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SCHEDULE-DEPENDENT INTERACTION OF DOXORUBICIN, PACLITAXEL AND GEMCITABINE IN HUMAN BREAST CANCER CELL LINES

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We showed previously that a sequential treatment with doxorubicin (4 hr) followed by paclitaxel (24 hr) (Dox→Pacl) induces a synergistic cytotoxic effect in the BRC-230 breast cancer cell line and in human primary breast cancer cultures. The validity of this experimental finding was confirmed in a clinical phase I/II study on advanced breast cancer patients. To improve the cytotoxic effect obtained by the Dox→Pacl sequence, we analyzed the effect of adding gemcitabine (Gem) to the Dox→Pacl sequence in a preclinical study. Our study was performed on BRC-230 and MCF-7 cell lines, and cytotoxic activity was evaluated by the sulforhodamine B assay and the type of drug interaction by Drewinko's test. When Gem (0.01 μg/ml for 24 hr) was given immediately or 24 hr after Dox→Pacl, an antagonistic cytotoxic effect was observed. Conversely, a synergistic effect was found when Gem was given 48 hr after Dox→Pacl. From results of flow cytometric analysis, the synergistic effect was attributed to cell cycle perturbation. Cells were arrested in G_2-M (95% in treated *vs.* 21% in control samples) 24 hr after Dox→Pacl treatment. The block progressively recovered thereafter, and after a further 24 hr, at the time of Gem treatment, the cells progressed into the G_1-S phase boundary (the cell cycle phase susceptible to the cytocidal effect of the drug). Our findings suggest that the interactions of Dox, Pacl and Gem are highly schedule- and time-dependent and should be taken into consideration in the planning of clinical protocols. *Int. J. Cancer* 80:413–416, 1999.
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Breast cancer is one of the most drug-sensitive tumors, and several polychemotherapy regimens are routinely used for advanced and adjuvant treatment. Doxorubicin (Dox) as a single agent induces a response rate of 30% and 50% in treated and previously untreated breast cancer patients, respectively. Combination chemotherapy protocols (Bonadonna, 1992; Bonadonna *et al.*, 1995; Harris *et al.*, 1997; Schumacher *et al.*, 1994) including or not anthracyclines have increased response rates and improved survival, thus becoming standard therapy for breast cancer.

Much interest has been focused on the search for new drugs with molecular targets other than DNA. Taxanes, which are mitotic spindle poisons that stabilize microtubules and inhibit their depolymerization to free tubulin, have been widely investigated in preclinical and clinical studies on different tumor types (Donehower and Rowinsky, 1993; Giannakakou *et al.*, 1998; Wani *et al.*, 1971; Zoli *et al.*, 1995). In particular, paclitaxel (Pacl) is highly active as a single agent in previously untreated breast cancers and in Dox-refractory breast cancers, producing objective response rates of more than 60% in previously untreated and about 50% in Dox-refractory breast cancers (O'Shaughnessy and Cowan, 1994; Swain *et al.*, 1995).

Gemcitabine (Gem), a new pyrimidine antimetabolite, has demonstrated an interesting cytotoxic activity against several solid tumors (Abbruzzese, 1996), including breast cancer.

We have evaluated the cytotoxic effects produced in human breast cancer cell lines by a combination of Dox, Pacl and Gem to analyze the different types of interaction of the 3 drugs as a function of different treatment schemes. We also tried to attribute the modulation of cytotoxic effect to the induction of cell cycle perturbations. The Dox→Pacl treatment that proved to be the most active (Amadori *et al.*, 1996; Frassineti *et al.*, 1997) was used in all experiments.

MATERIAL AND METHODS

Established cell lines

MCF-7 and BRC-230 human breast cancer cell lines were used. MCF-7 is an estrogen receptor (ER)-positive cell line. BRC-230 is an ER-negative cell line obtained in our laboratory from a ductal infiltrating breast carcinoma (Amadori *et al.*, 1993). Cells were maintained as a monolayer at 37°C and subcultured weekly. Culture medium was composed of DMEM/HAM F12 (1:1) supplemented with fetal calf serum (FCS) (10%), glutamine (2 mM), non-essential amino acids (1%), and (only for BRC-230) insulin (10 μg/ml). Cells were used in the exponential growth phase for all experiments.

In vitro *chemosensitivity test*

The sulforhodamine B (SRB) assay according to the method of Skehan *et al.* (1990) was used. Briefly, cells were collected during exponential growth phase culture by trypsinization, counted and plated in 96-well flat-bottomed microtiter plates (100 μl cell suspension per well). Each sample was run in octoplet, and each experiment was repeated 3 times. At 18–24 hr after plating (an adequate time for exponential growth recovery), 100 μl of culture medium containing or not the drugs were added to the wells. At the end of drug exposure, cells were fixed with 50% trichloroacetic acid (TCA) at 4°C (50 μl/well, final concentration 10%) for 1 hr. After 5 washes with tap water, cells were stained with 0.4% SRB dissolved in 1% acetic acid (50 μl/well) for 30 min and subsequently washed 4 times with 1% acetic acid to remove unbound stain. The plates were air-dried and solubilized with 150 μl of 10 mM unbuffered Tris base [tris(hydroxymethyl)aminomethane] solution. The optical density of treated cells was detected at 540 or 510 nm.

Flow cytometric analysis

Exponentially growing cells were trypsinized, rinsed and plated (3×10^5 cells per dish) in 60-mm Petri dishes and incubated for 18–24 hr at 37°C before drug exposure. Medium was aspirated from the plates, and different concentrations of drugs were added to the exponentially growing cells. Control dishes without drugs were cultured using the same conditions, with comparable media changes. After exposure to the drugs, cells were trypsinized, washed twice with phosphate-buffered saline (PBS) and resuspended in 1 ml of 4,6-diamino-2-phenylindole. Cells were then filtered through a disposable 40 μm filter assembly (Ratcom, Miami, FL). Human lymphocytes were utilized as internal stan-

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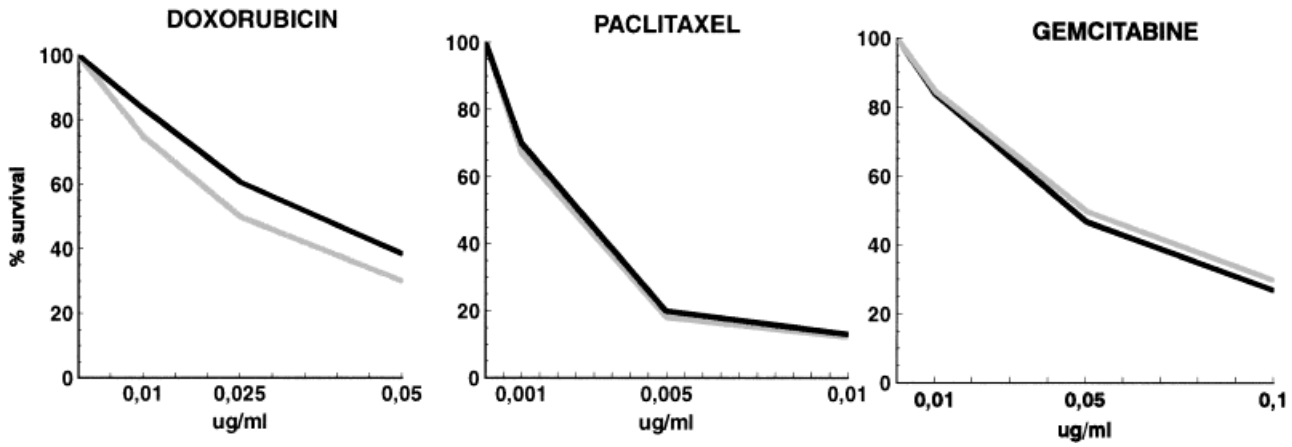

FIGURE 1 – Dose–response curves of cell lines treated with single drugs. ▬ : BRC-230 cell line; ▬ : MCF-7 cell line.

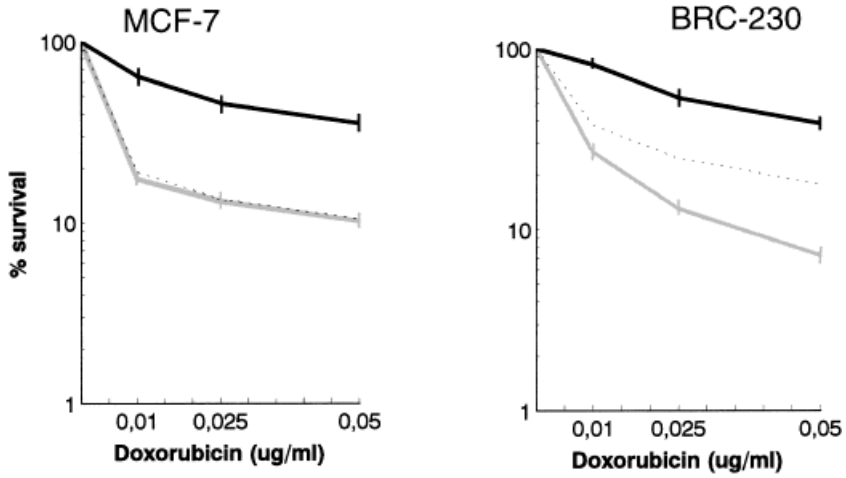

FIGURE 2 – Dose–response curves of cell lines treated with Dox→Pacl . ▬ : Dox (observed survival); - - : Dox→Pacl (expected survival); ▬ : Dox→Pacl (observed survival).

dard. For every sample, 30,000 cells were analyzed by flow cytometry (Ratcom). The data obtained were elaborated using Modfit (DNA Modeling System) software.

Drugs

Dox (Pharmacia, Milan, Italy) and Gem (Lilly, Sesto Fiorentino, Italy) were supplied as lyophilized powders, diluted with sterile physiological saline solution at a concentration of 1 mg/ml, divided into aliquots, and stored at −70°C. Pacl, supplied by the European Organization for Research and Treatment of Cancer (EORTC)/ Preclinical Therapeutic Models Group, was diluted with ethanol (95%) to obtain a concentration of 1 mg/ml and stored at −20°C until used. Drug stocks were freshly diluted in culture medium before any experiment. In the chemosensitivity assay, drugs tested singly were used at scalar concentrations of 0.01, 0.025 and 0.05 µg/ml for Dox, 0.001, 0.005 and 0.01 µg/ml for Pacl and 0.01, 0.05 and 0.1 µg/ml for Gem. From preliminary experiments in drug-combination studies, we tested Dox at all 3 concentrations and Pacl and Gem at intermediate concentrations of 0.005 µg/ml and 0.05 µg/ml, respectively. For flow cytometric analysis, cells were exposed during exponential growth to 0.025 µg/ml Dox, 0.005 µg/ml Pacl and 0.05 µg/ml Gem for 24 hr.

Statistical analysis

To quantify deviations from additive effects induced by Pacl combined with Dox and Gem, a statistical Student's *t*-test was devised (Drewinko *et al.*, 1976; Kendal and Stuard, 1983). For a given drug dose, we determined a surviving fraction (SF) of cells: SFa for Pacl, SFb for Dox and Sfc for Gem. Following the combined administration of Dox, Pacl and Gem, we determined SFabc. Additivity held, resulting in SFabc = SFa × SFb × SFc, so that our estimate of deviation from additivity was the quantity SFabc − (SFa × SFb × SFc). The ratio of differences between observed and expected survivals and the square root of the relative variances for all drug combinations examined were, in fact, distributed normally, with the average equaling 0 and the variance equaling 1. The results obtained were defined according the following criteria: SFabc = SFa × SFb × SFc indicates an additive effect; SFabc < SFa × SFb × SFc, a synergistic effect; and SFabc > SFa × SFb × SFc, an antagonistic effect.

RESULTS

The cytotoxic effects of individual drugs in the 2 established cell lines are shown in Figure 1. The cytotoxic activity of Dox was somewhat higher in the MCF-7 (IC_{50} = 0.025 µg/ml) than in the BRC-230 cell line (IC_{50} = 0.038 µg/ml), whereas a similar sensitivity to Pacl (IC_{50} = 0.0025 and 0.0027 µg/ml) and Gem (IC_{50} = 0.05 and 0.048 µg/ml) was observed in both lines.

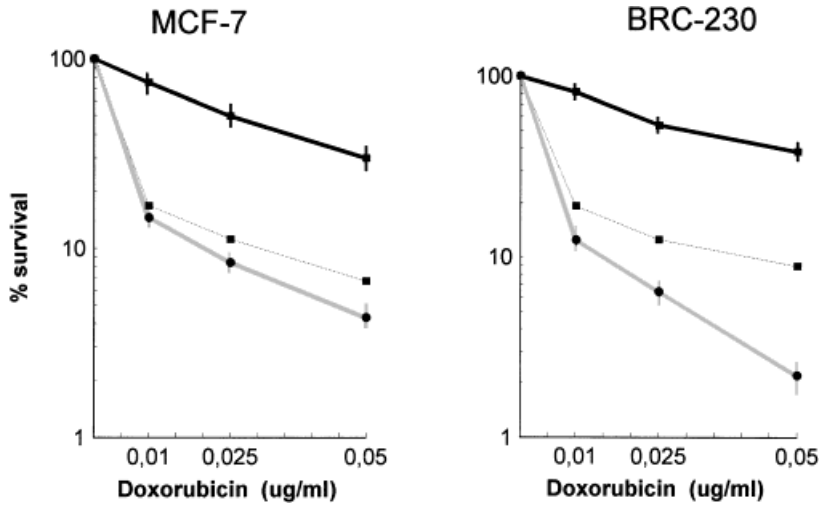

FIGURE 3 – Dose–response curves of cell lines treated with Dox→Pacl 48 hr washout→Gem sequence. ▬: Dox (observed survival); – –: Dox→Pacl→ 48 hr washout→Gem (expected survival); ▬: Dox→Pacl→48 hr washout→Gem (observed survival).

TABLE I – CELL CYCLE PERTURBATIONS INDUCED BY SINGLE DRUG EXPOSURE IN BRC-230 CELL LINE

Treatment	% Cells in G_0-G_1	% Cells in S phase	% Cells in G_2-M	% Debris
Control[1]	50.9 ± 1.2	28.1 ± 1.0	21.0 ± 1.3	3.0 ± 0.8
Doxorubicin (4 hr)	58.2	29.0	12.8	6.4
Gemcitabine (24 hr)	61.2	31.7	7.1	6.3
Paclitaxel (24 hr)	7.4	27.5	65.1	15.2
Paclitaxel (24 hr) → 24-hr washout	1.4	15.3	83.2	17.5

[1]Mean values ± standard deviation of experimental data detected at different treatment times in control samples.

TABLE II – CELL CYCLE PERTURBATIONS INDUCED BY DOX → PACL SEQUENCE IN BRC-230 CELL LINE

Treatment	% Cells in G_0-G_1	% Cells in S phase	% Cells in G_2-M	% Debris
Control[1]	50.9 ± 1.2	28.1 ± 1.0	21.0 ± 1.3	3.0 ± 0.8
Doxorubicin (4 hr) → paclitaxel (24 hr)	8.5	24.8	66.7	20.9
Doxorubicin (4 hr) → paclitaxel (24 hr) → 24-hr washout	0.7	3.9	95.4	31.8
Doxorubicin (4 hr) → paclitaxel (24 hr) → 48-hr washout	3.4	15.8	80.8	14.7
Doxorubicin (4 hr) → paclitaxel (24 hr) → 72-hr washout	13.8	26.2	60.0	25.1

[1]Mean values ± standard deviation of experimental data detected at different treatment times in control samples.

The Dox→Pacl sequence caused an additive cytocidal effect in the MCF-7 cell line and a synergistic effect in the BRC-230 cell line (Fig. 2), whereas the inverse sequence Pacl→Dox or simultaneous treatment with the 2 drugs produced an antagonistic effect (data not shown).

Cells were exposed to Gem for 24 hr to improve the cytotoxic effect obtained by the Dox→Pacl sequence. An antagonistic cytotoxic effect was observed in both cell lines when Gem was given immediately or 24 hr after Dox→Pacl treatment (data not shown). Conversely, a synergistic effect was seen when Gem was given 48 hr after the end of Dox→Pacl treatment (Fig. 3). The synergistic interaction was higher in the BRC-230 cell line than in the MCF7 cell line.

Flow cytometric analysis of cell cycle perturbations induced by single drugs or drug combinations was analyzed in BRC-230 cells. The 4-hr treatment with Dox induced a modest increase in the number of G_0-G_1 phase cells and a decrease in G_2-M phase cells (Table I). A similar but more evident cell cycle perturbation was observed after a 24-hr exposure to Gem.

The characteristic G_2-M accumulation of cells together with the total disappearance of cells in G_0-G_1 was evident after a 24-hr exposure to Pacl. The cell cycle perturbation was even greater after a 24-hr washout (Table I) and, when analyzed immediately after the end of Dox→Pacl treatment, was consistent (Table II) with a block of cells in the G2-M phase that increased after a 24-hr washout and progressively recovered after 48 and 72 hr.

The cell cycle perturbation induced by Dox→Pacl was not altered by a 24-hr treatment with Gem given immediately or after a 24-hr washout (Table III). Conversely, a 24-hr treatment with Gem after a 48-hr washout resulted in a considerable increase in S phase cells and in damage to about 40% of cells.

DISCUSSION

To date, the clinical design of polychemotherapeutic protocols has mainly taken into account information derived from experimental studies on single-agent mechanisms of action and has favored a combination of drugs with complementary mechanisms of action. As a result, sequential drug administration has always been based on weak or no experimental data. It is clear that sequencing is vitally important in order to avoid drug treatment when cells are not in the drug-sensitive phase.

In a previous *in vitro* study on human cell lines and primary breast cancer cultures, we showed (Amadori *et al.*, 1996) that the sequence Dox→Pacl, but not simultaneous drug administration or the inverse sequence (Pacl→Dox), produced a powerful synergistic interaction. Drug interaction becomes more complex with the increasing number of drugs included in clinical protocols. We thus attempted to define the Gem, Dox and Pacl interaction to formulate the most effective treatment schedule. We observed that the maximum cytocidal effect is achieved by the following sequence:

TABLE III – CELL CYCLE PERTURBATIONS INDUCED BY DOX → PACL → GEM SEQUENCE IN BRC-230 CELL LINE

Treatment	% Cells in G_0-G_1	% Cells in S phase	% Cells in G_2-M	% Debris
Control[1]	50.9 ± 1.2	28.1 ± 1.0	21.0 ± 0.3	3.0 ± 0.8
Doxorubicin (4 hr) → paclitaxel (24 hr)	8.5	24.8	66.7	20.9
Doxorubicin (4 hr) → paclitaxel (24 hr) → gemcitabile (24 hr)	9.5	26.3	64.2	15.8
Doxorubicin (4 hr) → paclitaxel (24 hr) → 24-hr washout gem-citabine (24 hr)	12.8	33.3	54.1	16.2
Doxorubicin (4 hr) → paclitaxel (24 hr) → 48-hr washout gem-citabine (24 hr)	11.3	51.0	37.3	36.3

[1]Mean values ± standard deviation of experimental data detected at different treatment times in control samples.

Dox→Pacl→ 48-hr washout and then 24-hr treatment with Gem. The synergistic effect was greater in ER^- than in ER^+ cell lines and would therefore probably be more effective in ER^- breast cancers, which are highly aggressive and often treated with polychemotherapy. The synergistic effect may be a result of cell cycle perturbation induced by the treatment schedules. In fact, according to this scheme, Gem attacks cells recovering from a G_2-M block as they progress to the S phase and produces a powerful cytocidal effect, as shown by the large amount of debris. Such a hypothesis is supported by the absence of a cytocidal effect of antimetabolite exposure on the cells still blocked in G_2-M immediately or 24 hr after Dox→Pacl treatment.

In view of the successful translation of results from preclinical studies on conventional and newly proposed cytocidal drugs (Amadori *et al.*, 1996; Savini *et al.*, 1992) and on modulators of drug resistance (Citro *et al.*, 1991; Silvestrini *et al.*, 1992, 1997) to a clinical setting, an advanced breast cancer protocol will shortly be activated in Italy based on our present findings.

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