Isoxyl activation is required for bacteriostatic activity against

*Mycobacterium tuberculosis*

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Running title: EthA-mediated activation of Isoxyl

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Abstract

Isoxyl (ISO), a thiourea derivative that was successfully used for the clinical treatment of tuberculosis during the 1960s, is an inhibitor of the synthesis of oleic and mycolic acids in *Mycobacterium tuberculosis*. Its effect on oleic acid synthesis has been shown to be attributable to its inhibitory activity on the stearoyl-CoA desaturase DesA3 but its enzymatic target(s) in the mycolic acid pathway remain(s) to be identified. With the goal of elucidating the mode of action of ISO, we have isolated a number of spontaneous ISO-resistant mutants of *M. tuberculosis* and undertaken their genotypic characterization. We report here the characterization of a subset of these strains carrying mutations in the monooxygenase gene *ethA*. Through complementation studies, we demonstrate for the first time that the EthA-mediated oxidation of ISO is absolutely required for this pro-drug to inhibit its lethal enzymatic target(s) in *M. tuberculosis*. An analysis of the metabolites resulting from the *in vitro* transformation of ISO by purified EthA revealed the occurrence of a formimidamide allowing the formulation of an activation pathway in which the oxidation of ISO catalyzed by EthA is followed by chemical transformations involving extrusion or elimination and, finally, hydrolysis.
**Introduction**

*Mycobacterium tuberculosis*, the etiologic agent of tuberculosis (TB), claims about 1.7 million lives annually and the global number of TB cases is still rising at a rate of 0.6% per year (26). Approximately 2% of new tuberculosis cases in the world are attributed to multi-drug-resistant (MDR) strains, defined as *M. tuberculosis* isolates resistant to at least isoniazid and rifampicin, the two most powerful anti-TB drugs. However, multi-drug resistance rates in some countries, particularly in the former Soviet Union, Asia, the Dominican Republic and Argentina, are much higher and may reach more than 22% of all isolates (16). Moreover, extensively drug resistant (XDR) strains that are resistant to three or more of the six classes of second line drugs in addition to rifampicin and isoniazid have been reported in all regions of the world (5) raising the possibility of future epidemics of virtually untreatable forms of TB. The continuing rise in disease incidence, the problem of drug resistance and the need to reduce treatment duration has prompted research on new drug developments (11). In this light, the re-examination of Isoxyl (ISO) (4,4’-diisoamyloxydiphenylthiourea; 4,4’-diisoamyloxythiocarbanilide; thiocarlide), a thiourea derivative that was successfully used for the clinical treatment of TB during the 1960s, may prove useful in identifying new therapeutic targets and in developing new therapeutic agents with greater potency and more desirable features than earlier thioureas (1,14,21,23-24).

An early note reported that ISO inhibits the synthesis of mycolic acids and free fatty acids in *M. bovis* BCG (28). We later demonstrated that ISO displays potent activity against other slow- and fast-growing species of *Mycobacterium*, including MDR clinical isolates of *M. tuberculosis*, and showed that the drug affects the synthesis of all types of mycolic acids in addition to that of shorter-chain fatty acids in *M. bovis* BCG, *M. tuberculosis* H37Rv and *M.*
aurum A+ (17). Our recent evidence indicates that the main effect of ISO in fatty acid metabolism is in the inhibition of the synthesis of oleic acid and that this effect is directly attributable to the inhibitory effect of the drug on the membrane-associated stearoyl-CoA (Δ9) desaturase DesA3 (Rv3229c) (18). Interestingly, sterculic acid, a known inhibitor of membrane-associated Δ9 desaturases, emulated the effect of ISO on oleic acid synthesis but did not affect mycolic acid synthesis, demonstrating that there is no relationship between the two effects of the drug (18). Therefore, ISO has at least one other enzymatic target in the mycolic acid biosynthetic pathway. This assumption is also supported by the fact that among the ISO derivatives that have been synthesized, some of the most potent ones still affected mycolic acid synthesis while having lost the ability to inhibit that of oleic acid (4).

Several lines of evidence suggest that ISO is a pro-drug requiring prior metabolic activation for antimycobacterial activity. ISO has a long history of cross-resistance with ethionamide (ETH) and thiacetazone (TAC), two second-line antituberculosis drugs that share with ISO a thiocarbonyl moiety which requires to be S-oxidized for expression of their toxicity. Genetic analysis of a subset of cross-resistant M. tuberculosis isolates identified the flavin-containing monooxygenase EthA (Rv3854c) as the likely common activator of all three drugs (6). This assumption was more recently substantiated by the demonstration that EthA catalyzes the direct enzymatic transformation of ETH, TAC and ISO in vitro and that of ETH in vivo (6,8,9,12,19,25). Furthermore, ethA overexpression was shown to increase the sensitivity of M. smegmatis or M. bovis BCG to ETH, TAC and ISO while overexpression of ethR (Rv3855), a repressor of ethA, conferred ETH resistance to M. tuberculosis and M. smegmatis and both ETH and TAC resistance to M. bovis BCG (2,6,8). Interestingly, however, ethR overexpression had no effect on the susceptibility of M. smegmatis, M. bovis BCG and M. tuberculosis to ISO and the
disruption of the *ethR* gene in *M. bovis* BCG only increased the sensitivity of the mutant strain to the drug two-fold as compared to ten-fold in the case of ETH and TAC (2,6,8). Consequently, in spite of clinical and experimental observations suggesting that ISO is a pro-drug that requires EthA for activation, the likely existence of multiple cellular targets of this drug, all of which may not be targeted by the same metabolite of ISO, and the modest effect of *ethR* expression levels on the susceptibility of mycobacteria to this compound raised questions about the absolute requirement of ISO activation for mycobacteriostatic activity and the role of EthA in this process. The identity of the metabolite(s) resulting from the activation of ISO by EthA also remained to be determined.

With the goal of elucidating the mode of action of ISO, we have isolated a number of *M. tuberculosis* strains displaying resistance to this drug and undertaken their genotypic and phenotypic characterization. We report here the characterization of a subset of these strains carrying mutations in the *ethA* gene and describe the effects of purified EthA on the metabolic transformation and activity of ISO in cell-free assays.

**Materials and Methods**

*Bacterial strains and growth conditions* - *M. tuberculosis* H37Rv (ATCC#25618), the *mutT1* transposon mutant of *M. tuberculosis* Mt103 (MT1K) (7), and *M. bovis* BCG 1173P2 (Pasteur strain) were grown in Middlebrook 7H9 medium (Difco) supplemented with ADC (0.2% dextrose, 0.5% bovine serum albumin fraction V, 0.085% NaCl, 0.0003% beef catalase) and 0.05% Tween 80 or on solid Middlebrook 7H11 medium (Difco) supplemented with OADC (0.005% oleic acid, 0.2% dextrose, 0.5% bovine serum albumin fraction V, 0.085% NaCl,
0.0003% beef catalase). *E. coli* XL1-blue, the strain used for cloning experiments, was propagated in Luria Bertani (LB) broth (pH 7.5) (Bactotryptone, 10 g/l, Bacto™ yeast extract, 5 g/l, NaCl, 5 g/l) (Becton Dickinson, Sparks, MD). *M. smegmatis* mc²155 was grown in Middlebrook 7H9 medium (Difco) supplemented with ADC and 0.05% Tween 80, Luria Bertani broth supplemented with 0.05% Tween 80 or MM63 minimal medium supplemented with 0.025% tyloxapol. Where indicated, kanamycin (Kan) and hygromycin B (Hyg) were added to final concentrations of 20 µg/ml and 50 µg/ml, respectively.

**Synthesis of Isoxyl** - The starting material in our synthesis of ISO was the commercially available *p*-nitrophenol. Alkylation of *p*-nitrophenol with 1-bromo-3-methyl-butane (isoamyl bromide) in the presence of 18-crown-6 ether and anhydrous potassium carbonate in acetone (20) and subsequent reduction with tin chloride in ethanol (3) gave *p*-isoamyloxy aniline. The yields in this two-step synthesis were high (87% in each case). Treatment of *p*-isoamyloxy aniline with carbon disulfide in pyridine in the presence of diphenyl phosphite and subsequent column chromatography of the crude product yielded the pure ISO. NMR and mass spectrometry data support the structure of the product.

**Drug susceptibility testing** - Minimal inhibitory concentration (MIC) values of ISO against *M. tuberculosis* wild-type and ISO-resistant strains were determined using the colorimetric resazurin microtiter assay (15) in 7H9-ADC broth at 37°C in the presence of 2 % DMSO. The susceptibility of the same strains to ethionamide was determined by the agar dilution method on 7H11-OADC medium containing 0, 1, 2.5 or 10 µg/ml of ethionamide (17). The MIC values of
ISO against *M. tuberculosis* H37Rv overexpressing ethA or ethR were determined by the resazurin microtiter assay in 7H9-ADC broth at 32°C (ethA) or 37°C (ethR).

**PCR amplification and DNA sequencing** - The entire promoter and coding sequences of desA3, ethA and ethR were amplified from *M. tuberculosis* genomic DNA of wild-type and mutant strains using standard PCR strategies with Pfu DNA polymerase (Stratagene) and sequenced on a capillary Applied Biosystems ABI 3100 Genetic Analyzer. The pairs of primers used for PCR were (5’-tggtgcacctggtggaaggc-3’)/ 5’-gtcaagtctggctgacactg-3’) for desA3, (5’-gtcgagtcggagcaatcc-3’)/ (5’-gcatgacgccagcacccgca-3’) for ethR and (5’-agcggacggtctcgagaagg-3’)/ (5’-acggcatctgctgtgac-3’) for ethA.

**Overexpression of ethA and ethR in *M. tuberculosis*** - The ethA and ethR genes were PCR amplified with Pfu DNA polymerase (Stratagene) from *M. tuberculosis* H37Rv genomic DNA using primers ethA.1 (5’-cgcccgcatatgaccgagcacctcgacgttg-3’)/ ethA.2 (5’-cgcaagcttaacccccaccggggcaggcc-3’) and ethR.1 (5’-gggaaacatatgaccacctccgcggccagtcag-3’)/ ethR.2 (5’-cccaagcttgcggttctcgccgtaaatgct-3’) and placed under control of the hsp60 transcription and translation signals in the mycobacterial expression plasmid pVV16 (13) yielding pVVethA and pVVethR. Recombinant proteins produced with this system carry a six-histidine tag at their carboxyl terminus allowing their analysis by immunoblotting with a mouse monoclonal anti-His antibody (Penta-His antibody, Qiagen) (13).

**Production and purification of EthA** - A recombinant His-tagged form of EthA was purified from *M. smegmatis* mc²155 overexpressing ethA from pVVethA. mc²155/pVVethA cells (3 g of wet
weight) resuspended in 3 ml of buffer A (25 mM Tris-HCl, pH 7.5, 300 mM NaCl) were disrupted by probe sonication for 8 min in the form of 60 s pulses with 90 s cooling intervals. The sonicate was centrifuged for 5 min at 2,000 x g at 4°C and the supernatant from this centrifugation was loaded onto a BD TALON™ Spin Column (Clontech). Unbound proteins were removed by washing the resin with buffer A containing 10 mM imidazole. His-tagged EthA bound to the resin was then gradually eluted with buffer A containing 50 and 300 mM imidazole. Fractions containing approximately 90% pure EthA were combined, desalted by PD-10 column (Amersham Pharmacia Biotech) and concentrated using a Vivaspin 6 (3 kDa MWCO) (Sartorius). The purified protein was immediately used in cell-free assays.

In vitro metabolism of ISO by purified EthA -

The in vitro activity of EthA on ISO was assayed as described by Vannelli and collaborators (25) with minor modifications. Briefly, the reaction mixture contained 100 mM NaCl, catalase (75 U ml⁻¹), superoxide dismutase (75 U/ml), bovine serum albumin (0.1 mg/ml), a NADPH regenerating system consisting of glucose-6-phosphate dehydrogenase (2.5 U/ml), glucose-6-phosphate (25 mM) and NADPH (1 mM), 40 µg of purified EthA protein, ISO dissolved in 40 µl of DMSO (2 µg/ml final concentration), and 25 mM Tris-HCl buffer (pH 7.5) in a final volume of 4 ml. Reactions were performed in pentaplicates. Reactions were stopped with 4 ml of chloroform after 60 min of incubation at 37°C and left rocking for 30 min at room temperature. Organic phases were removed, combined together, dried under a flow of nitrogen and stored at -20°C. Immediately before LC/MS analysis, samples were dissolved in DMSO and analyzed on an LC/MS apparatus (Agilent 1100 series) using a short C18 zorbax column, a methanol/water
gradient containing 0.07% of ammonium formate and an atmospheric pressure electrospray source (positive mode).

**Cell-free assays for mycolic acid and short-chain unsaturated fatty acid synthesis - M. bovis**

BCG cells (1 g of wet weight) resuspended in 25 mM Tris-HCl (pH 7.5) were disrupted by probe sonication as described above, centrifuged for 5 min at 2,000 x g and the resulting sonicate was used as the enzyme source for *in vitro* synthesis of mycolates. Reaction mixtures contained *M. bovis* BCG sonicate (3 mg of proteins), 1 μCi of [1,2-14C]acetic acid (specific activity, 113 Ci/mol, MP Biomedicals Inc.), partially purified EthA (up to 10 μg), ISO dissolved in 2.5 μl of DMSO (0, 2 or 5 μg/ml final concentrations) and 25 mM Tris-HCl buffer (pH 7.5) in a total volume of 250 μl. Reactions were pre-incubated on ice for 10 min, then incubated for 2 hrs at 37°C and stopped by the addition of 1 ml of 15% tetrabutylammonium hydroxide (Aldrich).

Mycolic acid methyl esters were then prepared as described (17) and analyzed by thin-layer chromatography (TLC) on silica gel 60-precoated plates F254 (E. Merck, Darmstadt, Germany) using *n*-hexane: ethyl acetate (95:5 by vol., three developments) as the eluent. TLC plates were exposed to Kodax Biomax MR films for 7 days at – 70°C.

The *in vitro* effect of ISO and EthA activation on the activity of the Δ9 acyl-CoA desaturase DesA3 was monitored using a recombinant form of DesA3 purified from *M. smegmatis* as the enzyme source and *M. smegmatis* membranes as a source of cofactors for the desaturation system (18). For the production of DesA3, the desA3 gene was PCR amplified from *M. tuberculosis* H37Rv genomic DNA using primers desA3.jam1 (5’-ccccgatccatggcagctgacg-3’) and desA3.jam2 (5’-cgctctagaggtgccatctg-3’) and placed under control of the inducible acetamidase promoter in the mycobacterial expression
plasmid pJAM2 (22). The resulting plasmid, pJAMdesA3, was electroporated into *M. smegmatis* mc²155 and the recombinant DesA3 protein was purified from mc²155/pJAMdesA3 cells following the same protocol as for EthA. Cell free reactions contained 0.5 mg of *M. smegmatis* membrane proteins, 3 µg of partially purified DesA3, 6 µg of partially purified EthA, 500 µM palmitoyl-CoA, 1 mM NADPH, ISO dissolved in 3.2 µl of DMSO (0 or 10 µg/ml final concentrations) and 25 mM Tris-HCl buffer (pH 7.5) in a final volume of 320 µl. Reactions were incubated for 75 min at 30°C and stopped with 2 ml of chloroform: methanol (2: 1, by vol.). The samples were left rocking for 30 min at room temperature, centrifuged and organic phases were removed and dried. Fatty acids methyl esters were prepared with the 3 N methanolic HCl kit from Supelco at 80°C overnight and analyzed by gas chromatography (GC) on a Shimadzu GC-14A chromatograph using a methyl silicone 5% phenyl column operating at a temperature of 175°C for 2 min followed by a programmed increase of 8°C/min to 300°C. The eluted peaks were identified by comparison of their retention time with those of fatty acid methyl ester standards (Supelco).

**Results and Discussion**

Isolation and characterization of ISO-resistant mutants of *M. tuberculosis*

With the goal of identifying the cellular targets of ISO in the tubercle bacillus, we have isolated spontaneous ISO⁸ mutants of *M. tuberculosis*. Two strains of *M. tuberculosis* were used in the screening: the laboratory strain H37Rv and a mutTI transposon mutant of *M. tuberculosis* Mt103 which we had shown earlier to display a hypermutator phenotype in the presence of rifampicin or isoniazid (7). Mutants were selected at 37°C on 7H11 plates supplemented with
OADC and 10 µg/ml ISO (four to five times the MIC value of ISO against wild-type *M. tuberculosis* in this medium) and scored for ISO-resistance 4 to 7 weeks post-inoculation. Selection was performed in the presence of oleic acid in the medium (in the form of OADC) to avoid selecting ISO\(^{R}\) mutants carrying mutations in the \(\Delta9\) acyl-CoA-desaturase DesA3, an already known target of the drug (18). Twenty-one independent mutants were isolated from the parent H37Rv and MT1K strains at a frequency of \(10^{-8}\) and \(2 \times 10^{-8}\), respectively. PCR-amplification and sequencing of the promoter regions and coding sequences of the *desA3*, *ethR* and *ethA* genes in these strains revealed that while none of them carried mutations in *desA3* or *ethR*, two had undergone mutations within the coding sequence of *ethA*. The nucleotide deletion at position 1218 in strain ISO-R33 results in a frameshift mutation while the three nucleotide-deletion at positions 164-166 of strain ISO-R22 results in a two amino acid change (SD \(\rightarrow\) Y). It is noteworthy that the mutation identified in ISO-R22 is located between two highly conserved motifs (DxxxGxGxxG and FxGxxxHxxxWP) of FAD- and NADPH-dependent Baeyer-Villiger monooxygenases known to be critical for catalysis and that it more particularly affects an amino acid residue that is conserved among other flavin-containing monooxygenases (D\(_{56}\)) (10,27). Data thus suggested that the activity of EthA on ISO might be affected if not abolished in those two mutants. Both strains displayed a high level of ISO-resistance with MIC values greater than 40 µg/ml (for solubility reasons, higher concentrations of ISO could not be tested) (Table 1). As expected, they also showed cross-resistance to ETH (MIC values > 10 µg/ml as compared to an MIC of ~ 5 µg/ml for the parent strains as determined by the agar dilution method).

To determine whether ISO-resistance in these strains arose from the mutations in *ethA*, a functional copy of the *ethA* gene carried by the multi-copy vector pVVethA was introduced into each of the mutants and the MIC of ISO against the complemented mutant strains was
determined. Complementation restored ISO-susceptibility in both mutants to levels comparable to those of wild-type \textit{M. tuberculosis} Mt103 and H37Rv (Table 1). The slight difference in drug sensitivity between H37Rv/pVVethA and the complemented mutants may be accounted for by the different background from which the mutant arise (the mutants were derived from the clinical isolate Mt103), the possible competition of the wild-type and mutated versions of EthA for ISO in the mutants, the total amount of active EthA produced by each strain or the deleterious effect of co-expressing a mutated and an active form of EthA on the oligomerization of this enzyme in the mutants (9). Nevertheless, it can be concluded from these results that ISO-resistance in the two mutants is solely due to the reduced or complete lack of activity of EthA on ISO and that this monooxygenase is thus absolutely required for ISO to inhibit its lethal enzymatic target(s) in \textit{M. tuberculosis}. Although the activation-dependent inhibitory effect of ISO on its cellular targets most likely explains this requirement, it is also possible that EthA serves to retain ISO or its metabolites inside the bacterial cell, as previously reported with ETH (12).

**Effect of overexpressing ethA and ethR on the susceptibility of \textit{M. tuberculosis} to ISO**

Overexpression of \textit{ethA} in \textit{M. tuberculosis} H37Rv increased two- to four-fold the sensitivity of this strain to ISO consistent with what had been reported earlier for \textit{M. bovis} BCG (8) (Table 1). However, in striking contrast with earlier work which showed only a modest to no effect of the level of expression of \textit{ethR} on the susceptibility of \textit{M. bovis} BCG, \textit{M. smegmatis} and \textit{M. tuberculosis} to ISO (2,8), we found that the overexpression of \textit{ethR} from pVVethR in \textit{M. tuberculosis} H37Rv resulted in more than an eight-fold increase in resistance to the drug (Table 1). Differences in the mycobacterial species and strains used in the different studies and/or in the
level of expression of ethR in the various overexpressors may account for this apparent discrepancy.

In vitro metabolism of ISO by purified EthA

To establish whether EthA interacted with ISO directly and identify the metabolites resulting from their interaction, we partially purified recombinant EthA from M. smegmatis overproducing an His-tagged version of this enzyme and incubated it with ISO as described under Materials and Methods. LC/MS analysis of the control experiment missing EthA revealed the presence of ISO (m/z = 401) along with traces amount of an oxidized species (m/z = 417). LC/MS analyses of the reaction mixtures containing EthA clearly pointed out the occurrence of several compounds (Fig. 1). Aside from unreacted ISO, the most important signals detected were: i) a species featuring a clear m/z signal at 385 compatible with the urea derivative (5) (M = 384); ii) a species featuring a clear m/z signal at 369 compatible with the formimidamide (3) (M = 368). Lesser species were observed, especially two unresolved compounds featuring m/z signals at 417 compatible with oxidized forms of ISO (M = 416). Other m/z signals observed (m/z = 474 and 546) are less easily explained and may be experimental artefacts.

The two other thio-bearing antituberculosis drugs ETH and TAC were demonstrated to undergo EthA-catalyzed successive oxidations of their sulfur atom. These unstable oxidized intermediates are then further transformed into reactive species thought to be responsible for the anti-mycobacterial activity of these drugs. In vitro, the occurrence of such reactive species was demonstrated by the identification of end products characteristic of their chemical reactivity (6,9,12,19,25). In our case, the in vitro action of EthA on ISO led to the formation of the formimidamide (3) as well as the urea derivative (5). As depicted in Figure 1, we suggest related
transformations leading to these two compounds. Oxidation reactions of the sulfur atom of ISO would lead to the intermediate (2) which would then undergo an extrusion reaction to give the formimidamide (3). Further chemical transformations of intermediate (2) (an EthA-based oxidation followed by an elimination) would lead to the reactive carbodiimide (4) which, in our experimental setting, would be hydrolyzed into the urea derivative (5).

In conclusion, LC/MS analysis of the reaction products resulting from the action of EthA on ISO in vitro revealed the occurrence of stable species arising from initial oxidation processes leading to reactive species. These results are in agreement with the previously reported occurrence of related reactive species following incubation of ETH or TAC with EthA and with the previously suggested inhibitory activity of these metabolites (6,12,19,25).

Effect of ISO activation on the synthesis of mycolic acids and unsaturated short-chain fatty acids by mycobacterial cell-free extracts

ISO inhibits the synthesis of mycolic acids through the inhibition of (an) as yet unidentified enzyme(s) and that of short-chain Δ9 mono-unsaturated fatty acids through the inhibition of the Δ9 desaturase DesA3 (17-18,28). We were thus interested in determining whether the in vitro activation of ISO by EthA stimulated the inhibitory effect of the drug on these two metabolic pathways. To this end, two cell-free assays were designed. Mycolic acid synthesis was monitored using *M. bovis* BCG sonicates as enzyme source and [1,2-\(^{14}\)C]acetic acid as the radiolabeled substrate. The catalytic activity of DesA3 was measured in an assay containing a partially purified form of the DesA3 protein, palmitoyl-CoA and *M. smegmatis* membranes as a further source of substrates and co-factors for the desaturation system. As expected, the addition of ISO to the first assay resulted in the inhibition of the synthesis of both...
alpha- and keto-mycolates in *M. bovis* BCG extracts (Fig. 2A). Consistent with earlier observations, the addition of ISO to the second assay also completely inhibited the stimulatory effect of DesA3 on the *in vitro* synthesis of palmitoleic and oleic acids (Fig. 2B) (18). Unexpectedly however, the addition of purified EthA to these assays almost totally abolished the inhibitory effect of the drug on mycolic acid, palmitoleic and oleic acid synthesis (Fig. 2). We conclude from these experiments that the addition of purified EthA to the reaction mixtures probably pushed the transformation of ISO towards the formation of downstream inactive metabolites. Thus, as shown earlier for ETH (25) and supporting the above hypothesis of an active - perhaps carbodiimide - intermediate, it is likely that the active metabolite(s) of ISO is (are) transient and/or unstable intermediate(s) formed in the process of the oxidative transformation and decomposition of the drug.

**Conclusion**

Through the selection of spontaneous ISO\(^R\) mutants of *M. tuberculosis* and complementation studies, we have shown that EthA is absolutely required for ISO to exert its lethal effect on *M. tuberculosis*. Based on our biological data, the identification of two metabolites resulting from the *in vitro* action of EthA on ISO and to what has been previously described for ETH and TAC, we thus conclude that ISO is a pro-drug activated by oxidation reactions catalyzed by EthA. Amongst the reaction products, the corresponding carbodiimide is likely to arise from these oxidations and we propose that this is the actual active form of ISO. As for ETH and TAC, it remains to be determined whether the reactive species derived from ISO act via covalent bond formation within the active site(s) of the enzymatic target(s) or through the formation of small adducts inside *M. tuberculosis* cells which would then become the actual inhibitors of these
enzymes. Work is now in progress in our laboratories to characterize the remaining ISO<sup>R</sup> mutants of <i>M. tuberculosis</i> that were isolated in the course of this study and to identify the enzymatic targets of the drug.

References


system permitting the efficient purification of a recombinant antigen from


   http://www.who.int/mediacentre/factsheets/fs104/en/print.html


Acknowledgements

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Figure legends

Figure 1: The proposed activation process of ISO

Figure 2: Effect of EthA on the activity of ISO in cell free assays.

A. Mycolic acid synthesis by *M. bovis* BCG cell free extracts. The incorporation of [1,2-\(^{14}\)C]acetate into the mycolic acids of *M. bovis* BCG sonicates incubated in the presence of different concentrations of ISO and in the presence (+) or absence (-) of partially purified EthA (10 \(\mu\)g) is shown. Mycolic acid methyl esters (MAMEs) and fatty acid methyl esters (FAMEs) were analyzed by TLC followed by autoradiography as described under Materials and Methods.

B. *In vitro* synthesis of unsaturated short-chain fatty acids. The activity of DesA3 was monitored in the presence or absence of ISO and EthA in a reaction containing *M. smegmatis* membranes, partially purified DesA3, palmitoyl-CoA, and NADPH. Fatty acids were extracted from the reaction mixtures, derivatized and analyzed by GC. Their relative percentages in the assay mixtures are shown. C16:0, palmitic acid; C16:1, palmitoleic acid; C18:1, oleic acid; C18:0, stearic acid; C19, tuberculostearic acid.
Table 1: MIC values (µg/ml) of ISO against *M. tuberculosis* H37Rv and Mt103, the complemented and non-complemented ISO^R^ mutants and *M. tuberculosis* H37Rv overexpressing ethA and ethR in 7H9 medium supplemented with ADC.

<table>
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<th>pVV16</th>
<th>pVVethA</th>
<th>pVVethR</th>
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<tbody>
<tr>
<td><em>M. tuberculosis</em> H37Rv</td>
<td>5-10</td>
<td>2.5</td>
<td>&gt; 40*</td>
</tr>
<tr>
<td><em>M. tuberculosis</em> Mt103</td>
<td>5</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td><em>M. tuberculosis</em> MT1K ISO-R22</td>
<td>&gt; 40*</td>
<td>5-10</td>
<td>nd</td>
</tr>
<tr>
<td><em>M. tuberculosis</em> MT1K ISO-R33</td>
<td>&gt; 40*</td>
<td>5-10</td>
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nd, not determined

* For solubility reasons, 40 µg/ml was the highest concentration of ISO that could be tested
Figure 1

ISO  
\( M = 400 \)

\[
\begin{align*}
&\text{R} \\
&\text{N} \quad \text{H} \quad \text{N} \\
&\text{S} \quad \text{OH} \\
&\text{R} \\
\end{align*}
\]

\( 1 \) (\( M = 416 \))

\[
\begin{align*}
&\text{R} \\
&\text{N} \quad \text{H} \quad \text{N} \\
&\text{S} \quad \text{OH} \\
&\text{R} \\
\end{align*}
\]

\( 2 \)

\[
\begin{align*}
&\text{R} \\
&\text{N} \quad \text{H} \quad \text{N} \\
&\text{S} \quad \text{OH} \\
&\text{R} \\
\end{align*}
\]

\( 3 \) (\( M = 368 \))

\[
\begin{align*}
&\text{R} \\
&\text{N} \quad \text{H} \quad \text{N} \\
&\text{S} \quad \text{OH} \\
&\text{R} \\
\end{align*}
\]

\( 4 \)

\[
\begin{align*}
&\text{R} \\
&\text{N} \quad \text{H} \quad \text{N} \\
&\text{S} \quad \text{OH} \\
&\text{R} \\
\end{align*}
\]

\( 5 \) (\( M = 384 \))

\[
\begin{align*}
&\text{R} \\
&\text{N} \quad \text{H} \quad \text{N} \\
&\text{O} \quad \text{H}_2 \text{O} \\
&\text{R} \\
\end{align*}
\]

Nota: \( R = \text{OCH}_2\text{CH}_2\text{CH(Me)}_2 \)
### Table

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<th>Condition</th>
<th>C16 :1</th>
<th>C16 :0</th>
<th>C18 :1</th>
<th>C18 :0</th>
<th>C19</th>
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<tbody>
<tr>
<td>Membranes alone</td>
<td>0</td>
<td>55.7</td>
<td>0.6</td>
<td>0.9</td>
<td>42.8</td>
</tr>
<tr>
<td>Membranes + DesA3</td>
<td>3.9</td>
<td>49.0</td>
<td>6.6</td>
<td>0.9</td>
<td>39.6</td>
</tr>
<tr>
<td>Membranes + DesA3 + ISO</td>
<td>0.1</td>
<td>54.1</td>
<td>0.1</td>
<td>1.0</td>
<td>44.7</td>
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<tr>
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<td>53.5</td>
<td>0.9</td>
<td>1.0</td>
<td>44.6</td>
</tr>
<tr>
<td>Membranes + DesA3 + EthA</td>
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<td>49.6</td>
<td>5.2</td>
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<td>41.4</td>
</tr>
<tr>
<td>Membranes + DesA3 + ISO + EthA</td>
<td>1.9</td>
<td>49.8</td>
<td>4.4</td>
<td>1.1</td>
<td>42.8</td>
</tr>
</tbody>
</table>

### Figure 2

**A.**

![FAMEs and MAMEs diagram](image)

- **FAMEs**
  - Alpha
  - Keto

- **MAMEs**

**B.**

<table>
<thead>
<tr>
<th>ISO (µg/ml)</th>
<th>EthA</th>
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<tbody>
<tr>
<td>0</td>
<td>-</td>
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<tr>
<td>2</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>+</td>
</tr>
</tbody>
</table>

**Table Legend:**
- **EthA:** - - - + + +