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ORIGINAL ARTICLE

Experimental study of combination therapy with S-1 against pancreatic cancer

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Abstract
Purpose To determine the most effective combination chemotherapy with S-1 against pancreatic cancer and to clarify the mechanism of synergy between S-1 and the partner drug.
Methods We tested a combination of S-1 with the following antitumor drugs in an in vitro MTT assay against pancreatic cancer cell line MIA PaCa-2: gemcitabine (GEM), cisplatin (CDDP), irinotecan (CPT-11), mitomycin C, adriamycin, and paclitaxel. The efficacy of S-1, GEM, and a combination of S-1 and GEM was also tested in vivo by administering S-1 (10 mg/kg) orally to nude mice five times a week for 3 weeks, and GEM (100 mg/kg) intravenously every 2–3 days for a total of six times. A treated-to-control ratio (T/C) of relative mean tumor weight values less than 50% was determined to be effective. Furthermore, we investigated the mechanism of the synergistic effect of S-1 and GEM on the cell cycle by flow cytometry, because both S-1 and GEM are known as antimetabolic drugs. To verify cell death induced by a change in the distribution of the cell cycle phases, we investigated apoptosis by sub-G1 analysis and a TUNEL assay.
Results From classical isobolography analysis of the in vitro MTT assay, the combination of S-1 plus GEM was found to be the most effective of the combinations tested. In vivo, T/C (percentage) with the combination of S-1 plus GEM was 48.2%, which was lower than that of S-1 or GEM alone, and the combination enhanced antitumor activity. Cell cycle analysis showed greater cell cycle delay with the combination treatment (S-1 plus GEM) than for each single drug treatment, and apoptotic cells were detected only in treatments including GEM.
Conclusion The combination chemotherapy of S-1 and GEM appears to be useful for pancreatic cancer. Both cycle delay by S-1 plus GEM and apoptosis induced by GEM are involved in this synergistic mechanism.

Keywords S-1 · Gemcitabine · Pancreatic cancer · Combination chemotherapy

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Introduction

Cancer is the second leading cause of death by rate of incidence, and estimated deaths from pancreatic cancer are ranked fourth according to anatomical region [1]. Pancreatic cancer usually invades locally or metastasizes to the liver, peritoneum, or lymph nodes even at the first medical examination. Therefore, its 5-year survival rate is only 5%,

and pancreatic cancer is considered intractable [1]. For many years, the key drug used to treat unresectable pancreatic cancer was 5-FU, but its response rate was only ~20% for both single-agent and combination chemotherapy. A phase III randomized trial conducted in 1997, concluded that gemcitabine (GEM) was more effective than 5-FU for the treatment of pancreatic cancer [2]. Since its approval in 2001 then, GEM has been the chemotherapy agent of choice pancreatic against cancer. However, there are cases that are unresponsive to treatment with GEM or that become tolerant to GEM [3]. TS-1 (S-1), which was approved in 2006 for pancreatic cancer, is the agent used most frequently in these cases.

S-1 is a new oral fluorinated pyrimidine that includes tegafur (FT), a prodrug of 5-FU [4]. Therefore, its main active antitumor compound is 5-FU, the nucleic acid analog of deoxythymidylic acid (dTMP) which causes arrest at S phase of cell cycle [5]. Unfortunately, 5-FU is rapidly catabolized by dihydropyrimidine dehydrogenase (DPD) in the liver. To solve this problem, antitumor activity of S-1 is enhanced effect by the inclusion of 5-chloro-2,4-dihydroxypyridine (CDHP), an inhibitor of DPD, which biochemically modulates 5-FU [5]. S-1 also contains potassium oxonate (Oxo), another 5-FU modulator, which decreases the phosphorylation of 5-FU in the gastrointestinal tract and leads to a reduction of gastrointestinal side effects [6]. Therefore, S-1 maintains a high blood level of 5-FU for an extended time with fewer gastrointestinal side effects.

S-1 is expected to overtake the outcome observed for therapy with 5-FU. In fact, S-1 reported a response rate of 37.5% and a median survival time of 8.8 months by single-agent chemotherapy in a phase II study [7]. With combination chemotherapy, S-1 is anticipated to provide improved treatment results. However, not enough is known about combination chemotherapy with S-1 against pancreatic cancer, and more evidence is needed to develop effective therapy strategies using S-1.

The purpose of this study was to determine the most effective combination chemotherapy with S-1 against pancreatic cancer cell line MIA PaCa-2 using an MTT assay in vitro and by administrating drugs to nude mice in an in vivo xenograft model. We also investigated cell cycle regulation by flow cytometry (FCM) and rates of cell death to identify the mechanisms of the synergic effect of combination therapy with S-1.

Materials and methods

Human pancreatic cancer cell line

MIA PaCa-2 was obtained from the American Type Culture Collection and cultured in DMEM (Wako Pure Chemical Industries, Ltd, Osaka, Japan) plus 10% fetal bovine serum (Invitrogen Corporation, Carlsbad, CA, USA) and 2.5% horse serum (Invitrogen Corporation).

Antitumor agents

The following chemotherapeutic agents were used: S-1 and CDHP (Taiho Pharmaceutical Co. Ltd, Tokyo, Japan), 5-FU (Kyowa Hakko Kogyo Co., Ltd, Tokyo, Japan), GEM (Eli Lilly Japan K.K., Kobe, Japan), cisplatin (CDDP; Bristol Myers K.K., Tokyo Japan), irinotecan (CPT-11; Yakult Honsha Co., Ltd, Tokyo, Japan), mitomycin C (MMC; Kyowa Hakko Kogyo Co., Ltd), adriamycin (ADM; Kyowa Hakko Kogyo Co., Ltd), and paclitaxel (PTX; Bristol Myers Japan K.K.).

In vitro MTT assay

The cells were plated into 96-well plates (Corning Inc., NY, USA) at a density of 2×10^4 cells/ml (100 µl per well) in serum containing medium and were allowed to grow for 24 h. An additional 100 µl of serum containing medium with various concentrations of antitumor agents ranging from 0.01 to 100 µg/ml (S-1), 0.01 to 100 µg/ml (5-FU), 0.0001 to 1 µg/ml (GEM), 0.1 to 50 µg/ml (CDDP), 0.1 to 100 µg/ml (CPT-11), 0.001 to 5 µg/ml (MMC), 0.001 to 5 µg/ml (ADM), and 0.0001 to 5 µg/ml (PTX), respectively, was added and incubated for another 72 h. Then, 20 µl/well of MTT reagent [0.4% MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, Sigma, St Louis, MO, USA] with 0.1 M disodium succinate hexahydrate (Kanto Chemical Co., Inc., Tokyo, Japan) was added and cells were incubated for 4 h. Formed formazans were dissolved in 150 µl of dimethyl sulfoxide (Kanto Chemical Co., Inc.), and the optical density (OD) for each well as read with a microplate reader (Elx800 Universal Microplate Reader, Bio-Tek Instruments, Inc., Winooski, VT, USA) at 540 nm. The Inhibition Index (I.I., %) was calculated from the formula below (*a* OD of cells, *b* OD of cells + MTT reagent, *c* OD of cells + MTT reagent + antitumor agent), and IC_{50} values were estimated for each antitumor agent.

$$\text{I.I.}(\%) = (b - c)/(b - a) \times 100.$$

The MTT assays for combination therapy with S-1 were done in the same way described above, except for the concentration of S-1 and the additive amount of each antitumor agent: S-1 was fixed at 0.05–0.5 µg/ml; the others were GEM (0.0015–0.01 µg/ml), CDDP (1–5 µg/ml), CPT-11 (1–5 µg/ml), PTX (0.0015–0.01 µg/ml), MMC (0.015–0.1 µg/ml), ADM (0.005–0.025 µg/ml), respectively, and 50 µl of each antitumor agent was added. I.I.(%) > 50% was determined to be effective.

Also, a classical isobologram was used to evaluate the synergism of each drug combination with S-1.

In vivo assay

Normal 6- to 8-week-old female BALB/c nu/nu mice were obtained from Japan Clea (Tokyo), maintained in a protected environment, and provided with autoclaved food and water. MIA PaCa-2 cells (5×10^6 cells, re-suspended in 200 μl of medium) were injected subcutaneously into the right back of each mouse. Three weeks after implantation, when the estimated tumor weight [$TW = (L \times W^2)/2$, where L is the tumor's major axis and W is the minor axis as measured by a micrometer caliper] increased to 100–300 mg, mice were divided into four groups (each group, $n = 5$–6). One group was a control; the others were administered S-1, GEM, or S-1 plus GEM. We administered S-1 (10 mg/kg as FT) orally five times a week for 3 weeks and GEM (100 mg/kg) intravenously every 2–3 days for a total of six times. The treated to control ratio (T/C) of relative mean tumor weight was calculated; values $< 50\%$ were determined to be effective.

Cell cycle analysis

Cells (1.4×10^5) were sowed into 60-mm culture dishes (Becton Dickinson, San Jose, CA, USA) in serum containing medium and allowed to grow for 24 h. The medium was replaced by medium including antitumor agents with concentrations of 0.4 μg/ml for S-1 and 0.002 μg/ml for GEM, either alone or in combination. The cells were incubated for another 72 h, then they were collected and the nuclei were bared with 0.1% TritonX-100/PBS. The cells were treated with RNase Type I-A (Sigma) at 37°C for 15 min to eliminate RNA and were stained with propidium iodide (PI) for 10 min. DNA histogram data were collected by CELLQuest ver.3.1 using a FACS calibur (Becton Dickinson) and the list mode files were analyzed with ModFit LT ver.3.0.

Synchronization

A double thymidine block was conducted to synchronize the cell cycle distribution at the G1/S phase boundary to determine the amount of cell cycle delay induced by antitumor agents. For the first block, 2 mM thymidine was added for 20 h to synchronize cells at S phase. Then, medium containing thymidine was replaced by a fresh medium to restart the cell cycle. After 10 h of incubation, 2 mM thymidine was added for a second block to synchronize the cells at the G1/S boundary; this point was considered to be 0 h. GEM (0.002 μg/ml) was added to cells at this point, while S-1 (0.4 μg/ml) was added to cells after the first thymidine block, the midstream of synchronization. The cells were collected eight times in 24 h and analyzed as described above.

Apoptosis detection

Sub-G1 assay

Cells were sowed and incubated as described for the cell cycle analysis method except the concentration of GEM was 0.008 μg/ml to more easily detect apoptosis. The cells were collected at 24, 48, and 72 h of incubation and were fixed in ethanol for more than 4 h. Phosphate–citrate buffer was used to extract DNA, and then RNase and PI were added. The percentage of sub-G1 cells was quantified by CELLQuest ver.3.1.

TUNEL assay

2.8×10^4 Cells were sowed into Lab-Tek chamber slides (Nalge Nunc International, Rochester, NY, USA) and incubated for 24 h. Antitumor agents were added with the same concentrations as for the sub-G1 assay, and cells were incubated for another 72 h. An In Situ Cell Death Detection kit, Fluorescein (Roche, Basel, Switzerland) was used following the manufacturer's instructions. Slides were observed using a fluorescence microscope (BZ-8000; Keyence Corporation, Osaka, Japan).

Statistical analysis

The data from the MTT assay, cell cycle analysis, and sub-G1 assay were expressed as mean ± SD. Differences between the IC_{50} values of S-1 and 5-FU were examined for statistical significance using Student's t test, and differences between the T/C ratios of GEM and S-1 plus GEM in vivo were examined by a Mann–Whitney U test. A P-value < 0.05 denoted a statistically significant difference. The statistical analysis was performed with STAT VIEW for Windows ver.5.

Results

MTT in vitro assay

Combination experiments of antitumor agents were conducted in vitro to identify the most effective partner agent for combination therapy with S-1. S-1 is a combination drug consisting of three compounds, but for our in vitro study, we omitted Oxo, which operates in the digestive tract, and used a combination of only 5-FU and CDHP (in a 1:2 ratio) as the S-1 drug. First, the IC_{50} values were

Table 1 IC_{50} values of each antitumor agent

Antitumor agent	IC_{50} (μg/ml)
S-1	0.39 ± 0.07
5-FU	0.67 ± 0.13
GEM	0.0081 ± 0.0021
CDDP	2.61 ± 0.36
CPT-11	2.66 ± 0.33
MMC	0.065 ± 0.004
ADM	0.020 ± 0.007
PTX	0.0058 ± 0.0007

Data are expressed as mean ± SD. Each IC50 value was determined from five to seven replicates of each experiment. The decrease in IC50 of S-1 relative to 5-FU was statistically significant ($P < 0.0001$), apparently due to the inhibition of DPD by CDHP

determined for each antitumor agent alone, as benchmarks for combination therapy with S-1; mean values are given for five to seven individual MTT assay experiments (Table 1). The IC_{50} value of S-1 was significantly lower than that of 5-FU ($P < 0.0001$).

When combining S-1 with other six antitumor agents, there were some effective partner agents. The IC_{50} values of the antitumor agents were connected with a dotted line in classical isobolograms to evaluate possible synergism for each combination (Fig. 1). From the data, GEM was determined to be the most effective agent against MIA PaCa-2 cells when combined with S-1. Therefore, we performed the rest of our experiments using this combination of drugs.

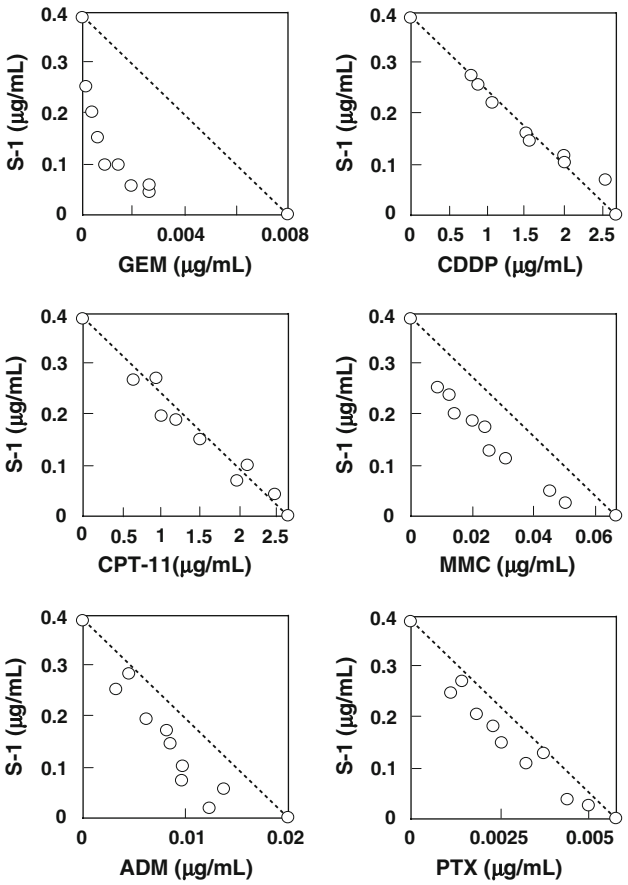

Fig. 1 Classical isobolograms of combination therapy with S-1 and other antitumor agents. IC_{50} values of S-1 and other antitumor agents were connected with a *dotted line* to distinguish the area of synergism and the area of antagonistic effect. Plots on the *dotted line* indicate an additive effect. S-1 plus GEM showed the most synergism compared with any of the other combinations

In vivo assay

An assay using nude mice was conducted to confirm the effectiveness of S-1 plus GEM in vivo. All of the groups treated with antitumor agents tended to suppress tumor growth compared with the control (Fig. 2). The lowest T/C ratios (%) observed for groups treated with S-1 or GEM alone were 71.7% (day 15) and 58.3% (day 22), respectively. However, the T/C ratios (%) for the group treated with a combination of S-1 and GEM were 49.8% (day 7), 48.2% (day 18), and 49.4% (day 22), indicating an enhancement of antitumor activity with the combination of these two drugs (Table 2).

Cell cycle delay was a mechanism for the synergism of combination therapy

To clarify the synergistic mechanism of antitumor activity induced by S-1 and GEM, we examined the change in the distribution of the cell cycle phase. As shown in Fig. 3, non-treated control cells were distributed throughout the cell cycle in similar ratio until 24 h, and then S phase gradually decreased until 72 h. S-1-treated cells started to accumulate in S phase after 24 h of exposure and remained in S phase until 72 h. GEM-treated cells began to accumulate at S phase starting at 12 h; this conversion achieved a peak at 24 h. Then, S phase gradually decreased, and at 72 h, the distribution of the cell cycle was similar to that of control cells. The combination of S-1 plus GEM showed changes similar to those of GEM-treated cells, but the accumulation of S phase remained until 72 h.

Synchronization at the G1/S boundary

To more thoroughly evaluate and compare the level of cell cycle delay induced by S-1, GEM, and the combination of these two agents, we synchronized the cell cycle of MIA PaCa-2 cells G1/S boundary by a thymidine double block (defined as 0 h), and then restarted the cell cycle to compare the distribution of each phase using a time course. GEM was added at 0 h, after synchronization was

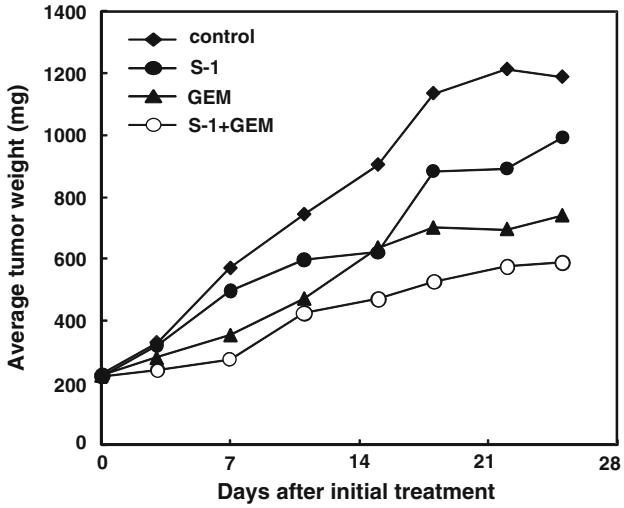

Fig. 2 Effect of combination therapy of S-1 and GEM against MIA PaCa-2 cells in vivo. The group treated with S-1 plus GEM was the most effective at suppressing tumor growth compared with groups treated with each agent alone

completed, while S-1 was applied to cells after the first thymidine block, midstream during synchronization, so that the effect of S-1 would appear by 0 h which reflected the cell cycle analysis. Six hours after synchronization, 71–83% of the drug-treated cells accumulated in S phase, while only 28% of control cells were in S phase, suggesting that the cell cycle was already delayed compared with control cells (Fig. 4). At 9 h, half of the S-1-treated cells were in the G2/M phase, but half of the cells treated with the combination of S-1 plus GEM still remained in the S phase. At 16 h, GEM-treated cells were starting to enter the second lap of the cell cycle, but this was not seen with cells treated with S-1 plus GEM. These results indicate that the combination of S-1 and GEM delays the cell cycle more than each agent alone.

Apoptosis detection assay

To reveal whether apoptosis is induced by blocking the S phase or by a delay in the cell cycle, two kinds of assays were conducted; a sub-G1 assay and a TUNEL assay. In PI staining and FCM analysis, cells in the sub-G1 phase are considered to be undergoing apoptosis. The relative percentage of sub-G1-phase cells in the non-treated control group was 2.2 ± 0.7% consistently from 24 to 72 h. In the S-1-treated group, the relative percentage of sub-G1 cells was 3.5 ± 2.6%, even after 72 h of exposure. On the other hand, in the GEM-treated and S-1 plus GEM-treated groups, although there were no differences at 24 h, gradually increased to 22.5 ± 16.2% (GEM) and 9.9 ± 8.3% (S-1 plus GEM) at 48 h and 43.7 ± 1.0% (GEM) and 37.2 ± 7.6% (S-1 plus GEM) at 72 h (Fig. 5). In the TUNEL assay, no apoptotic cell death was observed for the non-treated group and the S-1-treated group. However, apoptotic cell death was detected in MIA PaCa-2 cells treated with GEM or a combination of S-1 and GEM (Fig. 6).

Discussion

The S-1 is a new type of antitumor drug consisting of an improved 5-FU compound that is just beginning to be used against various cancers, including pancreatic cancer, mainly in Japan and Korea. For more than a decade, the only standard antitumor agent against pancreatic cancer has been GEM. The S-1, however, has displayed a comparable or superior antitumor effect to GEM [7]. In pancreatic cancer tissue, it is known that the activity of DPD, an enzyme that degrades 5-FU, is increased two- to threefold as compared with normal pancreas tissue [8]. Also, the mRNA expression of DPD is higher in pancreas tumors than in other digestive carcinomas, such as gastric cancer and colon cancers [9]. These facts suggest that S-1 might be more effective than 5-FU against pancreatic cancer because S-1 contains CDHP, an inhibitor of DPD. In fact, S-1 performed well in a phase II study of pancreatic cancer [7]. However, it is an undeniable fact that there is a limit on the outcome of single-agent chemotherapy. Now, to improve treatment results, the development of new types of antitumor agents and combination chemotherapy is underway worldwide. So, when combining S-1 with some other antitumor agents, it is likely to show much improvement in therapy for pancreatic cancer, which is known as an intractable cancer.

Table 2 Minimum T/C (%) for each treatment group

Antitumor agents	Administration schedule	Minimum T/C (%)
S-1	100 mg/kg 5 days × 3 weeks p.o.	71.1
GEM	100 mg/kg q 3–4 days × six times i.v.	58.3
S-1 + GEM	100 mg/kg 5 days × 3 weeks p.o. + 100 mg/kg q 3–4 days × six times i.v.	48.2

Combination therapy of S-1 plus GEM was the only group that showed T/C(%) < 50%. This was observed at day 18 after the initial treatment. The lowest T/C for S-1 and GEM alone were marked at day 15 and day 22, respectively

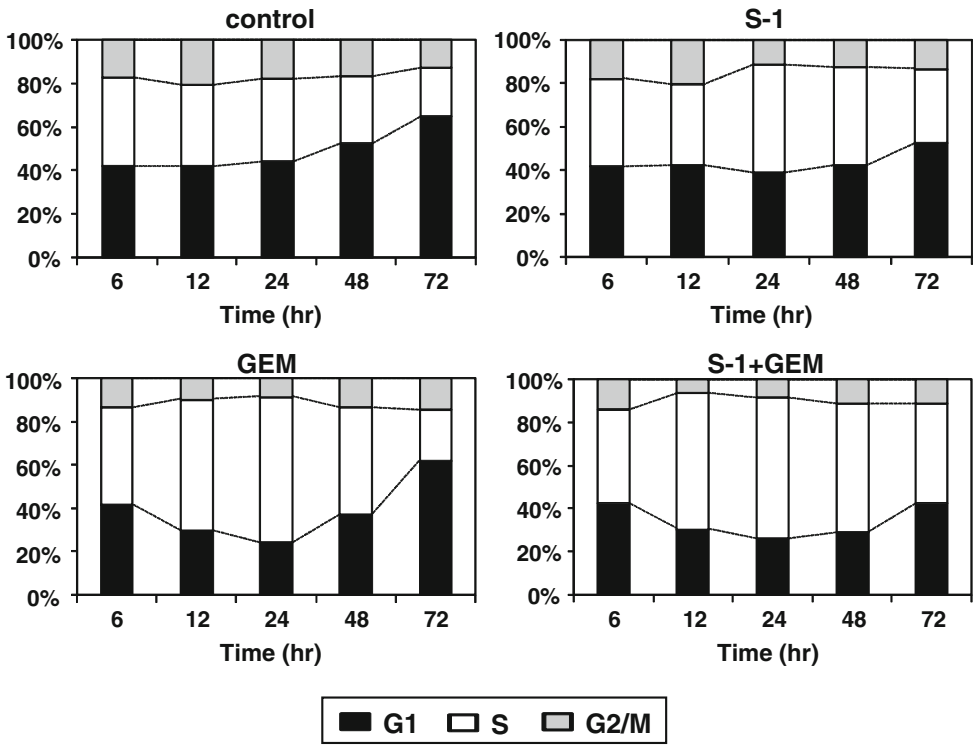

Fig. 3 The change of each phase of the cell cycle induced by S-1, GEM, and the combination of both drugs. The data (mean ± SD) are from three separate experiments. *Upper graph* was created by the mean data. Cells treated with S-1 started to accumulate in S phase after 24 h of exposure and sustained it until 72 h. Cells treated with GEM began to accumulate in S phase from 12 h, while the G2/M phase declined; this conversion peaked at 24 h. Then, S phase gradually decreased to the distribution of control cells by 72 h. Cells treated with a combination of S-1 plus GEM showed a change similar to that of GEM-treated cells, but accumulation of S phase remained at 72 h

control	G1	S	G2/M
6	42.2±1.5	40.6±1.2	17.2±2.3
12	42.1±0.9	37.3±1.8	20.7±2.3
24	44.3±3.4	38.0±5.0	17.6±6.5
48	52.6±2.2	30.8±3.3	16.7±2.0
72	65.1±2.4	22.0±1.1	12.9±1.3

S-1	G1	S	G2/M
6	41.8±1.5	40.4±2.5	17.8±2.4
12	42.5±0.8	36.9±1.5	20.5±1.1
24	38.8±0.4	49.9±3.4	11.3±3.4
48	42.3±0.6	45.0±3.1	12.7±2.4
72	52.5±1.2	34.2±2.1	13.3±0.9

GEM	G1	S	G2/M
6	41.6±1.8	44.9±0.2	13.5±1.7
12	29.8±4.4	60.3±4.9	9.9±1.7
24	24.3±2.6	66.9±1.6	8.9±1.1
48	36.8±2.8	49.7±4.2	13.5±1.5
72	61.6±9.0	23.7±11.3	14.7±3.3

S-1+GEM	G1	S	G2/M
6	42.3±0.8	43.7±1.9	14.0±2.6
12	30.4±5.2	63.1±10.7	6.4±5.6
24	26.2±2.0	65.2±3.6	8.6±1.6
48	28.9±4.4	59.6±2.8	11.4±1.8
72	42.3±4.7	46.5±4.7	11.2±0.5

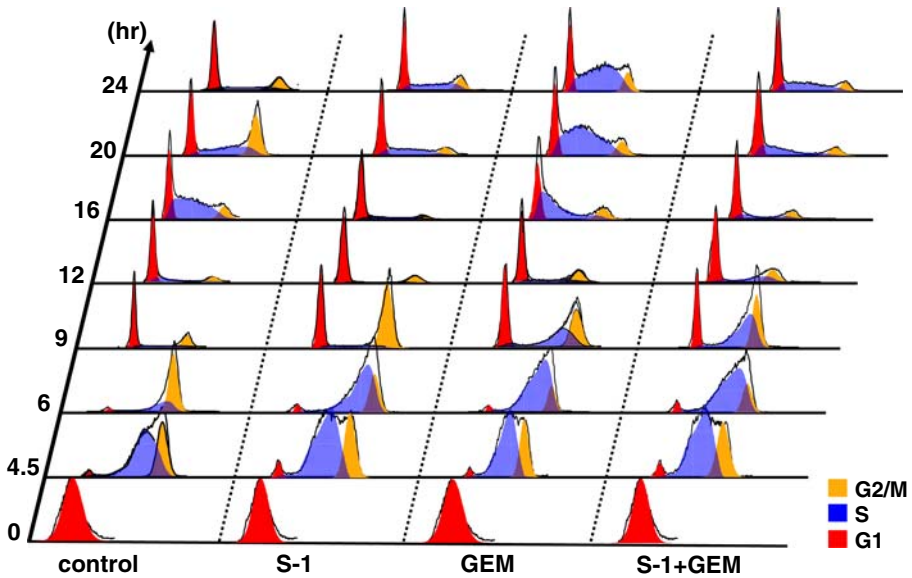

Fig. 4 Cell cycle progression of MIA PaCa-2 cells treated by S-1, GEM, and the combination of both drugs after synchronization with a thymidine double block. Treated cells were already delayed at 6 h from synchronization, which can be detected by comparison with the cells remaining in S phase. At 9 h, S-1 plus GEM-treated cells were delayed compared with the cells treated with S-1, and at 16 h, S-1 plus GEM-treated cells were delayed compared with the cells treated with GEM. Thus, combination therapy with S-1 plus GEM caused the most cell cycle delay compared with each agent alone

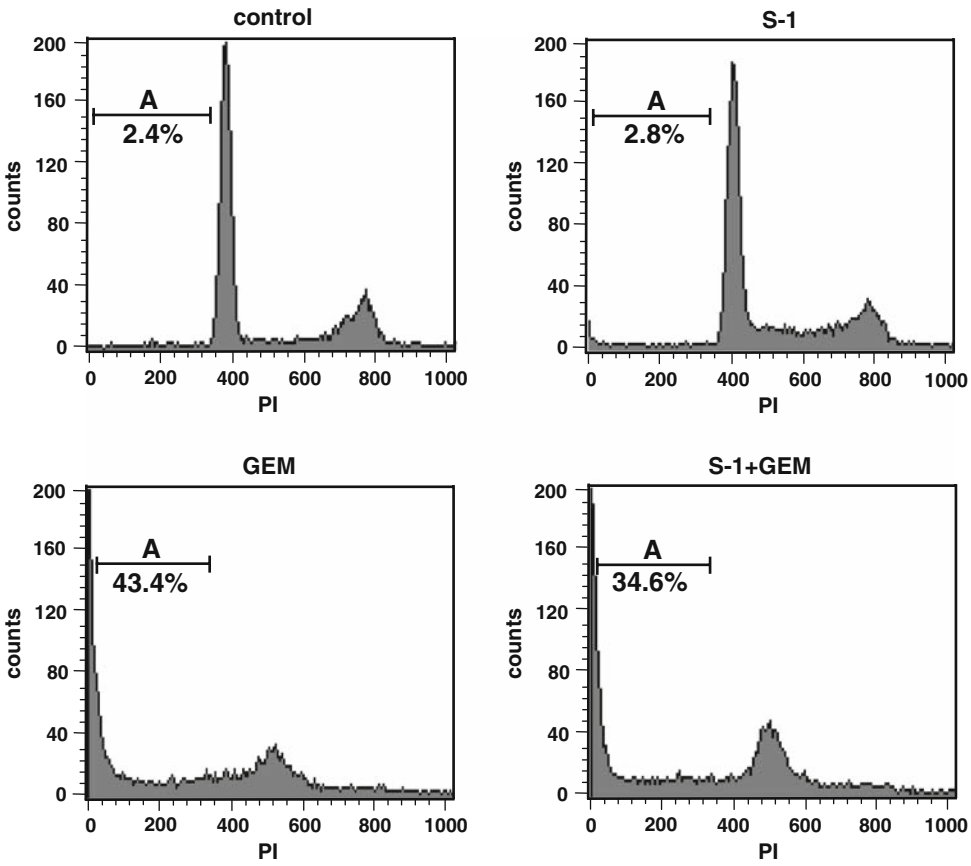

Fig. 5 Sub-G1 assay after 72 h of treatment of S-1, GEM, and the combination of both drugs. Cells in the sub-G1 phase (Area A) are undergoing apoptosis. Data are representative of three experiments. GEM- and S-1 plus GEM-treated cells generated sub-G1 phase cells, while control cells and S-1-treated cells did not

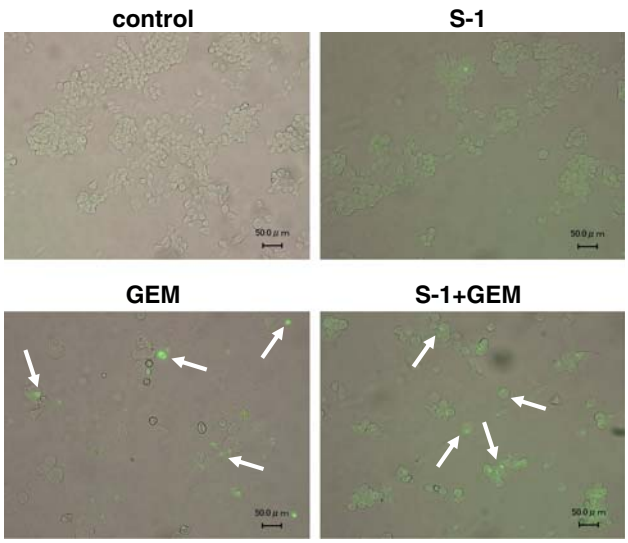

Fig. 6 Detection of apoptosis by a TUNEL assay after 72 h of treatment with S-1, GEM, and combination of both drugs. Apoptotic cell death was observed only after treatment with GEM and S-1 plus GEM. *Arrowheads* indicate TUNEL-positive nuclei in MIA PaCa-2 cells

To determine the most effective combination of antitumor agents with S-1, we conducted MTT assays. From classical isobolograms, S-1 plus GEM, S-1 plus MMC, and S-1 plus ADM showed higher synergism than three other combinations, but S-1 plus GEM was the most effective combination against MIA PaCa-2 cells (Fig. 1).

The efficacy of S-1 plus GEM was also tested in vivo using nude mice as a xenograft model. The S-1 (10 mg/kg) was given orally five times a week for 3 weeks, and GEM (100 mg/kg) was given intravenously every 2–3 days for a total of six times to nude mice. All three groups that were administered single or combined antitumor agents tended to exhibit suppression of the growth of tumors (Fig. 2). However, the lowest T/C (percentage) throughout the experiment was 71.1% for S-1 and 58.3% for GEM, while the combination of S-1 plus GEM had a T/C ratio of 48.2%. In addition, the difference between the T/C ratios of GEM and S-1 plus GEM was statistically significant ($P < 0.05$). These results indicate that the combination of S-1 and GEM enhanced antitumor activity and should be an effective therapy against pancreatic cancer.

Recently, the combination therapy of S-1 plus GEM has been assessed in a clinical study [10, 11]. Some regimens dictate S-1 followed by GEM, while others use a co-administration regimen; these administration schedules are the focus of the studies. Unfortunately, there is no consensus yet on this matter. However, an experimental study using UFT (another oral fluorinated pyrimidine that also

includes FT and uracil as a competitive antagonist of DPD) plus GEM has suggested that this combination is more effective when UFT is administered first, followed by GEM [12]. Our current study showed only the effectiveness of combination chemotherapy of S-1 and GEM. To develop effective therapy, the administration schedule of the two drugs needs to be considered further.

Meanwhile, we tried to clarify the synergistic mechanism induced by S-1 and GEM. S-1 and GEM are both nucleic acid analogs that are incorporated into DNA during its synthesis and stop chain elongation, which leads to the prolongation of the S phase [5, 13]. We weighed the effect of the cell cycle distribution changes caused by S-1, GEM, and the combination of two drugs, and by using cell cycle synchronization, we compared the speed of cell cycle progression. Furthermore, we investigated cell death, especially apoptosis, to elucidate whether cell death is associated with antitumor activity or not.

In the cell cycle analysis, S-1-treated cells began to accumulate in the S phase after 24 h of exposure. Although this behavior tended to decrease afterward, the high distribution in the S phase as compared with the control lasted until 72 h. On the other hand, the S phase in GEM-treated cells significantly increased starting at 12 h, and lasted until 48 h. The distribution was similar to control cells at 72 h. S-1 plus GEM-treated cells showed a similar change as cells treated with GEM only, but the accumulation of the S phase was sustained until 72 h. These results suggest that the effect of S-1 occurs 24 h after exposure and lasts until 72 h, and the effect of GEM appears at 12 h and concludes by 48 h. This result suggests that the cell cycle was delayed at the S phase following treatment with either drug.

In order to more thoroughly evaluate the cell cycle delay, we synchronized the cell cycle at the G1/S boundary by thymidine double block, restarted the cell cycle, and observed the distribution change by a time course. From the cell cycle analysis, S-1 needed 24 h to show its action; thus, S-1 was added midstream of the synchronization, while GEM was added just after the cell cycle was synchronized; 6 h after synchronization, cells treated with S-1, GEM, or S-1 plus GEM were already delayed relative to control cells (Fig. 4). Furthermore, S-1 plus GEM-treated cells were delayed relative to S-1-treated cells at 9 h and GEM-treated cells at 16 h (Fig. 4). These results indicate that by combining S-1 and GEM, more cell cycle delay will occur than is seen with each single agent. These results suggest that, even though S-1 and GEM are both nucleoside analogs, their mechanisms of antitumor action are different. In vitro, S-1 has a mild action whereby S phase arrests with a relatively long duration of cell cycle delay, while GEM has a more severe action of an S phase block with cell cycle delay, together with apoptosis induction.

It is known that DNA synthesis inhibition leads normal cells and even some cancer cells to initiate apoptosis [14]. Because this kind of cell death likely contributed to the synergism we observed, an apoptosis detection assay was conducted; the same results were obtained from both a sub-G1 assay and a TUNEL assay. Apoptotic cells were observed only in GEM-treated cells and S-1 plus GEM-treated cells (Figs. 5, 6). There was no significant difference in the number of apoptotic cells between the two groups. This result raises the possibility that apoptosis was induced by GEM, but not S-1. This difference in mechanism between two nucleoside analogs is probably due to some pharmacologic effect other than the inhibition of DNA chain elongation. Cancer cells tend to be resistant to apoptosis because they typically have mutated p53 [14]. The MIA PaCa-2 cells also have a mutation of the p53 gene [15]. Apoptosis, however, was observed only in groups treated with GEM in our experiment which suggests that GEM induced apoptosis on MIA PaCa-2 cells by a p53-independent pathway. It has been revealed that GEM reduces the membrane potential of mitochondria and releases cytochrome c, which induces apoptosis without activation of caspases in one case [16] and with activation of caspase 8 in another case [17]. It is known that 5-FU-induced apoptosis in cancer cells depends on the presence of wild-type p53, so mutation of p53 also inhibits apoptosis induction by 5-FU [18].

The 5-FU, which is the main component of S-1's antitumor activity, has two main mechanisms of action. First, 5-FU processed through the same pathway as uracil to be converted into fluorodeoxyuridine monophosphate (FdUMP). FdUMP forms a ternary complex with thymidylate synthase (TS) and 5, 10-methylenetetrahydrofolate (5, 10-CH_2-FH_4). This reaction leads to the inhibition of DNA synthesis because of the suppression of deoxythymidine monophosphate (dTMP) synthesis induced by the inactivation of TS. Therefore, it is known that cell cycle delay will occur at the S phase [5]. Secondly, 5-FU also converts to fluorouridine triphosphate and incorporates into RNA, which produces F-RNA, resulting in an RNA metabolic disorder [19–21]. Meanwhile, after GEM, a derivative of ara-C, is transported into the cells, it must be phosphorylated by deoxycytidine kinase to GEM diphosphate (dFdCDP) and GEM triphosphate (dFdCTP), an active form of GEM. The dFdCTP inhibits the chain elongation of DNA strands which produces the block at the S phase. Also, dFdCDP inhibits ribonucleotide reductase, which produces deoxycytidine triphosphate (dCTP), an antagonist of dFdCTP. This leads to a reduction of dCTP but augmentation of dFdCTP into cells and enhances its antitumor activity. This interaction is termed "self-potentiation" [22]. In this way, GEM is thought to have a greater ability to block the S phase than does S-1. In combination

therapy in vitro, the S phase block by S-1 and GEM leads to a synergistic delay in the cell cycle; in addition, the cell-killing effect induced by GEM might contribute to their high combinatorial antitumor effect. This mechanism is also thought to be associated with antitumor activity in vivo. Using molecular analysis, another group has reported that incorporation of GEM was enhanced by 5-FU [23]. It raises the possibility that the enhanced incorporation of GEM into cells by 5-FU causes the synergistic delay of the cell cycle.

To date, S-1 has been used to treat pancreatic cancer much less frequently than was GEM. Combination chemotherapy including S-1 and GEM is still in development. We believe that this study has demonstrated the effectiveness of combination therapy of S-1 and GEM at the experimental level. From here, we would like to clarify the synergism of the S-1 and GEM-induced cell cycle delay and apoptosis at the molecular level, and provide further insights into the synergy mechanism in vivo.

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