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Effects of the Tumor Inhibitor IKP-104, a 4(1*H*)-Pyridinone Derivative, on Cytoskeletal Microtubules of Cultured Tumor Cells

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The effects of IKP-104, a 4(1*H*)-pyridinone derivative, on the mitotic profile and cytoskeletal microtubule dynamics of cultured B16 melanoma cells were examined in order to investigate the mechanism of its antitumor activity. The exposure to IKP-104 caused accumulation of cells in abnormal metaphase with chromosomes scattered within the cytoplasm and induced polyploid and multinucleate cells as detected by differential staining microscopy with brilliant blue R and safranin O. An immunofluorescence study with monoclonal anti-α-tubulin antibody revealed that IKP-104 diminished cytoskeletal microtubules of both interphase and mitotic cells, resulting in induction of a few fragments resembling "microtubular bundles" induced by vinblastine (VLB). These results indicated that IKP-104 arrests cells in the mitotic phase by inhibition of polymerization and induction of depolymerization of cytoskeletal microtubules, similarly to VLB.

Key words: Mitotic arrest — IKP-104 — Cytoskeletal microtubule — Mitotic spindle — Anti-α-tubulin antibody

2-(4-Fluorophenyl)-1-(2-chloro-3,5-dimethoxyphenyl)-3-methyl-6-phenyl-4(1*H*)-pyridinone, IKP-104, is a new synthetic chemical which has antitumor activities against cultured and implanted tumors. IKP-104 arrested the mitosis of cultured B16 melanoma cells similarly to COL[4] or vindesine as revealed by morphological study and flow cytometric analysis, and it was suggested that the mode of action of IKP-104 is similar to that of the mitotic arrestants such as COL or vinca alkaloids.[1] Many substances act as mitotic arrestants, but they have different mechanisms of action.[2,3]

We have studied the effects of IKP-104 on the mitotic profile and cytoskeletal microtubules of B16 cells in comparison with several mitotic arrestants, COL, VLB, benomyl, griseofulvin, diazepam, CIPC, 2,5-hexanedione and methylmercury (II) chloride, of which the mechanisms of action are well known.[4–17] We used a differential staining technique with brilliant blue R and safranin O[18] and an indirect immunofluorescence technique[19] with monoclonal anti-α-tubulin antibody for detecting the differences in the effects of IKP-104 from those of the other chemicals. We found that IKP-104 caused similar changes of mitotic profile and microtubule dynamics to those induced by COL and VLB. The results are presented here.

MATERIALS AND METHODS

Chemicals and antitumor agents IKP-104 (purity 99.5%) was obtained from K-I Research Institute, Shizuoka, and dissolved in dimethyl sulfoxide.

Cell lines B16BL6 cell line established from murine melanoma was used in this study. The cells have been maintained and grown in Eagle's minimum essential medium supplemented with 10% fetal bovine serum. They were incubated at 37°C in a humidified 5% CO_2 atmosphere.

Treatment of cultured cells with chemicals Cells (6×10^3 cells for a differential staining observation and 1×10^4 cells for immunofluorescence observation) were seeded on sterile coverslips placed in 35 mm plastic petri dishes. The 3-day- (for a differential staining observation) or 24-h- (for immunofluorescence observation) cultured cells were exposed to 3.6 μg/ml IKP-104, a concentration equivalent to 70% of its 48-h IC_{50}.

Observations of mitotic figures of cells Cells were rinsed with PBS(−) at 1, 3, 6, 9, 12, 24 and 48 h after exposure to IKP-104, and fixed with fresh fixative (3:1 methanol/acetic acid) containing 4 m*M* $MgCl_2$ and 1.5 m*M* $CaCl_2$ for 15 min at room temperature. Following three 15-min fixative treatments, cells were stained with a mixture of brilliant blue R and safranine O, essentially according to

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[4] The abbreviations used are: COL, colchicine; VLB, vinblastine sulfate; CIPC, isopropyl *N*-(3-chlorophenyl) carbamate; IC50, 50% growth-inhibitory concentration; PBS(−), Ca^{2+}, Mg^{2+}-free phosphate-buffered saline; MES, 2-(*N*-morpholino)-ethanesulfonic acid; EGTA, ethyleneglycol-*O*,*O*′-bis(β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; GTP, guanosine-5′-triphosphate; MOPS, 3-(*N*-morpholino)propanesulfonic acid buffer; MI, mitotic index.

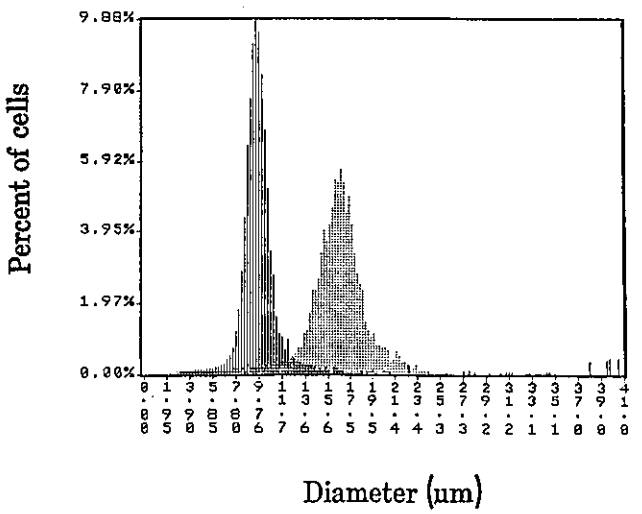

Fig. 1. Changes of diameter of B16 melanoma cells exposed to IKP-104. Cells exposed to 3.6 μg/ml IKP-104 were fixed with 70% ethanol at 0 (▥) and 48 h (▩), and their diameters were measured by a particle analyzer.

the procedure of Wissinger *et al.*[18] and Parry *et al.*[20] Two slides were prepared for each treatment and coded so that they could be scorded blind. Mitotic figures per 1,000 cells on a slide were alalyzed. Polyploid and multinucleate cells per 500 interphase cells on a slide were also analyzed. A cell with a nucleus more than 11 μm in diameter (or short diameter in the case of atypical nucleus) was classified as polyploid from the results of grading analysis with a particle analyzer (Multisizer, Coulter) (Fig. 1).

Indirect immunofluorescence observation of α-tubulin Immunofluorescent preparations were made at 20 min, 2 and 24 h after exposure to IKP-104 and at 22 h after the fresh medium following 2-h exposure, according to the modified method of Yoshida.[21] The cells on coverslips were rinsed for 30 s in A buffer (100 m*M* MES, pH 6.8; 1 m*M* EGTA; 0.5 m*M* $MgCl_2$ and 1 m*M* GTP) containing 0.5% Triton X, and were fixed for 30 min in 1% glutaraldehyde/1% Triton X/0.1 *M* MOPS buffer (pH 6.9). After being rinsed in 0.1 *M* MOPS buffer, the cells were dehydrated gradually through 70% or 90% ethanol for 10 min at 0°C and treated twice with freshly prepared $NaBH_4$ solution (0.5 mg/ml in 95% ethanol) at 0°C for 10 min each. Coverslips were rinsed in 0.1 *M* PBS(−), and treated with 500-fold monoclonal anti-α-tubulin (Biomakor, Israel) for 30 min at 37°C. The cells were rinsed three times for 10 min with 0.1 *M* PBS(−) and then incubated with 100-fold-diluted second antibody (FITC-labeled antimouse Fab fragment; Biomakor) for 30 min at 37°C. After extensive rinsing in PBS(−), the coverslips were mounted with 90% glycerol containing 5% *n*-propyl gallate on microscope slides. Cells were observed with a light fluorescence microscope.

Electron microscopic observation of microtubules Electron microscopic preparations were prepared at 2 h after exposure to the chemicals. Cells in a 35 mm plastic petri dish were fixed with 2.5% phosphate-buffered glutaraldehyde for 3 h and 2.5% glutaraldehyde/2% tannic acid for 1 h and then postfixed with 1% OsO_4 for 30 min. Cells were dehydrated with 50, 70, 90, 95 and 100% ethanol following block-staining with 2% aqueous uranyl acetate for 30 min, and embedded in Epon covered with gelatin capsules. After polymerization of Epon, the capsules were cut from the petri dish and shaken in xylol to dissolve the plastic. Thin sections were cut with an ultramicrotome and stained with uranyl acetate and lead citrate. They were examined with an electron microscope (JEM 1200EX, JEOL) at 100 kV.

RESULTS

Effects on mitotic figures of cells The conventional stages of mitosis, prophase, metaphase, anaphase and telophase, were observed in normal cells. The red chromosomes stained with safranine O and the blue bipolar mitotic spindles stained with brilliant blue R were clearly visible. The aberrant metaphase cells were analyzed according to the criteria of Lafi *et al.*[10]:

1) Chromosome cluster: Chromosomes were clustered in the center region of the cytoplasm. A few short spindles were observed, but bipolar spindles were not formed.

2) Scattered chromosomes: The spindle was completely absent, and chromosomes were scattered within the cytoplasm.

3) Chromatin damage: The real chromosome configuration was not observed. However, chromatin materials were condensed in a central region or dispersed throughout the cytoplasm.

The mitotic profiles of B16 melanoma cells exposed to IKP-104 are summarized in Table I. Mitotic cells accumulated at 1 h after exposure to 3.6 μg/ml IKP-104, and the MI of the cells increased in a time-dependent manner, reaching a maximum at 12 h. The conventional mitotic division figures were absent in the mitotic cells accumulated, and aberrant metaphase figures were observed. Aberrant metaphase cells had no mitotic spindles and had chromosomes scattered within the cytoplasm, or chromatin materials condensed in a central region or dispersed throughout the cytoplasm. The cells with scattered chromosomes decreased for 48 h after exposure, whereas those with chromatin damage increased. The exposure to IKP-104 also induced a lot of polyploid and multinucleate cells at 24 and 48 h after exposure.

Table I. Mitotic Profiles of B16 Melanoma Cells Exposed to IKP-104

Chemicals	Conc. (μg/ml)	Time (h)	Mitotic index[a]	Incidence of conventional mitotic phase (%)[b]				Aberrant mitotic phase (%)[c]				Cell morphology[d]		
				Pro	Meta	Ana	Telo	C. dam	C. clust unip	C. clust multp	S. chrom	mono	mult	poly
Control	0	1	3.9	13.0	39.0	13.0	35.0	0.0	0.0	0.0	0.0	100	0	0
		3	3.6	13.9	37.5	12.5	36.1	0.0	0.0	0.0	0.0	99.9	0.1	0
		12	3.6	16.9	39.4	11.3	31.0	0.0	1.4	0.0	0.0	99.7	0.3	0.2
		24	3.8	21.3	36.0	8.0	34.7	0.0	0.0	0.0	0.0	99.5	0.5	0
		48	3.9	26.0	33.8	6.5	31.2	0.0	2.5	0.0	0.0	99.5	0.5	0.1
IKP-104	3.6	1	6.4	0.0	0.0	0.0	0.0	2.3	0.0	0.0	97.7	100	0	0
		3	11.8	0.0	0.0	0.0	0.0	9.3	0.0	0.0	90.7	99.6	0.4	0.4
		12	30.9	1.3	0.0	0.0	0.0	14.2	0.0	0.0	84.5	94.2	5.8	4.0
		24	7.0	7.2	0.0	0.0	0.0	18.0	0.0	0.0	74.8	83.0	17.0	35.6
		48	13.1	8.8	0.0	0.0	0.0	20.2	0.0	0.0	71.0	77.5	22.5	62.5

a) Percent of dividing cells per 1,000 cells.
b) Pro: prophase, Meta: metaphase, Ana: anaphase, Telo: telophase.
c) C. dam: chromosome damage, C. clust: chromosome cluster, unip: uni polar, multp: multi polar, S. chrom: scattered chromosomes.
d) Percent of mono and multi nucleate or polyploid cells per 500 interphase cells., mono: mono nucleus, mult: multiple nuclei, poly: polyploid.

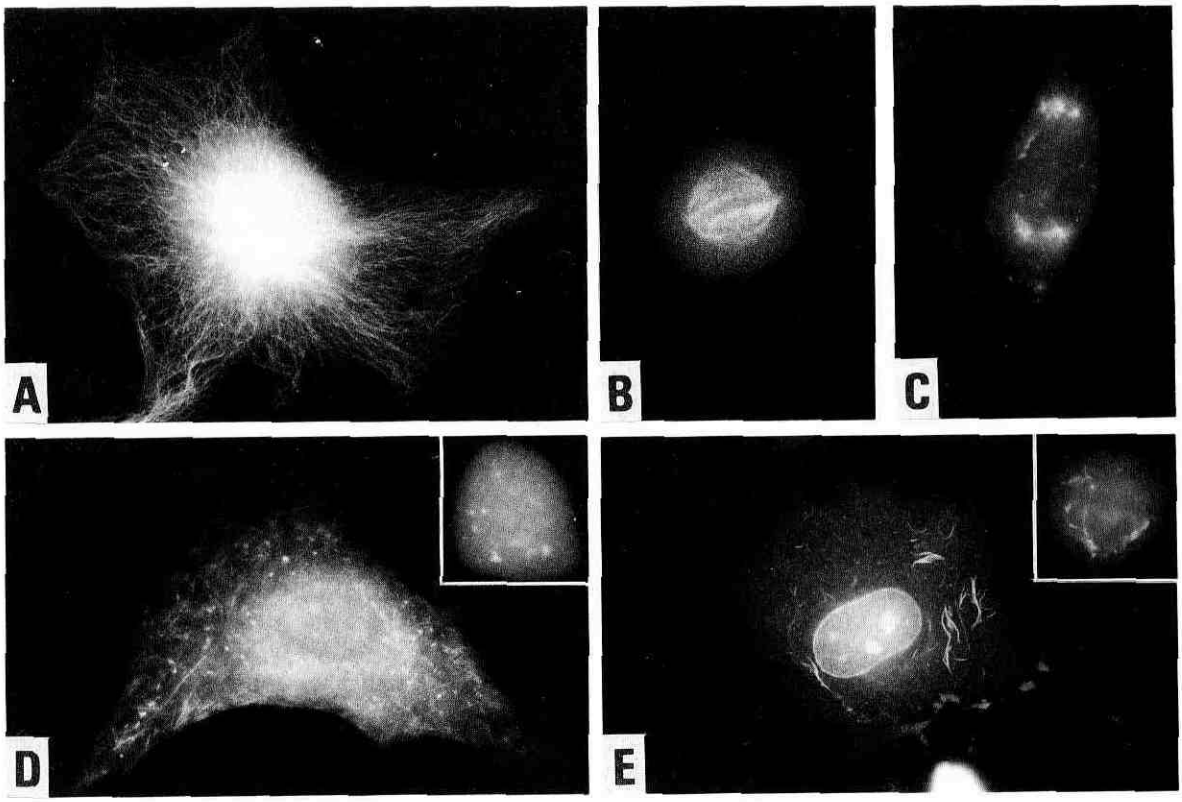

Fig. 2. Indirect immunofluorescence figures of B16 melanoma cells, which were exposed to IKP-104, and stained with anti-α-tubulin antibody. A and B: Normal figures. Interphase cells with fine network of microtubules (A) and metaphase cells with spindles are observed. C–E: Cells exposed to 3.6 μg/ml IKP-104. IKP-104 caused disappearance of mitotic spindles at 20 min after exposure (C), and then cytoplasmic or spindle microtubules, resulting in the appearance of a few fragments at 2 h (D). IKP-104 induced a few thick bundles in cytoplasm (E) at 24 h (direct ×600).

Effects on cellular microtubule dynamics The microtubule dynamics in normal B16 melanoma cells stained by indirect immunofluorescence (anti-α-tubulin) were as follows. A fine reticular network of microtubules (Fig. 2A) of interphase cells disappeared during prophase, and mitotic spindles (Fig. 2B) were formed in metaphase. Kinetochore microtubules shortened, astral or interzonal microtubules were well developed during anaphase and telophase, and the microtubule network was formed again in early interphase following cell division.

IKP-104 depolymerized spindle microtubules of mitotic cells at 20 min after exposure (Fig. 2C), but no change of the microtubule network was observed in interphase cells. The microtubule network in interphase cells exposed to IKP-104 disappeared gradually, and faint fluorescence accompanied with a few microtubular fragments diffused in the cytoplasms of mitotic and interphase cells appeared at 2 h (Fig. 2D). At 24 h, microtubules formed a few thick bundles in the cytoplasm, and in many cases the bundles surrounded the nucleus (Fig. 2E). When IKP-104 was removed by transferring the cells to fresh medium at 2 h after exposure, microtubule re-formation was observed at 22 h after transfer.

Electron microscopic observations of cellular microtubules The cellular microtubules of cells exposed to IKP-104 were examined by TEM. Though many normal microtubules forming a cytoplasmic network were observed in untreated cells (Fig. 3A), the exposure to 3.6 μg/ml IKP-104 for 2 and 24 h resulted in the induction of different sizes of bundles composed of a large number of wavy protofilaments (Fig. 3B).

DISCUSSION

In this study, we examined the effects of IKP-104 on the mitotic profile and cytoskeletal microtubules of B16 melanoma cells. IKP-104 induced accumulation of cells in aberrant metaphase, characterized by the appearance of scattered chromosomes and chromatin damage instead of the conventional mitotic division figures, at 1 h and more after exposure. Scattered chromosomes are considered to be the same as an abortive mitotic figure called "c-metaphase" induced by COL. Cells showing chromatin damage have severe aberrations to both spindle and chromosome, reflecting the lethality and toxicity of the chemicals.[10] So chromatin damage is considered not to be a specific change induced by IKP-104. IKP-104 also induced polyploid and multinucleate cells at 24 and 48 h. These findings were confirmed by previous flow cytometry analysis.[1] Terao[22] reported on the mechanism of

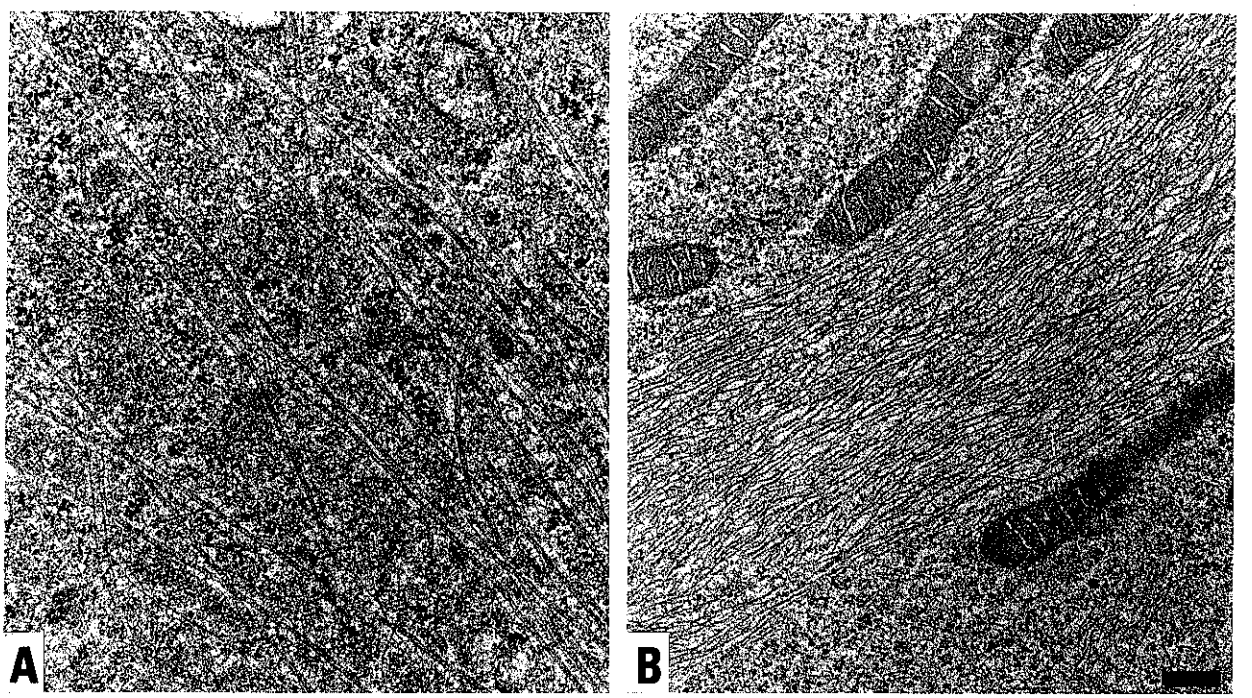

Fig. 3. Electron micrographs of cytoplasmic microtubules of B16 melanoma cells exposed to IKP-104. A: Normal cell with many microtubules. B: Cell exposed to 3.6 μg/ml IKP-104 for 24 h. Note different size of bundles composed of a large number of protofilaments (direct $\times$20,000; scale bar 200 nm).

multinucleation induced by mitotic arrestants, suggesting that the nuclear membranes formed around each chromosome united with each other in normal metaphase because of collectivity by mitotic spindles, but were not able to unite with each other in c-metaphase because of dispersion of chromosomes. Multinucleation by IKP-104 was considered to be induced by a similar mechanism.

The changes of mitotic profile of cells observed with differential staining could be explained by the results of immunofluorescence observation. The immunofluorescence study showed that IKP-104 diminished the cytoplasmic network of microtubules and mitotic spindles, resulting in a few fragments or bundles at 2 and 24 h after exposure. These fragments or bundles were observed as different sizes of bundles of wavy protofilaments, resembling those induced by 10^{-6} *M* VLB[23] in electron microscopic observation.

These changes of mitotic profile and cytoskeletal microtubules of B16 melanoma cells induced by IKP-104 were similar to those induced by COL or VLB, especially VLB, because COL did not induce any microtubule fragments and bundles, whereas VLB formed short microtubular bundles at low concentration (10^{-6} *M*) in immunofluorescence and electron microscopic observation. It is known that VLB or COL binds directly to soluble tubulin at one or more sites, though the sites for VLB and COL are not the same, resulting in inhibition of tubulin polymerization, and also depletes the soluble tubulin pool, consequently evoking depolymerization through the equilibrium with polymerized microtubules.[2] It is also known that VLB aggregates tubulin to form paracrystals.[23, 24] IKP-104 also inhibited polymerization of tubulin subunits at 1.31×10^{-6} *M in vitro* (unpublished data). Its mechanisms of action seem similar to those of VLB judging from the similarity of the effects on cytoskeletal microtubules found in this study.

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