

# Sequence-dependent synergism and antagonism between paclitaxel and gemcitabine in breast cancer cells: The importance of scheduling

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**Abstract.** The marked clinical anticancer activity of the paclitaxel (PTX) and gemcitabine (GEM) combination has suggested that the two drugs may interact more than additively. We have analyzed the *in vitro* growth and molecular interactions of the two chemotherapy drugs in a panel of human breast cancer cell lines (i.e., MCF-7, MDA-MB-231, MDA-MB-468, and SKBR3) that were treated with PTX and GEM combined either simultaneously (PTX + GEM) or sequentially (PTX→GEM; GEM→PTX). PTX-GEM interactions at the cellular level were assessed mathematically employing both the isobologram analysis (Berenbaum) and the combination index (Chou-Talalay) method. PTX-GEM molecular interactions on the apoptotic markers PARP, Bcl-2 and Bax were analyzed by immunoblotting procedures. Apoptosis was detected using a DNA ladder assay. We observed significant synergistic growth inhibitory interactions when PTX was administered before GEM. Additive interactions were observed when both the simultaneous regimen and the GEM followed by PTX regimen were used. DNA ladder and Western blotting results in the PTX followed by GEM sequence revealed a significant increase in the apoptotic cell death of breast cancer cells related to the Bax/Bcl-2 apoptotic pathway. In summary, the occurrence of clinically relevant synergism between PTX and GEM suggests a sequence-dependent nature in human breast cancer cells. This synergistic interaction on the PTX→GEM schedule appears to be related to an increase

in the Bcl-2-related mitochondrial apoptotic pathway. The synergism that we have observed may explain the favorable clinical responses that have been achieved in clinical studies, in which patients are administered PTX first, and then GEM.

## Introduction

Paclitaxel (PTX) is a member of the taxane family isolated from the Pacific yew (*Taxus brevifolia*) (1), known to inhibit cancer cell growth and trigger apoptosis. PTX is a microtubule-interfering agent, which causes the stabilisation of the mitotic spindle microtubules through the binding to the  $\beta$ -tubulin subunit leading to cell cycle arrest in the G<sub>2</sub>/M phase of the cell cycle, and apoptosis (2,3). It is used in the treatment of women with advanced breast cancer, with response rates ranging from 31% to 50% (4,5), and it is also used in the treatment of other cancers including ovarian and lung (6,7).

Gemcitabine (2'-2'-difluorodeoxycytidine; dFdCyd; GEM) is a nucleoside analogue used in solid tumor therapy, such as that of non-small cell lung, ovarian, pancreas and breast cancer (8). GEM is incorporated mainly within replicating DNA and leads to termination of DNA chain elongation. It also inhibits DNA synthesis through inhibition of DNA polymerases leading to cell cycle arrest in the G<sub>1</sub>/S phase of the cell cycle, which causes apoptosis. GEM toxicity can be increased on inhibition of the ribonucleotide reductase, which blocks *de novo* deoxyribonucleotide synthesis (9,10). As a single agent, GEM yields response rates ranging from 14 to 37% as a first-line treatment for advanced breast cancer (11,12) and 16-29% as a salvage therapy for patients previously treated with chemotherapy (13,14).

Combination chemotherapy offers the possibility of enhanced antitumor efficacy. Criteria for an effective combination include the use of drugs with different mechanisms of action, relative non-cross-resistance, and partially non-overlapping toxicities. PTX and GEM fulfil these criteria because PTX acts against microtubules inducing cell cycle arrest in the G<sub>2</sub>/M phase and GEM acts against DNA and causes cell cycle arrest in the G<sub>1</sub>/S phase, and they have

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non-overlapping toxicities. Excellent responses are achieved in experimental studies of this combination (15-18), but there is limited experience regarding which is the more appropriate sequence of administration. In this study we have evaluated the cellular and molecular interactions between PTX and GEM in cultured human breast cancer cells. In addition, we have investigated whether there is a more active treatment schedule.

## Materials and methods

**Cell culture.** The human breast cancer cell lines MCF-7, MDA-MB-231, MDA-MB-468 and SKBR3 were obtained from the American Type Culture Collection (ATCC), and were maintained in a humidified atmosphere containing 5% (v/v) CO<sub>2</sub> at 37°C. All the breast cancer cell lines were cultured in DMEM medium (Gibco), with the exception of the SKBR3 cell line that was cultured in McCoy's medium (Gibco). DMEM and McCoy media were supplemented with 10% synthetic foetal bovine serum (HyClone), 1% L-Glutamine (Biochrom AG), 1% sodium pyruvate (Biochrom AG) and 1% penicillin-streptomycin (Gibco), and routinely sub-cultured twice weekly, detaching them using trypsin 1X in PBS (Gibco). Trypsin activity was stopped using fresh culture medium.

**Cytotoxicity assay.** Cells (7x10<sup>3</sup>/well in the two-drug combination) in their exponential cell growth were plated in 96-well microdilution plates (Corning). Following cell adherence (24 h), experimental medium containing the chemotherapeutic drug(s) was added to triplicate wells (PTX, Bristol-Myers Squibb; GEM, Eli Lilly), and serial dilutions were performed to span the dose range suitable for isobologram analysis (Table I). We analysed the three possible schedules for the two-drug combination: PTX plus GEM (simultaneous), PTX followed by GEM and GEM followed by PTX. In all cases, time exposure to cytotoxic drugs was 72 h. For the sequential treatments, the first drug was used for 24 h, and the second for the following 48 h. The doses used to study drug combination were close to IC<sub>30</sub> values.

Following treatment, a cell viability assay was performed using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) method (19). Some changes on the protocol described by Mosmann were performed. Briefly, the medium was removed and replaced by fresh drug-free medium (100 µl/well), and MTT (5 mg/ml in PBS) was added to each well at a 1/10 volume. After incubation for 2-3 h at 37°C, the supernatants were carefully aspirated, 100 µl of DMSO was added to each well, and the plates were agitated to dissolve the crystal product. Absorbances were measured at 570 nm using a multi-well plate reader (Model Anthos Labtec 2010 1.7 reader). The cell viability effects from exposure of cells to each compound alone and their combination were analyzed as percentages of the control cell absorbances, which were obtained from control wells treated with appropriate concentrations of the compounds' vehicles that were processed simultaneously. For each treatment, cell viability was evaluated as a percentage using the following equation: (A570 of treated sample/A570 of untreated sample) x100.

**Synergy analysis: Berenbaum's isobologram and Chou-Talalay (median-effect plot) method.** This dose-oriented mathematical

Table I. Dose range suitable for the drug-combination analyses in each cell line.

Cell line	PTX (ng/ml)	GEM (ng/ml)
SKBR3	0.1-10	1-25
MCF-7	0.1-10	50-1000
MDA-MB-231	0.1-25	50-1000
MDA-MB-468	0.1-10	1-100

method to assess the nature of the interaction between therapeutic agents requires the determination of a given biological effect when the concentration ratio of two agents varies (20). In our experiments, the IC<sub>30</sub> value (i.e., the drug concentration needed to cause 30% reduction in cell viability) was chosen for comparisons. An interaction index (I<sub>x</sub>) was calculated using the following equation  $I_x = \sum E(y) = \sum (d_x/D_x)$  where d<sub>x</sub> is the dose needed to cause x% of growth inhibition when the drug is combined with another, and D<sub>x</sub> is the dose needed to cause the same effect when the drug is administered alone. Isoboles were constructed by plotting E(PTX) = d<sub>x</sub>/D<sub>x</sub> of PTX versus E(GEM) = d<sub>x</sub>/D<sub>x</sub> of GEM. If data points fall to the left of the additivity line (I<sub>x</sub> <1), synergy is indicated; if the data fall within the additivity line (I<sub>x</sub> =1), drug interaction is said to be additive; if the data points fall to the right of the additivity line (I<sub>x</sub> >1) then the combination is considered antagonistic.

Synergism, additivity or antagonism of the drugs was also determined by the median effect analysis (21). This involves plotting dose-effect curves for each agent and for multiply diluted, fixed ratio combinations of agents using the median-effect equation (a):  $f_a/f_u = (D/D_m)^m$ . In this equation, D is dose, D<sub>m</sub> is the dose required for 50% effect (e.g., 50% inhibition of cell growth, ED<sub>50</sub>), f<sub>a</sub> is the fraction affected by dose D (e.g., 0.9 if cell growth is inhibited by 90%), f<sub>u</sub> is the unaffected fraction (therefore, f<sub>u</sub>=1-f<sub>a</sub>), and m is a coefficient of sigmoidicity of the dose-effect curve; m=1, >1, and <1 indicate hyperbolic, sigmoid and negative sigmoid dose-effect curves, respectively, for an inhibitory drug.

Equation a may be rearranged as follows (b):  $D_x = D_m [f_a/(1-f_a)]^{1/m}$ . The parameters m and D<sub>m</sub> are easily determined by the median-effect plot  $x = \log(D)$  versus  $y = \log [f_a/(1-f_a)]$ , which is based on the logarithmic form of equation a and yield a straight line where m is the slope and log (D<sub>m</sub>) is the x intercept. IC<sub>50</sub> values (by interpolation) and D<sub>m</sub> values (by the median-effect plot) were usually similar. Equation b may thus be solved, providing the iso-effective dose (D<sub>x</sub>) for any effect level (e.g. ED<sub>80</sub> for f<sub>a</sub>=0.8; ED<sub>90</sub> for f<sub>a</sub>=0.9, and so forth). A combination index (CI) is then determined with the following equation (c):  $CI = (D)_1/(D_x)_1 + (D)_2/(D_x)_2 + \alpha(D)_1(D)_2/(D_x)_1(D_x)_2$  where (D<sub>x</sub>)<sub>1</sub> is the dose of agent 1 required to produce x percent effect alone, and (D)<sub>1</sub> is the dose of agent 1 required to produce the same x percent effect in combination with (D)<sub>2</sub>. Similarly (D<sub>x</sub>)<sub>2</sub> is the dose of agent 2 required to produce x percent effect alone, and (D)<sub>2</sub> is the dose required to produce the same effect in combination with (D)<sub>1</sub>. If the agents are

Table II. Single drug IC<sub>30</sub> values upon different PTX/GEM combination schedules.

Cell line	PTX (ng/ml)			GEM (ng/ml)		
	A	B	C	A	B	C
SKBR3	1.4±0.6	1.9±1	2.8±1.2	5.1±1.6	6.9±2.1	13.81±2.6
MCF-7	2.8±1.1	2.7±0.3	10.1±2.1	116±33.8	206.2±53.7	223.8±65.1
MDA-MB-231	3.6±0.6	9.7±5.7	18.59±3.8	70.1±14.6	137.3±66.6	351.1±73.3
MDA-MB-468	0.4±0.06	0.7±0.05	1.8±0.2	38.6±15	15.3±0.7	62.07±8

A, 72-h drug treatment; B, drug for 24 h → drug-free medium for 48 h; C, drug-free medium for 48 h → drug for 24 h.

mutually exclusive (e.g., similar mode of action), then  $\alpha$  is 0 (i.e., CI is the sum of 2 terms); if the agents are mutually non-exclusive (e.g., independent mode of action),  $\alpha$  is 1 (i.e., CI is the sum of 3 terms). If it is uncertain whether the agents act in a similar or an independent manner, the formula may be solved both ways. Different values of CI may be obtained by solving the equation for different values of  $f_a$  (e.g., different degrees of inhibition of cell growth). CI values of <1 indicate synergy (the smaller the value, the greater the degree of synergy), values >1 indicate antagonism and values equal to 1 indicate additive effects. In our current studies, CI profiles were compared to a preset null interval of 0.95-1.05 (addition), so that mean CI values >1.05 or <0.95 were interpreted as being suggestive of antagonism and synergism, respectively. Each experiment was carried out with triplicate cultures for each data and was repeated independently at least three times. The conformity of the experimental data to the median-effect principle of the mass-action law was automatically provided by the computer printout in terms of the linear correlation coefficient (r-value) of the median-effect plots. In our studies, the r-values for PTX, GEM and their combinations were all >0.95.

**Immunoblotting.** Cells (~6x10<sup>5</sup>) were seeded in 100-mm Petri dish plates (Corning). Following cell adherence (24 h), cells were treated with drugs in the different schedules described previously. Following treatment, cells were washed in PBS and lysed with lysis buffer (Tris 50 mM pH 7.5, NaCl 150 mM, EDTA 1 mM, Ortovanadate-Na 0.02 mM, 1% Triton X-100, PMSF 0.5 mM and protease cocktail). The quantification of the total protein was made by a Lowry assay (BioRad). Clarified protein lysates (30 to 50  $\mu$ g of protein of each sample) were electrophoretically resolved on 4-12% MOPS NuPage gels (Invitrogen), transferred to a 0.45- $\mu$ m pore size nitrocellulose membrane (Invitrogen), and then probed with anti-PARP (Oncogene), anti-Bax (Neomarkers), anti-Bcl-2 (Neomarkers), and anti- $\beta$ -actin (Santa Cruz Biotechnology) antibodies. Proteins were detected using peroxidase-conjugated anti-mouse or anti-rabbit immunoglobulines (Calbiochem) followed by incubation with Super-Signal West Pico chemiluminescence substrate (Pierce).

**DNA ladder.** Apoptosis was detected using the Suicide track DNA ladder kit (Calbiochem). Following treatments, DNA was extracted as per the manufacturer's instructions, and

equal amounts of DNA were electrophorated through a 1.5% agarose gel containing 0.3  $\mu$ g/ml ethidium bromide. Bands were visualized under UV Transilluminator Syngene (Bio Imaging Systems).

**Statistical analysis.** All experiments were performed in triplicate and were repeated at least three times. Data were expressed as mean values  $\pm$  SD, and were analysed by Student's t-test; the level of significance was set at  $p < 0.05$ .

## Results

**Single-agent activity.** When MCF-7, MDA-MB-231, MDA-MB-468, and SKBR3 human breast carcinoma cell lines were treated with graded concentrations of single-drug PTX or GEM, we observed a dose-dependent decrease in the percentage of cell viability. The IC<sub>30</sub> values (i.e., the concentrations of the drugs needed to reduce cell viability by 30%) ranged from 0.4 to 18.6 ng/ml in the case of PTX, and from 5 to 350 ng/ml in the case of GEM (Table II). The highly-metastatic MDA-MB-231 cell line was significantly more resistant to the tumoricidal effects of PTX and GEM when compared to MCF-7, SKBR3 and MDA-MB-468.

**Synergy analyses.** To evaluate potential synergistic or antagonistic interactions between PTX and GEM, we first performed combination studies at a non-fixed molar ratio by the isobologram method using drug-concentrations close to PTX and GEM IC<sub>30</sub> values.

**I. Simultaneous schedule (PTX + GEM).** Both the interaction indexes ( $I_{30} \approx 1$ ; Table III) and the isobolograms (Fig. 1a) revealed additive interactions when PTX and GEM were used concomitantly in SKBR3, MCF-7 and MDA-MB-468 breast cancer cell lines. An antagonistic interaction was apparent in MDA-MB-231 cells.

**II. Sequential schedule (PTX→GEM; GEM→PTX).** A different picture emerged upon sequential administration of PTX and GEM. When PTX was administered prior to GEM (Fig. 1b; Table III) a statistically significant synergy was observed in SKBR3, MCF-7 and MDA-MB-231 cell lines ( $I_{30} < 1$ ), while additive interactions occurred in MDA-MB-468 cells. When the drugs were administered in the sequence GEM followed by PTX, no synergism was observed (Fig. 1c; Table III).

Table III. Interaction index ( $I_x$ ) values for the combination of PTX/GEM Gemcitabine at 30% of cell death.

Cell line	PTX→GEM		GEM→PTX		PTX + GEM	
	$I_{30}$	Result	$I_{30}$	Result	$I_{30}$	Result
SK-Br3	0.862±0.47	Synergy	1.252±0.36	Addition	0.965±0.41	Addition
MCF-7	0.672±0.26	Synergy	0.984±0.38	Addition	1.303±0.67	Addition
MDA-MB-231	0.889±0.10	Synergy	1.05±0.41	Addition	3.244 <sup>a</sup> ±1.17	Antagonism
MDA-MB-468	1.137±0.26	Addition	1.238 <sup>a</sup> ±0.03	Antagonism	1.05±0.44	Addition

<sup>a</sup>Significance (p<0.05).

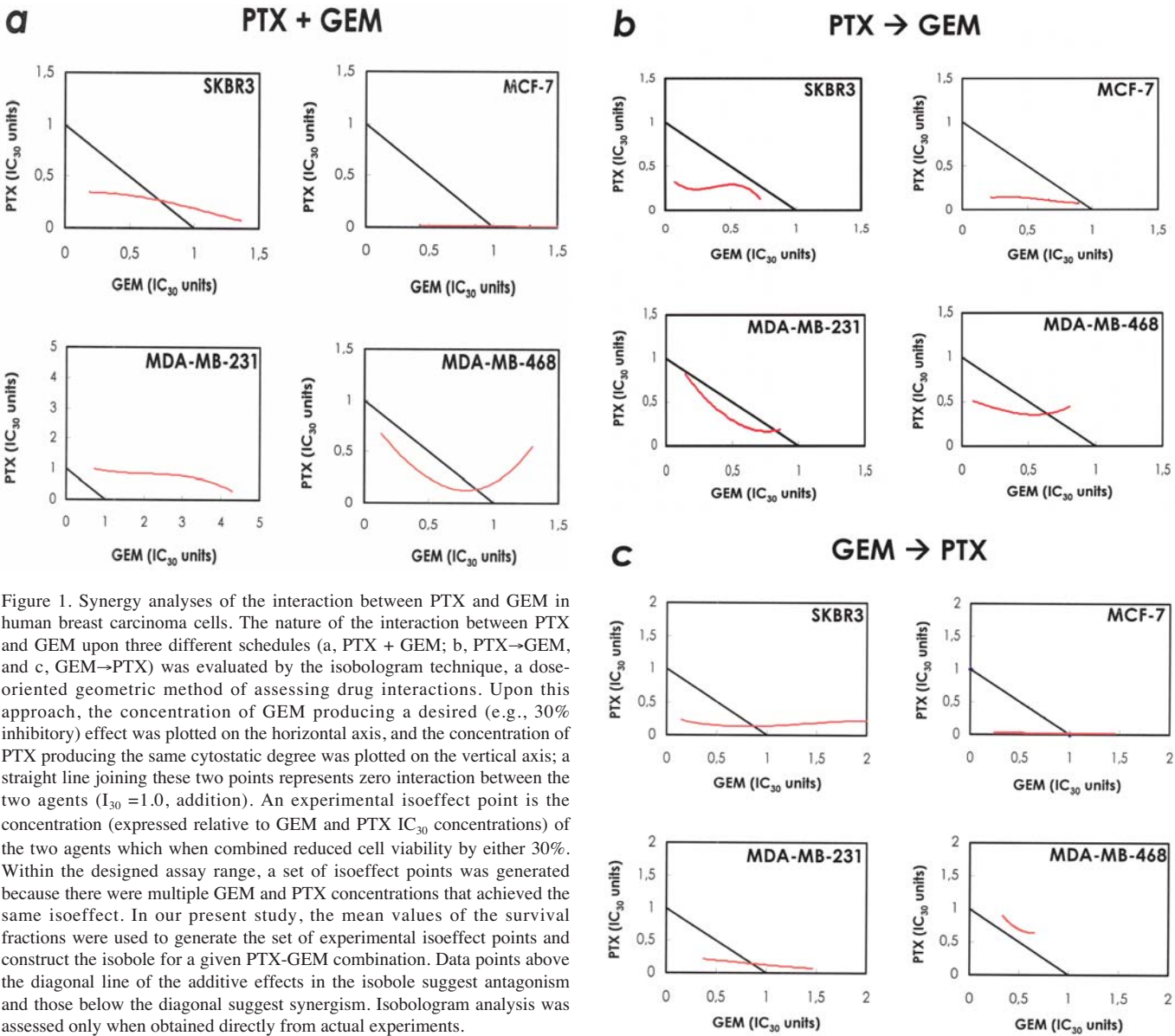


Figure 1. Synergy analyses of the interaction between PTX and GEM in human breast carcinoma cells. The nature of the interaction between PTX and GEM upon three different schedules (a, PTX + GEM; b, PTX→GEM, and c, GEM→PTX) was evaluated by the isobologram technique, a dose-oriented geometric method of assessing drug interactions. Upon this approach, the concentration of GEM producing a desired (e.g., 30% inhibitory) effect was plotted on the horizontal axis, and the concentration of PTX producing the same cytostatic degree was plotted on the vertical axis; a straight line joining these two points represents zero interaction between the two agents ( $I_{30} = 1.0$ , addition). An experimental isoeffect point is the concentration (expressed relative to GEM and PTX  $IC_{30}$  concentrations) of the two agents which when combined reduced cell viability by either 30%. Within the designed assay range, a set of isoeffect points was generated because there were multiple GEM and PTX concentrations that achieved the same isoeffect. In our present study, the mean values of the survival fractions were used to generate the set of experimental isoeffect points and construct the isobole for a given PTX-GEM combination. Data points above the diagonal line of the additive effects in the isobole suggest antagonism and those below the diagonal suggest synergism. Isobologram analysis was assessed only when obtained directly from actual experiments.

In order to confirm the synergistic interaction occurring when administered sequentially (i.e., PTX→GEM), the combined cytotoxic effect of PTX and GEM was further assessed using the (fixed molar ratio) median-effect plot analysis of Chou *et al* (21). This procedure allows the

characterization of drug interactions with a single number, the Combination Index (CI). The CI parameter indicates whether the doses of the two agents required to produce a given degree of cytotoxicity are greater than ( $CI > 1$  or antagonism), equal to ( $CI = 1$  or addition) or less than ( $CI < 1$  or synergism) the

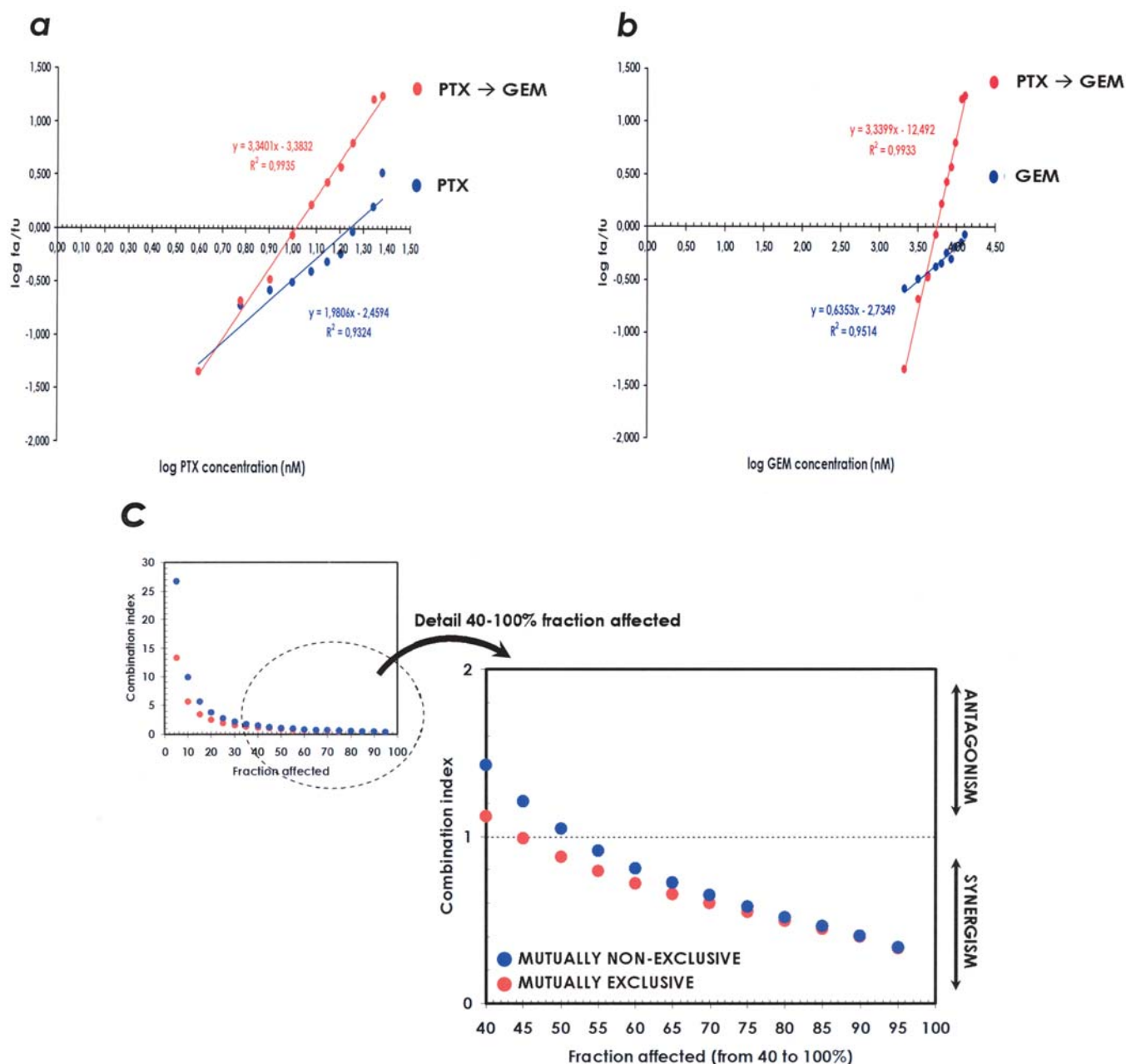


Figure 2. Synergistic effect of PTX and GEM on growth inhibition in MCF-7 breast cancer cells. (a and b) Median-effect plot. Effect of PTX, GEM or PTX→GEM at a constant ratio was measured by MTT assays as described in Materials and methods. The log values of the ratios of fraction affected to fraction unaffected ( $\log f_a/f_u$ ) were plotted against log values of concentrations of PTX and GEM single agents of PTX/GEM concentrations in combinations as shown in the median-effect plot.  $IC_{50}$  values of single drugs or the combination were determined from the x-intercepts where  $\log (f_a/f_u) = 0$ . (c) Combination Index (CI)/effect plot. Combination index values of combination treatment were analyzed as described in Materials and methods and plotted against fractional inhibition by the combination treatment. Significant synergism (Combination Index  $< 0.8$ ) was indicated for the PTX→GEM combination at cell growth inhibitory effects  $> 50\%$ .

doses that would be required if the two agents were strictly additive. For this type of analysis and for each drug separately (i.e., PTX and GEM), we measured how the fraction affected (i.e., the fractional cell toxicity) varied with differing doses. For two drugs in combination (i.e., PTX→GEM) we varied the doses of the two agents while monitoring the fraction affected; however, the doses were varied such that a constant ratio of agent 1 (PTX) to agent 2 (GEM) was maintained. Specifically, 1.5, 2.0- and 3.0-fold serial dilutions of PTX and GEM were prepared and combined with each other from the lowest to the highest concentration while assessing the

cell fraction affected (Fig. 2a and b). The combination ratio was designed to approximate the  $IC_{50}$  ratio of the drugs determined in preliminary experiments, so that the contribution of the effect for PTX and GEM in the mixture would be the same (i.e., equipotency ratio). Fig. 2c shows the CI plots at various effect levels (fraction affected) for the sequential combination PTX→GEM in MCF-7 breast cancer cells. The synergy observed with sequential PTX prior to GEM exposure for 72 h was apparent at levels exceeding the 50% cell kill boundary, with CI values ranging from 0.877 (moderate synergism) at the  $IC_{50}$  to 0.331 (strong synergism) at the  $IC_{95}$ .

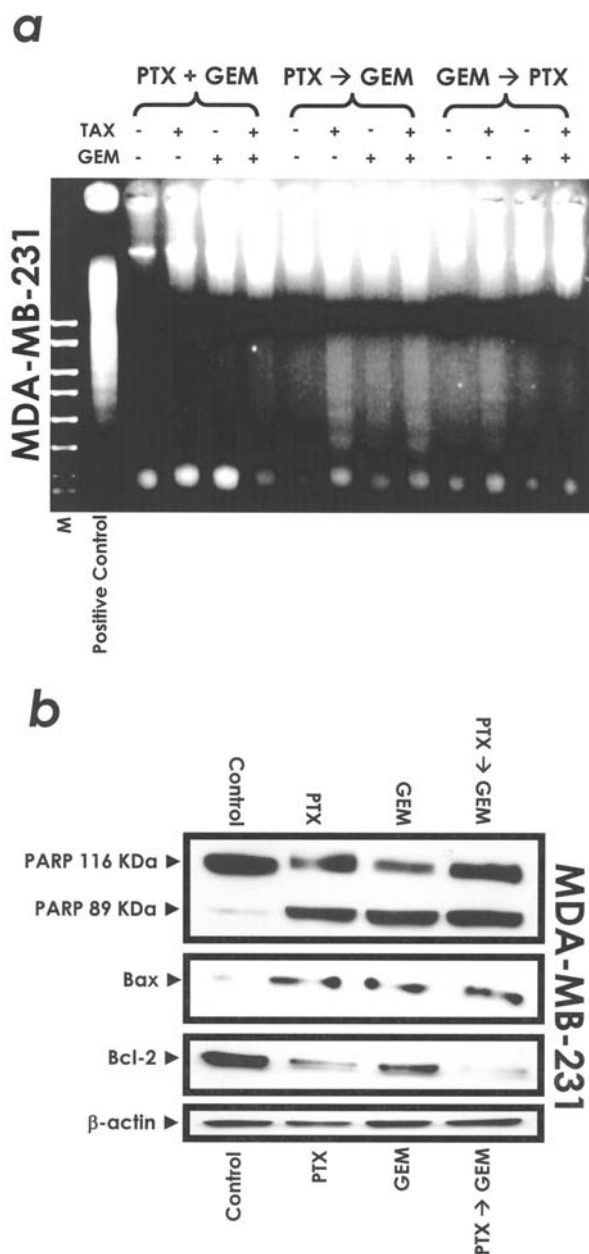


Figure 3. Assessment of apoptotic cell death. (a) DNA-laddering assay. Genomic DNA was prepared from MDA-MB-231 cells that were treated as specified. DNAs were fractionated on agarose gels, stained with ethidium bromide, and photographed. M, DNA molecular weight marker. (b) Western blot analyses for the PARP, Bax, and Bcl-2 proteins. Total whole-cell proteins were extracted from MDA-MB-231 cells treated with GEM alone with or without pre-exposure to PTX. Equal amounts of cellular protein were fractionated on a 4-12% MOPS NuPage gel and transferred to nitrocellulose membranes, followed by immunoblotting with anti-PARP, Bax, and Bcl-2 antibodies. PARP cleavage was analyzed using an anti-PARP antibody detecting both intact PARP (116 kDa) and the apoptotic marker PARP cleavage fragment (85 kDa).  $\beta$ -actin was used as a loading control. A representative Western blot analysis is shown. Equivalent results were obtained in three independent experiments.

These findings, altogether, reveal that sequential administration of PTX followed by GEM is necessary for maximal augmentation of cytotoxicity in breast cancer cells.

**Apoptosis.** To gain additional insight into the molecular mechanisms underlying the synergism/antagonism occurring

when combining PTX and GEM, we investigated the possible influence of the schedule treatment on PTX/GEM-induced apoptosis. Since an important hallmark of apoptotic cell death is the fragmentation of genomic DNA into integer multiples of 180-bp units, we first performed a DNA fragmentation assay (Fig. 3a). When MDA-MB-231 cells were exposed to GEM alone, essentially no clear DNA fragmentation was observed. However, on combination treatment with GEM and PTX, particularly when these cells were pre-exposed to PTX, GEM-induced DNA fragmentation was significantly increased, thus suggesting that the pre-treatment with PTX enhances GEM-induced apoptotic cell death in GEM-resistant MDA-MB-231 breast cancer cells.

We then performed immunoblotting on apoptotic markers such as Poly(ADP-ribose) polymerase (PARP), Bax and Bcl-2. PARP, a nuclear enzyme involved in DNA repair and activated in response to DNA-damage, is an early target of caspases during apoptosis (22-25). The specific cleavage of this protein by caspase-3 onto 89- and 24-kDa fragments is considered to be a hallmark of the apoptotic mode of cell death (22-25). Bax and Bcl-2 are members of the Bcl-2 protein family, which is involved in the apoptotic pathway (26-29). In fact, increases in the Bax/Bcl-2 ratio relate to increases in the extent of apoptotic cell death.

When PARP cleavage was evaluated using an antibody that recognizes both the intact 116-kDa (PARP p116) and the cleaved 89-kDa fragment (PARP p89), PARP was likewise cleaved as shown by the appearance of a signature 85-kDa fragment in whole cell lysates from PTX-, GEM-, and PTX→GEM-treated MDA-MB-231 cells. However, immunoblotting-based assessment of PARP cleavage did not reveal significant differences between treatment groups (PTX alone, GEM alone, or PTX→GEM sequential combination), indicating the existence of a threshold above which a rise in cell damage does not result in further PARP cleavage (Fig. 3b). Interestingly, a significantly higher Bax/Bcl-2 ratio was detected upon sequential treatment with PTX prior to GEM when compared to that obtained using single-drug treatments (Fig. 3a). Overall, the data show that the PTX→GEM sequential combination results in a significant activation of the mitochondrial damage pathway in GEM-resistant MDA-MB-231 breast cancer cells.

## Discussion

Although it is generally accepted that cell lines and preclinical data have limitations in their ability to accurately model the clinical picture, the use of preclinical evidence in designing chemotherapy combinations and schedules is not without value. In this regard, there is discordance between the potential antagonism between taxanes and Gemcitabine in some pre-clinical models and the positive clinical results obtained by the combinations of Gemcitabine and either Paclitaxel or Docetaxel. The present study demonstrates that, using *in vitro* experimentation, the combination of PTX and GEM can exhibit synergistic anticancer activity against breast cancer cells. Our experimental results may explain, at least in part, the good response rates that are achieved in clinical studies where the two drugs are combined in the treatment of metastatic breast cancer patients (15-18).

Our study demonstrates that the nature of the interaction between PTX and GEM (i.e., antagonism, addition, and synergism) is schedule-dependent. We analyzed three possible schedules (i.e., PTX + GEM, GEM→PTX, and PTX→GEM), and we found that synergistic interactions are mainly observed upon the sequence of PTX prior to GEM. This synergism is consistent with the results of Kroep *et al* (30), who showed that treatment with PTX significantly increases the cellular content on dFdCTP (i.e., the active form of GEM), thus improving GEM efficacy. Henley *et al* (31) suggested that the synergism occurring when combining PTX followed by GEM, rather than related to cell cycle progression arrest, might relate to the specific activation of the Bcl-2 apoptotic pathway. Here, we further clarify that exposure of breast cancer cells to PTX prior to GEM results in decreased expression of Bcl-2 with reciprocal increase in Bax protein. This increase in the Bax/Bcl-2 ratio can be proposed to drive the synergistic apoptotic cell death occurring in the PTX followed by GEM schedule.

In summary, through a series of *in vitro* assays including MTT-based cell viability assays, DNA fragmentation, and immunoblotting-based assessment of apoptotic markers, we provide evidence that antagonism can occur when breast carcinoma cells are exposed to PTX and GEM simultaneously or exposed to GEM before PTX. However, a clinically relevant synergism occurs when PTX is administered before GEM. These findings support earlier *in vitro* studies (32,33) and, more importantly, our own clinical results, in which patients with metastatic breast cancer treated with PTX and GEM demonstrated an overall response rate of 71% (34,35). This high response rate of the PTX/GEM regimen may be, at least in part, a clinical consequence of the synergism occurring at the cellular/molecular level.

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