Platensimycin Activity against Mycobacterial β-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. Herein, 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 μg/ml) and against Mycobacterium tuberculosis (MIC = 12 μg/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 β-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 μg/ml to 30 and 124 μg/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 μg/ml towards S. aureus, Enterococcus faecalis and Streptococcus pneumoniae [3]. Platensimycin targets fatty acid biosynthesis in these species by inhibiting FabF and FabH, two β-ketoacyl-ACP synthases (KAS) of the bacterial multienzyme fatty acid synthase complex FAS-II [2,3]. Mycobacterium tuberculosis, the causative agent of tuberculosis contains three distinct β-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 C16 and C26 fatty acids, and Fatty Acid Synthase-II (FAS-II) a bacterial-type multi-enzyme complex that extends FAS-I products to long chain C48-56 fatty acids termed meromeric acids. FAS-I derived C26 and meromeric acids then undergo a Claisen-type condensation to form mycolic acids [6,7], α-alkyl β-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 \( \mu \)g/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* mc² 155 which has been used in a number of studies as a surrogate for *M. tuberculosis*. The MIC99 of *M. smegmatis* in liquid medium was found to be 14 \( \mu \)g/ml (Table 1). We then monitored the growth of *M. smegmatis* in LB broth in the presence or absence of 14 \( \mu \)g/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 OD600 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 \( \mu \)g/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 \( \mu \)g/ml of platensimycin. In an effort to investigate the
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⁺TLG 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).

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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.

<table>
<thead>
<tr>
<th>Strain</th>
<th>MIC₉₀ (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M. tuberculosis CDC1551</td>
<td>12</td>
</tr>
<tr>
<td>M. tuberculosis H37Rv</td>
<td>12</td>
</tr>
<tr>
<td>M. smegmatis</td>
<td>14</td>
</tr>
<tr>
<td>M. smegmatis pVV16</td>
<td>14</td>
</tr>
<tr>
<td>M. smegmatis pVV16-KasA</td>
<td>30</td>
</tr>
<tr>
<td>M. smegmatis pVV16-KasB</td>
<td>124</td>
</tr>
<tr>
<td>M. smegmatis pVV16-KasAB</td>
<td>126</td>
</tr>
<tr>
<td>M. smegmatis pVV16-FabH</td>
<td>16</td>
</tr>
<tr>
<td>M. bovis BCG</td>
<td>&gt;128</td>
</tr>
<tr>
<td>M. bovis BCG pVV16</td>
<td>&gt;128</td>
</tr>
<tr>
<td>M. bovis BCG pVV16-KasA</td>
<td>&gt;128</td>
</tr>
<tr>
<td>M. bovis BCG pVV16-KasB</td>
<td>&gt;128</td>
</tr>
<tr>
<td>M. bovis BCGΔkasB</td>
<td>61</td>
</tr>
</tbody>
</table>

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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 [14C] 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 [14C]-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 [14C] malonate incorporation into fatty acids in cell free extracts of M. tuberculosis 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 [14C]malonyl-CoA is transacylated to AcpM via mtFabD prior to the addition of the relevant substrates (C16-AcpM and C16-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 IC50. Platensimycin was active against both Mt-KasA and Mt-KasB possessing IC50 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
Figure 5. TLC-autoradiography of FAMEs and MAMEs from \textit{M. smegmatis} strains overexpressing Mt-KasA, Mt-KasB and Mt-FabH following platensimycin treatment. Platensimycin (0–60 \textmu g/ml) was titred into \textit{M. smegmatis} cultures at an OD$_{600}$ nm of 0.4 prior to labelling with 1 \textmu Ci/ml \textsuperscript{1,2-}\textsuperscript{14}Cacetate for 12 h. \textsuperscript{14}C-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 \textsuperscript{14}Clabeled FAMES and MAMEs. \textit{(A)} \textit{M. smegmatis} \textit{pVV16}, \textit{(B)} \textit{M. smegmatis} \textit{pVV16-Mt-KasA}, \textit{(C)} \textit{M. smegmatis} \textit{pVV16-Mt-KasB}, and \textit{(D)} \textit{M. smegmatis} \textit{pVV16-Mt-KasAB}.

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Interestingly, the IC50 values obtained with platensimycin are significantly lower than those obtained with another mycobacterial KAS inhibitor TLM (KasA = 20 μM, KasB 90 μM) [25]. While studies by Wang et al. [2] demonstrated that S. aureus FabH activity was inhibited by platensimycin (IC50 = 67 μM), consistent with our overexpression studies, Mt-FabH activity was insensitive to platensimycin (IC50 > 150 μg/ml, 340 μM) in comparison to Mt-KasA and Mt-KasB (Table 2).

### Table 2. In vitro inhibition (IC50) of platensimycin against Mt-KasA, Mt-KasB, Mt-FabH and Ms-FAS-I and Cg-FAS-I.

<table>
<thead>
<tr>
<th></th>
<th>IC50 (μg/ml)</th>
</tr>
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<tbody>
<tr>
<td>Mt-KasA</td>
<td>2 (0.1)</td>
</tr>
<tr>
<td>Mt-KasB</td>
<td>4.2 (0.1)</td>
</tr>
<tr>
<td>Mt-FabH</td>
<td>&gt; 150</td>
</tr>
<tr>
<td>Ms-FAS-I</td>
<td>12 (0.1)</td>
</tr>
<tr>
<td>Cg-FAS-I</td>
<td>6.5 (0.2)</td>
</tr>
</tbody>
</table>

Figures in brackets represent calculated standard error.

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Interestingly, when crude cell free extracts of either M. smegmatis or the related C. glutamicum [26] were assayed for FAS-I activity, platensimycin inhibited FAS-I activity at an IC50 value of 12 μg/ml and 6.5 μg/ml, respectively (Table 2).

**Comparisons of in silico models of platensimycin-bound ketosynthases**

Despite only a moderate level of sequence identity (~36%) between Mt-KasB and 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 2GF0), 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 the ketolide group in Ec-FabF. The benzoic acid ring faces a structural environment that is virtually identical to that in Ec-FabF. The simulated complex structure suggests that the ketolide group in Ec-FabF would appear to be less exposed to solvent than the ketolide group in Ec-FabF. However, the simulated complex structure suggests that the ketolide group in Ec-FabF would appear to be less exposed to solvent than the ketolide group in Ec-FabF.
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 BCG‘kasB 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 at OD$_{600}$ 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 (OD$_{600}$) of 0.25. A 10 ml culture was aliquoted and platensimycin added at the MIC of 15 μg/ml. The OD$_{600}$ was recorded after 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$^{-2}$ and 10$^{-3}$ 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 MIC$_{50}$ 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 sterile 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$^{	ext{TM}}$, 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 MIC$_{50}$ calculations.

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

M. smegmatis cultures were grown to an OD$_{600}$ nm of 0.4 in the presence of 0.25% Tween 80 in Sauton 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/ml [1,2-14C]acetate (57 mCi/mmole, 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 CH$_3$OH/ 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 CH$_3$Cl$_2$ prior to thin-layer chromatography (TLC) and autoradiography [33]. Polar lipids were extracted by the addition of CHCl$_3$/CH$_3$OH/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 CHCl$_3$/ CH$_3$OH/0.3% NaCl (750 μl, 5:10:4, v/v/v). CHCl$_3$ (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 CHCl$_3$/CH$_3$OH (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 F$_{254}$ (Merck 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/v) to separate [14C]-labelled lipids (TDM and glucose monomycolate [GMG]). 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 know 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 CH$_3$Cl$_2$, 500 μl of CH$_3$I, 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 ethereal solution was evaporated to dryness and re-dissolved in 200 μl of CH$_3$Cl$_2$. Equivalent volumes of the resulting solution of FAMEs and MAMEs were subjected to TLC using silica gel plates (5735 silica gel 60F$_{254}$; 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. Ag$^{2+}$-TLC was performed as described previously using Ag$^{2+}$-impregnated TLC plates developed twice in direction I, hexane-ethyl acetate (95:5, v/v), and then thrice in direction II, petroleum ether-acetone (85:15, 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 M 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]-labeled fatty acids were extracted with diethyl ether, washed with 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 AB GF. Analyzed the data: AKB RCT AB GF KF. Contributed reagents/materials/analysis tools: KF. Wrote the paper: KF.

**References**


