LYSOBACTIN, A NOVEL ANTIBACTERIAL AGENT PRODUCED BY *LYSOBACTER* SP.

II. BIOLOGICAL PROPERTIES

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Lysobactin, an antibiotic isolated from a strain of *Lysobacter*, is 2 to 4-fold more active than vancomycin against aerobic and anaerobic Gram-positive bacteria. Included in the spectrum of lysobactin are Staphylococci, Streptococci, corynebacteria, clostridia and various other Gram-positive anaerobic bacteria. The activity of lysobactin against aerobic and anaerobic Gram-negative bacteria is poor. When given parenterally the compound was efficacious in systemic staphylococcal and streptococcal infections in mice. Similarly, when applied topically lysobactin was also curative in a staphylococcal wound infection in mice. Some studies on the mode of action of lysobactin are presented.

Gram-positive bacteria have traditionally been susceptible to a wide variety of antimicrobial agents including the β-lactams, macrolides and tetracyclines. However, in recent years, multiply-resistant strains have appeared with increasing frequency. Problem organisms include methicillin-resistant *Staphylococcus aureus* and *Staphylococcus epidermidis*, strains of Streptococci that are tolerant or relatively-resistant to the penicillins, enterococci, group JK corynebacteria and Gram-positive anaerobic bacteria such as *Clostridium difficile*, a major etiologic agent in pseudomembraneous colitis. The emergence of these resistant organisms has led to a dramatic increase in the usage of vancomycin, a glycopeptide antibiotic having a unique mode of action and first described by workers at Eli Lilly and Company in the 1950's.[1]

With this background in mind, a screen was initiated at Squibb to search for microbially produced compounds having a mode of action similar to vancomycin. One compound found in this screen, lysobactin, is a lipophilic, basic peptide antibiotic produced by *Lysobacter* sp. ATCC 53042. In the preceding paper[2] the screening method, and characteristics of lysobactin were described. Here we report on the biological properties of the compound and on some aspects of its mode of action.

Materials and Methods

Activity *In Vitro*

MICs were determined using the agar dilution method with 2-fold serial dilutions of the test compounds. In the primary screen, yeast - beef agar (BBL) was employed while for most other testing, Diagnostic Sensitivity Test (DST; Oxoid) agar was used. Methicillin-resistant Staphylococci were tested on DST agar plus 5% NaCl. For the more fastidious organisms, chocolate agar or DST agar supplemented with 5% sheep blood or 5% lysed sheep blood and vitamin K were the test media. Inoculation of the agar plates was with 10^4 cfu (primary screen), 10^4 cfu and 10^6 cfu (secondary Gram-positive screen), 5×10^5 cfu (Staphylococci, enterococci) or 10^5 cfu (other Streptococci, *Haemophilus*, *Neisseria*, anaerobes). Surface inoculum was applied using a multipronged inoculator (Denley In-

struments, Sussex, England). MICs were determined after incubation at 37°C for 18 hours under appropriate atmospheric conditions.

Acute Toxicity

Lysobactin and vancomycin were evaluated for acute toxicity in CD-1 female mice (Charles River Breeding Laboratories; 18~22 g) by both the ip and iv routes. Varying doses of the compound in 10% DMSO were given to mice (5/group) which were then observed for 7 days. At that time deaths were tabulated and LD_{50} values calculated.[3]

Efficacy Studies

Lysobactin was evaluated as a therapeutic agent for the treatment of systemic and wound infections in mice. For systemic infections, female CD-1 mice (18~22 g) from Charles River Breeding Laboratories were infected *via* the ip route with the test pathogen contained in 5% hog gastric mucin. Treatment was administered by the sc route in divided doses at 1 and 5 hours after infection. At least three concentrations of the test compounds were employed using 10 mice at each concentration. The median effective dose (ED_{50}) was calculated using the Reed and Muench procedure[3] from the number of survivors at the end of a 6-day observation period.

The procedures used to produce the experimental wound infections were those of McRIPLEY and WHITNEY.[4] Briefly, the backs of mice were shaved and under anaesthesia superficial surgical wounds were produced by making a 2-cm longitudinal incision. The skin on either side of the incision was retracted and the wound infected by insertion of a monocontaminated suture. Varying concentrations of the test compounds in a cream base were applied topically to the infected wounds at 1 and 5 hours after infection. At 18 hours after treatment, wounds were quantitatively cultured. The concentration of antibiotic resulting in a complete clearance of the infecting pathogen for 50% of the infected animals was designated the CD_{50}.

Mode of Action

The preparation of cell wall material was described in the preceding paper.[2] For binding studies, cell wall material was digested with trypsin (Sigma, type II), 0.1 mg/ml, pH 7, for 3 hours, 30°C. The trypsinized cell wall was recovered by centrifugation, washed and digested with lysozyme (Sigma, grade III), 0.1 mg/ml, pH 7, 15 hours, 30°C. This was then centrifuged to pellet the insoluble undigested walls which were washed and resuspended in water for testing. Binding assays were carried out by adding equal volumes of test substance to a solution of vancomycin or lysobactin and then assaying against *Bacillus subtilis* (SC 14019) in a plate assay. A zone diameter reduction of 4 mm when compared with unbound vancomycin was scored as a positive.

The estimation of cell wall precursors was by the method of STROMINGER.[5] The cell membrane integrity assay using *S. aureus* was the same as that described for *Candida* and other fungi.[6] Incorporation of radiolabel into cell polymers was carried out using a diaminopimelic acid requiring *Bacillus megaterium* (SC 11091).

Results and Discussion

Activity *In Vitro*

When tested for activity *in vitro* lysobactin exhibited an antimicrobial spectrum very similar to vancomycin. Lysobactin differed from vancomycin in showing increased potency. An overview of the activity is presented in Tables 1~3. As seen in Table 1, lysobactin was 2~4-fold more active than vancomycin against Gram-positive bacteria and even showed modest activity against some Gram-negative organisms. This increased activity was confirmed in the secondary Gram-positive screen (Table 2) where larger numbers of Gram-positive bacteria were examined including organisms resistant to a variety of other antibacterial agents. Table 3 highlights the superiority of lysobactin over vancomycin against anaerobic bacteria. Again, the primarily Gram-positive spectrum is evident. When

Table 1. Lysobactin: Antibacterial activity *in vitro* (primary screen).

Organism	SC No.	MIC (μg/ml) (10^4 cfu)	
		Lysobactin	Vancomycin
Staphylococcus aureus	1276	0.1	0.4
S. aureus	2399	0.2	0.8
S. aureus	2400	0.4	0.8
S. aureus	10165	0.4	0.8
Streptococcus faecalis	9011	0.8	0.4
S. agalactiae	9287	0.8	0.4
Micrococcus luteus	2495	0.1	0.4
Escherichia coli	8294	25.0	100.0
E. coli	10857	6.3	12.5
E. coli	10896	6.3	12.5
E. coli	10909	3.1	12.5
Klebsiella aerogenes	10440	25.0	>100.0
K. pneumoniae	9527	50.0	>100.0
Proteus mirabilis	3855	100.0	100.0
P. rettgeri	8479	100.0	100.0
P. vulgaris	9416	12.5	12.5
Salmonella typhosa	1195	25.0	>100.0
Shigella sonnei	8449	12.5	50.0
Enterobacter cloacae	8236	25.0	>100.0
E. aerogenes	10078	25.0	>100.0
Citrobacter freundii	9518	50.0	100.0
Serratia marcescens	9783	25.0	>100.0
Pseudomonas aeruginosa	9545	25.0	>100.0
P. aeruginosa	8329	25.0	>100.0
Acinetobacter calcoaceticus	8333	12.5	>100.0

tested against large numbers of Gram-positive clinical isolates the 2~4-fold superiority of lysobactin over vancomycin was further substantiated (Table 4). Staphylococci of various resistance types, enterococci and members of the major streptococcal groups all showed susceptibility to lysobactin as did corynebacteria. Strains of *Haemophilus influenzae* and *Neisseria gonorrhoeae* which are at times more sensitive to Gram-positive agents than typical aerobic Gram-negative bacteria were poorly susceptible to both lysobactin and vancomycin.

Acute Toxicity

When given to mice parenterally lysobactin showed an increase in toxicity over vancomycin (Table 5). The acute LD_{50} values of lysobactin were 77 and 132 mg/kg by the iv and ip routes, respectively.

Efficacy Studies

Lysobactin was found highly efficacious in Gram-positive systemic infections in mice caused by strains of *Streptococcus pyogenes* and *S. aureus* (Table 6). The ED_{50} values of lysobactin compared favorably with those of vancomycin with both compounds showing a slight superiority to cephalothin.

In Table 7 are the efficacy results with lysobactin in a *S. aureus* wound infection in mice. Lysobactin and gentamicin were very effective in clearing the pathogen at a concentration of 0.01~0.03%. Vancomycin was significantly less effective, not yielding complete clearance at a level 10-fold higher than either lysobactin or gentamicin.

Table 2. Lysobactin: Antibacterial activity *in vitro* (secondary Gram-positive screen).

Organism	SC No.	MIC (μg/ml)			
		Lysobactin		Vancomycin	
		10^4 cfu	10^6 cfu	10^4 cfu	10^6 cfu
Bacillus subtilis	3777	0.4	0.8	0.4	0.4
Staphylococcus epidermidis Pen^s	9052	0.8	3.1	1.6	3.1
S. epidermidis Pen^r	9083	0.8	3.1	1.6	3.1
S. epidermidis Pen^r	9087	0.8	1.6	3.1	6.3
S. epidermidis Pen^r	9607	0.8	1.6	1.6	3.1
S. epidermidis Pen^r	10547	0.8	1.6	3.1	6.3
S. saprophyticus	12875	1.6	1.6	3.1	6.3
S. aureus Pen^s	2399	0.4	0.8	1.6	3.1
S. aureus Tet^r	10016	0.4	0.8	1.6	3.1
S. aureus Pen^r	2400	0.4	3.1	1.6	6.3
S. aureus Pen^r	9593	0.8	3.1	1.6	6.3
S. aureus Pen^r	9998	0.8	1.6	1.6	3.1
S. aureus Meth^r	3184	1.6	3.1	3.1	3.1
S. aureus Meth^r	10014	0.2	0.8	1.6	3.1
S. aureus Meth^r	10020	0.4	3.1	1.6	3.1
S. aureus Gent^r	11239	0.4	3.1	1.6	3.1
S. aureus Eryth^r	10820	0.8	3.1	1.6	3.1
S. aureus Eryth^r	12691	0.8	1.6	1.6	3.1
Streptococcus faecalis	9011	0.8	3.1	1.6	1.6
S. faecalis	9376	1.6	3.1	3.1	3.1
S. faecalis	10938	1.6	3.1	1.6	1.6
S. agalactiae	9285	0.8	0.8	0.8	0.8
S. agalactiae	9287	0.2	0.4	0.8	0.8
Nocardia asteroides	2626	0.8	1.6	3.1	12.5
Listeria monocytogenes	8523	1.6	6.3	1.6	1.6

Pen: Penicillin, Tet: tetracycline, Meth: methicillin, Gent: gentamicin, Eryth: erythromycin.

Table 3. Lysobactin: Anaerobic bacterial screen.

Organism	SC No.	MIC (μg/ml) (10^5 cfu)	
		Lysobactin	Vancomycin
Bacteroides thetaiotaomicron	9005	12.5	100.0
B. thetaiotaomicron	10278	25.0	100.0
B. fragilis	9844	50.0	50.0
B. fragilis	10277	25.0	50.0
B. fragilis	10279	50.0	50.0
B. fragilis	10280	50.0	50.0
B. fragilis	11085	25.0	50.0
Fusobacterium necrophorum	10388	25.0	12.5
Clostridium histolyticum	8572	0.8	3.1
C. perfringens	11256	0.4	1.6
C. septicum	1780	0.1	3.1
C. sporogenes	2372	0.8	12.5
C. difficile	11251	0.2	1.6
Bifidobacterium dentium	11260	0.4	0.8
Eubacterium lentum	11261	0.4	1.6
Peptococcus variabilis	11264	0.2	0.4
Peptostreptococcus anaerobius	11263	0.8	0.8
Propionibacterium acnes	4020	0.1	0.8

Table 4. Lysobactin vs. clinical isolates.

Group (No. of strains)	Test compound	MIC (μg/ml)		
		MIC range	MIC_{50}[a]	MIC_{90}[a]
Staphylococcus aureus Pen^S (20)	Lysobactin	0.4~1.6	0.5	1.2
	Vancomycin	1.6~6.3	1.8	3.0
S. aureus Pen^R (20)	Lysobactin	0.4~1.6	0.6	1.3
	Vancomycin	1.6~3.1	1.3	2.6
S. aureus Meth^R (25)	Lysobactin	0.2~0.8	0.5	0.7
	Vancomycin	1.6~3.1	2.4	3.0
Streptococcus faecalis (25)	Lysobactin	0.4~1.6	0.6	1.1
	Vancomycin	1.6~6.3	3.1	5.6
Streptococci				
Group A (5)	Lysobactin	0.4~0.8	0.6	0.8
	Vancomycin	0.8	0.6	0.8
Group B (10)	Lysobactin	0.2~0.8	0.3	0.6
	Vancomycin	0.8~1.6	1.0	1.5
Viridans group (9)	Lysobactin	0.4~1.6	0.6	1.2
	Vancomycin	1.6~3.1	1.5	2.8
Streptococcus pneumoniae (2)	Lysobactin	<0.05~0.1		
	Vancomycin	0.4~1.6		
Corynebacteria (4)	Lysobactin	<0.05~0.4		
	Vancomycin	0.8~1.6		
Haemophilus influenzae (39)	Lysobactin	25~>100	72.1	>100
	Vancomycin	>100	>100	>100
Neisseria gonorrhoeae (25)	Lysobactin	100~>100	>100	>100
	Vancomycin	6.3~>100	30.7	87.5

[a] MIC_{50}, MIC_{90}: Concentrations necessary to inhibit 50% and 90% respectively, of the isolates tested.

Table 5. Acute toxicity of lysobactin in mice.

Compound	LD_{50} (mg/kg)	
	iv	ip
Lysobactin	77	132[a]
Vancomycin	>400	>1,000

[a] Mean of 2 determinations.

Table 6. Efficacy of lysobactin in systemic mouse infections.

Compounds	ED_{50} (mg/kg)	
	Streptococcus pyogenes[a]	*Staphylococcus aureus*[b]
Lysobactin	2.2	1.8
Vancomycin	2.0	1.9
Cephalothin	2.5	2.9

[a] 10 LD_{50}s given.
[b] 100 LD_{50}s given.

Table 7. Efficacy of lysobactin in a *Staphylococcus aureus* wound infection in mice.

Compound[a]	CD_{50} (%)[b]
Lysobactin	0.03
Vancomycin	>0.2
Gentamicin	0.01

[a] Compounds applied topically.
[b] % Compound in cream base.

Mode of Action

The screen[2] used to discover lysobactin was based on the ability of vancomycin to bind to acyl-D-alanyl-D-alanine (D-ala-D-ala) peptides in the cell wall. Relatively crude preparations of bacterial cell wall were used as a trapping agent thereby exploring the possibility that yet undiscovered binding sites or combinations of sites in the wall would act as targets for new antibacterial agents.

We examined this possibility with lysobactin by monitoring its binding to wall preparations from *S. aureus*, and *Bacillus cereus*. Table 8 shows that both vancomycin and lysobactin bind to trichloroacetic acid precipitated and trypsin-digested wall fragments of both organisms. When this wall

Table 8. The binding of lysobactin and vancomycin to cell wall preparations from *Staphylococcus aureus* and *Bacillus cereus*.

Sample	*Staphylococcus aureus*		*Bacillus cereus*	
	Lysobactin	Vancomycin	Lysobactin	Vancomycin
Crude wall	+	+	+	+
Trypsin digested wall	+	+	+	+
Lysozyme digest supernate	−	+	−	+
Lysozyme digest sediment	+	+	+	−

A + represents a zone diameter reduction of 4 mm or more when cell wall treated antibiotic was compared with untreated antibiotic in a disc diffusion assay using *B. subtilis* as test organism.

Table 9. Cell wall precursor accumulation in *Staphylococcus aureus*.

Sample	MIC (μg/ml)	Test concentration	*N*-Acetylamino sugar (mmol/l culture)
Control	—	—	11.9
Vancomycin	1.6	20×MIC	302
		10×MIC	193
Benzylpenicillin	0.05	20×MIC	124
Diumycin	0.05	20×MIC	193
Lysobactin	0.10	1×MIC	8.5
		10×MIC	5.2
		20×MIC	5.2
Fosfomycin	50	10×MIC	15.4
Bacitracin	25	10×MIC	23

material was digested by lysozyme, acyl-D-ala-D-ala-like peptides were solubilized. Vancomycin bound to this solubilized material but lysobactin did not. However, lysobactin bound to the insoluble material remaining after lysozyme digestion.

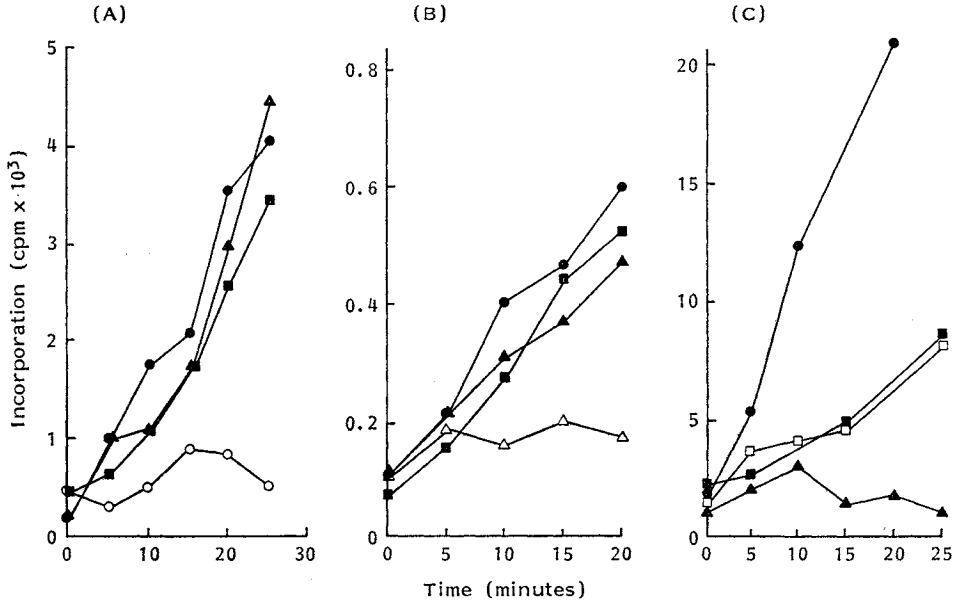
When cell wall-acting agents are tested against *S. aureus*, uridine 5′-pyrophosphate *N*-acetylamino sugar compounds accumulate in the cell.[5] In Table 9 it is seen that whereas benzylpenicillin, diumycin and vancomycin led to cell wall nucleotide precursor accumulation, lysobactin, when tested at 1 to 20 times the MIC did not. Bacitracin and fosfomycin were also inactive in this test.

In order to determine if lysobactin has an effect on membranes, a [^{14}C]aminoisobutyric acid leakage experiment was carried out.[6] This amino acid is not metabolized and its appearance in the medium can be taken as a measure of membrane damage. No evidence of leakage was seen at the MIC (0.1 μg/ml) or 10 times the MIC, however at concentrations above this, leakage increased markedly. Vancomycin at over 100 times the MIC was inactive in this test whereas gramicidin A was very active at its MIC concentration.

Lysobactin did not inhibit the incorporation of [^{14}C]uridine, or [^{14}C]thymidine into trichloroacetic acid precipitable cellular material but [^{14}C]diaminopimelic acid incorporation was inhibited at its MIC (Fig. 1). These data suggest that lysobactin is primarily a cell wall acting agent probably affecting a step prior to UDP-*N*-acetylglucosamine formation. A secondary effect on membrane integrity may contribute to its potent activity. Lysobactin therefore appears to have a biochemical profile similar to that of LY146032, a cyclic lipopeptide antibiotic[7] in that both are preferentially active against Gram-positive bacteria, they inhibit α-aminoadipic acid incorporation into the cell wall yet UDP-*N*-acetyl-

Fig. 1. The incorporation of [^{14}C]uridine (A), [^{14}C]thymidine (B) and [^{14}C]diaminopimelic acid (C) into trichloroacetic acid precipitable material by *Bacillus megaterium* SC 11091 in the presence of test substances at their MICs.

● Control, ▲ lysobactin, ■ vancomycin, ○ rifampicin, △ novobiocin, □ benzylpenicillin.



glucosamine does not accumulate. Neither compound has an effect on RNA and DNA biosynthesis or causes significant membrane damage.

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