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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

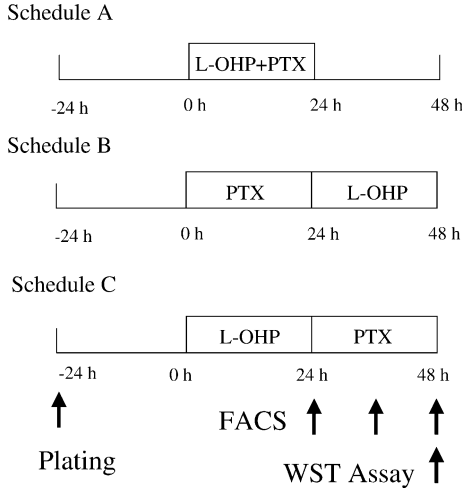

Fig. 1 The three combination schedules

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 ($\pm$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 $\pm$ 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

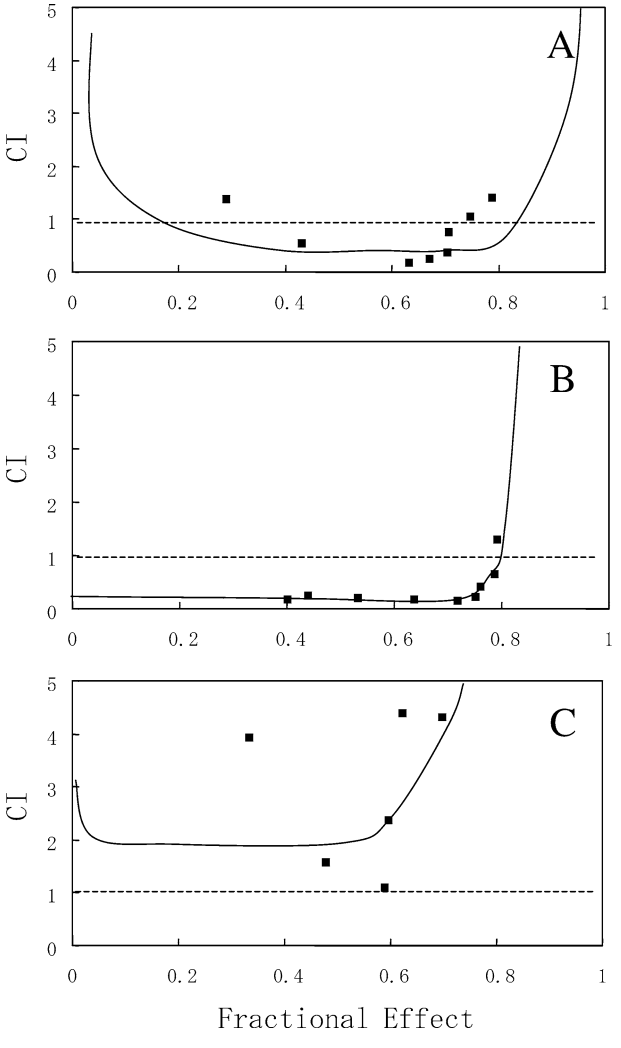

Fig. 2 Combination index (CI) plots of interactions between paclitaxel and oxaliplatin in AZ-521 cells. Cells were treated with (**a**) paclitaxel and oxaliplatin for 24 h simultaneously, (**b**) paclitaxel for 24 h followed by oxaliplatin for 24 h, or (**c**) oxaliplatin for 24 h followed by paclitaxel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

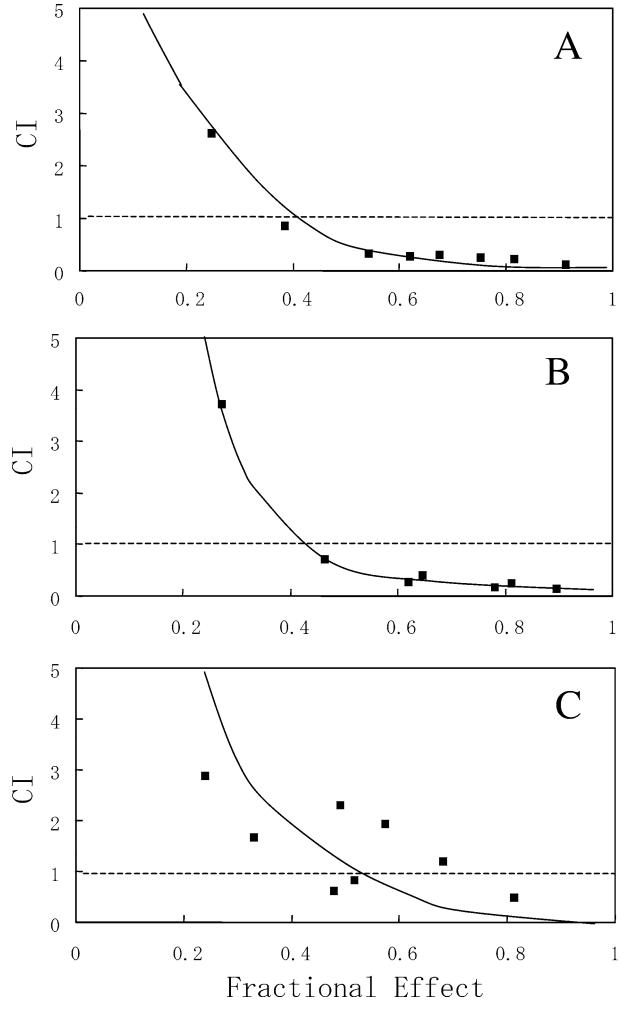

Fig. 3 Combination index (CI) plots of interactions between paclitaxel and oxaliplatin in HST-1 cells. Cells were treated with (**a**) paclitaxel and oxaliplatin for 24 h simultaneously, (**b**) paclitaxel for 24 h followed by oxaliplatin for 24 h, or (**c**) oxaliplatin for 24 h followed by paclitaxel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

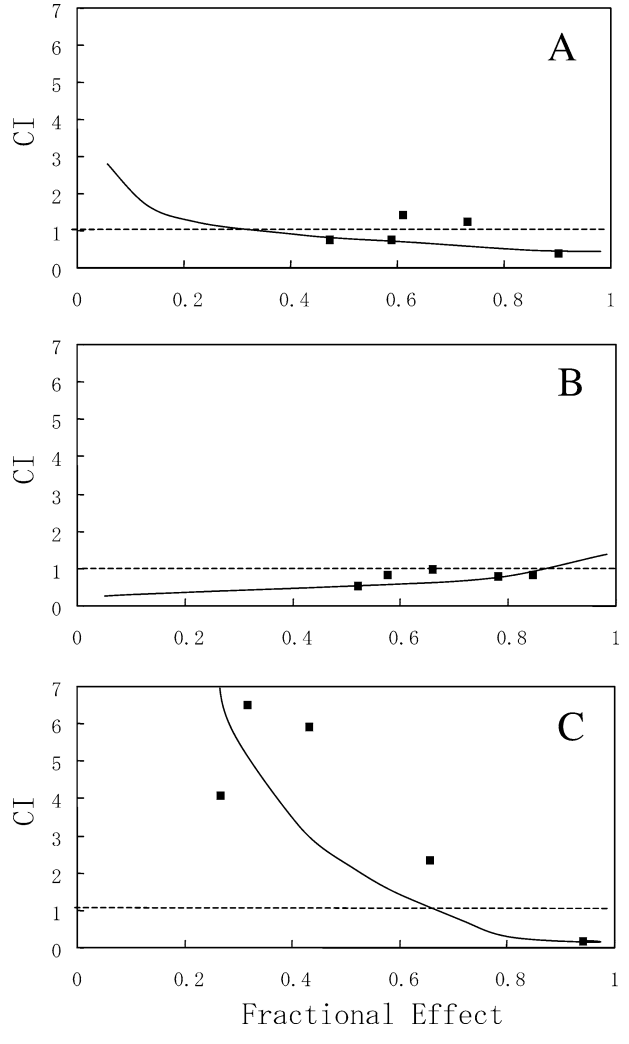

Fig. 4 Combination index (CI) plots of interactions between paclitaxel and oxaliplatin in KSE-1 cells. Cells were treated with (**a**) paclitaxel and oxaliplatin for 24 h simultaneously, (**b**) paclitaxel for 24 h followed by oxaliplatin for 24 h, or (**c**) oxaliplatin for 24 h followed by paclitaxel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