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In vitro schedule-dependent interaction between paclitaxel and cisplatin in human carcinoma cell lines

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Abstract The schedule-dependent interaction of paclitaxel and cisplatin was studied in four human carcinoma cell lines: non-small cell lung cancer, A549; breast cancer, MCF7; ovarian cancer, PA1; and colon cancer, WiDr cells. The cells were exposed simultaneously to the drugs for 24 h and sequentially to paclitaxel first for 24 h followed by cisplatin for 24 h, or vice versa, and then incubated in drug-free medium for 4 and 3 days, respectively. Cell growth inhibition was then determined by the 1-(4,5-dimethylthiazol-2-yl)-3,5-diphenyltetrazolium bromide (MTT) reduction assay. The effects of drug combinations at the IC_{80} level were analyzed by the isobologram method. On simultaneous exposure to paclitaxel and cisplatin, additive and sub-additive (slight antagonistic) effects were observed in A549, MCF7, and PA1 cells, while sub-additive and protective (antagonistic) effects were observed in WiDr cells. On sequential exposure to paclitaxel first, followed by cisplatin, additive effects were observed in all cell lines. On sequential exposure to cisplatin first, followed by paclitaxel, additive effects were observed in PA1 cells, while additive, sub-additive, and protective effects were observed in A549, MCF7, and WiDr cells.

These findings suggest that the interaction of paclitaxel and cisplatin is schedule- and cell line-dependent. The optimal schedule of this combination may be paclitaxel first followed by cisplatin.

Key words Paclitaxel · Cisplatin

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Introduction

cis-Diamminedichloro platinum(II) (cisplatin) is one of the most useful agents that has a broad spectrum of activity against solid tumors. Clinically, it has been used in combinations with other agents, producing high response rates in terms of remission. However, a significant percentage of patients have a poor prognosis, i.e., they fail to achieve remission or they relapse. Thus, new drugs with a higher therapeutic index are required for inclusion in combination regimens with cisplatin.

One of the most promising of such drugs is paclitaxel, an antimicrotubular agent isolated from the bark of the Pacific yew, *Taxus brevifolia* [30]. Unlike the action of vinca alkaloids, which inhibit microtubule assembly, paclitaxel promotes microtubule assembly and stabilizes tubulin polymer formation, resulting in loss of the dynamic balance of microtubule polymerization and depolymerization [18, 25, 26]. In clinical phase trials, the dose-limiting toxicity was found to be leucopenia, and other toxicities include hypersensitivity reactions, neuropathy, mucositis, mild nausea and vomiting, and cardiac toxicity [4, 5, 8, 31]. Paclitaxel has been shown to have significant activity against lung, breast, and ovarian cancers [4, 9, 20, 22]. Moreover, it has been shown to have antitumor activity against cisplatin-resistant ovarian cancer and doxorubicin-resistant breast cancer [4, 9].

Clinical studies of cisplatin in combination with paclitaxel have been initiated [10, 23]. Experimental findings on the combined cytotoxic effects of paclitaxel and cisplatin, however, have shown conflicting results [7, 11, 15, 19, 24, 28, 29]. In an effort to explore favorable schedules that could provide the basis for improved therapeutic benefit in combination chemotherapy, we have, therefore, studied the schedule-dependent interaction between paclitaxel and cisplatin in four human solid tumor cell lines.

Materials and methods

Cell lines

Experiments were conducted with the human lung cancer cell line, A549, breast cancer cells, MCF7, ovarian cancer cells, PA1, and colon cancer cells, WiDr. These cells were maintained in 75-cm^3 plastic tissue culture flasks containing RPMI1640 medium (Grand Island Biological, Grand Island, N.Y.) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Grand Island Biological, Grand Island, N.Y.) and antibiotics.

Drugs

Paclitaxel and cisplatin were provided by Brystol Myers Squibb Japan (Tokyo), and Nihon Kayaku (Tokyo), respectively. Paclitaxel was dissolved in dimethyl sulfoxide (Sigma Chemical, St Louis, Mo.). Cisplatin was dissolved in RPMI1640. Drugs were diluted with RPMI1640 plus 10% FBS.

Cell growth inhibiton by combined anticancer agents

On day 0, tumor cells growing in exponential phase were harvested with 0.05% trypsin, 0.02% EDTA and resuspended, to a final concentration of 5.0×10^3 cells/ml, in fresh medium containing 10% FBS and antibiotics. Cell suspensions (100 µl) were dispensed with a multichannel pipet into the individual wells of a 96-well tissue culture plate with lid (Falcon, Oxnard, Calif). Each plate had one 8-well control column containing medium alone and one 8-well control column containing cells but no drug. Four plates were prepared for each drug combination. The cells were reincubated overnight to allow for attachment.

Simultaneous exposure to paclitaxel and cisplatin

After 20–24 h incubation, solutions of paclitaxel and cisplatin (50 µl) at different concentrations were added to the individual wells (paclitaxel preceding cisplatin by about 10 min). The plates were then incubated under the same conditions for 24 h. The cells were then washed once with culture medium containing 1% FBS, and fresh medium containing 10% FBS (200 µl) and antibiotics were then added. The cells were incubated again for 4 days.

Sequential exposure to paclitaxel and cisplatin

After 20–24 h incubation, media containing 10% FBS (50 µl) and solutions of paclitaxel (or cisplatin) (50 µl) at different concentrations were added to individual wells. The plates were then incubated under the same conditions for 24 h. The cells were washed once with culture medium containing 1% FBS, and fresh medium containing 10% FBS (150 µl) and antibiotics were added, followed by the addition of cisplatin (or paclitaxel) (50 µl) at different concentrations. The plates were incubated again under the same conditions for 24 h. These cells were then washed once with culture medium, and fresh medium containing 10% FBS (200 µl) and antibiotics was added. The cells were then incubated again for 3 days.

The cells were exposed to the drugs for 24 h, since paclitaxel and cisplatin are often administered by infusion over a few hours or by continuous infusion, and cytotoxic plasma levels of these agents are maintained for more than 10 h.

MTT assay

Viable cell growth was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, as described previously [13]. Fifty microliters of MTT (1 mg/ml) was added to each well. After 4 h at 37°C, the supernatant was removed. Dimethyl sulfoxide (150 µl) was then added and the plates were vigorously shaken to solubilize the MTT-formazan product. Absorbance at 570 nm was measured with a Titertek multiscan. For all cell lines examined, we established a linear relationship between the MTT assay and cell number within the range of the experiments shown.

Isobologram analysis

Dose-response curves were plotted on a semilogarithmic scale as a percentage of the control, the cell number of which was obtained from samples not exposed to drugs that were processed simultaneously. Dose-response interactions between paclitaxel and cisplatin at the point of IC_{80} were evaluated by an isobologram [27]. The IC_{80} was defined as the concentration of drug which produced 80% cell growth inhibition; 80% reduction of absorbance. The theoretical basis of the isobologram method has been described in depth in previous studies [12, 14, 27]. Based upon the dose-response curves of paclitaxel and cisplatin, three isoeffect curves were constructed (Fig. 1):

1. Mode I line: When the dose of paclitaxel was selected, an incremental effect remained to be produced by cisplatin. The addition was calculated by taking the increment in doses, starting from zero, that produced log survivals that added up to IC_{80} (heteroaddition). If the agents are acting additively by independent mechanisms, combined data points would lie near the Mode I line.
2. Mode II (a) line: When the dose of paclitaxel was selected, an incremental effect remained to be produced by cisplatin. The addition was calculated by taking the increment in doses, starting from the point on the dose-response curve of paclitaxel where the effect of paclitaxel had ended, that produced log survivals that added up to IC_{80} (isoaddition).
3. Mode II (b) line: Similarly, when the dose of cisplatin was selected, an incremental effect remained to be produced by paclitaxel. The addition was calculated by taking the increment in doses, starting from the point on the dose-response curve of cisplatin where its effect had ended, that produced log survivals that added up to IC_{80} (isoaddition). If the agents are acting additively by a similar mechanism, combined data points would lie near Mode II lines.

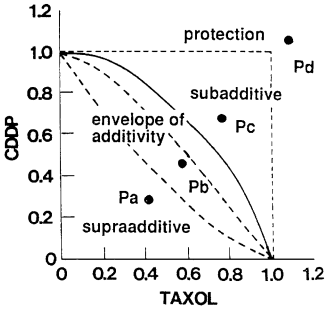

Fig. 1 Schematic representation of an isobologram [27]. Envelope of additivity, surrounded by Mode I (*solid line*) and Mode II (*dotted lines*) isobologram lines, was constructed from the dose-response curves of paclitaxel (*TAXOL*) and cisplatin (*CDDP*). The concentrations which produced 80% cell growth inhibition were expressed as 1.0 on the ordinate and the abscissa of isobolograms. Combined data points *Pa*, *Pb*, *Pc*, and *Pd* show supra-additive, additive, subadditive, and protective effects, respectively.

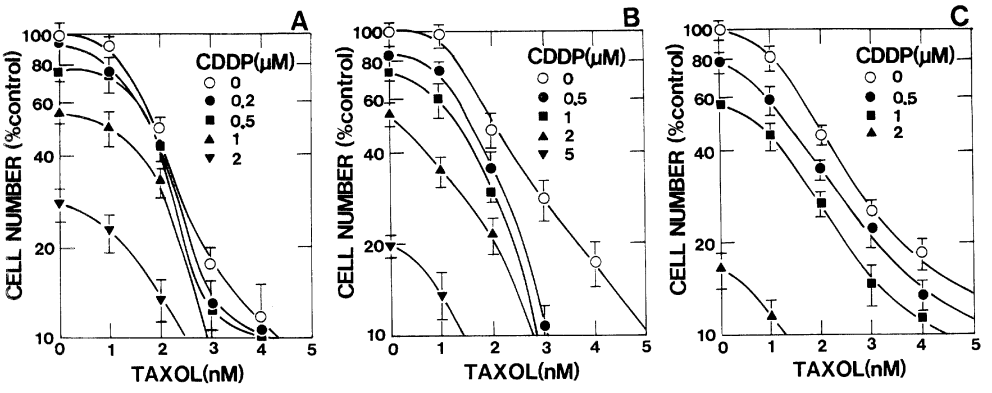

Fig. 2A–C Schedule dependence of the interaction between paclitaxel (*TAXOL*) and cisplatin (*CDDP*) in MCF7 cells. Cells were exposed to drugs simultaneously for 24 h (**A**), paclitaxel first for 24 h followed by cisplatin for 24 h (**B**), and the reverse sequence (**C**). After 5 days, the cell number determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, was plotted as a percentage of the control (cells not exposed to drugs). The concentrations of cisplatin for each symbol are shown in the upper right of each panel. Paclitaxel concentrations are shown on the abscissa. Data points represent the mean ± SD (*bars*) ($n = 4$)

When paclitaxel has a linear dose-response curve, the Mode II (b) line will be the same as Mode I, and vice versa. When both drug have linear dose-response curves, Mode I and Mode II lines will converge to make a straight line connecting 1.0 of the ordinate and abscissa.

Since we do not know whether the combined effects of two agents will be heteroadditive, isoadditive, or intermediate between these extremes, all possibilities should be considered. Thus, when the data points of the drug combination fell within the area surrounded by three lines (envelope of additivity), the combination was regarded as additive. We used this envelope not only to evaluate combinations for the simultaneous exposure to paclitaxel and cisplatin, but also to evaluate the combination for the sequential exposure to both agents, since the cytotoxicity of the first agent could be modulated by the second agent under our experimental conditions.

In this isobologram, an additive effect indicates greater superiority of the combination over a single agent, even though the combined data do not reach the supra-additive area. Thus, anticancer agents that show additive effects should have greater cytotoxic effects in combination than when used as single agents.

When the data points fell to the left of the envelope (i.e., the combined effect was caused by lower doses of the two agents than predicted), we regarded the drugs as having a supra-additive effect (synergism). When the points fell to the right of the envelope (i.e., the combined effect was caused by higher doses of the two agents than predicted), but within the square or on the line of the square, the two drugs had a sub-additive effect, that is, the combination was superior or equal to a single agent, but was less than additive. When the data points were outside the square, the combination was regarded as having a protective effect, i.e., the combination was inferior in cytotoxic action to a single agent. Both sub-additive and protective interactions were considered as antagonism.

Results

Experiments were repeated 3–5 times. The dose-response curves of paclitaxel and cisplatin were different in each experiment, but similar combined effects were observed on the same cells in three out of three, or four out of five experiments. Each point represents the mean value for the experiment performed in quadruplicate. We have chosen to present representative dose-response curves and isobolograms for MCF7 cells and to present only representative isobolograms for A549, PA1, and WiDr cells.

Figure 2 shows representative dose-response curves for MCF7 cells exposed to paclitaxel and cisplatin for 24 h at various schedules; simultaneous exposure to both drugs, sequential exposure to paclitaxel followed by cisplatin, and sequential exposure to cisplatin followed by paclitaxel. Isobolograms at IC_{80} were generated based upon these dose-response curves for the combinations.

Simultaneous exposure to paclitaxel and cisplatin

Figure 3 shows representative isobolograms of A549, MCF7, PA1, and WiDr cells. The combined effects of simultaneous exposure to the drugs differed depending upon the cell line. In A549, MCF7, and PA1 cells, the combined data points fell mainly in the envelope of additivity and in the area of sub-additivity, suggesting that the combination had slight antagonistic effects. In WiDr cells, the data points fell in the area of sub-additivity and protection, suggesting antagonistic effects.

Sequential exposure to paclitaxel first, followed by cisplatin

Figure 4 shows representative isobolograms of A549, MCF7, PA1, and WiDr cells. Under this experimental condition, all cell lines showed similar combined effects; most of the combined data points fell within the envelope of additivity. These findings suggest that sequential exposure to paclitaxel first, followed by cisplatin, produced additive effects.

Sequential exposure to cisplatin first, followed by paclitaxel

Figure 5 shows representative isobolograms of A549, MCF7, PA1, and WiDr cells. In PA1 cells, the

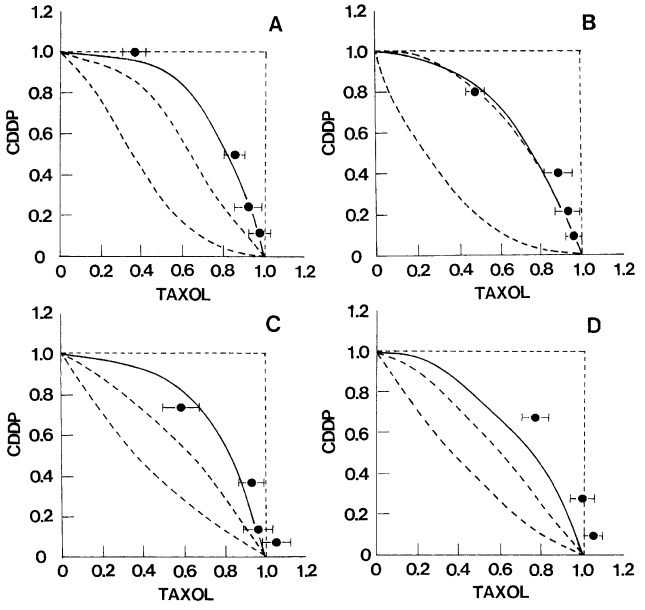

Fig. 3 Isobolograms of simultaneous exposure to paclitaxel (*TAXOL*) and cisplatin (*CDDP*) in A549 (**A**), MCF7 (**B**), PA1 (**C**), and WiDr (**D**) cells. In A549, MCF7, and PA1 cells, the combined data points fell mainly in the envlope of additivity and in the area of sub-additivity, suggesting that the combination showed slight antagonistic effects. In WiDr cells, the data points fell in the area of sub-additivity and protection, suggesting antagonistic effects. Data points represent the mean ± SD (*bars*) ($n = 4$)

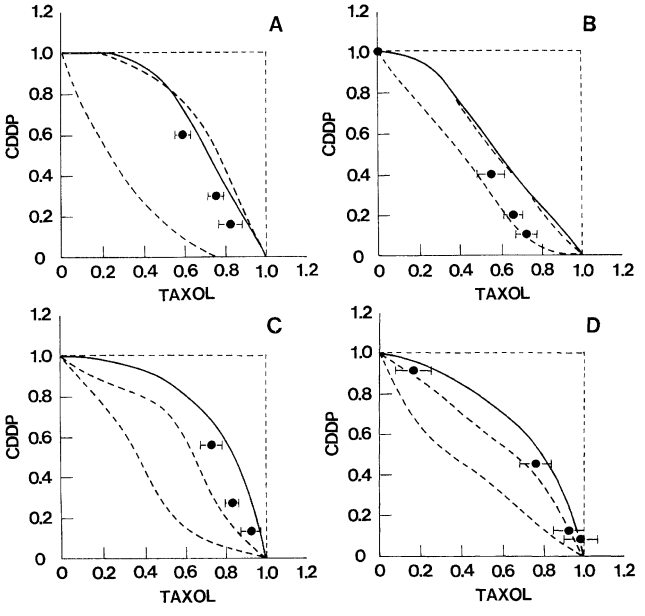

Fig. 4 Isobolograms of sequential exposure to paclitaxel (*TAXOL*) first, followed by cisplatin (*CDDP*), in A549 (**A**), MCF7 (**B**), PA1 (**C**), and WiDr (**D**) cells. The data points for the combination fell in the envelope of additivity for all four cell lines, suggesting an additive interaction

combined data points fell within the envelope of additivity, suggesting additive interaction. In A549, MCF7, and WiDr cells, the combined data points fell mainly in the envelope of additivity, and in the area of sub-additivity, suggesting slight antagonistic effects.

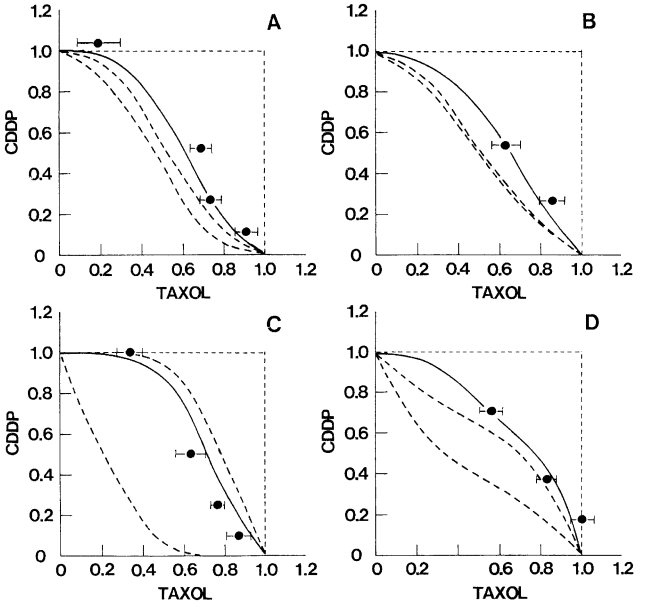

Fig. 5 Isobolograms of sequential exposure to cisplatin (*CDDP*) first, followed by paclitaxel (*TAXOL*) in A549 (**A**), MCF7 (**B**), PA1 (**C**), and WiDr (**D**) cells. In PA1 cells, the data points fell in the envelope of additivity, suggesting additive interaction. In A549, MCF7, and WiDr cells, the data points fell mainly in the additive to sub-additive area, suggesting a slight antagonistic interaction

Discussion

Clinical studies are now in progress to determine the optimal schedule of paclitaxel and cisplatin in combination. However, experimental data for this combination are conflicting and the optimal schedule for the combination is still controversial [7, 11, 15, 24, 29].

We studied the effects of schedule-dependent interaction between paclitaxel and cisplatin on four human carcinoma cell lines, A549, MCF7, PA1, and WiDr, in vitro. Analysis of the effects of drug-drug interaction was performed by the isobologram method, which was originally proposed for the evaluation of radiation-drug interactions (27), but which has also been developed for the evaluation of drug-drug interactions [12–14].

Our studies demonstrated that cytotoxic interaction between paclitaxel and cisplatin was schedule- and cell line-dependent. In A549, MCF7, and WiDr cells, antagonistic effects were observed for simultaneous exposure to paclitaxel and cisplatin and for sequential exposure to cisplatin first, followed by paclitaxel, while additive effects were observed for sequential exposure to paclitaxel first, followed by cisplatin. In PA1 cells, slight antagonistic effects were observed on simultaneous exposure to both agents, while additive effects were observed for the two sequential schedules. We

found no supra-additive (synergistic) interaction with any schedule or cell line.

In combination chemotherapy, schedules with antagonistic interaction are generally avoided, and, thus, simultaneous administration of both agents and the sequential administration of cisplatin followed by paclitaxel may not be favorable. Additive effects were observed in all four cell lines on sequential exposure to paclitaxel first, followed by cisplatin. Additive effects in our system show that the cytotoxic effect of the combination is greatly superior to that of each agent alone, even if the combination does not reach the synergistic area. We anticipated that sequential exposure to paclitaxel followed by cisplatin will exert a stronger cytotoxic effect than other schedules of the combination in both animal and clinical studies, and that this schedule would be suitable for the combination of these agents.

In vitro studies examining the interaction between paclitaxel and cisplatin have yielded conflicting results [7, 11, 15, 19, 24, 28, 29]. On simultaneous exposure to paclitaxel and cisplatin, some investigators have reported synergistic effects [7], while others have observed antagonistic effects [11, 15, 24, 29]. On sequential exposure, some investigators reported that cytotoxic effects with the paclitaxel/cisplatin sequence were not always superior to those for other schedules [7, 15], while others found that cytotoxic effects with the paclitaxel/cisplatin sequence were synergistic and superior to those for other schedules [11, 19, 24, 29]. These differences possibly derive from differences in experimental conditions, e.g., in the cell lines used, schedules for drug exposure, duration of exposure, and definitions of additivity. We found no synergistic effects in the paclitaxel/cisplatin sequence, probably due to differences in the definition of synergy [1], since the definition of synergy (supra-additive) adopted with the isobologram method is generally more stringent than that of others [27].

The clinical outcome of drug treatment includes both the antitumor effects and normal tissue toxicity that result from variable drug exposure, whereas in vitro models represent only antitumor effects at a constant drug exposure. Since paclitaxel and cisplatin display overlapping myelo- and neurotoxicities, we must be aware of the possibility of myelosuppression and neuropathy when these agents are used in combination [3, 23]. The regimen with cisplatin first followed by paclitaxel showed delayed paclitaxel clearance, resulting in profound myelosuppression, while the regimen with paclitaxel first followed by cisplatin was active and well tolerated [23]. Although recent clinical studies of paclitaxel and cisplatin have employed various administration schedules [2, 6, 16, 17, 21], the clinical pharmacokinetic findings in clinical trials and our results support the schedule of the sequential administration of paclitaxel first followed by cisplatin.

In conclusion, the interaction of paclitaxel and cisplatin was apparently dependent on schedule and cell type. In A549, MCF7, and WiDr cells, simultaneous exposure to paclitaxel and cisplatin and sequential exposure to cisplatin first, followed by paclitaxel, had antagonistic effects, while sequential exposure to paclitaxel first, followed by cisplatin, had additive effects. In PA1 cells, simultaneous exposure to both agents had antagonistic effects, while sequential exposure to paclitaxel first, followed by cisplatin, and the reverse sequence had additive effects. These findings suggest that the sequential administration of paclitaxel first, followed by cisplatin, may be the optimal schedule for this combination. However, since the interaction between paclitaxel and cisplatin is rather complex, further preclinical and clinical investigations are required to better understand the antitumor, toxic, and pharmacokinetic interactions of this combination.

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