Flavonoid inhibitors as novel antimycobacterial agents targeting Rv0636, a putative dehydratase enzyme involved in Mycobacterium tuberculosis fatty acid synthase II

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Flavonoids comprise a large group of bioactive polyphenolic plant secondary metabolites. Several of these possess potent in vivo activity against Escherichia coli and Plasmodium falciparum, targeting enzymes involved in fatty acid biosynthesis, such as enoyl-ACP-reductase, β-ketoacyl-ACP reductase and β-hydroxyacyl-ACP dehydratase. Herein, we report that butein, isoliquiritigenin, 2,2’,4’-trihydroxychalcone and fisetin inhibit the growth of Mycobacterium bovis BCG. Furthermore, in vitro inhibition of the mycolic-acid-producing fatty acid synthase II (FAS-II) of Mycobacterium smegmatis suggests a mode of action related to those observed in E. coli and P. falciparum. Through a bioinformatic approach, we have established the product of Rv0636 as a candidate for the unknown mycobacterial dehydratase, and its overexpression in M. bovis BCG conferred resistance to growth inhibition by butein and isoliquiritigenin, and relieved inhibition of fatty acid and mycolic acid biosynthesis in vivo. Furthermore, after overexpression of Rv0636 in M. smegmatis, FAS-II was less sensitive to these inhibitors in vitro. Overall, the data suggest that these flavonoids are inhibitors of mycobacterial FAS-II and in particular Rv0636, which represents a strong candidate for the β-hydroxyacyl-ACP dehydratase enzyme of M. tuberculosis FAS-II.

INTRODUCTION

Tuberculosis (TB) poses a global healthcare emergency with an estimated 8 million new cases and 1.8 million fatalities per annum worldwide (Dye, 2006). TB control has been made difficult in recent years by the apparent synergy between the causative agent, Mycobacterium tuberculosis, and HIV (Paolo & Nosanchuk, 2004). In addition, the length and complexity of current TB treatment regimens results in poor patient compliance, a major contributing factor in the emergence of multi-drug-resistant TB (MDR-TB) (Kaye & Frieden, 1996) and extensively drug-resistant (XDR)-TB (Centers for Disease Control and Prevention, 2006). Given this backdrop, the effective control of TB requires the identification of new drug targets and the discovery of novel drugs.

Mycolic acids are one of the most distinctive features of the mycobacterial cell wall. These unusual α-alkyl, β-hydroxy fatty acids are essential for bacterial survival and, when esterified to the non-reducing termini of the arabinogalactan-peptidoglycan cell wall core, form the inner leaflet of a formidable cell wall permeability barrier (Brennan & Nikaido, 1995; Dover et al., 2004). Mycobacteria are unusual in that they possess both a mammalian-type fatty acid synthase I (FAS-I), which carries all of the necessary enzymic activities and carrier functions on a single polypeptide (Smith et al., 2003), and a bacterial type fatty acid synthase II (FAS-II), in which disassociable enzymes interact with an acyl carrier protein (ACP) AcpM that tethers the growing fatty acyl chain between their active sites (Kremer et al., 2001). M. tuberculosis FAS-I conducts de novo synthesis of intermediate length (principally C_{16} and C_{24}) fatty acids. FAS-II, however, is incapable of de novo fatty acid synthesis and accepts short-chain (C_{16}) acyl-CoA primers from FAS-I via a condensation reaction carried out by β-ketoacyl-ACP synthase III (mFabH) (Brown et al., 2005). The newly formed β-ketoacyl-ACP is reduced by a β-ketoacyl-ACP reductase (MabA) (Banerjee et al., 1998) to form a β-hydroxyacyl-ACP intermediate. This product is then dehydrated by an unknown β-hydroxyacyl-ACP dehydratase, followed by further reduction by an enoyl-ACP reductase (InhA) to complete the FAS-II cycle (Kikuchi & Kusaka, 1984; Banerjee et al., 1998).

**Abbreviations:** ACP, acyl carrier protein; FAME, fatty acid methyl ester; FAS, fatty acid synthase; LC/SC, long-/short-chain; MAME, mycolic acid methyl ester; MDR-TB/XDR-TB, multi-/extensively drug-resistant tuberculosis.
1994). Subsequent FAS-II cycles are initiated by the acyl-ACP primed β-ketoacyl-ACP synthases KasA and KasB, respectively (Kremer et al., 2000; Mdluli et al., 1998; Schaeffer et al., 2001) to afford a meromyocyclic acid (C₅₆), which is then condensed with a C₂₆ fatty acid (Gande et al., 2004; Portevin et al., 2005; Takayama et al., 2005). The oxo-myocyclic acid intermediate is then reduced to form the mature mycocyclic acid (Lea-Smith et al., 2007).

A number of flavonoids have been shown to inhibit both fungal and human FAS-I (Li & Tian, 2004; Li et al., 2002; Wang et al., 2003). Flavonoids comprise a large group of polyphenolic secondary metabolites that are widespread throughout the plant kingdom (Koes et al., 1994). They are all based on a flavan skeleton, consisting of two aromatic rings interconnected by a three carbon atom heterocyclic ring (Tasdemir et al., 2006). More than 6400 flavonoids have been shown to have various interesting properties, including antibacterial, antiprotozoal, anti-inflammatory, dietary antioxidant, vascular and oestrogenic activities, mainly associated with inhibition of oxidases and NADH usage (Cos et al., 1998; Harborne & Williams, 2000).

Zhang & Rock (2004) have recently shown that epigallocatechin gallate and related flavonoids are potent inhibitors of Escherichia coli β-hydroxycetyl-ACP reductase and enoyl-ACP reductase (Table 1). More recently, Tasdemir et al. (2006) have shown that a number of flavonoids inhibit β-hydroxycetyl-ACP dehydratase of Plasmodium falciparum (Table 1).

In this study, for the first time the potential of flavonoids as antymycobacterial agents was explored. We observed inhibition of the growth of M. bovis BCG with four related flavonoid compounds that interfere with mycocyclic acid biosynthesis. We sought to employ these novel inhibitors to investigate our bioinformatics-driven hypothesis that the gene product of Rv0636 might represent the unknown β-hydroxycetyl-ACP dehydratase component of M. tuberculosis FAS-II. Overexpression of the gene in mycobacteria conferred resistance to selected flavonoids and rescued in vitro FAS-II activity in flavonoid-treated mycobacterial extracts.

**METHODS**

**Bacterial strains, growth conditions and minimum inhibition concentration (MIC₉₀).** All cloning steps were performed in E. coli TOP10 (Invitrogen). All reagents were of assay grade and purchased from Sigma-Aldrich. Restriction enzymes, Vent DNA polymerase and T4 DNA ligase were purchased from New England Biolabs.

Overexpression of pVV16-Rv0636 was conducted in M. bovis BCG on Middlebrook 7H10 agar supplemented with oleic-albumin-dextrose-catalase (OADC) enrichment (BD) containing 25 µg kanamycin ml⁻¹ and 50 µg hygromycin ml⁻¹ (Kremer et al., 1995). Liquid cultures of M. bovis BCG were grown at 37 °C in Sauton’s medium containing 25 µg kanamycin ml⁻¹ and 50 µg hygromycin ml⁻¹ (Sauton, 1912). The MIC required to inhibit the growth of tested cultures, MIC₉₀, was calculated to indicate the antymycobacterial potency of each flavonoid. MIC₉₀ values of flavonoids against M. bovis BCG, M. bovis BCG pVV16 and M. bovis BCG pVV16-Rv0636 were determined by Alamar Blue according to the manufacturer’s protocol (CellLighter-Blue; Promega) followed by MIC₉₀ calculations over the concentration range 0–200 µg ml⁻¹ (Framblau et al., 1998).

**Plasmids and DNA manipulation.** The E. coli mycobacterial shuttle vector pVV16 (a gift from Varalakshmi Vissa, CSU, CO, USA) containing the hsp60 promoter and encoding a 6-histidine C-terminal tag was used for overexpression of M. tuberculosis Rv0636. All DNA manipulations were performed using standard protocols, as described by Sambrook & Russell (2001). PCR amplification was performed using the upstream primer 5’-GATCTGACATATGGCGCTGCGT3’ and the downstream primer 5’-GATCGATCAAGCTGCCTAACCTGCAGGGCGGAC3’ (which contain Ndel and HindIII restriction sites, respectively (underlined)). The 430 bp PCR product was digested with Ndel and HindIII and ligated with similarly digested pVV16, giving rise to pVV16-Rv0636. The coding sequence of the recombinant gene was verified by DNA sequencing.

**Determination of the in vivo effects of flavonoids on fatty acid and mycocyclic acid synthesis.** M. bovis BCG cultures were grown to an OD₆₀₀ of 0.4 in the presence of 0.25 % Tween 80. The flavonoids were added at various concentrations followed by incubation at 37 °C for 8 h, and then 1 µCi 1,2-[³¹⁴C]acetate ml⁻¹ (57 mCi mmol⁻¹; GE Healthcare, Amersham Bioscience) was added to the cultures, followed by further incubation at 37 °C for 16 h. The ¹⁴C-labelled cells were harvested by centrifugation at 2000 g followed by washing with PBS. The ¹⁴C-labelled control and flavonoid-treated cells were then subjected to alkaline hydrolysis using 5 % aqueous tetrabutylammonium hydroxide (TBAH) at 100 °C overnight, followed by the addition of 4 ml CH₂Cl₂, 500 µl CH₃I and 2 ml water, followed by mixing for 30 min. The upper aqueous phase was discarded following centrifugation and the lower organic phase was washed thrice with water and evaporated to dryness. The resulting fatty acid methyl esters (FAMEs) and mycocyclic acid methyl esters (MAMEs) were dissolved in diethyl ether, insoluble residues were removed by centrifugation and the ether solution was evaporated to dryness and redissolved in 200 µl of CH₂Cl₂. An equivalent aliquot (20 µl) of the resulting solution of FAMEs and MAMEs was subjected to thin-layer chromatography (TLC) using silica gel plates (5735 silica gel 60F₂₅₄; Merck), 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.

**Preparation of cytosolic fractions, FAS-I and FAS-II assays.** Cytosolic extracts, enriched for FAS-I and FAS-II using ammonium sulfate precipitation, of M. smegmatis mc²155 pVV16 and M. smegmatis mc²155 pVV16-Rv0636 (approx. 10 g) were prepared as described by Kremer et al. (2002). The final extract containing the FAS-I and FAS-II activities, was dissolved in 5 ml buffer (50 mM MOPS, pH 7.9, 5 mM β-mercaptoethanol, 10 mM MgCl₂). Protein concentrations were determined using the BCA protein assay reagent kit ( Pierce). FAS-I and FAS-II experiments were conducted as described by Slayden et al. (1996) using the 40–80 % ammonium sulfate fraction (Kremer et al., 2002). Here, for FAS-II reactions, 0.1 mg protein, 10 µM palmitoyl-CoA and 45 nCi [¹⁴C]malonyl-CoA (52 mCi mmol⁻¹; GE Healthcare) were used.

**RESULTS**

**Antimycobacterial activity of flavonoids against M. bovis BCG.** A number of flavonoids inhibit the growth of E. coli and P. falciparum in vivo by targeting specific enzymes of fatty acid biosynthesis (Sharma et al., 2007; Tasdemir et al.,...
Butein, a product of *Rhus verniciflua*, quercetin from *Flaveria bidentis* and fisetin from *Rhus cotinus* target the enoyl-ACP reductases of both *P. falciparum* and *E. coli* at relatively low concentrations (Sharma *et al.*, 2007; Tasdemir *et al.*, 2006; Zhang & Rock, 2004). Isoliquiritigenin and 2,2′,4′-trihydroxychalcone, related products of *Dalbergia odorifera* inhibit both the enoyl-ACP and β-ketoacyl-ACP reductases of *E. coli* (Zhang & Rock, 2004). Interestingly, Tasdemir *et al.* (2006) also demonstrated that not only do these flavonoids target FAS-II reductases, but they also target the β-hydroxyacyl-ACP dehydratase (Table 1).

**Table 1. Structures and in vitro data of flavonoids against *P. falciparum* and *E. coli***

<table>
<thead>
<tr>
<th>Flavonoid</th>
<th>Structure</th>
<th><em>P. falciparum in vitro activity</em></th>
<th><em>E. coli in vitro activity</em></th>
</tr>
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<tbody>
<tr>
<td></td>
<td>[IC₅₀ (µM)]*</td>
<td>FabG</td>
<td>FabZ</td>
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<tr>
<td>Butein</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<tr>
<td>Isoliquiritigenin</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<tr>
<td>2,2′,4′-Trihydroxychalcone</td>
<td>–</td>
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<td>–</td>
</tr>
<tr>
<td>Quercetin</td>
<td>5.4</td>
<td>1.5</td>
<td>1.5</td>
</tr>
<tr>
<td>Fisetin</td>
<td>4.1</td>
<td>2</td>
<td>1</td>
</tr>
</tbody>
</table>

*Activities from Tasdemir *et al.* (2006).
†Activities from Sharma *et al.* (2007); Zhang & Rock (2004).
The five flavonoids as shown in Table 1 were initially tested for in vivo inhibitory properties against M. bovis BCG, a recognized surrogate for M. tuberculosis in drug susceptibility testing. The MIC99 was calculated to indicate the antimycobacterial potency for each flavonoid. Four of the five tested flavonoids inhibited M. bovis BCG growth. Butein was the most potent of the flavonoids with an MIC99 value of $43 \mu g ml^{-1}$ (157 \mu M); potency decreased in the series butein $>$ isoliquiritigenin (195 \mu M, 50 \mu g ml$^{-1}$) $>$ 2,2’,4’-trihydroxychalcone (214 \mu M, 55 \mu g ml$^{-1}$) $>$ fisetin (220 \mu M, 63 \mu g ml$^{-1}$). As quercetin did not affect bacterial growth even at high concentrations (>750 \mu M), it was not considered further.

**Effect of flavonoids on M. bovis BCG fatty and mycolic acid biosyntheses**

The biosynthesis of both fatty and mycolic acids in M. bovis BCG exposed to each of the four active flavonoids was followed by labelling with 1,2-$^{14}$Cacetate. Analyses of FAMEs and MAMEs extracted from the labelled cells by TLC (Fig. 1) revealed that exposure to all compounds coincided with decreases in the incorporation of label into both FAMEs and MAMEs (Fig. 1). Mycobacteria are unusual in that they produce a broad distribution of FAMEs in terms of hydrocarbon chain length, long-chain (LC-) FAMEs (>C$_{24}$), and short-chain (SC-) FAMEs (C$_{16}$–C$_{18}$). Two bands are often resolved in this TLC system. Essentially, the LC-FAMEs migrate faster, whilst SC-FAMEs are slightly retarded. These data suggest that the flavonoids tested inhibit de novo fatty acid biosynthesis in mycobacteria as well as mycolic acid biosynthesis. Interestingly, the populations of SC-FAMEs and LC-FAMEs, which both derive from FAS-I but with the latter being extended by FAS-II, appeared to be differentially affected by these compounds.

**In vitro flavonoid activity against M. smegmatis mc$^{2}$155 FAS-I and FAS-II**

To evaluate the effect of the flavonoids on biosynthesis of fatty and mycolic acids further, we studied their impact upon $^{14}$Cmalonate incorporation into fatty acids in extracts of M. smegmatis mc$^{2}$155 enriched for FAS-I and FAS-II activity (Kremer et al., 2002). Unlike its counterparts that were also active in vivo against M. bovis BCG pVV16 (Table 2), 2,2’,4’-trihydroxychalcone inhibited both FAS-I and FAS-II in vitro at low concentrations (Table 3), suggesting that its inhibition of fatty and mycolic acid biosynthesis is mediated by a common mechanism. Butein and fisetin inhibited FAS-II only, whereas isoliquiritigenin inhibited both FAS-I and FAS-II (Table 3), but the latter most acutely. These patterns of inhibition against FAS-I and FAS-II in vitro are consistent with the in vivo effects on fatty acid and mycolic acid biosynthesis, and support the findings that the flavonoids inhibit fatty acid and mycolic acid biosynthesis. The profiles of inhibition gained using butein, fisetin and isoliquiritigenin highlighted these as potential probes of FAS-II function.

**Identification of candidate (R)-specific hydratase/dehydratases in M. tuberculosis H37Rv**

Despite the classification of 21 gene products as belonging to the enoyl-CoA hydratase/isomerase superfamily of enzymes in the original annotation of the M. tuberculosis H37Rv genome (the majority being predicted to participate...
in fatty acid degradation) (Cole et al., 1998), no genes were strong candidates to encode the $\beta$-hydroxyacyl-ACP dehydratase activity required in FAS-II. BLASTP and TBLASTX analyses of the *M. tuberculosis* genome using FabZ and FabA from *E. coli* as query sequences failed to identify a specific mycobacterial dehydratase. Castell et al. (2005) identified seven possible (R)-specific hydratases in *M. tuberculosis* using a specific hydratase 2 motif [G-D-X-N-P-(LIV)-H-X$_5$-A] (Qin et al., 2000) which contains a histidine residue implicated in catalysis. These included protein products of *fas, Rv3538, Rv3389c, Rv0130, Rv0249, Rv0241* and *Rv0636*. The gene encoding FAS-I, *fas*, could be immediately discounted as encoding the unidentified FAS-II dehydratase. In addition, several of these candidates have been proposed to be non-essential through the existence of viable disrupted mutants generated by transposon mutagenesis (Sassetti et al., 2003). Using this logic, the potential of *Rv3538* and *Rv0636* as candidates encoding the FAS-II $\beta$-hydroxyacyl-ACP dehydratase was affirmed. Examination of the *Mycobacterium leprae* genome highlighted a high degree of ‘genetic decay’ with many genes being annotated as non-functional pseudogenes, and thus *M. leprae* has been thought of as maintaining a minimal gene set for mycobacterial pathogenesis (Eiglmeier et al., 2001). Despite this massive genetic decay, *M. leprae* possesses an intact cell wall and represents a useful reference when considering candidate genes for essential cell-wall-related enzymes (Vissa & Brennan, 2001). Taking this into consideration and the expected conservation of the $\beta$-hydroxyacyl-ACP dehydratase in *M. leprae*, we could exclude *Rv3538* as no orthologue appears to be present in the *M. leprae* genome. Thus the product of *Rv0636* remains as the most promising candidate for this essential enzyme.

Accordingly, CLUSTAL W alignment of the characterized (R)-enoyl-CoA hydratase of *Aeromonas caviae* (Fukui & Doi, 1997) and the $\beta$-hydroxyacyl dehydratase domain of the essential *C. glutamicum* FasA (Radmacher et al., 2005) with *M. tuberculosis* *Rv0636* and its supposed orthologue *M. bovis* Mb0655 showed that both mycobacterial polypeptides contain numerous similarities with these bona fide dehydratases. Particularly strong conservation is apparent in the immediate vicinity of the Asp$^{31}$ and His$^{36}$ catalytic dyad (numbering from PDB entry 1IQ6) (Hisano et al., 2003) (Fig. 2).

**In vivo effects of flavonoids and *M. bovis* BCG overexpressing *M. tuberculosis* *Rv0636***

The use of gene overexpression to identify the cellular targets of antimycobacterial drugs has been highly successful (Belanger et al., 1996; Kremer et al., 2000; Larsen et al., 2002). To investigate whether the putative *M. tuberculosis* dehydratase *Rv0636* is a target for flavonoids, we overexpressed *M. tuberculosis* *Rv0636* from plasmid *pVV16-Rv0636* in *M. bovis* BCG. This recombinant plasmid was constructed by cloning *Rv0636* downstream of the hsp60 promoter of the low-copy-number *E. coli-*
Mycobacterium shuttle vector pVV16. The use of this relatively strong promoter ensures constitutive expression of cloned genes in mycobacteria. In analyses of M. bovis BCG pVV16-Rv0636 we observed MIC\textsubscript{99} values of 59 \(\mu\)g ml\(^{-1}\) (216 \(\mu\)M) and 64 \(\mu\)g ml\(^{-1}\) (250 \(\mu\)M) for butein and isoliquiritigenin, respectively. In comparison to M. bovis BCG pVV16, which we used as a control, the overproduction of Rv0636 conferred some resistance to these agents (Table 3). A high level of resistance was not observed, indicating that the flavonoids might target several FAS-II enzymes. This was not surprising since flavonoids inhibit several FAS-II enzymes as shown in inhibition studies using E. coli and P. falciparum (Sharma \textit{et al.}, 2007; Tasdemir \textit{et al.}, 2006; Zhang & Rock, 2004) (Table 1). The overexpression of Rv0636 also had little or no effect on both 2,2',4',9'-trihydroxychalcone and fisetin activity \textit{in vivo} against M. bovis BCG (Table 3). Together, these data suggest that fisetin might inhibit FAS-I, but in a manner distinct from isoliquiritigenin and butein.

The resistance observed upon overexpression of Rv0636 against butein activity was further investigated in liquid culture (Fig. 3a). When butein was introduced at 100 \(\mu\)g ml\(^{-1}\) to exponentially growing shaken liquid cultures, growth was immediately depressed. In an identical experiment, however, the growth of M. bovis BCG pVV16-Rv0636 was not inhibited by butein (Fig. 3a). Furthermore, on overexpression of Rv0636 the incorporation of radiolabel into SC-/LC-FAMEs and MAMEs (Fig. 3b) was unaffected by exposure to butein up to 100 \(\mu\)g ml\(^{-1}\).

We also assessed the effect of Rv0636 overexpression on \textit{M. smegmatis} FAS-II activity \textit{in vitro} (Table 3). The over-representation of the Rv0636 protein afforded protection to FAS-II activity against both butein and isoliquiritigenin in ratios that were almost identical to the changes observed in MIC\textsubscript{99} values on Rv0636 overexpression in M. bovis BCG, which points towards the FAS-II lesion observed here bearing significance \textit{in vivo}. In accordance with our perception that these two agents exert their toxicity via a different route, we observed no protection of FAS-II activity by Rv0636 over-representation \textit{in vitro} when using 2,2',4',9'-trihydroxychalcone and fisetin.

Considering our \textit{in vivo} and \textit{in vitro} observations collectively, Rv0636 is an apparent cellular target of butein and isoliquiritigenin and its function seems intimately linked to the function of FAS-II, i.e. meromycolyl extension. Therefore, it represents an extremely strong candidate for the unidentified \(\beta\)-hydroxyacyl-ACP dehydratase of mycobacterial FAS-II.

**DISCUSSION**

The discovery of new antitubercular agents is essential. Not only does the World Health Organization estimate that TB is responsible for nearly 2 million deaths each year and that a third of the global population is infected with \textit{M. tuberculosis} (Dye, 2006), but recently the emergence of MDR-TB (Kaye & Frieden, 1996) and XDR-TB (Centers for Disease Control and Prevention, 2006) has come to the fore; an untreatable epidemic of XDR-TB cannot be excluded. In this worrying climate, the need for the discovery and characterization of key enzymes involved in essential mycobacterial biosynthetic pathways towards the definition of novel targets for chemical intervention and the testing of novel classes of potential inhibitors is apparent.

Previously, flavonoid compounds have been shown to be active against \textit{E. coli} and \textit{P. falciparum in vivo} and have been shown to inhibit FAS-II components (Sharma \textit{et al.}, 2007; Tasdemir \textit{et al.}, 2006; Zhang & Rock, 2004). Five flavonoids were evaluated for their potential as myco-

![Fig. 2. Alignment of amino acid sequences of A. caviae (R)-enoyl-CoA hydratase (1IQ6), C. glutamicum FasA (CgFasA), M. tuberculosis Rv0636 and M. bovis Mb0655 polypeptides. Filled boxes indicate strictly conserved residues and open boxes indicate similar residues. The hydratase 2 motif (G-D-X-N-P-LIV-H-X-5-A) is indicated by black triangles. The alignment was performed using CLUSTAL W and rendered using the ESPript Version 2.2.](http://mic.sgmjournals.org)
bacterial FAS-II inhibitors in vivo. Our results show that four of the five tested flavonoids compounds were active against M. bovis BCG. MICs for these compounds ranged between 150 to 220 μM, with butein being the most effective against M. bovis BCG at a concentration of 157 μM (43 μg ml⁻¹).

Analysis of the effects that exposure to these compounds exerted upon the biosyntheses of fatty and mycolic acids demonstrated that all four active compounds affected both systems with the inhibition of mycolate biosynthesis appearing more acute. Although 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. This is illustrated by the disparity between FAS-I and FAS-II inhibition caused by butein and isoliquirtigenin (Table 3). Although the effects of both inhibitors on mycolate biosynthesis are very similar, isoliquirtigenin appears to be a more potent inhibitor of fatty acid biosynthesis than butein. In general, the biosynthesis of all subtypes of mycolates appeared to be equally affected, and thus the inhibition of a common meromycolate precursor is likely, which is consistent with a lesion in FAS-II.

Confirmation of the activity of these flavonoids in vitro implied that they could be used as potential antitubercular agents, but also as a tool for the identification of the unidentified FAS-II dehydratase. In terms of target definition, a key resource was provided by the work of Sassetti et al. (2003) who applied high density transposon mutagenesis and mapping of insertion sites to identify non-essential genes for M. tuberculosis growth. The availability of several mycobacterial genome sequences, especially that of the obligate intracellular pathogen M. leprae, allows us to make useful, biologically informed comparisons across Mycobacterium. Historically, M. tuberculosis FAS-II has proven as a clinically relevant target (Banerjee et al., 1994; Dover et al., 2007; Kremer et al., 2000). Previous analyses by Castell et al. (2005) first suggested that Rv0636 of M. tuberculosis H37Rv is a good candidate for the unidentified β-hydroxyacyl-ACP dehydratase of FAS-II. The gene encoding Rv0636 is not only predicted to be essential (Sassetti et al., 2003), but is highly conserved over numerous mycobacterial species such as M. bovis, M. smegmatis and importantly M. leprae. We have shown that the specific hydratase 2 motif (Qin et al., 2000) which contains an Asp-His catalytic dyad is present in Rv0636. Furthermore, our attempts at homology modelling of Rv0636 using Aeromonas caviae (R)-enoyl CoA hydratase as a template suggest that Rv0636 might also adopt a comparable hot-dog fold like those of other FAS-II dehydratases E. coli FabA and P. falciparum FabZ. (A. K. Brown, unpublished results). Taken together, these obser-
vations suggest that Rv0636 represents an excellent candidate for the FAS-II dehydratase.

Consistent with its candidacy as the unidentified FAS-II dehydratase, the overexpression of M. tuberculosis Rv0636 in M. bovis BCG conferred resistance to butein and isoliquiritigenin, probably via flavonoid binding. Analysis of fatty acid and mycolic acid metabolism in vivo revealed the partial reversion to untreated FAME and MAME profiles in treated cells. These findings were echoed in our in vitro assays with over-representation of Rv0636 providing partial protection of FAS-II activity. When considered collectively, these observations suggest that elevated Rv0636 concentrations protect FAS-II from the action of these two agents that have previously been shown to inhibit β-hydroxyacyl-ACP dehydratases. As the other components of FAS-II are known, we can assert that Rv0636 represents the dehydratase of FAS-II. This however, requires formal proof; our ongoing studies involve purification of an active recombinant enzyme and the development of functional assays using relevant long-chain β-hydroxyacyl thioester substrates.

Interestingly, in consideration of the structure of anti-FAS-II flavonoids described here, a common structural feature became apparent. If one considers that the ketone group borne by each of these flavonoids emulates the carbonyl group of a substrate fatty acid, they all possess a 2,3 double bond consistent with a product mimic. Furthermore, the most potent inhibitors of FAS-II do not bear the oxygen-containing cycle possessed by fisetin and quercetin.

The activity of these compounds against M. bovis BCG identifies a new area for antitubercular drug development and the implication of Rv0636 as the unidentified β-hydroxyacyl-ACP dehydratase of FAS-II provides impetus towards characterization of this enigmatic enzyme, which surely represents an important potential target for future drug development studies.

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