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

Alistair K. Brown, Athina Papaemmanouil, Veemal Bhowruth, Apoorva Bhatt, Lynn G. Dover and Gurdyal S. Besra

School of Biosciences, University of Birmingham, Edgbaston, Birmingham B15 2TT, UK

Correspondence

Gurdyal S. Besra
g.besra@bham.ac.uk

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, isoliquirtigenin, 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 isoliquirtigenin, 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.

Received 18 May 2007

Revised 22 June 2007

Accepted 2 July 2007

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 C₁₆ and C₂₄) fatty acids. FAS-II, however, is incapable of *de novo* fatty acid synthesis and accepts short-chain (C₁₆) acyl-CoA primers from FAS-I via a condensation reaction carried out by β -ketoacyl-ACP synthase III (*mtFabH*) (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 -hydroxyl-acyl-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.*,

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 meromycolic acid (C_{56}), which is then condensed with a C_{26} fatty acid (Gande *et al.*, 2004; Portevin *et al.*, 2005; Takayama *et al.*, 2005). The oxo-mycolic acid intermediate is then reduced to form the mature mycolic 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 mediated through 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 -hydroxyacyl-ACP reductase and enoyl-ACP reductase (Table 1). More recently, Tasdemir *et al.* (2006) have shown that a number of flavonoids inhibit β -hydroxyacyl-ACP dehydratase of *Plasmodium falciparum* (Table 1).

In this study, for the first time the potential of flavonoids as antimycobacterial agents was explored. We observed inhibition of the growth of *M. bovis* BCG with four related flavonoid compounds that interfere with mycolic 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 -hydroxyacyl-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 (MIC₉₉). 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 μ g kanamycin ml⁻¹ and 50 μ g hygromycin ml⁻¹ (Kremer *et al.*, 1995). Liquid cultures of *M. bovis* BCG were grown at 37 °C in Sauton's medium containing 25 μ g kanamycin ml⁻¹ and 50 μ g hygromycin ml⁻¹ (Sauton, 1912). The MIC required to inhibit the growth of 99% of tested cultures, MIC₉₉, was calculated to indicate the antimycobacterial potency of each flavonoid. MIC₉₉ 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 (Celltiter-Blue; Promega) followed by MIC₉₉ calculations over the concentration range 0–200 μ g ml⁻¹ (Franzblau *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'-GATCGATCCATATGGCGCTGCGT GAGTTC-3' and the downstream primer 5'-GATCGATCAAGCTTC GCTTACTTCGCCGAG-3', which contain *Nde*I and *Hind*III restriction sites, respectively (underlined). The 430 bp PCR product was then digested with *Nde*I and *Hind*III 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 mycolic 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 μ Ci 1,2-[¹⁴C]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 μ l CH₃I and 2 ml 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 fatty acid methyl esters (FAMES) and mycolic 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 μ l of CH₂Cl₂. An equivalent aliquot (20 μ l) 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 μ M 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.*,

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

Flavonoid	Structure	<i>P. falciparum</i> <i>in vitro</i> activity [IC ₅₀ (μM)]*			<i>E. coli</i> <i>in vitro</i> activity [IC ₅₀ (μM)]†	
		FabG	FabZ	FabI	FabG	FabI
Butein		–	–	–	10	30
Isoliquirtigenin		–	–	–	20	40
2,2',4'-Trihydroxychalcone		–	–	–	25	40
Quercetin		5.4	1.5	1.5	20	20
Fisetin		4.1	2	1	30	50

*Activities from Tasdemir *et al.* (2006).†Activities from Sharma *et al.* (2007); Zhang & Rock (2004).

2006; Zhang & Rock, 2004). 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). Isoliquirtigenin 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).

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 µg ml⁻¹ (157 µM); potency decreased in the series butein > isoliquirtigenin (195 µM, 50 µg ml⁻¹) > 2,2',4'-trihydroxychalcone (214 µM, 55 µg ml⁻¹) > fisetin (220 µM, 63 µg ml⁻¹). As quercetin did not affect bacterial growth even at high concentrations (>750 µM), 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-[¹⁴C]acetate. 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 (>C₂₄), and short-chain (SC-) FAMES (C₁₆–C₁₈). 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* mc²155 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 [¹⁴C]malonate incorporation into fatty acids in extracts of *M. smegmatis* mc²155 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 isoliquirtigenin 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 isoliquirtigenin highlighted these as potential probes of FAS-II function.

Fig. 1. TLC autoradiography of *M. bovis* BCG FAMES and MAMES after flavonoid treatment. Flavonoids (0–250 µg ml⁻¹) were titrated into the *M. bovis* BCG cultures at an OD₆₀₀ of 0.4 prior to labelling with 1 µCi 1,2-[¹⁴C]acetate ml⁻¹ for 8 h. ¹⁴C-labelled SC-/LC-FAMES and MAMES were extracted and resolved by TLC. An equivalent aliquot (20 µl) of the resulting solution of SC-/LC-FAMES and MAMES was subjected to TLC using silica gel plates developed in petroleum ether/acetone (95:5). Autoradiograms were produced by overnight exposure to film to reveal ¹⁴C-labelled SC-/LC-FAMES and MAMES.

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

Table 2. Inhibition of *M. bovis* BCG growth by flavonoids

MIC₉₉ determinations were performed using the Alamar Blue cell viability assay (Zhang & Rock, 2004). Assays were performed in triplicate and the data presented represent typical results from three independent experiments with a variance of $\sim 2 \mu\text{g ml}^{-1}$.

Flavonoid	MIC ₉₉ ($\mu\text{g ml}^{-1}$)	
	pVV16	pVV16-Rv0636
Butein	43	59
Isoliquirtigenin	50	64
2,2',4'-Trihydroxychalcone	55	57
Fisetin	63	61

in fatty acid degradation) (Cole *et al.*, 1998), no genes were strong candidates to encode the β -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-X₅-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 (Sasseti *et al.*, 2003). Using this logic, the potential of *Rv3538* and *Rv0636* as candidates encoding the FAS-II β -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 β -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 β -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 Asp³¹ and His³⁶ 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*–

Table 3. Influence of Rv0636 overexpression on flavonoid inhibition of *M. smegmatis* fatty acid synthases *in vitro*

Cytosolic extracts were prepared from *M. smegmatis* mc²155 pVV16 and *M. smegmatis* mc²155 pVV16-Rv0636 and assayed for FAS-I and FAS-II activity in the presence of the flavonoids. The IC₅₀ figures represent the concentration of compound required to inhibit activity to 50 % of non-treated extracts. Assays were performed in triplicate and represent three independent experiments. Figures in parentheses represent standard errors. NI, No inhibition.

Flavonoid	IC ₅₀ ($\mu\text{g ml}^{-1}$)			
	FAS-I		FAS-II	
	pVV16	pVV16-Rv0636	pVV16	pVV16-Rv0636
Butein	NI	NI	22 (0.5)	30 (0.8)
Isoliquirtigenin	96 (1.1)	96 (1.2)	7 (0.4)	17 (1.7)
2,2',4'-Trihydroxychalcone	10 (0.4)	10 (0.3)	19 (0.6)	23 (0.7)
Fisetin	NI	NI	54 (1.0)	55 (1.0)

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-X₅-A) is indicated by black triangles. The alignment was performed using CLUSTAL W and rendered using the ESPript Version 2.2.

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 MIC₉₉ values of 59 µg ml⁻¹ (216 µM) and 64 µg ml⁻¹ (250 µM) for butein and isoliquirtigenin, 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 *et al.*, 2007; Tasdemir *et al.*, 2006; Zhang & Rock, 2004) (Table 1). The overexpression of Rv0636 also had little or no effect on both 2,2',4'-trihydroxychalcone and fisetin activity *in vivo* against *M. bovis* BCG (Table 3). Together, these data suggest that fisetin might inhibit FAS-I, but in a manner distinct from isoliquirtigenin 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 µg 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 µg ml⁻¹.

We also assessed the effect of Rv0636 overexpression on *M. smegmatis* FAS-II activity *in vitro* (Table 3). The overrepresentation of the Rv0636 protein afforded protection to FAS-II activity against both butein and isoliquirtigenin in ratios that were almost identical to the changes observed in MIC₉₉ values on Rv0636 overexpression in *M. bovis*

BCG, which points towards the FAS-II lesion observed here bearing significance *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 *in vitro* when using 2,2',4'-trihydroxychalcone and fisetin.

Considering our *in vivo* and *in vitro* observations collectively, Rv0636 is an apparent cellular target of butein and isoliquirtigenin 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 β-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 *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 *E. coli* and *P. falciparum* *in vivo* and have been shown to inhibit FAS-II components (Sharma *et al.*, 2007; Tasdemir *et al.*, 2006; Zhang & Rock, 2004). Five flavonoids were evaluated for their potential as myco-

Fig. 3. *In vivo* effect of butein against *M. bovis* BCG pVV16 and *M. bovis* BCG pVV16-Rv0636. (a) Cultures were grown to an OD₆₀₀ of 0.2 upon which 100 µg butein ml⁻¹ was added. ●, *M. bovis* BCG pVV16; ○, *M. bovis* BCG pVV16 + butein; ▼, *M. bovis* BCG pVV16-Rv0636; ▽, *M. bovis* BCG pVV16-Rv0636 + butein. (b) *M. bovis* BCG pVV16 and *M. bovis* BCG pVV16-Rv0636 were cultured in liquid medium at an OD₆₀₀ of 0.4 prior to labelling with 1 µCi 1,2-[¹⁴C]acetate ml⁻¹ for 8 h. ¹⁴C-labelled SC-/LC-FAMES and MAMES were extracted and resolved by TLC. An equivalent aliquot (20 µl) of the resulting solution of SC-/LC-FAMES and MAMES was subjected to TLC using silica gel plates developed in petroleum ether/acetone (95:5). Autoradiograms were produced by overnight exposure to film to reveal ¹⁴C-labelled SC-/LC-FAMES and MAMES.

bacterial FAS-II inhibitors *in vivo*. Our results show that four of the five tested flavonoid 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 isoliquiritigenin (Table 3). Although the effects of both inhibitors on mycolate biosynthesis are very similar, isoliquiritigenin 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-

variations suggest that Rv0636 represents an excellent candidate for the FAS-II dehydratase.

Consistent with its candidature 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.

ACKNOWLEDGEMENTS

G. S. B. acknowledges support in the form of a Personal Research Chair from Mr James Bardrick, a Royal Society Wolfson Research Merit Award as a former Lister Institute–Jenner Research Fellow, the Medical Research Council (UK) and The Wellcome Trust. A. B. acknowledges support from the Medical Research Council (UK) in the form of a Career Development Award.

REFERENCES

- Banerjee, A., Dubnau, E., Quemard, A., Balasubramanian, V., Um, K. S., Wilson, T., Collins, D., de Lisle, G. & Jacobs, W. R., Jr (1994). *inhA*, a gene encoding a target for isoniazid and ethionamide in *Mycobacterium tuberculosis*. *Science* **263**, 227–230.
- Banerjee, A., Sugantino, M., Sacchettini, J. C. & Jacobs, W. R., Jr (1998). The *mabA* gene from the *inhA* operon of *Mycobacterium tuberculosis* encodes a 3-ketoacyl reductase that fails to confer isoniazid resistance. *Microbiology* **144**, 2697–2704.
- Belanger, A. E., Besra, G. S., Ford, M. E., Mikusova, K., Belisle, J. T., Brennan, P. J. & Inamine, J. M. (1996). The *embAB* 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.

Brennan, P. J. & Nikaido, H. (1995). The envelope of mycobacteria. *Annu Rev Biochem* **64**, 29–63.

Brown, A. K., Sridharan, S., Kremer, L., Lindenberg, S., Dover, L. G., Sacchettini, J. C. & Besra, G. S. (2005). Probing the mechanism of the *Mycobacterium tuberculosis* β -ketoacyl-acyl carrier protein synthase III *mtFabH*: factors influencing catalysis and substrate specificity. *J Biol Chem* **280**, 32539–32547.

Castell, A., Johansson, P., Unge, T., Jones, T. A. & Backbro, K. (2005). Rv0216, a conserved hypothetical protein from *Mycobacterium tuberculosis* that is essential for bacterial survival during infection, has a double hotdog fold. *Protein Sci* **14**, 1850–1862.

Centers for Disease Control and Prevention (2006). Emergence of *Mycobacterium tuberculosis* with extensive resistance to second-line drugs – worldwide, 2000–2004. *MMWR Morb Mortal Wkly Rep* **55**, 301–305.

Cole, S. T., Brosch, R., Parkhill, J., Garnier, T., Churcher, C., Harris, D., Gordon, S. V., Eiglmeier, K., Gas, S. & other authors (1998). Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. *Nature* **393**, 537–544.

Cos, P., Ying, L., Calomme, M., Hu, J. P., Cimanga, K., Van Poel, B., Pieters, L., Vlietinck, A. J. & Vanden Berghe, D. (1998). Structure–activity relationship and classification of flavonoids as inhibitors of xanthine oxidase and superoxide scavengers. *J Nat Prod* **61**, 71–76.

Dover, L. G., Cerdano-Tarraga, A. M., Pallen, M. J., Parkhill, J. & Besra, G. S. (2004). Comparative cell wall core biosynthesis in the mycolated pathogens, *Mycobacterium tuberculosis* and *Corynebacterium diphtheriae*. *FEMS Microbiol Rev* **28**, 225–250.

Dover, L. G., Alahari, A., Gratraud, P., Gomes, J. M., Bhowruth, V., Reynolds, R. C., Besra, G. S. & Kremer, L. (2007). EthA, a common activator of thiocarbamide-containing drugs acting on different mycobacterial targets. *Antimicrob Agents Chemother* **51**, 1055–1063.

Dye, C. (2006). Global epidemiology of tuberculosis. *Lancet* **367**, 938–940.

Eiglmeier, K., Parkhill, J., Honore, N., Garnier, T., Tekai, F., Telenti, A., Klatser, P., James, K. D., Thomson, N. R. & other authors (2001). The decaying genome of *Mycobacterium leprae*. *Lepr Rev* **72**, 387–398.

Franzblau, S. G., Witzig, R. S., McLaughlin, J. C., Torres, P., Madico, G., Hernandez, A., Degnan, M. T., Cook, M. B., Quenzer, V. K. & other authors (1998). Rapid, low-technology MIC determination with clinical *Mycobacterium tuberculosis* isolates by using the microplate Alamar Blue assay. *J Clin Microbiol* **36**, 362–366.

Fukui, T. & Doi, Y. (1997). Cloning and analysis of the poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) biosynthesis genes of *Aeromonas caviae*. *J Bacteriol* **179**, 4821–4830.

Gande, R., Gibson, K. J., Brown, A. K., Krumbach, K., Dover, L. G., Sahm, H., Shioyama, S., Oikawa, T., Besra, G. S. & Eggeling, L. (2004). Acyl-CoA carboxylases (*accD2* and *accD3*), together with a unique polyketide synthase (Cg-pks), are key to mycolic acid biosynthesis in *Corynebacteriaceae* such as *Corynebacterium glutamicum* and *Mycobacterium tuberculosis*. *J Biol Chem* **279**, 44847–44857.

Harborne, J. B. & Williams, C. A. (2000). Advances in flavonoid research since 1992. *Phytochemistry* **55**, 481–504.

Hisano, T., Tsuge, T., Fukui, T., Iwata, T., Miki, K. & Doi, Y. (2003). Crystal structure of the (R)-specific enoyl-CoA hydratase from *Aeromonas caviae* involved in polyhydroxyalkanoate biosynthesis. *J Biol Chem* **278**, 617–624.

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

- Kikuchi, S. & Kusaka, T. (1984). Purification of NADPH-dependent enoyl-CoA reductase involved in the malonyl-CoA dependent fatty acid elongation system of *Mycobacterium smegmatis*. *J Biochem (Tokyo)* **96**, 841–848.
- Koes, R. E., Quattrocchio, F. & Mol, J. N. M. (1994). The flavonoid biosynthetic pathway in plants – function and evolution. *Bioessays* **16**, 123–132.
- Kremer, L., Baulard, A., Estaquier, J., Content, J., Capron, A. & Locht, C. (1995). Analysis of the *Mycobacterium tuberculosis* 85A antigen promoter region. *J Bacteriol* **177**, 642–653.
- Kremer, L., Douglas, J. D., Baulard, A. R., Morehouse, C., Guy, M. R., Alland, D., Dover, L. G., Lakey, J. H., Jacobs, W. R. & other authors (2000). Thiolactomycin and related analogues as novel antimycobacterial agents targeting KasA and KasB condensing enzymes in *Mycobacterium tuberculosis*. *J Biol Chem* **275**, 16857–16864.
- Kremer, L., Nampoothiri, K. M., Lesjean, S., Dover, L. G., Graham, S., Betts, J., Brennan, P. J., Minnikin, D. E., Locht, C., Besra, G. S. & other authors (2001). Biochemical characterization of acyl carrier protein (AcpM) and malonyl-CoA:AcpM transacylase (mtFabD), two major components of *Mycobacterium tuberculosis* fatty acid synthase II. *J Biol Chem* **276**, 27967–27974.
- Kremer, L., Dover, L. G., Carrere, S., Nampoothiri, K. M., Lesjean, S., Brown, A. K., Brennan, P. J., Minnikin, D. E., Locht, C., Besra, G. S. & other authors (2002). Mycolic acid biosynthesis and enzymic characterization of the beta-ketoacyl-ACP synthase A-condensing enzyme from *Mycobacterium tuberculosis*. *Biochem J* **364**, 423–430.
- Larsen, M. H., Vilcheze, C., Kremer, L., Besra, G. S., Parsons, L., Salfinger, M., Heifets, L., Hazbon, M. H., Alland, D. & other authors (2002). Overexpression of *inhA*, but not *kasA*, confers resistance to isoniazid and ethionamide in *Mycobacterium smegmatis*, *M. bovis* BCG and *M. tuberculosis*. *Mol Microbiol* **46**, 453–466.
- Lea-Smith, D. J., Pyke, J. S., Tull, D., McConville, M. J., Coppel, R. L. & Crellin, P. K. (2007). The reductase that catalyzes mycolic motif synthesis is required for efficient attachment of mycolic acids to arabinogalactan. *J Biol Chem* **282**, 11000–11008.
- Li, B. H. & Tian, W. X. (2004). Inhibitory effects of flavonoids on animal fatty acid synthase. *J Biochem (Tokyo)* **135**, 85–91.
- Li, X. C., Joshi, A. S., ElSohly, H. N., Khan, S. I., Jacob, M. R., Zhang, Z., Khan, I. A., Ferreira, D., Walker, L. A. & other authors (2002). Fatty acid synthase inhibitors from plants: isolation, structure elucidation, and SAR studies. *J Nat Prod* **65**, 1909–1914.
- Mdluli, K., Slayden, R. A., Zhu, Y., Ramaswamy, S., Pan, X., Mead, D., Crane, D. D., Musser, J. M. & Barry, C. E., III (1998). Inhibition of a *Mycobacterium tuberculosis* β -ketoacyl ACP synthase by isoniazid. *Science* **280**, 1607–1610.
- Paolo, W. F., Jr & Nosanchuk, J. D. (2004). Tuberculosis in New York city: recent lessons and a look ahead. *Lancet Infect Dis* **4**, 287–293.
- Portevin, D., de Sousa-D'Auria, C., Montrozier, H., Houssin, C., Stella, A., Laneelle, M. A., Bardou, F., Guilhot, C. & Daffe, M. (2005). The acyl-AMP ligase FadD32 and AccD4-containing acyl-CoA carboxylase are required for the synthesis of mycolic acids and essential for mycobacterial growth: identification of the carboxylation product and determination of the acyl-CoA carboxylase components. *J Biol Chem* **280**, 8862–8874.
- Qin, Y. M., Haapalainen, A. M., Kilpelainen, S. H., Marttila, M. S., Koski, M. K., Glumoff, T., Novikov, D. K. & Hiltunen, J. K. (2000). Human peroxisomal multifunctional enzyme type 2. Site-directed mutagenesis studies show the importance of two protic residues for 2-enoyl-CoA hydratase 2 activity. *J Biol Chem* **275**, 4965–4972.
- Radmacher, E., Alderwick, L. J., Besra, G. S., Brown, A. K., Gibson, K. J. C., Sahm, H. & Eggeling, L. (2005). Two functional FAS-I type fatty acid synthases in *Corynebacterium glutamicum*. *Microbiology* **151**, 2421–2427.
- Sambrook, J. & Russell, D. W. (2001). *Molecular Cloning: A Laboratory Manual*, 3rd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Sasseti, C. M., Boyd, D. H. & Rubin, E. J. (2003). Genes required for mycobacterial growth defined by high density mutagenesis. *Mol Microbiol* **48**, 77–84.
- Sauton, B. (1912). Sur la nutrition minérale du bacille tuberculeux. *C R Hebd Seances Acad Sci* **155**, 860–861.
- Schaeffer, M. L., Agnihotri, G., Volker, C., Kallender, H., Brennan, P. J. & Lonsdale, J. T. (2001). Purification and biochemical characterization of the *Mycobacterium tuberculosis* β -ketoacyl-acyl carrier protein synthases KasA and KasB. *J Biol Chem* **276**, 47029–47037.
- Sharma, S. K., Parasuraman, P., Kumar, G., Surolia, N. & Surolia, A. (2007). Green tea catechins potentiate triclosan binding to enoyl-ACP reductase from *Plasmodium falciparum* (PfENR). *J Med Chem* **50**, 765–775.
- Slayden, R. A., Lee, R. E., Armour, J. W., Cooper, A. M., Orme, I. M., Brennan, P. J. & Besra, G. S. (1996). Antimycobacterial action of thiolactomycin: an inhibitor of fatty acid and mycolic acid synthesis. *Antimicrob Agents Chemother* **40**, 2813–2819.
- Smith, S., Witkowski, A. & Joshi, A. K. (2003). Structural and functional organization of the animal fatty acid synthase. *Prog Lipid Res* **42**, 289–317.
- Takayama, K., Wang, C. & Besra, G. S. (2005). Pathway to synthesis and processing of mycolic acids in *Mycobacterium tuberculosis*. *Clin Microbiol Rev* **18**, 81–101.
- Tasdemir, D., Lack, G., Brun, R., Ruedi, P., Scapozza, L. & Perozzo, R. (2006). Inhibition of *Plasmodium falciparum* fatty acid biosynthesis: evaluation of FabG, FabZ, and FabI as drug targets for flavonoids. *J Med Chem* **49**, 3345–3353.
- Vissa, V. D. & Brennan, P. J. (2001). The genome of *Mycobacterium leprae*: a minimal mycobacterial gene set. *Genome Biol* **2**, 1–7.
- Wang, X., Song, K. S., Guo, Q. X. & Tian, W. X. (2003). The galloyl moiety of green tea catechins is the critical structural feature to inhibit fatty-acid synthase. *Biochem Pharmacol* **66**, 2039–2047.
- Zhang, Y. M. & Rock, C. O. (2004). Evaluation of epigallocatechin gallate and related plant polyphenols as inhibitors of the FabG and FabI reductases of bacterial type II fatty-acid synthase. *J Biol Chem* **279**, 30994–31001.

Edited by: W. Bitter