Schedule-dependent synergistic interaction between gemcitabine and oxaliplatin in human gallbladder adenocarcinoma cell lines

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To define the most effective combination schedule of gemcitabine and oxaliplatin (L-OHP), we investigated the in-vitro interaction between these drugs in a panel of four human gallbladder adenocarcinoma cell lines (HAG-1, GB-d1, NOZ, and G-415). Cytotoxic activity was determined by the WST-1 assay. Different schedules of the two drugs were compared and evaluated for synergism, additivity, or antagonism with a quantitative method based on the median-effect principle of Chou and Talalay. Cell cycle perturbation and apoptosis were evaluated by flow cytometry. Simultaneous and sequential treatments of gemcitabine followed by L-OHP exhibited synergistic effects in all four cell lines, whereas the reverse sequence largely showed an antagonism. Gemcitabine exclusively arrested cells at the G_0/G_1 phase, and L-OHP at the G_2/M phase, as measured by flow cytometric analyses. Apoptosis was most prominent when cells were treated simultaneously or in a sequence gemcitabine followed by L-OHP, producing apoptosis in treated cells (27–30%). In contrast, the reverse sequence yielded only 6–7% induction of apoptosis, the rate being not significantly different from those induced by each drug singly. Moreover, this sequence dependence was further confirmed by the experiment, which compared the number of HAG-1 cells 7 days after these combination schedules. These findings suggest that the interaction of gemcitabine and L-OHP is highly schedule dependent, with the most efficacious interaction observed in either simultaneous combination or in a sequence combination of gemcitabine followed by L-OHP. *Anti-Cancer Drugs* **20:123–130 © 2009 Wolters Kluwer Health | Lippincott Williams & Wilkins.**

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Introduction

Approximately 9500 cases of biliary tract cancer are reported annually in the United States [1]. Nearly two-third of these tumors arise in the gallbladder, making it the most common biliary tract cancer and fifth most common gastrointestinal tract cancer. As symptoms from gallbladder cancer manifest themselves late in the disease course, tumors often are detected only at an advanced stage. The prognosis for these patients is dismal, and the impact of existing chemotherapy is virtually negligible. Therefore, there is a clear need for new and effective chemotherapeutic regimens in the management of biliary tract cancer.

Gemcitabine (2′,2′-difluorodeoxycytidine) is a nucleoside analogue that inhibits cell growth by interfering with several pathways of nucleic acid metabolism. Gemcitabine is rapidly phosphorylated to its active forms of gemcitabine diphosphate (dFdCDP) and gemcitabine triphosphate (dFdCTP). They compete with deoxycytidine triphosphate for incorporation into DNA, resulting in an arrest of DNA polymerization [2]. Gemcitabine also affects deoxynucleotide pools by interfering with ribonucleotide reductase and inhibits RNA synthesis by incorporation into RNA [3]. Gemcitabine has shown broad activity in a variety of solid tumors [4]. Moreover, single-agent gemcitabine has been shown recently to be effective against gallbladder cancer in several phase II studies [5].

Oxaliplatin (trans-l-1,2-diaminocyclohexane oxalato platinum II, L-OHP) is a third-generation platinum compound that acts as an alkylating agent, inhibiting DNA replication by forming adducts between two adjacent guanines or guanine plus adenine [6]. L-OHP has been shown to exhibit antitumor activity against several cell lines with acquired cisplatin resistance as well as clinical tumors that are intrinsically resistant to cisplatin and carboplatin [7,8]. Phase II studies of single-agent L-OHP have shown the activity in a variety

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of solid cancers. Moreover, L-OHP has been shown recently to be effective against gallbladder cancer in several phase II studies [9].

Currently, combination therapy of gemcitabine combined with several platinum analogues including L-OHP has been regarded as promising regimen in various malignancies. It has been reported that the pooled analysis of clinical trials with these combinations increased survival in pancreatic carcinoma patients as compared with single-agent gemcitabine [10]. Although combination of gemcitabine and L-OHP has been also shown to be effective against the patients with advanced biliary tract cancer [11,12], the optimal combination schedule of gemcitabine and L-OHP still remains unclear. There is only one report that shows a greater additive effect in this combination, using colorectal cancer cell lines [13]. In this study, we investigated here the schedule-dependent interaction between gemcitabine and L-OHP using a panel of four human gallbladder cancer cell lines. We have found that the interaction of gemcitabine and L-OHP is highly schedule dependent.

Materials and methods

Cell lines and culture

Four human gallbladder cancer cell lines (HAG-1, NOZ, GB-d1, G-415) were used in this study. HAG-1 is a human cell line derived from moderately differentiated adenocarcinoma of the gallbladder and its cellular and molecular features were well characterized [14]. NOZ established from moderately differentiated adenocarcinoma of the gallbladder was purchased from the Japanese Cell Resource Bank (Tokyo, Japan) [15]. GB-d1 established from a metastatic lymphnode of poorly differentiated adenocarcinoma of the gallbladder was kindly provided by Dr H. Shimura, Department of surgery, Fukuoka University School of Medicine [16]. G-415 established from poorly differentiated adenocarcinoma of the gallbladder was obtained from Cell Resource Center for Biomedical Research of Tohoku University School of Medicine [17]. HAG-1 and GB-d1 were maintained in Dulbecco' minimum essential medium (Nissui, Tokyo, Japan), whereas NOZ and G-415 were maintained in William's E medium (MP Biomedicals, Morgan Irvine, California, USA). Each medium was supplemented with 10% heat-inactivated fetal bovine serum (Gibco, Grand Ireland, New York, USA), 100 IU/ml of penicillin, and 100 µg/ml of streptomycin at 37°C in a humidified incubator under an atmosphere containing 5% CO_2.

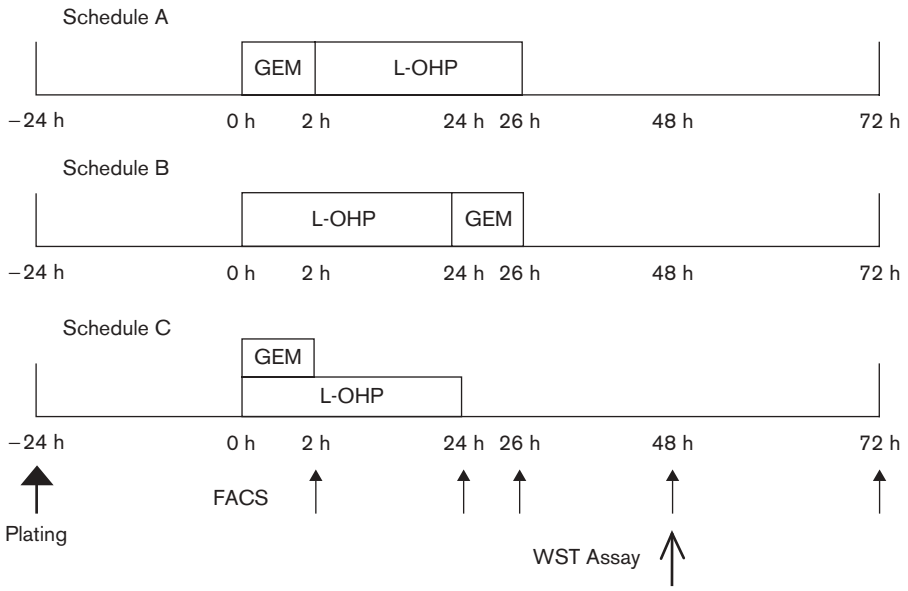
Drugs

L-OHP and gemcitabine were kindly provided by Yakult Co. (Tokyo, Japan) and Eli Lilly Co. (Kobe, Japan), respectively. Stock solutions of these drugs were prepared in sterile distilled water. Immediately before their use, gemcitabine and L-OHP were dissolved in culture medium.

Evaluation of cytotoxicity

Cytotoxic activity was measured by the WST-1 assay (Wako Chemicals, Osaka, Japan), following the manufacturer's instructions [18]. The WST-1 assay is a colorimetric method in which the intensity of the dye is proportional to the number of the viable cells. In brief, exponentially growing cells were plated into 96-well microplates at a density of 3000 cells/well in a volume of 100 µl/well and incubated for 24 h for sufficient cell growth. The cells

Fig. 1



Description of the three combination schedules (A, B and C). GEM, gemcitabine; L-OHP, oxaliplatin. Arrows indicate the harvest of samples for fluorescence-activated cell-sorting (FACS) analysis and WST-1 assay.

were then treated with graded concentrations of gemcitabine alone for 2 h or L-OHP alone for 24 h, or in simultaneous or sequential manner, as shown in Fig. 1. The sequence and exposure time of administration of these drugs would be important in determining the extent of therapeutic synergy. In this study, we incubated cells with L-OHP for 24 h instead of 2 h, not only because the pharmacokinetics of L-OHP administered at dose of 130 mg/m^2 for 4 h showed that the plasma half-life of L-OHP was approximately 27 h [19], but also because more than 1.5 mg/ml (3.8 mmol/l) of total plasma concentration of platinum lasted at least 24 h when patients were administered with 130 mg/m^2 of L-OHP for 2 h [20]. We incubated cells with gemcitabine for 2 h because the pharmacokinetics of gemcitabine administered at dose of 800–1250 mg/m^2 for 30 min showed that the plasma half-life of gemcitabine was about 1 h.

After treatment, the cells were washed twice with PBS, and cultured in drug-free medium for an additional 21 h. Then, 10 ml of WST-1 solution was added into each well and the plates were incubated at 37°C for 3 h. Absorbance values at 450 and 620 nm were measured using a Delta Soft Elisa analysis program for Macintosh computers interfaced with a microplate reader (Immuno-Mini NJ-2300; Bio-Tek, Winooski, Vermont, USA). Wells containing cells untreated with drugs were used as controls. Each experiment was carried out using six replicate wells for each drug concentration and carried out independently at least three times. The IC_{50} values were defined as the concentrations that inhibited 50% of cell growth.

Functional interactions between drugs

The combined drug effects were evaluated by using the Chou and Talalay analysis based on the median-effect principle [21,22]. This method involves plotting dose–effect curves for each drug and for multiply diluted, fixed-ratio combinations by using the median-effect equation: $f_a/f_u=(D/D_m)_m$, where D is the dose, D_m is the dose required for 50% effect (e.g. 50% inhibition of cell growth), f_a is the fraction affected by dose D (e.g. 0.9 if cell growth is inhibited by 90%), f_u is the unaffected fraction (therefore $f_a=1-f_u$) and 'm' is a coefficient of the sigmoidicity of the dose–effect curve. On the basis of slope of the dose–effect curves, it can be determined whether the drugs have mutually nonexclusive effects (e.g. independent or interactive mode of action).

The combination index (CI) is then determined by the equation: $CI=(D)_1/(D_x)_1+(D)_2/(D_x)_2+\alpha(D)_1(D)_2/(D_x)_1(D_x)_2$, where $(D_x)_1$ is the dose of drug 1 required to produce x percent effect alone and $(D)_1$ is the dose of drug 1 required to produce the same x percent effect in combination with $(D)_2$. If the mode of action of the drugs is mutually exclusive or nonexclusive, then α is 0 or 1, respectively. CI values were calculated by solving the equation for different values of f_α (i.e. different degrees of inhibition of cell growth). CI values below 1 indicate synergy, values equal to 1 indicate additive effects and values above 1 indicate antagonism.

Data analysis was performed automatically using the CalcuSyn software program (Biosoft, Cambridge, UK). The dose–effect relationships for the drugs tested alone or in combinations were subjected to the median-effect plot to determine their relative potency (IC_{50}), shape (m), and conformity (r) in each selected cell line. As defined previously, the IC_{50} and m values were used for calculating synergism or antagonism based on the CI equation.

Cell cycle analysis

HAG-1 cells were seeded at a density of 3×10^5/100-mm dish (3003; Falcon, Oxnard, California, USA). The cells were treated with gemcitabine or L-OHP singly, or concurrent or sequential combinations, as shown in Fig. 1. After medium change, the cultures were continued until cell cycle analyses 2, 24, 26, 48, and 72 h after the beginning of treatment. The cells were harvested by collecting floating and trypsinized adherent cells, and fixed in 70% ethanol in PBS for at least 30 min on ice. After removal of ethanol by centrifugation, cells were washed with ice-cold PBS and then incubated in PBS containing 45 mg/ml propidium iodide and 500 mg/ml ribonuclease A (Sigma, St Louis, Missouri, USA) for 30 min on ice in the dark. Cell cycle analysis was performed on a Becton Dickinson FACSCalibur flow cytometer using the CellQuest and ModFit 3.0 software packages (Becton Dickinson, San Jose, California, USA). The percentages of apoptotic populations were determined by measuring the sub-G_1 phase after collecting floating and trypsinized adherent cells at various times after drug exposure. Results were obtained from three separate experiments carried out in duplicate.

Statistical analysis

Statistical significance between these combination treatments was determined by Student's t-test. Significant differences were considered at P value of less than 0.05.

Results

Single-agent experiments

The cytotoxic activities of gemcitabine and L-OHP were tested individually on the four tumor cell lines. The cells were exposed to gemcitabine for 2 h and L-OHP for 24 h. The IC_{50} are summarized in Table 1. For gemcitabine, HAG-1 cells were most sensitive to gemcitabine (1.0 μg/ml) among the four tumor cell lines, and G-415 cells were markedly resistant (312.1 μg/ml). The IC_{50} values of GB-d1 and NOZ cells were 10.6 and 43.5 μg/ml, respectively. For L-OHP, the IC_{50} ranged from 8.2 μg/ml for HAG-1 cells to 2.9 μg/ml for GB-d1 cells. NOZ and G-415 cells showed IC_{50} values 2.4 and 9.9 μg/ml, respectively. These data indicate that cytotoxic activities of gemcitabine vary

Table 1 IC_{50} value of gemcitabine and oxaliplatin in four gallbladder cancer cell lines[a]

	HAG-1	GB-d15	NOZ	G-415
Gemcitabine 2 h (μg/ml)	1.00 ± 1.56	10.62 ± 17.14	43.53 ± 28.50	312.06 ± 14.72
Oxaliplatin 24 h (μg/ml)	8.22 ± 2.69	2.85 ± 1.96	2.37 ± 1.76	9.86 ± 8.47

The values are the means ± SD of three independent experiments.
[a]Cells were treated with various concentrations of gemcitabine for 2 h or oxaliplatin for 24 h.

significantly from cell to cell despite that the cytotoxic activities of L-OHP were largely similar in four tumor cell lines.

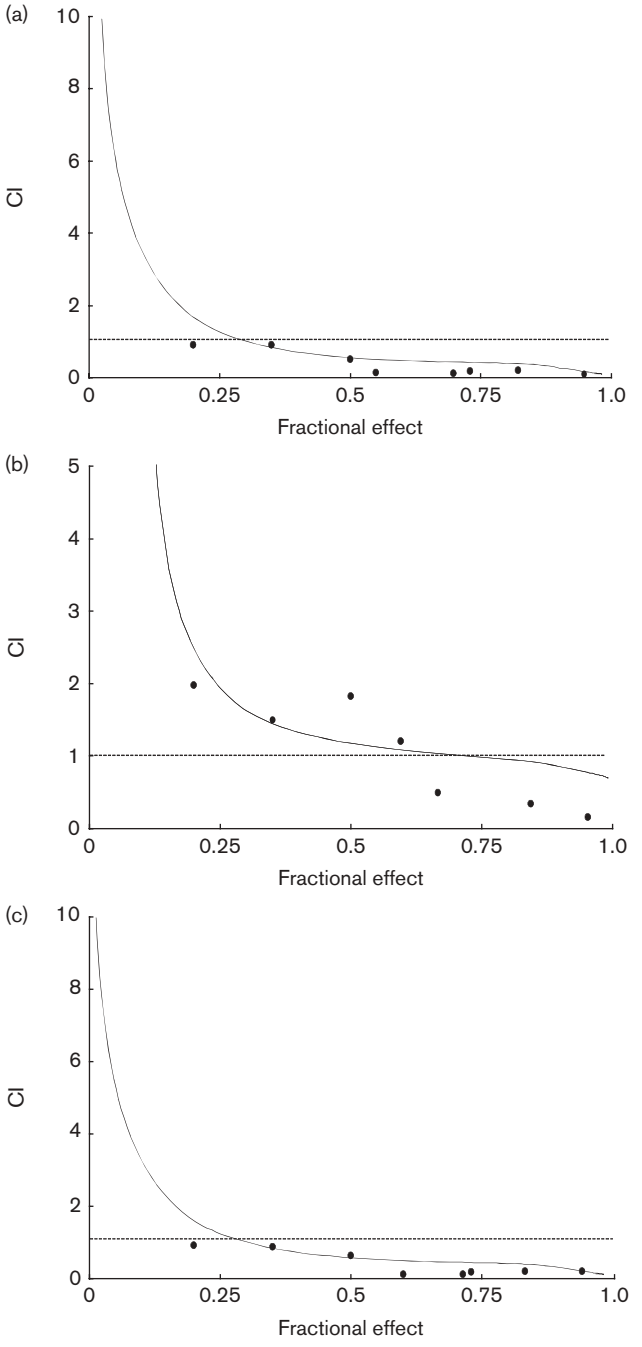
Median-effect analysis of gemcitabine and oxaliplatin combination *in vitro*

Gemcitabine and L-OHP were tested in different combinations to define the most effective schedule. Three different schedules (simultaneous and sequential drug exposures) were tested as shown in Fig. 1, with the exposure duration of 2 and 24 h for gemcitabine and L-OHP, respectively. In HAG-1 cells, both simultaneous treatment and the sequence gemcitabine followed by L-OHP showed synergistic effects (Fig. 2a and c), but the sequence L-OHP followed by gemcitabine exhibited an antagonism in the range of less than 65% cell kill fraction (Fig. 2b). In GB-d1 cells, simultaneous treatment and the sequence gemcitabine followed by L-OHP also showed a remarkable synergism at all cell kill fraction (Fig. 3a and c). In contrast, the reverse sequence (L-OHP followed by gemcitabine) showed an antagonism at the range of more than 60% cell kill fraction (Fig. 3b). In NOZ cells, simultaneous treatment and sequence gemcitabine followed by L-OHP produced a marked synergism in all ranges of cell kill fraction (Fig. 4a and c), whereas the opposite sequence L-OHP followed by gemcitabine indicated largely an antagonism (Fig. 4b). In G-415 cells, simultaneous treatment and the sequence gemcitabine followed by L-OHP yielded a marked synergism (Fig. 5a and c). When the reverse sequence was used, the combination effects became less, but greater than additive effects were still observed (Fig. 5b).

Effect of gemcitabine and oxaliplatin combination in long-term culture

To confirm the results obtained by median-effect analysis, we compared the total number of cells 7 days after the same number of HAG-1 cells (3×10^5) had been treated with various administration schedules of gemcitabine and L-OHP at the doses of IC_{50} (Table 2). The total cell numbers were lowest in simultaneous treatment, but there were no significant differences in the cell numbers between simultaneous treatment and the sequence gemcitabine followed by L-OHP. Notably, the number of cells in the sequence L-OHP followed by gemcitabine was significantly higher than for other schedules. These data seemed to be consistent with those obtained by median-effect and apoptosis analyses.

Fig. 2

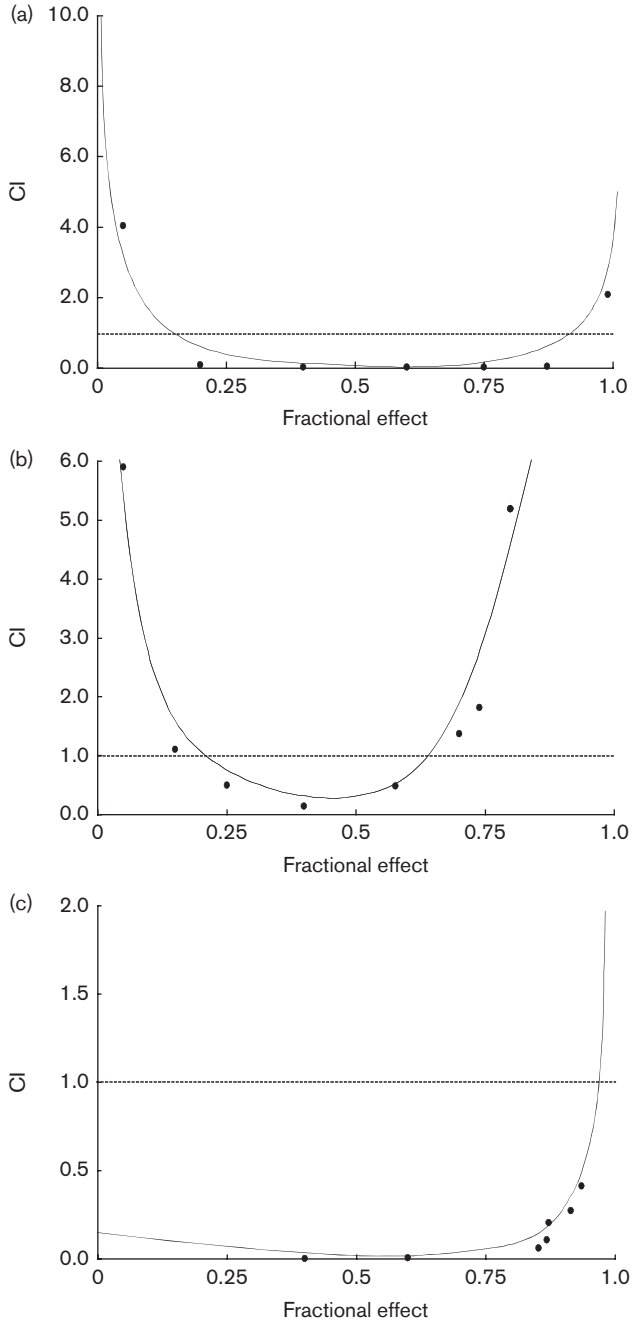


Combination index (CI) of interactions between gemcitabine and oxaliplatin (L-OHP) in HAG-1 cells. Cells were treated with (a) gemcitabine for 2 h followed by L-OHP for 24 h, (b) L-OHP for 24 h followed by gemcitabine for 2 h, (c) gemcitabine and L-OHP for 2 h simultaneously followed by L-OHP alone for 22 h. Dashed horizontal line indicates the level of 1.0 for CI.

Cell cycle perturbation and apoptosis

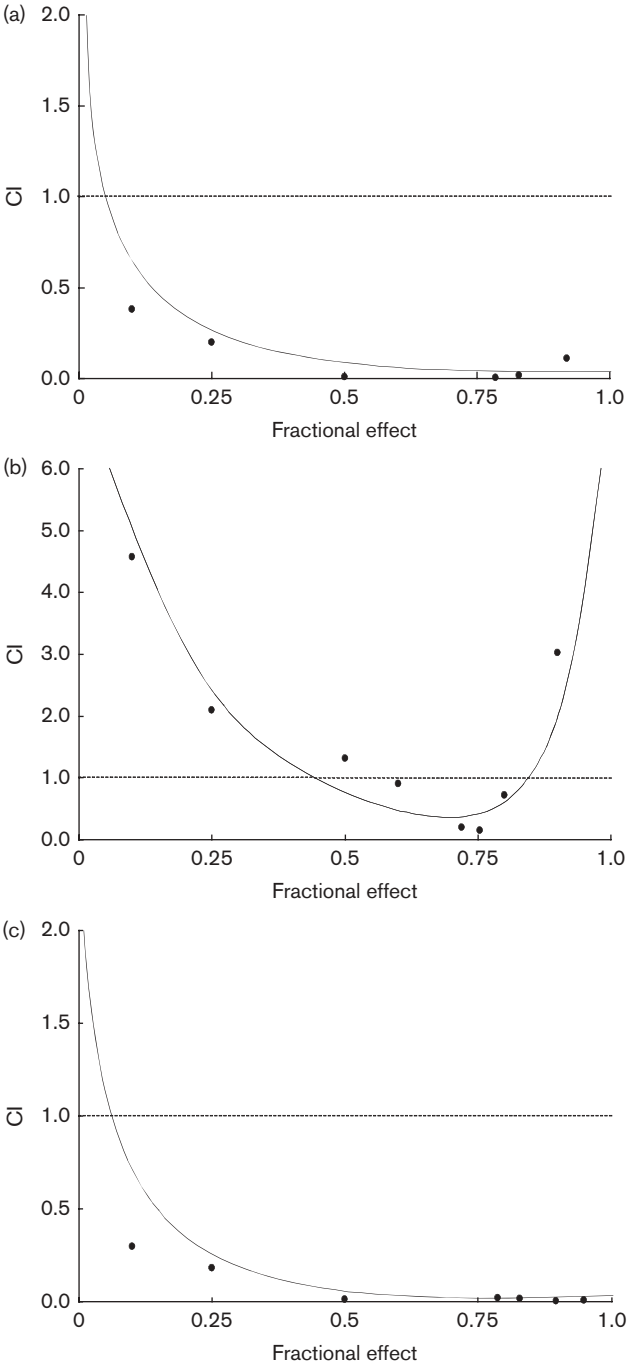
In an attempt to explain the mechanisms underlying the different types of interaction, the effects of gemcitabine and L-OHP on cell cycle distribution and apoptosis were

Fig. 3



Combination index (CI) of interactions between gemcitabine and oxaliplatin (L-OHP) in GB-d1 cells. Cells were treated with (a) gemcitabine for 2 h followed by L-OHP for 24 h, (b) L-OHP for 24 h followed by gemcitabine for 2 h, (c) gemcitabine and L-OHP for 2 h simultaneously followed by L-OHP alone for 22 h. Dashed horizontal line indicates the level of 1.0 for CI.

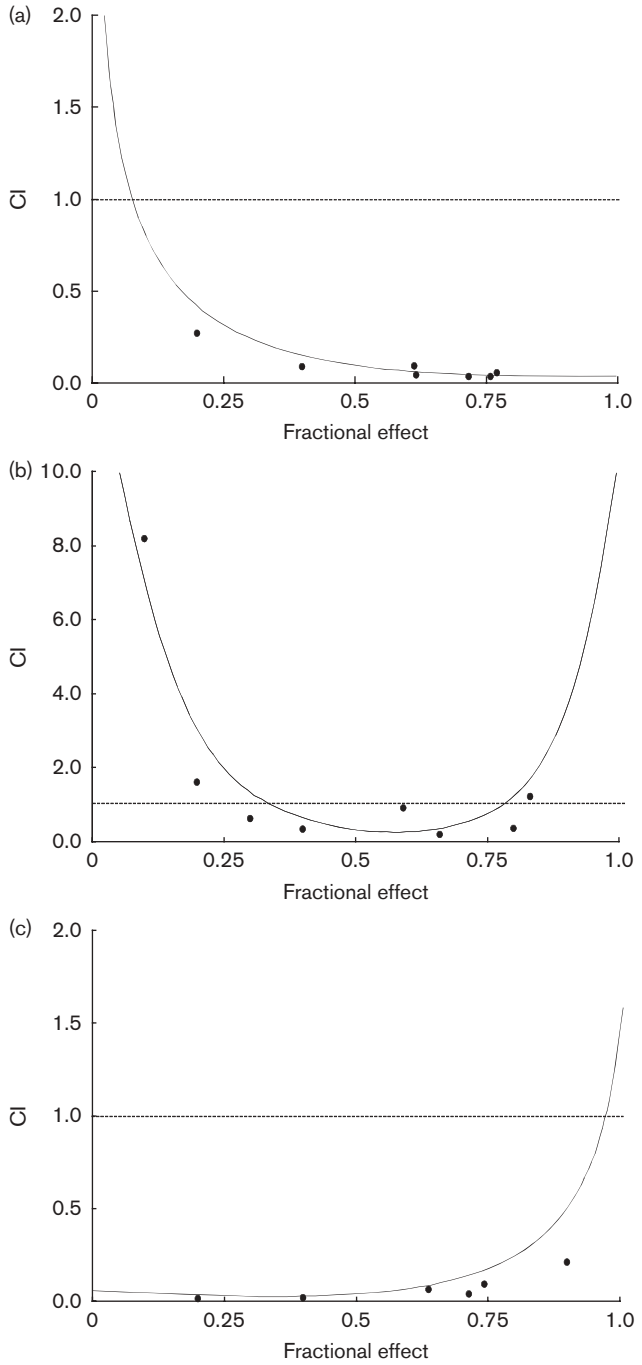
Fig. 4



Combination index (CI) of interactions between gemcitabine and oxaliplatin (L-OHP) in NOZ cells. Cells were treated with (a) gemcitabine for 2 h followed by L-OHP for 24 h, (b) L-OHP for 24 h followed by gemcitabine for 2 h, (c) gemcitabine and L-OHP for 2 h simultaneously followed by L-OHP alone for 22 h. Dashed horizontal line indicates the level of 1.0 for CI.

investigated in HAG-1 cells (Table 3). The cells were treated with either alone or in combination, with different schedules as shown in Fig. 1, and cell cycle distribution was analyzed 2, 24, 26, 48, 72 h after treatment using flow cytometry. Gemcitabine alone at a dose of IC_{50} induced accumulation of cells in the G_0/G_1 phase, lasting until 72 h. At a dose of IC_{50}, L-OHP alone caused an increase in G_2/M populations until 72 h with

Fig. 5



Combination index (CI) of interactions between gemcitabine and oxaliplatin (L-OHP) in G-415 cells. Cells were treated with (a) gemcitabine for 2 h followed by L-OHP for 24 h, (b) L-OHP for 24 h followed by gemcitabine for 2 h, (c) gemcitabine and L-OHP for 2 h simultaneously followed by L-OHP alone for 22 h. Dashed horizontal line indicates the level of 1.0 for CI.

a continuous decrease of G_0/G_1 block. Treatment with gemcitabine before L-OHP induced accumulation of cells in the G_0/G_1 phase, approximately similar distribution pattern to that observed in cells treated with gemcitabine alone. In contrast, the schedule of L-OHP before gemcitabine does not produced G_2/M block, but increase in S phase population almost different distribution pattern as that induced by L-OHP alone. These findings indicate that cell cycle distribution patterns with the sequential combinations were mostly influenced by the initial drug administered. Interestingly, simultaneous exposure led to accumulation of cells in G_0/G_1 phase, a pattern almost identical with that caused by gemcitabine alone, indicating that gemcitabine might have a dominant effect in cell cycle progression as compared with L-OHP, or that L-OHP might take more time to exert its activity for inducing apoptosis than gemcitabine.

To define the cytotoxic activities of combination schedules, drug-induced apoptosis was studied after treatment of HAG-1 cells by measuring the sub-G_1 population. The presence of hypodiploid DNA (sub-G_1) is associated with undergoing apoptosis of cells. As shown in Table 3, the schedule of gemcitabine before L-OHP and simultaneous treatment induced G_0/G_1 blockade, with induction of 27–30% apoptosis in the treated cells, significantly analogous to that induced by gemcitabine singly (29–30%). In contrast, the sequential administration of L-OHP followed by gemcitabine increased in S phase population, and the apoptosis was 6–7%, not significantly different from that induced by L-OHP singly (4–5%). These findings indicate that simultaneous treatment and sequential schedule of gemcitabine followed by L-OHP exhibited synergistic interaction in inducing apoptosis, but that sequential administration of L-OHP followed by gemcitabine is antagonistic.

Discussion

Gemcitabine and L-OHP combination would be potentially active in biliary tract cancer [11,12]. Optimal administration schedule of this combination, however, has not been determined in gallbladder cancer. In this study, we examined the sequence dependency of gemcitabine and L-OHP combination in four human gallbladder cancer cell lines *in vitro*. Both simultaneous combination and sequential treatment of gemcitabine followed by L-OHP exhibited synergistic effects in all four cell lines, with the most efficacious interaction observed in simultaneous combination. This is because simultaneous and the sequence of gemcitabine followed by oxaliplatin are considered to be fundamentally similar combination. In contrast, the reverse sequence yielded a clear antagonism except G-415 cell line. These observations were confirmed by the experiment that compared the cell number 7 days after various treatment schedules.

Despite the identical exposure times of respective drugs, our results are not consistent with the report by Faivre *et al.* [13] who showed that the gemcitabine and L-OHP combination was synergistic whatever the tested schedules

Table 2 Effects of treatment schedules of gemcitabine and oxaliplatin combination on total cell number yielded after 7 days[a]

	Total cell number		
Drug dose	GEM → L-OHP	L-OHP → GEM	GEM+L-OHP
GEM, 1 μg/ml	**	*	
	9.0 ± 1.5 (%)	19.3 ± 1.5 (%)	6.9 ± 1.7 (%)
L-OHP, 8 μg/ml	***		

GEM, gemcitabine; L-OHP, oxaliplatin.
[a]A fixed number of HAG-1 cells (5×10^5) were seeded and exposed to 1 μg/ml of GEM and 8 μg/ml of L-OHP in three combination schedules. The total yield of cells was determined after 7 days' incubation from the initiation of treatment. Data are means ± SD of five independent determinations.
*$P < 0.002$; **$P < 0.005$; ***$P > 0.05$; by Student's *t*-test.

Table 3 Cell cycle perturbation and apoptosis (%) induced by gemcitabine and oxaliplatin in HAG-1 cell line[a]

	2 h				24 h				26 h				48 h				72 h			
Treatment	G_0/G_1	S	G_2/M	Apo	G_0/G_1	S	G_2/M	Apo	G_0/G_1	S	G_2/M	Apo	G_0/G_1	S	G_2/M	Apo	G_0/G_1	S	G_2/M	Apo
Control	53.34	29.59	17.07	2.13																
GEM	53.95	28.88	17.17	1.89	58.96	25.84	15.20	3.16					74.38	14.90	10.72	14.28	83.06	12.14	4.80	29.39
L-OHP	52.52	29.17	18.32	1.98	38.90	42.34	18.76	3.00					22.56	54.89	22.55	4.70	10.17	57.38	32.45	4.87
GEM→ L-OHP					59.80	26.37	13.84	3.59					70.27	14.48	15.25	19.69	73.79	11.29	14.93	30.63
L-OHP→ GEM									33.15	50.13	16.72	3.46	33.26	49.14	17.60	5.68	27.59	58.18	14.23	6.96
GEM+ L-OHP	56.41	27.75	15.84	1.82	56.22	28.20	15.58	3.93					67.84	16.42	15.74	17.80	73.04	9.82	17.14	27.39

The data presented are the mean percentage values from three independent experiments.
FACS, fluorescence-activated cell-sorting; GEM, gemcitabine; L-OHP, oxaliplatin.
[a]Cells were treated with GEM or L-OHP singly, or in combination at the IC_{50} dose of GEM and L-OHP, and subjected to FACS analyses after collecting floating and trypsinized adherent cells at various times after drug exposure as described in Materials and methods. The apoptotic population percentages (Apo) were determined by measuring the sub-G_1 phase.

using human colon cancer cell lines. This may be explained by the difference of cellular origin from which cancer derived. Nonetheless, the observed antagonistic activity in the sequence L-OHP followed by gemcitabine in a variety of human gallbladder adenocarcinoma cell lines may provide a rationale for not using this sequence in the clinical trial.

To elucidate the possible mechanisms underlying the synergistic interaction, we further analyzed the perturbations induced in cell cycle by flow cytometric analyses using HAG-1 cells. First, we found that 2-h treatment with gemcitabine markedly affected the cell cycle distribution, producing a clear accumulation in the G_0/G_1 phase, and induced apoptosis in 29–30% of treated cells. L-OHP alone induced 4–5% of apoptosis by arresting cells in G_2/M and S phase. Simultaneous and sequence of gemcitabine followed by L-OHP led to 30–31 and 27–28% of apoptosis, respectively. In contrast, L-OHP followed by gemcitabine resulted in an antagonistic effect, reducing the rate of apoptosis to 6–7% with increment of S phase population. These results imply that L-OHP may kill the cells recovering from the gemcitabine-induced G_1/S blockade as they progress into G_2/M boundary, accounting for the synergistic interaction. In contrast, L-OHP followed by gemcitabine resulted in an antagonistic effect, reducing the rate of apoptosis to 6–7%. This would probably be explained by the decrease in the G_1/S population targeted by gemcitabine, because L-OHP pretreatment caused mitotic block in the G_2/M boundary, thereby reducing the number of cells entering the G_1 phase. This could be supported by the report that L-OHP treatment induced downregulation of the cell-cycle regulatory protein $p21^{waf1/cip1}$, allowing abrupt S-phase entry and resulting in G_2/M arrest [23].

In this study, simultaneous treatment and sequential schedule of gemcitabine followed by L-OHP exhibited synergistic interaction and was largely effective for G-415 cell line that was markedly resistant to gemcitabine in the single-agent experiment. According to the preclinical data on combination of gemcitabine and platinum analogues, gemcitabine increased the formation of platinum-DNA adducts and effectively inhibited the repair of L-OHP-induced platinum-DNA adducts [24]. Moreover, it has also been reported that cisplatin, a similar platinum compound, enhances the binding of gemcitabine

triphosphates to DNA and accordingly facilitates apoptosis of tumor cell lines [25,26]. Therefore, by combining gemcitabine with L-OHP, it might be possible to achieve a better therapeutic efficacy by overcoming resistance to either drug. Furthermore, according to the pharmacokinetics study, the active metabolites of gemcitabine remained significantly longer in the plasma when gemcitabine was administrated with L-OHP as compared with gemcitabine alone [27]. Such mechanisms may explain, at least in part, the synergistic interaction between gemcitabine and L-OHP.

The clinical phase III trial that administrated gemcitabine and L-OHP to patients with pancreas cancer failed to show a survival benefit, but this study showed significant advantage in terms of response rate, progression free survival, and clinical benefit [28]. In addition, in this trial oxaliplatin was administered with drug-free interval of nearly 24 h. Furthermore, this combination trial has been reported to have a potential activity against patients with bilialy tract cancer. Those reports, together with our present data, may warrant the trial with combination of gemcitabine and oxaliplatin in bilialy tract cancer.

Although the biochemical basis for the synergistic interaction between gemcitabine and L-OHP remains to be elucidated, the clear sequence-dependent activity of the combination of gemcitabine and L-OHP could provide a rationale for conducting clinical trials for the patients with gallbladder cancer.

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