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Antagonistic Effect of the Combination Gemcitabine/Topotecan in Ovarian Cancer Cells

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The in vitro interaction between the new antimetabolite gemcitabine (GEM) and topotecan (TPT) was analyzed in A2780 ovarian cancer cells. The growth inhibitory effect was assessed after 3 days of drug exposure. GEM and TPT obtained in vitro IC_{50} values of 2.1 ± 0.9 and 33.7 ± 10.2 nM, respectively. The interaction between GEM and TPT was evaluated by exposing cancer cells at increasing doses of GEM (0.1, 1, and 10 nM) and TPT (1, 10, 100, and 1000 nM). Analysis of data about the interaction between GEM and TPT was performed by applying the isobole method. An antagonistic effect was noticed when GEM was combined with TPT in the tested concentration range. DNA analysis was also performed and showed an augmentation of cells blocked in the G_2/M phase during TPT exposure, while an increase of blocked cells in the $G_{0/1}$ phase was observed after GEM treatment. This latter effect was predominant when the two drugs were used in combination. We also investigated the effect of sequential exposure to drugs, pretreating A2780 cells for 24 h with TPT and then for 48 h with GEM, and, conversely, pretreating A2780 cells with GEM for 24 h and thereafter with TPT for 48 h. Both these combined sequential treatments showed an antagonist effect of the drugs' combination. Long-term growth inhibition effect was established by clonogenic assay performed after 10 days of culture after drug treatment. Also these data confirmed the antagonistic effect between GEM and TPT in A2780 ovarian cancer cells.

Key words: Topotecan; Gemcitabine; Antagonism; Cell cycle

Despite advances in the treatment of ovarian cancer with chemotherapy and cytoreductive surgery, this tumor often develops drug resistance after first-line platinum-based therapy. The response rate of chemotherapeutics commonly used in refractory ovarian cancer (topotecan, gemcitabine, liposomal doxorubicin, oral VP16, and vinorelbine) ranged between 15% and 25% (1–7). An advantage in response rate may be hypothesized when combining drugs with different biological targets. To this aim, we focused our attention on antitumor activity of the new antimetabolite gemcitabine (GEM[2]) in combination with topotecan (TPT), new drugs introduced in treatment of advanced ovarian cancer.

GEM (2′,2′-difluorodeoxycytidine) is a new antimetabolite particularly active on solid tumors. It inhibits DNA synthesis through blocking DNA polymerization via GEM triphosphate incorporation instead of the natural nucleotide deoxycitidine. After the insertion of GEM triphosphate into DNA a further nucleotide is added before the arrest of DNA polymerization. This phenomenon determines "masking" of GEM with a longer action with respect to the other antimetabolite ara-C (8). Moreover, GEM indirectly blocks DNA synthesis by inhibiting the ribonucleotide-reductase enzyme, thus reducing the pool of natural nucleotides (8). GEM showed optimal tolerability and antitumor activity in clinical trials, demonstrating efficacy in advanced ovarian cancer (5,7,9) and in non-small-cell lung cancer (NSCLC) (10,11).

TPT is a semisynthetic analog of the alkaloid camptothecin, active in recurrent ovarian cancer previously treated with platinum-derived compounds or taxanes (2,4). It blocks both DNA and RNA synthesis, acting by trapping a covalent intermediate formed between DNA and DNA topoisomerase I. A stable, double-stranded break is formed, thereby arresting the DNA replication fork when approaching the site of breakage (12,13). The ultimate effect is a block in the cell cycle at the S-G_2 phase of cell cycle with the consequent triggering of programmed cell death (14).

In this study we tested in a human ovarian cancer cell line the combined antitumour effect of GEM and TPT, demonstrating an antagonistic effect in terms of growth inhibition, clonogenic ability, and DNA analysis.

MATERIALS AND METHODS

Chemicals

TPT (Hycamtin; SmithKline Beecham, King of Prussia, PA) and GEM (Gemzar; Eli Lilly and Co, Indianapolis, IN) were solubilized in sterile distillate water (stock solution 10 mM).

Cell Cultures

The ovarian cancer cell line A2780 purchased from the European Collection of Cell Cultures (Salisbury, Wiltshire SP4 OJG, UK) was used. Cells were grown in

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[2]Abbreviations used: GEM, gemcitabine; TPT, topotecan; NSCLC, non-small-cell lung carcinoma; PI, propidium iodide.

RPMI-1640 medium complemented with 10% fetal calf serum (FCS) and 200 U ml^{-1} penicillin (Sigma, St. Louis, MO). Cells, propagated as monolayer cultures in 75-cm^2 tissue culture flasks, were trypsinized twice weekly and plated at a density of 1×10^5 cells per milliliter. All cultures were incubated at 37°C under 5% carbon dioxide, 95% air in a high-humidity atmosphere.

Growth Experiments

Cells were plated in six-well flat-bottom plates (Falcon, Lincoln Park, NJ) at a density of 8×10^4 cells/ml in complete medium. After 24 h, the medium was replaced with fresh medium containing drug and incubated for additional 72 h. Control cells were treated with vehicle alone. Triplicate counts of triplicate cultures were performed after 3 days of exposure to the drugs. To calculate the growth inhibition effect, three independent experiments were performed for each drug/drug combination. The IC_{50} values were then calculated by fitting the concentration–effect curve data obtained in the three experiments with the sigmoid-E_{max} model using nonlinear regression, weighted by the reciprocal of the square of the predicted effect (15).

Evaluation of Drug Interaction

The effect of the combined treatment was analyzed by the isobole method (16) for a combination of drugs A and B, applying the equation: $A_c/A_e + B_c/B_e = D$, where A_c and B_c correspond to concentrations of drugs used in the combination treatment, and A_e and B_e correspond to the concentrations of drugs able to by themselves produce the same magnitude of effect. If D (combination index) < 1 the effect of combination is synergistic, whereas if D = 1 or D > 1 the effect is additive or antagonistic, respectively (16).

Flow Cytometric DNA Analysis

A2780 cells were plated in the specific medium supplemented as described above. After 24 h, the medium was replaced with fresh medium containing the compounds to be tested or vehicle alone. After 24 h of culture, cells were harvested and nuclei isolated and stained using a solution containing 0.1% (w/v) sodium citrate, 0.1% (v/v) NP40, 4 mM EDTA, and 50 μg/ml propidium iodide (PI) as a DNA dye (17). Cells were incubated with the staining solution for a minimum of 24 h at 4°C. Flow cytometric DNA analysis was performed by acquiring a minimum of 20,000 nuclei with an Epics-XL, flow cytometer (Coulter Immunology, Miami, FL). DNA fluorescence was collected in linear mode and pulse signal processing was used to set a doublet discrimination gate. Cell cycle analysis was performed using the Multicycle software package (Phoenix, San Diego, CA).

Clonogenic Assay

After the 3-day drug treatment, attached cells were counted and plated in six-well flat-bottom plates (Falcon, Lincoln Park, NJ) at a density of 5000 cells/ml in complete medium. After 10 days of culture, cells were washed with PBS and incubated for 1 h with staining solution containing 1% methylene blue and 70% ethanol. Staining solution was removed with several washings with distillate water and colonies were counted by visual inspection. Experiments were performed in triplicate.

Statistical Analysis

Statistical analysis was performed using MANOVA of the Statistica 5.0 software package (Statsoft, Tulsa, OK). Tukey test of the same program served to establish the significant interactions (at a value of $P < 0.05$).

RESULTS

The antiproliferative activity of GEM and TPT (range 0.01–100000 nM) was assessed after 72 h of single drug exposure in A2780 human ovarian cancer cell line (Fig. 1). The IC_{50} (the concentration giving the half-maximal growth inhibition) values were 2.1 ± 0.9 and 33.7 ± 10.2 nM for GEM and TPT, respectively. In order to evaluate the interaction between GEM and TPT, A2780 cells were cultured in the presence of increasing doses of

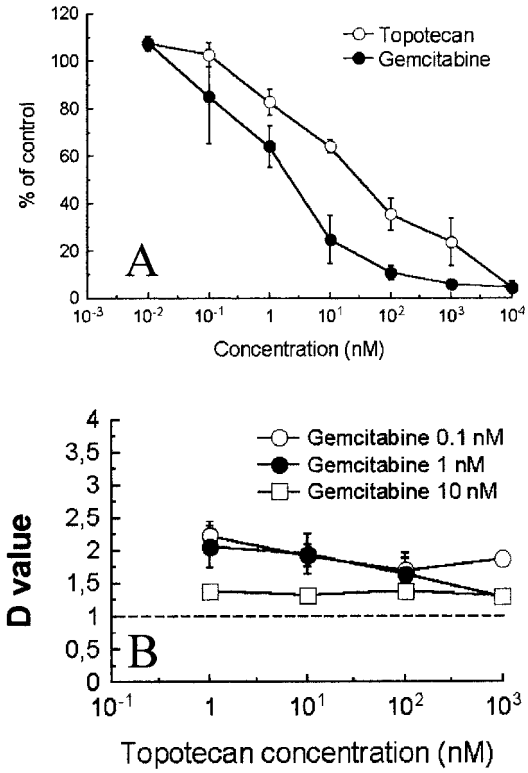

Figure 1. (A) Line chart reporting the growth inhibition effect of TPT and GEM on A2780 ovarian cancer cells after 72 h of treatment. Each point is the mean of three separate experiments performed in triplicate. Bars and circles represent SD and mean of the three experiments, respectively. (B) Line charts showing the antagonistic effect (D > 1) on A2780 ovarian cancer cells of the simultaneous treatment of TPT and GEM for 72 h. D values were obtained by the isobolic analysis of three independent experiments. Symbols and bars represent mean and SD, respectively.

GEM (0.1, 1, and 10 nM) in combination with increasing doses of TPT (1, 10, 100, and 1000 nM). The combination treatments did not potentiate the antiproliferative effect of each drug used alone; the isobole method demonstrated an antagonistic effect (D value > 1) (Fig. 1B).

On the basis of these data, we hypothesized that treatment schedule may interfere with the antiproliferative activity. To address this point, we pretreated A2780 cells for 24 h with TPT and then for 48 h with GEM; conversely, we pretreated A2780 cells with GEM for 24 h and thereafter with TPT for 48 h. Both these combined sequential treatments did not produce an augmentation in the antiproliferative effect, confirming the antagonist effect of the drug combination (Fig. 2A, B). In order to ascertain the presence of delayed antitumour effects in single drug exposure and their combinations, we treated A2780 cells for 72 h. After drug treatment, attached cells were plated and their clonogenic activity scored after an additional 10 days of culture. Results are expressed in Figure 3. TPT (at 10 nM) produced a statistically significant higher effect ($P < 0.05$) in clonogenic assays vs. the 3-day drug exposure. On the contrary, the opposite effect was determined by GEM (5 nM), with a statistically significant higher effect ($P < 0.05$) in 3-day drug exposure vs. clonogenic assays. At GEM 1 nM the same phenomenon was observed, but without attaining the threshold of statistical significance ($P = \text{NS}$). When combining the two drugs, a clear antagonistic effect was noticed, because the delayed antitumor effect of TPT (10 nM) either disappeared or subsided in combination with GEM 1 and 5 nM, respectively. Of note, the presence of antagonism is also evidenced by the fact that even at the highest tested dose, where we did not observe a clear antagonism, the statistically significant increase of the TPT activity in clonogenic assays vs. the 3-day drug exposure disappeared.

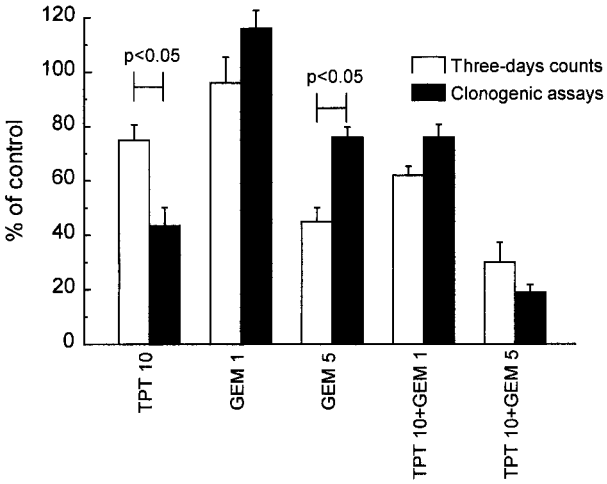

Figure 3. Bar charts showing the comparison among 3-day cell counts (open bars) and clonogenic assays (filled bars). After the 3-day drug treatment, viable A2780 cells were plated in the absence of drugs and their clonogenic activity scored after additional 10 days of culture. TPT (10 nM) showed a significantly higher activity in clonogenic assay than in 3-day culture, while GEM (1 and 5 nM) showed the opposite effect. The combination of the two drugs affects the delayed TPT activity in the clonogenic assays. Three independent experiments were performed. Bars and error bars are mean and SD, respectively.

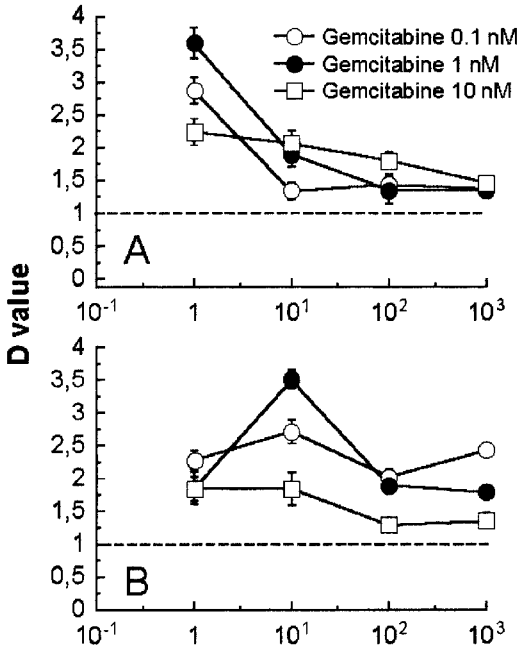

Figure 2. Line charts showing the antagonistic effect (D > 1) of the combination TPT/GEM on A2780 ovarian cancer cells. D values were obtained by the isobolic analysis of three independent exepriments. (A) Pretreatment with topotecan for 24 h, washing, and then treatment with gemcitabine for 48 h. (B) Pretreatment with gemcitabine for 24 h, washing, and treatment with topotecan for 48 h. Symbols and bars represent mean and SD, respectively.

Because the compounds tested have been reported to possess different cell cycle specificity, we assessed the cell cycle perturbations induced by the combination TPT/GEM. Figure 4 depicts DNA analysis of A2780 cells treated with TPT (1000 nM) and GEM (10 nM) after 24 h of exposure. An augmentation of cells blocked in the G_2/M phase of the cell cycle is evident with TPT, while an increase of cells blocked in the $G_{0/1}$ phase of the cell cycle is evident with GEM. This latter effect was predominant when we used the two drugs in combination after 24 h.

DISCUSSION

Recently, several authors focused their attention on the possible interactions among newly developed anticancer drugs such as topoisomerase I inhibitors (topotecan and irinotecan) and the antimetabolite gemcitabine (18–20). The clinical interest about possible positive interactions among these drugs was supported by their different mechanisms of resistance that cancer cells develop during drug exposure and by their nonoverlapping toxicities. However, conflicting results have been obtained in these previous studies. Indeed, all the three

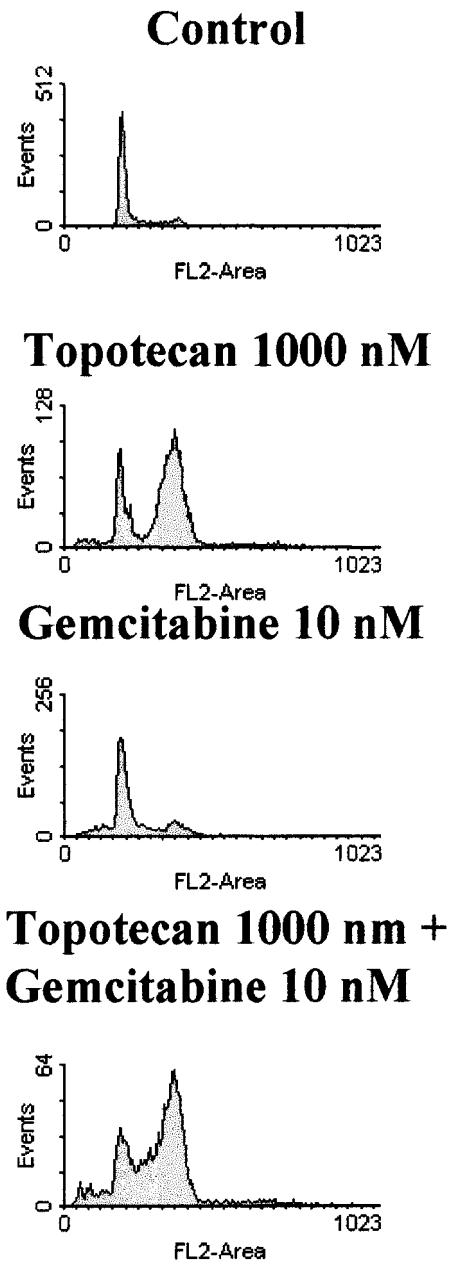

Figure 4. DNA histograms of A2780 cells after 24 h of drug exposure. Increase of cells blocked in G_2/M with TPT (1000 nM) and in $G_{0/1}$ with GEM (10 nM) when drugs are used alone. A prevalent block of the cell cycle at $G_{0/1}$ phase is evident when both drugs are used in combination.

possible effects have been reported: synergism (in breast and small-cell lung cancer cells) (18), additive effect (in non-small-cell lung cancer cells) (20), or antagonism (small-cell lung cancer cells) (19).

In this work, we demonstrated an antagonistic antitumor effect of the combination GEM/TPT in human ovarian cancer A2780 cells (expressing wild-type p53). This antagonistic effect is visible at three levels: 3-day cell counts, DNA analysis, and clonogenic assays. Remarkably, in clonogenic assays, TPT exhibited a long-lasting activity responsible for delayed additive effects, with a potentiation of the activity of near twofold in the 3-day counts vs. clonogenic assays. When combining the two drugs, this peculiar activity of TPT was lost, indicating a worrying decrease in the activity of the camptothecin analogue. In keeping with the view of Tolis et al. (20), the reason underlying such antagonism stems from DNA analysis. The $G_{0/1}$-specific cell cycle block induced by GEM prevailed over the S-G_2/M arrest induced by TPT. Thus, it appears likely that GEM could reduce the number of cells actively proliferating, susceptible to the S and G_2/M cytokinetic effect induced by TPT (14). Moreover, our preliminary results using an additional ovarian cancer cell line (OVCAR3 expressing mutant p53) confirm the antagonism among the two drugs. We also tried to assess if a sequential treatment of the two drugs could produce more effective results with respect to the simultaneous administration. Unfortunately, also when tested in a sequential treatment, the two drugs did not produce either an additive or synergistic effect. Although we cannot rule out that a longer interval between the two treatments could yield diverse results, it is likely that this schedule cannot be applied in clinical studies, because of the risk of treating patients too close to the nadir induced by the first drug in the sequential treatment. Thus, on the basis of these findings, we could suppose that the two drugs cannot be successfully administered in a strict temporary sequence. Results of a clinical trial that ended in our institution before the completion of our in vitro study confirmed this view, demonstrating that the concomitant treatment of TPT/GEM is not superior to using them as single agents in patients with ovarian refractory tumors (21). Moreover, also in terms of side effects, the combination of the two drugs did not reduce the hematological toxicity observed with the single drug treatment. In summary, the presence of antagonism, regardless of the administration sequence, and the diverse cell cycle blocking effects suggest that in designing new clinical studies the concomitant administration of TPT/GEM must be avoided.

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