

# Dual Inhibition of Mycobacterial Fatty Acid Biosynthesis and Degradation by 2-Alkynoic Acids

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## Summary

2-Hexadecynoic acid and 2-octadecynoic acid have cidal activity against *Mycobacterium smegmatis* and *Mycobacterium bovis* BCG. At subinhibitory concentrations, *M. smegmatis* rapidly transformed [<sup>1-14</sup>C]-2-hexadecynoic acid into endogenous fatty acids and elongated them into mycolic acids. Toxic concentrations of 2-hexadecynoic acid resulted in accumulation of 3-ketohexadecanoic acid, which blocked fatty acid biosynthesis, and 3-hexadecynoic acid, an inhibitor of fatty acid degradation. The combination of these two metabolites is necessary to achieve the inhibition of *M. smegmatis*. We conclude that 2- and 3-hexa/octadecynoic acids inhibit mycolic acid biosynthesis, fatty acid biosynthesis, and fatty acid degradation, pathways of significant importance for mycobacteria.

## Introduction

Drug-resistant tuberculosis (TB) is a worldwide problem in both human immunodeficiency virus-infected and immunocompetent populations [1]. Multidrug-resistant *Mycobacterium tuberculosis* strains are resistant to several first-line antituberculosis drugs, particularly isoniazid (INH) and rifampin [2]. Such strains are more difficult and expensive to treat than drug-susceptible TB, and they are more likely to be fatal, especially when diagnosis and appropriate therapy are delayed [3, 4]. It is clear

that new, effective antituberculosis drugs are urgently needed. Since its introduction in 1952, INH has been the most effective antituberculosis drug for both the treatment and prophylaxis of tuberculosis [5, 6]. Early studies indicated that INH kills mycobacteria by inhibiting mycolic acid biosynthesis [7]. Genetic studies determined that the target for INH is *InhA* [8], an enoyl-ACP reductase that catalyzes the NADH-specific reduction of 2-*trans*-enoyl-ACP [9]. *InhA* inhibition by INH or loss of function in an *inhA* temperature-sensitive mutant leads to mycobacterial cell death [10]. Altogether, these results make *InhA* an attractive target for further drug design. Based on a structural model of *InhA* with an acyl substrate [11], and the knowledge that this enzyme preferentially reduces long-chain substrates (16–24 carbons) [9], we reasoned that substrate analogs, such as long-chain 2-alkynoic acids, might competitively inhibit *InhA* activity and consequently lead to mycobacterial cell death. In this report, we present a study of the metabolism and toxic effect of 2-alkynoic acids in *M. smegmatis*.

## Results

### The Activity of 2-Alkynoic Acids Depends on Chain Length and Triple Bond Position

Previous work has shown that the enoyl-ACP reductase of the type II fatty acid synthase (FASII) system, an enzyme encoded by the *inhA* gene [9, 12], involved in mycolic acid biosynthesis and INH resistance in mycobacteria, is essential [10, 13]. In an effort to develop new antimycobacterial drugs, we synthesized 2-alkynoic acids, substrate analogs of *InhA*, which could presumably act as competitive inhibitors of the enzyme. As an initial screen for antimicrobial activity, 2-alkynoic acids of varying chain lengths (from 12 to 24 carbon atoms) were tested against the fast-growing, nonpathogenic *Mycobacterium smegmatis* and *Corynebacterium glutamicum*, a related actinomycete. *M. smegmatis* was inhibited by 2-hexadecynoic acid (2-HA), 2-octadecynoic acid (2-OA), and 2-nonadecynoic acid, with minimum inhibitory concentration (MIC) values of 2.5 µg/ml, 1 µg/ml, and 10 µg/ml (10, 4, and 35 µM), respectively. Notably, 2-HA and 2-OA activities against *M. smegmatis* are 4- and 10-fold higher than the activity of INH, a first-line antituberculosis drug (Table 1). The most active compounds, 2-HA and 2-OA, were also active against the slow grower *M. bovis* BCG, with MIC values of 4 µM and 9 µM, respectively (Table 1). Surprisingly, 2-HA was more active than 2-OA against *M. bovis* BCG. This trend was also observed against the slow-growing pathogenic *M. tuberculosis* H37Rv, with MICs for 2-HA and 2-OA of 20 µM and 25 µM, respectively. *C. glutamicum* was sensitive to 2-tetradecynoic acid and 2-HA, with MIC values of 66 µM and 30 µM, respectively (Table 1).

The effect of structural analogs of 2-HA/OA, the most potent compounds against *M. smegmatis*, was also studied by measuring the MIC values of isomers of HA and OA with a triple bond at positions 2, 3, 4, or 5. The

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Table 1. Minimum Inhibitory Concentration,  $\mu\text{M}$ , for Alkynoic Acids, Alkenoic Acids, and Isoniazid against *M. smegmatis*, *C. glutamicum*, and *M. bovis* BCG

Compound	Structure	<i>M. smegmatis</i> mc <sup>2</sup> 155	<i>M. bovis</i> BCG	<i>C. glutamicum</i> ATCC13032
2-Dodecynoic acid		>300	nd	>300
2-Tetradecynoic acid		268	nd	66
2-Hexadecynoic acid		10	4	30
3-Hexadecynoic acid		20	nd	nd
4-Hexadecynoic acid		30	nd	nd
5-Hexadecynoic acid		80	nd	nd
2-Octadecynoic acid		4	9	107
3-Octadecynoic acid		36	nd	nd
4-Octadecynoic acid		>360	nd	nd
5-Octadecynoic acid		360	nd	nd
2-Nonadecynoic acid		35	nd	204
2-Eicosynoic acid		>300	nd	nd
2-Docosynoic acid		>300	nd	nd
Isoniazid		40	0.7	>420
2- <i>trans</i> -Octadecenoic acid		>200	nd	>200
9- <i>cis</i> -Octadecenoic acid		>200	nd	>200

nd = not determined.

inhibitory action was found to decrease when the triple bond was located at a more distal position. Whereas 3-OA showed MIC values 10-fold higher than those for 2-OA, both 4-OA and 5-OA were almost inactive against *M. smegmatis* (Table 1). In contrast, 3- and 4-HA displayed MIC values only 2- to 3-fold higher than that of 2-HA, whereas 5-HA needed an 8-fold increase in its concentration to cause growth inhibition. Furthermore, 2-*trans*-octadecenoic acid, a long-chain fatty acid with 18 carbons like 2-OA, but a double bond instead of a triple bond at position 2, had no antimycobacterial activity against *M. smegmatis*, indicating that the triple bond is essential for the activity of these compounds (Table 1). Thus, comparison of the structure-function activity displayed by alkynoic acid analogs clearly shows that the inhibitory activity of this class of compounds depends on both the chain length and the presence and position of the triple bond.

#### The Activity of 2-Alkynoic Acids Is Not Due to Nonspecific Fatty Acid-Mediated Lysis

The effect of 2-alkynoic acids on mycobacteria was studied by growing cultures of *M. bovis* BCG or *M. smegmatis* in the presence of increasing amounts of 2-alkynoic acids. In *M. bovis* BCG, treatment with 2.5  $\mu\text{g/ml}$  2-HA or 2-OA resulted in a 3-log drop in col-

ony-forming units (CFU)/ml after 3 days and a 4-log drop in CFU/ml after 7 days (Figure 1A). In contrast, in *M. smegmatis*, growth and cell viability were not affected by low concentrations of 2-HA/OA (10 or 25  $\mu\text{g/ml}$ ), but high concentrations of 2-HA and 2-OA (50  $\mu\text{g/ml}$ ) resulted in a 3- and 6-log decrease in the viable counts over the course of one generation (Figure 1B) followed by cell lysis. Interestingly, 3-HA required a longer time to affect cell viability even at 50  $\mu\text{g/ml}$ , causing a 4-log drop in CFUs after 7 hr, while 3-OA (at 50  $\mu\text{g/ml}$ ) failed to significantly affect viability (Figure 1B). The results are consistent with the MIC data (Table 1), suggesting that the potency of these compounds is in the order 2-OA > 2-HA > 3-HA > 3-OA. Treatment of *M. smegmatis* with palmitic acid (at 50  $\mu\text{g/ml}$ ) had no effect on growth and cell viability. Since the concentration of 2-HA required for growth inhibition was 10- to 20-fold higher than the MIC determined in solid medium, we studied the role of the inoculum size on the inhibitory effect of 2-HA/OA in liquid medium. Low concentrations of 2-HA/OA (1–2  $\mu\text{g/ml}$ ) inhibited growth of the mycobacterial cultures started from small inoculum (10<sup>5</sup> CFU/ml), corresponding to the inoculum size used to determine the MIC on solid media. In contrast, cultures started from a larger inoculum size (10<sup>7</sup> CFU/ml) required a higher concentration of compounds (up to 50  $\mu\text{g/ml}$ ) to display

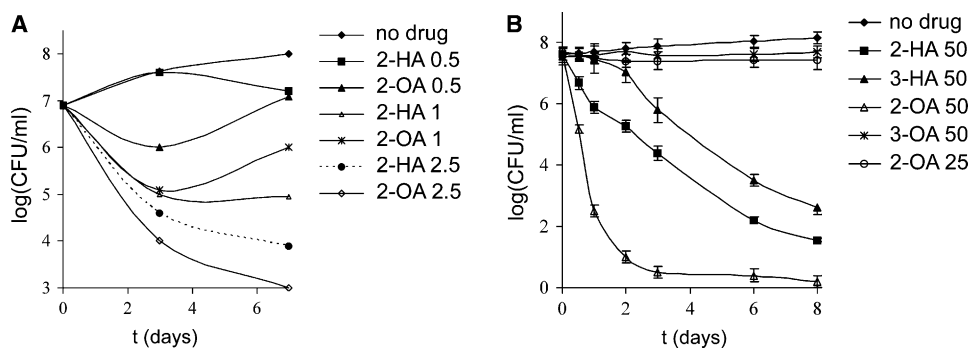


Figure 1. Effect of Hexadecynoic and Octadecynoic Acids on the Growth of *M. smegmatis*

(A and B) Early-log phase cultures of (A) *M. bovis* BCG and (B) *M. smegmatis* mc<sup>2</sup>155 were treated with DMSO solutions of 2-HA/OA (0.5–50 µg/ml) and 3-HA/OA (50 µg/ml) for *M. smegmatis*. The addition of DMSO did not affect culture viability. Aliquots were taken, diluted, and plated. CFUs were counted after 4 days of incubation for *M. smegmatis* or 3 weeks of incubation for *M. bovis* BCG at 37°C. For clarity, the data obtained for *M. smegmatis* treated with 2-HA (10 µg/ml and 25 µg/ml) and 2-OA (10 µg/ml) are not shown since they overlap with 2-OA (25 µg/ml). The data in (B) are representative of three independent experiments.

a comparable biological activity. Taken together, these results suggest that alkynoic acids do not merely disrupt the cell membrane by physical interaction, but act on *M. smegmatis* through a specific mechanism.

#### Activity of 2-Alkynoic Acids against the Enoyl-ACP Reductase InhA

Preliminary experiments examined the ability of long-chain 2-alkynoic acids to act as competitive inhibitors of InhA in vitro. The results obtained indicated that 2-HA and 2-OA, as their corresponding Coenzyme A thioesters, acted as linear competitive inhibitors versus 2-*trans*-dodecenoyl-CoA with  $K_{is} = 2.1 \pm 0.6$  µM (A. Quémard, personal communication), resulting in inhibition of mycolic acid biosynthesis [12].

Drugs that specifically inhibit InhA, such as INH, ethionamide, triclosan, or diazaborine, induce the formation of a 80 kDa protein consisting of a covalent complex containing the β-ketoacyl-ACP synthase KasA and the mycobacterial acyl carrier protein AcpM [14]. Immunoblot analysis with anti-KasA antibodies of *M. smegmatis* cells treated with INH (5 µg/ml) revealed the induction of this complex, in agreement with our previous observations (Figure 2). In order to assess whether *inhA* may also be an in vivo target for 2-HA and 2-OA, *M. smegmatis* cells were treated with increasing concentrations of 2-HA or 2-OA (1–50 µg/ml) for 30 min and were analyzed for the induction of the 80 kDa complex (Figure 2). Even the lowest concentration of 2-HA or 2-OA tested resulted in the formation of this complex, indicating that both compounds inhibit InhA. Similar results were obtained when cultures were treated for a longer period of time (6 hr) with both compounds (data not shown). Next, we determined the MIC values of 2-HA and 2-OA on *M. smegmatis* mc<sup>2</sup>2399, a strain bearing a multicopy plasmid in which the *inhA* gene was placed under the control of the *hsp60* promoter [15]. Since overexpression of a primary drug target leads to resistance via titration of the drug, we hypothesized that overproduction of InhA in this strain would confer enhanced resistance to 2-HA and 2-OA. Surprisingly, a low-level resistance to 2-HA and 2-OA was observed in mc<sup>2</sup>2399. The MICs against mc<sup>2</sup>2399 for 2-HA and 2-OA were 32 µM and

7 µM, respectively (a 3-fold and 2-fold increase, with respect to wild-type *M. smegmatis*), while resistance to INH, a specific inhibitor of InhA, increased up to 20-fold [15]. Thus, these results indicate that InhA is not the primary target in vivo of 2-alkynoic acids and suggest the existence of additional target(s).

#### Fatty Acid and Mycolic Acid Biosynthesis in Mycobacteria Is Inhibited by 2-Alkynoic Acids

Mycobacteria have a complex system for the synthesis of fatty acids, with prokaryotic (FASII)- and eukaryotic (FASI)-like fatty acid synthetases [16]. FASII carries on the synthesis of fatty acids (mainly C16:0 and C24:0), while FASII elongates C16:0 to long-chain fatty acids, which are the precursors of mycolic acids [16, 17]. To understand at which level the inhibition by 2-alkynoic acids takes place, the effect of increasing concentrations of 2-HA and 2-OA on the de novo synthesis of fatty

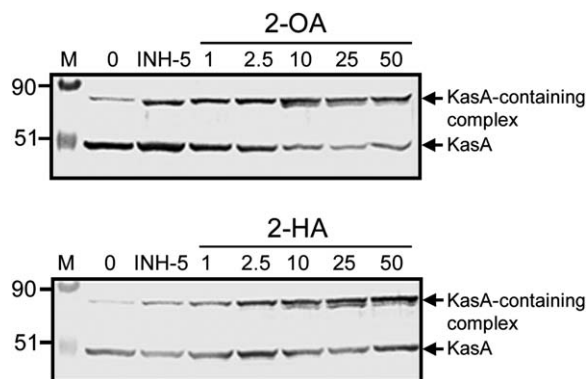
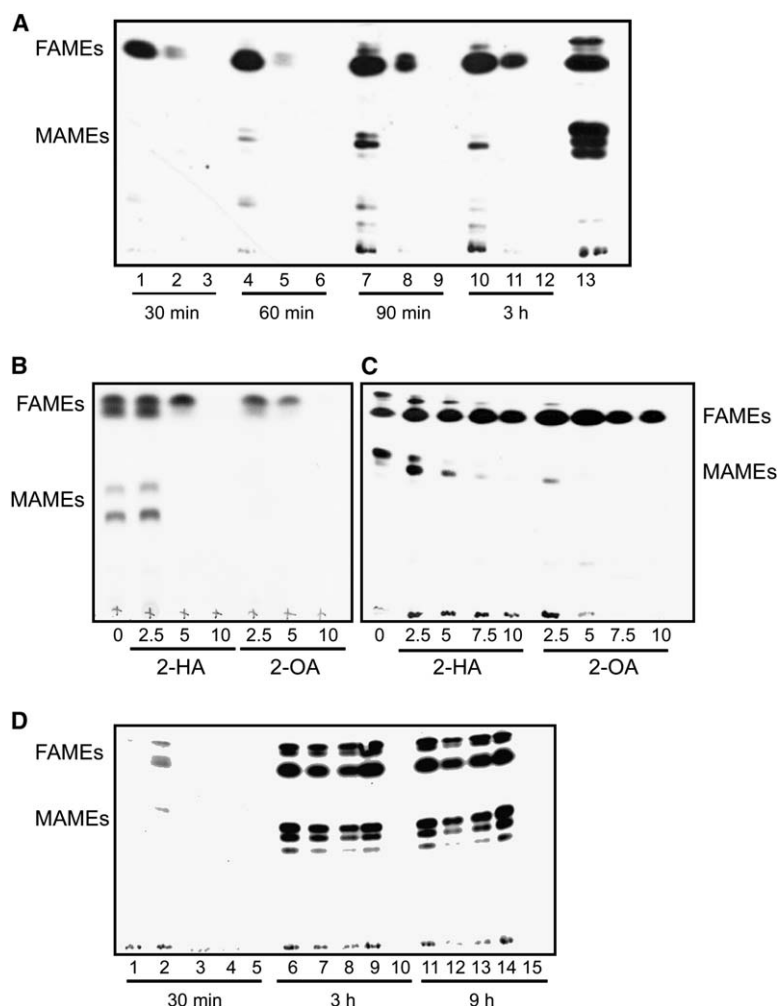


Figure 2. 2-HA and 2-OA Induce a KasA-Containing Complex

Mid-log phase cultures of *M. smegmatis* were treated with increasing concentrations of 2-HA (2.5–50 µg/ml) and 2-OA (1–50 µg/ml) for 30 min. INH (5 µg/ml) was used as a control for InhA inhibition. Crude lysates were loaded on a 12% acrylamide gel and transferred to a membrane for immunoblot analysis. For the detection of unbound KasA and the KasA-containing complex, the membranes were probed with rat anti-KasA antibodies and then incubated with anti-rat antibodies conjugated to alkaline phosphatase prior to detection.



**Figure 3. Dose-Response Effects of 2-HA, 3-HA, 2-OA, and 3-KHA on Fatty Acid and Mycolic Acid Biosynthesis**

(A) The effect of 2-HA on the incorporation of [ $^{14}\text{C}$ ]-acetate was assayed by labeling *M. smegmatis* in the presence of increasing concentrations of 2-HA (10  $\mu\text{g/ml}$  [lanes 1, 4, 7, and 10]; 25  $\mu\text{g/ml}$  [lanes 2, 5, 8, and 11], or 50  $\mu\text{g/ml}$  [lanes 3, 6, 9, and 12]) or in the absence of drug (lane 13). 10% of total counts were loaded in each lane.

(B) Mid-log phase cultures of *M. bovis* BCG were treated with 2-HA or 2-OA (0, 2.5, 5, and 10  $\mu\text{g/ml}$ ) and labeled with [ $^{14}\text{C}$ ]-acetate.

(C) Mid-log phase *M. smegmatis* cultures were treated with low concentrations of 2-HA or 2-OA and labeled with [ $^{14}\text{C}$ ]-acetate.

(D) Log-phase cultures of *M. smegmatis* were treated with 3-KHA (50  $\mu\text{g/ml}$ , lanes 1, 6, and 11), 3-HA (10  $\mu\text{g/ml}$ , lanes 2, 7, and 12; or 25  $\mu\text{g/ml}$ , lanes 3, 8, and 13), or a combination of 3-KHA (40  $\mu\text{g/ml}$ ) and 3-HA (10  $\mu\text{g/ml}$  [lanes 4, 9, and 14] or 25  $\mu\text{g/ml}$  [lanes 5, 10, and 15]) and labeled with [ $^{14}\text{C}$ ]-acetate. Fatty acid methyl esters (FAMES) and mycolic acid methyl esters (MAMES) were extracted, analyzed, and detected by autoradiography as described in [Experimental Procedures](#).

acids was studied. Mid-log phase cultures of *M. smegmatis* were grown in the presence of increasing amounts of 2-alkynoic acids and were labeled with [ $^{14}\text{C}$ ]-acetate. Fatty acids and mycolic acids were extracted and analyzed by TLC and HPLC. De novo biosynthesis of mycolic acids in cultures treated with either 2-HA or 2-OA (10, 25, or 50  $\mu\text{g/ml}$ ) was inhibited as early as 30 min after the addition of the compounds ([Figure 3A](#)). In parallel, the synthesis of long-chain, nonhydroxylated fatty acids was also severely diminished ([Figure 3A](#)). Increasing the concentration of 2-HA to 25  $\mu\text{g/ml}$  interfered with the de novo synthesis of fatty acids carried on by the FASI system, causing a decrease of 80% in the total incorporation of acetate into fatty acids. At 50  $\mu\text{g/ml}$ , the inhibition of fatty acid synthesis was complete ([Figures 3A and 4A](#)). The inhibition of the synthesis of both fatty acids and mycolic acids continued unaltered for up to 3 hr posttreatment for the higher concentrations of 2-alkynoic acids. However, at 10  $\mu\text{g/ml}$ , a partial restoration of mycolic acid biosynthesis was observed after 90 min of treatment ([Figure 3A](#)). This transient inhibition observed at 10  $\mu\text{g/ml}$  is in agreement with the small effect that this concentration has on the growth of the culture. Treatment of *M. bovis* BCG with increasing concentrations of 2-HA or 2-OA for 12 hr, followed by [ $^{14}\text{C}$ ]-acetate labeling for 1 hr, showed that

mycolic acid biosynthesis is first inhibited at 5  $\mu\text{g/ml}$  for 2-HA and at 2.5  $\mu\text{g/ml}$  for 2-OA. Fatty acid biosynthesis is totally abrogated in the presence of 10  $\mu\text{g/ml}$  of either 2-HA or 2-OA ([Figure 3B](#)).

The synthesis of mycolic acids and fatty acids was also analyzed in *M. smegmatis* cultures treated with subinhibitory concentrations of drug. Under these conditions, synthesis of mycolic acid was inhibited first without affecting synthesis of fatty acids ([Figure 3C](#)), which suggests that 2-alkynoic acids initially inhibit the FASII system. In conclusion, our results show that 2-HA and 2-OA inhibit both FASI and FASII systems in mycobacteria.

#### Alkynoic Acids Are Metabolized into Active Species

Previous studies on the effect of 2-HA on *Streptococcus mutans* showed the presence of 2-HA in the bacterial phospholipids [18]. Although 2-HA is less potent than 2-OA, the fact that [ $^{14}\text{C}$ ]-2-HA, but not [ $^{14}\text{C}$ ]-2-OA, is commercially available prompted us to choose 2-HA as a model by which to study the metabolism of 2-alkynoic acids in mycobacteria. Addition of [ $^{14}\text{C}$ ]-2-HA to *M. smegmatis* cultures yielded labeled mycolic acids, phospholipids, and glycolipids (data not shown). To confirm that these lipids incorporated unmodified 2-HA, the radiolabeled fatty acids extracted from

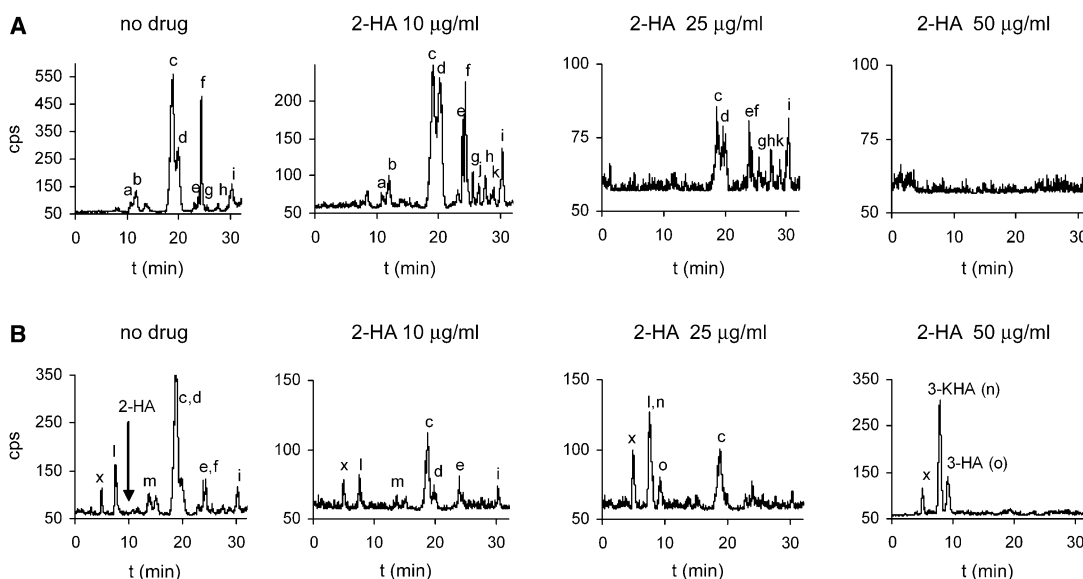


Figure 4. 2-HA Is Metabolized into Active Species that Inhibit Fatty Acid Biosynthesis

(A and B) *M. smegmatis* cultures treated with 0, 10, 25, or 50 µg/ml 2-HA were labeled with (A) [1-<sup>14</sup>C]-acetate or (B) [1-<sup>14</sup>C]-2-HA. [1-<sup>14</sup>C]-Labeled fatty acids were extracted, derivatized to the corresponding *p*-bromophenacyl esters, and analyzed by HPLC. The identification code and retention times for the principal *p*-bromophenacyl esters are as follows: (a) 10.9 min, C14:0; (b) 11.3 min, C16:1 *cis*-Δ<sup>9</sup>; (c) 18.0 min, C16:0; (d) 19.5 min, C18:1 *cis*-Δ<sup>9</sup>; (e) 23.6 min, C18:0; (f) 24.1 min, 10-methyl-C18:0; (g) 25.2 min, C20:0; (h) 27.4 min, C 22:0; (i) 29.8 min, C24:0; (j) 26.5 min, C22:1 *trans*-Δ<sup>2</sup>; (k) 28.8 min, C24:1 *trans*-Δ<sup>2</sup>; (l) 7.3 min, 3-hydroxyhexadecanoic acid; (m) 13.5 min, C16:1 *trans*-Δ<sup>3</sup>; (n) 7.5 min, 3-KHA; (o) 9.0 min, 3-HA; (x) 4.9 min, an unidentified compound.

*M. smegmatis* cultures treated with [1-<sup>14</sup>C]-2-HA were analyzed by HPLC. Surprisingly, instead of detecting [1-<sup>14</sup>C]-2-HA, we observed a rapid (less than 30 min) conversion of the label into endogenous fatty acids (Figure 4B). In order to examine the pathway acting upon 2-HA in mycobacteria, *M. smegmatis* cultures were treated with increasing concentrations of 2-HA for 30 min and labeled with [1-<sup>14</sup>C]-2-HA for another 30 min, and the extracted radiolabeled fatty acids were analyzed by HPLC (Figure 4B). In the absence of 2-HA, most of the label (68% of the total radioactivity) was present as saturated fatty acids (C16:0–C24:0) and oleic acid (11%). Two novel peaks identified as 3-hydroxyhexadecanoic acid (its stereochemistry was not determined) and 3-*trans*-hexadecenoic acid were also detected, representing 10% and 11%, respectively, of the total radioactivity. These two peaks were not present in fatty acids extracted from *M. smegmatis* cultures labeled with [1-<sup>14</sup>C]-acetate (Figure 4A), indicating that they were generated through metabolism of [1-<sup>14</sup>C]-2-HA. A low concentration (10 µg/ml) of 2-HA did not affect the distribution of the label. Increasing the concentration to 25 µg/ml caused a decrease in the amount of radiolabeled saturated (50%) and unsaturated (3-*trans*-hexadecenoic acid [4%], oleic acid [ $< 0.1\%$ ]) fatty acids and an increase in 3-hydroxyhexadecanoic acid (20%). At the same time, two new compounds, 3-ketohexadecanoic acid (3-KHA) (16%) and 3-HA (10%), were observed. When the concentration of 2-HA was raised to 50 µg/ml, the labeled fatty acids consisted of 3-KHA (70%), 3-HA (23%), and an unidentified, more polar compound (7%), with no trace of saturated fatty acids, unsaturated fatty acids, or 3-hydroxyhexadecanoic acid (Figure 4B).

Similar results were obtained when *M. bovis* BCG was treated with 2-HA and labeled with [1-<sup>14</sup>C]-acetate or [1-<sup>14</sup>C]-2-HA. [1-<sup>14</sup>C]-Acetate incorporation into fatty acids was inhibited by the addition of 2-HA, as seen for *M. smegmatis* (Figure 3B), while the metabolites derived from labeled 2-HA were comparable to those detected in *M. smegmatis* cultures.

As noted above, the activity of alkyneic acids decreased when the position of the triple bond was at a more distal position. To test if this reduction in biological activity correlates with a lack of metabolism of the isomeric alkyneic acids, mid-log phase cultures of *M. smegmatis* were treated with 2-, 3-, 4-, or 5-HA for 30 min, and the fatty acids were extracted and analyzed by HPLC. 2-HA and 3-HA were not detected, but 4-HA and 5-HA were found in the fatty acid extracts. The same pattern was observed for 2-, 3-, 4-, and 5-OA, confirming that the shift in the position of the triple bond is linked to the lack of activity. Incorporation of [1-<sup>14</sup>C]-acetate by cultures of *M. smegmatis* treated with 3-HA, 2-OA, or 3-OA showed inhibition of *M. smegmatis* FASI in a manner undistinguishable from that caused by 2-HA, indicating that these compounds also target FASI and FASII. Taken together, these results suggest that alkyneic acids require a specific combination of chain length and position of the triple bond and must be metabolized in order to display biological activity.

#### Both Fatty Acid Degradation and Fatty Acid Biosynthesis Systems Are Involved in 2-HA Metabolism

2-Alkyneic acids incorporated by the mycobacterial cell undergo reduction of the triple bond, yielding saturated



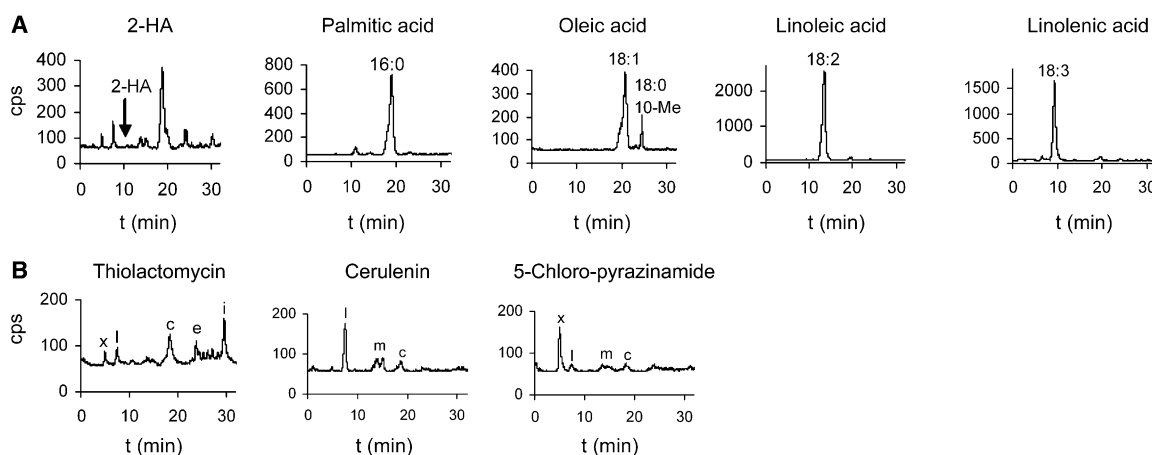


Figure 5. Metabolism of 2-HA Occurs via a Functional FASII System

(A and B) [ $1\text{-}^{14}\text{C}$ ]-Fatty acids obtained from (A) labeling *M. smegmatis* cultures with [ $1\text{-}^{14}\text{C}$ ]-2-HA, [ $1\text{-}^{14}\text{C}$ ]-palmitic acid, [ $1\text{-}^{14}\text{C}$ ]-oleic acid, [ $1\text{-}^{14}\text{C}$ ]-linoleic acid, or [ $1\text{-}^{14}\text{C}$ ]-linolenic acid or (B) after treatment of *M. smegmatis* cultures with cerulenin (5  $\mu\text{g/ml}$ ), 5-Cl-PZA (100  $\mu\text{g/ml}$ ), or TLM (50  $\mu\text{g/ml}$ ), followed by labeling with [ $1\text{-}^{14}\text{C}$ ]-2-HA, were analyzed by HPLC.

fatty acids (mainly palmitic acid) and oleic acid. The fact that labeled fatty acids longer than the added precursor (2-HA) are formed indicates that metabolites derived from 2-HA are substrates for elongation by FASI and FASII systems. The above-mentioned metabolites could arise either by complete  $\beta$ -oxidation of the 2-HA, yielding [ $1\text{-}^{14}\text{C}$ ]-acetate, which would be used in fatty acid biosynthesis, or by a partial modification of 2-HA, yielding primers for FASI. To discriminate between these possibilities, two approaches were undertaken. First, *M. smegmatis* cultures were labeled with [ $1\text{-}^{14}\text{C}$ ]-palmitic acid, [ $1\text{-}^{14}\text{C}$ ]-oleic acid, [ $1\text{-}^{14}\text{C}$ ]-linoleic acid, [ $1\text{-}^{14}\text{C}$ ]-linolenic acid, or [ $1\text{-}^{14}\text{C}$ ]-2-HA. Extraction and fractionation of the total cellular lipid extract demonstrated that the label was present in the glycolipid and phospholipid fractions (data not shown). Saponification and HPLC analysis of the fractions containing the free fatty acids, phospholipids, and neutral lipids showed that the radioactivity was present only as the original unsaturated fatty acid used, while, in the case of 2-HA, the label was associated with endogenous fatty acids (Figure 5A). Thus, intracellular metabolism of 2-HA is radically different from that of saturated and unsaturated (mono- and poly-) fatty acids in mycobacteria.

Second, the role of FASI in the metabolism of 2-HA was studied by using inhibitors of fatty acid biosynthesis. Thiolactomycin (TLM), a well-characterized inhibitor of the FASII  $\beta$ -ketoacyl-ACP synthetases [19], did not modify the metabolites that originated from [ $1\text{-}^{14}\text{C}$ ]-2-HA, while cerulenin, an inhibitor of mycobacterial FASI and FASII  $\beta$ -ketoacyl-ACP synthetases [20], abrogated the elongation of 2-HA-derived metabolites (Figure 5B). In this case, palmitic acid, 3-hexadecenoic acid, and 3-hydroxyhexadecanoic acid were the labeled metabolites detected. In a comparable manner, 5-chloro-pyrazinamide (5-Cl-PZA), a specific inhibitor of the *M. smegmatis* FASI enzyme [21, 22], inhibited elongation of 2-HA metabolites. Thus, a functional FASI is required for the conversion of 2-HA into longer-chain fatty acids.

### Long-Chain 3-Alkynoic Acids Are Potent Inhibitors of $\beta$ -Oxidation in Mycobacteria

In the previous sections, an analysis of the metabolism and mechanism of action of a set of isomeric long-chain alkynoic acids was described. The biological activities of alkynoic acids are far more diverse than the herein described inhibition of fatty acid biosynthesis, including inhibition of eukaryotic  $\omega$ -fatty acid hydroxylases [23] and enzymes of the  $\beta$ -oxidation pathway such as acyl-CoA dehydrogenases [24] and 3-ketoacyl thiolases [25]. Therefore, it was important to test the effect of long-chain 2- and 3-alkynoic acids on the  $\beta$ -oxidation pathway, since this pathway is a plausible candidate for the generation of 3-KHA. The observation that the concentration of [ $1\text{-}^{14}\text{C}$ ]-3-HA produced from [ $1\text{-}^{14}\text{C}$ ]-2-HA increased upon treatment of cell cultures with high concentrations of 2-HA led us to hypothesize that 3-HA might inhibit fatty acid degradation, thus allowing for the accumulation of 3-KHA seen in our samples. This hypothesis was tested by treating *M. smegmatis* (grown with palmitic or oleic acid as a carbon source) with 2- or 3-HA (50  $\mu\text{g/ml}$ ) for 1 hr before the addition of [ $1\text{-}^{14}\text{C}$ ]-palmitic acid. Inhibition of the  $\beta$ -oxidation pathway would impair the ability of the cells to produce  $^{14}\text{CO}_2$  from the radiolabeled fatty acid. A 60% reduction of the production of  $^{14}\text{CO}_2$  was observed in samples treated with 2-HA compared to untreated samples. The inhibition of 3-HA was more severe, reaching 90% inhibition. This confirms that 3-HA is a potent inhibitor of  $\beta$ -oxidation in *M. smegmatis*, which could lead to the accumulation of 3-KHA observed in our system.

### High Levels of 3-KHA Inhibit Fatty Acid Biosynthesis in *M. smegmatis*

At concentrations of 2-HA above 10  $\mu\text{g/ml}$ , *M. smegmatis* accumulates 3-hydroxyhexadecanoic acid and 3-KHA. In an attempt to identify the molecule(s) responsible for the inhibition of fatty acid biosynthesis, we analyzed the effect of both intermediates on growth and synthesis of fatty acids. Fatty acids from *M. smegmatis*

cultures treated with 3-hydroxyhexadecanoic acid (50  $\mu\text{g/ml}$ ) were extracted from the supernatant and cell pellet and analyzed by HPLC. 3-Hydroxyhexadecanoic acid was present in both fractions (data not shown), indicating that this compound was transported inside the cell but was not metabolized. The addition of 3-hydroxyhexadecanoic acid (50  $\mu\text{g/ml}$ ) to early log-phase cultures of *M. smegmatis* did not cause growth inhibition. At the same time, MIC determination showed that 3-hydroxyhexadecanoic acid had no effect on cell number or colony size at the highest concentration tested (120  $\mu\text{g/ml}$ ).

In contrast, 3-KHA was detected neither in the culture supernatant nor in the cell pellet. This result suggests that this molecule could have been transported and degraded or used for biosynthesis. Determination of its activity on solid media showed no change in cell viability or colony size at the highest concentration used (120  $\mu\text{g/ml}$ ). Surprisingly, treatment of early log-phase cultures with increasing concentrations of 3-KHA (10–50  $\mu\text{g/ml}$ ) showed a decrease in the growth rate. In order to test if an inhibition of fatty acid biosynthesis took place at the same time as the growth arrest, mid-log phase cultures of *M. smegmatis* were treated with 3-KHA at 10, 25, or 50  $\mu\text{g/ml}$  for 30 min, followed by the addition of [ $1\text{-}^{14}\text{C}$ ]-acetate for 30 min. Analysis of the labeled mycolic acids and fatty acids showed that their synthesis was inhibited when high concentrations of 3-KHA were used (Figure 3D). An equal concentration of 3-hydroxyhexadecanoic acid failed to inhibit fatty acid synthesis, in total agreement with its lack of effect on the growth of mycobacteria in both liquid and solid media. The block on the synthesis was relieved after 3 hr of incubation, as found by full restoration of the incorporation of the label (Figure 3D). Therefore, accumulation of 3-KHA transiently blocks fatty acid biosynthesis in mycobacteria.

#### Antimycobacterial Activity of 3-KHA Requires Inhibition of the $\beta$ -Oxidation Pathway

3-KHA temporarily inhibits both FASI and FASII systems in mycobacteria. 2-HA is partially converted into 3-HA, which inhibits fatty acid biosynthesis and the  $\beta$ -oxidation cycle, suggesting that the toxicity of 2-HA may be a consequence of the inhibition mediated by 3-HA, with the subsequent accumulation of 3-KHA. To test this hypothesis, we analyzed the effect of the addition of 3-HA on cultures of *M. smegmatis* treated with increasing concentrations of 3-KHA. Low concentrations of 3-HA (10–25  $\mu\text{g/ml}$ ) had a negligible effect on growth; however, in combination with increasing concentrations of 3-KHA, an inhibitory effect on the growth of *M. smegmatis* was observed (data not shown). Accordingly, incorporation of [ $1\text{-}^{14}\text{C}$ ]-acetate is severely decreased in cultures treated with 3-KHA (50  $\mu\text{g/ml}$ ), 3-HA (10 and 25  $\mu\text{g/ml}$ ), or a combination of both compounds for 30 min (Figure 3D). While the toxic effect is reversed within a doubling time for all of the compounds added independently, the treatment of the cultures with a 2:1 mixture of 3-KHA and 3-HA blocked fatty acid biosynthesis even at the longest time point analyzed (9 hr), which correlates with the biological action of 2-HA. In summary, our results indicate that 2-HA acts through the combined mechanism of action of the two major me-

tabolites, 3-HA and 3-KHA, which sets the stage for a novel concept for the design of antimycobacterial agents.

#### Discussion

In this report, the mechanism of action of alkynoic acids on mycobacteria is described. Long-chain 2-alkynoic acids are cidal antimycobacterial agents that inhibit the growth of *M. smegmatis* and *M. bovis* BCG. Like the first-line antituberculosis agents isoniazid and pyrazinamide, these compounds are not active as such, but they require metabolization. Wood and Lee showed that 2-HA inhibited the elongation of fatty acids longer than palmitic acid in liver microsomes [26]. Based on the metabolites detected, they postulated that, upon transport and esterification into acyl-CoA, 2-HA could be the substrate for a hydratase forming 3-KHA, or it could be isomerized by an acetylenic acid isomerase to a 2,3-allenyl-fatty acid, followed by conversion to 3-HA. We propose that a comparable pathway, schematically described in Figure 6, takes place in *M. smegmatis* since similar metabolites were detected. Although a  $\Delta^{2,3}$ -allenyl-containing fatty acid was not found in any of our fatty acid extracts, the identification of 3-hexadecenoic acid 10 and 3-HA 9 suggests that the 2,3-allenyl moiety 8 is formed in the mycobacterial cell. Isomerization of 8 would yield 3-HA, while reduction of the  $\Delta^2$  double bond would form 10. In fact, we detected these compounds in *M. smegmatis* fatty acid preparations. The fact that a 3-acetylenic acid isomerase activity has been found in *M. smegmatis* supports our hypothesis [27]. Furthermore, beef liver crotonase was reported to hydrate 2,3-allenic thioesters [28]. Moreover, a recent publication [29] showed that the liver mitochondrial enoyl-CoA hydratase (crotonase) was able to catalyze the hydration of 3-octynoyl-CoA, yielding 3-keto-octanoyl-CoA.

Treatment of *M. smegmatis* cultures with high concentrations of 2-HA leads to inhibition of fatty acid biosynthesis and fatty acid degradation, via accumulation of 3-HA and 3-KHA. The bacterial  $\beta$ -oxidation cycle degrades fatty acids by cleavage of two-carbon units that are released as acetyl-CoA. Components of this cycle include FadD (acyl-CoA synthetase), FadE (acyl-CoA dehydrogenase), and a tetrameric complex formed by two molecules of FadB (having activities of enoyl-CoA hydratase, enoyl-isomerase, and 3-hydroxyacyl-CoA dehydrogenase) and two molecules of FadA (having the activity of 3-ketoacyl-thiolase). One turn of the cycle thus results in the shortening of a fatty acyl chain by two carbons. *M. smegmatis* is unable to utilize 2-HA as the sole source of carbon and energy (H.R.M., unpublished data), raising the question of why 2-HA is not totally degraded to acetyl-CoA. Holland et al. showed that 3-butyryl-CoA irreversibly inhibits the activity of a mammalian 3-ketoacyl thiolase in vitro [30]. Our results are compatible with an inhibition of the degradation of 2-HA at the level of the thiolytic cleavage step. As we showed that 3-HA inhibits the degradation pathway, we suggest (although direct evidence is necessary) that 3-HA targets a long-chain 3-ketoacyl thiolase, a possibility compatible with the observed accumulation of 3-KHA.

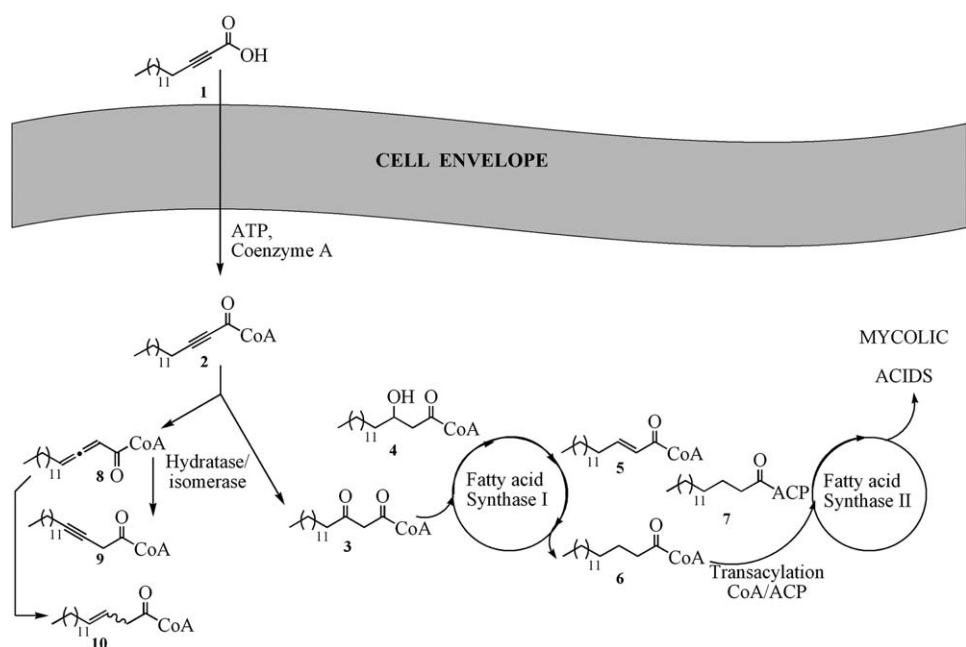


Figure 6. Metabolism of 2-HA in *M. smegmatis*

The proposed pathway is based on the metabolites derived from [1-<sup>14</sup>C]-2-HA and detected by HPLC. The identification key for the compounds shown is as follows: (1) 2-HA, (2) 2-hexadecynoyl-CoA, (3) 3-ketohexadecanoyl-CoA, (4) 3-hydroxyhexadecanoyl-CoA, (5) 2-*trans*-hexadecenoyl-CoA, (6) hexadecanoyl-CoA, (7) hexadecanoyl-ACP, (8) 2,3-hexadecadienoyl-CoA, (9) 3-hexadecynoyl-CoA, (10) 3-hexadecenoyl-CoA.

The major 2-HA-derived metabolite, 3-KHA, exerts a transitory toxic effect on the FASI- and FASII-mediated synthesis of fatty acids. In the absence of fatty acid elongation, cleavage of 3-KHA by a long-chain 3-ketoacyl thiolase would be the most likely explanation for the recovery of fatty acid biosynthesis observed in *M. smegmatis* treated with low concentrations of 2-HA. Although no long-chain 3-ketoacyl thiolase has been identified yet in mycobacteria, the *M. tuberculosis* genome encodes for three putative ketoacyl-CoA thiolases (*fadA3*, *Rv0914c*, *Rv3523*) [31]. Therefore, a conceivable mechanism for the 2-HA-mediated antimycobacterial effect is the accumulation of 3-KHA when the long-chain thiolase is inhibited, possibly by 3-HA. Our reconstitution experiments support this mechanism since neither 3-KHA nor 3-HA alone were able to significantly affect the mycobacterial growth or incorporation of [1-<sup>14</sup>C]-acetate. Both compounds added simultaneously at a 2:1 ratio yielded the same inhibitory effect displayed by 2-HA, demonstrating that growth inhibition is a consequence of the dual action of both 3-HA and 3-KHA. Therefore, we propose that the mechanism of action of 2-HA is based on the formation of the two major metabolites, 3-KHA and 3-HA, resulting in the dual inhibition of fatty acid biosynthesis and fatty acid degradation in mycobacteria, two pathways of extreme importance. Indeed, from a pharmacological point of view, fatty acid biosynthesis is the target of several antitubercular drugs, including pyrazinamide [22], isoniazid, or ethionamide [8, 10]. Physiologically, fatty acids are the preferred carbon source for mycobacteria isolated from animal lungs [32]. Deletion of *M. tuberculosis* isocitrate lyase 1 and 2 genes (key enzymes of the glyoxylate cycle) caused severe growth inhibition of *M. tuberculosis* in mice [33],

thus emphasizing the predominant role that fatty acid degradation plays in the intracellular survival capacity of this pathogen.

It is interesting to note that the effect of 2-HA on eukaryotic systems is different from that observed in mycobacteria [26]. Addition of 2-HA to rat liver microsomal preparations inhibited the synthesis of stearic acid (C18:0) and longer-chain fatty acids and had no effect on the synthesis of palmitic acid; the same effect was seen in rats fed with 2-HA [26, 34]. In *M. smegmatis*, 2-HA blocked the de novo synthesis of fatty acids as well as the synthesis of mycolic acids, which could be considered to be an "elongation" step. Based on our postulated metabolic pathway for 2-HA (Figure 6), we propose that the colocalization of both fatty acid synthesis and degradation is the key to explain these observations. If some of the enzymes of the  $\beta$ -oxidation cycle are required for the transformation of 2-HA, then they must be in the same compartment as the target system, the fatty acid biosynthesis system. The fatty acid degradation and FASI pathways are located in the mycobacterial cytoplasm, while FASII has been proposed to be loosely associated to the plasma membrane. Importantly none of these enzymatic complexes seems to be located in a subcellular compartment; therefore, the inhibitory metabolite has direct access to both FASI and FASII. This situation contrasts with the microsomal system, in which only the fatty acid elongation system colocalizes with the enzymes of the  $\beta$ -oxidation pathway. Since FASI is cytoplasmic, it is not inhibited. Thus, 2-alkynoic acids could be used specifically against bacteria without toxicity for the host. In this scenario, the mechanism of action of 2-alkynoic acids represents a reasonable approach for the rational design of new bifunctional



drugs. The isolation of mutants resistant to 2-alkynoic acids will confirm the hypothetical role of the mentioned enzymes and also identify other targets affected by 2- and 3-HA/OA, increasing our knowledge of the mycobacterial lipid metabolism.

## Significance

Long-chain alkynoic acids have antimycobacterial properties in the 10–40  $\mu$ M range. Their inhibitory effect on *M. smegmatis* requires a combination of a specific chain length (16 or 18 carbons) and the triple bond at position 2. We found that, at low concentrations, 2-hexadecynoic acid and 2-octadecynoic acid are metabolized to palmitic acid and elongated into endogenous fatty acids and mycolic acids in *M. smegmatis* and *M. bovis* BCG. At toxic concentrations, these compounds inhibit fatty acid degradation and fatty acid synthesis, resulting in cell death. This is the first example of an antibacterial compound inhibiting several pathways to achieve its effect. Furthermore, these compounds are another example of antimycobacterial agents, which require activation in order to be effective. We propose that the different effects seen in the eukaryotic versus mycobacterial systems stem from the different intracellular location of the enzyme components of the fatty acid degradation and fatty acid biosynthesis cycles. *M. tuberculosis* possesses a unique metabolism of lipids, ranging from the several pathways involved in the synthesis of complex lipids to the numerous enzymes taking part in fatty acid degradation. By inhibiting de novo fatty acid biosynthesis, fatty acid elongation, and fatty acid degradation, 2-alkynoic acids offer new tools by which to analyze these systems in mycobacteria.

## Experimental Procedures

### Experimental Reagents

Sources for the supplies were: Amersham Corp., [ $1\text{-}^{14}\text{C}$ ]-palmitic acid (specific activity 55 Ci/mol); NEN, [ $1\text{-}^{14}\text{C}$ ]-palmitic acid (850 Ci/mol) and [ $1\text{-}^{14}\text{C}$ ]-acetic acid (specific activity of 56 Ci/mol); American Radiochemical Company, [ $1\text{-}^{14}\text{C}$ ]-linoleic acid, [ $1\text{-}^{14}\text{C}$ ]-linolenic acid, and [ $1\text{-}^{14}\text{C}$ ]-oleic acid (specific activity of these three products was 55 Ci/mol). [ $1\text{-}^{14}\text{C}$ ]-2-Hexadecynoic acid (specific activity of 56 Ci/mol) was purchased from Moravak Biochemicals (Brea, CA). Thin-layer chromatography (TLC) plates and reagents for lipid detection were obtained from Alltech (Deerfield, IL), Fisher (Pittsburgh, PA), or Sigma (St. Louis, MO). Cerulenin was purchased from Sigma, and 5-chloro-pyrazinamide and thiolactomycin were the generous gifts from John Welsh (State University of New York, Albany, Albany, NY) and David Fidock (Albert Einstein College of Medicine, Bronx, NY), respectively. Fatty acid and lipid standards were obtained from Sigma or Indofine (Somerville, NJ). Microbiological media was purchased from Difco. All other biochemicals and solvents were reagent grade or better. The following compounds were prepared as described previously: 2-alkenoic acids [10], 3-hexadecenoic acid [35], and 3-KHA [36].

### Bacterial Strains and Growth Conditions

*M. smegmatis* strains and *Corynebacterium glutamicum* ATCC 13032 were grown at 37°C and 120 rpm in Mueller-Hinton or Sauton broth containing Tween 80 (0.02%, v/v) and were plated on Mueller-Hinton agar. mc<sup>2</sup>2399 was grown on Mueller-Hinton broth supplemented with 20  $\mu$ g/ml kanamycin. *M. bovis* var. BCG and *M. tuberculosis* H37Rv were grown at 37°C in Roller bottles (Corning) at 40 rpm in Sauton supplemented with Tween 80 (0.02%, v/v) and were plated on Middlebrook 7H10 medium with the addition of

10% OADC enrichment. Basal liquid medium for *M. smegmatis* was prepared according to Hartmans and de Bont [37], with the addition of methyl oleate or methyl palmitate (1.0 g/l) as carbon source.

### Synthesis of Alkynoic Acids

2-Alkynoic acids were synthesized as follows: (a) 1-hydroxyalkanes were converted into the corresponding 1-bromoalkanes by using phosphorus tribromide in diethyl ether; (b) the alkyl bromides were reacted with lithium acetylide ethylenediamine complex in dimethyl sulfoxide (DMSO) at room temperature; (c) the resulting 1-alkynes were converted to the lithium salts with *n*-butyllithium (*n*-BuLi) in tetrahydrofuran (THF) at –23°C [38]; and (d) the latter were quenched by bubbling CO<sub>2</sub> generated from dry ice into the solution containing the alkyne lithium. Acidification with HCl (6 N) and extraction with diethyl ether gave 2-alkynoic acids, which were purified by silica gel chromatography and recrystallization from hexane. 3-Alkynoic acids were obtained from 3-butyne-1-ol in four steps: (a) protection of the alcohol group as a tetrahydropyranyl (THP) ether; (b) treatment of the alkyne with *n*-BuLi in THF at –78°C, followed by coupling of the resulting lithium salt with 1-bromoalkane in the presence of hexamethylphosphoramide to form the 3-alkyn-1-OTHP [39]; (c) hydrolysis of the THP ether with acidic methanol; and (d) oxidation of the resulting alcohol with chromium trioxide. 4- and 5-Alkynoic acids were prepared in a similar way by using 4-pentyl-1-ol or 5-hexyn-1-ol as the starting material. The compounds were purified by silica gel chromatography, followed by recrystallization from hexane. The structures of all of the products were confirmed by <sup>1</sup>H- and <sup>13</sup>C-NMR spectroscopy.

### Determination of the Minimum Inhibitory Concentration

Liquid MIC values were determined by using a modified microbroth dilution assay [40]. The MIC was defined as the lowest concentration of alkynoic acid that prevented visible growth, and it was the average value of five cultures. Solid MIC values were obtained by using cultures of five independent colonies of *C. glutamicum* ATCC 13032 and *M. smegmatis*, grown to an OD<sub>600</sub> of 0.8–1.0 and diluted to give 10<sup>4</sup> CFU/ml. Aliquots (100  $\mu$ l) of each culture of *C. glutamicum* and *M. smegmatis* were plated on Mueller-Hinton plates containing increasing amounts of the compounds to be tested. In both solid and liquid MICs, the results were read after 4 days at 37°C. For *M. bovis* BCG and *M. tuberculosis*, the MIC was determined by growing the strains in Sauton media containing increasing concentrations of 2-HA and 2-OA and plating serial dilutions after 7 and 30 days of treatment. The MIC was defined as the concentration of drug causing a 99.9% reduction in the CFU/ml of the indicator strain.

### Immunoblotting

Aliquots (10 ml) of culture were harvested at mid-log phase. Cells were then resuspended in 0.8 ml phosphate-buffered saline (20 mM K<sub>2</sub>HPO<sub>4</sub> [pH 7.5], 0.15 M NaCl) and disrupted during 10 min by using a Branson Sonifier 450. Protein concentrations were determined by using the BCA Protein Assay Reagent kit (Pierce Europe, Oud-Beijerland, Netherlands). Equal amounts of proteins (25  $\mu$ g) were then separated on a SDS-polyacrylamide gel. Proteins were transferred onto a Hybond-C Extra membrane (Amersham). Membranes were then saturated with 5% dry milk in PBS-0.1% Tween 20 and incubated overnight with rat anti-KasA antibodies raised against the *M. tuberculosis* KasA protein (1/500 dilution), washed, and incubated with anti-rat antibodies conjugated to alkaline phosphatase (1/7000 dilution; Promega).

### Labeling of Bacterial Cultures

Early-log cultures of *M. smegmatis* or *M. bovis* BCG (OD<sub>600</sub> of ~0.4) were treated at 37°C with different concentrations of 2-HA for 30 min and 12 hr, respectively, and then labeled (0.5  $\mu$ Ci/ml) for 30 min and 1 hr, respectively, at 37°C. Cell pellets were washed twice with 1% bovine serum albumin in water to eliminate the carry-over of adsorbed fatty acids when polar lipids were analyzed. Cell pellets were used immediately or stored at –20°C before use.

### Mycolic Acid and Phospholipid Analysis

Mycolic acid methyl esters (MAMEs) and fatty acid methyl esters (FAMES) were prepared by alkaline treatment of the bacterial pellets as described previously [10]. Phospholipid extraction was done

according to Okuyama et al. [41]. The dried residue was resuspended in 100  $\mu$ l chloroform/methanol (1/1, v/v) prior to analysis by TLC.

#### TLC Systems

Radioactive aliquots (~20,000 cpm) of the different samples analyzed were separated on silica gel G plates. MAMEs and FAMEs were separated by two developments by using hexane/ethyl acetate (9/1, v/v) as a solvent system. Mycobacterial phospholipids were fractionated on silica gel G plates developed once in chloroform/methanol/water (60/24/1, v/v/v). Fractions containing nonpolar lipids and free fatty acids, obtained as an acetone-soluble fraction, were analyzed on silica gel G plates with hexane/ethyl acetate/acetic acid (80/20/0.5, v/v/v) as a solvent system. Bands were visualized by spraying with orcinol, phosphomolybdic acid, and ninhydrin (detecting glycolipids, phospholipids, and glycopeptidolipids, respectively). Incorporation of radiolabeled precursors was detected by autoradiography after exposure at  $-80^{\circ}\text{C}$  for 2 days.

#### Fatty Acid Analysis by High-Performance

##### Liquid Chromatography

Fatty acid *p*-bromophenacyl esters were prepared by alkaline treatment and derivatization as described previously [10]. The esters were analyzed on a Hewlett-Packard model HP1100 HPLC equipped with a thermostated column compartment set at  $45^{\circ}\text{C}$ , a diode array detector set at 260 nm, and an IN/US  $\beta$ -RAM flowthrough radioisotope  $\beta$ - $\gamma$  radiation detector, by using a reverse-phase  $\text{C}_{18}$  column ( $4.6 \times 150$  mm,  $3 \mu\text{m}$  column diameter, Alltima  $\text{C}_{18}$  from Alltech). The flow rate was 2 ml/min. The mobile phase was acetonitrile-water used as an isocratic elution (83/17, v/v) for the first 20 min, followed by a linear increase to 100% acetonitrile for 2 min and held at 100% acetonitrile for 18 min. In-Flow TM ES (IN/US Systems, Inc., Tampa, FL) was used to detect the  $^{14}\text{C}$ -labeled fatty acid esters (3:1 ratio with column eluant). The peaks were identified by comparison with chromatograms of fatty acid standards derivatized to their *p*-bromophenacyl esters.

#### Determination of Inhibition of $\beta$ -Oxidation by Alkynoic Acids

The ability of 2- and 3-HA to inhibit degradation of fatty acids was tested by measuring the production of  $^{14}\text{CO}_2$  from [ $^{14}\text{C}$ ]-palmitic acid. Briefly, *M. smegmatis* cultures were grown in basal medium with either methyl palmitate or methyl oleate (0.1%, v/v) as the carbon source. Early stationary phase cultures were centrifuged at room temperature, washed twice with warm basal medium devoid of a carbon source, concentrated 10-fold in the same medium, and treated with alkynoic acids at appropriate concentrations for 1 hr at  $37^{\circ}\text{C}$ . After this time, [ $^{14}\text{C}$ ]-palmitic acid ( $2 \mu\text{Ci/ml}$ ) was added, and the incubation was allowed to proceed overnight. The  $^{14}\text{CO}_2$  generated was trapped as  $\text{BaCO}_3$  in a filter paper disk impregnated with  $\text{Ba}(\text{OH})_2$  that was previously placed in the recessed lid of the vial. The filter disk was removed, and the trapped radioactivity was determined by liquid scintillation counting.

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#### References

- Corbett, E.L., Watt, C.J., Walker, N., Maher, D., Williams, B.G., Raviglione, M.C., and Dye, C. (2003). The growing burden of tuberculosis: global trends and interactions with the HIV epidemic. *Arch. Intern. Med.* 163, 1009–1021.
- Espinal, M.A. (2003). The global situation of MDR-TB. *Tuberculosis (Edinb.)* 83, 44–51.
- Park, M.M., Davis, A.L., Schluger, N.W., Cohen, H., and Rom, W.N. (1996). Outcome of MDR-TB patients, 1983–1993. Prolonged survival with appropriate therapy. *Am. J. Respir. Crit. Care Med.* 153, 317–324.
- Kent, J.H. (1993). The epidemiology of multidrug-resistant tuberculosis in the United States. *Med. Clin. North Am.* 77, 1391–1409.
- Fox, H.H. (1952). The chemical approach to the control of tuberculosis. *Science* 116, 129–134.
- Bernstein, J.W., Lott, A., Steinberg, B.A., and Yale, H.L. (1952). Chemotherapy of experimental tuberculosis. *Am. Rev. Tuberc.* 65, 357–374.
- Takayama, K., Wang, L., and David, H.L. (1972). Effect of isoniazid on the in vivo mycolic acid synthesis, cell growth, and viability of *Mycobacterium tuberculosis*. *Antimicrob. Agents Chemother.* 2, 29–35.
- Banerjee, A., Dubnau, E., Quemard, A., Balasubramanian, V., Um, K.S., Wilson, T., Collins, D., de Lisle, G., and Jacobs, W.R., Jr. (1994). *inhA*, a gene encoding a target for isoniazid and ethionamide in *Mycobacterium tuberculosis*. *Science* 263, 227–230.
- Quemard, A., Sacchettini, J.C., Dessen, A., Vilcheze, C., Bittman, R., Jacobs, W.R., Jr., and Blanchard, J.S. (1995). Enzymatic characterization of the target for isoniazid in *Mycobacterium tuberculosis*. *Biochemistry* 34, 8235–8241.
- Vilcheze, C., Morbidoni, H.R., Weisbrod, T.R., Iwamoto, H., Kuo, M., Sacchettini, J.C., and Jacobs, W.R., Jr. (2000). Inactivation of the *inhA*-encoded fatty acid synthase II (FASII) enoyl-acyl carrier protein reductase induces accumulation of the FASII end products and cell lysis of *Mycobacterium smegmatis*. *J. Bacteriol.* 182, 4059–4067.
- Rozwarski, D.A., Vilcheze, C., Sugantino, M., Bittman, R., and Sacchettini, J.C. (1999). Crystal structure of the *Mycobacterium tuberculosis* enoyl-ACP reductase, *InhA*, in complex with  $\text{NAD}^+$  and a  $\text{C}_{16}$  fatty acyl substrate. *J. Biol. Chem.* 274, 15582–15589.
- Marrakchi, H., Laneelle, G., and Quemard, A. (2000). *InhA*, a target of the antituberculous drug isoniazid, is involved in a mycobacterial fatty acid elongation system, FAS-II. *Microbiol.* 146, 289–296.
- Bhatt, A., Kremer, L., Dai, A.Z., Sacchettini, J.C., and Jacobs, W.R., Jr. (2005). Conditional depletion of *KasA*, a key enzyme of mycolic acid biosynthesis, leads to mycobacterial cell lysis. *J. Bacteriol.* 187, 7596–7606.
- Kremer, L., Dover, L.G., Morbidoni, H.R., Vilcheze, C., Maughan, W.N., Baulard, A., Tu, S.C., Honore, N., Deretic, V., Sacchettini, J.C., et al. (2003). Inhibition of *InhA* activity, but not *KasA* activity, induces formation of a *KasA*-containing complex in mycobacteria. *J. Biol. Chem.* 278, 20547–20554.
- Larsen, M.H., Vilcheze, C., Kremer, L., Besra, G.S., Parsons, L., Salfinger, M., Heifets, L., Hazbon, M.H., Alland, D., Sacchettini, J.C., et al. (2002). Overexpression of *inhA*, but not *kasA*, confers resistance to isoniazid and ethionamide in *Mycobacterium smegmatis*, *M. bovis* BCG and *M. tuberculosis*. *Mol. Microbiol.* 46, 453–466.
- Bloch, K. (1977). Control mechanisms for fatty acid synthesis in *Mycobacterium smegmatis*. *Adv. Enzymol. Relat. Areas Mol. Biol.* 45, 1–84.
- Barry, C.E., 3rd, Lee, R.E., Mdluli, K., Sampson, A.E., Schroeder, B.G., Slayden, R.A., and Yuan, Y. (1998). Mycolic acids: structure, biosynthesis and physiological functions. *Prog. Lipid Res.* 37, 143–179.
- Konhikamee, W., Gilbertson, J.R., Langkamp, H., and Gershon, H. (1982). Effect of 2-alkynoic acids on in vitro growth of bacterial and mammalian cells. *Antimicrob. Agents Chemother.* 22, 805–809.
- Kremer, L., Douglas, J.D., Baulard, A.R., Morehouse, C., Guy, M.R., Alland, D., Dover, L.G., Lakey, J.H., Jacobs, W.R., Jr., Brennan, P.J., et al. (2000). Thiolactomycin and related analogues as novel anti-mycobacterial agents targeting *KasA* and *KasB* condensing enzymes in *Mycobacterium tuberculosis*. *J. Biol. Chem.* 275, 16857–16864.

20. Parrish, N.M., Kuhajda, F.P., Heine, H.S., Bishai, W.R., and Dick, J.D. (1999). Antimycobacterial activity of cerulenin and its effects on lipid biosynthesis. *J. Antimicrob. Chemother.* 43, 219–226.
21. Boshoff, H.I., Mizrahi, V., and Barry, C.E., 3rd. (2002). Effects of pyrazinamide on fatty acid synthesis by whole mycobacterial cells and purified fatty acid synthase I. *J. Bacteriol.* 184, 2167–2172.
22. Zimhony, O., Cox, J.S., Welch, J.T., Vilcheze, C., and Jacobs, W.R., Jr. (2000). Pyrazinamide inhibits the eukaryotic-like fatty acid synthetase I (FASI) of *Mycobacterium tuberculosis*. *Nat. Med.* 6, 1043–1047.
23. CaJacob, C.A., and Ortiz de Montellano, P.R. (1986). Mechanism-based in vivo inactivation of lauric acid hydroxylases. *Biochemistry* 25, 4705–4711.
24. Powell, P.J., and Thorpe, C. (1988). 2-octynoyl coenzyme A is a mechanism-based inhibitor of pig kidney medium-chain acyl coenzyme A dehydrogenase: isolation of the target peptide. *Biochemistry* 27, 8022–8028.
25. Kunau, W.H., Dommès, V., and Schulz, H. (1995).  $\beta$ -oxidation of fatty acids in mitochondria, peroxisomes, and bacteria: a century of continued progress. *Prog. Lipid Res.* 34, 267–342.
26. Wood, R., and Lee, T. (1981). Metabolism of 2-hexadecynoate and inhibition of fatty acid elongation. *J. Biol. Chem.* 256, 12379–12386.
27. Miesowicz, F.M., and Bloch, K. (1979). Purification of hog liver isomerase. Mechanism of isomerization of 3-alkenyl and 3-alkynyl thioesters. *J. Biol. Chem.* 254, 5868–5877.
28. Branchini, B.R., Miesowicz, F.M., and Bloch, K. (1977). Allenic thioester hydration activity of beef liver crotonase. *Bioorg. Chem.* 6, 49–52.
29. Alipui, O.D., Zhang, D., and Schulz, H. (2002). Direct hydration of 3-octynoyl-CoA by crotonase: a missing link in Bloch's enzymatic studies with 3-alkynoyl thioesters. *Biochem. Biophys. Res. Commun.* 292, 1171–1174.
30. Holland, P.C., and Sherratt, H.S. (1972). Mechanisms of inhibition of fatty acid oxidation by pent-4-enoic acid: evidence against the coenzyme A-depletion hypothesis. *Biochem. J.* 127, 79P.
31. Cole, S.T., Brosch, R., Parkhill, J., Garnier, T., Churcher, C., Harris, D., Gordon, S.V., Eiglmeier, K., Gas, S., Barry, C.E., 3rd, et al. (1998). Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. *Nature* 393, 537–544.
32. Bloch, H., and Segal, W. (1956). Biochemical differentiation of *Mycobacterium tuberculosis* grown in vivo and in vitro. *J. Bacteriol.* 72, 132–141.
33. Munoz-Elias, E.J., and McKinney, J.D. (2005). *Mycobacterium tuberculosis* isocitrate lyases 1 and 2 are jointly required for in vivo growth and virulence. *Nat. Med.* 11, 638–644.
34. Wood, R., Lee, T., and Gershon, H. (1980). Effect of methyl 2-hexadecynoate on hepatic fatty acid metabolism. *Lipids* 15, 141–150.
35. Sampath Kumar, H.M., Subba Reddy, B.V., Jagan Reddy, E., and Yadav, J.S. (1999). SiO<sub>2</sub> catalysed expedient synthesis of [E]-3-alkenoic acids in dry media. *Tetrahedron Lett.* 40, 2401–2404.
36. Barnick, J.W.F.K., Van Der Baan, J.L., and Bickelhaupt, F. (1979). A convenient direct method for the preparation of  $\beta$ -keto-acids. *Synthesis (Mass.)* 787–788.
37. Hartmans, S., and de Bont, J.A.M. (1992). *The Genus Mycobacteria* (New York: Springer-Verlag).
38. Camps, F., Hospital, S., Rosell, G., Delgado, A., and Guerrero, A. (1992). Synthesis of biosynthetic inhibitors of the sex pheromone of *Spodoptera littoralis*. Part II: Acetylenic and cyclopropane fatty acids. *Chem. Phys. Lipids* 61, 157–167.
39. Valicenti, A.J., Pusch, F.J., and Holman, R.T. (1985). Synthesis of octadecynoic acids and [1-<sup>14</sup>C] labeled isomers of octadecenoic acids. *Lipids* 20, 234–242.
40. Swenson, J.M., Thornsberry, C., and Silcox, V.A. (1982). Rapidly growing mycobacteria: testing of susceptibility to 34 antimicrobial agents by broth microdilution. *Antimicrob. Agents Chemother.* 22, 186–192.
41. Okuyama, H., Kankura, T., and Nojima, S. (1967). Positional distribution of fatty acids in phospholipids from *Mycobacteria*. *J. Biochem. (Tokyo)* 61, 732–737.