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# In Vitro Activity of Levofloxacin, Singly and in Combination with Rifamycin Analogs, against *Mycobacterium leprae*

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**The in vitro susceptibility of *Mycobacterium leprae* to levofloxacin was studied by using two biochemical parameters to measure the metabolic activity of the organism. Levofloxacin consistently exhibited twofold greater bactericidal activity than ofloxacin, with the MIC being 0.75 μg/ml. When combined with one of the three rifamycin analogs, synergism was obtained with KRM-1648 and rifabutin but not with rifampin.**

The frontline drugs against leprosy since 1947 have been either dapsone, clofazimine, or rifampin. In 1981, the World Health Organization recommended the use of multidrug therapy consisting of these three drugs (20), and this has resulted in the reduction of the global prevalence of leprosy by about 57% (17). However, there are still 3.1 million cases of leprosy in the world, and about 600,000 new cases are being detected annually (17). Thus, the research will have to continue to develop new bactericidal drugs and also to formulate new multidrug therapy regimens for the treatment of multibacillary leprosy that will give better results in much shorter time.

The use of fluoroquinolones in the treatment of mycobacterial diseases, including tuberculosis, is now well accepted (13, 19, 21). Grosset and associates (10, 11) have found ofloxacin to be the most active agent against *Mycobacterium leprae* in both mice and patients with leprosy. They have shown that about 99.99%, or 4 logs, of *M. leprae* viable on day zero were killed by 22 doses of ofloxacin. Except for rifampin, no other drug thus far tested in humans with leprosy has demonstrated such a degree of bactericidal activity (14). Recently, Tomioka and Saito (18) have reported that use of the combination of ofloxacin and rifampin gave a significant increase in their efficacies. Using an in vitro system (7) and a mouse footpad model (5), we have observed a synergistic effect when ofloxacin was combined with rifabutin but not when it was combined with rifampin.

Ofloxacin exists as two optically active isomers because of the asymmetric center at the C-3 of the oxazine ring, and levofloxacin (the optically active L isomer of ofloxacin) has shown high levels of antibacterial activity against a number of bacterial species both in vitro and in mice (9). Recently, Heifets and associates (16) have observed a twofold greater inhibitory activity of levofloxacin over that of ofloxacin against extracellular as well as intracellular tubercle bacilli.

The aim of the present study was therefore to evaluate the activity of levofloxacin in comparison with that of ofloxacin against *M. leprae* in an in vitro system and also to study its synergism when it was combined with rifamycin analogs.

**Antimicrobial agents.** Levofloxacin and ofloxacin were obtained from the R. W. Johnson Pharmaceutical Research Institute (Raritan, N.J.). Stock solutions of levofloxacin and ofloxacin were prepared by first dissolving them in small amounts of 0.1 M NaOH and then diluting those solutions with distilled water to obtain appropriate working solutions. Three rifamycin analogs were tested: rifampin (Sigma Chemical Co., St. Louis, Mo.), rifabutin (Pharmacia-Adria Laboratories, Columbus, Ohio), and KRM-1648 (Kaneka Corp., Osaka, Japan). Stock solutions of the rifamycin analogs were prepared by dissolving them in small volumes of methanol and then diluting further with distilled water. Each working solution was then filter sterilized through a GA-6 membrane filter (pore size, 0.22 μm; Gelman Sciences, Inc., Ann Arbor, Mich.).

**Isolation of *M. leprae*.** *M. leprae* cells were harvested from the livers of nine-banded armadillos (*Dasypus novemcinctus*) that had previously been inoculated with human- or armadillo-derived *M. leprae*. Purification of the *M. leprae* suspension was carried out by using DNase and a Percoll gradient (3). Cell counts were determined microscopically by the pinhead method (4), and the cells were inoculated into DH medium to obtain $10^7$ cells per ml of medium.

**Growth medium.** The culture medium (DH medium) and the conditions used for the maintenance and growth of *M. leprae* were the same as those described earlier (8).

**Measurement of growth.** Two biochemical parameters were used to measure the metabolism (and growth) of *M. leprae*: the intracellular ATP content of *M. leprae* and the uptake of [$^3$H]thymidine by *M. leprae* cells (2). ATP determinations were carried out by the firefly bioluminescent technique described earlier (3), while for the measurement of thymidine uptake, the procedure of Khanolkar and coworkers (15) was followed.

Since the metabolic activity (and in vitro growth) of *M. leprae* is determined by using biochemical parameters, partial loss of metabolic activity does not mean that proportional numbers of the organisms in the aliquots taken have become nonviable. Thus, concentration of a drug that inhibits the metabolic activity of organism by 50% of its original activity cannot be taken as the MIC of that compound that inhibits 50% of isolates tested. Therefore, the MIC of the compound is defined here as the concentration of the compound that completely inhibits the metabolic activity of *M. leprae* in DH medium.

The effect of drug on *M. leprae* in primary culture was evaluated after incubating cultures for 4 weeks at 34°C. To determine if the effects were bacteriostatic or bactericidal, the cells from 4-week-old primary cultures were washed twice with fresh DH medium and were then suspended in fresh DH medium without drugs and incubated at 34°C for 4 more weeks. At this time, the ATP and [$^3$H]thymidine uptake assays were again performed.

**Evaluation of drug interactions.** The interactions between levofloxacin and either rifampin, rifabutin, or KRM-1648 against *M. leprae* were investigated by combining each drug at

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TABLE 1. Effect of levofloxacin (and ofloxacin) on metabolic activity (in vitro growth) of *M. leprae* in DH medium

| Agent and concn (μg/ml) | Metabolic activity of *M. leprae*: | | | |
|---|---|---|---|---|
| | In primary cultures at 4 wk[a] | | In subcultures at 4 wk | |
| | ATP (pg/$10^7$ cells) | [$^3$H]thymidine (pmol/5 × $10^7$ cells) | ATP (pg/$10^7$ cells) | [$^3$H]thymidine (pmol/5 × $10^7$ cells) |
| None | 352 ± 49 | 1.06 ± 0.13 | 524 ± 57 | 1.61 ± 0.22 |
| Levofloxacin | | | | |
| 0.0938 | 337 ± 40 | 0.99 ± 0.15 | 462 ± 69 | 1.43 ± 0.16 |
| 0.1875 | 303 ± 33 | 0.92 ± 0.09 | 376 ± 53 | 1.20 ± 0.18 |
| 0.375 | 189 ± 28 | 0.59 ± 0.08 | 217 ± 26 | 0.72 ± 0.09 |
| 0.75[b] | 0[c] | 0 | 0 | 0 |
| 1.5 | 0 | 0 | 0 | 0 |
| 3.0 | 0 | 0 | 0 | 0 |
| 6.0 | 0 | 0 | 0 | 0 |
| Ofloxacin | | | | |
| 0.75 | 181 ± 20 | 0.55 ± 0.06 | 228 ± 34 | 0.74 ± 0.10 |
| 1.5[b] | 0 | 0 | 0 | 0 |

[a] Zero hour values were as follows: ATP, 226 ± pg/$10^7$ cells; [$^3$H]thymidine, 0.64 ± 0.07 pmol/5 × $10^7$ cells.
[b] MIC of each compound.
[c] Indicates no metabolic activity.

concentrations lower than their respective MICs; the MICs of rifampin (0.4 μg/ml), rifabutin 0.2 μg/ml), and KRM-1648 (0.05 μg/ml) have been determined previously (6, 7). The bactericidal effect of each combination was estimated by calculating the fractional inhibitory concentration (FIC), also known as the interaction index, following determination of the MIC of each agent alone and in combination, as described above. The FIC was calculated by the following formula (12):

$$\text{FIC} = \frac{\text{MIC of A in the presence of B}}{\text{MIC of A alone}} + \frac{\text{MIC of B in presence of A}}{\text{MIC of B alone}}$$

where A and B are levofloxacin and a rifamycin analog, respectively. Synergy was defined as a FIC of ≤0.5, an additive effect was defined as a FIC of >0.5 and <2.0, and antagonism was defined as a FIC of ≥2.0.

Sampling for the assays from primary cultures and subcultures was done as described previously (7). The results presented here were derived from three separate experiments, with a different *M. leprae* strain used in each experiment. In each experiment, triplicate assays were done for each concentration of levofloxacin and also for each combination. The data were analyzed by the Student *t* test. *P* values of ≤0.05 were considered statistically significant.

**Susceptibility of *M. leprae* to levofloxacin.** In the control cultures, without drugs, the metabolic activity of *M. leprae* in primary cultures at 4 weeks was 162% of that at the zero hour (Table 1). When the cells from these primary cultures were transferred to fresh DH medium, the activity in the subcultures was 150% of the original at the zero hour of these subcultures (the 162% increase in primary cultures was adjusted to 100% at the zero hour for subcultures). With levofloxacin at concentrations of 0.375 μg/ml and lower, the metabolic activity of *M. leprae* in primary cultures as well as in subcultures was the same as that in control cultures. However, when the concentration of levofloxacin in primary cultures was 0.75 μg/ml and greater, no metabolic activity could be detected, thus suggesting that the MIC of levofloxacin for *M. leprae* was 0.75 μg/ml. When these cells from primary cultures were transferred to fresh drug-free DH medium, the cells failed to exhibit any metabolic activity, even after 4 weeks. This further suggests that levofloxacin has bactericidal activity against *M. leprae*. In each of the three experiments, ofloxacin was also included as an experimental control for comparison. For each of the three strains of *M. leprae*, the MICs of levofloxacin and ofloxacin were consistently 0.75 and 1.5 μg/ml, respectively.

**Susceptibility of *M. leprae* to drug combinations.** Levofloxacin at three different concentrations, all less than its MIC for *M. leprae*, was combined with either rifampin, rifabutin, or KRM-1648, each at concentrations less than their individual MICs (Table 2). With levofloxacin at 0.0938 μg/ml, the addition of any of the rifamycin analogs at levels below their individual MICs had no inhibitory effect on the metabolic activity of *M. leprae*. On the other hand, additive effects were observed when 0.2 μg of rifampin per ml was combined with 0.375 μg of levofloxacin per ml. However, 100% inhibition of the metabolic activity of *M. leprae* was observed when levofloxacin at 0.1875 μg/ml was combined with either rifabutin at 0.05 μg/ml or KRM-1648 at 0.0125 μg/ml.

Thus, the results of the present study indicate that levofloxacin at 0.75 μg/ml completely inhibits the metabolic activity of *M. leprae*. Furthermore, synergism was observed when levofloxacin was combined with either rifabutin or KRM-1648 but not when it was combined with rifampin. The superiority of rifabutin and KRM-1648 over rifampin against *M. leprae* has been shown earlier (6, 7). However, at this stage we cannot explain why levofloxacin exhibits synergism with rifabutin and KRM-1648 but not with rifampin. Similar observations have also been made in the case of ofloxacin (6, 7). Perhaps the structure-activity relationships of these rifamycin analogs with fluoroquinolones are worth investigating.

It has been demonstrated previously with *Mycobacterium lepraemurium* (4) and *Mycobacterium tuberculosis* (1) that increases in intracellular ATP levels parallel microscopic counts and viable CFU counts, respectively, during the in vitro growth of these organisms. Recently, we have also observed parallelism between the ATP levels, [$^3$H]thymidine uptake values, microscopic counts, and phenolic glycolipid (PGL-1) levels of *M. leprae* cells incubated in DH medium. Using this system, we have evaluated the antileprosy activities of several compounds, and the in vitro susceptibility results were confirmed by the established mouse footpad system (5–8). On the basis of these

TABLE 2. Effect of levofloxacin in combination with rifampin analogs on metabolic activity (in vitro growth) of *M. leprae* in DH medium

| Concn (μg/ml) | | | | Metabolic activity of *M. leprae*: | | | | FIC |
|---|---|---|---|---|---|---|---|---|
| | | | | In primary cultures at 4 wk[a] | | In subcultures at 4 wk | | |
| Levofloxacin | Rifampin | Rifabutin | KRM-1648 | ATP (pg/$10^7$ cells) | [$^3$H]thymidine (pmol/$5 \times 10^7$ cells) | ATP (pg/$10^7$ cells) | [$^3$H]thymidine (pmol/$5 \times 10^7$ cells) | |
| | | | | 309 ± 46 | 0.87 ± 0.12 | 402 ± 40 | 1.18 ± 0.13 | |
| 0.0938 | 0.1 | | | 287 ± 34 | 0.82 ± 0.09 | 396 ± 47 | 1.18 ± 0.14 | |
| 0.0938 | 0.2 | | | 239 ± 33 | 0.67 ± 0.10 | 287 ± 40 | 0.83 ± 0.10 | |
| 0.0938 | | 0.025 | | 311 ± 34 | 0.90 ± 0.12 | 395 ± 51 | 1.21 ± 0.12 | |
| 0.0938 | | 0.05 | | 291 ± 43 | 0.82 ± 0.09 | 346 ± 38 | 1.01 ± 0.12 | |
| 0.0938 | | 0.1 | | 252 ± 32 | 0.71 ± 0.11 | 262 ± 26 | 0.81 ± 0.09 | |
| 0.0938 | | | 0.0062 | 326 ± 45 | 0.93 ± 0.13 | 456 ± 58 | 1.38 ± 0.17 | |
| 0.0938 | | | 0.0125 | 300 ± 36 | 0.83 ± 0.09 | 369 ± 44 | 1.07 ± 0.11 | |
| 0.0938 | | | 0.025 | 258 ± 28 | 0.73 ± 0.09 | 284 ± 37 | 0.85 ± 0.12 | |
| 0.1875 | 0.1 | | | 311 ± 43 | 0.86 ± 0.13 | 404 ± 44 | 1.15 ± 0.12 | |
| 0.1875 | 0.2 | | | 249 ± 37 | 0.72 ± 0.07 | 266 ± 37 | 0.83 ± 0.09 | |
| 0.1875 | | 0.025 | | 291 ± 38 | 0.82 ± 0.11 | 367 ± 37 | 1.08 ± 0.14 | |
| 0.1875[b] | | 0.05[b] | | 0[c] | 0 | 0 | 0 | 0.5 |
| 0.1875 | | 0.1 | | 0 | 0 | 0 | 0 | 0.75 |
| 0.1875 | | | 0.0062 | 252 ± 30 | 0.71 ± 0.10 | 254 ± 30 | 0.74 ± 0.08 | |
| 0.1875[b] | | | 0.0125[b] | 0 | 0 | 0 | 0 | 0.5 |
| 0.1875 | | | 0.025 | 0 | 0 | 0 | 0 | 0.75 |
| 0.375 | 0.1 | | | 278 ± 41 | 0.80 ± 0.10 | 334 ± 40 | 1.01 ± 0.12 | |
| 0.375[b] | 0.2[b] | | | 0 | 0 | 0 | 0 | 1.0 |
| 0.375 | | 0.025 | | 261 ± 36 | 0.72 ± 0.11 | 264 ± 29 | 1.76 ± 0.08 | |
| 0.375 | | 0.05 | | 0 | 0 | 0 | 0 | 0.75 |
| 0.375 | | 0.1 | | 0 | 0 | 0 | 0 | 1.0 |
| 0.375 | | | 0.0062 | 0 | 0 | 0 | 0 | 0.625 |
| 0.375 | | | 0.0125 | 0 | 0 | 0 | 0 | 0.75 |
| 0.375 | | | 0.025 | 0 | 0 | 0 | 0 | 1.0 |
| 0.75 | | | | 0 | 0 | 0 | 0 | |
| | 0.4 | | | 0 | 0 | 0 | 0 | |
| | | 0.2 | | 0 | 0 | 0 | 0 | |
| | | | 0.05 | 0 | 0 | 0 | 0 | |

[a] Zero hour values were as follows: ATP, 219 ± 28 pg/$10^7$ cells; [$^3$H]thymidine, 0.59 ± 0.06 pmol/$5 \times 10^7$ cells.
[b] MICs for each combination.
[c] Indicates no metabolic activity.

results, it is safe to suggest that the increase in metabolic activity of *M. leprae* observed in the present studies is an indication of the increase in cell numbers in primary cultures, as seen in control cultures to which no drugs were added. Similar increases in the metabolic activity of *M. leprae* were also observed in cultures containing lower concentrations of levofloxacin as well as rifamycin analogs, implying normal growth of *M. leprae* and, thus, no growth inhibitory effects. On the other hand, when exposed to levofloxacin at 0.75 μg/ml or rifampin, rifabutin, and KRM-1648 at 0.4, 0.2, and 0.05 μg/ml, respectively, *M. leprae* lost all metabolic activity and, thus, its ability to multiply in such an environment.

In order to achieve success in controlling leprosy with multidrug therapy, there should be excellent compliance with the treatment regimen, which is best accomplished if the treatment is administered for a short period of time. For this purpose, only bactericidal drugs are to be included in the multidrug treatment regimen so that each drug will kill *M. leprae* isolates resistant to another drug. Levofloxacin has been shown here to be bactericidal and to inhibit the metabolic activity of *M. leprae* at a concentration lower than that at which ofloxacin inhibits *M. leprae* metabolic activity. Levofloxacin also exhibits synergistic activity against *M. leprae* when it is combined with rifabutin or KRM-1648. Thus, it seems that levofloxacin will be a better candidate than ofloxacin for inclusion in the multidrug regimen in the treatment of leprosy.

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