CHICAMYCIN*, A NEW ANTITUMOR ANTIBIOTIC

I. PRODUCTION, ISOLATION AND PROPERTIES

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Chicamycin is a new antitumor antibiotic produced by a strain of *Streptomyces albus*, No. J576-99. The antibiotic is extractable into organic solvents from the fermentation broth and is obtained in two active forms, chicamycins A and B, depending upon the isolation procedure used. Chicamycin A is not a natural antibiotic but the methanol adduct of naturally produced chicamycin B. Both forms of the antibiotic have weak antibacterial activity against some Gram-positive and acid-fast bacteria. They inhibit the growth of experimental tumors such as P388 mouse leukemia.

During the course of an antitumor screening program for microbial fermentation products, a *Streptomyces* strain (No. J576-99) was found to produce a novel antibiotic active against P388 mouse leukemia. The antitumor activity was extracted by organic solvents from the fermentation broth and purified by a series of chromatographies to afford a crystalline solid (chicamycin A). Subsequently, chicamycin A was found to be a methanol adduct form of the naturally occurring chicamycin B. The latter compound was isolated from the fermentation broth when the extraction and purification were carried out without using methanol. As described in a companion paper[1], chicamycin is a new member of the pyrrolobenzodiazepine group of antibiotics closely related to the neothramycins. This paper reports on the production, isolation, chemical characterization and biological activities of chicamycin.

Producing Organism

An actinomycete strain, No. J576-99, was isolated from a soil sample collected in Puerto Chicama, Peru. Strain J576-99 produces long, branched aerial mycelium (0.5 μm in width) which is not fragmented. Spore-chains are formed monopodially or at the hyphal tip of the aerial mycelium. Short, straight or hooked spore-chains containing 3 to 10 spores in a chain are produced on organic agar media such as Bennett's agar and oat meal agar. Long, irregularly coiled, open-spiralled or flexuous spore-chains containing 10 to 40 spores in a chain are formed on chemically defined media such as Czapek sucrose - nitrate agar. Tight coils or loops at the tip of spore-chain are often observed as a compact globose body. After maturation, a bead-like intermittent spore arrangement is commonly observed. The spores are spherical, oval or elliptical in shape ($0.6 \sim 1.0 \times 0.6 \sim 1.5$ μm) and have a smooth surface. Sporangium, motile spore and sclerotium are not produced.

Strain J576-99 grows well on ISP (International Streptomyces Project) media and other commonly used media. Aerial mycelia are formed abundantly on Czapek sucrose - nitrate agar, inorganic salts - starch agar and Bennett's agar, but poorly on yeast extract - malt extract agar and oat meal agar. The

* This antibiotic was originally designated as BBM-2040.

Table 1. Cultural characteristics* of strain No. J576-99.

Tryptone - yeast extract broth (ISP No. 1)	G**	Moderate growth and formation of floccose, pale yellow pellets
Sucrose - nitrate agar (Czapek agar)	G	Abundant
	R	Yellowish white (92)*** to moderate yellowish brown (77)
	A	Abundant, white (263) to yellowish white (92)
	D	None
Glucose - asparagine agar	G	Poor
	R	Yellowish white (92)
	A	None
	D	None
Glycerol - asparagine agar (ISP No. 5)	G	Moderate
	R	Yellowish white (92) to grayish yellow (90)
	A	Moderate, white (263) to yellowish white (92)
	D	None
Inorganic salts - starch agar (ISP No. 4)	G	Abundant
	R	Pale yellow (89) to moderate olive brown (95)
	A	Abundant, white (263) to yellowish white (92)
	D	None
Tyrosine agar (ISP No. 7)	G	Abundant
	R	Yellow white (92) to moderate yellowish brown (77)
	A	Moderate, white (263) to yellowish white (92)
	D	None
Nutrient agar	G	Poor to moderate
	R	Yellowish white (92) to pale yellow (89)
	A	Poor, white (263) to yellowish white (92)
	D	None
Yeast extract - malt extract agar (ISP No. 2)	G	Abundant
	R	Pale yellow (89) to dark orange yellow (72)
	A	Poor to moderate, white (263)
	D	Moderate yellowish brown (77)
Oat meal agar (ISP No. 3)	G	Poor to moderate
	R	Yellowish white (92)
	A	Poor, white (263)
	D	None
Bennett's agar	G	Abundant
	R	Dark orange yellow (72) to moderate yellowish brown (77)
	A	Abundant, white (263) to yellowish white (92)
	D	None
Peptone - yeast extract - iron agar (ISP No. 6)	G	Poor to moderate
	R	Grayish yellow (90)
	A	Poor, white (263) to yellowish white (92)
	D	Light olive brown (94)

* Observed after incubation at 28°C for 3 weeks.

** Abbreviation: G=growth; R=reverse color; A=aerial mycelium; D=diffusible pigment.

*** Color and number in parenthesis follow the color standard in KELLY, K. L. & D. B. JUDD: ISCC-NBS color-name charts illustrated with Centroid Colors. US Dept. of Comm. Cir. 553, Washington, D.C., Nov. 1975.

mass color of aerial mycelium is white to yellowish white. Substrate mycelia are yellowish to light brown. Melanoid and other diffusible pigment are not produced. Temperature for moderate growth ranges from 20°C to 47°C. No growth is seen at 50°C. It is highly tolerant to sodium chloride and

Table 2. Physiological characteristics of strain No. J576-99.

Test	Response	Method and medium
Range of temperature for growth	Maximal growth at 28~43°C Moderate growth at 20°C and 47°C No growth at 10°C and 50°C	Bennett's agar
Gelatin liquefaction	Liquefied	Glucose - peptone - gelatin medium
Starch hydrolysis	Hydrolyzed	Starch agar plate
Reactions in skimmed milk	Not coagulated and not peptonized	Difco skimmed milk
Formation of melanoid pigment	Not produced	Tyrosine agar, peptone - yeast - iron agar and Tryptone - yeast extract broth
Nitrate reduction	Negative	Czapek glucose - nitrate broth and glucose - yeast extract broth
pH tolerance	Growth at pH 5.0~10, no growth at 4.5	Yeast extract - malt extract agar
NaCl tolerance	Highly tolerant, growth at 15% or less	1% Yeast extract, 2% soluble starch, 1.5% agar
Lysozyme tolerance	Highly tolerant, growth at 0.1, 0.01, 0.001 and 0.0001%	Trypticase soy broth plus 1.5% agar

grows at NaCl concentrations of 15% or less. The cultural and physiological characteristics of strain J576-99 are shown in Tables 1 and 2, respectively. The pattern of carbohydrate utilization by the strain is shown in Table 3.

Purified cell-wall of strain J576-99 contains LL-diaminopimelic acid and glycine, and the whole cell hydrolysate contains ribose and mannose but lacks other diagnostic sugars. These chemical compositions of strain J576-99 indicate that it belongs to the actinomycete of cell-wall Type I.

Table 3. Utilization of carbon sources by strain J576-99.

Glycerol	+	Cellobiose	+
D(−)-Arabinose	+	Melibiose	−
L(+)-Arabinose	+	Trehalose	+
D-Xylose	+	Raffinose	+
D-Ribose	+	D(+)-Melezitose	−
L-Rhamnose	+	Soluble starch	+
D-Glucose	+	Cellulose	−
D-Galactose	+	Dulcitol	−
D-Fructose	+	Inositol	+
D-Mannose	+	D-Mannitol	+
L(−)-Sorbose	−	D-Sorbitol	+
Sucrose	+	Salicin	+
Lactose	+		

Basal medium: Pridham - Gottlieb inorganic medium. Observed after incubation at 28°C for 3 weeks.

Although the spore and spore-chain morphology of strain J576-99 resembles that of non-streptomycetes genera such as *Actinomadura*, the cultural and physiological characteristics of strain J576-99 and its Type I cell-wall composition indicate that strain J576-99 might be classified as belonging to the genus *Streptomyces*. According to the descriptions of BERGEY's Manual (8th ed., 1974), strain J576-99 should be placed in the species group, *spirales*, white series, non-chromogenic and smooth spore surface, which includes 17 species. Based on the ISP species descriptions[2~5], strain J576-99 resembles *S. albus* ATCC 3004, *S. almquisti* ATCC 618, *S. cacaoi* IMRU 3082 and *S. rangoon* ATCC 6860 in its predominant formation of short spore-chains, but differs in the carbon source utilization pattern. The carbohydrate utilization pattern of strain J576-99 is similar to that of *S. herbescens* INMI 1252 and *S. ochraceiscleroticus* ATCC 15814, but differences are seen in that *S. herbescens* has green-colored substrate mycelium and *S. ochraceiscleroticus* forms white, yellow, red or gray aerial mycelium and *Chainia* type

sclerotium. Strain J576-99 most resembles a recently reported strain of *S. albus* NRRL 11109, which produces polyether antibiotic A32887[6], in morphology and cultural characteristics. In addition, the carbon source utilization profiles of both strains are similar. Strain J576-99 was therefore determined to be a strain of *S. albus*, and the culture has been deposited in the American Type Culture Collection with the accession number ATCC 39143.

Antibiotic Production

A well grown agar slant of *Streptomyces* sp. strain No. J576-99 was used to inoculate seed medium containing 3.0% soybean meal, 2.0% corn starch, 1.0% $CaCO_3$ and 0.33% $MgSO_4 \cdot 7H_2O$, the pH being adjusted to 7.0 before sterilization. The seed culture was incubated at 28°C for 3 days on a rotary shaker (250 rpm) and 4 ml of the growth was transferred into a 500-ml Erlenmeyer flask which contained 100 ml of fermentation medium having the same composition as the seed medium. Fermentation was carried out on a rotary shaker at 28°C and the antibiotic activity in the fermentation broth was determined by paper disc-agar diffusion assay using *Bacillus subtilis* M45 (a Rec$^-$ mutant)[7] as a test organism. The pH of the broth gradually rose with the progress of fermentation and reached 8.0~8.2 after 120 hours when a peak antibiotic potency of 70~100 μg/ml was obtained.

Isolation and Purification

Chicamycin has been obtained in two different forms, A and B, according to the procedures used for the extraction and purification of the antibiotic. Both forms of chicamycins are bioactive. Structural studies[1] revealed that chicamycin A is a methanol adduct form of chicamycin B.

Isolation of Chicamycin A

The harvested broth (20 liters) was separated into mycelial cake and broth supernate by using a continuous centrifuge (Kokusan H-600). The mycelial cake was stirred with 3 liters of methanol for 30 minutes and the methanolic extract was concentrated to an aqueous solution (400 ml). The broth supernate was applied to a column of Diaion HP-20 (2 liters) and, after being washed with water (3 liters), the column was developed with a mixture of 1-butanol - methanol - water (2: 1: 1) to elute the antibiotic activity. The active eluate was evaporated *in vacuo* to an aqueous concentrate (400 ml) and combined with the aqueous concentrate derived from the mycelial extract. The mixture was washed with two 800-ml portions of ethyl acetate and extracted by two 800-ml portions of 1-butanol. The butanol extracts were combined and concentrated *in vacuo* to afford a crude preparation of chicamycin as a brownish solid (6.6 g). This solid was dissolved in a small amount of methanol and charged on a column of silica gel (Wakogel C-200, 100 g) which was developed with a mixture of ethyl acetate - methanol (9: 1). The elution was monitored by bioassay (*B. subtilis* M45) and by UV absorption at 254 nm. The chromatographic process was carried out in a cold room at 5°C. The active eluates were combined, concentrated *in vacuo* and lyophilized. Amorphous white solid thus obtained was crystallized from methanol to give a pure preparation of chicamycin A as colorless needles (1.10 g).

Isolation of Chicamycin B

The fermentation broth (50 liters) was centrifuged by a continuous centrifuge apparatus. The mycelial cake collected was homogenized with 80% aqueous acetone (14 liters) for 30 minutes and insoluble materials were removed by filtration. The filtrate was concentrated *in vacuo* to an aqueous solution which was combined with the broth supernate and passed through a column of Diaion HP-20 (2.5 liters). The column was washed with water (8 liters) and the activity eluted with 80% aqueous acetone. The

combined active eluates (8 liters) were concentrated *in vacuo* to an aqueous solution (2 liters), which was washed with two 2-liter portions of ethyl acetate and then extracted with two 2-liter portions of 1-butanol. The 1-butanol extracts were combined and evaporated *in vacuo* to give crude solid of chicamycin B (23.9 g). Since crude chicamycin B showed limited stability in solutions, the chromatographic process described below was operated in a cold room (5°C). The crude solid of chicamycin B (23 g) was dissolved in a small volume of aqueous acetonitrile and applied to a column of silica gel (200 g). The column was developed with 95% aqueous acetonitrile and the elution monitored by bioassay (*B. subtilis* M45) and TLC (SiO_2: EtOAc - MeOH, 4: 1). Appropriate fractions were collected and concentrated *in vacuo* below 35°C and lyophilized to afford a pure sample of chicamycin B as amorphous white powder (3.30 g).

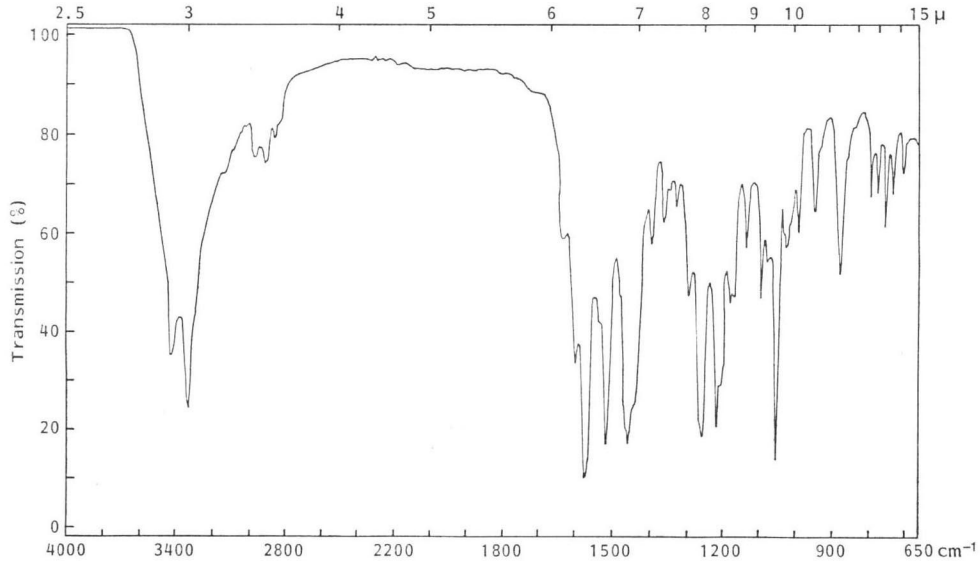
Physico-chemical Properties

Chicamycins A and B are readily soluble in methanol, ethanol, 1-butanol and pyridine, slightly soluble in ethyl acetate, acetone and water but practically insoluble in benzene, chloroform and *n*-hexane.

Table 4. Physico-chemical properties of chicamycins A and B.

	Chicamycin A		Chicamycin B	
Nature	Colorless needles		White amorphous powder	
Mp (dec)	161~163°C		134~136°C	
$[\alpha]_D^{26}$ (*c* 0.5, pyridine)	+350°		+552°	
Molecular formula	$C_{14}H_{18}N_2O_5$		$C_{18}H_{14}N_2O_4$	
Anal	Calcd ($C_{14}H_{18}N_2O_5$)	Found	Calcd ($C_{18}H_{14}N_2O_4 \cdot H_2O$)	Found
C (%)	57.13	56.85	55.70	54.91
H	6.16	6.16	5.76	5.56
N	9.52	9.33	10.00	9.33
MS *m/z*	294 (M^+), 262, 242, 219, 178, 150, 122, 86, *etc.*		262 (M^+), 242, 150, 122, 86, *etc.*	
UV $\lambda_{max}^{CH_3CN}$ nm (ε)	232 (24,900), 260 (sh. 7,700), 320 (3,900)		232 (20,200), 260 (sh. 6,900), 318 (3,000)	

Fig. 1. IR spectrum of chicamycin A.



They gave positive reactions with ferric chloride, RYDON-SMITH and ninhydrin (weak brownish pink) reagents, but were negative to SAKAGUCHI, EHRLICH and anthrone reactions. Molecular formulae of $C_{14}H_{18}N_2O_5$ and $C_{13}H_{14}N_2O_4$ were assigned to chicamycins A and B, respectively, based on the ^{13}C NMR and mass spectral data and microanalysis. Physico-chemical properties of chicamycins A and B are summarized in Table 4. The IR spectra of chicamycins A and B (in KBr pellet) are shown in Figs. 1 and 2.

Fig. 2. IR spectrum of chicamycin B.

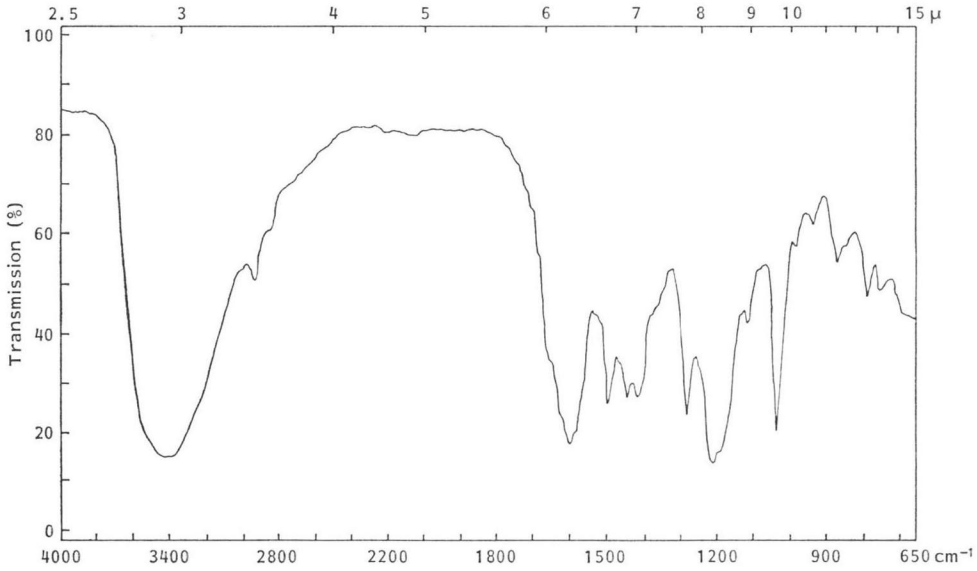
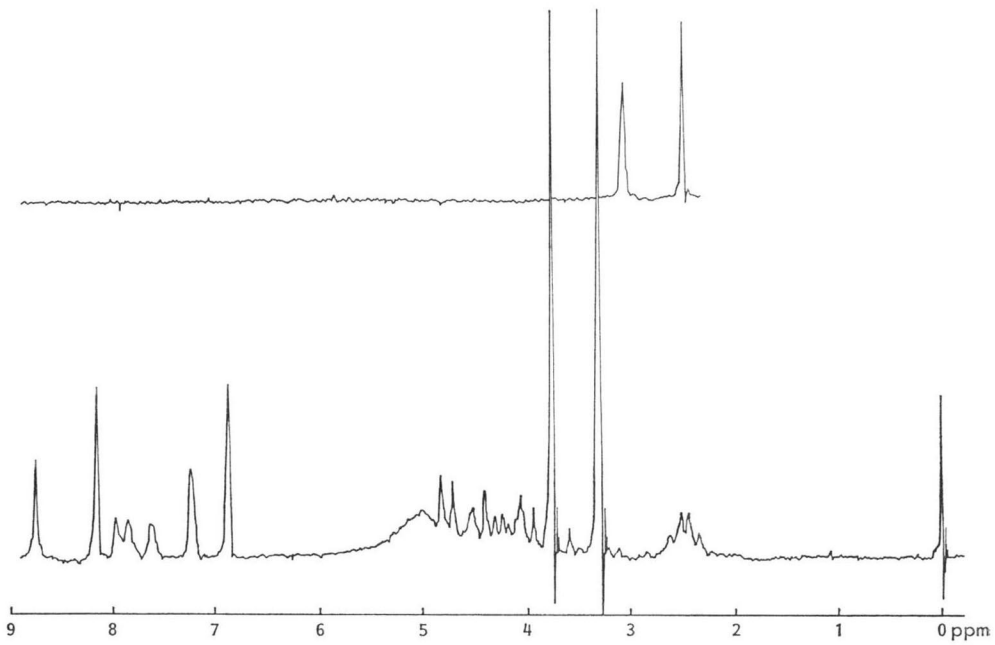


Fig. 3. ^1H NMR spectrum of chicamycin A (60 MHz, pyridine-d_5).



The ^{1}H NMR spectrum of chicamycin A (Fig. 3, 60 MHz, pyridine-d_5) involves two OCH_3 groups (δ 3.30 and 3.75 ppm), one high-field methylene group (2.1 ppm), five protons at around 3.9~4.8 ppm and two aromatic protons (6.82 and 8.10 ppm), along with one NH (7.84 ppm) and two OH (6.2 and 11.50 ppm) signals. The ^{1}H NMR spectrum of chicamycin B lacked the signals of higher-field OCH_3 and NH protons observed with chicamycin A, while a double bond proton (8.24 ppm) was present in the spectrum of chicamycin B. The physico-chemical properties of chicamycins A and B described above are similar to those of the neothramycins[8] and tomaymycin[9], belonging to the 1,4-benzodiazepine group of antibiotics. However, these antibiotics were readily distinguished from chicamycin by their TLC behavior (Table 5) and ^{1}H NMR spectra. Chicamycins A and B could not be differentiated by the three TLC systems examined. Chicamycins A and B were relatively stable in solution. At 25°C in 95% aqueous ethanol (5 mg/ml), more than 95% of the activity of chicamycins A and B remained after two weeks. Under the same condition, the activity of neothramycin* decreased to 25~30% of the original activity.

Antimicrobial Activity

The minimum inhibitory concentration (MIC) of chicamycin was determined for a variety of Gram-positive, Gram-negative and acid-fast bacteria by the serial two-fold agar dilution method. Nutrient agar medium was used for Gram-positive and Gram-negative organisms and No. 1001 medium (3% glycerol, 0.3% sodium L-glutamate, 0.2% peptone, 0.31% $Na_2HPO_4 \cdot 12H_2O$, 0.1% KH_2PO_4, 0.005% ammonium citrate, 0.001% $MgSO_4 \cdot 7H_2O$ and 1.5% agar) for acid-fast organisms. As shown in Table 6, chicamycins A and B showed weak antibacterial activity against *Streptococcus pyogenes, Micrococcus luteus, Micrococcus flavus* and *Mycobacterium* strains. The antibacterial spectrum of chicamycin is

Table 5. TLC of chicamycins A and B and related antibiotics.

Solvent system	Chicamycin A	Chicamycin B	Neothramycins	Tomaymycin
EtOAc - MeOH, 4:1	0.29	0.29	0.48, 0.40	0.51
$CHCl_3$ - MeOH, 5:1	0.24	0.24	0.42, 0.32	0.52
EtOAc - CH_3CN, 1:1	0.02	0.02	0.14, 0.08	0.18

Table 6. Antibacterial activity of chicamycins A and B.

Test organisms	MIC (μg/ml)		
	Chicamycin A	Chicamycin B	Neothramycin
Staphylococcus aureus FDA 209P	>100	>100	>100
〃 Smith	>100	>100	>100
Streptococcus pyogenes A20201	50	50	50
Micrococcus luteus PCI 1001	50	100	100
M. flavus D12	50	50	100
Bacillus subtilis PCI 219	>100	>100	50
Mycobacterium smegmatis 607	100	100	>100
M. phlei D88	100	100	>100
Escherichia coli NIHJ	>100	>100	50
〃 Juhl	>100	>100	>100
Klebsiella pneumoniae D-11	>100	>100	100
Proteus vulgaris A9436	>100	>100	100
Pseudomonas aeruginosa A9930	>100	>100	>100

* Neothramycin used in this paper is a mixture of neothramycins A and B.

Table 7. Antitumor activity of chicamycins A and B.

Tumor	Antibiotic	T/C(%) of MST*					
		Dose in mg/kg/day, ip					
		10	3	1	0.3	0.1	0.03
P388 leukemia	Chicamycin A	**152****	**152**	**128**	104	96	
	〃 B	**128**	**128**	112	96	104	
	Neothramycin		**152**	**136**	112	104	94
Sarcoma 180	Chicamycin A	**>444**	**>415**	**178**	104	111	
L1210 leukemia	Chicamycin A	110	90	90	90		
B16 melanoma	Chicamycin A	113	94	100	103	100	
Lewis lung carcinoma	Chicamycin A	100	105	90	110	115	

* Ratio of median survival time of test and control animals.
** Boldface type indicates significant antitumor effect.

similar to that of neothramycin. Chicamycin does not induce prophage in lysogenic bacteria up to a concentra-tion of 100 μg/ml.

Antitumor Activity and Toxicity

The antitumor activity of chicamycins A and B was determined in mice. Lymphocytic leukemia P388, lymphoid leukemia L1210, melanotic melanoma B16 and Lewis lung carcinoma were inoculated intraperitoneally into mice (male BDF_1 strain) with 10^6, 10^5, 5×10^5 and 10^6 cells per mouse, respectively. Sarcoma 180 ascites tumor was implanted intraperitoneally into mice (male *dd*Y strain) at an inoculum size of 5×10^6 cells per mouse. Graded doses of test compounds were administered to mice intraperitoneally 24 hours after tumor implantation. The treatments were given once daily for 9 days (qd 1→9) except for the mice bearing Lewis lung carcinoma which were treated for 11 days (qd 1→11). Neothramycin was comparatively tested as a reference compound in the leukemia P388 test.

The results are shown in Table 7. Chicamycin A and neothramycin were similarly active against P388 leukemia, while chicamycin B was somewhat less active than chicamycin A. Chicamycin A also showed high therapeutic activity against sarcoma 180. Chicamycin A was inactive against L1210 leukemia, B16 melanoma and Lewis lung carcinoma at the highest dose tested (10 mg/kg/day).

The acute toxicity of chicamycins A and B was determined in mice (male *dd*Y strain) by single intraperitoneal administration, the LD_{50} being 28 mg/kg and 57 mg/kg, respectively. The intraperitoneal LD_{50} of neothramycin has been reported to be 20~30 mg/kg[3].

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References

1) KONISHI, M.; H. OHKUMA, N. NARUSE & H. KAWAGUCHI: Chicamycin, a new antitumor antibiotic. II. Structure determination of chicamycins A and B. J. Antibiotics 37: 200~206, 1984
2) SHIRLING, E. B. & D. GOTTLIEB: Cooperative description of the type cultures of *Streptomyces*. II. Species descriptions from first study. Int. J. Syst. Bacteriol. 18: 69~189, 1968
3) SHIRLING, E. B. & D. GOTTLIEB: Cooperative description of the type cultures of *Streptomyces*. III. Additional species descriptions from first and second studies. Int. J. Syst. Bacteriol. 18: 279~392, 1968
4) SHIRLING, E. B. & D. GOTTLIEB: Cooperative description of the type cultures of *Streptomyces*. IV. Species descriptions from the second, third and fourth studies. Int. J. Syst. Bacteriol. 19: 391~512, 1969

5) SHIRLING, E. B. & D. GOTTLIEB: Cooperative description of the type cultures of *Streptomyces*. V. Additional descriptions. Int. J. Syst. Bacteriol. 22: 265~394, 1972
6) HAMILL, R. L. & M. M. HOEHN (Eli Lilly): Antibiotic A32887 and process for production thereof. U.S. Patent 4,133,876, Jan. 9, 1979
7) KADA, T.; K. TSUTIKAWA & Y. SADAIE: *In vitro* and hostmediated "*rec*-assay" producers for screening chemical mutagens; and phloxine, a mutagenic red dye detected. Mutation Res. 16: 165~174, 1972
8) TAKEUCHI, T.; M. MIYAMOTO, M. ISHIZUKA, H. NAGANAWA, S. KONDO, M. HAMADA & H. UMEZAWA: Neothramycins A and B, new antitumor antibiotics. J. Antibiotics 29: 93~96, 1976
9) ARIMA, K.; M. KOHSAKA, G. TAMURA, H. IMANAKA & H. SAKAI: Studies on tomaymycin, a new antibiotic. I. Isolation and properties of tomaymycin. J. Antibiotics 25: 437~444, 1972