ANTITUMOR ACTIVITY OF AN ANTIBIOTIC IDENTICAL WITH OR CLOSELY RELATED TO XANTHOMYCIN A

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An antibiotic, identical with or closely related to xanthomycin A, was isolated from a soil *Streptomyces*. The antibiotic displayed significant therapeutic activity by i.p. administration against i.p.-implanted mouse tumors: Ehrlich carcinoma, sarcoma 180 and P388 leukemia. Less therapeutic activity was observed by i.p. injection in mice bearing s.c. solid tumors of Ehrlich carcinoma and sarcoma 180. No significant activity was found against L1210 leukemia, B16 melanoma and Lewis lung carcinoma.

In vitro the antibiotic exhibited a potent cytotoxicity to human leukemia K562 and mouse lymphoblastoma L5178Y cells.

DNA strand scission of PM2 phage was caused by the antibiotic in the presence of dithiothreitol.

In a screening program for tumor-inhibitory antibiotics, we have isolated an antibiotic, identical with or closely related to xanthomycin A[1~7], from a soil *Streptomyces*. Since the detailed antitumor activity of xanthomycin A has not appeared in the literature, we have studied the tumor-inhibitory activity of this antibiotic.

Materials and Methods

Production and Isolation of an Antibiotic Resembling Xanthomycin A

Streptomyces strain IM7911T was grown in 2 sets of 15 liters of medium containing oat meal 20 g/liter and yeast extract 1 g/liter, pH 7.2, in a jar fermenter at 27°C for 48 hours. The stirrer was turned at 400 r.p.m., and the rate of aeration was 15 liters of air per minute. Celite was added to the culture broth, and the mycelial cake was removed by filtration. Antibiotic concentrations in fermentation and extraction samples were assayed by a disc method, using nutrient agar plates and *B. subtilis* PCI 219 as a test organism. The culture liquid of 21 liters, containing *ca.* 160 mg of antibiotic, was applied to a column of Amberlite IRC 50 (H^+ type), and the antibiotic was eluted with 0.1 N HCl. The eluate of 2.5 liters was adjusted to pH 7.7 with Amberlite IR 45, and extracted with 1.4 liters of ethyl acetate. The extract was concentrated to 44 ml, and treated with 11 ml of water at pH 1.5, adjusted with HCl. The antibiotic was further extracted with 3 ml of chloroform at pH 7.0, and transferred to 1 ml of water at pH 2.0, adjusted with HCl. The chloroform and water transfer was repeated, and the water layer was lyophilized. A reddish yellow powder of *ca.* 91 mg was obtained. The yield was approximately 56%.

Characterization of the Antibiotic

The antibiotic was a basic substance, and the hydrochloride salt was a crystalline reddish yellow powder; stable at acidic pH and labile at alkaline pH. The color was yellow in acidic solution, orange in neutral, and red in alkaline. The hydrochloride showed Rf 0.28 on TLC, using a solvent system of chloroform - ethanol (8:1).

The hydrochloride, mp 122~130°C (dec.), showed UV λ_{max} ($E^{1\%}_{1cm}$): 265 nm (311) and 358 nm (21)

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Table 1. Antimicrobial activity of the antibiotic.

Organism	MIC (μg/ml)	Organism	MIC (μg/ml)
Staphylococcus aureus FDA 209P	0.02	*Aspergillus niger*	>100
Micrococcus luteus IAM 1099	0.02	*Aspergillus oryzae*	>100
Bacillus subtilis PCI 219	0.1	*Botrytis cinerea*	>100
Bacillus cereus T	1.6	*Mortierea ramannianus*	>100
Corynebacterium xerosis	12.5	*Penicillium chrysogenum*	>100
Escherichia coli B	0.8	*Candida albicans*	>100
Escherichia coli Q 13	6.3	*Candida utilis*	>100
Enterobacter cloacae	12.5	*Cryptococcus neoformans*	>100
Enterobacter aerogenes	12.5	*Saccharomyces cerevisiae*	>100
Proteus vulgaris	12.5		
Klebsiella pneumoniae	12.5		
Salmonella enteritidis No. 11	12.5		
Shigella sonnei	25		
Pseudomonas aeruginosa IFO 3455	100		

in 0.1 N HCl, and 270 nm (201) and 504 nm (29) in 0.1 N NaOH. The elementary analysis of the hydrochloride was: C 50.99, H 5.98 and N 8.19. The free base showed IR ν_{max} 3380, 2940, 1660, 1635, 1615, 1510, 1450, 1375, 1315, 1230, and 1160 cm^{-1} in CCl_4.

The antibiotic inhibited growth of bacteria, but not that of fungi (Table 1). $LD_{100}=2$ mg/kg, and $LD_0=1$ mg/kg (*dd*Y mice, i.v.).

The physicochemical properties and antimicrobial activity indicated that the antibiotic may be identical with or closely related to xanthomycin A.

Cell Culture

Human leukemia cell line K562 was grown in PRMI 1640 medium, supplemented with 10% fetal calf serum, benzylpenicillin 100 units/ml and streptomycin 100 μg/ml, pH 7.2, at 37°C in a humidified atmosphere at 5% CO_2. Mouse lymphoblastoma L5178Y cells were cultured in FISCHER's medium supplemented with 10% horse serum, pH 7.2, at 37°C. The cells (2×10^4/ml) were inoculated into the medium with various concentrations of the antibiotic, and grown at 37°C for 3 days. The cell number was determined by a Coulter counter.

Chemotherapy Studies

The animals used in chemotherapy experiments were *dd*Y male mice, 6 weeks of age, for Ehrlich carcinoma and sarcoma 180; CDF_1 (BALB/cAnNCrj $\times$ DBA/2NCrj) female mice, 9~10 weeks of age, for L1210 and P388 leukemias; and BDF_1 (C57BL/6NCrj $\times$ DBA/2NCrj) female mice, 11~12 weeks of age, for B16 melanoma and Lewis lung carcinoma. Ehrlich carcinoma and sarcoma 180 were maintained by successive i.p. passage of 1 week interval in *dd*Y male mice, L1210 and P388 leukemias in CDF_1 female mice, and Lewis lung tumor and B16 melanoma by continuous s.c. passage of 2 week interval in the axillary region of C57BL/6NCrj male mice.

Ehrlich carcinoma and sarcoma 180 were transplanted i.p. at 2×10^6 cells/mouse or s.c. at 10^7 and 4×10^6, respectively. L1210 and P388 leukemias were implanted i.p. at 10^5 and 10^6 cells/mouse, respectively. B16 melanoma and Lewis lung carcinoma s.c. at 10^6 and 5×10^5 viable cells/mouse, respectively. The antibiotic was injected i.p. once a day, starting a day after tumor inoculation. Each group consisted of 10 animals with solid neoplasms, and 6 with ascitic tumors.

DNA Strand Scission

The cleavage of DNA was detected by agarose gel electrophoresis, following the procedure described previously[9]. PM2 phage DNA was incubated with the antibiotic at 37°C for 30 minutes in a mixture,

in 50 μl, containing: 50 mM tris-HCl, pH 7.6, 1 mM dithiothreitol, 0.1 OD_{260}/ml DNA, and various concentrations of the antibiotic.

Results

Chemotherapeutic Activity

When administered i.p. to mice inoculated i.p. with Ehrlich carcinoma or sarcoma 180, the antibiotic displayed significant therapeutic activity at doses more than 0.031 mg/kg/day. Some animals survived even 60 days after tumor inoculation (Tables 2 and 3). A maximum ILS (increase of life span), longer than 200 % with 50 % survival on day 60, was observed in Ehrlich tumor-bearing mice at a dose of 0.125 mg/kg/day × 9 days. A similar result was obtained with sarcoma 180.

Significant activity (over 25 % ILS) was also found by i.p. administration against P388 leukemia over a range of 0.125 ~ 1.0 mg/kg/day × 7 days (Table 4).

Less therapeutic activity was exhibited in mice bearing s.c. solid tumors of Ehrlich carcinoma or sarcoma 180 (Table 5). A maximum inhibition (60 %) was observed at a dose of 0.15 mg/kg/day × 9 days with sarcoma 180.

No significant activity was observed in L1210 leukemic mice over a dose range of 0.04 ~ 0.3 mg/kg/day × 9 days (16 % maximum ILS at 0.3 mg/kg/day).

No activity was observed against s.c.-implanted B16 melanoma and Lewis lung carcinoma over a dose range of 0.03 ~ 0.25 mg/kg/day × 9 days.

Table 2. Effect of i.p. administration of the antibiotic on the life span of mice inoculated i.p. with Ehrlich carcinoma.

Dose (mg/kg/day)	Schedule (days)	Median survival time (days)	Survival on 60 day	ILS (%)
Untreated control		21.3 ± 6.3	0	
0.031	1 ~ 7	28.7 ± 13.4	0	35
0.125		39.0 ± 19.3	2	>83
0.5		40.3 ± 16.0	1	>89
2.0	1	30.9 ± 10.6	0	45
Untreated control		14.8 ± 3.7	0	
0.031	1 ~ 9	21.5 ± 8.5	0	45
0.063		22.2 ± 4.5	0	52
0.125		44.4 ± 12.6	3	>200
0.25		36.0 ± 14.6	2	>143

Table 3. Effect of i.p. injection of the antibiotic on the life span of mice inoculated i.p. with sarcoma 180.

Dose (mg/kg/day)	Schedule (days)	Median survival time (days)	Survival on 60 day	ILS (%)
Untreated control		15.5 ± 2.7	0	
0.038	1 ~ 9	26.7 ± 18.0	1	>72
0.075		26.3 ± 4.7	0	69
0.15		38.0 ± 20.8	2	>145
0.3		34.0 ± 19.0	1	>119

Table 4. Effect of i.p. administration of the antibiotic on the life span of mice inoculated i.p. with P388 leukemia.

Dose (mg/kg/day)	Schedule (days)	Median survival time (days)	ILS (%)
Untreated control		11.2±1.2	
0.031	1~7	12.2±0.4	9
0.125		14.2±1.6	27
0.5		14.3±1.4	28
1.0		14.8±1.2	32

Table 5. Effects of i.p. injection of the antibiotic on tumor weight of sarcoma 180 and Ehrlich carcinoma.

Dose (mg/kg/day)	Sarcoma 180		Ehrlich carcinoma		Increase of body weight (g)
	Tumor weight (g)	Inhibition (%)	Tumor weight (g)	Inhibition (%)	
Untreated control	1.05±0.53		1.17±0.68		7.2
0.038	0.71±0.32	32	1.14±0.61	2	7.4
0.075	0.81±0.26	23	1.09±0.32	7	6.8
0.15	0.42±0.12	60	0.99±0.42	15	5.5
0.3	0.68±0.35	35	0.78±0.37	33	4.3

Doses were administered everyday from 1~9 days.

In Vitro Cytotoxicity

The *in vitro* studies using human leukemia K562 and mouse lymphoblastoma L5178Y cells revealed that the antibiotic shows a potent cytotoxicity, even at extremely low concentrations (Fig. 1). Approximately 50% growth inhibition was observed at a concentration of 2.0 ng/ml with K562 cells, and at 1.8 ng/ml with L5178Y cells.

Fig. 1. Effects of the antibiotic on growth of human leukemia K562 and mouse lymphoblastoma L5178Y cells.
100% = 3×10^5 cells/ml (K562) and 7×10^5 cells/ml (L5178Y).

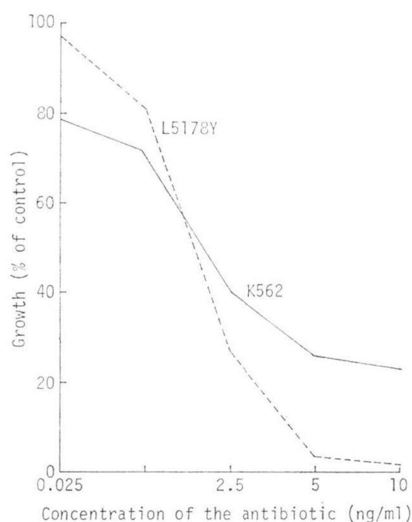
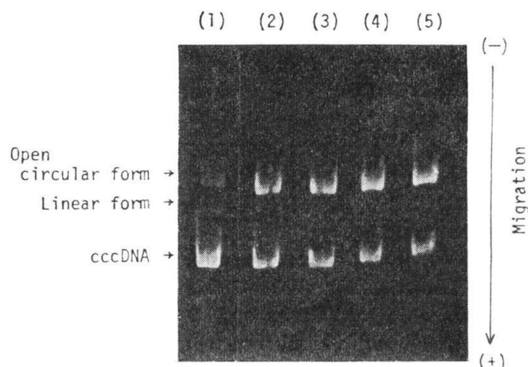


Fig. 2. Strand scission of PM2 phage DNA induced by the antibiotic: Drug concentration dependency.
The antibiotic 0 μg/ml (1), 3.65 μg/ml (2), 7.3 μg/ml (3), 14.6 μg/ml (4), and 29.2 μg/ml (5).



DNA Strand Scission

PM2 phage DNA showed 3 bands on agarose gel electrophoresis (Fig. 2). As reported by AAIJ and BORST[10], the fastest moving band corresponded to covalently closed circular (ccc)DNA, the most slowly migrating one the open circular form, and the intermediate the linear form. The electrophoretic analysis of the products of PM2 DNA and the antibiotic revealed that an antibiotic concentration-dependent decrease of the cccDNA and increase of open circular form DNA. The results indicated that the antibiotic caused a single strand scission of the cccDNA. The DNA cleavage was found at an antibiotic concentration of 3.65 μg/ml, with more breaks at higher concentrations.

Discussion

The structure of the xanthomycins has not been determined and the chemical characteristics have been not well established, probably because of its lability[1~7]. Therefore, identification of the antibiotic, which we have isolated from a *Streptomyces*, with xanthomycin A is incomplete. This antibiotic appears to be about 5 times less toxic than xanthomycin A. The difference in acute toxicity may be due to mouse strains and environment.

I.p. administration is more effective against i.p.-implanted tumors than s.c. solid tumors. I.p. administration resembles the *in vitro* effects, which are observed even at extremely low concentrations of the antibiotic. Less activity against s.c. solid tumors may be due to its poor absorption from the peritoneal cavity and distribution in s.c. tissues, and/or its metabolism in the body. The difference of the effects on P388 and L1210 leukemias seems to be attributed to their growth rates.

Quinone antibiotics, such as streptonigrin, adriamycin, daunorubicin, and aclacinomycin A, has been observed to induce DNA strand scission by forming free radicals[11~13]. RAO and PETERSON[4] have reported that xanthomycin A has quinoid properties. Therefore, the antibiotic may cleave the DNA strand in a similar manner to quinone antibiotics. HORVÁTH *et al.*[8], by using *E. coli*, have suggested that xanthomycin acts in two different ways, inhibiting both cell wall and DNA syntheses. The current results also support the assumption that DNA is the chemoreceptor in tumor cells.

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