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# Antitumor Effects due to Irreversible Stoppage of Tumor Tissue Blood Flow: Evaluation of a Novel Combretastatin A-4 Derivative, AC7700

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**The relation between tumor tissue blood flow (tBF) reduction and antitumor effects was investigated. Changes in tBF of normal tissues (liver, kidney cortex, bone marrow and brain cortex) and tumors (Yoshida sarcoma subline, LY80 and Sato lung carcinoma, SLC) due to i.v. administration of AC7700 (1, 3, 10 mg/kg), one of the combretastatin A-4 derivatives, were measured with the hydrogen clearance method. The change in blood flow in tumor microfoci was also observed directly using a rat transparent chamber. Chemotherapy against the solid tumors (LY80, SLC) was performed by administering AC7700 7 times at intervals of 3 days and the effect on the tumor growth, the histological effect, the effect on lymph node metastasis and the survival rate were investigated. Tumor tBF showed a dose-dependent response to AC7700. Although tumor tBF decreased markedly at a dose of 1 mg/kg, it tended to recover partly within several hours. At 10 mg/kg, however, tumor tBF completely stopped within approximately 30 min and never recovered in many regions. The irreversible stoppage of tumor tBF was observed in large s.c. tumors and in microfoci as well. On the other hand, in normal tissues, tBF changes due to AC7700 were not uniform. In the liver, although tBF decreased by approximately 50% at 10 mg/kg AC7700, it recovered within 8 h. In the brain, although the mean maximum reduction was 35%, the blood flow recovered to the original level within 24 h. The blood flow in the kidney cortex did not change at all. In the bone marrow, tBF decreased by approximately 80%. Generally, the blood flow reduction in normal tissues tended to be reversible. The effect on tumor growth and the histological effect were also dependent on the dose of AC7700. The tumor growth was markedly inhibited by 10 mg/kg AC7700 and extensive necrosis was induced. Lymph node metastases were significantly inhibited and survival was prolonged significantly. In the control group, all 8 SLC tumor-bearing rats died of cancer, the presence of which was verified by gross and microscopic evaluation, within 45 days after tumor implantation. On the other hand, in the treated group, 2 of 8 rats recovered completely and survived. No obvious side effects such as body weight loss, anemia or diarrhea were observed at the dose used in this experiment. From these results, we conclude that strong antitumor effects are obtained by stopping tumor tBF irreversibly and by shutting off the nutritional supply into tumor tissue. AC7700 has been demonstrated to be a promising anticancer compound which has such an action.**

Key words: Combretastatin A-4 — Tumor blood flow — Tumor vessel — Solid tumor — Antivascular

Combretastatin A-4 (CS A-4), a relatively simple stilbene compound isolated by Pettit *et al.*[1] from the African shrub *Combretum caffrum*, is a potent tubulin polymerization inhibitor and has shown cytotoxicity *in vitro*.[2] However, *in vivo* study of CS A-4 has not progressed because of its poor solubility in water. In recent years, many water-soluble derivatives have been synthesized[3–5] and their *in vivo* effects have been tested. CS A-4 compounds produced extensive hemorrhagic necrosis in experimental tumors and powerfully suppressed the tumor growth.[6,7] It is not yet completely understood how and why the extensive hemorrhagic necrosis arises in tumors. However, it was found that fluorescent dye hardly reached the tumor tissue when the dye was injected i.v. into tumor-bearing mice pretreated with CS A-4.[6] That is, tumor perfusion has been shown to be suppressed by CS A-4 compounds, a finding that suggests that the compounds might have an action which induces marked and prolonged decrease in tumor tissue blood flow (tBF). To date, however, there have been only a few reports concerning the effect of CS A-4 compounds on blood flow in normal and tumor tissue and more detailed investigation relating to this matter is necessary.

The aim of the present study was to analyze the relation between tumor tBF changes due to a CS A-4 compound and antitumor effects. For this study, we used AC7700, which is a soluble derivative of CS A-4 with higher antitu-

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mor activity and less toxicity. It was recently synthesized by our group.[5] AC7700 as well as CS A-4 has been shown to decrease tumor perfusion and to inhibit the growth of several kinds of mouse tumors significantly.[7] In the present study, using this compound, we investigated: a) the effect on tBF in normal and tumor tissues, b) the effect on the growth of solid tumors, c) the histological effect, d) the effect on lymph node metastasis and e) the effect on survival rate. The results suggest that compounds which can induce irreversible stoppage of tumor tBF are candidates as potent anticancer drugs.

## MATERIALS AND METHODS

**Rats and tumor** Male Donryu rats (Crj-Donryu; Nippon Charles-River, Yokohama) at 8–10 weeks of age and with average weight of 250–300 g were used for measurements of tissue blood flow. The same strain of rats, weighing 200–220 g each, was used for vital microscopic observation of tumor microcirculation using a transparent chamber[8] in a skinfold and for chemotherapy. The rats were bred and maintained within an air-conditioned and a specific pathogen-free room at a temperature of 24±1°C, with food and water freely available. They were usually housed two or three per cage. Every rat that was fitted with a transparent chamber was caged individually. The tumor cells used were LY80, a variant of the Yoshida sarcoma, established in 1966 by Dr. H. Satoh,[9] and Sato lung carcinoma (SLC), established in 1964 by Drs. R. Sato and Y. Shimosato.[10] LY80 and SLC are maintained by successive i.p. and s.c. transplantation, respectively, in our laboratory. Tumor volumes of LY80 and SLC became approximately 3–6 $cm^3$ by 8–10 days after s.c. implantation of $2\times10^6$ cells. The incidence of axillary and/or inguinal lymph node metastases of LY80 was usually 90% at 4 weeks after tumor cell implantation.[11] Lymph node metastasis was easily palpable. In SLC tumors, the incidence of lymph node metastases was approximately 20% in the natural course. For vital microscopic observation, a small fragment (approximately 0.1 $mm^3$) of solid tumor was implanted from a donor rat onto the tissue within the chamber when the transparent chamber was installed. Experiments were performed under anesthesia in a controlled-temperature box (25°C) fitted with a suction duct. All animal experiments including the survival study were conducted in accordance with the guidelines approved by the Animal Experiment Committee of our institute.

**Drugs** AC7700, a CS A-4 derivative, was synthesized by Ajinomoto Co., Inc. (Kawasaki). The properties of this compound have been reported elsewhere.[5, 7] The AC7700 powder was dissolved in 0.9% NaCl solution to a final concentration of 1, 3, or 10 mg/ml, just before use and injected i.v. at a volume of 1 ml/kg using an infusion pump (Compact Syringe Pump; Harvard Apparatus Co., Inc., Millis, MA). The infusion rate was 0.003 ml per second. Pentobarbital sodium (Nembutal; Abbott Laboratories, North Chicago, IL) and enflurane (Ethrane; Abbott Laboratories) were used for anesthesia. Pentobarbital sodium was administered i.m. at a dose of 25 mg/kg 10 min before the experiment, and supplemental doses (12.5 mg/kg i.m.) were given at 90-min intervals. The concentration of enflurane was maintained at 1% in the inhaled carrier gas (1 liter/min) by means of an anesthetic apparatus for small laboratory animals.[12]

**Measurements of mean arterial blood pressure** During tBF measurement, mean arterial blood pressure (MABP) was monitored in all of the rats. MABP was measured via a catheter (PE-50; Clay Adams, Persippany, NJ) inserted into the right femoral artery. The pressure in the catheter was recorded with a pressure transducer (TNF-R; Spectramed Medical Products, Singapore), the output of which was fed into an amplifier (6M82; NEC-Sanei Co., Tokyo) adapted for measurement of MABP.

**Measurement of tissue blood flow** Blood flow in normal and tumor tissue was measured with the hydrogen clearance method.[12–14] In brief, after saturation of the tissue with hydrogen following the inhalation of 7–9% hydrogen gas in air (at 1 liter/min), the blood flow value (in ml/min/100 g tissue) was calculated from the half-life of the clearance curve obtained. In the present experiment, a tissue blood flow meter with 2 separate amplifiers (PHG-201; Unique Medical Co., Tokyo) was used. Two hydrogen electrodes with 80 $\mu$m diameter (UHE-201C; Unique Medical) and the same number of rod-type Ag/AgCl reference electrodes (TT-98012; Unique Medical), which were inserted between the skin and the musculature in the caudal region, were used per rat, except for the blood flow measurement in the bone marrow. Measurement of tBF in both LY80 and SLC solid tumors was begun when the tumor volume reached approximately 3 $cm^3$ 8 days after tumor implantation. A small pin hole was made in the skin overlying a tumor using a syringe needle (23G; Termo Co., Tokyo) and a hydrogen electrode was inserted through the hole into the tumor tissue. The depth of the inserted electrode was less than 5 mm from the surface of the tumor nodule. To measure the tBF of the liver, the rats underwent laparotomy at the mid-line and electrodes were inserted into the median lobe. For measurements of tBF in the kidney, the right kidney was exposed after cutting the lower back region and electrodes were inserted into the cortex at the depth of 1.5–2 mm from the ventral surface. The tBF in the bone marrow was measured by means of one electrode inserted at the junction of the upper and middle thirds of the shaft of the left femur through a hole bored in the intercondyloid fossa with a dental drill. To measure the tBF of the brain cortex, a small hole was drilled at the right parietal bone and an electrode was introduced to the depth of 1.5–2 mm below the brain sur-

face. Throughout all blood flow measurements, rats were laid prone on a heated stage at 34°C. Rectal temperature was monitored with a thermistor for small animals (PTC-201; Unique Medical) and maintained at 33.5–35.5°C.

**Change in tissue blood flow due to AC7700** Before the administration of AC7700, tBF was measured 2 or 3 times at 30-min intervals. When the blood flow had stabilized, AC7700 was infused via the lateral tail vein by an infusion pump. tBF was measured at multiple time points (i.e., 10, 30, 60 min, and every subsequent 1 h) until 6 h after the administration. In some cases, the measurements continued up to a maximum of 8 h. In the case of the tumor, the dose of AC7700 administered was 1, 3, or 10 mg/kg. In the liver, the kidney, the bone marrow and the brain, 10 mg/kg AC7700 was administered.

**Tissue blood flow 24 h after AC7700 administration**

*Tumor*: Tumor tBF in the same region 24 h after AC7700 administration was measured using a rat fitted with a device for keeping electrodes within a tumor.[15] Changes in tumor tBF due to AC7700 were measured for 2 h, then the wire electrode outside the tumor was spiraled and kept in the hangar. It was taken from the hangar 24 h later and a rod-type reference electrode was inserted in the same region as 24 h earlier and tumor tBF in this region was again measured.

*Brain*: A small hole (400 $\mu$m in diameter) was drilled at the right parietal bone with a dental drill and an electrode was inserted into the brain cortex. The electrode was attached to the parietal bone with a synthetic resin glue (Aron alpha 201; Toagosei Chemical Industry Co., Tokyo) and the hole was closed with the resin. The change in tBF was measured for 30 min, and 1 h following AC7700 administration, the wire electrode outside the brain was kept in the s.c. pouch on the skull. The electrode was taken from the pouch 24 h later and the tBF in the same region was again measured.

**Vital microscopic observation on tBF changes due to AC7700** Rat transparent chambers were implanted in dorsal skin flaps under aseptic conditions for vital microscopic observation.[8] Each chamber consisted of two identical titanium frames containing a circular quartz glass window of 300 $\mu$m thickness. The rat with the transparent chamber was placed in the right lateral position on a heated stage (MATS-SFA; Tokai HIT Co., Ltd., Tokyo) at 34°C, attached to the mechanical stage of the microscope. AC7700 (10 mg/kg/ml) was administered via the lateral tail vein using an infusion pump. Tumor tBF change was observed directly through a light microscope (Fluophoto; Nippon Kogaku K.K., Tokyo) with a ×10 ocular and a ×4–20 objective. Tumor vessels within the chamber were transluminated by a 100 W tungsten lamp. The microscopic image was recorded using a closed-circuit video system consisting of a CCD video camera (CS-900; Olympus Kogaku K.K., Tokyo), a TV monitor (PVM-14M4J; Sony Corp., Tokyo) and an S-VHS video recorder (SVO-2100; Sony Corp.). A video timer (VTG-33; FOR.A Co., Ltd., Tokyo) was superimposed on the images for record keeping. Segments of the video tape that contained desired images were transferred to computer hard disk (Power Macintosh 8600/200; Apple Japan, Inc., Tokyo). Final images were output by a digital printer (Pictrography 4000; Fuji Photo Film Co., Ltd., Tokyo).

**Chemotherapy**

*Effect of AC7700 on the tumor growth and body weight*: In LY80 tumor-bearing rats, rats were divided into 3 groups: I, a group to which 0.9% NaCl solution was administered (6 rats); II, a group to which AC7700 (3 mg/kg) was administered (5 rats); and III, a group to which AC7700 (10 mg/kg) was administered (6 rats). The rats were anesthetized by light sedation with ether at the time of each treatment. The appropriate solution was injected into the tail veins. Treatments were performed 7 times at intervals of 3 days, starting on day 8 after tumor transplantation ($2\times10^6$ cells). The therapeutic efficacy and toxicity of AC7700 were evaluated by measuring the change of the tumor size and body weight every day for 25 days following the start of the treatments. In SLC tumor-bearing rats, rats were divided into 2 groups: I, a group to which 0.9% NaCl solution was administered (8 rats); and II, a group to which AC7700 (10 mg/kg) was administered (8 rats). The appropriate solution was injected into the tail veins. Treatments were performed 7 times at intervals of 3 days, starting on day 10 after tumor transplantation ($2\times10^6$ cells). Tumor size and body weight were measured every day for 25 days following the start of the treatments. Tumor size was measured with calipers and the volume ($V$) was calculated by means of the following formula:

$$V = (\pi/6)\times a\times b\times c$$

where $a$, $b$ and $c$ are the long axis, short axis and height of the tumor nodule, respectively.

*Effect on lymph node metastasis*: In the experiment using LY80 tumor-bearing rats, axillary and inguinal lymph nodes were examined by touch every day from the start of treatment. Any rat with a swelling of axillary and/or inguinal lymph nodes was defined as metastasis-positive. The incidence of metastasis-positive rats in each group was investigated as a function of the number of days elapsed.

*Survival effect*: After the experiment on the effect of AC7700 on the tumor growth was completed (on day 25 following the start of the treatments), observation of the tumor-bearing rats was continued. Several rats were killed when they became moribund.

*Histological examination*: LY80 tumor-bearing rats were divided into 4 groups: I, a group to which 0.9% NaCl solution was administered (8 rats); II, a group to which AC7700 (3 mg/kg) was administered (8 rats); III, a group

to which AC7700 (10 mg/kg) was administered (8 rats); and IV, an untreated group (5 rats). Each solution was injected as a single bolus into the tail vein on day 8 after tumor transplantation ($2\times10^6$ cells). Rats in group IV were killed with ether on day 9 after tumor transplantation. Each tumor was resected for routine histology, fixed in 15% formalin, processed and embedded in paraffin. Maximum sections of each tumor were cut (4 $\mu$m thick) and stained with hematoxylin and eosin. Rats in groups I–III were killed with ether 3 days after the administration (11 days after tumor transplantation) and treated in the same manner as group IV. Each section was projected with a profile projector (Nikon V-16; Nippon Kogaku K.K.) and necrotic regions were traced. The area and distribution ratio of the necrotic regions were calculated using an area analyzer (WT-4400SE, WACOM Co., Saitama).

**Statistics** All results were expressed as mean±SD. Comparisons of tumor size, body weight and the incidence of necrosis after the administration of AC7700 were made using unpaired two-group *t* tests. Since tBF of normal tissues and tumors did not change significantly with 0.9% NaCl solution during 6 h of experiment (data not shown), the statistical significance at each time point after the AC7700 administration was evaluated with paired *t* tests compared to the initial time point. Comparisons of the incidence of lymph node metastasis and the survival time were made using the Kaplan-Meier method and evaluated with the log rank test. *P* values of 0.05 or below were considered significant, and those of 0.001 or below were considered highly significant.

## RESULTS

**Effect of AC7700 on MABP** The effect of i.v. administration of 1, 3, or 10 mg/kg AC7700 on MABP is shown in Fig. 1. Although MABP decreased transiently from 103.3±11.2 to 74.8±11.9 mmHg after 10 mg/kg AC7700 ($n$=16), it soon increased to 145.1±9.7 mmHg. The increased MABP was maintained for 4–5 h. Approximately 6 h later MABP returned to the initial level (Fig. 1A). At 3 mg/kg ($n$=16), however, MABP increased gradually from 106.3±6.4 to 128.9±8.7 mmHg. The increased MABP was maintained for 2 h and returned to the initial level approximately 3 h later (Fig. 1B). At 1 mg/kg ($n$=15), MABP increased gradually from 105.2±10.9 to 115.0±13.9 mmHg and returned to the initial level within 2 h (Fig. 1C).

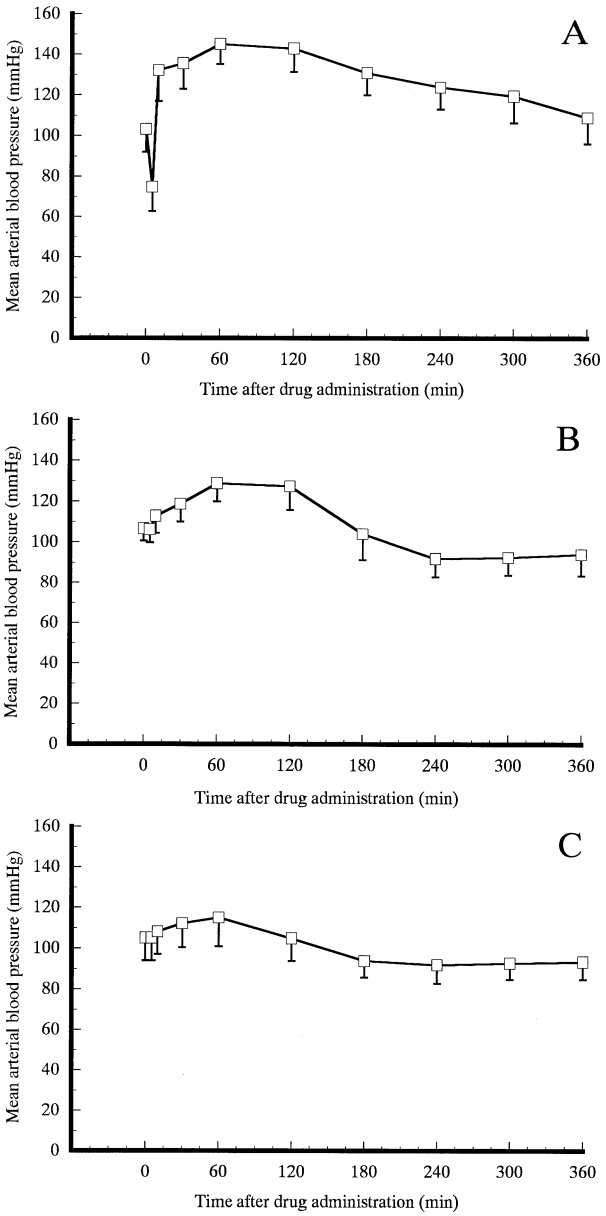


Fig. 1. The effect of AC7700 on MABP. A, 10 mg/kg AC7700 ($n$=16); B, 3 mg/kg AC7700 ($n$=16); C, 1 mg/kg AC7700 ($n$=15). □, mean value; bars, SD. AC7700 solution was administered i.v. by an infusion pump at 0 min.

**Effect of AC7700 on the blood flow in normal and tumor tissue** AC7700 significantly decreased tumor tBF of both LY80 (Fig. 2, A–C) and SLC (Fig. 2, D–F) tumors in a dose-dependent manner. Although tumor tBF was markedly decreased by 1, 3 mg/kg AC7700, it tended to recover after 2 and 4 h, respectively (Fig. 2, A, B, D, and E). At 10 mg/kg, however, tumor tBF stopped completely (Fig. 2, C and F) and dit not recover during the experiment (a maximum of 8 h).

The effect of AC7700 on the blood flow in normal tissue is shown in Fig. 3. At 10 mg/kg, tBF in the liver decreased by approximately 50%. However, it returned to its pretreatment level within 8 h (Fig. 3A). The tBF in the kidney cortex was not changed by AC7700 (Fig. 3B). The

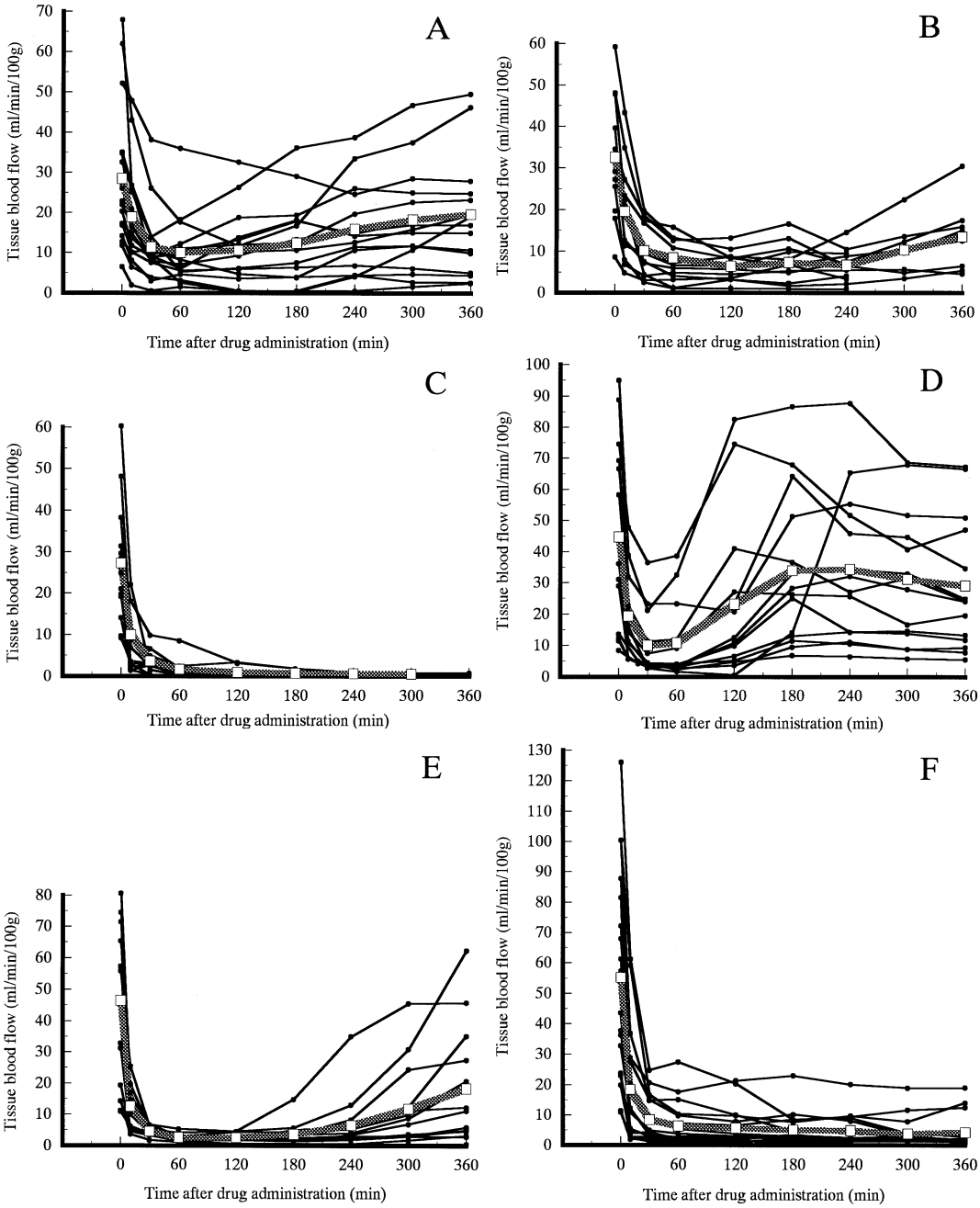


Fig. 2. The effect of AC7700 on tumor tBF. A, LY80 (1 mg/kg AC7700) ($n$=16); B, LY80 (3 mg/kg AC7700) ($n$=12); C, LY80 (10 mg/kg AC7700) ($n$=12); D, SLC (1 mg/kg AC7700) ($n$=14); E, SLC (3 mg/kg AC7700) ($n$=14); F, SLC (10 mg/kg AC7700) ($n$=18). □, mean value. AC7700 solution was administered i.v. by an infusion pump at 0 min.

tBF in the brain cortex was decreased by approximately 35% by AC7700 (Fig. 3C). In the bone marrow, the tBF markedly decreased by about 80% and did not recover during the measurement period of 6 h (Fig. 3D). The effects of AC7700 on blood flow of normal and tumor tissues are summarized in Table I.

**Tissue blood flow in tumor and brain 24 h after AC7700 administration** tBF in tumor and brain 24 h after i.v. administration of 10 mg/kg AC7700 is shown in Fig. 4. Although tumor tBF remained zero in almost all cases even 24 h later (Fig. 4A), in the brain (Fig. 4B) the blood flow returned to the original level within 24 h.

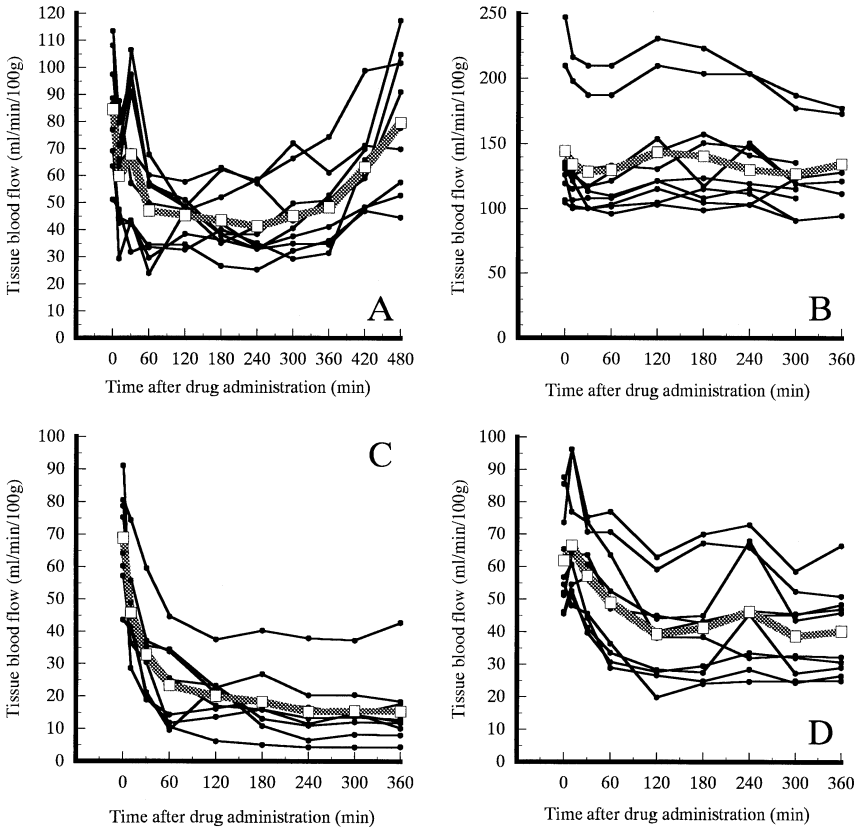


Fig. 3. The effect of AC7700 on the tBF of normal tissues. A, liver ($n$=10); B, kidney cortex ($n$=10); C, bone marrow ($n$=9); D, brain ($n$=10). □, mean value. AC7700 solution (10 mg/kg) was administered i.v. by an infusion pump at 0 min.

Table I. The Effect of AC7700 on Blood Flow of Normal Tissues and Tumors

| Tissue | | AC7700 (mg/kg) | $n$ | Tissue blood flow (ml/min/100 g) | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Time (min) | | | | | | |
| | | | | 0 | 30 | 60 | 120 | 240 | 360 | 480 |
| Tumor | A | 1 | 16 | 28.4±18.2 | 11.3±9.1[c)] | 9.9±8.5[c)] | 10.5±9.1[c)] | 15.8±10.9[c)] | 19.6±13.8[b)] | |
| | | 3 | 12 | 32.5±14.4 | 10.1±6.4[c)] | 8.4±4.6[c)] | 6.4±3.5[c)] | 6.7±3.8[c)] | 13.5±8.5[c)] | |
| | | 10 | 12 | 27.2±14.9 | 3.6±2.9[c)] | 1.6±2.2[c)] | 0.9±1.0[c)] | 0.6±0.2[c)] | 0.5±0.2[c)] | |
| | B | 1 | 14 | 44.7±30.1 | 10.0±10.1[c)] | 10.8±12.3[c)] | 23.1±26.0[b)] | 34.4±24.0[a)] | 29.1±21.1[b)] | |
| | | 3 | 14 | 46.5±26.3 | 4.5±1.4[c)] | 2.6±1.1[c)] | 2.5±1.4[c)] | 6.5±8.8[c)] | 18.3±18.5[b)] | |
| | | 10 | 18 | 55.2±34.1 | 8.4±7.3[c)] | 6.3±7.3[c)] | 5.6±6.3[c)] | 4.8±5.2[c)] | 4.2±5.3[c)] | |
| Normal | C | 10 | 10 | 84.5±19.6 | 67.9±27.9[a)] | 47.0±15.2[c)] | 45.4±7.7[c)] | 41.4±12.2[c)] | 48.4±13.1[c)] | 79.8±24.1 |
| | D | 10 | 10 | 144.5±46.5 | 128.6±38.5 | 129.9±38.5 | 143.7±43.7 | 130.1±55.2 | 134.6±33.6 | |
| | E | 10 | 9 | 68.9±14.3 | 32.8±11.9[c)] | 23.3±12.6[c)] | 20.0±8.6[c)] | 15.2±9.8[c)] | 15.4±11.1[c)] | |
| | F | 10 | 10 | 61.9±15.6 | 57.2±13.7 | 48.9±17.0[b)] | 39.3±14.1[b)] | 46.3±17.3[a)] | 40.3±13.5[b)] | |

A, LY80 tumor; B, SLC tumor; C, liver; D, kidney cortex; E, bone marrow; F, brain. Values, mean±SD. Drug or vehicle was injected i.v. at 0 min.

*a*) Significantly different from time 0 ($P$<0.05).
*b*) Significantly different from time 0 ($P$<0.001).
*c*) Significantly different from time 0 ($P$<0.0001).

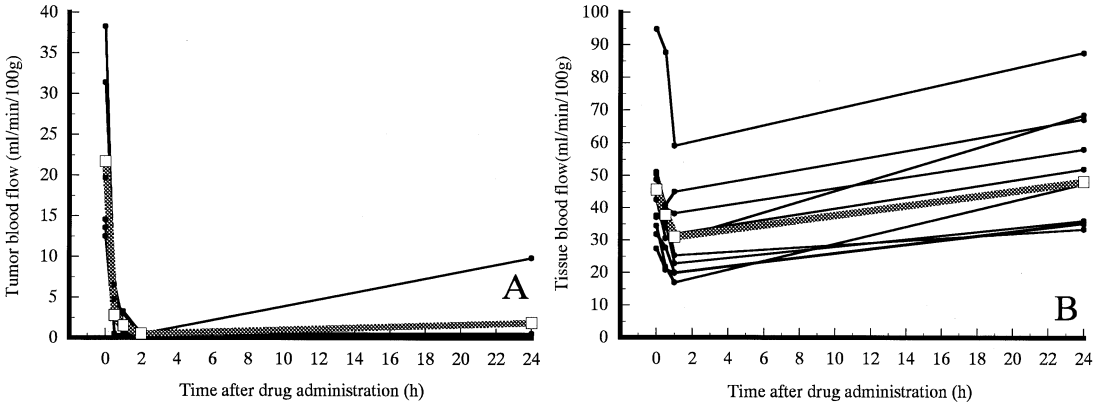


Fig. 4. tBF of the tumor and brain 24 h after AC7700 administration. A, LY80; B, brain. □, mean value. AC7700 solution (10 mg/kg) was administered i.v. by an infusion pump at 0 min.

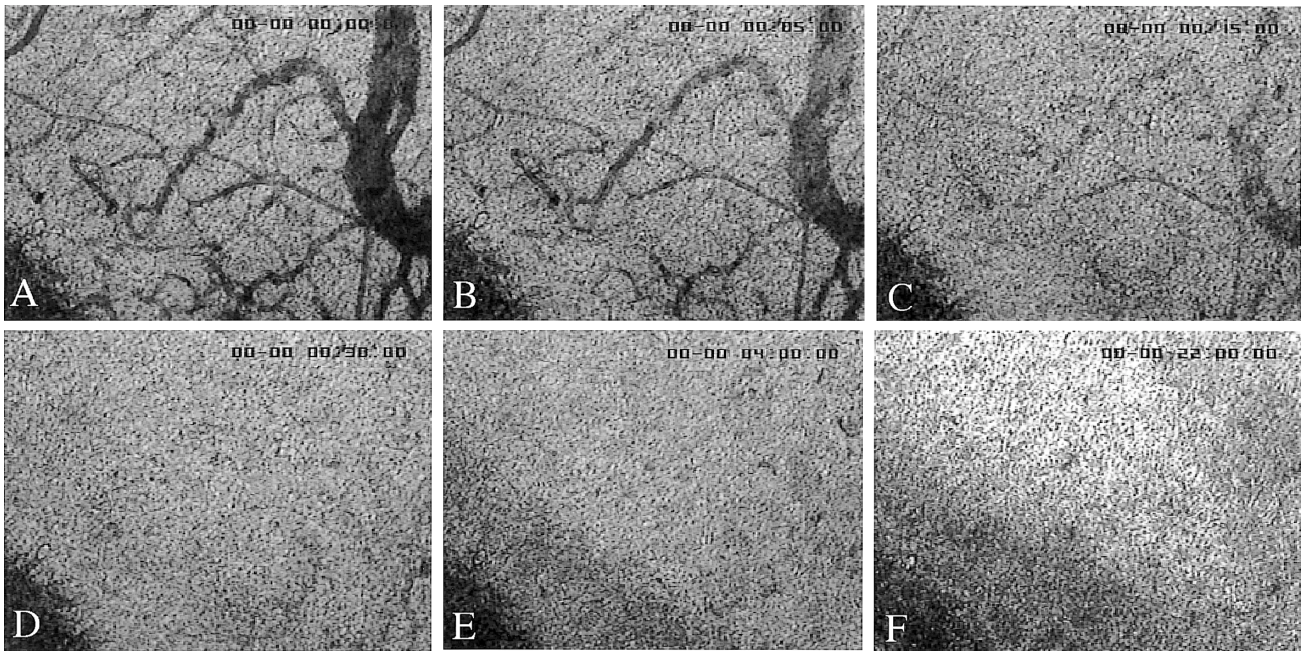


Fig. 5. Blood flow changes in tumor microfoci due to AC7700 administration. A, before 10 mg/kg AC7700 administration; B, 5 min after AC7700 administration; C, 15 min later; D, 30 min later; E, 4 h later; F, 22 h later. Tumor tBF began to decrease immediately after AC7700 administration and stopped completely at 30 min.

**Tumor tBF changes in microfoci** Fig. 5 shows a typical finding of the vital microscopic observation on tumor tBF changes in microfoci before and 5, 15, 30 min and 4, 22 h after administration of 10 mg/kg AC7700. Tumor tBF began to decrease immediately after the administration, and completely stopped within 30 min. Thrombi were never seen within tumor vessels and no bleeding was observed during the observation time. Tumor tBF in almost all areas observed did not recover even 22 h later and the tumor succumbed to extensive necrosis.

**Chemotherapy**

*Effects of AC7700 on the tumor growth and body weight*: Effects of AC7700 on the tumor growth and body weight are shown in Fig. 6. The growth of LY80 tumor showed a

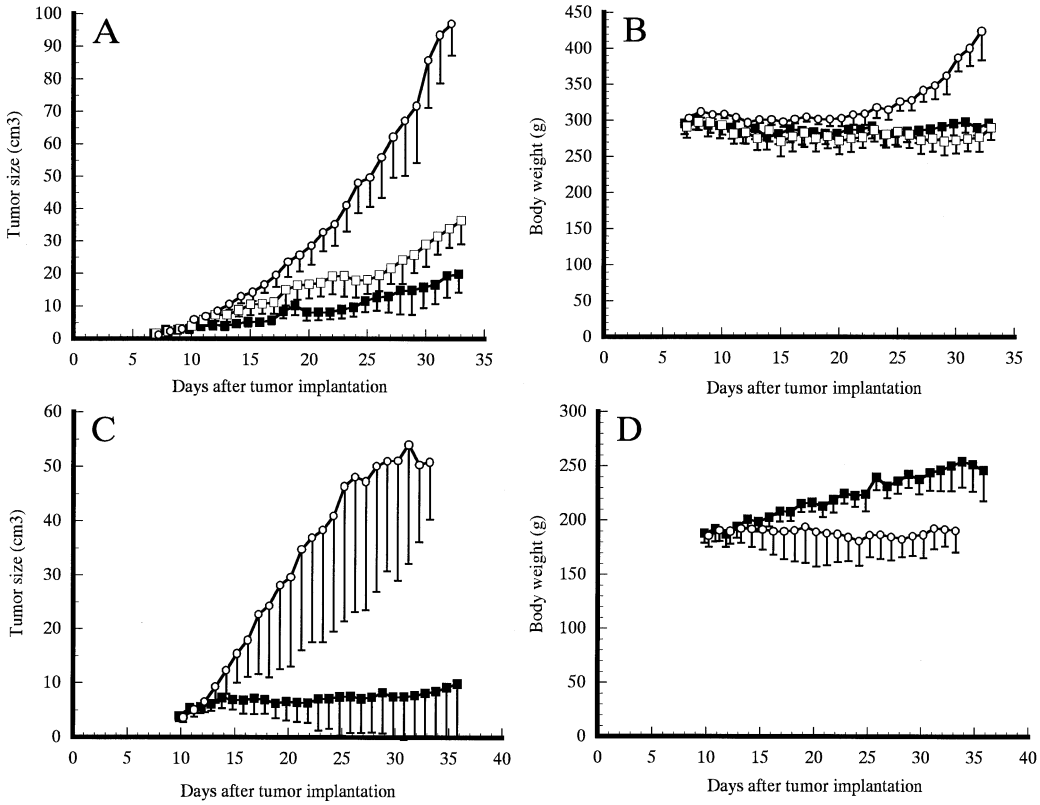


Fig. 6. The effect of AC7700 on the tumor growth and body weight. A, The growth of LY80 tumor; B, body weight of LY80 tumor-bearing rat; C, the growth of SLC tumor; D, body weight of SLC tumor-bearing rat. A. ○, group I (0.9% NaCl); □, group II (3 mg/kg AC7700); ■, group III (10 mg/kg AC7700). B. ○, group I (0.9% NaCl); ■, group II (10 mg/kg AC7700). Each point is the mean±SD. In LY80 tumor-bearing rats, AC7700 (3, 10 mg/kg) or 0.9% NaCl was administered i.v. on 8, 11, 14, 17, 20, 23 and 26 days after tumor implantation. In SLC tumor-bearing rats, AC7700 (10 mg/kg) or 0.9% NaCl was administered i.v. on 10, 13, 16, 19, 22, 25 and 28 days after tumor implantation. In LY80 tumor-bearing rats, the differences between group I (0.9% NaCl) and group III (10 mg/kg AC7700) on days 13−33 after tumor implantation were highly significant ($P<0.0001$). In SLC tumor-bearing rats, the differences between group I (0.9% NaCl) and group II (10 mg/kg AC7700) on days 15−33 were highly significant ($P<0.0001$).

dose-dependent response to AC7700. Although the tumor growth was markedly inhibited by 10 mg/kg AC7700 (Fig. 6A), the body weight did not decrease at all (Fig. 6B). The increase in body weight observed in the 0.9% NaCl group during the treatment was caused by an acute increase in tumor volume. The growth of SLC tumor was completely suppressed by 10 mg/kg AC7700 (Fig. 6C). No obvious side effect such as anemia or diarrhea was observed at the dose used in this experiment and the body weight of the rats continued to increase (Fig. 6D).

*Effect of AC7700 on lymph node metastases*: The incidence of regional lymph node metastases reached 100% in all groups by the end of the experiment. However, the time to development of metastasis was significantly different between the 0.9% NaCl group and the treatment groups [group I (0.9% NaCl) ($n$=6) vs. group III (10 mg/kg AC7700) ($n$=6), $P$ = 0.0072; group I vs. group II (3 mg/

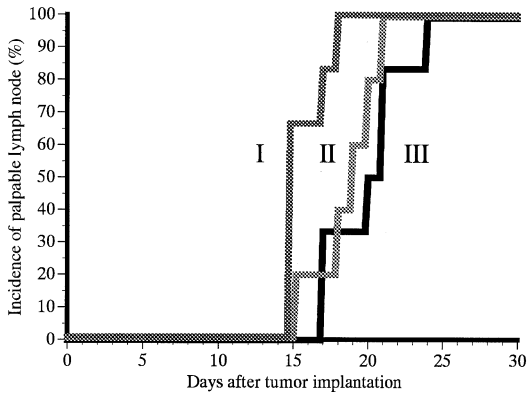


Fig. 7. The effect of AC7700 on lymph node metastases. The tumor used was LY80. I, 0.9% NaCl group ($n$=6); II, 3 mg/kg AC7700 group ($n$=5); and III, 10 mg/kg AC7700 group ($n$=6). I vs. II, $P$=0.0208; I vs. III, $P$=0.0072 (log rank test).

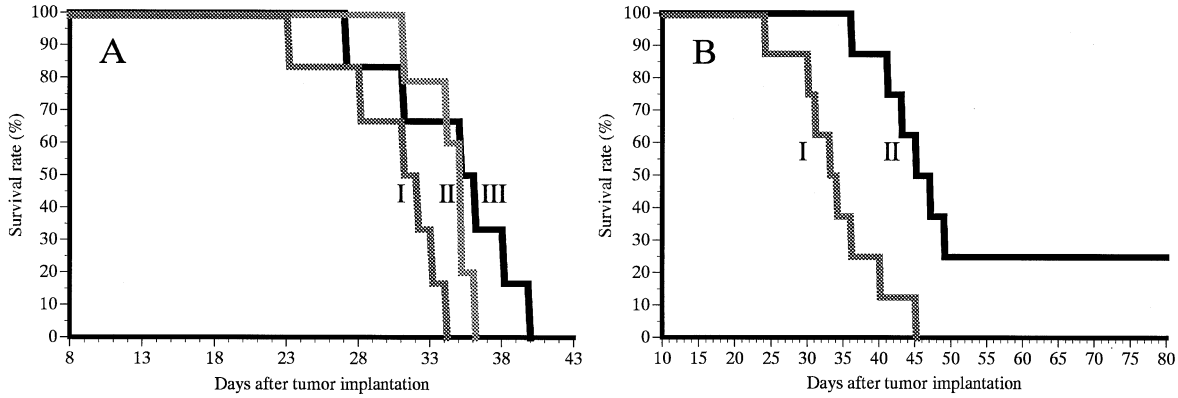



Fig. 8. The effect of AC7700 on the survival rate of tumor-bearing rats. A, LY80 tumor-bearing rats. AC7700 (3, 10 mg/kg) was administered i.v. on 8, 11, 14, 17, 20, 23 and 26 days after tumor implantation. Group I (0.9% NaCl) ($n$=6); group II (3 mg/kg AC7700) ($n$=5); group III (10 mg/kg) ($n$=6). I vs. III, $P$=0.045 (log rank test). B, SLC tumor-bearing rats. AC7700 (10 mg/kg) was administered i.v. on 10, 13, 16, 19, 22, 25 and 28 days after tumor implantation. Group I (0.9% NaCl) ($n$=8); group II (10 mg/kg AC7700) ($n$=8). I vs. II, $P$=0.0022 (log rank test). C, Scar formation (SLC tumor). Finding on day 7 after the last administration of AC7700 (35 days after tumor implantation). In SLC tumor-bearing rats, 2 out of 8 rats were cured completely through scab formation and deciduation.

kg AC7700) ($n$=5), $P$=0.0208] (Fig. 7). The growth of lymph node metastases was markedly inhibited by AC7700, as was that of s.c. tumors (data not shown).

*Survival effect*: The effect of AC7700 on the survival of tumor-bearing rats is indicated in Fig. 8. The survival of both LY80 tumor- (Fig. 8A) and SLC tumor-bearing rats (Fig. 8B) was significantly extended by AC7700 [LY80, group I (0.9% NaCl) ($n$=6) vs. group III (10 mg/kg AC7700) ($n$=6), $P$=0.045; SLC, group I (0.9% NaCl) ($n$=8) vs. group II (10 mg/kg AC7700) ($n$=8), $P$=0.0022]. In SLC tumor-bearing rats, all rats in group I died of cancer, the presence of which was verified by gross and microscopic evaluations. On the other hand, 2 out of 8 rats in group II recovered completely through scab formation (Fig. 8C) and deciduation. Although both surviving rats were killed with ether 60 days after the final treatment and various organs examined histologically, no tumors or metastases were observed.

*Histological examination*: Typical histology of an LY80 tumor 3 days after i.v. administration of 0.9% NaCl solution or 10 mg/kg AC7700 is shown in Fig. 9. No large necrosis was seen in the 0.9% NaCl group (Fig. 9, A and B). By contrast, extensive necroses were seen in the AC7700-treated group (Fig. 9, C and D). The percentage of necrosis in the maximum tumor section is shown in Fig. 10. The amount of necrosis depended on the dose of AC7700. The percentages of necrosis in I ($n$=8), II ($n$=8), III ($n$=8), and IV ($n$=5) were 18.0±9.5% (tumor area, 2.9 ±0.6 $cm^2$), 24.0±12.1% (tumor area, 2.4±0.5 $cm^2$), 44.8± 12.2% (tumor area, 1.6±0.3 $cm^2$) and 10.3±2.8% (tumor area, 1.8±0.4 $cm^2$), respectively. Highly significant necrosis was observed in the 10 mg/kg AC7700-treated group [group I vs. III, $P$<0.001; group III vs. IV, $P$<0.001].

## DISCUSSION

The present study clearly demonstrated that marked antitumor effects were obtained by stopping tumor tBF irreversibly and cutting off the nutrition to tumors. The antitumor effect was dependent on the extent of blood flow reduction and the duration of the effect. In the regions where tumor tBF had stopped irreversibly, both tumor cells and tumor vessels perished and the tumor tissue became necrotic.

The idea of trying to inhibit tumor growth by cutting off the nutrition supply into tumors is not new. Algire *et al.* observed that the blood flow in a mouse tumor transplanted within transparent chambers is completely cut off by podophyllotoxin, which binds to tubulin, resulting in an extensive hemorrhagic necrosis in the tumor.[16] This was the first report showing that tumor tBF could be cut off by a drug. However, podophyllotoxin has never been used

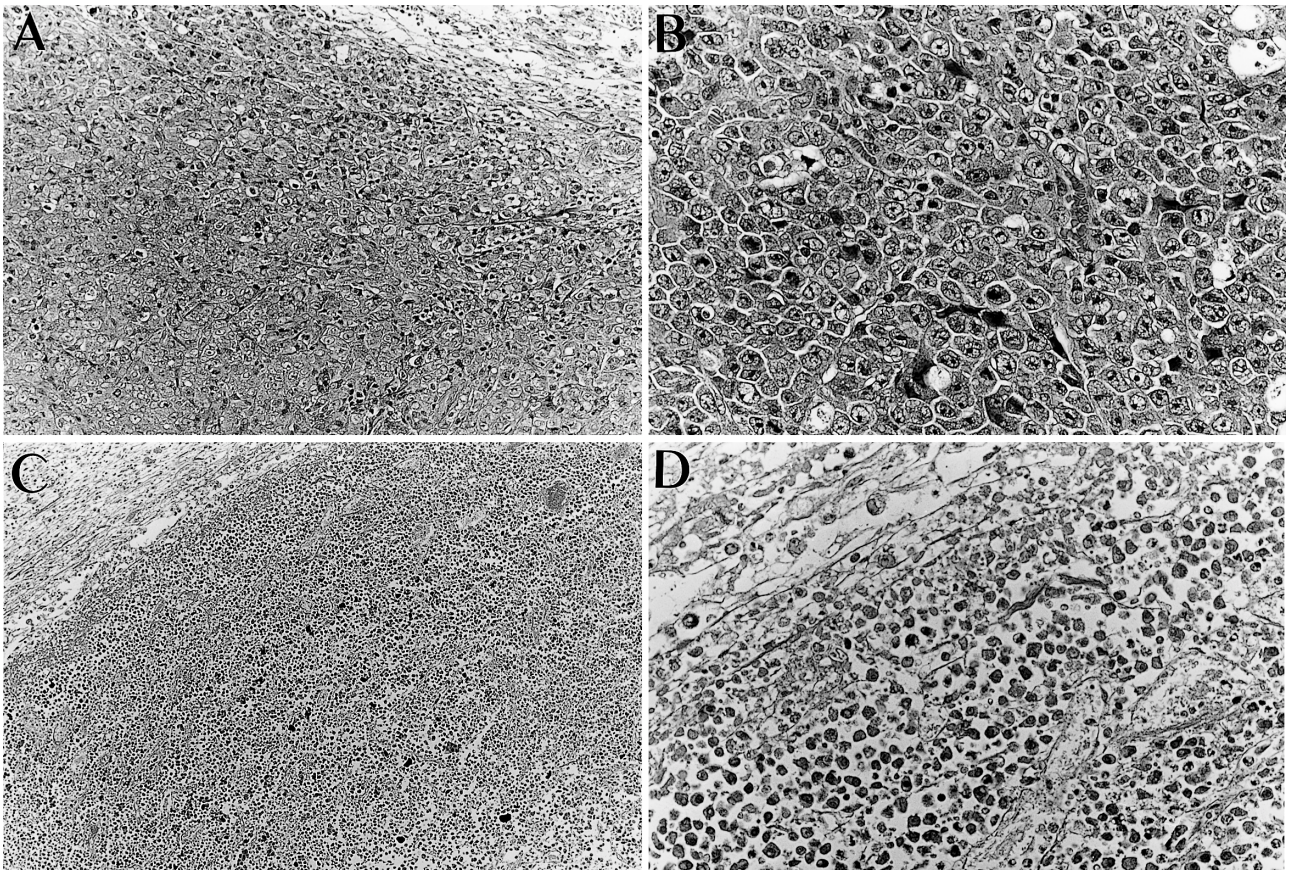


Fig. 9. The histological effect of AC7700. LY80 tumor-bearing rats were killed 3 days after the administration of AC7700 or 0.9% NaCl solution. A, 0.9% NaCl group (×100); B, 0.9% NaCl group (×400); C, 10 mg/kg AC7700 group (×100); D, 10 mg/kg AC7700 group (×400). Note that after AC7700 administration tumor cells have separated from each other and are undergoing pyknosis, and extensive necrosis occurs.

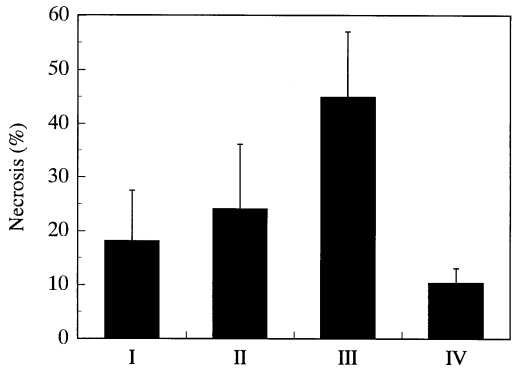


Fig. 10. The percentage of necrosis in the maximum tumor section. LY80 tumor-bearing rats were killed 3 days after the administration of AC7700 or 0.9% NaCl solution. I, 0.9% NaCl group ($n$=8); II, 3 mg/kg AC7700 group ($n$=8); III, 10 mg/kg AC7700 group ($n$=8); IV, untreated group ($n$=5) whose maximum section was almost the same as that in III. The values of mean±SD of percent of necrosis (tumor area) in I, II, III and IV are 18.0±9.5% (2.9±0.6 $cm^2$), 24.0±12.1% (2.4±0.5 $cm^2$), 44.8±12.2% (1.6±0.3 $cm^2$) and 10.3±2.8% (1.8±0.4 $cm^2$), respectively. The percent of necrosis was highly significantly enhanced in the AC7700-treated group compared to the 0.9% NaCl group [I vs. III, $P$<0.001].

clinically because of its high toxicity. In the last decade, a research group in the United Kingdom and New Zealand has discovered many potential tumor-selective vascular-damaging drugs, some of which are awaiting clinical evaluation.[17–23] To date it has been reported that serotonin,[24] hydralazine,[23] sodium nitroprusside,[25, 26] tumor necrosis factor (TNF)-α,[27] flavone acetic acid,[18, 20, 21] vinca alkaloids[19, 22] and an nitric oxide synthase (NOS)

inhibitor[28] decrease tumor tBF. With the exception of vinca alkaloids and the NOS inhibitor, however, it has been found in animal experiments that the decrease in tumor tBF is accompanied with a marked reduction of MABP. Drugs that induce a continuous decrease of MABP seem to be unsuitable for clinical use.

In 1989, a novel tubulin binding inhibitor, a combretastatin compound (CS A-4), was discovered.[1] Dark *et al.*[6] found that it suppressed tumor perfusion markedly. The AC7700 used in the present study is a soluble derivative of CS A-4. AC7700 completely cut off the tBF of LY80 and SLC tumors, which are different in origin and character, and it showed marked therapeutic effects against both tumors. After AC7700 administration, tumors formed a scab following coagulation necrosis and some tumor-bearing rats were cured completely with disappearance of the scab. Histologically, both tumor cells and tumor vessels were damaged and extensive necroses were induced within the tumors. In the natural course, the net body weight of SLC tumor-bearing rats decreases with the tumor growth because the tumor-bearing rats become cachectic. The fact that the body weight of SLC tumor-bearing rats continued to increase during the AC7700 treatment suggests that the compound severely damaged SLC cells.

AC7700 also showed strong anticancer effects against LY80 tumors, against which most anticancer drugs currently used in the clinic are in effective. In the natural course, LY80 cells metastasize to the regional lymph nodes in almost all of the tumor-bearing rats. Such metastases may even be promoted by administered drugs.[11] AC7700 inhibited the growth and the metastasis of such tumors, resulting in improved survival. This result raises the possibility that irreversible cutting off of tumor tBF might be effective not only against ordinary tumors but also against multidrug-resistant tumors. In addition, the fact that AC7700 irreversibly cut off tumor tBF in microfoci within transparent chambers strongly suggests that this compound might exhibit anticancer effects against micrometastatic foci.

On the other hand, the effects of AC7700 on the blood flow of normal tissue were not uniform. The blood flow in normal tissues except for kidney cortex tended to be decreased by the administration of 10 mg/kg AC7700. But the decrease was reversible in most cases. Although the tBF in the brain was decreased by 35% by 10 mg/kg AC7700, the tBF returned to the original level within 24 h and no abnormal symptom was observed during the reduction. In the bone marrow, tBF was decreased by approximately 80% by 10 mg/kg AC7700. Even after administration of 10 mg/kg AC7700 seven times, however, no anemia and no abnormal blood-picture were observed (data not shown). The reason why little bone marrow suppression was seen with AC7700 is not clear, but the remarkable regeneration capacity of the bone marrow might be a factor. Obvious side effects which are caused by many kinds of anticancer drugs, such as body weight loss, anemia and diarrhea, were not observed with AC7700 and the prognosis of the rats was very good. Thus, AC7700 seems to have very few side effects and little cumulative toxicity.

The mechanism by which CS A-4 compounds stop tumor tBF is not completely understood. Although *in vitro* studies have indicated potential antiangiogenic effects of CS A-4 and some investigators have assumed that the *in vivo* effects are caused in part by the antiangiogenic effects,[29] we think that the *in vivo* antitumor effects in the present study have no direct connection with the antiangiogenic effects. This conclusion is based on the fact that tumor tBF begins to decrease immediately following the start of AC7700 administration and the process of irreversible cutting off of tumor tBF is completed within 30 min. We consider that this time is too short for the mechanism of antiangiogenic action to work. Our conclusion is consistent with that reached by Dark *et al.*[6]

MABP decreased transiently following the start of AC7700 administration and soon increased markedly. The pattern of MABP change induced by AC7700 was similar to that by epinephrine administration. We previously reported that the sites of increased vascular resistance in the case of epinephrine were located predominantly in comparatively large arterioles and hence blood flow of both normal and tumor tissues was decreased markedly by epinephrine-induced hypertension.[30] Judging from the fact that MABP is increased and the blood flow in both normal and tumor tissues is decreased by AC7700, the acute effects of the compound might be due to contraction of the same arteriole that are affected by epinephrine. In addition, the finding that no tumor tBF change was induced by topical administration of AC7700 on the tumor blood vessels (data not shown) strongly suggests that the cutting off of tumor tBF by AC7700 might not be due to a direct effect on tumor vessels, but rather may be a secondary reaction via the contraction of host arterioles and/or preexisting arterioles in tumors.

The blood flow in normal tissues generally recovers to its original level eventually. By contrast, tumor tBF never recovers in many regions. The reason for this is not yet clear. The function of tumor microcirculation might be lost completely as a result of vascular shut down for over 24 h because of the fragile supporting structure. Whatever the mechanism, there is no doubt that the result of vascular stasis due to AC7700 is nutrient starvation, which in turn is probably the main cause of cancer cell death. Further investigations are needed to elucidate the mechanism by which tumor tBF is cut off by AC7700.

In conclusion, CS A-4 compounds are anticancer drugs of a new type, which take effect through the irreversible stoppage of tumor tissue blood flow. We believe that one

of the CS A-4 compounds, AC7700, is a promising candidate as an anticancer drug.

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