

## A Novel Combretastatin A-4 Derivative, AC-7700, Shows Marked Antitumor Activity against Advanced Solid Tumors and Orthotopically Transplanted Tumors

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**AC-7700, a novel combretastatin A-4 derivative, suppresses the growth of solid tumors by inhibiting tumor perfusion. We evaluated the antitumor activity of AC-7700 on solid tumors in two experimental models, an advanced tumor model (murine colon 26 (c26) adenocarcinoma, colon 38 (c38) adenocarcinoma, MethA fibrosarcoma, Sarcoma 180 (S180), Lewis lung carcinoma (3LL), human LS180 adenocarcinoma) and an orthotopically transplanted tumor model (c26), compared with that of cisplatin (CDDP). The maximum tolerable dose (MTD) of CDDP suppressed early-stage c26 and c38 tumor growth when treatment was started after the tumor volume (TV) reached 0.2–0.5 cm<sup>3</sup>, but it showed reduced activity against the same tumors at an advanced growth stage when TV exceeded 2 cm<sup>3</sup>. At its MTD, AC-7700 was active against all tumors tested except 3LL in both early and advanced growth stages, reducing the tumor mass and having a curative effect in advanced c38 tumors. AC-7700 was also effective on orthotopically transplanted c26 tumors, showing a comparable activity to that on subcutaneous tumors. Unlike flavon acetic acid, which damages tumor vasculature by inducing endogenous tumor necrosis factor- $\alpha$  production, AC-7700 potently suppressed the growth of advanced c26 tumors in athymic as well as euthymic mice. These results suggest that AC-7700 is a novel antivascular agent that may have potent activity against advanced-stage cancer in the clinical setting.**

**Key words:** Combretastatin — Tubulin-binding agent — Advanced solid tumor — Orthotopic transplantation — Host immune status

Despite the potent, direct suppressive effect chemotherapeutic agents have against solid tumor growth in experimental murine tumor models, their clinical application remains marginal, partly due to the progressive growth stage at which human tumors must often be treated. It has been reported that, as tumors grow, low- or no-flow areas are generated in malignant tissue and interstitial fluid pressure also increases.<sup>1–3</sup> Clinical tumors at advanced stage often grow slowly, and their tumor cell population in the growth phase decreases compared with that in subcutaneous (s.c.) rodent tumors. These factors markedly reduce drug accessibility to malignant tissue and tumor cell sensitivity to chemotherapeutic agents whose effectiveness depends on the cell cycle.<sup>4,5</sup>

Unlike chemotherapeutic agents directly affecting tumor cells, the efficacy of agents targeting tumor vasculature may not be affected by these factors in advanced tumors. Agents targeting tumor vasculature that were developed in

the last decade can be divided into (a) vascular damaging agents, e.g., tumor necrosis factor (TNF)- $\alpha$ ,<sup>6</sup> flavon acetic acid (FAA),<sup>7,8</sup> lipid A derivatives,<sup>9</sup> interferon (IFN),<sup>10</sup> and interleukin (IL)-1 $\beta$ ,<sup>11</sup> and (b) angiogenesis inhibitors, e.g., TNP-470,<sup>12–14</sup> 2-methoxyestradiol,<sup>15</sup> endostatin,<sup>16</sup> and angiostatin.<sup>17,18</sup> Many of these agents exhibit potent antitumor activity against murine and human s.c. solid tumors even at an advanced growth stage. However, most of the agents that have finished clinical trials, particularly the vascular damaging agents, have failed to achieve clinical responses of the same magnitude as chemotherapeutic agents directly acting on tumor cells.<sup>19–26</sup> This reduced response to vascular targeting agents may be partly caused by the differences of tumor vascular architecture, vascular response, and host responses to immunological stimulation between experimental s.c. tumors and primary human tumors.

Tubulin-binding agents are reported to reduce perfusion in s.c. murine tumors independently of TNF- $\alpha$  induction.<sup>27,28</sup> However, these agents also exert an antimetabolic effect and are highly toxic to normal tissue at doses which

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inhibit tumor perfusion. We have synthesized AC-7700, a combretastatin A-4 (CS A-4) derivative, which inhibits tubulin polymerization and potently suppresses solid murine tumor growth by reducing tumor perfusion rather than by mitotically arresting tumor cells in the tolerable dose range.<sup>29-31)</sup>

In the present study, we evaluated the antitumor activity of AC-7700 against two different tumor models, an advanced tumor model and an orthotopically transplanted tumor model which reflected human malignancy in growth stage and growth site. In the advanced tumor model, murine and human tumors were inoculated s.c. into mice and treatment was started after the tumor volume reached 2 cm<sup>3</sup>. The antitumor activity of AC-7700 was compared to that of cisplatin (CDDP), which acts directly on tumor cells and is widely used clinically. The other experiment employed an orthotopically transplanted tumor model, in which colon 26 (c26) was inoculated into mice in the cecum, the primary site of this cell line. We also investigated the relationship between antitumor activity of AC-7700 and host immune status using athymic and euthymic mice bearing c26 tumors, and compared the results with those of FAA. A preliminary study has been published as an abstract.<sup>32)</sup>

## MATERIALS AND METHODS

**Drugs** AC-7700 and FAA were synthesized by Ajinomoto Co., Inc. (Central Research Laboratories, Kawasaki). The chemical structures of AC-7739 and AC-7700 are shown in Fig. 1. AC-7700 is a serine prodrug of AC-7739 and is cleaved by aminopeptidase, releasing the active form *in vitro* as well as *in vivo*.

AC-7700 and CDDP (Nippon Kayaku Co., Inc., Tokyo) were dissolved in and diluted with saline. FAA was dissolved in and diluted with 5% NaHCO<sub>3</sub>. AC-7700 was administered intravenously (i.v.) or s.c., CDDP was administered i.v., and FAA was administered intraperitoneally (i.p.).

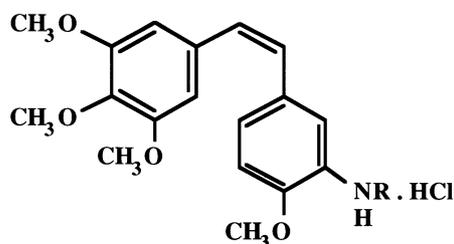


Fig. 1. Chemical structures of AC-7739 and AC-7700. AC-7739: (Z)-2-methoxy-5-[2-(3,4,5-trimethoxyphenyl)viny]phenylamine hydrochloride. AC-7700: (Z)-N-[2-methoxy-5-[2-(3,4,5-trimethoxyphenyl)viny]phenyl]-L-serinamide hydrochloride. AC-7739: R=H. AC-7700: R=COCHNH<sub>2</sub>CH<sub>2</sub>OH.

**Animals** Female BALB/c×DBA/2F1 (CD2F1), C57BL/6×DBA/2F1 (BD2F1), Balb/c mice, Balb/c *nu/nu*, and ICR *nu/nu* athymic nude mice were obtained from Charles River Japan, Inc. (Yokohama). CD2F1, BD2F1, and Balb/c mice were given access to food (CRF1; Charles River Japan, Inc.) and water *ad libitum*. The athymic nude mice diet consisted of autoclaved food and water *ad libitum*. Each strain was 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 colon 26 (c26) adenocarcinoma was supplied by Simonsen Laboratories (Gilroy, CA) under the auspices of the National Cancer Institute, NIH (Bethesda, MD), and was maintained by the Japanese Foundation for Cancer Research (Tokyo). Murine colon 38 (c38) adenocarcinoma, MethA fibrosarcoma, and Lewis lung carcinoma (3LL) were supplied by the Japanese Foundation for Cancer Research (Tokyo). Murine Sarcoma 180 (S180) and p388 leukemia were purchased from Dainihon Pharmaceutical Co., Inc. (Osaka). Human colon adenocarcinoma LS180 was purchased from the American Type Culture Collection (Rockville, MD).

**Antitumor effects on solid tumor models *in vivo*** Tumor fragments consisting of 5 mg of c26, c38, LS180, and 3LL or 3×10<sup>6</sup> cells of MethA and S180 were inoculated s.c. into 5- to 6-week-old CD2F1 (c26, MethA), BD2F1 (c38, 3LL), ICR (S180) and ICR *nu/nu* (LS180) mice on day 0. In early tumor models, drugs were administered on day 7, 11, and 15 when the tumor volume (TV) was 0.2–0.5 cm<sup>3</sup>. In advanced tumor models, drugs were administered 3 times at 3-day intervals (q4d×3) after the TV exceeded 2 cm<sup>3</sup>.

After treatment started, tumor size (long and short diameter) and body weight were measured two or three times a week. TV was calculated by use of the following formula

$$TV (\text{cm}^3) = (DL \times DS^2) \times 1/2 \times 1/1000$$

where *DL* is the tumor's long diameter and *DS* the short diameter. The inhibition ratio (IR, %) of TV was calculated by use of the following formula

$$IR (\%) = (1 - T/C) \times 100$$

where *T* is the TV in treated mice and *C* that in controls. Antitumor effects were evaluated on the day when IR peaked after the treatment was completed. Body weight change (BWC) was calculated by means of the following formula

$$BWC (\%) = (Wdx/Wds - 1) \times 100$$

where *Wdx* is body weight on the day when the antitumor effect was evaluated and *Wds* is body weight on the day treatment was started.

**Antitumor effects on ascitic tumor model *in vivo*** p388

Table I. Antitumor Activity of AC-7700 and CDDP against Colon 26 Adenocarcinoma<sup>a)</sup>

Drugs	Dose (mg/kg/day) q4d×3 <sup>b)</sup>	Route	Early tumor model				Advanced tumor model			
			IR <sup>c)</sup> (%)	BWC <sup>d)</sup> (%)	Dead/total	Comment <sup>e)</sup>	IR <sup>c)</sup> (%)	BWC <sup>d)</sup> (%)	Dead/total	Comment <sup>e)</sup>
AC-7700	40	i.v.	70.4	-3.2	0/6	Active	57.9	-6.4	0/6	Active
	80	i.v.	78.7	-7.6	0/6	MTD, <sup>f)</sup> active	84.1	-4.6	0/6	MTD, <sup>f)</sup> highly active
AC-7700	5	s.c.	17.2	-1.3	0/5	Inactive	2.4	-6.6	0/5	Inactive
	10	s.c.	38.0	3.3	0/5	Inactive	25.8	-12.4	0/5	Inactive
	20	s.c.	67.8	-0.2	0/5	Active	31.8	-5.3	0/5	Inactive
	40	s.c.	77.4	-4.0	0/5	MTD, <sup>f)</sup> active	75.7	-1.9	0/5	MTD, <sup>f)</sup> active
CDDP	2.5	i.v.	24.1	-6.0	0/5	Inactive	29.9	-6.6	0/5	Inactive
	5	i.v.	63.7	-6.4	0/5	MTD, <sup>f)</sup> active	34.5	-14.3	0/5	MTD, <sup>f)</sup> inactive
FAA	150	i.p.	52.1	-2.5	0/5	Active	68.4	-7.2	0/6	Active
	200	i.p.	65.6	-5.2	0/5	MTD, <sup>f)</sup> active	95.7	4.0	0/6	MTD, <sup>f)</sup> highly active

- a) Colon 26 adenocarcinoma was inoculated s.c. into female CD2F1 mice on day 0.
- b) Treated on day 7, 11 and 15 in early tumor model, and on day 14, 18 and 22 in advanced tumor model.
- c) IR, inhibition ratio of tumor volume.
- d) BWC, body weight change.
- e) Activity rating is indicated in Table VI.
- f) MTD, maximum tolerable dose.

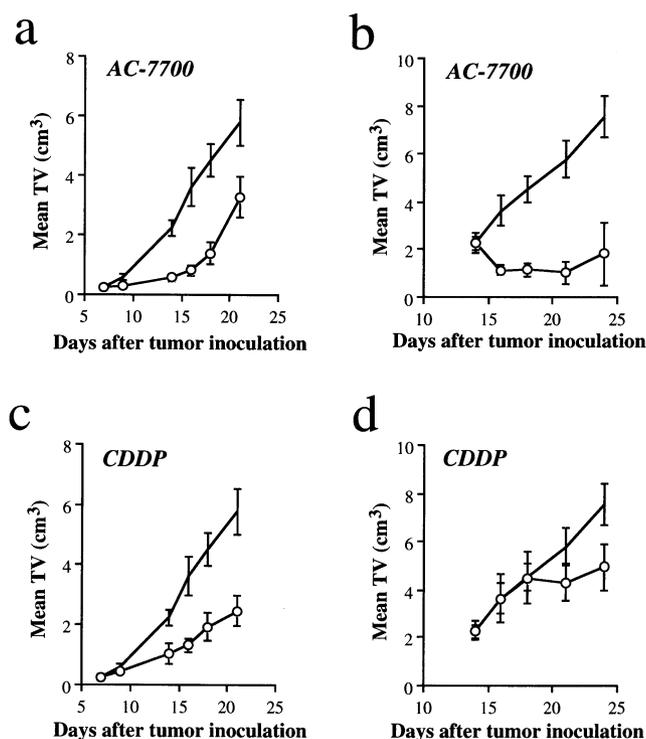


Fig. 2. Growth curves of c26 tumors in early (a, c) and advanced (b, d) growth stage treated with AC-7700 (40 mg/kg, s.c.) and CDDP (5 mg/kg, i.v.). c26 was inoculated s.c. into CD2F1 mice on day 0. Drugs were administered on day 7, 11 and 15 in the early tumor model and on day 14, 18 and 22 in the advanced tumor model. Solid line, mean TV of nontreated mice. Open circles, TV of treated mice. Bars, mean±SD (n=5).

leukemia ( $3 \times 10^6$  cells) was inoculated i.p. into 6-week-old female CD2F1 mice on day 0. Drugs were administered on day 1, 5, and 9 and antitumor activity was evaluated in terms of increased life span in mice, calculated by using the following formula

$$\%T/C = LT \times 1 / Ln \times 100$$

where  $Ln$  is the mean survival days of nontreated and  $LT$  is that of drug-treated mice.

**Antitumor effects on orthotopically transplanted tumors** c26 cells ( $5 \times 10^5$ ) were inoculated intracemumly (i.c.) into 6-week-old female CD2F1 mice on day 0. Drugs were administered on day 9, 13, and 17. Mice were killed by cervical dislocation on day 19. Local i.c. tumor size was measured and TV was calculated as described above. **Antitumor activity of AC-7700 in immune-deficient mice** Tumor fragments of c26 were inoculated s.c. into 7-week-old Balb/c or Balb/c *nu/nu* mice on day 0. FAA and AC-7700 were administered on day 8 and 15. After treatment started, tumor size and body weight were measured two or three times a week. Antitumor activity was calculated as described above.

## RESULTS

### Antitumor activity against advanced solid murine tumor models

**Colon 26 adenocarcinoma:** AC-7700 and CDDP were tested using an intermittent schedule (q4d×3) in early- and advanced-stage s.c. c26 (Table I). I.v. and s.c. injection of AC-7700 exerts strong antitumor activity against early-

Table II. Antitumor Activity of AC-7700 and CDDP against Colon 38 Adenocarcinoma<sup>a)</sup>

Drugs	Dose (mg/kg/day) q4d×3 <sup>b)</sup>	Route	Early tumor model				Advanced tumor model			
			IR (%)	BWC (%)	Dead/total	Comment	IR (%)	BWC (%)	Dead/total	Comment
AC-7700	5	s.c.	31.0	2.4	0/5	Inactive	31.0	-14.7	1/5	Inactive
	10	s.c.	51.3	4.8	0/5	Active	69.1	3.2	0/5	Active
	20	s.c.	82.1	11.4	0/5	Highly active	84.2	3.2	0/5	Highly active
	40	s.c.	ND <sup>c)</sup>	ND <sup>c)</sup>	ND <sup>c)</sup>		92.8	1.7	0/5	MTD, highly active
CDDP	2.5	i.v.	46.4	-10.3	2/5	Inactive	46.3	-10.6	3/5	Inactive
	5	i.v.	57.2	-9.9	0/5	MTD, active	14.5	-22.2	4/5	MTD, inactive

a) Colon 38 adenocarcinoma was inoculated s.c. into female BD2F1 mice on day 0.

b) Treated on day 7, 11 and 15 in early tumor model, and on day 14, 18 and 22 in advanced tumor model.

c) ND, not determined.

stage c26 tumors at half the MTD (maximum tolerable dose) and at the MTD. AC-7700 also suppressed advanced-stage c26 tumor growth, reducing the tumor mass (Fig. 2). AC-7700 did not induce body weight loss due to drug toxicity, with a BWC of -4.6% (80 mg/kg/day, i.v.) (BWC of nontreated mice bearing advanced c26 tumors: -15.3%). AC-7700 was also highly effective against advanced c26 tumors in s.c. administration.

Although MTD (5 mg/kg/day) of CDDP suppressed early-stage c26 tumor growth (IR: 63.7%), it showed reduced activity in the advanced tumor model (IR: 34.5%). MTD of CDDP did not suppress the body weight reduction caused by tumor bearing, producing a BWC of -14.3%.

*Colon 38 adenocarcinoma:* Early- and advanced-stage s.c. c38 tumors were highly sensitive to AC-7700 (Table II). In the advanced tumor model, a potent antitumor activity was observed even at one-fourth of the MTD and s.c. injection of 20 or 40 mg/kg/day AC-7700 produced a curative effect (2/5) and markedly increased the life span of mice with %T/C values of 194% and 187%, respectively (Fig. 3).

The CDDP dose tested (5 mg/kg/day) was active against early-stage tumors with an IR of 57.2%, but had no effect against advanced-stage tumors, causing loss of body weight due to drug toxicity.

*MethA fibrosarcoma:* AC-7700 at the tested doses moderately inhibited early-stage MethA tumor growth (IR: 52.9–64.7%) (Table III). In the advanced tumor model, one-fourth the MTD (5.5 mg/kg/day) produced suppressive activity on tumor growth comparable to the MTD (43.6 mg/kg/day) in the early tumor model, and higher AC-7700 doses strongly suppressed advanced tumor growth.

CDDP administration of 5 mg/kg/day was ineffective in early- and advanced-stage MethA tumors (IR: <50%). The effect was marginal and equivalent to that of AC-7700 at a dose of less than one-fourth the MTD in each model.

*Sarcoma 180:* In advanced-stage S180 tumors (Table IV), AC-7700 suppressed tumor growth moderately with an IR

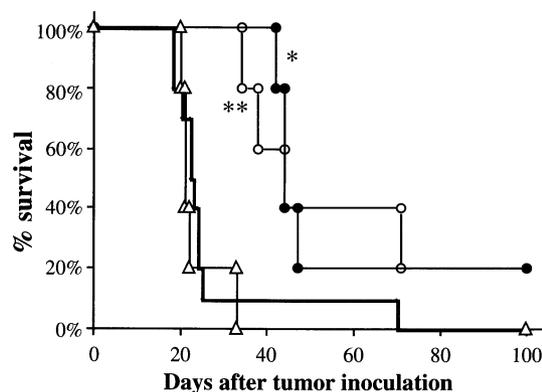


Fig. 3. Increase of life span in mice bearing advanced colon 38 adenocarcinoma by AC-7700. A tumor fragment of colon 38 was inoculated s.c. on day 0 and drugs were administered on day 14, 18 and 22. Solid line, nontreated mice; open circles, AC-7700 20 mg/kg/day, s.c.; closed circles, AC-7700 40 mg/kg/day, s.c.; triangles, CDDP 5 mg/kg/day, i.v. \*  $P < 0.05$  or \*\*  $P < 0.01$ , significantly different from the control group (generalized Wilcoxon test).

of 64.7% (43.6 mg/kg/day, s.c.). CDDP was also effective against advanced S180 tumors.

*Lewis lung carcinoma:* AC-7700 and CDDP were marginally effective against advanced 3LL tumors (Table IV). In this tumor model, mice died of tumor growth and metastasis during drug treatment.

#### Antitumor activity against advanced-stage human tumor xenograft

*Human colon carcinoma LS180:* AC-7700 was highly effective against advanced-stage human colon adenocarcinoma LS180 in two types of intermittent schedule (q4d×3, q2d×6) (Table V). The MTD of CDDP was not effective in either early- or advanced-stage tumors.

AC-7700 induced hemorrhagic necrosis in a tumor mass 6 h after injection and peak tumor growth suppression was

Table III. Antitumor Activity of AC-7700 and CDDP against MethA Fibrosarcoma<sup>a)</sup>

Drugs	Dose (mg/kg/day) q4d×3 <sup>b)</sup>	Route	Early tumor model				Advanced tumor model			
			IR (%)	BWC (%)	Dead/total	Comment	IR (%)	BWC (%)	Dead/total	Comment
AC-7700	5.5	s.c.	ND	ND	ND	ND	56.3	-1.9	0/6	Active
	10.9	s.c.	52.9	-0.9	0/6	Active	74.8	4.3	0/6	Active
	21.8	s.c.	56.3	-3.8	0/6	Active	88.7	3.0	0/6	MTD, highly active
	43.6	s.c.	64.7	-7.2	0/6	MTD, active	93.0	2.9	2/6	Toxic
CDDP	2.5	i.v.	ND	ND	ND	ND	27.9	-9.5	0/6	MTD, inactive
	5	i.v.	45.9	-0.6	0/6	MTD, inactive	47.0	-8.3	2/6	Toxic

a) MethA fibrosarcoma was inoculated s.c. into female CD2F1 mice on day 0.

b) Treated on day 11, 15 and 19 in early tumor model, and on day 17, 21 and 25 in advanced tumor model.

Table IV. Antitumor Activity of AC-7700 and CDDP against Sarcoma 180 and Lewis Lung Carcinoma at Advanced Growth Stage<sup>a)</sup>

Drugs	Dose (mg/kg/day) q4d×3 <sup>b)</sup>	Route	S180				3LL			
			IR (%)	BWC (%)	Dead/total	Comment	IR (%)	BWC (%)	Dead/total	Comment
AC-7700	5.5	s.c.	28.0	-2.8	0/6	Inactive	13.9	-5.9	0/6	Inactive
	10.9	s.c.	50.9	-0.4	0/6	Active	35.9	-3.1	1/6	Inactive
	21.8	s.c.	40.5	-4.5	0/6	Inactive	24.3	-6.8	2/6	MTD, inactive
	43.6	s.c.	64.7	-9.0	0/6	MTD, active	Toxic	Toxic	6/6	Toxic
CDDP	2.5	i.v.	25.4	-11.5	0/6	Inactive	-11.1	-4.7	2/6	Inactive
	5	i.v.	55.3	-6.1	0/6	MTD, active	18.1	-9.4	1/6	MTD, inactive

a) Sarcoma 180 was inoculated s.c. into female ICR mice on day 0. Lewis lung carcinoma was inoculated s.c. into female BD2F1 mice on day 0.

b) Treated on day 17, 21 and 25 in Sarcoma 180, and on day 14, 18 and 22 in Lewis lung carcinoma.

Table V. Antitumor Activity of AC-7700 and CDDP against Human Colon Adenocarcinoma LS180<sup>a)</sup>

Drugs	Dose (mg/kg/day)	Route	Early tumor model				Advanced tumor model			
			IR (%)	BWC (%)	Dead/total	Comment	IR (%)	BWC (%)	Dead/total	Comment
AC-7700	60×3 (q4d) <sup>b)</sup>	i.v.	60.4	-6.0	0/8	MTD, active	67.8	-9.4	0/7	MTD, active
AC-7700	30×6 (q2d) <sup>c)</sup>	i.v.	ND	ND	ND		70.5	-2.1	0/7	Active
	60×6 (q2d) <sup>c)</sup>	i.v.	74.5	-4.0	0/8	MTD, active	81.4	0.4	0/7	MTD, highly active
CDDP	5×3 (q4d) <sup>b)</sup>	i.v.	29.7	0.4	0/8	MTD, inactive	40.7	-17.6	0/7	MTD, inactive

a) Colon adenocarcinoma LS180 was inoculated s.c. into female ICR *nu/nu* mice on day 0.

b) Treated on day 10, 14 and 18 in early tumor model, and on day 27, 31 and 35 in advanced tumor model.

c) Treated on day 10, 12, 14, 17, 19 and 21 in early tumor model, and on day 27, 29, 31, 34, 36 and 38 in advanced tumor model.

attained a few days after drug treatment in all tumors tested above.

**Antitumor activity against murine ascitic tumor models** AC-7700 and CDDP activity against the p388 leukemia ascitic tumor model was evaluated using an intermittent schedule (q4d×3) (Fig. 4). The MTD of AC-7700 was marginally effective with a %T/C of 119%, while the MTD of CDDP potentially increased the mouse

life span with a %T/C of 333%. A vascular damaging agent, FAA was inactive on the ascitic tumor model with a %T/C of 88%.

The antitumor activities of AC-7700 and CDDP are summarized in Table VI, together with the criteria of activity.

**Antitumor activity against orthotopically transplanted tumors** Antitumor activity of AC-7700 against c26

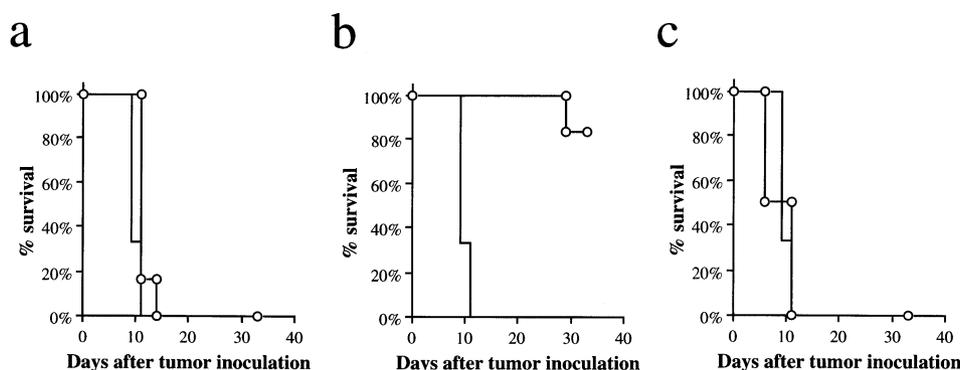


Fig. 4. Survival curves of mice bearing murine p388 leukemia ascitic tumor. p388 cells ( $3 \times 10^6$ ) were inoculated i.p. into CD2F1 mice on day 0 ( $n=6$ ). (a) AC-7700 (80 mg/kg/day, i.v.), (b) CDDP (5 mg/kg/day, i.v.), and (c) FAA (200 mg/kg/day, i.p.) were administered on day 1, 5, 9. Solid line, nontreated mice; open circles, treated mice.

Table VI. Comparison of Antitumor Activity of AC-7700 and CDDP at Optimal IV Treatment Schedule

Tumor system	Model activity rating			
	Early tumor models		Advanced tumor models	
	AC-7700	CDDP	AC-7700	CDDP
i.p. p388 leukemia	–	+++	ND	ND
s.c. colon 26 adenocarcinoma	++	+	++	–
s.c. colon 38 adenocarcinoma	+++	+	+++	–
s.c. MethA fibrosarcoma	+	–	+++	–
s.c. Sarcoma 180	ND	ND	+	+
s.c. Lewis lung carcinoma	ND	ND	–	–
s.c. human colon adenocarcinoma LS180	+	–	++	–
Activity rating	Rating	Leukemias (%T/C)	Solid tumors (IR)	
Inactive	–	<120%	<50%	
Active	+	120–160%	50–65%	
	++	160–200%	65–80%	
	+++	>200%	>80%	

tumors inoculated orthotopically into murine cecum was evaluated using an intermittent schedule (q4d $\times$ 3) (Fig. 5). The MTD of AC-7700 strongly suppressed the growth of i.c. c26 tumors with an IR of TV of 76%, comparable to that in s.c. c26 tumors.

#### Comparison with antivascular agents

**Advanced colon 26 tumors:** FAA is a TNF- $\alpha$  inducer reported to be effective against advanced-stage solid murine tumors.<sup>8)</sup> The MTD (200 mg/kg/day) of FAA suppressed tumor growth to the same extent as AC-7700 at the MTD in early- and advanced-stage c26 tumors (Table I).

**Colon 26 tumors in immune-deficient mice:** Euthymic and athymic mice bearing c26 tumors were treated with drugs 2 times at 7-day intervals, on day 8 and 15. The MTD of

AC-7700 was effective against c26 tumors in both euthymic balb/c mice and nude balb/c mice without a significant difference (Fig. 6). Although the MTD of FAA suppressed c26 tumor growth in the euthymic host comparably to AC-7700, it was ineffective in a nude mouse host.

#### DISCUSSION

Chemotherapeutic agents directly affecting tumor cells have only limited clinical value for solid tumor treatment. This is due in part to the difficulties related to progression of tumor growth, e.g., reduction of tumor cell population in the growth stage, drug delivery depression, cachexia induction, and generation of mutant tumor cells exhibiting multidrug resistance.

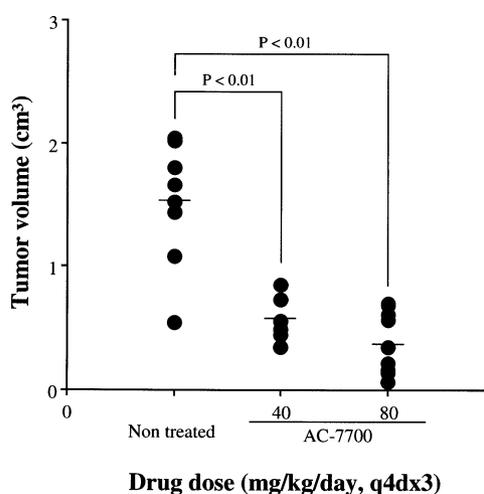


Fig. 5. Antitumor activity of AC-7700 on orthotopically transplanted c26 tumors. c26 cells ( $5 \times 10^5$ ) were inoculated i.c. into CD2F1 mice on day 0. AC-7700 was administered i.v. on day 9, 13, 17 and antitumor activity was evaluated on day 19. Non-treated mice,  $n=8$ ; AC-7700 40 mg/kg/day,  $n=6$ ; AC-7700 80 mg/kg/day,  $n=10$ . The significance of differences was examined using the Dunnett two-tailed test.

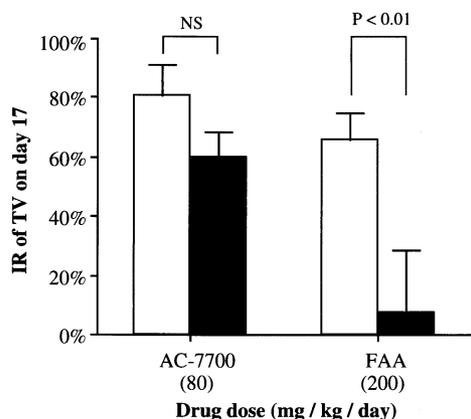


Fig. 6. Antitumor activity of AC-7700 in immune-deficient mice. A tumor fragment of c26 was inoculated s.c. into Balb/c (open column) or Balb/c *nu/nu* (solid column) mice on day 0. AC-7700 (80 mg/kg/day, i.v.) and FAA (200 mg/kg/day, i.p.) were administered on day 8 and 15. Antitumor activity was evaluated on day 17 when the IR of TV peaked after the treatment was completed. Significant differences were examined using the Tukey compromise test. Bars, mean  $\pm$  SD ( $n=5$ ); NS, no significant difference.

We found that CDDP prolonged the life span of mice bearing p388 ascitic tumors and inhibited early-stage solid tumor growth but showed reduced activity against an advanced solid tumor model. The efficacy of other cancer

chemotherapeutic agents, such as 5'-deoxy-5-fluorouridine (5'DFUR) and E7010, which also exert an effect dependent on tumor cell mitosis, was also decreased against advanced tumors (data not shown). These results confirmed clinical observations that chemotherapeutic agents directly targeting tumor cells have reduced efficacy against advanced-stage tumors.

In contrast, although AC-7700 was only marginally effective against ascitic tumors, it markedly suppressed growth in almost all early- and advanced-stage solid tumors in multiple doses. In previous studies, AC-7700 exhibited antitumor activity by inhibiting perfusion within solid tumors, inducing hemorrhagic necrosis.<sup>30</sup> Dark *et al.* also reported that CS A-4, a lead compound of AC-7700, had antivascular activity on solid tumors.<sup>29</sup> Indirect action of AC-7700 on tumor cells may account for the prominent effect in the advanced tumor model. In fact, the immunopotentiator FAA, which disrupts tumor vasculature by inducing TNF- $\alpha$  production within the tumor mass, was also active on advanced-stage c26 tumors although it was inactive on ascitic tumors. Moreover, it was reported that an antibody-tissue factor conjugate which produced thrombosis in malignant tissue vessels is active against advanced-stage solid tumors,<sup>33</sup> and that inhibiting tumor perfusion by clamping tumor vessels can delay solid tumor growth.<sup>34</sup> These results show that indirect killing of tumor cells has advantages over direct killing in the treatment of advanced-stage solid tumors.

Hori *et al.* reported that blood flow in rat s.c. tumor was greatly slowed or even stopped transiently in large tumors, and that chemotherapeutic agents had reduced effectiveness due to poor drug delivery.<sup>1,2</sup> This observation was consistent with our results described above. However, why did AC-7700 exhibit antitumor activity in the advanced tumor model despite poor drug delivery? By means of the hydrogen gas clearance technique, complete inhibition of tumor perfusion was observed within 30 min after AC-7700 injection (Hori *et al.*, unpublished data). Thus, the drug concentration in malignant tissue may have been sufficient to decrease tumor perfusion immediately after bolus injection and then the reduced perfusion may have kept the drug concentration high enough to prolong the cessation of tumor perfusion even after the plasma drug level decreased. In fact, our preliminary study showed that a high concentration of AC-7739, the active form of AC-7700 was maintained in c26 tumors 24 h after AC-7700 injection.

In many patients, advanced-stage malignant tissue grows slowly or is static with diminished populations of endothelial cells as well as tumor cells in the growth phase. It was reported that the vessel density in rat solid tumors changed with progression of tumor growth.<sup>35</sup> In high-growth phases just after tumor inoculation, the vessel density increases in proportion to tumor growth. In slow-

growth phases after a large tumor mass has developed, the vessel density starts to decrease, producing necrosis in malignant tissue, as in clinical tumors. AC-7700 was effective against all early- and advanced-stage tumors tested except 3LL, which has different vessel density and angiogenic properties.

Field *et al.* reported that vascular response caused by hydralazine differed between primary murine skin tumors and s.c. transplanted tumors.<sup>36)</sup> It was also reported that orthotopically transplanted tumors showed much higher and more physiological expression of tumor progression and metastatic capability than did s.c. tumors.<sup>37,38)</sup> We observed that AC-7700 inhibited growth of orthotopically as well as s.c. transplanted c26 tumors, suggesting that AC-7700 may be active against clinical primary tumors whose tumor vascular architecture is different from that of experimental s.c. tumors. The effect of AC-7700 on perfusion of orthotopic c26 tumors is now being assessed.

Vascular damaging agents that disrupt malignant tissue perfusion are classified into tubulin-binding agents and agents acting via the host immune system, e.g., TNF- $\alpha$ , TNF- $\alpha$  inducer (FAA and lipid A derivatives), IFNs and IL-1 $\beta$ . Lymphocytes partly contribute to the antitumor activity of these agents acting via the immune system.<sup>39,40)</sup> In normal mice, FAA potently suppresses c26 tumor growth at an advanced growth stage in a manner comparable to AC-7700, but it shows decreased efficacy in athymic mice, suggesting that its activity is dependent on lymphocytes.<sup>39)</sup> AC-7700, however, potently suppressed tumor growth independently of host immune status. Our preliminary studies have also indicated that AC-7700 (a) maintained activity against solid tumor growth when combined with dexamethasone, and (b) did not induce expression of mRNAs for cytokines with anti-vascular activity

(TNF- $\alpha$ , IL-1 $\beta$ , and IFN- $\gamma$ ) during reduced tumor perfusion (data not shown). Vinblastine and vincristine are also reported to reduce perfusion of solid murine tumors through some mechanism other than the induction of TNF- $\alpha$ .<sup>28,41)</sup> These results show that AC-7700 has pharmacological properties different from those of immunopotentiators affecting the tumor vasculature.

Although human recombinant TNF- $\alpha$  and the endogenous TNF- $\alpha$  inducer FAA showed dramatic preclinical activity, they produced no demonstrable clinical response. This was because doses could not be escalated to the effective range due to adverse side effects (hypotension, fever, and warmth induced by prostaglandin release). In preliminary studies using rats, we found that AC-7700 induced moderate hypertension rather than hypotension in the effective dose range (data not shown). The myelotoxicity of AC-7700 was low enough for it to exert a curative effect against advanced c38 tumors. Dose-limiting toxicities of AC-7700 are diarrhea and pulmonary edema. These toxicological differences between AC-7700 and TNF- $\alpha$  have encouraged us to examine the effectiveness of AC-7700 in clinical trials.

To summarize, we found that AC-7700 is highly effective against advanced-stage solid murine and human tumors, and against orthotopically transplanted solid tumors through an antivascular effect that is independent of the host immune status. The pharmacological and toxicological properties of AC-7700 are different from those of vascular damaging agents which have already been tested in clinical trials, suggesting that AC-7700 may be useful to treat advanced-stage cancer patients.

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## REFERENCES

- 1) Hori, K., Suzuki, M., Tanda, S. and Saito, S. Characterization of heterogeneous distribution of tumor blood flow in the rat. *Jpn. J. Cancer Res.*, **82**, 109–117 (1991).
- 2) Hori, K., Suzuki, M., Tanda, S., Saito, S., Shinozaki, M. and Zhang, Q.-H. Circadian variation of tumor blood flow in rat subcutaneous tumors and its alteration by angiotensin II-induced hypertension. *Cancer Res.*, **52**, 912–916 (1992).
- 3) Jain, R. K. Transport of molecules in the tumor interstitium: a review. *Cancer Res.*, **47**, 3039–3051 (1987).
- 4) Horsman, M. R., Chaplin, D. J. and Overgaard, J. The use of blood flow modifiers to improve the treatment response of solid tumors. *Radiother. Oncol.*, **20** (Suppl.), 47–52 (1991).
- 5) Suzuki, M., Hori, K., Abe, I., Saito, S. and Sato, H. A new approach to cancer chemotherapy: selective enhancement of tumor blood flow with Angiotensin II. *J. Natl. Cancer Inst.*, **67**, 663–669 (1981).
- 6) Carswell, E. A., Old, L. J., Kassel, R. L., Green, S., Fiore, N. and Williamson, B. An endotoxin-induced serum factor which causes necrosis of tumors. *Proc. Natl. Acad. Sci. USA*, **72**, 3666–3670 (1975).
- 7) Mahadevan, V., Malik, S. T. A., Meager, A., Fiers, W., Lewis, G. P. and Hart, I. R. Role of tumor necrosis factor in flavone acetic acid-induced tumor vasculature shutdown. *Cancer Res.*, **50**, 5537–5542 (1990).
- 8) Pratesi, G., Rodolfo, M., Rovetta, G. and Parmiani, G. Role of T cells and tumor necrosis factor in antitumor activity and toxicity of flavon acetic acid. *Eur. J. Cancer*, **26**, 1079–1083 (1990).
- 9) Yang, D., Satoh, M., Ueda, H., Tsukagoshi, S. and Yamazaki, M. Activation of tumor-infiltrating macrophages by a synthetic lipid A analog (ONO-4007) and its implication in antitumor effects. *Cancer Immunol. Immunother.*, **38**, 287–293 (1994).

- 10) Dvorak, H. F. and Gresser, I. Microvascular injury in pathogenesis of interferon-induced necrosis of subcutaneous tumor in mice. *J. Natl. Cancer Inst.*, **81**, 497–502 (1989).
- 11) Belardelli, F., Proietti, E., Ciolli, V., Sestili, P., Carpinelli, G., Vito, M. D., Ferretti, A., Woodrow, D., Boraschi, D. and Podo, F. Interleukin-1 beta induces tumor necrosis and early morphologic and metabolic changes in transplantable mouse tumors. Similarities with the anti-tumor effects of tumor necrosis factor alpha or beta. *Int. J. Cancer*, **44**, 116–123 (1989).
- 12) Yanase, T., Tamura, M., Fujita, K., Kodama, S. and Tanaka, K. Inhibitory effect of angiogenesis inhibitor TNP-470 on tumor growth and metastasis of human cell lines *in vitro* and *in vivo*. *Cancer Res.*, **53**, 2566–2570 (1993).
- 13) Yamaoka, M., Yamamoto, T., Masaki, T., Ikeyama, S., Sudo, K. and Fujita, T. Inhibition of tumor and metastasis of rodent tumors by the angiogenesis inhibitor O-(chloroacetyl-carbamoyl)fumagillol (TNP-470; AGM-1470). *Cancer Res.*, **53**, 4262–4267 (1993).
- 14) Gutterman, J. and Kavanagh, J. A phase I study of the toxicity, pharmacokinetics, and activity of TNP-470 administered to patients with advanced or recurrent squamous cell cancer of the cervix. *Proc. Am. Soc. Clin. Oncol.*, **14**, 281 (1995).
- 15) Fotsis, T., Zhang, Y., Pepper, M. S., Adlercreutz, H., Montesano, R., Nawroth, P. P. and Schweigerer, L. The endogenous oestrogen metabolite 2-methoxyoestradiol inhibits angiogenesis and suppresses tumor growth. *Nature*, **368**, 237–239 (1994).
- 16) Boehm, T., Folkman, J., Browder, T. and O'Reilly, M. S. Antiangiogenic therapy of experimental cancer does not induce acquired drug resistance. *Nature*, **390**, 404–407 (1997).
- 17) O'Reilly, M. S., Holmgren, L., Chen, C. and Folkman, J. Angiostatin induces and sustains dormancy of human primary tumors in mice. *Nat. Med.*, **2**, 689–692 (1996).
- 18) Sim, B. K. L., O'Reilly, M. S., Liang, H., Fortier, A. H., He, W., Madsen, J. W., Lapcevic, R. and Nacy, C. A. A recombinant human Angiostatin protein inhibits experimental primary and metastatic cancer. *Cancer Res.*, **57**, 1329–1334 (1997).
- 19) Hieber, U. and Heim, M. E. Tumor necrosis factor for the treatment of malignancies. *Oncology*, **51**, 142–153 (1994).
- 20) Feinberg, B., Kurzorck, R., Talpaz, M., Blick, M., Saks, S. and Gutterman, J. U. Phase I trial of intravenously-administered recombinant tumor necrosis factor-alpha in cancer patients. *J. Clin. Oncol.*, **6**, 1328–1334 (1988).
- 21) Kerr, D. J., Maughan, T., Newlands, E., Rustin, G., Bleehen, N. M., Lewis, C. and Kaye, S. B. Phase II trials of flavone acetic acid in advanced malignant melanoma and colorectal carcinoma. *Br. J. Cancer*, **60**, 104–106 (1989).
- 22) Siegenthaler, P., Kaye, S. B., Monfardini, S. and Renard, J. Phase II trial with flavon acetic acid (NSC.347512, LM975) in patients with non-small cell lung cancer. *Ann. Oncol.*, **3**, 169–170 (1992).
- 23) Gradisher, W. J. An overview of clinical trials involving inhibitors of angiogenesis and their mechanism of action. *Invest. New Drugs*, **15**, 49–59 (1997).
- 24) Pluda, J. M., Wyvill, K., Figg, W. D., Whitcup, S. M., Lietzau, J., Saville, M. W., Cohen, R., Feigal, E., Parks, D., Foli, A., Bailey, J., Broder, S. and Yarchoan, R. A phase I study of an angiogenesis inhibitor, TNP-470 (AGM-1470), administered to patients with HIV-associated Kaposi's sarcoma. *Proc. Am. Soc. Clin. Oncol.*, **13**, 51 (1994).
- 25) Zukiwski, A., Gutterman, J., Bui, C., Sella, A., Ellerhorst, J., Tu, S., Amato, R., Figg, W., Kilbourn, R. and Logothetis, C. Phase I trial of the angiogenesis inhibitor TNP-470 (AGM-1470) in patients with androgen independent prostate cancer. *Proc. Am. Soc. Clin. Oncol.*, **13**, 252 (1994).
- 26) Kudelka, A., Edwards, C., Freedman, R., Girtanner, R., Kaplan, A., Fishman, A., Balat, O., Tresukosol, D., de Leon, C. G., Hord, M., Finnegan, M., Calayag, M., Hunter, C., Gutterman, J. and Kavanagh, J. A phase I study of the toxicity, pharmacokinetics, and activity of TNP-470 administered to patients with advanced or recurrent squamous cell cancer of the cervix. *Proc. Am. Soc. Clin. Oncol.*, **14**, 281 (1995).
- 27) Chaplin, D. J., Pettit, G. R., Parkins, C. S. and Hill, S. A. Antivascular approaches to solid tumor therapy: evaluation of tubulin binding agents. *Br. J. Cancer*, **74** (Suppl. XXVII), S86–S88 (1996).
- 28) Hill, S. A., Sampson, L. E. and Chaplin, D. J. Anti-vascular approaches to solid tumor therapy: evaluation of Vinblastine and flavone acetic acid. *Int. J. Cancer*, **63**, 119–123 (1995).
- 29) Dark, G. G., Hill, S. A., Prise, V. E., Tozer, G. M., Pettit, G. R. and Chaplin, D. J. Combretastatin A-4, an agent that displays potent and selective toxicity toward tumor vasculature. *Cancer Res.*, **10**, 1829–1834 (1997).
- 30) Nihei, Y., Suzuki, M., Okano, A., Tsuji, T., Akiyama, Y., Saito, S., Hori, K. and Sato, Y. Anti-vascular effects of AC-7700 on solid tumors; comparison with other tubulin binding agents. *Proc. Am. Assoc. Cancer Res.*, **39**, #324 (1998).
- 31) Ohsumi, K., Nakagawa, R., Fukuda, Y., Hatanaka, T., Morinaga, Y., Nihei, Y., Ohishi, K., Suga, Y., Akiyama, Y. and Tsuji, T. Novel combretastatin analogues effective against murine solid tumors; design and structure-activity relationships. *J. Med. Chem.*, **41**, 3022–3032 (1998).
- 32) Nihei, Y., Suga, Y., Morinaga, Y., Ohishi, K., Suzuki, M., Okano, A., Ohsumi, K., Nakagawa, R., Tsuji, T., Akiyama, Y. and Tsuruo, T. A novel combretastatin-A4 derivative AC-7700 shows marked antitumor activity against advanced solid tumors. *Proc. Am. Assoc. Cancer Res.*, **39**, #1143 (1998).
- 33) Huang, X., Molema, G., King, S., Watkins, L., Edgington, T. S. and Thorpe, P. E. Tumor infarction in mice by antibody-directed targeting of tissue factor to tumor vasculature. *Science*, **275**, 547–550 (1997).
- 34) Denekamp, J., Hill, S. A. and Hobson, B. Vascular occlusion and tumor cell death. *Eur. J. Cancer Clin. Oncol.*, **19**,

- 271–275 (1983).
- 35) Yamamura, H. and Sato, H. Quantitative studies on the developing vascular system of rat hepatoma. *J. Natl. Cancer Inst.*, **53**, 1229–1240 (1974).
- 36) Field, S. B., Needham, S., Burney, I. A., Maxwell, R. J., Coggle, J. E. and Griffiths, J. R. Differences in vascular response between primary and transplanted tumors. *Br. J. Cancer*, **63**, 723–726 (1991).
- 37) Fu, X., Besterman, J. M., Monosov, A. and Hoffman, R. M. Models of human metastatic colon cancer in nude mice orthotopically constructed by using histologically intact patient specimens. *Proc. Natl. Acad. Sci. USA*, **88**, 9345–9349 (1991).
- 38) Togo, S., Shimada, H., Kubota, T., Moossa, A. R. and Hoffman, R. M. Host organ specifically determines cancer progression. *Cancer Res.*, **55**, 681–684 (1995).
- 39) Bibby, M. C., Phillips, R. M., Double, J. A. and Pratesi, G. Anti-tumor activity of flavone acetic acid (NSC 347512) in mice — influence of immune status. *Br. J. Cancer*, **63**, 57–62 (1991).
- 40) Ching, L.-M., Joseph, W. R. and Baguley, B. C. Antitumor responses to flavone-8-acetic acid and 5,6-dimethylxanthone-4-acetic acid in immune deficient mice. *Br. J. Cancer*, **66**, 128–130 (1992).
- 41) Baguley, B. C., Holdaway, K. M., Thomsen, L. L., Zhuang, L. and Zwi, L. J. Inhibition of growth of colon38 adenocarcinoma by vinblastine and colchicine: evidence for a vascular mechanism. *Eur. J. Cancer*, **27**, 482–487 (1991).