JANIEMYCIN, A NEW PEPTIDE ANTIBIOTIC

EDWARD MEYERS, FRANK L. WEISENBORN, FELIX E. PANSY,
DOROTHY S. SLUSARCHYK, MALCOLM H. VON SALTZA*,
MARLENE L. RATHNUM and WILLIAM L. PARKER

The Squibb Institute for Medical Research
New Brunswick, New Jersey, 08903, U. S. A.

(Received for publication September 21, 1970)

Janiemycin is a new basic peptide antibiotic produced by a strain of *Streptomyces macrosporeus* ATCC 21,388. It is a bactericidal compound, active primarily against gram-positive bacteria. In model murine infections with *Streptococcus pyogenes* C 203 and *Diplococcus pneumoniae* Type 3, janiemycin is as active as penicillin G ; moreover, a single subcutaneous dose provides prolonged protection (at least 48 hours) to mice infected with *S. pyogenes* C 203. Janiemycin and enduracidin are apparently related, but they are easily distinguished by the absence of α-amino-3, 5-dichloro-4-hydroxyphenylacetic acid from the hydrolysate of janiemycin.

Janiemycin is a new peptide antibiotic produced by a strain of *Streptomyces macrosporeus* isolated from soil. The antibiotic is similar in amino acid composition to enduracidin[1], but lacks a chlorinated amino acid found in enduracidin. The isolation, characterization and biological properties of this new antibiotic form the subject of this report.

Production and Isolation

Janiemycin is produced by a strain of *Streptomyces macrosporeus*, ATCC 21,388, a member of the gray spore color series of PRIDHAM. The culture was maintained on tomato paste-oatmeal agar slants, prepared by adding one volume of water containing tomato paste (4 %) and oatmeal (4 %) to one volume of boiling water containing 3.0 % agar.

To prepare germinators, the growth from well-sporulated slants was suspended in 0.01 % sodium lauryl sulfate solution and used to inoculate 100-ml portions of medium contained in cotton-plugged 500-ml Erlenmeyer flasks. The medium had the following composition : soybean meal, 15.0 g ; dehydrated mashed potato, 15.0 g ; glucose, 50.0 g ; $CoCl_2 \cdot 2H_2O$, 0.005 g ; $CaCO_3$, 10.0 g ; agar, 2.5 g and distilled water to 1,000 ml. The flasks were incubated at 25°C for 96 hours on a rotary shaker, operating at 280 rpm with a 2-inch (5.08 cm) throw, after which a 5 % (v/v) transfer of the germinator culture was made to fermentor vessels. Fermentations were done in a medium consisting of soybean meal, 30.0 g ; glucose, 20.0 g ; $CaCO_3$, 10.0 g ; Ucon LB 625, 0.05 %

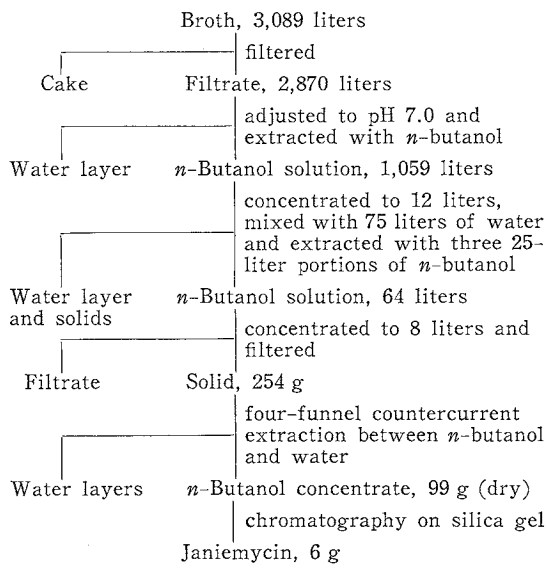
* Present address : Biological Sciences Department, University of Connecticut, Storrs, Connecticut.

(Union Carbide, New York) and water to 1,000 ml. The fermentation was allowed to proceed for 144 hours at 25°C before being harvested.

Conventional twofold tube-dilution assays and paper disc-agar diffusion assays, using *Staphylococcus aureus* FDA 209P, were used to follow the production of the antibiotic, as well as the degree of purification achieved during isolation. Partition paper chromatography and thin-layer chromatography also were used to analyze samples. Bioautography with *S. aureus* FDA 209P[2] was used to locate janiemycin on the chromatograms.

The antibiotic was sometimes found in both the mycelia and the fermentation broth filtrate and at other times primarily in the broth filtrate. The mycelia, if they contained sufficient bioactivity, were collected by filtration and processed; otherwise they were discarded. Thus, when appropriate, the mycelia were extracted with methanol and the extract was concentrated *in vacuo* to remove the organic solvent. The broth filtrate was extracted with *n*-butanol, and the butanol layer was concentrated *in vacuo*. The concentrate was combined with the mycelial extract, when obtained, and the bioactivities were extracted into *n*-butanol saturated with water. Partial purification was achieved by further distribution between butanol and water at pH 7.0. Material thus obtained was purified by chromatography on silica gel. Inactive impurities were eluted with *n*-propanol - *n*-butanol (2 : 3, by volume), and janiemycin was subsequently eluted with the upper phase of *n*-propanol - *n*-butanol - water (2 : 3 : 4, by volume). The isolation and purification of janiemycin from an 800-gallon (3,040-liter) tank fermentation is outlined in Chart 1. Material obtained by this process was used for biological testing. A small increase in activity could be obtained by preparative thin-layer chromatography on silica gel, using the upper phase of *n*-propanol - *n*-butanol - 1 N ammonia (2 : 3 : 4, by volume) as the solvent system. Material with Rf values between 0.04 and 0.15 was collected.

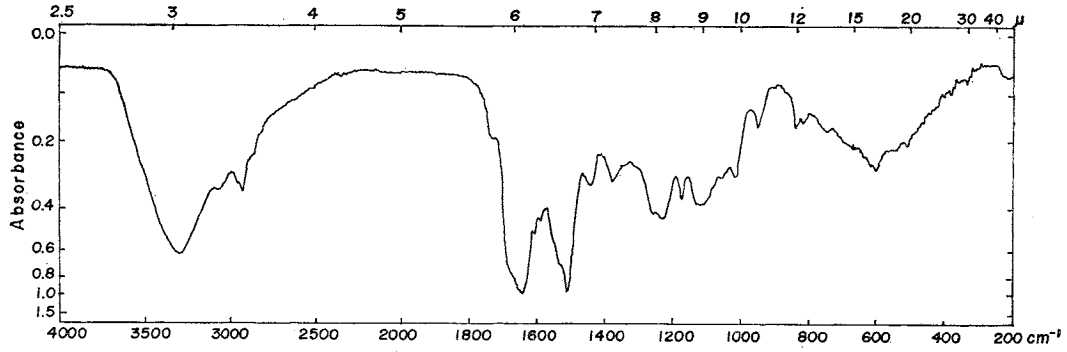
Chart 1. Isolation and purification of janiemycin



Physical and Chemical Properties

Janiemycin, an amorphous, light-tan powder, chars without softening at 260~315°C *in vacuo*. Three antibiotic components, two major and one minor, could be resolved by electrophoresis at pH 3.3 in the presence of 40 % formamide. The electrophoretic mobilities, using safranin O as cathodic indicator and apalon as electroosmotic indicator, are +85 and +44 for the major components and +12 for the minor one. Partition chromatography on Whatman No. 1 paper with *n*-butanol - acetic acid - water (4 : 3 : 7, by volume) and with *n*-butanol - pyridine - water (4 : 3 : 7, by volume) gave Rf

Fig. 1. Infrared spectrum of janiemycin (KBr).



values of 0.13 and 0.85, respectively, for the complex.

The ultraviolet spectrum of janiemycin has maxima at 239 and 272 nm in dimethylsulfoxide and at 253 nm in 0.15 N ammonia solution. The ir spectrum is shown in Fig. 1.

The elemental analysis of two independent preparations purified by thin-layer chromatography gave the following compositions: C 47.81, 47.49; H 5.48, 5.30; N 13.07, 13.07; Cl –, 0.79; ash, 2.8, 2.4 %.

The nitrogen content, as well as the amide band in the infrared spectrum, indicated that janiemycin is a peptide antibiotic. Consequently, a sample of janiemycin (2 mg) was hydrolyzed in 6 N HCl (1 ml, ammonia-free) at 110°C for 48 hours in a sealed tube. The resulting solution was evaporated to dryness *in vacuo* and the mixture of amino acids was analyzed by the conventional STEIN-MOORE technique. The results are shown in Table 1. Seven of the ten major ninhydrin-positive components were tentatively identified by comparison with standard amino-acid mixtures.

Table 1. The amino acid composition of janiemycin

Amino acid	Molar amount based on alanine
Aspartic acid	0.93
Threonine and/or allothreonine	2.46
Serine	0.73
Citrulline	0.08
Glycine	0.99
Alanine	(1.00)
α-Amino-4-hydroxyphenylacetic acid	5.70
Ammonia	2.43
Ornithine	1.38
A	*
B	*

* No ninhydrin color yield could be calculated since standards were not available.

Both the ultraviolet absorption spectrum and the results of the STEIN-MOORE analysis suggest a relationship of janiemycin to the basic peptide antibiotic, enduracidin[1]. This proposed relationship is further supported by the identification of α-amino-4-hydroxyphenylacetic acid[3], a reported constituent of enduracidin[1], in the hydrolysate of janiemycin. Two components of the hydrolysate (A and B in Table 1) were not identified, but their elution times in the STEIN-MOORE analysis indicated that they may be enduracididine and alloenduracididine[1,4]. Traces of citrulline could be found and the quantity could be increased by reducing the hydrolysis time. The amino acid composition of janiemycin and enduracidin differ with respect to α-amino-3, 5-dichloro-4-hydroxyphenylacetic acid, a major constituent of enduracidin[1]. None of this amino acid could be detected in the hydrolysate of janiemycin. This difference is reflected in the chlorine content of the two antibiotics. A sample of the chlorine-

containing amino acid was prepared for chromatographic comparison by treating α-amino-4-hydroxyphenylacetic acid (44 mg) in 4.4 N HCl (0.6 ml) with Cl_2. The hydrochloride, which crystallizes from the reaction mixture, was neutralized with ammonia, giving the free amino acid. The properties were in agreement with those reported for this substance[1,5].

Biological Characterization

In Vitro:

The antimicrobial spectrum of janiemycin, obtained by conventional twofold tube dilution assays, is shown in Table 2. The activity is primarily against gram-positive bacteria, with little or no activity against gram-negative bacteria, yeasts and fungi. Janiemycin is not cross-resistant with a variety of antibiotics, as shown by the minimal inhibitory concentration (M. I. C.) values against a number of variants of *Staphylococcus aureus* resistant to such antibiotics as penicillin G, the tetracyclines, erythromycin, oleandomycin, methymycin, carbomycin, ristocetin, streptomycin, neomycin, bacitracin, thiostrepton, actinomycin and chloramphenicol (Table 3).

Table 2. Antimicrobial spectrum of janiemycin *in vitro*

Organism	M.I.C. (μg/ml)
Streptococcus pyogenes C203	0.01
Staphylococcus aureus FDA 209P	0.31
Bacillus subtilis ATCC 6633	0.09
Bacillus cereus ATCC 10876	0.60
Sarcina lutea ATCC 9341	0.12
Diplococcus pneumoniae Type 3 ATCC 6303	0.80
Corynebacterium sp. ATCC 13959	0.10
Listeria sp. SC 8523*	1.2
Lactobacillus casei ATCC 393	1.2
Micrococcus lysodeikticus ATCC 4698	0.01
Clostridium tetanomorphum SC 3103*	1.0
Mycobacterium tuberculosis BCG SC 5516*	0.78
Escherichia coli ATCC 10536	>100.0
Klebsiella-Aerobacter sp. SC 8411*	>100.0
Serratia marcescens SC 1468*	>100.0
Pseudomonas aeruginosa SC 8329*	>100.0
Salmonella schottmuelleri SC 3850*	37.5
Proteus vulgaris SC 8504*	>100.0
Pasteurella multocida SC 8739*	50.0
Herellea sp. SC 8333*	12.5
Hemophilus influenzae ATCC 9333	> 12.5
Candida albicans SC 5314*	>100.0
Fusarium bulbigenum SC 5273*	>100.0
Trichophyton mentagrophytes SC 2637*	>100.0

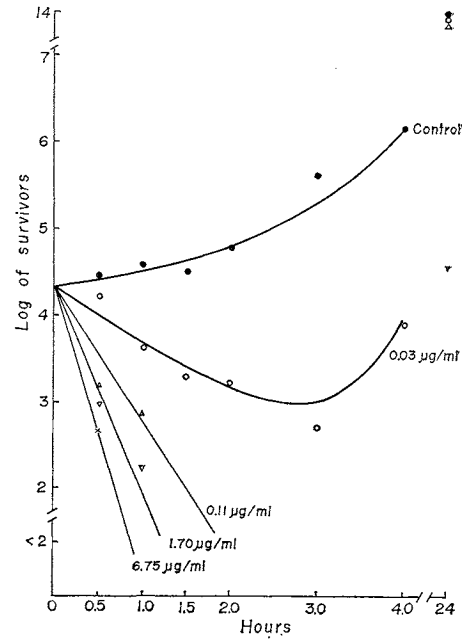
* Squibb Culture Collection

Table 3. Activity of janiemycin against antibiotic-resistant variants of *Staphylococcus aureus* FDA 209P *in vitro*

Organism			M.I.C. (μg/ml)
Staphylococcus aureus		FDA 209P	0.31
Staphylococcus aureus		SC 2961*	0.28
〃	〃	SC 2664	0.48
〃	〃	SC 2661	0.21
〃	〃	SC 2957	0.37
〃	〃	SC 3538	0.42

* Squibb Culture Collection
SC 2961 : resistant to thiostrepton, erythromycin, oleandomycin, methymycin and carbomycin.
SC 2664 : resistant to streptomycin, neomycin, tetracyclines.
SC 2661 : resistant to thiostrepton.
SC 2957 : resistant to actinomycin, chloramphenicol, bacitracin.
SC 3538 : resistant to penicillin, ristocetin.

Fig. 2. Bactericidal action of janiemycin against *S. aureus* FDA 209P.



The bactericidal nature of janiemycin is shown in Fig. 2. At levels above 0.1 μg/ml, janiemycin markedly reduced the population of growing cells of *Staphylococcus aureus* FDA 209P, but did not accomplish sterilization until the antibiotic concentration had reached 6.75 μg/ml. At levels of 6.75 μg/ml and above, no viable cells were recovered at any time after 1 hour of incubation. This lack of growth is not attributable to antibiotic carryover to the Petri dishes used for cell counting, since the 0.5-hour samples had substantial numbers of viable cells.

At levels between 0.1 and 1.7 μg/ml, janiemycin did not completely eliminate the bacteria. Although a rapid and drastic reduction in the number of viable cells occurred within 4 hours, high cell counts were obtained after 24 hours of incubation. This outgrowth was not the result of a rapid development of substantial resistance to the antibiotic, nor was it due to instability of the antibiotic during the incubation period. Subcultures made from the 24-hour samples in which growth had occurred were assayed against a freshly prepared solution of janiemycin. The M. I. C. value was the same for these cultures as for the parent culture. Solutions of janiemycin incubated at 37°C for 24 hours (*i. e.*, the conditions of the test) were as active as freshly prepared solutions of the antibiotic.

The concentration of cells in the inoculum used in the tube dilution assay had little effect on the response of *Staphylococcus aureus* FDA 209P to the antibiotic. Thus, varying the population density in the inoculum from approximately 10 cells up to 1×10^7 cells per ml resulted in only a twofold increase in M. I. C. values at the highest inoculum level used. In the presence of 50% human serum, the M. I. C. of janiemycin was increased eightfold, from 0.09 to 0.78 μg/ml.

In Vivo:

Janiemycin, when given subcutaneously in divided doses at 1 and 5 hours after infection, protected mice against death due to experimental infections with *Streptococcus pyogenes* C203 and *Diplococcus pneumoniae* Type 3 ATCC 6303 (Table 4). Oral administration was not effective when doses of 400 mg/kg were given. It is noteworthy that a single, subcutaneous dose of janiemycin, given either 24 or 48 hours prior to infection with *Streptococcus pyogenes* C203, was capable of protecting the mice. The PD_{50} values were 2.59 and 4.88 mg/kg, respectively.

Table 4. Activity of janiemycin *in vivo**

Infection**	Antibiotic	PD_{50} (mg/kg)
Streptococcus pyogenes C203	Janiemycin	0.62
	Penicillin G	0.61
Diplococcus pneumoniae Type 3 ATCC 6303	Janiemycin	0.5
	Penicillin G	1.5

* Both janiemycin and penicillin G were given subcutaneously to mice.
** See ref. 6 for a description of the model infections.

Thus, janiemycin is active *in vivo* against gram-positive bacteria at levels equal to or slightly superior to those required with penicillin G. In addition, janiemycin exhibits a prolonged protective activity, with a duration of at least 48 hours.

Acknowledgements

We thank Messrs. J. Alicino, F. Russo-Alesi and O. Kocy for the elemental, Stein-Moore and electrophoretic analyses, respectively, H. I. Basch for the *in vitro* spectrum study, W. P. Jambor and Mrs. J. Wilber-Murphy for technical assistance with the *in vivo* studies, W. H. Trejo for indentification of the culture, and H. Moes and F. F. Moser for their contributions in the pilot plant.

References

1) Asai, M.; M. Muroi, N. Sugita, H. Kawashima, K. Mizuno & A. Miyake : Enduracidin, a new antibiotic. II. Isolation and characterization. J. Antibiotics 21 : 138～146, 1968.
2) Meyers, E. & R. C. Erickson : Bioautography of antibiotics on thin-layer chromatograms. J. Chromatogr. 26 : 531～532, 1967.
3) Bognar, R.; S. Makleit, F. Sztaricskai, N. N. Lomakina & M. S. Yurina : Synthesis of amino acids from the hydrolysis of actinoidin. Antibiotiki 9 : 875～880, 1964
4) Horii, S. & Y. Kameda : Enduracidin, a new antibiotic. V. Structures of new basic amino acids, enduracididine and alloenduracididine. J. Antibiotics 21 : 665～667, 1968
5) Kamiya, K.; M. Nishikawa, H. Matsumaru, M. Asai & K. Mizuno : X-ray analysis of an unusual amino acid isolated from the hydrolysate of a new antibiotic, enduracidin. Chem. Pharm. Bull. 16 : 2303～2304, 1968
6) Miraglia, G. J. & H. I. Basch : Activity of selected penicillins *in vitro* and in experimental bacterial infections in mice. Appl. Microbiol. 15 : 566～568, 1967