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Reaction of Roxithromycin and Clarithromycin with Macrolide-Inactivating Enzymes from Highly Erythromycin-Resistant *Escherichia coli*

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The activities of two new 14-membered-ring macrolide antibiotics, roxithromycin (RXM) and clarithromycin (CAM), against highly erythromycin (EM)-resistant *Escherichia coli* strains were evaluated. Pretreatment of macrolide phosphotransferase (MPH) (2′) I-producing strains with EM increased the MICs of EM and CAM without any noticeable change in the MIC of RXM. The MPH (2′) II-producing strain was more susceptible to CAM, while the EM esterase-producing strains were more susceptible to RXM than EM. Pretreatment of these latter two strains with EM did not alter their susceptibility to either RXM or CAM. In addition, the compounds were assessed as substrates for inactivation by crude enzyme preparations. Of the 14-membered-ring macrolides, RXM was the least favored substrate for MPH (2′) I or II. CAM and RXM were substrates for the EM esterase but were the least preferred of the 14-membered-ring macrolides.

Erythromycin (EM) and other macrolides are important antibiotics that are mainly used for the treatment of infections due to gram-positive bacteria, especially *Staphylococcus* and *Streptococcus* species, in Japan. Macrolides have been used against a wide range of infections in Europe as well (2–4).

In recent years, *Escherichia coli* strains that are highly resistant to EM have been reported, and the enzymes that inactivate macrolide antibiotics have also been described (10, 11). EM esterase was first identified in Europe (1, 5, 6, 15), while macrolide phosphotransferase (MPH) (2′) I was initially found in Japan (13, 14, 16–19). MPH (2′) II was found in *E. coli* isolated in Europe (9).

Both roxithromycin (RXM) and clarithromycin (CAM), two new 14-membered-ring macrolide antibiotics, have recently been placed on the market and are frequently used in clinical treatment. In this study, the antibiotic activities of RXM and CAM against EM-resistant *E. coli* and inactivation of these two macrolides by MPH (2′) I and II and EM esterase A from clinical isolates were compared.

The macrolides used in this study were EM, oleandomycin (OL; Sigma Chemical Co., St. Louis, Mo.), RXM (powder; a gift of Eizai Co., Ltd.), CAM (Taisho Pharmaceutical Co., Ltd.), and leucomycin (LM; Wako Pure Chemical Industries Ltd., Osaka, Japan). The bacterial strains used were two highly EM-resistant strains of *E. coli* isolated in Japan, Tf481A (17) and L441D (14); an EM esterase gene (*ereA*)-bearing strain, BM694/pAT63 (an *E. coli* strain with a plasmid-encoded EM esterase) (6); and clinical isolate BM2506 (9), kindly provided by P. Courvalin, Pasteur Institute (Paris, France). *E. coli* K12W3110rif (rifampin resistance mutant) (9) was also used as a comparator strain lacking any of the three inactivating enzymes.

MICs were determined by the agar double-dilution method (7, 9), as follows. An overnight bacterial culture grown in Mueller-Hinton broth (Difco Laboratories, Detroit, Mich.) was diluted 100 times with the same broth, and a loopful of suspension was inoculated onto Mueller-Hinton agar plates containing serial twofold dilutions of each antibiotic. The MIC was determined after overnight culture at 37°C.

Crude enzyme solutions of MPH (2′) I (17), MPH (2′) II (9), and washed bacterial cells containing EM esterase (6, 15) were used as sources of macrolide-inactivating enzymes. Briefly, strains Tf481A, L441D, and BM2506 were grown in nutrient broth medium (400 ml) and disrupted by sonication. After centrifugation to remove broken cell fragments and unbroken cells, the resultant supernatant fluids constituted the MPH (2′) I and II crude enzyme preparations, respectively. A total of 0.1 ml of each crude enzyme solution, 0.1 ml of 40 mM ATP, 0.1 ml of macrolide antibiotic (125 μg/ml), and 0.7 ml of TMK solution (0.01 M $MgCl_2$, 0.06 M KCl, and 6 mM 2-mercaptoethanol in 0.1 M Tris-HCl buffer, pH 7.8) (16) were mixed and incubated at 37°C for 0, 0.5, 1, 2, 4, or 20 h, following which each sample was heated at 100°C for 3 min. The supernatant, whose protein had been removed by centrifugation, was used to measure the residual activity of the macrolide antibiotic by microbioassay with *Bacillus subtilis* ATCC 6633. The protein concentration was measured by the method of Lowry et al. (12). In order to prepare the EM esterase in washed bacteria, bacteria selected on bromthymol blue-lactose agar medium (Eiken Co., Ltd., Tokyo, Japan) containing 200 μg of EM per ml were immediately suspended and shaken overnight in 10 ml of nutrient broth medium. The centrifuged bacteria were washed with physiological saline and then suspended in 1 ml of a 0.1 M phosphate buffer solution (pH 7.0). For measurement of the esterase reaction, 0.1 ml of the washed bacterial cell suspension or the diluted one with same buffer was mixed with 0.1 ml of macrolide antibiotic (125 μg/ml) and 0.8 ml of 0.1 M phosphate buffer solution (pH 7.0), incubated at 37°C for 0, 0.5, 1, 2, 4, or 20 h, and then heated at 100°C for 3 min. The supernatant, whose cells had been removed by centrifugation, was used to measure the residual macrolide antibiotic activity by microbioassay.

Table 1 shows a comparison of the MICs of different macrolides for each strain. Resistance was induced by treatment with 100 μg of EM per ml in the broth overnight. Induction with EM has already been observed with strain Tf481A, which

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TABLE 1. MICs of macrolide antibiotics for *E. coli* strains

Strain	MIC (μg/ml)			
	EM	CAM	RXM	OL
Tf481A	800[a]	400	800	>800[a]
L441D	800	400	400	>800
BM2506	800	200	800	>800[a]
BM694/pAT63	>800[a]	>800	200	>800
K12W3110rif	50	25	50	200

[a] From reference 9.

produces MPH (2′) I (17). Pretreatment of the MPH (2′) I-producing strains Tf481A and L441D with EM increased the EM MICs from 800 and 800 μg/ml to >800 and >800 μg/ml and the CAM MICs from 400 and 400 μg/ml to 800 and >800 μg/ml without any noticeable change in the MICs of RXM. The MPH (2′) II-producing strain was more susceptible to CAM, while the EM esterase-producing strains were more susceptible to RXM than EM. Pretreatment of these latter two strains with EM did not alter their susceptibility to either RXM or CAM.

Table 2 presents the substrate specificity for inactivation of EM, RXM, CAM, OL, and LM by the bacterial enzymes. The specific activities of MPH (2′) from strains Tf481A (noninduced), L441D (noninduced), and BM2506 and of EM esterase from strain BM694/pAT63 were 16.0, 20.5, and 20.6 nmol of inactivated OL per mg of protein and 860 nmol of inactivated OL per mg (dry weight) of cells per h, respectively.

The residual potency of each drug after the inactivation reaction was calculated, using a set of standard curves for macrolides, from the zone of inhibition by microbioassay. It was translated to the inactivation activity and then to the number of inactivated moles by each molecular weight of macrolides as shown in Table 2. The degree of inactivation of the other macrolides was then expressed relative to the degree of inactivation of OL by each sample. The MICs and macrolide inactivation rates for each strain for EM and LM were almost the same as the data reported in the past (9, 14, 17).

CAM was inactivated by MPH (2′) I, MPH (2′) II, and EM esterase. However, compared with EM, CAM tended to be more resistant to inactivation by these three macrolide-inactivating enzymes. CAM reacted slightly with EM esterase, while the 16-membered-ring macrolide LM was not a substrate. These findings suggest that O methylation of the OH group of the C-6 position in 14-membered-ring macrolides affects not only the activities of MPH (2′) I and MPH (2′) II but also the activity of EM esterase to quite a degree.

RXM was inactivated by MPH (2′) I but to a lesser degree than EM and CAM. This is regarded as the reason that the MIC of RXM did not change even for strains in which resistance had been induced. It was reported that 50% of highly EM-resistant intestinal bacteria isolated in Europe owe their resistance to an unknown mechanism (5). MPH (2′) II was detected in strain BM2506 (9), which is considered one of the above-mentioned highly EM-resistant intestinal bacteria, and RXM was not inactivated at all by this enzyme. This suggests that the C-9 side chain of RXM has a strong inhibitory effect on MPH (2′) II activity as well as on the activities of MPH (2′) I and EM esterase. Both RXM and CAM were inactivated by EM esterase to only half the degree that EM was inactivated.

TABLE 2. Substrate specificity of inactivation by bacterial enzymes

Inactivating enzyme	Strain used	Relative % inactivation with substrate[a]				
		EM	CAM	RXM	LM	OL
MPH (2′) I	Tf481A	87[b]	64	37	1[b]	100
	L441D	82[c]	73	47	1	100
MPH (2′) II	BM2506	23	17	0	61[d]	100
EM esterase	BM694/pAT63	15	8	7	0[e]	100

[a] Relative to inactivation of OL per hour, which was considered 100%.
[b] From reference 17.
[c] From reference 14.
[d] From reference 9.
[e] From reference 15.

In summary, it was found that RXM and CAM are less inactivated than EM by the three macrolide-inactivating enzymes described above. The results of this study will be helpful for the future development of macrolide derivatives (8). The experimental system using highly EM-resistant strains and the reaction system of the macrolide-inactivating enzymes [MPH (2′) I and II and EM esterase] will be very useful for studies of new macrolide-resistant, gram-positive bacterial strains, especially staphylococci, which are a major therapeutic target of macrolide antibiotics.

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