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Mechanistic Studies and Biological Activity of Bioxalomycin α_2, a Novel Antibiotic Produced by *Streptomyces viridodiastaticus* subsp. "*litoralis*" LL-31F508

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The bioxalomycins, a novel complex of broad-spectrum antibiotics, were isolated from fermentations of *Streptomyces viridodiastaticus* subsp. "*litoralis*" LL-31F508. Bioxalomycin α_2, the major component of this complex, exhibited antibacterial activity. The MICs ranged from ≤0.002 to 0.008 μg/ml for gram-positive organisms and from 0.50 to 4 μg/ml for gram-negative organisms. Bioxalomycin α_2 was found to be bactericidal and to inhibit bacterial DNA synthesis preferentially. Bioxalomycin α_2 protected mice from a lethal challenge with *Staphylococcus aureus* Smith. The 50% effective dose of bioxalomycin α_2 administered orally was 10 times greater than that when the drug was given subcutaneously or intravenously. These data suggest a stability or bioavailability problem when the compound is administered orally.

The evolution and spread of antibiotic-resistant pathogens remain major clinical problems (15). Although the discovery of new antimicrobial agents has become increasingly more difficult, the search for unique metabolites from microorganisms remains an attractive venture (2, 13).

During the course of our screening program for novel antibacterial agents, fermentation samples of culture LL-31F508 exhibited activity against methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant *Enterococcus faecium*. Culture LL-31F508 was isolated from an intertidal soil sample collected in Key West, Fla., and was identified as *Streptomyces viridodiastaticus* subsp. "*litoralis*" (1). Chemical analyses of fermentation broths revealed the presence of a novel antibiotic complex, the bioxalomycins (18, 22). Among the bioxalomycins, bioxalomycin α_2 was the most active, abundant, and stable component (22). Although the chemical structure is similar to those of quinocarcins, cyanocyclins, and saframycin A, bioxalomycin α_2 is more closely related to naphthyridinomycin (5, 19, 21, 23). Here we report the results from in vitro and in vivo studies on the antibacterial activities of bioxalomycin α_2 (Fig. 1).

MATERIALS AND METHODS

Bacterial strains. Clinical isolates were collected between 1987 and 1993 from various medical centers in the United States, and quality control strains were obtained from the American Type Culture Collection, Rockville, Md. *Bacillus subtilis* BGSC1A1 (*trpC2*) and *Escherichia coli* BAS849 (*imp*) were obtained from the Bacillus Genetic Stock Center and S. A. Benson (14), respectively. Identification of each culture was done by conventional methods: gram-negative bacilli by the API 20E (Analytab Products, Plainview, N.Y.) and NF (Remel, Lenexa, Kans.) systems and staphylococci by Staph Trac (Analytab Products). All isolates were stored frozen in skim milk at −70°C.

Media. All media were prepared in distilled deionized (DI) water. Mueller-Hinton (MH) medium was purchased from Becton Dickinson Microbiology Systems, Cockeysville, Md. Luria-Bertani (LB) broth was purchased from Difco Laboratories, Detroit, Mich. Modified minimal medium contained the following, per liter: glucose, 4 g; NH_4Cl, 1 g; KH_2PO_4, 3 g; Na_2HPO_4, 6 g; $MgSO_4 \cdot 7H_2O$, 0.25 g; $FeSO_4 \cdot 7H_2O$, 0.5 mg; vitamin-free Casamino Acids, 2 g; arginine, 0.1 g; and threonine, 0.1 g. All ingredients used in the minimal medium were purchased from Sigma Chemical Co., St. Louis, Mo., with the exception of Casamino Acids, which were purchased from Difco.

Chemicals. [^{3}H]thymidine ([^{3}H]Tdr; TRK.686; 90 Ci/mmol), [^{3}H]uridine ([^{3}H]Udr; TRK.410; 49 Ci/mmol), and ^{3}H-labeled amino acids (^{3}H-AA; TRK.550; a mixture of leucine, lysine, phenylalanine, proline, and tyrosine, with specific activities of 135, 83, 123, 103, and 118 Ci/mmol, respectively) were purchased from Amersham Corporation, Arlington Heights, Ill. All DNA and control antimicrobial agents except aztreonam (Bristol-Myers Squibb Co., Syracuse, N.Y.) and piperacillin (American Cyanamid Company, Pearl River, N.Y.) were purchased from Sigma Chemical Co. Bioxalomycin α_2 and cinodine were provided by J. Zaccardi and G. Ellestad, American Cyanamid Company.

In vitro susceptibility testing. The in vitro antibacterial activities were determined by the agar or broth microdilution method as recommended by the National Committee for Clinical Laboratory Standards (11). Mueller-Hinton II agar (MHA) was used for nonfastidious aerobic bacteria, and the medium was supplemented with 5% sheep blood for *Streptococcus* spp. and for the determination of the effect of blood on the antibacterial activity. Inocula were adjusted to a density of 10^7 CFU/ml, and approximately 3 μl was applied to the agar surface with a Steers replicator. The test plates were incubated at 35°C for 18 h. The agar MIC was defined as the lowest concentration of antimicrobial agent that completely inhibited visible growth of the organism. The MICs obtained by the broth microdilution method were determined by adding 5 μl of

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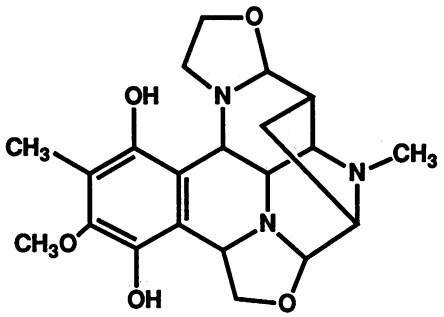

FIG. 1. Chemical structure of bioxalomycin α_2.

an exponential-phase bacterial culture (1×10^7 to 5×10^7 CFU/ml) to 0.1 ml of minimal medium or Mueller-Hinton broth (MHB) containing the drug at 0.002 to 128 μg/ml. The MIC was defined as the lowest concentration of antibiotic which prevented turbidity after 18 h of incubation at 37°C.

Bactericidal activity. An exponential-phase culture of *S. aureus* (10 ml in a 250-ml Erlenmeyer flask) was incubated at 37°C and 200 rpm in the presence of bioxalomycin α_2. At the indicated times, aliquots (100 μl) of the treated culture and an untreated control were removed, serially diluted in saline (0.9%), and plated onto MHA (9). After 24 h at 37°C, viable cell counts were determined.

Incorporation of radiolabeled precursors. Macromolecular synthesis in *E. coli* was studied by measuring the incorporation of the appropriate radiolabeled precursors into trichloroacetic acid (TCA)-precipitable material. *E. coli* (*imp*) was grown at 37°C and 200 rpm in modified minimal medium (50 ml of medium per 250-ml Erlenmeyer flask) to an A_{450} of 0.20. Aliquots of 100 μl were dispensed into microtiter wells containing antibacterial agents, and the plates were incubated for 5 to 30 min at 37°C with vigorous agitation. Cells were pulse-labeled for 5 min by adding the following radiolabeled precursors at the indicated final concentrations: [^{3}H]Tdr, 0.5 μCi/ml with 0.1 μg of unlabeled thymidine per ml; [^{3}H]Udr, 0.5 μCi with 0.5 μg of unlabeled uridine per ml; or ^{3}H-AA, 10 μCi/ml. To determine the specific incorporation into DNA, RNA, and protein, 100 μl of chilled (4°C) TCA (10%) supplemented with 0.5 mg of unlabeled precursors per ml was added to each well, and the plate was immediately refrigerated for 1 h. The precipitate was collected on a glass fiber filter (Wallac filtermat B, Wallac 1205-404) by using a Skatron 96-well cell harvester (model 11050) programmed for a 3-s prewet with chilled DI water, a 10-s wash with 5% chilled TCA, a 10-s wash with chilled DI water, and a 10-s drying cycle. To assess the effects of the drugs on the uptake of radiolabeled precursors, the contents of each well were harvested onto a glass fiber filter by using the Skatron 96-well cell harvester programmed for a 3-s prewet, a 10-s wash with chilled normal saline, and a 10-s drying cycle (17). Filter mats were dried for 7 min at high power in a microwave oven (700 W; Quasar), solid scintillant (MeltilexB; 1205-402; Pharmacia) was applied, and the isotope that was retained on the filter was quantitated in an LKB Betaplate scintillation counter (Wallac 1205). The levels of incorporation of [^{3}H]Tdr, [^{3}H]Udr, and ^{3}H-AA were expressed as the percentage of that of the untreated control.

Morphological effects on *E. coli*. An overnight culture of *E. coli* (*imp*) was diluted 1:1,000 into fresh LB medium and was incubated at 37°C and 200 rpm until an A_{450} of 0.20 was reached. The log-phase culture was treated with bioxalomycin α_2, cinodine, ciprofloxacin, aztreonam, or water (untreated control). After 2 h, the cells were examined by phase-contrast microscopy (×1,000 magnification; Olympus BH2 microscope) and photographed by using Ektachrome 160ASA film (Kodak) aided by an Olympus PM10 AD autoexposure device.

Effect of exogenous DNA on antibacterial activity. Antibacterial agents were serially diluted in minimal medium and were mixed with equal volumes of DNA solution (400 μg/ml of minimal medium). After 20 min at 37°C, broth MICs were determined as described previously.

In vivo efficacy against murine infection. The in vivo antibacterial activity of bioxalomycin α_2 was determined against acute lethal infections in mice infected with *S. aureus* Smith or *E. coli* 311. Female mice, strain CD-1, from Charles River Laboratories, Charles River, N.Y., weighing 20 ± 2 g each were challenged by intraperitoneal injection of 0.5 ml of the bacterial suspension in broth containing 5% hog gastric mucin (10 to 100 50% lethal doses [LD_{50}s]). Six dose levels of the antibiotic in phosphate-buffered saline (pH 7.4, 0.01 M) were administered intravenously (0.2 ml), subcutaneously (0.5 ml), or orally (0.5 ml) at 30 min postinfection. Each dose group had five animals. All untreated animals died within 48 h of infection. The median effective dose (ED_{50}) was estimated from the survival ratios by computerized probit analysis (3).

Maximum tolerated dose and LD_{50} in mice. Six dose levels of the antibiotic in phosphate-buffered saline (pH 7.4, 0.01 M)

TABLE 1. Antibacterial activity of bioxalomycin α_2 against gram-positive clinical isolates[a]

Organism (no. of strains)	MIC (μg/ml)				
	Bioxalomycin α_2	Piperacillin	Gentamicin	Vancomycin	Erythromycin
MSSA (4)	≤0.002–0.015	1–4	0.5–128	1	0.25–>128
MRSA (33)	0.004–0.015	>128	0.5–>8	1–2	4–>128
SCN (6)	≤0.002–0.004	1–>128	≤0.06–128	1–2	≤0.06–>128
Staphylococcus haemolyticus (1)	≤0.002	>128	8	1	>128
Streptococcus pyogenes (1)	≤0.002	≤0.06	NT	0.25	≤0.06
Streptococcus agalactiae (1)	≤0.002	≤0.06	NT	0.50	≤0.06
Streptococcus pneumoniae (1)	0.015	≤0.06	NT	0.25	≤0.06
Enterococcus faecalis VS (4)	≤0.002–0.25	1–>128	4–8	0.50–1	2–>128
Enterococcus faecalis VR (1)	0.03	8	>128	>128	>128
Enterococcus faecium VS (2)	≤0.002–0.12	0.12–2	0.12–8	1	0.06–>128
Enterococcus faecium VR (2)	0.03–0.06	128–>128	8–16	>128	>128
Bacillus cereus (1)	0.12	2	0.25	1	≤0.06

[a] The agar dilution method was used. MSSA and MRSA, methicillin-susceptible and methicillin-resistant *Staphylococcus aureus*, respectively; SCN, coagulase-negative staphylococci; VS, vancomycin-susceptible; VR, vancomycin-resistant; NT, not tested.

TABLE 2. Antibacterial activity of bioxalomycin α_2 against gram-negative clinical isolates[a]

Organism (no. of strains)	MIC (μg/ml)				
	Bioxalomycin α_2	Piperacillin	Gentamicin	Vancomycin	Erythromycin
Escherichia coli (2)	0.5–1	≤0.06–32	0.25–0.5	0.5–>128	≤0.06–128
Klebsiella pneumoniae (2)	0.25–0.5	64–>128	0.25–1	>128	8–>128
Enterobacter cloacae (1)	1	128	0.5	>128	>128
Enterobacter aerogenes (1)	8	1	0.5	>128	>128
Serratia marcescens (2)	0.5–1	16–32	0.5–8	>128	>128
Citrobacter diversus (1)	1	16	0.5	>128	>128
Morganella morganii (2)	0.5–1	0.12–0.5	0.5	>128	>128
Providencia stuartii (2)	2	2–4	2–8	>128	>128
Salmonella sp. strain 2806 (1)	0.5	2	1	>128	64
Pseudomonas aeruginosa (3)	0.5–2	4–16	2–16	>128	128–>128
Xanthomonas maltophilia (2)	2	32–>128	128–>128	>128	64–>128

[a] The agar dilution method was used.

were administered intravenously (0.2 ml), subcutaneously (0.5 ml), or orally (0.5 ml) to healthy female mice weighing 20 ± 2 g (strain CD-1 from Charles River Laboratories). Each dose group had five animals. The number of survivors was counted after 7 days. The maximum tolerated dose (in milligrams per kilogram of body weight) was the highest level at which none of the animals died. The LD_{50} (in milligrams per kilogram of body weight) was the level at which ≥50% of the animals died.

RESULTS

In vitro activity. Bioxalomycin α_2 exhibited excellent activity against gram-positive organisms, with MICs being between ≤0.002 and 0.25 μg/ml (Table 1). The presence of 5% sheep blood in MHA increased the MICs by one- to twofold (data not shown). Bioxalomycin α_2 was less active against gram-negative organisms (Table 2). Similar results were obtained when the MICs were determined by the broth microdilution method (Table 3). The addition of exogenous DNA (200 μg/ml) increased the MICs of bioxalomycin α_2 and adriamycin from 0.03 to 0.12 and 8 to 64 μg/ml, respectively, whereas the MIC of penicillin G (4 μg/ml) was unaffected.

Bioxalomycin α_2 was bacteriostatic against a strain of *S. aureus* when it was tested at its MIC. At concentrations of ≥5× the MIC, cell viability decreased by 3 log units over a 4-h period (Fig. 2), and a further decrease in viability was observed after 6 h (data not shown). During this period of decreasing viability, exposure to bioxalomycin α_2 also induced filamentation in *E. coli* (20 to 40 μm long); the filaments were similar in appearance to those induced by cinodine or ciprofloxacin (Fig. 3). Aztreonam, in contrast, produced longer filaments (40 to 60 μm) because of its inhibitory effect on penicillin-binding protein 3 (PBP 3) (6).

TABLE 3. Activities of bioxalomycin α_2 and other antimicrobial agents

Compound	MIC (μg/ml)[a]	
	S. aureus (MSSA)	*E. coli* (*imp*)
Bioxalomycin α_2	0.008	0.03
Ciprofloxacin	0.25	0.015
Rifampin	0.008	0.015
Chloramphenicol	4	2
Polymyxin B	>64	2

[a] MICs were determined by the broth microdilution method. Minimal medium was used for *E. coli* and MHB was used for methicillin-susceptible *S. aureus* (MSSA).

Incorporation of radiolabeled precursors. Inhibitions of DNA, RNA, and protein syntheses were determined by measuring the incorporation of [^{3}H]Tdr, [^{3}H]Udr, and ^{3}H-AA, respectively, into the TCA-precipitable material of a logarithmic-phase culture of *E. coli* (*imp*). The effects of the drugs on the cellular uptake of radiolabeled precursors were determined by measuring the radioactivity retained in saline-washed cells exposed to the same experimental conditions. For each drug tested, uptake of the three radiolabeled precursors was unaffected relative to the specific inhibition of incorporation into TCA-precipitable material.

Within 10 min of bioxalomycin α_2 treatment, DNA synthesis was reduced by 56%, whereas RNA and protein syntheses were inhibited by only 2 and 11%, respectively (Table 4). During the same period, control drugs, i.e., ciprofloxacin, rifampin, and chloramphenicol, predominantly inhibited DNA, RNA, and protein syntheses, respectively. Polymyxin B inhibited incorporation into all three macromolecules.

The concentrations of bioxalomycin α_2 required for 50% inhibition (IC_{50}s) of DNA, RNA, and protein syntheses within 5 min were 0.025, 0.175, and 0.69 μg/ml, respectively (Fig. 4). After 10 min, the IC_{50}s for RNA and protein syntheses decreased to 0.095 and 0.275 μg/ml, respectively, whereas the IC_{50} for DNA synthesis (0.023 μg/ml) was unchanged (data not

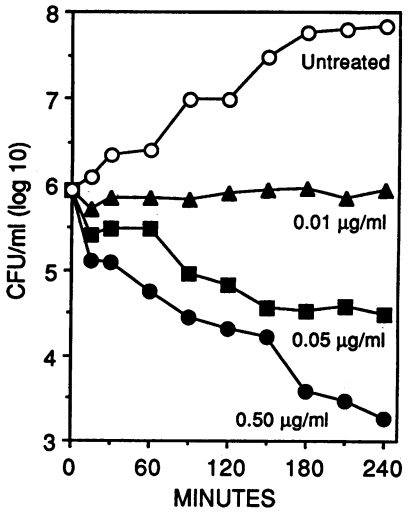

FIG. 2. Bactericidal activity of bioxalomycin α_2 against *S. aureus*.

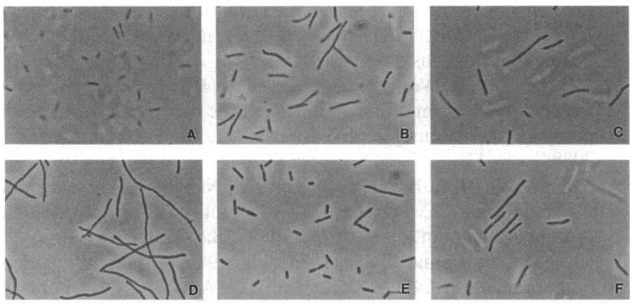

FIG. 3. Morphological effects on *E. coli*. (A) Untreated control; (B) cinodine (100 μg/ml); (C) ciprofloxacin (1 μg/ml); (D) aztreonam (1 μg/ml); (E and F) bioxalomycin α_2 (1 and 2 μg/ml, respectively).

shown). Although inhibition of RNA and protein syntheses increased after 30 min of treatment with bioxalomycin α_2, DNA synthesis remained the primary target (data not shown). Similar secondary effects on macromolecular processes were also observed with control drugs after prolonged exposures.

In vivo efficacy and toxicity. The ability of bioxalomycin α_2 to protect mice from lethal challenges with *S. aureus* Smith or *E. coli* 311 was compared with that of minocycline (Table 5). The maximum tolerated dose and LD_{50}s varied from 0.25 to 2 mg/kg, depending on the route of administration. Bioxalomycin α_2 administered by any of the three routes protected the mice from a lethal challenge with *S. aureus* Smith in a dose-dependent manner. However, it was unable to protect mice from a lethal challenge with *E. coli*. The ED_{50} of bioxalomycin α_2 against *S. aureus* by the oral route was threefold greater than that by either the intravenous or subcutaneous route. Similarly, the ED_{50}-to-LD_{50} ratio for the parenteral and oral routes ranged from 1:16 to 1:32.

DISCUSSION

The bioxalomycins are broad-spectrum antibacterial agents with excellent in vitro activity against gram-positive organisms and good activity against gram-negative organisms. The decreased activity against gram-negative bacteria may be due to the presence of the outer membrane, since bioxalomycin α_2 exhibited four- to fivefold better activity against an *E. coli imp* strain. The *imp* mutation increases the permeability of the cell membrane and renders the bacteria much more susceptible to many antibacterial agents, especially larger molecules (14). Since the presence of blood increased the MICs only one- to twofold, binding of this drug to blood components may be low or reversible. Cross-resistance to other classes of antibacterial agents including β-lactams, tetracyclines, macrolides, quinolones, and aminoglycosides was not observed in the bacteria tested. Bioxalomycin α_2 was also found to have excellent in vivo activity against gram-positive infections.

Mechanistic studies suggested that DNA synthesis is the primary target of bioxalomycin α_2. Bioxalomycin α_2 preferentially inhibited DNA synthesis over a wide concentration range. By interpolating the data for 5 min of treatment, the IC_{50}s for inhibition of RNA and protein syntheses were estimated to be 7 and 28 times greater, respectively, than the IC_{50} for DNA synthesis (Fig. 4). The decrease in IC_{50}s for RNA and protein syntheses after a 10-min exposure to bioxalomycin α_2 is consistent with a secondary effect of the drug on these processes. In contrast, the IC_{50} for DNA synthesis remained the same, supporting DNA synthesis as the primary target.

The related antibiotic naphthyridinomycin has also been reported to inhibit DNA synthesis in *E. coli* (8, 16), and its interactions with bacterial and animal DNAs have been estab-

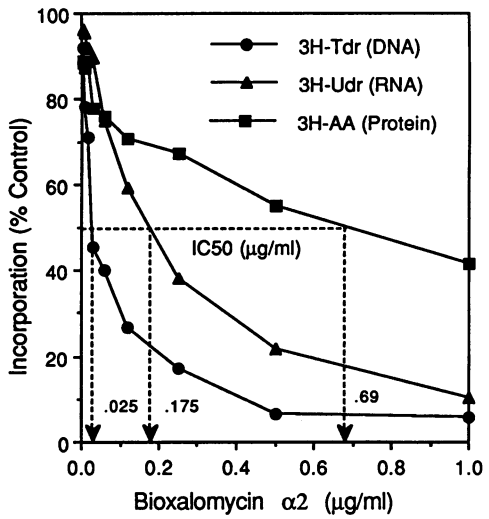

FIG. 4. Effect of bioxalomycin α_2 on the incorporation of radiolabeled precursors into *E. coli* (*imp*). Exponential-phase cells were preincubated with drug for 5 min and were then pulse-labeled. The arrows indicate the IC_{50}s (concentrations at which incorporation was inhibited by 50%) for each macromolecular process.

TABLE 4. Effects of antimicrobial agents on macromolecular synthesis in *E. coli* (*imp*)[a]

Compound	Concn (μg/ml)	% Inhibition		
		DNA	RNA	Protein
Bioxalomycin α_2	0.03	56	2	11
	0.015	40	0.5	5
Ciprofloxacin	0.25	97	7	15
Rifampin	0.25	10	98	85
Chloramphenicol	8	20	0	90
Polymyxin B	8	99	98	97

[a] Exponential-phase cells were preincubated with the drug for 10 min and were then pulse-labeled for 5 min. The levels of incorporation of [^{3}H]Tdr, [^{3}H]Udr, and ^{3}H-AA into the untreated controls were 3,139, 1,019, and 3,317 cpm/100-μl aliquot, respectively.

TABLE 5. In vivo activity and toxicity of bioxalomycin α_2 in mice

Compound	Route[a]	ED_{50} (mg/kg)		Maximum tolerated dose (mg/kg)	LD_{50} (mg/kg)
		S. aureus Smith	*E. coli*		
Bioxalomycin α_2	SSC	0.03–0.06	>1	1	1–2
	SIV	0.03–0.06	>0.25	0.25	0.50–1
	SOD	0.25–0.50	>2	2	4–8
Minocycline	SSC	0.25–0.50	2–4	512	>512
	SIV	0.25–0.50	2–4	32	64–128
	SOD	0.50–2	8–16	512	>512

[a] SSC, single subcutaneous dose; SIV, single intravenous dose; SOD, single oral dose.

lished (7, 23). Several other related compounds, such as quinocarcin, saframycin A, and cyanocycline A, are also DNA-binding agents with potent cytotoxic activities (5, 19, 21). The cytotoxicity of bioxalomycin α_2 and its mode of action in eukaryotic cells are under investigation.

Inhibition of cell division in *E. coli* leads to filamentation (10). The β-lactam antibiotics demonstrating strong affinities for PBP 3 are known to produce long filaments in *E. coli* (6). Inhibitors of DNA synthesis, such as ciprofloxacin and cinodine, indirectly inhibit cell division by inducing the SOS response and produce filaments (4, 12, 20). The filamentation induced by bioxalomycin α_2 in *E. coli* (shorter filaments) is consistent with a DNA-damaging mode of action.

Agents which bind strongly to DNA show a considerable decrease in biological activity in the presence of exogenous DNA (5). The twofold decrease in the antibacterial activity of bioxalomycin α_2 upon the addition of exogenous DNA suggests that this antibiotic may bind to DNA; however, a direct DNA-binding assay would be required to confirm this property. These data are consistent with published reports on the related compound, naphthyridinomycin (7, 23).

Although bioxalomycin α_2 protected mice from a lethal challenge with *S. aureus*, it failed to protect mice against an *E. coli* infection. The latter can be explained by the poor therapeutic index against gram-negative organisms, for which the LD_{50} is equivalent to the ED_{50}. Bioxalomycin α_2 was more effective when administered by the parenteral route than by the oral route, and the ED_{50}s correlated well with the MICs.

In conclusion, bioxalomycin α_2 is a novel, potent antibiotic with excellent in vivo activity against gram-positive bacteria. On the basis of the results of mechanistic studies, the primary cellular target appears to be DNA. In view of its in vivo toxicity, bioxalomycin α_2 will require further structural modification before the clinical potential of this class of antibacterial agents can be assessed.

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