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## In Vitro Activity of Roxithromycin against the *Mycobacterium tuberculosis* Complex

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Roxithromycin has recently been shown to possess significant in vitro activity against a variety of atypical mycobacteria such as the *M. avium* complex, *M. scrofulaceum*, *M. szulgai*, *M. malmoense*, *M. xenopi*, *M. marinum*, and *M. kansasii* and rare pathogens like *M. chelonae* and *M. fortuitum*. In the present investigation, screening of its in vitro activity was further extended by testing it against 34 strains belonging to the *M. tuberculosis* complex (including *M. tuberculosis*, *M. africanum*, *M. bovis*, and *M. bovis* BCG). The MICs were determined by the radiometric BACTEC 460-TB methodology at pHs of both 6.8 and 7.4, as well as with 7H10 agar medium by the 1% proportion method. With the exception of *M. bovis* BCG (MIC ranges, 0.5 to 4 µg/ml at pH 6.8 and 0.25 to 2 µg/ml at pH 7.4), MICs for all of the isolates were significantly greater (MIC ranges, 32 to >64 µg/ml at pH 6.8 and 16 to >32 µg/ml at pH 7.4) than those reported previously for atypical mycobacteria. Roxithromycin MICs of 64 or >64 µg/ml for all of the *M. tuberculosis* isolates screened were found by the 7H10 agar medium method. Roxithromycin, however, showed a pH-dependent bactericidal effect against *M. tuberculosis* because the drug was relatively more active when it was used at pH 7.4 than when it was used at pH 6.8. We conclude that roxithromycin per se is not a drug of choice for the treatment of *M. tuberculosis* infection or disease; however, considering its pharmacokinetics, eventual anti-tubercle bacillus activity in an in vivo system cannot yet be excluded. We suggest that the use of roxithromycin in chemoprophylactic regimens for the prevention of opportunistic infections (including *M. avium* complex infections) in patients with AIDS should be carefully monitored, and patients should be enrolled in such a regimen only after it has been excluded that the patient has an underlying infection or disease caused by *M. tuberculosis*.

Macrolides are relatively safe antibiotics with a fairly broad antimicrobial spectrum of activity (19), and potential cooperation between this class of antibiotics and the immune system is particularly important in patients with impaired immune functions, such as those afflicted with AIDS (1, 8). Some of the newer macrolides like clarithromycin and roxithromycin are useful in the treatment of infections caused by organisms such as *Pneumocystis carinii*, *Toxoplasma gondii*, and *Mycobacterium avium* complex (MAC), all of which are among the major opportunistic diseases associated with AIDS (19). Roxithromycin, a semisynthetic 14-membered ring macrolide has recently been shown to possess significant in vitro activity against a variety of atypical mycobacteria such as the MAC, *M. scrofulaceum*, *M. szulgai*, *M. malmoense*, *M. xenopi*, *M. marinum*, and *M. kansasii* and rare pathogens like *M. chelonae* and *M. fortuitum* (11). Its extracellular anti-MAC activity was further enhanced in two- or three-drug combinations with ethambutol, rifampin, amikacin, ofloxacin, and clofazimine (12). More recently, by using human monocyte-derived macrophages, the intracellular activity of roxithromycin alone or in combination with other drugs against clinical isolates of MAC was assessed (14), and the results showed that roxithromycin-ethambutol used with rifampin or clofazimine was the most uniformly bactericidal combination against all of the isolates, with an overall intracellular killing of 1 to 3 log units (depending on the individual MAC isolate or drug combination used) within 5 days of drug addition.

Although roxithromycin has been the subject of increased interest among scientists as far as its activity against atypical

mycobacteria is concerned (2, 3, 9, 11, 12, 14), its activity against the organisms of the *M. tuberculosis* complex has not previously been explored in detail. This aspect of research appeared particularly urgent to us, especially because human immunodeficiency virus (HIV)-infected patients are also prone to the development of tuberculosis, which is a problem early in the course of AIDS, whereas MAC emerges only in the later stages of disease, when CD4 levels fall to less than 100 cells per mm<sup>3</sup> (15). Recently, roxithromycin has been used successfully for simultaneous chemoprophylaxis of *P. carinii*, *T. gondii*, and MAC infections in HIV-infected patients with CD4 cell counts of less than 200 cells per mm<sup>3</sup> (5), but this raises the intriguing question of the eventual effect of such a chemoprophylactic regimen on any underlying *M. tuberculosis* infection. The aim of the present investigation was therefore to compare the in vitro activity of roxithromycin against the *M. tuberculosis* complex, which includes *M. tuberculosis*, *M. africanum*, *M. bovis*, and *M. bovis* BCG; the MICs were determined by using the BACTEC 460-TB methodology at pHs of both 6.8 and 7.4, as well as with Middlebrook 7H10 agar medium by the 1% proportion method at a routine pH of 6.8.

The study was performed on a total of 34 strains (see Table 1) belonging to the *M. tuberculosis* complex, as follows: 25 strains of *M. tuberculosis* (12 drug-susceptible isolates including the type strain H37Rv, 8 multidrug-resistant [MDR] isolates from HIV-negative patients, and 5 MDR isolates from HIV-positive patients), the type strain ATCC 25420 and 2 clinical isolates of *M. africanum*, the type strain ATCC 19210 and 2 clinical isolates of *M. bovis*, and 3 strains of *M. bovis* BCG (strains Pasteur, Denmark, and Russia). Bacteria were scraped from Löwenstein-Jensen slants, resuspended in 3 ml of BACTEC diluting fluid, and homogenized with glass beads (2

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TABLE 1. Comparative MICs of roxithromycin for organisms of the *M. tuberculosis* complex determined radiometrically at pH 6.8 and 7.4 and on Middlebrook 7H10 agar

Strain (no. tested)	MIC ( $\mu\text{g/ml}$ )					
	BACTEC method <sup>a</sup>				7H10 agar method <sup>b</sup>	
	pH 6.8		pH 7.4		50%	Range
50% <sup>c</sup>	Range	50%	Range			
<i>M. tuberculosis</i> (25)						
Susceptible strain (12)	64	32->64	32	16->32	>64	64->64
MDR strains from HIV-negative patients (8)	64	64->64	16	16->32	>64	64->64
MDR strains from HIV-positive patients (5)	>64	32->64	32	16-32	>64	64->64
<i>M. africanum</i> (3)	>64	64->64	>32	32->32	ND <sup>d</sup>	ND
<i>M. bovis</i> (3)	>64	64->64	32	32->32	ND	ND
<i>M. bovis</i> BCG (3)	2.0	0.5-4.0	0.5	0.25-2.0	ND	ND

<sup>a</sup> MICs were determined radiometrically by the BACTEC 460-TB methodology. The drug-containing vials were inoculated with 0.1 ml of a preculture grown to a BACTEC GI of about 500.

<sup>b</sup> MICs were determined by the 1% proportion method.

<sup>c</sup> 50%, MIC at which 50% of isolates tested are inhibited.

<sup>d</sup> ND, not done.

mm in diameter). The suspension was allowed to stand for a few minutes to permit sedimentation of the bacterial clumps. The homogeneous supernatant was taken, and the turbidity was adjusted to a McFarland no. 1 standard with diluting fluid. A BACTEC 12B vial (Becton-Dickinson, Towson, Md.) was injected with 0.1 ml of this suspension. This vial was used as a primary inoculum after the growth index (GI) reached a value of about 500. The radiometric determination of MICs was performed as reported earlier (13, 16). Briefly, 0.1 ml of bacterial suspension from the preculture vial (GI, 500) was injected into drug-containing vials as well as a control vial. A second control vial (the 1:100 control) containing an initial bacterial inoculum diluted 100-fold was also prepared. GIs were followed once daily. When the GI of the control vial diluted 1:100 reached 30, the results of the test were read for at least 1 additional day before it was terminated. The results were interpreted as follows: if the difference in the GIs from the previous day (called  $\Delta\text{GI}$ ) in the case of drug-containing vials was less than the  $\Delta\text{GI}$  of the 1:100 control, then the bacteria were considered susceptible to the drug concentration tested.

The MICs of roxithromycin were determined at two different pHs, i.e., 6.8 and 7.4 (11). Drug concentrations of 0.5, 1, 2, 4, 8, 16, 32, and 64  $\mu\text{g/ml}$  were screened at pH 6.8, whereas those of 0.25, 0.5, 1, 2, 4, 8, 16, and 32  $\mu\text{g/ml}$  were screened at pH 7.4. Experiments in a non-weekend schedule were started on a Friday and in this case, the GI readings were obtained from Monday onward, i.e., on days 3, 4, 5, 6, and 7. Although experiments were mostly terminated within 7 days, because of delayed growth at pH 7.4, in rare cases, the experiments were continued for longer periods. The pH-dependent effect of roxithromycin on bacterial viability was determined by plating the bacterial suspensions from individual BACTEC vials at the beginning and the end of the experiments onto 7H10 agar medium for viable count enumeration, and the results were expressed as the mean  $\pm$  standard error viable count. MIC determinations with 7H10 agar by the 1% proportion method were performed as reported previously at a routine pH of 6.8 (13).

The results obtained in the present investigation are summarized in Table 1 and Fig. 1 and 2. Only with exception of *M.*

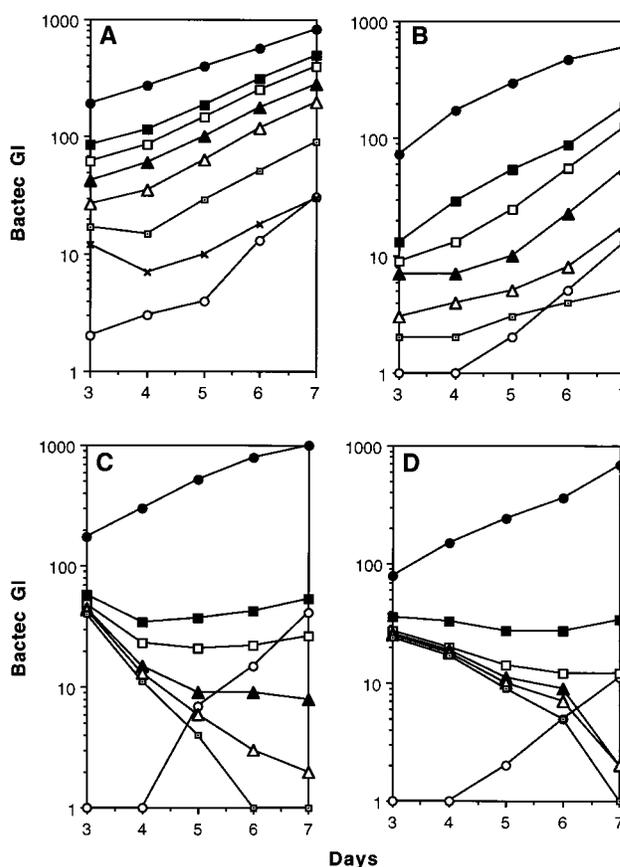


FIG. 1. Typical example of the dose-dependent response of roxithromycin on growth of *M. tuberculosis* clinical isolate 90-0240 (A and B) and *M. bovis* BCG strain Pasteur (C and D). The growth was monitored radiometrically by the BACTEC 460-TB methodology at pH 6.8 (A and C) and pH 7.4 (B and D). Symbols in panels A and B: ●, direct control; ○, 1:100 control; ■, roxithromycin at 2  $\mu\text{g/ml}$ ; □, roxithromycin at 4  $\mu\text{g/ml}$ ; ▲, roxithromycin at 8  $\mu\text{g/ml}$ ; △, roxithromycin at 16  $\mu\text{g/ml}$ ; ☆, roxithromycin at 32  $\mu\text{g/ml}$ ; \*, roxithromycin at 64  $\mu\text{g/ml}$ . Symbols in panels C and D: ●, direct control; ○, 1:100 control; ■, roxithromycin at 0.25  $\mu\text{g/ml}$ ; □, roxithromycin at 0.5  $\mu\text{g/ml}$ ; ▲, roxithromycin at 1  $\mu\text{g/ml}$ ; △, roxithromycin at 2  $\mu\text{g/ml}$ ; ☆, roxithromycin at 4  $\mu\text{g/ml}$ .

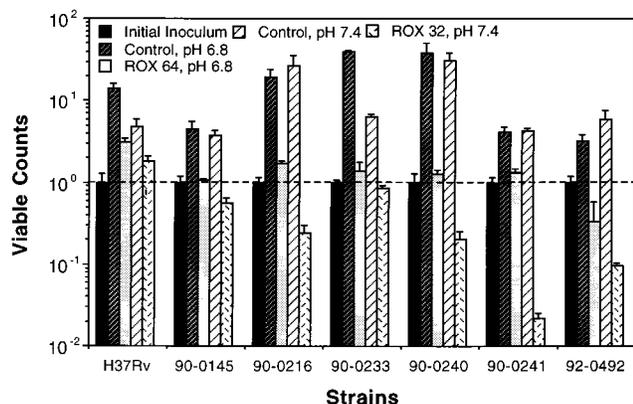


FIG. 2. pH-dependent effects of roxithromycin on bacterial viability against seven *M. tuberculosis* strains. The viable counts in this set of experiments were screened only at fixed MICs of 64 and 32  $\mu\text{g/ml}$  at pH 6.8 and 7.4, respectively. The results are shown as the mean  $\pm$  standard error change in viable counts of inoculum containing drug versus drug-free control at each pH at the end of the test period. The dotted line represents the initial inoculum added to BACTEC vials (taken as 1 in this illustration). The length of incubation was 7 days for all test points except for the vials containing strains 90-0216, 90-0240, and 90-0492 at pH 7.4, which were incubated for 12 days because growth in the controls was delayed at alkaline pH. ROX 64, roxithromycin at 64  $\mu\text{g/ml}$ ; ROX 32, roxithromycin at 32  $\mu\text{g/ml}$ .

*bovis* BCG (Table 1), radiometric MICs of roxithromycin for all of the isolates belonging to the *M. tuberculosis* complex were much greater than those reported previously for atypical mycobacteria including MAC (2, 3, 11, 12). At pH 6.8, MICs of 32  $\mu\text{g/ml}$  were found for only 2 of 31 isolates, whereas MICs of 64 and  $>64$   $\mu\text{g/ml}$  were found for the remaining 14 of 31 and 15 of 31 of the isolates, respectively. Although the MICs at pH 7.4 were lower by 1 to 2 dilutions (MICs, 16, 32, and  $>32$   $\mu\text{g/ml}$  for 9 of 31, 17 of 31, and 5 of 31 of the isolates, respectively), none of these MICs were below the reported maximum concentration of the drug in serum ( $C_{\text{max}}$ ; 7.9, 10.82, and 12.24  $\mu\text{g/ml}$  after administration of single oral doses of 150, 300, and 450 mg, respectively [8]). This is quite unlike the previous results with 28 strains of atypical mycobacteria belonging to 16 distinct mycobacterial species (11), for which the drug had MICs below its mean  $C_{\text{max}}$  of 10.8  $\mu\text{g/ml}$  for 21 of 28 strains representing 13 of 16 species tested at pH 6.8 and for 24 of 28 strains representing 15 of 16 species tested at pH 7.4. Similarly, unlike the high MICs for *M. tuberculosis* in the present investigation (Table 1), the drug had MICs at which 90% of isolates tested are inhibited of as low as of 8 and 4  $\mu\text{g/ml}$  at pH 6.8 and 7.4, respectively, for MAC clinical isolates (12). A typical dose-dependent response of roxithromycin on *M. tuberculosis* growth monitored radiometrically by the BACTEC 460-TB methodology at pH 6.8 and 7.4 is illustrated in Fig. 1A and B, respectively.

The roxithromycin MICs obtained by the conventional methodology (1% proportion method with Middlebrook 7H10 agar medium at a routine pH of 6.8) followed the same picture described above. For 25 isolates of *M. tuberculosis* screened, MICs were less than 64  $\mu\text{g/ml}$  for none of the isolates and MICs were 64  $\mu\text{g/ml}$  for only 6 of 25 isolates, whereas for the remaining 19 of 25 isolates MICs were  $>64$   $\mu\text{g/ml}$  (Table 1). Thus, irrespective of the methodologies, media, or pH used in the present investigation, roxithromycin MICs for all of the *M. tuberculosis* isolates were significantly greater than the mean reported  $C_{\text{max}}$  of roxithromycin of 10.8  $\mu\text{g/ml}$ .

As mentioned earlier, the only exception to the above rule was the relatively high degree of activity of roxithromycin

against *M. bovis* BCG (Table 1; MICs, 0.5 to 4  $\mu\text{g/ml}$  at pH 6.8 and 0.25 to 2  $\mu\text{g/ml}$  at pH 7.4). Typical radiometric data against BCG strain Pasteur at both pHs are illustrated in Fig. 1C and D. The relative inactivity of roxithromycin against *M. tuberculosis*, *M. africanum*, and *M. bovis* and its high degree of activity against *M. bovis* BCG are in agreement with the results of previous studies with another macrolide drug, clarithromycin (10), and in our opinion, it may not only contribute to an extremely powerful taxonomic marker that can be used to separate *M. bovis* BCG from *M. bovis* (and even from other members of the *M. tuberculosis* complex) but it also suggests the potentials of both of these macrolides for use in the treatment of BCG-related adenopathies. Studies to further define the taxonomic utility of macrolides by both BACTEC and conventional methodologies are in progress among members of our group.

The pH-dependent effects of roxithromycin on bacterial viability against the type strain *M. tuberculosis* H37Rv, five drug-susceptible clinical isolates of tubercle bacilli, and a single MDR clinical isolate (92-0492) are illustrated in Fig. 2. The viable counts in this set of experiments were screened only at fixed MICs of 64 and 32  $\mu\text{g/ml}$  at pH 6.8 and 7.4, respectively. A relatively strong pH effect on viability was evident for strains 90-0216, 90-0240, 90-0241, and 92-0492, but this effect was not so obvious for strains H37Rv, 90-0145, and 90-0233. Thus, contrary to the bacteriostatic activity of roxithromycin at pH 6.8, the drug was considerably more active when it was used at pH 7.4.

Our results therefore show that roxithromycin per se is not a drug of choice for the treatment of *M. tuberculosis* infection or disease. However, roxithromycin has a bimodal distribution within the eucaryotic cell, with higher concentrations achieved within the lysosomes than in the cytosol, resulting in intracellular/extracellular drug concentration ratios as high as 30 in polymorphonuclear cells and 60 to 190 in alveolar macrophages (6). Thus, considering the ability of roxithromycin to concentrate many-fold inside host macrophages and bacteria-loaded phagolysosomes, lower MICs of macrolide drugs at alkaline pH (7, 10), the increased bactericidal activity of roxithromycin against *M. tuberculosis* at pH 7.4 (Fig. 2), and recent debate in the literature concerning the lack of acidification in mycobacteria-containing phagosomes (4, 17, 18), we conclude that although roxithromycin is not a drug of choice for the treatment of *M. tuberculosis* infections per se, its eventual activity against tubercle bacilli in an in vivo system cannot yet be excluded. Our results also tend to suggest that the use of macrolide drugs in chemoprophylactic regimens for the prevention of opportunistic infections (including MAC) in patients with AIDS should be carefully monitored, and patients should be enrolled in such a regimen only after it has been clearly excluded that the patient has an underlying infection or disease caused by *M. tuberculosis*.

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