CHEMICAL MODIFICATION OF ERYTHROMYCINS

III. *IN VITRO* AND *IN VIVO* ANTIBACTERIAL ACTIVITIES OF NEW SEMISYNTHETIC 6-*O*-METHYLERYTHROMYCINS A, TE-031 (CLARITHROMYCIN) AND TE-032

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The *in vitro* and *in vivo* antibacterial activities of 6-*O*-methylerythromycin A (TE-031, A-56268, or clarithromycin) and 6,11-di-*O*-methylerythromycin A (TE-032) have been compared with those of erythromycin A (EM) and josamycin (JM). TE-031 and TE-032, having the same antibacterial spectra as EM, are active against aerobic Gram-positive bacteria, some Gram-negative bacteria, anaerobic bacteria, L-form bacteria and *Mycoplasma pneumoniae*. The activity of TE-031 against clinical isolates is equal to or two times more potent than that of EM, whereas TE-032 is slightly less active than EM. The activities of TE-031 and TE-032 are pH dependent (more active at pH 8 than at 5) and are increased by adding serum to medium. TE-031 and TE-032 show dose-related bactericidal activities against *Haemophilus influenzae*. The therapeutic efficacies of TE-031 and TE-032 against systemic and subcutaneous infections provoked by Gram-positive bacteria in mice are 4- to 35-fold superior to those of EM and JM. TE-031 and TE-032 have demonstrated higher and longer-lasting plasma levels than EM when administered orally to mice, rats or dogs.

Erythromycin A (EM) has been a clinically useful macrolide antibiotic for over three decades. EM is particularly effective against most Gram-positive bacteria; some Gram-negative bacteria, including *Neisseria*, *Bordetella*, *Brucella*, *Campylobacter* and *Legionella*; and *Treponema*, *Chlamydia* and *Mycoplasma*.[1,2] One of limitations of EM is poor absorption after po administration because of its lability at gastric pH.

6-*O*-Methylerythromycin A (TE-031, clarithromycin) and 6,11-di-*O*-methylerythromycin A (TE-032) are new semisynthetic macrolides prepared by the first chemical modification[3] on the C-6 hydroxyl group of EM; they are more stable to acid than EM.[4] In this paper we describe the antibacterial activities of TE-031 and TE-032 which are compared with those of EM and josamycin (JM) *in vitro and in vivo*. Additionally, the pharmacokinetic profiles of these compounds in several species of animals are presented.

Materials and Methods

Bacterial Strains

Clinically isolated strains (198 strains) used in this study were obtained from several Japanese hospitals. The other strains (80 strains) were from the Taisho culture collection and ATCC strains.

Antibacterial Agents

TE-031 and TE-032 were prepared at Taisho Pharmaceutical Co., Ltd. EM and JM were obtained from Abbott Laboratories and Yamanouchi Pharmaceutical Co., Ltd., respectively. Stock solutions of the drugs were prepared for *in vitro* tests by dissolving 10 mg of drug in 5 ml of methanol. The drugs were

suspended in physiological saline containing 5% gum arabic for po administration *in vivo* tests. For iv injection of drugs, the test drugs were suspended in distilled water and then dissolved by the addition of the required amount of dilute hydrochloric acid keeping pH over 7.

Susceptibility Studies

Antibiotic susceptibility was determined by an agar dilution technique using Sensitivity Test Agar (STA, Eiken) as a basal test medium. This medium was supplemented with 5% horse blood for *Streptococcus pyogenes* and *Streptococcus pneumoniae*, and with heat inactivated 5% horse blood for *Haemophilus influenzae* and *Neisseria gonorrhoeae*. GAM Agar (Nissui) was used for anaerobic bacteria. Brain Heart Infusion Broth (Eiken) was supplemented with agar 1%, horse serum 10%, NaCl 4% and yeast extract 0.5% for L-form bacteria. PPLO Broth (Eiken) was supplemented with agar 1%, malt extract 0.2% and horse serum 20% for *Mycoplasma pneumoniae*. Inoculum sizes were 10^6 cfu/ml for both aerobic and anaerobic bacteria and 10^5cfu/plate for *M. pneumoniae*. Each serial dilution of the test drugs was mixed with the medium. The overnight culture broths of bacteria or their dilutions were seeded onto agar plates with a spot replicating device. MIC end point, which is defined as the lowest antibiotic concentrations showing less than four colonies per spot, was read after 18 hours incubation for aerobic bacteria, 48 hours incubation for anaerobes and 7 days incubation for *M. pneumoniae*. All incubations were at 37°C.

Effect of Various Factors on Antibacterial Activity

The effects of pH of the medium, inoculum size and addition of horse serum on the antibacterial activities were assayed by determining the MICs of test drugs against *Staphylococcus aureus* 209P-JC, *Staphylococcus epidermidis* IID 866, *S. pneumoniae* IID 553, *Bacillus subtilis* ATCC 6633, and *Escherichia coli* NIHJ JC-2 by use of STA as a basal test medium. The pH of the medium was adjusted to 8, 7, 6 or 5 with 1 N NaOH or 1 N HCl. Horse serum was added to the medium to obtain a final serum concentration of 20%. Inoculum sizes tested were 10^9, 10^8, 10^7, 10^6 and 10^5 cfu/ml.

Time-kill Curve Studies

The bactericidal activities of TE-031, TE-032, EM and JM over time against *S. aureus* Smith 4 and *H. influenzae* IID 984 were measured in Sensitivity Test Broth (Eiken). The medium was supplemented with 5% Fildes enrichment for *H. influenzae*. An overnight culture of each strain was seeded into an appropriate broth and incubated at 37°C. When the bacteria were grown to a concentration of $10^5 \sim 10^6$ cells/ml, one-fourth to 4-fold the MIC of test drug was added. The number of viable cells was counted at 2, 4, 6, 8 and 24 hours after the addition of drugs. For counting bacteria, STA and STA supplemented with heat inactivated 5% horse blood were used as the media for *S. aureus* and *H. influenzae*, respectively.

In Vivo Therapeutic Activity

Acute Systemic Infection: Male ICR mice, 4 weeks of age, were infected intraperitoneally with 0.5 ml of a bacterial suspension containing 100% or more of minimal lethal doses of bacteria. Hog gastric mucin (5%, w/v) was added to the suspensions of organisms before injection. The test drugs in 5% gum arabic were administered orally in 0.2 ml volumes to groups of 20 mice at 1 hour after the inoculation. Mortality of the animals was recorded daily over a period of 5 to 7 days and the ED_{50} values were calculated by the methods of probit[5] or VAN DER WAERDEN.[6]

Subcutanous Infection: Male *dd*Y mice, 4 weeks of age, were infected subcutaneously with 0.2 ml of the suspension containing 5×10^7 cfu/ml of *S. aureus* BB into the dosal area. The drugs were administered orally 1 hour after inoculation. A diameter of absess was measured at 48 hours after the infection. The amount of drug (mg/mouse) required for a 50% reduction of the diameter of abscess (ID_{50}) as compared with untreated controls was estimated.

Determination of Plasma Level

The antibiotics were administered orally or intravenously to animals. Plasmas were collected at regular time intervals after administration and the concentrations of the antibiotic were measured by the bioassay method using *Micrococcus luteus* ATCC 9341 as a test organism. Animals used and the doses (route) of

drug administration were as follows: Male ICR mice, 100 mg/kg (po); male Wister rats, 50 mg/kg (po) and 10 mg/kg (iv); male New Zealand White rabbits, 10 mg/kg (iv); male Beagle dogs, 10 mg/kg (po and iv) and 2 mg/kg (iv).

Results

In Vitro Susceptibility Studies

Antibacterial Spectrum

In vitro activities (MICs) of TE-031 and TE-032 were compared with those of EM and JM against standard strains of bacteria. The results are shown in Tables 1, 2 and 3. The potency of TE-031 is generally equal to or 2-fold greater than that of EM and 2- to 8-fold greater than that of JM against aerobic Gram-positive bacteria, including *S. aureus*, *S. epidermidis* and *S. pneumoniae*; the potency of TE-032 against these organisms is equal to or 2-fold less than that of EM. The MICs against the EM-sensitive organisms ranged from ≦0.012 to 0.10 μg/ml for TE-031 and from ≦0.012 to 0.39 μg/ml for TE-032. TE-031 and TE-032 were found to be inactive against EM-resistant strains which were sensitive to JM (Table 1). The potency of TE-031 against *N. gonorrhoeae* (MIC 0.10 μg/ml) and *H. influenzae* (MIC

Table 1. *In vitro* antibacterial activity of TE-031, TE-032, EM and JM against Gram-positive bacteria.

Strain	MIC (μg/ml)			
	TE-031	TE-032	EM	JM
Bacillus subtilis ATCC 6633	0.10	0.20	0.20	0.39
B. cereus ATCC 9634	0.05	0.20	0.05	0.78
Micrococcus luteus NIHJ	0.025	0.05	0.025	0.10
M. flavus	0.10	0.20	0.10	0.10
Staphylococcus aureus 209P-JC	0.10	0.20	0.10	0.20
S. aureus Terajima	0.10	0.20	0.10	0.78
S. aureus Smith 4	0.10	0.20	0.20	0.78
S. aureus BB	0.10	0.20	0.10	0.39
S. aureus J-74	>100	>100	>100	1.56
S. aureus J-65	100	>100	>100	0.39
S. aureus J-70	>100	>100	>100	0.78
S. aureus J-109	>100	>100	>100	>100
S. aureus T-98	0.10	0.39	0.39	6.25
S. epidermidis IID 866	0.10	0.20	0.20	0.39
S. epidermidis sp-al-1	0.10	0.20	0.20	1.56
Streptococcus pneumoniae IID 552[a]	0.025	0.05	0.025	0.10
S. pneumoniae IID 553[a]	0.025	0.05	0.05	0.10
S. pneumoniae IID 554[a]	0.025	0.05	0.025	0.10
S. pneumoniae J-4[a]	≦0.012	0.025	≦0.012	0.10
S. pyogenes IID 689[a]	≦0.012	0.05	0.025	0.10
S. pyogenes J-1[a]	0.05	0.10	0.05	0.20
S. pyogenes A-14[a]	0.025	0.05	0.025	0.20
Streptococcus A group 4[a]	>100	>100	>100	>100
Streptococcus B group 1[a]	0.025	0.05	0.025	0.10
Streptococcus B group 2[a]	0.05	0.10	0.05	0.20
Streptococcus C group 1[a]	0.05	0.10	0.05	0.39
Streptococcus C group 2[a]	0.05	0.10	0.05	0.39
Streptococcus G group 1[a]	0.025	0.05	0.05	0.20
Corynebacterium xerosis IID 551	0.025	0.05	0.05	0.39
C. diphtheriae IID 526	≦0.012	≦0.012	≦0.012	≦0.012

Inoculum size: 10^6 cfu/ml.
Medium: Sensitivity Test Agar (STA, Eiken).
[a] STA + 5% horse blood.

Table 2. *In vitro* antibacterial activity of TE-031, TE-032, EM and JM against Gram-negative bacteria.

Strain	MIC (μg/ml)			
	TE-031	TE-032	EM	JM
Escherichia coli NIHJ JC-2	100	>100	100	>100
Salmonella typhi IID 610	3.13	6.25	3.13	3.13
S. paratyphi-B B-79	25	50	25	>100
S. enteritidis KB-21	25	100	50	100
Shigella flexneri Type 2a	12.5	12.5	12.5	100
S. sonnei EW 33	12.5	50	25	100
Klebsiella pneumoniae IFO 3317	50	100	50	>100
K. pneumoniae 3K-2	25	50	25	>100
Serratia marcescens IID 618	100	>100	50	>100
S. liquefaciens MCNH-3	100	>100	100	>100
Enterobacter aerogenes IFO 12010	50	>100	50	>100
Proteus vulgaris IID 874	100	>100	100	>100
P. mirabilis IFO 3849	>100	>100	>100	>100
Morganella morganii IID 602	100	>100	100	>100
Providencia rettgeri TCP 3232	100	>100	>100	>100
Pseudomonas aeruginosa GNB 1-1-1	50	>100	100	>100
P. aeruginosa P 32	12.5	100	25	>100
Neisseria gonorrhoeae J-1[a]	0.10	0.39	0.20	0.78
N. gonorrhoeae J-4[a]	0.10	0.39	0.20	0.78
Haemophilus influenzae IID 984[a]	6.25	12.5	3.13	25
H. influenzae J-13[a]	6.25	12.5	3.13	25

Inoculum size: 10^6cfu/ml.
Medium: Sensitivity Test Agar (Eiken).
[a] Chocolate agar.

6.25 μg/ml) was similar to that of EM and greater than that of JM, while TE-032 was 2- to 4-fold less active than EM against these organisms (Table 2). TE-031 had approximately 2-fold more potent activity than EM against Gram-positive anaerobes and *Bacteroides*; the MICs of TE-031 against these organisms were ≦0.012~1.56 μg/ml and 0.05~1.56 μg/ml, respectively. The potency of TE-032 was equal to or less than that of EM against these organisms (Table 3). TE-031 and TE-032 had low activities against *E. coli*, *Klebsiella*, *Proteus*, *Pseudomonas* and *Fusobacterium* as low as EM (Tables 2 and 3). Table 4 shows the activities of TE-031 and TE-032 against L-forms of *S. aureus*, *S. pyogenes* and *E. coli*. The activity of TE-031 (MIC 0.05~0.10 μg/ml) against these bacteria was 2- to 4-fold and 4- to 8-fold more active than those of EM and JM, respectively, whereas the activity of TE-032 was equal to that of EM against these bacteria.

Susceptibility of Clinical Isolates

The *in vitro* antibacterial activities of TE-031 and TE-032, compared with EM and JM, were tested against 198 strains of clinical isolates, including *S. aureus* (48), *S. epidermidis* (50), *Enterococcus faecalis* (45), *S. pyogenes* (11), *S. pneumoniae* (10), *H. influenzae* (19) and *Mycoplasma pneumoniae* (15). As shown in Table 5, the potency of TE-031 was equal to or slightly greater than that of EM against all groups of test organisms, whereas TE-032 was 2-fold less potent than EM. In particular, TE-031 was effective against 100% of all the isolates, for *S. pneumoniae* (MIC≦0.012~0.05 μg/ml), *S. pyogenes* (MIC≦0.012~0.05 μg/ml), *M. pneumoniae* (MIC 0.0020~0.0156 μg/ml), *H. influenzae* (MIC 1.56~6.25 μg/ml); the $MIC_{s_{50}}$ of TE-031 against these organisms were ≦0.012, 0.025, 0.0078 and 3.13 μg/ml,

Table 3. *In vitro* antibacterial activity of TE-031, TE-032, EM and JM against anaerobic bacteria.

Strain	MIC (μg/ml)			
	TE-031	TE-032	EM	JM
Gram-positive:				
Peptostreptococcus asaccharolyticus 10-2	0.78	1.56	1.56	0.78
P. prevotii ATCC 9321	0.78	1.56	1.56	0.39
P. magnus ATCC 14955	1.56	3.13	3.13	0.78
P. micros 1194	0.78	3.13	1.56	0.78
Propionibacterium acnes ATCC 11827	≦0.012	≦0.012	≦0.012	0.05
P. acnes ATCC 11828	≦0.012	0.025	0.025	0.05
P. granulosum ATCC 25564	≦0.012	0.025	0.025	0.05
Bifidobacterium adolescentis ATCC 15703	≦0.012	0.025	0.025	0.05
Eubacterium lentum H-1	≦0.012	0.025	0.05	0.20
E. limosum ATCC 8486	0.05	0.10	0.20	0.20
E. aerofaciens ATCC 25986	≦0.012	≦0.012	≦0.012	≦0.012
Clostridium perfringens 8329	0.39	0.78	0.78	0.78
C. botulinum Type B	0.05	0.20	0.10	0.39
C. bifermentans	0.05	0.20	0.10	0.20
C. sordellii 6559	0.20	0.39	0.39	0.39
C. tetani	0.10	0.20	0.20	0.10
Gram-negative:				
Bacteroides fragilis FA-32	0.05	0.20	0.20	0.10
B. thetaiotaomicron 46	1.56	6.25	3.13	0.39
B. distasonis HR-122	0.05	0.20	0.10	0.20
B. distasonis 7007	0.78	1.56	1.56	0.39
B. ovatus 4999	0.39	3.13	1.56	0.20
B. praeacutus ATCC 25539	0.39	1.56	0.39	0.39
Fusobacterium varium B-1083	>100	>100	>100	>100
F. necrophorum S-45	>100	>100	>100	>100
F. mortiferum WA-1-4	>100	>100	>100	100
F. mortiferum ATCC 9817	>100	>100	>100	>100

Inoculum size: 10^6 cfu/ml.
Medium: GAM Agar (Nissui).

Table 4. Antibacterial activity of TE-031, TE-032, EM and JM against L-forms and standard strains.

Strain	MIC (μg/ml)			
	TE-031	TE-032	EM	JM
L-Form:				
Staphylococcus aureus 209PL	0.10	0.20	0.20	0.39
Streptococcus pyogenes 124L	0.05	0.10	0.10	0.39
Escherichia coli EcL-N5	0.05	0.20	0.20	0.39
Standard strain:				
S. aureus 209P-JC	0.10	0.20	0.10	0.20
S. pyogenes IID 689[a]	≦0.012	0.05	0.025	0.10
E. coli NIHJ JC-2	100	100	100	>100

Medium: L-Form, Brain Heart Infusion Broth (Eiken)+agar 1%+horse serum 10%+NaCl 4%+yeast extract 0.5%; Standard strain, Sensitivity Test Agar (Eiken).
[a] Supplemented with 5% horse blood.

respectively. TE-031, TE-032 and EM showd no potency (MIC>100 μg/ml) against 54, 40 and 51% of the test strains of *S. aureus*, *S. epidermidis* and *E. faecalis*, respectively.

Effect of Various Factors on Antibacterial Activity

As shown in Table 6, the decrease of the medium pH from 8 to 5 resulted in a 128- to 256-fold

Table 5. Comparative antibacterial activities of TE-031 and TE-032 and reference compounds (EM and JM) against various clinical isolates of bacteria.

Organism (No. of strain)	Drug	MIC range (μg/ml)	MIC_{50}	MIC_{80}
Staphylococcus aureus (meth^s 22)	TE-031	0.10～>100	0.10	0.20
	TE-032	0.39～>100	0.39	0.39
	EM	0.20～>100	0.20	0.20
	JM	0.78～>100	1.56	1.56
S. aureus (meth^r 26)	TE-031	0.10～>100	>100	>100
	TE-032	0.39～>100	>100	>100
	EM	0.20～>100	>100	>100
	JM	0.78～>100	1.56	>100
S. epidermidis (50)	TE-031	0.10～>100	0.20	>100
	TE-032	0.20～>100	0.39	>100
	EM	0.10～>100	0.20	>100
	JM	0.39～>100	0.78	>100
Enterococcus faecalis (45)	TE-031	0.10～>100	100	>100
	TE-032	0.20～>100	>100	>100
	EM	0.10～>100	>100	>100
	JM	1.56～>100	100	>100
Streptococcus pyogenes[a] (11)	TE-031	≦0.012～0.05	0.025	0.025
	TE-032	0.025～0.39	0.05	0.05
	EM	≦0.012～0.05	0.025	0.025
	JM	≦0.012～0.39	0.10	0.20
S. pneumoniae[a] (10)	TE-031	≦0.012～0.05	≦0.012	0.025
	TE-032	0.025～0.05	0.05	0.05
	EM	≦0.012～0.05	0.05	0.05
	JM	0.05～0.39	0.20	0.39
Haemophilus influenzae[b] (19)	TE-031	1.56～6.25	3.13	6.25
	TE-032	6.25～12.5	6.25	12.5
	EM	0.78～6.25	3.13	3.13
	JM	6.25～25	25	25
Mycoplasma pneumoniae[c] (15)	TE-031	0.0020～0.0156	0.0078	0.0078
	TE-032	0.0156～0.0313	0.0156	0.0313
	EM	0.0039～0.0313	0.0156	0.0156
	JM	0.0313～0.125	0.0625	0.125

meth^s: Methiciline-susceptible strains. meth^r: Methiciline-resistant strains.
Inoculum size: 10^6 cfu/ml.
Medium: Sensitivity Test Agar (STA, Eiken).
[a] STA+5% horse blood, [b] Chocolate agar, [c] PPLO broth (Eiken)+malt extract (Nissui) 0.2%+horse serum 20%+agar 1% (Inoculum size: 10^5 cfu/plate).

decrease of the observed activities of TE-031 and TE-032 against the Gram-positive bacteria. For EM over the same pH range, there was 1,024- to 2,048-fold decrease in measured activity. In addition, the activities of TE-031 and TE-032 against *E. coli* increased with an increase in the medium pH as well as EM. Like EM and JM, the MICs of TE-031 and TE-032 were slightly affected by changes in inoculum size between $10^5 \sim 10^9$ cfu/ml (Table 7). The addition of horse serum to the medium, to provide a serum content of 20%, resulted in a 2- to 16-fold increase in the antibacterial activities of TE-031 and TE-032, as well as in that of EM (Table 8).

Time-kill Curve Studies

The bactericidal activities of TE-031 and TE-032 against *S. aureus* Smith 4 and *H. influenzae* IID 984 were compared with those of EM and JM (Figs. 1 and 2). At concentrations equal to the MIC, TE-031 and TE-032 did not cause a significant reduction in the number of viable cells during the first 8 hours

Table 6. Effect of medium pH on the antibacterial activity of TE-031, TE-032, EM and JM.

Organism	pH	MIC (μg/ml)			
		TE-031	TE-032	EM	JM
Staphylococcus aureus 209P-JC	5	3.13	6.25	25	6.25
	6	0.78	1.56	1.56	0.78
	7	0.10	0.39	0.10	0.39
	8	≦0.012	0.025	≦0.012	0.20
S. epidermidis IID 866	5	3.13	12.5	50	12.5
	6	0.78	1.56	0.78	1.56
	7	0.10	0.20	0.20	0.39
	8	≦0.012	0.05	0.025	0.39
Streptococcus pneumoniae IID 553[a]	6	0.10	0.20	0.20	0.39
	7	0.025	0.05	0.05	0.20
	8	≦0.012	0.025	≦0.012	0.20
Bacillus subtilis ATCC 6633	5	1.56	6.25	25	6.25
	6	0.78	1.56	0.78	1.56
	7	0.05	0.20	0.10	0.39
	8	≦0.012	0.05	0.025	0.39
Escherichia coli NIHJ JC-2	5	>100	50	>100	>100
	6	>100	100	>100	>100
	7	50	12.5	100	>100
	8	6.25	6.25	12.5	>100

Inoculum size: 10^6 cfu/ml.
Medium: Sensitivity Test Agar (Eiken).
[a] Supplemented with 5% horse blood.

Table 7. Effect of inoculum size on the antibacterial activity of TE-031, TE-032, EM and JM.

Strain	Inoculum size (cfu/ml)	MIC (μg/ml)			
		TE-031	TE-032	EM	JM
Staphylococcus aureus 209P-JC	10^5	0.10	0.39	0.10	0.20
	10^6	0.10	0.39	0.10	0.20
	10^7	0.10	0.39	0.20	0.20
	10^8	0.20	0.78	0.39	0.78
	10^9	0.39	0.78	0.39	0.78
S. epidermidis IID 866	10^5	0.10	0.20	0.10	0.20
	10^6	0.10	0.39	0.20	0.39
	10^7	0.10	0.39	0.20	0.39
	10^8	0.20	0.78	0.39	0.78
	10^9	0.39	0.78	0.39	1.56
Streptococcus pneumoniae IID 553[a]	10^5	0.012	0.025	0.025	0.10
	10^6	0.012	0.05	0.025	0.10
	10^7	0.025	0.05	0.05	0.10
	10^8	0.025	0.05	0.05	0.10
Bacillus subtilis ATCC 6633	10^5	0.05	0.20	0.10	0.39
	10^6	0.10	0.20	0.20	0.39
	10^7	0.10	0.39	0.20	0.39
	10^8	0.10	0.39	0.20	0.39
	10^9	0.20	0.39	0.20	0.39
Escherichia coli NIHJ JC-2	10^5	100	>100	100	>100
	10^6	100	>100	100	>100
	10^7	100	>100	100	>100
	10^8	100	>100	100	>100
	10^9	>100	>100	>100	>100

Medium: Sensitivity Test Agar (Eiken).
[a] Supplemented with 5% horse blood.

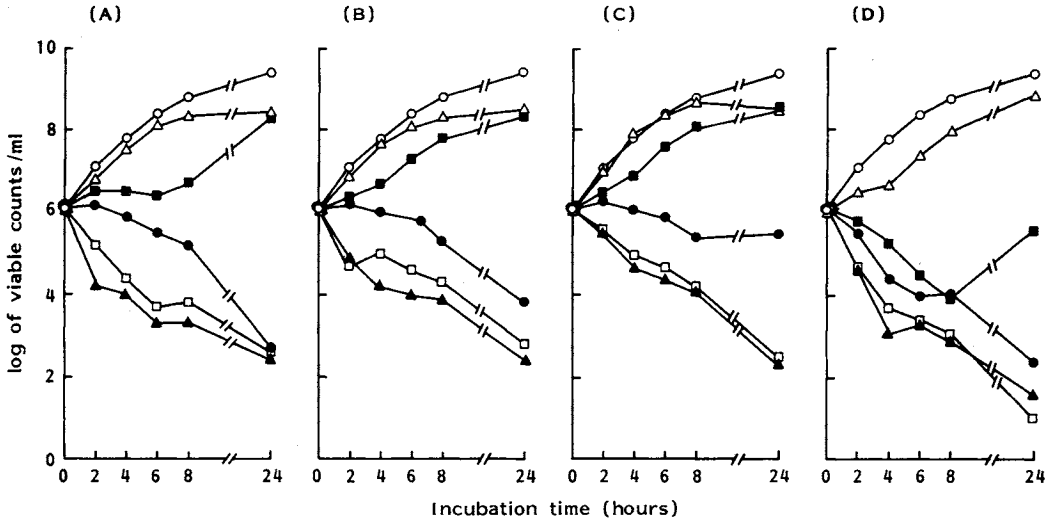
Table 8. Effect of horse serum on the antibacterial activity of TE-031, TE-032, EM and JM.

Strain	MIC (μg/ml)							
	TE-031		TE-032		EM		JM	
	0%[a]	20%	0%	20%	0%	20%	0%	20%
Staphylococcus aureus 209P-JC	0.10	0.05	0.39	0.10	0.20	0.05	0.39	0.20
S. epidermidis IID 866	0.10	0.025	0.39	0.05	0.20	0.025	0.39	0.20
Bacillus subtilis ATCC 6633	0.10	0.025	0.20	0.05	0.10	0.05	0.39	0.39
Streptococcus pneumoniae IID 553	0.025	≦0.012	0.05	≦0.012	0.05	≦0.012	0.20	0.10
Escherichia coli NIHJ JC-2	100	6.25	>100	25	100	6.25	>100	>100

Inoculum size: 10^6 cfu/ml.
Medium: Sensitivity Test Agar (Eiken).
[a] % of horse serum supplemented.

Fig. 1. Bactericidal activities of TE-031(A), TE-032(B), EM(C) and JM(D) against *Staphylococcus aureus* Smith 4.

○ No drug, △ 1/4 MIC, ■ 1/2 MIC, ● 1 MIC, □ 2 MIC, ▲ 4 MIC.
(A) MIC: 0.20 μg/ml, (B) MIC: 0.39 μg/ml, (C) MIC: 0.20 μg/ml, (D) MIC: 0.78 μg/ml.



incubation of *S. aureus*. On the other hand, TE-031 was highly bactericidal against *H. influenzae* IID 984 as well as EM, showing dose-related killing kinetics; TE-031 caused a significant reduction (*ca.* 1/1,000) in the number of viable cells during the first 8 hours at the concentration of MIC. Moreover, TE-031 and TE-032 inhibited the growth of *S. aureus* and *H. influenzae* at the concentrations of one to four times the MIC after 24 hours of incubation.

In Vivo Therapeutic Activity

Table 9 shows activities of TE-031, TE-032, EM and JM against experimental systemic infections provoked by *S. aureus* Smith 4, *S. aureus* BB, *S. pyogenes* J-1 and *S. pneumoniae* IID 553 in mice. When administered orally, TE-031 was 6 to 15 times and 11 to 35 times more active than EM and JM, respectively; the ED_{50} values for TE-031 varied from 0.055 to 0.340 mg/mouse against the test organisms. TE-032 was

Fig. 2. Bactericidal activities of TE-031 (A), TE-032 (B), EM (C) and JM (D) against *Haemophilus influenzae* IID 984.

○ No drug, △ 1/4 MIC, ■ 1/2 MIC, ● 1 MIC, □ 2 MIC, ▲ 4 MIC.
(A) MIC: 12.5 μg/ml, (B) MIC: 12.5 μg/ml, (C) MIC: 6.25 μg/ml, (D) MIC: 12.5 μg/ml.

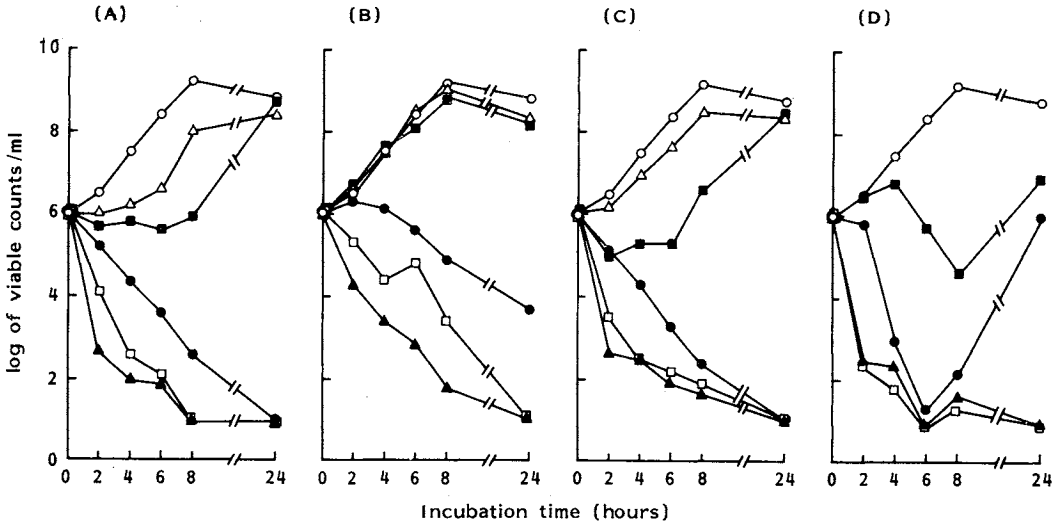


Table 9. *In vivo* antibacterial activity of TE-031, TE-032, EM and JM against experimental systemic infections in mice.

Strain	Challenge dose (cfu/mouse)	Drug	ED_{50} (mg/mouse)[a]	MIC (μg/ml)[b]
Staphylococcus aureus Smith 4	2×10^7 (5% Mucin)	TE-031	0.212(0.168~0.279)	0.10
		TE-032	0.285(0.245~0.333)	0.20
		EM	1.23 (0.979~1.54)	0.20
		JM	3.01 (2.27 ~3.87)	0.78
S. aureus BB	2×10^7 (5% Mucin)	TE-031	0.340(0.281~0.411)	0.10
		TE-032	0.663(0.539~0.813)	0.20
		EM	3.32 (2.73 ~4.41)	0.10
		JM	11.9 (10.1 ~14.0)	0.39
Streptococcus pyogenes J-1	3.9×10^7 (5% Mucin)	TE-031	0.055(0.045~0.067)	0.05
		TE-032	0.162(0.140~0.189)	0.10
		EM	0.837(0.669~1.04)	0.05
		JM	1.92 (1.36 ~2.28)	0.20
S. pneumoniae IID 553	3.3×10^6	TE-031	0.332(0.254~0.430)	0.025
		TE-032	0.352(0.223~0.512)	0.05
		EM	3.31 (2.36 ~4.42)	0.05
		JM	3.74 (2.98 ~4.70)	0.10

Administration of drug: po, 1 hour after infection.
Mouse: Male ICR mouse, 4 weeks, 20 mice/group.
[a] 95% confidence limits.
[b] Inoculum size: 10^6 cfu/ml.

4 to 9 times more potent than EM and equal to or 3-fold less potent than TE-031. As shown in Table 10, TE-031 and TE-032 were approximately 5 and 7 times more active than EM, respectively, against the subcutaneous abscess infection with *S. aureus* BB in mice.

Pharmacokinetic Studies in Animals

The plasma levels of TE-031 and TE-032, compared with EM, were measured after single po or iv

administrations to mice, rats, rabbits or dogs. The peak concentration in plasma (Cmax), AUC and $t_{1/2}$ are shown in Table 11. The Cmax and $t_{1/2}$ values for TE-031 and TE-032 were 1- to 4-fold higher than those of EM after po administration. The AUCs for TE-031 and TE-032 were 5- to 10-fold greater than those of EM. In addition, when administered intravenously, TE-031 demonstrated 2 to 3 times longer-lasting plasma levels as compared with EM.

Discussion

The biological properties of TE-031 and TE-032 have been compared with those of EM and JM *in vitro* and *in vivo*.

TE-031 and TE-032, having the same antibacterial spectrum as EM, have demonstrated an excellent *in vitro* activity against standard strains and clinical isolates, including aerobic and anaerobic Gram-positive bacteria and *M. pneumoniae*. In general, the *in vitro* potency of TE-031 against those organisms is equal to or greater than that of EM, whereas TE-032 is slightly less potent than EM. Among Gram-positive bacteria including *S. aureus*, *S. epidermidis* and *E. faecalis*, a significant number are resistant to TE-031 and TE-032 as well as to EM.

TE-031 and TE-032 are bactericidal against *H. influenzae* IID 984, but exert a bacteriostatic effect against *S. aureus* Smith 4.

Like EM, activities of TE-031 and TE-032 increased by the addition of serum to the medium but are not greatly affected by inoculum size. The higher decrease of the antibacterial activity in EM at the medium pH 5, as compared with those in

Table 10. *In vivo* antibacterial activity of TE-031, TE-032, EM and JM against experimental subcutaneous infection with *Staphylococcus aureus* BB in mice.

	TE-031	TE-032	EM	JM
ID_{50}[a] (mg/mouse)	0.40	0.30	2.1	3.7

Challenge: *S. aureus* BB, 1×10^7 cfu (sc).
Administration: 1 hour after infection (po).
Mouse: Male *ddY* mouse, 4 weeks, 5 mice/group.
[a] The amount of drug required for a 50% reduction of the diameter of the abscess compared with untreated controls.

Table 11. Pharmacokinetics of TE-031 and TE-032 in laboratory animals.

Compound	Animal (Number)	Dose (mg/kg)	Route[b]	Plasma level[a]		
				Cmax (μg/ml)	AUC (μg·hour/ml)	$T_{1/2}$ (hour)
TE-031	Mouse (3)	100	po	10.77	26.89	1.44
TE-032	Mouse (3)	100	po	5.48	24.38	2.89
EM	Mouse (3)	100	po	3.40	3.87	0.91
JM	Mouse (3)	100	po	2.04	4.54	1.17
TE-031	Rat (3)	50	po	2.40	11.17	2.47
TE-032	Rat (3)	50	po	2.53	15.07	3.52
EM	Rat (3)	50	po	0.64	2.37	2.81
TE-031	Dog (4)	10	po	3.15	33.09	5.68
EM	Dog (4)	10	po	2.52	3.35	1.35
TE-031	Rat (6)	10	iv	—	3.648	2.54
EM	Rat (4)	10	iv	—	0.991	1.01
TE-031	Rabbit (3)	10	iv	—	3.931	2.32
EM	Rabbit (3)	10	iv	—	2.27	1.06
TE-031	Dog (3)	2	iv	—	7.848	3.14
TE-031	Dog (1)	10	iv	—	57.955	2.45
EM	Dog (3)	10	iv	—	3.271	0.99

[a] Plasma levels were determined by bioassay method using *Micrococcus luteus* ATCC 9341.
[b] Drugs were administered orally (po) or intravenously (iv).

TE-031 and TE-032, can be explained by the differences of acid-stabilities among these drugs.[4]

The peak plasma levels of TE-031 and TE-032 are significantly higher than those of EM in several kinds of animals after the po or iv administrations of drugs.

When administered orally, TE-031 and TE-032 exhibit several times more potent *in vivo* activity than EM against the systemic infection and the subcutaneous abscess infection in mice. The higher therapeutic efficacy provided by TE-031 and TE-032 in these models cannot be explained by differences in MICs, but may be related to the higher and longer-lasting plasma levels of both drugs.

Thus, 6-*O*-methyl derivatives of EM have demonstrated excellent *in vivo* antibacterial activity and improved pharmacokinetic profiles: Especially, TE-031, which is more potent than TE-032, is interesting enough for further investigations.

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