In Vitro Schedule-dependent Interaction between Docetaxel and Gemcitabine in Human Gastric Cancer Cell Lines

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ABSTRACT

Purpose: The purpose of this study was to assess the activity of clinically used drugs and to define the most effective treatment scheme in human gastric cancer cell lines.

Experimental Design: Cytotoxic activity was evaluated by sulforhodamine B assay, potential clinical activity was estimated by relative antitumor activity, and the type of drug interaction was assessed using the method of Chou and Talalay. Cell cycle perturbations and apoptosis were evaluated by flow cytometry, mitotic index by microscopic analysis, bax, bcl-2, and p53 by immunohistochemistry, and cyclin B expression by Western blot.

Results: Gemcitabine (GEM) and docetaxel (DOC) were the most potent of the seven drugs tested, with maximum relative antitumor activity values in all of the cell lines. Simultaneous treatment with GEM and DOC, and the sequence GEM → DOC caused an antagonistic interaction, as shown by the combination index > 1, at all levels of killed cell fraction. Conversely, the sequential treatment DOC → GEM produced a synergistic interaction (combination index < 1).

On the basis of cell cycle perturbations, it can be hypothesized that the antimetabolite (GEM) attacks cells recovering rapidly from an M block induced by DOC as they progress to the S phase, producing a powerful cytoidal effect, as shown by the increase from 15 to 75% of apoptotic cells.

Conclusions: Our findings suggest that the interaction of DOC and GEM is highly schedule dependent and has been used recently to plan a Phase I-II clinical protocol.

INTRODUCTION

Adenocarcinoma of the stomach was the leading cause of cancer death worldwide throughout most of the twentieth century. Although the incidence of gastric cancer has gradually decreased in many parts of the world, the prognosis remains poor, with a 5-year survival from 5% to 15% in most of the Western world (1, 2).

Prognosis is mainly dependent on the stage of the disease at diagnosis and on the possibility of radical surgery. However, even after curative surgery, patients with locally advanced cancers without nodal metastases have ~50% probability of dying within 5 years, and patients with lymph node metastases have an even poorer prognosis (3).

Neoadjuvant chemotherapy is a promising approach in diseases such as gastric cancer, which present with systemic dissemination at the time of diagnosis and for which complete resection of the primary tumor is often difficult or impossible. Postoperative adjuvant therapy is the traditional approach for a disease with a probability of relapse in a high percentage of patients (3).

Tens of drugs belonging to different classes have proven active in patients with gastric cancer. Alkylating agents, antimetabolites, and anthracyclines used in polychemotherapy have induced tumor regression in some patients. Clinical results have also been reported for DNA topoisomerase inhibitors or platinum-based agents and, more recently, for taxanes, Vinca alkaloids, and epipodophyllotoxins, such as etoposide. However, about half of all patients are resistant to chemotherapy, and less than half with locally advanced disease are amenable to surgical resection after neoadjuvant chemotherapy (3). For these reasons, new and hopefully more effective drugs or innovative treatment strategies are needed.

A great deal of interest has been focused on the search for new drugs with molecular targets other than DNA. Taxanes, mitotic spindle poisons that stabilize microtubules and inhibit their depolymerization to free tubulin, have been investigated widely in preclinical and clinical studies on different tumor types (4–7). In particular, DOC is active as a single agent in gastric cancer (8, 9).

Gemcitabine (GEM), a new pyrimidine antimetabolite, has demonstrated an interesting cytotoxic activity against several solid tumors (10, 11), including gastric cancer.

In the present study we tested the cytotoxic effect of DOC and GEM used in different combinations on three human gastric cancer cell lines. Moreover, we investigated the mechanisms underlying the different types of drug interactions after various

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3 The abbreviations used are: DOC, docetaxel; GEM, gemcitabine; PACL, paclitaxel; MIT, mitomycin C; OXA, oxaliplatin; EPI, epipodophyllotoxin; 5-FU, 5-fluouracil; RAA, relative antitumor activity; CI, combination index.
association and sequence schemes by analyzing cell cycle perturbations and the apoptosis process.

MATERIALS AND METHODS

Cell Lines. The study was performed on three human gastric adenocarcinoma cell lines established in our laboratory, AKG, GK2 (12), and KKP (13), and characterized by 36, 30, and 32 h doubling times, respectively. Cells were maintained as a monolayer at 37°C in a humidified atmosphere with 5% CO₂ and subcultured weekly. Culture medium was composed of DMEM supplemented with 10% FCS, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 2 mM l-glutamine.

All of the experiments were performed during exponential cell growth.

Drugs. DOC (Rhône-Poulenc Rorer, Milan, Italy), GEM (Lilly, Sesto Fiorentino, Florence, Italy), PACL (Bristol Meyers Squibb, Rome, Italy), MIT (Kyowa, Milan, Italy), OXA (Sanofi-Synthelabo, Paris, France), EPI (Pharmacia & Upjohn, Milan, Italy), and 5-FU (Roche, Milan, Italy) were diluted at concentrations of 10, 40, 6.1, 3.33, 2, and 50 μg/ml in saline solution (0.9%), respectively, then divided into aliquots and stored at −70°C. Drug stocks were freshly diluted in culture medium just before each experiment.

In Vitro Chemosensitivity Assay. The sulforhodamine B assay according to the method of Skehan et al. (14) was used. Cells in the exponential growth phase were trypsinized, counted, and seeded in 96-well flat-bottomed microtiter plates at a density of 10,000 cells/well. Experiments were run in octuplet, and each experiment was repeated three times. Eighteen to 24 h after plating (a sufficient time for exponential growth recovery), 100 μl of culture medium containing or not containing the drugs was added to each well. At the end of drug exposure, cells were fixed with 50% trichloroacetic acid at 4°C (50 μl/well, final concentration 10%) for 1 h. After five washes with tap water, cells were stained with 0.4% sulforhodamine B (Sigma-Aldrich, St. Louis, MO), dissolved in 1% acetic acid (100 μl/well) for 30 min, and subsequently washed four times with 1% acetic acid to remove unbound stain. Plates were air-dried, and protein-bound stain was solubilized with 100 μl of 10% unbuffered Tris base. The cell density was determined at a wavelength of 540 or 510 nm at a fluorescence plate reader.

DOC, GEM, PACL, MIT, OXA, and EPI were tested singly at scalar concentrations of 0.001, 0.01, 0.1, and 1 μg/ml, and 5-FU at concentrations of 0.01, 0.1, 1, and 10 μg/ml. A 24-h exposure to single agents was chosen from the dose inhibition rate curves as the time that produced the maximum effect. In sequence or association experiments, the exposure time to each drug was 24 h, and drugs were combined at a constant ratio of 1:1 μg/ml on the basis of their activity.

Dose response curves were created by Excel software, and IC₅₀ values were determined graphically from the plots. Drug activity was defined as RAA, that is, the ratio between peak plasma level and in vitro IC₅₀ value (15). We used the clinically achievable peak plasma concentrations reported in the literature of 3.67 μg/ml for DOC (16), 3.2 μg/ml for GEM (17), 0.5 μg/ml for PACL (18), 1.7 μg/ml for MIT (19), 3 μg/ml for EPI (20), 25 μg/ml for 5-FU (21), and 3.25 μg/ml for OXA (22).

The type of interaction between drug activities was determined by the median effect principle according to the method of Chou and Talalay (23). On the basis of this approach, the interaction between the two drugs was quantified by determining a CI at increasing levels of cell kill.

CI values lower, equal, or higher than 1 indicated synergy, additivity, or antagonism, respectively. Samples were run in octuplet, and each experiment was repeated three times. Therefore, each experimental value in the graphs represents the median of 24 samples.

Cell Cycle Perturbations. Two × 10⁵ cells were seeded in 60-mm Petri dishes. After an 18–24 h incubation at 37°C medium was replaced with fresh medium containing or not containing (control) the drugs at a concentration of 1 μg/ml for 24 h. After drug exposure, cells were washed out for 24 h, then trypsinized, washed twice with PBS, and stained in a solution containing RNAase (10 Ku/ml; Sigma-Aldrich), NP40 (0.01%; Sigma-Aldrich) and propidium iodide (1 μg/ml; Sigma-Aldrich). The samples were stored for 30–60 min, then filtered through a disposable 40-μm filter assembly (RATCOM, Miami, FL) and immediately analyzed at a fluorescence-activated cell sorter Vantage flow cytometer (Becton Dickinson, San Jose, CA). For each sample, 10,000 events were collected for subsequent analysis. Data acquisition was performed using CELLQuest software (Becton Dickinson). Data were elaborated using Modfit (DNA Modeling System) software (Verity Software House Inc., Topsham, ME) and expressed as fractions of cells in the different cycle phases. Samples were run in triplicate, and each experiment was repeated three times. The values of treated samples are expressed as a percentage of those of controls.

Apoptosis. At the end of single or sequential drug treatments and after a 24-h washout, cells were trypsinized, washed twice with PBS, and fixed in 1% paraformaldehyde in PBS on ice for 15 min. After two washes in PBS cells were resuspended in ice-cold ethanol 70%, stored overnight at −20°C, then washed in PBS and incubated in 50 μl of solution containing terminal deoxynucleotidyltransferase and FITC-conjugated dUTP deoxynucleotides 1:1 in reaction buffer (Boehringer Mannheim, Mannheim, Germany) for 60 min at 37°C in the dark. After washing in PBS containing 0.1% Triton X-100, the cells were stained with 5 μg of propidium iodide (Sigma-Aldrich) and 10 Ku RNAase (Sigma-Aldrich) in 10 ml of PBS for 1 h at 4°C in the dark.

Flow cytometric analysis was performed on a fluorescence-activated cell sorter Vantage flow cytometer (Becton Dickinson). Data acquisition and analysis were performed using CELLQuest software (Becton Dickinson). For each sample, 10,000 events were recorded.

Oncogene Expression. Cells were trypsinized, washed with PBS, and fixed in 10% formalin. Three-μm sections from paraffin-embedded blocks were deparaffinized in xylene, rehydrated, and treated with 3% hydrogen peroxide solution. The sections were then processed for nonspecific binding with 3% BSA in PBS for 20 min and incubated for 1 h at room temperature with anti-p53 (clone Pab 1801, dilution 1:200 in PBS; NeoMarkers, Union City, CA), anti-bcl-2 (clone 124, dilution 1:50 in PBS; Dako, Santa Barbara, CA), or anti-bax (polyclonal rabbit antihuman bax, dilution 1:1000 in PBS; PharMingen, San Diego, CA). After incubation the specimens were washed twice...
with PBS, incubated with biotinylated antimonosecondary antibody rinsed in PBS, and incubated with avidin-biotin conjugate (LSAB+ kit; Dako). Sections were then rinsed in PBS, and antibody binding was detected by staining with diaminobenzidine (DAB+ Substrate chromogen system; Dako). The cell nuclei were counterstained blue by Mayer’s Hemalum, and sections were mounted in Faramount (Dako).

Positive cells were quantified by evaluating at least $3 \times 10^5$ cells and were expressed as the percentage ratio over the total number of scored cells. All of the samples were evaluated blind by two separate observers at light microscope. Negative controls were obtained by omission of the primary antibody.

Mitotic Index. Control and treated cells were trypsinized and washed twice with PBS. Approximately $0.5\cdot 10^5 \times 10^5$ cells were plated onto microscope slides using Cytospin cell preparation system (Shandon, Pittsburgh, PA). Fixed samples were stained with H&E. Mitotic index was defined as the percentage of mitosis in total cells by scoring 1500 cells on each slide for a total of 4500 cells.

Cyclin B1. Cell proteins were isolated by lysing the cells in 10 mm Tris-HCl (pH 7.1), 50 mm NaCl, 59 mm EDTA, 1% and Triton X-100, and adding 1 mm phenylmethylsulfonyl fluoride and a protease inhibitor mixture (Sigma-Aldrich). They were determined by the micro-bacteriochlorine a (BCA) protein assay reagent kit (Pierce, Rockford, IL), and 60 mg were denatured and separated on 12% SDS-polyacrylamide gel, then electroblotted to Hybond-C extra membrane (Amersham Pharmacia Biotech, Piscataway, NJ). The membrane was stained with Ponceau S (Sigma-Aldrich), then incubated overnight at 4°C in T-PBS containing 5% nonfat dry milk. The membrane was probed for 2 h at room temperature with cyclin B1 antibody (monoclonal V152, Ab-1; Bio Optica, Milan, Italy), diluted 1:200 in T-PBS 5% nonfat dry milk, then incubated in horseradish peroxidase-conjugated second antibody (Dako, Santa Barbara, CA) 1:1000 in the same buffer for 1 h at room temperature. The bound antibodies were detected by enhanced chemiluminescence using enhanced chemiluminescence kit (Amersham Pharmacia Biotech), and the samples were analyzed using QuantiScan software (Biosoft, Cambridge, United Kingdom).

RESULTS

Drug Activity. The cytotoxic activity of individual drugs on AKG, KKP, and GK2 cells is shown in Table 1. GEM and DOC were the most potent of the seven drugs tested and induced the maximum RAA in all of the cell lines, indicating a potentially high clinical activity. Good antitumor activity was also observed in all of the cell lines for EPI, OXA, MIT, and 5-FU, and a much lower, not statistically significant activity, for PACL. A modulation in drug sensitivity was observed for the three cell lines, with the highest sensitivity for GK2, the lowest for AKG, and an intermediate one for KKP line. Therefore, this panel of cell lines adequately reproduces the variability in drug sensitivity of clinical tumors. Moreover, it must be highlighted that all of the IC_{50} values were under the peak plasma levels.

The two most effective drugs, DOC and GEM, were tested in different combinations to define the most effective schedule. Dose-effect curves were observed for DOC and GEM singly or in different combination schemes in all of the cell lines (Fig. 1). Three different treatment schedules were tested, simultaneous or sequenced drug exposure, and exposure time to each drug was 24 h. Simultaneous treatment with the two drugs for 24 h, as well as the treatment sequence GEM→DOC, caused antagonistic effects in all of the cell lines, as shown by the CI > 1 at all levels of killed cell fraction (Fig. 2). Conversely, the inverse sequence DOC→GEM produced a marked synergistic interaction (CI < 1), which was more pronounced in the GK2 line.

Cell Cycle Perturbations and Apoptosis. Cell cycle perturbations and the induction of apoptotic cells were investigated after the most active sequence in all of the cell lines. Moreover, because G2-M phase is the most critical target for taxanes, flow cytometric analysis was paralleled by microscopic analysis to distinctly quantify the cells that belong to the G2 or M phase, and which, together, contribute to flow cytometric peak.

GEM did not produce any significant variation in cell cycle distribution pattern after a 24-h treatment (Table 2). Conversely, a 24-h exposure to DOC caused a significant decrease of cells in G1 and S phases, and a marked increase of mitosis in all of the cell lines. Twenty-four h after drug removal, taxane-arrested mitotic cells moved, and the partially synchronized wave repopulated the various cycle phases. Conversely, when the 24-h DOC treatment was followed by a 24-h exposure to GEM, a maximum accumulation was observed in S phase.

In parallel, cyclin B1 protein expression was not influenced by a 24-h treatment with GEM. Conversely, it increased 3-fold after a 24-h exposure to DOC and returned to control levels after a 24-h washout or a 24-h exposure to GEM in GK2 line (Fig. 3). These variations were similar in all of the cell lines, although basal cyclin B1 expression was lower in AKG and KKP than in the GK2 cell line.

Apoptotic cells increased moderately after a 24-h treatment

**Table 1** RAA and IC_{50} of drugs after 24-h exposure

<table>
<thead>
<tr>
<th>Concentrations tested (µg/ml)</th>
<th>GK2</th>
<th>KKP</th>
<th>AKG</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RAA IC_{50} µg/ml</td>
<td>RAA IC_{50} µg/ml</td>
<td>RAA IC_{50} µg/ml</td>
</tr>
<tr>
<td>Gemcitabine 0.001-0.01-0.1-1</td>
<td>40.00 0.08</td>
<td>10.30 0.31</td>
<td>4.32 0.74</td>
</tr>
<tr>
<td>Docetaxel 0.001-0.01-0.1-1</td>
<td>36.70 0.10</td>
<td>8.15 0.45</td>
<td>4.82 0.76</td>
</tr>
<tr>
<td>Epidoxorubicin 0.001-0.01-0.1-1</td>
<td>8.57 0.35</td>
<td>4.68 0.64</td>
<td>3.33 0.90</td>
</tr>
<tr>
<td>Oxaliplatin 0.001-0.01-0.1-1</td>
<td>6.50 0.50</td>
<td>4.92 0.66</td>
<td>3.25 1.00</td>
</tr>
<tr>
<td>Mitomycin C 0.001-0.01-0.1-1</td>
<td>4.14 0.41</td>
<td>3.09 0.55</td>
<td>1.77 0.96</td>
</tr>
<tr>
<td>Paclitaxel 0.001-0.01-0.1-1</td>
<td>1.92 0.26</td>
<td>0.83 0.60</td>
<td>0.56 0.88</td>
</tr>
<tr>
<td>5-FU 0.01-0.1-1-10</td>
<td>2.94 8.50</td>
<td>2.77 9.00</td>
<td>2.31 10.8</td>
</tr>
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Research.
with GEM, increased markedly after a 24-h exposure to DOC, and persisted after a 24-h drug removal, but more importantly, dramatically increased after the treatment sequence DOC→GEM (Table 3).

The analysis of apoptosis-related markers showed a dramatic decrease in bax expression and unchanged values of bcl-2 expression after a 24-h exposure to GEM. Conversely, a 24-h exposure to DOC again produced a strong decrease in bax but also a significant increase in bcl-2 expression. These changes, especially those related to bax, were still present after a 24-h washout. In contrast, when the 24-h exposure to DOC was followed by a 24-h exposure to GEM, we observed an apoptosis-related marker profile similar to that of untreated cells in the fraction of surviving cells. p53 expression was not influenced by any treatment (data not shown).

**DISCUSSION**

Chemotherapy plays a central role in every phase of cancer treatment. To date, clinical protocols for cancer chemotherapy...
Table 3  bax, bcl-2 positive cells, and apoptosis (%) after DOC and GEM treatments

<table>
<thead>
<tr>
<th></th>
<th>GK2</th>
<th>KKP</th>
<th>AKG</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Bax</td>
<td>Bcl-2</td>
<td>Apoptosis</td>
</tr>
<tr>
<td>Untreated cells</td>
<td>95</td>
<td>15</td>
<td>1</td>
</tr>
<tr>
<td>GEM (24 h)</td>
<td>21*</td>
<td>21</td>
<td>7*</td>
</tr>
<tr>
<td>DOC (24 h)</td>
<td>18*</td>
<td>60*</td>
<td>15*</td>
</tr>
<tr>
<td>DOC (24 h) + 24 h washout</td>
<td>14*</td>
<td>35*</td>
<td>17*</td>
</tr>
<tr>
<td>DOC (24 h) → GEM (24 h)</td>
<td>96</td>
<td>10</td>
<td>75*</td>
</tr>
</tbody>
</table>

* P < 0.05 by t test.

often combine two or more drugs based on their different mechanisms of action. Moreover, it has been documented recently that different drug sequences or combinations induce different interactions, ranging from antagonistic to synergistic, even when using the same two-drug combination (24, 25). This makes it of the utmost importance to define the best timing and sequence at preclinical level.

In our study, GEM and DOC proved to be the most potent drugs among the panel tested, and showed a potentially high clinical activity, as indicated by the RAA values.

The additional contribution from our study is that exposure to DOC followed by GEM produced a synergistic interaction, whereas an antagonistic interaction was caused by the inverse sequence or simultaneous exposure to the two drugs. These findings indicate that simultaneous exposure or the sequence GEM → DOC is not efficacious or advisable and that the sequential administration of DOC → GEM should be used.

To explain the possible mechanism underlying the synergistic interaction of this sequence, we analyzed the perturbations induced on cell cycle, apoptosis, and its related markers by flow cytometric, morphological, and immunohistochemical techniques.

Flow cytometric analysis showed that a 24-h treatment with DOC markedly affected cell cycle distribution, producing a relevant accumulation in G2-M phases, as already reported by others (26, 27). In the present study, the morphological observation allowed us to attribute the block maximally to mitotic phase with the typical taxane-induced metaphase.

The block was reversed after drug removal, and a partially synchronized cell wave progressed from mitosis to repopulate the different cell cycle compartments.

Moreover, in agreement with the results published recently by Motwani et al. (28) for PACL on MKN-74 gastric and MCF-7 breast cancer cell lines, we observed an increase in cyclin B1 expression in gastric cell lines after DOC exposure, paralleled by a strong accumulation of cells in mitosis. After a 24-h washout, cyclin B1 expression had totally recovered, mitotic block had decreased to values observed in untreated cells, and the fraction of apoptotic cells was not relevant. Conversely, exposure to GEM immediately after treatment with DOC led to apoptosis in the majority of cells, whereas the surviving cell fraction exhibited a profile of apoptotic-related markers similar to that observed in untreated cells. These results all suggest that GEM kills the majority of cells recovering from the mitotic block produced by DOC as they progress to S phase, accounting for a synergistic interaction. At the same time, the increased bax expression, similar to the level observed in untreated cells, may make the residual surviving fraction sensitive to successive treatments.

In conclusion, the interaction between the taxane and the antimitabolite is highly schedule-dependent and, in the absence of combination clinical protocols and more importantly, of clinical results for these two drugs in gastric cancer, the most effective sequence DOC → GEM is currently under investigation in a Phase I-II clinical study.

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