In Vitro Chemotherapeutic Combinations Against Isoniazid-Resistant *Mycobacterium tuberculosis* and *Mycobacterium fortuitum*

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It is an acceptable medical practice to use second-line antimycobacterial drugs in combination with isoniazid in treatment of isoniazid-resistant tuberculosis. Recent investigations have demonstrated the importance of determining chemotherapeutic interaction in instances of multiple antibiotic use. We studied the inhibitory effect of combinations of isoniazid with ethambutol, rifampin, ethionamide, cycloserine, viomycin, and kanamycin against three isoniazid-resistant strains of *Mycobacterium tuberculosis* and three strains of *M. fortuitum*. The isobologram technique with drug concentrations of 0.4 to 100 μg/ml was used. With the exception of single instances in which kanamycin plus isoniazid (*M. tuberculosis* strain 9999) and ethionamide plus isoniazid (*M. fortuitum* strain 2080) seemed to have a synergistic effect, neither synergy nor antagonism was noted for any of the combinations. These studies show that the combined use of isoniazid and a second line antimycobacterial agent results in vitro in indifferent inhibitory activity.

It is an acceptable medical practice to use second-line antimycobacterial drugs in combination with isoniazid in the treatment of isoniazid-resistant tuberculosis. The rational for this seemingly contradictory practice is that resistance within a mycobacterial population varies and some of the microorganisms remain susceptible to isoniazid. This argument presupposes that the antibacterial action of isoniazid is unaffected by the presence of other antimicrobials. Recent investigations have demonstrated the importance of proving this assumption since chemotherapeutic interaction in instances of multiple antibiotic use may produce either a synergistic or an antagonistic effect rather than an indifferent (or additive) one (13, 21). In the present study, we evaluated the inhibitory effect of combinations of isoniazid with ethambutol, rifampin, ethionamide, cycloserine, viomycin, and kanamycin against three isoniazid-resistant strains of *Mycobacterium tuberculosis* and three strains of *M. fortuitum*.

MATERIALS AND METHODS

Isoniazid, ethambutol, ethionamide, cycloserine, viomycin, kanamycin, and rifampin were kindly provided by the respective manufacturers. With the exception of rifampin, each drug was dissolved in sterile water and appropriately diluted to provide concentrations of 100, 50, 25, 16.6, 12.5, 6.2, 3.1, 1.6, 0.8, and 0.4 μg/ml when dissolved in 15 ml of Middlebrook 7H-10 media. Rifampin was dissolved in 10% methanol, and serial dilutions were made with distilled water. A maximum drug concentration of 100 μg/ml was arbitrarily chosen since data obtained from higher concentrations would be unlikely to have clinical relevance.

**Microorganisms.** Three isoniazid-resistant strains of *M. tuberculosis* and three of *M. fortuitum* were used. Two strains of *M. tuberculosis* (303 and 326) were kindly supplied by Hyun K. Kim of the Trudeau Institute at Saranac Lake. The third (9999) was from a patient with pulmonary tuberculosis at Mattapan Chronic Disease Hospital. Fifty per cent or more of the microorganisms present in each strain were capable of growing in media containing 10.0 μg of isoniazid per ml and more than 1% grew in media containing 100 μg/ml. Two strains of *M. fortuitum* (A3 and A10) were from the Battey Hospital Collection at Rome, Ga. The third (2080) was isolated from an apparently noninfected patient at Mattapan Chronic Disease Hospital. All three isolates of *M. fortuitum* were not pigmented and grew on Middlebrook 7H-10 and MacConkey agar within 5 days. They were niacin test-negative and reduced both nitrate and tellurite in 3 days; the arylsulfatase test was positive in 3 days. More than 1% of the microorganisms of strain 2080

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grew in media containing 1.6 μg of isoniazid per ml; a similar percentage of cells from strain A10 were resistant to 25 μg of isoniazid per ml; and almost all cells of A3 were resistant to 100 μg of isoniazid per ml. The strains were maintained by monthly transfer on Lowenstein-Jensen Medium.

Isobologram technique. The isobologram technique used in these experiments measured the combined antimycobacterial activity of isoniazid and a second line antimycobacterial drug against M. tuberculosis or M. fortuitum. A 0.5-ml amount of each dilution of isoniazid and the appropriate second-line drug dilution were pipetted into petri dishes in such a manner that each petri dish contained a particular concentration of the two drugs, and all possible drug combinations were represented. A 14-ml amount of Middlebrook 7H-10 media was added to each petri dish to make a final concentration of 0 to 100 μg/ml for each drug.

Each strain of M. tuberculosis was inoculated into 45 ml of Middlebrook 7H-9 broth containing Tween 80 and incubated at 37°C for 2 weeks. On the day of the experiment, 1 ml of the culture was diluted to 100 ml with sterile saline, and 0.1 ml of this dilution was inoculated into each petri dish. The concentration of the mycobacterial inoculum was determined by serial dilution techniques. The cultures containing different concentrations of antibiotics were incubated for 15 days before mycobacterial growth was assessed. Minimal inhibitory concentrations of the drugs were considered to be present when growth was less than 1% of that of control plates.

The same procedures were followed in the experiments with M. fortuitum with the exceptions that 5- to 7-day-old Middlebrook 7H-9 broth cultures were used, and the culture plates were examined after 5 days of incubation.

The isobologram data were interpreted by using the criteria mentioned by Jawetz (13) and Sabath (21). Synergy was present if the effect produced by the combination was greater than the sum of effects produced by each of the components. Antagonism was present if the effect of the combination was less than that produced by the sum of the effects of the components. Indifference occurred when the effect of the combination equaled the effect of the single more active component of the mixture or the arithmetic sum of the effects of the two individual drugs. To take into account errors in dilution and plating inherent in these techniques for determining drug activity, a greater than twofold difference in concentration from the theoretical additive effect was considered the minimal level for significance.

RESULTS

Combining isoniazid with a second line antimycobacterial agent resulted in an indifferent effect in almost all instances. With two exceptions, neither a synergistic nor an antagonistic effect was noted in these experiments.

Figure 1 shows the isobolograms for M. tuberculosis strains 303, 326, and 9999, isolates resistant to 100 μg of isoniazid per ml. The susceptibility of strain 303 to second line antimicrobial agents varied from 0.4 μg/ml for rifampin to 25 μg/ml for cycloserine. Indifference with the effect equaling that of the more active component occurred when kanamycin or rifampin was combined with isoniazid. An additive effect occurred when isoniazid was combined with ethambutol, viomycin, ethionamide, and cycloserine. The inhibitory concentrations of these combinations were less than the minimal inhibitory concentration of the second-line antibiotic alone (Fig. 1).
The susceptibility of *M. tuberculosis* strain 326 to second-line antimicrobial agents varied from 0.4 µg/ml for ethambutol and rifampin to 12.5 µg/ml for cycloserine and ethionamide. Indifference was noted for each combination with the antibacterial effect equal to the effect of the second-line antimicrobial agent.

*M. tuberculosis* strain 9999 was resistant to 100 µg of isoniazid per ml. Susceptibility to the other antimicrobials varied from 1.6 µg of rifampin per ml to 100 µg/ml for ethionamide and viomycin. With the exception of the kanamycin plus isoniazid isobologram, antibacterial activity was due to the second-line agent alone (isoniazid plus ethambutol, cycloserine, viomycin) or an additive effect of the two antimicrobials (isoniazid plus rifampin or ethionamide). The inward bowing in the kanamycin plus isoniazid isobologram suggests a synergistic interaction. However, since this increase in antimycobacterial activity is a twofold dilution difference, it is difficult to be certain that the enhancement in activity of the combination is not an additive one.

*M. fortuitum* strain 2080 was susceptible to 3.1 or 6.2 µg of isoniazid per ml. Susceptibility to the other antimicrobials varied from 3.6 µg of ethambutol per ml to more than 100 µg/ml for cycloserine. The isobolograms obtained with strain 2080 showed an additive effect for all combinations except isoniazid plus cycloserine, in which the extreme resistance of the strain to cycloserine prevented determination of antibiotic interaction at the concentrations studied, and isoniazid-ethionamide, in which the combination demonstrated a small degree of synergy. Intermediate points were present in each isobologram, illustrating the additive effect of the combinations (Fig. 2).

Similar results were obtained with strain A10 which was susceptible to 50 µg of isoniazid per ml and showed an additive effect when isoniazid was combined with a second-line antimycobacterial agent. Strain A3 was resistant to more than 100 µg of isoniazid and cycloserine per ml, 50 µg of kanamycin, viomycin, and ethionamide per ml, 17 µg of ethambutol per ml, and 1.6 µg of rifampin per ml. Combination of isoniazid with these agents resulted either in no antibacterial effect or an indifferent effect which equaled that of the second-line drug. Synergy or antagonism did not occur in any of these experiments. The inoculum of *M. tuberculosis* and *M. fortuitum* varied between 10^6 and 6 × 10^8 colony-forming units for each of the isobologram studies.

**DISCUSSION**

The present study demonstrates in vitro that combining isoniazid with a second-line antimycobacterial agent results in an indifferent antibacterial effect against isoniazid-resistant mycobacteria in almost all instances. With the exception of the isoniazid plus ethionamide isobologram for *M. fortuitum* strain 2080 and the possible exception of the isoniazid plus kanamycin isobologram for *M. tuberculosis* strain 9999, the second-line drug effect was independent of the isoniazid effect. Hence none of the combinations can be considered more or less advantageous than any other. We do not have an explanation for the two instances of synergy, but this type of individual strain variation has been noted with other microorganisms (5, 6).

A few in vivo investigations utilizing isoniazid-susceptible tubercle bacilli have been reported in which comparisons of antituberculous activity of
isoniazid alone and isoniazid in combination with streptomycin, p-aminosalicylic acid, or pyrazinamide have been made (16, 17). With the exception of the isoniazid-pyrazinamide combination which showed some enhancement of antituberculous activity when compared with the activity of either agent alone, interacting drug effects were not noted for any of the combinations.

Information concerning the mechanisms of action of the antituberculous drugs varies from considerable detail for cycloserine to virtually nothing for viomycin. These agents have usually been studied empirically for antimycobacterial effect. Only after demonstration of activity has the biochemical basis for the activity been explored. The history of isoniazid illustrates this point (18).

The compound was synthesized in 1912 (19). In the late '40s and early '50s antituberculous activity was noted and intensive pharmacodynamic experimentation was initiated (1, 2). These subsequent studies have shown that isoniazid is bactericidal for mycobacteria that are actively multiplying (22). Various mechanisms, such as chelation of metallic ions necessary for enzyme function (25), interference with nucleic acid synthesis (9), or combining with an essential enzyme necessary for cellular metabolism (26) have been proposed to explain the activity of isoniazid, but none has been proven.

Of the second-line antimycobacterial agents, the mechanism of action of cycloserine is best understood. This compound is a structural analogue of D-alanine, an important component of the cell walls of mycobacteria. Physicochemical similarity appears to allow cycloserine to interfere competitively with the racemization of L-alanine to produce the D-alanine necessary for cell wall synthesis (23). The specificity of this inhibition is such that the presence of D-alanine reverses the effect of cycloserine (11, 12). The mechanism of action of kanamycin also appears to be well understood. This drug is an aminoglycoside similar to streptomycin and like the latter it causes a misreading of the genetic code (4, 10). The misreading occurs at the soluble ribonucleic acid-ribosome level resulting in defective protein synthesis. Rifampin is a new semisynthetic derivative of rifamycin which is active against M. tuberculosis at extremely low concentrations (15). This antimicrobial inhibits protein synthesis through interference with ribonucleic acid polymerase (24).

The mechanism of action of ethambutol is unknown. This drug is chemically unrelated to any other antimycobacterial agent. It has been shown to affect actively multiplying cells (8). Preliminary evidence has been presented which may support inhibition of ribonucleic acid synthesis as the primary mode of action (7, 14).

Ethionamide resembles isoniazid in chemical structure, whereas viomycin has a distinct structure. At present too little information is available to assess the mode of action of these antimicrobics.

It is apparent from the above discussion that present knowledge concerning the mechanisms of action of these chemotherapeutic agents is insufficient to allow accurate prediction of isoniazid interaction with second-line agents. However, it would appear that with the exception of ethionamide, differences in structure and sites of action mitigate against direct interaction. This view receives indirect support from empirical evidence that development of resistance to isoniazid does not confer cross-resistance to second-line agents. Also consistent are the present data since, in vitro, an indifferent type of antibiotic interaction was demonstrated with isoniazid-resistant mycobacteria.

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LITERATURE CITED

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