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Antitumor Activities of IKP-104, a 4(1*H*)-Pyrizinone Derivative, on Cultured and Implanted Tumors

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Antitumor activities of IKP-104, a 4(1*H*)-pyrizinone derivative, were investigated with cultured tumor cell lines and implanted tumors in mice. IKP-104 inhibited the growth of cultured murine tumor cell lines (L1210 leukemia, Lewis lung carcinoma and B16 melanoma) and human tumor cell lines (K562 leukemia and HeLa cervical carcinoma). It also had antitumor effects on implanted murine ascitic tumors (L1210 leukemia and sarcoma 180) and a murine solid tumor (Lewis lung carcinoma). IKP-104 could be classified as a phase-dependent cytostatic drug based on the mode of growth inhibition of cultured B16 melanoma cells compared with those of several other antitumor agents. The effect of IKP-104 on the cell cycle traverse of cultured B16 melanoma cells was estimated by morphological and flow cytometric analyses. Cells accumulated in the mitotic phase, and abortive mitosis or polyploidy or multinucleation was induced from 6 h after exposure to IKP-104. Based on these results, IKP-104 is expected to be useful for the treatment of tumors, and its mode of action seemed to be similar to that of metaphase arrestants such as colchicine or vinca alkaloids.

Key words: Antitumor activity — IKP-104 — 4(1*H*)-Pyrizinone derivative — Cultured tumor cell — Implanted tumor

We have found that 4(1*H*)-pyrizinone derivatives, which were originally prepared in the course of a search for new fungicides at Kumiai Chemical and K-I Research Institutes, have antitumor activities. In the process of safety evaluation in mammals, it was found that the 4(1*H*)-pyrizinone derivatives had similar cytotoxic effects on cultured mammalian cells to those of antitumor agents which are widely used. We have therefore attempted to develop them as medicinal drugs. A hundred derivatives were tested for cytocidal activity against L1210 leukemia and B16 melanoma cells. We analyzed the structure-activity relationships by the Free-Wilson method[1)] to select the most effective 4(1*H*)-pyrizinone derivatives against the tumor cells while retaining low toxicity to mammals. 2-(4-Fluorophenyl)-1-(2-chloro-3,5-dimethoxyphenyl)-3-methyl-6-phenyl-4(1*H*)-pyridinone (IKP-104) was selected on the basis of the results of structure-activity relationship analysis and toxicity studies as the most promising candidate. The purpose of this communication is to report the antitumor activities of IKP-104 *in vitro* and *in vivo*, and to present some information on the mode of action.

MATERIALS AND METHODS

Chemicals and antitumor agents IKP-104 (purity 99.5%) was synthesized at K-I Research Institute (Fig. 1). Reagent-grade colchicine, hydroxyurea and 5-fluorouracil (5-FU) were purchased from Wako Pure Chemical Industry Co., Osaka. Mitomycin C (titer: 2 mg/vial) and adriamycin (10 mg/vial) were purchased from Kyowa Hakko Industry Co., Tokyo, vindesine (3 mg/vial) from Shionogi & Co., Osaka, and cisplatin (10 mg/ 20 ml) from Nihon Kayaku Co., Tokyo. Each agent was dissolved with dimethyl sulfoxide (DMSO) or saline, and suspended in saline containing 0.5% Tween 80.

Cell lines The cell lines used in this study were L1210 leukemia (L1210), B16-BL6 melanoma (B16), sarcoma 180 (S 180), Lewis-LL/2 lung carcinoma (Lewis) established from murine tumors, and K562 myelogenous leukemia (K562), HeLa-S3 cervical carcinoma (HeLa) established from human tumors. L1210 and K562 cells have been maintained and grown in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS). B16 and HeLa cells have been maintained and grown in Eagle's minimum essential medium (MEM) supplemented with 10% FBS. Lewis cells have been maintained and grown in Dulbecco's modified MEM supplemented with 10% FBS. The cells were grown in a humidified 5% CO_2 atmosphere at 37°C.

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Assay for cytotoxicity to cultured tumor cells L1210 and K562 cells (4×10^5), B16 (1.2×10^4), Lewis (2×10^3), and HeLa cells (1.6×10^4) in 2 ml of medium were seeded in 35 mm plastic Petri dishes (Falcon 3001; Becton Dickinson Labware, Oxnard, California). The 3-day-cultured cells, which were growing exponentially, were exposed to a test agent at several doses for 48 h. Relative percentage of surviving cells in each dose with respect to the control was calculated by measuring color absorption with a photodensitometer (Monocellator; Olympus Co., Tokyo) for B16 and HeLa cells according to the method reported by Ishidate and Odashima,[2] and also by counting total cells per ml of medium with a Bürker-Türk plate for L1210, Lewis and K562 cells according to the method reported by Ikekawa *et al.*[3] The 50% growth-inhibitory concentrations (IC_{50}) were determined by a probit diagraming analysis based on the results of cytotoxicity assays.

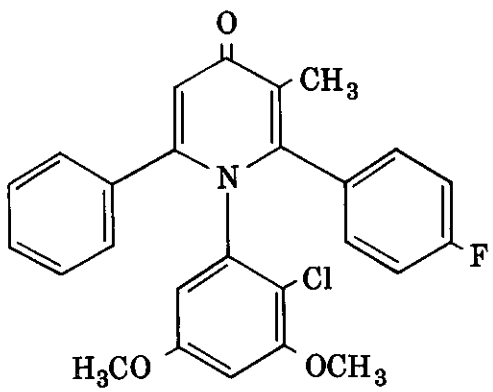

Fig. 1. Chemical structure of IKP-104.

Animals and cell maintenance slc:ICR, slc-CDF_1 and C57BL/6 Cr Slc male mice for cell maintenance and assay were purchased from Japan SLC Inc., Shizuoka, and used at 5 weeks of age. S 180 and L1210 have been maintained by weekly intraperitoneal (ip) transplantation into ICR and CDF_1 mice, respectively. Lewis cells have also been maintained by monthly subcutaneous (sc) transplantation into C57BL/6 mice.

Assay for antitumor effects on murine implanted tumor S 180 (10^6) and L1210 (10^5) cells were implanted ip in groups of six mice and IKP-104, 5-FU or vindesine was given ip successively for 5 days from the day after cell implantation. Lewis (2×10^5) cells were implanted sc in a dorsal region of mice, and IKP-104 or 5-FU was given sc for 9 days from the next day. The antitumor effects were evaluated on the basis of the increase of life span (ILS) in the assays for L1210 and Lewis, or the total packed cell volume (TPCV) in the assay for S 180.[4]

Flow cytometry B16 cells exposed to a concentration of about 70% of IC_{50} of the agents were stained with propidium iodide by a modified Krishan method[5] at 3, 6, 9, 12, 24 and 48 h after exposure to the agents. The cells were harvested by trypsinization, and rinsed with Ca^{2+}, Mg^{2+}-free phosphate-buffered saline (PBS(−)). The cells were resuspended in PBS(−) after centrifugation at 200*g* and fixed in 70% ethanol at 0°C for 2 h. The cells were incubated in RNase solution (1 mg/ml in PBS(−)) at 37°C for 1 h after being rinsed with PBS(−). Following centrifugation, the samples were stained with propidium iodide solution (50 μg/ml in PBS(−)) containing 0.2% Nonidet P-40 for 10 min, and filtered through a 37 μm nylon mesh. Propidium iodide-stained cells were analyzed by using a flow cytometer (EPICS CD, Coulter) with a 488 nm laser. The specific fluorescence of DNA in 10^4 cells was measured for each histogram at a rate of 750 cells/min.

Table I. Comparison of Cytotoxicity of IKP-104 and Several Other Antitumor Agents against Various Cell Lines *in vitro*

Antitumor agent	IC_{50} (μg/ml)				
	L1210	B16	Lewis	K562	HeLa
IKP-104	0.0025	5.2	0.0017	0.0012	0.015
Hydroxyurea	3.2	19.0	5.0	8.6	54.0
5-Fluorouracil	0.11	11.0	0.011	4.8	28.0
Mitomycin C	0.13	3.0	0.011	0.14	1.7
Adriamycin	0.035	0.5	0.027	0.022	0.50
Cisplatin	0.18	5.5	0.21	0.31	0.97
Colchicine	0.010	0.8	0.0074	0.0016	0.010
Vindesine	0.030	0.6	0.0032	0.015	0.0023

Cells were incubated with the agents for 48 h at 37°C in 5% CO_2. Numbers of cells of each cell line seeded were 4×10^5 (L1210), 1.2×10^4 (B16), 2×10^3 (Lewis), 4×10^5 (K562), and 1.6×10^4 (HeLa) cells.

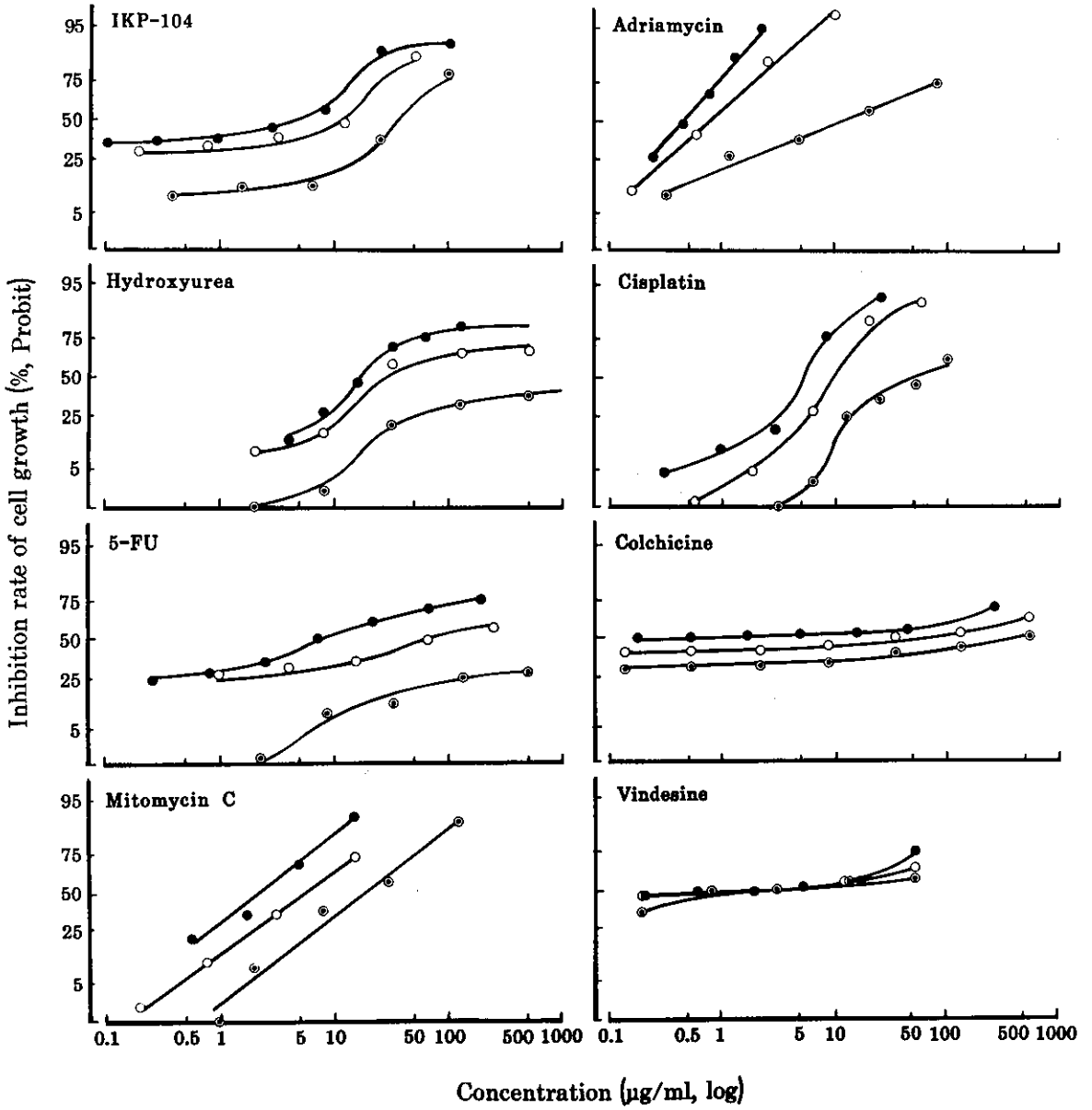

Fig. 2. Dose-response curves for IKP-104 and several antitumor agents in B16 melanoma cells. Cells were incubated for 12 (◉), 24 (○) and 48 h (●) with the agents at different concentrations at 37°C in 5% CO_2.

RESULTS

Cytotoxicity to cultured tumor cells The cytotoxic effects of IKP-104 on cultured murine and human tumor cells were compared with those of several other antitumor agents. The IC_{50} values are shown in Table I. The IC_{50} values of IKP-104 ranged from 0.0012 to 0.015 μg/ml against the cell lines tested except B16 cells. These values are lower than those of the antitumor agents used for comparison, and it was found that IKP-104 was more effective against these murine and human tumors *in vitro* than the other antitumor agents tested. The IC_{50} value of IKP-104 against B16 cells was about 300 to 4000 times larger than those against the other cell lines. The IC_{50} values of the antitumor agents against B16 cells were also about 10 to 500 times larger than those against the other

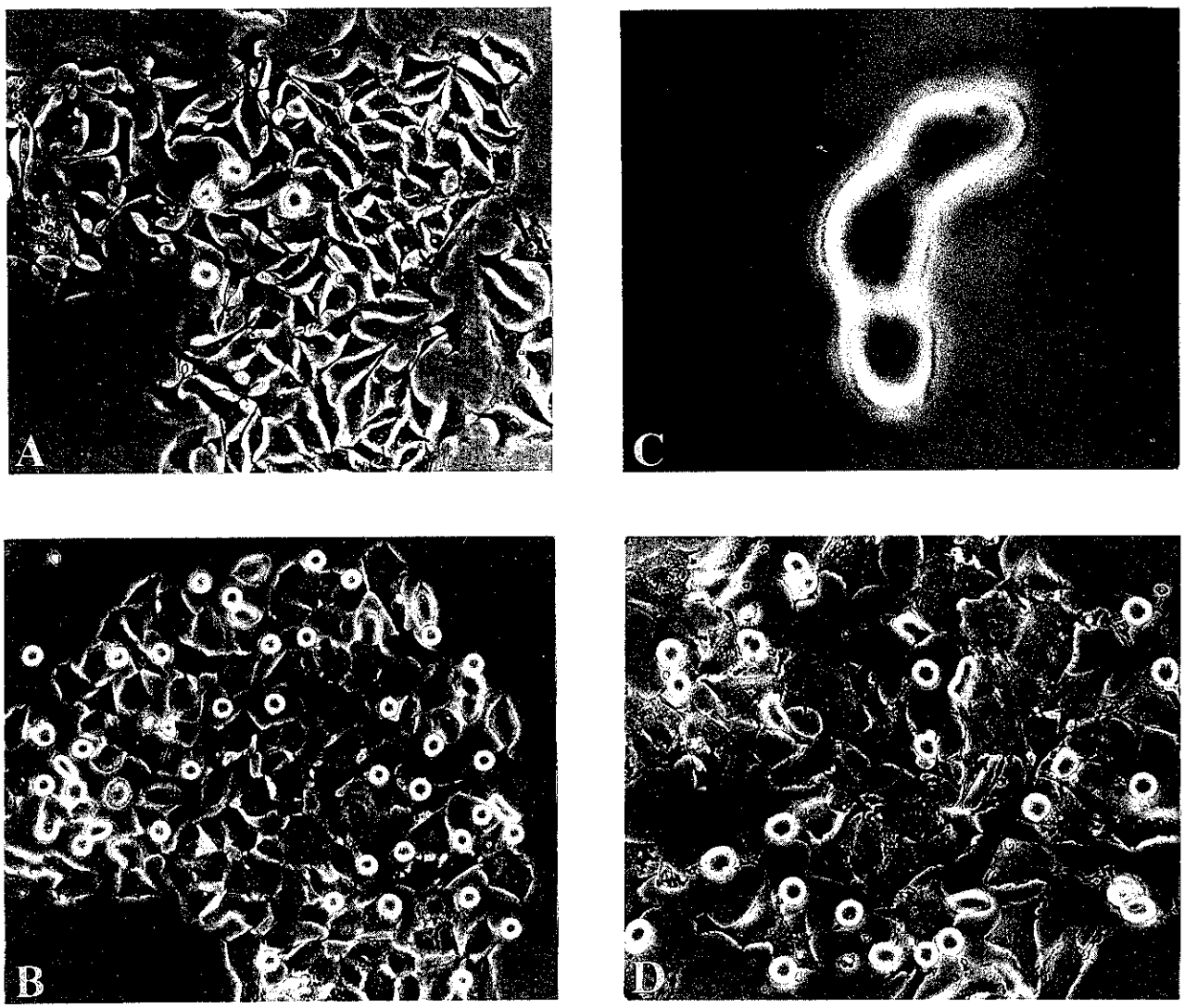

Fig. 3. Morphological changes of B16 melanoma cells which were exposed to IKP-104 at 3.6 μg/ml for 9 and 48 h. A: Control cells ($\times$100). B: Cells exposed to IKP-104 for 9 h. Cell accumulation in the mitotic phase was observed ($\times$100). C: Cells exposed to IKP-104 for 9 h. Abortive mitosis was observed ($\times$700). D: Cells exposed to IKP-104 for 48 h. Tetraploid or octaploid (see arrow) or multinuclear cells were observed ($\times$100).

lines, so that all the antitumor agents were less effective against B16 cells than other cell lines.

The dose-response relation and morphological changes of cells by IKP-104 and the antitumor agents were examined with B16 cells. The dose-response curve of B16 cells exposed to IKP-104 had a flat sigmoidal shape, and 0 or 100% growth inhibition doses were not included in this dose range (Fig. 2). Similar curves were obtained with colchicine, vindesine and 5-FU. The curves for hydroxyurea or cisplatin were sigmoidal, and linear plots were obtained with mitomycin C or adriamycin. Zero or 100% growth inhibition doses could be estimated from these curves.

IKP-104 increased the proportion of round-shaped cells, which were arrested in mitotic phase (M phase), from 3 h after exposure and accumulated mitotic cells with abortive mitosis to the maximum extent at 9 to 12 h (Fig. 3B and 3C). At 24 or 48 h, polyploid and multinuclear cells were induced (Fig. 3D). Similar changes were also observed in cells exposed to colchicine and

Table II. Antitumor Effects of IKP-104, 5-FU and Vindesine against a Murine Ascitic Tumor, Sarcoma 180

Agent	Dose (mg/kg)	No. of animals	No. of dead animals	No. of animals with ascites	T/C[a] (%)	Activity[b]
Control	0	6	0	6	—	
IKP-104	0.5	6	0	6	124	−
	1.0	6	0	2	15	++
	5.0	6	0	0	0	+++
	10.0	6	0	0	0	+++
5-FU	0.5	6	0	5	74	−
	1.0	6	0	5	45	+
	5.0	6	0	0	0	+++
	10.0	6	0	0	0	+++
Vindesine	0.1	6	0	6	62	+
	0.3	6	0	0	9	+++
	1.0	6	2	0	0	+++

Tumor cells (10^6 cells) were implanted ip into ICR mice, and the agents were administered ip for 5 days from the next day. Antitumor effects were evaluated in terms of total packed cell volume (TPCV).
a) T/C: TPCV of treatment group/TPCV of control.
b) Criteria: −, 66–100; +, 41–65; ++, 11–40 and +++, 0–10%.

Table III. Antitumor Effects of IKP-104, 5-FU and Vindesine against a Murine Ascitic Tumor, L1210 Leukemia

Agent	Dose (mg/kg)	No. of animals	ILS[a] (%)	No. of animals surviving for 30 days	Activity[b]
Control	0	6	0	0	
IKP-104	0.5	6	0	0	−
	1.0	6	24	0	++
	5.0	6	43	0	+++
5-FU	1.0	6	8	0	−
	5.0	6	19	0	+
	10.0	6	34	0	+++
	20.0	6	77	0	+++
Vindesine	0.1	6	8	0	−
	0.3	6	28	0	++

Tumor cells (10^5 cells) were implanted ip into CDF_1 mice, and the agents were administered ip for 5 days from the next day.
a) ILS: Increase in life span (T/C − 1).
b) Criteria: −, 0–9; +, 10–19; ++, 20–29 and +++, more than 30%.

Table IV. Antitumor Effects of IKP-104 and 5-FU against a Murine Solid Tumor, Lewis Lung Carcinoma

Agent	Dose (mg/kg)	No. of animals	ILS[a] (%)	No. of animals surviving for 30 days	Activity[b]
Control	0	6	—	0	
IKP-104	1.0	6	49	0	+++
	5.0	6	56	3	+++
5-FU	10.0	6	73	4	+++

Tumor cells (2×10^5 cells) were implanted sc into C57BL/6 mice, and the agents were administered sc for 9 days from the next day.
a) ILS: Increase in life span (T/C − 1).
b) Criteria: −, 0–9; +, 10–19; ++, 20–29 and +++, more than 30%.

vindesine. In contrast with these drugs, hydroxyurea, 5-FU, mitomycin C, adriamycin and cisplatin decreased mitotic cells.

Antitumor effects on murine ascitic tumors The antitumor effects of IKP-104 on murine ascitic tumors, S180 and L1210, were compared with those of 5-FU and vindesine. The results of the study with S 180 are shown in Table II. The effects of IKP-104 were dose-dependent in the range of 1.0 to 10.0 mg/kg. 5-FU at doses of 5.0 to 10.0 mg/kg and vindesine at 0.1 to 1.0 mg/kg were also effective. The antitumor potency of IKP-104 against S 180 was higher than that of 5-FU and lower than that of vindesine. However, IKP-104 was less toxic to mice than vindesine, which caused some deaths at 1.0 mg/kg.

The results with L1210 are shown in Table III. IKP-104 at doses of 1.0 and 5.0 mg/kg showed effects comparable to those of 5-FU at 10.0 and 20.0 mg/kg and vindesine at 0.3 mg/kg.

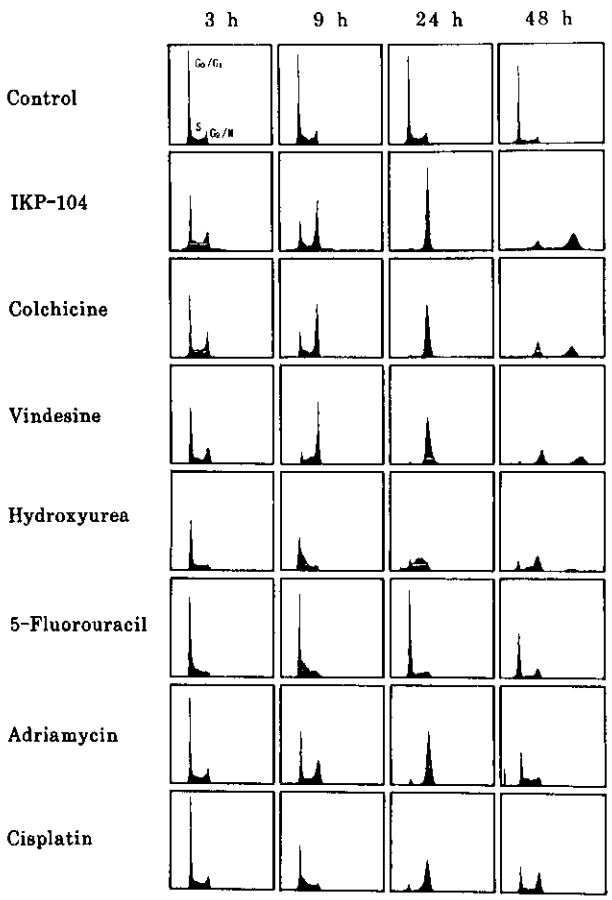

Fig. 4. Changes of the DNA histograms of B16 melanoma cells which were exposed to IKP-104 and other antitumor agents. Cells were treated at the concentrations (μg/ml) of 3.6 (IKP-104), 0.56 (colchicine), 0.42 (vindesine), 13.3 (hydroxyurea), 7.7 (5-FU), 0.35 (adriamycin) and 3.9 (cisplatin), which are equivalent to 70% of the respective IC_{50} values. Cells were stained with propidium iodide after fixation with 70% ethanol.

Antitumor effects on murine Lewis lung carcinoma The antitumor effects of IKP-104 on murine solid tumor (Lewis) were compared with those of 5-FU. As shown in Table IV, IKP-104 at doses of 1.0 and 5.0 mg/kg, and 5-FU at 10.0 mg/kg were similarly effective.

Cell cycle analysis The effects of IKP-104 and the antitumor agents on cell cycle traverse of cultured B16 cells were analyzed by preparing DNA histograms at 3, 6, 9, 12, 24 and 48 h after exposure, as shown in Fig. 4. The percentage of cells in G_2-M phase (really considered to be M phase because of the increase of mitotic cells found morphologically) was increased at 3 h after exposure to IKP-104 and cells clearly accumulated in M phase at 6 to 9 h. The proportion of cells in M phase reached a maximum at 9 to 24 h. Tetraploid or octaploid cells were induced at 48 h. Similar changes of cell cycle traverse were observed in colchicine and vindesine. In contrast with these drugs, hydroxyurea, 5-FU, adriamycin and cisplatin showed different changes of cell cycle traverse from IKP-104. A delay of the synthetic phase (S phase) in the case of hydroxyurea and an accumulation of G_1 phase in the case of 5-FU and an accumulation of G_2 phase in the case of adriamycin or cisplatin were observed.

DISCUSSION

The cell lines and the assay system used in this study were based on the screening panel of the National Cancer Institute.[6] Antitumor agents with different modes of action were selected for comparison with IKP-104. IKP-104 exhibited significant antitumor activities against implanted tumors in mice as well as against cultured murine and human cell lines *in vitro*. Median lethal dose value (LD_{50}; ip) of IKP-104 in rats was 44 mg/kg (unpublished results), and IKP-104 was less toxic than mitomycin C (2.92 mg/kg),[7] adriamycin (16.0 mg/kg),[8] cisplatin (8.3 mg/kg),[9] colchicine (6.1 mg/kg)[10] and vindecine (0.81 mg/kg).[11] However, hydroxyurea (5780 mg/kg po)[12] and 5-Fu (1025 mg/kg)[13] were less toxic than IKP-104. From the results of these studies, IKP-104 was more active than or as active as antitumor agents which have been widely used for the chemotherapy of neoplastic diseases.

B16 cells were not so sensitive to IKP-104 or the other antitumor agents. This tendency was apparent in the data reported by Shimomura *et al.*,[14] and generally, adhesive cell lines have low sensitivities to antitumor agents. Gupta[15] studied the sensitivities of cell lines derived from different species to mitotic arrestants, and suggested that the differences in cytotoxicity were caused by differences in the cellular transport of these drugs. Cellular transport might be one of the factors causing the differences in cytotoxicity in this study too.

The dose-response curve of cultured B16 cells exposed to IKP-140 was flat-sigmoidal, and the growth inhibition rates reached constant values with increasing dosage (becoming dose-independent). IKP-104 was classified a phase-dependent drug according to the criteria of Bruce *et al.*[16, 17] and as a cytostatic drug after Shimoyama and Kimura.[18] So its cytocidal action was considered to resemble that of vinca alkaloids, which are typical phase-dependent and cytostatic drugs.

The morphological changes of cells exposed to IKP-104 in the cytotoxicity study were characterized by accumulation of mitotic cells with abortive mitosis and induction of polyploid and multinuclear cells. Similar morphological findings are observed in cultured L1210 cells

exposed to vinca alkaloids and are considered to be induced as a result of mitotic arrest.[19] Mitotic arrest by IKP-104 was confirmed by the DNA histogram obtained by flow cytometry, which corresponds to those of metaphase arrestants such as vinca alkaloids.[20, 21]

From the results of the above studies, IKP-104 might be useful as an antitumor drug. Its action against tumor cells is similar to that of metaphase arrestants such as colchicine or vinca alkaloids, which have been reported to bind to tubulin and affect its polymerization to microtubules.[22, 23] Thus, IKP-104 might have a similar mechanism of action.

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