GARDIMYCIN, A NEW ANTIBIOTIC FROM *ACTINOPLANES*

III. BIOLOGICAL PROPERTIES

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The new antibiotic gardimycin has an interesting *in vitro* antibacterial activity against Gram-positive bacteria and *Neisseria gonorrhoeae*. Parenteral administration gives a high degree of protection against experimental infections in mice. It also shows some chemotherapeutic activity when given rectally.

Gardimycin is a new polypeptide antibiotic produced by a strain of *Actinoplanes garbadinensis*[1,2]. Its mechanism of action consists of inhibition of synthesis of cell wall[3]. In this paper we report the results of bacteriological and chemotherapeutic studies.

Materials and Methods

In vitro studies

The antimicrobial activity of gardimycin was assayed in broth cultures, by the serial dilution technique. The cultures were inoculated with 10^2 cells/ml for Gram-negative bacteria and with 10^8 cells/ml for *Neisseria* and Gram-positive bacteria. For yeasts, *Mycoplasma* and *Mycobacterium*, the number of cells was between 10^4 and 5×10^5/ml, depending on the particular species.

Penassay broth was the medium used for *Staphylococci* and Gram-negative bacteria. The medium for *Streptococci* and *Diplococci* was Brain Heart infusion (Difco) plus 1 % bovine serum; for *Neisseria* it was Tryptose Phosphate broth (Difco) plus 3 % bovine serum; for yeasts, SABOURAUD broth (Difco); for *Mycoplasma*, PPLO broth (Difco) plus 10 % horse serum; for *Mycobacterium*, KIRSCHNER's medium[4] plus 10 % horse serum. The influence of serum on the minimal inhibitory concentration (MIC) values was determined by adding increasing concentrations of bovine serum to the test media.

To assess the influence of the pH of the medium on the MIC, media with several pH values were prepared by addition of 1N HCl or 1N NaOH.

The influence of the size of the inoculum on the *in vitro* activity was evaluated in broth cultures inoculated with $10^3\sim10^8$ cells per ml.

The bactericidal activity was determined by adding different concentrations of gardimycin to cultures in the exponential phase of growth. The cultures were incubated and aliquots were taken at different times after addition of the gardimycin to determine on agar plates the numbers of colony-forming units (c.f.u.).

The frequency of mutants resistant to gardimycin was determined by plating about 10^9 cells on plates containing the antibiotic at a concentration 5 times that of the MIC for the particular strain being studied. To isolate a resistant mutant of *Streptococcus haemolyticus* the strain was grown in Brain Heart infusion with increasing concentrations of drug (training). The degree of resistance was determined by the serial dilution test. Cross-resistance was determined by evaluating the *in vitro* activities of different antibiotics against a mutant of *Streptococcus haemolyticus* which was resistant to gardimycin. The stability of the antibiotic at different pH's was studied by preparing solutions in buffers at pH 2.2, 3.6, 7.38, 8.5 and 10 which were then maintained at room temperature for 48 hours. At different times during that period, aliquots

were assayed against *Sarcina lutea* ATCC 9341 by the agar diffusion method and gardimycin activity determined from the standard curve.

Experimental infections in mice

Male and female albino CF_1 mice weighing 18~22 g were used. The animals were infected by intraperitoneal injection of 16-hour broth cultures of the following strains of bacteria: *Diplococcus pneumoniae*, Felton UC 41 strain; *Streptococcus haemolyticus*, C 203 ISM strain; *Staphylococcus aureus*, Tour strain.

The cultures were diluted so as to produce death of the control animals within 48 hours due to septicemic spreading of the infection. The therapeutic treatment was begun 30 minutes after infection with the organisms and was continued for 2 additional days, at intervals of 24 hours. On the 10th day, the value for the ED_{50} in mg/kg/day was calculated by the method of SPEARMAN and KÄRBER[5], on the basis of the percentage of surviving animals at each dose of product.

Results

Spectrum of Activity:

As shown in Table 1, the spectrum of activity includes some Gram-positive organisms, *Neisseria gonorrhoeae* and *Clostridium perfringens*. Gram-negative bacteria, yeasts, *Mycoplasma* and *Mycobacterium* were not sensitive to levels of gardimycin up to 100 μg/ml.

Table 1. *In vitro* activity of gardimycin on laboratory strains.

Organism	M.I.C. μg/ml
Staphylococcus aureus ATCC 6538	100
Staphylococcus aureus Tour	50
Staphylococcus aureus ATCC 9144	100
Staphylococcus albus ATCC 12228	>100
Staphylococcus sp. SKF 24390	100
Staphylococcus Sackman 10B CIBA	50
Micrococcus flavus ATCC 10240	1
Streptococcus faecalis ATCC 10541	50
Streptococcus bovis ATCC 9809	100
Streptococcus haemolyticus C 203	2
Diplococcus pneumoniae UC 41	50
Neisseria gonorrhoeae ATCC 9826	20
Clostridium perfringens ISS 30543	2
Proteus vulgaris X 19H ATCC 881	>100
Escherichia coli ATCC 10536	>100
Pseudomonas aeruginosa ATCC 10145	>100
Candida albicans SKF 2270	>100
Trichophyton mentagrophytes SKF 17410	>100
Mycobacterium tuberculosis H37Rv ATCC 9360	>100
Mycoplasma gallisepticum S 6 Weybridge	>100

The most interesting activity appeared to be that against *Streptococcus haemolyticus*. To make sure that this activity was not restricted only to the laboratory strain used, the sensitivities of other strains of *Streptococcus*, isolated clinically and including *S. haemolyticus* and *S. viridans* species, were also determined (Table 2). All strains tested were sensitive to gardimycin, but with considerable differences in sensitivity from one strain to another.

Influence of Serum on the MIC:

When concentrations of bovine serum were increased from 0 to 70%, there were no differences in the *in vitro* activity of gardimycin (Table 3).

Influence of pH of the Medium on the MIC:

The *in vitro* activity of gardimycin appears to be slightly influenced by the pH of the medium (Table 4). The antibiotic is more active at acid pH's than at basic pH's.

Influence of the Size of the Inoculum on *in vitro* Activity:

The *in vitro* activity of gardimycin is only very slightly affected by increasing the size of the inoculum from 10^3 to 18^8 cells/ml (Table 5).

Bactericidal Action:

Gardimycin possess a moderate bactericidal activity against *Streptococcus haemolyticus* (Fig. 1).

Table 2. *In vitro* activity of gardimycin on clinical isolates of *Streptococcus* species

Organism		M.I.C. μg/ml
Streptococcus haemolyticus	2073	20
Streptococcus haemolyticus	2078	1
Streptococcus haemolyticus	2087	1
Streptococcus viridans	2083	10
Streptococcus viridans	2057	5
Streptococcus viridans	2085	2
Streptococcus viridans	2063	20

Table 4. Influence of the pH of the medium on the M.I.C. of gardimycin on *S. haemolyticus* C 203

pH	M.I.C. μg/ml
6	0.78
6.5	0.78
7	0.78
7.5	0.78
8	1.56
9	3.12

After one hour in contact with all of the doses used, there was only some slowing of the rate of growth. After more prolonged times of contact, the number of c.f.u. was decreased. After 3 hours, with the highest dose used (20 μg/ml, corresponding to 10 times the MIC), 18.2 % of the cells could still give rise to colonies.

Resistance:

The frequency of mutants of *Streptococcus haemolyticus* resistant to gardimycin was less than 10^{-10}. When 10^9 cells were plated on Brain-Heart infusion agar plus 1 % bovine serum containing 5 μg/ml of antibiotic, no mutants were obtained. The MIC of the strain under these conditions is 1 μg/ml. It was possible to isolate a resistant mutant of *Streptococcus haemolyticus* only after "training". The level of resistance obtained was to 20 μg/ml, 10 times the MIC.

Cross-resistance:

When tested against a series of antibiotics (Table 6), no cross-resistance was seen in the

Table 3. Influence of serum concentrations on the M.I.C. of gardimycin

% Bovine serum	M.I.C. μg/ml	
	S. haemolyticus C 203	*S. aureus* Tour
0	1.56	50
10	1.56	—
20	0.78	—
30	0.78	20
40	0.78	—
50	0.78	—
70	0.78	—

Table 5. Influence of the size of inoculum on the M.I.C. of gardimycin

Size of inoculum	M.I.C. μg/ml	
	S. haemolyticus C 203	*M. flavus* ATCC 10240
10^3	1.56	0.78
10^4	1.56	1.56
10^5	1.56	1.56
10^6	1.56	1.56
10^7	1.56	3.12
10^8	3.12	3.12

Fig. 1. Bactericidal activity of gardimycin on *Streptococcus haemolyticus* C 203.

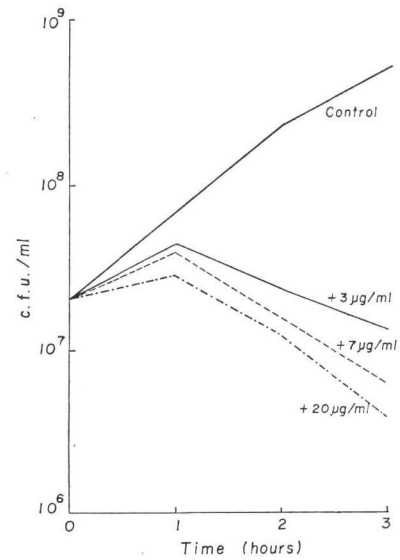

Table 6. Activity of some antibiotics against a *S. haemolyticus* mutant resistant to gardimycin

Antibiotics	M.I.C. μg/ml	
	S. haemolyticus C 203	*S. haemolyticus* C 203 gdm-r*
Penicillin	0.02	0.01
Bacitracin	0.01	0.01
Ristocetin	2	2
Vancomycin	1	1
Rifampicin	0.05	0.1
Erythromycin	0.05	0.05
Gardimycin	2	20

* gdm-r=Gardimycin-resistant.

Table 7. Stability of gardimycin at different pH

Hours	pH 2.2	pH 3.6	pH 7.38	pH 8.5	pH 10
0	268.80*	271.80	246.60	242.40	261.80
5	272.60	237.60	242.60	240.90	226.00
24	262.20	293.40	249.00	242.40	101.85
48	267.60	265.30	254.00	241.00	51.50

* Level expressed in μg/ml of gardimycin.

Table 8. Activity of gardimycin in experimental infections in mice

Organisms	Strains	ED_{50} mg/kg (confidential limits)
Diplococcus pneumoniae	Felton UC 41	9.23 (11~7.7)
Streptococcus haemolyticus	C 203 ISM	0.61 (0.75~0.53)
Staphylococcus aureus	Tour	~120

Streptococcus haemolyticus mutant isolated after training. Therefore, the mutation to resistance to gardimycin was not accompanied by any change in sensitivity of the strain even to antibiotics with similar mechanism of action, such as bacitracin, ristocetin, vancomycin or penicillin.

Stability:

Gardimycin was very stable for 48 hours at room temperature over a pH range of 2.2~8.5 (Table 7). At pH 10, an 80% inactivation was found in the 48th hour.

Experimental Infections:

Gardimycin showed a high therapeutic effectiveness when given subcutaneously to mice infected with *D. pneumoniae* or *Streptococcus haemolyticus* (Table 8). It was less active against infection with *S. aureus*. Oral administration of doses as high as 250 mg/kg was completely non-protective. When given rectally, the ED_{50} of gardimycin against *Streptococcus haemolyticus* infection was about 200 mg/kg. Even at the higher doses employed orally, subcutaneously and rectally no signs of toxicity were noted.

Conclusions

Gardimycin has *in vitro* activity against Gram-positive bacteria, Gram-negative cocci (*N. gonorrhoeae*) and obligate anaerobes (*C. perfringens*).

In vitro activity is not influenced by increasing concentrations of serum in the test medium. Increasing the size of the bacterial inoculum has some effect, but a very minor one. Gardimycin is more active when assayed in media of acid pH than in those of basic pH. This is probably due to the fact that gardimycin is less stable at highly basic pH. A gardimycin-resistant mutant did not show any cross-resistance to all the other antibiotics tested, even those having a similar mechanism of action. The frequency of mutants resistant to gardimycin is very low (less than 10^{-10}). Gardimycin's therapeutic activity when administered parenterally to mice experimentally infected with *Streptococcus* or *Diplococcus* is comparable to those of ampicillin and cephaloridin, under our experimental conditions. Its activity against experimental infection with *S. aureus* is lower.

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