ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Nov. 1990, p. 2061–2064
0066-4804/90/112061-04$02.00/0

Vol. 34, No. 11

Action of 1-Isonicotinyl-2-Palmitoyl Hydrazine against the *Mycobacterium avium* Complex and Enhancement of Its Activity by *m*-Fluorophenylalanine

NALIN RASTOGI* AND KHYE SENG GOH

Unité de la Tuberculose et des Mycobactéries, Institut Pasteur, 75724 Paris Cedex 15, France

Received 23 May 1990/Accepted 13 August 1990

In the present work, we investigated whether resistance to isoniazid (INH) of organisms belonging to the *Mycobacterium avium* complex was caused by the bacterial cell envelope, with the cell wall and the outer layer acting as an exclusion barrier. We observed that this exclusion barrier was most efficient in excluding the hydrophilic drug INH, as this drug could not penetrate a wall matrix formed of various polymethylated lipidic or amphipathic substances. Two main strategies were proposed for circumventing this drug resistance: (i) synthesis of amphipathic derivatives of otherwise highly hydrophilic drugs and (ii) inhibition of synthesis of the bacterial outer layer. The purpose of this work was to demonstrate that attaching a palmitic acid side chain to INH rendered it growth inhibitory against *M. avium* complex bacteria and that the concomitant use of this amphipathic INH derivative with *m*-fluorophenylalanine (an inhibitor of mycoside C biosynthesis which causes the disruption of the bacterial outer layer) resulted in further enhancement of its activity, leading to a bactericidal effect.

Treatment of human infections caused by organisms of the *Mycobacterium avium* complex (MAC) still remains a challenge, as this opportunistic human pathogen is multiply drug resistant (5). This problem is one of the main handicaps in the successful chemotherapy of opportunistic infections in immunosuppressed patients (18).

It has been proposed that the multiple drug resistance of MAC organisms can not be caused by genetic factors or membrane-associated permeability factors alone and that it is rather caused by an exclusion barrier situated at the level of the cell wall architecture (1, 4, 13, 17). This proposition was recently supported by the work of Jarlier and Nikaido (8) and by our own work (15), in which we demonstrated that inhibition of the synthesis of the cell wall or the outer layer (OL) in MAC bacteria by specific inhibitors resulted in enhanced antibacterial activity of a variety of drugs. However, none of the cell wall inhibitors used could potentiate the anti-MAC activity of isoniazid (INH), which remained nil as usual (15). We attempted to circumvent this problem by synthesizing an amphipathic derivative of INH (1-isonicotinyl-2-palmitoyl hydrazine [INH-PALM]) and using it in association with *m*-fluorophenylalanine (FL-PHE), an inhibitor of mycoside C biosynthesis which causes the disruption of the bacterial OL (3).

MATERIALS AND METHODS

Bacteria and growth. The 17 MAC strains (14 laboratory-maintained strains, including the type strain, ATCC 15769, and 3 recent clinical isolates) used were from our own culture collection. The bacteria were grown in complete 7H9 medium (Difco Laboratories, Detroit, Mich.) containing 0.05% (vol/vol) Tween 80 to an optical density of 0.15 (measured at 650 nm with a Coleman Junior II spectrophotometer), which corresponded to about 10^8 viable counts per ml. Tween 80 was used to avoid clumping of the bacteria in the initial inoculum (preculture), 0.1 ml of which was then injected into a BACTEC vial to prepare the final inoculum (growth index [GI] [see below], 500); only the latter was used in the radiometric studies reported here.

Drugs and inhibitors. INH-PALM was synthesized from palmitic acid and commercially available INH (Aldrich-Chemie, Strasbourg, France) and characterized as reported earlier (16). Other amphipathic derivatives of INH synthesized included 1-isonicotinyl-2-(12-hydroxydodecanoyl) hydrazine and 1-isonicotinyl-2-[12(1αD-mannopyranosyl)dodecanoyl] hydrazine (16). All the above-stated INH derivatives were kindly provided by B. Moreau and M.-L. Capmau, Cercoa, Centre National de la Recherche Scientifique, Thiais, France.

MICs. MICs were determined by the 1% proportion method on 7H11 agar plates as follows. Appropriate dilutions of the various strains were plated on control 7H11 agar as well as on drug-containing agar, and the CFU per milliliter were counted after 21 days of incubation at 37°C. The MIC was defined as the lowest concentration of a drug which caused a 2-log reduction in the viable counts as compared with the respective controls.

Radiometric drug susceptibility testing. Radiometric drug susceptibility testing with the BACTEC 460-TB apparatus (Becton Dickinson, Towson, Md.) was performed as reported recently (14, 15). Bacterial growth in a confined atmosphere was measured as a function of the release of ^{14}C-labeled CO_2 and expressed as the GI, which ranged from 1 to 999. The primary bacterial culture was stopped at a GI of 500 (see above), and 0.1 ml of this suspension diluted 10-fold was used for inoculating control and drug (or inhibitor-)-containing vials, which were incubated at 37°C. The GI readings were recorded daily and compared with parallel control readings.

The GI values for vials containing INH alone were also compared with that for second control vial (1:1,000 final dilution) to interpret any resultant growth inhibition by the 1% proportion method criterion (14). However, as all the

* Corresponding author.

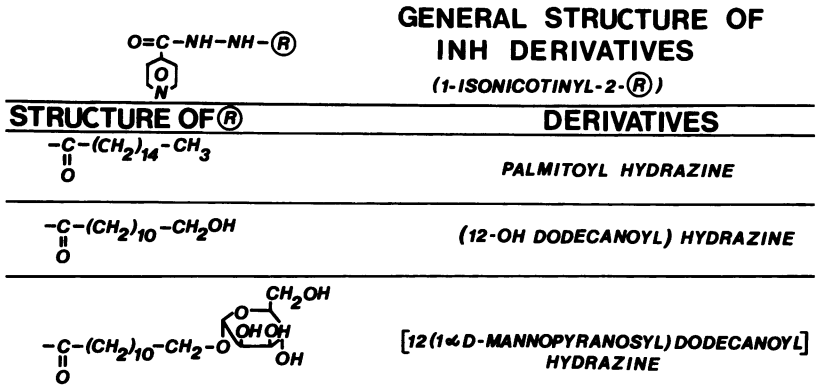

FIG. 1. Chemical structures of various synthetic amphipathic derivatives of INH.

MAC organisms tested were 100% resistant to INH in the standard BACTEC protocol, this comparison was omitted in later drug enhancement protocols, in which we used the method of *X*/*Y* quotient calculation by using only the vials inoculated with the 10-fold-diluted inoculum and viable count determinations (15).

Combined action. The action of the combination of drug plus inhibitor against MAC strains was evaluated as reported recently (6, 7, 15). In brief, the combined action was equal to *X*/*Y*, where *X* was the BACTEC GI obtained with a combination of cell wall inhibitor plus drug and *Y* was the lowest GI obtained at the same time for the drug or the inhibitor used alone. An *X*/*Y* of 1 indicated that there was no interaction between the two, an *X*/*Y* of <0.5 indicated enhanced drug action, and an *X*/*Y* of >2.0 indicated the presence of antagonism between the drug and the inhibitor. In the present work, combined action was calculated 6 and 8 days after the drug and the inhibitor were added to MAC cultures. In addition, counts of viable bacteria in the control vial at the time of inoculation and after 8 days of incubation were determined by plating serial dilutions on 7H11 agar medium and measuring the CFU per milliliter after 21 days of incubation at 37°C. These control values were compared with the values obtained for the test vials containing drug and cell wall inhibitor after 8 days of incubation. CFU were determined in all cases in which the *X*/*Y* quotient was <0.5, permitting us to obtain drug activity enhancement data not only by BACTEC radiometry but also by conventional viable count determinations.

For studies of the bactericidal effect of INH, concentrations of 0.2 and 4 μg/ml were used; the first corresponded to the critical concentration in routine drug susceptibility testing for tuberculosis (14), and the latter corresponded to the concentration achieved in human serum after a single oral administration of INH (12). All the amphipathic INH derivatives were used at concentrations representing their INH moieties alone.

RESULTS AND DISCUSSION

MICs determined by the 1% proportion method on 7H11 agar plates confirmed the high resistance of MAC organisms to the native, hydrophilic INH molecule: among the 17 strains studied, none was susceptible to INH, even at 10 μg/ml. However, when a C_{16} fatty acid side chain was attached to the essentially hydrophilic INH molecule, the resulting amphipathic derivative (INH-PALM; Fig. 1) was much more active against the MAC bacilli: among the above-mentioned 17 INH-resistant strains, 4 became susceptible to as little as 1, 12 to 5.0, and 14 to 10, μg of INH-PALM per ml. The other amphipathic derivatives of INH synthesized, 1-isonicotinyl-2-(12-hydroxydodecanoyl) hydrazine and 1-isonicotinyl-2-[12(1αD-mannopyranosyl) dodecanoyl] hydrazine (16; Fig. 1), were, respectively, less active against MAC bacteria than INH-PALM was (among 14 MAC strains tested, only 3 became susceptible to 5 and to 8 to 10 μg/ml) and ineffective. These two derivatives were consequently not used in further studies.

We recently showed enhancement of drug susceptibility of MAC bacteria by inhibitors of cell envelope synthesis (15). Consequently, we decided to investigate whether inhibition of the bacterial OL by FL-PHE could further potentiate the activity of the INH-PALM molecule.

Typical experiments showing the effect of the amphipathic derivative of INH alone and in association with FL-PHE (an inhibitor of the bacterial OL; 5, 15) and various control experiments (using the 10-fold-diluted bacterial inoculum) are shown in Fig. 2. INH-PALM used alone at 4 μg/ml was highly growth inhibitory against *M. avium* ATCC 15769 (a final GI of only 12 at day 8 in the test vial as compared with a final GI of 999 in the parallel control vial); INH-PALM used in association with FL-PHE was bactericidal. That FL-PHE enhanced the action of INH-PALM against *M. avium* was clear from our observations that in the vial containing FL-PHE and 4 μg of INH-PALM per ml, the daily GI remained at 0 during the course of the investigation (8 days). In contrast, FL-PHE had no effect on the activity of the native INH molecule (15), and it only inhibited the growth of bacteria (but to a much lesser extent than with INH-PALM) when used with the palmitate control.

We confirmed these BACTEC radiometric data by determining bacterial viable counts (Fig. 3). The bacterial counts in the control BACTEC vial at the time of drug addition were assigned a value of 1, and the growth inside control and test vials was compared after 8 days of incubation at 37°C by plating the serially diluted bacterial suspensions from the vials onto 7H11 agar medium. Both the radiometric and viable count data (Fig. 2 and 3) showed the INH-PALM used alone at 4 μg/ml was highly bacteriostatic and that INH-PALM used in combination with 50 μg of FL-PHE per ml was bactericidal.

We next investigated the enhancement of INH-PALM action by FL-PHE against seven MAC strains (ATCC 15769, three laboratory-maintained strains, and three recent clinical isolates). For drug-cell wall inhibitor combinations, the criteria for evaluating enhanced action by BACTEC radiom-

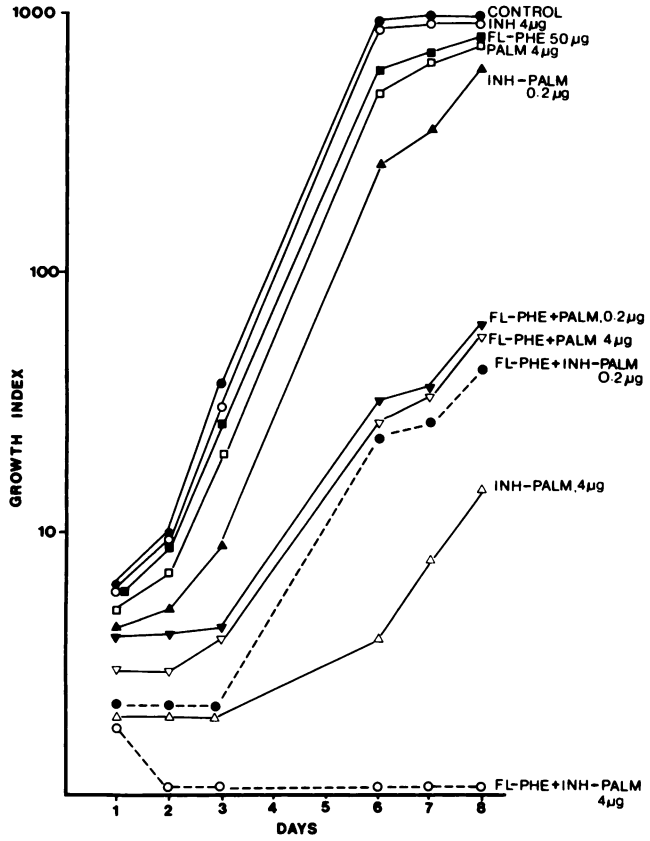

FIG. 2. Radiometric data showing the effects of the native INH molecule (0.2 [control] and 4 μg/ml), its amphipathic derivative INH-PALM (0.2 and 4 μg/ml, in terms of the INH moiety only), and FL-PHE (50 μg/ml) used alone and in combinations against *M. avium* ATCC 15769. Parallel controls included palmitate (PALM) used alone and in association with FL-PHE. Bacterial growth is represented in terms of BACTEC GI values measured for 8 days after the addition of drugs and inhibitors to 7H12a broth vials inoculated with 0.1 ml of a 10-fold-diluted inoculum (see the text for further details).

etry were the same as those defined earlier (15). The INH-PALM–FL-PHE combination was highly active against MAC bacteria, as all seven strains tested were found to be susceptible to this combination. In contrast, FL-PHE could not potentiate the activity of the native INH molecule (15; this study), suggesting that inhibition of the MAC OL alone was not sufficient for the native INH molecule to attain the bacterial membrane; it also needed to be rendered amphipathic.

These results are in agreement with our earlier proposition that the *M. avium* cell envelope acts as an exclusion barrier, not permitting the standard antituberculosis drugs to attain the bacterial membrane (1, 13). It was shown earlier that most of the drugs which are effective against MAC bacteria are essentially hydrophobic molecules that interact with the surface amphiphiles and that are capable of dissolving in the lipids forming the OL (2). However, increased liposolubility of a drug in the OL alone is not sufficient to make it active against the bacteria, as penetration through the periplasmic space and ultimate drug activity would depend on the size of the molecule and the inherent drug susceptibility or resistance characteristics of the bacteria (2, 16).

MAC bacteria utilize palmitic acid during their active multiplication (9, 10). It is therefore possible that INH-PALM was first dissolved in the bacterial OL and that palmitic acid was then used as a source of energy, thus liberating the native INH molecule inside the bacteria, where it could exert its natural antimycobacterial effect. In this connection, one should reinvestigate the earlier observations of McCarthy (11), who showed variations in the INH susceptibility of *M. avium* bacteria during the cell cycle.

The present investigation thus underlines the following points. (i) INH, which is completely ineffective against MAC bacteria but highly bactericidal against *M. tuberculosis*, may be rendered active against MAC bacteria by conversion into new amphipathic derivatives. (ii) The use of antituberculosis drugs in association with various cell wall inhibitors may be a strategy of choice for combating diseases caused by *M. avium*.

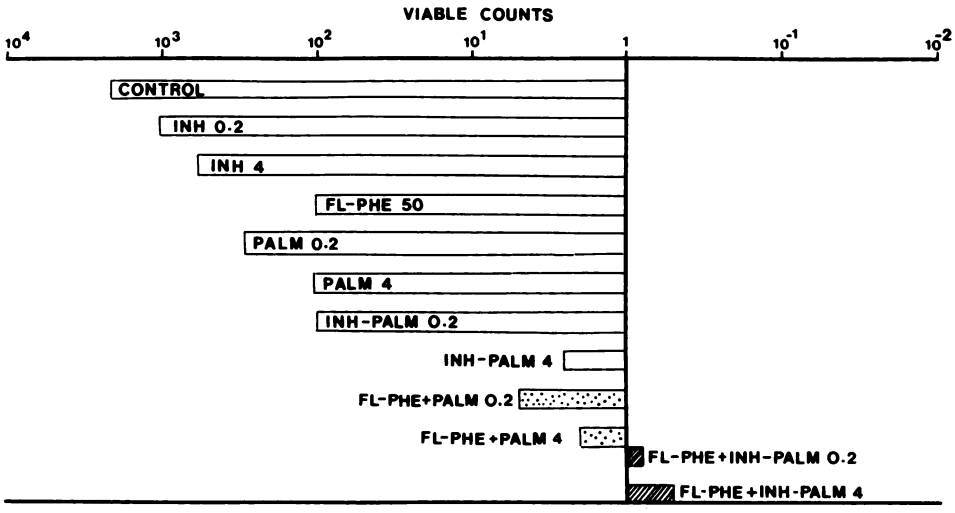

FIG. 3. Viable count data showing the antibacterial activity of the amphipathic derivative of INH (INH-PALM) as compared with that of the native molecule and enhancement of the anti-*M. avium* action in the presence of FL-PHE. Also shown are data for parallel controls done with palmitate (PALM) alone and in association with FL-PHE. All the vials were inoculated with the same number of bacilli (about 10^4 CFU, represented as 1 on the figure). The control bar shows the total bacterial growth after 8 days of incubation at 37°C, the other bars on the left-hand side show the relative growth inhibition, and the bars on the right-hand side show the relative bactericidal effects. Concentrations are in micrograms per milliliter.

Recently, Jarlier and Nikaido (8) reported a similar barrier for hydrophilic solutes in *M. chelonei*. Our observations (15; this study) further confirm that circumventing the exclusion barrier for hydrophilic solutes at the cell envelope level may be the strategy of choice in fighting the battle against opportunistic infections caused by the multiple-drug-resistant atypical mycobacteria.

This report is the first showing the anti-MAC activity of an INH derivative used at achievable concentrations in serum and showing that the synthesis of new amphipathic derivatives of INH may now be undertaken by the pharmaceuticals industry. The clinical relevance of such an approach should now be evaluated: e.g., the likelihood of oral absorption, intravenous solution, stability in serum, etc. It would also be interesting to attach to the INH molecule lipidic side chains which are readily absorbed by hosts but which are only specifically catabolized by the invading bacteria.

ACKNOWLEDGMENTS

We thank B. Moreau and M.-L. Capmau for kindly providing INH-PALM and B. Quiviger (Becton-Dickinson France SA) for kindly lending the BACTEC 460-TB apparatus and 7H12a medium. We are grateful to H. L. David for helpful discussions.

LITERATURE CITED

1. **David, H. L.** 1981. Basis for the lack of drug-susceptibility of atypical mycobacteria. Rev. Infect. Dis. **3**:878–884.
2. **David, H. L., S. Clavel-Sérès, F. Clément, and K. S. Goh.** 1987. Uptake of selected antibacterial agents in *Mycobacterium avium*. Zentralbl. Bakteriol. Mikrobiol. Hyg. Ser. A **265**:385–392.
3. **David, H. L., N. Rastogi, S. Clavel-Sérès, and F. Clément.** 1988. Alterations in the outer wall architecture caused by the inhibition of mycoside C biosynthesis in *Mycobacterium avium*. Curr. Microbiol. **17**:61–68.
4. **David, H. L., N. Rastogi, S. Clavel-Sérès, F. Clément, and M.-F. Thorel.** 1987. Structure of the cell envelope of *Mycobacterium avium*. Zentralbl. Bakteriol. Mikrobiol. Hyg. Ser. A **264**:49–66.
5. **Etzkorn, E. T., S. Aldarondo, C. K. McAllister, J. Matthews, and A. J. Ognibene.** 1986. Medical therapy of *Mycobacterium avium-intracellulare* pulmonary disease. Am. Rev. Respir. Dis. **134**:442–445.
6. **Hoffner, S. E., G. Källenius, A. E. Beezer, and S. B. Svenson.** 1989. Studies on the mechanisms of synergistic effects of ethambutol and other antimycobacterial drug combinations on *Mycobacterium avium* complex. Acta Leprol. **7**(Suppl. 1):195–199.
7. **Hoffner, S. E., S. B. Svenson, and G. Källenius.** 1987. Synergistic effects of antimycobacterial drug combinations on *Mycobacterium avium* complex determined radiometrically in liquid medium. Eur. J. Clin. Microbiol. **6**:530–535.
8. **Jarlier, V., and H. Nikaido.** 1990. Permeability barrier to hydrophilic solutes in *Mycobacterium chelonei*. J. Bacteriol. **172**: 1418–1423.
9. **McCarthy, C.** 1971. Utilization of palmitic acid by *Mycobacterium avium*. Infect. Immun. **4**:199–204.
10. **McCarthy, C.** 1974. Effect of palmitic acid utilization on cell division in *Mycobacterium avium*. Infect. Immun. **9**:363–372.
11. **McCarthy, C.** 1981. Variation in isoniazid susceptibility of *Mycobacterium avium* during the cell cycle. Am. Rev. Respir. Dis. **123**:450–453.
12. **McClatchy, J.** 1980. Antituberculosis drugs: mechanisms of action, drug resistance, susceptibility testing, and assays of activity in biological fluids, p. 135–169. *In* V. Lorian (ed.), Antibiotics in laboratory medicine. The Williams & Wilkins Co., Baltimore.
13. **Rastogi, N., C. Fréhel, A. Ryter, H. Ohayon, M. Lesourd, and H. L. David.** 1981. Multiple drug resistance in *Mycobacterium avium*: is the wall architecture responsible for the exclusion of antimicrobial agents? Antimicrob. Agents Chemother. **20**:666–677.
14. **Rastogi, N., K. S. Goh, and H. L. David.** 1989. Drug susceptibility testing in tuberculosis: a comparison of the proportion methods using Löwenstein-Jensen, Middlebrook 7H10 and 7H11 agar media and a radiometric method. Res. Microbiol. **140**:405–417.
15. **Rastogi, N., K. S. Goh, and H. L. David.** 1990. Enhancement of drug susceptibility of *Mycobacterium avium* by inhibitors of cell envelope synthesis. Antimicrob. Agents Chemother. **34**:759–764.
16. **Rastogi, N., B. Moreau, M.-L. Capmau, and H. L. David.** 1988. Antibacterial action of amphipathic derivatives of isoniazid against the *Mycobacterium avium* complex. Zentralbl. Bakteriol. Mikrobiol. Hyg. Ser. A **268**:456–462.
17. **Woodley, C. L., and H. L. David.** 1976. Effect of temperature on the rate of the transparent to opaque colony type transition in *Mycobacterium avium*. Antimicrob. Agents Chemother. **9**:113–119.
18. **Young, L. S., C. B. Inderlied, O. G. W. Berlin, and M. S. Gottlieb.** 1986. Mycobacterial infections in AIDS patients with an emphasis on the *M. avium* complex. Rev. Infect. Dis. **8**:1024–1033.