Jpn. J. Cancer Res. 83, 211–218, February 1992

Interaction of the Tumor Inhibitor IKP-104, a 4(1*H*)-Pyridinone Derivative, with Microtubule Proteins

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The effects of a mitotic arrestant, IKP-104, which has an antitumor activity, on the *in vitro* polymerization and depolymerization of rat brain microtubules were investigated. IKP-104 inhibited microtubule polymerization at concentrations greater than 0.71×10^{-6} *M*, and its IC_{50} value was determined to be 1.31×10^{-6} *M* by probit analysis. Fifty-two percent of pre-polymerized microtubules depolymerized at 1.31×10^{-6} *M* IKP-104. Electron micrographs of microtubules taken immediately after treatment with 1×10^{-3} *M* IKP-104 revealed a fraying of microtubule ends into elongated coil-like filaments, which were composed of 2 or 3 protofilaments. When microtubule protein treated with 1×10^{-3} *M* IKP-104 was cleaved by trypsin, fragments of 41, 36, 34, 23, 21, 19 and 16 kilodaltons (kDa) derived from α-tubulin were produced. In particular, the 19, 23 and 34 kDa fragments were characteristically observed in the trypsin cleavage of microtubules treated with IKP-104, and these fragments were not observed with untreated microtubules. The effects of IKP-104 on microtubule protein mentioned above were mostly similar to those of vinblastine (VLB) and we suggest that IKP-104 bound to the site or sites near "VLB-binding site or sites" of α-tubulin subunit, resulting in induction of conformational changes.

Key words: Inhibition of polymerization — IKP-104 — Microtubule — Tubulin

Morphological and flow cytometric changes of cultured tumor cells treated with the antitumor agent IKP-104, 2-(4-fluorophenyl)-1-(2-chloro-3,5-dimethoxyphenyl)-3-methyl-6-phenyl-4(1*H*)-pyridinone, are similar to those induced by mitotic arrestants such as COL[4] or vinca alkaloids.[1] Microscopic observations using brilliant blue R-safranin O double staining showed that IKP-104 arrested the cell cycle of cultured B16 melanoma cells in the mitotic phase and accumulated abnormal metaphase cells called "c-metaphase."[2] Immunofluorescence microscopy using monoclonal tubulin antibody revealed that IKP-104 diminished cytoplasmic microtubules in the interphase and spindle microtubules in the mitotic phase of cultured B16 melanoma cells, resulting in the formation of a few short microtubule bundles resembling those induced by VLB.[3,4] It is known that both COL and VLB inhibit microtubule polymerization and cause microtubule depolymerization.[5,6] It is also known that COL binds at the COOH-terminal one-third of the α-tubulin subunit,[7] while VLB binds at two sites of α-tubulin[8] and stabilizes the COL binding to α-tubulin.[9,10] From the similarity of the effects of IKP-104 on cytoskeletal microtubules of B16 cells to those of VLB, it is suggested that IKP-104 could inhibit microtubule polymerization.

In this paper, we compared the interaction of IKP-104 with rat brain microtubule protein to that of COL or VLB in order to characterize its binding mode to microtubule protein.

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[4] The abbreviations used are: COL, colchicine; VLB, vinblastine sulfate; MES, 2-(*N*-morpholino)ethanesulfonic acid; EGTA, ethyleneglycol-*O,O'*-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; GTP, guanosine-5'-triphosphate; PMSF, phenylmethylsulfonyl fluoride; NCDC, 2-nitro-4-carboxyphenyl-*N,N*-diphenyl carbamate; SDS, sodium dodecyl sulfate; BPB, bromophenol blue; TCA, trichloroacetic acid; PVDF, polyvinylidene difluoride; DAB, 3,3'-diaminobenzidine; IC_{50}, 50% inhibitory concentration; pp(Ch2)pG, guanyl-5'-methylene diphosphonate.

MATERIALS AND METHODS

Chemicals and antitumor agents IKP-104 (purity 99.5%) was obtained from K-I Research Institute, Shizuoka. Reagent grade COL and VLB were purchased from Wako Pure Chemical Industry Co., Osaka. Trypsin (diphenyl carbamyl chloride-treated) and chymotrypsin (N^{α}-*p*-tosyl-L-lysine chloromethyl ketone-treated) were purchased from Sigma, St. Louis, MO.

Purification of microtubules Microtubule protein from Wistar rat brain was purified by 2 cycles of assembly and disassembly in buffer A (100 m*M* MES, pH 6.8; 1 m*M* EGTA; 0.5 m*M* $MgCl_2$ and 1 m*M* GTP), essentially according to the procedure of Shelanski *et al.*[11] Microtubule protein was adjusted to 4 *M* glycerol and stored at −80°C until required. Protein concentration was deter-

mined by the method of Lowry *et al.*[12] with bovine serum albumin as a standard.

Polymerization and depolymerization assay The polymerization and depolymerization of microtubules *in vitro* was measured by turbidity assay[13] at 350 nm using a Hitachi double-beam spectrophotometer equipped with a thermostatically controlled cell holder. Microtubule polymerization was initiated in buffer A by warming the sample (1.4 mg/ml protein) from 0 to 37°C, and depolymerization was initiated by cooling the sample from 37 to 4°C. The agents were dissolved in dimethylsulfoxide, and were treated in the reaction samples at 0.5% (final concentration).

Electron microscopy Samples (1.4 mg/ml protein) were diluted 10-fold into buffer A. Carbon-coated collodion 200 mesh copper grid (Ohken Trading Co., Tokyo) was placed on a drop of diluted sample solution, and after 30 s excess solution was withdrawn from the grid with filter paper. The samples were then negatively stained with 2% uranyl acetate solution for 1 min and air-dried. Specimens were examined with a JEOL 1200 EX electron microscope at 100 kV.

Limited proteolysis Limited proteolysis[14, 15] of samples (1.4 mg/ml protein) with trypsin and chymotrypsin was carried out by incubation at 25°C. The protease concentration was 2% (w/w) in each case. The reaction was arrested by the addition of 1 m*M* PMSF to trypsin and NCDC to chymotrypsin, and samples were analyzed immediately by gel electrophoresis.

Gel electrophoresis Proteolytic samples were boiled for 2 min in the presence of 0.25 *M* Tris-HCl buffer (pH 6.8), 10% glycerol, 5% 2-mercaptoethanol, 2% SDS and 0.01% BPB. Electrophoresis was performed in 5–15% gradient acrylamide-SDS slab gels containing 6 *M* urea according to the procedure of Laemmli[16] with a constant current of 20 mA/gel, and the gels were fixed with 15% TCA and stained with 0.25% Coomassie brilliant blue R-250.

Immunoblotting Proteins were transferred from gels to PVDF membrane according to the procedure of Hirano[17] and Phelps.[18] Proteins were treated with monoclonal anti-α- and anti-β-tubulins (Biomakor, Israel), detected with peroxidase-labeled anti-mouse Fab fragment (Biomakor) and visualized with DAB.[19]

Indirect immunofluorescence observation of α-tubulin B16BL6 murine melanoma cells (1×10^4 cells) were seeded on sterile coverslips placed in 35 mm petri dishes. The 24-h-cultured cells were treated with mixtures of 3.6 μg/ml IKP-104 and 0.56 μg/ml COL, or IKP-104 and 10 μg/ml VLB for 2 h, and with IKP-104 alone for 2 h after 2-h-exposure to COL or VLB. Immunofluorescence observations were conducted according to the previous report.[2]

RESULTS

Effects on polymerization and depolymerization of microtubules The inhibitory effect of IKP-104 on microtubule polymerization was compared with that of COL or VLB. IKP-104 inhibited both the initial rate and extent of microtubule polymerization in a concentration-dependent manner (Fig. 1A). From the data on the extent of microtubule polymerization, the IC_{50} value of IKP-104 was determined by probit analysis[20] to be 1.31×10^{-6} *M*. COL and VLB also inhibited microtubule polymerization (Figs. 1B and 1C). The IC_{50} values of COL and VLB were 1.20×10^{-6} *M* and 0.75×10^{-6} *M*, respectively.

The effects of IKP-104 on depolymerization of prepolymerized microtubules are shown in Fig. 2. The agents were added at concentrations equivalent to IC_{50} for microtubule polymerization after 30 min incubation of microtubules at 37°C. Depolymerization was initiated by chilling to 4°C. IKP-104 depolymerized 52% of the microtubules at 1.31×10^{-6} *M*. COL and VLB depolymerized 95% at 1.20×10^{-6} *M* and 65% at 0.75×10^{-6} *M*, respectively.

Electron microscopic observation Morphological changes of microtubules treated with IKP-104 were examined with an electron microscope. In samples taken 30 min after the treatment with 1×10^{-3} *M* IKP-104 at 37°C, no polymerized microtubules were observed. However, elongated coil-like filaments (Fig. 3A), which were composed of 2 or 3 protofilaments, were observed. In samples taken at 2 min after IKP-104 treatment, polymerized microtubules, some of which contained curled filaments at their ends, were observed (Fig. 3A). On the other hand, a few small aggregates were observed in the sample treated with 1×10^{-3} *M* COL (Fig. 3B), and a large number of coil and ring aggregates were observed in the sample treated with 1×10^{-3} *M* VLB (Fig. 3C).

Limited proteolysis and immunoblot analysis Microtubule protein treated with 1×10^{-3} *M* IKP-104, COL or VLB was digested with trypsin or chymotrypsin for 10 or 20 min at 25°C. It is known that trypsin and chymotrypsin selectively cleave α- and β-tubulin, respectively, under suitably controlled conditions.[14, 15] Cleaved products were analyzed by slab gel polyacrylamide electrophoresis. The molecular weights of fragments obtained in this analysis were estimated from a standard curve obtained with markers (Bio-Rad Labs., Richmond, CA).[21] The origins of cleaved products were confirmed by immunoblotting analysis. The results of limited proteolysis of microtubules treated with IKP-104 are shown in Fig. 4. Bands due to α- and β-tubulin were present at 57 and 55 kDa in the electrophoretic pattern of normal microtubules. The major tryptic products of normal microtubules appeared as fragments of 41 and 16 kDa after 10

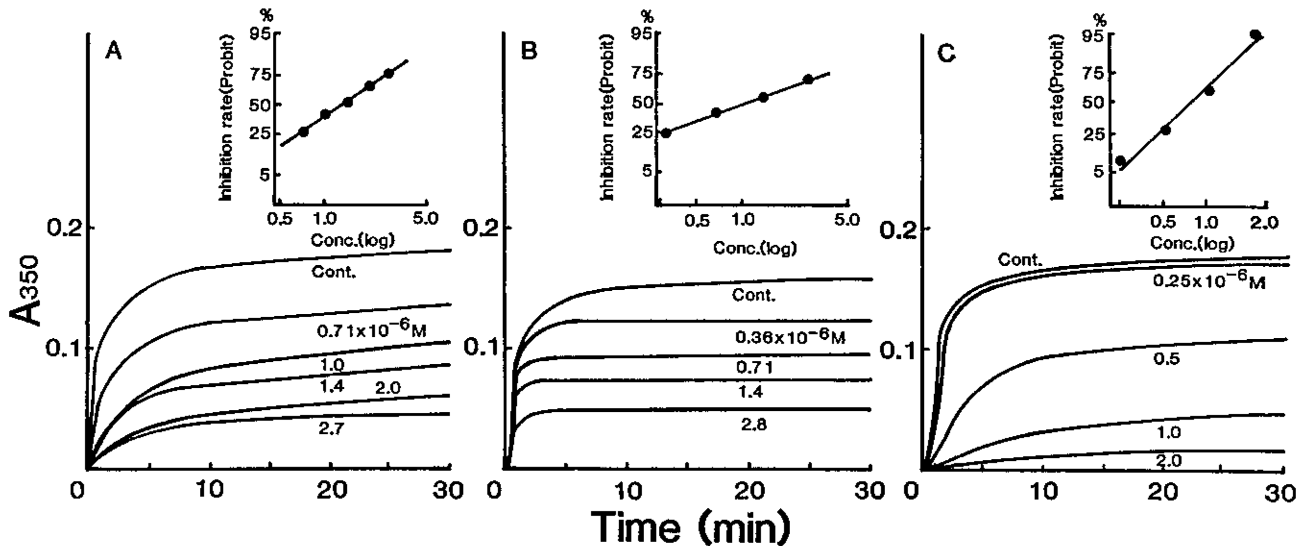

Fig. 1. Inhibitory effects of mitotic arrestants on microtubule polymerization. Microtubule protein (1.4 mg protein/ml) in buffer A was mixed with different concentrations of chemicals at 0°C. Polymerization was initiated by warming to 37°C, and was monitored by turbidimetric assay. A, IKP-104; B, COL; C, VLB.

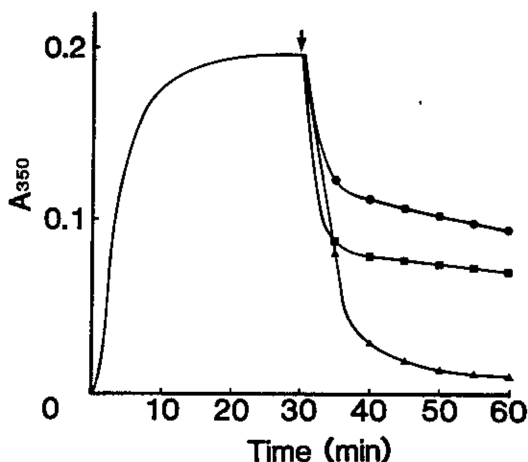

Fig. 2. Effects of mitotic arrestants on microtubule depolymerization. Chemicals were added to microtubule protein (1.4 mg protein/ml) at the termination of pre-incubation for 30 min at 37°C. Depolymerization was initiated by chilling to 4°C. Final concentrations of chemicals were 1.31×10^{-6} *M* IKP-104 (●), 1.20×10^{-6} *M* COL (▲) and 0.75×10^{-6} *M* VLB (■). The arrow indicates the addition of chemicals.

min incubation, and additional fragments of 36 and 21 kDa were observed after 20 min incubation. When tryptic digestion was performed in the presence of 1×10^{-3} *M* IKP-104, α-tubulin was less susceptible to digestion after 10 min incubation. After 20 min treatment with IKP-104, the production of 36- and 21-kDa fragments increased, and additional peptides of 34, 23 and 19 kDa were observed besides the same fragments as those of normal microtubules. However 41- and 16-kDa fragments were produced at low levels. In the presence of 1×10^{-3} *M* COL, the pattern of tryptic digestion was similar to that of normal microtubules. On the other hand, in the presence of 1×10^{-3} *M* VLB, a similar pattern to that in the case of microtubules treated with IKP-104 was obtained. However, α-tubulin treated with VLB was less susceptible to digestion than that treated with IKP-104, and the fragment of 34 kDa was absent.

The major chymotryptic products of normal microtubules appeared as fragments of 34 and 21 kDa, which were derived from β-tubulin, after 10 and 20 min incubation. When chymotryptic digestion was performed in the presence of 1×10^{-3} *M* IKP-104, COL or VLB, a similar digestion pattern to that of normal microtubules was obtained, and no difference among the electrophoretic patterns was observed.

Immunoblotting analysis of the tryptic and chymotryptic fragments of microtubules treated with IKP-104 for 20 min were conducted in order to reveal their origins (Fig. 5). Because the tryptic fragments that reacted to monoclonal α-tubulin antibody were 16, 19, 21 and 23 kDa, it was confirmed that these fragments and their paired fragments, 41, 36 and 34 kDa, respectively, were derived from α-tubulin. Further, the chymotryptic

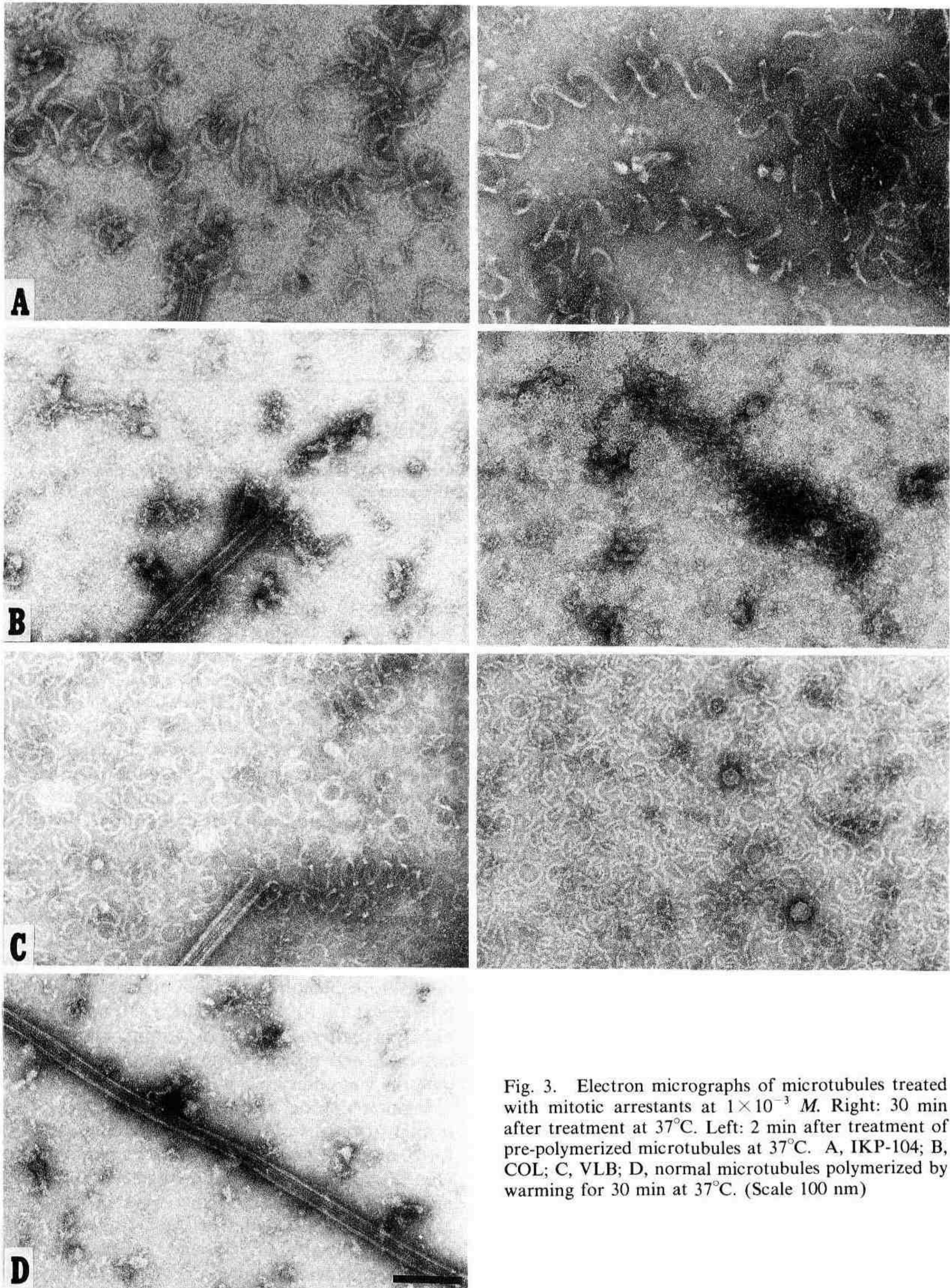

Fig. 3. Electron micrographs of microtubules treated with mitotic arrestants at 1×10^{-3} *M*. Right: 30 min after treatment at 37°C. Left: 2 min after treatment of pre-polymerized microtubules at 37°C. A, IKP-104; B, COL; C, VLB; D, normal microtubules polymerized by warming for 30 min at 37°C. (Scale 100 nm)

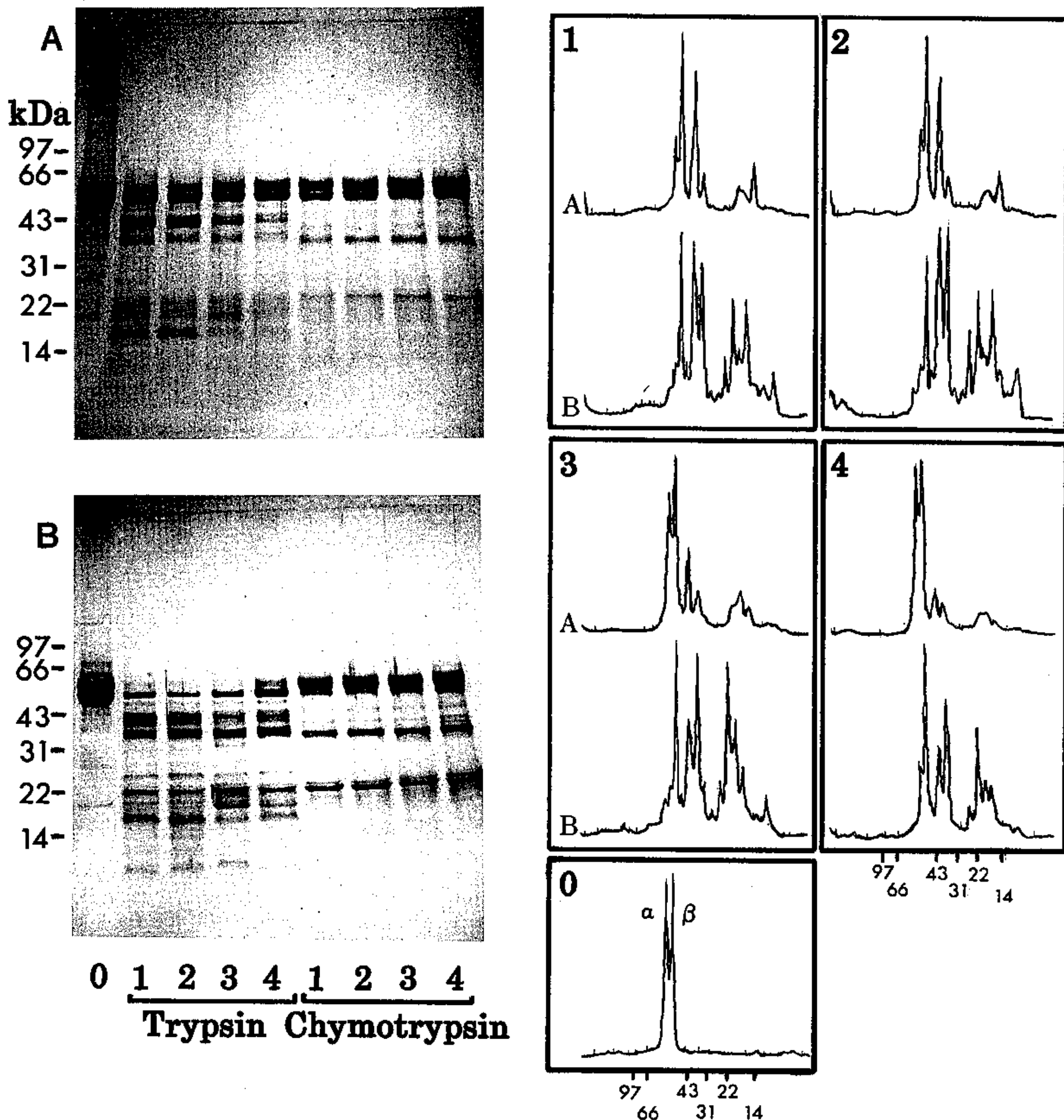

Fig. 4. Polyacrylamide slab gel electrophoresis of microtubules digested with trypsin and chymotrypsin. Microtubules (1.4 mg/ml) were incubated with 2% (w/w) trypsin or chymotrypsin at 25°C in the absence of chemicals (lane 1) and in the presence of 1×10^{-3} *M* COL (lane 2) or IKP-104 (lane 3) or VLB (lane 4). Lane 0 was undigested microtubules. A: Microtubules incubated for 10 min. B: Microtubules incubated for 20 min. The electrophoresis was performed in 5–15% gradient acrylamide gels containing 6 *M* urea. Densitometric charts are also shown. Numbers in the charts correspond to the lane numbers in figures showing electrophoretic analysis of trypsin digests.

fragments, 21 and 34 kDa, were confirmed to be derived from β-tubulin.

Immunofluorescence observation of cellular microtubules Effects of COL and VLB on the changes of cytoplasmic microtubules of B16 cells treated with IKP-104 were examined. In the cells treated with mixtures of IKP-104 and COL, or VLB, the microtubule network disappeared, and a few bundles composed of large numbers of proto-

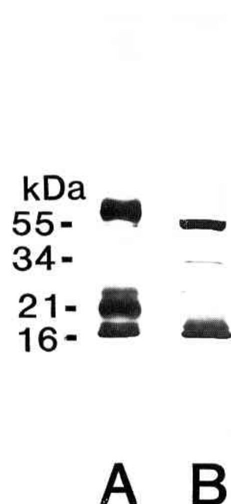

Fig. 5. Immunoblotting of proteolytic microtubules treated with IKP-104. After transfer from gels to PVDF membrane, proteolytic proteins were treated with monoclonal anti-α- and anti-β-tubulins. A: Tryptic microtubules treated with anti-α-tubulin. B: Chymotryptic microtubules treated with anti-β-tubulin.

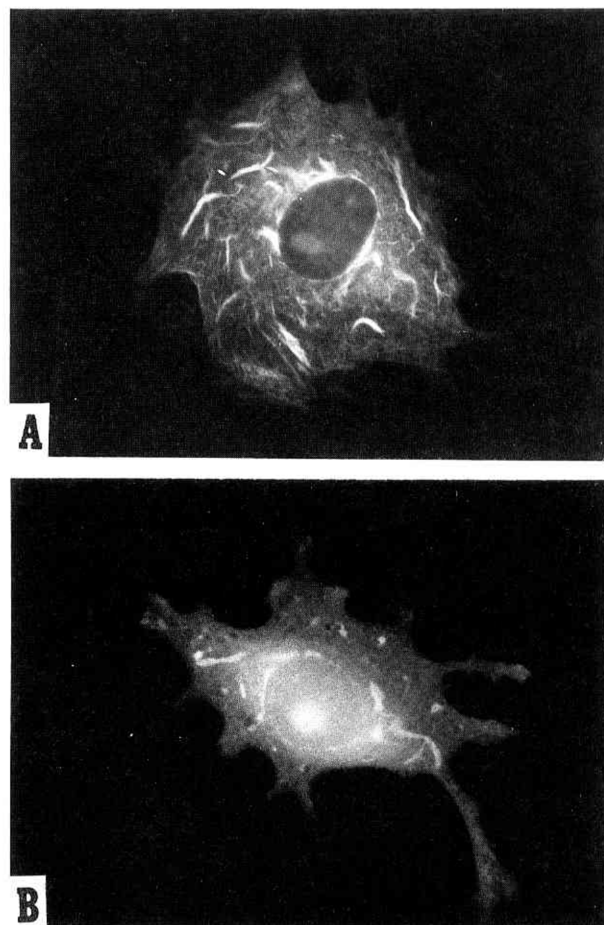

Fig. 6. Indirect immunofluorescence examination of B16 melanoma cells treated with IKP-104. A: Cells treated with a mixture of 3.6 μg/ml IKP-104 and 0.56 μg/ml COL for 2 h. B: Cells treated with 3.6 μg/ml IKP-104 for 2 h following 2-h exposure to 10 μg/ml VLB. (Direct $\times$600)

filaments were formed (Fig. 6A). Similar changes of cellular microtubules were observed in the cells treated with IKP-104 following 2-h exposure to COL. When the cells were treated with IKP-104 for 2 h following 2-h exposure to VLB, disaggregating paracrystals and a few bundles of protofilaments were observed in the cytoplasm (Fig. 6B).

DISCUSSION

We have studied the interaction of a mitotic arrestant, IKP-104, with rat brain microtubules by turbidimetry, electron microscopy and limited proteolysis in order to elucidate its mode of action.

IKP-104 inhibited microtubule polymerization and its inhibitory activity was almost the same as that of known mitotic arrestants such as COL and VLB. IKP-104 also caused the depolymerization of pre-polymerized microtubules. Fifty-two percent of microtubules depolymerized at 1.31×10^{-6} M IKP-104 (equivalent to the IC_{50} value of polymerization) and the depolymerization activity is similar to that of VLB. In contrast, 95% of microtubules were depolymerized at 1.2×10^{-6} M COL.

Under the electron microscope, the microtubules treated with 1×10^{-3} M IKP-104 revealed ends fraying into elongated coil-like filaments, each composed of 2 or 3 protofilaments. These structures were also observed in microtubules treated with IKP-104 at concentrations of more than 8×10^{-6} M. These structures were considered to be similar to ribbon structures, which are transient intermediates formed during normal polymerization of microtubules, though they are usually detected in abnormal processes of polymerization.[22] For example, ribbon structures were formed from microtubules in the presence of diethylstilbestrol[22] or pp(CH_2)pG instead of GTP.[23] It was considered that these structures formed short bundles, composed of a large number of wavy protofilaments, in the cells treated with IKP-104 and examined by immunofluorescence microscopy.[2] The microtubules treated with the same concentration of VLB revealed coil and ring structures attributed to ag-

gregation, and the appearance of these structural changes is tubulin concentration-dependent.[24,25] Microtubules treated with IKP-104 resembled electron-microscopically those treated with VLB. However, in the former case, the structural changes were independent of the concentration of tubulin (data not shown). COL-treated microtubules, on the other hand, showed only a few small aggregates.

Limited proteolysis of tubulin has been studied to clarify the interaction and binding site of GTP or microtubule-associated proteins (MAPs) or mitotic arrestants.[26-28] It is known that trypsin mainly cleaves Lys or Arg bonds at the COOH-terminal, and chymotrypsin cleaves Tyr, Phe, Trp or Leu.[29] Brown and Erickson[14] reported that α-tubulin was preferentially digested by trypsin, and the β-subunit was digested by chymotrypsin. They also reported that tryptic digestion of microtubules resulted in the production of the fragments of 41, 35, 14 and 5 kDa. Serrano *et al.*[7] reported that normal α-tubulin was digested into 2 fragments of 36 and 16 kDa, and in the presence of COL, digestion yielded additional fragments of 41, 11 and 5 kDa. Our data indicated that both normal and COL-treated α-tubulin were digested by trypsin to fragments of 41 and 16 kDa mainly, and 36 and 21 kDa secondarily, in accordance with the finding of Brown and Erickson. In contrast, when 1×10^{-3} *M* IKP-104 was present α-tubulin was less susceptible to digestion after 10 min incubation, while the production of 36- and 21-kDa fragments increased and additional peptides of 34, 23 and 19 kDa were observed after 20 min incubation. The tryptic digestion pattern of purified tubulin in the presence of IKP-104 was the same as that of microtubules (data not shown). The effect of IKP-104 on the tryptic digestion of microtubules was similar to that of VLB except for the formation of the 34-kDa fragment. Chymotryptic digestion patterns of β-subunit were the same. In Fig. 7, we propose a cleavage map in the presence or absence of IKP-104 based on the results of limited proteolysis. No- or COL-treated α-tubulin was digested by trypsin at site a and then at site b secondarily (Fig. 7A). α-Tubulin of microtubules treated with IKP-104 was resistant to the tryptic digestion at site a, but it was markedly digested by trypsin at site b and new sites, c and d, because of the binding of IKP-104 to α-tubulin (Fig. 7B). The chymotryptic digestion of β-tubulin was not affected by the binding of IKP-104.

From the changes of tryptic cleavage sites in the tubulin molecule when the microtubules were digested in the presence of IKP-104 and the similarity of most findings on its effects on microtubules to those of VLB, we suggest that IKP-104 mainly binds to α-tubulin near the region of 41 kDa from the N-terminal, near where VLB binds, resulting in induction of a conformational change of tubulin. However, in the immunofluorescence study, induction of the morphological changes of cellular microtubules by IKP-104 was observed in the presence of COL or VLB, suggesting that binding of IKP-104 on the microtubules is not competitive with COL or VLB.

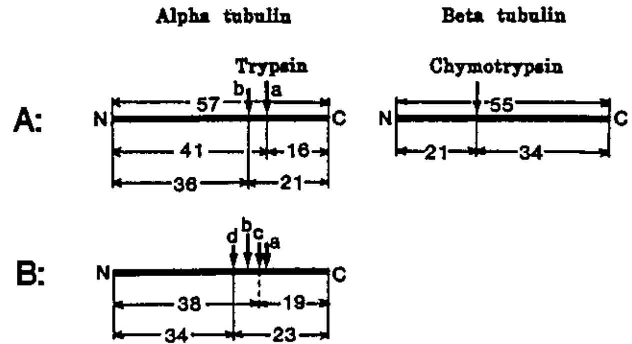

Fig. 7. Schematic diagram of the proteolytic sites of microtubules in the absence or in the presence of IKP-104. Trypsin or chymotrypsin attacks only α- or β-tubulin, respectively. Alpha tubulin: When microtubules were digested with trypsin in the absence of IKP-104, α-tubulin is cleaved at site a, producing 41- and 16-kDa fragments, and is also cleaved secondarily at site b producing 36- and 21-kDa fragments (A). In the presence of 1×10^{-3} *M* IKP-104, α-tubulin is cleaved at sites b and c, producing 36-, 21- and 19-kDa fragments, and is also cleaved at site a (B). The 23- and 34-kDa fragments were observed characteristically in the presence of IKP-104, and it is suggested that α-tubulin is cleaved at site d. Beta tubulin: β-Tubulin is cleaved at only one site, producing 34- and 21-kDa fragments. No differences were observed in proteolytic patterns of microtubules in the presence or absence of IKP-104.

We examined the antitumor activities of IKP-104 against VCR-resistant P388 cells *in vivo*; its ILS values were 41% and 68% at 5 and 10 mg/kg (ip-ip, single injection at 1 day), respectively, in contrast with VCR (ILS 10% at 1.5 mg/kg), VLB (14% at 2 mg/kg) and COL (20% at 1 mg/kg). So we consider that IKP-104 has a different binding mode from vinca alkaloids or COL, and it might be expected that IKP-104 will be effective against tumors resistant to other spindle poisons.

ACKNOWLEDGMENTS

The authors thank the staff of JEOL Ltd. for technical assistance in the electron microscopic observations.

(Received July 24, 1991/Accepted November 9, 1991)

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