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Ester-prodrugs of ethambutol control its antibacterial activity and provide rapid screening for mycobacterial hydrolase activity

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Abstract

M. tuberculosis, contains an unusually high number of serine hydrolases by proteome percentage compared to other common bacteria or humans. This letter describes a method to probe the global substrate specificity of mycobacterial serine hydrolases with ester-protected prodrugs of ethambutol, a first-line antibiotic treatment for TB. These compounds were synthesized directly from ethambutol using a selective *o*-acylation to yield products in high yield and purity with minimal workup. A library of derivatives was screened against *M. smegmatis*, a non-infectious model for *M. tuberculosis*, which displayed significantly lowered biological activity compared to ethambutol. Incubation with a general serine hydrolase reactivated each derivative to near-ethambutol levels, demonstrating that esterification of ethambutol should provide a simple screen for mycobacterial hydrolase activity.

Graphical abstract

Keywords

Hydrolases; *M. tuberculosis*; Prodrugs; Antibiotics; Tuberculosis; ethambutol

Tuberculosis (TB) is the deadliest infectious disease worldwide.¹ The complex bioavailability and cell permeability of *Mycobacterium tuberculosis*, the causative agent of TB, complicates traditional drug discovery pathways.² Among the methods used to overcome the impermeability of *M. tuberculosis* is the utilization of prodrugs, which can be designed with the proper polarity balance for high cell permeability before being activated

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by internal enzymes.³ TB prodrugs have additional advantages, including trapping the activated drug within the mycobacterial cell⁴ and introducing additional disease specificity.⁵ Pyrazinamide and isoniazid, two frontline medications for TB, are both prodrugs that are metabolized by *M. tuberculosis* enzymes as part of their respective mechanisms of action (Figure 1).^{6, 7} The threat posed by TB has led to considerable investment into prodrug strategies,⁵ and several novel TB treatments, such as lansoprazole⁸ and pretomanid (formerly PA-824)⁹, are currently in clinical development.

A classic strategy for prodrug development is the conversion of alcohol or carboxylic acid functionalities, which can limit cell permeability, into less-polar ester groups.³ Ester-based prodrugs increase the oral bioavailability of TB therapeutics in preclinical studies, and potentially offer additional benefits over other prodrug strategies. For example, pyrazinoic acid esters have been found to be effective against pyrazinamide-resistant strains of *M. tuberculosis*,^{10–14} with some compounds demonstrating dual effects through inhibition by both the masked drug and the masking group itself.¹⁵ A drawback to ester-prodrugs is the assumption that nonspecific human hydrolases will remove any ester protecting groups prior to cell internalization, removing the additional therapeutic selectivity of the ester-prodrug. Certain simple ester-protected derivatives of molecular probes are, however, resistant to activation by a broad range of mammalian hydrolases, suggesting the potential for orthogonal ester masking groups.¹⁶ *M. tuberculosis* contains a significantly higher number of serine hydrolases (186 predicted serine hydrolases; ~4% of the proteome)¹⁷ by proteome percentage than other common bacteria¹⁸ or even humans (>200 predicted serine hydrolases; ~1% of the proteome).^{19, 20} We hypothesized that this large, diverse family of mycobacterial serine hydrolases might encode orthogonal ester reactivity for endowing mycobacterial specificity into ester-based prodrugs.

As a proof of principle for a TB-specific prodrug, we chose the simple antibiotic ethambutol. Ethambutol is a bacteriostatic, antimycobacterial drug that has been part of front-line TB treatment since the 1960s. While effective, ethambutol usage can present with several serious side effects, which include hepatotoxicity²¹ and ocular neuritis.²² SAR studies undertaken to ameliorate these effects have found ethambutol to be a poor lead structure for analogues. In particular, modification of its alcohol functionalities, including to a methyl ester, completely abolished antimycobacterial activity.²³ Thus, ester-protection of ethambutol should create a simple screen for mycobacterial hydrolase activity where the protected version is nontoxic, but the activated version has TB specific activity (Figure 1). In addition to serving as a useful tool for evaluating hydrolase activity, ester prodrug derivatives of ethambutol could be a novel strategy for decreasing in vivo toxicity. Herein, we report the synthesis and biological evaluation of a series of acylated ethambutol derivatives.

With the goal of probing global serine hydrolase activity in *Mycobacteria*, compounds **3a–h** were chosen for the initial library to test simple ester features (Figure 2). Branching patterns (**3f**, **3g**, and **3h**) were chosen to mimic the complex mycolic acids adorning the outside of the mycobacterial cell wall.^{24, 25} Tertiary ester functionalities (**3d** and **3e**) were also added, as these esters were not hydrolyzed in many mammalian cell types.^{16, 26} Double-protected derivatives were also targeted over single-protected variants, as they were found to be more

stable and compatible with cellular activation. Compounds were prepared from commercially-available (S,S)-ethambutol dihydrochloride **1** and a corresponding acid chloride as outlined in Figure 2, following a procedure developed for selective *O*-acylation of amino alcohols.²⁷ Acid chlorides were either commercially sourced or prepared from the precursor carboxylic acid using oxalyl chloride in dichloromethane and catalytic DMF. Ethambutol derivatives were prepared by adding the desired acid chloride to a solution of **1** dissolved in trifluoroacetic acid, which was stirred at room temperature for ten minutes. Diethyl ether was added to the mixture to precipitate the products as hydrochloride salts, which were collected using vacuum filtration. These proved to be soluble in CDCl₃, which allowed for NMR characterization without conversion to the free base. IR spectroscopy of products showed the disappearance of the ethambutol O-H stretch at 3300 cm⁻¹ while NMR indicated the presence of ammonium protons, demonstrating that ester products were synthesized instead of amides (see Supplemental Information).

Compounds **3a–h** were screened for their ability to inhibit the growth of *M. smegmatis*, a non-infectious model mycobacterium for TB infection. *M. smegmatis* has a high conservation of serine hydrolases with *M. tuberculosis*, including 22/24 Lip family hydrolases, all cutinases, and other hydrolases identified as therapeutic targets.^{28–30} Initial screening was performed using disc diffusion measurements (Figure 3). By comparing the relative sizes of the inhibition zones across each compounds, disc diffusion provided a qualitative assessment of the relative MIC values of ester prodrugs in comparison to ethambutol. As expected, ester protection significantly decreased the toxicity of ethambutol, with significantly higher concentrations of compounds **3b** (25 µg/mL), **3e** (6.25 µg/mL), and **3h** (3.125 µg/mL) required for similar zones of inhibition as **1** (0.39 µg/mL). Levels of inhibition, however, varied across ester-protected derivatives, with **3h** showing significantly greater inhibition zones across all concentrations. Matching with previous observations about the high stability of methyl cyclopropyl esters,¹⁶ compound **3e** showed the lowest relative toxicity.

To quantitate the relative activation of these prodrugs and the dependence of this activation on serine hydrolase activity, values for MIC₉₉ were determined for compounds **3a–h** under a variety of conditions (Table 1 and Figure 4). For each compound, the MIC₉₉ for *M. smegmatis* was compared with the MIC₉₉ after pre-incubation of the compounds with pig liver esterase (PLE), a general serine hydrolase, to confirm that the prodrugs could be activated by serine hydrolases. If compounds were fully activated, MIC₉₉ values +PLE should be comparable to the MIC₉₉ value for the parent compound **1**. Additionally, pre-incubation of each compound with PLE in tandem with the general serine hydrolase inhibitor, phenylmethylsulfonyl fluoride (PMSF), was performed to demonstrate that ester-prodrug activation was dependent on the serine hydrolase activity of PLE rather than nonspecific hydrolysis. If ester activation was dependent on the serine hydrolase activity of PLE, the MIC₉₉ values for +PLE +PMSF should be comparable to the ester-protected compound.

Similar to the measurements with the disc diffusion, ester protection of ethambutol increased the MIC₉₉ values from compound **1** between 8- and 130-fold (Table 1). The methyl cyclopropyl ester (**3e**) again showed the greatest stability and least toxicity with an MIC₉₉

value at or close to the solubility limit for this compound (Figure 4), whereas the propyl ester (**3a**) showed the greatest activation. This profile matches the relative stability of other carboxylic ester libraries where small, unbranched esters like **3a** were activated in a variety of cell types and conditions, but tertiary esters like **3d** and **3e** required either specialized enzymes¹⁶ or enzyme engineering^{31, 32} to accomplish these reactions. Relative activation varied 16-fold across all ester-protected derivatives, suggesting differential activation by mycobacterial serine hydrolases.

Pre-incubation with PLE to confirm serine hydrolase-dependent activation of the ester prodrugs found that each compound was activated by PLE either to nearly equivalent MIC₉₉ values with **1** (Figure 4A) or within 8-fold of **1** for the stable tertiary esters (Figure 4B). Propyl ester **3a** showed no further activation, suggesting that this compound was already fully activated either by mycobacterial hydrolases or was inherently hydrolytically unstable. Pre-incubation of PLE along with PMSF (+PLE +PMSF) decreased biological activity compared to that of PLE alone, confirming that ester prodrug activation was dependent on serine hydrolase activity (Table 1 and Figure 4). To determine any potential contributions from nonspecific hydrolysis, each compound was dissolved in water and Middlebrook media and incubated at 37°C for 10 days, using comparative TLC to track degradation. After 72h, no ethambutol was observed in either medium (Figure S1), although **3a**, **3b**, **3c** and **3g** showed some breakdown in Middlebrook based on the disappearance of the parent compound. Degradation products only began appearing in compounds **3a**, **3b**, **3c** and **3f** in water after a full 10 days of exposure (Figure S2). Additionally, the observed TLC changes suggest that only one of the two ester functionalities is being hydrolyzed rather than reverting completely to ethambutol, which may also explain the observed incomplete PLE activation of all derivatives. This high hydrolytic stability strongly implicates differences in mycobacterial hydrolase activity in the shifted MIC values of the ester prodrugs.

Overall, the esterified derivatives of ethambutol displayed significantly lowered biological activity towards *M. smegmatis* compared to the parent compound, confirming that the alcohol is necessary for the biological activity of ethambutol. The MIC₉₉ values were also dependent on the structure of the ester functionality, with **3a** and **3h** displaying the greatest activity. Incubation of each compound with PLE also resulted in the restoration of biological activity, demonstrating that the ester functionality can be cleaved by a broad-spectrum serine hydrolase. Thus, esterification of ethambutol successfully attenuates its biological activity while remaining susceptible to activation by serine hydrolase, creating a simple screen for determining the substrate specificity of mycobacterial hydrolases. Ester-protected ethambutol derivatives were easily synthesized and their susceptibility to hydrolases such as PLE makes them excellent biological probes for mycobacteria. Building on this proof of principle, efforts to expand this library and explore the specificity of mycobacterial hydrolases are ongoing.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- A library of ester-prodrugs of ethambutol was synthesized
- Selective o-acylation was used to generate derivatives in one step
- Esterified derivatives were deactivated relative to ethambutol
- Incubation with a general serine hydrolase restored ethambutol-like activity

Figure 1.

Tuberculosis prodrugs and their activated forms. Isoniazid is converted to the acyl radical by KatG , which reacts with NAD(H) to form the prodrug complex. Pyrazinamide is converted to the active pyrazinoic acid by reaction with pyrazinamidase. We proposed that ester-protected prodrugs of ethambutol would regulate its native antibacterial activity and provide a simple screen for mycobacterial hydrolase activity.

Figure 2.

Esterified derivatives of ethambutol. All reactions were run at RT for 15 minutes with stirring, before cooling to 0°C and adding Et₂O to precipitate products as hydrochloride salts.

Figure 3. Disc diffusion measurements for ethambutol and selected derivatives in Middlebrook agar media against *M. smegmatis*. Derivatives were serially diluted from 25 µg/mL to 0.781 µg/mL, while ethambutol was diluted from 0.391 µg/mL to 0.0244 µg/mL. Inhibition was measured after two days of growth at 37°C.

Figure 4.

Dose-dependency curves for branched (3h, A) and tertiary (3e, B) ethambutol derivatives, along with PLE treatment (middle) and PLE + PMSF (right). Derivatives were serially diluted in Middlebrook media and further diluted 1:1 with *M. smegmatis* in Middlebrook (final concentrations 25 $\mu\text{g/mL}$ to 0.012 $\mu\text{g/mL}$). Pig liver esterase treatment was performed at the highest dilution (50 $\mu\text{g/mL}$) by incubating with PLE (0.1 mg/mL) or PLE + PMSF (1 mM) for 1h at RT. Plates were incubated at 37°C for 3 days, and cell growth was measured at A_{600} .

Table 1MIC₉₉ for ester-protected ethambutol derivatives.

Compound	MIC ₉₉ (µg/mL)*	MIC ₉₉ + PLE (µg/mL)	MIC ₉₉ + PLE + PMSF (µg/mL)
1	0.098	0.098	0.098
3a	0.78	0.78	0.78
3b	3.1	0.39	6.2
3c	6.3	0.098	1.6
3d	6.3	0.78	6.3
3e	13	0.78	25
3f	6.3	0.39	6.3
3g	6.3	0.20	0.39
3h	1.6	0.20	0.78

* MIC₉₉ was measured against *M. smegmatis*. The highest concentration sample in the MIC₉₉ dilutions was preincubated with PMSF (1mM), PLE (0.1 mg/mL), or the combination of PLE and PMSF for 1 hour at RT before dilution. The A₆₀₀ of the growth plates were measured after 72 h of culture growth at 37 °C and 225 rpm.