In-vitro schedule-dependent interaction between oxaliplatin and 5-fluorouracil in human gastric cancer cell lines

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In order to define the most effective combination schedule of oxaliplatin (L-OHP) and 5-fluorouracil (5-FU), we investigated the in vitro interaction between these drugs in a panel of four human gastric adenocarcinoma cell lines (MKN-1, NUGC-3, NUGC-5 and AZ-521). 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 L-OHP followed by 5-FU exhibited synergistic effects in all four cell lines, whereas the reverse sequence yielded a clear antagonism. 5-FU exclusively arrested cells at the G_0/G_1 phase, and L-OHP at the G_0/G_1 and G_2/M phases. Apoptosis was most prominent when cells were treated simultaneously or in a sequence of L-OHP followed by 5-FU, producing apoptosis in the majority of treated cells (55.5–61.5%). In contrast, the reverse sequence yielded only 20% 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 total number of NUGC-3 cells 7 days after these combination schedules. These findings suggest that the interaction of 5-FU and L-OHP could be highly schedule dependent, with the most efficacious interaction observed in simultaneous combination and that 5-FU followed by L-OHP would not be recommended in clinical trials for patients with advanced gastric cancer. *Anti-Cancer Drugs* **17:445–453**

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Introduction

Gastric cancer is the second most common cause of cancer death worldwide, with most cases being diagnosed at the stage of advanced disease [1,2]. The prognosis for this disease is extremely poor, with a median survival time ranging between 6 and 8 months [3,4]. Only 10% of patients with advanced gastric cancer can survive 2 years with chemotherapy [5]. Although gastric cancer appears to be a chemotherapy-sensitive disease, there have been no standard chemotherapeutic regimens for this incurable disease. Therefore, it is of paramount importance to develop the most beneficial chemotherapeutic regimens that improve survival of patients.

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 and adenine [6]. L-OHP has been demonstrated to exhibit anti-tumor 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 activity in colorectal [9], ovarian [10], breast [11] and non-small cell lung cancers [12]. Moreover, L-OHP has been shown recently to be effective against gastric cancer in several phase II studies [13,14]. L-OHP has a different toxicity profile from that of cisplatin, with mild nausea/vomiting and, in contrast to carboplatin, mild to moderate hematological toxicity. The dose-limiting toxicity of L-OHP is a dose-dependent and reversible peripheral neuropathy [15].

Currently, the combination of 5-fluoruracil (5-FU)/leucovorin and L-OHP is regarded as a standard regimen for patients with advanced colorectal cancer. This combination was administered with the sequence L-OHP/leucovorin followed by 5-FU [16]. However, the optimal combination schedule of 5-FU and L-OHP still remains unclear. Fischel *et al.* demonstrated that the combination of L-OHP and 5-FU is synergistic whatever the tested schedules using four human colorectal cancer cell lines [17]. Since this combination has been shown to be effective against patients with advanced gastric cancer [14,18–20], we investigated the schedule-dependent interaction between 5-FU and L-OHP using a panel of

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human gastric cancer cell lines. We have found that the interaction of 5-FU and L-OHP could be highly schedule dependent.

Materials and methods

Cell lines and culture

Four human gastric cancer cell lines (AZ-521, MKN-1, NUGC-3 and NUGC-5) were purchased from the Japanese Cell Resource Bank (Tokyo, Japan). The cells were maintained in DMEM (Nissui, Tokyo, Japan) supplemented with 10% heat-inactivated FBS (Gibco, Grand Island, New York, USA), 100 IU/ml penicillin and 100 μg/ml streptomycin at 37°C in a humidified incubator under an atmosphere containing 5% CO_2.

Drugs

5-FU and L-OHP were kindly provided by Kyowa Hakko (Tokyo, Japan) and Yakult (Tokyo, Japan), respectively. Stock solutions of these drugs were prepared in sterile distilled water. Immediately before their use, 5-FU and L-OHP were dissolved in culture medium.

Evaluation of cytotoxicity

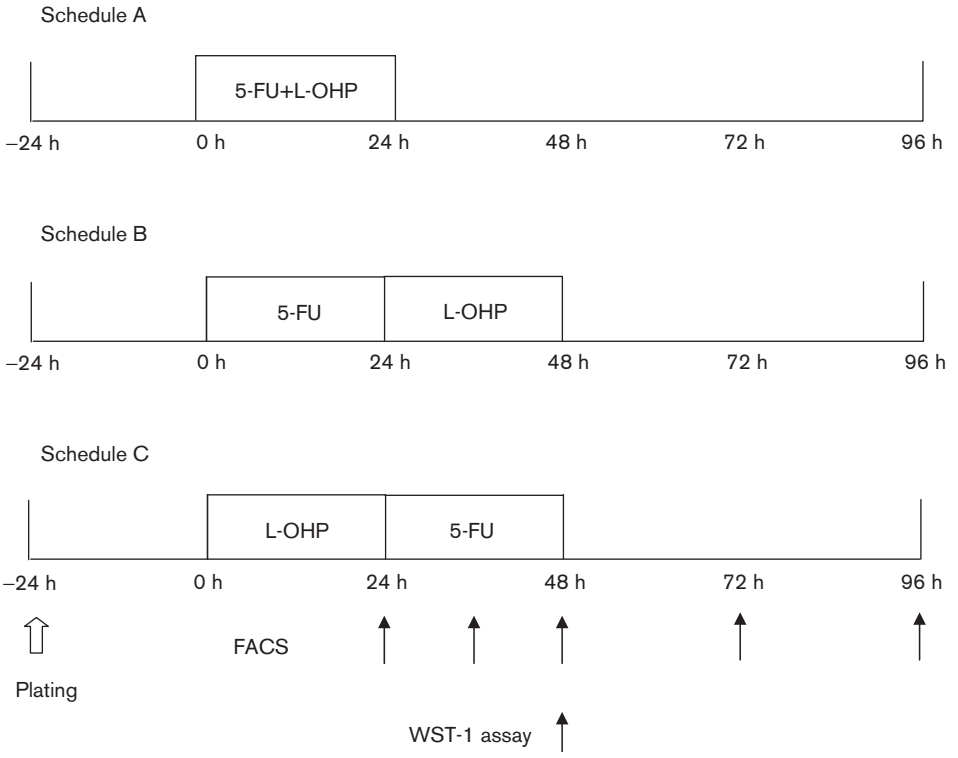
Cytotoxic activity was measured by the WST-1 assay (Wako Chemicals, Osaka, Japan), following the manufacturer's instructions [21]. The WST-1 assay is a colorimetric method in which the intensity of the dye is proportional to the number of viable cells. Briefly, exponentially growing cells were plated 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 were then treated with graded concentrations of 5-FU or L-OHP alone for 24 h, or in simultaneous or sequential fashion, as shown in Fig. 1.

After treatment, the cells were washed twice with PBS, and cultured in drug-free medium for an additional 21 h. Then, 10 μl 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 performed using six replicate wells for each drug concentration and carried out independently at least 3 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

Fig. 1



Description of the three combination schedules. Closed arrows indicate the harvest of samples for FACS analysis and WST-1 assay.

principle [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. Based on the slope of the dose–effect curves, it can be determined whether the drugs have mutually non-exclusive 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 non-exclusive, then α is 0 or 1, respectively. CI values were calculated by solving the equation for different values of f_a (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 in order 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

AZ-521 cells were seeded at a density of 3×10^5 per 100-mm dish (3003; Falcon, Oxnard, California, USA). Then the cells were treated with 5-FU 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 24, 36, 48, 72 and 96 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 μg/ml propidium iodide (PI) and 500 μg/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 following drug exposure. Results were obtained from three separate experiments performed in duplicate.

Statistical analysis

Statistical significance between these combination treatments was determined by Student's t-test. Significant differences were considered at $P < 0.05$.

Results

Single-agent experiments

The cytotoxic activities of 5-FU and L-OHP were tested individually on the four tumor cell lines. The cells were exposed to each drug for 24 h. The IC_{50} are summarized in Table 1. For 5-FU, the IC_{50} ranged from 16.1 μmol/l for AZ-521 cells to 344 μmol/l for MKN-1 cells. NUGC-3 and NUGC-5 cells showed IC_{50} values of 115 and 129 μmol/l, respectively. For L-OHP, AZ-521 cells were the most sensitive to L-OHP (3.27 μmol/l) among the four tumor cell lines and MKN-1 cells were the least sensitive (15.4 μmol/l). The IC_{50} values of NUGC-3 and NUGC-5 cells were 13.3 and 14.1μM, respectively.

Median-effect analysis of 5-FU and L-OHP combination *in vitro*

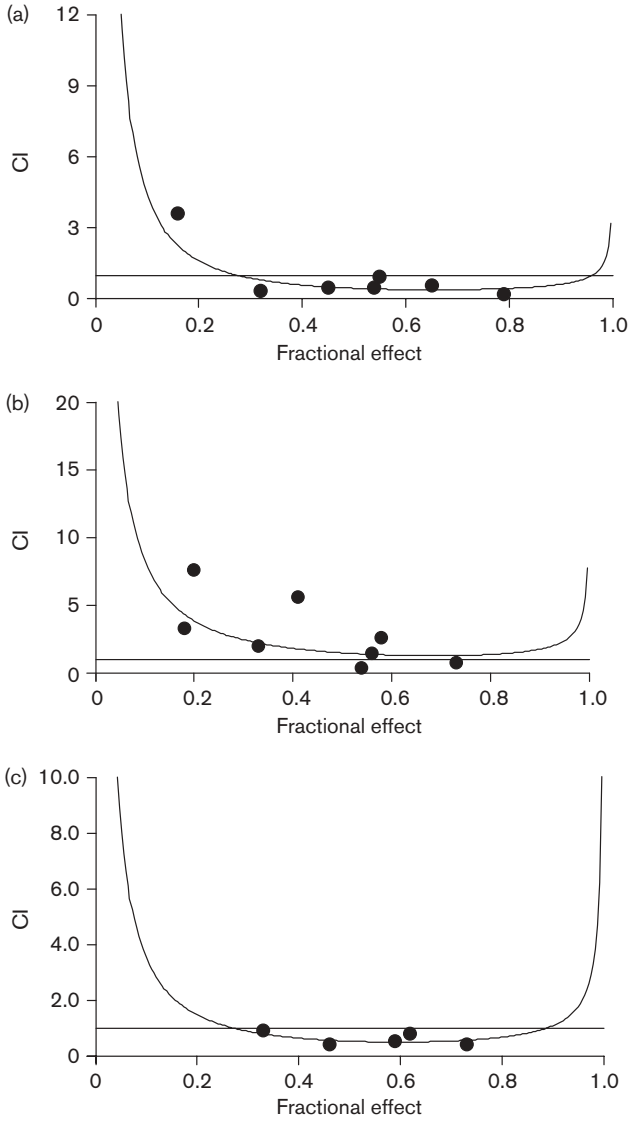
5-FU and L-OHP were tested in different combinations to define the most effective schedule. Three different schedules (simultaneous and sequential drug exposures) were tested as shown in Fig. 1 and the exposure duration to each drug was 24 h. In MKN-1 cells, both simultaneous treatment and the sequence L-OHP followed by 5-FU showed synergistic effects (Fig. 2a and c), while the sequence 5-FU followed by L-OHP exhibited an antagonistic effect at almost all ranges of the cell kill fractions (Fig. 2b). In NUGC-3 cells, simultaneous treatment and the sequence L-OHP followed by 5-FU also showed a remarkable synergism at all cell kill fractions (Fig. 3a and c). In contrast, the reverse sequence (5-FU followed by L-OHP) demonstrated a clear antagonism at all cell kill fractions (Fig. 3b). In NUGC-5 cells, simultaneous treatment and sequence L-OHP followed by 5-FU produced a marked synergism at all ranges of cell kill fractions (Fig. 4a and c), whereas the opposite sequence 5-FU followed by L-OHP produced an antagonism at all cell kill fractions (Fig. 4b). In AZ-521 cells, simultaneous treatment and the sequence L-OHP followed by 5-FU yielded a synergism (Fig. 5a and c).

Table 1 **IC_{50} values of 5-FU and L-OHP in four gastric cancer cell lines**

Drug	MKN1	NUGC-3	NUGC-5	AZ-521
5-FU (μmol/l)	344 ± 43.8	115 ± 70.0	129 ± 45.4	16.1 ± 0.77
Oxaliplatin (μmol/l)	15.4 ± 9.06	13.3 ± 7.3	14.1 ± 11.6	3.27 ± 0.76

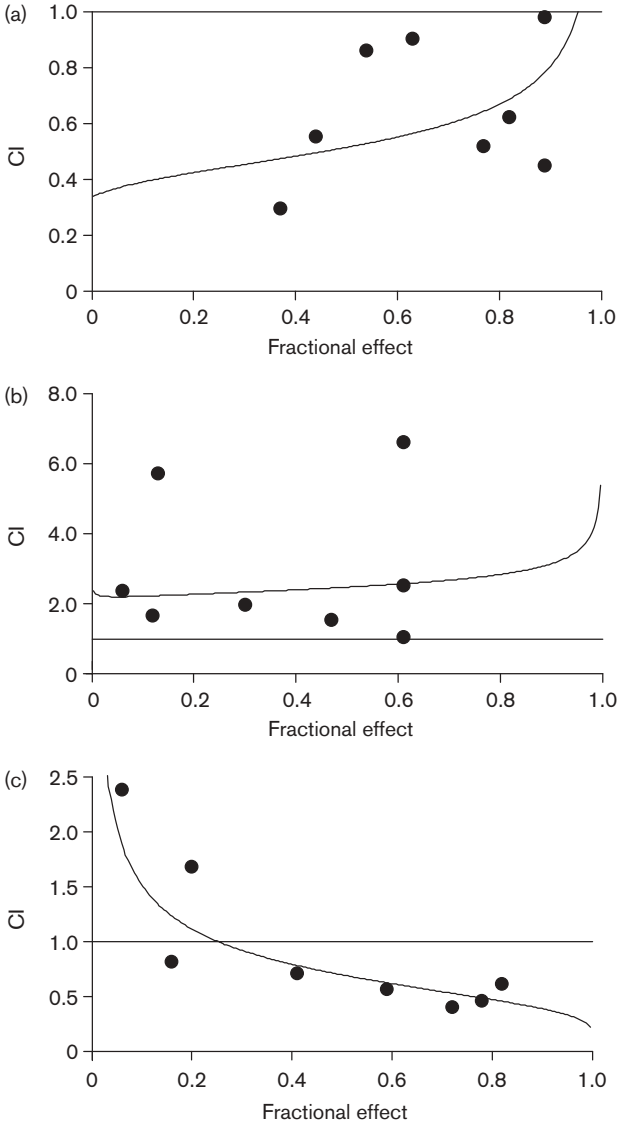
Cells were treated with various concentrations of 5-FU for 24 h or L-OHP for 24 h, and assayed for cytotoxicity as described in Materials and methods. The values are the means ± SD of three independent experiments.

Fig. 2



CI of interactions between 5-FU and L-OHP in MKN-1 cells. Cells were treated with (a) 5-FU and L-OHP for 24 h simultaneously, (b) 5 FU for 24 h followed by L-OHP for 24 h or (c) L-OHP for 24 h followed by 5-FU for 24 h. The horizontal line indicates the level of 1.0 for the CI.

Fig. 3



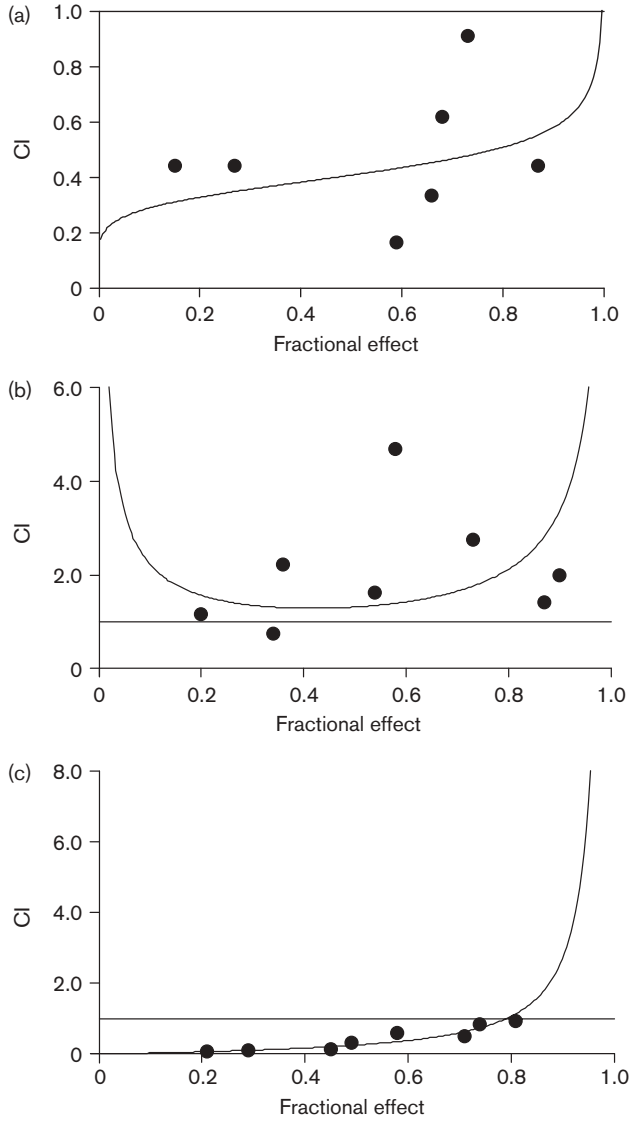
CI of interactions between 5-FU and L-OHP in NUGC-3 cells. Cells were treated with (a) 5-FU and L-OHP for 24 h simultaneously, (b) 5 FU for 24 h followed by L-OHP for 24 h or (c) L-OHP for 24 h followed by 5-FU for 24 h. The horizontal line indicates the level of 1.0 for the CI.

Conversely, when the reverse sequence was used, a distinct antagonism was observed (Fig. 5b).

Effect of 5-FU and L-OHP combination in long-term culture

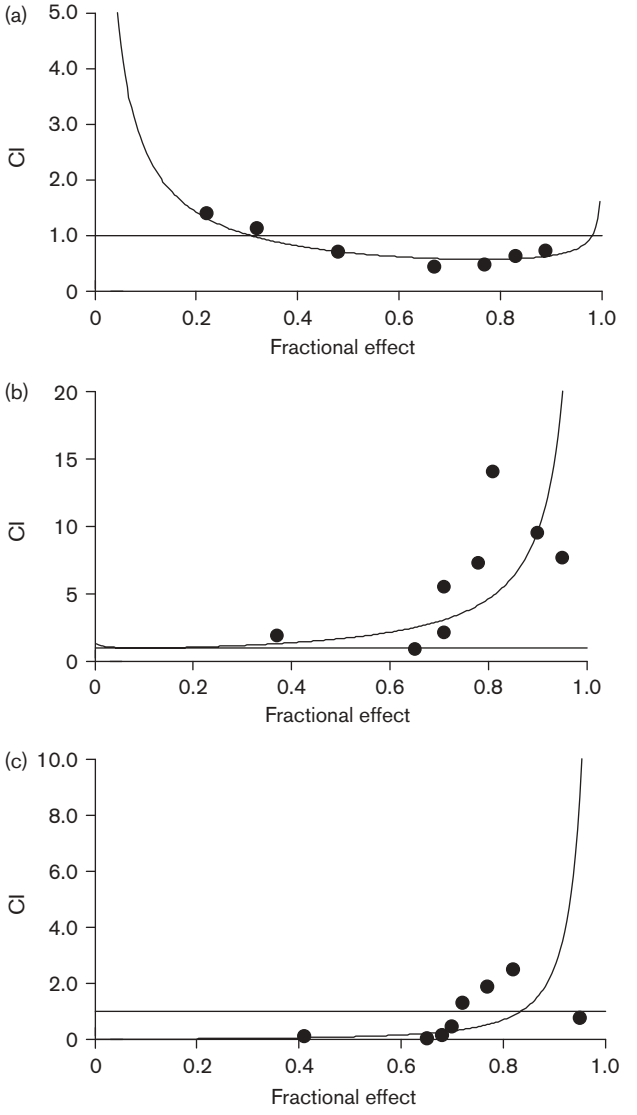
To confirm the results obtained by median-effect analysis, we compared the total number of cells 7 days after the same number of NUGC-3 cells (5×10^5) had been treated with various administration schedules of 5-FU and L-OHP at two fixed doses of 5-FU and L-OHP around the IC_{50} (Table 2). For both doses, 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 L-OHP followed by 5-FU. Of note is that the number of cells in the sequence 5-FU followed by L-OHP was significantly higher than for other schedules. These data appeared to be consistent with those obtained by median-effect and apoptosis analyses.

Fig. 4



CI of interactions between 5-FU and L-OHP in NUGC-5 cells. Cells were treated with (a) 5-FU and L-OHP for 24 h simultaneously, (b) 5 FU for 24 h followed by L-OHP for 24 h or (c) L-OHP for 24 h followed by 5-FU for 24 h. The horizontal line indicates the level of 1.0 for the CI.

Fig. 5

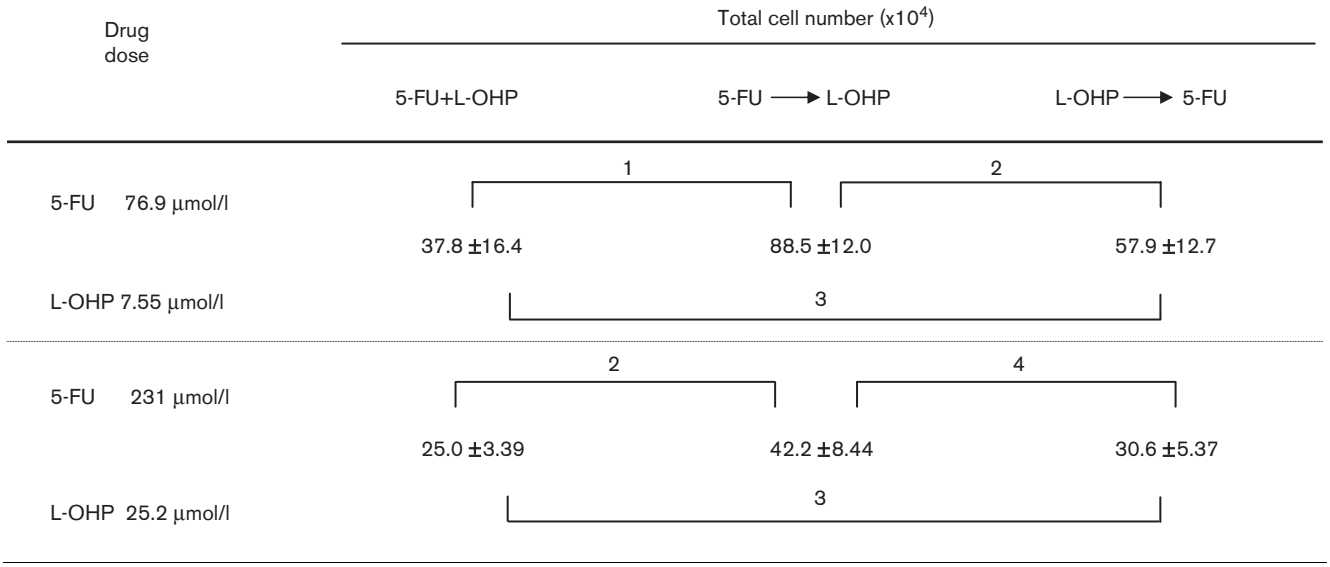


CI of interactions between 5-FU and L-OHP in AZ-521 cells. Cells were treated with (a) 5-FU and L-OHP for 24 h simultaneously, (b) 5 FU for 24 h followed by L-OHP for 24 h or (c) L-OHP for 24 h followed by 5-FU for 24 h. The horizontal line indicates the level of 1.0 for the CI.

Cell cycle perturbation and apoptosis

In an attempt to explain the mechanisms underlying the different types of interaction, the effects of 5-FU and L-OHP on cell cycle distribution and apoptosis were investigated in AZ-521 cells (Table 3). The cells were treated with these drugs for 24 h either alone or in combination, with different schedules, and cell cycle distribution was analyzed 24, 36, 48, 72 and 96 h after treatment using flow cytometry. 5-FU alone at a dose of IC_{50} induced accumulation of cells in the G_0/G_1 phase, lasting until 96 h. At a dose of $IC_{50,}$ L-OHP alone caused an increase in both G_0/G_1 and G_2/M populations, and G_0/G_1 arrest increased gradually until 96 h with the continuous decrease of G_2/M block. Treatment with 5-FU prior to L-OHP induced accumulation of cells in the G_0/G_1 phase, with an approximately similar distribution pattern to that observed in cells treated with 5-FU alone. In contrast, the schedule of L-OHP before 5-FU produced both G_0/G_1 and G_2/M block, with almost the same distribution pattern as that induced by L-OHP alone. These findings indicate that cell cycle distribution patterns with the sequential combinations could be mostly influenced by the initial drug administered. Interestingly, simultaneous exposure led to accumulation

Table 2 Effects of treatment schedules of 5-FU and L-OHP combination on total cell number after 7 days



5-FU, 5-fluorouracil; L-OHP, oxaliplatin.
*A fixed number of NUGC-3 cells (5×10^5) were seeded and exposed to 76.9 μmol/l 5-FU and 7.55 μmol/l L-OHP or 231 μmol/l 5-FU and 25.2 μmol/l L-OHP in three combination schedules. The total yield of cells was determined after 7 days of incubation from the initiation of treatment. Data are means ± SD of five independent determinations.
[1]$P < 0.001$,
[2]$P < 0.005$,
[3]$P > 0.05$,
[4]$P < 0.05$ by Student's *t*-test.

of cells in both G_0/G_1 and G_2/M phase – a pattern almost identical with that caused by L-OHP alone – indicating that L-OHP might have a dominant effect in cell cycle progression as compared to 5-FU or that 5-FU might take more time to exert its activity than L-OHP.

To define the cytotoxic activities of combination schedules, drug-induced apoptosis was studied after treatment of AZ-521 cells by measuring the sub-G_1 population. The presence of hypodiploid DNA (sub-G_1) is associated with cells undergoing apoptosis. As shown in Table 3, simultaneous treatment induced both G_0/G_1 and G_2/M blockade, with induction of 61.5% apoptosis in the treated cells. The induction rate of apoptosis by this combination was the highest among those induced by these combination schedules and much greater than that of 5-FU alone (1.61–26.9%) or L-OHP alone (1.9–20%). Sequential administration of L-OHP followed by 5-FU also caused both G_0/G_1 and G_2/M block with the apoptotic population of 55.5%. In contrast, the reverse sequence resulted in G_0/G_1 block and the apoptosis was 20% – not significantly different from that induced by 5-FU or L-OHP singly. These findings indicate that simultaneous treatment and sequential schedule of L-OHP followed by 5-FU exhibited synergistic interaction in inducing apoptosis, but that sequential administration of 5-FU followed by L-OHP is antagonistic. These results are consistent with those obtained by combination assays.

Discussion

In this study, we examined the sequence dependency of 5-FU and L-OHP combination in four human gastric cancer cell lines *in vitro*. Both simultaneous combination and sequential treatment of L-OHP followed by 5-FU exhibited synergistic effects in all four cell lines, with the most efficacious interaction observed in simultaneous combination, whereas the reverse sequence yielded a clear antagonism. This observation was confirmed by the experiment that compared the cell number 7 days after various treatment schedules. However, our results are not consistent with the report by Fischel *et al.* who showed that the clinically relevant L-OHP and 5-FU combination was synergistic whatever the tested schedules using human colorectal cancer cell lines [17]. The different exposure time of L-OHP may explain the difference. In our study, we incubated cells with L-OHP for 24 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 [23], but also because more than 1.5 μg/ml (3.8 μmol/l) of total plasma concentration of platinum lasted at least 24 h

Table 3 Cell cycle perturbation and apoptosis (%) induced by 5-FU and L-OHP in AZ-521 cells

Treatment	24 h				36 h				48 h				72 h				96 h			
	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	49.5	36.5	14.0	1.82																
5-FU	86.6	4.49	8.91	1.61	87.8	4.16	8.04	3.11	86.0	7.19	6.81	4.60	82.4	6.00	11.6	12.2	80.6	6.50	12.9	26.9
L-OHP	63.6	3.60	32.8	1.89	64.5	7.30	28.2	7.74	68.2	6.70	25.1	14.7	70.5	7.10	22.4	14.8	70.5	14.4	15.1	20.0
L-OHP + 5-FU	64.9	3.50	31.6	2.27	65.3	6.70	28.0	8.05	68.7	5.50	25.8	14.6	73.4	6.50	20.1	42.7	73.9	9.30	16.8	61.5
5-FU → L-OHP					87.3	5.29	7.41	3.93	86.5	5.96	7.54	7.73	85.7	7.68	6.62	20.0	85.6	7.74	6.66	16.2
L-OHP → 5-FU					64.7	8.80	26.5	7.23	68.3	5.80	25.9	13.7	72.5	6.70	20.8	37.7	73.4	10.0	16. 6	55.5

Cells were treated with 5-FU or L-OHP singly or in combination at the IC_{50} doses, and subjected to FACS analyses after collecting floating and trypsinized adherent cells at various times following drug exposure as described in Materials and methods. The apoptotic population percentages (Apo) were determined by measuring the sub-G_1 phase. The data presented are the mean percentage values from three independent experiments.

when patients were administered with 130 mg/m^2 of L-OHP for 2 h [24]. Therefore, the sequence and exposure time of administration of these drugs might be important in determining the extent of therapeutic synergy.

To elucidate the possible mechanisms underlying the synergistic interaction, we further analyzed the perturbations induced in the cell cycle by flow cytometric analyses using AZ-521 cells. First, we found that 24-h treatment with 5-FU markedly affected the cell cycle distribution, producing a clear accumulation in the G_0/G_1 phase and induced apoptosis in 26.9% of treated cells. L-OHP alone induced 20% of apoptosis by arresting cells in both G_0/G_1 and G_2/M phases. Simultaneous 24-h exposure to 5-FU and L-OHP and sequential 24-h exposure to 5-FU immediately after L-OHP treatment led to 61.5 and 55.5% apoptosis, respectively, without affecting cell cycle distribution induced by L-OHP. These results imply that 5-FU may kill the cells recovering from the mitotic block produced by L-OHP as they progress into S phase, accounting for the synergistic interaction. In contrast, 5-FU followed by L-OHP resulted in an antagonistic effect, reducing the rate of apoptosis to 20%. This would probably be explained by the decrease in the G_2 population targeted by L-OHP, because 5-FU pre-treatment caused accumulation of cells at the G_1/S boundary, thereby reducing the number of cells entering the G_2 phase.

In our study, simultaneous treatment and a sequential schedule of L-OHP followed by 5-FU exhibited synergistic interaction. It has been shown that cisplatin can inhibit methionine uptake into tumor cells, resulting in increased methionine synthesis and subsequent expansion of the reduced folate pool [25,26]. In the presence of 5-FU, these biochemical events lead to greater stabilization of the ternary complex formed between 5-fluoro-dUMP-thymidylate synthetase and 5-10-methylenetetrahydrofolate [27]. Such mechanisms may explain, at least in part, the synergistic interaction presently observed between L-OHP and 5-FU. In addition, recent pharmacokinetic investigations have suggested that L-OHP can alter 5-FU clearance [28]. L-OHP can inhibit dihydropyrimidine dehydrogenase, which is the rate-controlling enzyme of 5-FU catabolism [29]. Conversely, 5-FU may also influence L-OHP cytotoxic effects. Previously, we reported a significant reduction of the repair of cisplatin-induced DNA interstrand crosslinks in cells exposed to 5-FU/cisplatin [30], presumably through 5-FU-induced reduction of ERCC1 mRNA expression [31]. It is thus likely that 5-FU may induce similar molecular effects when combined with L-OHP. Moreover, experiments in colon cancer cell lines have demonstrated that L-OHP treatment results in downregulation of both thymidylate synthase (TS) mRNA level and free TS protein expression [32]. This TS modulation and downregulation may

provide a basis for explaining synergism in the simultaneous and sequence L-OHP followed by 5-FU treatment.

Unlike cisplatin, L-OHP appears to arrest cells at both the G_0/G_1 and G_2/M phases, indicating an action distinct from that of cisplatin, which causes an accumulation of cells in the G_2/M phase [33,34]. Therefore, the different patterns of DNA damage induced [35] and distinct cell cycle perturbations between L-OHP and cisplatin may induce different interactions with other drugs. Accordingly, 5-FU followed by L-OHP exhibited a clear antagonism, as opposed to the 5-FU and cisplatin combination, where sequential treatment of 5-FU followed by cisplatin shows a synergistic activity [36–39]. The sequence-dependent synergy exhibited by the 5-FU and cisplatin combination can be explained by the mechanism of DNA damage repair and detoxification processes, i.e. pre-treatment of 5-FU increased cisplatin cytotoxicity and even circumvents cisplatin resistance by inhibiting repair of platinum–DNA interstrand crosslinks as well as by reducing the cellular GSH levels [30,40]. For the combination with 5-FU followed by L-OHP, such mechanisms may not be involved. In spite of many similarities between L-OHP and cisplatin, there are important differences in their targets and mechanisms of action that may be related to their different activity profiles. Reciprocal interference of drug binding to nucleic acid might underlie this antagonism, since it has been shown that DNA binding of L-OHP is significantly reduced by the presence of 5-FU and *vice versa* [41].

Although the biochemical basis for the synergistic interaction between 5-FU and L-OHP remains to be elucidated, an antagonistic activity observed in the sequence 5-FU followed by L-OHP in a variety of human gastric cancer cell lines may provide a rationale for avoiding this sequence in clinical trials.

References

1 Tsai JY, Safran H. Status of treatment for advanced gastric carcinoma. *Curr Oncol Rep* 2003; **5**:210–218.
2 Ho D. Epidemiologic studies in gastric cancer. In: Ho D (editor): Gastric cancer. New York: Churchill Livingstone; 1988. pp. 1–25.
3 Kohne CH, Wils JA, Wilke HJ. Developments in the treatment of gastric cancer in Europe. *Oncology* 2000; **14** (**12 Suppl**):22–25.
4 Wils J. Treatment of gastric cancer. *Curr Opin Oncol* 1998; **10**:357–361.
5 Janunger KG, Hafstrom L, Nygren P. Swedish Council of Technology Assessment in Health Care. A systematic overview of chemotherapy effects in gastric cancer. *Acta Oncol* 2001; **40**:309–326.
6 Jennerwein MM, Eastman A, Khokhar A. Characterization of adducts produced in DNA by isomeric 1,2-diaminocyclohexane platinum (II) complexes. *Chem Biol Interact* 1989; **70**:39–49.
7 Dunn T, Schmoll HJ, Grunwald V, Bokemeyer C, Casper J. Comparative cytotoxicity of oxaliplatin and cisplatin in non-seminomatous germ cell cancer cell lines. *Invest New Drugs* 1997; **15**:109–114.
8 Fukuda M, Ohe Y, Kanzawa F, Oka M, Hara K, Saijo N. Evaluation of novel platinum complexes, inhibitors of topoisomerase I and II in non-small cell lung cancer (NSCLC) sublines resistant to cisplatin. *Anticancer Res* 1995; **15**:393–398.
9 Becouarn D, Ychou M, Ducreux M, Borel C, Bertheault-Cvitkovic F, Seitz JF, *et al.* Phase II trial of oxaliplatin as first-line chemotherapy in metastatic colorectal cancer patients: Digestive Group of French Federation of Cancer Centers. *J Clin Oncol* 1998; **16**:2739–2744.
10 Piccart MJ, Green JA, Lacave AJ, Reed N, Vergote I, Bendetti-Panici P, *et al.* Oxaliplatin or paclitaxel in patients with platinum-pretreated advanced ovarian cancer: a randomized phase II study of the European Organization for Research and Treatment of Cancer Gynecology Group. *J Clin Oncol* 2000; **18**:1193–1202.
11 Garufi C, Nistico C, Brienza S, Vaccaro A, D'Ottavio A, Zappala AR, *et al.* Single-agent oxaliplatin in pretreated advanced breast cancer patients:a phase II study. *Ann Oncol* 2001; **12**:179–182.
12 Monnet I, Brienza S, Hugret F, Voisin S, Gastiaburu J, Saltiel JC, *et al.* Phase II study of oxaliplatin in poor-prognosis non-small cell lung cancer (NSCLC): Association pour le Traitement des Tumeurs Intrathoraciques. *Eur J Cancer* 1998; **34**:1124–1127.
13 Kim DY, Kim JH, Lee SH, Kim TY, Heo DS, Bang YJ, Kim NK. Phase II study of oxaliplatin, 5-fluorouracil and leucovorin in previously platinum-treated patients with advanced gastric cancer. *Ann Oncol* 2003; **14**:383–387.
14 Louvet C, André T, Tigaud JM, *et al.* Phase II study of oxaliplatin, fluorouracil, and folinic acid in locally advanced or metastatic gastric cancer patients. *J Clin Oncol* 2002; **20**:4543–4548.
15 Extra JM, Espie M, Calvo F, Ferne C, Mignot L, Marty M. Phase I study of oxaliplatin in patients with advanced cancer. *Cancer Chemother Pharmacol* 1990; **25**:299–303.
16 de Gramont A, Figer A, Seymour M, Homerin C, Hmissi A, Cassidy J. Leucovorin and fluorouracil with or without oxaliplatin as first-line treatment in advanced colorectal cancer. *J Clin Oncol* 2000; **18**:2938–2947.
17 Fischel JL, Etienne MC, Formento P, Milano G. Search for the optimal schedule for the oxaliplatin/5-fluorouracil association modulated or not by folinic acid: preclinical data. *Clin Cancer Res* 1998; **4**:2529–2535.
18 Al-Batran SE, Atmaca A, Hegewisch-Becker S, Jaeger D, Hahnfeld S. Phase II trial of biweekly infusional fluorouracil, folinic acid, and oxaliplatin in patients with advanced gastric cancer. *J Clin Oncol* 2004; **22**: 658–663.
19 De Vita F, Orditura M, Matano E. A phase II study of biweekly oxaliplatin plus infusional 5-fluorouracil and folinic acid (FOLFOX-4) as first-line treatment of advanced gastric cancer patients. *Br J Cancer* 2005; **92**:1644–1649.
20 Chao Y, Yeh KH, Chang CJ, Chen LT, Chao YY, Wu MF, *et al.* Phase II study of weekly oxaliplatin and 24-h infusion of high-dose 5-fluorouracil and folinic acid in the treatment of advanced gastric cancer. *Br J Cancer* 2004; **91**:453–458.
21 Ishiyama M, Shiga M, Sasamoto K, Mizogichi M, He P. A new sulfonated tetrazolium salt that produces a highly water-soluble formazan dye. *Chem Pharm Bull* 1993; **41**:1118.
22 Chou TC, Talalay P. Quantitative analysis of dose–effect relationships: the combined effects of multiple drugs or enzyme inhibitors. *Adv Enzyme Regul* 1984; **22**:27–55.
23 Kern W, Braess J, Bottger B, Kaufmann CC, Hiddeman W, Schleyer E. Oxaliplatin pharmacokinetics during a four-hour infusion. *Clin Cancer Res* 1999; **5**:761–765.
24 Takimoto CH, Remick SC, Sharma S, Mani S, Ramanathan RK, Doroshow J, *et al.* Dose-escalating and pharmacological study of oxaliplatin in adult cancer patients with impaired renal function: a National Cancer Institute Organ Dysfunction Working Group Study. *J Clin Oncol* 2003; **21**: 2664–2672.
25 Scanlon KJ, Safirstein RL, Thies H, Gross RB, Waxman S, Guttenplan JB. Inhibition of amino acid transport by *cis*-diamminedichloroplatinum(II) derivatives in L1210 murine leukemia cells. *Cancer Res* 1983; **43**: 4211–4215.
26 Krebs HA, Hems R, Tyler B. The regulation of folate and methionine metabolism. *Biochem J* 1976; **158**:341–353.
27 Scanlon KJ, Newman EM, Lu Y, Priest DG. Biochemical basis for cisplatin and 5-fluorouracil synergism in human ovarian carcinoma cells. *Proc Natl Acad Sci USA* 1986; **83**:8923–8925.
28 Gamelin E, Boisdron-Cell M, Allain P, Turcant A, Delva R, Cailleux A, *et al.* Pharmacokinetic interference between oxaliplatin (LOHP) and fluorouracil: delayed 5-FU metabolism inhibition by LOHP. *Proc Am Soc Clin Oncol* 1997; **38**:223.
29 Milano G, Etienne MC. Potential importance of dihydropyrimidine dehydrogenase (DPD) in cancer chemotherapy. *Pharmacogenetics* 1994; **4**:301–306.
30 Esaki T, Nakano S, Tatsumoto T, Kuroki-Migita M, Mitsugi K, Nakmura M, *et al.* Inhibition by 5-fluorouracil of *cis*-diamminedichloroplatinum(II)-induced DNA interstrand cross-link removal in a HST-1 human squamous carcinoma cell line. *Cancer Res* 1992; **52**:6501–6506.
31 Fujishima H, Nakano S, Esaki T, Tatsumoto T, Kondo T, Niho Y. Inhibition by 5-fluorouracil of ERCC1 and gamma-glutamylcysteine synthetase messenger RNA expression in a cisplatin-resistant HST-1 human squamous carcinoma cell line. *Oncol Res* 1997; **9**:167–172.

32 Yeh KH, Cheng AL, Wan JP, Lin CS, Liu CC. Down-regulation of thymidylate synthase expression and its steady-state mRNA by oxaliplatin in colon cancer cells. *Anticancer Drugs* 2004;**15**:371–376.
33 Ma J, Maliepaard M, Nooter K, Boersma AW, Verweij J, Stoter G, *et al.* Synergistic cytotoxicity of cisplatin and topotecan or SN-38 in a panel of eight solid-tumor cell lines *in vitro*. *Cancer Chemother Pharmacol* 1998; **41**:307–316.
34 Sorenson CM, Eastman A. Mechanism of *cis*-diamminedichloroplatinum(II)-induced cytotoxicity: role of G_2 arrest and DNA double-strand breaks. *Cancer Res* 1988; **48**:4484–4488.
35 Page JD, Husain I, Sancar A, Chaney SG. Effect of the diaminocyclohexane carrier ligand on platinum adduct formation, repair, and lethality. *Biochemistry* 1990; **29**:1016–1024.
36 Pratesi G, Gianni L, Manzotti C, Zunino F. Sequence dependence of the antitumor and toxic effects of 5-fluorouracil and *cis*-diamminedichloroplatinum combination on primary colon tumors in mice. *Cancer Chemother Pharmacol* 1988; **21**:237–240.
37 Trave F, Rustum YM, Goranson J. Synergistic antitumor activity of cisplatin (DDP) and 5-fluorouracil (FUra) in mice bearing leukemia L1210 cells. *Proc Am Assoc Cancer Res* 1985; **26**:1270.
38 Vietti T, Coulter D, Valeriote F. Interval and sequence dependent lethal effects of *cis*-diammine dichloroplatinum (Cis-Pt) in coordination with other agents. *Proc Am Assoc Cancer Res* 1979; **20**:818.
39 Kuroki M, Nakano S, Mitusgi K, Ichinose I, Anzai K, Nakamura M, *et al. In vivo* comparative therapeutic study of optional administration of 5-fluorouracil and cisplatin using a newly established HST-1 human squamous-carcinoma cell lines. *Cancer Chemother Pharmacol* 1992; **29**:273–276.
40 Esaki T, Nakano S, Masumoto N, Fujishima H, Niho Y. Schedule-dependent reversion of acquired cisplatin resistance by 5-fluorouracil in a newly established cisplatin-resistant HST-1 human squamous carcinoma cell line. *Int J Cancer* 1996; **65**:479–484.
41 Fischel JL, Formento P, Ciccolini J, Rostagno P, Etienne MC, Catalin J, *et al.* Impact of the oxaliplatin–5 fluorouracil–folinic acid combination on respective intracellular determinants of drug activity. *Br J Cancer* 2002; **86**:1162–1168.