

## Evaluation of Antivascular and Antimitotic Effects of Tubulin Binding Agents in Solid Tumor Therapy

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**Tubulin binding agents (TBAs) reduce tumor perfusion and inhibit mitosis of tumor cells in solid tumors, but it is not clear which effects contribute to the suppression of solid tumor growth. We evaluated the antivascular and antimitotic effects of several TBAs, combretastatin A-4 (CS A-4) phosphate, AC-7700, a novel CS A-4 derivative, colchicine, E7010, and vinblastine, on subcutaneous (s.c.) murine colon26 adenocarcinoma (c26). Tolerable doses of vinblastine and E7010 strongly inhibited tumor growth and induced mitotic arrest of tumor cells without affecting tumor perfusion. Colchicine had no effect on tumor growth and perfusion. When the injected dose was increased to the lethal range, however, these drugs markedly reduced tumor perfusion and caused necrosis of tumor tissue. Within the tolerable dose range, AC-7700 both strongly suppressed tumor growth and reduced tumor perfusion, and CS A-4 phosphate also exhibited a moderate antivascular effect. To evaluate the contribution of antivascular activity of TBAs to tumor growth suppression, excluding their direct cytotoxic effect on tumor cells, we established c26/acr, which is resistant to TBAs *in vitro*. Although E7010 showed a reduced suppressive effect on s.c. c26/acr tumor growth as compared with its effect on wild-type c26, AC-7700 remained potent against both cell lines. These results indicate that TBAs exert antivascular and antimitotic effects on solid tumors with marked differently effective dose ranges from agent to agent, and that the antivascular effect of TBAs inhibits solid tumor growth independently of the direct cytotoxic effect on tumor cells.**

Key words: Tubulin binding agent — Combretastatin — Tumor vessel — Antimitotic — Antivascular

Tumor vasculature is a promising target for novel anti-cancer agents that would have several theoretical advantages over agents affecting tumor cells, e.g., such drugs could easily gain access to target cells and development of resistance would be unlikely because endothelial cells are genetically stable, homogenous and have a low mutational rate.

Over the last decade, several investigators have reported that tubulin binding agents (e.g. vincristine, vinblastine, colchicine) reduced perfusion and induced hemorrhagic necrosis in subcutaneous (s.c.) murine tumors.<sup>1–3</sup> This effect occurs through an unknown mechanism other than the induction of tumor necrosis factor (TNF)- $\alpha$ <sup>4</sup> and may be closely related to microtubule disruption. However, only a few attempts have been made to adapt this “antivascular effect” to clinical use because (a) tumor perfusion is inhibited only when drugs are administered at the maximum tolerable dose (MTD), and (b) it is not clear whether the antivascular activity of tubulin binding agents contributes to the suppression of solid tumor growth, since an

antimitotic effect on tumor cells is induced through inhibition of spindle formation at doses lower than those required to reduce tumor perfusion.

A novel tubulin binding agent, combretastatin A-4 (CS A-4), was isolated from the bark of the South African tree *Combretum caffrum*.<sup>5–7</sup> This compound and its disodium phosphate salt (CS A-4 prodrug) were reported to inhibit strongly the perfusion of solid murine and human tumors at doses less than one-tenth of the MTD.<sup>8</sup> However, single doses of these compounds have limited potential for inhibiting tumor growth in the tolerable dose range.<sup>9</sup> In a previous study, by replacing the phenolic OH of CS A-4 with NH<sub>2</sub>, we obtained AC-7739, which potently suppressed solid murine tumor growth.<sup>10</sup> We also improved the water solubility of this agent by synthesizing AC-7700, a serine AC-7739 prodrug.<sup>11</sup>

In the present study, we investigated how the two activities of tubulin binding agents, the antivascular and antimitotic effects, contribute to the suppression of solid tumor growth by using multiple doses of agents which interact with the colchicine-binding site (CS A-4 phosphate, AC-7700, colchicine, E7010) and vinca alkaloid-binding site (vinblastine) of tubulin. Moreover, we investigated

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whether the antivasular effect of tubulin binding agents can suppress solid tumor growth independently of the direct cytotoxic effect on tumor cells.

## MATERIALS AND METHODS

**Drugs** AC-7700, AC-7739, CS A-4 phosphate, and E7010<sup>12)</sup> were synthesized by Ajinomoto Co., Inc. (Pharmaceutical Research Laboratories, Kawasaki). AC-7700 is a serine prodrug of AC-7739 and is cleaved by aminopeptidase, immediately releasing an active form *in vitro* as well as *in vivo*. E7010 is a novel oral microtubule inhibitor which has been tested in clinical trials.

AC-7700, AC-7739, CS A-4 phosphate, vinblastine (Kyorin Co., Inc., Tokyo), colchicine (Sigma Chemical Co., St Louis, Missouri), and *cis*-diamminedichloroplatinum (cisplatin, Nippon Kayaku Co., Inc., Tokyo) were dissolved in and diluted with saline. E7010 was suspended in and diluted with 0.5% carboxymethylcellulose. AC-7700 and AC-7739 were administered intravenously (i.v.) or subcutaneously (s.c.), CS A-4 phosphate was injected intraperitoneally (i.p.), vinblastine, colchicine, and cisplatin were injected i.v., and E7010 was administered *per os* (p.o.). **Animals** Female BALB/c×DBA/2F1 (CD2F1) mice were obtained from Charles River Japan, Inc. (Yokohama), and were given access to food (CRF1; Charles River Japan, Inc.) and water *ad libitum*. Mice were maintained under specific pathogen-free conditions at 23±2°C and 50±10% relative humidity. Lighting was automatic on a 12-h light/dark cycle.

**Tumor cells** Murine colon26 adenocarcinoma (c26) was supplied by Simonsen Laboratories (Gilroy, California) under the auspices of the National Cancer Institute, NIH (Bethesda, Maryland), and was maintained by the Japanese Foundation for Cancer Research (Tokyo). C26/acr was established from c26 by maintenance with AC-7739 for 1.5 years. This cell line is resistant only to tubulin polymerization inhibitors. MethA fibrosarcoma was supplied by the Japanese Foundation for Cancer Research. Tumor cell lines were maintained in RPMI1640 medium supplemented with 10% fetal calf serum, 100 units/ml penicillin, 100 µg/ml streptomycin, and 0.2% NaHCO<sub>3</sub> under 5% CO<sub>2</sub>, at 37°C.

**MTD** The MTD of tubulin binding agents in an intermittent schedule (q4d×3) was determined using CD2F1 mice as follows: AC-7700, 80 mg/kg/day (i.v.), 40 mg/kg/day (s.c.); CS A-4 phosphate, 500 mg/kg/day (i.p.); vinblastine, 10 mg/kg/day (i.v.); E7010, 500 mg/kg/day (p.o.); colchicine, 1 mg/kg/day (i.v.). We defined the tolerable dose range as doses under the MTD and defined the lethal dose range as doses above the MTD.

**Antitumor effects on solid tumor models *in vivo*** Tumor fragments consisting of 5 mg of c26, 5×10<sup>6</sup> cells of c26/acr, and 3×10<sup>6</sup> cells of MethA fibrosarcoma were inocu-

lated s.c. into 5- to 6-week-old CD2F1 mice on day 0. Drugs were administered 3 times at 3-day intervals (q4d×3) after the tumor volume (TV) reached 0.2–0.5 cm<sup>3</sup>. Tumor size (long and short diameter) and body weight were measured two or three times a week. TV was calculated using the following formula

$$TV \text{ (cm}^3\text{)} = (D_L \times D_S^2) \times 1/2 \times 1/1,000$$

where  $D_L$  is the tumor's long diameter and  $D_S$  the short diameter. Antitumor activity was calculated using the following formula

$$T/C \text{ (\%)} = T/C \times 100$$

where  $T$  is the TV in treated mice and  $C$  that in controls. Antitumor effects were evaluated on the day when  $T/C$  reached the minimum after treatment was completed.

**Histopathological analysis** Histological analysis was conducted using mice bearing c26 or MethA (9–10 days after implantation). Mice were injected with a single drug dose and killed by cervical dislocation 6 h after drug injection. Local s.c. tumor masses were removed and fixed with 10% neutral buffered formalin, pH 7.4. Micrometer sections were made and stained with hematoxylin and eosin (H&E).

Mitosis of tumor cells was scored in coded slides by microscopic examination at 200× magnification. The morphological features were used for histological identification of mitotic tumor cells. Nine fields were randomly selected in each histological specimen, and in each field the number of mitotic tumor cells was recorded as number per 5000 cells. The mitotic index was calculated by using the following formula

$$\text{Mitotic index} = Mt/Mc$$

where  $Mt$  is the number of tumor cells at metaphase in mice treated with drugs and  $Mc$  is that in controls.

Necrotic area within a tumor mass was calculated by microscopic examination at 200× magnification using "Photoshop" (version 4.0, Adobe).

**Tumor perfusion measurement** Relative tumor perfusion was evaluated by using the Evans blue extraction technique.<sup>13)</sup> Evans blue (125 mg/kg) was injected i.v. into mice bearing c26 or MethA tumors (9–10 days after tumor inoculation). Mice were killed 30 min later, then the tumors were removed and weighed. Evans blue in tumors was extracted into formamide (3 volumes per tumor weight) at room temperature. The formamide solution was added to a 96-well microplate (50 µl/well) and the concentration of Evans blue was determined from the optical density (630 nm). The content of Evans blue in tumor tissue is proportional to tumor perfusion, and relative perfusion in tumors was expressed as the percentage optical density of treated relative to non-treated tumors.

Tissue content of Evans blue is also influenced by vascular permeability. We evaluated the vascular permeability

in tumor tissue by using the FITC-dextran leakage technique.<sup>14</sup> The administration of tubulin binding agents did not change the vascular permeability in tumor tissue. Values obtained by the Evans blue extraction technique thus mainly reflected tumor perfusion in these models.

**Cytotoxic effects against murine tumor cell line** Tumor cells ( $5 \times 10^3$  in  $50 \mu\text{l}$ /well) were seeded into 96-well microplates and allowed to attach at  $37^\circ\text{C}$ , 5%  $\text{CO}_2$ , for 24 h. Identical plates were then exposed to drugs for 72 h. Cell growth was assessed by MTT assay, for which  $10 \mu\text{l}$  of 5 mg/ml 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT, Sigma Chemical Co.) solution was added to each well and the plates were incubated for 4 h under 5%  $\text{CO}_2$ , at  $37^\circ\text{C}$ . The formed formazan was dissolved in  $100 \mu\text{l}$  of 10% sodium dodecyl sulfate+0.01 N HCl solution. The optical density/well was measured at 570–630 nm using a  $V_{\text{max}}$  microplate reader (Molecular Device Co., Inc., Menlo Park, CA). Cell growth was determined from the percentage ratio of optical density in treated wells to that in non-treated wells. The compound concentration required for 50% inhibition of cell growth ( $\text{IC}_{50}$ ) was determined from the dose-response curve.

## RESULTS

**Inhibitory effect on solid tumor growth** The antitumor activity of tubulin binding agents, AC-7700, CS A-4 phosphate, colchicine, E7010, and vinblastine, was examined in mice bearing subcutaneous c26 tumors using an intermittent schedule (q4d $\times$ 3) (Fig. 1). AC-7700 (s.c.), E7010 (p.o.), and vinblastine (i.v.) strongly suppressed tumor growth in a dose-dependent manner. CS A-4 phosphate showed a moderate growth-suppressive effect at the MTD, whereas colchicine had no effect on c26 tumor growth. The s.c. injection of AC-7700 (40 mg/kg/day) was as effective as i.v. injection of 80 mg/kg/day (MTD) with % treated/control value (T/C) of TV of 31.2 and 21.0, respectively.

**Histological analysis of solid tumors treated in the tolerable dose range** AC-7700 (40 mg/kg, s.c.) produced prominent diffuse hemorrhagic necrosis at both the periphery and center of s.c. c26 tumor tissue with pyknosis of tumor cells 6 h after injection (Fig. 2b). Hemorrhagic necrosis of tumor sections was observed from 3 h after injection in the tolerable dose range of AC-7700 (20–40

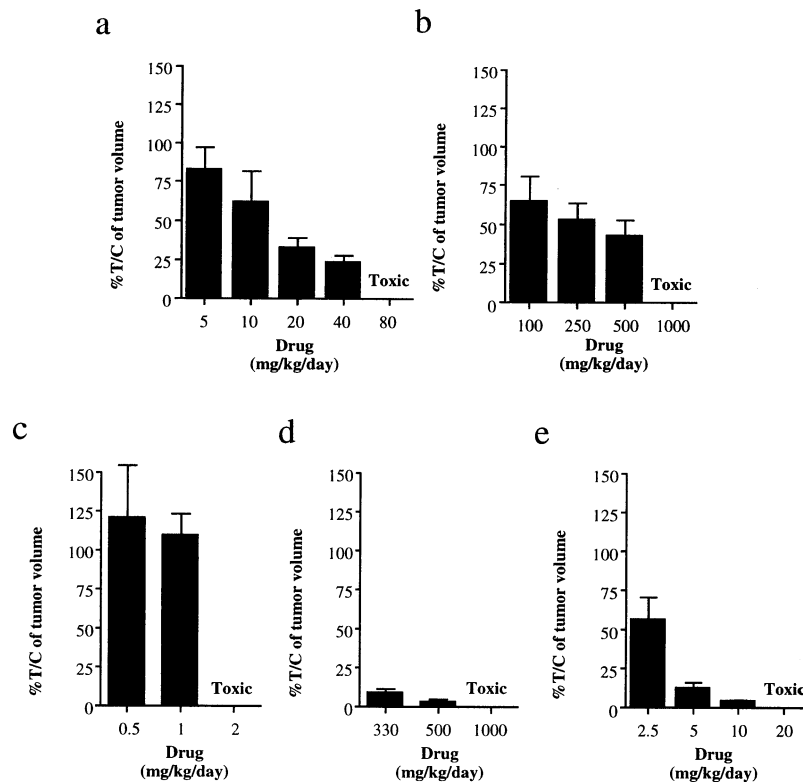


Fig. 1. Antitumor activity of tubulin binding agents in mice bearing colon26 adenocarcinoma; 5 mg of c26 tumor fragment was inoculated s.c. into CD2F1 mice on day 0 and AC-7700 (s.c.) (a), CS A-4 phosphate prodrug (i.p.) (b), colchicine (i.v.) (c), E7010 (p.o.) (d), and vinblastine (i.v.) (e) were administered on day 7, 11 and 15. Antitumor activity was evaluated on the day when the growth-suppressive effect peaked after treatment finished and is expressed as %T/C of tumor volume. Bars: mean  $\pm$  SD ( $n=5-6$ ).

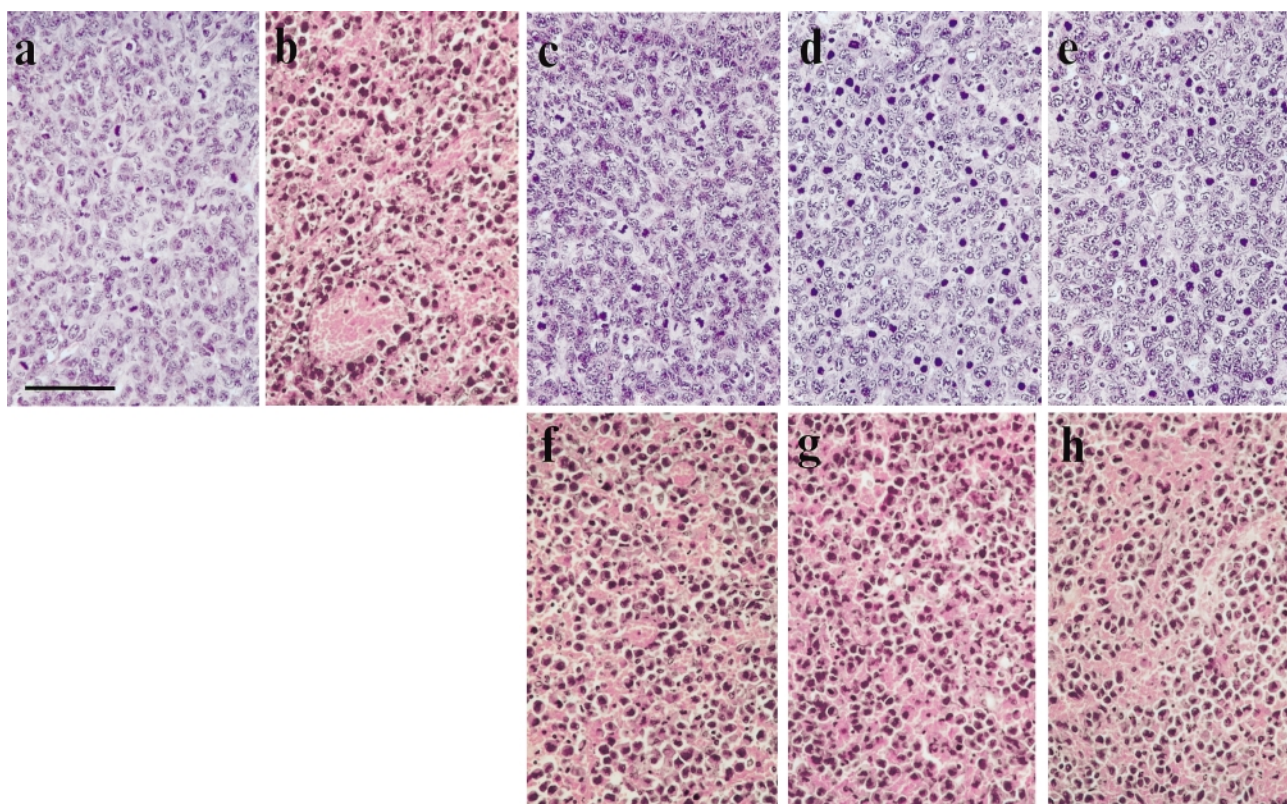


Fig. 2. Histopathological appearance of colon26 adenocarcinoma (H&E) 6 h after treatment with a tolerable (b, c, d, e) or lethal dose (f, g, h) of tubulin binding agents. (a) Non-treated tumor, (b) AC-7700 (40 mg/kg, s.c.), (c) colchicine (1 mg/kg, i.v.), (d) E7010 (330 mg/kg, p.o.), (e) vinblastine (5 mg/kg, i.v.), (f) colchicine (20 mg/kg, i.v.), (g) E7010 (2000 mg/kg, p.o.), (h) vinblastine (40 mg/kg, i.v.). Bar: 100  $\mu$ M.

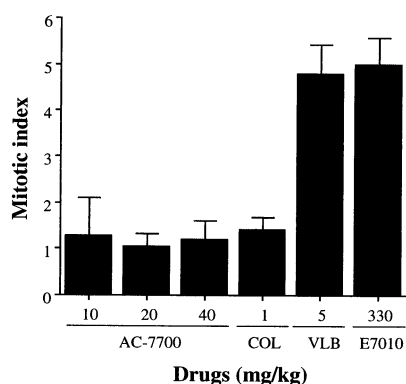


Fig. 3. Mitotic index of tumor cells in c26 tumors treated with tubulin binding agents. Mitotic index was scored from histological sections made from tumors at 6 h after treatment. The mitotic index of non-treated tumor is 1. Bars: mean  $\pm$  SD ( $n=3$ ).

mg/kg, s.c.) and this effect was also induced by i.v. injection of AC-7700 (40–80 mg/kg). CS A-4 phosphate pro-drug (100–500 mg/kg, i.p.) also induced necrotic changes

within the tumor mass, but the affected area was limited to the center of the malignant tissue. The tolerable doses of E7010 (330 mg/kg/day) and vinblastine (5 mg/kg/day) which markedly suppressed tumor growth with %T/C of TV of 12 and 8, respectively, increased the tumor cell population arrested in the metaphase (Fig. 2, d and e). Colchicine has no effect on the histopathology of s.c. c26 tumors (Fig. 2c).

The proportion of tumor cells arrested at metaphase within the c26 tumor mass was quantitatively evaluated as the mitotic index (Fig. 3). Vinblastine and E7010 at tolerable doses increased the tumor cell population arrested in the metaphase by about five times as compared with non-treated tumors 6 h after injection. In contrast, AC-7700 (10–40 mg/kg/day, s.c.) had no influence on the mitotic index of solid c26 tumors.

**Effect of AC-7700 on relative tumor perfusion** A single dose of AC-7700 (40 mg/kg, s.c.) reduced the relative perfusion of c26 tumors from 2 h postinjection; relative tumor perfusion continued to fall 6 h later (Fig. 4). Histological analysis of c26 tumors showed that inhibition of tumor

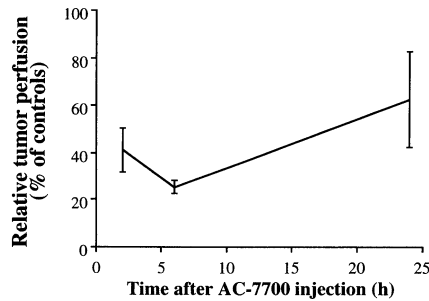


Fig. 4. Influence of AC-7700 on relative tumor perfusion of subcutaneous c26 tumors. A single dose of AC-7700 (40 mg/kg) was administered s.c. and changes in relative tumor perfusion were measured over time. Relative tumor perfusion was measured using the evans blue extraction technique and expressed as the percentage ratio of the optical density of treated to non-treated tumors. Bars: mean $\pm$ SD ( $n=6$ ).

perfusion preceded tumor necrosis. The inhibitory effect of AC-7700 on solid tumor perfusion was also observed in subcutaneous MethA fibrosarcoma (Fig. 5, b and c)

We found inhibition of tumor perfusion at 6 h following a single dosing to be closely correlated with relative TV after AC-7700 treatment in an intermittent schedule (q4d $\times$ 3) in s.c. colon26 adenocarcinoma (Fig. 5a) and MethA fibrosarcoma (Fig. 5b). The inhibitory effect of AC-7700 on perfusion of MethA fibrosarcoma was also correlated with the necrotic area of the tumor section 6 h after a single AC-7700 treatment (Fig. 5c). Although AC-7700 did not affect tumor perfusion at 5 mg/kg, it slightly increased the proportion of tumor cells arrested at the metaphase in c26 tumors (mitotic index=2.74 $\pm$ 0.46).

**Inhibition of tumor perfusion by tubulin binding agents** We evaluated the antivasular activities of tubulin binding agents in the tolerable to lethal dose range. AC-7700 strongly reduced tumor perfusion dose-dependently below the MTD (Fig. 6a). CS A-4 phosphate also showed a moderate inhibitory effect on tumor perfusion at the MTD (Fig. 6b). In contrast, the tolerable doses of colchicine, E7010 and vinblastine had only marginal or no effect on tumor perfusion (Fig. 6, c, d and e).

When the dose was elevated to a lethal level, vinblastine and colchicine potently inhibited tumor perfusion, their effects becoming comparable to those of tolerable AC-7700 doses (Fig. 6, c and e). Vinblastine, colchicine and E7010 also induced diffuse hemorrhagic necrosis with congested tumor vessels in c26 tumors (Fig. 2, f, g and h). Tumor sections resembled those of mice treated with AC-7700 in the tolerable dose range. In contrast, cisplatin which inhibits DNA replication of tumor cells by binding to nucleosides did not affect tumor perfusion or the histopathology of c26 tumors even at the lethal doset. Mice

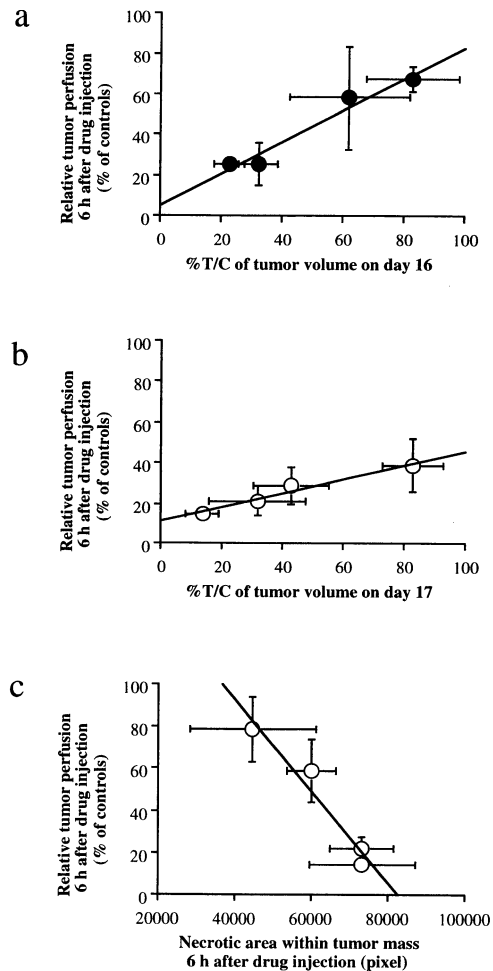


Fig. 5. Correlation between inhibition of tumor perfusion and tumor growth suppression or necrosis induction within the tumor mass by AC-7700 in colon26 adenocarcinoma (a) and MethA fibrosarcoma (b, c). AC-7700 was injected s.c. at doses of 5, 10, 20 and 40 mg/kg/day. Relative tumor perfusion and the necrotic area within the tumor mass were measured 6 h after a single AC-7700 injection and suppression of tumor growth was evaluated on the day when the growth suppressive effect peaked after treatment finished (q4d $\times$ 3). The correlation was examined by linear regression analysis and the regression coefficient was determined as follows: (a)  $y=0.052+0.78x$ ,  $R^2=0.96$ ; (b)  $y=0.11+0.35x$ ,  $R^2=0.97$ ; (c)  $y=1.8-0.00002x$ ,  $R^2=0.95$ . Bars: mean $\pm$ SD ( $n=3-6$ ).

treated with a lethal dose of tubulin binding agents or cisplatin died a few days after drug injection.

Antivasular and antimetabolic activities of tubulin binding agents are summarized in Table I, together with the suppressive effect on tumor growth.

**C26/acr cell line resistant to tubulin binding agents** AC-7739, an active form of AC-7700, inhibited the polymerization of tubulin extracted from the bovine brain with

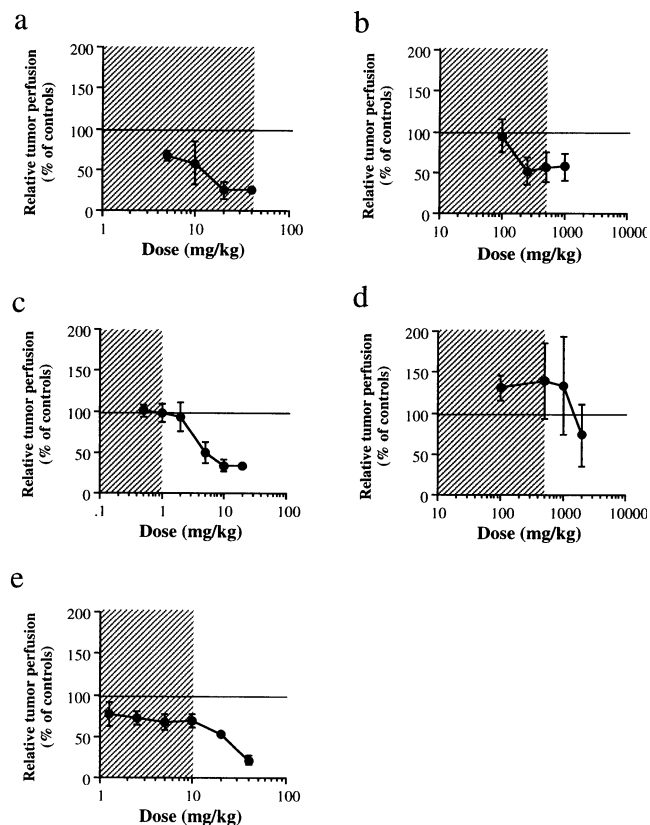


Fig. 6. Effect of AC-7700 and tubulin binding agents on tumor perfusion in mice bearing colon26 adenocarcinoma. Relative tumor perfusion in c26 tumors was measured by the Evans blue extraction technique 6 h after drug injection. Relative tumor perfusion was expressed as the percentage ratio of the Evans blue optical density of treated to non-treated tumors. (a) AC-7700 (s.c.), (b) CS A-4 phosphate prodrug (i.p.), (c) colchicine (i.v.), (d) E7010 (p.o.), (e) vinblastine (i.v.). Hatched column: tolerable dose range. Bars: mean±SD (n=3–6).

Table I. Summary of Antivascular and Antimitotic Activities of Tubulin Binding Agents at a Tolerable Dose Range<sup>a)</sup>

Tubulin binding agent	Antitumor activity (%T/C of TV)	Relative tumor perfusion (% of controls)	Mitotic index
AC-7700	22.7	25.6	1.2
CS A-4 phosphate	42.4	56.4	ND <sup>b)</sup>
Colchicine	109.6	97.9	1.4
E7010	7.9	135.2	4.8
Vinblastine	12.1	67.2	5.0

a) AC-7700 (40 mg/kg, s.c.), CS A-4 phosphate (500 mg/kg, i.p.), colchicine (1 mg/kg, i.v.), E7010 (330 mg/kg, p.o.), vinblastine (5 mg/kg, i.v.).  
 b) ND, not determined.

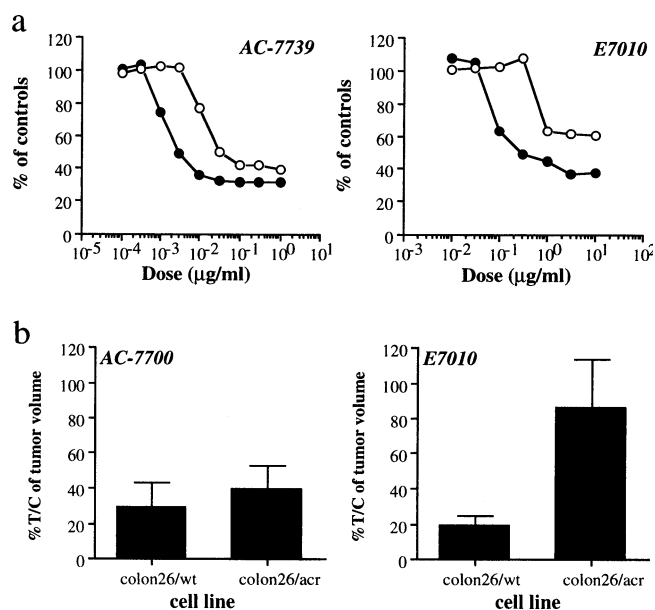


Fig. 7. Effects of AC-7700 and E7010 on c26/acr cell line resistant to tubulin binding agents. (a) *In vitro*; cytotoxicity of AC-7739, the active form of AC-7700 and E7010 was evaluated by using MTT assay, involving contact with drugs for 72 h. Closed circles: c26/wt. Open circles: c26/acr. (b) *In vivo*; inhibitory activity against tumor growth was examined in s.c. tumors. Treatment was initiated when the tumor volume reached about 0.2–0.5 cm<sup>3</sup>. Drugs were administered s.c. (AC-7700, 40 mg/kg/day) or p.o. (E7010, 330 mg/kg/day) in an intermittent schedule (q4d×3) and antitumor activity was evaluated on the day when the %T/C value of tumor volume reached a minimum after treatment was completed. Bars: mean±SD (n=5–6).

an IC<sub>50</sub> of 4 μM, comparable to that of CS A-4 (IC<sub>50</sub>=4 μM), and arrested tumor cells in the metaphase *in vitro*.<sup>10)</sup> To evaluate the contribution of the direct cytotoxic effect of AC-7700 on tumor cells to the suppression of solid tumor growth, c26/acr, a cell line derived from c26 (c26/wt) resistant to tubulin binding agents, was established *in vitro*. C26/acr is about ten times more resistant to AC-7739 than c26/wt *in vitro*, with an IC<sub>50</sub> of 13 ng/ml for c26/acr as compared with 1.4 ng/ml for wild cells (Fig. 7a). E7010, an agent that binds to the colchicine site of tubulin, was also resistant to c26/acr. In contrast to tubulin binding agents, the cytotoxic activities of hydroxyurea, cycloheximide, actinomycin D and oligomycin towards the two cell lines were identical.

The inhibition of solid tumor growth by AC-7700 and E7010 was studied using mice inoculated s.c. with the two cell lines, c26/wt and c26/acr. E7010 showed reduced growth suppression against c26/acr compared to c26/wt with %T/C of TV of 18.9 for c26/wt and 85.4 for c26/acr,



as also observed *in vitro* (Fig. 7b). AC-7700 was equivalently effective against both resistant and wild-type solid tumors, with %T/C of TV; 28.7 for c26/wt and 39.4 for c26/acr. Hemorrhagic necrosis was observed in c26/acr tumors at 6 h after AC-7700 injection.

## DISCUSSION

Tubulin binding agents affect the growth of solid murine tumors in two ways; inhibiting tumor cell mitosis by disrupting the nucleospindle and reducing tumor perfusion through an unknown mechanism. However, the extent to which the antivasular or antimitotic effects of each agent contribute to suppression of solid tumor growth remains to be clarified.

We found that AC-7700, a novel combretastatin A-4 derivative, produced diffuse hemorrhagic necrosis of tumors and reduced tumor tissue perfusion in proportion to tumor growth suppression in the tolerable dose range. We also showed, using the murine tumor cell line c26/acr, which is resistant to tubulin binding agents, that AC-7700 did not act directly on tumor cells *in vivo*. These results suggested that some tubulin binding agents suppressed solid tumor growth through an antivasular effect and that the antivasular effect could potentially inhibit solid tumor growth independently of the direct cytotoxic effect on tumor cells.

Unlike AC-7700, tolerable doses of vinblastine and E7010 that potentially suppressed tumor growth increased the tumor cell population arrested at the metaphase within c26 tumors. These drugs had only a marginal or no effect against tumor perfusion and did not induce hemorrhagic necrosis in tumor tissue at the same doses; tumors treated with vincristine at the MTD also showed the same changes. Therefore, these tubulin binding agents suppressed tumor growth mainly by arresting tumor cells at the metaphase. These observations conflict with previous reports that vinblastine and vincristine potentially inhibited the perfusion of s.c. murine tumors in the tolerable dose range, to about 10–20% of the level in non-treated tumors.<sup>1–3</sup> This discrepancy may be due to differences in the methods of measuring tumor perfusion or in the kind of experimental solid tumors. The Evans blue extraction technique tends to overestimate tissue perfusion as compared with other measurements, such as the <sup>86</sup>RbCl extraction technique and the double-label fluorescence staining technique. The Evans blue methods may detect only larger changes of perfusion which are sufficient to induce tumor necrosis through hypoxic stress. In fact, the hydrogen gas clearance technique showed that AC-7700 completely blocked tumor perfusion in a rat tumor system.<sup>15</sup> In any event, vinblastine had a marginal effect on tumor perfusion compared to AC-7700 using the same measurement method and tumor system. These results suggest that tubu-

lin binding agents can be divided into antimitotic and antivasular agents based on how they suppress solid tumor growth.

Vinblastine, E7010, and colchicine strongly reduced tumor perfusion at the lethal doses. Their inhibition of tumor cell mitosis disappeared and hemorrhagic necrosis was induced in s.c. tumors. A single dose of AC-7700 (5 mg/kg, s.c.), which did not affect tumor perfusion, caused a slight increase of the tumor cell population arrested at the metaphase. Cisplatin, an inhibitor of DNA replication, neither increased tumor cell population in the metaphase nor affected tumor perfusion in the tolerable to lethal dose range. These observations indicate that antivasular and antimitotic effects against solid tumors are common biological activities of tubulin binding agents and the effective dose ranges for these two activities are markedly different for each agent.

The antivasular effect of tubulin binding agents is not mediated by TNF- $\alpha$  induction.<sup>4</sup> The inhibition of angiogenic factor production may also not be related to the action mechanism of the antivasular effect, because shutdown of tumor perfusion commences only 30 min after microtubule inhibitor injection.<sup>15</sup> Dark *et al.* reported that vascular resistance was increased in solid tumors treated with CS A-4 phosphate.<sup>8</sup> Our preliminary study also indicated that AC-7700 elevated the mean arterial blood pressure (MABP) slightly in rats and dogs when given at a tolerable dose. (a) Induction of endogenous vasoconstrictive factors, (b) inhibition of production of vasodilative factors (e.g., constitutive nitric oxide synthesis from endothelial cells), or (c) alteration of endothelial cell shape by AC-7700 may increase peripheral vascular resistance and finally elevate MABP as well as reduce perfusion specifically in tumor tissue. Combining AC-7700 with inhibitors of endogenous vasoactive factors and anticoagulants had no effect on the antitumor activity of AC-7700. This observation is consistent with previous reports that CS A-4 increased vascular resistance in rat tumors perfused with a cell-free buffered solution *ex vivo*.<sup>8</sup> Although the vascular actions of AC-7700 resemble those of a nitric oxide synthase inhibitor, e.g., its tissue selectivity, kinetics of perfusion inhibition, and the effect against MABP,<sup>16, 17</sup> AC-7700 did not inhibit vasodilation of rat aorta dependent upon NO synthesis in an *ex vivo* system. We have observed that human umbilical vein endothelial cells (HUVEC) changed rapidly from flat to round within 1 h of exposure to microtubule inhibitors *in vitro*. Dark *et al.* speculated that endothelial cell detachment could occlude tumor vessels,<sup>8</sup> but we observed no thrombi consisting of vascular endothelial cells or any other component in c26 tumor vessels treated with tubulin binding agents. It thus appears most likely that endothelial cell shape changes narrowed the vessels, and reduced perfusion within malignant tissue.

The mechanism of impairment of solid tumor perfusion by the vasodilative agent hydralazine may serve as a reference for one more hypothesis. Hydralazine reportedly reduces systemic perfusion pressure and causes an imbalance between the intravascular and extravascular pressures in malignant tissue, selectively reducing tumor perfusion.<sup>18,19)</sup> This action depends on high interstitial fluid pressure within the tumor tissue derived from abnormalities of tumor vessels, e.g., high vascular permeability, poor lymph drainage, and a lack of basal lamina and smooth muscle cell lining.<sup>20,21)</sup> Tubulin binding agents may alter this balance by weakening the structural durability of the tumor vasculature through destruction of the cytoskeleton of endothelial cells or by increasing the vascular permeability within malignant tissue. Vascular permeability was increased *in vitro* (HUVEC monolayers on Transwell plates) and *in vivo* (Miles assay using dorsal mouse skin) by treatment with concentrations of AC-7700, vincristine and colchicine that are attainable in plasma when tolerable to lethal doses of these drugs are administered. This observation is consistent with results obtained by Watts *et al.* using CS A-4.<sup>22)</sup> However, histopathological analysis using FITC-dextran leakage methods indicated that AC-7700 did not affect vascular permeability in c26 tumors.

The difference in mode of action between AC-7700 and other tubulin binding agents is also not related to the binding site on the tubulin molecule, because colchicine, E7010, and AC-7700 all bind to the same tubulin "colchicine site."<sup>23-25)</sup> Because almost all tubulin binding agents showed antivasular activity at doses above the MTD, differences in their mode of action may be due to their different abilities to disrupt microtubules and to lethal effects on normal tissue. The hydrogen gas clearance technique

showed that the perfusion of subcutaneous rat tumors was shut down only 30 min after AC-7700 injection.<sup>15)</sup> Mitotic arrest of tumor cells, however, peaked about 6 h later. Potent microtubule disruption over a short period may make it possible to reduce tumor perfusion without the lethal toxicity caused by mitotic inhibition. It is reported that CS A-4, the lead compound of AC-7700, is highly effective among natural products in disrupting microtubules and binds irreversibly to plasma protein (albumin) to be rapidly inactivated.<sup>5,9)</sup> These biological and pharmacokinetic properties may enable CS A-4 and its derivatives to disrupt microtubules for a short period, exerting antivasular actions in the tolerable dose range. This speculation is consistent with our hypothesis that reduction of tumor perfusion was caused by changes in the endothelial cells or tumor vessels, not by endothelial cell death. In our preliminary experiments, the myelotoxicity of AC-7700 was milder than that of vinblastine or colchicine.

In the present study, we showed that tubulin binding agents exert antivasular and antimetabolic effect on solid tumors with markedly different effective dose ranges for each agent, and we demonstrated that the antivasular effect of tubulin binding agents could potentially suppress solid tumor growth independently of direct cytotoxic action on tumor cells. AC-7700 is a unique tubulin binding agent, which exerts antivasular activity at tolerable doses with potent antitumor activity on solid tumors. Further investigation is needed to elucidate the mechanism by which tubulin binding agents reduce solid tumor perfusion.

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