RADIOIMMUNO ASSAY FOR ERYTHROMYCIN DERIVATIVES

YOSHITAKE TANAKA, KEIKO KIMURA, YASUKO KOMAGATA, KAZUO TSUZUKI, HIROSHI TOMODA and SATOSHI ŌMURA*

The Kitasato Institute, and
School of Pharmaceutical Sciences
of Kitasato University,
Minato-ku, Tokyo 108, Japan

(Received for publication September 3, 1987)

Erythromycin A (EM-A), a 14-membered macrolide antibiotic, is clinically useful for treatment of infections by Gram-positive bacteria and mycoplasmas. Although occurring rather infrequently, side effects of the drug such as diarrhea and vomiting suggest certain adverse reactions to digestive organs. According to the recent finding by ITOH *et al.*, this side effect of EM-A is due to its gastro intestinal motor-stimulating (GMS) activity, which resembles closely that caused by the digestive hormone motilin.[1~3]

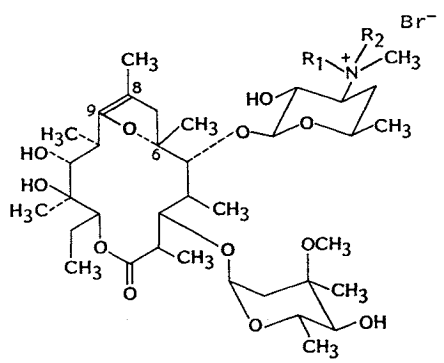
We have reported the synthesis of EM derivatives with an increased GMS activity and a decreased antibacterial activity.[4] As described elsewhere,[5] our subsequent trials led to the discovery of several newer useful derivatives, which exhibited no detectable antibacterial activity, but stronger GMS activity than EM-A, and increased acid stability. We proposed to designate this series of compounds as "motilide" after their motilin-like activity and macrolide structure. Because motilides have virtually no antimicrobial activity, a new assay system is required other than conventional microbiological methods.

This paper describes the development of a radioimmuno assay for sensitive and selective monitoring of motilides (Fig. 1) as well as EM-A.

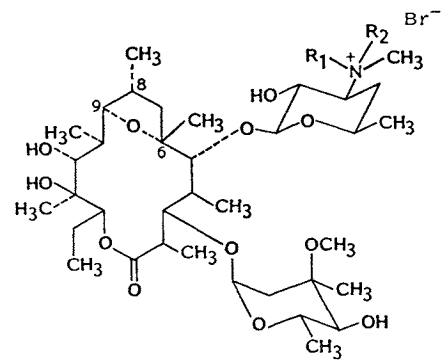
The immunogen to EM was prepared by the methods of KITAGAWA *et al.*, using *N*-maleoyl aminobutyric acid (MABA) as a heterobifunctional cross-linker.[6,7] MABA was synthesized by the method of RICH *et al.*[8] To a solution containing 258 mg of MABA and 500 mg of *N*-demethyl EM-A in 15 ml of dry tetrahydrofuran, 1.5 g of *N,N'*-dicyclohexylcarbodiimide was added, and incubated first at 27°C for 1 hour, then at 4°C for 48 hours. After evaporation, the reaction mixture was made alkaline with $NaHCO_3$, and extracted with $CHCl_3$. The solvent layer was evaporated, and the residue was applied to a silica gel column, developed with $CHCl_3$ - MeOH (30 : 1) to yield MABA-acylated EM (400 mg). The structure was identified by ^1H NMR spectroscopy.

Acetylmercaptosuccinyl bovine serum albumin (AMS-BSA, 150 mg), was obtained as described by FUJIWARA *et al.*[7] A portion of AMS-BSA (10 mg) was incubated in 0.2 ml of 0.1 M hydroxylamine, pH 7.2, at room temp for 30 minutes. The deacetylated product mercapto-

Fig. 1. Structure of motilides.



EM-201 $R_1 = CH_3$ $R_2 = —$
EM-523 $R_1 = C_2H_5$ $R_2 = —$
EM-536 $R_1 = CH_3$ $R_2 = CH_2C{\equiv}CH$



EM-501 $R_1 = CH_3$ $R_2 = —$
EM-610 $R_1 = C_2H_5$ $R_2 = —$
EM-507 $R_1 = CH_3$ $R_2 = CH_2C{\equiv}CH$

succinyl BSA (MS-BSA) was mixed immediately with 15 mg of MABA-acylated EM in 3 ml of 0.1 M sodium phosphate buffer (pH 7.0), and incubated for 2 hours with stirring. The resulting conjugate was purified by column chromatography on Sephadex G-100 developed with distilled water. The fractions containing the conjugate were collected and concentrated *in vacuo*. Several repetitions of the above coupling steps, followed by lyophilization, yielded 216 mg of the conjugate. SDS-polyacrylamide gel electrophoresis revealed that the purified conjugate contains about 3 molecules of EM per BSA molecule.

An aliquot of aqueous conjugate (2 mg/ml) was emulsified with an equal volume of FREUND's complete adjuvant. Three male white rabbits were given an intramuscular injection of 1 ml of the emulsified immunogen on the footpads. Booster injections, given subcutaneously on the back at a 2-fold amount of the first dose, were repeated 7 times biweekly. One week after the final immunization, all rabbits were sacrificed, and the serum were taken. The antiserum was fractionated with ammonium sulfate (0~30%) followed by dialysis at 4°C against several changes of 0.1 M sodium phosphate buffer (pH 7.0). To remove the antibody to BSA, the dialyzate was passed through a column of BSA-Cellulofine, which was prepared by mixing formyl-Cellulofine (Seikagaku Co., Tokyo) with BSA according to the supplier's manual. The purified antibody to EM was stored frozen at −20°C until use.

The radioimmuno assay was performed by the double-antibody method.[9] Antibody solution was diluted with 0.01 M sodium phosphate buffer containing 0.5% BSA, 25 mM EDTA, and 0.9% NaCl, pH 7.4 (PBS-BSA) to bind about 50% of [^{14}C]EM-A used for assay. The reaction mixture in a total volume of 0.5 ml contained; diluted antibody solution 0.1 ml, PBS-BSA 0.2 ml, reference or sample solution 0.1 ml, and [^{14}C]EM-A (0.05 μCi/ml, New England Nuclear Co.) 0.1 ml. The mixture was incubated at 37°C for 2 hours. Then, 0.1 ml of a goat anti-rabbit IgG solution was added. After a further incubation for 1 hour, the immune precipitate was washed with 1.0 ml of 0.1 M sodium phosphate buffer by centrifugation (3,000 rpm, 20 minutes). The radioactivity of the precipitate was determined after addition of 5 ml of a xylene-based scintillant (ACS II, Amersham).

Fig. 2. Standard curve for erythromycin A.

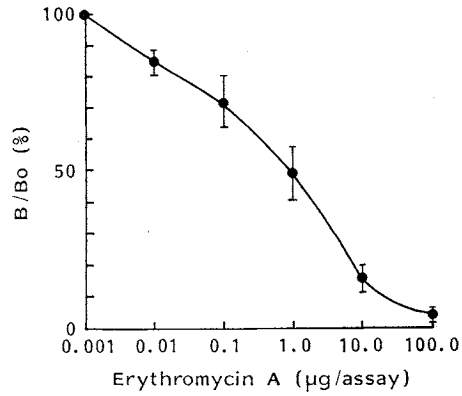


Table 1. Cross-reactivities of naturally occurring erythromycins (A) and motilides (B).

Compound (A)	CR_{50}[a] (%)	Compound (B)	CR_{50}[a] (%)
EM-A	100.0	EM-201	6.0
EM-B	8.6	EM-523	7.8
EM-C	17.1	EM-536	10.0
EM-104[b]	<0.1	EM-501	27.7
EM-107[b]	85.7	EM-610	36.0
EM-202[b]	>100.0	EM-507	95.6

[a] Defined as: $\frac{\text{Amount of unlabeled EM-A at midpoint of standard curve}}{\text{Amount of test compound at midpoint}} \times 100$

[b] EM-104; 5-*O*-Desosaminylerythronolide A, EM-107; di-*N*-demethyl EM-A, EM-202; anhydro EM-A. The cross-reactivities of the compounds listed below were less than 0.1%.

Other macrolides: Methymycin, pikromycin, oleandomycin, megalomicin A, lankamycin, tylosin, spiramycin, leucomycin, lankacidin C, leucanicidin and avermectin. Other antibiotics: Penicillin, streptomycin, kanamycin, chloramphenicol, tetracycline, lincomycin, novobiocin, doxorubicin, amphotericin B, rifampicin, nalidixic acid and nanaomycin A.

In the present radioimmuno assay system, the detection range of EM-A was 0.001 to 100 μg/assay, *i.e.* 0.01 to 1,000 μg/ml of sample (Fig. 2). As shown in Table 1, the antibody exhibited weak cross-reactivity against EM-B and EM-C, and good cross-reactivity against the naturally occurring EM metabolites EM-107 (di-*N*-demethyl EM-A) and EM-202 (anhydro EM-A) except for EM-104 which lacks cladinose moiety. The three metabolites are antibiotically inactive. The antibody did not cross-react with other macrolides such as methymycin (12-membered ring), oleandomycin (14-membered ring), tylosin, leucomycin (16-membered ring), and other classes of antibiotics, *e.g.* β-lactams. Among motilides tested, EM-201, EM-523 and EM-536, all excellent motilides with a C-8, C-9 double bond, and a C-6, C-9 hemiketal together with a modified amino group on the desosamine moiety (Fig. 1), exhibited weak cross-reactivity. They could be detected at more than 0.1 μg/assay. Whereas, EM-501, EM-610 and EM-507, which are among best motilides with saturated C-8 and C-9 carbons and with similar modifications as the above three, showed good to moderate cross-reactions. They were detectable in the range of 0.01 to 100 μg/assay. It is suggested that this antibody recognizes the aglycone and cladinose moieties with a little nonspecific binding to the desosamine moiety. This may be related to the fact that the immunogen were prepared by coupling BSA with EM-A at the amino group of the desosamine moiety. The sensitivity of EM-A in the present radioimmuno assay system was nearly equal to that by microbial assay using *Micrococcus luteus*. Nevertheless, this system is expected to be useful for the qualitative and quantitative determination of several motilides with no antimicrobial activity, in addition to EM-A and its metabolites.

References

1) ITOH, Z.; T. SUZUKI, M. NAKAYA, M. INOUE & S. MITSUHASHI: Gastrointestinal motor-stimulating activity of macrolide antibiotics and analysis of their side effects on the canine gut. Antimicrob. Agents Chemother. 26: 863~869, 1984
2) ITOH, Z.; M. NAKAYA, T. SUZUKI, H. ARAI & K. WAKABAYASHI: Erythromycin mimics exogenous motilin in gastrointestinal contractile activity in the dog. Am. J. Physiol. 247: G 688~694, 1984
3) ITOH, Z.; T. SUZUKI & M. NAKAYA: Structure-activity relation among macrolide antibiotics in initiation of interdigestive migrating contractions in the canine gastrointestinal tract. Am. J. Physiol. 248: G 320~325, 1985
4) ŌMURA, S.; K. TSUZUKI, T. SUNAZUKA, H. TOYOTA, I. TAKAHASHI & Z. ITOH: Gastrointestinal motor-stimulating activity of macrolide antibiotics and the structure-activity relationship. J. Antibiotics 38: 1631~1632, 1985
5) ŌMURA, S.; K. TSUZUKI, T. SUNAZUKA, S. MARUI, H. TOYODA, N. INATOMI & Z. ITOH: Macrolides with gastrointestinal motor-stimulating activity. J. Med. Chem. 30: 1941~1943, 1987
6) KITAGAWA, T.: Recent progress in the methods for selective modification of proteins. Yūki Gousei Kagaku 42: 283~292, 1984
7) FUJIWARA, K.; H. SAIKUSA, M. YASUNO & T. KITAGAWA: Enzyme immunoassay for the quantification of mitomycin C using β-galactosidase as a label. Cancer Res. 42: 1487~1491, 1982
8) RICH, D. H.; P. D. GESELLCHEN, A. TONG, A. CHEUNG & C. K. BUCKNER: Alkylating derivatives of amino acids and peptides. Synthesis of *N*-maleoylamino acids, [1-(*N*-maleoylglycyl)-cysteinyl]oxytosin. Effects on vasopressin-stimulated water loss from isolated toad bladder. J. Med. Chem. 18: 1004~1010, 1975
9) SHIMADA, N.; T. UEDA, T. YOKOSHIMA, K. UMEMURA & T. SHOMURA: Radioimmunoassay for metabolites of 9,3″-diacetylmidecamycin, a macrolide antibiotic. Antimicrob. Agents Chemother. 22: 976~980, 1982