A NEW ANTITUMOR ANTIBIOTIC, CHROMOXYMYCIN

II. PRODUCTION, ISOLATION, CHARACTERIZATION AND ANTITUMOR ACTIVITY

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Chromoxymycin is a new antitumor antibiotic produced by a new actinomycete named *Streptomyces libani* subsp. *rubropurpureus* No. 6362.

Chromoxymycin is active against P388 leukemia and B16 melanoma in mice, and has weak antibacterial activity against some Gram-positive bacteria.

In our screening program for antitumor compounds, *Streptomyces libani* subsp. *rubropurpureus* No. 6362 was found to produce a novel antitumor antibiotic, which was extracted from the fermentation broth and purified by a series of chromatographies to afford a crystalline solid named chromoxymycin.

The present paper describes the production, isolation, physico-chemical properties and biological activities of chromoxymycin. The taxonomy of the producing strain *Streptomyces libani* subsp. *rubropurpureus* No. 6362 and the structural studies on chromoxymycin are reported in separate papers[1,2].

Fermentation

A loopful of mature slant culture of *Streptomyces libani* subsp. *rubropurpureus* No. 6362 was inoculated into a seed medium (160 ml) containing corn starch 1%, glycerol 1%, glucose 0.5%, cotton seed flour 1%, dried yeast 0.5%, corn steep liquor 0.5% and $CaCO_3$ 0.2% (pH 6.5) in a 500-ml Erlenmeyer flask and cultured at 30°C on a rotary shaker with 7.6 cm-throw at 220 rpm for 72 hours.

Fermentation studies were carried out in tank fermentors. A seed culture was shaken in the above mentioned Erlenmeyer flasks and then transferred at the rate of 2% to 80 liters of the same seed medium in a 200-liter jar fermentor, which was stirred at 265 rpm at 30°C for 48 hours. A 35-liter portion of the seed jar fermentor was inoculated into 1,700 liters of production medium containing sucrose 2%, peanut powder 1.5%, molatein 0.5%, NaI 0.00005% and $CoCl_2 \cdot 12H_2O$ 0.0004% in a 2,000-liter stainless steel fermentor which was operated at 30°C for 96 hours under aeration of 1,700 liters/minute and agitation of 180 rpm.

The antitumor antibiotic level in the fermentation broth was assayed by both high pressure liquid chromatography and cytotoxic activity against P388 murine leukemia cells in tissue culture.

Isolation and Purification

The fermentation broth (1,700 liters) was filtered with the aid of diatomaceous earth (5 kg). The

filtrate was adjusted to pH 7.0 with 6 N NaOH and passed through a column of Diaion HP-20 (75 liters). The HP-20 column was washed with water (150 liters) and then with 25% aqueous acetone (150 liters), and eluted with 50% aqueous acetone (150 liters). The active fraction was concentrated *in vacuo* to a volume of 20 liters and adjusted to pH 10 with 28% NH_4OH. The concentrate was added to 40 liters of butanol and stirred for 10 minutes. This extraction procedure was carried out twice and the extracts were combined. The extracts were concentrated *in vacuo* to a volume of 500 ml. The oily materials obtained were mixed with silica gel (2 liters) and applied to a chromatography column using silica gel (5 liters). The column was washed with 12 liters of methanol and then eluted with 18 liters of methanol. The eluates were concentrated *in vacuo* to dryness. The crude sample was dissolved in 100 ml of 60% aqueous methanol containing 10 mM ammonium acetate, subjected to chromatography on a NS gel column (750 ml) and developed with 5 liters of 60% aqueous methanol containing 10 mM ammonium acetate. The active fractions eluted were concentrated *in vacuo* to a volume of 1 liter and passed through a column of HP-20 (300 ml). After washing with water, the column was eluted with 50% aqueous acetone (1 liter). The active fraction was evaporated to dryness under reduced pressure to yield purified active materials as free base (5.7 g). Yellow crystals were obtained from hot methanol (4 g).

Physico-chemical Properties

Chromoxymycin was readily soluble in water, slightly soluble in methanol and acetone, and insoluble in chloroform, hexane and diethyl ether. Chromoxymycin gave positive reactions to cerium sulfate, iodine and potassium permanganate reagents, though negative to Ehrlich, Dragendorff, Molisch and ninhydrin reactions.

Fig. 1. ^1H NMR spectrum of chromoxymycin in pyridine-d_5 (400 MHz).

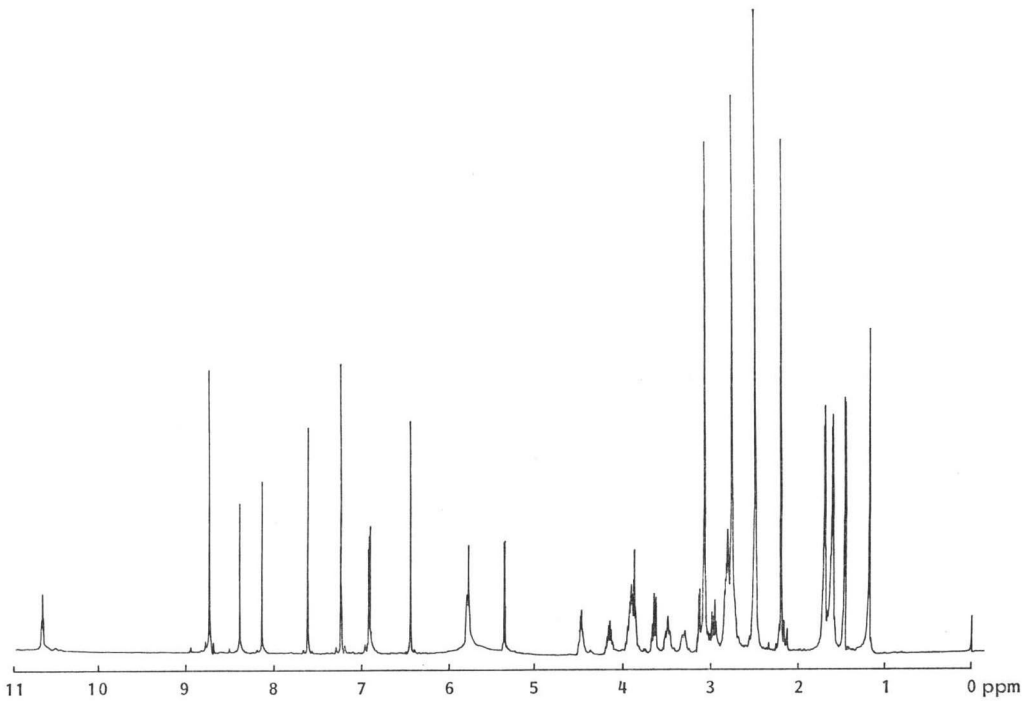

Fig. 2. ^{13}C NMR spectrum of chromoxymycin in pyridine-d_5 (67.8 MHz).

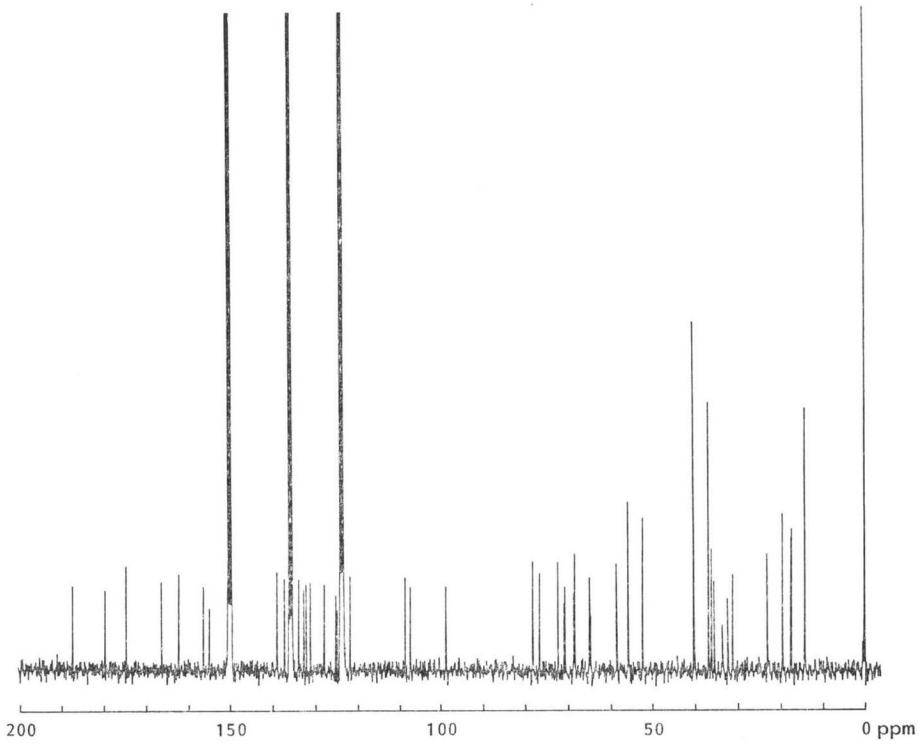

Table 1. Physico-chemical properties of chromoxymycin.

Melting point	85°C (dec)
$[\alpha]_D^{23}$ (c 0.675, H_2O)	+291°
UV $\lambda_{max}^{H_2O}$ nm (ε)	276 (36,200), 340 (7,400) sh*
$\lambda_{max}^{H_2O+HCl}$ nm (ε)	272 (37,100), 330 (7,500) sh
$\lambda_{max}^{H_2O+NaOH}$ nm (ε)	243 (25,500), 278 (32,500), 410 (2,900)
IR ν_{max}^{KBr} cm^{-1}	3400, 2950, 1650, 1560, 1465, 1435, 1395 (sh), 1375, 1280, 1150, 1075, 1040 (sh), 960, 895, 855, 840
Elemental analysis (%)	
Found:	C 58.56, H 6.85, N 5.54, H_2O 6.71
Calcd for $C_{49}H_{60}N_4O_{14}\cdot 4H_2O$:	C 58.79, H 6.85, N 5.60, H_2O 7.20
SI-MS (m/z)	929 $(M+H)^+$
TLC** Rf[a]	0.5
Rf[b]	0.3

* Some absorption persists up to 500 nm.

** Stationary phase, silica gel sheet (Merck); developing solvent, [a] 2-propanol - H_2O - 28% NH_4OH (70: 30: 1), [b] BuOH - AcOH - H_2O (4: 1: 2).

The ^1H and ^{13}C NMR spectra of chromoxymycin are shown in Figs. 1 and 2, respectively. The other physico-chemical properties are summarized in Table 1. From the data of the ^{13}C NMR and SI-MS spectra, together with the elemental analysis, the molecular formula of chromoxymycin was determined to be $C_{49}H_{60}N_4O_{14}$. Two singlet signals at 187.0 and 179.5 ppm in the ^{13}C NMR spectrum and a strong IR absorption at 1650 cm^{-1} indicated the presence of conjugated ketones in the chromoxymycin molecule. It was apparent that two dimethylamino groups are present in the molecule

from the ^{13}C NMR [37.2 (two carbons, q) and 40.6 (two carbons, q) ppm] and ^{1}H NMR [δ 2.51 (6H, s) and 2.76 (6H, s)] spectral data. This spectroscopic evidence suggests structural features similar to those of hedamycin[3]. However, the structure of chromoxymycin seems to be significantly different from that of hedamycin in view of UV spectral data and solubilities. Further structural studies on chromoxymycin are currently underway and will be published soon[2].

Biological Activity

Antitumor Activity

The antitumor activity of chromoxymycin was determined in mice. Lymphocytic leukemia P388 and melanotic melanoma B16 were implanted intraperitoneally into BDF_1 mice (female, 8 weeks old) at an inoculum size of 1×10^6 cells per mouse. Twenty-four hours after the implantation of the tumor cells, graded doses of chromoxymycin were administered to mice intraperitoneally. Treatments were given on day 1, 2, 3 and 4 (qd 1~4). Chromoxymycin was solubilized in physiological saline solution (0.9% saline). Control animals received intraperitoneal doses of physiological saline solution. The injection volume was 0.2 ml in all experiments. Five mice were used for each experimental group. Doxorubicin hydrochloride (Adriacin-Kyowa) was comparatively tested simultaneously as a reference compound.

Antitumor activity was evaluated by the mean survival time of a group of mice and also expressed by the T/C % value (mean survival time of treated group/mean survival time of control group, $\times100$).

The results are shown in Tables 2 and 3. Chromoxymycin was quite active against leukemia P388 and melanoma B16. Doses between 12.5~400 mg/kg against P388 and 6.0~200 mg/kg against B16 resulted in significant increase in the life span of mice, respectively. Doxorubicin was also active against P388 at doses between 0.04~2.5 mg/kg and against B16 at doses between 0.03~2.0 mg/kg on the same schedule, respectively.

Antimicrobial Activity

The antimicrobial activity of chromoxymycin was determined by a serial broth dilution method in bouillon medium for bacteria and in Sabouraud medium for fungi and yeasts. The minimum inhibitory concentration (MIC) was expressed in terms of μg/ml after overnight incubation at 37°C for bacteria

Table 2. Antitumor activity of chromoxymycin against P388 leukemia.

Drug	Dose (mg/kg/day)	Mean survival time (days)	T/C (%)
Chromoxymycin	400	20.5	192
	200	20.7	193
	100	16.1	150
	50	16.1	158
	25	15.1	141
	12.5	14.1	131
Doxorubicin	2.5	26.9	251
	0.6	22.3	208
	0.15	18.3	171
	0.04	13.7	128
Control		10.7	100

Table 3. Antitumor activity of chromoxymycin against B16 melanoma.

Drug	Dose (mg/kg/day)	Mean survival time (days)	T/C (%)
Chromoxymycin	200	22.4	149
	100	25.2	168
	50	27.6	184
	25	25.2	168
	12.5	25.4	169
	6.0	19.8	132
Doxorubicin	2.0	35.0	233
	0.5	35.0	233
	0.125	34.8	232
	0.03	22.8	152
Control		15.0	100

and 48~72 hours incubation at 28°C for fungi and yeasts. Chromoxymycin had weak antimicrobial activity against *Staphylococcus aureus* 209P (MIC 100 μg/ml) and *Bacillus subtilis* ATCC 6633 (MIC 25 μg/ml).

Acute Toxicity

The acute toxicity of chromoxymycin was determined in *dd*Y mice (5 weeks old, female) by a single intravenous injection of graded doses of test compound into 5 mice. The LD_{50} was 1,000 mg/kg.

Discussion

From the ^{1}H and ^{13}C NMR spectral data, chromoxymycin was classified as being of the hedamycin type[3]. The UV spectra showed, however, that the main chromophore of chromoxymycin is different from that of the hedamycin group of antibiotics such as hedamycin[3], pluramycin[4], and kidamycin[5,6]. The structural elucidation of chromoxymycin is now in progress and will be published soon[2]. Another characteristic of chromoxymycin in its physico-chemical properties is that the antibiotic is readily soluble in water. This property is also distinct from those of the hedamycin group of compounds and may be of greater advantage for developing the antibiotic as a clinical agent.

Chromoxymycin was approximately 80 times less toxic than pluramycin A in the LD_{50} determination in mice by single administration[6,7].

In addition, chromoxymycin was quite active in murine tumor systems including P388 and B16 over a wide range of extremely high doses. *In vivo* activity was reproducibly demonstrated at doses much more than 100 mg/kg. Further *in vivo* evaluation of the antitumor activities of this compound is now progress in the Division of Cancer Treatment, National Cancer Institute (USA).

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