Effect of a combination of S-1 and gemcitabine on cell cycle regulation in pancreatic cancer cell lines

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In a previous study, we showed that a combination of an oral fluoropyrimidine anticancer agent (S-1) and gemcitabine (GEM) had synergistic effects on cell growth and cell cycle arrest in the pancreatic cancer cell line MIA PaCa-2. Therefore, we conducted further mechanistic studies using the pancreatic cancer cell lines MIA PaCa-2 and SUIT-2. The combined effect of S-1 and GEM in SUIT-2 cells was evaluated using an 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, and the effects of S-1, GEM and S-1 plus GEM on cell cycle regulation were assessed using flow cytometry. We also examined the expression of several cell cycle regulatory proteins in both MIA PaCa-2 and SUIT-2 cells by western blotting. Classical isobolographic analysis of the MTT assay results showed that the combination of S-1 and GEM had a synergistic effect in SUIT-2 cells, and flow cytometric analysis of the cell cycle showed that the combination of S-1 plus GEM induced S-phase arrest to a greater degree than did either S-1 or GEM alone. Also, the combination of S-1 and GEM resulted in the downregulation of cyclin D1 expression and upregulation of cyclin A, p21 and p27 expression levels. Treatment of MIA PaCa-2 and SUIT-2 cells with a combination of both drugs also led to the increased phosphorylation of checkpoint kinase 1. Combined treatment with S-1 and GEM resulted in more prolonged S-phase arrest than with either treatment alone. This difference is shown to be potentially due to the higher levels of phosphorylated checkpoint kinase 1 in pancreatic cancer cell lines treated with the two agents. *Anti-Cancer Drugs* 23:505–514 © 2012 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Introduction

Pancreatic cancer is a very aggressive neoplasm with a short median survival time. It is the fifth leading cause of cancer-related death in Japan, as well as in the US and Europe [1]. Because of delays in clinical diagnosis, pancreatic cancer is often detected at an advanced stage, and the prognosis is extremely poor. Therefore, further improvements in systemic chemotherapy are needed to increase the survival of patients with pancreatic cancer.

Gemcitabine (GEM), a pyrimidine nucleoside analogue, is the standard treatment modality for advanced pancreatic cancer. However, the clinical benefit provided by GEM is poor, with a median survival of only 5–7 months [2]. This is because pancreatic cancer cells rapidly become tolerant to the drug [3].

S-1 is a new oral fluoropyrimidine developed by Taiho Pharmaceutical Co. Ltd (Tokyo, Japan). The drug contains a combination of tegafur (FT), 5-chloro-2, 4-dihydroxypyridine (CDHP) and oteracil potassium (Oxo) at a molar ratio of 1 : 0.4 : 1 [4]. Tegafur is a prodrug of 5-FU and is catabolized by dihydropyrimidine dehydrogenase in the liver and tumour tissues [5,6]. CDHP is a competitive inhibitor of dihydropyrimidine dehydrogenase and acts to maintain effective concentrations of 5-FU in both plasma and tumour tissues [7]. Oxo, a competitive inhibitor of orotate phosphoribosyltransferase, inhibits the phosphorylation of 5-FU in the gastrointestinal tract, thus reducing the serious gastrointestinal toxicity associated with this drug [8]. The antitumour effect of S-1 has already been demonstrated in a variety of solid tumours [9–11]. With regard to pancreatic cancer, S-1 had a reported response rate of 37.5% and a median survival time of 8.8 months when used for single-agent chemotherapy in a phase II study [12]. Therefore, the combined use of S-1 and GEM to treat pancreatic cancer is eagerly anticipated in Japan.

Good therapeutic efficacy was reported by two representative clinical trials [13,14]; however, the experimental and clinical evidence for combination therapy is not yet sufficient, and there is little information regarding the mechanisms underlying the combined effects of these drugs.

A previous study using MIA PaCa-2 cells showed that a combination of S-1 and GEM was more effective than

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either drug alone, both *in vitro* and *in vivo*. Moreover, a combination of S-1 and GEM induced a greater degree of S-phase arrest than did either agent alone [15].

The purpose of this study was to investigate the relationship between S-phase arrest and the expression of cell cycle regulatory proteins such as cyclin D1, cyclin E, cyclin A, p21, p27 and checkpoint kinase 1 (Chk1) following treatment of two pancreatic cancer cell lines, MIA PaCa-2 and SUIT-2, with S-1 plus GEM.

Materials and methods

Cell culture

The human pancreatic cancer cell lines MIA PaCa-2 and SUIT-2 were obtained from the American Type Culture Collection (Rockville, Maryland, USA) and Japanese Collection of Research Bioresourse (Osaka, Japan), respectively. MIA PaCa-2 cells were grown in Dulbecco's Modified Eagle Medium (Wako Pure Chemical Industries, Ltd, Osaka, Japan) supplemented with 10% fetal bovine serum (Invitrogen Corporation, Carlsbad, California, USA), 2.5% horse serum (Invitrogen Corporation), penicillin and streptomycin. SUIT-2 cells were grown in RPMI1640 (Wako Pure Chemical Industries) supplemented with 10% fetal bovine serum (Invitrogen Corporation), penicillin and streptomycin.

Antitumour agents

S-1 was obtained from Taiho Pharmaceutical Co. Ltd (Tokyo, Japan), and GEM was from Eli Lilly Japan K.K. (Kobe, Japan).

Antibodies

Polyclonal antibodies against cyclin D1 (H-295), cyclin E (C-19), cyclin A (H-432), p27 (C-19), Chk1 (FL-476) and glyceraldehyde-3-phosphate dehydrogenase (FL-335) were purchased from Santa Cruz Biotechnology (Santa Cruz, California, USA). Monoclonal antibodies against p21 (12D1) and phosphorylated-Chk1 (Ser345) were purchased from Cell Signaling Technology (Beverly, Massachusetts, USA).

Cell proliferation assay [3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide assay]

Cells were seeded in 96-well plates at a density of 1×10^3 cells/well. Twenty-four hours later, the culture medium was replaced with 0.2 ml of fresh medium containing S-1 (0.1–10 μg/ml) and/or GEM (0.00025–0.005 μg/ml). After a further 72 h, 20 μl of 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) reagent [3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (Sigma, St Louis, Missouri, USA) 0.4% in 0.1 mol/l disodium succinate hexahydrate (Kanto Chemical Co. Inc., Tokyo, Japan)] was added to each well. The plate was then incubated for 4 h, after which 0.15 ml of dimethyl sulfoxide (Kanto Chemical Co. Inc.) was added to each well to dissolve the formazan crystals. The absorbance of each well was then measured using a microplate reader (Elx800 Winooski, Vermont, USA) at a wavelength of 540 nm. The inhibition index (II, %) was calculated using the following formula:

$$\mathrm{II}\,(\%) = (b - c) / (b - a) \times 100,$$

where a is the optical density (OD) of cells, b the OD of cells + MTT reagent, c the OD of cells + MTT reagent + antitumour agent. Inhibitory concentration (IC_{50}) values were then calculated. A classical isobologram was used to evaluate the synergistic effects of S-1 and GEM [16,17]. All experiments were repeated at least four times.

Cell cycle analysis

Cells (7.0×10^4) were seeded in dishes and cultured for 24 h. The medium was replaced by fresh medium containing the antitumour agents (S-1, 0.3 μg/ml or GEM, 0.006 μg/ml) and the cells were incubated for the indicated times. The same concentrations were also used when the two drugs were combined. For flow cytometry, cells were trypsinized, pelleted by centrifugation, and resuspended in 0.3 ml of 0.1% Triton X-100 (Sigma)/PBS. Cells were treated with RNase Type I-A (Sigma) at 37°C for 15 min and stained with propidium iodide for 10 min. DNA content was determined using a FACSCalibur (Becton Dickinson Biosciences, San Jose, California, USA). Cell cycle distribution was analysed using ModFit LT (version 3.0) (Becton Dickinson Biosciences) cell cycle analysis software.

Western blot analysis

Cells (6.0×10^5) were incubated as described above for cell cycle analysis. Cells were rinsed twice with PBS and scraped into lysis buffer [25 mmol/l Tris HCL (pH 7.6), 150 mmol/l NaCl, 1% nonidet P-40, 1% sodium deoxycholate, 0.1% SDS, complete protease inhibitor cocktail (Roche, Mannheim, Germany) and PhosSTOP phosphatase inhibitor cocktail (Roche)]. After incubation on ice for 20 min, the cell lysates were cleared by centrifugation at 15 000*g* at 4°C for 15 min. Protein concentration was determined using the bicinchoninic acid protein assay reagent (Pierce, Rockford, Illinois, USA). Samples were boiled for 15 min and diluted in 2× loading buffer. Equal amounts of total protein were separated on 12% SDS polyacrylamide gels at a constant current of 20 mA. Separated proteins were then transferred to polyvinylidene difluoride membranes (Millipore, Billerica, Massachusetts, USA) at 30 V. The membrane was blocked for 1 h with Block Ace (DS Pharma Biomedical, Osaka, Japan) and hybridized overnight at 4°C with various primary antibodies. Membranes were then probed with a horseradish peroxidase-conjugated anti-rabbit antibody (Santa Cruz Biotechnology or Cell Signaling Technology) and chemiluminescence was developed using an enhanced chemiluminescence detection kit (GE Healthcare UK Ltd, Amersham, UK), followed by exposure to hyper film (GE Healthcare). Band intensities were analysed using Image J 1.41 (National Institutes of Health, Bethesda, Maryland, USA).

Statistical analysis

The data from the MTT assays and cell cycle analyses were expressed as means ± SD, and western blotting data were expressed as means ± SEM. Differences between groups were examined for statistical significance using analysis of variance followed by Fisher's protected least significant difference analysis. A *P*-value of less than 0.05 was considered statistically significant. Statistical analysis was performed using STATVIEW for Windows version 5 (SAS Institute, Cary, North California, USA).

Results

A combination of S-1 and GEM has a synergistic, antiproliferative effect in SUIT-2 cells

An MTT assay was used to investigate whether S-1 and GEM exhibited cooperative effects in inhibiting cell growth in SUIT-2 cells. S-1 is a combination drug comprising three compounds, but in our study we omitted the Oxo component (Oxo is used to reduce gastrointestinal side effects) and used instead a combination of 5-FU and CDHP at a molar ratio of 1 : 2. First, we calculated the IC_{50} values for each drug. The IC_{50} values for S-1 and GEM were 0.69 ± 0.13 and 0.0011 ± 0.0003 μg/ml, respectively.

Simultaneous administration of various concentrations of S-1 and GEM resulted in an increased inhibition of SUIT-2 cell growth (Table 1). The IC_{50} values of S-1 and GEM were connected using a dotted line in a classical isobologram to evaluate any possible synergistic effects (Fig. 1). The data showed that these drugs had a synergistic effect in both SUIT-2 and MIA PaCa-2 cells [15].

The combination of S-1 and GEM induces S-phase arrest in SUIT-2 cells

We next performed flow cytometric analyses to examine changes in cell cycle progression when cells were exposed to S-1 and/or GEM (Fig. 2). The results showed that the percentage of untreated control cells in the S-phase gradually decreased for up to 72 h after treatment with both agents. The distribution of cell cycle phases in S-1-treated cells was similar to that in control cells up to 24 h after treatment. At 48 h, cells in S-phase began to accumulate and remained in S-phase up to 72 h after treatment. Meanwhile, there was a marked accumulation of GEM-treated cells in S-phase between 12 and 24 h after treatment, but this decreased after 48 h. However, cells treated with a combination of S-1 and GEM showed a greater accumulation in S-phase between 12 and 72 h than did cells treated with either agent alone. The combined treatment group exhibited characteristics seen in both the S-1-treated group (S-phase accumulation occured late in the 72 h incubation period) and the GEM-treated group (S-phase accumulation occurred early). A significant difference in the percentage of cells arrested in S-phase between the combination group and both single agent groups was observed at 48 h ($P < 0.05$).

The combination of S-1 and GEM affects the expression of cyclins D1, E and A

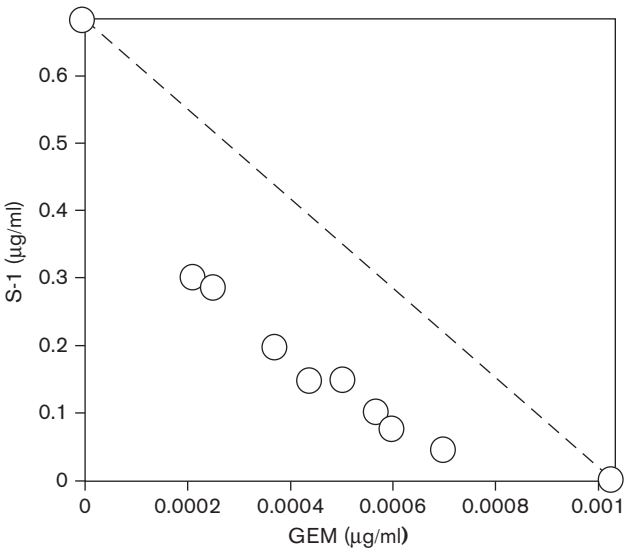
To further explore the mechanisms underlying S-phase arrest caused by the combination of S-1 and GEM, we investigated the levels of cyclin D1, cyclin E and cyclin A in the two cell lines. We previously found that the combination of S-1 and GEM induced S-phase arrest in MIA PaCa-2 cells after 72 h [15]. In the present study, cell cycle analyses indicated that the combination of S-1 and GEM caused SUIT-2 cells to accumulate in S-phase at 48 h. Therefore, we examined the expression of cyclin D1, cyclin E and cyclin A during cell cycle arrest at 72 h in MIA Paca-2 cells and at 48 h in SUIT-2 cells. In MIA PaCa-2, combined treatment with S-1 and GEM reduced the levels of cyclin D1 and cyclin E protein at 72 h

Table 1 **Effect of combination therapy of S-1 and GEM against SUIT-2**

	GEM (μg/ml)					
S-1 (μg/ml)	0	0.00025	0.0005	0.0006	0.0007	0.001
0	–	2.7	21.9	42.0	52.7	72.8
0.1	11.2	19.7	41.1	52.7	58.6	75.0
0.15	27.9	30.9	52.1	61.5	68.2	77.9
0.2	37.5	39.8	54.9	64.3	69.4	77.7
0.3	49.6	54.6	65.6	70.7	73.8	80.4
0.5	62.6	64.7	71.4	75.6	77.2	82.3

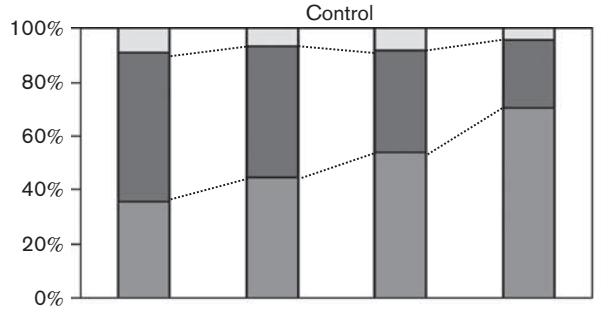
Data are shown as the mean inhibition index (%) from four separate experiments. Numbers on a grey background are greater than or equal to 50.
GEM, gemcitabine; S-1, oral fluoropyrimidine anticancer agent.

Fig. 1

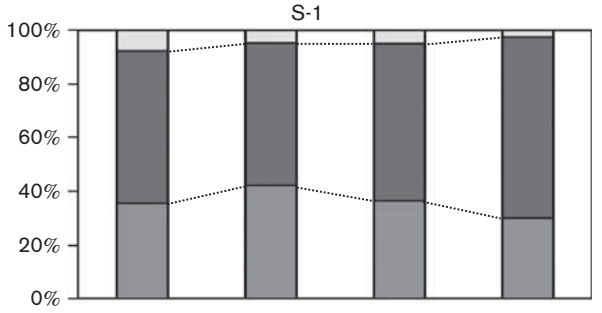


Classical isobologram showing treatment of SUIT-2 cells with a combination of S-1 and GEM. The IC_{50} values for S-1 and GEM were connected with a dotted line to distinguish the area of synergism and the area of antagonistic effect. Plots on the dotted line indicate an additive effect. S-1 plus GEM shows a synergistic effect. GEM, gemcitabine; IC_{50}, inhibitory concentration; S-1, oral fluoropyrimidine anticancer agent.

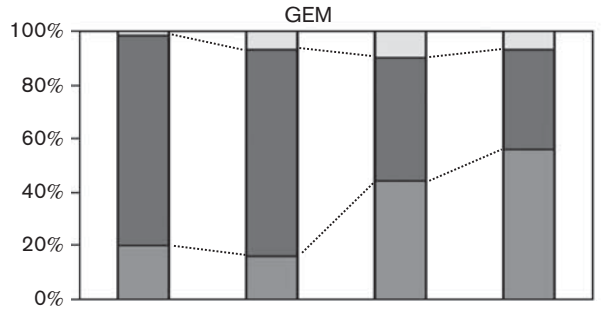
Fig. 2



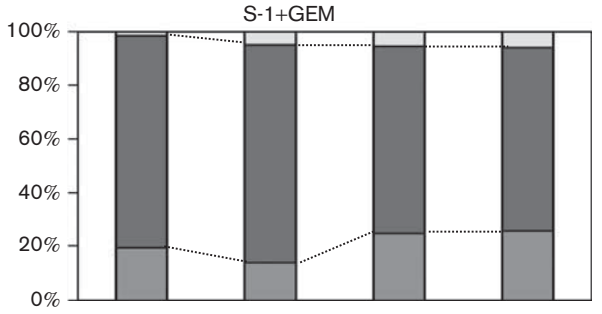
Time	12 h	24 h	48 h	72 h
G2/M	9.1±0.9	6.7±0.5	8.2±0.7	4.4±0.7
S	55.1±1.3	48.6±1.7	37.7±0.3	25.1±1.3
G1	35.8±0.4	44.7±1.5	54.1±0.7	70.5±1.1



Time	12 h	24 h	48 h	72 h
G2/M	7.7±0.4	4.6±0.3	5.0±0.3	2.5±1.9
S	56.8±0.4	53.4±0.7	58.5±0.6	67.6±0.9
G1	35.5±0.6	42.0±0.5	36.5±0.9	29.9±2.0



Time	12 h	24 h	48 h	72 h
G2/M	1.7±0.2	6.9±0.4	9.9±1.4	7.0±0.2
S	78.3±0.1	77.1±0.4	46.0±0.7	37.1±1.5
G1	20.0±0.3	16.0±0.6	44.1±0.9	55.9±1.6



Time	12 h	24 h	48 h	72 h
G2/M	1.7±0.3	5.2±0.2	5.6±0.3	6.0±0.2
S	78.8±0.1	80.9±0.2	69.6±1.2	68.1±0.9
G1	19.5±0.3	13.9±0.3	24.8±0.9	25.9±1.1

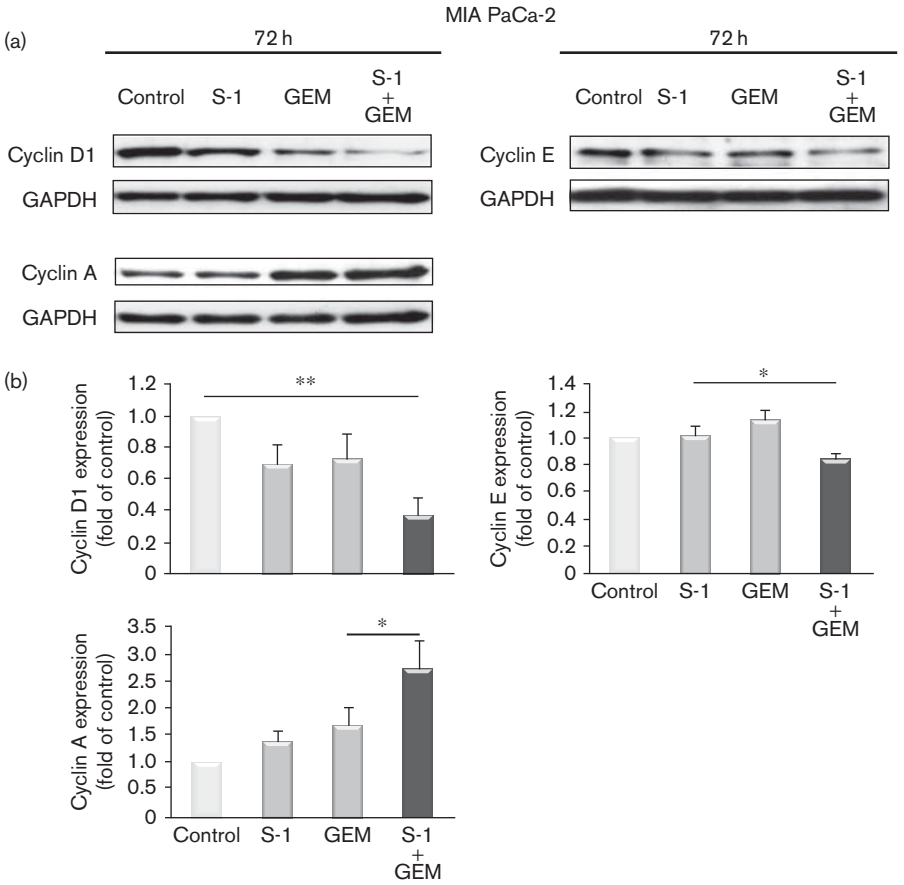
Changes at each phase of the cell cycle induced in SUIT-2 cells by S-1, GEM, or a combination of both drugs. Data (mean ± SD) are from three separate experiments ($n=3$). GEM, gemcitabine; S-1, oral fluoropyrimidine anticancer agent.

(Fig. 3a and b). The expression of cyclin E was also reduced. In contrast, combined treatment with S-1 and GEM resulted in maximal expression of cyclin A. In SUIT-2 cells, S-1 plus GEM and S-1 as a single agent significantly reduced the levels of cyclin D1. In contrast, the expression of cyclin E was significantly increased at 48 h (Fig. 4a and b).

The combination of S-1 and GEM reduces the expression of the cyclin-dependent kinase inhibitor proteins p21 and p27

We next investigated the expression of p21 and p27, which are cyclin-dependent kinase (CDK)-inhibiting factors that function in cell cycle progression. In MIA PaCa-2 cells, the combination of S-1 and GEM resulted in reduced levels of both p21 and p27 compared with the control group at 72 h (Fig. 5a and b). Quantitative densitometry showed that the combination of S-1 and GEM induced 0.4-fold and 0.3-fold reductions in p21 and p27 expression levels, respectively. In SUIT-2 cells, the combination of S-1 and GEM reduced the levels of p21 and p27 compared with the GEM-treated group at 48 h (Fig. 5c and d). Quantitative densitometry showed that the combination of S-1 and GEM induced 0.4-fold and 0.7-fold reductions in p21 and p27 expression levels, respectively.

Fig. 3



Effect of S-1, GEM and a combination of both drugs on cyclin D1, cyclin E and cyclin A levels in MIA PaCa-2 cells (a and b). MIA PaCa-2 cells were treated for 72 h either without (control) or with S-1, GEM or a combination of both. The relative band intensities of cyclin D1, cyclin E and cyclin A were quantified using densitometric analysis (b). Values are expressed as the mean ± SEM of three independent experiments ($n=6$). *P less than 0.05, **P less than 0.01 by Fisher's PLSD. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GEM, gemcitabine; PLSD, protected least significant difference; S-1, oral fluoropyrimidine anticancer agent.

The combination of S-1 and GEM induces checkpoint kinase 1 phosphorylation

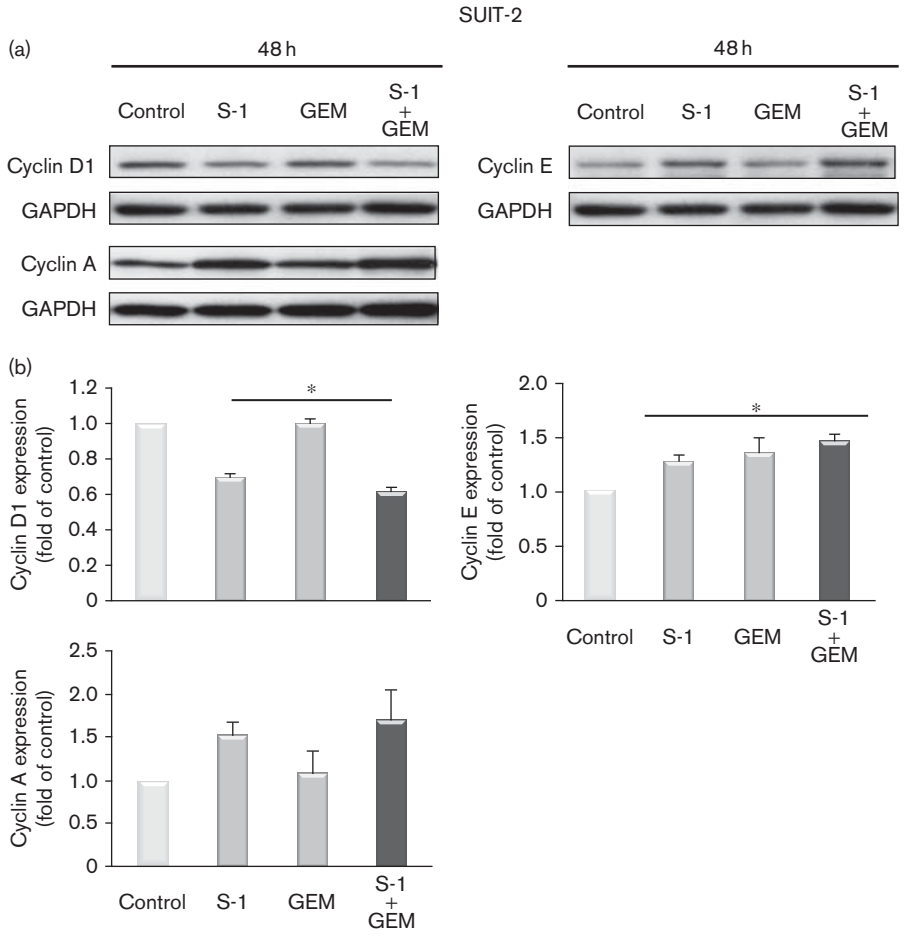
We also examined the phosphorylation of the DNA damage-induced cell cycle checkpoint protein Chk1. Western blotting was used to assess the levels of phosphorylated Chk1 (P-Chk1) in the two cell lines. MIA PaCa-2 cells treated with S-1 alone, or with a combination of S-1 plus GEM, showed a greater accumulation of phosphorylated Chk1 between 48 and 72 h than did the control group, or cells treated with GEM alone, without any simultaneous changes in total Chk1 protein levels (Fig. 6a and b). At 72 h, quantitative densitometry showed that P-Chk levels in cells treated with either S-1 or a combination of S-1 and GEM were 2.0-fold and 2.5-fold higher than those in the control. There was a significant difference between the S-1 group and the combination group. In SUIT-2 cells, greater levels of P-Chk1 accumulation were observed in cells treated with a combination of S-1 plus GEM at 24 h compared with the control group and the GEM-treated group, without any simultaneous changes in total Chk1 protein levels (Fig. 6c and d). The combination of S-1 and GEM resulted in increased levels of P-Chk1 protein compared with GEM treatment alone at 48 h.

Discussion

GEM is a first-line antitumour drug used to treat pancreatic cancer, but its response rate is poor and cells quickly acquire resistance. Therefore, improvements in combination chemotherapy are urgently needed.

S-1 is a new type of antitumour drug that comprises three components that act to enhance the effect of 5-FU [14]. The antitumour effects of this drug have been shown against gastric cancer [9], colon cancer [10], advanced non-small-cell lung cancer [11], and pancreatic and biliary tract cancer in both Japan and Korea [12,18]. Because treatment with GEM alone has a poor response rate, various clinical trials involving combination chemotherapy have been conducted in the search for a more efficient

Fig. 4



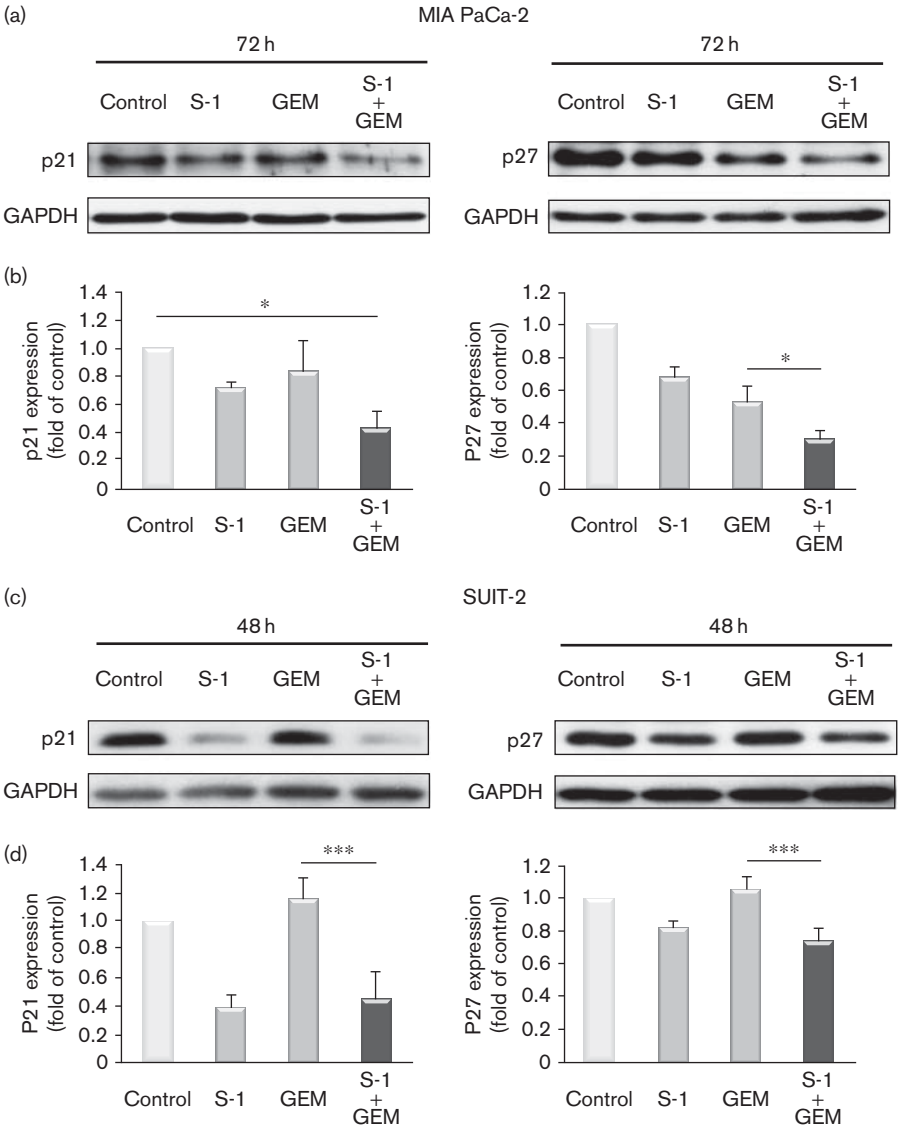
Effect of S-1, GEM and a combination of both drugs on cyclin D1, cyclin E and cyclin A levels in SUIT-2 cells (a and b). SUIT-2 cells were treated for 48 h either without (control) or with S-1, GEM or a combination of both. The relative band intensities of cyclin D1, cyclin E and cyclin A were quantified using densitometric analysis (b). Values are expressed as the mean ± SEM. of three independent experiments ($n = 6$). *P less than 0.05 by Fisher's PLSD. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GEM, gemcitabine; PLSD, protected least significant difference; S-1, oral fluoropyrimidine anticancer agent.

therapy. In a previous study, we examined various combinations of GEM and other antitumour drugs using the pancreatic cell line MIA Paca-2 and discovered the superior antitumour effects of a combination of S-1 and GEM [15]. Furthermore, we showed that the mechanism underlying their combined effect was related to cell cycle arrest and apoptosis in the treated cells.

In the present study, we showed the synergistic effect of S-1 and GEM against MIA Paca-2 and SUIT-2 cells using an MTT assay (Fig. 1). Using cell cycle analysis, we also showed that S-1 plus GEM induced a greater accumulation of SUIT-2 cells in S-phase at 48 and 72 h after treatment than did either agent alone (Fig. 2). In contrast, the greatest S-phase accumulation was observed at 12 and 72 h after treatment with GEM and S-1, respectively (Fig. 2). These results reveal that the combination of S-1 plus GEM leads to prolonged and increased S-phase accumulation, which may reflect the synergistic effect of the two agents, as shown in the MTT assay.

To confirm the accumulation of cells in S-phase, we analysed the expression of cyclins and CDK inhibitors in the two cell lines. Cyclin is a cell cycle oscillator protein that is repeatedly expressed and degraded throughout the cell cycle. Cyclin D1, cyclin E and cyclin A appear in the G1, late G1 or early S, and the S or G2 phases, respectively. Cyclins function by binding CDKs, which are cell cycle engines [19,20]. CDK inhibitors act as 'cell cycle brakes' that bind complexes of cyclin and CDK and inhibit their cell cycle accelerator function. We analysed two CDK inhibitors, $p21^{CIP1/WAF}$ (hereafter referred to as p21) and $p27^{KIP1}$ (hereafter referred to as p27), both of which are deeply involved in the repair of DNA damage and in cell cycle arrest [21,22]. p21 is controlled by p53

Fig. 5

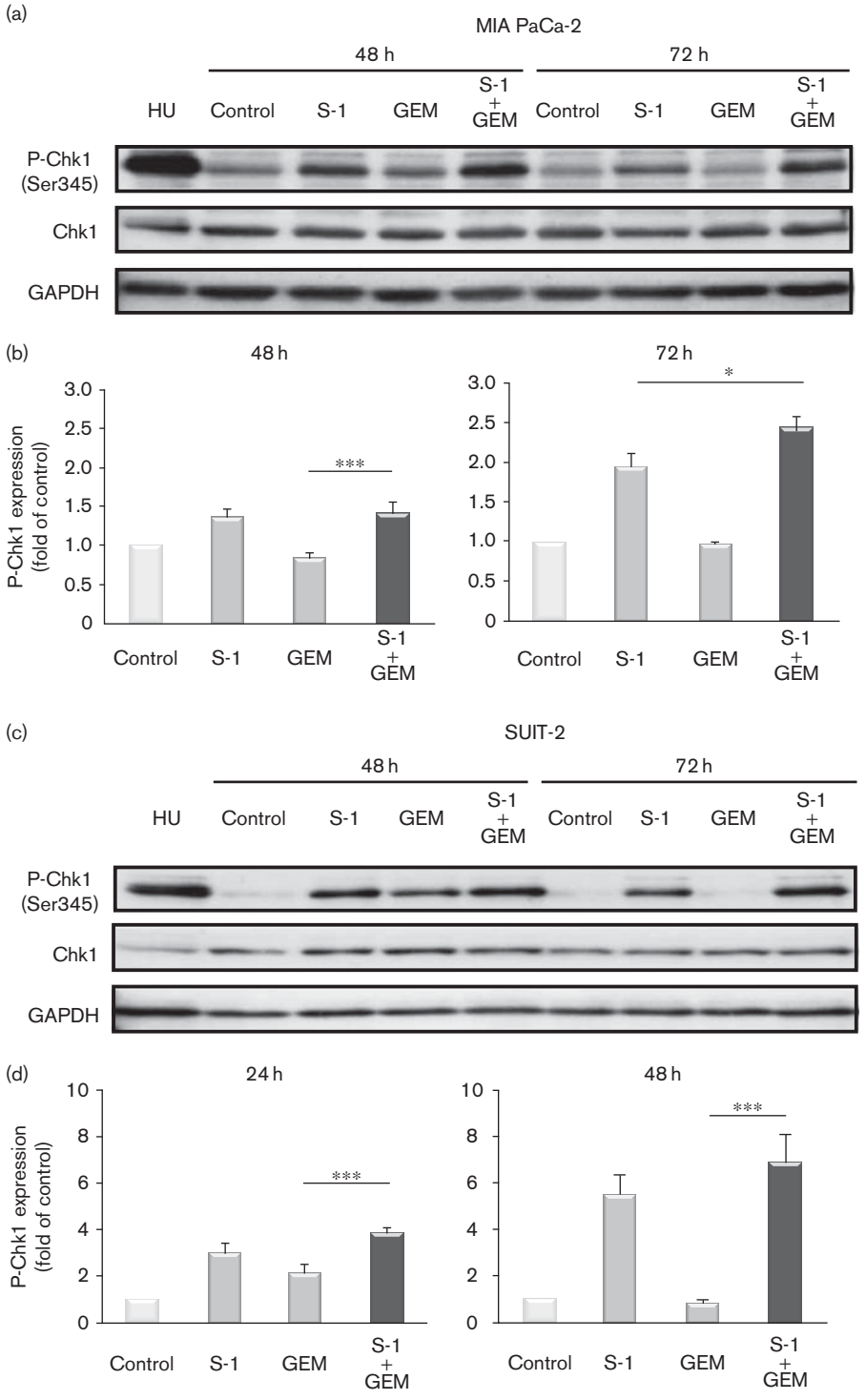


Effect of S-1, GEM and a combination of both drugs on p21 and p27 expression in MIA PaCa-2 (a and b) and SUIT-2 (c and d) cells. MIA PaCa-2 and SUIT-2 cells were treated for 72 h and 48 h, respectively, with S-1, GEM or a combination of both drugs. Control cells received no treatment. The relative band intensities for p21 and p27 were quantified using densitometric analysis (b and d). Values are expressed as the mean ± SEM of three independent experiments ($n=6$). *P less than 0.05, ***P less than 0.005 by Fisher's PLSD. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GEM, gemcitabine; PLSD, protected least significant difference; S-1, oral fluoropyrimidine anticancer agent.

and stops the cell cycle to allow time for DNA damage repair. p27 is activated by a defect in growth signals. Both p21 and p27 are ubiquitinylated by a Skp1-Cullin 1-F-box protein:ubiquitin ligase complex, which contains the F-box protein S-phase kinase-associated protein 2. S-phase kinase-associated protein 2-mediated p21 and p27 degradation is induced during S-phase [23,24].

The expression level of cyclin D1 in MIA PaCa-2 cells treated with each agent alone showed a tendency to decrease compared with that in control cells; however, no significant difference was detected 72 h after treatment with the antitumour drugs (Fig. 3a and b). Cyclin D1 levels in cells treated with S-1 plus GEM were significantly decreased compared with those in control cells and seemed to be lower than those in cells treated with either agent alone. Meanwhile, the level of cyclin E (increased during the late G1-phase and early S-phase) in S-1 plus GEM-treated cells was lower than that in cells treated with either agent alone. The level of cyclin A, which increases during the middle-to-late S-phase, was maximal in cells treated with a combination of S-1 plus

Fig. 6



Effect of S-1, GEM and a combination of both drugs on Chk1 expression in MIA PaCa-2 (a and b) and SUIT-2 (c and d) cells. MIA PaCa-2 and SUIT-2 cells were treated for the indicated times either without (control) or with S-1, GEM or a combination of both drugs. The relative band intensities for phosphorylated Chk1 (P-Chk1) were quantified using densitometric analysis (b and d). Values are expressed as the mean ± SEM of three independent experiments ($n=6$). HU (hydroxy urea) was used as a positive control for P-Chk1. *P less than 0.05, ***P less than 0.005 by Fisher's PLSD. Chk1, checkpoint kinase 1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GEM, gemcitabine; PLSD, protected least significant difference; S-1, oral fluoropyrimidine anticancer agent.

GEM. The increase in cyclin A protein levels suggests that, after treatment with a combination of S-1 plus GEM, cells accumulate in the middle-to-late S-phase. Takeda *et al.* [25] and Crescenzi *et al.* [26] showed that 5-FU or GEM induced cells to accumulate in S-phase and also increased the expression of cyclin A. At the same time points, MIA-Paca2 cells treated with S-1 plus GEM expressed lower levels of p21 and p27 than did control cells (or cells treated with either agent alone) (Fig. 5a and b). These observations suggest that the combination of S-1 plus GEM induced cell cycle arrest in late S-phase after the degradation of p21 and p27 in MIA Paca-2 cells.

Cyclin D1 protein levels in SUIT-2 cells were reduced to a greater extent after combined treatment than after treatment with either agent alone (Fig. 4a and b). There were no significant differences in the expression of cyclin A among the treatment groups, but the expression of cyclin E protein was more strongly upregulated in SUIT-2 cells treated with S-1 plus GEM than in the control group. At the same time points, the combination of GEM plus S-1 resulted in significant decreases in p21 and p27 levels compared with GEM alone and in untreated controls (Fig. 5c and d).

These results suggest that, in SUIT-2 cells, the combination of S-1 plus GEM induced cell cycle arrest in early S-phase before the degradation of cyclin E and after degradation of p21 and p27.

The low levels of p21 and p27 observed in both cell lines after combined treatment suggest that degradation of these proteins occurred as the cells entered S-phase.

We also examined Chk1 phosphorylation. Chk1 is the major activator of cell cycle checkpoints, including the intra-S-phase checkpoint. Phosphorylation of Chk1 (to yield P-Chk1) arrests the cell cycle. On DNA damage caused by various genotoxic stresses, Chk1 is activated by phosphorylation, and P-Chk1 inactivates the protein phosphatase Cdc25, thereby preventing the activation of its downstream target Cdc2 kinase. When Cdc2 kinase, which is responsible for the G2/M transition, is inactive, cells are arrested in S-phase [27]. Recently, it was shown that 5-FU or GEM induced S-phase arrest through activation of the Chk1 pathway [28,29].

Treatment with a combination of S-1 plus GEM for 72 h resulted in maximum accumulation of P-Chk1 protein levels in MIA PaCa-2 cells (Fig. 6a and b). In SUIT-2 cells, combination therapy induced greater accumulation of P-Chk1 at 48 h than did treatment with GEM alone or no treatment (Fig. 6c and d). In both MIA PaCa-2 and SUIT-2 cells, the time points at which P-Chk1 levels increased after treatment with a combination of S-1 plus GEM coincided with cell cycle arrest in S-phase (Figs 2 and 6) [15].

5-FU, which is the main antitumour agent within S-1, is converted intracellularly to several active metabolites: fluorodeoxyuridine monophosphate, fluorodeoxyuridine triphosphate and fluorouridine triphosphate. These active metabolites deplete deoxythymidine monophosphate, which is necessary for DNA synthesis. They are also incorporated into RNA, disrupting normal DNA synthesis and RNA processing and function [30]. GEM undergoes complex intracellular conversion to yield the nucleotides gemcitabine diphosphate and triphosphate. These compounds mediate its cytotoxic effects, which are induced after the incorporation of dCTP into the growing DNA strand and the subsequent arrest of DNA strand elongation [31]. Our results suggest that the superior antitumour effects mediated by the combination of S-1 and GEM are produced through increased S-phase arrest. This occurs through Chk1 activation, which is triggered by severe damage to the mechanisms involved in DNA synthesis.

S-1 seems to be capable of inducing Chk1 activation more strongly than GEM in MIA-Paca2 and SUIT-2 cells (Fig. 6). However, GEM is effective at much lower doses than S-1 and is, therefore, more tolerable for patients. The combination of GEM and S-1 is thought to be more effective than single-agent therapies, not only because of their synergic antitumour effects but also because of supplementary effect to sensitivities of each tumour to these two drugs. All tumours have genetical heterogeneity and heterogenic (or 'different') chemosensitivity even if they occur in the same organ and are classified into the same category [32]. Therefore, combination chemotherapy of multiple drugs can be expected to cover wider cases of patients with tumour than single-drug chemotherapy.

The prognosis for pancreatic cancer remains dismal, and chemotherapy results in only limited improvement [33]. The unsatisfactory results seen for chemotherapy in pancreatic cancer may be due to various mutations in genes involved in the control of cellular proliferation, cell cycle, and apoptosis [34]. In fact, most pancreatic cancer cell lines are resistant to apoptosis induced by antitumour drugs [35]. Our previous study showed that treatment with a combination of S-1 and GEM had no effect on apoptosis [15]. Therefore, the present study suggests that the effectiveness of S-1 plus GEM against pancreatic cancer is related to the inhibition of cell proliferation mediated by cell cycle arrest.

In conclusion, this study shows the synergistic effects of a combination of S-1 and GEM at the experimental level and has elucidated, at least in part, the molecular mechanism underlying these combined effects. We hope that these results lead to the development of more effective chemotherapy treatments for pancreatic cancer.

Acknowledgements

Conflicts of interest

There are no conflicts of interest.

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