ANTITUMOR ACTIVITY OF PYRINDAMYCINS A AND B

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Pyrindamycins A(**1**) and B(**2**) exhibited stronger cytotoxic activities than doxorubicin towards murine and human tumor cell lines and especially towards doxorubicin-resistant cells. Pyrindamycins A and B were also active *in vivo* against P388/ADR, a multidrug-resistant tumor cell line. Intracellular accumulation of pyrindamycins A and B in P388/ADR was the same as in P388. These antibiotics strongly inhibited DNA synthesis compared with RNA or protein synthesis. They showed significant therapeutic effects towards murine leukemia, but not to solid tumors.

In the course of our screening for new antitumor antibiotics, pyrindamycins A(**1**) and B(**2**) were isolated in the culture broth of *Streptomyces* sp. SF2582. In the previous papers[1,2], we reported the fermentation, isolation, physico-chemical properties, structure elucidation including X-ray analysis of pyrindamycin A, antimicrobial activity and antitumor activity against P388 leukemia of pyrindamycins.

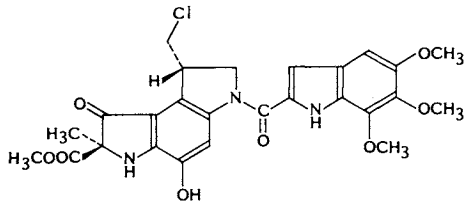
In this paper, we describe the *in vitro* cytotoxic activities, effects on macromolecular syntheses and *in vivo* antitumor activities of pyrindamycins against various murine tumors.

Materials and Methods

Agents

Pyrindamycins A and B were prepared in our laboratories. Doxorubicin was purchased from Kyowa Hakko Kogyo Co., Ltd., Tokyo, RPMI-1640 medium from Nissui Pharmaceutical Co., Ltd., Tokyo, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), TCA and 2-hydroxyethyldisulfide (2-HEDS) from Wako Pure Chemical Industries, Ltd., Osaka, L-[*U*-^{14}C]leucine (317.8 mCi/mmol), [*methyl*-^{3}H]thymidine (6.7 Ci/mmol) and [5-^{3}H]uridine (28.9 Ci/mmol) from New England Nuclear, Boston, Mass., U.S.A.

Pyrindamycins A and B were dissolved in dimethyl sulfoxide and diluted with distilled water. MTT was dissolved in phosphate buffered saline (PBS).



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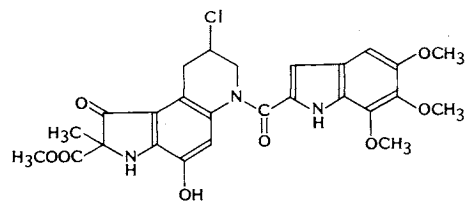

2

Table 1. Cell lines used in this study.

Cell line	Origin	Inoculum size ($\times 10^3$ cells/well)	Growth form
Mouse			
P388	Leukemia	2.5	Susp.
P388/ADR	Leukemia[a]	2.5	Susp.
L1210	Leukemia	2.5	Susp.
B16	Melanoma	2.5	Adh.
Meth-A	Fibrosarcoma	5.0	Susp.
Human			
K-562	Leukemia (Myelocytic)	5.0	Susp.
MOLT-3	Leukemia (T-cell)	5.0	Susp.
CCRF-CEM	Leukemia (T-cell)	5.0	Susp.
CCRF-SB	Leukemia (B-cell)	5.0	Susp.
J-111	Leukemia (Monocytic)	5.0	Susp.
KB	Nasopharynx carcinoma	5.0	Adh.
PC-14	Lung carcinoma	5.0	Adh.
MKN-1	Gastric carcinoma[b]	5.0	Adh.
MKN-28	Gastric carcinoma[c]	5.0	Adh.
MKN-45	Gastric carcinoma[d]	5.0	Adh.
MKN-74	Gastric carcinoma[c]	5.0	Adh.
GOTO	Neuroblastoma	5.0	Adh.
YT/nu	Neuroblastoma	5.0	Adh.
T-24	Urinary bladder carcinoma	5.0	Adh.
HeLa S_3	Uterine cervix carcinoma	5.0	Adh.
HMV-1	Melanoma in vagina	5.0	Adh.

[a] Multidrug-resistant subline of P388, [b] adenosquamous, [c] well differentiated, [d] poorly differentiated.
Susp.: Suspension cultured cells. Adh.: adherent growing cells.

Cell Culture

All cells were cultivated in RPMI-1640 medium supplemented with 10% fetal calf serum and 10 μM of 2-HEDS at 37°C under 5% CO_2 in air. Cell lines and inoculum size are shown in Table 1.

Animals

Specific pathogen free (SPF) BDF_1 male mice, 5 weeks old, were supplied from Shizuoka Agricultural Cooperative Association for Experimental Animals, Hamamatsu. Mice were fed with commercial diet (NMF, Oriental Yeast Co., Tokyo) and water *ad libitum*.

Cytotoxic Activity

Cells were cultivated with 96-well flat-bottomed microplate (Falcon, No. 3002). In the case of suspension culture, drugs were added into the culture medium immediately after cell inoculation and cells were cultivated for 72 hours. The viable cell fraction was measured by the modified MTT assay[3,4]. The modified MTT assay was carried out as follows: After the addition of 10 μl of MTT solution (5 mg/ml), the microplate was incubated at 37°C for 6 hours. Then a 100-μl of 0.01 N HCl-10% SDS mixture was added to each well and further incubated at 37°C for 16 hours for dissolution of MTT-formazan and cell debris. Absorbance of MTT reaction mixture in well was measured at 577 nm with the reference absorbance at 630 nm using a ELISA analyser (ETY-96, Oriental Instruments Ltd.). In the case of adherent growth, drugs were added into the culture medium after 24 hours.

Fifty % growth inhibitory concentrations (IC_{50} values) were calculated by the probit method[5].

Intracellular Accumulation of Drugs

Intracellular amounts of pyrindamycins A, B and doxorubicin were measured by flow-cytometric method utilizing their fluorescence. After 5×10^5 cells of P388 or P388/ADR were incubated with 5 μg/ml of a drug for 2 hours, cells were collected and washed with PBS. Fluorecence intensity of cells was measured with the cell sorter (FACStar, Becton-Dickinson).

Inhibition of Macromolecular Synthesis

DNA, RNA or protein syntheses were measured by the incorporation of radioactive precursors into the cell fraction. In the case of DNA synthesis, exponentially growing CCRF-CEM cells (5×10^4 cells/well) were treated with pyrindamycin A or B. After 1 hour, 0.2 μCi of [^{3}H]thymidine was added and cells were incubated further 2 hours. In the case of RNA or protein synthesis, 0.2 μCi of [^{3}H]uridine or 0.1 μCi of [^{14}C]leucine was used instead of [^{3}H]thymidine and the procedure was the same as in the case of DNA synthesis. After incubation, cells were harvested, washed with 5% TCA and their radioactivity was measured by liquid scintillation counter (TRI-CARB 2000CA, Packard).

In Vivo Antitumor Activity

All *in vivo* experiments were carried out under SPF conditions. For implantation, cells of P388, P388/ADR, M5076 or EL-4 was suspended in HANKS' solution and implanted intraperitoneally to mice at 1×10^6 cells/mouse and 1×10^5 cells/mouse for L1210. B16 cells were implanted intraperitoneally to each mouse with 0.5 ml of 1 : 10 tumor brei. Drugs were administered intraperitoneally once a day on days 1, 3 and 5. Each experiment was terminated on the 60th day after inoculation. Antitumor activity was assessed as the percent increase in life span (ILS %) from mean survival time of the drug-treated group and of the control group.

Results

Cytotoxic Activity

As shown in Table 2, pyrindamycins A and B exhibited stronger cytotoxic activity than doxorubicin towards all cells used in this study. Especially, pyrindamycins A and B showed strong cytotoxic activity towards doxorubicin-resistant cells, such as Meth-A fibrosarcoma, PC-14 lung carcinoma, MKN-28 gastric carcinoma and HeLa S_3 uterine cervix carcinoma, and IC_{50} values of pyrindamycin A or B towards these cells were 5 to 30 times smaller than that of doxorubicin.

IC_{50} values of pyrindamycins A and B in P388 and P388/ADR, a multidrug-resistant subline of P388[6], were almost the same. On the other hand, the IC_{50} value of doxorubicin in P388/ADR was about 30 times greater than that in P388.

Intracellular Accumulation

The results of intracellular accumulation of pyrindamycins A, B and doxorubicin are shown in Fig.

Table 2. *In vitro* cytotoxic activity.

Cell line	IC_{50} value (ng/ml)			Cell line	IC_{50} value (ng/ml)		
	PDMA	PDMB	DXR		PDMA	PDMB	DXR
P388	3.9	10	21	PC-14	11	22	360
P388/ADR	3.9	11	600	MKN-1	33	32	94
L1210	4.9	13	82	MKN-28	16	26	150
B16	27	17	52	MKN-45	17	25	75
Meth-A	48	13	200	MKN-74	14	25	60
K-562	14	13	30	GOTO	7.6	11	50
MOLT-3	3.1	12	16	YT/nu	8.8	11	64
CCRF-CEM	16	28	46	T-24	5.9	15	65
CCRF-SB	7.2	25	24	HeLa S_3	10	18	120
J-111	7.0	21	50	HMV-1	8.4	17	80
KB	19	27	98				

Cells were incubated with each sample for 72 hours in RPMI-1640 medium supplemented with 10% of fetal calf serum and 10 μM of 2-HEDS. Survived cell fraction was measured with modified MTT assay and IC_{50} values were calculated by the probit method.

Abbreviations: PDMA, pyrindamycin A; PDMB, pyrindamycin B; DXR, doxorubicin.

Fig. 1. The ratios of intracellular accumulation in P388 (open) and P388/ADR (closed) cells.

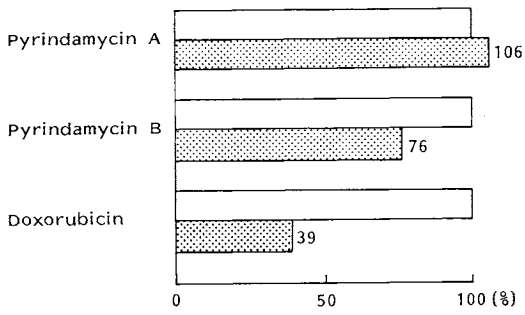

Table 3. Effects on macromolecular syntheses in CCRF-CEM cells.

Drug	IC_{50} value (ng/ml)		
	DNA	RNA	Protein
PDMA	25.6	194	2,030
PDMB	4.08	146	2,300

CCRF-CEM cells (5×10^4 cells/well) were cultured with each sample for 1 hour and 0.2 μCi of [^{3}H]thymidine, 0.2 μCi of [^{3}H]uridine or 0.1 μCi of [^{14}C]leucine was added. After 2 hours, radioactivity of cells was measured by liquid scintillation counter.

Abbreviations: See footnote in Table 2.

Table 4. *In vivo* antitumor effects.

Tumor cell line		ILS (%)					
Dose	(mg/kg/day)	P388	P388/ADR	L1210	EL-4	B16	M5076
PDMA	0.2	−16	−35	12	−54	−66	−73
	0.1	39	63	46	26	12	−52
	0.05	37	63	39	17	−6.4	21
	0.025	29	39	32	14	0.7	9.0
	0.013	8.2	33	22	28	9.3	2.1
	0.0063	12	15	20	16	7.1	0.7
PDMB	0.8	49	−5.6	34	−25	−64	−59
	0.4	53	57	39	28	−8.6	28
	0.2	49	70	42	32	2.1	10
	0.1	39	56	34	23	3.6	6.3
	0.05	25	30	29	19	6.4	7.6
	0.025	14	24	27	19	13	10

Tumors were transplantated intraperitoneally and each sample was administered intraperitoneally once a day starting the day after tumor transplantation for three times on days 1, 3 and 5. ILS was calculated by the following equation;

$$\text{ILS (\%)} = \left(\frac{\text{mean survival days of test group}}{\text{mean survival days of control group}} - 1 \right) \times 100$$

Abbreviations: See footnote in Table 2.

1. The ratios of intracellular accumulation in P388/ADR compared to P388 for pyrindamycins A, B and doxorubicin were 106, 76 and 39%, respectively.

Effect on Macromolecular Synthesis

Effects of pyrindamycins A and B on DNA, RNA and protein synthesis are shown in Table 3. IC_{50} values for DNA, RNA and protein synthesis of pyrindamycin A were 25.6, 194 and 2,030 ng/ml, and those of pyrindamycin B were 4.08, 146 and 2,300 ng/ml, respectively. These results showed that both pyrindamycins A and B were specific inhibitors of DNA synthesis.

In Vivo Antitumor Activity

In vivo antitumor activity of pyrindamycins A and B are shown in Table 4. Pyrindamycin A was active against P388, P388/ADR and L1210, showing maximum ILS (ILS_{max}) towards those tumors of 39, 63 and 46%, respectively. Pyrindamycin B was active against P388, P388/ADR, L1210 and EL-4, showing

ILS_{max} towards them of 53, 70, 42 and 32%, respectively. Both antibiotics were inactive against B16 and M5076.

Discussion

Pyrindamycins A and B exhibited stronger cytotoxic activities to several tumor cell lines than doxorubicin, and especially to cell lines resistant to doxorubicin, such as Meth-A, PC-14, MKN-28 and HeLa S_3.

Pyrindamycins A and B also showed therapeutic efficacies to multidrug-resistant cell line, P388/ADR. Furthermore, pyrindamycins A and B were accumulated in P388 and P388/ADR at similar degree. The results suggested that those agents did not undergo rapid efflux from resistant cell lines[7,8].

Pyrindamycins A and B inhibited DNA synthesis more than RNA or protein synthesis and the antibiotics were specific inhibitors of DNA synthesis. Although pyrindamycin B inhibited DNA synthesis in CCRF-CEM cells more strongly than pyrindamycin A, the cytotoxic activity of pyrindamycin B against CCRF-CEM was weaker than that of pyrindamycin A. The results suggested that pyrindamycin A possessed another cytotoxic mechanism.

Both pyrindamycins A and B showed antitumor activities against murine leukemia. Since pyrindamycins A and B exhibited antitumor activity against multidrug-resistant tumor, they might be a candidate as useful chemotherapeutic agents for severe multidrug-resistant tumors[9].

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