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NOTES

Intracellular Activities of Roxithromycin Used Alone and in Association with Other Drugs against *Mycobacterium avium* Complex in Human Macrophages

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Recent reports have shown that roxithromycin possesses significant activity against atypical mycobacteria, including the *Mycobacterium avium* complex (MAC), and that its extracellular anti-MAC activity is further enhanced in two- or three-drug combinations with ethambutol, rifampin, amikacin, ofloxacin, and clofazimine. In accordance with the above data, the anti-MAC potential of roxithromycin used alone and in combination with the above-mentioned antituberculous drugs was screened intracellularly against five clinical MAC isolates (from both human immunodeficiency virus-positive and human immunodeficiency virus-negative patients), phagocytized by human monocyte-derived macrophages. The results showed that roxithromycin used alone and within clinically achievable levels was active against all of the MAC isolates tested. Screening of two-drug combinations showed that both rifampin and clofazimine further increased the intracellular activity of roxithromycin against all five isolates by 35 to 80% (ethambutol, ofloxacin, and amikacin resulted in increased intracellular activity against one, two, and four isolates, respectively). For the three-drug combinations, the combination of roxithromycin plus ethambutol used with rifampin or clofazimine was the most uniformly active against all five MAC isolates, with activity increases of 42 to 90%, followed by roxithromycin plus ethambutol used with amikacin, which resulted in activity increases of 15 to 90%. The overall level of intracellular killing after 5 days of drug addition, in comparison with growth in untreated controls, varied from 1 to 3 log units depending on the individual MAC isolate and/or drug combination used.

With the advent of the global AIDS pandemic, disseminated *Mycobacterium avium* complex (MAC) disease has emerged as one of the most difficult-to-treat complications among terminally ill patients, considerably affecting the quality of life and resulting in increased morbidity and mortality (13). One of the major clinical aims in the management of AIDS patients is the prevention and treatment of MAC infections. This opportunistic pathogen poses one of the most formidable challenges to clinicians and scientists alike, not only because of its multiple drug resistance (12) but also because our understanding of MAC pathogenicity is limited (13). Furthermore, recent reports have shown that AIDS patients with MAC infection not only were highly immunosuppressed but also consistently showed much lower levels of oral antimycobacterial agents in serum than expected (7, 13), two factors which further complicate the efficacy of MAC therapeutic regimens (3).

Resistance in *M. avium* is apparently associated with the cell envelope and its refractoriness toward drug penetration (12, 14). We have previously attempted to circumvent this problem by breaking the MAC permeability barrier using combined drug treatment, which resulted in increased bactericidal activity against both extracellular and intracellular organisms (17). We have recently shown that roxithromycin possessed significant in vitro activity against atypical mycobacteria (15). Moreover, its extracellular anti-MAC activity was further enhanced in two- or three-drug combinations with ethambutol, rifampin, amikacin, ofloxacin, and clofazimine (16), all of which have been previously included in various therapeutic regimens to treat MAC infections (1, 2, 10, 13, 20). In the present investigation, we decided to screen the intracellular activity of roxithromycin used alone and in combination with above-mentioned potential anti-MAC drugs against clinical isolates from human immunodeficiency virus-positive (HIV^+) and HIV-negative (HIV^-) patients in a human macrophage system.

MAC strains used in this investigation (MAC1 through MAC3 from patients with HIV^+ serology and MAC4 and MAC5 from patients with HIV^- serology) were from our own culture collection. Bacteria were grown in complete 7H9 broth (supplemented with Middlebrook ADC enrichment; Difco Laboratories, Detroit, Mich.), containing 0.05% (vol/vol) Tween 80 to avoid clumping, at 37°C and were harvested in their mid-logarithmic phase at an optical density of 0.15 (measured at 650 nm with a Coleman Junior II spectrophotometer), which corresponded to about 10^8 CFU/ml (15–17).

Cultures of human macrophages were prepared from adherent peripheral blood monocytes (from healthy donors) and infected with various MAC strains as reported previously (19). The monolayers (about 1 million macrophages per well) were allowed to phagocytize bacteria for 4 h at 37°C, after which all the extracellular bacilli were thoroughly washed away with

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TABLE 1. Intracellular activities of roxithromycin used alone and in combination with other antituberculous drugs against MAC isolates from HIV^+ and HIV^- patients[a]

Experimental conditions[b]	Result with clinical isolates from:				
	HIV^+ patients			HIV^- patients	
	MAC1	MAC2	MAC3	MAC4	MAC5
Control D0	1.000 ± 0.120	0.580 ± 0.140	1.000 ± 0.250	0.780 ± 0.035	0.940 ± 0.100
Control D5	10.000 ± 1.000	3.900 ± 0.500	11.750 ± 1.000	4.000 ± 0.450	23.000 ± 2.000
EMB 6	0.232 ± 0.015	1.130 ± 0.230	0.480 ± 0.040	0.275 ± 0.049	0.580 ± 0.100
OFLO 5	0.047 ± 0.010	0.575 ± 0.073	0.456 ± 0.080	2.100 ± 0.280	0.140 ± 0.040
RIF 15	0.019 ± 0.005	0.270 ± 0.050	0.146 ± 0.014	0.273 ± 0.033	0.160 ± 0.040
AMIK 20	0.044 ± 0.016	0.096 ± 0.010	0.382 ± 0.100	0.210 ± 0.040	0.100 ± 0.010
ROX 10	0.055 ± 0.010	0.160 ± 0.010	0.613 ± 0.240	0.131 ± 0.025	0.160 ± 0.010
CLOFA 2.5	0.057 ± 0.010	0.035 ± 0.004	0.158 ± 0.020	0.126 ± 0.013	0.100 ± 0.010
ROX + EMB	0.127 ± 0.014	0.140 ± 0.023	0.295 ± 0.060	0.188 ± 0.024	0.160 ± 0.020
ROX + OFLO	0.100 ± 0.010	0.125 ± 0.010	0.278 ± 0.032	0.156 ± 0.015	0.190 ± 0.012
ROX + RIF	0.013 ± 0.002	0.040 ± 0.004	0.033 ± 0.010	0.096 ± 0.005	0.064 ± 0.009
ROX + AMIK	0.033 ± 0.005	0.084 ± 0.014	0.14 ± 0.014	0.094 ± 0.010	0.130 ± 0.026
ROX + CLOFA	0.036 ± 0.010	0.012 ± 0.001	0.058 ± 0.008	0.025 ± 0.002	0.036 ± 0.003
ROX + EMB + OFLO	0.113 ± 0.02	0.120 ± 0.012	0.277 ± 0.056	0.150 ± 0.016	0.220 ± 0.040
ROX + EMB + RIF	0.011 ± 0.002	0.031 ± 0.002	0.051 ± 0.002	0.067 ± 0.008	0.048 ± 0.006
ROX + EMB + AMIK	0.038 ± 0.008	0.044 ± 0.008	0.019 ± 0.030	0.075 ± 0.007	0.076 ± 0.010
ROX + EMB + CLOFA	0.020 ± 0.005	0.013 ± 0.004	0.035 ± 0.003	0.013 ± 0.003	0.054 ± 0.015

[a] Results are expressed as viable counts ± standard errors (in millions).
[b] For control D0 (control at day 0), the number of bacteria initially phagocytized at day 0 was determined; for control D5, growth in the control after 5 days of incubation was determined. EMB 6, ethambutol at 6 μg/ml; OFLO 5, ofloxacin at 5 μg/ml; RIF 15, rifampin at 15 μg/ml; AMIK 20, amikacin at 20 μg/ml; ROX 10, roxithromycin at 10 μg/ml; CLOFA 2.5, clofazimine at 2.5 μg/ml. The same concentrations were used for drug combinations.

Hanks balanced salt solution and the number of bacteria effectively phagocytized was determined by lysing the macrophages with 0.25% (wt/vol) sodium dodecyl sulfate, doing immediate serial dilutions, and plating the lysates on 7H11 agar medium for viable-count determinations (19). After phagocytosis, fresh medium containing the desired antibiotics was refed to macrophage-containing wells and the bacteria were enumerated after lysing of the macrophages 5 days after drug addition (17, 19). The results (expressed as mean viable counts ± standard errors) were compared with the growth of bacteria in control, untreated macrophages. In accordance with previously defined criteria for evaluating intracellular drug activity against MAC isolates (12, 17–19), a drug was considered bactericidal if it reduced the bacterial viable counts in the test samples by 1 log unit or more in comparison with the initial inoculum added at the time of drug addition. Increase in intracellular bactericidal activity of drug combinations in comparison with activity of the drugs used alone was calculated by the following equation (18):

$$\text{increase} = 1 - \left(\frac{\text{CFU with drug combination}}{\text{lowest CFU with either drug used alone}} \right) \times 100$$

In accordance with our experimental model for determining the intracellular action of drugs (17–19), all drugs were used at their reported maximum concentrations in serum in humans as follows: roxithromycin, 10 μg/ml (4); ethambutol, 6 μg/ml (11); ofloxacin, 5 μg/ml (21); rifampin, 15 μg/ml (11); amikacin, 20 μg/ml (5); and clofazimine, 2.5 μg/ml (9). Roxithromycin and ofloxacin (Roussel-Uclaf), ethambutol (Lederle), amikacin (Bristol-Myers Squibb), and clofazimine (Ciba-Geigy) were kindly provided by their manufacturers, whereas rifampin was purchased from Sigma Chemical Co., St. Louis, Mo.

The results obtained in this investigation are summarized in Table 1. During 5 days of incubation, the *M. avium* isolates grew from a low of $5.8 \times 10^5 \pm 1.4 \times 10^5$ to $3.9 \times 10^6 \pm 0.5 \times 10^6$ CFU per macrophage monolayer (strain MAC2) to a high of $9.4 \times 10^5 \pm 1.0 \times 10^5$ to $2.3 \times 10^7 \pm 0.2 \times 10^7$ CFU per macrophage monolayer (strain MAC5). In general, all organisms grew by about 1 log unit during 5 days of intracellular growth. Roxithromycin used alone and within the clinically achievable level of 10 μg/ml was active against all five MAC isolates, although to a somewhat lesser extent with strain MAC3 (Table 1). Both rifampin and clofazimine further increased the intracellular activity of roxithromycin against all five isolates by an order of 35 to 80% (Table 1; Fig. 1), in comparison with amikacin, which resulted in about a 13 to 64% increase against four of five strains. Both clofazimine and amikacin have been previously included in MAC treatment regimens with rifamycins and/or macrolide drugs, yielding favorable results (1, 13, 20). On the other hand, addition of ethambutol and ofloxacin resulted in increased intracellular killing of only one and two isolates, respectively.

For three-drug combinations, roxithromycin plus ethambutol used with rifampin or clofazimine was the most uniformly active combination against all five MAC isolates, with an overall activity increase of 42 to 90% in comparison with the activity of any of the drugs used alone at its peak concentration in serum (Table 1; Fig. 1). The combination of roxithromycin, ethambutol, and amikacin resulted in activity increases of 15 to 90% in comparison with the activity of any of the drugs used alone. On the other hand, no significant enhancement of the intracellular activity by ofloxacin in three-drug combinations was noticed against any of the five clinical isolates (Fig. 1).

When our viable-count data are compared with published evidence about the refractoriness of MAC isolates to most antimicrobial agents (3, 12–14), it is noteworthy that the combination of roxithromycin plus ethambutol used with rifampin, clofazimine, or amikacin resulted in overall inhibition of some of the MAC isolates of 1 to 3 log units in comparison with their growth in untreated controls at day 5 (Table 1). However, despite an increase in intracellular bactericidal activity of up to 1 log unit with certain two- or three-drug combinations over the level achieved with individual drugs (Fig. 1), it is not possible to choose one particular combination over others because

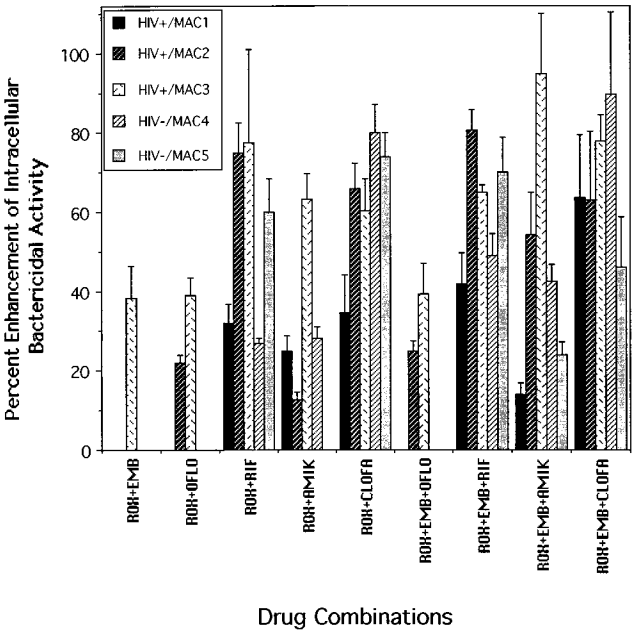

FIG. 1. Percent enhancement of intracellular bactericidal activity of various drug combinations against MAC organisms inside human macrophages. The increase in intracellular bactericidal activity of drug combinations in comparison with activity of the drugs used alone was calculated by the following equation (18):

$$\text{increase} = \left(1 - \frac{\text{CFU with drug combination}}{\text{lowest CFU with either drug used alone}}\right) \times 100$$

EMB, ethambutol; OFLO, ofloxacin; RIF, rifampin; AMIK, amikacin; ROX, roxithromycin; CLOFA, clofazimine.

of wide strain-to-strain variations in the drug susceptibility of MAC isolates (12, 16, 19). Overall, the results obtained in this investigation are comparable to those reported previously for another macrolide drug, clarithromycin, against MAC organisms growing intracellularly in murine (17) or human (18) macrophages.

On the basis of the significant anti-*M. avium* activity of roxithromycin in human macrophages (this paper), as well as its favorable pharmacokinetic properties (4, 6, 8), we conclude that this drug warrants further assessment in controlled clinical trials. We suggest that the three-drug combination of roxithromycin, ethambutol, and rifampin may be successfully used in combination with other drugs, which may include either clofazimine or amikacin, in future attempts to treat *M. avium* infections.

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