

Pretreatment with S-1, an Oral Derivative of 5-Fluorouracil, Enhances Gemcitabine Effects in Pancreatic Cancer Xenografts

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Abstract. *Background:* The systemic administration of gemcitabine (GEM) has been accepted as a standard treatment for patients with advanced pancreatic cancer. The major mediator of cellular uptake of GEM is the human equilibrative nucleoside transporter 1 (hENT1) whose expression is up-regulated by thymidylate synthase inhibitors, such as 5-fluorouracil (5-FU). S-1 is a novel oral derivative of the 5-FU prodrug tegafur combined with two modulators. Recent clinical trials have reported the promising effect of S-1 in pancreatic cancer. The purpose of this study was to evaluate the relationship between different schedules and the effects of GEM/S-1 combination therapy on pancreatic cancer xenograft models. *Materials and Methods:* Human pancreatic tumor xenografts were prepared by subcutaneous implantation of MiaPaCa-2 into nude mice. Expression of hENT1 was determined by quantitative RT-PCR. GEM cellular uptake was determined using [³H] GEM. *Results:* Significant increases in hENT1 expression and GEM cellular uptake were observed after S-1 treatment. Six different treatment schedules (no treatment, single agent of GEM or S-1, combination treatment with GEM either before, simultaneously or following administration of S-1) were compared. Significant tumor growth inhibition was observed in the mice treated with S-1 followed by GEM compared to either untreated mice or the

mice treated with the other schedules. *Conclusion:* Based on the effects of S-1 on the uptake of GEM, S-1 should be used before GEM treatment. The GEM/S-1 combination therapy in patients with pancreatic cancer may be promising and should be tested in clinical trials.

Gemcitabine (2',2'-difluorodeoxycytidine (dFdC); Gemzar), accepted as a standard chemotherapeutic agent for patients with advanced pancreatic cancer, is a cell cycle-dependent deoxycytidine analogue of the antimetabolic class. It must first be transported into the cell and then be phosphorylated to its active triphosphate form. Incorporation of gemcitabine triphosphate into DNA is most likely the major mechanism through which gemcitabine exerts its cytotoxic actions (1).

Cells can synthesize nucleotides either through a *de novo* synthesis pathway or a salvage pathway. 5-Fluorouracil (5-FU), one of the thymidylate synthase (TS) inhibitors, is known to act as a *de novo* synthesis inhibitor (2). In the salvage pathway, nucleosides and nucleobases must first be transported across the cell membrane by nucleoside transporter proteins. In addition to nucleosides, nucleoside analogues are also taken up into the cell *via* these specific transporters (3). Gemcitabine is a substrate for five of the nucleoside transporters found in humans (4). These are human equilibrative nucleoside transporter 1 (hENT1), hENT2, human concentrative nucleoside transporter 1 (hCNT1), hCNT2 and hCNT3. The most active gemcitabine uptake is *via* hENT1 (4). It has been reported that 5-FU leads to an increase in cell surface hENT1 (5, 6). An increase in hENT1 can potentially augment the effect of gemcitabine because this agent enters the cell *via* hENT1. In fact, it has also been reported that pretreatment of pancreatic cancer cell lines *in vitro* and *in vivo* with 5-FU augmented the effects of single-agent gemcitabine

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treatment, whereas concurrent treatment or gemcitabine prior to 5-FU did not (7, 8). These results suggest that the effect of gemcitabine/5-FU combination therapy could be dependent on the selected treatment schedule.

S-1 (TS-1) is an oral fluorinated pyrimidine which contains tegafur (FT), 5-chloro-2,4-dihydropyridine (CDHP) and potassium oxonate (Oxo) in a molar ratio of FT:CDHP:Oxo of 1:0.4:1, based on the biochemical modulation of 5-FU (9, 10). FT, a prodrug of 5-FU, is gradually converted to 5-FU and is rapidly catabolized by dihydropyrimidine dehydrogenase (DPD) in the liver. CDHP is a competitive inhibitor of 5-FU catabolism, being about 180 times more potent than uracil in inhibiting DPD (11). When combined with 5-FU, this results in the prolonged maintenance of 5-FU concentrations, both in plasma and in tumors. In addition, it has been suggested that CDHP has the potential to enhance the antitumor activity of 5-FU against subcutaneous tumors in nude mice, using human pancreatic carcinoma cells with a high tumoral DPD activity (12). Oxo is a selective inhibitor of phosphoribosyl pyrophosphate transferase in normal gastrointestinal tissues, resulting in decreased drug incorporation into cellular RNA and, therefore, in the reduction of gastrointestinal toxicity (13). Recent Phase II clinical trials using S-1 as a single agent have shown promising results in various solid tumors, particularly gastric and colorectal cancers, with a response rate of 31.6 to 53.6% and 16.7 to 39.5%, respectively (14). In patients with metastatic pancreatic cancer, a Phase II clinical trial showed the safety and efficacy of S-1, with a response rate of 37.5% and a median survival time of 8.8 months (15).

The relatively mild toxicity profile of gemcitabine has allowed for the development of gemcitabine-based combination chemotherapy regimens (16, 17). The combination of gemcitabine and 5-FU has been shown to have a marked synergistic cytotoxic effect including against pancreatic carcinoma cells in an *in vitro* assay (18, 19). The administration of oral S-1 is more convenient and simulates the effect of continuous infusion of 5-FU. However, no randomized Phase III trial has yet established the survival benefits of a combination of gemcitabine and 5-FU compared to gemcitabine alone (16, 17). Recently, two clinical trials of gemcitabine and S-1 have demonstrated favorable response and tolerability with different dose and schedule (20, 21). Therefore, we focused on optimizing the efficacy of gemcitabine through modification of the schedule of these two agents.

The purpose of the present study was to evaluate the relationship between the schedules of gemcitabine/S-1 combination therapy and their effects in pancreatic cancer. We hypothesized that pretreatment with S-1 would increase hENT1 and thus increase the cytotoxicity of gemcitabine, which enters the cell *via* hENT1, in pancreatic cancer.

Materials and Methods

Cell culture. In a previous study, we selected three human pancreatic carcinoma cell lines which showed a higher rate of 5-FU-induced increase in hENT1 mRNA, however, we failed to establish subcutaneous tumors except for MiaPaCa-2, hence the human pancreatic carcinoma cell line MiaPaCa-2 was used in the present study (8). MiaPaCa-2 was obtained from the Japanese Collection of Research Bioresources (JCRB, Japan) and was cultured at 37°C under 5% CO₂ in Dulbecco's modified Eagle's medium (DMEM) (Sigma Chemical Co., St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS) (Hyclone Laboratories, Inc., Rockville, MD, USA) and 100 units/ml each of penicillin and streptomycin.

Drugs and reagents. S-1 (TS-1) and gemcitabine (GEMZAR) were kindly provided by Taiho Pharmaceutical Co., Ltd (Tokyo, Japan) and Eli Lilly, Japan (Indianapolis, IN, USA) respectively. [³H] Gemcitabine was purchased from Moravек Biochemicals, Inc. (Brea, CA, USA). All drugs were dissolved in distilled water.

Pancreatic cancer xenograft model and treatment. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Graduate School of Medicine, Osaka University. Four-week-old female BALB/c *nu/nu* mice were purchased from Japan Clea (Tokyo, Japan) and maintained in specific pathogen-free conditions. Human pancreatic tumor xenografts were prepared by subcutaneous implantation of MiaPaCa-2 cells (5x10⁶ cells/ 100 µl phosphate-buffered saline) into the right back of nude mice. When the tumors reached a volume between 100 and 200 mm³, mice were randomly divided into treatment groups (n=5 for each group) according to tumor volume (day 0). S-1 was orally administrated at a dose of 10 mg/kg/day as described elsewhere (22), and gemcitabine was injected into the peritoneal cavity at a dose of 240 mg/kg as described elsewhere (23).

Quantitative reverse transcription-polymerase chain reaction (Q-RT-PCR). RNA extraction was carried out with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) using a single-step method, and cDNA was generated with avian myeloblastosis virus reverse transcriptase (Promega, Madison, WI, USA), as described elsewhere (24). In this assay, porphobilinogen deaminase (PBGD) mRNA was used as an internal control (25). Q-RT-PCR was performed in a LightCycler apparatus using LightCycler Fast Start DNA Master SYBR Green I (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. The PCR primers used for detection of PBGD and hENT1 cDNAs were synthesized as described elsewhere (25, 26). PCR was performed with cycling conditions of 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 10 s, annealing at 62°C for 10 s, and extension at 72°C for 20 s. To quantify the data, hENT1 mRNA levels were normalized by PBGD mRNA levels.

hENT1 mRNA expression and [³H] gemcitabine uptake assay *in vivo*. The BALB/c mice bearing distinct MiaPaCa-2 tumors were divided into the following three groups of 6 mice each: (a) no treatment; (b) daily oral administration of S-1 for 5 consecutive days at a dose of 10 mg/kg/day (day 1 to 5); (c) daily oral administration of S-1 for 5 consecutive days at the dose of 50 mg/kg/day (days 1 to 5). On day 6, MiaPaCa-2 tumors in three of the mice killed by cervical dislocation of each group were removed and homogenized, and

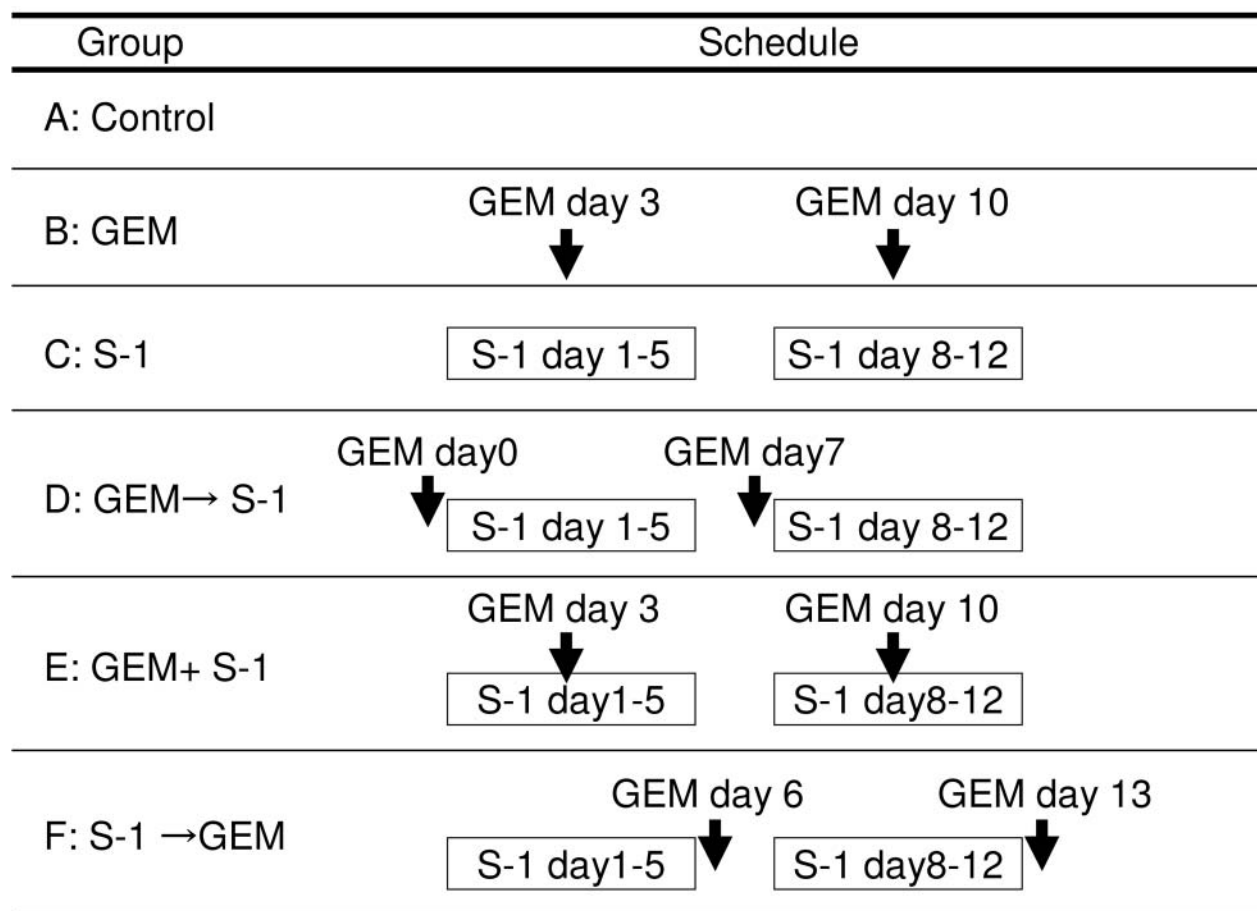


Figure 1. Treatment schedule. MiaPaCa-2 cells (5×10^6 cells/100 μ l phosphate-buffered saline) were injected subcutaneously into the right back of BALB/c mice. The mice were divided into 6 groups of 5 mice according to tumor volume. Gemcitabine (240 mg/kg) was administered by intraperitoneal injection and S-1 (10 mg/kg) by oral gavage. GEM: gemcitabine.

quantitative gene expression of hENT1 mRNA in tumors was analyzed using LightCycler. The remaining three mice of each group were given a single intraperitoneal injection of [3 H] gemcitabine (240 mg/kg). Thirty minutes after gemcitabine injection, mice were killed by cervical dislocation, tumors were removed, weighed, and homogenized in Lumasolve solubilizer (LUMAC*LSC, Landgraaf, Netherlands). Gemcitabine concentrations in tumors were analyzed by quantitation of radioactivity as described elsewhere (27). The [3 H] gemcitabine uptakes were normalized by tumor weight.

Antitumor experiments in vivo. Mice bearing distinct tumors were randomly divided into the following six groups of 5 mice each (Figure 1): (A) no treatment; (B) weekly intraperitoneal injections of gemcitabine as a single agent (day 3, 10); (C) daily oral administrations of S-1 as a single agent for 5 consecutive days a week (day 1 to 5, day 8 to 12); (D) sequential combination treatment with gemcitabine (day 0, 7) prior to S-1 (day 1 to 5, day 8 to 12); (E) coadministrations of gemcitabine (day 3, 10) and S-1 (day 1 to 5, day 8 to 12); (F) sequential combination treatment with S-1 (day 1 to 5, day 8 to 12) prior to gemcitabine (day 6, 13). The

animals were monitored for activity, physical condition, determination of body weight, and measurement of tumor volume [$1/2 \times$ (the major axis) \times (the minor axis) 2] every other day. Statistical analysis of the data for the comparison of different groups was carried out using tumor volumes on day 18.

Statistical analysis. Statistical analysis was performed using the StatView J-5.0 program (Abacus Concepts, Inc., Berkeley, CA, USA). Data were expressed as the average of experiments and represented as mean \pm SD. Differences between groups were examined for statistical significance using ANOVA. In all analyses, values of $p < 0.05$ were considered statistically significant.

Results

hENT1 mRNA expression in pancreatic cancer xenograft tumors. The effect of S-1 on the hENT1 mRNA expression in xenograft tumors of MiaPaCa-2 was examined using quantitative RT-PCR (Figure 2). After the daily oral administrations of S-1 for 5 consecutive days, the tumors in

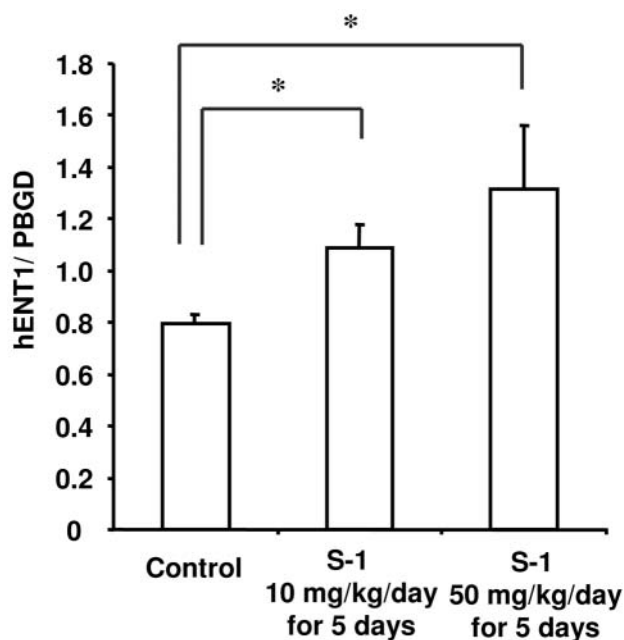


Figure 2. Quantification of hENT1 mRNA expression in MiaPaCa-2 xenograft tumors treated with or without S-1. To quantify the data, hENT1 mRNA levels were normalized by PBGD mRNA levels. Results represent mean \pm SD (each group, n=3). The tumors in S-1 treated mice showed increased hENT1 levels. * p <0.05.

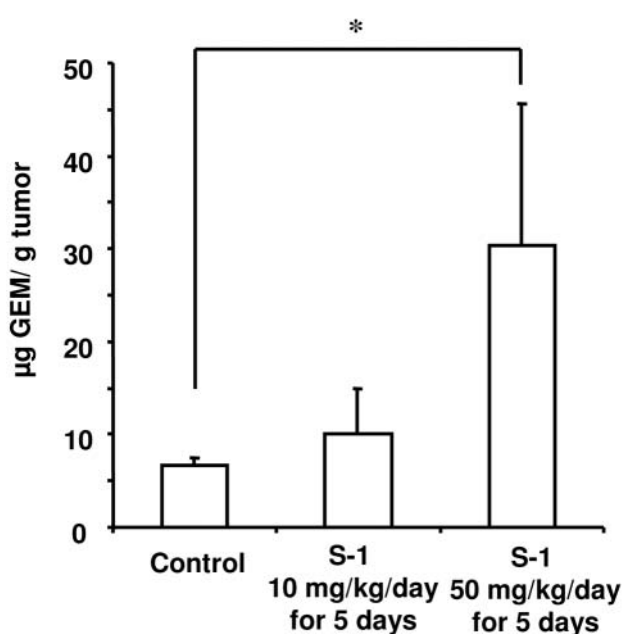


Figure 3. Uptake of [³H] gemcitabine after pretreatment with S-1 in pancreatic cancer xenograft tumors. The [³H] gemcitabine uptakes were normalized by tumor weight. Results represent mean \pm SD (each group, n=3). The tumoral gemcitabine uptakes in S-1 treatment group at the dose of 50 mg/kg/day were significantly higher than the uptakes in control group. * p <0.05.

S-1-treated mice showed increased hENT1 levels. The tumoral hENT1 expression in the S-1 treatment groups was significantly up-regulated compared with those in the control group.

Uptake of [³H] gemcitabine after pretreatment with S-1 in pancreatic cancer xenograft tumors. The uptakes of gemcitabine were quantified using a [³H] gemcitabine uptake assay (Figure 3). Although the S-1 treatment group at the dose of 10 mg/kg/day tended to show increased [³H] gemcitabine uptake level, it was not significantly higher than that of the control group. However, the tumoral gemcitabine uptakes in S-1 treatment group at the dose of 50 mg/kg/day were significantly higher than the uptakes in the control group.

The schedule-dependent antitumor effect of gemcitabine and S-1 on human pancreatic tumor xenografts. The nude mice with the subcutaneous tumor were divided into six groups and treated as described in "Materials and Methods" and the antitumor effects were evaluated with the tumor volume at the day 18. The mean tumor volumes on day 18 were 4.2 \pm 1.5, 2.0 \pm 0.2, 2.3 \pm 1.0, 2.8 \pm 0.9, 2.1 \pm 0.7 and 1.0 \pm 0.3 cm³ in Groups A, B, C, D, E and F, respectively (Figures 4 and 5). Tumor volumes of each treatment group were

significantly smaller than those of the control group except for group D (GEM \rightarrow S-1). Although there was not difference in tumor volumes among treatment groups B, C, D and E, statistically significant tumor growth inhibition was observed in Group F (S-1 \rightarrow GEM) compared with all the other treatment groups (p <0.05). Although the mice which were given S-1 first or simultaneously with gemcitabine showed stronger weight loss compared with other groups during and after the treatment, no mice died from the side-effects of the drugs or any other reasons up to the end of the study (day 18) (Figure 6).

Discussion

Although the systemic administration of gemcitabine is currently considered the standard first-line treatment for patients with advanced pancreatic cancer, single-agent gemcitabine has provided limited benefit, with objective response rates of less than 15% and a median survival of less than 6 months (28-30). Owing to the activity of gemcitabine, a variety of studies have now assessed its activity in combination with other agents. These studies have shown varying degrees of success, with no combination showing clear evidence of significantly superior activity (28). Combination of anticancer agents

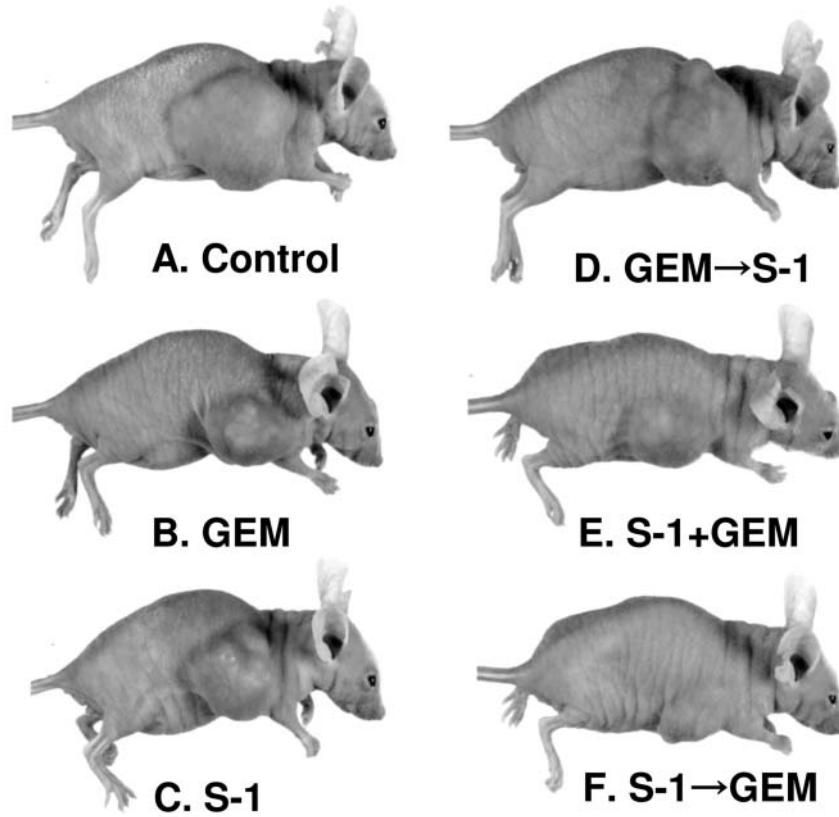


Figure 4. BALB/c mice with subcutaneous human pancreatic tumor after treatment.

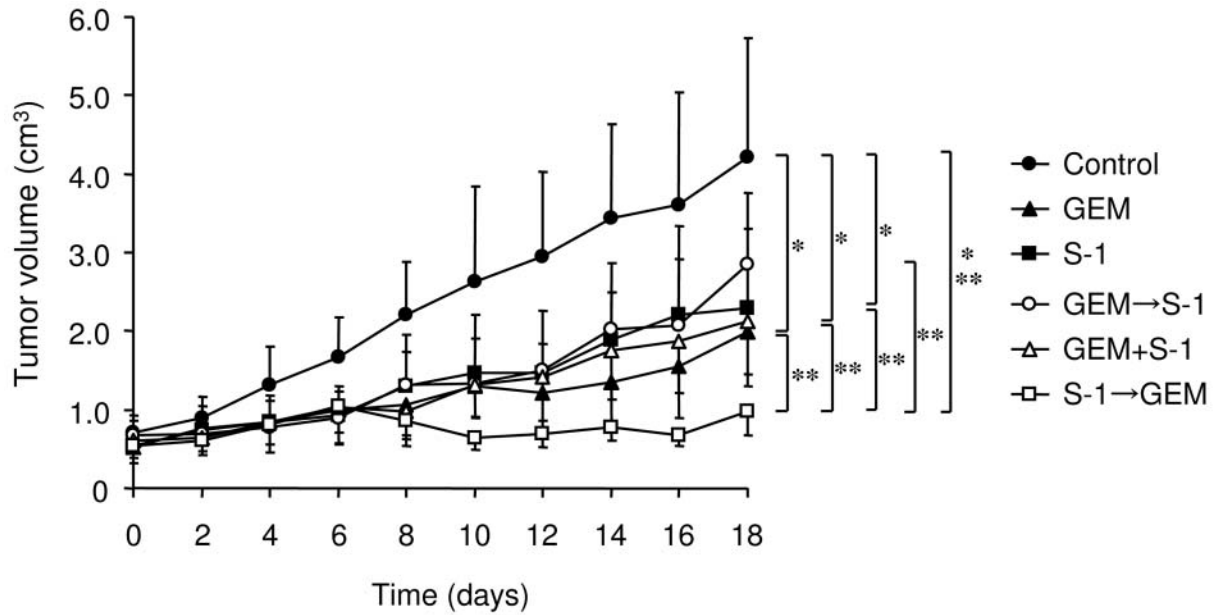


Figure 5. Antitumor effect of the combination of gemcitabine with S-1 in vivo. Six different groups of the therapeutic experiments were carried out (Figure 1). Statistical analysis for the comparison of different groups was performed using tumor volumes on day 18. Results represent mean \pm SD (each group, $n=5$). Tumor volumes of each treatment group were significantly smaller than those of the control group except for group D ($*p<0.05$). Statistically significant growth inhibition was observed in Group F compared to all other treatment groups ($**p<0.05$). $*$, $**p<0.05$.

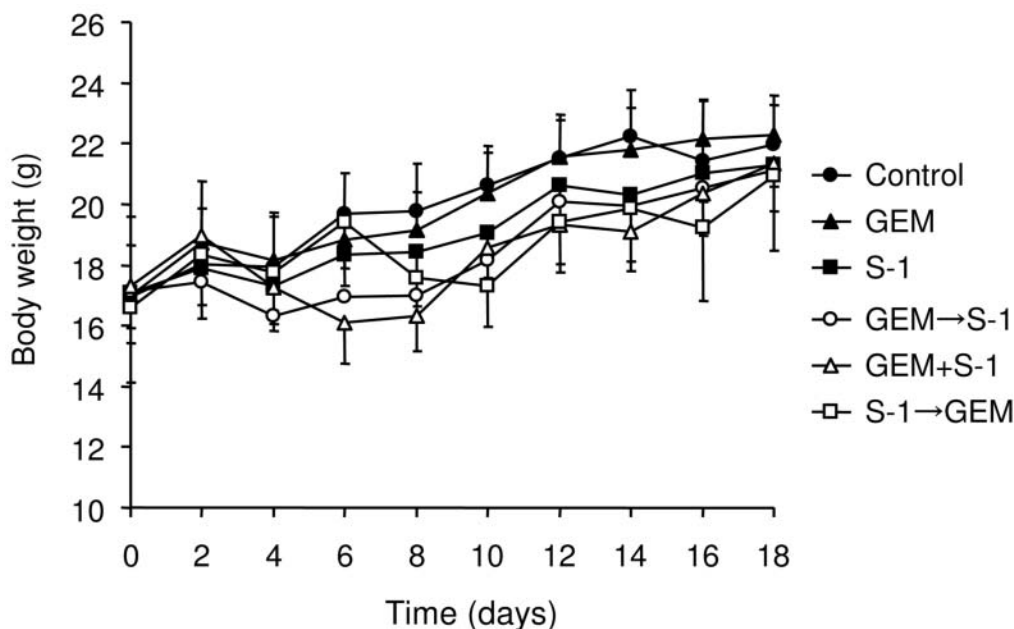


Figure 6. Body weight curves of the mice. The corresponding tumor growth curves are shown in Figure 3. Results represent mean \pm SD (each group, $n=5$). There was no statistically significant difference between groups.

has become a highly important modality for the treatment of cancer. Furthermore, combination chemotherapy is influenced by the employment of optimal scheduling (31). Gemcitabine is considered to be attractive for combination chemotherapy due to its mild toxicity profile at an active dose (16, 17).

However, in one study, the combination of 5-FU with gemcitabine did not improve the median survival of patients with advanced pancreatic carcinoma compared to single-agent gemcitabine (30). On the other hand, a novel oral derivative of 5-FU, S-1, was reported to have favorable results in pancreatic cancer patients (15), and a Phase II trial of S-1 combined with gemcitabine demonstrated promising efficacy with objective response rates of 48% and a median survival of 12.5 months (21). Therefore, we evaluated the effect of gemcitabine/S-1 combination therapy on human pancreatic cancer especially in terms of the schedule of the two drugs. Our study shows that the sequence can be an important factor in the antitumor activity of the combination of gemcitabine and S-1, and that hENT1 might play an important role in this effect.

The sequence-dependent effects of the combination of TS inhibitors and gemcitabine on human pancreatic cancer cells were reported to be seen with maximum effect when the TS inhibitors preceded gemcitabine *in vitro* (7). Moreover, this effect was not associated with basal hENT1 levels but with a significant increase in

hENT1 levels caused by the TS inhibitors (7). Therefore, for *in vivo* experiments, we decided to use the cell lines which showed the highest rate of 5-FU induced increase in hENT1 mRNA (8).

We first examined the effects of S-1 on expression of hENT1 mRNA and gemcitabine cellular uptake in pancreatic cancer xenografts. 5-FU, known as a TS inhibitor, blocks the formation of 2'-deoxythymidine-5'-monophosphate (dTMP) and depletes intracellular nucleoside pools so that proliferating cells then depend on salvage of preformed nucleosides from extracellular fluid. 5-FU also up-regulates the amount of cell surface hENT1 as confirmed by flow cytometric analysis (7). In this study, we demonstrated that treatment with S-1 resulted in hENT1 up-regulation at the mRNA level and increased tumoral gemcitabine uptake.

To clarify the optimal schedule of treatment, we investigated the effect of six different schedules of treatment of S-1 and/or gemcitabine on a xenograft model of MiaPaCa-2 tumors. We did not detect any significant differences among the monotherapy groups and combination groups except for the S-1 followed by gemcitabine group. However, significant tumor growth inhibition was observed in mice treated with S-1 followed by gemcitabine compared with either untreated mice or the other treatment groups. Moreover, the synergistic tumor growth inhibitory effect was observed only in the S-1 followed by gemcitabine group and not in the other

combination groups. These results suggest that the schedule in which the gemcitabine is administered after S-1 could be the optimal combination of these two agents in the treatment of pancreatic cancer. The up-regulation of hENT1 induced by S-1 may play an important role in the enhanced growth inhibitory effect of gemcitabine.

Moreover, only the gemcitabine followed by S-1 treatment group did not show any efficacy compared with the control group. Previous reports showed that treatment of gemcitabine increased hENT1 expression and reduced 5-FU sensitivity in human pancreatic cancer cell lines (7). Therefore, the pretreatment with gemcitabine might reduce the effects of 5-FU due to the increasing supply of nucleosides and nucleobases *via* the salvage pathway. Indeed, the expression level of basal hENT1 was inversely associated with 5-FU sensitivity in human pancreatic cancer cell lines (8). These data suggested that the lack of benefit with sequences other than that of S-1 followed by gemcitabine *in vivo* might be due to gemcitabine-induced up-regulation of hENT1 and subsequent reduced 5-FU sensitivity.

It has been reported that mouse ENT1 (mENT1) mRNA was highly expressed in the heart, spleen, lung, liver and testes, and that lower levels of expression were detected in the brain and kidney (32). Therefore, enhanced gemcitabine effects induced by S-1 may also aggravate gemcitabine-induced side-effects. However, in this study, neither severe side-effects nor mortality were observed, although the mice treated with S-1 first or simultaneously with gemcitabine showed greater weight loss than other groups.

In conclusion, our results showed that the administration of S-1 followed by gemcitabine provides greater inhibitory effects than other gemcitabine/S-1 schedules in the treatment of pancreatic cancer.

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