Antimycobacterial Activity and Mechanism of Action of NAS-91[▽]

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The antimalarial agents NAS-91 and NAS-21 were found to express potent antimycobacterial activity, NAS-91 being more active than NAS-21. They partially inhibited mycolic acid biosynthesis and profoundly altered oleic acid production. The development of a cell-free assay for $\Delta 9$ -desaturase activity allowed direct demonstration of the inhibition of oleic acid biosynthesis by these compounds.

The type II fatty acid synthase (FAS-II) plays an essential role in fatty acid biosynthesis and is found in most bacteria and plants as well as in the malarial parasite *Plasmodium falciparum* (13, 25). FAS-II is typified by the existence of distinct enzymes encoded by unique genes for catalyzing each of the four individual reactions required to complete successive cycles of fatty acid elongation. This is in contrast with the type I fatty acid synthase (FAS-I) found in mammals, which is characterized by a multifunctional enzyme bearing all the necessary enzymatic activities for fatty acid elongation (20). Since FAS-II is absent from humans, this pathway has recently received considerable attention as a good target for the development of new and selective inhibitors (5, 26). The third step of the elongation cycle is carried out by the β -hydroxyacyl-acyl carrier protein (ACP) dehydratase (FabZ), which catalyzes the dehydration of β -hydroxyacyl-ACP to *trans*-2-enoylacyl-ACP. However, until recently, FabZ was completely unexplored as a potential inhibitor target. Two lead compounds, NAS-91 and NAS-21 (Fig. 1A), were designed as inhibitory molecules toward *P. falciparum* FabZ and represent the first FabZ inhibitors known to date (19). They have also been shown to inhibit the intraerythrocytic growth of *P. falciparum*, opening new avenues for treating malaria (16, 19). However, the efficacy of these compounds against bacteria remains unknown.

Mycobacteria are unusual in that they possess both FAS-I and FAS-II (3, 10, 21), and numerous antitubercular inhibitors have been shown to inhibit mycolic acids by targeting the FAS-II enzymes (10, 23). Thiolactomycin inhibits the β -ketoacyl ACP synthases KasA and KasB (11), whereas isoniazid (INH) and ethionamide inhibit the enoyl-ACP reductase InhA (1, 23); KasA/KasB and InhA are enzymes that catalyze the first and last steps of the repetitive FAS-II cycle, respectively. Although no orthologue genes of *fabZ* have yet been identified in mycobacterial genomes, two recent studies have reported

Rv0636 as the gene encoding the FAS-II β -hydroxyacyl-ACP dehydratase in *Mycobacterium tuberculosis* (4, 17).

In this study, we evaluated the antimycobacterial potential of NAS-91 and NAS-21, which were synthesized as described earlier (19). The activity of these molecules was first assessed against *Mycobacterium bovis* BCG 1173P2 on Middlebrook 7H11 agar plates supplemented with oleic acid, albumin, dextrose, and catalase (OADC) enrichment with increasing inhibitor concentrations. Serial 10-fold dilutions of actively growing cultures were plated and incubated at 37°C for 10 to 14 days. The MIC was defined as the minimum concentration required to inhibit 99% of the growth. As shown in Fig. 1B, NAS-91 exhibited potent antimycobacterial activity, with an MIC of 10 to 25 μ g/ml. NAS-21 also inhibited *M. bovis* BCG growth, although less efficiently than NAS-91, with an MIC of 50 μ g/ml (data not shown). We next determined the activity of NAS-91 against *Mycobacterium tuberculosis* H37Rv using the agar proportion method. The culture was grown in Middlebrook 7H9 medium at 37°C with shaking until the optical density at 600 nm reached 1.0. Serial dilutions of the logarithmically growing culture were made, and an aliquot of the diluted culture expected to give $\sim 1,000$ CFU on Middlebrook 7H11 agar plates supplemented with OADC was used for plating on both control plates and drug-containing plates and incubated at 37°C. Colonies were counted after 15 to 20 days. NAS-91 appeared to be a far better inhibitor than NAS-21, exhibiting 99% growth inhibition at 10 μ g/ml. Conversely, NAS-91 did not show any inhibition activity against *Mycobacterium smegmatis* even at high concentrations (up to 100 μ g/ml) (data not shown).

The similar growth inhibitory effects observed in *M. bovis* BCG and *M. tuberculosis* prompted us to investigate the mechanism of action of NAS-91 in mycobacteria. Since this inhibitor has been shown to target *P. falciparum* FabZ (19), we examined whether this compound would also inhibit mycolic acids, which are known to be the end products of FAS-II in mycobacteria. Mid-log-phase cultures of *M. bovis* BCG (4 ml) were treated with various drug concentrations, followed by further incubation at 37°C for 8 h. At this point, 1 μ Ci/ml of [2-¹⁴C]acetate (56 mCi/mmol; Amersham Biosciences) was added to the

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FIG. 1. NAS-91 inhibits *M. bovis* BCG growth. (A) Structures of NAS-91 and NAS-21. (B) Antimycobacterial effect of NAS-91 against *M. bovis* BCG. The susceptibility of *M. bovis* BCG strains to NAS-91 was determined on Middlebrook 7H11 solid medium containing OADC enrichment with increasing inhibitor concentrations ($\mu\text{g/ml}$). Serial 10-fold dilutions (indicated on the plates) of actively growing culture were plated and incubated at 37°C for 10 to 14 days. The MIC, defined as the minimum concentration required to inhibit 99% of the growth, was estimated to be around 10 to 25 $\mu\text{g/ml}$.

cultures, followed by further incubation at 37°C for 16 h. The ^{14}C -labeled cells were harvested by centrifugation, washed once with phosphate-buffered saline, and subjected to alkaline hydrolysis using 15% aqueous tetrabutylammonium hydroxide at 100°C overnight, followed by the addition of 4 ml of CH_2Cl_2 , 300 μl of CH_3I , and 2 ml of water. The entire reaction was then mixed for 1 h. The upper aqueous phase was discarded, and the lower organic phase was washed twice with water and evaporated to dryness. Fatty acid methyl esters (FAMES) and mycolic acid methyl esters (MAMES) were redissolved in diethyl ether, and the solution was again evaporated to dryness. The final residue was then dissolved in 200 μl of CH_2Cl_2 . Equal counts of the resulting solution were subjected to one-dimensional (1-D) thin-layer chromatography (TLC) using silica gel plates (5735 Silica Gel 60F₂₅₄; Merck). Labeled mycolates were resolved in petroleum ether-acetone (95:5, vol/vol), and autoradiograms were obtained by exposure to Kodak Biomax MR film to reveal ^{14}C -labeled FAMES and MAMES. As shown in Fig. 2A, mycolic acid biosynthesis was partially inhibited by NAS-91. In order to quantitate the percentage of mycolic acid inhibition, spots corresponding to labeled mycolates (α - and keto-mycolates) were scraped and counted. Figure 2A (lower panel) demonstrates that around 55% of mycolic acid inhibition occurred in the presence of 100 $\mu\text{g/ml}$ NAS-91, but that synthesis was completely abolished when cells were treated with INH. NAS-91 inhibited both α - and keto-mycolates to similar extents, supporting the fact that the elongation step was affected by the inhibitor. These results suggest that FAS-II activity was affected by NAS-91, presumably through inhibition of the FAS-II β -hydroxyacyl-ACP dehydratase. Vilchèze et al. (22) have shown that INH treatment of *M. bovis* BCG caused a rapid cessation of mycolic acid biosynthesis with a concomitant accumulation of the FAS-I end products, notably saturated hexacosanoic acid (C_{26}), which corresponds to the α -branch of mycolic acids. In order to compare the fatty acid profiles in untreated and NAS-91-treated cells, FAMES were

resolved by reverse-phase TLC on C_{18} -silica gel plates (KC18; Whatman) using CHCl_3 -MeOH (2:3, vol/vol). Figure 2B shows the presence of all fatty acids from *M. bovis* BCG ranging from C_{16} to C_{26} . INH-treated cells clearly showed an accumulation of C_{26} , in agreement with earlier work (22). Treatment with NAS-91 also led to a rapid increase in C_{26} , consistent with the fact that this compound inhibits FAS-II activity. Interestingly, treatment with subinhibitory concentrations of NAS-91 (5 $\mu\text{g/ml}$) also led to an accumulation of C_{18} , presumably corresponding to saturated stearic acid. This was even more evident at higher doses (Fig. 2B). Since stearic acid is a direct precursor of monounsaturated oleic acid, we reasoned that the accumulation of stearic acid in NAS-91-treated cells may directly result from the inhibition of oleic acid production. To test this hypothesis, radiolabeled lipids from NAS-91-treated *M. bovis* BCG were separated by 2-D TLC on silver nitrate-impregnated plates, which allows for discrimination between saturated and unsaturated fatty acids (12). ^{14}C -labeled samples were run in the first dimension along the narrow strip without silver impregnation by developing the plates twice with hexane-ethyl acetate (19:1, vol/vol). The plates were then dried, turned, and run into the silver layer by developing them three times with petroleum ether-diethyl ether (17:3, vol/vol). As shown in Fig. 3A and B, synthesis of oleic acid methyl esters was significantly inhibited in the presence of either NAS-21 or NAS-91 at 100 $\mu\text{g/ml}$. The inhibition of oleic acid was dose dependent, as observed in cultures treated with increasing inhibitor concentrations (data not shown). As reported above, a significant decrease in α - and keto-mycolates was also observed.

The Δ^9 -stearoyl-coenzyme A (CoA) desaturase catalyzes the insertion of the double bond at carbon 9 of stearic acid. To investigate whether NAS-91 and NAS-21 inhibit the Δ^9 -desaturase activity, we have developed a cell-free assay based on that described by Fulco and Bloch (8). *M. bovis* BCG was grown in Sauton's medium supplemented with 20 $\mu\text{g/ml}$ FeSO_4

FIG. 2. Effect of NAS-91 on mycolic acid and fatty acid biosynthesis. *M. bovis* BCG cultures were grown in Sauton's medium and incubated for 8 h with increasing inhibitor concentrations prior to the addition of [14 C]acetate. Labeling was done for an additional 16 h, and FAMES and MAMES were then extracted and analyzed by TLC. (A) NAS-91 partially inhibits mycolic acid biosynthesis. Equal counts (50,000 cpm) of each sample were loaded on a normal TLC plate, and α -mycolates and keto-mycolates were separated using petroleum ether-acetone (95:5, vol/vol). Cultures treated with INH (used at 1 μ g/ml) were also included in the experiment as a positive control of mycolic acid inhibition (upper panel). Radiolabeled MAMES were then scraped from the TLC plate and counted to allow direct quantification of mycolic acid biosynthesis inhibition by NAS-91 (lower panel). (B) Accumulation of C_{18} and C_{26} fatty acids in *M. bovis* BCG-treated cells. Equal counts (50,000 cpm) of each sample were loaded on a reverse-phase TLC plate, and the fatty acids were separated using chloroform-methanol (2:3, vol/vol). Radiolabeled C_{16} -methyl ester was used as a standard. Autoradiograms were obtained by overnight exposure to Kodak Biomax MR film to reveal 14 C-labeled FAMES and MAMES. Results are from one representative experiment out of three independent experiments. Values shown are means $\pm$ standard errors of the means (SEM) from triplicates.

(9). Following centrifugation, cells were resuspended in 0.25 M sucrose and disrupted using a French pressure cell. Conversion of stearic acid into oleic acid was assayed in a reaction mixture containing 1 mg of *M. bovis* BCG crude lysate, 1 μ mol of

NADPH (Sigma) in 0.1 mM potassium phosphate buffer (pH 7.2), and 0.25 μ Ci [14 C]stearoyl-CoA (55 mCi/mmol; American Radiolabeled Chemicals) in a final volume of 1 ml. After incubation at 37°C for 1 h, reactions were terminated by the

FIG. 3. NAS-91 and NAS-21 inhibit oleic acid biosynthesis in *M. bovis* BCG. (A) Cultures were grown in Sauton's medium and incubated for 8 h with 100 μ g/ml of either NAS-91 or NAS-21 prior to the addition of [14 C]acetate and labeling for an additional 16 h. FAMES and MAMES were then extracted, and equal counts (50,000 cpm) were applied to 2-D, 10% AgNO_3 -impregnated TLC plates. The plates were developed in the first direction by using two developments of hexane-ethyl acetate (19:1, vol/vol) and in the second direction by using a triple development of petroleum ether-diethyl ether (17:3, vol/vol). Autoradiograms were obtained by overnight exposure to Kodak Biomax MR film to reveal 14 C-labeled FAMES and MAMES. OAME, oleic acid methyl ester. SFAMES, saturated FAMES. (B) Radiolabeled α -mycolic acid, keto-mycolic acid, and oleic acid methyl esters were scraped from the 2-D TLC plates, and the bands were counted to allow direct quantification following exposure to NAS-91 or NAS-21. Results are from one representative experiment out of three independent experiments. Values shown are means $\pm$ SEM from triplicates. The statistical significance of differences between NAS-treated cells and untreated cells was calculated by using Student's *t* test (*, $P \leq 0.05$).

FIG. 4. Inhibition of oleic acid synthesis by NAS-91 and NAS-21 in a cell-free assay. Crude extracts of *M. bovis* BCG were incubated in the presence of NADPH and [14 C]stearoyl-CoA. Following incubation at 37°C, radiolabeled fatty acids were saponified and methyl esterified. The radiolabeled products were then resolved by argentation-TLC in petroleum ether-diethyl ether (17:3, vol/vol). Autoradiograms were obtained by overnight exposure to Kodak Biomax MR film. (A) Synthesis of oleoyl-CoA from stearoyl-CoA in the presence of *M. bovis* BCG lysates (lane 3). The appropriate radiolabeled standards used were stearic acid (lane 1) and oleic acid (lane 2). (B) The reaction mixtures were preincubated in the presence of increasing concentrations (μ g/ml) of either NAS-21 (left panel) or NAS-91 (right panel) at 37°C for 10 min prior to the addition of [14 C]stearoyl-CoA. (C) The inhibition of Δ 9-desaturase activity was evaluated by scraping and counting the bands corresponding to [14 C]oleic acid shown in panel B. Results are from one representative experiment out of two independent experiments. Values shown are means $\pm$ SEM from triplicates.

addition of 2 ml of tetrabutylammonium hydroxide, saponified, and methylated. The radiolabeled products were resolved by 10% argentation-TLC in petroleum ether-diethyl ether (17:3, vol/vol). Figure 4A shows the presence of a band comigrating with the oleic acid methyl ester standard, thus reflecting the conversion of stearoyl-CoA to oleoyl-CoA. More importantly, when the reaction mixtures were preincubated with NAS-21 or NAS-91, there was a dose-dependent inhibition of oleic acid formation, indicating that both compounds are capable of inhibiting Δ 9-desaturase activity (Fig. 4B). Quantification of the production of [14 C]oleic acid was performed by scraping and counting the bands corresponding to the methyl ester of oleate. NAS-91 appeared to be more active than NAS-21, as 25 μ g/ml of NAS-91 or NAS-21 inhibited 75% or 25% of the Δ 9-desaturase activity, respectively (Fig. 4C). These data are consistent with the higher efficiency (lower MIC) of NAS-91 than that of NAS-21 against whole mycobacteria. In *M. tuberculosis*, DesA3 has been reported to be the stearoyl-CoA desaturase leading to the formation of oleic acid (14). However, DesA3 appears not to be essential (18), suggesting that it is probably not the lethal target of NAS-91. Interestingly, bioinformatics studies conducted by Raman et al. (15) revealed that the two other desaturases, DesA1 and DesA2, classified as essential for mycobacterial survival, may participate in mycolic acid synthesis. Whether DesA1 and/or DesA2 is a target of NAS-91 remains to be demonstrated. In addition, experimental evidence regarding their functional roles in the mycolic acid pathway is not presently available.

Oleic acid represents the most abundant unsaturated fatty acid in mycobacteria and is a crucial constituent of mycobacterial membrane phospholipids (24). It is also a direct precur-

sor of tuberculostearic acid (10-methyl stearic acid), which is a major fatty acid of phosphatidylinositol mannosides, lipomannan, and lipoarabinomannan. Therefore, one expects the inhibition of oleic acid biosynthesis to also directly affect the synthesis of these major lipoglycans. All together, these results indicate that, in addition to inhibiting mycolic acid production, NAS-91 and NAS-21 affect oleic acid production in mycobacteria. Despite their structural dissimilarities (Fig. 1A), the present data suggest that both inhibitors share common targets.

We report here for the first time the potent antimycobacterial activities of NAS-91 and NAS-21 as well as their molecular mechanism of action. Inhibition of oleic acid by NAS-91 in mycobacteria, although rather unusual, is reminiscent of the mode of action of isoxyl, which has been shown to inhibit both mycolic acid and oleic acid formation in *M. tuberculosis* (14). Thiocarbamide-containing drugs, such as isoxyl, ethionamide, and thiacetazone, are proinhibitors that must be activated by the mycobacterial monooxygenase EthA (6, 7). In contrast, NAS-21 and NAS-91 are not thiocarbamide-containing inhibitors and, thus, unlikely to be activated by EthA. This is also supported by the fact that an *M. bovis* BCG strain overproducing EthA appears to be 10-fold-more sensitive to ethionamide (2, 7), but not NAS-91, compared to the wild-type strain (data not shown). The present data suggest that NAS-91 and NAS-21 have multiple targets, which is particularly desirable for avoiding the emergence of resistant strains of *M. tuberculosis*. Therefore, NAS-91 represents a potent pharmacophore and appears to be a promising lead compound for future inhibitor development against tuberculosis.

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P.G. and L.K. designed and carried out the experiments, A.S. and N.S. designed, synthesized, and supplied NAS-21 and NAS-91, and G.S.B. and L.K. wrote the manuscript.

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