Inhibition of Angiogenesis and Intrahepatic Growth of Colon Cancer by TAC-101

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Abstract
We demonstrated in this study that inhibition of intrahepatic growth of colon cancer by TAC-101 is mediated by inhibition of angiogenesis. In vitro experiments showed that TAC-101 inhibited the proliferation of murine hepatic sinusoidal endothelial (HSE) cells accepted by coculture with murine colon 26-L5 (L5) cells. HSE cell proliferation was also enhanced by conditioned medium of L5 cells (CM-L5), and this enhancement of proliferation was abrogated by anti-vascular endothelial growth factor antibody. CM-L5 also enhanced in vitro tube formation of HSE cells on Matrigel, and this activity of CM-L5 was abrogated by TAC-101 in a concentration-dependent manner. On the other hand, p.o. administration of TAC-101 inhibited tumor-induced angiogenesis in vivo and decreased the weights of L5 tumors in the mouse liver. Reverse transcriptase-PCR analysis using in vivo tumor tissue suggested that repression of vascular endothelial growth factor expression by TAC-101 was associated with the antiangiogenic activity. TAC-101 alone and 5-fluorouracil (5-FU)/D,L-leucovorin (LV) significantly inhibited the intrahepatic growth of L5 tumors (P < 0.002 and 0.001, respectively), whereas 5-FU alone did not (P = 0.088). When TAC-101 was administered with 5-FU/LV, marked enhancement of antitumor activity was observed (95% inhibition; P < 0.001). This enhanced antitumor effect was also observed in experiments using Co-3 human colon adenocarcinoma. Concurrent treatment with TAC-101 and 5-FU/LV and sequential treatment with 5-FU/LV followed by TAC-101 resulted in significant augmentation of antitumor activity against Co-3 (overall P = 0.007 and 0.015, respectively). These findings indicate that TAC-101 inhibits tumor angiogenesis and suggest that it may be effective against hepatic metastasis of colon cancer.

Introduction
Liver metastasis is one of the common malignancies in patients with gastrointestinal cancer (1). Surgical resection is the curative treatment for liver-metastasized cancer as well as for the primary colon cancer (2). Because not all patients are, in fact, candidates for resection, more potent treatment modalities are needed to improve the prognosis of patients with hepatic metastasis. Recent studies have indicated that antiangiogenic therapy can inhibit the progression and recurrence of metastatic disease, which depends on adequate vasculature and, therefore, on angiogenesis (3–6). Synthetic or endogenous inhibitors of angiogenesis, such as TNP-470 (AGM-1470), SU-5416, several retinoids, angiostatin, and endostatin, have been reported to exhibit antiangiogenic and antmitotic activities (6–9).

We previously reported that 4-[3,5-bis(trimethylsilyl)benzamido]benzoic acid (project code name: TAC-101) inhibited liver metastasis in several experimental models (10–12). Like some other benzoic acid derivatives (13), TAC-101 can bind to RARs,2 especially to RAR-α (14, 15). Because the antiangiogenic activity of some retinoids has been suggested to be mediated by RAR-α (16), we attempted to determine whether the antitumor activity of TAC-101 was associated with inhibition of angiogenesis.

The experimental model of liver metastasis produced by intrasplenic injection of tumor cells has frequently been used to assess the antimitotic activities of novel inhibitors. However, it is difficult to quantitatively evaluate the growth of established metastatic tumors in the liver because multiple tumor nodules result from intrasplenic injection. In this study, we directly implanted a small fragment of colon cancer into the liver of mice to form a single tumor nodule in the liver, similar to a liver-metastatic nodule, and examined the efficacy of TAC-101 against intrahepatic growth and angiogenesis in vivo. We also examined the combined inhibitory effect of TAC-101 and 5-FU/LV on intrahepatic tumor growth of murine and human colon cancers because fluorinated pyrimidine-based combination therapy is used to treat metastatic colorectal cancer (17).

Materials and Methods

Chemicals. TAC-101 {4-[3,5-bis(trimethylsilyl)benzamido]benzoic acid} was synthesized by Taiho Pharmaceutical Co., Ltd. (Saitama, Japan). 5-FU and LV were purchased from

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2 The abbreviations used are: RAR, retinoic acid receptor; 5-FU, 5-fluorouracil; LV, D,L-leucovorin; L5, colon 26-L5; HSE, hepatic sinusoidal endothelial; EM, endothelial mitogen; CM-L5, conditioned medium of L5; VEGF, vascular endothelial growth factor; RT-PCR, reverse transcriptase-PCR; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
Wako Pure Chemical Industries, Ltd., and Takeda Pharmaceutical Co., Ltd., respectively. For \textit{in vivo} experiments, TAC-101 was suspended in 0.5% hydroxy-propoxy methyl cellulose. 5-FU was dissolved in physiological saline. For \textit{in vitro} experiments, TAC-101 and 5-FU were dissolved in DMSO at a concentration of 20 mM for the stock solutions and kept at $-20^\circ\text{C}$ until use.

**Cell Lines.** L5 murine colon cancer cells (18) and murine HSE cells (19) were maintained as monolayer cultures in RPMI 1640 supplemented with 5% FCS and DMEM/F-12 supplemented with 5% FCS and bovine EM (Biomedical Technologies, Inc.), respectively. Human colon adenocarcinoma Co-3 was supplied by the Central Institute for Experimental Animals (Tokyo, Japan) and maintained by serial s.c. transplantation of cubic fragments ($\sim 2 \times 2 \times 2$ mm) in the right subaxillary region of athymic nude mice. L5 was also maintained by serial s.c. transplantation for \textit{in vivo} use.

**Mice.** Specific pathogen-free female BALB/c mice and male KSN mice (6 weeks old) were purchased from Japan SLC, Inc. The mice were handled and housed according to institutional guidelines in a protected environment and maintained in a 12-h lighting cycle at a temperature of 22–25$^\circ\text{C}$. Following tumor inoculation in acclimatized animals, 10 animals were assigned to the control group, and 7 animals were assigned to each treatment group. In all experiments, the day of implantation was regarded as day 0.

**Assay for \textit{in Vitro} Proliferation of HSE and L5 Cells.** Trypsinized HSE cells were resuspended in DMEM/F-12 supplemented with 5% FCS and EM and seeded into 96-well plates (2 $\times$ $10^5$/well) precoated with attachment factor (Cell Systems, Inc.). L5 cells were resuspended in RPMI 1640 supplemented with 5% FCS and seeded into the plates (2 $\times$ $10^5$/well). After 24 h preincubation, various concentrations of TAC-101 and 5-FU were added to the cultures, and cultures were incubated for 72 h. Crystal violet staining was performed to evaluate the activity, as described previously (20).

**Preparation of CM-L5.** L5 cells (5 $\times$ $10^5$/well) were seeded into 24-well plates and incubated for 48 h. The medium was replaced with fresh RPMI 1640 with 5% FCS in the absence or presence of TAC-101. After 24 h of incubation, the medium was harvested and filtered through a 0.2-$\mu$m pore membrane.

**Assay for \textit{in Vitro} Proliferation of HSE Cells Induced by CM-L5 or by Coculture with L5 Cells.** Proliferation of HSE cells was examined by a previously described method with some modifications (21). HSE cells (1 $\times$ $10^5$/well) were seeded into attachment factor-coated 96-well plates. After 24 h of preincubation, the medium was replaced with fresh medium containing various concentrations of CM-L5. The culture were incubated with or without anti-VEGF antibody (R&D Systems, Inc.) for 72 h, and crystal violet staining was performed as described above.

**Assay for \textit{in Vitro} Tube Formation by HSE Cells Induced by CM-L5.** HSE cells (2 $\times$ $10^3$/well) were incubated for 3 h on 48-well plates coated with Matrigel (Collaborative Biochemical Products, Inc.) with 50% CM-L5, which was prepared in the absence or presence of TAC-101 (1, 3, or 10 $\mu$m), as described previously (22). The cultures were fixed with 2.5% glutaraldehyde and stained with hematoxylin to observe morphological changes.

**In Vivo Angiogenesis Assay.** A fragment of L5 tumor ($\sim 1$ mm$^3$) was implanted in the left lobe of the liver of BALB/c mice to form a single tumor nodule in the liver as described previously (10). TAC-101 was administered daily p.o. for 7 days daily, initiated on day 1. Vehicle (0.5% HPMC) was administered p.o. to the control group. The tumors in both groups ($n = 3$) were collected on day 8, weighed, and used to prepare frozen sections. Intratumoral vasculature was detected by using anti-CD31 antibody (Pharminogen), as described previously (23). The angiogenesis index was determined by visually estimating the CD31-positive area in three fields of triplicate samples and represented as T/C (%).

**In Vivo Antitumor Activity against Intrahepatic Growth of L5 and Co-3 Tumors.** A 1-mm$^3$ fragment of L5 or Co-3 tumor was implanted in the left lobe of the liver of BALB/c or KSN mice. In the experiments using L5 tumors, animals were allocated to groups on day 1. TAC-101 (optimal dose of 8 mg/kg/day; Ref. 10) was administered p.o. for 15 days daily, beginning on day 1. 5-FU (20 mg/kg/day; Ref. 24), alone or in combination with LV (maximal dose of 30 mg/kg/day; 5FU/LV) was administered i.v. for 5 days, beginning on day 1. For treatment with 5-FU/LV, LV was given i.v. 1 h prior to 5-FU injection. In the group concurrently administered both TAC-101 and 5-FU/LV, TAC-101 was given p.o. $\sim 3$ h prior to 5-FU/LV. The vehicle for TAC-101 was given p.o. for 15 days to the control group.

In the experiments using Co-3 tumors, mice were allocated to groups on day 7 when tumor nodules were macroscopically observed. 5-FU (25 mg/kg/day; maximum tolerated dose for KSN mice) and LV (30 mg/kg/day) were administered i.v. with the same timing as in the L5 tumor experiment, beginning on day 7. TAC-101 (8 mg/kg/day) was administered p.o. for 21 days on two different schedules initiated on days 7 and 14. The mice implanted with L5 and Co-3 tumors were sacrificed on days 16 and 35, respectively. The tumor in the liver was removed and weighed. After evaluation of antitumor activity, total RNA was isolated from the L5 tumor in vehicle- or TAC-101-treated animals for RT-PCR.

**Detection of VEGF Transcription by RT-PCR.** Isolated total RNA was reverse-transcribed to form cDNA, as described previously (11). The PCR amplification was performed by denaturation at 94°C for 30 s, annealing at 60°C for 1 min, and extension at 72°C for 1 min and 30 s, using template cDNA and a Takara Ex Taq PCR kit (Takara Shuzo Co., Ltd.). The sequences of the VEGF primers were follows: 5’-GGCTGGTGTAACGATGAAGC-3’ (VEGF sense) and 5’-TTAACACGCTGCTCGC-3’ (VEGF anti-sense). The sequences of the GAPDH primers were as described previously (25). The PCR products were electrophoresed on 1.5% agarose gels and detected by ethidium bromide staining.
Results

In Vitro Antiproliferative Activity of TAC-101 against HSE and L5 Cells. Incubation with 10 μM TAC-101 for 72 h inhibited proliferation of HSE and L5 cells by 22.5 and 42.7%, respectively. In contrast, 5-FU inhibited proliferation of HSE and L5 cells by 86.9 and 93.1%, respectively (Fig. 1). EM is generally required for the proliferation of vascular endothelial cells. However, incubation with CM-L5 induced the proliferation of HSE cell without EM in a concentration-dependent manner (Fig. 2A). The enhancement of HSE cell proliferation by CM-L5 was inhibited by anti-VEGF antibody (Fig. 2B).

Effect of TAC-101 on Tube Formation by HSE Cells Induced by CM-L5 in Vitro. The incubation of HSE cells with 50% CM-L5 in Matrigel-coated wells caused tube-like formations by HSE cells within 3 h. Addition of CM-obtained L5 cells cultured with various concentrations of TAC-101 inhibited the tube formation in a concentration-dependent manner (Fig. 3). The incubation of HSE cells cocultured with L5 cells cultured with various concentrations of TAC-101 in-
of mice that had received p.o. administration of vehicle or TAC-101 for 15 days. The expression of VEGF mRNA was markedly reduced in TAC-101-treated tumors, as compared with vehicle-treated tumors (Fig. 7).

**Effect of TAC-101 and Combined Treatment with 5-FU/LV on the Intrahepatic Growth of L5 and Co-3 Colon Cancer.** The mean weight of the L5 tumors was \( \sim 1.5 \text{ g} \) (1.48 ± 0.72 g) on day 16 after implantation (Fig. 8). p.o. administration of TAC-101 for 15 days significantly inhibited tumor growth (0.49 ± 0.33 g; \( P = 0.002 \)), whereas i.v. administration of 5-FU did not cause significant inhibition (0.98 ± 0.41 g; \( P = 0.088 \)). Combined treatment of 5-FU with LV (5-FU/LV) showed significant antitumor activity (0.38 ± 0.42 g; \( P = 0.001 \)). Furthermore, the combination of TAC-101 and 5-FU/LV markedly reduced the tumor weight (0.07 ± 0.11 g; \( P < 0.001 \)). No significant decrease in body weight was observed in this combined treatment group (data not shown).

We also investigated the effect of TAC-101 against intrahepatic growth of Co-3 human colon adenocarcinoma. As shown in Fig. 9, the weight of the Co-3 tumor in the control group reached \( \sim 2.6 \text{ g} (2.58 \pm 0.38 \text{ g}) \) on day 35. Treatment with TAC-101 for 21 days initiated on days 7 and 14 inhibited tumor growth (1.43 ± 0.37 g; \( P < 0.001 \); and 1.24 ± 0.23 g; \( P < 0.001 \), respectively). Treatment with 5-FU/LV for 5 days initiated on day 7 also reduced tumor weight (1.70 ± 0.40 g; \( P = 0.006 \)). Concurrent treatment with TAC-101 (days 7–27) and 5-FU/LV (days 7–11) significantly enhanced antitumor activity, as compared with either treatment alone (0.85 ± 0.38 g; overall \( P = 0.007 \)). Furthermore, sequential administration of 5-FU/LV (on days 7–11) and TAC-101 (on days 14–34) also significantly enhanced antitumor effect (0.86 ± 0.33 g; overall \( P = 0.015 \)). Thus, the combination of TAC-101 and 5-FU/LV in both treatment modalities significantly enhanced antitumor activity.

**Fig. 4** Tumor-induced morphological change of HSE cells. HSE cells \((2 \times 10^4/\text{well})\) were seeded into Matrigel-coated 48-well plates in the absence (A) or presence (B–E) of 50% CM-L5. B, control; C, 1 \( \mu \text{M} \) TAC-101; D, 3 \( \mu \text{M} \) TAC-101; and E, 10 \( \mu \text{M} \) TAC-101. After 3 h of incubation, the plates were fixed with glutaraldehyde and stained with hematoxylin. Magnification, \( \times 200 \).

**Fig. 5** Detection of intratumoral angiogenesis. Vehicle and TAC-101 \((8 \text{ mg/kg/day})\) were administered p.o. for 7 days, beginning on day 1 after the intrahepatic implantation of L5 tumor. The mice were sacrificed on day 8, and frozen sections of tumor were prepared on the day after the implantation. To evaluate angiogenesis, we immunohistochemically stained frozen sections of tumor from animals treated with vehicle (A, magnification, \( \times 200 \)). C, magnification, \( \times 400 \)) and TAC-101 (B, magnification, \( \times 200 \); D, magnification, \( \times 400 \)) with anti-CD31 antibody.
Discussion

Our previous studies (10–12) showed that TAC-101 inhibited multiple liver metastases caused by intrasplenic or intraportal vein injection of tumor cell lines such as AZ-521 (human gastric adenocarcinoma), A549 (human lung adenocarcinoma), and colon 26-L5 (metastatic variant of murine colon cancer). TAC-101 prolonged the life span of mice with liver metastasis. In this study, a small fragment of L5 tumor was directly implanted into the left lobe of the liver to form a single tumor nodule in the liver for evaluation of antitumor and antiangiogenic activities. p.o. administration of TAC-101 significantly inhibited tumor angiogenesis and growth on day 8 after intrahepatic implantation of L5 tumor (Figs. 5 and 6). This finding indicates that the inhibition of intrahepatic tumor growth by TAC-101 may be associated with suppression of tumor-induced neovascularization. Neovascularization toward and into tumor is a crucial step for nutrient delivery to tumor and for hematogenous metastasis of tumor cells, and it consists of proliferation, migration, and capillary tube formation of endothelial cells (3).

In vitro experiments revealed that incubation with CM-L5 or coculture with L5 cells induced HSE cell proliferation (Figs. 2 and 3). Addition of anti-VEGF antibody to culture reduced HSE proliferation that was induced by CM-L5. Although 30 μM TAC-101 showed marked antiproliferative activity against cell lines (Fig. 1), a much higher concentration of TAC-101...
compared with 5-FU was required to inhibit cell proliferation directly. TAC-101 inhibited the induction of HSE cell proliferation at the noncytotoxic concentrations of <10 μM (Figs. 1 and 3). RT-PCR analysis using in vivo tumor tissue indicated that p.o. administration of TAC-101 repressed the expression of VEGF mRNA in tumor tissue on day 16 after implantation (Fig. 7). These findings suggest that VEGF derived from L5 tumor is related to the stimulation of endothelial cell proliferation and that the inhibitory effect of TAC-101 is due to suppression of VEGF expression in L5 tumor. VEGF is known to induce capillary tube formation of vascular endothelial cells in vitro as well as cell proliferation (30, 31). As shown in Fig. 4, TAC-101 eliminated the ability of CM-L5 (containing VEGF) to induce tube formation at the noncytotoxic concentrations of 10⁻⁷ M (Figs. 1 and 20). This observation is consistent with previous reports of in vitro inhibition of tube formation by TAC-101. The inhibitory effect of TAC-101 is due to suppression of VEGF expression in L5 tumor. VEGF is known to induce capillary tube formation of vascular endothelial cells in vitro as well as cell proliferation (30, 31). As shown in Fig. 4, TAC-101 eliminated the ability of CM-L5 (containing VEGF) to induce tube formation at the noncytotoxic concentrations of 10⁻⁷ M (Figs. 1 and 20). This observation is consistent with previous reports of in vitro inhibition of tube formation by TAC-101. The inhibitory effect of TAC-101 is due to suppression of VEGF expression in L5 tumor.

Several angiogenesis inhibitors have been shown to inhibit tumor progression in experimental models and are currently being tested in clinical trials. However, antiangiogenic therapy is thought to require long-term treatment with low-toxicity inhibitors and/or combination with other cytotoxic agents to inhibit the progression of metastatic tumors or the recurrence of metastatic disease (9). Because fluorinated pyrimidine-based combination therapy has been used to treat metastasized colorectal cancer (17), we examined the antitumor activity of TAC-101 in combination with 5-FU/LV on our experimental model. Concurrent treatment with TAC-101 and 5-FU/LV significantly inhibited intrahepatic growth of L5 tumors, compared with either treatment alone (Fig. 8). In addition to concurrent administration, sequential administration of 5-FU/LV followed by TAC-101 significantly increased antitumor activity against intrahepatic growth of colon cancer (Fig. 9).

In conclusion, this study demonstrated that TAC-101 inhibited intrahepatic growth of colon cancer through inhibition of angiogenesis via down-regulation of VEGF mRNA. Combined treatment with TAC-101 and 5-FU/LV significantly increased antitumor activity. For the evaluation of the therapeutic potential of TAC-101, prospective clinical studies will be needed.

References


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