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

CRISTINA OLIVERAS-FERRAROS1,2, ALEJANDRO VAZQUEZ-MARTIN1,2, RAMON COLOMER1,2,3, RAFAEL DE LLORENS4, JOAN BRUNET1,2 and JAVIER A. MENENDEZ1,2

1Metabolism and Cancer Laboratory, Girona Biomedical Research Institute (IdIBGi), Dr Josep Trueta University Hospital of Girona; 2Catalan Institute of Oncology (ICO)-Health Services Division of Catalonia; 3M.D. Anderson International Madrid, Madrid; 4Biochemistry and Molecular Biology of Cancer, Faculty of Sciences, University of Girona (UdG), Girona, Catalonia, Spain

Received May 29, 2007; Accepted August 12, 2007

Correspondence to: Dr Javier A. Menendez, Metabolism and Cancer Laboratory, Girona Biomedical Research Institute (IdIBGi), Dr Josep Trueta University Hospital of Girona, Avenida de Francia s/n; 17007 Girona, Catalonia, Spain
E-mail: jmenendez@ico.scs.es

Key words: paclitaxel, gemcitabine, chemotherapy, breast cancer, Bcl-2

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 cells. We evaluated cell viability in four 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 ß-tubulin subunit leading to cell cycle arrest in the G2/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 G1/S 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).

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 fulfill these criteria because PTX acts against microtubules inducing cell cycle arrest in the G2/M phase and GEM acts against DNA and causes cell cycle arrest in the G1/S phase, and they have
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) CO₂ at 37°C. All the breast cancer cell lines were 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 (7x10³/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 isobolographic 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 combinations were close to IC₅₀ 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 Labtech 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: \( \text{A}_{\text{sample/A}_{\text{untreated}}} \times 100 \).

Synergy analysis: Berenbaum’s isobologram and Chou-Talalay (median-effect plot) method. This dose-oriented mathematical 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 IC₅₀ value (i.e., the drug concentration needed to cause 50% reduction in cell viability) was chosen for comparisons. An interaction index \( I_x \) was calculated using the following equation:

\[
I_x = \frac{E_{\text{GEM}}}{E_{\text{PTX}}} = \frac{d_1}{D_1} \frac{d_2}{D_2}
\]

where \( E_x = d_1 \frac{d_2}{D_1} \) is the dose needed to cause \( x\% \) of growth inhibition when the drug is combined, \( D_1 \) and \( D_2 \) are the dose needed to cause the same effect when the drug is administered alone. Isoboles were constructed by plotting \( E_{\text{PTX}} = d_1 / D_1 \) of PTX versus \( E_{\text{GEM}} = d_2 / D_2 \) of GEM. If data points fall to the left of the additivity line \( (I_x < 1) \), synergy is indicated; if the data fall within the additivity line \( (I_x = 1) \), drug interaction is said to be additive; if the data points fall to the right of the additivity line \( (I_x > 1) \) 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 \( a \) : \( f_a = \frac{D}{D_m} \). In this equation, \( D \) is dose, \( D_m \) is the dose required for 50% effect (e.g., 50% inhibition of cell growth, \( E_{D_2} \)), \( f_a \) is the fraction affected by dose \( D \) (e.g., 0.9 if cell growth is inhibited by 90%), \( f_u \) is the unaffected fraction (therefore, \( f_a = 1 - f_u \)), 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 \( b \) : \( D_a = \frac{D_{1a}}{D_{1b}}[1 - f_a]^{1/m} \). The parameters \( m \) and \( D_{1b} \) are easily determined by the median-effect plot \( x = \log (D) \) versus \( y = \log \left[ (1 - f_a) \right] \), which is based on the logarithmic form of equation \( a \) and yields a straight line where \( m \) is the slope and \( \log (D_{1a}) \) is the \( x \) intercept. IC₅₀ values (by interpolation) and \( D_{1b} \) values (by the median-effect plot) were usually similar. Equation \( b \) may thus be solved, providing the iso-effective dose \( (D_a) \) for any effect level (e.g. \( D_{1b} = 0.8; E_{D_0} = 0.9 \), and so forth). A combination index \( (CI) \) is then determined with the following equation:

\[
CI = \frac{E_1(D_1) + E_2(D_2) + a(D_1)(D_2)(D_1)(D_2)}{(D_1)(D_2)}
\]

where \( D_{1b} \) is the dose of agent 1 required to produce \( x \) percent effect alone, and \( D_{2b} \) is the dose of agent 2 required to produce the same \( x \) percent effect in combination with \( D_1 \). Similarly \( D_{2b} \) is the dose of agent 2 required to produce \( x \) percent effect alone, and \( (D_2) \) is the dose required to produce the same effect in combination with \( D_1 \). If the agents are

<table>
<thead>
<tr>
<th>Cell line</th>
<th>PTX (ng/ml)</th>
<th>GEM (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SKBR3</td>
<td>0.1-10</td>
<td>1-25</td>
</tr>
<tr>
<td>MCF-7</td>
<td>0.1-10</td>
<td>50-1000</td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td>0.1-25</td>
<td>50-1000</td>
</tr>
<tr>
<td>MDA-MB-468</td>
<td>0.1-10</td>
<td>1-1000</td>
</tr>
</tbody>
</table>

Table I. Dose range suitable for the drug-combination analyses in each cell line.
<table>
<thead>
<tr>
<th>Cell line</th>
<th>PTX (ng/ml)</th>
<th>GEM (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>B</td>
</tr>
<tr>
<td>SKBR3</td>
<td>1.4±0.6</td>
<td>1.9±1</td>
</tr>
<tr>
<td>MCF-7</td>
<td>2.8±1.1</td>
<td>2.7±0.3</td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td>3.6±0.6</td>
<td>9.7±5.7</td>
</tr>
<tr>
<td>MDA-MB-468</td>
<td>0.4±0.06</td>
<td>0.7±0.05</td>
</tr>
</tbody>
</table>

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 α is 0 (i.e., CI is the sum of 2 terms); if the agents are mutually non-exclusive (e.g., independent mode of action), α 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 f1 (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 (~6×10^6) 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, Orthovanadate-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 μg of protein of each sample) were electrophoretically resolved on 4-12% MOPS NuPage gels (Invitrogen), transferred to a 0.45-μm pore size nitrocellulose membrane (Invitrogen), and then probed with anti-PARP (Oncogene), anti-Bax (Neomarkers), anti-Bcl-2 (Neomarkers), and anti-ß-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 μg/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 ± 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_{50} values (i.e., the concentrations of the drugs needed to reduce cell viability by 50%) 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 tumorcidal 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_{50} values.

I. **Simultaneous schedule (PTX + GEM).** Both the interaction indexes (I_{50} =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_{50} <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).
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
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 IC₅₀ 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 IC₅₀ to 0.331 (strong synergism) at the IC₉₅.
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 preclinical 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 metastatic breast cancer treated with PTX and GEM. However, a clinically relevant synergism occurs when PTX is administered before GEM, 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.

Acknowledgments

JAM is the recipient of a Basic Clinical and Translational Research Award (BCTR0600004) from the Susan G. Komen Breast Cancer Foundation (Dallas, TX, USA). This study was also supported by the Instituto de Salud Carlos III (Ministerio de Sanidad y Consumo, Fondo de Investigación Sanitaria -FIS-, Spain, grants C06/0090 and PI06-0778 to JAM, and grant RD06-0020-0026 to JAM, JB, EdL, and RC).

References