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Antimicrotubule Effects of the Novel Antitumor Benzoylphenylurea Derivative HO-221

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Abstract The antitumor action of HO-221, a novel benzoylphenylurea derivative, was studied. The in vitro cytotoxic strength of HO-221 was investigated, as measured by IC_{50} values, compared with those of other drugs with different action mechanisms, using Chinese hamster lung (CHL) cells, mouse leukemia L1210 cells and human promyelocytic leukemia HL-60 cells. Morphological alterations following treatment were observed under a phase contrast microscope, and the mitotic index was determined at regular intervals to check for accumulation of metaphase cells. HO-221 was found to have a very strong toxic effect on all cell types, equal to that of the spindle poisons used as controls. HO-221 also produced the same specific morphological changes as the spindle poisons, with a significant accumulation of metaphase cells. A chromosome analysis of treated cells showed that HO-221 frequently induced polyploid and aneuploid cells, but without accompanying chromosome-breaking activity. An in vivo mouse bone marrow micronucleus assay was also carried out. The assay allowed the in vivo identification of a chromosome breaker or a spindle poison through the measurement of the relative sizes of micronuclei produced and erythrocytes. HO-221 was found frequently to induce relatively large micronuclei, an action regarded as specific to spindle poisons. It was thus demonstrated that HO-221 acts as a spindle poison both in vitro and in vivo. In order to investigate the mechanism of this action, a study of tubulin assembly using purified calf brain tubulin was carried out, which demonstrated clearly that HO-221 inhibits microtubule assembly. A detailed investigation of the action mechanism of HO-221 as a spindle poison is now called for.

Key words: HO-221 · Benzoylphenylurea derivative · Antimicrotubule effect

Introduction

HO-221 is a newly developed antitumor compound chosen from among many related compounds [1] because of its excellent antitumor action and its lack of particular toxicities in animal experiments. It has been reported to show significant activity against various murine tumors and human xenografts and appears to be especially effective against solid tumors [2]. It has also been reported that HO-221 shows no cross-resistance to ten antitumor agents used clinically in the treatment of human cancer. In the present study, the antimicrotubule effect of HO-221 was investigated.

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Materials and methods

Chemicals

HO-221 (*N*-[4-(5-bromo-2-pyrimidinyloxy)-3-chlorophenyl] -*N'*-(2-nitrobenzoyl) urea; molecular mass 492.67 Da) was synthesized (Fig. 1). For the in vitro study, it was dissolved in dimethyl sulfoxide (DMSO) or in dimethyl-β-cyclodextrin. For the in vivo micronucleus study of its oral administration, it was pulverized by being shaken together with glass beads in a 5% (w/v) HC060 (polyoxyethylene-hydrogenated castor oil 60) solution supplemented with Dynomill (Willy A. Bachofen Co.).

Other substances used were: the spindle poisons vincristine sulfate (VCR), vinblastine sulfate (VBL), colchicine (COL), taxol (TAX) and

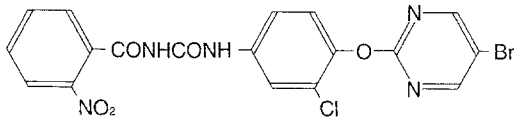

Fig. 1 Chemical structure of HO-221

podophyllotoxin (POD); the metabolic inhibitors cytosine arabinoside (Ara-C), and 5-fluorouracil (5FU); the DNA synthesis inhibitor aphidicolin (APH); the antitumor antibiotic mitomycin C (MMC); the cytokinesis inhibitor cytochalasin B (CYT). VCR, VBL, TAX., Ara-C, APH, MMC and CYT were purchased from Sigma Chemical Co. (St. Louis, Mo.); COL, POD and 5FU from Wako Chemical Co. (Osaka). VCR, COL, Ara-C, 5FU and MMC were dissolved in saline solution; VBL, TAX, POD, APH and CYT in DMSO.

Cells and animals

Eagle's minimum essential medium with 10% calf serum was used for the CHL Chinese hamster lung cells, and RPMI-1640 with 10% fetal calf serum was used for the mouse leukemia L1210 cells and the human promyelocytic leukemia HL-60 cells. All cells were cultured in an atmosphere containing 5% CO_2 in an incubator at 37°C. The CHL cells (modal chromosomal number 25), used extensively for chromosomal aberration tests [3], were obtained from the Japanese Cell Research Bank (JCRB) of the National Institute of Hygienic Sciences (Tokyo). The L1210 and HL-60 cells were purchased from the Department of Laboratory Products of Dainippon Pharmaceutical Co. (Osaka). Male ICR mice (body weight 31.3–37.3 g, age 8 weeks) were purchased from Charles River Japan (Kanagawa). Feed and drinking water were provided ad libitum.

50% growth inhibition concentrations (IC_{50}), morphological observations and mitotic index

CHL cells seeded at a density of 1.2×10^4/plate were treated with the test drugs after 3 days of culture. L1210 and HL-60 cells were seeded at a density of 2×10^5/plate and treated immediately. The cells were cultured for 24 h (L1210) or 48 h (CHL, HL-60) after treatment with the test drugs, during which time morphological observations were carried out under a phase contrast microscope. At the end of this period, the number of viable cells was counted with a Burker-Turk hematometer using the dye-incorporation method and IC_{50} values were calculated. In addition, cells were sampled at intervals, stained with aceto-orcein and the mitotic index calculated by examining them under a microscope in batches of 500 and determining the proportion of metaphase cells.

Chromosome analysis

CHL cells seeded at 2×10^4/plate were treated with the test drug after 3 days of culture. Chromosome specimens were prepared 48 h after treatment. Specimen cells were treated with 0.1 μg/ml colcemid for 2 h and incubated after trypsinization in 75 m*M* KCl hypotonic solution for 15 min at 37°C. They were then fixed with fixative (methanol/glacial acetic acid, 3:1). A few drops of the cell suspension were placed on slide glasses and stained with Giemsa solution. The number of cells with a chromosomal aberration was determined in 50 or 200 well-spread metaphase cells. Structural aberrations were classified into five types: chromatid gap (ctg), chromatid break (ctb), chromatid exchange (cte), chromosome break (csb) and chromosome exchange (cse). Numerical aberrations were determined by counting the chromosomes under a microscope or on a microphotograph. Cells with over 38 chromosomes (triploid) were judged to be polyploid.

Mouse bone marrow micronucleus assay

HO-221 was administered orally to mice as a single dose of 0 (vehicle), 10, 50, 100, 200, 500, or 1000 mg/kg. Doses were assessed to be in the range of 1/200 to 1/2 LD_{50}. As positive controls, MMC was administered intraperitoneally at a dose of 2 mg/kg and VCR intravenously at a dose of 0.25 mg/kg. Dosages of MMC and VCR represented 1/4 LD_{50} and 1/30 LD_{50}, respectively [4]. Non-treated mice were used as negative controls. Each group consisted of five animals. The mice were sacrificed 24 h after treatment by cervical dislocation, a femur was removed and bone marrow smears prepared. Slides were air-dried, fixed in methanol and stained with Giemsa solution. Under the microscope, the frequency of micronucleated polychromatic erythrocytes (MNPCEs) was measured using samples of 1000 polychromatic erythrocytes (PCEs) from each animal. The number of PCEs per 1000 erythrocytes was also recorded.

Statistical significance was determined using Kastenbaum and Bowman's tables [5] for MNPCEs and Student's *t*-test for PCEs.

In order to analyze the site of action of micronucleus-inducing agents, the diameter of cytoplasm (D) and micronucleus (d) of 100 randomly selected MNPCEs was measured with calipers on a microphotograph. It was found that chromosome-breaking agents induced relatively small ($d < D/4$) micronuclei in over 90% of cases, and spindle poisons relatively large ($d \geqq D/4$) micronuclei in over 50% of cases [6].

Inhibition assay of microtubule assembly and disassembly

Purified tubulin was prepared from fresh calf brain using the twofold operation of microtubule assembly and disassembly [7]. The protein concentration of the prepared tubulin was measured using a protein assay kit (Bio-Rad) and was found to be 2.7 mg/ml. These proteins were determined as a single band on SDS gradient electrophoresis and their purity was estimated at over 95%.

The inhibitory effects of HO-221 on microtubules were examined under the conditions of microtubule assembly and disassembly using an adaptation of the method of Schiff et al. [8]. In the assembly inhibition assay, 100 μl tubulin protein with 285.6 μl 0.1 *M* PIPES buffer (pH 6.8), 50 μl 30 m*M* GTP and 13.5 μl drug solution were mixed in a tube and, after being gently shaken for 50 min at 35°C, the absorbance at 350 nm was measured, and the 50% assembly inhibition concentration (IC_{50}) was determined. In the disassembly inhibition assay, after repolymerization of tubulin using the above method, the test drugs were added along with 4 m*M* $CaCl_2$. This mixture was gently shaken for 30 min at 4°C, since low temperature is a precondition of disassembly. The absorbance at 350 nm was again measured to determine the 50% disassembly inhibition concentration (IC_{50}). DMSO and dimethyl-β-cyclodextrin, which were used as solvents, had no effect on the results of the assay.

Results

Cytotoxicity of HO-221

As shown in Table 1, HO-221 exhibited significant cytotoxicity towards the three cell types used. The cytotoxicity of metabolic inhibitors was comparatively mild. In descending order of cytotoxic strength, the test drugs ranked approximately thus: VCR, COL, HO-221, MMC, APH, Ara-C, 5FU, CYT. A strong cytotoxic effect is typical of spindle poisons.

Table 1 IC_{50} values of HO-221 in CHL, L1210 and HL-60 cells

Drug	IC_{50}					
	CHL[a] mol/l	μg/ml	L1210[b] mol/l	μg/ml	HL-60[a] mol/l	μg/ml
HO-221	2.0×10^{-7}	0.098	5.1×10^{-8}	0.025	7.3×10^{-8}	0.036
VCR	7.6×10^{-8}	0.070	5.7×10^{-9}	0.005	4.8×10^{-9}	0.004
COL	2.4×10^{-7}	0.094	5.7×10^{-8}	0.023	1.3×10^{-8}	0.005
Ara-C	1.4×10^{-6}	0.338	Not tested		1.4×10^{-6}	0.333
5FU	6.2×10^{-6}	0.803	1.9×10^{-6}	0.249	4.3×10^{-6}	0.553
APH	4.5×10^{-7}	0.153	6.3×10^{-7}	0.213	2.0×10^{-7}	0.069
MMC	2.4×10^{-7}	0.080	3.6×10^{-7}	0.121	2.7×10^{-7}	0.091
CYT	1.4×10^{-6}	0.676	2.8×10^{-6}	1.355	2.3×10^{-6}	0.786

[a] Cells exposed to drug for 48 h
[b] Cells exposed to drug for 24 h

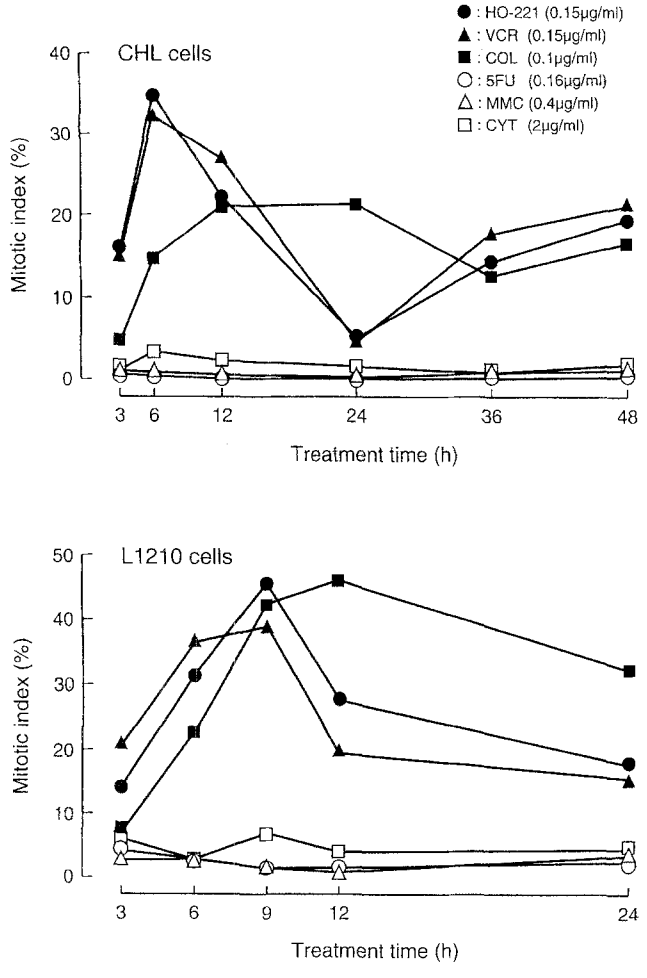

Fig. 2 Accumulation of metaphase cells in CHL and L1210 cells after HO-221 treatment

Morphological alteration by HO-221

In CHL cells, HO-221, VCR and COL induced suspended round cells and multinucleated cells. The latter were also induced by CYT, although the morphological effects of this drug were different to those of the others. With the other drugs, cell squamation and nuclear swelling were observed.

In L1210 cells, cell elongation was typically observed after treatment with HO-221 and spindle poisons. The clear visibility of chromosomes in elongated cells demonstrates that these cells were in metaphase and that elongation was induced during the mitotic phase. Multinucleation was also evident. No characteristic morphological alterations were observed with the other drugs apart from cell swelling.

Accumulation of metaphase cells after HO-221 treatment

Time-dependent changes in mitotic index are shown in Fig. 2. HO-221, VCR and COL produced marked mitotic arrest. However, 5FU, MMC, APH and CYT did not exhibit a metaphase-arresting action. A two-phase accumulation of metaphase cells was clearly manifest, especially in CHL cells. Mitotic index patterns with COL differed from those with HO-221 and VCR.

Chromosome analysis of CHL cells after HO-221 treatment

As shown in Table 2, HO-221, VCR, COL and CYT were found to frequently induce numerical chromosomal aberrations in the form of polyploid cells. With the other drugs, however, a chromosome-breaking action rather than numerical aberrations was observed. The histograms of chromosomal numbers shown in Fig. 3 indicate that aneuploid cells were found predominantly at low concentrations of HO-221. HO-221 seemed to cause atypical mitosis.

Table 2 Chromosome analysis of CHL cells treated with HO-221 for 48 h

Drug	Dose (μg/ml)	Number of cells analyzed	Structural aberration (%)						Polyploid (%)
			ctg	ctb	cte	csb	cse	Total	
DMSO	(0.5%)	200	1	0.5	0	0	0	1.5	0
Saline	(10%)	200	1	0	0	0	0	1	0
HO-221	0.08	200	0	0	0	0	0	0	2.5
	0.10	200	1	0	0	0	2.5	3.5	41
	0.12	200	0	0	0	0	6	6	85
	0.14	200	0.5	0	0	0	1.5	1.5	100
	0.16	200	0	0	0	0	1.5	1.5	100
VCR	0.15	50	0	0	0	0	4	5	96
COL	0.15	50	0	0	0	0	2	2	84
Ara-C	0.4	50	30	64	62	0	2	84	0
5FU	1.6	50	12	40	12	0	0	50	0
APH	0.4	50	8	48	46	0	4	76	0
MMC	0.1	200	8.5	51.5	91.5	0	0	96.5	0
CYT	1.0	50	0	0	0	0	2	2	68

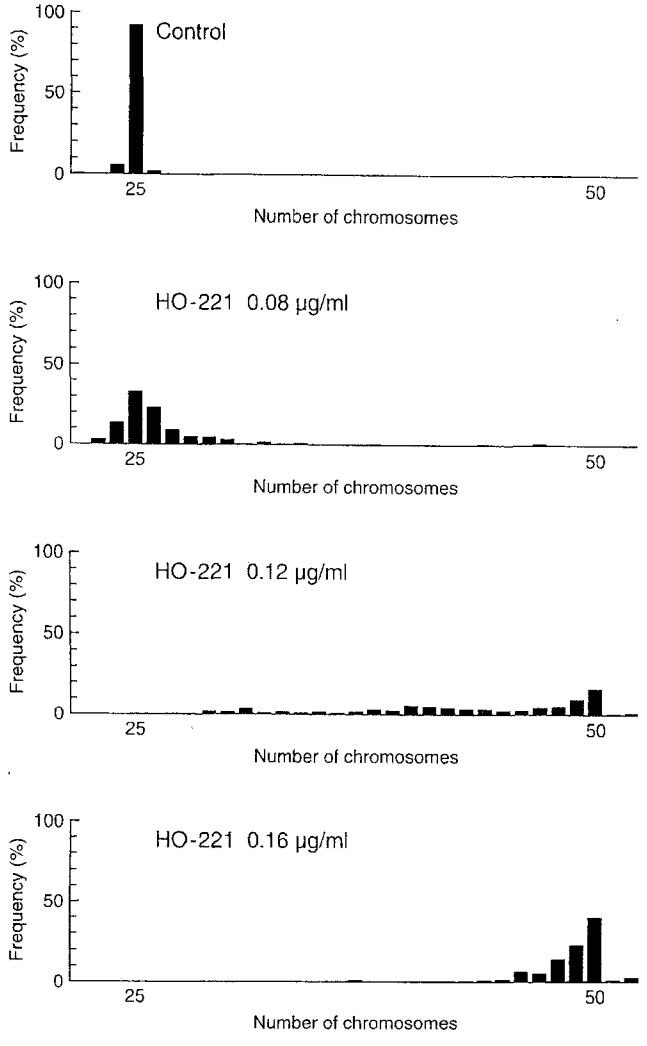

Fig. 3 Histograms showing chromosome numbers in CHL cells treated with HO-221 for 48h

Mouse bone marrow micronucleus assay

The results of the micronucleus assay are shown in Table 3 and MNPCE data regarding the relative size of micronuclei in Table 4. HO-221 appeared to have micronucleus-inducing activity in common with VCR and MMC, which were used as positive controls. HO-221 and VCR frequently induced the formation of relatively large micronuclei, while the chromosome-breaking agent MMC typically produced small micronuclei.

Inhibitory effects of HO-221 on microtubule assembly and disassembly

IC_{50} values and dose-response curves are shown in Table 5 and Fig. 4, respectively. HO-221, as well as VBL and POD, significantly inhibited microtubule assembly. TAX, however, had no such effect. Moreover, HO-221 appeared to have an inhibitory effect on microtubule disassembly similar to that of TAX, which is a specific microtubule disassembly-inhibiting agent. The strength of HO-221 was one-tenth that of TAX. VBL exhibited rapid aggregation of microtubules at low temperatures in the disassembly inhibition assay.

Discussion

In this morphological and cytogenetic study of cultured cells, HO-221, in common with the other spindle poisons, caused an accumulation of metaphase cells, with the appearance of multinucleated cells, elongated cells, and aneuploid and polyploid cells. Elongation of L1210 cells has also been reported by Oguro et al. [9].

Table 3 Micronucleus induction of HO-221 in mouse bone marrow 24 h after single treatment. Values are means ± SD

Drug	Route	Dose (mg/kg)	Incidence (%)	
			MNPCEs	PCEs
Non-treated	—	—	0.12 ± 0.11	56.6 ± 6.3
HO-221	oral	0	0.18 ± 0.11	57.8 ± 9.4
		10	0.34 ± 0.21*	49.0 ± 4.7
		50	0.48 ± 0.19**	47.0 ± 7.6
		100	0.60 ± 0.23**	50.3 ± 7.6
		200	3.56 ± 0.98**	35.1 ± 10.4**
		500	5.46 ± 3.37**	20.2 ± 2.8**
		1000	4.28 ± 1.34**	21.2 ± 5.4**
VCR	i.v.	0.25	6.34 ± 2.89**	40.5 ± 11.4**
MMC	i.p.	2	10.76 ± 2.59**	27.2 ± 10.4**

*$P < 0.05$, **$P < 0.01$

Table 4 Incidence of MNPCEs containing relatively large micronuclei induced by HO-221 treatment (*D* diameter of erythrocyte, *d* diameter of micronucleus)

Drug	Dose (mg/kg)	Incidence of MNPCE types (%)	
		Large ($d \geqq D/4$)	Small ($d < D/4$)
MMC	2	4	96
HO-221	500	51	49
	1000	55	45
VCR	0.25	65	35

Table 5 IC_{50} values of HO-221 on microtubule assembly and disassembly (*ND* not determined)

Drug	$IC_{50}(M)$ Assembly	Disasembly
HO-221	6.6×10^{-6}	4.5×10^{-5}
TAX	ND	4.4×10^{-6}
VBL	3.9×10^{-7}	ND
POD	1.4×10^{-6}	ND

This is thought to be a morphological alteration resulting from a lack of dynamic equilibrium between dysfunctional microtubules and the spindle poles. CYT has also been seen to produce multinucleated cells, but these cells also show morphological differences, and there is no accumulation of metaphase cells, indicating a clear difference between the action mechanisms of CYT and HO-221. CYT is known to specifically prevent cytokinesis by blocking the function of the actin filaments which play the chief part in this process [10]. In connection with the accumulation of metaphase cells, agents also exist which specifically cause an accumulation of prometaphase cells by inhibiting the division of centrioles rather than blocking the function of microtubules [11]. Regarding the cytotoxic property of spindle poisons, metabolic disturbance through an excess or a deficiency of genetic information brought about by an uneven distribution of chromosomes offers the readiest explanation. However, it also seems certain that apoptosis plays a part in this process, as has been shown in the case of the chromosome breaker cisplatin [12].

A micronucleus test was carried out as an in vivo study, using mouse bone marrow. This experiment was designed to indirectly assess chromosomal aberration in bone marrow erythroblasts on the basis of micronuclei appearing in erythrocytes following nuclear expulsion. This is regarded as a simple method for in vivo testing of chromosomal aberration. It was found that HO-221, like VCR, very frequently induced relatively large micronuclei. Therefore HO-221 induced chromosomal aberration in association with spindle inhibition in the in vivo environment also. With chromosome breakers such as MMC, a very small acentric chromosomal fragment forms a micronucleus, while with spindle poisons, one or a number of chromosomes as a unit form a micronucleus. It is believed to be because of this phenomenon that spindle poisons induce relatively large micronuclei. Nonuniform division of bone marrow erythroblasts is also thought to be a contributing factor.

In the present study, HO-221 did not show any greater tendency to produce micronuclei than the positive controls MMC and VCR, which were found to induce micronuclei at relatively low dosages (MMC 1/4 LD_{50}; VCR 1/30 LD_{50}). This suggests that HO-221 would have extremely little systemic toxicity or myelotoxicity as an anticancer drug. The CYT used earlier in the tests on cultured cells is now being

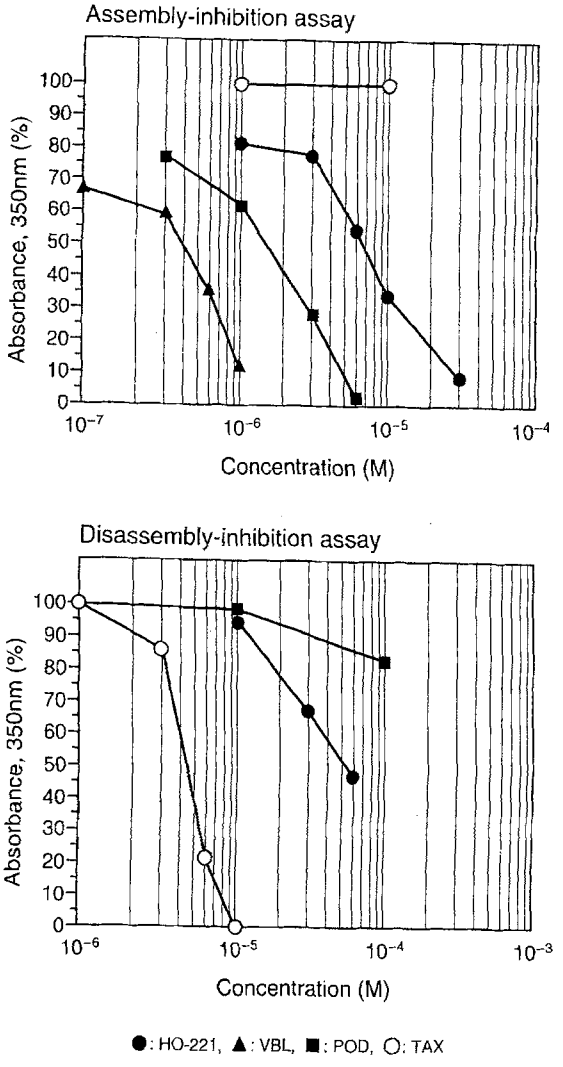

Fig. 4 Inhibitory effect of HO-221 on tubulin assembly and disassembly

adapted so that it can be used not only in vitro but also in the in vivo micronucleus test. Combined exposure to CYT and the test drug, using only immediate post-metaphase binuclear cells as the basis of micronucleus observation, is expected to improve the capacity to detect chromosomal aberration and will allow easy determination in the course of the test of whether a chromosome-breaking action or a spindle-poisoning action is involved through staining of the centromeres in the micronuclei formed [13]. There have been no reports so far on whether CYT itself induces micronuclei, but this seems unlikely considering the mechanism of its action.

In a further test using purified calf brain tubulin protein, HO-221 was found to have a microtubule assembly-inhibiting action as well as a disassembly-inhibiting action, albeit slight. Screening of novel anti-mitotic agents, using the reproduction rate of HL-60 cells and the mitotic index as indicators, has been carried out by Paull et al. [14]. Among the agents tested was the benzoylphenylurea compound NSC62544, which has a similar structure to HO-221. This substance was found to display an assembly-inhibiting action and to bind specifically to the COL binding site of tubulin. An investigation of the binding site of HO-221 to tubulin protein is now needed.

A number of contradictions have appeared in relation to the disassembly-inhibiting action of HO-221. Although the data are not presented here, electron microscopic observation of microtubules in HL-60 cells has clearly shown the formation of semicrystalline tubulin aggregates after treatment with VCR [15]. After treatment with HO-221 however, not only were no such aggregates observed, but no microtubules appeared to be present either, thus negating the possibility of an intracellular disassembly-inhibiting action. Further, the formation of non-specific tubulin polymers has been reported with COL in the presence of high concentrations of magnesium [16], and with phomopsin A when it is added to reconstituted microtubules [17]. These results suggest the necessity of a thorough reexamination of the results of the present disassembly inhibition test with regard to both assay method and electron microscopic observation. Finally, an Ames test [18] using *Salmonella* showed that HO-221 had no mutagenicity and no noxious effect on DNA (data omitted).

In summary, HO-221 was demonstrated through a range of investigations to be an assembly-inhibiting agent acting upon spindle structures. This may, however, represent the elucidation of no more than one aspect of the complex action mechanism of this compound. It is hoped that continued research as well as clinical application will shed further light on this extremely interesting substance.

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