

## A Potential Use of a Synthetic Retinoid TAC-101 as an Orally Active Agent That Blocks Angiogenesis in Liver Metastases of Human Stomach Cancer Cells

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TAC-101 (4-[3,5-bis(trimethylsilyl)benzamido]benzoic acid) is a novel, synthetic retinoid that is effective against liver metastases of human gastrointestinal cancer cells such as the human stomach carcinoma line AZ-521 in animal models, and is currently in use in phase I cancer trials. However, the mechanism of its antimetastatic action is still poorly understood. Tumor metastasis depends on angiogenesis, and various retinoids have been found to exhibit antiangiogenic activity. Based on these findings we here examined the antiangiogenic effects of TAC-101. Oral administration of TAC-101 (2–8 mg/kg/day) resulted in a drastic suppression of the AZ-521 cell-induced angiogenesis in a mouse dorsal air sac assay system, compared to the vehicle alone. Immunohistochemical analysis with antibody against the endothelial marker CD31 revealed a significant reduction in microvessel density in liver metastases from animals treated with TAC-101 (8 mg/kg p.o.), compared to liver metastases from the untreated control animals. The ability of TAC-101 (8 mg/kg p.o.) to prevent experimental liver metastasis of AZ-521 cells in athymic nude mice was comparable with that of the known angiogenesis inhibitor TNP-470 (30 mg/kg s.c.). TAC-101 also affected angiogenesis in chorioallantoic membranes and some functions of endothelial cells associated with angiogenesis, whereas the retinoid failed to suppress AZ-521 cell proliferation directly. These data suggest that the TAC-101 is an orally active antiangiogenic agent and that this antiangiogenic property may contribute to its efficacy against liver metastasis of human stomach cancer cells.

Key words: Antiangiogenic activity — TAC-101 — Synthetic retinoid — Antimetastatic activity — Angiostatic therapy

How patients with refractory and/or disseminated cancers, including primary hepatomas and metastatic liver tumors from gastrointestinal cancers such as stomach carcinoma, are treated is an important problem. Recent studies have shown that the growth and metastasis of tumors, which are major causes of the difficulty in cancer therapy, are all dependent on neovascular formation from host tissues, suggesting that angiogenesis is a useful target for improving cancer treatment.<sup>1,2)</sup> This is the reason why angiogenesis has recently received considerable attention in cancer research. For the establishment of an angiostatic therapeutic strategy, it is very important to develop useful antiangiogenic agents because they are highly likely to improve cancer treatment. The use of several inhibitors of angiogenesis, including TNP-470 and inhibitors of matrix metalloproteinases (MMPs), in cancer treatment is being evaluated in a clinical trial. In addition, clinical trials of two endogenous angiogenesis inhibitors, endostatin and angiostatin, were quite recently started.

We postulate that a cell differentiation modulator could also interfere with neovascularization, on the basis of the finding that differentiation (or dedifferentiation) of angiogenic endothelial cells is a critical event in the angiogenesis process.<sup>3,4)</sup> Studies concerning this hypothesis have shown that different agents with cell differentiation-modulating activity exhibit antiangiogenic effects in a chick embryo chorioallantoic membrane (CAM) assay, including natural and synthetic retinoids.<sup>4)</sup> Independently, other groups found that some retinoids such as all-*trans*-retinoic acid (ATRA) exhibited antiangiogenic activity.<sup>5–9)</sup> Furthermore, our recent studies have proven that orally administered TAC-101 (4-[3,5-bis(trimethylsilyl)benzamido]benzoic acid; also known as Am 555S), a novel synthetic retinoid,<sup>10)</sup> is more effective against human primary hepatocellular carcinomas, and liver metastases of human stomach cancer cells and colon adenocarcinoma cells than *cis*-diamminedichloroplatinum (CDDP) and 5-fluorouracil (5-FU) in animal models.<sup>11,12)</sup> Based on these facts, a clinical trial of TAC-101 was started in patients with cancer in the United States. However, the precise mechanism of the antitumor action of TAC-101 is not fully understood,

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although it has been shown to suppress the production of urokinase-type plasminogen activator (uPA) and MMP-9 by phorbol myristate acetate-stimulated tumor cells.<sup>13)</sup> Overall, these findings suggest the hypothesis that TAC-101 inhibits angiogenesis, and if this is correct, its antiangiogenic action might be involved in its previously observed efficacy against liver metastasis of human gastrointestinal cancer cells, including the human stomach carcinoma line AZ-521, in animal models. However, there is no direct evidence supporting this hypothesis. Thus, we investigated the effects of TAC-101 on angiogenesis in *in vivo* and *in vitro* model systems, and also directly compared its antimetastatic activity with that of the known angiogenesis inhibitor TNP-470.

## MATERIALS AND METHODS

**Materials** TAC-101 and TNP-470 were synthesized by Taiho Pharmaceutical Co., Ltd. The chemical structure of TAC-101 is shown in Fig. 1. ATRA and CDDP were purchased from Sigma (St. Louis, MO) and Nippon Kayaku Co., Ltd. (Tokyo), respectively. For *in vivo* experiments, TAC-101 and ATRA were suspended in 0.5% hydroxypropyl methylcellulose and 5% ethanol/95% olive oil, respectively, and administered by oral gavage in a volume of 0.1 ml per 10 g body weight. For immunohistochemical and antimetastatic experiments, an untreated control was used as a common reference control because previous studies showed virtually no difference in survival time between vehicle-treated and -untreated groups in the experimental liver metastatic model of a human stomach cancer.<sup>12)</sup> A human stomach cancer cell line AZ-521 was obtained from Japanese Cancer Research Resources Bank (Tokyo). Human dermal microvascular endothelial cells (HDMECs) were obtained from Cell Systems (Kirkland, WA).

**Mice** Female ICR mice aged 7 to 9 weeks were purchased from Charles River Japan (Atsugi). Male Balb/c athymic nude mice aged 5 weeks were purchased from Japan Clea, Inc. (Tokyo). They were housed in a temperature-, air- and light-controlled room with free access to laboratory rodent chow and water. The animal experiments were approved by the Committee on the Ethics of Animal Experiments of the Tokyo Metropolitan Institute of Medi-

cal Science (for the mouse dorsal air sac assay) or Taiho Pharmaceutical Co., Ltd. (for the experiments on immunohistochemistry and antimetastasis), and were carried out in accordance with the Guidelines for Animal Experiments of the Tokyo Metropolitan Institute of Medical Science or Taiho Pharmaceutical Co., Ltd.

**Mouse dorsal air sac assay** The effect of TAC-101 on tumor-related angiogenesis was examined in the mouse dorsal air sac assay.<sup>14)</sup> A Millipore chamber containing AZ-521 cells ( $1 \times 10^6$  cells) or calcium- and magnesium-free phosphate-buffered saline (PBS) alone was implanted into an air sac formed artificially in the back of a 10-week-old ICR mouse on day 0. The treated animals received oral administration doses of TAC-101 ranging from 0 to 8 mg/kg/day once a day from day 0 to day 4. On day 5 the angiogenic response was examined under a dissecting microscope by counting neovessels with a zig-zag character of 3 mm or more in length within an area encircled by a black ring with the same inner diameter as the Millipore ring. Angiogenesis indexes 0, 1, 2, 3, 4 and 5 represented neovessel numbers of 0, 1, 2, 3, 4 and 5 or more, respectively. Either 6 or 13 animals were used per experiment.

**CD31 immunohistochemistry** According to the previous paper,<sup>11)</sup> an experimental liver metastasis model was produced by intrasplenic injection of AZ-521 cells ( $1 \times 10^6$  cells/mouse) into male Balb/c athymic nude mice ( $n=3$  for each) on day 0. On day 1 the animals were divided into three groups and treated as follows: the three groups received no agent (control), TAC-101 (8 mg/kg/day p.o.), and ATRA (8 mg/kg/day p.o.), respectively. TAC-101 and ATRA were administered for 5 consecutive days a week for 6 weeks, initiated on day 1. On day 43 the animals were sacrificed, the liver weight was determined for evaluation of the tumor metastasis,<sup>13)</sup> and then the metastases in the livers were immediately removed from each of the three animal groups and frozen at  $-80^\circ\text{C}$ . The liver metastases were subjected to CD31 immunohistochemistry, as reported previously.<sup>15)</sup> In brief, 5- $\mu\text{m}$  frozen tissue sections were fixed in acetone, incubated in 0.3%  $\text{H}_2\text{O}_2$  in methanol, and then rinsed in PBS containing 0.02% Tween 80. The sections were then treated with 1.5% normal rabbit serum, with 25% Block ace (Dainippon Pharm., Tokyo) and finally with a rat anti-mouse CD31 antibody (1:200 dilution; PharMingen, San Diego, CA). They were then rinsed in PBS containing 0.02% Tween 80 and incubated with a biotin-labeled anti-rat IgG antibody (Vector Laboratories, Burlingame, CA). After incubation in the presence of an avidin-biotin-peroxidase complex, the sections were stained with 3,3-diaminobenzidine. Microvessel density was evaluated by counting CD31-positive vessels/1  $\text{mm}^2$  in the areas that were considered to be most active as to neovascularization. The counting was performed in three fields and the average was calculated.

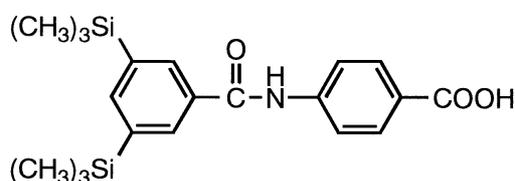


Fig. 1. Chemical structure of TAC-101. Formula:  $\text{C}_{20}\text{H}_{27}\text{NO}_3\text{Si}_2$ ; mol. wt.: 385.61.

**Antimetastatic assay** Experimental liver metastasis models involving AZ-521 cells were prepared as described above. Treatment started on day 1, TAC-101 at 8 mg/kg/day ( $n=7$ ) being administered orally for 5 consecutive days weekly for 6 weeks. TNP-470 at 30 mg/kg/day ( $n=7$ ) was administered subcutaneously every other day for 42 days, this dosing schedule being effective in antitumor or antimetastatic models.<sup>16</sup> CDDP at 7 mg/kg/day ( $n=6$ ) was intravenously administered once, this regimen being optimal for the agent in athymic nude mice.<sup>17</sup> Eleven animals were used for the control untreated group. On day 43 the animals were sacrificed simultaneously and their liver weights were measured for evaluation of tumor metastasis.<sup>13</sup>

**CAM assay** A CAM assay was performed as described previously.<sup>3</sup> The 5-day-old CAMs were treated with an ethylene-vinyl acetate copolymer 40 (EV; a generous gift from Mitsui-Du Pont Polychemicals, Tokyo) impregnated with the indicated doses of TAC-101 at 37°C in a humidified egg incubator. After incubation for 2 days, an appropriate volume of a 20% fat emulsion was injected into the chorioallantois to further visualize the vascular network against the white background of the fat emulsion. When the avascular zone in a treated CAM was 3 mm or more in diameter, the antiangiogenic response was assessed as being effective.

**AZ-521 cell proliferation** In the presence of indicated concentrations of TAC-101, AZ-521 cells ( $1 \times 10^4$  cells/well) were cultured in 12-multiwell dishes containing 1 ml of Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% fetal bovine serum (Moregate, Mel-

bourne, Australia), 1% antibiotic and 25 mM HEPES at 37°C in a humidified 5% CO<sub>2</sub>-95% air incubator. After 72-h culture, the cells were trypsinized and then counted with a Coulter counter Z1 (Coulter Japan, Tokyo).

**PA production** The complete medium for HDMECs comprised MCDB-131 medium (Sigma) supplemented with 1% antibiotic, 15 mM HEPES, 10 µg/ml endothelial cell growth supplement (Upstate Biotechnology, Lake Placid, NY), 10 µg/ml heparin (Sigma) and 10 ng/ml epidermal growth factor (Sigma). The effect of TAC-101 on PA production by HDMECs was examined as described previously.<sup>18</sup> HDMECs ( $1 \times 10^5$  cells per cm<sup>2</sup>) were seeded

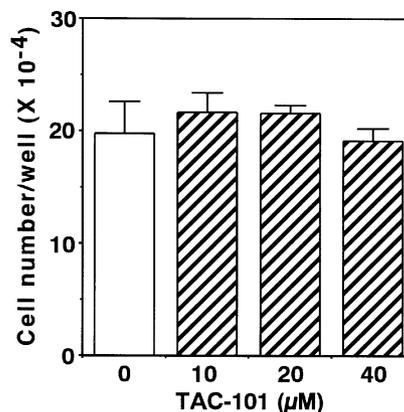


Fig. 2. Effect of TAC-101 on proliferation of AZ-521 human stomach cancer cells *in vitro*.

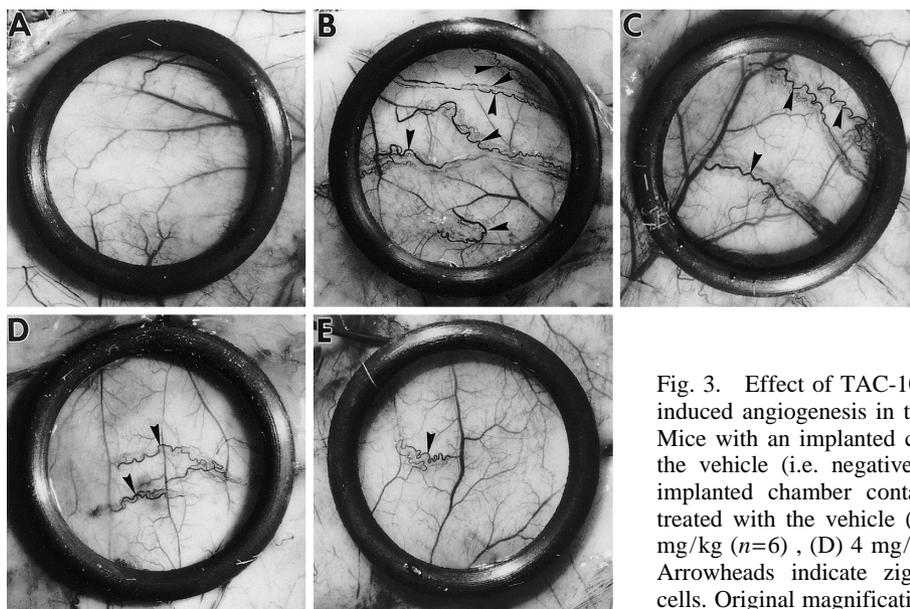


Fig. 3. Effect of TAC-101 on AZ-521 human stomach cancer cell-induced angiogenesis in the mouse dorsal air sac assay system. (A) Mice with an implanted chamber containing PBS were treated with the vehicle (i.e. negative control group;  $n=6$ ); (B) mice with an implanted chamber containing AZ-521 cells ( $1 \times 10^6$  cells) were treated with the vehicle (i.e. positive control group;  $n=13$ ), (C) 2 mg/kg ( $n=6$ ), (D) 4 mg/kg ( $n=6$ ) or (E) 8 mg/kg ( $n=6$ ) TAC-101. Arrowheads indicate zigzagging neovessels induced by AZ-521 cells. Original magnification  $\times 2.8$ .

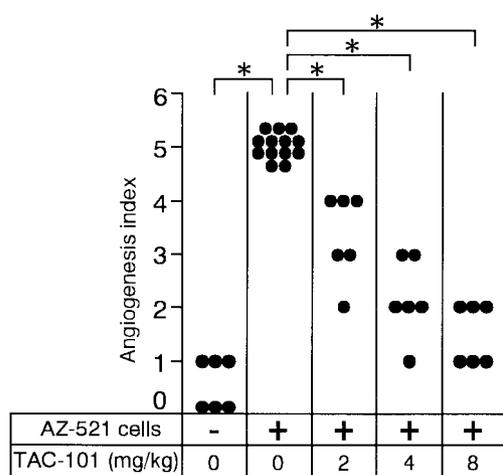


Fig. 4. Dose-dependent inhibition of AZ-521 cell-induced angiogenesis by TAC-101. \*,  $P < 0.01$  compared with the positive control group (two-tailed Mann-Whitney's  $U$  test).

onto gelatin-coated culture plates and incubated at 37°C for 24 h in 5% CO<sub>2</sub> in complete medium with 10% fetal bovine serum (Moregate), and then incubated in serum-free complete medium containing 0.1% bovine serum albumin (BSA; Sigma) in the presence or absence of various concentrations of TAC-101 for 18 h. Cell supernatants and extracts were prepared as described previously.<sup>18</sup> PA activity was determined using plasminogen (Daiichi Chemicals, Tokyo) and S-2251 (Chromogenix, Mölndal, Sweden). PA activity was expressed in milliunits (mU)/μg protein or mU/10<sup>5</sup> cells. Protein concentrations were determined with BSA as a standard according to the manufacturer's instructions (DC protein assay; Bio-Rad, Hercules, CA).

**Cell migration assay** HDMEC migration was determined by a wound migration assay.<sup>19</sup> Two 5-mm scratches were made in gelatin-coated 35-mm culture dishes containing confluent monolayers of HDMECs using a razor blade, washed twice with MCDB-131 containing 0.1% BSA and

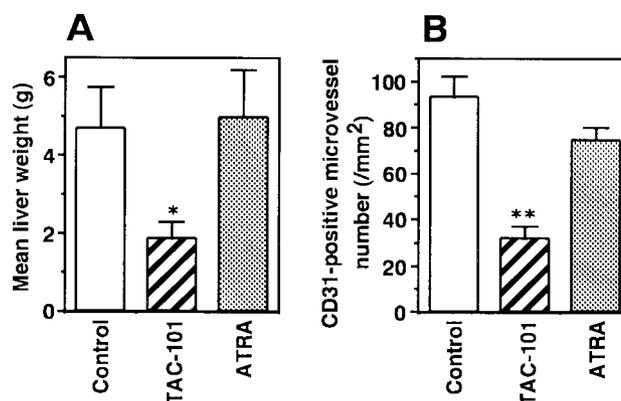


Fig. 5. Suppression of both liver metastasis of AZ-521 human stomach cancer cells and microvascularization of AZ-521 cell liver metastases on TAC-101 administration. Values are means ± SD ( $n=3$ ). (A) At the dose of 8 mg/kg/day, p.o., autopsy was done on day 43 after injection of AZ-521 cells into the spleens of nude mice. (B) Immunohistochemical determination of CD31-positive microvessels in liver metastases from the non-treated control, TAC-101- and ATRA-treated groups in the experiments shown in (A). \*,  $P < 0.05$ ; \*\*,  $P < 0.01$  compared with the non-treated control group (two-tailed Welch's  $t$  test).

then further incubated for 18 h in complete medium containing 0.1% BSA in the presence of the indicated concentrations of TAC-101. Cells were stained with Giemsa, and those that migrated across the edge of the wound were recorded at ×100 magnification by a color video camera recorder (TK-1280U; Victor, Tokyo). The total cells migrated in ten randomly chosen microscopic fields per dish were measured with a microcomputer running NIH Image (Version 1.58).

**Tube formation assay** Tube formation by HDMECs on Matrigel (Becton Dickinson Labware, Bedford, MA) was performed as described previously.<sup>18</sup> Gels were prepared in 24-well culture dishes by incubating Matrigel (2 mg/0.2 ml/well) at 37°C for 60 min. HDMECs at 1×10<sup>5</sup> cells/well were seeded onto the gels in 24-well culture dishes

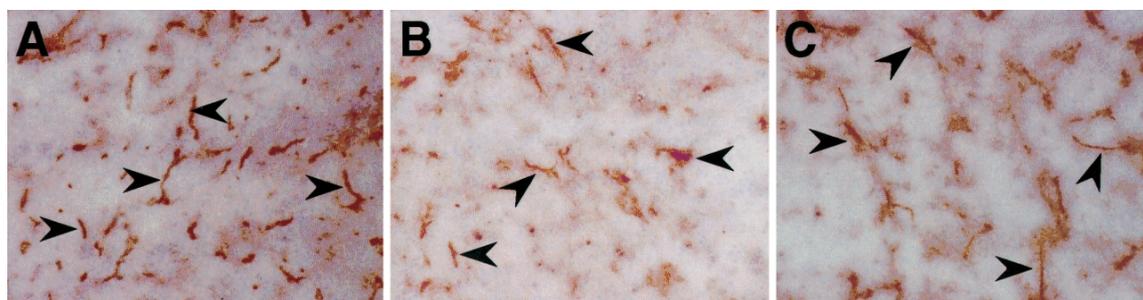


Fig. 6. Immunohistochemical stain for CD31 in liver metastases from the non-control (A), TAC-101-treated (B) and ATRA-treated (C) groups in the experiments shown in Fig. 5. Original magnification ×75.

containing 1 ml of complete medium including 1% fetal bovine serum and the indicated concentrations of TAC-101, and incubated at 37°C under 5% CO<sub>2</sub>. After incubation for 18 h, the formation of tube-like structures by HDMECs was analyzed by phase contrast microscopy at ×100 magnification, and the total lengths of the tubular structures in nine randomly chosen microscopic fields per well were determined using a Leica Q500MC image analyzer equipped with Qwin image analysis software (Cambridge, UK).

**Statistics** Data on the mouse dorsal air sac assay were analyzed by means of the Mann-Whitney's *U* test. The results of immunohistochemical experiments were analyzed by using Welch's *t* test. The results of antimetastatic experiments were analyzed by Welch's *t* test or Dunnett's multiple comparisons post-hoc test. Data on the CAM assay were analyzed by Fisher's exact probability test. A *P* value of <0.05 was considered significant for all analyses.

## RESULTS

**Effect of TAC-101 on proliferation of AZ-521 human stomach cancer cells** *In vitro* experiments were conducted to examine whether or not TAC-101 interfered directly with AZ-521 cell proliferation. TAC-101 had no effect on AZ-521 cell growth at concentrations up to 40 μM (Fig. 2), suggesting that it did not cause direct inhibition of the cancer cell growth.

**Effect of TAC-101 on tumor-related angiogenesis induced by AZ-521 human stomach cancer cells** The effect of oral administration of TAC-101 on angiogenesis induced by AZ-521 human stomach cancer cells was examined in a mouse dorsal air sac assay system, because our previous study revealed that orally administered TAC-101 effectively prevented experimental liver metastases of AZ-521 cells in a nude mouse model.<sup>11)</sup> The observations obtained in typical experiments are presented in Fig. 3. In a negative control group, in which PBS-containing chambers were implanted, and which received the vehicle alone, there was little or no formation of neovessels (Fig. 3A). In a positive control group, which had had AZ-521 cell-containing chambers implanted and which were given the vehicle alone, there was drastic induction of the formation of new blood vessels, characterized by zigzag lines (Fig. 3B). The formation of such neovessels was dramatically prevented by increasing doses of TAC-101 from 2 to 8 mg/kg/day p.o. (Fig. 3, C–E). This angiogenic response was assessed under a dissecting microscope by counting the tumor cell-induced neovessels showing the characteristic zigzag lines (Fig. 4). The median angiogenesis index was 0.5 (range, 0–1; *n*=6) in the negative control group. Compared to the negative control group, the positive control group significantly induced angiogenesis (*P*<0.001), the median angiogenesis index being 5 (*n*=13). Significant

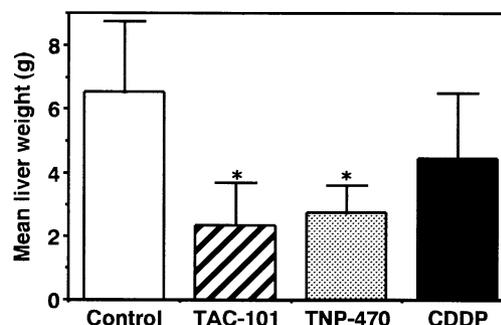


Fig. 7. In the treatment of a liver metastasis model of AZ-521 cells, the oral administration of TAC-101 exhibited significant efficacy comparable to that of parenteral administration of TNP-470. \*, *P*<0.01 compared with the non-treated control group (two-tailed Dunnett's *t* test). Values are means±SD (*n*=10 for control group; *n*=7 for TAC-101- and TNP-470-treated groups; *n*=6 for CDDP-treated group).

and dose-dependent suppression of the AZ-521 cell-triggered angiogenic activity was observed in groups treated with increasing doses of TAC-101, compared to the positive control group (*P*<0.01). The median angiogenesis indexes were 3.5 (range, 2–4; *n*=6) for 2 mg/kg, 2 (range, 1–3; *n*=6) for 4 mg/kg, and 1.5 (range, 1–2; *n*=6) for 8 mg/kg TAC-101, respectively.

**Immunohistochemical evaluation of microvascularization of liver metastases** We further investigated whether or not TAC-101 treatment interfered with angiogenesis in liver metastases in an experimental metastasis model of AZ-521 cells. This liver metastasis model is characterized by colony-forming ability and increased weight of the liver. Indeed our previous study showed that in this metastasis model TAC-101 produced significant reduction both in the number of the metastatic colonies on the liver surface and in the liver weight of mice treated.<sup>11)</sup> So, in this study the liver weight was determined for the evaluation of tumor metastasis. TAC-101 exhibited significant suppression of liver metastasis of AZ-521 cells (*P*<0.05), as determined by measuring liver weight (Fig. 5A), which reconfirmed our previous observation.<sup>11)</sup> Such an effect was not observed with ATRA with the same dosing schedule as for TAC-101 (*P*>0.05), consistent with our previous finding.<sup>11)</sup> Angiogenesis was determined by doing immunohistochemistry for CD31, a specific marker antigen for vascular endothelial cells.<sup>20)</sup> The observations of typical experiments are presented in Fig. 6. Immunohistochemical staining with antibody against CD31 revealed a 66% decrease in the microvessel density in TAC-101-treated animals (31.7±5.6 microvessels/mm<sup>2</sup>, *n*=3, *P*<0.01) versus untreated animals (93.3±8.8 microvessels/mm<sup>2</sup>, *n*=3), but only a 20% decrease in the microvessel density was observed with ATRA treatment (74.7±5.2 microvessels/

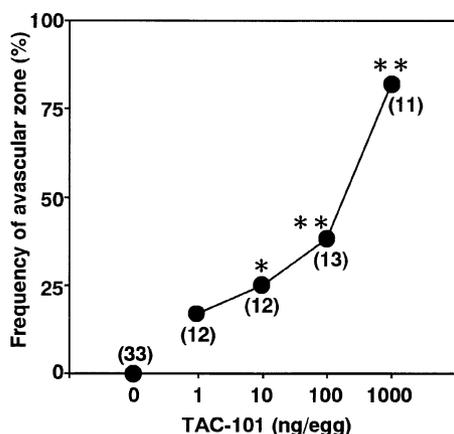


Fig. 8. Dose-dependent inhibition of 5-day-old CAM angiogenesis by TAC-101. The points indicate the frequencies (%) of avascular zones showing an antiangiogenic response. The values in parentheses are the numbers of CAMs examined. \*,  $P < 0.05$ ; \*\*,  $P < 0.001$  compared with the value for control CAMs ( $n = 33$ ) not showing an avascular zone (Fisher's probability exact test).

mm<sup>2</sup>,  $n = 3$ ,  $P > 0.05$ ) (Fig. 5B). Overall, there is a good correlation between antiangiogenesis and inhibition of metastasis.

**Antimetastatic abilities of TAC-101, TNP-470 and CDDP** To compare directly the antimetastatic ability of TAC-101 with that of TNP-470 in the experimental metastasis model of AZ-521 cells, antimetastatic activity was determined by measuring the liver weight of treated mice (Fig. 7). A 64% reduction in mean liver weight was found with oral administration of TAC-101 (8 mg/kg) versus the untreated control ( $P < 0.01$ ). This effect was comparable to that of subcutaneously administered TNP-470 (30 mg/kg), which caused a 58% reduction in mean liver

weight compared to the control ( $P < 0.01$ ). Intravenous administration of CDDP exhibited less antimetastatic activity than these two angiogenesis inhibitors, the extent of inhibition being 32%.

**Effect of TAC-101 on 5-day-old CAM angiogenesis**

The antiangiogenic activity of TAC-101 was also examined in a CAM assay. At 10 ng/egg or more TAC-101 inhibited embryonic neovascularization (Fig. 8). This inhibition was dose-dependent and statistically significant ( $P < 0.05$ ), the ID<sub>50</sub> value being 210 ng (540 pmol) per egg. Representative observations are shown in Fig. 9. Treatment of the 5-day-old CAMs with 1000 ng/egg of TAC-101 for 2 days inhibited angiogenesis, and thereby produced a significant avascular zone (Fig. 9B), whereas the formation of a significant avascular zone was not observed for any of the control CAMs tested ( $n = 33$ ) (Fig. 9A).

**Effects of TAC-101 on endothelial cell functions associated with *in vivo* angiogenesis**

*In vitro* experiments were conducted to determine which functions of endothelial cells related to *in vivo* angiogenesis were affected by TAC-101. Treatment of HDMECs with TAC-101 resulted in a marked reduction in the cell-associated PA level, migration or tube formation of these cells (Fig. 10). These inhibitory effects were concentration-dependent, the IC<sub>50</sub> values for PA production, migration and tube formation being 13, 18 and 13 μM, respectively. At concentrations up to 20 μM TAC-101 caused little or no inhibition of proliferation of HDMECs (data not shown).

**DISCUSSION**

TAC-101 has been through phase I clinical evaluation. This stage of clinical evaluation was mainly performed in patients with lung cancer, and the results will be published after completion of data processing. So far we can say that

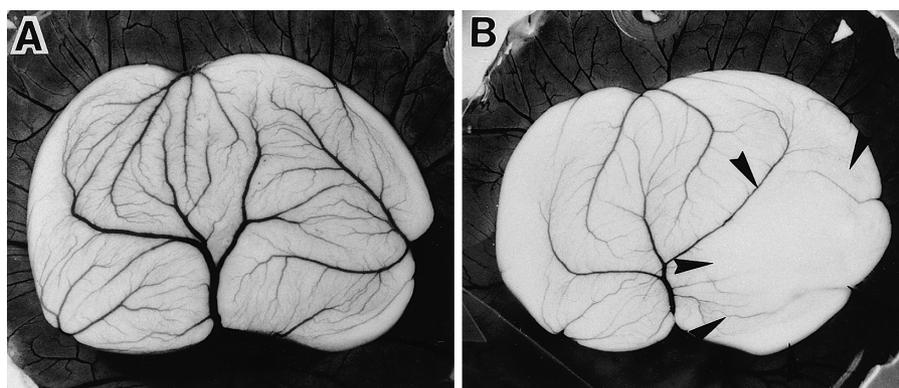


Fig. 9. Effect of TAC-101 on 5-day-old CAM angiogenesis. The CAM angiogenesis assay was performed with an empty EV pellet alone (i.e. control) (A) or a TAC-101-containing EV pellet (1 μg per egg) (B). The results shown are for a representative experiment. Arrowheads indicate an avascular zone. Original magnification ×2.1.

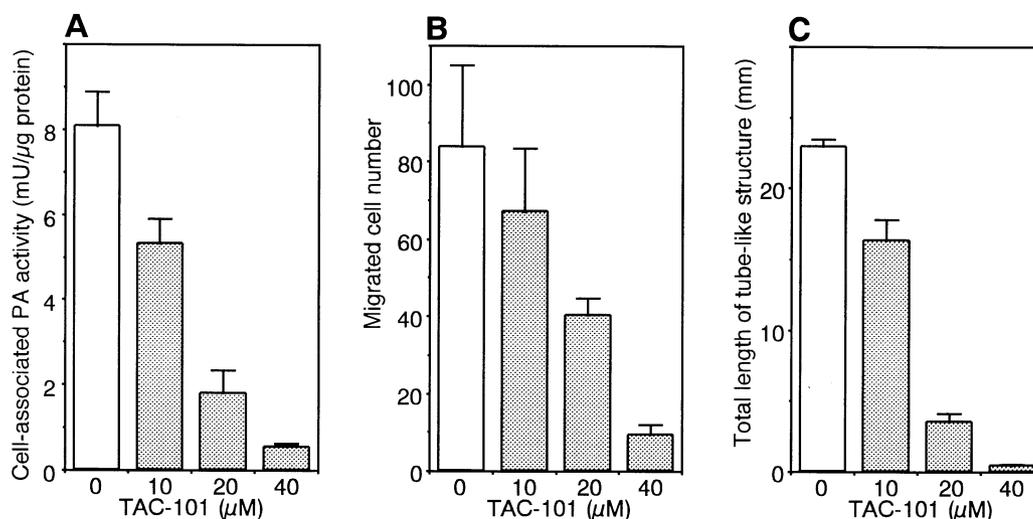


Fig. 10. Prevention of vascular endothelial cell functions associated with *in vivo* angiogenesis by TAC-101. TAC-101 treatment resulted in concentration-dependent suppression of the cell-associated PA level (A), cell migration (B) or formation of tube-like structures on Matrigel (C) in microvascular endothelial cells. Values are means  $\pm$  SD ( $n=4$  for A and B;  $n=3$  for C).

there were many cases with stable disease, and one complete response was observed, which is an event seldom observed in phase I studies. Based on our preclinical data, we anticipate that TAC-101 will show good efficacy in the treatment of patients with liver cancer. The next trial is at the organizational stage, and the results will have very important implications for the future developmental schedule.

The present study provides novel evidence supporting the ability of TAC-101 to act as an orally active antiangiogenic agent that causes suppression of liver metastasis of human stomach cancer cells *in vivo*. Specifically, our mouse dorsal air sac assay confirmed the efficacy of orally administered TAC-101 against AZ-521 cell-induced tumor angiogenesis. This finding indicated the possible involvement of the antiangiogenic activity of TAC-101 in its previously (and presently) observed effectiveness on a liver metastasis model of AZ-521 cells,<sup>11</sup> because angiogenesis plays important roles in metastasis of tumor cells and because different inhibitors of angiogenesis, such as TNP-470 and angiostatin,<sup>21,22</sup> have been proven to be effective against tumor metastasis via their antiangiogenic actions in animal models. Therefore, we examined whether or not the antiangiogenic activity of TAC-101 is actually involved in its efficacy against the liver metastasis model of AZ-521 cells, angiogenesis being determined as the microvessel density by immunohistochemistry using antibody against CD31, a specific endothelial cell marker.<sup>20</sup> This staining demonstrated that a significant decrease in microvessel density occurred in the suppressed liver metastases of AZ-521 cells from the TAC-101-treated animals. In contrast, ATRA did not have a significant effect

on the microvessel density in the liver metastases of AZ-521 cells from the ATRA-treated animals, nor did ATRA exhibit antimetastatic activity against these tumor cells. Overall, it seems reasonable to consider that the antiangiogenic activity of TAC-101 contributes to its antimetastatic effect. The antiangiogenic mechanism of TAC-101 may also be involved in its previously observed effect on liver metastasis of human colon adenocarcinoma cells or on the growth of human primary hepatocellular carcinomas in animal models.<sup>11,12</sup> Further studies are needed using other metastatic tumor cell lines.

The *in vivo* antitumor activity of TAC-101, however, may not be exclusively accounted for by its antiangiogenic activity because it cannot be completely ruled out that the retinoid has an additional antitumor mechanism, although it is unlikely that it directly suppresses the growth of AZ-521 cells, because it had no effect on the proliferation of these cells in *in vitro* experiments. In this regard, our previous study revealed that TAC-101 exerted a significant antimetastatic effect even when treatment started on day 7, 14, or 21 after inoculation of AZ-521 cells in the experimental liver model.<sup>11</sup> There appeared to be no significant difference in the antimetastatic effect among these three regimens, which is consistent with the possibility that besides the antiangiogenic activity, other properties of TAC-101 are involved in the mechanism of its antimetastatic action. It has been shown that TAC-101 causes the induction of apoptosis of human hepatocellular carcinoma cells or murine colon 26-L5 carcinoma cells *in vitro*, as well as the inhibition of hepatocyte growth factor-stimulated invasion of these hepatocellular carcinoma cells

*in vitro*.<sup>12,13</sup>) It also prevented the production of uPA, MMP-2 or MMP-9,<sup>13</sup>) proteases that also assist tumor cell invasion. Thus, further studies are needed to examine the effects of TAC-101 on AZ-521 cell invasion and the production of these proteases in AZ-521 cells. In addition, we did not examine the effects of TAC-101 on the *in vitro* characteristics of AZ-521 cells, such as production of angiogenic cytokines, expression of adhesion molecules, etc.

The present finding that systemic administration of ATRA had neither antiangiogenic nor antimetastatic activity against AZ-521 human stomach cancer cells seemed to conflict with previous observations obtained with this retinoid. One report showed that peritoneal administration of ATRA leads to antitumor and antiangiogenic activities in a nude mouse model of human head and neck squamous cell carcinomas.<sup>9</sup>) Another report demonstrated inhibitory effects of orally administered ATRA on tumor growth and angiogenesis in nude mice with human cervical squamous cell carcinoma xenografts.<sup>8</sup>) At present, the reason for the apparent discrepancy between our study and others is unknown, but it may reflect differences in the sensitivity to ATRA of the tumor cell types used. Indeed, previous studies suggested that squamous cell carcinomas may exhibit relatively high sensitivity to ATRA.<sup>8, 9, 23, 24</sup>)

The differential activities of TAC-101 and ATRA on the present liver metastasis model of AZ-521 cells seem a key problem. Our experimental system involves chronic administration of both compounds. Under such circumstances the biotransformation of ATRA can be very rapid, resulting in a dramatic decrease of its effective concentration in the target organ, which is the main eliminating organ of the drug. Indeed, this phenomenon was reported as a reason for the relapse of leukemia patients treated with ATRA.<sup>25, 26</sup>) In our preclinical studies in monkeys, no acceleration of biotransformation of TAC-101 occurred even after prolonged treatment for several weeks. So we suspect that TAC-101 is well retained in the liver of treated animals. The differences of these properties between the two retinoids might, at least in part, explain why TAC-101 showed more potent antiangiogenic activity than ATRA when administered systemically. In contrast, topical treatment of ATRA was found to have antiangiogenic activity in a CAM assay,<sup>3-5</sup>) where biotransformation of test substances including ATRA is highly unlikely to occur. Alternatively, it might be possible that higher doses of ATRA could have antiangiogenic activity in the dorsal air sac assay and antimetastatic activity in the metastasis model of AZ-521 cells. However, this possibility was not examined in the present study because our previous studies had shown that even the same dose of ATRA as that used in this study caused some reduction of liver weight in normal mice.<sup>11</sup>) The effects of ATRA on angiogenic endothelial cells *in vitro* also remain to be determined, though other groups have shown that the agent affects some

endothelial cell functions involved in *in vivo* angiogenesis, such as endothelial cell migration.<sup>3-6, 24</sup>)

TAC-101 belongs to a class of compounds generally not showing cytotoxic effects over the range of concentrations observed in the plasma of experimental animals during treatment with effective doses. Under our assay conditions in the present study, TAC-101 at 20  $\mu$ M inhibited PA activity, cell migration and tube formation of HDMECs by more than 50% of the control in the absence of any drug, but did not affect proliferation of HDMECs or stomach cancer AZ-521 cells. We observed no inhibition of cell proliferation under these assay conditions, although some effect cannot be completely excluded. The exact mechanism by which TAC-101 shows such specific action on the endothelial cell-induced angiogenic process remains unclear; however, one may speculate as to the involvement of the cell differentiation-modulating activity of TAC-101, because the agent is a synthetic retinoid that may exhibit this activity. Differentiation (or dedifferentiation) of angiogenic endothelial cells is an important event in the process of angiogenesis. Overall, it appears reasonable to assume that TAC-101 could show specific actions on angiogenic endothelial cells by modulating differentiation (or dedifferentiation) of these cells during angiogenesis. This idea is supported by the present finding that TAC-101 affected vascular tube formation, which is an *in vitro* model of endothelial cell differentiation.

It is likely that an antiangiogenic agent would have to be administered continuously to cancer patients for a long time, suggesting that oral administration might be the route of choice.<sup>14, 27, 28</sup>) The development of an antiangiogenic agent equivalent to aspirin in convenience would be highly desirable.<sup>29</sup>) Therefore, it is noteworthy that orally administered TAC-101 exhibits an antimetastatic ability similar to that of TNP-470 administered subcutaneously. Also, one should note that our previous and present studies have shown that long-term multiple administration of TAC-101 is highly likely to show only mild toxicity *in vivo*.<sup>11, 12</sup>) Namely, there were no significant adverse effects of TAC-101, except for some body weight reduction at doses higher than 16 mg/kg/day.

In conclusion, although further research is required to clarify the mechanism of the antiangiogenic action of TAC-101, it is expected that this drug will prove useful as an orally active antiangiogenic agent for the treatment of cancers, at least liver tumors.

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