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ORIGINAL ARTICLE

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In vitro schedule-dependent interaction between paclitaxel and oxaliplatin in human cancer cell lines

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Abstract *Purpose*: In order to define the most effective administration schedule of the combination of paclitaxel and oxaliplatin, we investigated the in vitro interaction between these drugs in a panel of three human cancer cell lines (AZ-521 gastric adenocarcinoma cell line, HST-1 tongue squamous carcinoma cell line, and KSE-1 esophageal squamous carcinoma cell line). *Materials and methods*: Cytotoxic activity was determined by the WST-1 assay. Different administration 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. *Results*: Simultaneous treatment of cells with paclitaxel and oxaliplatin showed greater than additive effects. Upon 24-h sequential exposure, the sequence of paclitaxel followed by oxaliplatin showed synergistic effects in AZ-521 and HST-1 cells, and greater than additive effects in KSE-1 cells, while the opposite sequence yielded marked antagonistic effects in all three cell lines. Flow cytometric analysis indicated that paclitaxel induced G_2/M arrest with subsequent induction of apoptosis in the sub-G_1 phase. Apoptosis was most prominent when paclitaxel preceded oxaliplatin, which produced apoptosis in the majority of treated cells (75%). By contrast, the reverse sequence yielded only 39% induction of apoptotic cells, the rate being not different from those induced by each drug singly. *Conclusions*: Our findings suggest that the interaction of paclitaxel and oxaliplatin is highly schedule-dependent and that the sequential administration of paclitaxel followed by oxaliplatin should thus be incorporated into the design of a clinical trial.

Keywords Oxaliplatin · Paclitaxel · Drug interaction · Sequence-dependence

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Introduction

In view of the limited effectiveness of the currently available cytotoxic drugs for solid tumors such as gastric cancer, esophageal cancer, and head and neck cancer, there is an urgent need of new and better therapeutic approaches to improve the clinical outcome of these diseases. Multiple novel agents have been investigated in the treatment of patients with these cancers. Among these, paclitaxel and oxaliplatin are two anticancer drugs used increasingly in monotherapy or in combination with other drugs in the clinic.

Paclitaxel is a chemotherapeutic agent that induces apoptosis by arresting the cell cycle at the G_2/M phase through tubulin polymerization [28]. This agent has demonstrated clinical efficacy in the treatment of ovarian cancer, non-small-cell lung cancer, breast cancer, and head and neck cancer [26]. Paclitaxel produces peripheral neuropathy, dose-limiting bone marrow suppression, and alopecia [5]. Oxaliplatin (trans-l-1,2-diaminocyclohexane oxalato platinum II) 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 [12]. Oxaliplatin has been demonstrated to exhibit antitumor activity against cell lines with acquired cisplatin resistance as well as clinical tumors that are intrinsically resistant to cisplatin and carboplatin [6, 9, 26]. Phase II studies of single-agent oxaliplatin have shown activity in colorectal [1], ovarian [23], breast [10], and untreated non-small-cell lung cancers [19]. Oxaliplatin has a different toxicity profile from that of cisplatin, with mild nausea and vomiting and, in contrast to

carboplatin, mild to moderate hematological toxicity. The dose-limiting toxicity of oxaliplatin is a dose-dependent and reversible peripheral neuropathy [7].

Paclitaxel has shown synergism with cisplatin both in vitro and in vivo [13, 18]. The combination of oxaliplatin and paclitaxel appears to show substantial activity in ovarian cancer patients previously treated with cisplatin or carboplatin, even in those with platinum resistance [8]. Although the combination of oxaliplatin and paclitaxel would be expected to have potent activity similar to that of the combination of paclitaxel and cisplatin, few preclinical data for the interaction between these drugs are currently available. In order to obtain the clinical rationale for the optimal administration schedule of this combination. we investigated the interaction between oxaliplatin and paclitaxel using an in vitro model of human cancer cell lines using a quantitative method which assessed the synergism or antagonism between these two agents.

Materials and methods

Cell lines and culture

The human AZ-521 gastric adenocarcinoma cell line was kindly provided by the JCRB Cell Bank (Tokyo, Japan) and the cells were maintained in Dulbecco's minimum essential medium (DMEM) (Nissui, Tokyo, Japan) supplemented with 10% heat-inactivated fetal calf serum (GIBCO, Grand Ireland, N.Y.) in an incubator at 37°C and 100% humidity in air containing 5% CO_2. The human HST-1 tongue squamous carcinoma cell line [20] and the human KSE-1 esophageal squamous carcinoma cell line [17] were established in our laboratory and the cells were maintained under the same conditions as the AZ-521 cells.

Drugs

Paclitaxel was a gift from Bristol-Myers (Tokyo, Japan) and oxaliplatin was a gift from Yakult (Tokyo, Japan). Stock solutions of paclitaxel were prepared in DMSO and those of oxaliplatin were prepared in distilled water. Both solutions were stored at –4°C prior to use. The final concentration of DMSO for all experiments and treatments was maintained at less than 0.02%. These conditions were found to be non-cytotoxic.

Cytotoxicity assay

Cytotoxic activity was measured by the WST-1 assay (Wako Chemicals, Osaka, Japan) following the manufacturer's instructions [11]. The WST-1 assay is a colorimetric method in which the intensity of the dye is proportional to the number of viable cells. Briefly, cells were plated into 96-well microtiter plates at a density of 5×10^3 cells/well, and incubated for 24 h for sufficient cell growth. Cells were then treated with graded concentrations of paclitaxel (0.3–1000 ng/ml) or oxaliplatin (0.3–1000 μg/ml) alone for 24 h, and were incubated with drug-free medium for an additional 24 h. Cells were washed with PBS and 100 μl medium, and 10 μl WST-1 solution was added to each well and the plates were incubated at 37°C for another 3 h. Absorbance at 450 nm and 640 nm was measured using a Delta Soft ELISA analysis program for Macintosh computers interfaced with a Bio-Tek microplate reader (Immuno-Mini NJ-2300). Wells containing only DMEM and WST-1 were used as controls. Each experiment was performed 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 reduced the absorbance in each test by 50%.

For the combination experiments, three different schemes were used to investigate the interaction of paclitaxel and oxaliplatin as shown in Fig. 1: in schedule A, paclitaxel and oxaliplatin were exposed simultaneously for 24 h and incubated for additional 24 h with drug-free medium; in schedule B, paclitaxel was administered for 24 h followed by oxaliplatin for 24 h; and in schedule C, oxaliplatin was administered for 24 h followed by paclitaxel. Immediately after these treatments, the cytotoxic effects were evaluated by WST-1 assay.

Analysis of combination effects

On the basis of the growth inhibition curve for each single drug, we analyzed the effects of the drug combinations using the method described by Chou and Talalay and the Calcusyn software program for automated analysis (Biosoft, Cambridge, United Kingdom) [2, 3]. The effect of combining the two drugs was evaluated by comparing the results of the sequential assays with those of the assays involving oxaliplatin or paclitaxel exposure

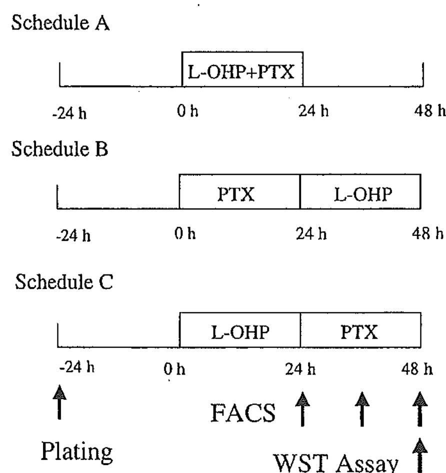

Fig. 1 The three combination schedules

alone. The combination effect was evaluated from isoeffect analysis (Cis), calculated as follows: CI = $C_{paclitaxel}/Cx_{paclitaxel} + C_{oxaliplatin}/Cx_{oxaliplatin}$, where $Cx_{paclitaxel}$ and $Cx_{oxaliplatin}$ are the concentrations of paclitaxel and oxaliplatin alone, respectively, needed to achieve a given effect (x%) and $C_{paclitaxel}$ and $C_{oxaliplatin}$ are the concentrations of paclitaxel and oxaliplatin needed for the same effect (x%) when the drugs are combined. These concentrations were calculated for each experiment and for each combination experiment at a fixed concentration ratio. The combination was considered as positive (synergistic) when the combination index was < 1 and negative (antagonistic) when it was > 1, and values of 1 were considered to indicate additivity.

Cell cycle determination

AZ-521 cells were cultured at 1×10^5 cells per 60-mm dish. The same protocols as described in the growth inhibition assay were used. After treatment, the cells were harvested, washed twice in ice-cold PBS (pH 7.4), and then fixed in 100% ethanol and stored at 4°C for up to 3 days prior to cell cycle analysis. After the removal of ethanol by centrifugation, cells were washed with PBS and stained with a solution containing propidium iodide and RNase (Sigma-Aldrich, St. Louis, Mo.) on ice for 30 min. Cell cycle analysis was performed on a Becton Dickinson FACS/Calibur flow cytometer using the CELLQuest or ModFit 3.0 software packages (Becton Dickinson, San Jose, Calif.), and the percentages of apoptotic populations were determined by measuring the sub-G_1 phase using FACS analysis after collecting floating and trypsinized adherent cells at various times following drug exposure. Each experiment was performed in triplicate.

Results

Single-agent experiments

The cytotoxic activities of paclitaxel and oxaliplatin were tested individually on the three tumor cell lines. The cells were exposed to each drug for 24 h. The IC_{50} values (±SD) are summarized in Table 1. For paclitaxel, the IC_{50} ranged from 14.0 ng/ml (0.016 n*M*) for AZ-521 cells to 26.0 ng/ml (0.03 n*M*) for HST-1 cells. HST-1 cells were more resistant than AZ-521 or KSE-1 cells. AZ-521 cells were the most sensitive to oxaliplatin (0.95 µg/ml, 2.39 *µM*) among the three tumor cell lines, and KSE-1 cells were the least sensitive (11.9 µg/ml, 29.7 *µM*).

Median-effect analysis of paclitaxel and oxaliplatin combination in vitro

Paclitaxel and oxaliplatin were tested in different combinations to define the most effective schedule. Three different schedules were tested (simultaneous or sequential drug exposure as shown in Fig. 1) and the exposure time to each drug was 24 h. In AZ-521 cells, simultaneous treatment with the two drugs for 24 h caused largely additive effects in the moderate cytotoxic range (Fig. 2a). Sequential treatment with paclitaxel followed by oxaliplatin produced great synergy in all the ranges of cell kill fraction (CIFig. 2b). Conversely, when

Table 1 IC_{50} values of paclitaxel and oxaliplatin in three cell lines. Cells were treated with various concentrations of paclitaxel for 24 h or oxaliplatin for 24 h. The values are the means ± SD of three independent experiments

	AZ-521	HST-1	KSE-1
Paclitaxel (ng/ml)	14 ± 1.9	26 ± 2.6	18.9 ± 0.9
Oxaliplatin (µg/ml)	0.95 ± 0.4	3.5 ± 2.0	11.9 ± 5.6

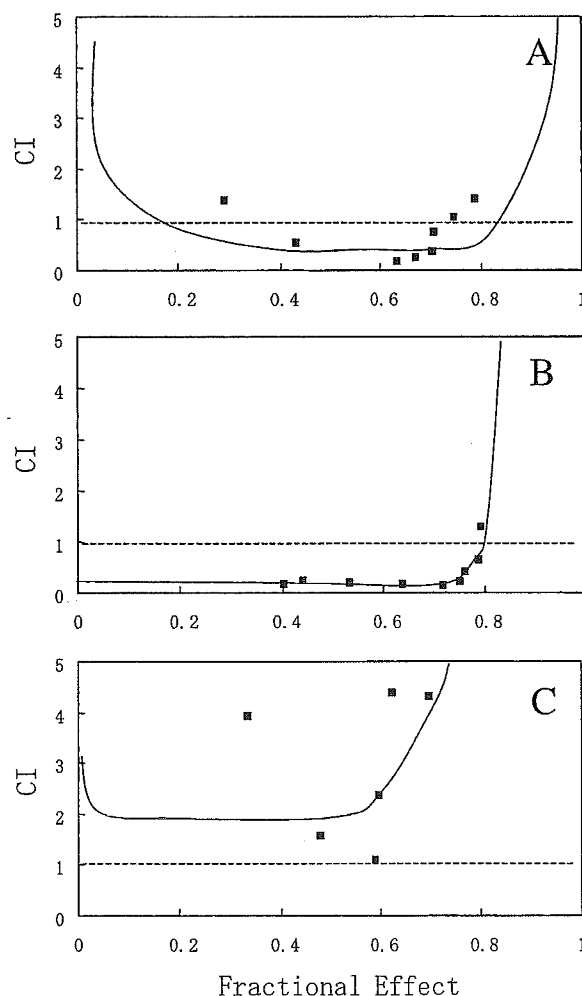

Fig. 2 Combination index (CI) plots of interactions between paclitaxel and oxaliplatin in AZ-521 cells. Cells were treated with (**a**) paclitaxel and oxaliplatin for 24 h simultaneously, (**b**) paclitaxel for 24 h followed by oxaliplatin for 24 h, or (**c**) oxaliplatin for 24 h followed by paclitaxel for 24 h

the inverse sequence (oxaliplatin followed by paclitaxel) was used, antagonistic effects were observed at all levels of cell kill fraction (CI > 1; Fig. 2c).

In HST-1 cells, simultaneous treatment and sequence paclitaxel followed by oxaliplatin yielded similar synergistic effects at the same cytotoxic ranges corresponding to greater than 40% inhibition of cell growth (Fig.3a, b), whereas the opposite sequence showed antagonism (Fig. 3c). In the KSE-1 cells, simultaneous treatment and sequence paclitaxel followed by oxaliplatin showed additive to synergistic effects (Fig. 4a, b). In contrast, oxaliplatin followed by paclitaxel showed antagonistic effects (Fig. 4c).

Cell cycle perturbation and apoptosis

In an attempt to explain the mechanisms underlying the different types of interaction, the effects of paclitaxel and oxaliplatin on cell cycle distribution and apoptosis were studied in AZ-521 cells (Table 2). The cells were treated with these drugs either alone or in combination with different schedules, and cell cycle distribution was analyzed 24, 36 and 48 h after the beginning of treatment using flow cytometry. Paclitaxel alone at a dose of 12.5 ng/ml induced accumulation of cells in the G_2/M phase. At 1 μg/ml, oxaliplatin alone caused an increase in the G_1 population and a decrease in the S-phase population, showing that it inhibited $G_1$to S progression. Treatment with paclitaxel prior to oxaliplatin induced accumulation of cell in the G_2/M phase as well as a reduction in the G_1 cell population, a similar distribution patterns to that observed in cells treated with paclitaxel alone, although a slight increase in the G_0/G_1 population and a decrease in the G_2/M population were observed compared with cells treated with paclitaxel alone. In contrast, oxaliplatin prior to paclitaxel caused almost identical distribution patterns to those observed

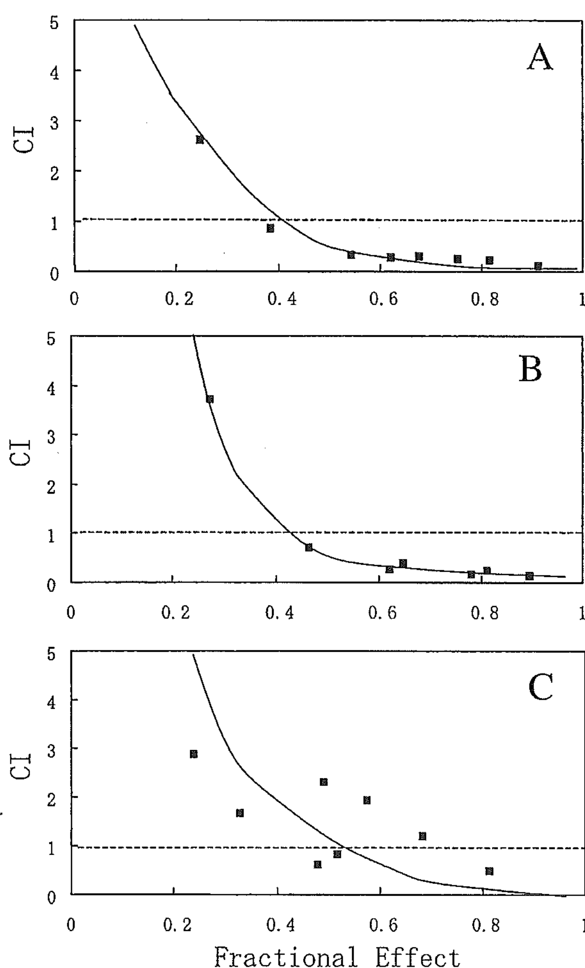

Fig. 3 Combination index (CI) plots of interactions between paclitaxel and oxaliplatin in HST-1 cells. Cells were treated with (**a**) paclitaxel and oxaliplatin for 24 h simultaneously, (**b**) paclitaxel for 24 h followed by oxaliplatin for 24 h, or (**c**) oxaliplatin for 24 h followed by paclitaxel for 24 h

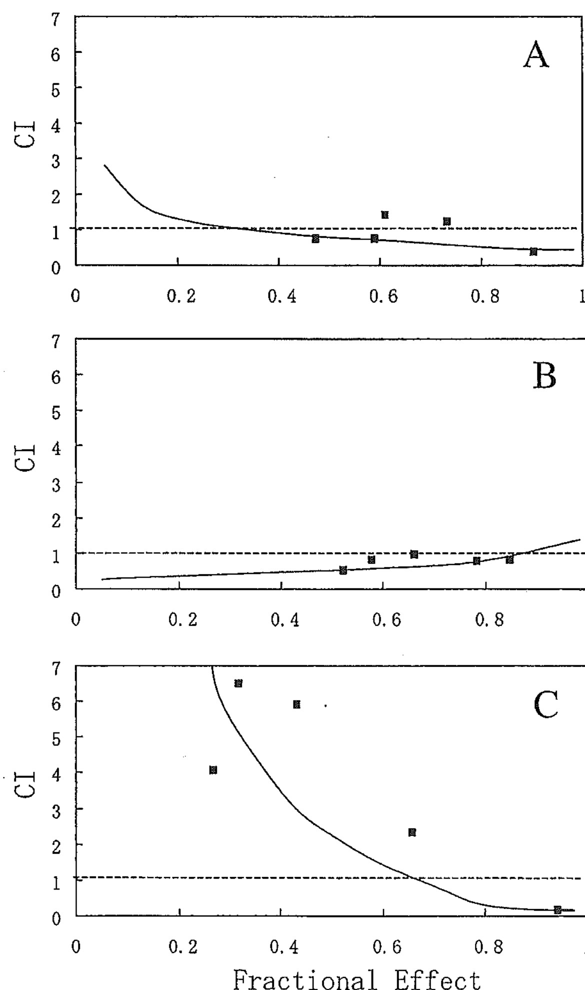

Fig. 4 Combination index (CI) plots of interactions between paclitaxel and oxaliplatin in KSE-1 cells. Cells were treated with (**a**) paclitaxel and oxaliplatin for 24 h simultaneously, (**b**) paclitaxel for 24 h followed by oxaliplatin for 24 h, or (**c**) oxaliplatin for 24 h followed by paclitaxel for 24 h

with oxaliplatin alone, although a slight decrease in the G_0/G_1 population and an increase in the G_2/M population were observed compared with cells treated with oxaliplatin 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 the G_2/M phase, a pattern similar to that caused by paclitaxel alone, indicating that paclitaxel might have a dominant effect in cell cycle progression as compared to oxaliplatin, or that oxaliplatin might take more time to exhibit its activity than paclitaxel.

To confirm the activities of sequential combinations, the apoptotic activity was investigated after treatment of AZ-521 cells by measuring the sub-G_1 population by FACS analysis. The presence of hypodiploid DNA (sub-G_1) is associated with cells undergoing apoptosis. As shown in Table 2, paclitaxel followed by oxaliplatin induced a G_2/M block, with substantial induction of apoptosis in the majority of the treated cells (75%). The induction rate of apoptosis by this sequential treatment was greater than that of paclitaxel alone (56–66%) or oxaliplatin alone (35–38%). By contrast, the reverse sequence caused G_1 block, and the apoptotic population was 38–41%, that is less than that induced by paclitaxel alone (56–66%), and similar to that induced by oxaliplatin alone (35–38%). These findings indicate the sequence of oxaliplatin followed by paclitaxel is antagonistic in inducing apoptosis.

Discussion

In this study, we examined in vitro the sequence dependency of the paclitaxel and oxaliplatin combination in three human cancer cell lines derived from tongue, esophagus, and stomach. Simultaneous treatment with these two drugs resulted in mostly additive effects. With the sequence paclitaxel followed by oxaliplatin, either synergism or additivity was observed in all three cell lines, indicating that this sequence would be the most effective schedule. By contrast, a clear antagonism was observed with the sequence oxaliplatin followed by paclitaxel in all of the cell lines.

To explain the possible mechanism underlying the synergistic interaction of paclitaxel followed by oxaliplatin, we further analyzed the perturbations induced in cell cycle by flow cytometric analysis using AZ-521 cells. First, we found that a 24-h treatment with paclitaxel markedly affected the cell cycle distribution, producing a relevant accumulation in the G_2/M phase, and induced apoptosis in 56% of treated cells. Oxaliplatin alone induced apoptosis (35%) by arresting cells in the G_1 phase. Exposure to oxaliplatin immediately after treatment with paclitaxel led to apoptosis in the majority of cells (75%) without affecting cell cycle distribution induced by paclitaxel. These results suggest that oxaliplatin may kill the cells recovering from the mitotic block produced by paclitaxel as they progress into S phase, accounting for the synergistic interaction. By contrast, oxaliplatin followed by paclitaxel had an antagonistic effect, reducing the rate of apoptosis to 39%. This would probably be explained by the decrease in the G_2 population targeted by paclitaxel, because pretreatment with oxaliplatin caused accumulation of cells at G_1/S boundary, thereby reducing the number of cells entering G_1 phase.

Unlike cisplatin, oxaliplatin, a new type of platinum derivative containing a diaminocyclohexane carrier ligand, appears to arrest the cells mainly at G_1 phase, suggesting an action distinct from that of cisplatin causing accumulation of cells in the G_2/M phase [16, 29]. It has been consistently demonstrated that oxaliplatin exhibits activity in cell lines with acquired cisplatin resistance and is active even in tumor types that are intrinsically resistant to cisplatin as well as carboplatin [6, 9, 25]. Therefore, this non-cross-resistance might be due to the differential patterns of DNA damage induced [21] and distinct cell cycle perturbations between oxaliplatin and other platinum compounds. With regard to a synergistic or additive interaction observed when paclitaxel preceded oxaliplatin, a similar sequence-dependent interaction has been reported with the combination of paclitaxel and CDDP. Synergistic or additive effects have been observed when paclitaxel precedes cisplatin [4, 18, 22, 27], whereas antagonistic interactions have been observed with the reverse sequence [15, 30]. There are several explanations for the increased activity of the sequence paclitaxel followed by cisplatin: cisplatin hastens the exit from mitosis in paclitaxel-treated cells

Table 2 Cell cycle perturbation (%) and apoptosis induced by paclitaxel and oxaliplatin in AZ-521 cells. The apoptotic population percentages were determined by measuring the sub-G_1 phase by FACS analysis after collecting floating and trypsinized adherent cells at various times following drug exposure. The data presented are the mean percentage values from three independent experiments

	24 h				36 h				48 h			
	G_0/G_1	S	G_2/M	Apoptosis	G_0/G_1	S	G_2/M	Apoptosis	G_0/G_1	S	G_2/M	Apoptosis
Control	52.64	31.75	15.61	3.52								
Paclitaxel	15.02	16.66	68.32	39.07	20.09	19.88	60.02	66.61	23.53	25.07	51.40	56.47
Oxaliplatin	71.87	3.12	25.01	11.01	76.96	9.51	13.53	37.80	86.89	5.22	7.89	35.12
Oxaliplatin + paclitaxel	21.56	12.05	66.39	38.57	24.93	8.05	67.02	57.70	29.34	5.31	65.35	65.32
Paclitaxel → oxaliplatin					28.32	26.01	45.67	75.83	15.02	34.13	50.85	74.91
Oxaliplatin → paclitaxel					68.68	3.42	27.91	40.78	81.16	2.57	16.27	37.93

[18]; paclitaxel induces an increase in intracellular uptake of cisplatin [4]; and paclitaxel inhibits repair of cisplatin-induced DNA damage [22]. Therefore, we hypothesize that similar, if not identical, mechanisms to those demonstrated for the interaction between cisplatin and paclitaxel may operate for the combination of oxaliplatin and paclitaxel.

Clinically, oxaliplatin is frequently used in combination to improve its efficacy. Over the past years, oxaliplatin combinations have been explored preclinically and clinically, mainly with thymidylate synthase inhibitors [24], other platinum compounds [25], and topoisomerase I inhibitors [31]. Based on the fact that paclitaxel exhibits synergism with cisplatin, combinations of oxaliplatin and paclitaxel would be expected to show potent activity similar to that of paclitaxel and cisplatin. Recently, clinical activity of the oxaliplatin and paclitaxel combination has been shown in platinum-pretreated patients with ovarian cancer [8]. The combination of oxaliplatin and docetaxel has also been reported to be a feasible and well-tolerated outpatient regimen as front-line chemotherapy in patients with non-small-cell lung cancer or advanced breast cancer [14]. Although the biochemical basis for their interaction remains unknown, the clear sequence-dependent activity of the combination of oxaliplatin and paclitaxel should be incorporated into the design of a clinical trial.

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Synergistic interaction between oxaliplatin and SN-38 in human gastric cancer cell lines *in vitro*

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Abstract. The interaction between CPT-11 and oxaliplatin, a new platinum derivative that has a great antitumor activity against colon cancer, has not been determined in gastric cancer cells. In this study, we investigated *in vitro* cytotoxic activity of oxaliplatin alone or in combination with SN-38, an active metabolite of CPT-11, using different exposure schedules in three human gastric cancer cell lines (AZ-521, MKN-45, and NUGC-4). Cytotoxicity was determined by WST-1 assay. Different treatment 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 was evaluated by flow cytometry. In 24-h exposure, simultaneous administration of oxaliplatin and SN-38 showed a synergistic effect in AZ-521 and NUGC-4 cells, and an additive effect in MKN-45 cells. Greater than additive effects were observed in all of the cell lines when cells were treated with oxaliplatin followed by SN-38, whereas such effects were observed only in NUGC-4 cells in the reverse sequence. Flow cytometric analyses at IC_{50} indicated that apoptosis was most prominent in simultaneous exposures with accumulation of cells in both G_0/G_1 and S phases. These results suggest that SN-38 may kill the cells recovering from the G_1 block produced by oxaliplatin as they progress into the S phase. Simultaneous administration appears most active in gastric cancer cell lines. These results may provide important information for a clinical trial of oxaliplatin and CPT-11 combination for patients with gastric cancer.

Introduction

Gastric cancer remains one of the leading causes of cancer death worldwide and the prognosis of patients with unresectable and recurrent gastric cancer is extremely poor. Although complete resection is the only curative approach, randomized trials demonstrated that fluorouracil-based chemotherapy improves survival and quality of life in patients with advanced gastric cancer (1-3). Nevertheless, none of these regimens can be regarded as standard treatment because of their low activity (4). Therefore, it is of extreme importance to develop new strategies with better clinical efficacy in the treatment of advanced gastric cancer.

Oxaliplatin is a new platinum analogue that exhibits a wide spectrum of antitumor activity against tumors resistant to cisplatin as well as carboplatin (5-7), and has shown to be more active in preclinical models compared with cisplatin, in that it requires fewer DNA adducts to achieve an equal level of cytotoxicity (8). In comparison to other platinum compounds, oxaliplatin lacks the nephrotoxicity of cisplatin and myelosuppression of carboplatin, but it produces a reversible cold-sensitive peripheral neuropathy (9). Oxaliplatin has shown antitumor activity against colon cancer both *in vitro* and *in vivo*, and is now used in the chemotherapeutic treatment of metastatic colorectal cancer (10). Recently, it has been demonstrated that adding oxaliplatin to a standard adjuvant treatment (fluorouracil and leucovorin) improves the efficacy of adjuvant treatment of colon cancer (11). It has also been demonstrated that oxaliplatin is active in gastric cancer. In phase II studies in patients with advanced gastric cancer, the combination of oxaliplatin and 5-fluorouracil had a significant activity with a favorable toxicity profile (12,13).

Irinotecan hydrochloride (CPT-11) is rapidly converted *in vivo* to its active metabolites, SN-38, by carboxylesterase and shows the potent antitumor activity against various solid tumors, including colorectal, lung and ovarian cancers (14-17), through the inhibition of DNA topoisomerase-I (18,19). Recently, CPT-11 has also been demonstrated to be active against gastric cancer, with the response rate of 18% in a phase II study (20), and has produced a much higher response rate than conventional chemotherapy when combined with cisplatin (21). Moreover, we previously reported that maximal synergy is achieved *in vitro* when cisplatin and SN-38 is applied simultaneously (22,23).

Because of a different mechanism of action and non-overlapping toxicity profile, the combination of oxaliplatin and CPT-11 is currently under clinical investigation in patients with colorectal cancer (24). Although preclinical studies have shown a schedule-dependent additivity and synergism

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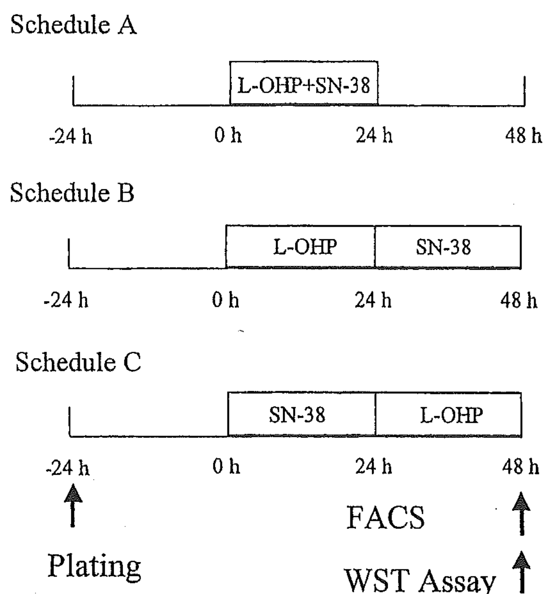

Figure 1. Description of the three combination schedules.

Table I. IC_{50} values of oxaliplatin and SN-38 in a panel of three cell lines.

	AZ-521	MKN45	NUGC-4
Oxaliplatin (μg/ml)	0.95±0.4	1.7±0.9	1.49±0.9
SN-38 (ng/ml)	14.2±0.4	19.5±0.5	17.83±0.9

Cells were treated for 24 h with various concentrations of SN-38 (0.3-1000 ng/ml) and oxaliplatin (0.3-1000 μg/ml). Results are expressed as the concentration that inhibits 50% of growth in comparison with controls (IC_{50}). The values are mean ± SD of three independent experiments.

between these drugs in colon cancer cell lines (25,26), such studies have yet to be determined in gastric cancer cells where both drugs are also active. In this study, we investigated *in vitro* cytotoxic activity of oxaliplatin alone or in combination with SN-38, an active metabolite of CPT-11, using different exposure schedules in three human gastric cancer cell lines, including AZ-521, MKN-45 and NUGC-4.

Material and methods

Cell line and culture. The three human gastric cancer cell lines, AZ-521, MKN-45 and NUGC-4, were purchased from Japanese Cell Resource Bank. The cells were propagated in D-MEM supplemented with 10% heat-inactivated FCS in an incubator at 37°C and 100% humidity with 5% CO_2 and air.

Drugs. SN-38 and oxaliplatin were a gift from Yakult (Tokyo, Japan). Stock solutions of SN-38 were prepared in DMSO and stored at -4°C prior to use. Oxaliplatin was prepared in distilled water. The final concentration of DMSO for all experiments and treatments was maintained at <0.02%. These conditions were found to be non-cytotoxic.

Growth inhibition assay. Cytotoxic activity was measured using the WST-1 assay (Wako-chemical Co. Japan) according to the manufacturer's instructions (27). The WST-1 assay is a colorimetric method in which the intensity of the dye is proportional to the number of viable cells. Briefly, AZ-521, MKN-45 and NUGC-4 cells were plated into 96-well microtiter plates at a density of $5x10^3$ cells/well. After overnight incubation, the cells were treated for 24 h with graded concentrations of SN-38 (0.3-1000 ng/ml), or oxaliplatin (0.3-1000 μg/ml). To define the best schedule for the combination, either simultaneous or sequential 24-h exposures to the two agents were tested. Forty-eight hours after the beginning of the treatment, cells were washed with PBS and 100 μl medium, and the plates were incubated at 37°C for another 3 h after addition of 10 μl WST solution. Absorbance at 480 and 640 nm was measured using a Delta Soft ELISA analysis program for Macintosh computer interfaced with Bio-Tek microtiter reader (immuno Mini NJ-2300). Wells containing only DMEM and WST solution were used as controls. Each experiment was performed using six replicated wells for each drug concentration and carried out independently at least three times. The IC_{50} was defined as the concentration that reduced the absorbance in each test by 50%. For the combination experiments, three different schemes were used to investigate the interaction of SN-38 and oxaliplatin, as shown in Fig. 1; (A) oxalipatin and SN-38 were exposed simultaneously for 24 h and incubated for an additional 24 h with drug-free medium, (B) oxaliplatin was administered for 24 h followed by SN-38 for 24 h, or (C) SN-38 was administered for 24 h followed by oxaliplatin. Immediately after these treatments, the cytotoxic effects were evaluated by WST-1 assay.

Analysis of combination effects. Combination analysis was performed using the method described by Chou and Talalay (28,29), using the Calcusyn software program for automated analysis (Bio-soft, Ferguson, MO). The influence on the combination of the two drugs was evaluated by comparing the sequential assays with assays involving oxaliplatin or SN-38 exposures alone. The combination effect was evaluated from isoeffect analysis (CIs), calculated as follows: $CI=C_{oxaliplatin}/Cx_{oxaliplatin}+C_{SN-38}/Cx_{SN-38}$, where $Cx_{oxaliplatin}$ and Cx_{SN-38} are the concentrations of oxaliplatin and SN-38 alone, respectively, needed to achieve a given effect (x%), and $C_{oxaliplatin}$ and C_{SN-38} are the combined concentrations of oxaliplatin and SN-38 needed for the same effect (x%). These concentrations were calculated for each experiment and for each combination experiment at a fixed concentration ratio. The CIs were calculated under the assumption of a mutually exclusive drug interaction, i.e. that the effect of SN-38 may influence the effect of oxaliplatin and vice versa. The combination is considered as synergistic when the CI is <1, and antagonistic when it is >1, whereas a value of 1 indicates additivity.

Cell cycle determination. Human gastric cancer cell line, AZ-521, cells were cultured at $1x10^5$ cells per 60 mm dish. The same protocols as described in the growth inhibition assay were used. After treatment, the cells were harvested,

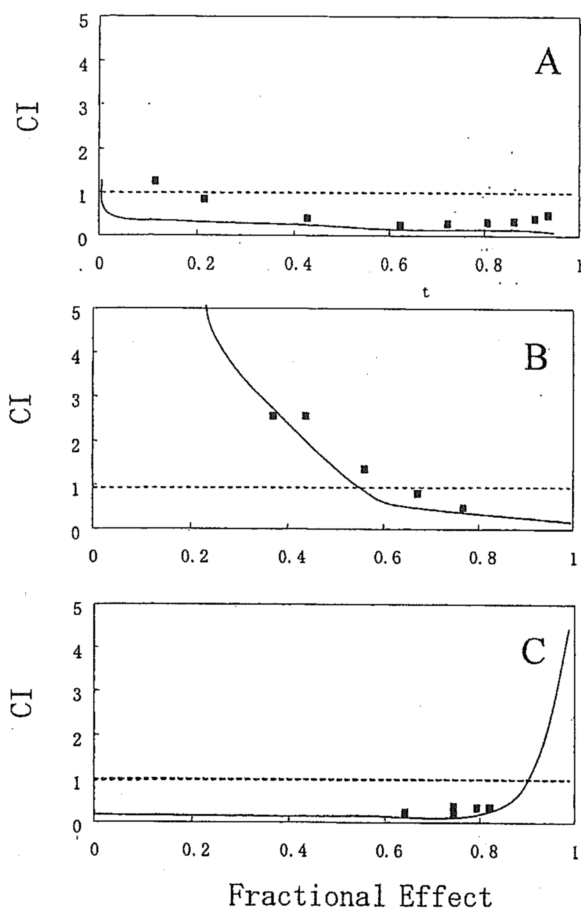

Figure 2. Combination index (CI) plots obtained from three gastric cancer cell lines exposed simultaneously to oxaliplatin and SN-38 for 24 h. (A) AZ-521, (B) MKN-45, (C) NUGC-4.

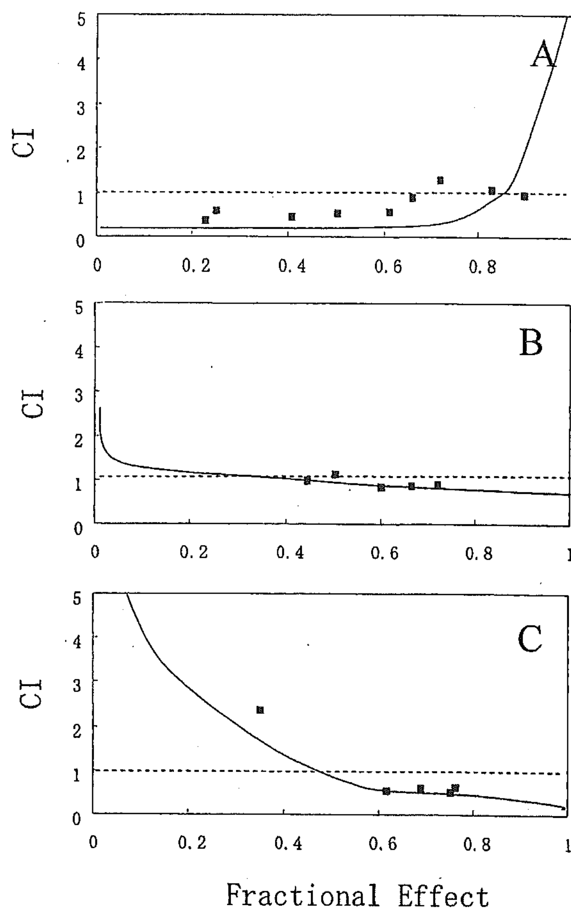

Figure 3. Combination index (CI) plots obtained from three gastric cell lines exposed to oxaliplatin for 24 h followed by SN-38 for 24 h. (A) AZ-521, (B) MKN-45, (C) NUGC-4.

washed twice in ice-cold PBS (pH 7.4), fixed in 100% ethanol and stored at 4˚C for up to 3 days, prior to cell cycle analysis. After the removal of ethanol by centrifugation, cells were washed with PBS and stained with a solution containing propidium iodide and RNase (Sigma-Aldrich, St. Louis, MO, USA) on ice for 30 min. Cell cycle analysis was performed on a Becton Dickinson FACS/Calibur Flow Cytometer using the CELLQuest or ModFit 3.0 software packages (Becton Dickinson, San Jose, CA, USA), and the percentages of apoptotic populations were determined by measuring the sub-G_1 phase using FACS analysis at various times following drug exposure. Each experiment was performed in triplicate.

Results

Determination of IC_{50} value. Three gastric cancer cell lines were exposed to oxaliplatin or SN-38, an active metabolite of CPT-11, for 24 h, and assayed for growth using the WST-1 assay. The IC_{50} for each drug and each cell line are shown in Table I. For oxaliplatin, the IC_{50} for 24-h exposure ranged from 0.95 μg/ml (2.39 μM) for AZ-521 cells to 1.7 μg/ml (4.3 μM) for MKN45 cells. The AZ-521 cell line was 2-fold more sensitive than MKN45 or NUGC-4 cell lines. AZ-521 cells were the most sensitive to SN-38 (14.2 ng/ml; 36 nM) among the three tumor cell lines, MKN45 cells being the least sensitive (19.5 ng/ml; 49 nM). These IC_{50} values were used to select concentration ranges for the combination studies.

Median-effect analysis of oxaliplatin and SN-38 combination in vitro. Oxaliplatin and SN-38 were tested in different combinations to define the most effective schedule. Three different schedules were tested, simultaneous or sequential drug exposures, as shown in Fig. 1, and exposure time to each drug was 24 h. Fig. 2 illustrates the CI plot obtained from three gastric cancer cell lines exposed simultaneously to oxaliplatin and SN-38 for 24 h. When either AZ-521 or NUGC-4 cells were treated with oxaliplatin and SN-38 simultaneously, the CI values were <1 at all levels of killed cell fraction, indicating a marked synergistic effect. This did not apply to MKN45 cells, where greater than additive effects were seen at the ranges corresponding to >50% inhibition of cell growth. A similar effect was observed when cells were treated with oxaliplatin for 24 h followed by SN-38 for 24 h and showed a greater than additive effect in all of the cell lines (Fig. 3), although the cytotoxic effects of this sequential administration appear less than those of simultaneous treatment. As shown in Fig. 4, when cells were treated with SN-38 followed by oxaliplatin, a greater than additive effect was observed only in NUGC-4 at all levels of killed cell fraction. However, in the MKN45 cell line, a greater than additive effect was observed at higher levels of

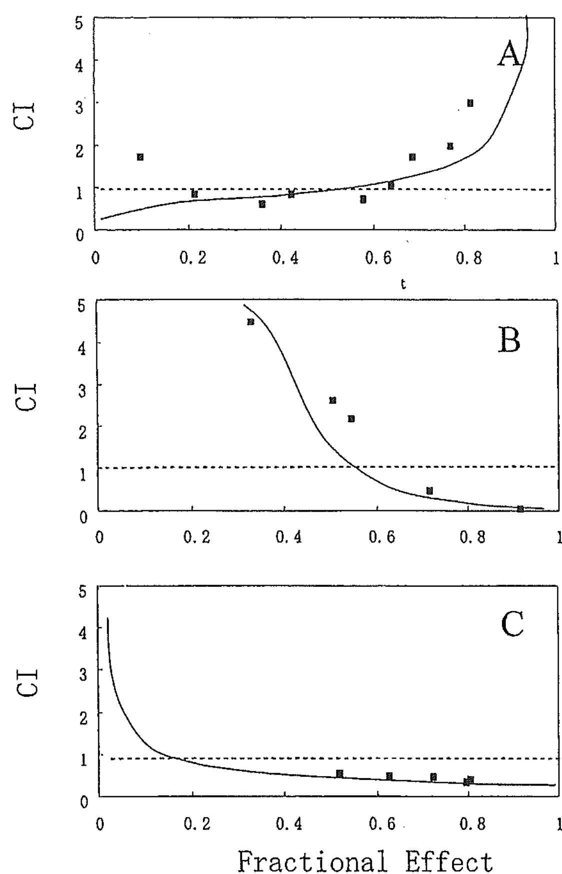

Figure 4. Combination index (CI) plots obtained from three gastric cell lines exposed to SN-38 for 24 h followed by oxaliplatin for 24 h. (A) AZ-521, (B) MKN-45, (C) NUGC-4.

killed cell fraction. Conversely, this sequence is antagonistic in AZ521 cells at the range corresponding to >70% killed cell fraction (Fig. 4).

Cell cycle perturbation and apoptosis. In an attempt to explain the mechanisms underlying the different types of interaction, the effects of SN-38 and oxaliplatin on cell cycle distribution and apoptosis were studied in AZ-521 cells (Table II). The cells were treated with these drugs at the IC_{50} concentration, either alone or in combination with different schedules, and cell cycle distribution was analyzed at 36 and 48 h after the beginning of the treatment, using flow-cytometric analysis. SN-38 alone induced the accumulation of cells in the S phase and markedly decreased the population of G_0/G_1 and G_2/M phases. By contrast, oxaliplatin alone caused an increase in the G_0/G_1 and G_2/M population and a decrease in the population of the S-phase. Oxaliplatin prior to SN-38 caused almost identical distribution patterns to those observed with oxaliplatin alone, indicating that the activity of oxaliplatin is unaffected by SN-38 in this treatment period. In contrast, the treatment with SN-38, prior to oxaliplatin, induced the accumulation of cells in the S phase as well as the reduction of the G_2/M cell population, showing similar distribution patterns to those observed in the cells treated with SN-38 alone, although a slight increase in G_0/G_1 and decrease in S population were observed as compared to those treated with SN-38 alone, indicating a modest influence of oxaliplatin. Therefore, in sequential combination, cell cycle distribution patterns for the initial drug appear mostly unaffected by the second drug administered, indicating a dominant effect of the initial drug. Interestingly, simultaneous exposures led to the accumulation of cells in both G_0/G_1 and S phases as well as a marked reduction of G_2/M population, indicating that the activities of individual drugs were exhibited with a substantial interaction.

To confirm the activities of these combinations, the apoptotic activity was investigated after treatment of AZ-521 cells by measuring the population of the sub-G_1 phase using FACS analyses. The presence of hypodiploid DNA (sub-G_1) is associated with cells undergoing apoptosis. As shown in Table II, SN-38 followed by oxaliplatin and the reverse sequence induced apoptosis in 12 and 8% of treated cells, respectively, being comparable to the rates of apoptosis induced by SN-38 (7.5%) or oxaliplatin alone (5.9%). By contrast, the simultaneous treatment induced apoptosis in 20.9% of treated cells.

Discussion

Cisplatin and CPT-11 are widely used anticancer drugs that act against gastric cancer (21), and a significant synergy was observed experimentally when combining them *in vitro* (23,30-32). As oxaliplatin has a markedly different spectrum of activity to cisplatin (7), we examined the interaction of oxaliplatin and SN-38 in a panel of gastric cancer cell lines *in vitro*,

Table II. Cell cycle perturbation (%) and apoptosis induced by L-OHP and SN-38 in the AZ-521 cell line.[a]

	36-h				48-h			
	G_0/G_1 (%)	S (%)	G_2/M (%)	sub-G_1 (%)	G_0/G_1 (%)	S (%)	G_2/M (%)	sub-G_1 (%)
Control					57.57	21.10	21.32	
SN-38	33.08	66.75	0.18	4.98	14.04	85.48	0.48	7.49
L-OHP	60.37	10.85	28.75	5.23	59.55	15.58	24.86	5.89
SN-38+L-OHP	56.31	41.06	2.69	11.85	55.86	44.14	0.00	20.87
L-OHP→SN-38	59.38	12.89	27.73	5.07	62.23	13.84	23.93	7.58
SN-38→L-OHP	21.64	73.81	4.54	6.82	26.12	64.19	9.69	12.14

[a]Data represent mean percentage values from three independent eperiments. L-OHP, oxaliplatin.

with special reference to cell- and schedule-dependency of this combination. Simultaneous exposure to oxaliplatin and SN-38 for 24 h produced synergistic interaction in AZ-521 and NUGC-4 cell lines, whereas only an additive effect was observed in MKN-45. A greater than additive effect was observed in all of the cell lines when cells were treated with oxaliplatin followed by SN-38. However, a greater than additive effect was observed only in NUGC-4 cells when treated with SN-38 followed by oxaliplatin. Either sequence produced no more than additive effects in MKN-45 cell lines. Therefore, simultaneous treatment appears most active at least in these three gastric cancer cell lines.

It has been shown *in vitro* that the combination of oxaliplatin and SN-38 produces a strong synergism in the HT-29 human colon cancer cell line, regardless of the sequence of administration or the exposure time of drugs (26). Among them, 1-h exposure of oxaliplatin followed by SN-38 with a 3-h interval was most cytotoxic. Based on these results, clinical trials were studied by the schedule of sequential administration of oxaliplatin followed by CPT-11. As CPT-11 administered by 90-min infusion resulted in a long-term half-life of SN-38 in humans (33) and oxaliplatin is a highly time-dependent drug (26), 24-h exposure rather than 2-h exposure seems to be better for translating these results into the clinical setting. Arnould and coworkers reported, when using the HT-29 colon cancer cell line, that a synergism was observed when cells were simultaneously exposed to oxaliplatin and CPT-11 for 24 h or when cells were first exposed to CPT-11 for 24 h and then oxaliplatin for 24 h, whereas the reverse sequence showed only an additive effect (26). Our data support their results in the gastric cancer cell line, in that simultaneous treatment is most active. However, as opposed to their sequential results, we found that oxaliplatin followed by SN-38 was much more active than the reverse sequence. Correspondingly, we reported that synergistic interactions were mostly exhibited by concurrent and sequential schedules in which CDDP precedes SN-38 in HST-1 human squamous carcinoma cells (23). The discrepancy in the activities of the sequential combinations may be caused by the difference of pharmacokinetics *in vitro* between SN-38 and CPT-11, a prodrug needed to be converted to SN-38 by carboxylesterase.

To explain the possible mechanism underlying the synergistic interaction of oxaliplatin and SN-38, we further analyzed the cell cycle perturbations using the AZ-521 human gastric cancer cell line. We found that a 24-h treatment with SN-38 markedly affected the cell cycle distribution, producing a relevant accumulation in the S phase, and induced apoptosis in 7% of treated cells. Oxaliplatin alone induced apoptosis (6%) by arresting cells in the G_0/G_1 and G_2/M phases. In sequential combinations, distribution patterns of the cell cycle for the initial drug were unaffected by the second drug administered, with comparable rates of apoptosis (8-12%). By contrast, simultaneous exposures led to the accumulation of cells into both G_0/G_1 and S phases and a marked reduction of G_2/M population, with subsequent apoptosis in 21% of treated cells, indicating that the activities of individual drugs were exhibited with a substantial interaction. These results suggested that SN-38 may kill the cells recovering from the G_1 block produced by oxaliplatin as they progress into S phase, accounting for a synergistic interaction.

The exact mechanism for the synergistic interaction remains unclear. Previously, we demonstrated that synergistic interaction of CDDP and SN-38 would be due to the inhibition by SN-38 of the repair of CDDP-induced DNA interstrand cross-links (22). Since a great synergy between oxaliplatin and SN-38 has been shown in the same treatment schedules as in the CDDP and SN-38 combination, the inhibition by SN-38 of the repair of oxaliplatin-induced DNA interstrand cross-links may cause the synergy. However, the synergistic mechanism may vary from cell to cell, depending on the cell type, because it has been reported that clearly opposite interactions exist among human colon cancer cell lines in the combination of oxaliplatin and CPT-11 (25). Moreover, as shown in the present study, simultaneous combination of oxaliplatin and SN-38 at IC_{50} for 24 h accumulates AZ-521 cells almost exclusively into G_0/G_1 and S phases 24 h after treatment, whereas simultaneous combination of oxaliplatin and CPT-11 at IC_{50} for 24 h has been reported to accumulate HT-29 cells into S and G_2/M phases in the same period of time (26). Further experiments would be necessary for clarifying this discrepancy. Nonetheless, the present study highlights the importance of a treatment schedule for the combination of oxaliplatin and CPT-11. These *in vitro* findings might provide important information for a future clinical trial of the combination of oxaliplatin and CPT-11 for gastrointestinal cancer.

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ORIGINAL ARTICLE

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In vitro sequence-dependent interaction between nedaplatin and paclitaxel in human cancer cell lines

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Abstract *Purpose*: To define the most effective combination schedule of paclitaxel and nedaplatin, a new platinum derivative, we investigated the in vitro interaction between these drugs in AZ-521 and NUGC-4 gastric adenocarcinoma and KSE-1 esophageal squamous carcinoma cell lines. *Materials and methods*: Cytotoxic activity was determined by the WST-1 assay. Different treatment schedules of the two drugs were compared and evaluated for synergism, additivity, or antagonism using a quantitative method based on the median-effect principle of Chou and Talalay. Cell-cycle perturbation and apoptosis were evaluated by means of flow cytometry. *Results*: Upon 24-h sequential exposure, the sequence paclitaxel followed by nedaplatin induced greater than additive effects in all of the cell lines, with synergistic interactions in NUGC-4 and KSE-1 cells. By contrast, antagonistic effects were observed with the reverse sequence. Simultaneous treatment resulted in either a synergistic or antagonistic effect, depending on the cell line. Therefore, the sequence paclitaxel followed by nedaplatin appears most active, at least in these three cell lines. Flow cytometric analyses at IC_{50} indicated that paclitaxel induced G2/M arrest with subsequent induction of apoptosis (56%) in the sub-G1 phase. When paclitaxel preceded nedaplatin, apoptosis was most prominent (70%) with pronounced G2/M arrest. By contrast, the reverse sequence yielded only 28% induction of apoptotic cells, with almost identical cell-cycle distribution patterns to those observed with nedaplatin alone, indicating that the activity of paclitaxel is abolished by pretreatment with nedaplatin. *Conclusions*: Our findings suggest that the interaction of nedaplatin and paclitaxel is highly schedule dependent and that the sequential administration of paclitaxel followed by nedaplatin should be thus incorporated into the design of a clinical trial.

Keywords Nedaplatin · Paclitaxel · Sequence dependence · Drug interaction

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Introduction

Cisplatin has played a major role in the chemotherapy of a variety of solid tumors over the past two decades. However, the clinical usefulness of cisplatin is limited due to its toxicity to many normal tissues, such as kidney. Nedaplatin is a new platinum derivative, selected from a series of platinum analogues based on its pronounced preclinical antitumor activity against various solid tumors with lower nephrotoxicity [10]. Preclinical studies indicate that nedaplatin has an antitumor activity comparable to cisplatin [2, 12] and has been shown experimentally to overcome cisplatin resistance in a cisplatin-resistant K562 cell line [12]. Clinically, single-agent nedaplatin has shown a wide spectrum of antitumor activity, producing the favorable response rates in head and neck [9], esophagus [24], non-small cell lung [7], and cervical cancers [18]. The activity of nedaplatin against gastric cancer, however, still remains unclear, despite the fact that nedaplatin has a spectrum of antitumor activity similar to that of cisplatin in phase-I and phase-II studies.

Paclitaxel has demonstrated broad clinical efficacy in a variety of malignancies including ovarian, non-small-cell lung [22], esophageal [1], head and neck [6], gastric [21] and cervical [16] cancers. Paclitaxel in combination with cisplatin is well known for its sequence-dependent synergy in vitro and in vivo [11, 17], and the sequence of paclitaxel followed by cisplatin has been recommended

for clinical studies. Although combination of paclitaxel and nedaplatin is expected to have a potent activity similar to that of paclitaxel and cisplatin combination, few preclinical data for the interaction between these drugs are currently available. Moreover, the efficacy of nedaplatin against gastric cancer cell lines has yet to be determined in vitro. In order to obtain the clinical rationale for the optimal administration schedule of this combination for the treatment of gastric and esophageal cancers, we investigated the interaction between nedaplatin and paclitaxel using an in vitro model of human cancer cell lines derived from esophagus and stomach, using a quantitative method that assesses the synergism or antagonism between these two agents.

Materials and methods

Cell lines and culture

The human AZ-521 and NUGC4 gastric adenocarcinoma cell lines were kindly provided by JCRB Cell Bank (Tokyo, Japan) and maintained in Dulbecco's minimum essential medium (DMEM) (Nissui, Tokyo, Japan) supplemented with 10% heat-inactivated fetal calf serum (GIBCO, Grand Ireland, NY, USA) in an incubator at 37°C and 100% humidity with 5% CO_2 and air. The human KSE-1 esophageal squamous carcinoma cell line [15] was established in our laboratory and maintained under the same conditions as were AZ-521 cells.

Drugs

Nedaplatin was a gift from Shionogi (Osaka, Japan) and paclitaxel was a gift from Bristol-Myers (Tokyo, Japan). Stock solutions of nedaplatin were prepared in distilled water and those of paclitaxel were prepared in dimethylsulfoxide (DMSO). Both solutions were stored at −4°C prior to use. The final concentration of DMSO for all experiments and treatments was maintained at less than 0.02%. These conditions were found to be non-cytotoxic.

Cytotoxicity assay

Cytotoxic activity was measured by means of the WST-1 assay (Wako Chemicals, Osaka, Japan) using manufacturer's instructions [8]. The WST-1 assay is a colorimetric method in which the intensity of the dye is proportional to the number of the viable cells. Briefly, cells were plated into 96-well microtiter plates at a density of 5×10^3 cells/well, and incubated for 24 h for sufficient cell growth. Cells were then treated with graded concentrations of nedaplatin (0.3–1,000 μg/ml) or paclitaxel (0.3–1,000 ng/ml) alone for 24 h, and were incubated with drug-free medium for an additional 24 h. Cells were washed with PBS, and 100 μl medium and 10 μl WST-1 solution were added to each well; then the plates were incubated at 37°C for another 3 h. Absorbances at 450 nm and 620 nm were measured using a Delta Soft ELISA analysis program for Macintosh computer interfaced with a Bio-Tek microplate reader (Immuno-Mini NJ-2300). Wells containing only DMEM and WST-1 were used as controls. Each experiment was performed using six replicated wells for each drug concentration and carried out independently at least three times. The IC_{50} was defined as the concentration that reduced the absorbance in each test by 50%.

For the combination experiments, three different schemes were used to investigate the interaction of paclitaxel and oxaliplatin as shown in Fig. 1: (a) nedapaltin and paclitaxel were exposed simultaneously for 24 h and incubated for an additional 24 h with drug-free medium, (b) nedaplatin was administered for 24 h followed by paclitaxel for 24 h, or (c) paclitaxel was administered for 24 h followed by nedaplatin. Immediately after these treatments, the cytotoxic effects were evaluated by WST-1 assay.

Analysis of combination effects

On the basis of the growth inhibition curve for each single drug, we analyzed the effects of the drug combinations using the method described by Chou and Talalay and a Calcusyn software program for automated analysis (Biosoft, Cambridge, UK) [3, 4]. The influence on the combination of the two drugs was evaluated by comparing the sequential assays with assays involving nedaplatin or paclitaxel exposure alone. The combination effect was evaluated from isoeffect analysis (Cis), calculated as follows: CI $= C_{nedaplatin}/Cx_{nedaplatin} + C_{paclitaxel}/Cx_{paclitaxel}$ where $Cx_{paclitaxel}$ and $Cx_{paclitaxel}$ are

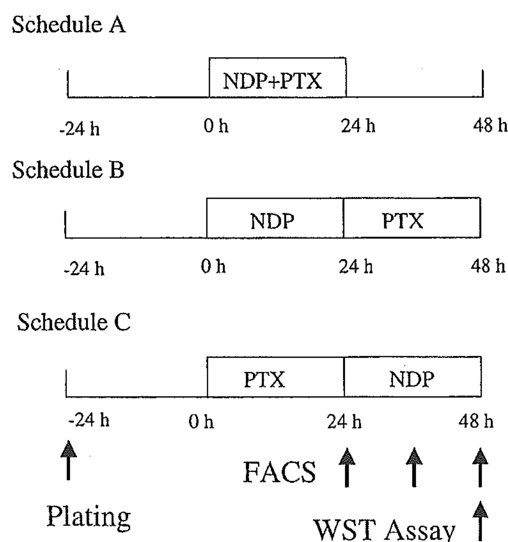

Fig. 1 Description of the three combination schedules. *NDP* nedaplatin, *PTX* paclitaxel

the concentrations of nedaplatin and paclitaxel alone, respectively, needed to achieve a given effect (x%) and $C_{nedaplatin}$ and $C_{paclitaxel}$ are the concentrations of nedaplatin and paclitaxel needed for the same effect (x%) when the drugs were combined. These concentrations were calculated for each experiment and for each combination experiment at a fixed concentration ratio. The combination was considered as positive (synergistic) when the combination index was <1 and negative (antagonistic) when it was >1.

Cell-cycle determination

Human gastric cancer cell line, AZ-521 cells were cultured at 1×10^5 cells per 60-mm dish. The same protocols as described in the growth inhibition assay were used. After treatment, the cells were harvested, washed twice in ice-cold PBS (pH 7.4), and then fixed in 100% ethanol and stored at 4°C for up to 3 days prior to cell-cycle analysis. After the removal of ethanol by centrifugation, cells were then washed with PBS and stained with a solution containing propidium iodide and RNase (Sigma-Aldrich, St. Louis, MO, USA) on ice for 30 min. Cell-cycle analysis was performed on a Becton Dickinson FACS/Calibur Flow Cytometer using the CELLQuest or ModFit 3.0 software packages (Becton Dickinson, San Jose, CA, USA), and the percentages of apoptotic populations were determined by measuring the sub-G1 phase using FACS analysis after collecting floating and trypsinized adherent cells at various times following drug exposure. Each experiment was performed in triplicate.

Results

Single-agent experiments

The cytotoxic activities of nedaplatin and paclitaxel were tested individually on the three tumor cell lines. Each drug was exposed to the cells for 24 h. The IC_{50} values ($\pm$SD) are summarized in Table 1. The IC_{50} value of nedaplatin for KSE-1 (3.4 μg/ml) was not significantly different from those for AZ-521 (3.8 μg/ml) and NUGC-4 (4.6 μg/ml) gastric cancer cells, indicating that nedaplatin appears to be equally effective against these esophageal and gastric cancer cell lines. By contrast, the IC_{50} of paclitaxel for these cell lines varied, depending on the cell type. AZ-521 gastric cancer cells were most sensitive to paclitaxel (14 ng/ml) among the three tumor cell lines, NUGC-4 gastric cancer cells being least sensitive (26 ng/ml).

Table 1 IC_{50} values of nedaplatin and paclitaxel in a panel of three cell lines. Cells were treated with various concentrations of nedaplatin or paclitaxel for 24 h. Results are expressed as the concentration that inhibits 50% of growth in comparison with controls (IC_{50}). The values are mean ± SD of three independent experiments

	AZ-521	NUGC-4	KSE-1
Nedaplatin (μg/ml)	3.8 ± 1.3	4.6 ± 1.3	3.4 ± 0.3
Paclitaxel (ng/ml)	14.0 ± 1.9	26.0 ± 2.6	18.9 ± 0.9

Median-effect analysis of paclitaxel and oxaliplatin combination in vitro

Nedaplatin and paclitaxel were tested in different combinations to define the most effective schedule. Three different schedules were tested (simultaneous exposure or sequential drug exposures) as shown in Fig. 1. When cells were treated with nedaplatin and paclitaxel simultaneously (Fig. 2), the CI values were below 1 at all levels of killed cell fraction in NUGC-4 and at higher levels of killed cell fraction in KSE-1 cells (Fig. 2b, c), indicating a marked synergistic effect, while moderately antagonistic effects (CI > 1) were seen in AZ-521 cells at the ranges corresponding to less than 70% killed cell fraction (Fig. 2a). When cells were treated with paclitaxel followed by nedaplatin, greater than additive effects were obtained in all of the cell lines, with synergistic interactions observed in NUGC-4 and KSE-1 cells at all ranges (Fig. 3). By contrast, largely antagonistic effects were seen in all of the cell lines when cells were treated with the reverse sequence (Fig. 4), although this sequence appeared to be synergistic in KSE-1 cells at the higher cytotoxic ranges (Fig. 4c). Therefore, the sequence paclitaxel followed by nedaplatin appears most active at least in these three cell lines.

Cell-cycle perturbation and apoptosis

In an attempt to explain the mechanisms underlying the different types of interaction, the effects of paclitaxel and nedaplatin on cell-cycle distribution and apoptosis were studied in AZ-521 cells (Table 2). The cells were treated with these drugs either alone or in combination, with different schedules, and cell-cycle distribution was analyzed 36 h and 48 h after the beginning of treatment, using flow cytometric analysis. Paclitaxel alone at a dose of 12.5 ng/ml induced the accumulation of cells in the G2/M phase. At a concentration of 5 μg/ml, nedaplatin alone caused an increase in the S population and a decrease in the G0/G1 population without affecting the population of G2/M, indicating that it inhibited both S to G2 and G2/M to G1 progression. The simultaneous exposure led to the accumulation of cells in the S and G2/M phase and a decrease in the population of G1, showing the combined activity of both drugs. The treatment with paclitaxel prior to nedaplatin led to the accumulation of cells more exclusively into the G2/M phase, showing similar distribution patterns to those observed in the cells treated with paclitaxel alone, although a significant increase in G2/M population was

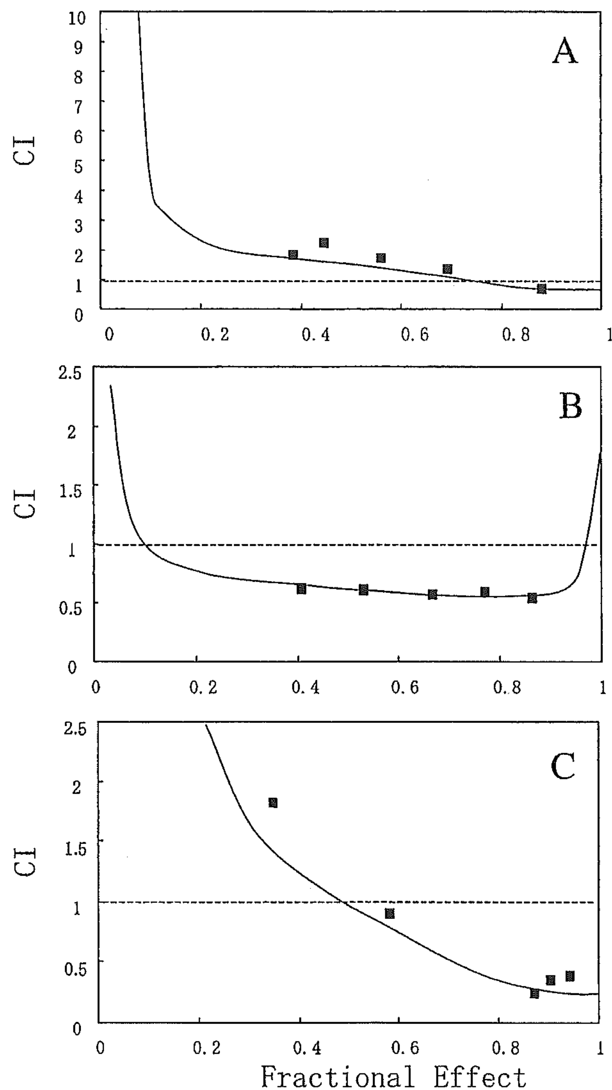

Fig. 2 Combination index (*CI*) plots obtained from three cancer cell lines exposed simultaneously to nedaplatin and paclitaxel for 24 h. **a** AZ-521; **b** NUGC-3; **c** KSE-1

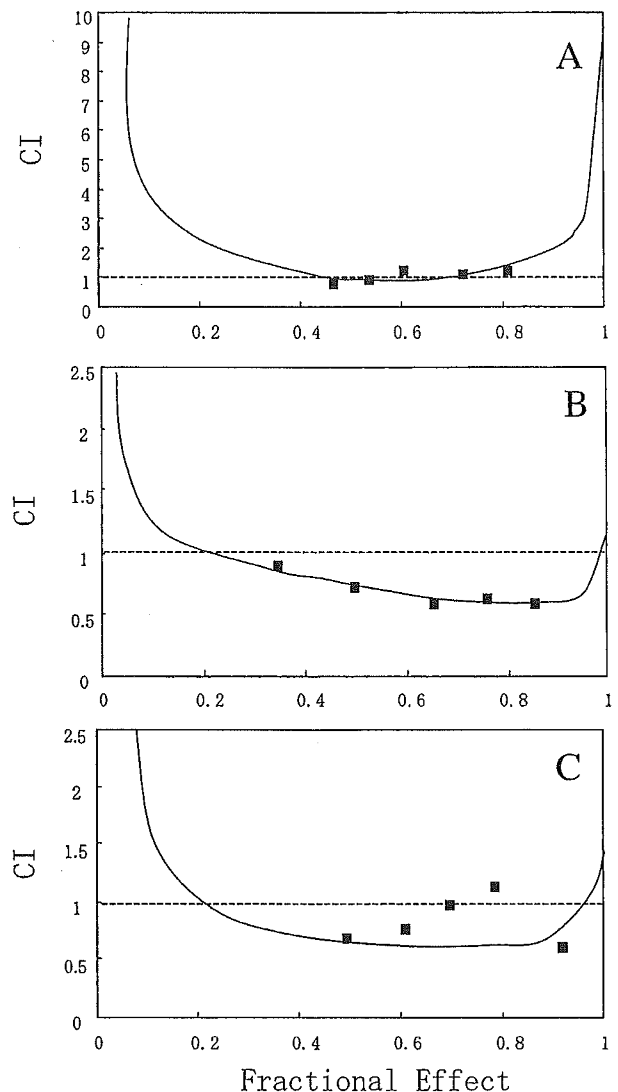

Fig. 3 Combination index (*CI*) plots obtained from three gastric cell lines exposed to paclitaxel for 24 h followed by nedaplatin for 24 h. **a** AZ-521, **b** NUGC-3, **c** KSE-1

observed when compared with treatment with paclitaxel alone. In contrast, nedaplatin prior to paclitaxel caused almost identical distribution patterns to those observed with nedaplatin alone. These data indicate that cell-cycle distribution patterns in sequential combination were mostly influenced by the initial drug administered.

To confirm the activities of sequential combination, the apoptotic activity was investigated after treatment of AZ-521 cells by measuring the population of sub-G1 phase using FACS analyses. The presence of hypodiploid DNA (sub-G1) is associated with cells undergoing apoptosis. As shown in Table 2, paclitaxel followed by nedaplatin induced the G2/M block, with substantial induction of apoptosis in the majority of the treated cells (70%). The induction rate of apoptosis by this sequential administration was greater than those of paclitaxel alone (56–66%) or nedaplatin alone (14–17%). By contrast, the reverse sequence caused S-phase block, and apoptotic population was 24–28%, being less than those induced by paclitaxel alone (56–66%), and slightly more than those induced by nedaplatin alone (14–17%). These data indicate that sequence nedaplatin followed by paclitaxel is antagonistic in inducing apoptosis.

Discussion

In this study, we examined the schedule-dependent interaction of nedaplatin and paclitaxel in a panel of three human cancer cell lines derived from stomach and esophagus in vitro. First, we compared the sensitivity of

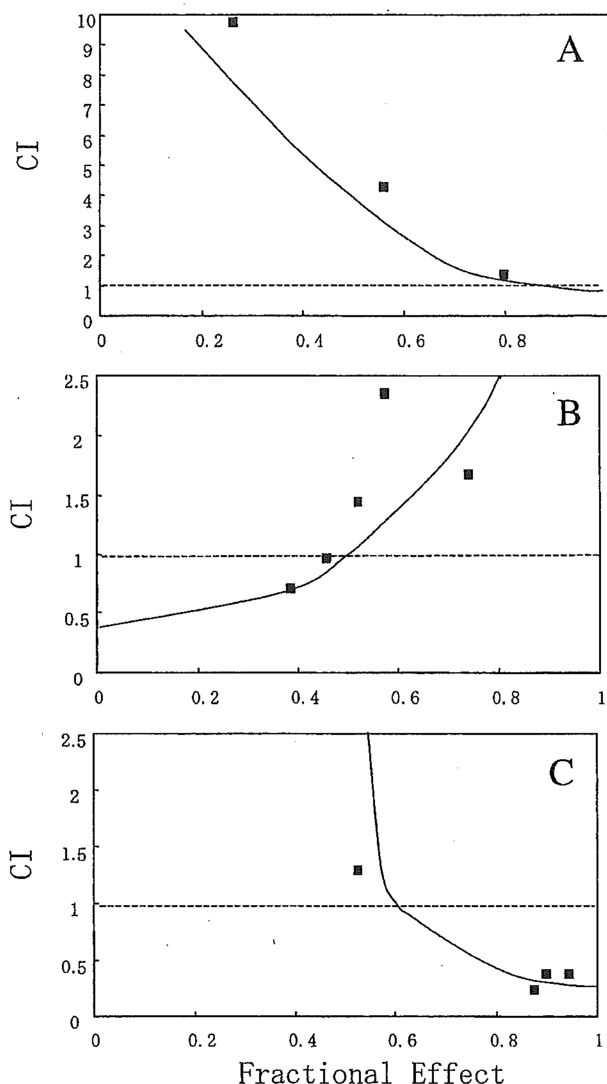

Fig. 4 Combination index (*CI*) plots obtained from three cell lines exposed to nedaplatin for 24 h followed by paclitaxel for 24 h. **a** AZ-521, **b** NUGC-3, **c** KSE-1

nedaplatin between these esophageal and gastric cancer cell lines, because nedaplatin has been clinically shown to be active against esophageal cancer [24], but its clinical efficacy against gastric cancer has not been determined. We have found that the IC_{50} of nedaplatin is not significantly different between these gastric and esophageal cancer cell lines, indicating that nedaplatin may be potentially effective against gastric cancer. Second, we found that the sequence paclitaxel followed by nedaplatin resulted in either synergism or additivity in all three cell lines. By contrast, a clear antagonism was observed in the reverse sequence in two of three cell lines. Simultaneous treatment with these two drugs resulted in either synergistic or antagonistic effects, depending on the cell line. Therefore, the sequence paclitaxel followed by nedaplatin appears to be the most active, at least in these three cell lines. A similar sequence-dependent antitumor activity of this combination was demonstrated in a preclinical in vivo mouse tumor model [26].

To explain the possible mechanism underlying the synergistic interaction of paclitaxel following nedaplatin sequence, we further analyzed the perturbations induced on cell cycle by flow cytometric analyses using the AZ-521 human gastric cancer cell line. We found that a 24-h treatment with paclitaxel markedly affected the cell-cycle distribution, producing a relevant accumulation in the G2/M phase with subsequent induction of apoptosis (56%) in the sub-G1 phase. Nedaplatin alone caused an increase in the S population and a decrease in the G0/G1 population without affecting the population of G2/M, suggesting that it inhibited both S to G2/M and G2/M to G1 progression. The treatment with paclitaxel prior to nedaplatin accumulated cells almost exclusively into the G2/M phase with prominent apoptosis (70%). By contrast, the reverse sequence yielded only 28% induction of apoptotic cells with almost identical cell-cycle distribution patterns to those observed with nedaplatin alone, indicating that the activity of paclitaxel is abolished by pretreatment of nedaplatin, accounting for an antagonistic interaction. The inhibition of paclitaxel-induced cytotoxicity by nedaplatin would probably be explained by the decrease in the number of G2 population targeted by paclitaxel, because pretreatment with

Table 2 Cell-cycle perturbation (%) and apoptosis induced by nedaplatin and paclitaxel in the AZ-521 cell line. Data are presented as mean percentage values from three independent experiments. *NDP* nedaplatin, *PTX* paclitaxel

Treatment	36-h				48-h			
	G0/G1(%)	S(%)	G2/M(%)	Apoptosis[a]	G0/G1(%)	S(%)	G2/M(%)	Apoptosis[a]
Control					58.34	19.91	21.76	3.52
NDP	40.85	31.89	27.26	14.52	37.81	39.91	22.28	17.05
PTX	16.33	24.65	58.42	66.61	19.45	18.02	62.53	56.47
NDP + PTX	12.85	41.48	45.66	49.53	16.98	38.93	44.09	54.76
PTX → NDP	10.91	20.28	68.81	66.10	11.70	13.88	74.42	70.14
NDP → PTX	37.31	44.97	17.72	24.05	36.99	40.40	22.60	28.46

[a] The percentages of apoptotic populations were assessed by measuring sub-G1 phase using FACS analyses after collecting floating and trypsinized adherent cells at various times following drug exposure

nedaplatin accumulates cells mostly in the phases before G2/M, thereby reducing the number of cells entering G2/M phase.

We have found that nedaplatin can arrest the cells mainly at both G1 and S phases, suggesting a distinct action from that of cisplatin or oxaliplatin, because cisplatin and oxaliplatin mainly accumulate cells into G2/M [14, 23] and G1 phases [25], respectively. However, sequence-dependent interactions between paclitaxel and these three platinum compounds did not differ; a synergistic or additive interaction was observed when paclitaxel precedes CDDP [13, 17, 20, 27] or oxaliplatin [25], whereas there were antagonistic interactions in the reverse sequence [13, 25, 27]. Several explanations for increased activity of the sequence paclitaxel followed by cisplatin are shown: cisplatin hastens the exit from mitosis in paclitaxel-treated cells [17]; paclitaxel induces an increase in intracellular uptake of cisplatin [5]; and paclitaxel inhibits repair of cisplatin-induced DNA damage [19]. Therefore, we hypothesize that the similar mechanisms, if not identical, to those as demonstrated in the interaction between CDDP and paclitaxel may also operate in the combination of nedaplatin and paclitaxel.

Clinically, single-agent nedaplatin produced promising response rates in phase-II trials for the treatment of head and neck, lung, esophagus and cervical cancers [7, 9, 18, 24]. In this study, we have shown that nedaplatin is effective against gastric cancer cells and exhibits a significant synergy with paclitaxel. Since these drugs have an overlapping spectrum of clinical efficacies, this combination is a promising chemotherapeutic regimen for the treatment of patients with these cancers. Although the biochemical basis for their interaction remains unknown, a clear sequence-dependent activity of nedaplatin and paclitaxel combination should be thus incorporated into the design of a clinical trial.

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