KUNDRYMYCIN, A NEW TUMOR-INHIBITORY ANTIBIOTIC. II

ISOLATION, CHEMICAL CHARACTERIZATION AND BIOLOGICAL ACTIVITY

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Kundrymycin, a new antibiotic, was isolated from fermentations of *Streptomyces metachromogenes*, sp. n., ATCC 21,440, by methylene chloride extraction. It is an acid-base indicator with a molecular weight of 878.9. Biologically it is inhibitory to gram-positive bacteria, moderately cytotoxic to HeLa cells and inhibitory *in vivo* to WALKER 256 tumors in rats.

Kundrymycin was first detected in crude streptomyces culture filtrates tested for tumor-inhibitory effects against WALKER 256 carcinosarcoma transplanted in the intramuscular site of rats. This tumor test system has been designated as WM. Subsequently, it was noted that these filtrates had various *in vitro* activities, with inhibition of gram-positive bacteria being a useful property to facilitate assay during purification procedures. The producing culture *Streptomyces metachromogenes*, sp. n., ATCC 21,440, is a novel strain and is described, along with fermentation and production information, in the preceding paper[1]. The isolation procedure, chemical characterization and biological properties, both *in vitro* and *in vivo*, of kundrymycin are presented below.

Isolation

Kundrymycin was isolated from a 10-gallon (38-liter) tank fermentor by the following procedure. Diatomaceous earth filter-aid (3 kg) was added to 15 liters of harvested broth and the mixture filtered on a 24″-precoated Nutsche filter followed by water wash (8 liters) of the cake. The combined filtrate and wash (23 liters) was adjusted to pH 5.4 with 30 % aqueous sulfuric acid and extracted by agitation with 23 liters methylene chloride for 30 minutes. The organic phase (16 liters) was separated, concentrated to 890 ml, and diluted with 2.67 liters Skellysolve B to precipitate 9 g crude kundrymycin.

The cake from the broth filtration was stirred with 20 liters acetone for 30 minutes and filtered on a 24″-Nutsche filter, another 27 liters acetone being used as wash. The combined filtrate and wash were evaporated to 12 liters, at which point solids began to precipitate. The concentrate was diluted with 6 liters water, adjusted to pH 5.2 with 30 % sulfuric acid, and extracted for 30 minutes with 18 liters methylene chloride under agitation. The organic phase (16 liters) was evaporated to 600 ml and diluted with 3.5 liters Skellysolve B to precipitate 72 g crude kundrymycin.

Crude solids (10 g) were dissolved in 50 ml boiling benzene, the volume reduced

slightly, and the solution allowed to cool slowly to room temperature and then seeded if necessary. After standing overnight at 8°C, 9.1 g pure crystalline kundrymycin was collected.

Characterization

The pure antibiotic, when recrystallized from benzene, exists in a platelet crystalline form which melts at 183~185°C (capillary). From methanol, ethanol, or *n*-butanol it crystallizes as fine needles, melting at 213~214°C (block), 234~235°C (capillary). It is very soluble in dioxane, acetone, ethyl acetate, chloroform, methylene chloride and hot benzene, and essentially insoluble in water, carbon tetrachloride and petroleum ether.

Fig. 1 a. Ultraviolet spectrum of kundrymycin
Concentration : 20 μg/ml in 0.1 N NaOH or HCl in ethanol

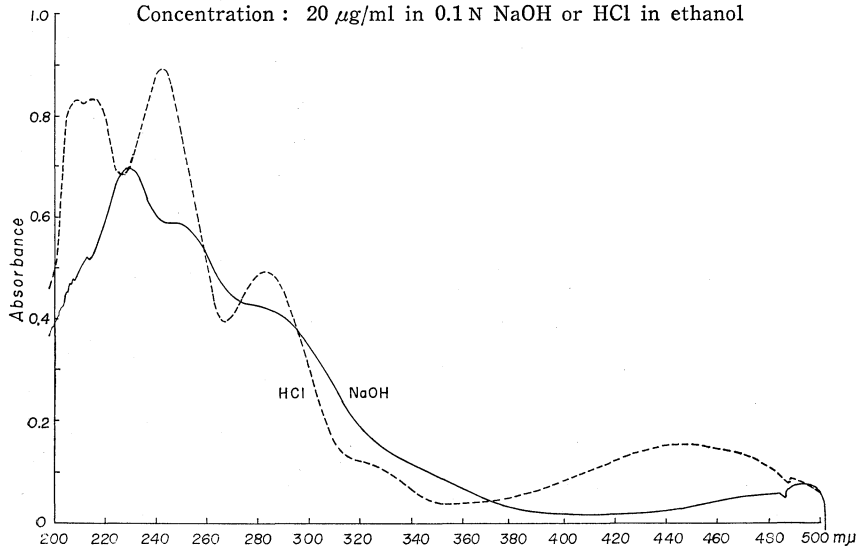


Fig. 1 b. Visible spectrum of kundrymycin
Concentration : 100 μg/ml in 0.1 N NaOH or HCl in ethanol

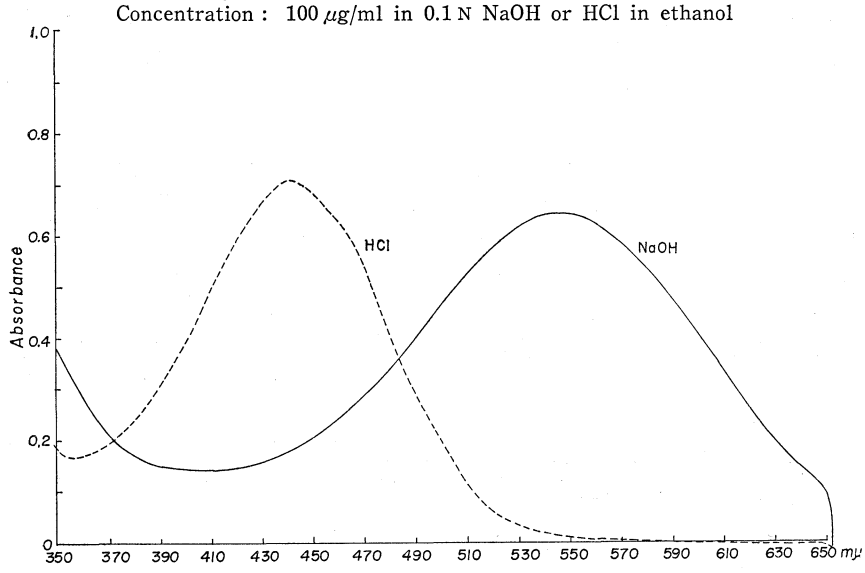


Fig. 2. Infrared spectrum of kundrymycin

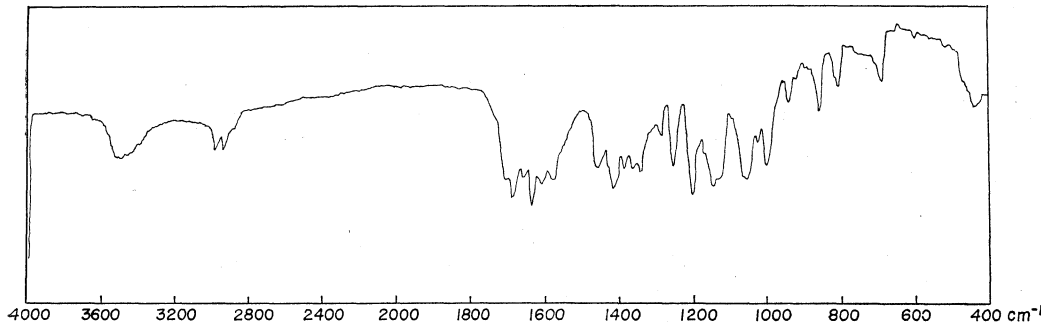
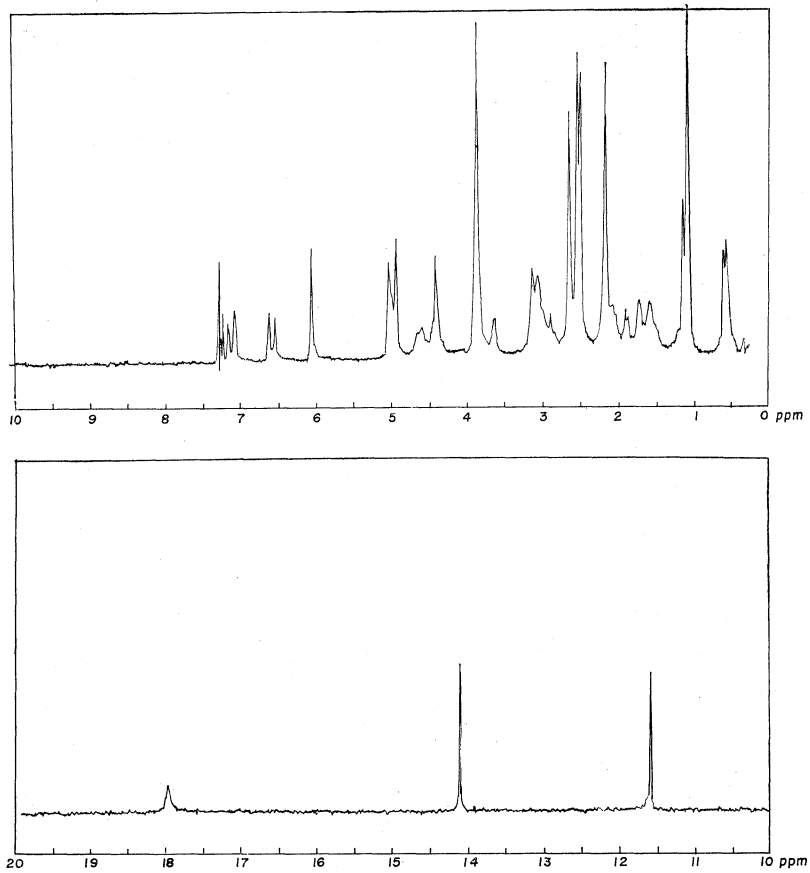


Fig. 3. Nuclear magnetic resonance spectrum of kundrymycin



Kundrymycin is very stable in neutral solution (1 mg/ml methanol). No loss of activity could be detected by antibacterial assay after 6 weeks at 37°C. Solutions are unstable below pH 5 and above pH 8.

The results of various energy measurements are shown in the accompanying figures. The ultraviolet and visible spectra are shown in Fig. 1a and 1b. Absorption peaks (0.1 N HCl in ethanol) were 214 mμ (a=43.5), 241 mμ (a=44.0), 282 mμ (a=24.5), and 442 mμ (a=7.05). Using 0.1 N NaOH in ethanol there was a slight shift to 229 mμ (a=34.8), 243~249 mμ (shoulder) (a=29.3), 278~286 mμ (shoulder) (a=21.0) and 548 mμ (a=6.35).

The IR spectrum is shown in Fig. 2 and the nuclear magnetic resonance spectrum in Fig. 3. The latter shows the presence of 50 protons. The optical rotation is $[\alpha]_D^{25}$ $-70°$ (*c* 0.1 in ethanol), $[\alpha]_D^{25}$ $-123.2°$ (*c* 0.335 in dioxane).

Osmometric determination of the molecular weight of kundrymycin gave a value of 894 in $CHCl_3$, and elemental analysis suggested a molecular formula of $C_{45}H_{50}O_{18}$ (mol. wt. 878.9). There were no other elements or ash.

Calc'd: C 61.52, H 5.70. Found: C 61.69, 61.41, H 5.87, 5.70.

In Vitro Biological Effects

Antimicrobial Activity

The minimum inhibitory concentration (MIC) of kundrymycin for a number of microorganisms was determined by the tube dilution procedure (see ref. 2 for details of methods). Some gram-positive organisms were sensitive to the antibiotic (Table 1); gram-negative bacteria, yeasts, and protozoa were generally insensitive.

Table 1. Antimicrobial spectrum of kundrymycin

	Test organism	Minimum inhibitory concn. (μg/ml)
Bacteria	*Staphylococcus aureus* A-9537	0.2
	Mycobacterium bovis BCG	25.0
	Escherichia coli A-15119	>50.0
	Pseudomonas aeruginosa A-9843	>50.0
	Proteus mirabilis A-9900	>50.0
	Streptococcus faecalis A-9536	0.05
	Klebsiella pneumoniae A-9977	>50.0
	Salmonella enteritidis A-9531	>50.0
Fungi	*Candida albicans* A-9540	50.0
	Trichophyton mentagrophytes A-9870	>50.0
	Microsporum canis A-9872	>40.0
	Histoplasma capsulatum A-15056	>50.0
Protozoa	*Trichomonas vaginalis* A-20074	5.0
	Trichomonas foetus A-20075	>10.0

Induction of Lysogenic Bacteria

Kundrymycin was tested for its ability to induce bacteriophage production in the lysogenic strain of *Escherchia coli* W 1709[3]. There was no evidence of induction at 12.5 μg/ml, and toxicity to the host organism was observed at 50 μg/ml.

Tissue Culture Cytotoxicity

Tube dilution protein tests to determine cytotoxic effects of kundrymycin on HeLa cells in tissue culture gave a 50% end-point (ED_{50}) of 10 μg/ml[2].

Table 2. Acute toxicity of kundrymycin in mice

LD_{50} mg/kg	(19/20 confidence limits)	Slope	(19/20 confidence limits)	Days of death	
				Median	Last
13	(7.65~22.1)	1.86	(1.283~2.697)	Day 1	Day 11

Doses: 2 to 128 mg/kg in 0.5 ml saline, intraperitoneally 5 mice per dose.
Evaluation: LITCHFIELD and WILCOXON, 1949[11].
Observation: 35 Days.

In Vivo Biological Effects

Acute Toxicity

The acute intraperitoneal LD_{50} of kundrymycin was determined in Swiss Ha/ICR female mice (Table 2). During the 35-day observation interval, 19 mice died, 17 on Day 1, and one each on Days 2 and 11. Thus the antibiotic seemed generally free of delayed toxic effects.

Mouse Protection Test

An attempt was made to establish the minimum dose of kundrymycin which

Table 3. Effect of kundrymycin on transplanted mouse tumors

Daily dose mg/kg	Leukemia 1210 (ascitic)			Leukemia P-388 (ascitic)			Sarcoma 180 (solid)		
	Av Wt* difference (T-C, g)	T/C % MST	Survivors Day 5	Av Wt difference (T-C, g)	T/C % MST	Survivors Day 6	Av Wt difference (T-C, g)	T/C Av. Diam.	Survivors Day 8
8	0	107	6/6	−0.5	105	6/6	−4.6	0.44	3/5
4	+1.0	100	6/6	−1.5	100	6/6	−1.4	0.80	5/5
2	+1.0	100	6/6	−1.0	105	6/6	−0.9	0.77	5/5
1	−1.5	100	6/6	−0.5	100	6/6	−1.0	0.98	5/5

Treatment schedules: All injected once daily intraperitoneally in 0.5 ml volume starting day 1. Leukemia 1210, Leukemia P-388 and Sarcoma 180, 13, 10 and 7 injections respectively.
* Evaluation: T/C % MST=Median survival time in days, Treated/control×100. A T/C ≧ 125 considered significant prolongation of survival with L-1210 and P-388. Average diameter measurements T/C ≦ 0.75 considered significant tumor inhibition with S-180.

would protect 50 % of mice challenged with a lethal dose of a pathogenic bacterium. Swiss mice were given *Staphylococcus aureus* SMITH cells equivalent to 5,000 LD_{50} intraperitoneally and treated at 0 and 2 hours after challenge with doses up to the maximum tolerated dose of 4 mg/kg injection intramuscularly. A curative dose was not achieved as all of the mice died of infection.

Table 4. Effect of kundrymycin on WALKER 256 carcinosarcoma

Daily dose mg/kg	Av Wt difference T-C, g	Tumor weight treated/control g	T/C index	Effect	Survivors day 7
8	−18.8	0.9/7.5	0.12	+	7/10
6	−15.6	1.5/7.5	0.20	+	9/10
4	−10.4	3.0/7.5	0.40	+	10/10
3	− 8.4	3.0/7.5	0.40	+	10/10
2	− 2.4	4.6/7.5	0.61	±	10/10
1.5	− 1.3	5.6/7.5	0.75	−	10/10
1	− 0.7	5.8/7.5	0.77	−	10/10
0.75	− 1.3	6.9/7.5	0.92	−	10/10

Treatment schedule: Once daily, intraperitoneally, 4 injections, days 3~6, 2 ml volume.
Evaluation: +=Tumor inhibition P ≦ 0.01
±= 〃 〃 P ≦ 0.05
−=No tumor inhibition

Antitumor Effects

The effect of kundrymycin treatment was studied in several transplanted rodent tumor systems. Details of methods used have been previously described for the mouse tumors Sarcoma 180 (S-180)[4], Leukemia 1210 (L-1210)[5] and Leukemia P-388[5], and for the rat tumor WALKER 256 intramuscular carcinosarcoma (WM)[5]. The results with the mouse tumors are shown in Table 3. There was no effect on the 2 leukemias and inhibition only at a toxic dose (2/5 deaths) on S-180. The rat tumor, WM, which originally responded to material in crude fermentation broths, was more sensitive to treatment with pure kundrymycin. A typical dose-response titration is shown in Table 4. If the LD_{10} is considered the maximum tolerated dose (MTD) and the lowest dose giving tumor inhibition ($P \leqq 0.05$) the minimum effective dose (MED) the therapeutic ratio, MTD/MED=3.

Discussion

Kundrymycin appears to be a polynuclear quinone indicator antibiotic based on the physical and chemical measurements reported, and on structure studies which will be the subject of a future publication. It can be differentiated from other non-nitrogen containing indicator antibiotics on the basis of molecular weight, empirical analysis, melting point and/or energy absorption spectra. Examples of antibiotics in this category which have been reported to have antitumor or mammalian cell crytotoxic effects are aquayamycin[6], ayamycin A[7], julimycin[8], minomycin[9], and pillaromycin[10].

Although the antitumor effects of kundrymycin are limited with respect to the number

of tumor systems sensitive to it, the fact that it is a novel antibiotic which clearly inhibits one tumor, WALKER 256 carcinosarcoma, and dose not appear to have delayed lethal toxicity suggests that further pharmacological studies are warranted as a prelude to possible clinical trial.

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