

Schedule-Dependent Cytotoxic Interaction between Epidoxorubicin and Gemcitabine in Human Bladder Cancer Cells *in Vitro*

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ABSTRACT

Purpose: The aim of the study was to evaluate the activity of epidoxorubicin (EPI) and gemcitabine (GEM) and to define the most effective schedule in human bladder cancer cells.

Experimental Design: The study was performed on HT1376 and MCR cell lines. Cells were exposed for 1 and 24 h to drugs used in different schemes. Cytotoxic activity was evaluated by the 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 assessed by flow cytometry; BAX, BCL-2, and P53 expression was evaluated by Western blot; and DNA damage was assessed using the alkaline Comet assay.

Results: EPI and GEM produced a cytotoxic effect in both cell lines, with 50% inhibitory concentration and relative antitumor activity values suggestive of a high clinical activity. Simultaneous treatment with EPI and GEM and the sequence GEM→EPI caused an antagonistic interaction (combination index > 1) after both 1- and 24-h treatments. Conversely, the inverse sequence, EPI→GEM, produced a synergistic interaction that was more pronounced in MCR cells than in HT1376 cells. The increase in DNA-damaged cells from 10% to 20% after single-drug exposure to 40–60% at the end of EPI→GEM treatment may explain the synergistic interaction produced by the anthracycline-anti-metabolite sequence.

Conclusions: Our findings show that the efficacy of the EPI and GEM combination is highly schedule dependent

and indicate that the most active scheme is EPI followed by GEM, which is currently being validated in an ongoing intravesical Phase I-II clinical protocol.

INTRODUCTION

Bladder cancer is an important cause of morbidity and mortality, and, in contrast to the United States, its incidence and mortality rates are still increasing in European countries, even though the majority of new cases are diagnosed as early and superficial tumors (1, 2).

Transitional cell carcinomas of the bladder can be classified into two groups with distinct behavior and different molecular profiles: (a) low-grade tumors, which include papillary and usually superficial lesions; and (b) high-grade tumors, which comprise papillary and nonpapillary, often invasive lesions.

Superficial cancers (T_a-T₁ stages) account for 70–80% of newly diagnosed bladder cancers. First-line treatment is transurethral resection, fulguration, or laser coagulation, but approximately two-thirds of patients develop recurrences, with a progression of grade and stage despite the macroscopically complete eradication of the primary lesion (3–5).

The high rate of recurrence and stage progression represents an important challenge for the correct management of these patients. Intravesical therapy is usually given, but, up to now, no treatment has resulted in a significant improvement in overall survival.

Intravesical immunotherapy with *Bacillus Calmette-Guerin* (BCG) is currently the most effective approach for the therapy and prophylaxis of superficial bladder cancer (3, 5–7), but it is associated with serious morbidity (5, 8, 9). Moreover, only two-thirds of patients respond to BCG treatment, and, among these, one-third are destined to relapse (1, 4). An improvement in overall survival has also never been demonstrated (5).

Up to now, antitumor agents such as thiotepa, doxorubicin, epidoxorubicin (EPI), and mitomycin C used intravesically in clinical trials on large case series have not proven capable of significantly reducing disease progression or improving overall survival (6, 10, 11). Valrubicin, which was used recently as a second-line intravesical chemotherapy in BCG-refractory patients, produced complete responses in only 20% of cases (12, 13). Therefore, cystectomy represents the treatment of choice for these patients.

A great deal of interest has been focused on research into new drugs or new drug combinations for intravesical chemotherapy. Gemcitabine (GEM) has proven to be effective when systemically administered to advanced bladder cancer patients (14–16) and is also active, with minimal bladder irritation, in instillation treatment (4, 17). EPI has been shown to be active as a single agent in intravesical therapy (18–20).

In the present study, we investigated the cytotoxic activity of GEM and EPI and their interaction in two human bladder cancer cell lines by reproducing local infusion or systemic

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treatment used in clinical practice. We also investigated the mechanisms underlying the different types of interactions, paying particular attention to DNA damage stabilization, apoptotic process, and cell cycle perturbations.

MATERIALS AND METHODS

Cell Lines. The study was performed on two human bladder cancer cell lines: (a) one commercial cell line (HT1376; 37-h doubling time); and (b) one established in our laboratory (MCR; 48-h doubling time). The MCR cell line was established from a metastatic s.c. lesion of a 51-year-old male who had been diagnosed with grade III transitional cell carcinoma of the bladder. Cells presented an abnormal DNA content with a DNA index of 1.35 and an abnormal karyotype with a modal chromosome number of 53. HT1376 cells are known to harbor *p53* gene mutations (21), and both cell lines express BAX and BCL-2 at different levels.

Cells were maintained as a monolayer in culture medium (DMEM) supplemented with 10% FCS, 100 IU/ml penicillin, 100 µg/ml streptomycin, and 2 mM L-glutamine and subcultured weekly. All of the experiments were performed during exponential cell growth.

Drugs. EPI (Pharmacia & Upjohn, Milan, Italy) and GEM (Lilly, Sesto Fiorentino, Florence, Italy) were diluted in saline solution (0.9%), stocked at -70°C , and freshly diluted in culture medium before each experiment.

In Vitro Growth Inhibition Assay. The sulforhodamine B assay was used according to the method of Skehan *et al.* (22). Cells were seeded in 96-well flat-bottomed microtiter plates at a density of 10,000 cells/well. Eighteen to 24 h after plating, 100 µl of culture medium alone or culture medium containing the drugs were added to each well. At the end of drug exposure, cells were fixed with 50% trichloroacetic acid and 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. Protein-bound stain was solubilized with 100 µl of 10 mM unbuffered Tris base, and cell density was determined using a fluorescence plate reader (wavelength, 540 or 510 nm). 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.

Single-Drug Exposure. Cells were treated for 1 and 24 h to reproduce clinical situations of endovesical and systemic treatments. Drugs were tested at 1, 2, and 3 µg/ml in the short-exposure scheme and at 0.01, 0.1, 1, and 10 µg/ml in the long-exposure scheme, taking into account that peak plasma levels are 3 µg/ml for EPI (23) and 3.2 µg/ml for GEM (24). Control samples were processed in the same way as treated samples but in drug-free medium, and the evaluation of the cytotoxic effect was performed immediately after the end of drug exposure. Relative growth was calculated as described previously by Monks *et al.* (25): $[(\text{OD}_{\text{TREATED}} - \text{OD}_{\text{ZERO}}) / (\text{OD}_{\text{CONTROL}} - \text{OD}_{\text{ZERO}})] \times 100\%$, when $\text{OD}_{\text{TREATED}}$ is $\geq \text{OD}_{\text{ZERO}}$. If $\text{OD}_{\text{TREATED}}$ is $< \text{OD}_{\text{ZERO}}$, cell killing has occurred. OD_{ZERO} depicts the cell number at the moment of drug addition, $\text{OD}_{\text{CONTROL}}$ reflects the cell number in untreated wells, and $\text{OD}_{\text{TREATED}}$ reflects the cell number in treated wells on the day

of the drug treatments. Drug activity was defined as relative antitumor activity, which is the ratio between peak plasma levels and IC_{50} *in vitro*. Dose-response curves were created by Excel software, and the IC_{50} values were determined graphically from plots.

Drug Combinations. The drugs were combined at a constant ratio of 1:1 at 1, 2, and 3 µg/ml (1 h) or 0.01, 0.1, 1, and 10 µg/ml (24 h) in both sequence and association schemes. In experiments with simultaneous drug treatments, cells were exposed to the two drugs for 1 or 24 h. In drug sequence experiments, cells were exposed for 1 or 24 h to the first drug, which was then removed before exposure to the second drug for 1 or 24 h. Control samples were processed in the same way, but in drug-free medium. Evaluation of the cytotoxic effect was performed immediately after the end of drug exposure.

Data Analysis. The type of interaction between the two drugs was determined by the median effect principle according to the method of Chou and Talalay (26). The interaction between the two drugs was quantified by determining a combination index at increasing levels of cell growth inhibition. Combination index values less than, equal to, or greater than 1 indicated a synergistic, additive, or antagonistic interaction, respectively.

Cell Cycle Perturbations and Apoptosis. Cells (2×10^5) were cultured in medium alone (control) or in medium containing the cytotoxic drugs in combination or sequence at a concentration of 0.1 µg/ml. At the end of drug exposure (24 h for single or simultaneous drug treatments and 48 h for sequence treatments), cells were harvested and stained in a solution containing RNase (10 Kunits/ml; Sigma-Aldrich), NP40 (0.01%; Sigma-Aldrich), and propidium iodide (1 µg/ml; Sigma-Aldrich). After 30–60 min, samples were analyzed by flow cytometry (Becton Dickinson, San Jose, CA). Data acquisition (10,000 events for each sample) 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 cell cycle phases. Samples were run in triplicate, and each experiment was repeated three times. Therefore, each experimental value represents the median of nine samples. The values of treated samples are expressed as a percentage of those of controls.

Apoptosis was evaluated in cells fixed in 1% paraformaldehyde in PBS on ice for 15 min, suspended in ice-cold ethanol (70%), and stored overnight at -20°C . Cells were then incubated for 60 min at 37°C in the dark in 50 µl of a solution containing terminal deoxynucleotidyltransferase and FITC-conjugated dUTP deoxynucleotides (1:1) in reaction buffer (Boehringer Mannheim, Mannheim, Germany). After washing in PBS containing 0.1% Triton X-100, the cells were stained with 5 µg of propidium iodide (Sigma-Aldrich) and 10 Kunits of RNase (Sigma-Aldrich) in 10 ml of PBS for 1 h at 4°C in the dark and then analyzed by flow cytometry. Data acquisition and analysis were performed using CELLQuest software (Becton Dickinson). For each sample, 10,000 events were recorded.

Comet Assay. The assay was performed according to the Trevigen protocol (27). At the end of 1- or 24-h single-drug or sequential drug treatments, 10^4 cells were suspended in 0.5% (w/v) solution of low melt agarose in PBS (pH 7.4) at 37°C and trans-

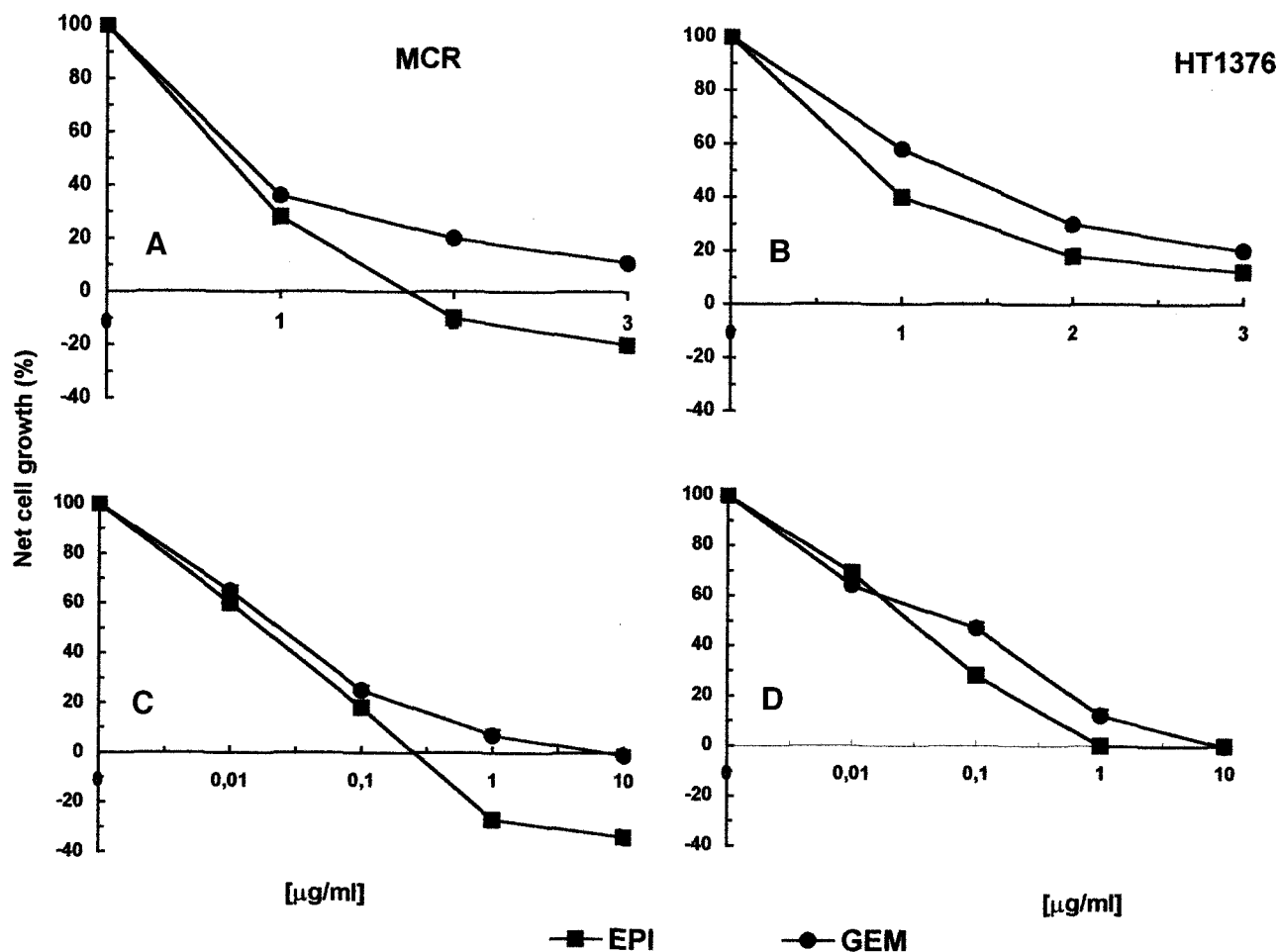


Fig. 1 Antiproliferative and cytotoxic activity induced by a 1-h treatment (A and B) or a 24-h treatment (C and D) with epidoxorubicin or gemcitabine in bladder cancer cell lines. Each data point represents the percentage of proliferating cells in relation to untreated control and is the average of at least three independent experiments performed in octuplet. Bars, SD never exceeded 5%.

ferred immediately onto a frosted microscope slide (Trevigen, Gaithersburg, MD). The samples were incubated for 1 h at 4°C in a solution of 2.5 M NaCl, 100 mM EDTA (pH 10), 10 mM Tris base, 1% sodium lauryl sarcosinate, and 0.01% Triton X-100 to remove cellular proteins and isolate DNA nucleoids. Samples were then washed three times in formamidopyridine glycosylase buffer [100 mM Tris (pH 7.5), 10 mM EDTA (pH 8.0), and 500 mM NaCl] and tapped dry. The agarose-embedded cells were covered with formamidopyridine DNA glycosylase (0.1 unit/gel; Trevigen), incubated for 1 h at 37°C in a moist atmosphere, and immersed in prechilled denaturing buffer [0.3 M NaOH and 0.001 M EDTA (pH 12.1)] for 30 min before electrophoresis at 25 V for 30 min. After washing in 0.4 M Tris-HCl (pH 7.5), buffer cells were stained with SYBR Green dye (1:10 dilution). The comets were scored in 100 cells/sample using a fluorescence microscope. Each experiment was repeated twice, and the results are expressed as the total number of comets detected.

Oncogene Mutation and Expression. DNA was extracted from the MCR cell line by using the QIAamp DNA Mini Kit (Qiagen, Qiagen-Gruppe, Hilden, Germany). Exons 2–11 of *p53* gene were amplified by PCR, and DNA sequencing was

performed using Big Dye Terminator v3.0 Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA). The sequence products were analyzed by 3100 Avant Sequencer (Applied Biosystems).

Cell proteins from HT1376 and MCR were isolated by lysing the cells in 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% Triton X-100, and 0.1% SDS supplemented with 1 mM phenylmethylsulfonyl fluoride and a protease inhibitor mixture (Sigma-Aldrich). The protein concentration was determined by the micro bacteriochlorine *a* protein assay reagent kit (Pierce, Rockford, IL). Identical amounts of total proteins were denatured and separated on 12% SDS-polyacrylamide gel and then electroblotted onto Hybond-C extra membrane (Amersham Pharmacia Biotech, Piscataway, NJ). The membrane was stained with Ponceau S (Sigma-Aldrich), incubated overnight at 4°C in Tween 20-PBS containing 5% nonfat dry milk, and probed with monoclonal anti-p53 antibody (1:400; PAB 1801; Bioptica, Milan, Italy), anti-bcl-2 antibody (1:50; clone 124; Dako, Santa Barbara, CA), and polyclonal anti-bax antibody (1:1000; PharMingen, San Diego, CA). Actin polyclonal antibody (Sigma-Aldrich) was used as a control to demonstrate equal loading.

Table 1 Cytotoxic effect^a of drugs on bladder cancer cell lines

Drugs	MCR		HT1376	
	Mean IC ₅₀ (μg/ml)	RAA ^b	Mean IC ₅₀ (μg/ml)	RAA
EPI	0.03 ± 0.007	100	0.06 ± 0.005	50
GEM	0.05 ± 0.008	64	0.09 ± 0.007	36

^a After a 24-h treatment with 0.01, 0.1, 1, or 10 μg/ml of drug.

^b RAA, relative antitumor activity; EPI, epidoxorubicin; GEM, gemcitabine.

The membranes were then incubated in horseradish peroxidase-conjugated secondary antibody (1:1000; Dako) for 1 h at room temperature. The bound antibodies were detected using an enhanced chemiluminescence kit (ECL; Amersham Pharmacia Biotech), and the samples were analyzed using QuantiScan software (Biosoft, Cambridge, United Kingdom).

RESULTS

The cytotoxic activity of GEM and EPI at different concentrations and exposure times is shown in Fig. 1. Dose-effect

curves were observed in both cell lines after 1- and 24-h exposures, with a generally higher sensitivity seen for MCR cells than HT1376 cells. GEM induced an antiproliferative effect on both cell lines at all tested concentrations and at all exposure times. EPI produced an antiproliferative effect on HT1376 cells and a killing effect on MCR cells at concentrations of >1.5 μg/ml after 1 h or 0.1 μg/ml after a 24-h exposure. It must be pointed out that the IC₅₀ values observed for each drug were under the peak plasma levels reported in clinical trials. Moreover, EPI and GEM induced a growth inhibition in both cell lines, with relative antitumor activity values suggestive of a high clinical activity (Table 1).

The effect on cell growth produced by different simultaneous or sequential treatments is shown in Fig. 2. Simultaneous exposure to the two drugs, as well as treatment with GEM followed by EPI, showed similar dose-dependent growth inhibitions. Conversely, a significantly higher inhibition was observed at all drug concentrations when cells were treated with the schedule EPI → GEM. Identical results were obtained after a 1- or 24-h exposure to the drugs. The analysis of the drug activity interaction (Table 2) revealed antagonistic effects (com-

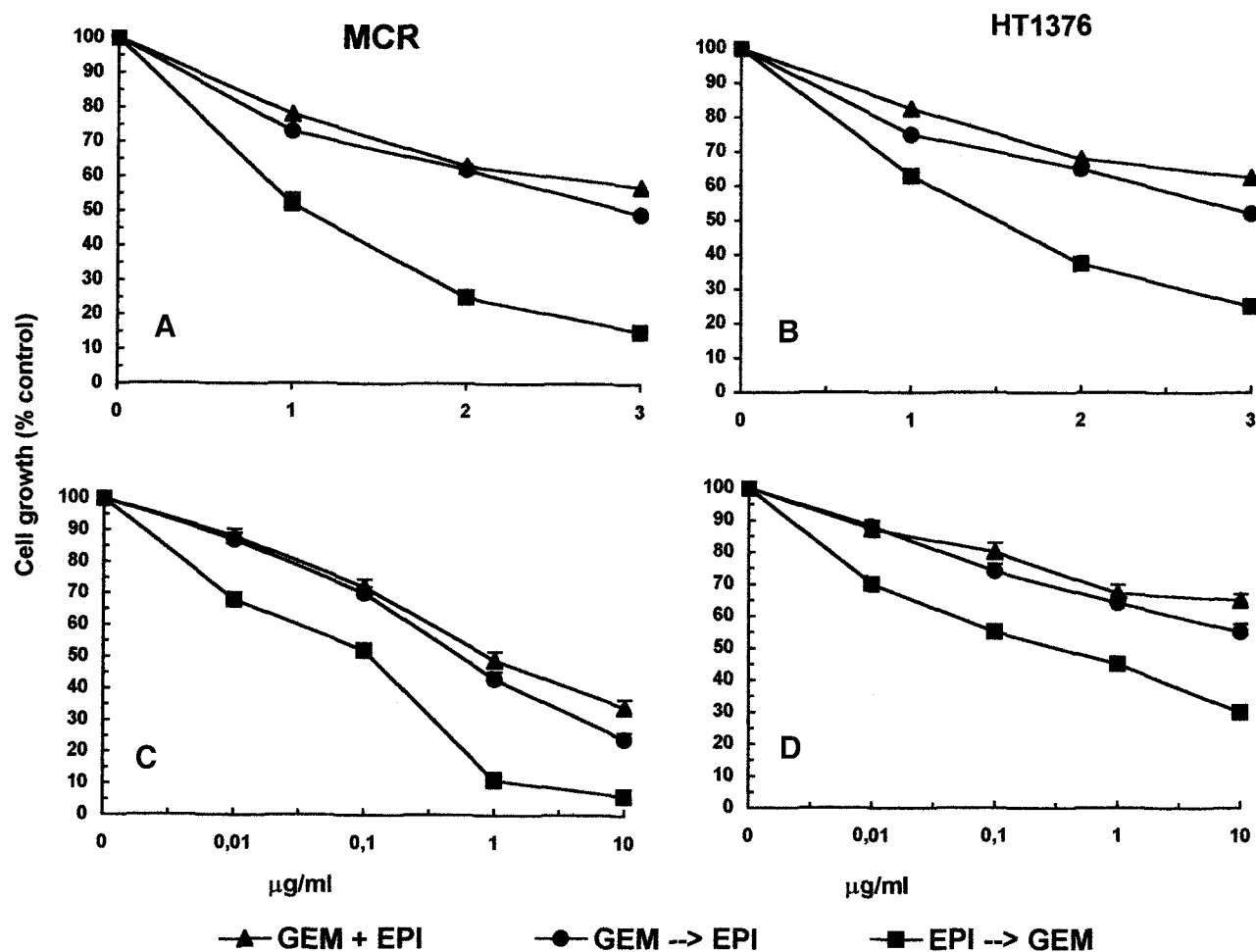


Fig. 2 Dose-response cytotoxicity of epidoxorubicin and gemcitabine after different treatment schemes in MCR and HT1376 bladder cancer cell lines (A and B, 1-h treatment; C and D, 24-h treatment). Each data point represents the percentage of proliferating cells in relation to untreated control and is the average of at least three independent experiments performed in octuplet. Bars, SD never exceeded 5%.

Table 2 Combination index^a after different treatment times and schemes

Treatment	MCR	HT1376
EPI ^b + GEM (1 h)	2.1 ± 0.08	2.2 ± 0.50
GEM (1 h)→EPI (1 h)	1.6 ± 0.09	2.0 ± 0.10
EPI (1 h)→GEM (1 h)	0.2 ± 0.03	0.3 ± 0.08
EPI + GEM (24 h)	2.9 ± 0.10	9.0 ± 1.80
GEM (24 h)→EPI (24 h)	1.5 ± 0.05	1.7 ± 0.06
EPI (24 h)→GEM (24 h)	0.04 ± 0.01	0.1 ± 0.02

^a Calculated at the drug concentration value inducing 90% cell growth inhibition.

^b EPI, epidoxorubicin; GEM, gemcitabine.

combination index > 1) of simultaneous exposure to the two drugs or the sequence GEM→EPI after both 1- and 24-h schemes in both cell lines. Conversely, the inverse sequence, EPI→GEM, produced a synergistic interaction (combination index < 1) in both cell lines.

Cell cycle analysis (Table 3) did not show any perturbations after a 24-h exposure to EPI in either cell line. Conversely, a 24-h exposure to GEM caused a statistically significant increase of cells in G₀-G₁ phase (+45%) and a similar decrease in both S (-45%) and G₂-M (-50%) phases. Similar and statistically significant perturbations were observed when cells were exposed to EPI with or after GEM, but not after the sequence EPI→GEM.

A point mutation from C to T, which produces a stop codon (CAG→TAG) with a consequent loss of 2 exons and formation of a truncated protein, was detected in the *p53* gene of MCR cells (Fig. 3). Moreover, the expression of apoptosis-related genes *bcl-2* and *bax* was not influenced (Fig. 4), and only a small number of apoptotic cells were observed after any treatment in both cell lines.

Conversely, DNA damage, evaluated as single-stranded breaks and alkali-labile sites by Comet assay, showed important changes after different treatments. In particular, in the MCR cell line, the occasional DNA-damaged cells observed in control samples increased to 10% and 20% after a 24-h exposure to GEM or EPI, respectively. The percentage of damaged cells persisted 24 h after EPI removal and dramatically increased to about 65% when anthracycline treatment was followed by GEM. Similar behavior was observed in the HT1376 cell line,

albeit starting from a lower number of Comet cells in untreated samples (Fig. 5).

DISCUSSION

Treatment of superficial bladder cancer aims to eradicate existing disease and to prevent tumor recurrence and/or muscle invasion and metastasis. Despite the fact that treatment of superficial bladder cancer permits an immediate and direct contact between tumor cells and antitumor agents, intravesical instillation of drugs or biological molecules has not yet resulted in a significant improvement in overall survival.

Anthracyclines are DNA-intercalating agents that cause the inhibition of topoisomerase II-mediated DNA resealing and stabilize DNA breaks. Among these, EPI, in particular, has proven active in bladder cancer cell lines and effective and generally well tolerated in clinical intravesical treatment (18–20, 28). Few systemic adverse events have been reported, and local side effects are limited to chemical cystitis, which occurs in a low percentage of cases compared with that caused by BCG (29).

GEM is a nucleoside analog that, after intracellular activation, inhibits DNA synthesis by directly terminating DNA strand elongation and by decreasing cellular deoxynucleotide triphosphates through inhibition of ribonucleotide reductase activity. GEM has shown cytotoxic activity in preclinical studies on some solid tumors, including bladder cancer (28–31). Moreover, a recent work by Kilani *et al.* (32) reported that GEM selectively induces a cytotoxic effect against rat and human bladder cancer cell lines but does not affect cocultured normal human and rat fibroblasts. This observation may explain the minimal bladder irritation and tolerable myelosuppression observed in a recent Phase I trial of intravesical GEM in patients with BCG-refractory transitional cell carcinoma (4)

The ability of GEM to interact with RNA and DNA, which results in masked chain termination and inhibition of DNA repair, may be of importance for drug combinations with DNA-interacting agents such as anthracyclines. Moreover, the low systemic and local toxicity exerted by GEM and EPI, used singly, supports the feasibility of a combined intravesical treatment, once the most effective scheme reproducing locoregional treatment has been defined through direct contact between drugs and tumor cells *in vitro*.

In our experiments, we used cell lines with mutated *p53*

Table 3 Cell cycle perturbations (%) and apoptotic cells (%) induced by EPI^a and GEM on bladder cancer cell lines^b

	MCR				HT1376			
	G ₀ -G ₁	S	G ₂ -M	Apoptotic cells	G ₀ -G ₁	S	G ₂ -M	Apoptotic cells
Untreated cells	46.0 ± 0.7	40.0 ± 0.5	14.0 ± 0.4	3	50.0 ± 0.6	38.0 ± 0.2	12.0 ± 0.2	2
EPI (24 h)	40.0 ± 0.6	41.0 ± 0.5	19.0 ± 0.8	5	47.3 ± 0.9	40.5 ± 0.9	12.3 ± 0.8	3
GEM (24 h)	67.7 ± 1.5 ^c	25.0 ± 1.1 ^c	7.3 ± 1.1 ^c	5	70.0 ± 1.2 ^c	22.0 ± 1.4 ^c	8.0 ± 0.8 ^c	4
GEM + EPI (24 h)	70.0 ± 1.5 ^c	21.0 ± 1.1 ^c	9.0 ± 1.1 ^c	4	62.2 ± 1.2 ^c	18.0 ± 1.4 ^c	13.8 ± 0.8	3
GEM (24 h)→EPI (24 h)	65.5 ± 1.7 ^c	25.5 ± 1.2 ^c	8.0 ± 1.3 ^c	4	70.0 ± 1.1 ^c	21.0 ± 1.1 ^c	9.0 ± 0.4 ^c	3
EPI (24 h)→GEM (24 h)	45.5 ± 1.2	40.0 ± 0.9	14.5 ± 0.7	8	47.5 ± 1.1	41.0 ± 0.8	11.5 ± 0.4	6

^a EPI, epidoxorubicin; GEM, gemcitabine.

^b 0.1 μg/ml for each drug. Data represent mean percentage values ± SD on nine samples from three independent experiments.

^c P < 0.05 by *t* test.

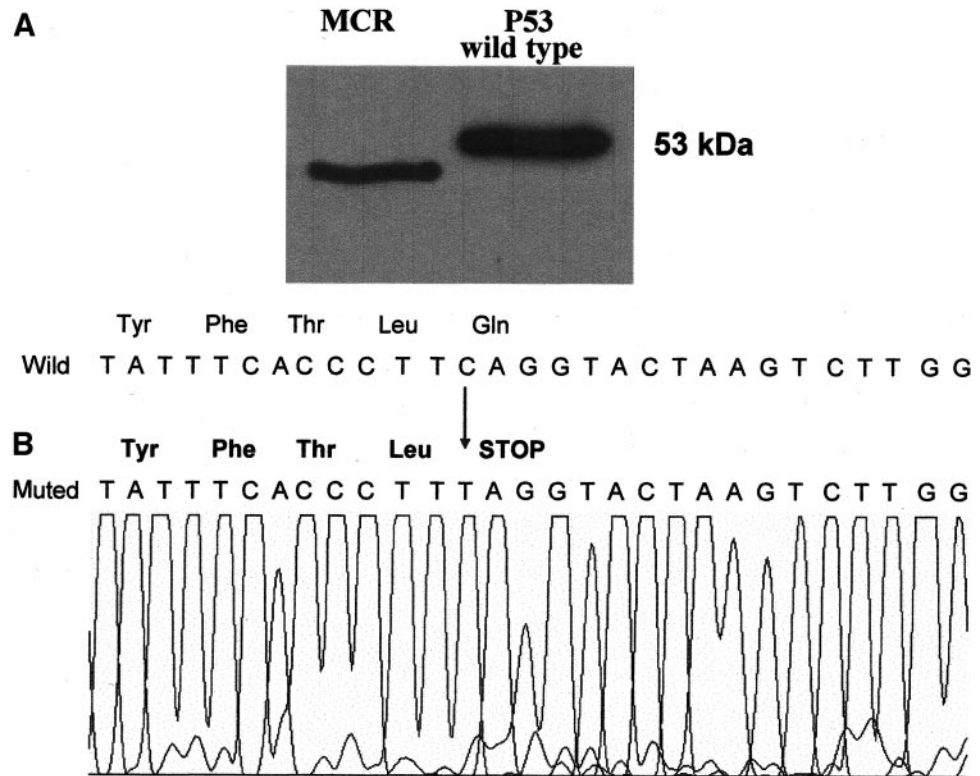


Fig. 3 A, P53 expression of MCR cells. A P53 protein with a molecular mass of <53 kDa was detected, suggesting the presence of a truncated form of the protein. B, sequence analysis of *p53* gene (exons 2–11). A point mutation (arrow) from C to T is present on the sequence of exon 9.

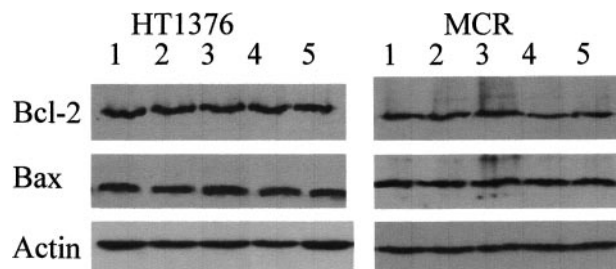


Fig. 4 BAX and BCL-2 protein expression after different treatment schemes in MCR and HT1376 bladder cancer cell lines. Lane 1, untreated cells; Lane 2, epidoxorubicin (EPI; 24 h); Lane 3, gemcitabine (GEM; 24 h); Lane 4, EPI (24 h) + washout (24 h); Lane 5, EPI (24 h)→GEM (24 h).

because altered expression of this oncosuppressor gene is detected in nearly half of bladder cancers and plays a role in multistep tumor progression and sensitivity to drugs (33–36).

Our results confirm the cytotoxicity of EPI and GEM as single agents in the *p53*-mutated cell lines and show a high schedule dependency. Simultaneous treatment with the two drugs as well as the sequence GEM→EPI produced antagonistic effects, whereas a synergistic interaction was observed when treatment with EPI was followed by GEM exposure after both 1- and 24-h treatment schemes. The synergistic interaction cannot be attributed to cell cycle perturbation or the induction of apoptosis. In fact, at the end of the EPI→GEM treatment, the cell cycle profile was identical to that observed in control

samples, with few apoptotic cells and no changes in BCL-2 or BAX expression. Conversely, it is probable that the synergism is a consequence of the relevant increase in cells with damaged DNA produced by GEM on EPI-treated cells.

The clinical activity recently reported in Phase II studies based on the i.v. infusion of a combination of GEM and anthracycline derivatives in advanced breast (37), pancreatic (38–40), bladder (41, 42), and non-small cell lung cancers (43) could be further improved by using the most effective *in vitro* scheme. Moreover, the synergistic interaction observed after 1- and 24-h

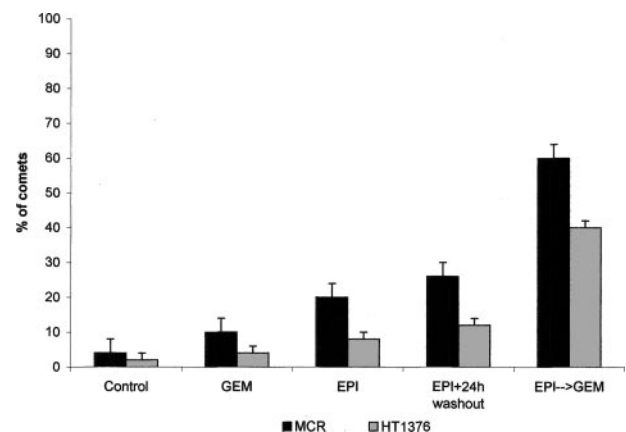


Fig. 5 Percentage of cells with damaged DNA (comets) evaluated after drug treatments (1 h) in MCR and HT1376 bladder cancer cell lines.

exposures to drugs opens up interesting perspectives for infusional or systemic clinical treatments, and the scheme defined as the most active in this preclinical study has provided the basis for the rationale in an ongoing intravesical Phase I-II clinical protocol.

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