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Anti-tumor Effect of N-[3,4-dimethoxycinnamoyl]-anthranilic Acid (tranilast) on Experimental Pancreatic Cancer

Makoto Hiroi, Masahiko Onda, Eiji Uchida and Takayuki Aimoto

First Department of Surgery, Nippon Medical School

Abstract

The anti-tumor effect of N-[3,4-dimethoxycinnamoyl]-anthranilic acid (tranilast) was examined in experimental pancreatic cancer. Proliferation of PGHAM-1 cells was inhibited by tranilast in a dose-dependent manner, showing a significant difference at a concentration of 25 $\mu\text{g}/\text{ml}$ ($p < 0.05$). In colony formation, tranilast reduced the number of colonies at a concentration of 25 $\mu\text{g}/\text{ml}$ ($p < 0.01$). DNA synthesis for 12 hours was attenuated dose-dependently and a significant difference was observed at concentrations of greater than 50 $\mu\text{g}/\text{ml}$ ($p < 0.05$). From cell cycle analysis, a dose-dependent increase in the distribution of G0-G1 phase was observed. In the dorsal air sac model, the mean angiogenesis indices in PGHAM-1 chambers were 4.17 ± 0.22 (control group) and 2.33 ± 0.84 (treatment group), and in VEGF chambers they were 3.60 ± 0.67 (control group) and 1.92 ± 0.42 (treatment group). In the peritoneal dissemination model, the quantity of sanguineous ascites, the number and the size of diaphragmatic nodules and the microvessel density (MVD) of the metastatic site were reduced by tranilast significantly. In conclusion, the anti-tumor effect of tranilast on proliferation and on tumor-angiogenesis was confirmed in experimental pancreatic cancer.

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Key words: tranilast, hamster pancreatic cancer, proliferation, angiogenesis

Introduction

Ductal pancreatic adenocarcinoma, a lethal disease with an increasing incidence worldwide, carries mortality close to 100%¹. Chance for surgical resection is provided only for a limited numbers of cases with proof of tumor localization and resectability without any distinct metastasis at diagnosis^{1,2}. However, prognosis after curative resection is quite poor because of local recurrence^{3,4}, liver metastasis³ and peritoneal dissemination⁵, which arise in a relatively short time after operation, and consequently a 5-year survival rate after curative resection is achieved in only 10%

–25% of cases^{6–8}. Current therapeutic approaches, including extended surgical resection, chemotherapy and irradiation⁹, failed to prolong the survival rate significantly. In these circumstances, a new therapeutic agent, which has a possibility to induce a dormant state of tumor progress without side effects, is longed for to improve the prognosis of this disease.

Tranilast is an orally active anti-allergic compound for the systemic and topical treatment of bronchial asthma, atopic dermatitis and allergic conjunctivitis¹⁰. The clinical effectiveness in these allergic diseases is brought about by its inhibitory effect on the release of chemical mediators from mast cells^{11,12}. Tranilast is also effective in diseases with excessive fibrosis,

Correspondence to Makoto Hiroi, First Department of Surgery, Nippon Medical School, 1-1-5 Sendagi, Bunkyo-ku, Tokyo 113-8603, Japan

E-mail: hiroi-surg 1@nms.ac.jp

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such as keloid scars^{13,14} and restenosis after percutaneous transluminal coronary angioplasty (PTCA)¹⁵, suppressing the proliferation of fibroblasts in the former and smooth muscle cells¹⁶ in the latter. As for angiogenesis¹⁷, tranilast inhibits proliferation, VEGF-induced chemotaxis and tube formation of vascular endothelial cells *in vitro* and consequently suppresses VEGF-induced angiogenesis *in vivo*¹⁰. In addition, anti-tumor effects on malignant glioma cells were introduced for the first time referring to the effectiveness of tranilast alone on neoplasms¹⁸.

PGHAM-1, a hamster pancreatic cancer cell line which we established, was derived from BOP (N-nitrosobis(2-oxopropyl) amine) – induced hamster pancreatic cancer cells^{20,21}. It is quite similar to human pancreatic cancer biologically and histologically¹⁹, showing a high malignant potential of metastasis, invasion and dissemination as we previously reported^{19,20}. Recently, we reported the effectiveness of angiostatin, a potent anti-angiogenic substance, on experimental pancreatic cancer²¹. In this study, the anti-tumor effect of tranilast on experimental peritoneal dissemination model was examined.

Materials and Methods

Animals

Five-week old female Syrian golden hamsters (Shizuoka Laboratory Animal Center, Shizuoka, Japan) were used in this study. The animals were housed in groups of 5 under standard conditions (temperature; $20 \pm 5^\circ\text{C}$, humidity; $40\% \pm 10\%$, light/dark cycle; 12 h/12 h) and given food (MF-1, Oriental Yeast Co., Tokyo, Japan) and water. Protocols for these animal experiments were discussed and approved by the Animal Experiment Committee of Nippon Medical School.

Cell culture

PGHAM-1 cells were maintained in Dulbecco's modified Eagle medium (DMEM; Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (Cytosystems FBS; IBL, Maebashi, Japan), penicillin-streptomycin (100 unit/ml; Gibco), kanamycin (100 $\mu\text{g}/\text{ml}$; ICN) and amphotericin B (100 $\mu\text{g}/\text{ml}$; Gibco) at 37°C in 75 cm² tissue culture flask (Corning Glass Works, Corning, NY) in a humidified incubator gassed with 5% CO₂. The cells, grown to approximately

80% confluency after 3 to 5 culture-passages, were used in experiments.

Reagent

N- [3,4-dimethoxycinnamoyl] -anthranilic acid (tranilast) (MW 327.24) was kindly provided by Kissei Pharmaceutical Co., Ltd. (Nagano, Japan) and solubilized with 1% NaHCO₃ solution at 70°C and diluted with assay buffer to final concentrations. In *vivo* experiments, when the dose of tranilast was far beyond its solubility, it was administered by suspension in 1% NaHCO₃¹⁰.

Proliferation, colony formation and cytotoxicity

PGHAM-1 cells (2×10^4 cells in 800 μl DMEM per well) were seeded on Collagen type-I coated 6-well multi plates (Iwaki Glass Co. Ltd., Tokyo, Japan) at 0 hour. Then the medium was aspirated and replaced with the fresh medium containing various concentrations of tranilast (final concentrations: 0, 12.5, 25, 50 and 100 $\mu\text{g}/\text{ml}$ per plate) at 24 hours. After aspiration of the medium, cells were harvested by adding 0.25% trypsin containing 0.02% EDTA (Gibco) and the number of cells in each well was counted with a hemacytometer at 48 hours and at 72 hours. Mean and SEM of 6 wells was obtained to make a growth curve under various concentrations of tranilast. Colony formation was observed under $40 \times$ magnification after fixing cells with methanol for 2 min. and then staining with thiazine and eosin using DiffQuick (Dade Behring, Duedingen, Switzerland) 48 hours after exposure to tranilast. A cell-mass which consists of more than 50 cells was regarded as a colony, and the number of colonies was counted as a colony index. Cytotoxicity was assessed after 48 hours' duration of exposure to tranilast by measuring lactate dehydrogenase (LDH) activity of the culture supernatant (A) and the total LDH activity after cell-lysis by 0.2% Tween 20 (Sigma Chemical Co. St. Louis, MO, USA) (B)¹⁰. As a positive control of cytotoxic agent, 500 ng of cisplatin was also applied to this experiment. LDH release due to cell injury caused by reagents was defined as $A/(A+B) \%$. To measure LDH activity by ELISA, a Wako LDH C-IItest kit (Wako Pure Chemical Industries, Ltd, Osaka, Japan) was used.

DNA synthesis and cell cycle analysis

PGHAM-1 cells (5×10^3 cells in 100 μ l DMEM per well) were seeded on 96-well multi plates at 0 hour, and the medium was aspirated and replaced with the fresh medium containing various concentrations of tranilast (final concentrations: 0, 25, 50, 100 and 200 μ g/ml) at 24 hours. Then [Methyl- 3 H] thymidine (1 μ Ci) (PerkinElmer Life Sciences, Inc., Boston) was added to each well at 36 hours. After 12 hours' duration of co-incubation with [Methyl- 3 H] thymidine, cells were harvested on glass fiber filters at 48 hours for liquid scintillation counting.

For cell cycle analysis, PGHAM-1 cells (1×10^6) were seeded in 75 cm² culture flask at 0 hour, exposed to various concentrations of tranilast (final concentrations: 0, 25, 50, 100 and 200 μ g/ml) at 24 hours and prepared for analysis on FACs-caliber flow cytometer at 72 hours using Cell Quest acquisition and analysis software (Becton Dickinson, Heidelberg, Germany). PGHAM-1 cells were treated using Cycle TESTTMPLUS (Becton Dickinson, Heidelberg, Germany) as manufacturers instructions as follows: the surface of the culture was washed gently with PBS, then cells were harvested after incubation with 0.25% trypsin for 2 min at 37°C. The number of cells were readjusted to 1×10^6 per one sample after washing and centrifuging at 70 G in PBS 3 times. Then trypsin inhibitor was added to the samples and they were incubated for 10 min at room temperature. Further RNase-A was added, and they were incubated for 10min at room temperature. Finally the isolated nucleus was fixed with 70% ice-cold ethanol and was stained with propidium iodide. The samples were kept in ice for 30 min before FACS-caliber flow cytometer analysis.

Angiogenesis in dorsal air sac (DAS) model

The DAS assay, a method described by Oikawa *et al*²², was performed on hamsters to examine the angiogenic response triggered by VEGF and the effect of tranilast on tumor angiogenesis *in vivo*. Briefly, a rubber column chamber, both sides of which were covered with Millipore filters of 0.4- μ m pore size (ISOPORETM MEMBRANE FILTERS, Millipore Corporation, Bedford, MA, USA), was made to contain PGHAM-1 cell suspension (10^6 cells in 150 μ l of PBS) or VEGF (Recombinant Human VEGF,

Genzyme-Techne, USA) (30 ng/ml) solution or only 150 μ l of PBS as negative control. Dorsal air sac was performed by injecting a 10 ml volume of air subcutaneously in the dorsum of hamster under general anesthesia. Then the chamber was implanted into the sac of the dorsum. The animals were divided into a treatment group (n=5), a control group (n=5) and a negative control group (n=5). The animals in the treatment group were administered 400 mg/kg of tranilast *per os* from day 0 to day 4. On day 5, the implanted chambers were removed and the angiogenic response was assessed by the number of newly formed blood vessels of > 3 mm in length. The extent of angiogenesis was scored as an index of 0, 1, 2, 3, 4, or 5, indicating the numbers of newly formed blood vessels (just index 5 includes 5 or more vessels). The blood vessels newly formed by an angiogenic substance were morphologically distinct from the preexisting background vessels by the zigzagging characteristics, as described previously²² and as shown in Figure 6b.

Estimation of LD₅₀ tranilast (L. P.) Hamster

To determine the adequate dose by intra peritoneal injection, estimation of LD₅₀ in the hamsters was essential. Various doses of tranilast in the volume of 1 ml suspension were administered by intra peritoneal injection as follows, Group 1 (n=5); 200 mg/kg, Group 2 (n=5); 600 mg/kg, Group 3 (n=5); 1,000 mg/kg and Group 4 (n=5); 1,400 mg/kg. The number of acute deaths in one week after administration of tranilast was counted and 50% of the lethal dose (LD₅₀) in hamsters was estimated by the Behrens-Kärber method, which equation is given as $LD_{50} = LD_{100} - (\Sigma zd) / m$ (z: the half numeric of the summation of the number of dead animals in the two continuous doses, d: differential between the two continuous doses, m: the number of animals in one group).

Peritoneal dissemination model

A PGHAM-1 cell suspension (1×10^6 cells in 100 μ l PBS) was inoculated into the peritoneal cavity under general anesthesia by ether inhalation on day 0. The treatment group (n=15) was administered tranilast (400 mg/kg/1 ml of 1%NaHCO₃) by intra peritoneal injection every other day from day 1 to day 19. The control group (n=15) was administered 1 ml

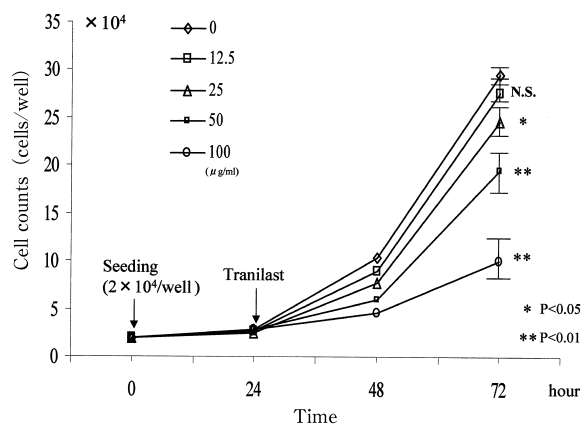


Fig. 1 Effect of tranilast on proliferation
Proliferation of PGHAM-1 cells was inhibited by tranilast significantly at a concentration of 25 $\mu\text{g/ml}$ and at concentrations greater than 50 $\mu\text{g/ml}$ [control: $(29.5 \pm 2.96) \times 10^4$ cells/well, tranilast 25 $\mu\text{g/ml}$: $(24.5 \pm 4.75) \times 10^4$ cells/well ($p < 0.05$), tranilast 50 $\mu\text{g/ml}$: $(19.4 \pm 4.51) \times 10^4$ cells/well ($p < 0.01$)].

of 1% NaHCO_3 in the same way. All animals were sacrificed on day 21 and all the tumors were served for routine histological examination. Treatment effectiveness was evaluated as follows: (1) The incidence and the quantity of sanguineous ascites; (2) The incidence, the number and the size of disseminated nodules in the omentum, mesenterium and diaphragm; and (3) Micro vessel density (MVD) of metastatic site.

Microvessel density (MVD)

Paraffin embedded sections of disseminated tumors were stained for factor VIII related antigen (von Willebrand factor) by the avidin-biotin-peroxidase complex (ABC)-immunoperoxidase method as described previously²³. Rabbit polyclonal antibody against factor VIII-related antigen (Dako Japan Co. Kyoto, Japan) and ABC kit (Nichirei Co. Tokyo, Japan) were used to detect vascular endothelial cells of micro vessels induced by tumor-angiogenesis. The area of highest microvascular density of the tumor was focused, and the highest number of microvessels at $200\times$ magnification in a single field (0.78 mm^2 per field) was counted and MVD was obtained as the number of microvessels in 1 mm^2 field of section. The MVD of each section was assessed independently 3 times, and the average of the 3 counts was taken as the MVD of each mass.

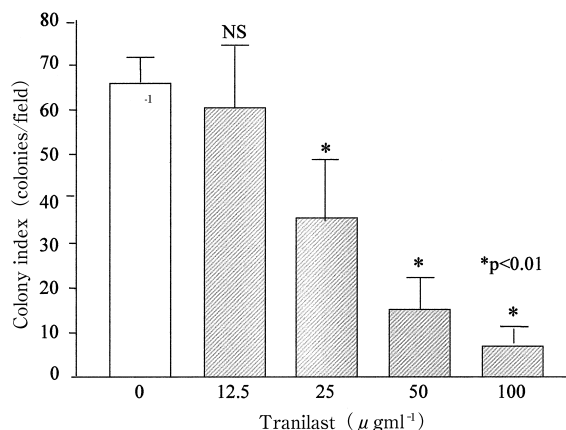


Fig. 2 Effect of tranilast on colony formation
Colony formation was more sensitively inhibited by tranilast than proliferation at the same minimal effective concentration of 25 $\mu\text{g/ml}$ with a significant difference [control: 65.4 ± 7.70 colonies/field, tranilast 25 $\mu\text{g/ml}$: 35.2 ± 9.62 colonies/field ($p < 0.01$)].

Statistical analysis

The significance of the observed effects was evaluated by t-test or ANOVA at $p < 0.05$.

Results

Effect of tranilast on proliferation of PGHAM-1 cells

In the 48 hours duration of exposure to tranilast, proliferation of PGHAM-1 cells was inhibited in a concentration-dependent manner as shown in **Fig. 1**. A significant difference in proliferation was observed at a concentration of 25 $\mu\text{g/ml}$ ($p < 0.05$) and at concentrations greater than 50 $\mu\text{g/ml}$ ($p < 0.01$). In colony formation, tranilast was powerful enough even at concentration of 25 $\mu\text{g/ml}$ to reduce the number of colonies with a significant difference ($p < 0.01$) as shown in **Fig. 2**. No apparent cytotoxicity to PGHAM-1 cells was found in cytotoxic assay by LDH release even at 100 $\mu\text{g/ml}$ (LDH release; $13.5 \pm 2.8\%$) compared to the control (LDH release; $12.1\% \pm 2.8\%$) as shown in **Fig. 3**. Thus, the inhibitory effect without cytotoxicity of tranilast on proliferation and colony formation of PGHAM-1 cells was ascertained.

Effect of tranilast on DNA synthesis and cell cycle in PGHAM-1 cells

PGHAM-1 cells were exposed to tranilast for 24 hours (from 24 hours to 48 hours after seeding) and [$\text{Methyl-}^3\text{H}$] thymidine was pulsed with PGHAM-1

cells during the final 12 hours (from 36 hours to 48 hours after seeding) before liquid scintillation counting. The quantity of incorporated [Methyl-³H] thymidine (cpm) was significantly attenuated by tranilast at concentrations greater than 50 μg/ml (p<0.05) and 200μg/ml (p<0.01) as shown in **Fig. 4**. From cell cycle analysis by using FACs-caliber system, increases in distribution of cells in G0-G1 phase and decreases in G2-M and total S phases were observed in dose-dependent manners as shown in **Fig. 5**. The distribution of cells staying at G0-G1 phase was

80.31%, 93.33% and 96.03%, in the control, 100μg/ml and 200μg/ml respectively. At concentrations greater than 100 μg/ml, there were no cells in G2-M phase.

Angiogenesis in DAS model

Newly formed blood vessels, showing the zigzagging characteristics, were induced by PGHAM-1 and VEGF (**Fig. 6 b**). The implantation of the chambers

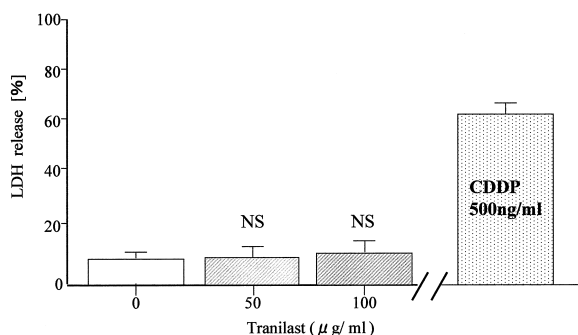


Fig. 3 Cytotoxicity of tranilast to PGHAM-1 cells
No apparent cytotoxicity to PGHAM-1 cells was observed in cytotoxic assay measured by LDH release even at a concentration of 100 μg/ml of tranilast (LDH release; 13.5 ± 2.8%) compared to the control (LDH release; 12.1 ± 2.8%).

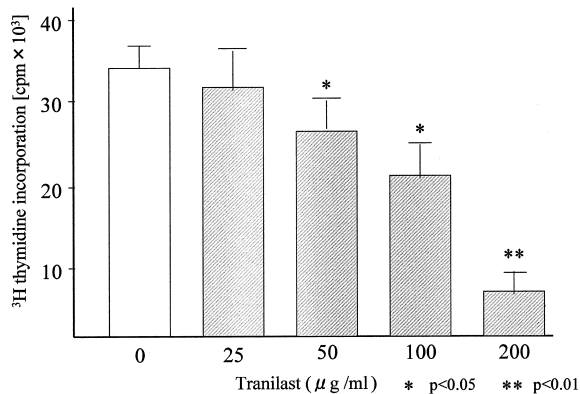


Fig. 4 Effect of tranilast on DNA synthesis of PGHAM-1 cells

[Methyl-³H] thymidine incorporation to PGHAM-1 cells for 12 hours, measured by liquid scintillation counting, was attenuated significantly by tranilast at concentrations greater than 50μg/ml [control:34.2 × 10³cpm, tranilast 50 μg/ml : 27.0 × 10³cpm (p<0.05)].

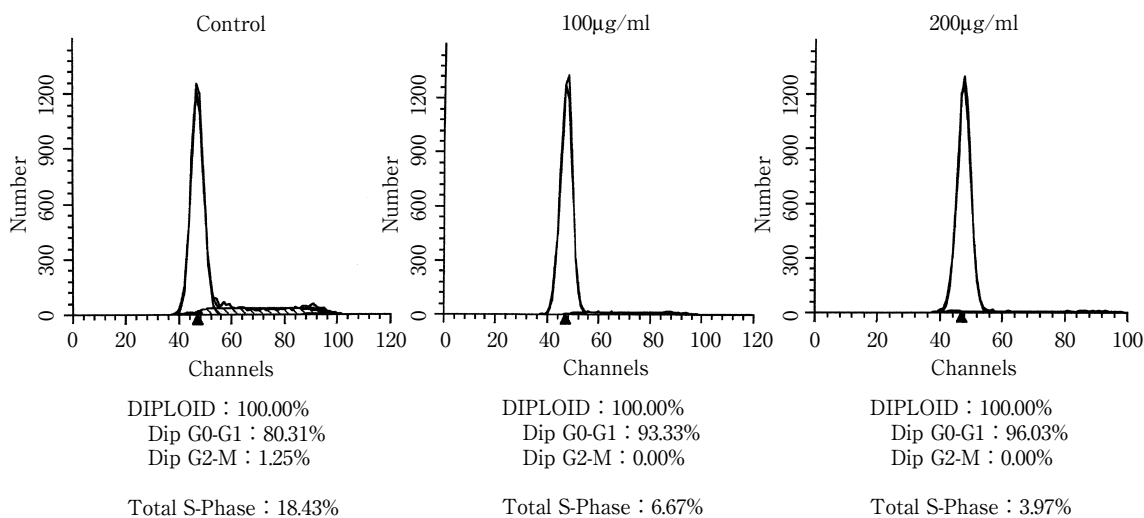


Fig. 5 Effect of tranilast on cell cycle of PGHAM-1 cells

Cell cycle under various concentrations of tranilast was analyzed by FACs-caliber system. The distribution of cells staying at G0-G1 phase increased in a concentration-dependent manner, 80.31%, 93.33% and 96.03% as in control, 100 μg/ml and 200 μg/ml respectively. No cells in G2-M phase were observed at concentrations greater than 100 μg/ml.

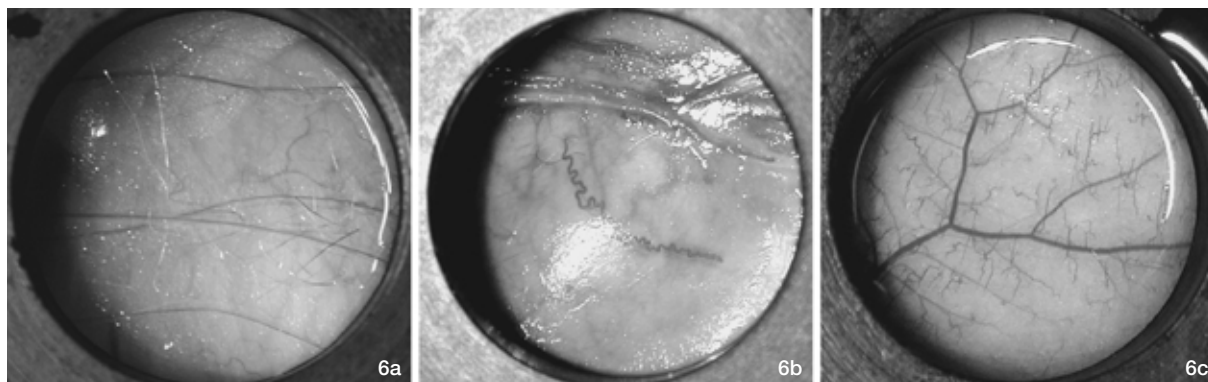


Fig. 6 6a, 6b, 6c Dorsal air sac (DAS) model

6a: Negative control (PBS)
 6b: Blood vessels newly formed by PGHAM-1 or VEGF show the zigzagging characteristics
 6c: Attenuated zigzagging vessels (PGHAM-1 or VEGF chamber) in the treatment group

Table 1 Effect of tranilast on tumor-induced and VEGF-induced angiogenesis in the dorsal air sac model

The number of newly formed blood vessels by PGHAM-1 cells and VEGF were significantly less in the treatment group [PGHAM-1: 4.17 ± 0.22 (control), 2.33 ± 0.84 (treatment) ($p < 0.05$) VEGF: 3.60 ± 0.67 (control), 1.92 ± 0.42 (treatment) ($p < 0.05$)].

	Control (n=5)	Treatment group (n=5)
PGHAM-1	4.17 ± 0.22	2.33 ± 0.84 *

* ($p < 0.05$) vs. control

	Control (n=5)	Treatment group (n=5)
VEGF	3.60 ± 0.67	1.92 ± 0.42 *

* ($p < 0.05$) vs. control

containing only PBS (negative control) (Fig. 6 a) was associated with a minimal angiogenesis (angiogenesis index; 0.67), indicating that the experimental manipulation and healing process did not induce a significant angiogenic response. Zigzagging vessels were attenuated in the treatment group (Fig. 6 c) The mean angiogenesis indices in the chamber containing PGHAM-1 were 4.17 ± 0.22 in the control group and 2.33 ± 0.84 in the treatment group ($p < 0.05$). In the VEGF chamber, the angiogenesis indices were 3.60 ± 0.67 in the control group and 1.92 ± 0.42 in the treatment group ($p < 0.05$), as shown in Table 1.

Estimation of LD₅₀ tranilast_{(I.P.)Hamster}

There were no acute deaths one week after administration of tranilast by intra-peritoneal injection

Table 2 Estimation of LD50 IP in hamster

Dose (mg/kg)	Death rate	z	d
200	0/5		
600	0/5	0	400
1,000	3/5	1.5	400
1,400	5/5	4	400

$$LD_{50(IP)} = 1,400 - \{(400 \times 0) + (400 \times 1.5) + (400 \times 4)\} / 5 = 960 \text{mg/kg}$$

z: the half neumeric of the summation of the number of dead animals in the two continuous dose
 d: differential between the two continuous dose
 m: the number of animals in one group

at doses of 200 mg/kg and 600 mg/kg. Three deaths in 5 animals were observed at a dose of 1,000 mg/kg, and all 5 died at a dose of 1,400 mg/kg, as shown in Table 2. Thus, we estimated LD₅₀ tranilast_{(I.P.)Hamster} as 960 mg/kg from the Behrens-Kärber method and the dose of tranilast by intra-peritoneal injection in one shot was determined as 400 mg/kg, that is half of a summation of continuous two doses, 200 mg/kg and 600 mg/kg, which yielded no acute deaths in this experiment.

Effects of tranilast in peritoneal dissemination model

As shown in Table 3, the effects of tranilast in the peritoneal dissemination model were observed in the categories as follows:

(1) The incidence and the quantity of sanguineous ascites

The incidence of sanguineous ascites was 100% in both the treatment group and in the control group.

Table 3 Effects of tranilast in peritoneal dissemination

Quantity of sanguineous ascites*, number** and size* of diaphragmatic nodules and microvessel density (MVD) of metastatic site* were significantly less in the treatment group than in the control (* $p < 0.05$, ** $p < 0.01$).

	Control (n = 15)	Treatment group (n = 15)
Sanguineous ascites		
Incidence	100 % (15/15)	100 % (15/15)
Quantity (ml)*	7.89 ± 4.36	5.00 ± 3.35
Omental nodule		
Incidence	100 % (15/15)	100 % (15/15)
Number of nodules	Mass formation	Mass formation
Mesenterial nodule		
Incidence	93.3% (14/15)	73.3% (11/15)
Number of nodules	10.0 ± 9.4	6.9 ± 3.2
Size (mm)	2.8 ± 1.2	2.4 ± 1.0
Diaphragmatic nodule		
Incidence	73.3% (11/15)	66.6% (10/15)
Number of nodules**	12.3 ± 10.4	4.5 ± 4.2
Size (mm)*	2.6 ± 1.4	1.5 ± 0.6
Microvessel density (MVD)*	48.3 ± 24.6	33.4 ± 17.6

* $p < 0.05$ ** $p < 0.01$

However, the quantity of ascites was significantly less in the treatment group (5.00 ± 3.35 ml) than in the control group (7.89 ± 4.36 ml) ($p < 0.05$).

(2) The incidence and the number of disseminated nodules in omentum, mesenterium and diaphragm (**Table 3**)

Omental dissemination (**Fig. 7a**) was observed as a mass commonly in all hamsters of the two groups. Mesenterial dissemination (**Fig. 7b**) was observed as metastatic nodules in mesenterium. The incidence, the number and the average size of nodules were 93.3% (14/15), 10.0 ± 9.4, 2.8 ± 1.2 mm in the control group and 73.3% (11/15), 6.9 ± 3.2, and 2.4 ± 1.0 mm in the treatment group. There were no significant differences between the two groups. Diaphragmatic dissemination (**Fig. 7c**) was observed as metastatic nodules on the diaphragms and histologically proven as well-differentiated adenocarcinoma (**Fig. 8**). The incidences of diaphragmatic dissemination in the control and the treatment groups were 73.3% (11/15) and 66.6% (10/15), respectively. Significant differences in the number of nodules (control 12.3 ± 10.4 vs. treatment 4.5 ± 4.2) and the average size (control 2.6 ± 1.4 mm vs. treatment 1.5 ± 0.6 mm) were induced ($p < 0.05$).

(3) Microvessel density (MVD)

MVD of metastatic site (**Table 3**) was significantly

lower in the treatment group (33.4 ± 17.6/mm², **Fig. 9b**) than in the control group (48.3 ± 24.6/mm², **Fig. 9a**) ($p < 0.05$).

Discussion

Tranilast is an orally active anti-allergic compound that is clinically effective in the control of IgE-mediated diseases such as bronchial asthma and atopic dermatitis by inhibiting the release of various chemical mediators from mast cells¹¹. Tranilast also has been used for the treatment of hypertrophic scars and keloids, because it suppresses collagen deposition by its inhibition of fibroblast proliferation. Recently, it was reported that tranilast inhibits the vascular permeability elevation and angiogenesis induced by vascular endothelial growth factor (VEGF)¹⁰. As VEGF is closely related to tumor growth and angiogenesis, tranilast may have a benefit for the treatment of solid tumors. Actually, human malignant glioma cells were successfully treated by tranilast¹⁸.

The inhibitory effect of tranilast on proliferation was confirmed in various kinds of cells. In non-neoplastic cells, tranilast suppresses proliferation of fibroblasts (by inhibiting the release of TGFβ, interleukin (IL)-1 β, prostaglandin (PG) E₂ and IL-2 from human

monocytes and macrophages), smooth muscle cells, human dermal microvascular endothelial cells (HDMECs)¹⁰, vascular smooth muscle cells²⁴ and coronary arterial smooth muscle cells²⁵. In neoplasms, tranilast inhibits proliferation, migration and invasiveness of malignant glioma cells by reduction of the release of TGF- β ¹⁸.

In the present study, tranilast inhibited the proliferation of PGHAM-1 cells significantly at a concentration of 25 μ g/ml both 24 hours and 48 hours

after exposure. Colony formation, which is a basic and essential cell biological action in keeping stable proliferation, is more sensitively suppressed than proliferation with significant difference ($p < 0.01$) at the same minimum effective concentration of 25 μ g/ml. No apparent cytotoxicity compared to CDDP (500 ng/ml) was detected in LDH release assay even at the maximum dose (100 μ g/ml) in the series, which means the effect of tranilast solely depends on its character of tumor inhibition. DNA synthesis

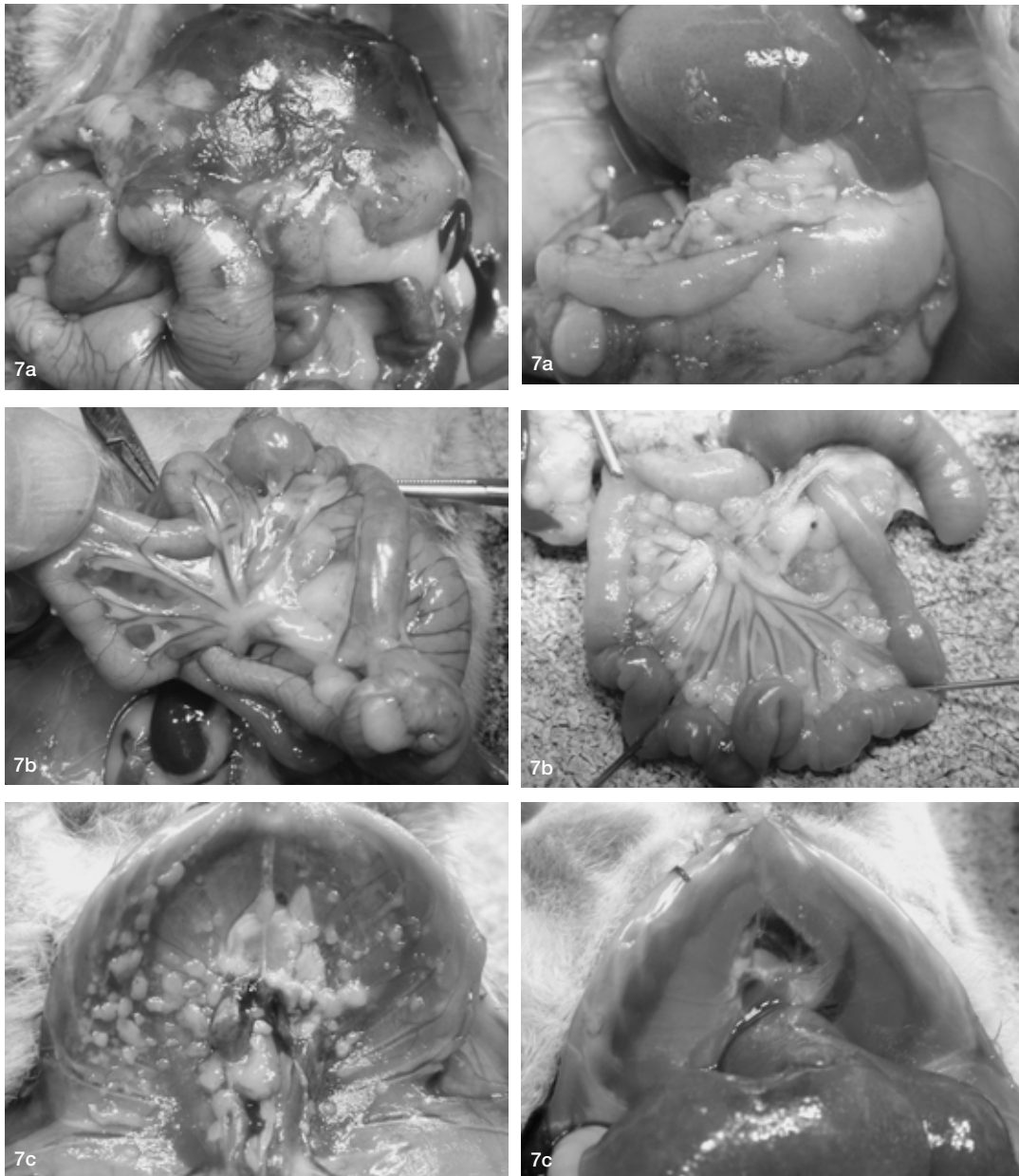


Fig. 7a, 7b, 7c Macroscopic findings of peritoneal dissemination

7a: Omental disseminated mass in the control group (left) and the treatment group (right).

7b: Mesenteric nodules in the control group (left) and the treatment group (right).

7c: Diaphragmatic nodules in the control group (left) and the treatment group (right).

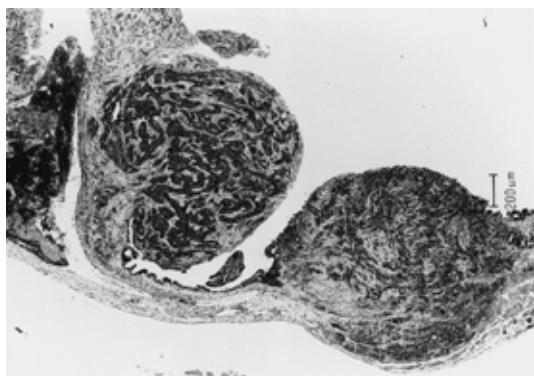


Fig. 8 Photomicrograph of diaphragmatic disseminated nodules

Diaphragmatic dissemination was observed as metastatic nodules on the diaphragm and histologically proven as well-differentiated adenocarcinoma (HE staining, 40×).

measured by incorporated [Methyl-³H] thymidine in the duration of 12 hours was attenuated by tranilast in dose dependent manner. From cell cycle analysis, increases in the distribution of G0-G1 phase were observed in a dose-dependent manner. These observations confirmed that the inhibitory effect of tranilast on proliferation was caused by G0-G1 slowing in the cell cycle, which consequently results in attenuation of DNA synthesis. Attenuation of DNA synthesis by S-phase slowing with accumulation of p 21 was observed in malignant glioma cells¹⁸. Thus, the inhibitory effect of tranilast alone on PGHAM-1 cells without cytotoxicity was ascertained.

Angiogenesis is a complex biologic process involved in the development and formation of new blood vessels, which is known to play a critical role in tumor growth and also in metastasis²⁶. Such angiogenesis is known to be regulated by a number of growth factors secreted by tumor cells, such as VEGF and transforming growth factor (TGF)²⁷. VEGF is a vascular permeability factor and is now believed to be a potent mitogen specific for endothelial cells. The correlation between the expression of VEGF and either metastasis or a poor prognosis has been reported for various cancers²⁸⁻³⁰. The tumor angiogenesis as reflected by the microvessel density (MVD) has been reported to have possible prognostic implications in a various tumors²⁶. In our study using DAS model, the mean angiogenesis index both in PGHAM-1 chamber and VEGF chamber in the

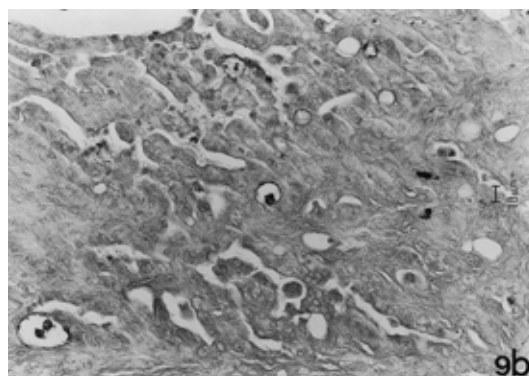
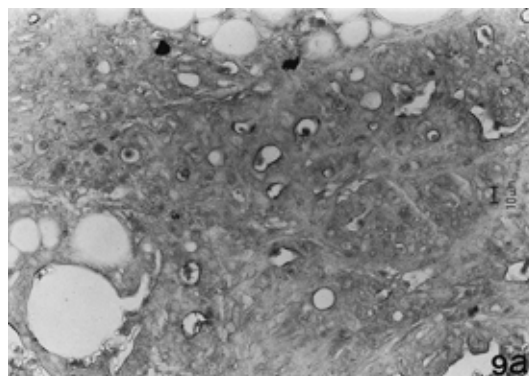


Fig. 9a, 9b Immunohistochemical staining of factor VIII

Vascular endothelial cells were positively stained by anti-factor VIII related antigen antibodies. Microvessels were observed as circular structures, of which the diameter was approximately 10 μm. Microvessel density (MVD) of metastatic site was significantly lower in the treatment group ($33.4 \pm 17.6/\text{mm}^2$, 9 b) than in the control group ($48.3 \pm 24.6/\text{mm}^2$, 9 a) (Methyl green staining, 400×).

tranilast-treatment group was clearly decreased when compared with the control group. These results suggested that PGHAM-1 induced angiogenesis was suppressed by tranilast via the same mechanism in which VEGF was involved.

Patients with ductal pancreatic adenocarcinoma often develop peritoneal dissemination, which is the most unfavorable and fatal state in the clinical progression of this disease. Treatment and prevention of peritoneal dissemination is necessary for improving the prognosis of patients⁵. In recent studies, VEGF-induced angiogenesis²⁸ is also important in producing malignant ascites²⁹⁻³¹ like other factors such as adhesion molecules^{35,36}, and matrixolytic enzymes³⁷, aberrant oncogene/tumor suppressor gene

expression^{38,39} and cytokines/growth factors⁴⁰ in the development of peritoneal dissemination. In this study, sanguineous ascites induced by peritoneal implant were significantly reduced by tranilast. The effect of tranilast was also proven by reduction of numbers and sizes of diaphragmatic nodules with decreasing MVD. TNP-470, a potent anti-angiogenesis substance, was reported to reduce the peritoneal nodules in pancreatic cancer⁴¹. These data suggested that anti-angiogenic drugs can possibly suppress the peritoneal implants of pancreatic cancer.

Many angiogenesis inhibitors were identified, but most of these inhibitors have problems concerning therapeutic applications because of toxicity and/or limited efficacy. Dozens of angiogenesis inhibitors, including TNP-470, thalidomide, and angiostatin, are currently being studied in clinical trials as treatments for solid tumors⁴². For clinical use, the toxicity of any anti-angiogenic drug should be low because of a prolonged treatment period. Thus, attempts have been made to find agents having anti-angiogenic activity among drugs being already clinically used for other purpose. As for tranilast, few side effects, except eosinophilic cystitis⁴³, which is well known and recognized among clinical practitioners, were observed in long-term treatment of patients with hypertrophic scars and keloids, and its safety is well established. Practical use of this drug in the treatment of patients with ductal pancreatic adenocarcinoma would be realized as the supplemental agent in combination with other therapeutic modalities.

In conclusion, we have demonstrated here that tranilast inhibited the proliferation of experimental pancreatic cancer cells without cytotoxicity *in vitro* and suppressed the tumor angiogenesis in peritoneal dissemination model of pancreatic cancer.

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