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Activities of Roxithromycin Used Alone and in Combination with Ethambutol, Rifampin, Amikacin, Ofloxacin, and Clofazimine against *Mycobacterium avium* Complex

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Preliminary studies showed that roxithromycin possessed significant *in vitro* activity against a variety of atypical mycobacteria such as the *Mycobacterium avium* complex, *M. scrofulaceum*, *M. szulgai*, *M. malmoense*, *M. xenopi*, *M. marinum*, and *M. kansasii* and rare pathogens such as *M. chelonae* and *M. fortuitum*. In this investigation, radiometric MICs of roxithromycin, ethambutol, rifampin, amikacin, ofloxacin, and clofazimine for 10 clinical isolates of the *M. avium* complex (5 each from human immunodeficiency virus [HIV]-positive and HIV-negative patients) were determined. Roxithromycin MICs against all the isolates were below the reported maximum concentration of drug in serum at the routine pH of 6.8, and the MICs were further lowered by 1 to 2 dilutions at a pH of 7.4. *In vitro* enhancement of roxithromycin activity against all strains was further investigated by the previously established Bactec 460-TB method by combining the drugs at sub-MIC levels. Antibacterial activity of roxithromycin was enhanced in all 10 strains by ethambutol, in 3 strains each by rifampin and clofazimine, in 2 strains by amikacin, and in 1 strain by ofloxacin. *In vitro* screening of three-drug combinations showed that combinations of roxithromycin, ethambutol, and a third potential anti-*M. avium* drug (rifampin, amikacin, ofloxacin, or clofazimine) resulted in further enhancement of activity in 13 out of 20 drug combinations screened.

Two of the major clinical aims in the management of patients with AIDS are prevention and treatment of opportunistic infections. Opportunistic strains of the *Mycobacterium avium* complex (MAC) pose some of the greatest difficulties in treatment of human immunodeficiency virus (HIV)-infected individuals, not only because of their multiple drug resistance but also because our understanding of their pathogenicity is limited (11). It is clear that the major problem in the treatment of MAC infections is the resistance of these strains to various antimycobacterial agents (6, 12). Unlike the genetically related multiple drug resistance that develops as the result of inadequate therapy for *M. tuberculosis* infection (21), drug resistance in MAC is apparently associated with the cell envelope and its resistance to penetration by drugs (12, 13). We have attempted to circumvent this problem in the past by using a combination of drugs chosen for their potential to inhibit the biosynthesis of components that contribute to the cell envelope structure, thus breaking the permeability barrier in MAC (16). More recently, we showed that combined drug treatment with the macrolide clarithromycin, ethambutol (a cell envelope-inhibiting drug), and rifampin was highly bactericidal against both extracellular and intracellular MAC (18).

We have recently shown that roxithromycin possesses significant *in vitro* activity against atypical mycobacteria (15). Given the observations described above and keeping in mind the difficulty of treating MAC infections, we thought it desirable to test roxithromycin against MAC. As recent reports have indicated higher activity levels of macrolides at alkaline pH than at acidic pH (7, 14), we also decided to determine the MICs of roxithromycin at both pH 6.8 and pH 7.4; 6.8 was taken as the standard pH used for drug screening in mycobacteria, whereas

pH 7.4 was based on the physiological pH prevailing in the plasma. Further potentiation of the anti-MAC activity of roxithromycin by other antituberculous drugs (ethambutol, rifampin, amikacin, ofloxacin, and clofazimine) was also investigated radiometrically by the previously described *x/y* quotient method (18-20).

The MAC strains used in this investigation (see Tables 1 and 2) 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 10⁸ CFU/ml.

Radiometric determination of roxithromycin MICs was performed with a Bactec 460-TB apparatus (Becton Dickinson, Towson, Md.) as described earlier (14, 18-20). The MICs were determined in parallel at pHs of 6.8 and 7.4. For the former pH, the medium used was commercially available Bactec 12B broth; in the latter case, the pH in the 12B vials was adjusted to 7.4 as previously described (14, 15). The MICs of other antituberculous drugs were determined only at a routine pH of 6.8 with Bactec 12B vials. For drug-combination studies, all of the drugs were used at sub-MIC levels. The concentrations chosen were (depending on the individual MICs indicated in Table 1) 0.5 to 2 µg/ml for roxithromycin, 0.25 to 1 µg/ml for rifampin, 0.25 to 2 µg/ml for ofloxacin, 0.025 to 0.05 µg/ml for clofazimine, and 1 µg/ml each for ethambutol and amikacin. The reason for this choice was that at these concentrations, the drugs used alone were unable to significantly reduce the initial inoculum in the Bactec vials. In such a case, any significant drug enhancement observed according to the radiometric *x/y* quotient criterion may indicate potential activity in infected

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TABLE 1. Radiometric MICs of selected drugs, including roxithromycin, for 10 clinical isolates of MAC

Drug	MIC ^a ($\mu\text{g/ml}$) of indicated clinical isolate									
	Isolates from HIV-positive patients					Isolates from HIV-negative patients				
	733	551	804	827	1423	711	969	1110	1257	1295
Roxithromycin	2	8	8	8	8	8	4	8	8	2
pH 6.8	1	4	4	2	4	2	2	4	4	0.5
pH 7.4	2	2	<2	2	2	2	4	<2	2	2
Ethambutol	2	8	2	2	8	16	8	<2	2	2
Rifampin	4	2	2	4	2	4	<2	2	4	2
Amikacin	16	8	16	4	8	>16	>16	8	4	4
Ofloxacin	0.12	0.25	0.25	0.25	0.25	0.25	0.12	0.12	0.5	0.12

^a All MICs were determined only at pH 6.8 except that of roxithromycin.

TABLE 2. In vitro enhancement of anti-MAC activity of roxithromycin by selected drugs in two- and three-drug combinations

Drugs ^a	Enhancement of drug activity (<i>xy</i> quotient) for indicated isolate ^b									
	Isolates from HIV-positive patients					Isolates from HIV-negative patients				
	733	551	804	827	1423	711	969	1110	1257	1295
ROX + EMB	+	+	+	+	+	+	+	+	+	+
ROX + RIF	(0.22)	(0.15)	(0.26)	(0.02)	(0.04)	(0.04)	(0.02)	(0.19)	(0.35)	(0.24)
ROX + AMIK	-	+	-	-	-	-	-	-	-	(0.4)
ROX + OFLO	-	+	-	-	-	-	-	-	-	(0.38)
ROX + CLOFA	+	-	-	-	+	-	-	-	-	(0.5)
					(0.34)					(0.014)
ROX + EMB + RIF	+	+	+	+	+	+	+	+	+	-
ROX + EMB + AMIK	+	+	+	+	+	+	+	+	+	(0.025)
ROX + EMB + OFLO	-	+	+	+	+	+	+	+	+	(0.08)
ROX + EMB + CLOFA	+	+	+	+	+	+	+	+	+	(0.014)

^a All of the drugs were used at sub-MIC levels. The concentrations chosen on the basis of MICs of each drug for individual isolates were as follows: ethambutol (EMB), 1 $\mu\text{g/ml}$ each; roxithromycin (ROX), 0.5 $\mu\text{g/ml}$ for strains 733, 969, and 1295, 1 $\mu\text{g/ml}$ for strains 804, 827, 711, and 1257, and 2 $\mu\text{g/ml}$ for strains 551, 1423, and 1110; rifampin (RIF), 0.5 $\mu\text{g/ml}$ for strains 551 and 1423, 1 $\mu\text{g/ml}$ for strains 711 and 1110, and 0.25 $\mu\text{g/ml}$ for the other strains; ofloxacin (OFLO), 1 $\mu\text{g/ml}$ for strains 733 and 804, 2 $\mu\text{g/ml}$ for strains 711 and 969, and 0.5 $\mu\text{g/ml}$ for the other strains; and clofazimine (CLOFA), 0.025 $\mu\text{g/ml}$ for strains 969 and 1110, and 0.05 $\mu\text{g/ml}$ for the other strains.

^b A radiometric *xy* quotient of <0.5 (two-drug combinations) or <0.33 (three-drug combinations) indicates enhanced drug action. The scores for *xy* quotients are given as follows: +, <0.5; ++, <0.1; and +++, <0.05 (two-drug combinations); and +, <0.33; ++, <0.06; and +++, <0.03 (three-drug combinations).

^c ND, not done.

host cells, where the drugs are available in much higher concentrations.

The radiometric x/y enhancement data were also confirmed by plating the bacterial suspensions from individual vials at the beginning and at the end of the experiment on 7H10 agar medium for viable-cell count enumeration, and the results were expressed as mean viable cell counts \pm standard errors.

Roxithromycin and ofloxacin (Roussel-Uclaf), ethambutol (Lederle), amikacin (Bristol), and clofazimine (Ciba-Geigy) were kindly provided by their manufacturers, and rifampin was purchased from Sigma Chemical Co., St. Louis, Mo.

The results obtained in the present investigation are summarized in Tables 1 and 2 and Fig. 1 to 4. When the radiometric MICs of roxithromycin as well as those of potential anti-MAC drugs (ethambutol, rifampin, amikacin, ofloxacin, and clofazimine) for 10 clinical isolates of MAC were compared at a routine pH of 6.8 (Table 1), roxithromycin MICs for all of the isolates (irrespective of the HIV status of the patients) were found to be below its reported maximum concentration in serum (10.8 $\mu\text{g/ml}$; time to peak, 1.5 h; observed after administration of a single oral dose of 300 mg). When roxithromycin was tested at pH 7.4, the MICs were further lowered by 1 to 2 dilutions.

In vitro enhancement of roxithromycin activity determined with the x/y quotient data is summarized in Table 2. The anti-MAC activity of roxithromycin was enhanced in all 10 strains by ethambutol, in 3 strains each by rifampin and clofazimine, in 2 strains by amikacin, and in 1 strain by ofloxacin. On the basis of the above results, we further decided to test three-drug combinations against five MAC strains. In this case the two-drug combination roxithromycin-ethambutol was kept constant, but the third drug was changed (Table 2). The results obtained showed that the anti-MAC activity of roxithromycin-ethambutol was further enhanced in four of five strains by amikacin and in three of five strains by rifampin, ofloxacin, and clofazimine, i.e., in a total of 13 of 20 drug combinations screened.

Figures 1 and 2 illustrate representative radiometric data showing the enhancement of anti-MAC activity of roxithromycin in two- and three-drug combinations against isolates 733 and 551, respectively. As reported previously (18, 20), the radiometric x/y quotient data were further verified in all cases by viable-cell count determinations. A typical example of such viable-cell count data is shown in Fig. 3. A careful examination indicates a satisfactory correlation between the radiometric data in Fig. 1 and 2, the corresponding x/y quotients in Table 2, and the viable-cell count data in Fig. 3. To prevent any misinterpretation of our data, it must be emphasized that the enhancement in this method is intentionally performed with sub-MIC levels of the drugs; this explains the fact that the overall effect observed on the basis of the viable-cell count data is only rarely bactericidal. However, a comparison of the viable-cell count data with published evidence about the resistance of MAC to most of these antimicrobial agents, even when the drugs are at much higher concentrations (6, 12, 17), indicates that roxithromycin and ethambutol used in combination with a third potentially antituberculous drug (which was used at a much lower sub-MIC concentration) was able to significantly reduce MAC growth relative to untreated controls (often by 1 to 2 log units or more).

To provide rapid results as well as to avoid any drug decay with prolonged incubations, the Bactec experiments were designed by calibrating the bacterial inoculum so that the experiments could be finished within 4 days. However, in some rare cases the experiments were performed for an additional 4 days to determine whether the flattened lines in the inhibition

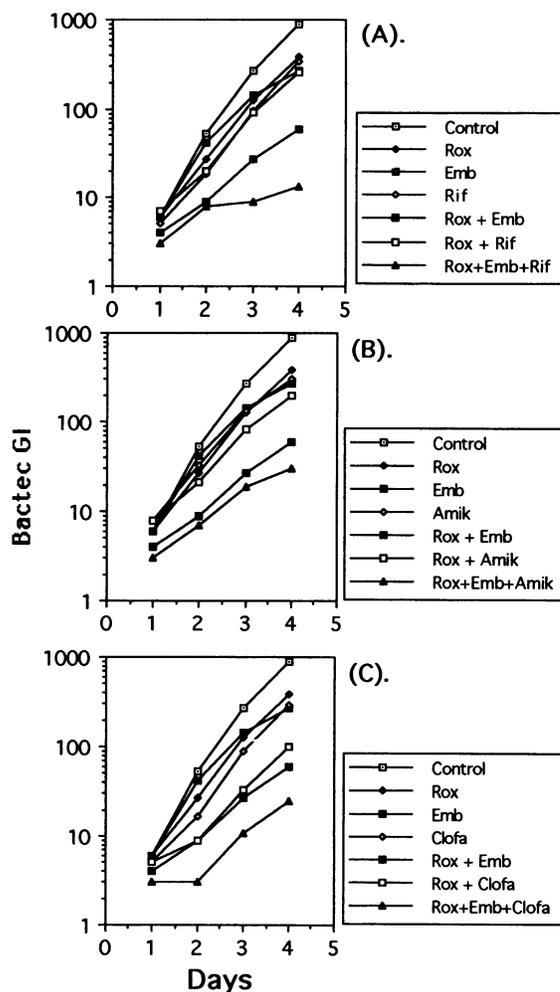


FIG. 1. Radiometric data showing enhancement of anti-MAC activity of roxithromycin used in two- and three-drug combinations against HIV-associated MAC clinical isolate 733. All of the drugs were used at sub-MIC levels (see text for further details). Rox, roxithromycin; Emb, ethambutol; Rif, rifampin; Amik, amikacin; Oflo, ofloxacin; Clofa, clofazimine; GI, growth index.

curves obtained with various two- or three-drug combinations would remain flattened after prolonged incubations or would converge towards the maximal growth index. With sustained drug potency, such a test would be a good indication of the in vitro efficacy of successful drug combinations, permitting elimination of the possibility that bacterial growth inhibition was actually due to the loss of a susceptible subpopulation which would then have regrown if the incubation had continued for a longer time. Typical radiometric data illustrating the sustained effects of successful and less successful drug combinations against two clinical isolates are illustrated in Fig. 4. Thus, strain 551, against which roxithromycin-ethambutol-rifampin, roxithromycin-ethambutol-amikacin, and roxithromycin-ethambutol-ofloxacin had enhanced action (Table 2; x/y quotients of <0.001 , <0.001 , and 0.003 , respectively), also had consistently flattened inhibition curves despite prolonged incubation periods of up to 8 days (Fig. 4A and B). On the other hand, use of the combination roxithromycin-ethambutol-clofazimine, whose x/y quotients did not indicate synergism (Table 2), resulted in a flattened inhibition curve initially for 3 days;

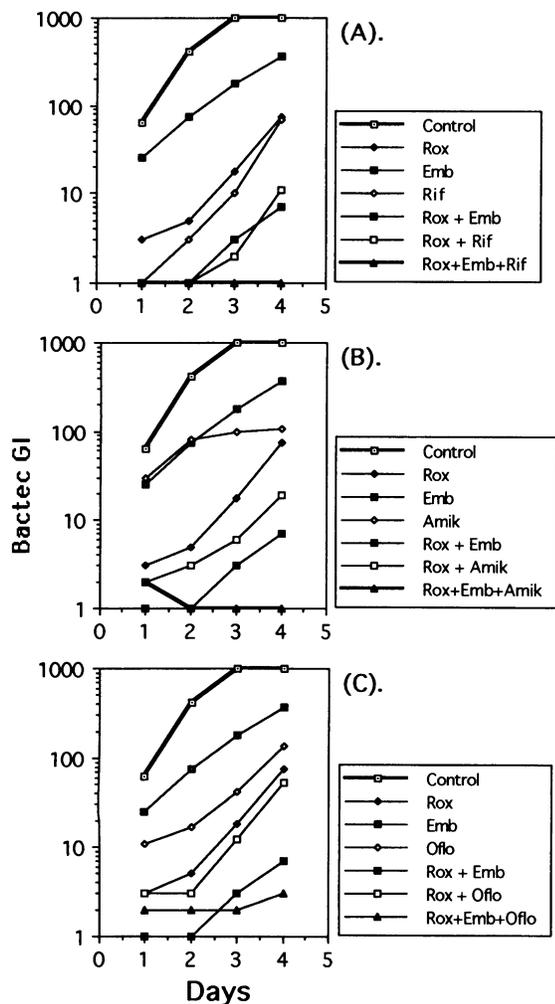


FIG. 2. Radiometric data showing enhancement of anti-MAC activity of roxithromycin used in two- and three-drug combinations against HIV-associated MAC clinical isolate 551. All of the drugs were used at sub-MIC levels. Rox, roxithromycin; Emb, ethambutol; Rif, rifampin; Amik, amikacin; Oflo, ofloxacin; Clofa, clofazimine; GI, growth index.

however, the strain grew exponentially thereafter (Fig. 4A). Similar observations for strain 827 are illustrated in Fig. 4C and D.

We have previously shown that roxithromycin has significant *in vitro* activity against atypical mycobacteria, as radiometric MICs significantly below the reported peak levels of the drug in serum were found with such potential pathogens as MAC, *M. scrofulaceum*, *M. szulgai*, *M. malmoense*, *M. xenopi*, *M. marinum*, and *M. kansasii*, as well as rare pathogens such as *M. chelonae* and *M. fortuitum* (15). Our present results further show that roxithromycin MICs for all of the MAC isolates tested (irrespective of the patient's HIV status) were below the reported maximum drug concentration in serum at the routine pH of 6.8 and that the MICs were further lowered by 1 to 2 dilutions at pH 7.4. Moreover, the anti-MAC activity of roxithromycin was enhanced for all 10 tested clinical isolates by ethambutol, for 3 isolates each by rifampin and clofazimine, for 2 isolates by amikacin, and for 1 isolate by ofloxacin. Screening of three-drug combinations (roxithromycin and

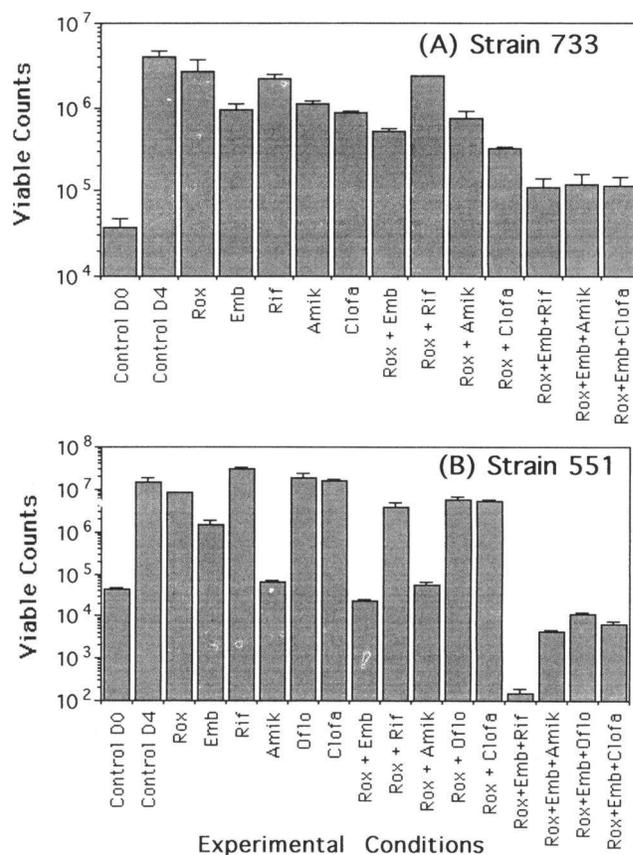


FIG. 3. Viable-cell count data showing anti-MAC activity of roxithromycin used in two- and three-drug combinations against strains 733 (A) and 551 (B). The viable-cell counts were done by plating the cultures from the experiments whose results are shown in Fig. 1 and 2, respectively, at the beginning and at the end of the experiment. All of the drugs were used at sub-MIC levels. Rox, roxithromycin; Emb, ethambutol; Rif, rifampin; Amik, amikacin; Oflo, ofloxacin; Clofa, clofazimine; D, day.

ethambutol with a third potential anti-MAC drug, i.e., rifampin, amikacin, ofloxacin, or clofazimine) resulted in further enhancement of activity in 13 out of 20 drug combinations screened. Thus, in agreement with our previous results (18–20), these observations confirm that ethambutol was able to break the drug exclusion barrier located in the MAC cell wall by inhibiting specific components of the barrier.

Since combined drug therapy is the only way to prevent the emergence of resistant strains, in view of the large bacterial populations in patients with disseminated MAC infections, many investigators have tried a variety of drug combinations. Synergistic effects of ethambutol against MAC with clarithromycin and/or rifampin (9, 10, 18, 24), with sparfloxacin and/or rifampin (20), and with amikacin (19) have been found to be more effective than any of the drugs used alone or other two-drug combinations that do not include ethambutol. Clarithromycin-ethambutol-rifampin was first shown to be the most bactericidal combination against both extracellularly and intracellularly growing MAC by Rastogi and Labrousse (18), and these results were later confirmed by Stauffer et al. (22). In their experiments, this combination was able to effectively kill 16 of 20 MAC isolates tested. Ethambutol was also included with rifabutin in the three most successful reported treatment

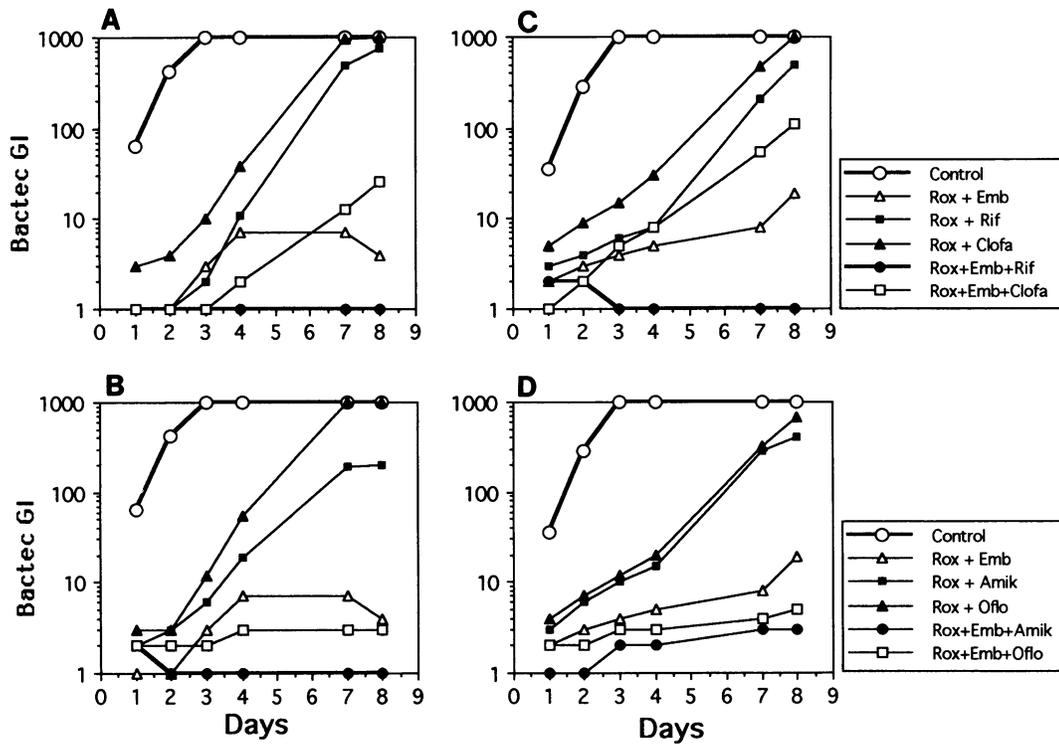


FIG. 4. Radiometric data illustrating sustained effect of drug combinations against HIV-associated MAC clinical isolates. (A and B) Bactec growth curves of strain 551; (C and D) Bactec growth curves of strain 827. All of the drugs were used at sub-MIC levels. Rox, roxithromycin; Emb, ethambutol; Rif, rifampin; Amik, amikacin; Oflo, ofloxacin; Clofa, clofazimine; GI, growth index.

regimens for MAC infection in patients with AIDS and was exceedingly well tolerated (1, 3, 8). Apart from ethambutol, both clofazimine (1, 8) and amikacin (2, 3), which we used in the present study, have been previously included in MAC treatment regimens and have yielded favorable results. This brief literature survey clearly indicates that apart from developing individual drugs for the treatment of MAC infections, one of the major issues today is the development of appropriate drug combination regimens.

Another major clinical aim in the management of patients with AIDS is the primary prevention of opportunistic infections. Recently, in an open, comparative, and randomized pilot study involving 52 HIV-infected patients with CD4 cell counts below 200/ml and no history of *Pneumocystis carinii* infections or cerebral toxoplasmosis, three chemoprophylactic regimens were compared. Eighteen patients received pentamidine alone (300 mg/month by aerosol), 17 received only roxithromycin (300 mg three times a week), and 17 received these doses of pentamidine and roxithromycin (5). After a 16- to 18-month of follow-up, the numbers of episodes of *P. carinii* pneumonia and cerebral toxoplasmosis were significantly lower for patients treated with roxithromycin; furthermore, in contrast to the pentamidine group, none of the patients in the roxithromycin group developed mycobacterial infections.

Roxithromycin is characterized by high concentrations in serum resulting in a maximum concentration in serum of 10.8 $\mu\text{g/ml}$ (time to peak, 1.5 h) after administration of a single 300-mg dose (15) and has been reported to concentrate more than 20-fold in a model of *Staphylococcus aureus*-infected macrophages (23). By comparing projected intracellular concentrations of roxithromycin with its MIC for 90% of MAC

strains (8 and 4 $\mu\text{g/ml}$ at pH 6.8 and 7.4, respectively [Table 1]), it can be concluded that this drug possesses a high level of anti-MAC activity as well as favorable pharmacokinetic properties, making it an immediate choice for future assessment in controlled clinical trials. On the basis of our results, it may be successfully combined with other potential anti-MAC drugs such as rifampin, clofazimine, amikacin, and fluoroquinolones, provided that ethambutol is maintained as an essential component in the therapeutic regimens to be tested.

In conclusion, roxithromycin possesses an antimycobacterial spectrum similar to that of clarithromycin (14, 18, 19). It has been suggested that the efficacy of clarithromycin in eliminating MAC from the blood of patients with AIDS is associated with its high inhibitory activity at pH 7.4 (7). Considering that intracellular accumulation of roxithromycin is higher under nonacidic conditions than under acidic conditions (23) and that, contrary to previous dogma, vesicles containing living, virulent MAC in experimentally infected human macrophages are indeed not acidic (4), we are currently evaluating the intracellular activity of roxithromycin used alone and in association with other antituberculous drugs in MAC-infected human macrophages.

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