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Activities of WIN-57273, Minocycline, Clarithromycin, and 14-Hydroxy-Clarithromycin against *Mycobacterium avium* Complex in Human Macrophages

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The activities of the fluoroquinolone WIN-57273, 14-OH clarithromycin (a human metabolite of clarithromycin), and minocycline against two virulent strains of *Mycobacterium avium* complex were evaluated in a model of intracellular infection and compared with that of clarithromycin. Human monocyte-derived macrophages were infected at day 6 of culture. Intracellular CFU at 60 min and intracellular and supernatant CFU on days 4 and 7 were counted after inoculation. The concentrations used, which were equal to peak levels in serum, were 3 μg of WIN-57273 per ml (MICs for the two strains, 1 μg/ml), 4 μg of 14-OH clarithromycin per ml (MICs, 8 and 2 μg/ml, respectively, at pH 7.4), 4 μg of minocycline per ml (MICs, 64 and 32 μg/ml, respectively), and 4 μg of clarithromycin per ml (MICs, 2 and 0.5 μg/ml, respectively, at pH 7.4). On day 7, compared with controls, WIN-57273, minocycline ($P < 0.02$), clarithromycin, or different combinations of clarithromycin and the other drugs ($P < 0.001$) slowed the intracellular replication of strain MO-1. 14-OH clarithromycin ($P < 0.02$), clarithromycin ($P < 0.02$), 14-OH clarithromycin plus clarithromycin ($P < 0.01$), clarithromycin plus minocycline, or clarithromycin plus minocycline plus 14-OH clarithromycin ($P < 0.001$) slowed the intracellular replication of strain LV-2. WIN-57273 was less effective than clarithromycin against strain MO-1 ($P < 0.05$). Clarithromycin plus 14-OH clarithromycin plus minocycline ($P < 0.02$) was more effective than clarithromycin alone against strain LV-2. Thus, clarithromycin plus minocycline, which corresponds in humans to three active molecules, may exhibit a better efficacy than clarithromycin in this model.

Mycobacterium avium complex, a primarily intracellular bacterium which multiplies within phagocytic cells, frequently causes infection in patients with AIDS (7, 20). Among new macrolides, clarithromycin was reported to exhibit a good activity against *M. avium* complex in human macrophages (15, 16), in the beige-mouse model of *M. avium* complex infection (8, 12), and in the blood of patients with AIDS, in which it decreases bacterial counts (6).

In addition to the new macrolides, new fluoroquinolones exhibit an in vitro efficacy against *M. avium* complex. Sparfloxacin, the more effective fluoroquinolone in vitro, has been shown to be effective in human macrophages infected with *M. avium* complex (16). New antimicrobial agents and new drug combinations are needed to eradicate *M. avium* complex and to avoid the emergence of resistant strains (5). WIN-57273, a new fluoroquinolone, has been shown to be effective in vitro against *M. avium* complex (9), with a slight efficacy in the beige mouse model (14). Minocycline is a longer-acting tetracycline derivative whose in vitro activity against *M. avium* complex was reported by Tsukamura in 1980 (18). Recently, in view of the high activity of a clarithromycin and minocycline combination against *Mycobacterium leprae* (13), it seemed interesting to test the activity of this combination against *M. avium* complex. In patients with AIDS treated with clarithromycin (2 g/day), the peak level of clarithromycin in serum was accompanied by a very similar concentration of 14-hydroxy-clarithromycin (1, 4). This human metabolite has a longer half-life than clarithromycin, and its trough level is higher than that of clarithromycin. Thus, 14-OH clarithromycin could participate in the therapeutic efficacy of clarithromycin in humans. The efficacy of this metabolite, which is not present in mice, cannot be assessed by clarithromycin treatment of infected mice.

The aim of the present study was to evaluate, in a cell model, the activities of WIN-57273, minocycline, 14-OH clarithromycin alone, and 14-OH clarithromycin in combination with other drugs against *M. avium* complex multiplication within human macrophages. These activities were compared with that of clarithromycin, which was studied in the same model (15).

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MATERIALS AND METHODS

Bacteria. Two strains of *M. avium* complex, MO-1 and LV-2, used in our previous study (16) were isolated from patients with AIDS and used after a single subculture on mycobacterium 7H11 agar (Difco Laboratories, Detroit, Mich.) supplemented with Middlebrook OADC enrichment (Difco). One flat transparent colony of each strain was picked and cultivated at 37°C in Middlebrook 7H9 broth (Difco) supplemented with ADC enrichment (Difco) in Falcon tissue culture flasks (Becton Dickinson Labware, Oxnard, Calif.). After 21 days of culturing, the bacterial sus-

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pension was adjusted to a density of 1 mg/ml with a turbidimeter (Institut Pasteur Production, Paris, France). Counts of CFU on 7H11 agar correlated this density to bacterial concentrations of 1.5×10^8 and 5×10^8 CFU/ml for strains MO-1 and LV-2, respectively. Aliquots of the bacterial suspension were frozen at −80°C.

Antibiotics. The following antibiotics were used: WIN-57273 (Sterling-Winthrop Research Institute, Rensselaer, N.Y.), clarithromycin (Abbott France, Rungis, Val de Marne, France), 14-OH clarithromycin (Abbott France), and minocycline (Lederle laboratories, Oullins, Rhône, France). Stock solutions of each drug were prepared in accordance with the instructions of the manufacturers. From these stock solutions, working solutions were made in distilled water to be incorporated into the media that were used.

The concentration of each antibiotic used was close to the peak concentration obtained in the serum of humans given the antibiotic orally. This concentration was 3 μg of WIN-57273 per ml, 4 μg of clarithromycin per ml, 4 μg of 14-OH clarithromycin per ml, and 4 μg of minocycline per ml.

The following combinations were tested: clarithromycin plus 14-OH clarithromycin, clarithromycin plus minocycline, and clarithromycin plus 14-OH clarithromycin plus minocycline. The concentrations used for the different combinations were the same as those used for the single drugs.

MIC determination by the agar macrodilution method. Serial twofold dilutions of each antibiotic were incorporated into 7H11 agar medium (pH 6.6) plated into quadrant petri dishes. The inoculum was made from a 7-day-old culture in Dubos-Tween medium adjusted to 1 mg/ml (wet weight) and diluted to 10^{-3} and 10^{-5}. From each dilution, 0.05 ml was plated on one quadrant. Every assay was duplicated. Plates were incubated at 37°C, and colonies were counted after 14 days of culture. The lowest concentration of drugs that inhibited more than 99% of the bacterial population was considered the MIC (14).

Since the pH of the medium modifies the MICs of new macrolides, the MIC of clarithromycin was also determined in Mueller-Hinton agar medium (pH 7.4) supplemented with Middlebrook OADC enrichment (16, 17).

Monocyte-derived macrophages. Human monocyte-derived macrophages were obtained from healthy donors. Briefly, 60 ml of peripheral blood was drawn, heparinized with 1 ml of heparin (5,000 IU/ml; Leo), and mixed with 30 ml of RPMI 1640 tissue culture medium (GIBCO Laboratories, Grand Island, N.Y.). This suspension was centrifuged on Ficoll-Hypaque to obtain purified leukocytes. These cells were washed twice in RPMI 1640 medium, and monocytes were counted by the esterase stain method (19). The cell suspension was distributed into Lab-Tek chambers (Miles Scientific, Div. Miles Laboratories, Inc., Naperville, Ill.) to obtain 5×10^5 monocytes per chamber. After sedimentation for 3 h at 37°C, the supernatant containing nonadherent cells was removed, and adherent cells corresponding to monocytes were incubated in RPMI 1640 medium containing 10% human normal serum in a 5% CO_2 atmosphere. This nutrient medium was changed on days 1 and 3. On day 6, a homogeneous monolayer of macrophages was obtained.

Infection of macrophage monolayers. For the inoculum, an aliquot of the frozen suspension of *M. avium* complex was thawed in warm water and diluted with RPMI 1640 tissue culture medium containing 10% of human normal serum to obtain a concentration of 3×10^7 CFU/ml. Macrophages were inoculated on day 6 of culture with 1 ml of *M. avium* complex suspension and were incubated for 60 min at 37°C in a 5% CO_2 atmosphere. After ingestion, extracellular bacteria were removed by four washings with 2 ml of phosphate-buffered saline containing Ca^{2+} ions (pH 7.2) per wash. In control chambers, intracellular bacteria were counted by enumeration of CFU. In other chambers, fresh RPMI 1640 medium containing 10% normal human serum and one of the antibiotics tested was added, and macrophages were incubated as described above.

On day 4 after inoculation, the CFU in supernatants were counted. In half of the chambers, the intracellular CFU were counted; in the other half, culturing was prolonged to day 7. In these latter chambers, supernatant was replaced on day 4 by the same fresh medium containing the antibiotic at the same concentration (see above). On day 7 after inoculation, the intracellular and supernatant CFU were counted separately.

The density of the macrophage monolayer was checked with a microscope and a micrometric ocular before inoculation and at 60 min, day 4, and day 7 after inoculation. Macrophages did not significantly detach during the ingestion phase. The proportions of macrophages that detached from the bottom of the chambers after inoculation were 17% between 60 min and day 4 and 22% between 60 min and day 7.

Extracellular bacteria were removed by four washings with 2 ml of phosphate-buffered saline per wash, and serial dilutions of the bacterial suspension were plated onto 7H11 agar medium. Intracellular bacteria were recovered by disruption of macrophages by introduction of 2 ml of distilled water over 30 min and mechanical shaking, and serial dilutions of this bacterial suspension were plated onto 7H11 agar medium. Plates were incubated at 37°C, and colonies were counted after 14 days of culturing.

Expression of CFU count results. At 60 min after inoculation, only intracellular CFU counts, corresponding to the intracellular inocula, were expressed. In this model, the multiplication of bacteria is mainly intracellular (2, 3). The bacterial load of macrophages that detached from the chambers remained in the supernatant and was counted with the extracellular bacteria. Thus, the total multiplication of bacteria on day 4 was expressed by adding intracellular and supernatant CFU counts on day 4. Since supernatant bacteria were removed from the chambers on day 4 to replace the medium, the total multiplication of bacteria on day 7 was expressed by adding intracellular and supernatant CFU counts on day 7 plus supernatant CFU counts on day 4. For each strain and each antibiotic, the results of CFU counts were expressed as the mean of at least three experiments ± the standard error of the mean.

Statistical analysis. For all strains, results of CFU counts in control experiments and each drug experiment were compared on days 4 and 7 by a one-way analysis of variance. If the F value was significant, a multiple t test was used to compare the means two by two.

RESULTS

MICs of antibiotics for the two strains of *M. avium* complex. The MICs of the antibiotics for strains MO-1 and LV-2 of the *M. avium* complex were, respectively, 1 and 1 μg of WIN-57273 per ml, 64 and 32 μg of minocycline per ml, 8 and 2 μg of clarithromycin per ml (at pH 6.6), and 32 and 8 μg of 14-OH clarithromycin per ml (at pH 6.6). At pH 7.4, the MICs of clarithromycin decreased, respectively, to 2 and 0.5 μg/ml, and the MICs of 14-OH clarithromycin decreased, respectively, to 8 and 2 μg/ml.

Efficacies of the various antibiotics evaluated by CFU

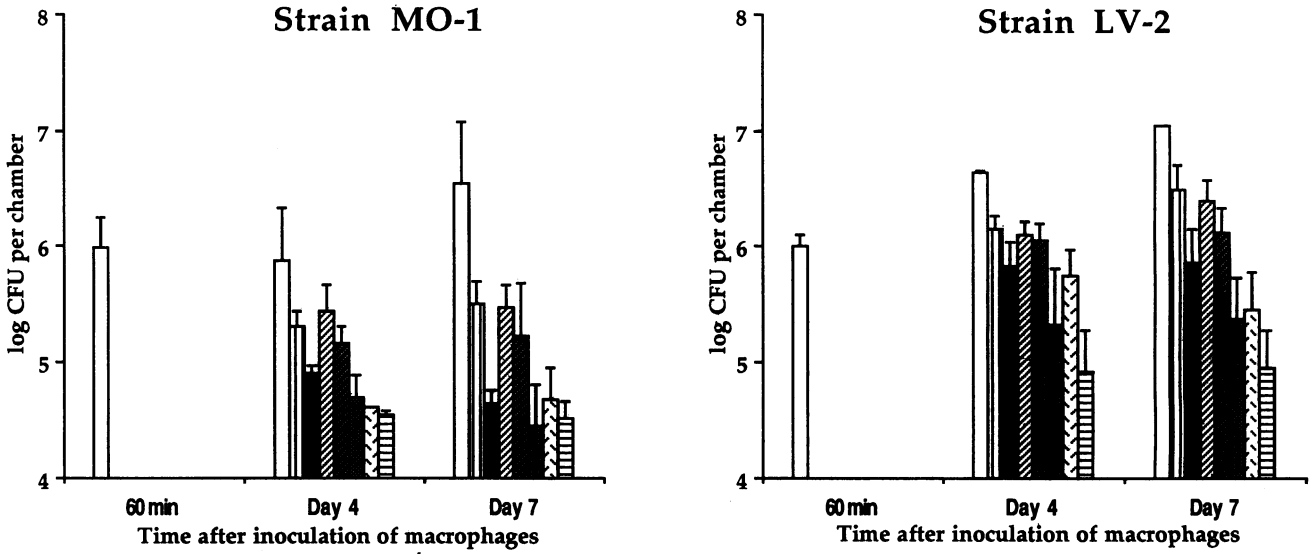

FIG. 1. Comparison of intracellular growth of two strains of the *M. avium* complex in Lab-Tek chambers without antibiotics (controls) and in the presence of different single antibiotics (WIN-57273, 3 μg/ml; clarithromycin, 4 μg/ml; 14-OH clarithromycin, 4 μl/ml; minocycline, 4 μg/ml) or the combinations clarithromycin and 14-OH clarithromycin, clarithromycin and minocycline, and clarithromycin plus 14-OH clarithromycin plus minocycline. The concentrations used for the combinations were the same as those used for single drugs. Results on day 4 represent intracellular plus supernatant CFU counts on day 4. Results on day 7 represent intracellular plus supernatant CFU counts on day 7 plus supernatant CFU counts on day 4, since these bacteria were removed for medium replacement. Results are the mean of at least three experiments ± standard errors of the means. Symbols: □, controls; ▥, WIN-57273; ■, clarithromycin; ▨, minocycline; ■, 14-OH clarithromycin; ▩, clarithromycin plus minocycline; ⧅, clarithromycin plus 14-OH clarithromycin; ⧇, clarithromycin plus minocycline plus 14-OH clarithromycin.

counts. Results of the efficacies of the various antibiotics evaluated by CFU counts are shown in Fig. 1. The mean proportions of the total CFU counts in the supernatant were 21% on day 4 and 23% on day 7. The analysis of variance permitted comparisons between control experiments and each drug experiment for each strain. For both strains, bacillary replication between 60 min and day 7 was observed in control experiments without antibiotic. For strain MO-1, the CFU counts decreased significantly between 60 min and day 7 with clarithromycin ($P < 0.001$), clarithromycin plus 14-OH clarithromycin ($P < 0.01$), clarithromycin plus minocycline ($P < 0.001$), and clarithromycin plus 14-OH clarithromycin plus minocycline ($P < 0.01$) but did not decrease significantly with WIN-57273, 14-OH clarithromycin, or minocycline. For strain LV-2, the CFU counts did not decrease significantly between 60 min and day 7 with any drug, reflecting the bacteriostatic activities of the drugs.

On day 4, compared with controls, WIN-57273 ($P < 0.05$), 14-OH clarithromycin ($P < 0.01$), clarithromycin ($P < 0.001$), and the various combinations with clarithromycin ($P < 0.001$) significantly slowed the intracellular replication of strain MO-1, while minocycline did not.

On day 4, compared with controls, clarithromycin plus 14-OH clarithromycin ($P < 0.05$), clarithromycin plus minocycline ($P < 0.01$), and clarithromycin plus 14-OH clarithromycin plus minocycline ($P < 0.001$) significantly slowed the intracellular replication of strain LV-2, while WIN-57273, clarithromycin, minocycline, and 14-OH clarithromycin did not.

On day 7, compared with controls, WIN-57273 ($P < 0.02$), minocycline ($P < 0.02$), 14-OH clarithromycin ($P < 0.01$), and clarithromycin ($P < 0.001$) and the combinations with clarithromycin ($P < 0.001$) significantly slowed the intracellular replication of strain MO-1.

On day 7, compared with controls, 14-OH clarithromycin ($P < 0.02$), clarithromycin ($P < 0.02$), 14-OH clarithromycin plus clarithromycin ($P < 0.01$), clarithromycin plus minocycline ($P < 0.001$), and clarithromycin plus minocycline plus 14-OH clarithromycin ($P < 0.001$) significantly slowed the intracellular replication of strain LV-2, while WIN-57273 and minocycline did not.

When the efficacies of the regimens against strain MO-1 were compared two by two, no drug alone or in combination was significantly better than clarithromycin alone on days 4 and 7. WIN-57273 was less effective than clarithromycin ($P < 0.05$) on day 7.

When the efficacies of the regimens against strain LV-2 were compared two by two, clarithromycin plus minocycline plus 14-OH clarithromycin was more effective than clarithromycin alone on day 4 ($P < 0.05$) and day 7 ($P < 0.02$), while the other drugs and combinations did not exhibit higher efficacies than clarithromycin alone.

DISCUSSION

In our cell model, clarithromycin or 14-OH clarithromycin slowed the intracellular multiplication of two virulent strains of the *M. avium* complex. Minocycline or the fluoroquinolone WIN-57273 slowed the intracellular multiplication of strain MO-1 but not that of strain LV-2. WIN-57273, at a concentration equal to three times the MIC, was less effective than clarithromycin against strain MO-1. Clarithromycin plus 14-OH clarithromycin plus minocycline was more effective than clarithromycin alone against LV-2.

Among the new fluoroquinolones, we have shown in this model, with the same strains, that temafloxacin was less active than clarithromycin and that sparfloxacin, as active as clarithromycin, was an interesting drug (16).

It was important to assess the activity of 14-OH clarithromycin against *M. avium* complex, since this human metabolite achieves a high concentration in the serum of AIDS patients treated for *M. avium* complex infection with clarithromycin (1). In our study, in which the bacteria were inside macrophages, 14-OH clarithromycin was effective against the two strains of *M. avium* complex at a concentration of one-eighth the MIC for strain MO-1 and one-half the MIC for strain LV-2, probably because of the intracellular concentrations of the molecule. Despite a slight difference in favor of clarithromycin, the difference between 14-OH clarithromycin and clarithromycin did not reach statistical significance, and their combination was as effective as clarithromycin alone. Recently, Inderlied et al. reported a lower efficacy of 14-OH clarithromycin than of clarithromycin against *M. avium* complex in vitro (11). In conclusion, this metabolite with a longer half-life than clarithromycin allows prolonged efficacy, with an amount of active drug (clarithromycin plus 14-OH clarithromycin) in the serum close to twice the concentration of clarithromycin. The pharmacokinetic properties of clarithromycin in humans suggest that treatment of patients for *M. avium* complex infection could be more effective than treatment in mice, which do not produce 14-OH clarithromycin.

Minocycline is a tetracycline analog that exhibits in vivo activity against *M. leprae* in a murine model (13). In our study, minocycline alone or in combination with clarithromycin and 14-OH clarithromycin exhibited efficacy against *M. avium* complex, despite its use at a concentration equal to 1/16 or 1/8 the MIC, respectively. Clarithromycin plus 14-OH clarithromycin plus minocycline, which was more effective than clarithromycin alone against strain LV-2, corresponds in fact to a clarithromycin-plus-minocycline combination in humans.

In clinical trials for the treatment of *M. avium* complex infection in patients with AIDS, combinations of antimycobacterial agents were often disappointing, since they did not achieve bacterial eradication. In order to decrease the emergence of resistant strains and to have an additive action against *M. avium* complex, new combinations are needed. Thus, while WIN-57273 exhibited a moderate activity in this model, the combination of two drugs, clarithromycin and minocycline, which corresponds in humans to three active molecules, seems to be of interest for further evaluation. This combination may exhibit a better efficacy than clarithromycin alone in this model.

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