cite{2} demonstrated that \textit{S. aureus} FabH activity was inhibited by platensimycin (IC\textsubscript{50} = 67 \textmu M), consistent with our overexpression studies, Mt-FabH activity was insensitive to platensimycin (IC\textsubscript{50} > 150 \textmu g/ml, 340 \textmu M) in comparison to Mt-KasA and Mt-KasB (Table 2).

![Figure 6. TLC-autoradiography of \textit{M. smegmatis} lipid extracts and cell wall bound mycolates following platensimycin treatment.](image)

Platensimycin (0, 20, 30, 40 \textmu g/ml) were added to \textit{M. smegmatis} cultures at an OD\textsubscript{600} of 0.4 for 8 h prior to labelling with 1 \muCi/ml \textit{[1,2-\textsuperscript{14}C]}acetate for 12 h. (A) 2D-Ag\textsuperscript{2+} TLC using silica gel plates developed twice in hexane-ethyl acetate (95:5) (direction I) then thrice in petroleum ether-diethyl ether (85:15) (direction Ag II). (B) Cell wall bound mycolate profiles were revealed following two developments in petroleum ether-acetone (95:5). (C) \textsuperscript{[14C]}-Apolar lipids were extracted and resolved by TLC; direction 1, chloroform-methanol-water (100:14:0.8); direction 2, chloroform-acetone-methanol-water (50:60:2.5:3). DAT, diacyltrehalose; GMM, glucose monomycolate; TDM, trehalose dimycolate. Autoradiograms were produced by overnight exposure to film to reveal \textsuperscript{[14C]}-labelled lipids.

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\begin{table}[h]
\centering
\caption{In vitro inhibition (IC\textsubscript{50}) of platensimycin against Mt-KasA, Mt-KasB, Mt-FabH and Ms-FAS-I and Cg-FAS-I.}
\begin{tabular}{|c|c|}
\hline
Enzyme & IC\textsubscript{50} (\textmu g/ml) \\
\hline
Mt-KasA & 2 (0.1) \\
Mt-KasB & 4.2 (0.1) \\
Mt-FabH & >150 \\
Ms-FAS-I & 12 (0.1) \\
Cg-FAS-I & 6.5 (0.2) \\
\hline
\end{tabular}
\end{table}

Figures in brackets represent calculated standard error.

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Interestingly, when crude cell free extracts of either \textit{M. smegmatis} or the related \textit{C. glutamicum} \cite{26} were assayed for FAS-I activity, platensimycin inhibited FAS-I activity at an IC\textsubscript{50} value of 12 \textmu g/ml and 6.5 \textmu g/ml, respectively (Table 2).

Comparisons of \textit{in silico} models of platensimycin-bound ketosynthases

Despite only a moderate level of sequence identity (~36\%) between Mt-KasB and \textit{E. coli} FabF, the two enzymes display identical folds (Suppl. Figure 1A). To ascertain whether the active site of Mt-KasB would be compatible with the steric requirements of platensimycin, we generated a hypothetical structural model of platensimycin-bound Mt-KasB (Suppl. Figure 1B, C). Utilising the Mt-KasB (PDB code 2GP6) superposition with platensimycin-bound Ec-FabF (PDB code 2GFX), subsequent conjugate-gradient energy minimization relieved mild steric clashes between protein and ligand and resulted, compared to ligand-free Mt-KasB, in minor to moderate shifts of side chains located within a 4 Å-radius of platensimycin (root mean square displacement 0.93 Å for 186 main and side chain atoms, maximum displacement 3.5 Å).

The model illustrates steric compatibility between platensimycin and the active site of Mt-KasB, but hints at subtle differences in
protein-inhibitor interactions between Ec-FabF and Mt-KasB. The benzoic acid ring faces a structural environment that is virtually identical to that in Ec-FabF (Suppl. Figure 1B). However, the ketolide group would appear to be less exposed to solvent than it is in the Ec-FabF:platensimycin complex (Figure S1 B,C). Indeed, the simulated complex structure suggests that the ketolide group makes close hydrophobic interactions with Met212 and Ile214 (Figure S1 B,C), contacts that are not seen in platensimycin-bound Ec-FabF, where these residues correspond to alanine side chain. Such differences provide scope for design efforts with the aim to enhance inhibitor affinity in a species-specific manner.

Discussion

Mycolic acid biosynthesis is essential for mycobacterial survival and many antituberculosis drugs like isoniazid, ethionamide and thiolactomycin target enzymes of this exclusive pathway [8,21]. The identification and functionality of novel lead antitubercular agents are essential in the present times due mainly to the emergence of MDR-TB [27] and more recently, XDR-TB [28]. In this worrying climate where untreatable strains of XDR-TB may become apparent the impetus for the discovery of new antitubercular agents becomes crucial. The discovery and characterization of new drugs targeting key enzymes involved in essential mycobacterial biosynthetic pathways is paramount but without the introduction of novel lead compounds, such as platensimycin antitubercular therapy will not proceed fast enough to cope with the increasing resistance observed today. Not only should drug development focus on the synthesis of active analogues of existing anti-mycobacterials, but also on identification of new classes of drugs, such as platensimycin.

Platensimycin, a natural product produced by Streptomyces platensis represents a new chemical class of antibiotics [3]. In this study we have shown that platensimycin is active against M. tuberculosis and through the use of the M. smegmatis surrogate system we were able to investigate and elucidate the mode of action of platensimycin against Mycobacterium spp. We observed inhibition of both mycobacterial FAS-I and FAS-II in vitro in cell free assays and resultant inhibition of both fatty acid and mycolic acid biosynthesis in whole cells treated with the antibiotic. Although, in Mycobacterium the fatty acyl-products of FAS-I provide primers for extension to meromycolate precursors of mycolic acids, the effects on FAS-II appear to be more complex than a simple deprivation of primer supply brought about via FAS-I inhibition. Therefore the combination of activity observed against both FAS-I and the FAS-II components KasA/KasB contributed to the inhibitory effect of platensimycin against M. smegmatis.

While from the point of view of drug development FAS-II inhibition, which causes cessation of the essential mycolic acids, is desirable, inhibition of FAS-I is not since mammalian fatty acid synthases are similar to FAS-I. Indeed, human and rat FAS-I were found to be inhibited in vitro by platensimycin [29]. However, platensimycin does not have the same effect when used on whole cells as it was previously demonstrated by the patent applicants Wang et al. [3] that platensimycin had low mammalian cell toxicity (IC50 of HeLa MTT >1000 µg/ml). Additionally, the authors also confirmed a lack of antifungal activity against Candida albicans which synthesises fatty acids via a type I fatty acid synthase (>64 µg/ml). Furthermore, our own studies with the related C. glutamicum, showed that while corynebacterial FAS-I activity was inhibited in vitro (Table 2), platensimycin failed to inhibit growth of cultures of C. glutamicum (data not shown). These findings suggested that, contrary to data obtained with in vitro inhibition assays with eukaryotic FAS-I, platensimycin selectively inhibits mycobacterial FAS-II in vivo.

Previous studies by Wang et al. [2] demonstrated that platensimycin poorly inhibited FabH in other bacteria. Interestingly, it was shown that platencin (Figure 1B) a related antibiotic was more active against S. aureus FabII (9.17 µM). Both platensimycin and platencin are structurally similar. Platensimycin contains a pentacyclic motif with a cyclic ether ring, whereas platencin contains a unique tetracyclic motif without the ether ring [2]. The chemical modification between platensimycin and platencin has been proposed to be responsible for the change in activity in S. aureus FabII. While platensimycin failed to inhibit mycobacterial Mt-FabH, it would be interesting to examine whether platencin is active against Mt-FabH. Altogether, the culture inhibition studies and in vitro assay data indicate that platensimycin targets Mt-KasA and Mt-KasB. Given that overexpression of Mt-KasA or Mt-KasB increased the MIC of the host M. smegmatis strain for platensimycin, it was surprising that platensimycin was relatively inactive against M. bovis BCG. Poor permeability may have been one of the factors responsible for the observed resistance. Indeed, deletion of kasB from M. bovis BCG resulted in a strain which was more permeable to lipophilic antibiotics [10] and thus sensitive to platensimycin (Table 1). However, it remains unclear whether poor permeability was the sole factor responsible for the resistance of BCG to the antibiotic. This data also suggests that in order to realise the full potential of this compound as an anti-tuberculosis agent, it is imperative that any future modifications to platensimycin include designs that render it more diffusible into the lipid-rich envelope of mycobacteria. Furthermore, in silico modelling of platensimycin-bound Mt-KasB suggested novel molecular interactions that were not seen in platensimycin-bound Ec-FabF. Such differences provide scope for design efforts with the aim to enhance inhibitor affinity in a species-specific manner. Our results highlight the potential of platensimycin as an inhibitor of the essential fatty acid and mycolic acid biosynthesis pathways in mycobacteria.

Materials and Methods

Plasmids, strains and DNA manipulation

The Escherichia coli-mycobacteria shuttle vector pVV16 (a gift from Varalakshmi Vissa, Colorado State University, CO, USA) containing the hip60 promoter and encoding a 6-histidine C-terminal tag was used for the over-expression of Mt-KasA and Mt-KasB. Mt-kasA PCR amplification was performed using the upstream primer 5’-gatcgatcagtgatagctcctcagc-3’ and the downstream primer 5’-gtacgtcagcttcgagcaagcgcgcg-3’, which contain HindIII and HindIII restriction sites, respectively (underlined). The 1251 bp PCR product was then digested with HindIII and ligated with similarly digested pVV16, giving rise to pVV16-Mt-KasA. Mt-kasB was cloned similarly using the upstream primer 5’-gatcgatcagtgatagctcctcagc-3’ and the downstream primer 5’-gtacgtcagcttcgagcaagcgcgcg-3’, which contain NdeI and HindIII restriction sites, respectively (underlined). The 1317 bp PCR product was then digested with NdeI and HindIII and ligated with similarly digested pVV16, giving rise to pVV16-Mt-KasB. Mt-FabH was cloned similarly using the upstream primer 5’-gatcgatcagtgatagctcctcagc-3’ and the downstream primer 5’-gtacgtcagcttcgagcaagcgcgcg-3’, which contain NdeI and HindIII restriction sites, respectively (underlined). The 1033 bp PCR product was then digested with NdeI and HindIII and ligated with similarly digested pVV16, giving rise to pVV16-Mt-FabH. The pVV16-KasAB was constructed using the Mt-kasA upstream primer and the Mt-kasB downstream primer, the 2623 bp PCR product was then digested with NdeI and HindIII and ligated with similarly digested pVV16,
giving rise to pVV16-Mt-KasAB. The kasB-knockout phage pHAE404 [10] was utilized to construct a kasB deletion in M. bovis BCG. Specialized transduction was performed as described in Bardarov et al. [30]. The validity of the M. bovis BCGΔkasB was confirmed by Southern blot analysis (Figure 4). The coding sequences of all the recombinant genes were verified by DNA sequencing.

Whole cell effects of platensimycin on Mycobacterium spp

M. tuberculosis CDC1551 was grown in 7H9 broth supplemented with 10% OADC enrichment and 0.05% Tween-80 to OD600 of 0.4. Following serial 10 fold dilutions, 20 ml of each dilution was spotted on 7H10 agar plates containing 0–128 µg/ml platensimycin. The minimum concentration of platensimycin required to inhibit growth of single colonies was noted as the minimum inhibitory concentration (MIC).

M. smegmatis-pVV16 and overexpression strains were grown in Luria-Bertani Broth (LB) (Difco) with 25 µg/ml kanamycin and 0.05% Tween 80 at 37°C to an optical density of 600 nm (OD600) of 0.25. A 10 ml culture was aliquoted and platensimycin added at the MIC of 15 µg/ml. The OD600 was recorded over 72 h and 100 µl samples were taken periodically and stored at 4°C for viable count analysis. After 72 h the cells were pelleted by centrifugation and washed with 8 ml of PBS buffer to remove platensimycin and the pellet resuspended in fresh LB media. The OD was recorded over 55 h and 100 µl samples taken and viable counts determined at each time point [31]. Briefly, the 100 µl samples were serially diluted to 10–7 and 10 µl samples in triplicate, were spotted on to LB selective agar thrice. Following incubation at 37°C, the colonies were counted and converted into colony forming units (CFU) (CFU/ml). The MIC99 of platensimycin against M. smegmatis and M. bovis BCG were calculated by Alamar Blue testing as previously described [32]. Briefly, 200 µl of sterile deionized water was added to all outer-perimeter wells of a 96-well plate (Corning Incorporated, Corning, NY, USA) to minimize evaporation of the medium in the test wells during incubation. The wells in rows B to G in columns 3 to 11 received 100 µl of 7H9 medium containing 25 µg/ml kanamycin, 50 µg/ml hygromycin and ADC (Beckton Dickinson, Sparks, MD).

Platensimycin was added to rows B–G followed by 1:2 serial dilutions across the plate to column 10, and 100 µl of excess medium was discarded from the wells in column 10. A bacterial culture (100 µl) was added to the wells in rows B to G in columns 2 to 11, where the wells in column 11 served as drug-free controls.

The plates were sealed with parafilm and were incubated at 37°C for 24 h for M. smegmatis strains or 5 days for M. bovis BCG strains.

A freshly prepared 1:1 mixture of Alamar Blue (Celltiter-Blue™, Promega Corp, Madison, WI, USA) reagent and 10% Tween 80 (50 µl) were added to well B11. The plates were reincubated at 37°C for 24 h. The cell viability assay was carried out as per the manufacturer’s protocol followed by MIC99 calculations.

Determination of the in vivo effects of platensimycin on cell envelope lipid synthesis

M. smegmatis cultures were grown to an OD600 nm of 0.4 in the presence of 0.25% Tween 80 in Sautons medium at 37°C. Platensimycin was added at various concentrations followed by incubation at 37°C for 16 h for M. bovis BCG and 8 h for M. smegmatis at which point 1 µCi/g [1,2-14C]acetate (57 mCi/mmol, GE Healthcare, Amersham Bioscience) was added to the cultures. The M. bovis BCG and M. smegmatis cultures were further incubated at 37°C for 24 h and 12 h, respectively. The [14C]-labelled cells were harvested by centrifugation at 2000×g, washed with PBS and processed as described below.

The [14C] labelled cells were initially resuspended in CH2Cl2/0.3% NaCl (2 ml, 100:10, v/v) and mixed with 1 ml of petroleum ether (60-80°C) for 15 min. The upper petroleum ether layer was removed and a further 1 ml of petroleum ether added, followed by further mixing for 15 min. The petroleum ether extracts were combined and evaporated under nitrogen using a heating block.

The dried apolar lipid extract was resuspended in 200 µl of CH2Cl2 prior to thin-layer chromatography (TLC) and autoradiography [33]. Polar lipids were extracted by the addition of CHCl3/CH3OH/0.3% NaCl (2.5 ml, 9:10:3, v/v/v) to the lower methanolic saline phase and mixed for 1 h. The mixture was centrifuged and the pellet re-extracted twice with CHCl3/CH3OH/0.3% NaCl (750 µl, 5:10:4, v/v/v). CHCl3 (1.3 ml) and 0.3% NaCl (1.3 ml) were added to the combined extracts and the mixture centrifuged. The lower layer containing the polar lipids recovered and dried. The polar lipid extract was resuspended in CHCl3/CH3OH (2:1, v/v). The apolar lipid extract (50,000 cpm) was applied to the corners of 6.6×6.6 cm plates of silica gel 60 F254 (Merk 5554) TLC plates. The plates were then developed using direction 1, chloroform-methanol-water (100:14:0.8, v/v/v) and direction 2, chloroform-acetone-methanol-water (50:60:2.5:3, v/v/v) to separate [14C]-labelled lipids (TDM and glucose monomycolate [GMM]). Lipids were visualized by autoradiography by overnight exposure of Kodak X-Omat AR film to the TLC plates to reveal [14C]-labelled lipids and compared to known standards [33].

Determination of the in vivo effects of platensimycin on mycolic acid synthesis

The delipidated cells and whole cell pellets were similarly subjected to alkaline hydrolysis using 5% aqueous tetrabutylammonium hydroxide (TBAH) at 100°C overnight, followed by the addition of 4 ml of CH2Cl2, 500 µl of CH3I, 2 ml of water, followed by mixing for 30 min. The upper aqueous phase was discarded following centrifugation and the lower organic phase washed thrice with water and evaporated to dryness. The resulting FAMEs and MAMEs were dissolved in diethyl ether and insoluble residues removed by centrifugation. The etheral solution was evaporated to dryness and re-dissolved in 200 µl of CH2Cl2. Equivalent volumes of the resulting solution of FAMEs and MAMEs were subjected to TLC using silica gel plates (5735 silica gel 60F254, Merck, Darmstadt, Germany), developed in petroleum ether-acetone (95:5). Autoradiograms were produced by overnight exposure of Kodak X-Omat AR film to the plates to reveal [14C]-labelled FAMEs and MAMEs. Ag2+-TLC was performed as described previously using Ag2+-impregnated TLC plates developed twice in direction I, hexane-ethyl acetate (95:5, v/v/v), and then thrice in direction II, petroleum ether-acetone (85:15, v/v/v) [34].

Determination of the in vitro effects of platensimycin using crude cell-free extracts and purified proteins Mt-KasA, Mt-KasB and Mt-FabH

FAS-I extracts from M. smegmatis and C. glutamicum were prepared as described previously [35]. FAS-I experiments were conducted as described [18] using the 40–80% ammonium sulfate fraction [34]. Briefly, platensimycin was titred (0.1–150 µg/ml) into the standard reaction as follows: 100 mM potassium phosphate pH 7.0, 5 mM EDTA, 5 mM dithiothreitol, 300 µM acetyl-CoA, 100 µM NADPH, 100 µM NADH, 1 µM flavin mononucleotide, 300 µM α-cyclodextrin, 20 µM malonyl-CoA, 100,000 cpm of [2-14C]malonyl-CoA, and 100 µl of the cytosolic
enzyme preparation (1 mg of protein) in a total volume of 500 µl. Reactions were performed in triplicate at 37°C for 1 h and terminated by the addition of 500 µl of 20% potassium hydroxide in 50% methanol at 100°C for 30 min. Following acidification with 300 µl of 6 M HCl, the resultant [14C]-labelled fatty acids were extracted three times with petroleum ether. The organic extracts were pooled, washed once with an equal volume of water, and dried in a scintillation vial prior to scintillation counting using 5 ml of EcoScintA (National Diagnostics, Hull, U.K.).

Mt-KasA, Mt-KasB and Mt-FabH proteins were purified and assayed as described previously [21,24]. Briefly, platensimycin was titrated (0.1–150 µg/ml) into the standard reaction as follows: Holo-AcpM (40 µg) was incubated on ice for 30 min with β-mercaptoethanol (0.5 mmol) in a total volume of 40 µl of [2,14C]malonyl-CoA (100,000 cpm, 6.78 nmol, 1.66 kBq; Amer sham), Mt-FabD (40 ng) and 25 µl of 1 M potassium phosphate buffer, pH 7.0, were added followed by incubation at 37°C for 30 min. C14-AcpM/holo-AcpM heterogeneous mix (22.5 pmol) was added bone followed by incubation at 37°C for 30 min. C14-AcpM/holo-AcpM heterogeneous mix (22.5 pmol) was added to obtain a final volume of 89 µl. Mt-KasA or Mt-KasB (0.25 µg) was added to initiate the reaction and was held at 37°C for 1 h. The reaction was quenched by the addition of 2 ml of a NaBH4 reducing solution (5 mg/ml NaBH4 in 0.1 M K2HPO4, 0.4 M KCl and 30% v/v THF). The reaction was held at 37°C for a further 1 h followed by two extractions with 2 ml of water-saturated toluene. The combined organic phases were pooled and washed using 2 ml of toluene-saturated water. The organic layer was concentrated under vacuum, diluted to 1 ml of 6 M HCl, the resultant [14C]-labelled fatty acids were extracted three times with petroleum ether. The organic extracts were pooled, washed once with an equal volume of water, and dried in a scintillation vial prior to scintillation counting using 5 ml of EcoScintA (National Diagnostics, Hull, U.K.).

The activity of Mt-FabH was determined as previously described [24]. Platensimycin was titrated (0.1–150 µg/ml) into the standard reaction as follows. The assays contained 50 µM holo-ACP/AcpM, 1 mM β-mercaptoethanol, 0.1 M sodium phosphate buffer, pH 7.0, 50 µM malonyl-CoA, 45 nCi of [2-14C]malonyl-CoA (100,000 cpm, 6.78 nmol, 1.66 kBq; American), 12.5 µM acyl-CoA primer, and Mt-FabD (0.3 µg of protein) in a volume of 50 µl and incubated at 37°C for 30 min. The reaction was initiated by the addition of 0.5 µg of Mt-FabH followed by incubation at 37°C for 40 min. The Mt-FabH assays were quenched and processed as described earlier for the Mt-KasA/B assays.

**Supporting Information**

**Figure S1**

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**Author Contributions**

Conceived and designed the experiments: AKB GSB. Performed the experiments: AKB RCT ABKF. Analyzed the data: AKB RCT ABKF GSB. Contributed reagents/materials/analysis tools: KF. Wrote the paper: KF. GSB.

**References**

1. Singh SB, Jayasuriya H, Ondeyka JG, Herath KB, Zhang C, et al. (2006) Isolation, structure, and absolute stereochemistry of platensimycin, a broad spectrum antibiotic discovered using an antisense differential sensitivity strategy. J Am Chem Soc 128: 11916–11920.

2. Wang J, Kodali S, Lee SH, Galgoci A, Painter R, et al. (2007) Discovery of platensimycin, a dual FabF and FabH inhibitor with in vivo antibiotic properties. Proc Natl Acad Sci U S A 104: 7612–7616.

3. Wang J, Soisson SM, Young K, Sheop W, Kodali S, et al. (2006) Platensimycin is a selective FabF inhibitor with potent antibiotic properties. Nature 441: 358–361.

4. Bhattacharya A, Badger D, Jacobs WR Jr, Kremer L (2007) The Mycobacterium tuberculosis FabH-II condensing enzymes: their role in mycolic acid biosynthesis, acid-fastness, pathogenesis and in future drug development. Mol Microbiol 64: 1442–1454.

5. Cole ST, Brosch R, Parkhill J, Garnier T, Churcher C, et al. (1998) Deciphering the biology of Mycobacterium tuberculosis from the complete genome sequence. Nature 393: 537–544.

6. Ganje R, Gibson KJ, Brown AK, Krumhach K, Dover LG, et al. (2004) Acyl-CoA carboxylases (accd2 and acdc3), together with a unique polyketide synthase (Cg-pks), are key to mycolic acid biosynthesis in Corynebacterium glutamicum. Mol Microbiol 27: 4487–4497.

7. Portevin D, De Sousa-D’Auria C, Housain C, Grimard C, Chami M, et al. (2004) De novo polyketide synthase catalyzes the last condensation step of mycolic acid biosynthesis in mycobacteria and related organisms. Proc Natl Acad Sci U S A 101: 314–319.

8. Banerjee A, Dinhaia E, Quemard A, Balasubramanian V, Usp KS, et al. (1994) inlA, a gene encoding a target for isoniazid and ethionamide in Mycobacterium tuberculosis. Science 263: 227–230.

9. Banerjee A, Sugamato M, Sasatchettin JC, Jacobs WR Jr. (1998) The inlA gene from the inl operon of Mycobacterium tuberculosis encodes a 3-ketoacyl reductase that fails to confer isoniazid resistance. Microbiology 144:10: 2697–2704.

10. Bhattacharya A, Fujiwara N, Bhattacharya G, Kachra SS, Kremer L, et al. (2007) Deletion of kasB in Mycobacterium tuberculosis causes loss of acid-fastness and subclinical latent tuberculosis in immunocompetent mice. Proc Natl Acad Sci U S A 104: 5157–5162.

11. Bhattacharya A, Kremer L, Dai AZ, Sasatchettin JC, Jacobs WR Jr. (2005) Conditional depletion of kasA, a key enzyme of mycolic acid biosynthesis, leads to mycobacterial cell lysis. J Bacteriol 187: 7596–7606.

12. Brown AK, Bhattacharya A, Sugarm A, Evans AF, et al. (2007) Identification of the dehydratase component of the mycobacterial mycolic acid-synthesizing fatty acid synthase-II complex. Microbiology 153: 4166–4173.

13. Kremer L, Dover LG, Carrere S, Namputhi KM, Lesjean S, et al. (2002) Mycolic acid biosynthesis and enzymic characterization of the beta-ketoacyl-ACP synthase A-condensing enzyme from Mycobacterium tuberculosis. Biochem J 364: 423–430.

14. Parnis T, Roberts G, Laval F, Schaefer M, Duff M, et al. (2007) Functional complementation of the essential gene fabG1 of Mycobacterium tuberculosis by Mycobacterium smegmatis fabG. J Bacteriol 189: 3721–3728.

15. Quemard A, Sasatchettin JC, Dessen A, Vilcheze C, Bittman R, et al. (1995) Enzymatic characterization of the target for isoniazid in Mycobacterium tuberculosis. Biochemistry 34: 8235–8241.

16. Sacco E, Covarrubias AS, O’Hare HM, Carroll P, Eynard N, et al. (2007) The missing piece of the type II fatty acid synthase system from Mycobacterium tuberculosis. Proc Natl Acad Sci U S A 104: 14620–14623.

17. Gao LY, Laval F, Lawson EH, Groger RK, Woodruff A, et al. (2003) Requirement for kasB in Mycobacterium acidophilus mycolic acid biosynthesis, cell wall impermeability and intracellular survival: implications for therapy. Mol Microbiol 49: 1547–1563.

18. Skleden RA, Lee RE, Armour JW, Cooper AM, Orme IM, et al. (1996) Antimycobacterial action of thioclyocin: an inhibitor of fatty acid and mycolic acid synthesis. Antimicrob Agents Chemother 40: 2813–2819.

19. Badger AR, Betts JC, Eng涨幅-Ndong J, Quan S, McAdam RA, et al. (2000) Activation of the pro-drug ethionamide is regulated in mycobacteria. J Biol Chem 275: 20326–20331.

20. Belanger AE, Badger DS, Ford ME, Mikasza K, Belisle JT, et al. (1996) The embA2 genes of Mycobacterium avium encode an arabinosyl transferase involved in cell wall arabinan biosynthesis that is the target for the antimycobacterial drug ethambutol. Proc Natl Acad Sci U S A 93: 11919–11924.

21. Kremer L, Douglas JD, Baulard AR, Morehouse C, Guy MR, et al. (2000) Requirement for kasA and kasB condensing enzymes in Mycobacterium tuberculosis. J Biol Chem 275: 16857–16864.

22. Larsen MH, Vilcheze C, Kremer L, Besra GS, Parsons L, et al. (2007) Overexpression of inlA, but not kasA, confers resistance to isoniazid and ethionamide in Mycobacterium smegmatis, M. bovis BCG and M. tuberculosis. Mol Microbiol 46: 453–460.
23. Choi KH, Kremer L, Besra GS, Rock CO (2000) Identification and substrate specificity of beta-ketoacyl (acyl carrier protein) synthase III (mtFabH) from *Mycobacterium tuberculosis*. J Biol Chem 275: 28201–28207.

24. Brown AK, Sritharan S, Kremer L, Lindenberg S, Dover LG, et al. (2005) Probing the mechanism of the *Mycobacterium tuberculosis* beta-ketoacyl-acyl carrier protein synthase III mtFabH: factors influencing catalysis and substrate specificity. J Biol Chem 280: 32539–32547.

25. Schaeffer ML, Agnihotri G, Volker C, Kallender H, Brennan PJ, et al. (2001) Purification and biochemical characterization of the *Mycobacterium tuberculosis* beta-ketoacyl-acyl carrier protein synthases KasA and KasB. J Biol Chem 276: 47029–47037.

26. Radmacher E, Alderwick LJ, Besra GS, Brown AK, Gibson KJ, et al. (2005) Two functional FAS-I type fatty acid synthases in *Corynebacterium glutamicum*. Microbiology 151: 2421–2427.

27. Kaye K, Frieden TR (1996) Tuberculosis control: the relevance of classic principles in an era of acquired immunodeficiency syndrome and multidrug resistance. Epidemiol Rev 18: 52–63.

28. Wright A, Bai G, Barrera L, Boulahbal F, Martin-Casabona N, et al. (2006) Emergence of *Mycobacterium tuberculosis* with extensive resistance to second-line drugs - Worldwide, 2000-2004 (Reprinted from MMWR, vol 55, pg 301–305, 2006), Jama-Journal of the American Medical Association 295: 2349–2351.

29. Singh SB, Tota MR, Wang J (2008) Method of treatment using fatty acid synthesis inhibitors. US: World Intellectual Property Organization. pp 1–49. PCT/US2007/020226.

30. Bardarov S, Bardarov S Jr., Pavelka MS Jr., Sambandamurthy V, Larsen M, et al. (2002) Specialized transduction: an efficient method for generating marked and unmarked targeted gene disruptions in *Mycobacterium tuberculosis*, *M. bovis* BCG and *M. smegmatis*. Microbiology 148: 3007–3017.

31. Vilecheza C, Morbidoni HR, Weisbrod TR, Isomoto H, Kao M, et al. (2000) Inactivation of the inhA-encoded fatty acid synthase II [FASII] enoyl-acyl carrier protein reductase induces accumulation of the FASI end products and cell lysis of *Mycobacterium smegmatis*. J Bacteriol 182: 4059–4067.

32. Franzblau SG, Winzig RS, McLauglin JC, Torres F, Madico G, et al. (1998) Rapid, low-technology MIC determination with clinical *Mycobacterium tuberculosis* isolates by using the microplate Alamar Blue assay. J Clin Microbiol 36: 362–366.

33. Dobson G, Minnikin DE, Minnikin SM, Parlett JH, Goodfellow M, et al. (1985) Systematic analysis of complex mycobacterial lipids. In: Goodfellow M, Minnikin DE, eds. Chemical Methods in Bacterial Systematics. London: Academic Press. pp 237–263.

34. Kremer L, Guerardel Y, Gurcha SS, Locht G, Besra GS (2002) Temperature-induced changes in the cell-wall components of *Mycobacterium thermoresistibile*. Microbiology 148: 3145–3154.

35. Brown AK, Papaemmanouil A, Bhowerth V, Bhatt A, Dover LG, et al. (2007) Flavonoid inhibitors as novel antimycobacterial agents targeting Rv0636, a putative dehydratase enzyme involved in *Mycobacterium tuberculosis* fatty acid synthase II. Microbiology 153: 3314–3322.