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Mechanism of Action and In Vitro and In Vivo Activities of S-6123, a New Oxazolidinone Compound

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The in vitro activity of S-6123, a synthetic antimicrobial compound of the new oxazolidinone series, was compared with those of other orally administered agents against 328 clinical isolates. The compound was moderately active (MICs, 16 to 64 μg/ml) against 90% of staphylococci, nonenterococcal streptococci, and *Haemophilus influenzae*, including strains resistant to beta-lactam antibiotics. S-6123 was minimally active against enterococci and facultative gram-negative bacilli. Nevertheless, the compound had significant activity in a lethal rat *Escherichia coli* peritonitis model at serum concentrations of one-tenth the MIC against the infecting organism. The drug demonstrated only bacteriostatic activity against susceptible organisms. Studies to define the mechanism of antibacterial action revealed that S-6123 inhibited ribosomal protein synthesis without inhibiting DNA or RNA synthesis. This compound represents a new series of antibacterial agents not related to any other antibacterial compound of natural or synthetic origin.

The oxazolidinone compounds are recently developed, oral, synthetic antibacterial compounds unrelated to any other known antibacterial agents of natural or synthetic origin. Among those synthesized are S-6123, the first compound available, DuP 105, and DuP 721 (3). S-6123 has the structure (*R*)-4[5-(hydroxymethyl)-2-oxo-3-oxazolidinyl]-benzene sulfonamide (Fig. 1).

In this study, we describe the in vitro antibacterial spectrum of S-6123 in comparison with currently available oral antibacterial agents and the in vivo activity of the compound in a lethal peritonitis model in the rat. The mechanism of antibacterial action of S-6123 was explored by examining its effects on the syntheses of DNA, RNA, and protein in whole cells and on polypeptide synthesis in isolated bacterial ribosomes.

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MATERIALS AND METHODS

Organisms. Bacterial strains used in this study were unique clinical isolates collected at the New England Deaconess and Massachusetts General Hospitals, Boston, Mass., with the exceptions of the penicillin-resistant pneumococci and viridans group streptococci, which had been collected in South Africa (4, 6).

Antimicrobial agents and chemicals. Standard antimicrobial reference powders were obtained from the following sources: S-6123, E. I. du Pont de Nemours & Co., Inc., Wilmington, Del.; ampicillin, Bristol Laboratories, Syracuse, N.Y.; cephalexin, Eli Lilly & Co., Indianapolis, Ind.; ciprofloxacin, Miles Pharmaceuticals, West Haven, Conn.; clindamycin, The Upjohn Co., Kalamazoo, Mich.; trimethoprim and sulfamethoxazole, 1:19, with MIC data reported in terms of trimethoprim concentration, Burroughs Wellcome Co., Research Triangle Park, N.C. Tetracycline was purchased from Sigma Chemical Co., St. Louis, Mo. All antibiotic solutions were prepared on the day of use.

Agar dilution susceptibility studies. Susceptibility testing was performed by a standard agar dilution technique (11), using Mueller-Hinton agar (BBL Microbiology Systems, Cockeysville, Md.), which was supplemented with 5% defibrinated sheep blood when nonenterococcal streptococci were tested or IsoVitaleX (BBL) and 5% chocolatized horse blood for testing *Haemophilus influenzae*. Inocula of approximately 10^4 CFU were applied to plates with a 32-prong inoculating device. Inocula were prepared by appropriate dilution of overnight broth cultures (gram-negative bacilli) or suspensions prepared from fresh plates (gram-positive organisms and *H. influenzae*) in fresh Mueller-Hinton broth (BBL). Suspensions were standardized to a 0.5 McFarland standard before dilution. Inoculated plates were examined for growth after 18 to 20 h of incubation at 37°C in room air. *Campylobacter jejuni* were tested on brucella agar (Difco Laboratories, Detroit, Mich.) with 10% sheep blood, and plates were read after 24 h of incubation in a microaerophilic atmosphere (BBL). To evaluate effects of different media and supplements on the in vitro activity of S-6123, susceptibility studies against selected isolates were performed with dextrose phosphate broth (GIBCO Laboratories, Grand Island, N.Y.) plus 1.5% Bacto-agar (Difco) and Mueller-Hinton agar supplemented with 5% defibrinated sheep blood, horse blood, pooled human serum, rat serum, or thymidine phosphorylase (Burroughs Wellcome). Thymidine phosphorylase was added to examine whether thymidine in the media affected the in vitro activity of the new compound.

Broth dilution studies. Activity of S-6123 was examined by a broth dilution technique against selected strains. Tubes containing serial twofold dilutions of S-6123 in Mueller-Hinton broth, or Todd-Hewitt broth (BBL) for streptococci, were inoculated beneath the surface with log-phase cultures of test organisms to yield a final inoculum of 10^5 to 10^6 CFU/ml in a final volume of 2 ml. Tubes were swirled on a Vortex mixer after 20 h of incubation, and the MIC was determined

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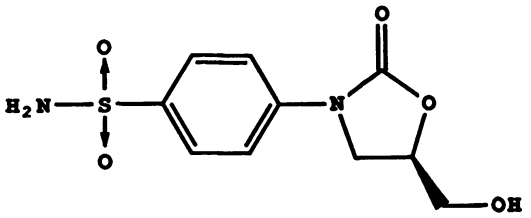

FIG. 1. Chemical structure of S-6123.

by visual inspection for lack of turbidity. Tubes were reincubated for 4 h, and 0.01 ml was transferred from clear tubers to antibiotic-free plates, which were incubated for 24 h at 37°C. The MBC, as defined by 99.9% reduction in viable bacteria relative to the initial inoculum, was determined by the method of Pearson et al. (8). MICs were also determined by using a chemically defined "minimal salts" medium containing K_2HPO_4, sodium citrate, NH_4SO_4, and glucose with and without Ca^{2+} (50 mg/liter) and Mg^{2+} (50 mg/liter) for *Escherichia coli* ATCC 25922.

Selection of resistant organisms. Agar plates with increasing subinhibitory concentrations of S-6123 were used in an effort to select resistant organisms (10). Briefly, heavy inocula of *E. coli* ATCC 25922 or *Enterococcus faecalis* E-1 were applied to agar plates containing S-6123 at a concentration equal to one-half the MIC. Colonies arising at 24 to 72 h were then serially transferred to plates containing twofold incremental concentrations of the drug until a concentration was reached that prevented further growth. The stability of resistance selected by this method was studied after passage of strains on drug-free medium. The frequency of resistant subpopulations or of single-step mutations was assessed by application of 10^9 CFU to plates containing S-6123 at four times the MIC.

Effects on synthesis of macromolecules in *E. coli*. The effect of S-6123 on DNA, RNA, and protein syntheses in *E. coli* was studied by using the methods of Crumplin and Smith (2). In brief, log-phase cells were grown in Mueller-Hinton broth containing [^{3}H]thymidine, [^{3}H]uridine, or [^{3}H]leucine (0.1 μCi/ml, final concentration). S-6123 at a concentration of 250 μg/ml was added to one set of tubes, while a second set without antimicrobial agent served as a control. Samples were withdrawn at 30-min intervals over 2 h. Trichloroacetic acid (TCA)-precipitable material from samples labeled with [^{3}H]thymidine was collected over a filter (0.45 μm; Millipore Corp., Boston, Mass.) presoaked in cold thymidine, washed, and counted in a scintillation counter (Beckman Instruments, Inc., Fullerton, Calif.) to measure DNA synthesis. RNA synthesis was calculated by subtracting incorporation of [^{3}H]uridine into alkali-stable material from total [^{3}H]uridine incorporation into TCA-precipitable material. Protein synthesis was measured by incorporation of [^{3}H]leucine into TCA-precipitable macromolecules. Chloramphenicol (25 μg/ml) was used in comparison as a positive control. Colony counts were determined at each sampling time in parallel specimens, prepared as above but without radiolabeled compounds.

Studies of protein synthesis in a cell-free system. The direct effect of S-6123 on protein synthesis, using ribosomes prepared from a clinical strain of *Enterococcus faecalis*, was assessed in a cell-free system by the method of Nirenberg (7) with minor modifications as described by Farber et al. (6). Ribosomal protein synthesis was measured by using [^{14}C]phenylalanine (54 mCi/mmol; Dupont, NEN Research Products, Boston, Mass.) incorporation into TCA-precipitable material, with poly(U) (Sigma) as the messenger. Inhibition of protein synthesis by S-6123 was assessed by the reduction of counts per minute in TCA-precipitable material after the addition of S-6123 at final concentrations of 0.2, 2.0, 20, and 200 μg/ml.

Rat peritonitis model experimental design. The in vivo activity of S-6123 was examined with male Sprague-Dawley rats, 150 to 250 g (Charles River Breeding Laboratories, Wilmington, Mass.). The animals were kept under standard conditions of chow and water. The organism used to produce infection was a clinical isolate of *E. coli* from the New England Deaconess Hospital.

Peritonitis was established in the following manner: a gelatin capsule containing 0.5 ml of a mixture of equal volumes of an overnight culture of *E. coli* and autoclaved rat cecal contents with 10% (wt/vol) barium sulfate was implanted intraperitoneally in rats under ether anesthesia. Capsules contained a final inoculum of approximately 10^8 CFU. One group of rats was treated with S-6123, 50 mg/kg (5-mg/ml solution in 3% dimethyl sulfoxide–water). A dose of 50 mg/kg was chosen since preliminary studies had shown a protective effect of this dose in a lethal mouse infection model. Another group received ampicillin, 25 mg/kg (5-mg/ml solution in 3% dimethyl sulfoxide–water), a dosing regimen which yielded serum concentrations below the in vitro MIC. Both drugs were administered by oral-gastric intubation immediately prior to and 4 h after implantation of the inoculum. A third group of untreated animals served as controls. Mortality was noted at 24 and 48 h, and the animals were observed for late mortality for at least 7 days and up to 3 weeks posttherapy. At the end of the observation period, animals were sacrificed and the peritoneal cavity was inspected for residual infection.

To determine the time of peak serum concentrations, blood was obtained with a capillary tube from the retroorbital sinus in noninfected rats at 15- to 30-minute intervals after oral dosing with either ampicillin (25 mg/kg) or S-6123 (50 mg/kg). Blood for assay of peak serum concentrations was similarly obtained in infected rats in both treatment groups, and mean peak serum concentrations were calculated. In four noninfected animals, a 24-h urine collection beginning at the time of initial dosing of S-6123 was done to determine urinary concentrations. Serum for assay of bactericidal activity was obtained by cardiac puncture 90 min after dosing with S-6123 by oral-gastric intubation in noninfected rats. Serum and urine concentrations of S-6123 were determined by a high-pressure liquid chromatographic assay method at the laboratories of E. I. duPont de Nemours & Co., Inc., Glenolden, Pa., and ampicillin levels were measured by using a *Micrococcus luteus* bioassay (1). Serum bactericidal activity against *E. coli* was determined by a standard method (9).

RESULTS

Susceptibility studies. Results of agar dilution susceptibility studies are shown in Tables 1, 2, and 3. S-6123 demonstrated greatest activity against nonenterococcal streptococci and *H. influenzae*, with MICs for 90% of the strains of 16 to 32 μg/ml. There was no significant difference in activity against the penicillin-resistant or penicillin-susceptible viridans streptococci, or the β-lactamase-producing or non-β-lactamase-producing *H. influenzae*. Staphylococci were inhibited at concentrations between 8 and 64 μg/ml. *Enterococcus faecalis* and *E. coli* were inhibited at concentrations of ≤128 μg/ml, a level which may be achiev-

TABLE 1. In vitro activities of S-6123 and comparative antimicrobial agents against gram-positive bacteria

Strain (no.)	Antibiotic	MIC (μg/ml)[a]		
		50%	90%	Range
Streptococcus pyogenes (10)	S-6123	16	32	8–32
	Ampicillin	≤0.06	≤0.06	≤0.06
	Ciprofloxacin	0.5	0.5	0.25–1.0
	Clindamycin	≤0.06	≤0.06	≤0.06
	Tetracycline	0.25	0.5	0.25–0.5
	Trimethoprim-sulfamethoxazole	>5	>5	≤0.01–>5
Streptococcus agalactiae (10)	S-6123	32	32	32
	Ampicillin	≤0.06	0.125	≤0.06–0.125
	Ciprofloxacin	0.5	0.5	0.5–1.0
	Clindamycin	≤0.06	≤0.06	≤0.06–0.125
	Tetracycline	16	32	0.25–32
Group C and G streptococci (10)	S-6123	16	32	8–32
	Ampicillin	≤0.06	≤0.06	≤0.06–1.0
	Ciprofloxacin	0.5	0.5	0.25–0.5
	Clindamycin	≤0.06	0.125	≤0.06–0.125
	Tetracycline	0.125	0.125	0.125–32
	Trimethoprim-sulfamethoxazole	0.08	0.16	0.04–1.25
Viridans group streptococci, penicillin susceptible (30)	S-6123	16	32	8–32
	Ampicillin	≤0.06	0.125	≤0.06–1.0
	Ciprofloxacin	0.5	2	0.125–4
	Clindamycin	≤0.06	≤0.06	≤0.06–64
	Tetracycline	0.25	16	0.25–>128
	Trimethoprim-sulfamethoxazole	0.01	0.625	0.01–1.25
Viridans group streptococci, penicillin resistant (15)	S-6123	8	16	8–32
	Ampicillin	8	8	≤0.06–32
	Ciprofloxacin	2	4	0.25–4
	Clindamycin	8	64	≤0.06–64
	Tetracycline	64	128	0.5–128
Streptococcus pneumoniae, penicillin resistant (10)	S-6123	16	32	8–32
	Ampicillin	0.5	4	≤0.06–4
	Ciprofloxacin	0.5	2	0.25–2
	Clindamycin	≤0.06	8	≤0.06–32
	Tetracycline	0.5	128	0.125–128
	Trimethoprim-sulfamethoxazole	1.25	1.25	0.16–5.0
Staphylococcus aureus, methicillin susceptible (14)	S-6123	64	64	32–64
	Cephalexin	4	16	2–16
	Ciprofloxacin	0.5	1.0	0.25–1.0
	Clindamycin	0.125	0.125	≤0.06–0.125
	Tetracycline	0.5	0.5	0.5–32
	Trimethoprim-sulfamethoxazole	0.15	0.31	0.15–0.31
Staphylococcus aureus, methicillin resistant (16)	S-6123	64	64	32–64
	Cephalexin	>128	>128	>128
	Ciprofloxacin	1.0	1.0	0.5–1.0
	Clindamycin	>128	>128	>128
	Tetracycline	0.5	0.5	0.5
	Trimethoprim-sulfamethoxazole	0.31	0.31	0.15–0.31
Staphylococcus epidermidis (28)	S-6123	64	64	8–64
	Ciprofloxacin	0.25	0.5	≤0.06–1.0
	Clindamycin	0.125	>128	≤0.06–>128
	Tetracycline	1.0	128	0.125–128
	Trimethoprim-sulfamethoxazole	0.15	5.0	0.04–5
Enterococcus faecalis (30)	S-6123	128	128	32–128
	Ampicillin	1.0	1.0	0.5–2
	Ciprofloxacin	1.0	2	0.25–2
	Clindamycin	32	>128	0.25–>128
	Tetracycline	128	>128	0.125–>128

[a] 50% and 90%, MIC for 50 and 90% of the strains, respectively.

TABLE 2. In vitro activity of S-6123 and comparative antibiotics against selected gram-negative bacilli

Strain (no.)	Antibiotic	MIC (μg/ml)[a]		
		50%	90%	Range
Haemophilus influenzae, β-lactamase negative (17)	S-6123	16	32	8–32
	Ampicillin	1.0	1.0	0.25–1.0
	Ciprofloxacin	≤0.06	≤0.06	≤0.06
	Tetracycline	0.5	1.0	0.25–16
Haemophilus influenzae, β-lactamase positive (9)	S-6123	32	32	16–32
	Ampicillin	32	128	32–128
	Ciprofloxacin	≤0.06	≤0.06	≤0.06
	Tetracycline	0.5	0.5	0.5–16
E. coli (50)	S-6123	128	128	64–128
	Ampicillin	4	>128	0.5–>128
	Cephalexin	4	8	4–16
	Ciprofloxacin	≤0.06	≤0.06	≤0.06
	Tetracycline	2	>128	1.0–>128
	Trimethoprim-sulfamethoxazole	0.08	2.5	0.02–>5

[a] 50% and 90%, MIC for 50 and 90% of the strains, respectively.

able in the urine after oral dosing. Occasional strains of other gram-negative organisms were resistant to the drug at this concentration (Table 3). Testing in dextrose phosphate broth agar or in Mueller-Hinton agar with supplemental sheep blood, horse blood, human serum, rat serum, or thymidine phosphorylase did not significantly alter MICs of S-6123 against five strains each of *E. coli*, *Klebsiella pneumoniae*, *Enterobacter aerogenes*, *Staphylococcus aureus*, *Enterococcus faecalis*, or viridans streptococci (data not shown).

Against six strains of representative staphylococci and streptococci, MBCs exceeded broth dilution MICs by at least fourfold. In all instances, MBCs were >128 μg/ml. There was no significant difference in the MIC of S-6123 against *E. coli* ATCC 25922 whether the compound was tested in minimal salts media with or without Ca^{2+} or Mg^{2+} or in Mueller-Hinton broth.

Selection of resistance. No resistant colonies of *E. coli* could be selected by stepwise passage on agar plates containing twofold incremental concentrations of S-6123. However, with serial passage on incremental concentrations of S-6123, colonies of *Enterococcus faecalis* resistant to levels as high as 1,000 μg/ml could be selected. The faint haze of growth of *Enterococcus faecalis* which occurred at high concentrations of S-6123 required 72 h of incubation for detection, and the resulting colonies returned to base-line susceptibility after passage in drug-free agar. No resistant colonies were noted when a high inoculum of *E. coli* or *Enterococcus faecalis* was applied to agar containing S-6123 at four times the MIC.

TABLE 3. Activity of S-6123 against other gram-negative bacilli

Strain (no.)	MIC (μg/ml)[a]		
	50%	90%	Range
Klebsiella pneumoniae (20)	128	>128	32–>128
Citrobacter freundii (9)	128	128	64–128
Acinetobacter anitratus (10)	>128	>128	>128
Enterobacter aerogenes (10)	128	128	32–128
Enterobacter cloacae (10)	128	128	128
Serratia marcescens (10)	128	>128	≥128
Campylobacter jejuni (10)	128	128	≥128

[a] 50% and 90%, MIC for 50 and 90% of the strains, respectively.

Mechanism of action. Studies of macromolecular synthesis showed no direct effect of S-6123 on DNA or RNA synthesis, but marked inhibition of protein synthesis in *E. coli* (Fig. 2). The effect of S-6123 on protein synthesis was similar to that seen with chloramphenicol (Fig. 3). S-6123 at concentrations of 0.2 to 200 μg/ml caused a 33 to 75% decrease in poly(U)-directed synthesis of polyphenylalanine in the cell-free system (Fig. 4).

Rat peritonitis model. The MICs of S-6123 and ampicillin against the strain of *E. coli* used in this model were 64 and 2.0 μg/ml, respectively. Pharmacokinetic studies performed in the rats showed that peak serum concentrations of S-6123 occurred 60 to 120 min after oral dosing, and those of ampicillin occurred 30 min after oral dosing. Mean peak serum concentrations of S-6123 were 3.8 μg/ml at 1 h after the initial dose and 7.5 μg/ml at 1 h after the second oral dose. The mean peak concentration of ampicillin, 30 min

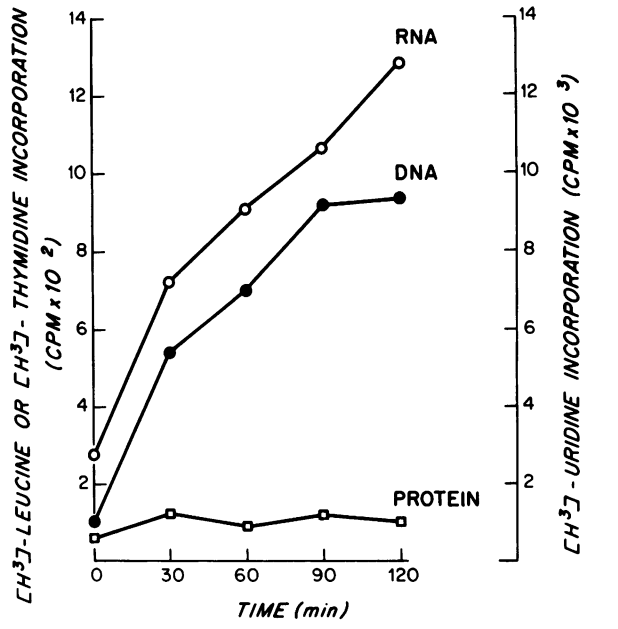

FIG. 2. Effect of S-6123 on DNA, RNA, and protein syntheses in *E. coli*.

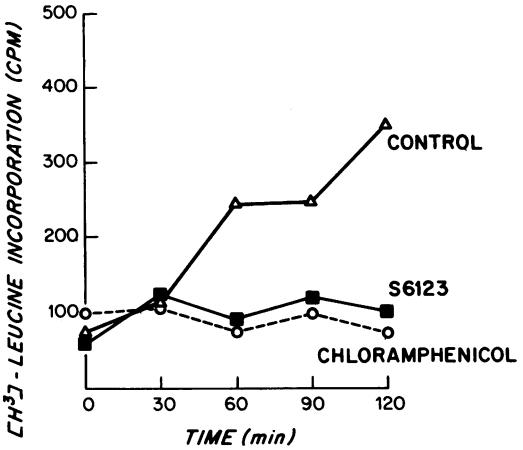

FIG. 3. Effects of S-6123 and chloramphenicol on protein synthesis in *E. coli*.

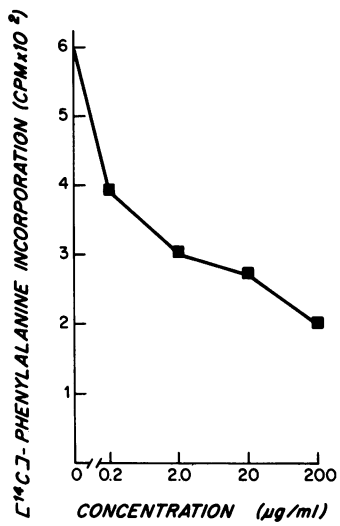

FIG. 4. Effect of S-6123 on protein synthesis in cell extracts of *Enterococcus faecalis*.

after dosing, was 0.93 μg/ml, with concentrations of ≤0.17 μg/ml immediately prior to the second dose. Thus, the peak serum levels of both drugs in infected rats were less than the MIC of the drugs for the test organism. The average urinary concentration of S-6123 was 169 μg/ml in a 24-h urine specimen. Serum obtained from the animals at the time of peak drug concentrations had no in vitro bacteriostatic or bactericidal activity against *E. coli*. In addition, time-kill studies showed that there was no in vitro bactericidal activity of the drug at concentrations of 16 to 64 μg/ml or at ampicillin concentrations of 0.5 to 2.0 μg/ml against the test strain.

Twenty-three rats were given S-6123 and 21 animals received ampicillin, while 23 animals served as untreated controls. Thirteen percent (3 of 23) of the S-6123-treated, 20% (6 of 21) of the ampicillin-treated, and 96% (22 of 23) of the control animals were dead at 48 h. The differences were statistically significant between S-6123 and control (χ^2 = 28.39; $P < 0.05$) and between ampicillin and control (χ^2 = 18.5; $P < 0.05$), but there was no statistically significant difference between S-6123 and ampicillin (χ^2 = 0.812). All surviving animals examined in both treatment and control groups showed the presence of large intraabdominal abscesses on the intraperitoneal surface at 3 weeks after inoculation despite the lack of apparent morbidity. The abscesses grew *E. coli* in pure culture.

DISCUSSION

The oxazolidinone, S-6123, is an early representative of a new series of antimicrobial agents. This agent was found to have greatest in vitro activity against streptococci and staphylococci. Inhibition of enterococci and *E. coli* required somewhat higher concentrations, while other gram-negative bacilli appeared to be relatively resistant to the drug. Although actual levels attainable in humans are still incompletely defined, animal studies suggest that the more susceptible of these organisms would be inhibited by concentrations of the drug achievable in the urine after oral administration.

Susceptibility to the compound did not appear to correlate with susceptibility to comparative agents within any species studied. While it was possible to select for resistance to the drug in enterococci by repetitive passage on increasing concentrations of the compound, this resistance was unstable after passage on antimicrobial agent-free media. This, together with the fact that detection frequencies for spontaneous resistance to fourfold the MIC of the drug were $<10^{-9}$, suggests that emergence of drug resistance during therapy would not likely be a major problem.

The new drug demonstrated only bacteriostatic activity against representative gram-positive isolates. On the basis of studies in both whole cells and cell-free systems, the antimicrobial effect of the drug was found to be exerted primarily through inhibition of protein synthesis. Attempts to isolate a resistant mutant to pinpoint the ribosomal subunit specificity of the compound were unsuccessful. Studying another representative of the oxazolidinone class of new antimicrobial agents, Eustice et al. (5) have also shown this drug to inhibit protein synthesis in *Bacillus subtilis*. Using a series of mutants with known resistance markers at specific target levels, these authors concluded that the action of the drug occurs at a target prior to chain elongation.

Of great interest was the observation that serum concentrations of S-6123 equivalent to one-tenth the MIC for the infecting strain of *E. coli* substantially reduced mortality in a rat model of lethal peritonitis. Reasons for this discrepancy between in vitro and in vivo effects are unknown, but do not appear due to medium dependency of susceptibility determinations. A similar discrepancy occurred in the ampicillin group. These results indicate that the new drug does possess in vivo activity that is worthwhile to pursue.

A number of compounds in this series are orally administered and excreted in the urine in animals, suggesting that they may be useful in the treatment of urinary tract infections. Even if S-6123 does not prove useful for human therapeutic purposes, it is representative of a new class of antimicrobial agents that shows a great deal of promise. Further studies with S-6123 and its analogs will be of great interest.

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