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Original Paper

Schedule-dependent Interaction Between Paclitaxel and Doxorubicin in Human Cancer Cell Lines *In Vitro*

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The schedule-dependent interaction of paclitaxel and doxorubicin was evaluated in four human cancer cell lines. The cells were exposed simultaneously or sequentially to the two agents for 24 h, and were then incubated in drug-free medium for 4 and 3 days, respectively. The cell growth inhibitions were determined by the MTT assay. The cytotoxic interactions at the IC_{80} level were evaluated by the isobologram method of Steel and Peckham. In non-small cell lung cancer A549, breast cancer MCF7 and colon cancer WiDr cells, antagonistic effects were observed for the paclitaxel and doxorubicin combination on simultaneous exposure to the two agents and on sequential exposure to doxorubicin followed by paclitaxel, while additive effects were observed for the combination on sequential exposure to paclitaxel followed by doxorubicin. In ovarian cancer PA1 cells, additive effects were observed for all schedules. These findings suggest that sequential administration of paclitaxel followed by doxorubicin may be the most suitable sequence, while the simultaneous administration of the two agents and the sequential administration of doxorubicin followed by paclitaxel may result in less tumour cell kill than anticipated. Further preclinical and clinical studies are required to elucidate the relationship between paclitaxel and doxorubicin with regard to both antitumour activity and toxicity.

Key words: paclitaxel, doxorubicin, drug combination
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INTRODUCTION

PACLITAXEL IS a new antimicrotubular agent isolated from the bark of the Pacific yew, *Taxus brevifolia* [1]. This drug has a novel mechanism of action, promoting the polymerisation and stabilisation of tubulin to microtubules, thereby inhibiting the dynamic reorganisation of the microtubulus network required for mitosis and cell proliferation [2–4]. This is in contrast to the mechanism of action of the vinca alkaloids, which induce microtubule disassembly. The broad preclinical activity and unique mechanism of action of paclitaxel have been attracting increased attention, despite the scarcity of its natural source and its insolubility in water. In clinical phase studies, granulocytopenia was the principal dose-limiting toxicity. Hypersensitivity reactions, neuropathy, mucositis, mild nausea and vomiting, and cardiac injury were also observed [5–8]. Paclitaxel has shown significant activity against lung, breast and ovarian cancers [8–12]. Further, paclitaxel has proven to be useful against cisplatin-resistant ovarian cancer and doxorubicin-resistant breast cancer [8, 10]. Doxorubicin is one of the most useful agents with a broad spectrum of activity against solid tumours and haematological malignancies. Because of its clinical importance, there has been interest in the combination of paclitaxel and doxorubicin. However, experimental data on the combination are limited and controversial [13–17].

The aim of this present study was to elucidate the cytotoxic effects of combinations of paclitaxel and doxorubicin given at various schedules on four human carcinoma cell lines, attempting to avoid antagonistic interaction and determining those schedules best suited to provide the basis for improved therapeutic benefit in combination chemotherapy.

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MATERIALS AND METHODS

Cell lines

Experiments were conducted with the human non-small cell lung cancer cell line, A549, the breast cancer cells, MCF7, the ovarian cancer cells, PA1, and the colon cancer cells, WiDr. These cells were obtained from the American Type Culture Collection (Rockville, Maryland, U.S.A.) and were maintained in 75-cm^3 plastic tissue culture flasks containing RPMI 1640

medium (Grand Island Biological Co., Grand Island, New York, U.S.A.) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Grand Island Biological Co.) and antibiotics. The doubling times of A549, MCF7, PA1 and WiDr cells in our experimental conditions were 30, 27, 24 and 27 h, respectively.

Drugs

Paclitaxel was provided by Brystol Myers Squibb Japan Co. Ltd (Tokyo), and doxorubicin (Kyowa Hakko Co. Ltd Tokyo) was obtained from our hospital pharmacy. Paclitaxel was dissolved in dimethyl sulphoxide (Sigma Chemical Co., St Louis, Missouri, U.S.A.) and doxorubicin was dissolved in RPMI 1640. The drugs were diluted with RPMI 1640 plus 10% FBS.

Cell growth inhibition by combined anticancer agents

On day 0, exponentially growing cells were harvested with trypsin (0.05%):EDTA (0.02%) 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 into the individual wells of a 96-well covered tissue culture plate (Falcon, Oxnard, California, U.S.A.). Each plate had one eight-well control column containing medium alone and one eight-well control column containing cells but no drug. Four plates were prepared for each drug combination schedule in each cell line. The cells were reincubated overnight to allow for attachment.

Simultaneous exposure to paclitaxel and doxorubicin. After 20- to 24-h incubation, solutions of paclitaxel and doxorubicin (50 μl each) at different concentrations were added to individual wells containing cell suspensions (paclitaxel preceding doxorubicin by approximately 10 min). The plates were then incubated under the same conditions for 24 h. After treatment, the drug-containing medium was removed, and fresh medium was provided. The cells were incubated again for 4 days.

Sequential exposure to paclitaxel and doxorubicin. After a 20- to 24-h incubation, medium containing 10% FBS (50 μl) and solutions of paclitaxel (or doxorubicin) (50 μl) at different concentrations were added to individual wells containing the cell suspensions. The plates were then incubated under the same conditions for 24 h. The cells were washed once with culture medium containing 1% FBS, and then, fresh medium containing 10% FBS (150 μl) and solutions of doxorubicin (or paclitaxel) (50 μl) at different concentrations were added. The plates were incubated again under the same conditions for 24 h. After treatment, the drug-containing medium was removed, and fresh medium was provided. The cells were incubated again for 3 days.

MTT assay

Viable cell growth was determined by a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazonium bromide (MTT) assay, as described previously [18]. Fifty microlitres of MTT (1 mg/ml) were added to each well. After 4 h at 37°C, the supernatant was removed. Dimethyl sulphoxide (150 μl) was then added and the plates were vigorously shaken to solubilise the MTT-formazan product. Absorbance at 570 nm was measured with a Titertek multiscan. For all cell lines examined, we established a linear relation between the MTT assay and cell number within the range of the experiments shown.

Isobologram analysis

Dose–response curves were plotted on a semilog 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 doxorubicin at the point of IC_{80} were evaluated by the isobologram method of Steel and Peckham [19]. The theoretical basis of this method has been described in depth previously [19–21].

Based upon the dose–response curves of paclitaxel and doxorubicin, three isoeffect curves were constructed (Figure 1).

(1) Mode I line. When the dose of paclitaxel was selected, an incremental effect remained to be produced by doxorubicin. The addition was calculated by taking the increment in doses, starting from zero, that produced log survivals that summated to IC_{80} (heteroaddition). If the agents are acting additively by independent mechanism, 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 doxorubicin. 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 summated to IC_{80} (isoaddition).

(3) Mode II (b) line. Similarly, when the dose of doxorubicin 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 doxorubicin where its effect had ended, that produced log survivals that summated to IC_{80} (isoaddition). If the agents are acting additively by similar mechanism, combined data points would lie near Mode II lines.

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

Since we can 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

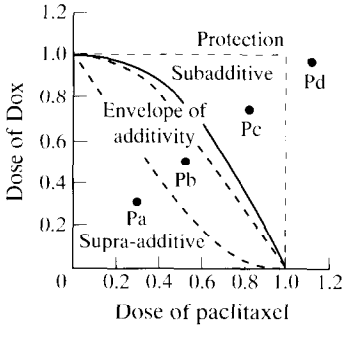

Figure 1. Schematic presentation of the isobologram [19]. Envelope of additivity, surrounded by Mode I (solid line) and Mode II (broken lines), was constructed from the dose–response curves of paclitaxel and doxorubicin (Dox). Combined data points within the envelope show additive interaction. Data points Pa, Pb, Pc and Pd show supra-additive, additive, subadditive, and protective effects, respectively (see text).

to paclitaxel and doxorubicin, 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 great superiority of the combination to 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 combinations 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 subadditive 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 subadditive and protective interaction were considered as antagonism.

Experiments were repeated three to five times. The dose–response curves of paclitaxel and doxorubicin were different in each experiment, but similar combined effects were observed on the same cells in three of three or four of five experiments. We have chosen to present representative dose–response curves for MCF7 cells and to present representative isobolograms for A549, MCF7, PA1 and WiDr cells.

RESULTS

The IC_{50} and IC_{80} values of paclitaxel and doxorubicin against A549, MCF7, PA1 and WiDr cells are shown in Table 1. PA1 cells were most sensitive to both paclitaxel and doxorubicin, while WiDr was the most resistant.

Figure 2 shows representative dose–response curves for MCF7 cells exposed to paclitaxel and doxorubicin for 24 h at various schedules; simultaneous exposure to drugs (Figure 2a), sequential exposure to paclitaxel followed by doxorubicin (Figure 2b), and sequential exposure to doxorubicin followed by paclitaxel (Figure 2c), respectively. Isobolograms at the IC_{80} level were generated based upon these dose–response curves.

Simultaneous exposure to paclitaxel and doxorubicin

Figure 3(a–d) shows representative isobolograms of A549, MCF7, PA1 and WiDr cells, respectively, for the simultaneous exposure to paclitaxel and doxorubicin. The combined effects of simultaneous exposure to drugs differed with the different cell lines. In PA1 cells, the combined data points fell within the envelope of additivity (Figure 3c), indicating that simultaneous exposure produced additive effects. In A549 and MCF7 cells, the combined data points fell within the envelope and in the area of sub-additivity or protection (Figure 3a,b), suggesting that the combination had slight antagonistic effects. In WiDr cells, the data points fell in the area of subadditivity and protection (Figure 3d), suggesting antagonistic effects.

Sequential exposure to paclitaxel first followed by doxorubicin

Figure 4(a–d) shows representative isobolograms of A549, MCF7, PA1 and WiDr cells, respectively. In 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 followed by doxorubicin produced additive effects.

Sequential exposure to doxorubicin first followed by paclitaxel

Figure 5(a–d) shows representative isobolograms of A549, MCF7, PA1 and WiDr cells, respectively. In PA1 cells, the combined data points fell within the envelope of additivity (Figure 5c), suggesting additive interaction. In WiDr cells, the combined data fell within the envelope of additivity and in the area of subadditivity (Figure 5d), suggesting slight antagonistic effects in this condition. In A549 and MCF7 cells, the combined data fell in the area of subadditivity and protection (Figure 5a, b), suggesting antagonistic effects.

DISCUSSION

Combination chemotherapy in disseminated cancer has developed from an empirical to a reasonably scientific treatment modality. The agents used for combinations were generally selected on the basis of single-agent activity, non-overlapping toxicity, and lack of crossresistance. Furthermore, demonstration that two or more agents have synergistic or additive interaction has been used as a rationale for their inclusion in clinical regimens. Synergy is defined as the production of an effect by agents in combination greater than that which would be expected from their individual effects, additive is the production of the expected effect, while antagonism is the production of an effect less than expected. However, a confusion has arisen because dose–response curves of anticancer agents are variable and different investigators have disagreed as to what constitutes an "expected" level of effect when two agents are combined [22]. Although dose–response curves of anticancer agents are important factors in the analysis of the effects of drug combinations, many studies lack full dose–response data for each drug, either alone or in combination. The isobologram method of Steel and Peckham [19] can be used to calculate additive interaction of any combination, irrespective of the shapes of the dose–response curves of the agents and of whether they have independent or overlapping damage.

Table 1. IC_{50} and IC_{80} *values of paclitaxel alone and doxorubicin alone were the mean ± S.D. for three to five experiments in simultaneous exposure to both agents without the addition of doxorubicin and paclitaxel, respectively*

	Paclitaxel (nM)		Doxorubicin (10 nM)	
	IC_{50}	IC_{80}	IC_{50}	IC_{80}
A548	3.2 ± 0.5	4.6 ± 0.9	2.0 ± 0.4	3.7 ± 0.7
MCF7	2.0 ± 0.4	3.2 ± 0.8	2.0 ± 0.3	3.2 ± 0.3
PA1	1.9 ± 0.2	3.0 ± 0.2	0.8 ± 0.2	1.2 ± 0.4
WiDr	2.8 ± 0.4	5.6 ± 1.2	4.7 ± 2.1	9.3 ± 3.0

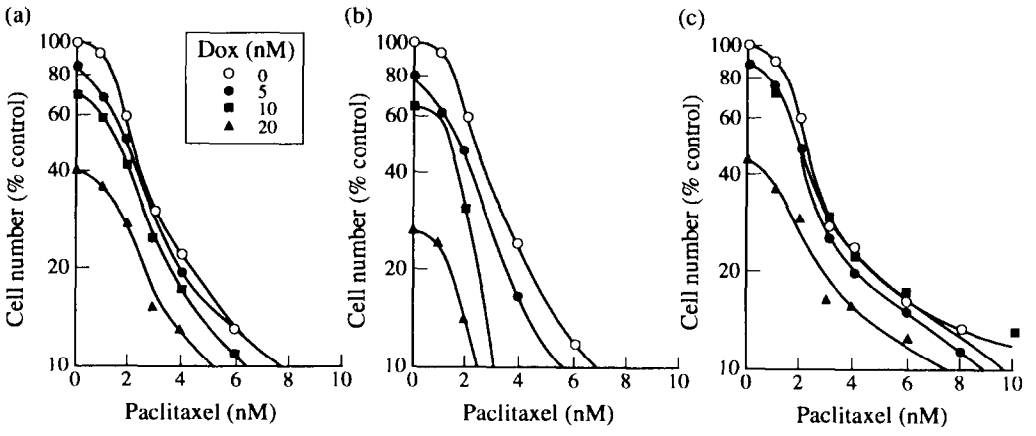

Figure 2. Dose–response curves for paclitaxel and doxorubicin (Dox) combinations in MCF7 cells. (a) Simultaneous exposure (24 h) to paclitaxel and doxorubicin; (b) sequential exposure to paclitaxel (24 h) followed by doxorubicin (24 h); (c) sequential exposure to doxorubicin (24 h) followed by paclitaxel (24 h). The cell number was measured by MTT assay after 5 days and was plotted as a percentage of the control (cells not exposed to drugs). Doxorubicin concentrations for each symbol are shown in the upper right of panel (a). Paclitaxel concentrations are shown on the abscissa. Each point represents the mean value for the experiments in quadruplicate; the S.D. of the means were less than 15% and were omitted.

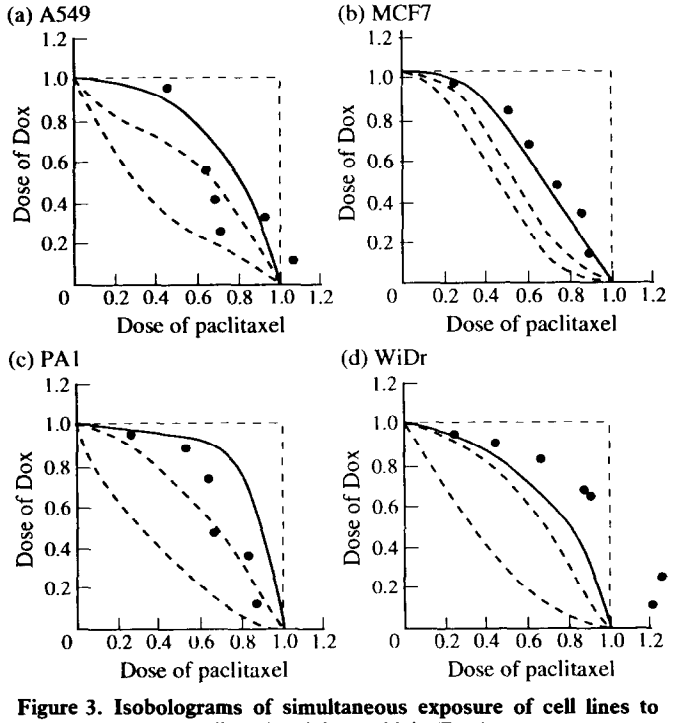

Figure 3. Isobolograms of simultaneous exposure of cell lines to paclitaxel and doxorubicin (Dox).

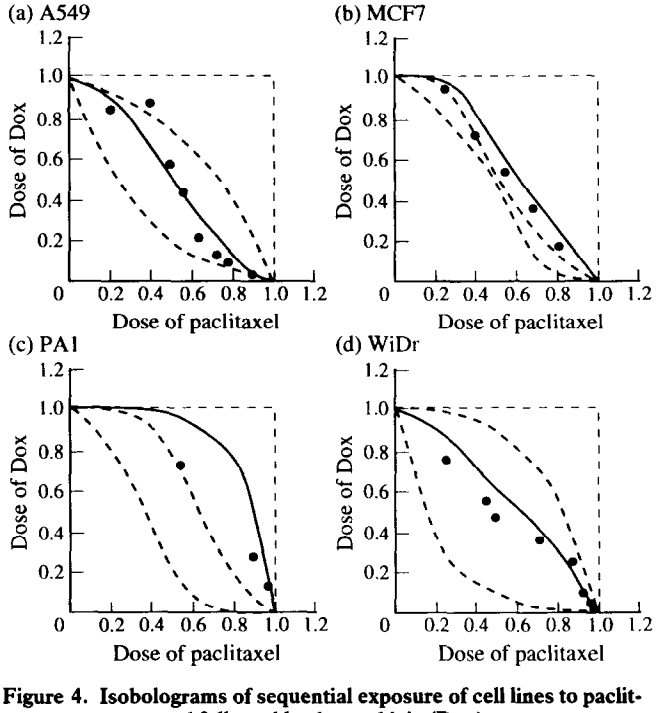

Figure 4. Isobolograms of sequential exposure of cell lines to paclitaxel followed by doxorubicin (Dox).

Using this method, we studied schedule-dependent interaction between paclitaxel and doxorubicin in four human carcinoma cell lines, A549, MCF7, PA1 and WiDr, *in vitro*. We demonstrated that cytotoxic interaction between paclitaxel and doxorubicin was schedule- and cell line-dependent. On simultaneous exposure to paclitaxel and doxorubicin and sequential exposure to doxorubicin followed by paclitaxel, additive effects were observed in PA1 cells, while antagonistic effects were observed in A549, MCF7 and WiDr cells. On sequential exposure to paclitaxel followed by doxorubicin, additive effects were observed in all four cell lines.

These findings suggest that sequential exposure to paclitaxel followed by doxorubicin may be the optimal schedule for this combination, while simultaneous administration of paclitaxel and doxorubicin and sequential administration of doxorubicin followed by paclitaxel may be less cytotoxic, and inadequate. Supra-additive (synergistic) interaction was not found with any schedules of this combination. Our definition of synergism is quite different from that of clinical synergism, which is defined as the combination producing a better tumour response than either drug alone, with toxicity being acceptable. In our isobologram method, the definition of synergistic effect is much more stringent, and even a subadditive effect means that the cytotoxic effect of the drug combination is equal or superior to that of a single agent alone.

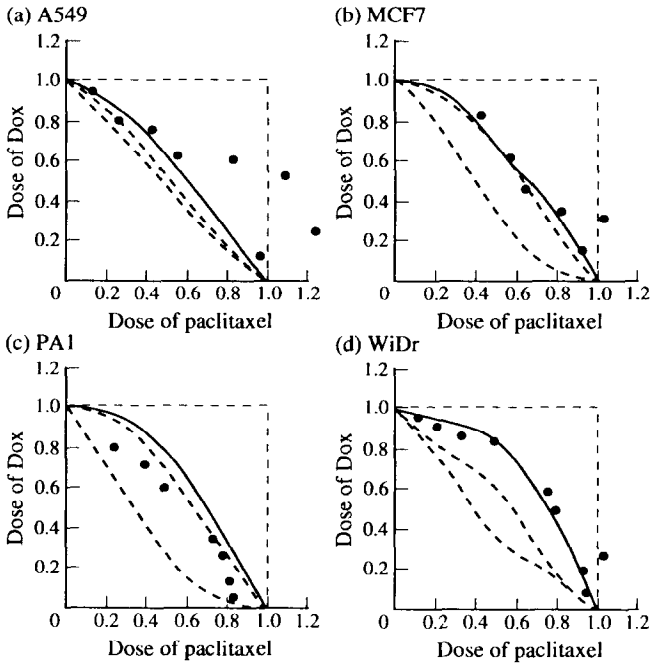

Figure 5. Isobolograms of sequential exposure of cell lines to doxorubicin (Dox) followed by paclitaxel.

Combination protocols containing doxorubicin have become standard therapy for variety of cancers. In addition to broad antitumour activity and flexibility as to dose and schedule, doxorubicin is considered to have no documented antagonistic interactions with most anticancer agents [23]. These make doxorubicin very useful in the design of drug combination. The majority of combination protocols containing doxorubicin have employed simultaneous administration of doxorubicin and other agents. Doxorubicin had additive effects with most commonly used antitumour agents in simultaneous exposure in our previous study [24]. However, the present findings suggest that the optimal schedule of paclitaxel and doxorubicin is sequential administration; paclitaxel followed by doxorubicin, but not simultaneous administration of the two agents.

Both experimental and clinical studies on the combination of paclitaxel and doxorubicin have been clearly reviewed [25]. Previous experimental studies on the interaction between paclitaxel and doxorubicin have produced conflicting results [13–17]. LoRusso and associates [13] found that separation of these agents may yield superior antitumour effects to concomitant administration in the 16/C mammary carcinoma model. Rose [14] observed less than additive effects of concomitant administration of paclitaxel and doxorubicin in a murine M109 lung carcinoma model. Our findings, that simultaneous exposure to paclitaxel and doxorubicin had antagonistic effects, support their findings. Hahn and associates [16] reported the antagonistic effects in A549, MCF7 and human ovarian carcinoma OVG1 cells with two schedules; 24 h exposure to paclitaxel followed by 1 h exposure to doxorubicin in the presence of paclitaxel, and 1 h exposure to doxorubicin followed by 24 h exposure to paclitaxel. In their experiments using the paclitaxel/doxorubicin sequence, the tumour cells were exposed to doxorubicin in the presence of paclitaxel. Thus, simultaneous exposure to the two agents might act antagonistically and influence the cytotoxic effects in this sequence. By contrast, Waud and associates [15] observed that the doxorubicin/paclitaxel sequence was more cytotoxic than the reverse sequence and concomitant exposure to the two agents in MCF7 cells. Koechli and associates [17] reported that simultaneous 90-min exposure to paclitaxel and doxorubicin had some synergistic effects in three breast cancer cell lines, T47D, MCF7 and BT20. The observations of Waud and colleagues and Koechli and associates are quite different from ours. The discrepancy may be due to the differences in culture schedules, cell lines used and assay methods used to determine viable cells. Furthermore, the method for evaluating the effects of drug combination are different, indicating that definitions of additivity, synergy and antagonism also differ with each study. This may also contribute to the differences in results [19, 22]. In general, the isobologram method entails stricter criteria for synergism and antagonism than the other methods since the additive area is expanded as much as possible [19].

Clinical combination trials of paclitaxel and doxorubicin have been initiated [25–28]. In contrast to simple *in vitro* models, the end points to be measured in clinical combination trials include therapeutic, toxic and pharmacokinetic outcomes. Berg and associates [26] observed that the steady state concentration of paclitaxel and doxorubicin was not affected when the drugs were administered as simultaneous 72-h infusions compared with results for single agent administration. The modest complete response rate is in agreement with our observation that simultaneous exposure to both agents had antagonistic effect. Holmes and associates [27] reported that 24-h paclitaxel infusion immediately prior to the initiation of 48-h doxorubicin infusion decreased doxorubicin clearance, resulting in higher toxicity than the reverse sequence. Sledge and colleagues [28] also observed higher toxicity in the paclitaxel/doxorubicin sequence. Consequently, they recommended sequential administration of doxorubicin followed by paclitaxel. However, an antagonistic interaction may have contributed to weaker toxicity in the doxorubicin/paclitaxel sequence, and the optimal doses of paclitaxel and doxorubicin in the paclitaxel/doxorubicin sequence may be lower than those in the reverse sequence. In contrast to these clinical findings, no differences in the toxicity have been observed with the combination of intravenous bolus doxorubicin and 3 h infusion paclitaxel administration by either sequence in an ongoing phase I study at the Istituto Nazionale Tumori in Milan, Italy (reviewed in ref. 25). Thus, at present, it is not obvious which schedule is optimal for the clinical trial of this combination.

In conclusion, our findings show that the interaction of paclitaxel and doxorubicin is schedule-dependent. Exposure to paclitaxel followed by doxorubicin had additive effects, and would therefore be a suitable schedule, whereas simultaneous exposure to the two agents and sequential exposure to doxorubicin followed by paclitaxel had antagonistic effects and should thus be avoided. Our findings are not similar to all of those reported previously. Since preclinical and clinical studies of the paclitaxel and doxorubicin combination have shown conflicting results, further intensive studies are required to better understand the antitumour, toxic and pharmacokinetic interactions of this combination.

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