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Evaluation of Three 4″-Deoxy-4″-Sulfonamido-Oleandomycin Derivatives with Erythromycin-Like Antibacterial Potency

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Three derivatives of oleandomycin in which the C″-4 hydroxyl moiety was replaced for the first time with a nitrogen functionality have been compared with erythromycin base and oleandomycin base. The minimum inhibitory concentrations of these derivatives for 90% of a group of clinical isolates of *Staphylococcus aureus* were one-half to one-fourth those of erythromycin. The minimum inhibitory concentrations of the experimental macrolides for 50% of a group of *S. aureus* isolates resistant to >12.5 μg of erythromycin per ml ranged from 0.2 to 0.39 μg/ml. The activities of these experimental compounds were equivalent to the activities of erythromycin against *Staphylococcus epidermidis*, *Bacteroides fragilis*, and *Haemophilus influenzae* isolates. In general, erythromycin was more active against *Streptococcus* species. Each experimental macrolide was superior to erythromycin in inhibiting RNA-directed, cell-free polypeptide synthesis. The three experimental compounds were markedly more active than erythromycin base after oral administration to mice infected with *S. aureus*. The 50% protective doses of the experimental compounds ranged from 27.4 to 45.7 mg/kg; that of erythromycin was approximately 100 mg/kg.

Oleandomycin, a 14-member ring macrolide, has an antibacterial spectrum similar to that of erythromycin but is less active in vitro. However, oleandomycin has much greater acid stability than erythromycin base. Because of this key advantage, chemical modification of oleandomycin was initiated in an attempt to increase potency (1). It was found that the substitution of a nitrogen functionality for the C″-4 hydroxyl conferred increased antibacterial potency in the derivatives. A comparison of the in vitro and in vivo activities of these derivatives with the activities of oleandomycin and erythromycin is reported here.

MATERIALS AND METHODS

Antibiotics and chemicals. The three derivatives and oleandomycin were prepared by members of the Department of Medicinal Chemistry in the Central Research Division, Pfizer Inc., Groton, Conn., by previously described methods (1). Erythromycin was purchased from commercial sources. All compounds were used as the free base. The oleandomycin derivatives were unique in that the C″-4 hydroxyl moiety was replaced with a nitrogen functionality, i.e., *p*-chlorobenzene-sulfonamide (P-CBS), 3-methyl-thiophene-sulfonamide (3-MeTPS), or thiophene-sulfonamide (TPS) (Fig. 1).

MS2 viral RNA and tRNA were from Miles Laboratories, Elkhart, Ind. GTP, ATP, phosphenol pyruvate, and DNase (RNAase) were from C. F. Boehringer & Söhne GmbH, Mannheim, Federal Republic of Germany. Dithiothreitol, imidazole, and EDTA were from Eastman Kodak Co., Rochester, N.Y. Pyruvate kinase, β-mercaptoethanol, and Tris-hydrochloride were from Calbiochem-Behring, La Jolla, Calif. Radioactive amino acid mixture was from New England Nuclear Corp., Boston, Mass. L-Amino acids were from U.S. Biochemical Corp., Cleveland, Ohio. 3A70 scintillation solution was from Research Products International Corp., Mount Prospect, Ill.

Microorganisms. The microorganisms used were recent clinical isolates obtained from hospitals in several areas within the eastern United States. Upon receipt, brain heart infusion agar slants or appropriately adjuncted brain heart infusion agar slants were inoculated, and the resulting growth was layered with sterile mineral oil. These were maintained at refrigerator temperature until used.

MICs. Minimal inhibitory concentrations (MICs) were determined by a standard dilution method as described previously (7). The basal medium, brain heart infusion agar, was enriched with 5% (vol/vol) sheep blood for studies with *Streptococcus pyogenes* and with 5% Fildes–2% IsoVitaleX for studies with *Haemophilus influenzae*. Incubation at 37°C was in an atmosphere of 5% CO_2, 10% H_2, 85% N_2, and a trace of O_2. The inoculum delivered by a multiprong device was approximately 20,000 cells. Studies with *Bacteroides fragilis* were carried out in prereduced brain heart infusion broth. Incubation at 37°C was in a mixture of 80% N_2, 10% CO_2, and 10% H_2 in an anaerobic chamber.

Experimental infections in mice. Outbred ICR mice having an average weight of 13 g were purchased from Blue Spruce Farms, Altamont, N.Y. Acute systemic infections were produced by intraperitoneal injection of 1 to 10 100% lethal doses of bacterial culture suspended in 5% hog gastric mucin. Mice were treated orally via gavage 0.5 h after challenge and again at 4 and 24 h. The dosage range consisted of four different antibiotic concentrations in a twofold dilution series administered to 10 mice per dosage level. The percentage of survival was recorded after a 4-day observation period. After three or four experiments were completed, survival data were averaged, and a 50% protective dose (PD_{50}) expressed in milligrams per kilogram per dose was calculated (2).

Assay of inhibition of polypeptide synthesis. Cell extract (S30) was prepared as described previously (6). mRNA was obtained by partially unfolding the infectious MS2 viral RNA by a mild reaction with formaldehyde as described by Lodish (3). After treatment, the MS2 RNA was redissolved in 0.3 M Tris (pH 7.4)–0.02 M β-mercaptoethanol buffer and was stored at −70°C. Cell-free polypeptide synthesis was carried out in a final reaction mixture of 100 μl that was prepared in several steps. First, a preincubation mix of S30 cell extract (0.3 mg of protein), 0.2 M imidazole (pH 7.4), 0.27 M NH_4Cl, 37 mM $MgCl_2$, and 13 mM β-mercaptoethanol in a volume of

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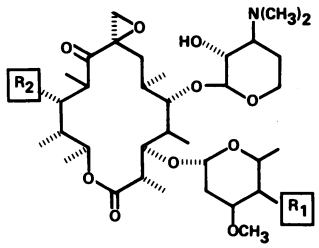

		R_1	R_2
Oleandomyin		OH	OH
P-CBS	p-chlorobenzene-sulfonamide	NH—SO_2—C$_6$H$_4$—Cl	CH_3—C(=O)—O
Me-TPS	3-methyl-thiophene-sulfonamide	NH—SO_2—(thiophene)—H_3C	CH_3—C(=O)—O
TPS	thiophene-sulfonamide	NH—SO_2—(thiophene)	CH_3—C(=O)—O

FIG. 1. Chemical structures.

30 μl was incubated for 20 min at 37°C. Second, 63 μl of a solution consisting of 81 mM Tris (pH 7.4), 5.4 mM β-mercaptoethanol, 0.34 mM GTP, 4 mM ATP, 7 mM phosphenol pyruvate, 15 IU of pyruvate kinase, 50 absorbance units of tRNA (260 nm), 14 μM concentrations of 20 L-amino acids, and 5 μCi of a mixture of 15 L-^{3}H-amino acids (concentration, 1 μCi/ml) was added to the preincubation mixture. Finally, antibiotics were added at the indicated concentrations, followed by 15 μg of formyltetrahydropteroylglutamic acid and 0.125 absorbance units (260 nm) of treated MS2 RNA. Volumes were adjusted with water to give a final volume of 100 μl. All additions were carried out at 4°C. The reaction was initiated immediately after the addition of the MS2 RNA by placing the tubes in a shaking water bath at 37°C for 15 min. The reaction was terminated by the addition of 3 ml of 5% trichloroacetic acid containing

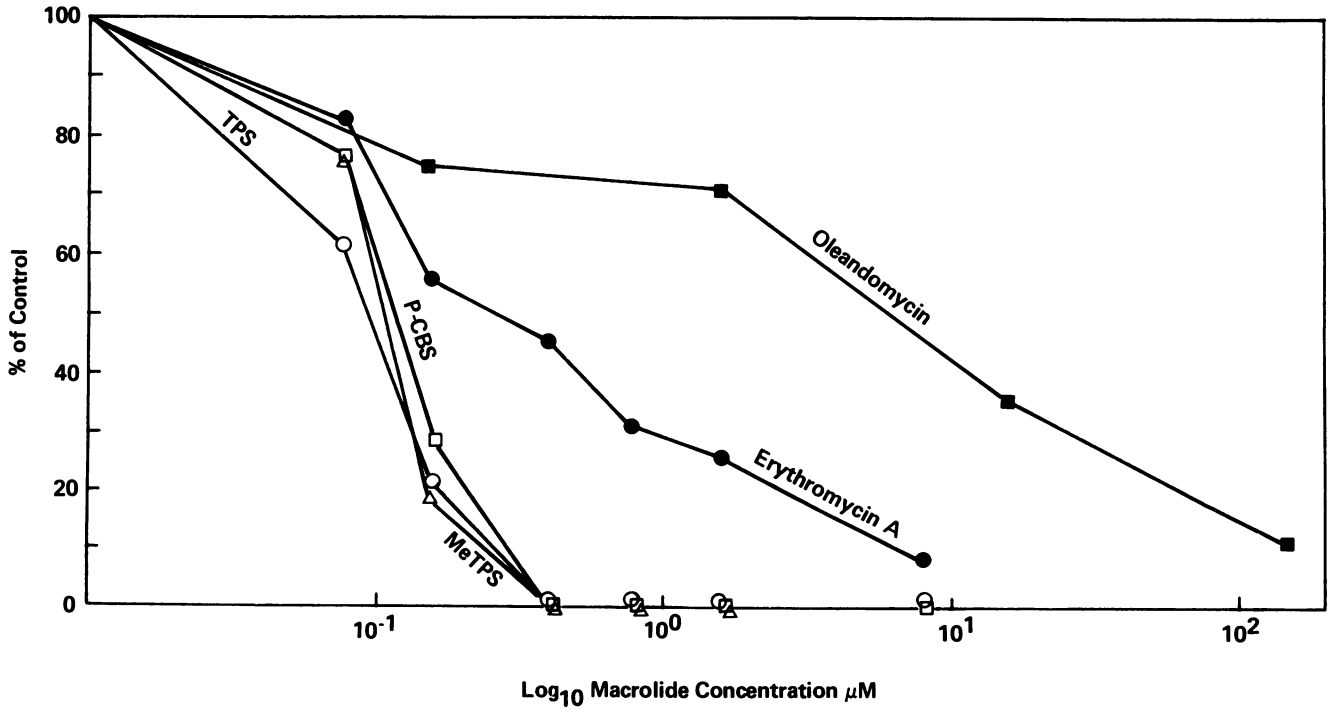

FIG. 2. Capacities of three 4″-deoxy-4″-sulfonamido-oleandomycin derivatives to inhibit MS2 viral RNA-directed polypeptide synthesis in cell extracts of *E. coli* MRE600. Symbols: □, P-CBS; △, MeTPS; ○, TPS; ●, erythromycin; ■, oleandomycin.

TABLE 1. In vitro activity of three 4″-deoxy-4″-sulfonamido-oleandomycin derivatives, erythromycin, and oleandomycin against gram-positive and gram-negative clinical isolates

Organism (no. of isolates)	Compound	MIC (μg/ml)		
		Range	50%	90%
Staphylococcus aureus (22)	P-CBS	0.10–0.39	0.20	0.39
	3-MeTPS	0.10–0.39	0.20	0.20
	TPS	0.10–0.39	0.20	0.39
	Erythromycin	0.10–1.56	0.20	0.78
	Oleandomycin	0.39–1.56	1.56	1.56
S. aureus resistant to erythromycin (25)	P-CBS	0.20–>12.5	0.20	>12.5
	3-MeTPS	0.20–>12.5	0.39	>12.5
	TPS	0.20–>12.5	0.20	>12.5
	Erythromycin	3.12–>12.5	12.5	>12.5
	Oleandomycin	1.56–>12.5	1.56	>12.5
S. epidermidis (28)	P-CBS	0.10–>12.5	0.20	>12.5
	3-MeTPS	0.20–>12.5	0.39	>12.5
	TPS	0.20–>12.5	0.39	>12.5
	Erythromycin	0.20–>12.5	0.20	>12.5
	Oleandomycin	0.78–>12.5	0.78	>12.5
Streptococcus pyogenes (18)	P-CBS	0.025–>12.5	0.05	0.20
	3-MeTPS	0.39–>12.5	0.78	1.56
	TPS	0.39–>12.5	0.78	1.56
	Erythromycin	0.05–12.5	0.10	0.20
	Oleandomycin	0.39–>12.5	0.78	0.78
S. agalactiae (9)	P-CBS	ND[a]		
	3-MeTPS	0.78[b]	0.78	0.78
	TPS	0.78–1.56	0.78	1.56
	Erythromycin	0.05–0.10	0.05	0.10
	Oleandomycin	0.39–0.78	0.39	0.78
S. faecalis (15)	P-CBS	0.78–>12.5	1.56	>12.5
	3-MeTPS	1.56–>12.5	3.12	>12.5
	TPS	1.56–>12.5	3.12	>12.5
	Erythromycin	0.20–>12.5	0.78	>12.5
	Oleandomycin	1.56–>12.5	1.56	>12.5
S. viridans (9)	P-CBS	0.10–0.20	0.10	0.20
	3-MeTPS	0.20–0.78	0.78	0.78
	TPS	0.39–0.78	0.78	0.78
	Erythromycin	0.05–0.10	0.10	0.10
	Oleandomycin	0.20–0.78	0.78	0.78
H. influenzae (32)	P-CBS	1.56–12.5	6.25	6.25
	3-MeTPS	1.56–12.5	6.25	6.25
	TPS	1.56–12.5	6.25	6.25
	Erythromycin	1.56–12.5	0.78	6.25
	Oleandomycin	25–>50	50.0	>50.0
B. fragilis (24)	P-CBS	0.2–12.5	0.78	6.25
	3-MeTPS	0.39–12.5	0.78	6.25
	TPS	1.56–50	1.56	6.25
	Erythromycin	0.39–50	0.78	3.12
	Oleandomycin	1.56–100	3.12	3.12
E. coli (3)	P-CBS	25[b]		
	3-MeTPS	25[b]		
	TPS	25[b]		
	Erythromycin	100[b]		
	Oleandomycin	>200[b]		

[a] ND, Not done.
[b] Mean value.

a 2.5 mM concentration of each amino acid present in the ^{3}H-amino acid mixture. The samples were then placed in a 95°C water bath for 5 min, at which time they were removed, allowed to cool, and then put on ice water for 1 h. Precipitated protein was removed by filtering through a type BD 0.6-μm Polyvic Millipore filter prewashed with 3 ml of the above 5% solution. The filters were then washed three times with 3 ml of ice-cold 5% trichloroacetic acid solution, removed

TABLE 2. Activities of three 4″-deoxy-4″-sulfonamido-oleandomycin derivatives against experimental infections in mice

Antibiotic	*Staphylococcus aureus*				*Streptococcus pyogenes*	
	Normal		Erythromycin-resistant			
	MIC (μg/ml)	PD_{50} (mg/kg)[a]	MIC (μg/ml)	PD_{50} (mg/kg)	MIC (μg/ml)	PD_{50} (mg/kg)
P-CBS	0.05	27.4 ± 3.7	0.05	48.7 ± 5.4	0.025	12.7 ± 2.1
3-Me-TPS	0.10	27.9 ± 3.1	0.10	100	0.10	2.5 ± 0.7
TPS	0.20	45.7 ± 3.8	0.38	141.7	0.10	15.7 ± 2.9
Erythromycin	0.05	94.5 ± 10	6.25	200	0.025	21.6 ± 2.6

[a] Values show size of each of three doses administered orally 0.5, 4, and 24 h after infection; 95% confidence limits.

from holders, allowed to air dry, and placed in scintillation vials. 2-Methoxyethanol (1 ml) and 3A70 counting cocktail (5 ml) were added to each vial, and radioactivity was counted.

RESULTS

The MICs of the experimental macrolides P-CBS, 3-MeTPS, and TPS for 90% of erythromycin-susceptible *Staphylococcus aureus* isolates ranged from 0.2 to 0.39 μg/ml; this was one-half to one-eighth the corresponding MICs for erythromycin and the parent compound, oleandomycin. The 50% MICs (MIC_{50}s) of the experimental macrolides for *S. aureus* isolates resistant to erythromycin ranged from 0.2 to 0.39 μg/ml, compared with MIC_{50}s of 12.5 μg/ml for erythromycin and 1.6 μg/ml for oleandomycin (Table 1).

The MIC_{50}s of P-CBS, 3-MeTPS, and TPS for clinical isolates of *Staphylococcus epidermidis* were equivalent to those of erythromycin. Erythromycin had the lowest MIC_{50} against *Streptococcus faecalis* and, except for P-CBS, was the most active compound against *S. pyogenes* and *Streptococcus viridans* (Table 1).

The activities of the experimental compounds against clinical isolates of *H. influenzae* and *B. fragilis* (Table 1) were equivalent to those of erythromycin and were markedly superior to those of oleandomycin. Although the MICs of the experimental compounds against *Escherichia coli* isolates were not within a clinically useful range, these agents were four times as active as erythromycin (Table 1).

Derivatives P-CBS and 3-MeTPS had identical oral activities against the experimental infection produced by the susceptible *S. aureus* strains in mice (Table 2). Based on PD_{50}s, both were twice as active as TPS and more than three times as active as erythromycin base. P-CBS was two to three times more active than 3-MeTPS and TPS against infections produced by erythromycin-resistant *S. aureus*; erythromycin was inactive against this organism at a dosage of 220 mg/kg.

Based on PD_{50}s against experimental *S. pyogenes* infection, 3-MeTPS was five to nine times more active than TPS, P-CBS, and erythromycin (Table 2).

TPS, P-CBS, and 3-MeTPS were equally active in inhibiting MS2 viral RNA-directed polypeptide synthesis in *E. coli* cell extracts (Fig. 2). At a concentration of 0.3 μM, polypeptide synthesis was inhibited about 90% by the experimental macrolides. Erythromycin effected about 50% inhibition, and oleandomycin effected about 26% inhibition, at this concentration.

DISCUSSION

The rank-order relationship between MICs and in vivo activities of the experimental macrolides in the infections produced by the erythromycin-susceptible and erythromycin-resistant *S. aureus* was good (Table 2). P-CBS, for example, had the lowest MIC against erythromycin-resistant *S. aureus* and demonstrated the lowest PD_{50}. The MICs and PD_{50}s of 3-MeTPS and TPS were two to four times greater than those of P-CBS against this resistant *S. aureus*.

Given the improved activity of these new derivatives against *E. coli* (Table 1), we sought to identify a mechanistic basis for the distinction from erythromycin and oleandomycin. It has been known for several years that the weak activity shown by erythromycin against the *Enterobacteriaceae* results from poor penetration of the outer cell wall. The erythromycin molecule is too large to pass easily through the porins (5, 8). When tested against L-forms, i.e., cells lacking a cell wall, erythromycin demonstrated excellent activity (9). Because P-CBS, 3-MeTPS, and TPS have greater molecular weights than oleandomycin and erythromycin, they should also be too large to penetrate the *E. coli* outer cell wall readily. It has been demonstrated that erythromycin binds to the 50S subunits of *S. aureus* and *E. coli* ribosomes with equal affinity (4, 10). Thus, by measuring the ability of 14-member ring macrolides to inhibit protein synthesis in a cell extract obtained from *E. coli*, we sought to discriminate between increased intrinsic efficacy and an effect of the molecular modifications on increased transport.

The data presented in Fig. 2 indicate that the experimental macrolides are much more active than erythromycin and oleandomycin in the cell-free system. This suggests that their greater potency against intact cells as compared with the parent oleandomycin compound is probably due to their superior ability to interact with the ribosome. Interestingly, the concentration of the oleandomycin derivatives required to inhibit polypeptide synthesis (0.26 μg/ml; Fig. 2) is very similar to the concentration required to inhibit susceptible *S. aureus* (Table 1).

The above studies indicate that both biological and chemical properties of oleandomycin, e.g., increased potency and increased affinity for the ribosome, can be profoundly altered by chemical replacement of the C″-4 hydroxyl group of the oleandrose sugar with various nitrogen functionalities.

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