Enhancement of in vivo Antitumor Activity of a Novel Antimitotic 1-Phenylpropenone Derivative, AM-132, by Tumor Necrosis Factor-α or Interleukin-6

Yasuaki Tatsumi,1, 4 Hitoshi Arioka,1 Shun-ichi Ikeda,3 Hisao Fukumoto,1 Ken-ichi Miyamoto,4 Kazuya Fukuoka,1 Yuichiro Ohe,3 Nagahiro Saijo2 and Kazuto Nishio1, 5

1Pharmacology Division, National Cancer Center Research Institute and 2Medical Oncology, National Cancer Center Hospital, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, 3Tokyo Research Laboratories, Kyowa Hakko Kogyo Co., Ltd., 3-6-6 Asahi-machi, Machida-shi, Tokyo 194-0023, 4Department of Pharmacology and Pharmaceutics, Graduate School of Natural Science and Technology, Kanazawa University, 13-1 Takara-machi, Kanazawa 920-0934

TK5048 and its derivatives, AM-132, AM-138, and AM-97, are recently developed antimitotic (AM) compounds. These 1-phenylpropenone derivatives induce cell cycle arrest at the G2/M phase of the cell cycle. TK5048 inhibited tubulin polymerization in human lung cancer PC-14 cells in a concentration-dependent manner. In a polymerization assay using bovine brain tubulin, AM-132 and AM-138 were quite strong, AM-97 was moderately strong, and TK5048 was a relatively weak inhibitor of tubulin polymerization. A murine leukemia cell line resistant to a sulfonamide antimitotic agent, E7010, which binds to colchicine-binding sites on tubulin, was cross-resistant to the in vitro growth-inhibitory effect of AM compounds. Inhibition of tubulin polymerization is therefore one of the mechanisms of action of these AM compounds against tumor cells. To profile the antitumor effect of AM compounds, the in vivo antitumor effect of AM-132 was evaluated against cytokine-secreting Lewis lung carcinoma (LLC). Tumor-bearing mice were treated with intravenous AM-132 using three different treatment schedules. LLC tumors expressing tumor necrosis factor-α (TNF-α), granulocyte macrophage colony-stimulating factor (GM-CSF), or interleukin (IL)-6 were very sensitive to AM-132. In particular, LLC tumors expressing IL-6 were markedly reduced by AM-132 treatment, and showed coloring of the tumor surface and unusual hemorrhagic necrosis. These results suggest a combined effect of AM-132 and cytokines on the blood supply to tumors.

Key words: Antimitotic — Tubulin — Cytokine — TNF-α — IL-6

The antimitotic (AM) agents are among the most widely used drugs in chemotherapy. Both vinca alkaloids and taxanes have specific binding sites on β-tubulin.1, 2) In addition, the colchicine-binding site on β-tubulin is of increasing interest because several agents that act at this site have been developed, including the podophytoxins. However, none of these compounds that bind to the colchicine-binding site has proved to be a potent antitumor agent in clinical use. Etoposide and its derivatives are major antitumor podophytoxins, but their mechanism of action has been demonstrated to be the inhibition of DNA topoisomerase II.3) E7010, developed by Eisai Co., Ltd. (Tokyo), is a colchicine-binding type AM agent for which clinical phase I trials are now underway.4) AM compounds are 1-phenylpropenone derivatives that were developed by Kyowa Hakko Kogyo Co. (Tokyo) and these compounds show potent antitumor activity in vivo (unpublished data). Here we report the cytotoxicity profile against human cancer cells and the mechanisms of action of these agents.

5To whom reprint requests should be sent.
E-mail: knishio@gan2.res.ncc.go.jp

Tumors often secrete cytokines, and this can influence the performance status, survival, and tumor response of a patient.5–12) Small cell lung cancers frequently secrete several growth factors and cytokines, which can cause paraneoplastic syndromes.13–15) Some cytokines such as tumor necrosis factor (TNF-α), and interleukin (IL)-2 have antitumor actions, but the effects are relatively weak and do not generally influence patients’ survival.16, 17) Various combinations of cytokines with antitumor effects are currently being considered for clinical use.

The purpose of this study was to clarify the mechanisms of action of the AM compounds in vivo and to examine factors that influence sensitivity to antimitotic agents in vivo. We report here that the AM compounds exert antitumor effects through the inhibition of tubulin polymerization. In a preliminary experiment, we found that the surface of a cytokine-secreting colon tumor darkened after administration of an AM compound. A similar effect may be seen in tumors treated with TNF-α. We suggest that there may be a combination effect of cytokines and AM compounds on murine lung cancer cells ectopically expressing several cytokines.16, 18–22)
MATERIALS AND METHODS

AM compounds TK5048 and the related compounds AM-97, AM-132 and AM-138, were donated by Kyowa Hakko Kogyo Co. (Fig. 1).

Cell lines and culture The human small cell lung cancer H69 cell line was established at the National Cancer Institute (Bethesda, MD), and a stock was provided by Dr. Y. Shimosato (National Cancer Center, Tokyo). We also used the non-P-glycoprotein-mediated paclitaxel- and vinodesine-resistant H69/Txl and H69/VDS cell lines that were established in our laboratory.19, 20 The human non-small cell lung cancer PC-14 cell line was provided by Dr. Y. Hayata, Tokyo Medical University, and a P-glycoprotein-mediated docetaxel-resistant subline called PC-14/TXT was also established in our laboratory.21 The human non-small cell lung cancer SBC-3 cell line was obtained from the Japanese Cancer Research Resources Bank (Tokyo), and a multidrug-resistant subline, SBC-3/ADM100, which is approximately 100-fold more resistant to doxorubicin than the parental line, was donated by Dr. T. Ohnoshi (Okayama University, Okayama).22 A murine lung cancer cell line, Lewis lung carcinoma (LLC) was obtained from the Japanese Cancer Research Resources Bank (Tokyo), and a multidrug-resistant subline, LLC/ADM100, which is approximately 100-fold more resistant to doxorubicin than the parental line, was donated by Dr. T. Ohnoshi (Okayama University, Okayama).22

Growth inhibition assay To determine the growth-inhibitory effects of AM compounds, we utilized the tetrazolium dye assay developed by Mosmann with some modifications.19, 28 Briefly, 180 µl of exponentially growing cell suspensions containing 10⁴ H69, H69/Txl and H69/VDS, 10³ PC-14 and PC-14/TXT, or 10³ P388, P388/4.0r-M and LLC/IL2, LLC/IL6, LLC/TNF, LLC/GM-CSF, LLC/IGIF, and LLC/IL12, were seeded in 96-well microtiter plates (Becton Dickinson & Co., Lincoln Park, NJ), and incubated for about 12 h, then 20 µl aliquots of drug solutions at the indicated concentrations were added. After a 72-h exposure to the drug, 20 µl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma Chemical, St. Louis, MO) solution (5 mg/ml in phosphate-buffered saline, PBS) was added to each well. The plates were incubated for a further 4 h, then centrifuged at 800g for 15 min. The medium was removed, 200 µl of dimethylsulfoxide was added to each well and the optical density of each resulting solution was measured at 562 and 630 nm using DeltaSoft II (BioMetallics Inc., Princeton, NJ) and a Bio Kinetics Reader EL 340 (Bio-
Teck Instruments Inc., Winooski, NJ). Each experiment included 6 replicate wells for each drug concentration and three independent experiments were carried out. Wells containing only RPMI-FBS and MTT were used as controls. The IC_{50} was defined as the drug concentration required for 50\% reduction of the optical density in each test, determined by interpolation of a growth curve drawn by plotting [(mean absorbance of 6 wells containing the drug–mean absorbance of 6 control wells)/mean absorbance of 6 drug-free wells–mean absorbance of 6 control wells]×100 against drug concentration. The relative resistance was defined as (IC_{50} of the resistant subline)/IC_{50} of the parental cell line.

**Flow-cytometric analysis** PC-14 cells were exposed to 1 to 100 nM TK5048 or 0.1 to 10 nM of its derivatives, AM-97, AM-132, and AM-138 for 24 h. Three million cells were washed with cold PBS, collected and resuspended in cold 50\% ethanol, and stored at 4°C. After removal of ethanol, cell pellets were resuspended in 0.2 M Na_{2}HPO_{4}, 0.1 M citrate, pH 7.4, 25 μg/ml RNase A, and 50 mg/ml propidium iodide and subjected to flow cytometry using a FACScan flow cytometer (Becton Dickinson, San Diego, CA).

**In vitro tubulin polymerization assay** The effect of AM compounds on in vitro tubulin polymerization was evaluated by the turbidity method. Bovine brain tubulin protein in G-PEM (1 mM GTP, 80 mM piperazine-N,N'-bis[2-ethanesulfonic acid], 1 mM ethylene glycol-bis(α-aminoethyl ether)-N,N',N'-tetraacetic acid, EGTA, and 1 mM MgCl_{2}, pH 6.8) containing 10% glycerol was purchased from Cytoskeleton (Santa Barbara, CA). A 100-μl aliquot of 2.5 mg/ml tubulin protein was mixed with various concentrations of each agent in a 96-well microtiter plate, and incubated for 30 min on ice. Polymerization was evaluated by measuring changes in the absorbance at 340 nm every minute for 20 min with EL-340. An aliquot without the drug was used as a control. The inhibition of tubulin polymerization was expressed as % inhibition using the formula [1-(maximum absorbance with drug)/(maximum absorbance without drug)]×100. Total and polymerized tubulin levels in the cells were determined by western blot analysis. Total and polymerized tubulin proteins were isolated by the methods of Thower et al. and Minotti et al. with some modifications. PC-14 cells were exposed to 0, 1.0, 10 and 100 nM TK5048 for 24 h. Total tubulin was prepared by washing the cells twice with cold PBS, centrifuging cell suspensions containing 10^7 cells at 200g for 5 min, resuspending the cells in 0.3 ml of depolymerization buffer (0.1 M 2-[N-morpholino]ethanesulfonic acid, 1 mM MgSO_{4}, 10 mM CaCl_{2}, 5 mM GTP, pH 6.9), and lysing the cells on ice by sonication with a Branson Sonifier 450 (Branson Ultrasonics Corp., Danbury, CT) at an output of 15 W for two periods of 15 s each. Polymerized tubulin was prepared by washing exponentially growing cells with warm PBS, centrifuging cell suspensions containing 4×10^7 cells at 200g for 5 min, and resuspending the pellets in 1 ml of stabilization buffer (20 mM Tris-HCl, pH 6.8, 0.14 M NaCl, 0.5% (v/v) Nonidet P-40, 1 mM MgCl_{2}, 2 mM EGTA, 4 mg/ml paclitaxel), followed by incubation at 37°C for 30 min, and centrifugation at 50,000g for 15 min at 37°C using a TL-100 centrifuge (Beckman Instruments Inc., Fullerton, CA) with a TL-45 rotor. The resulting supernatants were aspirated; the pellets were resuspended in 0.3 ml depolymerization buffer and homogenized. Following these steps, lysates of both total and polymerized tubulin proteins in depolymerization buffer were treated as described below. The lysates were incubated in depolymerization buffer for 1 h on ice, and centrifuged at 50,000g for 15 min at 4°C. Each supernatant was mixed with an equal volume of Tris sodium dodecyl sulfate (SDS), 2-mercaptoethanol, Sepranol (Emprotech, Hyde Park, MA) and applied to a 10% (w/v) SDS-polyacrylamide gel (Multigel 10, Daiichi Pure Chemicals, Tokyo). After electrophoresis, blotting on a polyvinylidene fluoride membrane (Immobilon, Nilohn Millipore Ltd., Tokyo) and blocking with 5% (w/v) skim milk in PBS, the membrane was incubated with a mouse anti-α-tubulin monoclonal antibody (1:500, Sigma Chemical Co.). It was washed with PBS containing 0.1% (v/v) polyoxyethylene sorbitan monolaurate (Tween 20, Sigma Chemical Co.), then biotinylated anti-mouse IgG (Vector Laboratories Inc., Burlingame, CA) was added. The membrane was incubated, and washed with PBS-Tween 20, and the protein bands were detected with ECL western blotting detecting reagents (Amersham International plc, Buckinghamshire, UK). The bands were quantified densitometrically using ImageMaster DTS (Pharmacia Biotech, Tokyo). The polymerized tubulin ratio was calculated as 1/4×(density of the polymerized tubulin band)/(density of the total tubulin band). Multiplication by 1/4 was carried out to adjust the polymerized tubulin content according to the ratio of the numbers of cells used for preparation of total and polymerized tubulin.

**In vivo evaluation of AM-132-induced antitumor activity and histological examination** B16F10, Colon 26, LLC, LLC/IL6 and LLC/TNF cells (1×10^6) were subcutaneously injected into the back of mice at day 1. AM-132 was injected into the tail vein following three different schedules from day 7: schedule 1, 25 mg/kg/day from day 7 to 11; schedule 2, 50 mg/kg/day once at day 7; schedule 3, 12.5 mg/kg/day×3 days at day 7, 9, and 11. The antitumor effects were evaluated in terms of the lowest T/C and body weight of mice as described previously. The inoculated tumors were thereafter frozen, and 4-μm sections were stained using hematoxylin and eosin (HE) or immunohistochemically using anti-CD31 antibody.
RESULTS

Flow cytometry  Flow cytometry was used to determine whether these AM agents induce cell cycle arrest. We exposed PC-14 cells to 1 to 100 nM TK5048, or to 0.1 to 10 nM of the derivatives, since TK5048 has an approximately 10-fold greater IC$_{50}$ value for these cells than other agents. Exposure of cells for 24 h to concentrations up to 10 nM TK5048 and 1 nM of the other compounds had no apparent effect on the cell cycle distribution (Fig. 2). However, at the highest concentrations of these agents, cells accumulated in the G2/M phase. Among the derivative agents AM-132 induced the most marked G2/M block, while AM-97 blocked the majority of cells at G1 phase. Morphological experiments revealed mitotic arrest with chromosome condensation in the cells treated with AM compounds (data not shown).

Effect of TK5048 and derivatives on tubulin polymerization in vivo and at the cellular level  We investigated the effect of TK5048 on intracellular tubulin polymerization. As shown in Fig. 3A, TK5048 inhibited tubulin polymerization in a concentration-dependent manner in cultured cells. We then evaluated the effects of AM compounds on in vitro tubulin polymerization using purified bovine brain tubulin. The magnitude of inhibition of tubulin polymerization varied among compounds. TK5048 (ED$_{50}$ (effective dose 50): 10.0 µM) and colchicine (ED$_{50}$: 10.1 µM) showed weak inhibition, AM-97 (ED$_{50}$: 1.43 µM) moderate inhibition, and AM-132 (ED$_{50}$: 1.12 µM) and AM-138 (ED$_{50}$: 1.19 µM) potent inhibition (Fig. 3B). These results suggest that AM compounds inhibit tubulin polymerization in vivo and in vitro.

Growth-inhibitory effects of TK5048 and derivatives  The growth-inhibitory effects of these AM compounds on...
effective on multidrug-resistant cells, we examined the colchicine. To evaluate whether these compounds are less and AM-138 (×3.6), these cells were highly resistant to colchicine. To evaluate whether these compounds are less effective on multidrug-resistant cells, we examined the cytotoxicities of these agents against another multidrug resistant cell line, SBC-3/ADM100. This cell line showed cross-resistance to colchicine, and a lower degree of resistance to the AM compounds. To elucidate the mechanisms of action of TK5048 on typical multidrug-resistant cells, we examined their effects on cell lines resistant to other antimitotic agents. Paclitaxel- and vindesine-resistant cells were not cross-resistant to TK5048 nor its derivatives. P388/4.0r-M, a non-P-glycoprotein-mediated resistant subline which is approximately 100 times more resistant than the parent cell line to E7010, a colchicine-binding antimitotic agent, showed cross-resistance to all the AM compounds (Table I). These results suggest that TK5048 inhibits cell growth via binding to the colchicine-binding site on tubulin.

### Table I. Growth-inhibitory Effects of AM Compounds in Several Cancer Cells and Resistant Cell Lines

|                | TK5048 | AM-97 | AM-132 | AM-138 | Colchicine |
|----------------|--------|-------|--------|--------|------------|
| H69            | 84.9±8.3×10⁻⁶ | 22.1±5.5 | 11.5±3.5 | 23.8±4.2 | 34.0±7.1   |
| H69/Txl        | 97.0±1.4×10⁻³ | 7.5±1.1×10⁻³ | 7.0±0.1×10⁻³ | 10.4±3.0×10⁻³ | 36.7±5.5×10⁻³ |
| H69/VDS        | 74.0±5.7×10⁻³ | 10.0±2.8×10⁻³ | 14.7±0.7×10⁻³ | 18.0±0.5×10⁻³ | 32.0±4.2×10⁻³ |
| PC-14          | 53.5±6.4 | 6.5±0.4 | 5.9±0.4 | 7.9±1.7 | 18.7±0.5   |
| PC-14/TXT      | 103.0±21.2×10⁻³ | 10.0±1.7×10⁻³ | 29.1±0.7×10⁻³ | 28.7±0.1×10⁻³ | 330.0±155.6×10⁻³ |
| P338           | 42.0 | 7.3 | 6.7 | 11.0 | ND         |
| P338/4.0r-M    | >3000×10⁻³ | 68.0±9.3 | 60.3±9.0 | 175.7±16.0 | ND         |
| SBC-3          | 28.0±9.9 | 10.4±7.3 | 4.2±2.3 | 6.9±0.2 | 7.3±0.3    |
| SBC-3/ADM100   | 21.5±3.5×10⁻³ | 9.2±4.0×10⁻³ | 6.6±3.7×10⁻³ | 11.7±3.9×10⁻³ | 20.9±1.2×10⁻³ |

*a* Cells were exposed to TK5048, AM compounds, and colchicine, followed by incubation for 3 days. Results are presented as means±SD (RR) of 3 independent experiments of MTT assay. **RR**, relative resistance. **RR** = IC₅₀ of resistant cells/IC₅₀ of parental cells.

### Table II. Antitumor Effect of AM-132 against Murine Carcinoma Cells in vitro and in vivo

#### A

| Cell lines | Schedule | Dose (mg/kg) | Mn T/C |
|------------|----------|--------------|--------|
| Colon 26   | q1d×5 i.v. | 25 | 0.14 |
| B16F10     | q1d×5 i.v. | 25 | 0.27 |
| LLC        | q1d×5 i.v. | 25 | 0.46 |

#### B

| Cells        | LLc | LLc/neo | LLc/IL6 | LLc/TNF |
|--------------|-----|--------|--------|--------|
| IC₅₀ of AM-132 (µM)* | 0.41 | 0.51 | 0.18 | 0.45 |

A: Colon 26 (human colon carcinoma), B16F10 (murine melanoma cells), and LLC (murine lung carcinoma) cells were inoculated subcutaneously into mice. AM-132 (25 mg/kg) was administered intravenously into the tail vein every 5 days (from day 1 to day 5). Antitumor effects were evaluated as the minimum ratio of tumor volume (T/C). AM-132 showed high antitumor activity against Colon 26 tumors (T/C = 0.14).

B: Growth-inhibitory effects of AM-132 in LLC and the cytokine-gene transfectants.

a) Cells were exposed to AM-132, followed by incubation for 3 days. Results are presented as means of 3 independent experiments of MTT assay.

**In vivo activity of AM-132** The in vivo antitumor effect of AM-132 was evaluated against Colon 26 (human colon carcinoma), B16F10 (murine melanoma), and LLC (murine lung carcinoma) tumors. A 25 mg/kg dose of AM-132 was administered into the tail vein every 5 days (from day 1 to 5). Antitumor effects were evaluated as minimum T/C. AM-132 showed the highest antitumor activity against Colon 26 (T/C = 0.14). Colon 26 cells have been reported to secrete cytokines such as IL-6 and TNF-α, suggesting that the antitumor effect of AM-132 might be related to cytokines.\(^{32, 33}\)

**In vitro activity of AM-132 against LLC cells expressing various cytokines** The in vitro growth-inhibitory activities of several antimitotic agents were evaluated by MTT assay. The IC₅₀ values were determined after a 72-h exposure. IC₅₀ values for AM-132 against LLC/IL2, LLC/IL6, LLC/TNF, LLC/IGIF, and LLC/IL12 cells were not different from those for LLC/neo and parental LLC cells (Table IIB). However LLC/GM-CSF cells were resistant to AM-132. These results suggest that cytokines may not modulate the antitumor activity of antimitotic agents directly.
Enhanced Activity of AM-132 in Cytokine-secreting Tumor

Fig. 4. In vivo activity of AM-132 against cytokine-secreting lung cancer tumors. Treatment schedule is shown in A. In vivo antitumor effect of AM-132 on tumor volume (B, D, F) and body weight (C, E, G) of mice bearing LLC tumors expressing various cytokines: Schedule 1, 25 mg/kg×5 days i.v. (B and C); schedule 2, 50 mg/kg single injection i.v. (D and E); schedule 3, 12.5 mg/kg×3 days i.v. on every other days (F and G).
In vivo activity of AM-132 against cytokine-secreting LLC tumors

The effects of AM-132 on LLC tumors expressing various cytokines were then evaluated using three different schedules (Fig. 4A). In vivo experiments with LLC/IL6 tumors in mice showed no significant difference in the tumorigenicity and growth rate as compared with those of LLC/neo-transplanted mice. LLC/IL6 transplantation shortened survival and caused body weight loss in mice as reported previously.22) A 25 mg/kg/day dose of AM-132 given on days 7–11 (5 days, schedule 1) reduced the size of LLC/TNF tumors 2 weeks after treatment. This treatment schedule also markedly reduced the size of LLC/IL6 tumors compared to LLC and LLC/neo cells (Fig. 4B). Mice carrying LLC/TNF and LLC/IL6 tumors that were treated with AM-132 showed weight loss (Fig. 4C). The enhanced tumor reduction and body weight loss by AM-132 were not observed in the mice inoculated with other cytokine-secreting tumors. Mice with LLC/IL6 tumors were dead by day 8 after the initiation of treatment.

Bolus injection of 50 mg/kg/day AM-132 (schedule 2, single injection, Fig. 4, D and E) had no effect on mice bearing LLC/IL6, all of which died on day 1. The LLC/TNF tumors responded to AM-132 with a marked reduction in size and disappearance of the tumor by 14 days.

The third administration schedule was designed to minimize the toxicity of AM-132 on LLC/IL6-bearing mice (schedule 3, administration on every other day, Fig. 4, F and G). Bolus administration of 12.5 mg/kg AM-132 three times every 2 days led to marked shrinkage of LLC/IL6 tumors, but these mice had no significant loss of body weight. The in vivo activities of AM-132 are summarized in Table III.

During the evaluation, darkening of the surface of some tumors was observed after AM-132 administration. Pathological analysis of these tumors by HE staining showed marked leakage of hematocytes from capillaries in the LLC/IL6 tumors in AM-132-treated mice (Fig. 5B). Vessel staining with anti-CD31 antibody demonstrated enhanced destruction of tumor vessels in AM-132-treated LLC/IL6 tumors (Fig. 5D).

DISCUSSION

Since colchicine is a potent antimitotic agent, colchicine analogues were expected to exert potent antimitotic effects via binding to the colchicine-binding site of tubulin. However, to date no agent that binds to the colchicine-binding site has shown clinically promising effects. E7010, which binds at this site, has been evaluated in a clinical study.4) Other antimitotic agents, such as vinca alkaloids,2) rhizoxin,34) and taxanes,1) exert strong antitumor activities and their mechanisms of action are considered to involve binding to tubulin sites other than that for colchicine. Herein, we have demonstrated that TK5048 and its derivatives have potent growth-inhibitory effects on human cancer cell lines in vitro, and that they probably exert these potent antitumor effects via the colchicine-binding site on tubulin.

Colchicine has a high affinity for P-glycoprotein, which is an efflux pump for various antitumor agents, and shows suppressed activity in multidrug-resistant phenotypes.35) The 1-phenylpropenone derivatives effectively inhibit the growth of P-glycoprotein-mediated multidrug-resistant cell lines, such as SBC-3/ADM100.

The mechanisms of resistance to antimitotic agents include: 1) decreased drug accumulation, 2) changes in tubulin, 3) altered microtubule-associated proteins and subsequent effects on signal transduction, and 4) changes in intracellular drug metabolism.36) The H69/Txl and H69/VDS cell lines are, respectively, paclitaxel- and vindesine-resistant, via mechanisms not mediated by either increased efflux pump activity or decreased intracellular accumulation. In these cell lines, altered tubulin status is considered to be at least one of the major causes of drug resistance.19, 20) Even for these resistant cell lines, our novel AM compounds showed marked antitumor activity (Table II), suggesting that the mechanisms of action of these novel compounds are different from those of vindesine and paclitaxel. However, E7010-resistant cell lines showed cross-resistance to TK5048 and all of its derivatives (Table II), suggesting similar cytotoxic mechanisms. A previous report demonstrated marked accumulation of E7010 not only in P388 cells but also in P388/4.0r-M resistant cells, which do not overexpress P-glycoprotein.26, 37) This suggests that altered expression of β-tubulin isotypes in the resistant cell lines may play an important role in the resistance to E7010 and our AM compounds.

AM-132 and AM-138 were the most potent inhibitors of polymerization of tubulin and in vitro cytotoxicity. These

---

Table III. In vivo Effect of AM-132 on Cytokine cDNA-transfected LLC Cells Inoculated Subcutaneously into Mice

| Cell line       | Schedule | 1          | 2          |
|-----------------|----------|------------|------------|
|                 | T/C      | Evaluation day | T/C      | Evaluation day |
| LLC/neo         | 0.66     | 8          | 0.72      | 9           |
| LLC (LLC/IL2)   | 0.31     | 8          |           |             |
| LLC/IL6         | 0.21     | 8          | 0.18      | 9           |
| LLC/GM-CSF      | 0.31     | 10         |           |             |
| LLC/IGIF        | 0.69     | 8          |           |             |
| LLC/TNF         | 0.03     | 14         | 0.07      | 9           |

Antitumor effect was expressed as the lowest T/C during the evaluation. Schedule 1: 25 mg/kg i.v. every 5 days from day 1 to 5. Schedule 3: 12.5 mg/kg i.v. three times at days 1, 3, 5.
data correlated with the results of *in vitro* growth inhibition of P388 (*r*=0.965) and P388/4.0r-M (*r*=0.984) cells (data not shown). Thus, our AM compounds are considered to be potent colchicine binders, and this action appears to be related to the cytotoxic effect of these compounds.

Antimitotic agents such as taxanes and estramustine are reported to act on tumor vessels and angiogenesis. Vinca alkaloids also influence the tumor vessels and change the color of the tumor surface. Therefore, blood vessels are one of the possible targets for these antimitotic agents.

TNF-α secreting tumors (LLC/TNF) showed high sensitivity to AM-132 *in vivo*. Pathological findings showed necrosis of tumors (data not shown). It is possible that the TNF-α acts cooperatively with AM-132 on the tumor vessels.

Cytokine production by tumors may be a good predictive marker for response to an antimitotic agent. We previously reported that subcutaneous transplantation of IL-6-secreting tumors (LLC/IL6) into mice caused body weight loss. We observed in this study that AM-132 administration enhanced the IL-6-induced body weight loss, with associated high tumor shrinkage. A dose reduction of AM-132 improved the body weight loss of mice, but retained a good T/C. These results suggest that IL-6 could be useful in combination with antimitotic agents including AM-132.

**ACKNOWLEDGMENTS**

This research was supported in part by Grants-in-Aid from the Ministry of Health, Labour and Welfare for the 2nd Term of Comprehensive 10-Year Strategy for Cancer Control, from the Ministry of Health, Labour and Welfare of Japan, and from the trust fund under government regulation for research on AM compounds from Kyowa Hakko Kogyo Co., Ltd.

(Received January 10, 2001/Revised April 17, 2001/Accepted April 23, 2001)
REFERENCES

1) Rao, S., Orr, G. A., Chaudhary, A. G., Kingston, D. G. and Horwitz, S. B. Characterization of the taxol binding site on the microtubule. 2-(m-Azidobenzoxy) taxol photolabels a peptide (amino acids 217–231) of β-tubulin. J. Biol. Chem., 270, 20235–20238 (1995).

2) Safa, A. R., Hamel, E. and Felsted, R. L. Photoaffinity labeling of tubulin subunits with a photosensitive analogue of vinblastine. Biochemistry, 26, 97–102 (1987).

3) van Maanen, J. M., Retel, J., de Vries, J. and Pinedo, H. M. Mechanism of action of antitumor drug etoposide: a review. J. Natl. Cancer Inst., 80, 1526–1533 (1988).

4) Koyanagi, N., Nagasu, T., Fujita, F., Watanabe, T., Tsukahara, K., Funahashi, Y., Fujita, M., Taguchi, T., Yoshino, H. and Kitoh, K. In vivo tumor growth inhibition produced by a novel sulfonamide, E7010, against rodent and human tumors. Cancer Res., 54, 1702–1706 (1994).

5) De Vita, F., Orditura, M., Galizia, G., Romano, C., Roscigno, A., Lieto, E. and Catalano, G. Serum interleukin-10 levels as a prognostic factor in advanced non-small cell lung cancer patients. Chest, 117, 365–373 (2000).

6) De Vita, F., Orditura, M., Auriemma, A., Infusino, S., Roscigno, A. and Catalano, G. Serum levels of interleukin-6 as a prognostic factor in advanced non-small cell lung cancer. Oncol. Rep., 5, 649–652 (1998).

7) De Vita, F., Orditura, M., Auriemma, A., Infusino, S. and Catalano, G. Serum concentrations of proinflammatory cytokines in advanced non small cell lung cancer patients. J. Exp. Clin. Cancer Res., 17, 413–417 (1998).

8) Hickish, T. F., Smith, I. E., O’Brien, M. E., Ashley, S. and Middleton, G. Clinical benefit from palliative chemotherapy in non-small-cell lung cancer extends to the elderly and those with poor prognostic factors. Br. J. Cancer, 78, 28–33 (1998).

9) De Vita, F., Orditura, M., Galizia, G., Romano, C., Lieto, E., Iodice, P., Tuccillo, C. and Catalano, G. Serum interleukin-10 is an independent prognostic factor in advanced solid tumors. Oncol. Rep., 7, 357–361 (2000).

10) Zhang, G. J. and Adachi, I. Serum interleukin-6 levels correlate to tumor progression and prognosis in metastatic breast carcinoma. Anticancer Res., 19, 1427–1432 (1999).

11) Ando, M., Ando, Y., Sugiuira, S., Minami, H., Saka, H., Sakai, S., Shimokata, K. and Hasegawa, Y. prognostic factors for short-term survival in patients with stage IV non-small cell lung cancer. Jpn. J. Cancer Res., 90, 249–253 (1999).

12) Shinkai, T., Eguchi, K., Sasaki, Y., Tamura, T., Ohe, Y., Kojima, A., Oshita, F., Miya, T., Okamoto, H., Iemura, K. and Saijo, N. A prognostic-factor risk index in advanced non-small-cell lung cancer treated with cisplatin-containing combination chemotherapy. Cancer Chemother. Pharmacol., 30, 1–6 (1992).

13) Parma, M., Diament, M., Garcia, C., Piccinni, E., Mondelo, N. and Klein, S. Mechanisms of paraneoplastic syndromes in mice bearing a spontaneous lung adenocarcinoma.
25) Kunikane, H., Krishan, A., Ohta, S., Nishio, K., Kubota, N., Takeda, Y., Oyama, H., Nomura, K. and Saijo, N. Flow cytometric analysis of cellular Adriamycin accumulation in small-cell lung cancer cell lines: correlation of P-glycoprotein expression, cytotoxicity and intracellular distribution. *Cell. Pharmacol.*, 1, 293–300 (1994).

26) Yoshimatsu, K., Yamaguchi, A., Yoshino, H., Koyanagi, N. and Kitoh, K. Mechanism of action of E7010, an orally active sulfonamide antitumor agent: inhibition of mitosis by binding to the colchicine site of tubulin. *Cancer Res.*, 57, 293–300 (1997).

27) Yoshimatsu, K., Yamaguchi, A., Yoshino, H., Koyanagi, N. and Kitoh, K. Mechanism of action of E7010, an orally active sulfonamide antitumor agent: inhibition of mitosis by binding to the colchicine site of tubulin. *Cancer Res.*, 57, 3208–3213 (1997).

28) Iwamoto, Y., Nishio, K., Fukumoto, H., Yoshimatsu, K., Yamakido, M. and Saijo, N. Preferential binding of E7010 to murine β3-tubulin and decreased β3-tubulin in E7010-resistant cell lines. *Jpn. J. Cancer Res.*, 89, 954–962 (1998).

29) Mosmann, T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assay. *J. Immunol. Methods*, 65, 55–63 (1983).

30) Minotti, A. M., Barlow, S. B. and Cabral, F. Resistance to antimitotic drugs in Chinese hamster ovary cells correlates with changes in the level of polymerized tubulin. *J. Biol. Chem.*, 266, 3987–3994 (1991).

31) Thrower, D., Jordan, M. A. and Wilson, L. Quantitation of cellular tubulin in microtubules and tubulin pools by a competitive ELISA. *J. Immunol. Methods*, 136, 45–51 (1991).

32) Fukumoto, H., Nishio, K., Ohta, S., Hanai, N., Fukuoka, K., Ohe, Y., Sugihara, K., Kodama, T. and Saijo, N. Effect of a chimeric anti-ganglioside GM2 antibody on ganglioside GM2-expressing human solid tumors in vivo. *Int. J. Cancer*, 82, 759–764 (1999).

33) Soda, K., Kawakami, M., Kashii, A. and Miyata, M. Manifestations of cancer cachexia induced by colon 26 adenocarcinoma are not fully ascribable to interleukin-6. *Int. J. Cancer*, 62, 332–336 (1995).

34) Koyanagi, N., Nagasu, T., Fujita, F., Watanabe, T., Tsukahara, K., Funahashi, Y., Fujita, M., Taguchi, T., Yoshino, H. and Kitoh, K. In vivo tumor growth inhibition produced by a novel sulfonamide, E7010, against rodent and human tumors. *Cancer Res.*, 54, 1702–1706 (1994).

35) Arioka, H., Nishio, K., Heike, Y., Abe, S. and Saijo, N. In vitro and in vivo modulation by rhizoxin of non-P-glycoprotein-mediated vinblastine resistance. *J. Cancer Res. Clin. Oncol.*, 123, 195–200 (1997).

36) Kartner, N., Shales, M., Riordan, J. R. and Ling, V. Daunorubicin-resistant Chinese hamster ovary cells expressing multidrug resistance and a cell-surface P-glycoprotein. *Cancer Res.*, 43, 4413–4419 (1983).

37) Nishio, K. and Saijo, N. Cytoskeletons and antimitotic agents developed in Japan. *Anticancer Drug Des.*, 14, 133–141 (1999).

38) Iwamoto, Y., Nishio, K., Fukumoto, H., Yoshimatsu, K., Yamakido, M. and Saijo, N. Preferential binding of E7010 to murine β3-tubulin and decreased β3-tubulin in E7010-resistant cell lines. *Jpn. J. Cancer Res.*, 89, 954–962 (1998).

39) Lau, D. H., Xue, L., Young, L. J., Burke, P. A. and Cheung, A. T. Paclitaxel (Taxol): an inhibitor of angiogenesis in a highly vascularized transgenic breast cancer. *Cancer Biother. Radiopharm.*, 14, 31–36 (1999).

40) Belotti, D., Vergani, V., Drudis, T., Borsotti, P., Picelli, M. R., Viale, G., Giavazzi, R. and Taraboletti, G. The microtubule-affecting drug paclitaxel has antiangiogenic activity. *Clin. Cancer Res.*, 2, 1843–1849 (1996).

41) Vacca, A., Iurlaro, M., Ribatti, D., Minischetti, M., Nico, B., Ria, R., Pellegrino, A. and Dammacco, F. Antiangiogenesis is produced by nontoxic doses of vinblastine. *Blood*, 94, 4143–4155 (1999).