Thiolactomycin and Related Analologues as Novel Anti-mycobacterial Agents Targeting KasA and KasB Condensing Enzymes in Mycobacterium tuberculosis*

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Prevention efforts and control of tuberculosis are seriously hampered by the appearance of multidrug-resistant strains of Mycobacterium tuberculosis, dictating new approaches to the treatment of the disease. Thiolactomycin (TLM) is a unique thiolactone that has been shown to exhibit anti-mycobacterial activity by specifically inhibiting fatty acid and mycolic acid biosynthesis. In this study, we present evidence that TLM targets two β-ketoacyl-acyl-carrier protein synthases, KasA and KasB, consistent with the fact that both enzymes belong to the fatty-acid synthase type II system involved in fatty acid and mycolic acid biosynthesis. Overexpression of KasA, KasB, and KasAB in Mycobacterium bovis BCG increased in vivo and in vitro resistance against TLM. In addition, a multidrug-resistant clinical isolate was also found to be highly sensitive to TLM, indicating promise in countering multidrug-resistant strains of M. tuberculosis. The design and synthesis of several TLM derivatives have led to compounds more potent both in vitro against fatty acid and mycolic acid biosynthesis and in vivo against M. tuberculosis. Finally, a three-dimensional structural model of KasA has also been generated to improve understanding of the catalytic site of mycobacterial Kas proteins and to provide a more rational approach to the design of new drugs.

Tuberculosis, in terms of infectious diseases, is the leading cause of morbidity and mortality worldwide, infecting 8 million and killing 3 million people annually (1). The situation has recently been exacerbated by the human immunodeficiency virus pandemic and the increased prevalence of multidrug-resistant strains of Mycobacterium tuberculosis (2). Vaccine prophylaxis, using Mycobacterium bovis BCG, has proven unsatisfactory in many parts of the world (3). Recent research has focused on understanding the molecular basis of drug resistance in M. tuberculosis, and a great deal of progress has been made in this regard in relation to several major anti-tubercular drugs, including rifampicin (4), streptomycin (5), pyrazinamide (6), ethambutol (7), and isoniazid (INH)1 (8–10).

Mycolic acids are high molecular weight α-alkyl, β-hydroxy fatty acids with the general structure R-CH(OH)-CH(R′)-COOH, where R is a “meromycolate” chain consisting of 50–56 carbons and R′ is a shorter aliphatic branch possessing 22–26 carbons (11). Mycolic acids are key components of the mycobacterial cell wall and may play a role as an effective lipophilic barrier to the penetration of some antibiotics (11). Considering the importance of mycolic acids in bacterial survival, enzymes involved in the metabolism of these specific molecules represent attractive targets for the design of new anti-mycobacterial agents. Although there has been controversy about the mechanism of action of INH, it is clear that disruption of mycolic acid biosynthesis is one of its earliest detectable effects. This is apparently achieved through inhibition of inhA, an enoyl-acyl carrier protein (ACP) reductase, a key enzyme involved in the biosynthesis of fatty acids and mycolic acids (9). However, mutations within katG, which encodes a catalase-peroxidase enzyme, lead to the majority of INH-resistant isolates (8), demonstrating that INH is a produg and that an activated metabolite is responsible for its mode of action (12). Presumably, inhA is the primary target for the activated form of INH, and indeed, mutations in the inhA gene account for some cases of INH resistance in M. tuberculosis. More recently, mutations have also been observed within clinical M. tuberculosis isolates resistant to INH, traceable to kasA, which encodes a β-ketoacyl-ACP synthase, another key enzyme involved in mycolic acid biosynthesis (10).

Earlier studies have demonstrated that thiolaactomycin (TLM) selectively inhibits bacterial and plant type II fatty-acid synthases (FAS-II) through inhibition of β-ketoacyl-ACP synthase (Kas) (13–17). In addition, we have previously demon-

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1 The abbreviations used are: INH, isoniazid; ACP, acyl-carrier protein; CEB, cerulenin; FAS, fatty-acid synthase; Kas, β-ketoacyl-ACP synthase; MAS, mycolate-synthesizing activity; MIC, minimal inhibitory concentration; TLM, thiolaactomycin; OADC, oleic-albumin-dextrrose-catalase; PCR, polymerase chain reaction.
strated that TLM acts as a potent anti-tuberculosis agent by inhibiting both fatty acid and mycolic acid biosynthesis in mycobacteria (18). In light of this clinical aspect and of recent genomic information, we have reexamined the mode of action of TLM in mycobacteria. In this study, we have established that KasA and KasB, which both possess a high degree of similarity with other Kas enzymes, are targets for TLM. In addition, we have extended this study in the search of new anti-tuberculosis agents by generating a hypothetical structure of KasA to assist in future drug design and synthesized several lipophilic TLM derivatives. These analogues possess improved activities both in vitro against fatty acid and mycolic acid biosynthesis and in vivo against M. tuberculosis. This opens up new avenues for exploring the development of novel anti-mycobacterial agents based on TLM inhibition of Kas proteins in M. tuberculosis.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Growth Conditions**—All cloning steps were performed in Escherichia coli XLI-Blue (Stratagene, La Jolla, CA). Mycobacterium smegmatis mc^155 is an electroproportion-efficient mutant of mc^6 (19). Expression of KasA and KasB was conducted in the M. bovis BCG vaccine strain 1173P2 (World Health Organization, Stockholm, Sweden). M. bovis BCG was transformed as described previously (20), and recombinant M. bovis BCG clones selected on Middlebrook 7H10 agar supplemented with oleic-albumin-dextrose-catalase (OADC) enrichment DiFCo, Detroit, MI) containing 25 μg/ml kanamycin. Liquid cultures of M. smegmatis, M. bovis, and M. tuberculosis H37Rv were grown at 37°C in Sauton’s medium (21); Large-scale cultures of M. bovis BCG were grown to mid-log phase (10–14 days), harvested, washed with phosphate-buffered saline, and stored at −20°C until further use.

**Plasmids and DNA Manipulation**—The E. coli mycobacterial shuttle vector pMV261 containing the hsp60 promoter was used as described previously (22). Analysis of plasmids from mycobacteria was achieved by electrodution in

**Preparation of Cytochrome and Cell Wall Enzyme Fractions**—M. bovis BCG and recombinant strains (approximately 10 g) were washed and resuspended in 30 ml of buffer containing 100 mM potassium phosphate (pH 7.0), 1 mM EDTA, 5 mM dithiothreitol, 5 mM MgCl₂, and 2 mM phenylmethylsulfonyl fluoride at 4°C and subjected to probe sonication (1 cm probe, Soniprep 150, MSE Ltd., Crawley, Sussex, United Kingdom) for 15 cycles of 60s pulses with 90-s cooling intervals between pulses. The disrupted cells were then centrifuged at 27,000 × g for 30 min at 4°C, and the resulting supernatant fraction was recenterfuged at 100,000 × g for 1 h at 4°C to yield the soluble cytosolic fraction. This fraction was then adjusted to 40% ammonium sulfate, and the supernatant obtained after centrifugation was adjusted to 80% ammonium sulfate. The 40–80% ammonium sulfate precipitate, containing the FAS-II activity, was collected after centrifugation, dissolved in 3 ml of buffer (100 mM potassium phosphate (pH 7.0), 1 mM EDTA, 1 mM dithiothreitol, and 5 mM MgCl₂), and dialyzed overnight (18). The P60 particulate cell wall fraction possessing mycolate-synthesizing activity (MAS) was prepared as described previously (18, 27). Protein concentrations were determined using the BCA protein assay reagent kit (Pierce).

**FAS-II and Mycolate-synthesizing Assays**—The standard reaction mixture for incorporation of radioactivity from [2-14C]malonyl-CoA into C₆₀-C₆₃ fatty acids catalyzed by FAS-II consisted of 5 mM EDTA, 5 mM dithiothreitol, 100 μM palmitoyl-CoA (Sigma), 140 μM NADH (Sigma), and 140 μM NADPH (Sigma), 112 μM of [1-14C]acetate (50–62 mCi/mmol, Amersham Pharmacia Biotech), and 200 μM of cytosolic enzyme preparation with the final volume adjusted to 250 μl using 1× potassium phosphate (pH 7.0). TLM (0.1 mM) was added with the other assay components prior to the addition of protein. Reactions were performed in triplicate at 37°C for 30 min and stopped by the addition of 250 μl of 20% potassium hydroxide in 50% methanol at 100 °C for 45 min. Following acidification using 150 μl of 6 M HCl, the resulting 14C-labeled fatty acids were extracted with three portions (2 ml) of petroleum ether. The organic extracts were pooled, washed once with an equal volume of water, and dried in a scintillation vial, prior to scintillation counting (18, 27). Incubations utilizing the mycolate-synthesizing P60 cell wall fraction contained 50 mM potassium phosphate (pH 5.0), 10 mM NaHCO₃, 2.5 μCi of [1,2-14C]acetate (50–62 μCi/mmol, Amersham Pharmacia Biotech), and 500 μg of P60 preparation adjusted to a final volume of 1 ml with 50 mM potassium phosphate (pH 7.0). TLM (0.2 mM) was mixed with the other assay components prior to the addition of protein. Reaction mixtures were performed in triplicate, incubated at 37°C for 1 h, and stopped by the addition of 2 ml of 15% aqueous tert-butylammonium hydroxide at 100°C overnight. The 14C-labeled fatty acid and mycolic acid methyl esters were isolated as described previously (19, 20, 26). Methyl esters were redissolved in diethyl ether, and the solution was again evaporated to dryness. The final residue was then dissolved in 200 μl of CHCl₃. An aliquot of the resulting solution of fatty acid methyl esters and mycolic acid methyl esters was subjected to TLC using silica gel plates (5735 silica gel 60F254; Merck, Darmstadt, Germany), developed in petroleum ether-acetone (95:5). Autoradiograms were exposed to Kodak XAR film to reveal 14C-labeled fatty acid and mycolic acid methyl esters.

**Drug Susceptibility Testing of Recombinant M. bovis BCG**—The kasA, kasB, and kasAB—The kasA open reading frame (Rv2245) was cloned into the mycobacterial overexpression vector pMV261. PCR amplification and performing transformation into a particulate cell wall fraction possessing mycolate-synthesizing activity (MAS) was prepared as described previously (18, 27). Protein concentrations were determined using the BCA protein assay reagent kit (Pierce).
the Quanta Molecular Graphics package (MSI, Cambridge, United Kingdom). The input data were the coordinates (1KAS) for the x-ray crystallographic model of the β-ketoacyl-acyl carrier protein synthase II monomer (FabF) from \textit{E. coli} (29) and an alignment based upon the 41% identity between the two proteins. Due to the precision of the alignment, the fully automated Modeler routine was used to produce three models employing maximum refinement.

**Synthesis of TLM and TLM-related Analogues—**TLM and thiolactone (1) were synthesized as described previously by Slayden et al. (18). The synthesis of the remaining aliphatic TLM-related analogues (2–8) will be documented separately.

**RESULTS**

**Sequence Comparisons of Various Kas Proteins—**Mycobacteria, in contrast to most bacteria, are unusual in that they possess two FASs: a multifunctional FAS-I and a dissociated FAS-II (30, 31). We have previously demonstrated that TLM inhibited only FAS-II and not FAS-I of \textit{M. smegmatis} (18). In addition, TLM is a known potent inhibitor of FAS-II type β-ketoacyl-ACP synthases in \textit{E. coli} (32–34). Therefore, we postulated that the corresponding FAS-II β-ketoacyl-ACP synthases (Kas) of \textit{M. tuberculosis} may be potential targets of TLM. To test this hypothesis, we have examined the \textit{M. tuberculosis} H37Rv genome (35) for the presence of potential kas-like genes.

Analysis of the \textit{M. tuberculosis} genome data base revealed the presence of two genes, designated Rv2245 (kasA) and Rv2246 (kasB), which were present in a single operon downstream of an acyl carrier protein (acpM) (10). Subsequent data base searches using BLAST demonstrated that both \textit{M. tuberculosis} gene products were highly related (67% identity); displayed 40% homology with other Kas enzymes, such as the FabB and FabF proteins of \textit{E. coli}; and were highly homologous to the Kas proteins from \textit{Mycobacterium leprae} (Fig. 1). Indeed, FabB and FabF, which are also known as KasI and KasII, respectively, are involved in \textit{E. coli} fatty acid elongation and biosynthesis (32, 36). Interestingly, a high degree of homology was also observed with other Kas proteins, such as those from \textit{Streptomyces glaucescens} (Fab-Sgl) (37) and \textit{Aquifex aeolicus} (FabF-Aae) (38). Biosynthetically, Kas proteins elongate specific fatty acyl precursors, generating new carbon-carbon bonds via a condensation reaction. Mechanistically, such reactions involve 1) transfer of a pantotheine bound acyl-primer to a cysteine residue of the condensing kas enzyme, 2) decarboxylation of an acyl carrier bound donor unit (malonate) to yield a carbanion, and 3) condensation of the carbanion with the carbonyl carbon of the enzyme bound primer. Recent sequence comparisons among various Kas and related enzymes, \textit{i.e.} chalcone and thiolas, by Siggaard-Anderson and co-workers (39, 40) has led to the identification of a number of conserved amino acid residues. For instance, FabB from \textit{E. coli} possesses a key Cys-163 and constitutes the active site residue. Other residues, such as His-298, Lys-328, and His-333, were also identified; when these residues were replaced by Ala, decarboxylation and overall elongation activity were completely abolished. This suggests the importance of these amino acids in catalysis of Kas-related proteins (39, 40). The alignment presented in Fig. 1 illustrates that these residues are also highly conserved in the mycobacterial KasA and KasB proteins.

**Overexpression of KasA, KasB, and KasAB in \textit{M. bovis} BCG—**In order to establish the relationship between the \textit{M. tuberculosis} open reading frames Rv2245 and Rv2246 and TLM boxes, and conserved residues are indicated in gray. Dots illustrate residues conserved in all sequences and known to be involved in the catalytic reaction mechanism in FabB from \textit{E. coli} (Eco), \textit{S. glaucescens} (Sgl), \textit{A. aeolicus} (Aae), \textit{M. leprae} (Mlp), or \textit{M. tuberculosis} (Mtb).
Fig. 2. Overexpression of kasA in M. bovis BCG. A, the enriched cytosolic fraction (40–80% ammonium sulfate fraction, 20 μg of protein) from M. bovis BCG harboring pMV261::kasA or the control plasmid pMV261 was analyzed by SDS-polyacrylamide gel electrophoresis and Coomassie Blue staining. The numbers on the left indicate the size of the molecular weight standards, and the arrow on the right indicates the overexpressed KasA protein. B, effects of KasA overexpression on the morphology of M. bovis BCG in the presence of TLM. M. bovis BCG and pMV261::kasA were grown to mid-log phase, and serial dilutions were spotted on plates in the absence (-) or presence (+) of TLM at 80 μg/ml of protein. Growth and morphology of the cells were scored after 10–14 days of incubation at 37 °C.

Resistance to TLM is Determined by the Amount of Functional KasA and KasB Produced in M. bovis BCG—To evaluate whether overexpression of KasA or KasB could affect the overall level of sensitivity against other powerful anti-mycobacterial drugs that inhibit fatty acid and/or mycolic acid synthesis, we determined the MICs of the above recombinant strains against INH, ethionamide, isoxyl, and CER. As shown in Table I, no significant differences were observed between M. bovis BCG and the two recombinant strains regardless of the drug used. Thus, these results suggest that the mode of action of TLM involves a separate and distinct mechanism from INH, ethionamide, isoxyl, and CER. The fact that overexpression of KasA did not generate increased resistance against INH is intriguing, because it has been reported that INH inhibits the M. tuberculosis KasA protein (10). In addition, because it is well known that CER constitutes a potent Kas inhibitor in relation to FAS-II and FAS-I, the results suggest that CER exhibits its potent in vivo anti-mycobacterial action primarily through inhibition of de novo fatty acid synthesis via FAS-I. Taken together, the preliminary data suggest that the two condensing enzymes, KasA and KasB, are targets for TLM. In addition, expression of the kasAB genes produced an additive effect (MIC, 150–200 μg/ml, Table I). However, to determine whether KasA and KasB do represent valid TLM targets, we decided to conduct in vivo pulse labeling experiments and examine in vitro FAS-II and mycolate synthesizing activities in the different recombinant M. bovis BCG strains, in the absence and presence of TLM.

Resistance to TLM Is Determined by the Amount of Functional KasA and KasB Produced in M. bovis BCG—To evaluate the effect of TLM on both mycolic acid and fatty acid synthesis in M. bovis BCG, purified methyl mycolates and fatty acid methyl esters were prepared from cultures in the presence of increasing concentrations of TLM and analyzed by TLC. As shown in Fig. 3, TLM affects the synthesis of all classes of mycolates in M. bovis BCG (α-mycolates and ketomycolates) and also fatty acid methyl esters in a dose-dependent manner. This inhibitory effect was detectable at low concentrations of TLM (10 μg/ml) for M. bovis BCG (Fig. 3). In contrast, synthesis of fatty acid methyl esters and both classes of mycolates in...
the KasA (Fig. 3) and KasB (data not shown) overproducing strains were more refractory to inhibition by TLM.

Formation of long-chain (C\textsubscript{18}-C\textsubscript{34}) fatty acids produced by an \textit{in vitro} FAS-II assay (30, 31) was also highly sensitive to TLM inhibition using \textit{M. bovis} BCG(pMV261) (Table II). Indeed, 71% inhibition was obtained in the presence of 0.1 mM TLM using extracts prepared from \textit{M. bovis} BCG(pMV261). However, only 18% inhibition of FAS-II activity was obtained in the case of \textit{M. bovis} BCG overproducing KasA. It was also interesting to note an overall increase in FAS-II specific activity using extracts from \textit{M. bovis} BCG(pMV261::kasA). The effect of TLM on mycolic acid synthesis was examined using a particulate cell wall (P60) extract (18, 27) (Table II). As predicted, mycolic acid synthesis was strongly inhibited by TLM in \textit{M. bovis} BCG (pMV261), resulting in approximately 73% of inhibition in the presence of 0.2 mM TLM. Interestingly, the P60 mycolate-synthesizing activity of \textit{M. bovis} BCG overexpressing KasA remained highly sensitive to TLM inhibition (79%). In contrast to KasA, overexpression of KasB did not lead to a significant protective effect in relation to FAS-II activity (Table II). However, the P60 mycolate synthesizing extracts from \textit{M. bovis} BCG overexpressing KasB possessed an increased level of specific activity and were less sensitive to TLM inhibition compared with \textit{M. bovis} BCG(pMV261) extracts.

The above \textit{in vivo} and \textit{in vitro} data clearly demonstrate that KasA and KasB are TLM targets and probably catalyze two different fatty acid elongation steps. Thus, it is likely that KasA catalyzes the condensation steps from C\textsubscript{18} to C\textsubscript{34} long-chain fatty acids, via the classical mycobacterial FAS-II system (30, 31); these may then be used as substrates utilized by KasB within the MAS extract for further elongation, leading to longer chain fatty acids (perhaps meromycolic acids, which are precursors of mycolic acids (see Ref. 43)).

\textit{Anti-mycobacterial Activity of TLM against \textit{M. tuberculosis} Isolates}—We examined the efficiency of TLM against INH-sensitive and INH-resistant \textit{M. tuberculosis} strains (Table III). TLM exhibited a potent activity against wild-type clinical \textit{M. tuberculosis} strains (MIC = 20–40 \mu g/ml), which was similar to those obtained for \textit{M. bovis} BCG and \textit{M. tuberculosis} H37Rv (18). Although the G269S mutation within KasA has been suggested to be associated with resistance against INH in clinical isolates (10), we found that this mutation did not affect sensitivity against INH in strains M262 and M269 (MIC = 0.2 \mu g/ml). However, these two strains were more resistant to TLM, suggesting that the G269S mutation may affect targeting of TLM to KasA. Surprisingly, the multidrug-resistant strain M307 (KatG, S315I), which is resistant to six different frontline drugs, including INH, appears to be very sensitive to TLM (MIC < 10 \mu g/ml). The fact that an INH-resistant mutant is highly sensitive to TLM is particularly interesting in relation to TLM as a potential anti-tuberculosis agent in a clinical setting.

\textit{Structure of TLM-related Analogues and Evaluation of Their in Vivo and in Vitro Inhibitory Activities against FAS-II and Mycolic Acid Synthesis}—A library of TLM analogues, with various substituents at the C-4 position of the thiolactone ring, were synthesized in order to evaluate their potential as TLM mimics in inhibiting fatty acid and mycolic acid biosynthesis. These compounds were tested both \textit{in vivo} with liquid cultures and \textit{in vitro} with FAS-II and P60 particulate cell wall fractions (18, 27). Liquid cultures of \textit{M. tuberculosis} H37Rv were treated with a range of concentrations of analogues 1-8 and incubated at 37 °C for 10–14 days, and the MIC\textsubscript{50} and MIC\textsubscript{90} values were obtained (Table IV). It is interesting to note that thiolactone (analogue 1) itself is insufficient to produce an inhibitory effect both \textit{in vivo} and \textit{in vitro}. Analogues 2 and 3, although they possess a five-carbon lipid backbone similar to that in TLM, produce rather weak \textit{in vitro} and whole cell activities (MIC\textsubscript{50} > 300 \mu M). In contrast, increasing the length of the side chain up to C\textsubscript{10}/C\textsubscript{15} clearly enhanced whole cell activity (analogues 6 and 7, MIC\textsubscript{90} = 29 \mu M; cf. to TLM of 125 \mu M). Although, the transgeranyl analogue (analogue 4) itself appears to be relatively inactive (MIC\textsubscript{90} = 200 \mu M), saturation of the internal double bond (5, MIC\textsubscript{90} = 56 \mu M) and both double bonds (6, MIC\textsubscript{90} = 29 \mu M) increased activity even further. In contrast to TLM, analogues 4-7 possessed very poor inhibitory effects against \textit{in vitro} FAS-II activity but strongly inhibited mycolic acid biosynthesis (Table IV). The benzophenone analogue (8) displayed equal \textit{in vivo} activity to TLM but was only a modest inhibitor of mycolic acid biosynthesis. A preliminary analysis of the TLM
anallogues, in comparison to TLM, suggests that the more hydrophobic inhibitors were more potent against mycolic acid synthesis rather than against the mycobacterial FAS-II system (Table IV). Overall, these inhibitors, especially 6 and 7, produced a much more potent in vitro effect, in comparison to TLM. Thus, it appears that altering the hydrophobicity of the side chain via length and saturation generates more potent TLM derivatives against \(M.\) \(\text{tuberculosis}\).

Three-dimensional Structure of KasA—Models of the three-dimensional structure of the \(M.\) \(\text{tuberculosis}\) KasA protein (Fig. 4) were generated by homology modeling of aligned sequences, based on the recently determined x-ray structure of the \(E.\) \(\text{coli}\) FabF protein (29). The position of Cys-171 is identical to the active site Cys (Cys-163) of FabF, whereas the active site His-311, Lys-340, and His-345 (corresponding to His-304, Lys-338, and His-341 of FabF) also superimpose (Fig. 4A).

DISCUSSION

The antibiotic TLM is a selective inhibitor of type II fatty acid biosynthesis, is not toxic to mice, and affords significant protection against urinary tract and intraperitoneal bacterial infections (13–17). TLM has moderate in vitro activity against a broad spectrum of pathogens, including Gram-positive cocci and enteric, acid-fast, and anaerobic bacteria (15, 17). More recently, TLM has shown encouraging anti-malarial activity via inhibition of type-II fatty acid biosynthesis in apicoplasts (41). In \(E.\) \(\text{coli}\), all three condensing enzymes (FabB, FabF, and FabH) are inhibited by TLM both in vivo and in vitro (42). In contrast, TLM has no effect on type I fatty acid biosynthesis in \(Saccharomyces\) \(\text{cerevisiae}\), \(Candida\) \(\text{albicans}\), or rat liver (17).

The effect of TLM in mycobacteria has recently been investigated and shown to inhibit specifically the type II but not type I fatty-acid synthases (18). In this study, we present evidence that overexpression of KasA or KasB individually or co-expression of both enzymes in \(M.\) \(\text{bovis}\) BCG results in increased resistance levels against TLM. Subsequent MIC determinations using a variety of agents that require penetration through the mycobacterial cell wall (such as rifampicin and other well characterized mycolic acid inhibitors, including INH) suggest that TLM sensitivity is not likely to be mediated by cell wall permeability and that its mechanism of action is dissimilar to other known mycolic acid inhibitors (8–10). Overexpression studies provided indirect evidence that KasA and KasB are TLM targets. Further in vivo metabolic labeling and, more importantly, in vitro analyses demonstrated clearly that overexpression of KasA and KasB mediated a protective effect in both a mycobacterial FAS-II assay system (30, 31) and a mycolic acid synthesizing extract to TLM (18, 27). It was also interesting to note that TLM inhibition studies using extracts from \(M.\) \(\text{bovis}\) BCG carrying either pMV261::kasA or pMV261::kasB suggest that KasA may participate in the synthesis of \(C_{18}\)–\(C_{34}\) fatty acids, whereas KasB may be involved in later steps of mycolic acid biosynthesis. These results have recently been supported by independent studies conducted by Slayden et al. (43).
INH-susceptible and INH-resistant strains. However, 10 strains with mutations in codon 269 were found, 5 among INH-susceptible strains and 5 among INH-resistant strains. Interestingly, these mutations in kasA (Fig. 4A) do not reside within the catalytic site. Two possible explanations with regard to INH susceptibility/resistance include, first, their influence on the relative degree of acyl-AcpM binding, and second, the stabilization of the dimerization of the KasA protein. As a consequence, these mutations may affect the KasA-INH-AcpM complex, a scenario that remains to be further investigated. We describe two distinct clinical isolates of M. tuberculosis displaying the G269S substitution that are still sensitive to INH, although resistant to TLM. Overall, these results suggest that mutations within kasA do not confer significant INH resistance. Considering that the serum levels of INH are 35–60 times above the MIC (45), the results suggest that, clinically, point mutations in KasA are not significant in terms of INH resistance. However, because INH has been proven to be one of the most effective anti-tuberculosis agents that targets FAS-II through InhA (9), it would be anticipated that TLM would be of therapeutic value to INH-sensitive and INH-resistant strains through synergistic INH therapy. This is reinforced by the observation that the multidrug-resistant M307 isolate (its resistance is due in part to mutations in KatG) appears to be highly susceptible to TLM, suggesting that TLM may be suitable for the treatment of tuberculosis.

During the course of our studies, several elegant structural reports have appeared defining the key catalytic amino acids involved in the elongation and decarboxylation process involved in fatty acid elongation by Kas enzymes (29, 39, 40). We have taken advantage of this available structural information to predict a model structure for mycobacterial KasA. It is clear that the key catalytic amino acids described for FabB by Sigggaard-Anderson and co-workers (39, 40) are highly conserved and that a deep hydrophobic pocket surrounds the catalytic Cys-163 residue (Fig. 4). It should also be pointed out that the mycobacterial FAS-II and meromycolate system elongate C₁₆ fatty acid primers, initially through Kas enzymes, whereas E. coli FAS-II performs de novo synthesis from acetyl-CoA/ACP and malonyl-ACP (42). As a consequence, we decided to investigate whether a range of TLM inhibitors differing in hydrophobicity, and possibly resembling more the nature of the condensing enzyme substrates, would be more potent than TLM itself. Clearly, there was a strict requirement for a C-4 side chain as the parent thiolactone moiety was inactive both in vivo and in vitro. Analogues 2 and 3 were very similar in relation to the overall structure of TLM and possessed similar in vitro properties to TLM but were very poor in vivo inhibitors. Analogues 3–7 were influenced by the overall chain length and degree of saturation of the C-4 side chain in terms of in vivo activity, with 6 and 7 possessing a 4-fold increase in potency. It was interesting to observe that there was a lack of inhibition against mycobacterial FAS-II by analogues 4–8 but excellent inhibitory properties against the mycolic-acid synthase extract. However, it should be pointed out that further studies are required to determine true structure-activity relationships for TLM analogues and FAS-II inhibition. For instance, studies using chloroplasts from peas demonstrated that analogue 4 (inactive in the mycobacterial FAS-II system) was a potent inhibitor of FAS-II within the pea FAS-II assay system (46).

Overall, the results suggest that TLM targets both KasA and KasB, whereas the more hydrophobic derivatives may target KasB and meromycolate synthesis. Studies examining recombinant E. coli expression of soluble Kas proteins (i.e. KasA and KasB) and site-directed mutants and studies of structural and binding/co-crystallization using TLM are currently in progress. In this regard, although it is a weak inhibitor, the benzophenone analogue 8 may provide a useful photoprobe for labeling investigations and covalent modification of the Kas enzymes.

TLM remains an interesting but rather unexploited antibiotic sharing little or no cross-resistance with other classes of antibiotics. The TLM family are confirmed as members of a group of mycobacterial mycolic acid inhibitors (INH, ethionamide, and isoxyl) that target various fatty acid biosynthetic genes. The selective enhanced activity of a number of analogues of TLM demonstrates the potential for antibiotic development. A key feature of TLM (and its analogues) is its selectivity for type-II FAS systems, which promotes its effectiveness as a broad spectrum antibiotic. The added value in the context of M. tuberculosis is the presence of multiple Kas enzymes. It can be envisaged that a suitable TLM derivative would block multiple condensing enzymes, i.e. both KasA and KasB, and thus reduce the frequency of appearance of TLM-resistance in M. tuberculosis. In this regard, several attempts to generate a laboratory-cultured M. bovis BCG strain resistant to TLM has failed (data not shown), supporting this notion. In summary, further characterization of genes involved in the biosynthesis of mycolic acids, such as KasA and KasB, may lead to the development of new therapeutic anti-tubercular agents, especially in the context of more potent TLM mimics.

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