Biochemical characterization of acyl-coenzyme A synthetases involved in mycobacterial steroid side-chain catabolism and molecular design: synthesis of an anti-mycobacterial agent

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Abstract

The metabolism of host cholesterol by *Mycobacterium tuberculosis* is an important factor for both its virulence and pathogenesis. However, the rationale for this cholesterol metabolism has not been fully understood yet. In the present study, we characterized several previously undescribed acyl-CoA synthetases that are involved in the steroid side-chain degradation in *Mycobacterium smegmatis*, and an analogue of intermediate from steroid degradation, 5'-O-(lithocholoyl sulfamoyl) adenosine (LCA-AMS), was successfully designed and synthesized to be used as a specific anti-mycobacterial agent. The acyl-CoA synthetases exhibited strong preferences for the length of side chain. FadD19 homologs, including FadD19 (MSMEG_5914), FadD19-2 (MSMEG_2241), and FadD19-4 (MSMEG_3687), are unanimously favorable cholesterol with a C8 alkanoate side chain. FadD17 (MSMEG_5908) and FadD1 (MSMEG_4952) showed high preferences for steroids, containing a C5 alkanoate side chain. FadD8 (MSMEG_1098) exhibited specific activity toward cholestenoate with a C8 alkanoate side chain. An acylsulfamoyl analogue of lithocholate, 5'-O-(lithocholoyl sulfamoyl) adenosine (LCA-AMS), was designed and synthesized. As expected, the intermediate analogue not only specifically inhibited those steroid-activated acyl-CoA synthetases, but also selectively inhibited the growth of mycobacterial species, including *M. tuberculosis*, *M. smegmatis*, and *Mycobacterium neoaurum*. Overall, our research advanced our understanding of mycobacterial steroid degradation and provided new insights to develop novel mechanism-based anti-mycobacterial agents.

Keywords Biochemical characterization · Acyl-coenzyme A synthetases · Steroid side-chain degradation · Anti-mycobacterial agent

Introduction

The *Mycobacterium* genus of a pathogenic species causes a variety of infectious diseases in a range of hosts, from humans to animals (Shinnick and Good 1994). The well-known infectious diseases, such as tuberculosis (TB) and

leprosy, are caused by *Mycobacterium tuberculosis* (Mtb) and *Mycobacterium leprae*, respectively. In addition, the *Mycobacterium* genus consists of several non-pathogenic species (e.g., *Mycobacterium smegmatis* and *Mycobacterium neoaurum*). A growing body of evidence suggests that cholesterol plays a pivotal role in the infectivity and virulence of Mtb (Abuhammad 2017; Ouellet et al. 2011; Pandey and Sasseti 2008; Soto-Ramirez et al. 2017; Lovewell et al. 2016; Nesbitt and Yang 2010; Meng et al. 2015), and the catabolism of cholesterol contributes to the pool of propionyl-CoA, a precursor that is incorporated into lipid virulence factors (Yang et al. 2009; Griffin et al. 2012; Vandervan et al. 2015). A better understanding of the molecular mechanism of the cholesterol degradation process in mycobacteria is critical for completely comprehending the biology of the infection, which may provide new targets for the development of new anti-TB drugs.

Yang Niu, Fanglan Ge and Yongzhi Yang have contributed equally to this work.

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To date, a large steroid degradation gene locus has been described in actinobacteria, especially *Mtb*, *M. smegmatis* and *Rhodococcus* species; however, the majority of the genes encoding these enzymes involved in steroids degradation should be precisely mapped and functionally characterized (Nesbitt and Yang 2010; Vandervyn et al. 2015; Mohn et al. 2008; Fernández Cabezón et al. 2017; Geize et al. 2007; Wilbrink et al. 2011; Crowe et al. 2017). The alkyl side-chain substituent on the D ring of steroids is degraded by reactions similar to fatty acid β -oxidation reactions (Wilbrink et al. 2011; Gibson et al. 1966). The catabolism of the steroid alkyl side chain initially requires oxidation by a cytochrome P450 to form cholestenoate, with a carboxylate group at position C-26 (Capyk and Kalscheuer 2009; Johnston et al. 2010). The free terminal carboxylate group of cholestenoate or cholate sidechain is activated by an AMP-forming acyl-coenzyme A (acyl-CoA) synthetase, to form an acyl-CoA thioester, that is then degraded via β -oxidation (Geize et al. 2007; Capyk and Kalscheuer 2009; Johnston et al. 2010; Casabon et al. 2014).

Acyl-CoA synthetases belong to a large family of acyl-adenylate/thioester-forming enzyme, and represent the primary route for activation of fatty acid in microorganisms (Johnston et al. 2010; Casabon et al. 2014; Arora et al. 2009). The enzymatic mechanism is a two-step reaction, which is proceeded via formation of an acyl-adenylate (acyl-AMP) intermediate (Wilbrink et al. 2011; Arora et al. 2009; Groot et al. 1976) (Fig. 1). Based on their primary sequence similarity, acyl-CoA synthetases have been divided into two classes: fatty acyl-CoA ligases (FAAL) and fatty acyl-AMP ligase (FACL) enzymes (Arora et al. 2009; Trivedi et al. 2004). Analysis of the available genome sequences of mycobacterial species has revealed a large family of acyl-CoA synthetases (annotated as *FadD*) in the genome (Fernández Cabezón et al. 2017; Geize et al. 2007; Kendall et al. 2007; Mohn et al. 2012; Ge et al. 2011; Rodríguezgarciá et al. 2016). The cholesterol catabolic cluster contains three *fadD* genes: *fadD17*, *fadD19*, and *fadD3* in mycobacterial species (Fernández Cabezón et al. 2017; Kendall et al. 2007). Recently, biochemical characterization of purified *FadD19*

Fig. 1 Chemical structures of steroid compounds investigated as potential substrates for *FadD*s and the intermediate mimic LCA-AMS. **a** Two-step reactions catalyzed by acyl-CoA synthetases. **b** Steroid compounds, 1: 3β-hydroxy-5-cholesten-26-oate; 2: cholate;

3: lithocholate; 4: 3-oxo-23,24-bisnorchole-4-en-22-oate (4-BNC); 5: the acyl-CoA synthetic reaction intermediate (lithocholoyl-AMP, LCA-AMS) of lithocholate; 6: 5'-O-(lithocholoyl sulfamoyl) adenosine (LCA-AMS)

and FadD17 from Mtb, as well as Cas G and Cas I from *Rhodococcus jostii* RHA1, was reported (Casabon et al. 2014; Mohn et al. 2012), and FadD19, FadD17, and Cas I were demonstrated to specifically catalyze steroids with C8, C5 and C3 side chain, respectively (Casabon et al. 2014). In addition, FadD3 was identified essential for the catabolism of cholesterol and cholate rings C and D in *R. jostii* RHA1 (Casabon et al. 2013).

In the present study, we aimed to report the biochemical characterization of several novel acyl-CoA synthetases (FadDs) involved in the steroid side-chain degradation of *M. smegmatis*. A range of predicted steroid substrates that differed in side-chain length were examined with the enzymes and the enzymes were also characterized using steady-state kinetic analysis. Based on the information about the specificity of the FadDs, a reaction intermediate analogue was designed and synthesized, that is the first potent inhibitor of FadDs involved in steroid side-chain degradation. In addition, the inhibitory effects on FadDs and on the growth of mycobacterial species were studied.

Materials and methods

Chemicals

Adenosine triphosphate (ATP), adenosine monophosphate (AMP), CoA, cholesterol, various fatty acids, cholate (CA), lithocholate (LCA), and 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) were purchased from Sigma-Aldrich (St. Louis, MO, USA). In the manner described in our previous study (Liu et al. 2011), 3-oxo-23,24-bisnorchol-4-en-22-oate (4-BNC) was prepared and characterized on the basis of spectroscopic features [^1H NMR, ^{13}C NMR, infrared (IR), and time-of-flight (TOF)–mass spectrometry (TOF–MS)]. The purity of 4-BNC was 99%, as well. Furthermore, 3 β -hydroxy-5-cholesten-26-oate was prepared from diosgenin in our laboratory and characterized on the basis of the spectroscopic features (Sun et al. 2017). Restriction enzymes and T4 DNA ligase were purchased from Takara (Dalian, China). Expand High Fidelity polymerase chain reaction (PCR) system and Taq Master Mix PCR kit were purchased from Omega (Guangzhou, China). Other molecular biology reagents were purchased from Sangon Biotech Co. Ltd. (Shanghai, China).

Microbial strains and growth conditions

Mycobacterium tuberculosis H37Rv, *M. smegmatis* mc²155, and *M. neoaurum* NRRL B-3805 were maintained on Middlebrook 7H10 agar or 7H9 broth supplemented with 10% oleic albumin dextrose catalase (OADC) enrichment (Becton–Dickinson, Franklin Lakes, NJ, USA). *Escherichia*

coli (*E. coli*) DH5 α and BL21 (DE3) cells were grown in Luria–Bertani (LB) broth or on LB agar, and *Saccharomyces cerevisiae* CICC 1308 was maintained on YPD broth.

5'-O-(Lithocholoyl sulfamoyl) adenosine (LCA-AMS) synthesis

The acyl-AMP analogue 5'-O-(lithocholoyl sulfamoyl) adenosine (LCA-AMS) (Fig. 1) was synthesized according to the procedure described previously (Ferrerias et al. 2005; Arora et al. 2009) (see Supplementary Fig. S1). The synthesis was accomplished in five steps with a yield of about 30%, and the synthesized LCA-AMS was purified by chromatography on a silica gel (Florisil) column. At the end, about 1 g LCA-AMS with purity of 96% has been prepared. The structure of the final product was determined using spectroscopic techniques, including ^1H NMR, ^{13}C NMR, and TOF–MS. The TOF–MS map is shown in Fig. S2. Data are as follows: TOF–MS m/z : 705.36 $[\text{M}+\text{H}]^+$, 727.35 $[\text{M}+\text{Na}]^+$ for a calculated mass of 705.36. ^1H NMR spectrum (300 MHz, methanol- d_4) δ : 8.53 (s, 1H, 1-H), 8.19 (s, 1H, 5-H), 6.07 (d, 1H, 6-H), 4.69–4.65 (m, 1H, N-H), 4.39–4.33 (m, 2H, 7-H, 8-H), 4.29–4.24 (m, 2H, 9-H, 10-H). ^{13}C NMR (300 MHz, methanol- d_4) δ : 183.77 (11-C), 153.84 (2-C), 141.17 (1-C), 89.04 (4-C), 84.64 (5-C), 76.15 (3-C), 72.45 (6-C), 72.38 (9-C), 69.10 (7-C), 59.46 (30-C), 57.78 (8-C), 57.65 (10-C), 43.89 (17-C), 43.56 (22-C), 41.85 (18-C), 41.51 (19-C), 37.36 (15-C), 37.20 (14-C), 36.93 (26-C), 36.50 (20-C), 35.66 (31-C), 33.75 (28-C), 31.20 (23-C), 29.23 (27-C), 28.36 (16-C), 27.63 (24-C), 25.22 (29-C), 24.78 (13-C), 23.94 (25-C), 21.92 (12-C), 20.68 (21-C), 18.95 (32-C), 13.94 (33-C), 12.49 (34-C).

Cloning, protein expression, and purification

DNA was propagated, digested, ligated, and transformed using the standard protocols (Sambrook et al. 1989). The genes *MSMEG_4952* (*fadD1*), *MSMEG_5908* (*fadD17*), *MSMEG_5914* (*fadD19*), *MSMEG_2241* (*fadD19-2*), *MSMEG_2231* (*fadD19-3*), *MSMEG_1098* (*fadD8*), *MSMEG_4772* (*fadD3*), *MSMEG_3687* (*fadD19-4*), *MSMEG_5086* (*fadD6*), *MSMEG_5348* (*fadD14*), and *MSMEG_0131* (*fadD5*) were PCR amplified from *M. smegmatis* mc²155 genomic DNA, and the primers are listed in Table S1. The genes were inserted into plasmid pET28a+ (Novagen), replicated in *E. coli* DH5 α , and then transformed into *E. coli* BL21(DE3) for protein expression.

Recombinant *E. coli* BL21(DE3) was cultured at 37 °C and shaken at 200 rpm in LB medium to the optical density at 600 nm (OD₆₀₀) of 0.8. The expression of the recombinant proteins was induced with 1 mM isopropyl- β -D-thiogalactopyranoside (IPTG). Cells were grown for a further 6 h at 18 °C and then were shaken at 200 rpm. All

cells were harvested by centrifugation at 10,000 rpm for 5 min at 4 °C. The harvested cell pellets were washed in Tris–NaCl (50 mM Tris–HCl pH7.5, 500 mM NaCl) plus 1 mM phenylmethylsulfonyl fluoride (PMSF), and were then re-suspended in 10 mL of Tris–NaCl. The cell-free extract was prepared by sonication for 10 min and centrifugation at 11,000 rpm for 30 min at 4 °C. The supernatant was passed through a 0.45- μ m filter and supplemented with 10 mM Mimidazole, and then loaded onto 2.5 mL Ni-Sepharose 6 Fast Flow resin (GE Healthcare, Chicago, IL, USA). The His-tagged proteins were eluted with elution buffer, containing Tris–NaCl and 300 mM imidazole (pH 8.0). The eluted fractions were dialyzed against 20 mM Tris/HCl (pH 8.0) and 50 mM NaCl, and then digested by thrombin to remove the N-terminal His-tag. The cleaved protein was separated from cleaved tag by passing the mixture through a 0.4-mL Ni-Sepharose column equilibrated with ice-cold 20 mM sodium phosphate (pH 8.0), 300 mM NaCl, and 10 mM Mimidazole. The protein sample was recovered in the flow-through and dialyzed overnight at 4 °C against 20 mM Tris/HCl (pH 8.0) and 50 mM NaCl, and then concentrated at 4 °C by ultra-filtration to a final concentration of 5.0 mg/mL for further biochemical studies.

Preparation, purification, and analysis of CoA thioester

Reactions of producing thioesters were performed in 2 mL of HEPES (0.1 M, pH 7.5) with 5 mM of MgCl_2 , 1 mM of CoA, 2.5 mM of ATP, 1 mM substrate, and 1 μ g of purified enzymes incubated for 4 h at 30 °C. CoA thioesters (cholest-4-en-26-oyl-CoA and LCA-CoA) were purified by high-performance liquid chromatography (HPLC) on a C_{18} 5 μ m 250 $\times$ 4.6 mm column (Welchrom column; Welch Materials Inc., Shanghai, China) (Casabon et al. 2013). CoA thioesters were eluted using a linear gradient of 5–95% acetonitrile in 0.1 M ammonium acetate (pH 4.5) for within 20 min at 1 mL/min, and detected at the wavelength of 254 nm. Purified samples of CoA thioesters were subjected to electron spray ionization tandem mass spectrometry (ESI–MS/MS) in the positive-ion mode.

Biochemical assays

The FadD activity was determined using a DTNB colorimetric assay (Zhuravleva et al. 2012; Guo et al. 2014; Bernson 1976). During this process, free CoA in reduced form of CoA-SH reacted with DTNB to form 5-thionitrobenzoic acid could be measured at the wavelength of 412 nm (Zhuravleva et al. 2012; Guo et al. 2014). Reactions were undertaken in 200 μ L of HEPES (0.1 M, pH 7.5), containing 10 mM of KCl, 10 mM of MgCl_2 , 50 μ M of CoA, 200 μ M of ATP, and 100 μ M of substrate. These reactions were initiated by

adding 1 μ g of purified FadD enzymes, followed by a reaction time of 10 min at 30 °C, which was then stopped by heating the mixture at 80 °C for 5 min. When the samples were cooled to room temperature, 4 μ L of DTNB (5 mM) was added, and the color was allowed to develop for 5 min. Samples were measured by a microplate reader (Thermo Fisher Scientific, Waltham, MA, USA) at 412 nm. Serially diluted concentrations of CoA were assayed and used as standard curves for calculating the amounts of CoA reduced in experimental samples. Enzyme kinetic parameters were also determined using varied substrate concentrations (i.e., 3 β -hydroxy-5-cholesten-26-oate, cholate, and 4-BNC at 0–600 μ M, respectively).

To evaluate the effects of LCA-AMS on the activity of FadDs, 0–300 μ M of LCA-AMS was used to determine the half-maximal inhibitory concentration (IC_{50}) value. First, the inhibitor was incubated with FadDs at 30 °C for 10 min. Then, 100 μ M substrate was added to start the reaction. In each experiment, a sample without the LCA-AMS was used as the control. The measured data were analyzed using GraphPad Prism 4.03 software. A log inhibitor versus normalized response-variable slope model was utilized to calculate IC_{50} . A competitive enzyme-inhibition model was used for estimation of K_i .

The effects of inhibition on the growth of microbial strains

The susceptibility of several kinds of microbial strains used in the present study to LCA-AMS (0–300 μ M) was assayed using colony-forming units (CFUs) on plate. The effects of inhibition on the growth of *M. smegmatis* mc²155 and *M. neoaurum* NRRL B-3805 were measured by subcultures in Middlebrook 7H9 broth medium (supplemented with OADC) and liquid minimal medium (0.5 g/L NaCl, 1.5 g/L NO_3NH_4 , 0.2 g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.4 g/L K_2HPO_4 , 0.8 g/L KH_2PO_4 , 5.0×10^{-4} g/L $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 2.0×10^{-4} g/L $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 5.0×10^{-5} g/L $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, pH 7.5) with cholesterol or cholate as the only carbon source, for 2 days at 37 °C at 200 rpm. The effects of inhibition on the growth of *M. tuberculosis* H37Rv were assessed by subculture in liquid minimal medium with cholesterol as the only carbon source at 37 °C. The effects of inhibition on the growth of *S. cerevisiae* were analyzed by subculture in Yeast Extract Peptone Dextrose (YEPD) medium at 30 °C and 200 rpm. The effects of inhibition on the growth of *E. coli* were evaluated by subcultures in LB medium at 37 °C and 200 rpm.

Quantitative reverse transcription PCR (RT-qPCR) analysis

To investigate the expressions of the FadD-encoded genes, *M. smegmatis* mc² 155 was cultured for 2 days in minimal

medium plus cholesterol or glycerol as the carbon source at 37 °C and 200 rpm. Total RNA was extracted using RNeasy® Mini Kit (Qiagen, Valencia, CA, USA). A Prime Script II First Strand cDNA Synthesis kit (Takara, Dalian, China) was used for cDNA synthesis and RNA digestion. Reactions were conducted for RT-qPCR using SYBR Green I Mastermix (Takara, Dalian, China) run in a MiniOpticon Thermal Cycler (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The constitutively expressed house-keeping gene 16S ribosomal RNA was used as an internal standard. The primers are listed in Table S2.

Results

Phylogenetic analysis of acyl-coenzyme A (acyl-CoA) synthetases involved in mycobacterial steroid degradation

To evaluate the occurrence of acyl-CoA synthetases (FadDs) involved in degradation of steroid side chain in mycobacteria, we conducted a phylogenetic analysis using the following as query sequences: FadD17 and FadD19, which are acyl-coenzyme A synthetases involved in the cholesterol degradation of *M. smegmatis*, as well as CasG_{RHA1} and CasI_{RHA1}, which are acyl-CoA synthetases involved in the cholate catabolism of *Rhodococcus* sp. RHA1 (Wilbrink et al. 2011; Casabon et al. 2014; Mohn et al. 2012). A predicted acyl-CoA synthetase gene *MSMEG_1098* (*fadD8*), induced with cholesterol and containing a KstR operator sequence, was used as a fifth query sequence (Uhía et al. 2012). As subject sequences, the proteins encoded in the genomes of several mycobacterial species were utilized, including *M. smegmatis*, *M. neoaurum*, *Mycobacterium fortuitum*, *M. tuberculosis*, *Mycobacterium marinum*, and *Mycobacterium avium*. Orthologous genes were identified by obtaining reciprocal best BLAST hits. The final analysis was carried out using 49 sequences, from 6 mycobacterial species, with a minimum of 34% identity at amino acid level (Table S3). According to the results, the *M. smegmatis* genome contains four *fadD19* homologous genes (Table S3), namely, *MSMEG_5914* (*fadD19*), *MSMEG_2241* (*fadD19-2*), *MSMEG_2231* (*fadD19-3*), and *MSMEG_3687* (*fadD19-4*), sharing 34–57% identity at amino acid level. In addition to *fadD17* (*MSMEG_5908*), *M. smegmatis* has another homologous gene *MSMEG_4952* (with 52% identity at amino acid level), whose *M. tuberculosis* homolog was annotated as *fadD1*. It appears that no CasG ortholog exists in the *M. smegmatis* genome, although there is a gene *MSMEG_4065* showing 34% identity at amino acid level with CasG. *MSMEG_4952* (*fadD1*) and *MSMEG_5908* (*fadD17*) show 44% and 43% identities with CasI (ro05822) at amino acid level, whereas *XA26_18490* from *M. fortuitum* and *D174_08270* from *M.*

neoaurum were found to be highly homologous to Cas I with 70% identity at the amino acid level.

Phylogenetic analysis of the 49 sequences revealed that they could be grouped into five main classes on the basis of enzyme function (Fig. 2). FadD19, FadD8 (*MSMEG_4952*), and CasG_{RHA1} belong to classes I, III, and IV, respectively, while CasI_{RHA1} and FadD17 both belong to class II (Fig. 2). Moreover, two sequences, including *XA26_18490* from *M. fortuitum* and *D174_08270* from *M. neoaurum* shared greater identity (70%) at amino acid level with CasI compared with FadD17_{Msm} (about 45%) and FadD1_{Msm} (*MSMEG_4952*; about 50%) (Table S3). The orthologs of CasG_{RHA1} in class IV shared as little as 34% identity at amino acid level, and they found to be the least divergent in class II, whose members shared 82% identity. Several *fadD19* homolog genes were noted in the genome of the most mycobacterial species, while only one *fadD19* homolog gene was found in the *M. tuberculosis* genome. Those extra *fadD19* homolog genes composed of the class I of phylogenetic tree. The orthologs of *MSMEG_1098* (*fadD8*), which were assumed to be FadD8, shared up to 80% identity at amino acid level; however, their biological functions have largely remained unknown.

The enzymatic characterization and substrate specificity of acyl-CoA synthetase

To investigate the in vitro activities of the homologs of FadD17, FadD19, Cas I, as well as FadD8, we attempted to clone and express these proteins in *E. coli*. FadD1 (encoded by *MSMEG_4952*, MW = 59 kD), FadD17 (encoded by *MSMEG_5908*, MW = 57 kD), FadD19 (encoded by *MSMEG_5914*, MW = 62 kD), FadD19-2 (encoded by *MSMEG_2241*, MW = 62 kD), FadD8 (*MSMEG_1098*, MW = 60 kD), and FadD19-4 (MW = 62 kD, *MSMEG_3687*), which have been successfully expressed and purified to above 95% purity (see Fig. 3). The activities of the purified recombinant proteins were assessed using several cholesterol metabolites, cholate and its analogues, which included substrates with four-ringed nuclei harboring either a C3, C5, or C8 side chain (see Fig. 1). In addition, variable carbon chain-length fatty acids, such as acetic acid (C2:0), butyric acid (C4:0), hexanoic acid (C6:0), caprylic acid (C8:0), capric acid (C10:0), lauric acid (C12:0), myristic acid (C14:0), palmitic acid (C16:0), stearic acid (C18:0), arachidic acid (C20:0), behenic acid (C22:0), lignoceric acid (C24:0), cerotic acid (C26:0), and octacosanoic acid (C28:0), were also used as substrates.

We adapted the DTNB colorimetric assay to assess enzyme activity by measuring the reduction of thiol group of free CoA-SH in the reactions (Zhuravleva et al. 2012; Guo et al. 2014). These six enzymes showed distinct preferences for particular side-chain lengths (Fig. 3). FadD8

Fig. 2 Phylogenetic tree of mycobacterial acyl-CoA synthetases. Coding sequences are identified by name or number and the strain in which they occur. MSMEG, *Mycobacterium smegmatis* mc²155;

RHA1, *Rhodococcus jostii* RHA1; D174, *Mycobacterium marinum*; MAP, *Mycobacterium avium* sub sp., *paratuberculosis* K-10; XA, *Mycobacterium fortuitum*; Rv, *Mycobacterium tuberculosis*

(MSMEG_1098) and FadD19 (MSMEG_5914), as well as the FadD19 homologs, FadD19-2 (MSMEG_2241) and FadD19-4 (MSMEG_3687), only showed activity with compound that had a C8 side chain (see Fig. 3). The identity of FadD8_{Msm} and FadD19_{Msm} products was confirmed to be cholest-4-en-26-oyl-CoA by ESI-MS/MS (see Fig. S3A). In contrast, FadD17_{Msm} detectably

converted compounds with a C3, C5, and C8 side chain at C-17, demonstrating the highest activity with compounds that had a C5 side chain, while insignificant activity was observed with compounds that had a C3 or C8 side chain. FadD1_{Msm} (MSMEG_4952) expressed activity with compounds that had a C5 side chain. The confirmation of the identity as cholyl-CoA from cholate by FadD1_{Msm} and

Fig. 3 Activation of steroids by different FadDs of *M. smegmatis*. Substrate preferences of recombinant FadD proteins toward different steroids and fatty acids as determined by 5,5'-dithiobis-(2-nitrobenzoate) (DTNB) assay

FadD17 was achieved by the ESI-MS/MS analysis (see Fig. S3B).

For instance, when the thioesterification reaction of cholate was tested with FadD17 (MSMEG_5908), the HPLC peaks for ATP and CoA-SH decreased the intensity as two new peaks appeared (see Fig. S4). The first peak had the same retention time (2.8 min) as the AMP standard, and the second peak eluted at 11.5 min, similar to that of a CoA thioester (Casabon et al. 2013; Casabon et al. 2014). As for 3β-hydroxy-5-cholesten-26-oate, the second peak eluted at 14.5 min. To further substantiate the formation of steroid-CoA thioesters, the products were purified by HPLC, and the obtained CoA thioesters were detected by TOF-MS (see Fig. S3). The results confirmed that cholate was converted into cholyl-CoA and 3β-hydroxy-5-cholesten-26-oate was converted into cholest-4-en-26-oyl-CoA, respectively. In addition, MSMEG_4952 (fadD1), MSMEG_2241 (fadD19-2), and MSMEG_3687 (fadD19-4) showed high activity with compounds with fatty acids, ranging from C8 to C16 side chain. However, MSMEG_5914 (fadD19), MSMEG_5908 (fadD17), and MSMEG_1098 (fadD8) showed insignificant activity with these fatty acids (Fig. S5). In contrast, genes that shared low identity at amino acid level with *fadD8*, *fadD17* and *fadD19*, MSMEG_0131 (fadD5), MSMEG_4772 (fadD3), MSMEG_5086 (fadD6), MSMEG_5348 (fadD14) were also expressed in *E. coli*. As we expected, the purified proteins encoded by those four genes did not detectably catalyze the thioesterification of cholate, lithocholate, or cholestenic acid. Among these FadDs, the purified FadD5 showed preference toward fatty acids with chain lengths

ranging from C6 to C16, and purified FadD6 exhibited high activity for fatty acids with chain lengths ranging from C10 to C16. Purified FadD14 showed a preference for fatty acids with chain lengths from C4 to C12 (data were not shown). Unexpectedly, purified FadD3 demonstrated no activity with fatty acids that had chain lengths ranging from C2 to C28 (data were not shown).

Steady-state kinetics

Using a DTNB assay, we determined the kinetic parameters of FadD19 (MSMEG_5914), FadD19-2 (MSMEG_2241), FadD19-4 (MSMEG_3687), FadD17 (MSMEG_5908), FadD8 (MSMEG_1098), and FadD1 (MSMEG_4952) toward various steroid substrates (see Table 1). FadD17 exhibited typical features of Michaelis–Menten kinetics toward 3β-hydroxy-5-cholesten-26-oate, lithocholate, cholate, and 4-BNC (see Fig. 4). It possessed a very low K_m value (9.85 μM) with cholate, containing a C5 side chain (see Table 1). Importantly, the ratio of K_{cat}/K_m for lithocholate and cholate was remarkably higher than that for both 3β-hydroxy-5-cholesten-26-oate (with a C8 side chain) and 4-BNC (with a C3 side chain). The value for cholate ($1.19 \times 10^5/M/s$) was 45 times greater than that for 4-BNC and 10 times higher than that for 3β-hydroxy-5-cholesten-26-oate. FadD17 illuminated similar specificity constants for lithocholate and cholate, demonstrating that the configuration of the steroid nucleus had a notably less important influence than side-chain length on enzyme–substrate preferences and catalytic efficiency.

Table 1 Kinetic parameters for acyl-CoA synthetases

Enzyme and substrate	Mean K_m (μM) (SE)	Mean K_{cat} (s^{-1}) (SE)	Mean K_{cat}/K_m ($\text{m}/\text{M}/\text{s}$)
FadD17 (MSMEG_5908)			
3 β -Hydroxy-5-cholesten-26-oate	31.84 (3.5)	3.76 (0.09)	118.09
Lithocholic acid	11.47 (1.50)	10.62 (0.28)	925.89
Cholate	9.85 (1.47)	11.77 (0.34)	1194.92
4-BNC	68.42 (12.45)	1.75 (0.09)	25.58
FadD1 (MSMEG_4952)			
3 β -Hydroxy-5-cholesten-26-oate	48.89 (9.47)	3.25 (0.17)	66.48
Lithocholic acid	9.97 (0.89)	10.80 (0.18)	1083.25
Cholate	11.54 (0.93)	9.86 (0.16)	854.41
FadD8 (MSMEG_1098)			
3 β -Hydroxy-5-cholesten-26-oate	9.54 (0.96)	9.785 (0.19)	1025.68
FadD19-2 (MSMEG_2241)			
3 β -Hydroxy-5-cholesten-26-oate	32.10 (3.31)	4.05 (0.10)	126.17
FadD19-4 (MSMEG_3687)			
3 β -Hydroxy-5-cholesten-26-oate	22.23 (2.9)	7.61 (0.22)	342.33
FadD19 (MSMEG_5914)			
3 β -Hydroxy-5-cholesten-26-oate	6.88 (0.61)	12.34 (0.19)	1793.6

Values in parentheses represent standard errors. Data show the mean of three replicates

Fig. 4 Steady-state kinetic analysis of FadD17_{Msm} with different substrates. FadD17 activity was determined using a DTNB colorimetric assay. (cholate: $K_m = 9.86 \pm 1.47 \mu\text{M}$, $V_{max} = 1.04 \pm 0.029 \text{ mmol/mg/min}$; lithocholic: $K_m = 11.47 \pm 1.51 \mu\text{M}$, $V_{max} = 0.93 \pm 0.024 \text{ mmol/mg/min}$; 3 β -hydroxy-5-cholesten-26-oate: $K_m = 31.84 \pm 3.51 \mu\text{M}$, $V_{max} = 0.33 \pm 0.008 \text{ mmol/mg/min}$; 4-BNC: $K_m = 68.42 \pm 5.45 \mu\text{M}$, $V_{max} = 0.15 \pm 0.008 \text{ mmol/mg/min}$)

FadD1 (MSMEG_4952) also followed Michaelis–Menten kinetics toward 3 β -hydroxy-5-cholesten-26-oate, lithocholate, and cholate (Table 1). Similar to FadD17, FadD1 (MSMEG_4952) displayed higher specificity constants for lithocholate and cholate compared with 3 β -hydroxy-5-cholesten-26-oate. Of the 6 FadD enzymes and 4 substrates tested, 3 β -hydroxy-5-cholesten-26-oate was the favorable substrate of FadD19_{Msm} with a K_m of 6.88 μM and a specificity constant of $1.79 \times 10^5 \text{ M/s}$. Besides, 3 β -hydroxy-5-cholesten-26-oate was the preferred substrate of FadD8 (MSMEG_1098) with a K_m of 9.54 μM (Table 1).

Class-selective multi-target inhibitor

Since acyl-AMP analogues were found to be potent inhibitors of related adenylate-forming enzymes (Arora et al. 2009; Léger et al. 2009), we postulated that acyl-sulfamoyl analogues of lithocholate could selectively inhibit the steroid-activated acyl-CoA synthetases. Therefore, acholoyl-AMP analogue was designed and synthesized, and the structure was checked by MS and nuclear magnetic resonance (see Fig. S2). The inhibitory profile of LCA-AMS was investigated with purified FadDs by examining the enzymatic activity in the presence of increasing inhibitor concentrations. The results indicated that LCA-AMS exhibited significantly different inhibitory influences on those Fads, as illustrated in Fig. 5. For instance, as calculated using a competitive inhibition model, LCA-AMS inhibited FadD17_{Msm} and FadD1_{Msm} enzyme activities at low IC_{50} values (0.081 and 1.4 $\mu\text{mol/L}$), which were corresponding with inhibitory constant (K_i) values at 23 and 67 nmol/L (see Fig. 5b), respectively. The IC_{50} of LCA-AMS for FadD19_{Msm} (MSMEG_5914), FadD8_{Msm} (MSMEG_1098), FadD19-2 (MSMEG_2241), and FadD19-4 (MSMEG_3687) were found to be remarkably higher than those for FadD17_{Msm} and FadD1_{Msm} (see Fig. 5a). Similar to the substrate preferences, this compound had no significant influences on FadD5 (MSMEG_0131), FadD3 (MSMEG_4772), FadD6 (MSMEG_5086), or FadD14 (MSMEG_5348), even up to a concentration of 300 μM .

Fig. 5 Inhibitory effect of LCA-AMS on different FadDs' activity. FadDs' activity in the presence of increasing concentrations of LCA-AMS was determined using DTNB assay. U_T and U_C are activities in inhibitor and DMSO-containing reactions. **a** FadD19, FadD19-2,

FadD19-4, and FadD8 used β -hydroxy-5-cholesten-26-oate as substrate; **b** FadD1 and FadD17 used cholate as substrate. Data shown are representative of three independent experiments

The potent inhibitory activity of LCA-AMS with purified proteins motivated us to investigate whether the analogue could influence mycobacterial growth in different culture media. For this purpose, we determined its ability to inhibit the growth of *M. tuberculosis* H37Rv, *M. smegmatis* mc²155, *M. neoaurum* NRRL B-3805, *S. cerevisiae*, and *E. coli* DH5 α . The latter two strains were included as controls, as illustrated in Fig. 6. As expected, LCA-AMS could not inhibit the growth of *E. coli* or *S. cerevisiae*, even in concentration up to 400 μM (Fig. 6a). Meanwhile, the addition of LCA-AMS to *M. tuberculosis* H37Rv, *M. smegmatis* mc²155, and *M. neoaurum* NRRL B-3805 induced a dose-dependent inhibition of growth with more than 80% loss of viability at 59, 40, and 50 μM of the inhibitor, indicating that LCA-AMS specifically inhibits mycobacteria. The growth media also seem to have obvious influences on the inhibition efficiency. For *M. smegmatis* mc²155, IC_{50} is about 31.58 μM during growth on cholesterol (Fig. 6a). However, with growth of mycobacterial strains in a nutrient-rich

medium (Middlebrook 7H9 broth medium), the IC_{50} value was increased to $41.42 \pm 0.36 \mu\text{M}$, as illustrated in Fig. 6a.

Discussion

The first important finding of the current study is that, in *M. smegmatis* genome, aside from the previously reported FadD17 and FadD19, at least six FadDs capable of activating steroid side chains for subsequent degradation were observed, advancing our understanding of mycobacterial steroid degradation. The different isoforms of acyl-CoA synthetase exhibited strong preferences for particular chain lengths, while tolerating variable steroid nucleus structures. Based on the annotation of the genomes of several mycobacterial species and results of a BLAST search with FadD17, FadD19, CasG and CasI, and FadD8 (MSMEG_1098), we observed that they all had one or more homologous genes with 34–90% identity at the amino acid level.

Fig. 6 Selective inhibition of mycobacterial growth. **a** Efficacy of LCA-AMS in inhibiting the growth of *M. smegmatis* mc²155, *E. coli* DH5a, *S. cerevisiae*, *M. smegmatis* mc²155 when were cultured in several different media. **b** Efficacy of LCA-AMS in inhibiting the

growth of *M. tuberculosis* H37Rv, *M. neoaurum*. CFU_T and CFU_C are the CFU of inhibitor and DMSO-containing cultures. Data show the mean of three replicates

In addition, the genomes of most mycobacterial species contain four FadD19 homologous genes, while *M. tuberculosis* has only one FadD19 homologous gene. In addition, *fadD19* homologous genes, including *fadD19-1*, *fadD19-2*, and *fadD19-3*, showed 34–57% identity at amino acid level with one another (Table S3). Analysis of the substrate specificity and kinetic data revealed that the FadD19, FadD19-1, and FadD19-2 were preferable cholestenoate with a C8 alkanoate side chain. The RT-qPCR analysis showed that MSMEG_5914 (*fadD19*) and MSMEG_3687 (*fadD19-2*) were upregulated by 36.0- and 12.1-fold change, respectively, suggesting that they are cholesten-26-oyl-CoA synthetases.

The ortholog of MSMEG_1098 in *M. tuberculosis* was annotated as FadD8, and it contains KstR operator sequences (Geize et al. 2007; Uhía et al. 2012; Pei et al. 2007). Although FadD8 shared as low as 26% identity at amino acid level with FadD19, unexpectedly, FadD8 was observed to specifically catalyze the thioesterification of the cholestenoate possessing a C8 side chain, in which the favorable substrate was similar to that of FadD19. Compared with FadD19-1 and FadD19-2, FadD8 exhibited low activity toward fatty acids (Fig. S5). The results of RT-PCR showed that MSMEG_1098 (*fadD8*) was remarkably upregulated in cholesterol compared with glycerol, which was consistent with a previous microarray analysis of *M. smegmatis* (Uhía et al. 2012) (see Fig. S6).

The data of this study demonstrated that FadD17 exhibited high activity with steroids, which had a C5 side chain (Fig. S5). Based on its occurrence in the actinobacterial cholesterol catabolic cluster, FadD17 was believed to be involved in the activation of cholesterol metabolites with a C5 side chain (Casabon et al. 2014). However, in accordance with the previous studies (Geize et al. 2007; Uhía et al. 2012), our RT-qPCR analyses also showed that *fadD17* (MSMEG_5908) was upregulated to a lower level during the growth of *M. smegmatis* in cholesterol (see Fig. S6). Besides, *fadD1* (MSMEG_4952) was upregulated to a lower level during the growth of *M. smegmatis* in a cholesterol or cholate medium compared with that of cells growing in media with glycerol as the sole carbon source (see Fig. S6). Therefore, the activation of steroids with a C5 alkanoate side chain may be performed by other redundant Fads, as well as FadD17 and FadD1. It has been previously reported that CasI is a steroid-22-oyl-CoA synthetase, activating steroids with a C3 isopropanoate side chain (Casabon et al. 2014), with the favorable substrate differing from that of FadD17 and FadD1. Moreover, XA26_18490 and 174_08270 should be the orthologs of Cas I, as both shared identity up to 70% at amino acid level with Cas I. In spite of exhibiting activity with various fatty acids with different lengths, FadD5 (MSMEG_0131), FadD6 (MSMEG_5086), and FadD14

(MSMEG_5348) were not able to transform cholate, lithocholate, or cholestenoic acid.

The second significant finding of the current study is that acholoyl-AMP mimic, LCA-AMS, can inhibit specific mycobacteria, justifying why it was designed and synthesized. To date, various acylsulfamoyl analogues that resemble the common intermediate reaction of acyl-CoA synthetases have been synthesized and used for fatty acyl-AMP ligases or fatty acyl-CoA ligases (Arora et al. 2009; Léger et al. 2009; Ferreras et al. 2005; Ferreras et al. 2008). Among them, lauroyl-sulfamate adenosine was reported to express inhibitory effect against FadD19 and FadD28 (Arora et al. 2009). Leger et al. reported that the half-maximal effective concentration (EC₅₀) of the adenosine 5'-dodecylphosphate against FadD32 was 0.11 μ M (Léger et al. 2009). Both adenosine 5'-dodecylphosphate and lauroyl-sulfamate adenosine are analogues of transient intermediate lauroyl-AMP, which can be generated from lauric acid by various acyl-CoA synthetases (Arora et al. 2009). Therefore, such inhibitors exhibit an inhibition with a very broad range of acyl-CoA synthetase targets, including those from human hosts. The lack of specificity might limit their further practical application. Therefore, we hypothesized that non-hydrolyzable analogues of steroid metabolites (e.g., cholestenoic acyl-AMP or choloyl-AMP) could act as a class-specific inhibitor of the acyl-CoA synthetases being responsible for steroid side-chain degradation (Fig. 1). Based on this assumption, we designed and synthesized acholoyl-AMP mimic, LCA-AMS, and 11 FadDs, which were used as research subjects to examine the inhibitory effect of the synthesized LCA-AMS. The results showed that LCA-AMS inhibits FadD17_{Msm} and FadD1_{Msm} enzyme activities with IC₅₀ values of 0.081 and 1.4 μ mol/L, respectively. However, no inhibition against FadD5 (MSMEG_0131), FadD3 (MSMEG_4772), FadD6 (MSMEG_5086), or FadD14 (MSMEG_5348) was evident, correlating exactly with the favorable substrate of these FadDs.

Importantly, LCA-AMS exhibited highly selective inhibition toward mycobacterial strains, such as *M. tuberculosis* H37Rv, *M. smegmatis* mc²155, and *M. neoaurum* NRRL B-3805, with low concentration, whereas *E. coli* and *S. cerevisiae* were almost insensitive to LCA-AMS (see Fig. 6). Overall, although there has been considerable effort to develop specific inhibitors for a mycobacterial lipid metabolism pathway, a limited number of these inhibitors have demonstrated the functionality and specificity being necessary for clinical practice. To our knowledge, an inhibitor that is highly specific to steroids activating FadDs has not been previously reported. Further studies are in progress in our laboratory to achieve more transient intermediate analogues, such as 5'-O-(cholestenoyl sulfamoyl) adenosine, which would inhibit specific steroid-activating FadDs.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest with the contents of this article.

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