*Original Research*

# Cell cycle arrest and apoptosis in *TP53* subtypes of bladder carcinoma cell lines treated with cisplatin and gemcitabine

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## Abstract

**Currently, the combination of cisplatin and gemcitabine is considered a standard chemotherapeutic protocol for bladder cancer. However, the mechanism by which these drugs act on tumor cells is not completely understood. The aim of the present study was to investigate the effects of these two antineoplastic drugs on the apoptotic index and cell cycle kinetics of urinary bladder transitional carcinoma cell lines with wild-type or mutant *TP53* (RT4: wild type for *TP53*; 5637 and T24: mutated *TP53*). Cytotoxicity, cell survival assays, clonogenic survival assays and flow cytometric analyses for cell cycle kinetics and apoptosis detection were performed with three cell lines treated with different concentrations of cisplatin and gemcitabine. $G_1$ cell cycle arrest was observed in the three cell lines after treatment with gemcitabine and gemcitabine plus cisplatin. A significant increase in cell death was also detected in all cell lines treated with cisplatin or gemcitabine. Lower survival rates occurred with the combined drug protocol independent of *TP53* status. *TP53*-wild type cells (RT4) were more sensitive to apoptosis than were mutated *TP53* cells when treated with cisplatin or gemcitabine. Concurrent treatment with cisplatin and gemcitabine was more effective on transitional carcinoma cell lines than either drug alone; the drug combination led to a decreased cell survival that was independent of *TP53* status. Therefore, the synergy between low concentrations of cisplatin and gemcitabine may have clinical relevance, as high concentrations of each individual drug are toxic to whole organisms.**

**Keywords:** apoptosis, cisplatin, gemcitabine, *TP53*, urinary bladder transitional cell carcinoma

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## Introduction

Urinary bladder cancer is the fourth most common malignancy in the Western world,[1] with transitional cell carcinoma (TCC) comprising approximately 90% of all primary bladder tumors.[2] Clinically, the problem associated with these tumors is their highly unpredictable potential for recurrence and progression to muscle-invasive disease.[3] Early-stage bladder tumors have been classified into two groups with distinct behaviors and unique molecular profiles: (1) low-grade tumors (always papillary and usually superficial) and (2) high-grade tumors (either papillary or non-papillary and often invasive). Clinically, superficial bladder tumors (stages Ta and Tis) account for 75–85% of neoplasms, while the remaining 15–25% are invasive (T1, T2–T4) or metastatic lesions at the time of initial presentation.[4] The most important risk factor for bladder cancer seems to be cigarette smoking,[5] although other risk factors, including certain occupational exposures (arylamines) or dietary components,[6] may also be relevant.

Combined chemotherapeutic protocols have been studied extensively with the hope of treating urinary bladder cancer and improving overall survival.[7] Current treatment protocols include the drugs methotrexate, vinblastine, doxorubicin and cisplatin (MVAC). Because of its relative success, gemcitabine has also been included to treat urothelial tumors.[8] Von der Maase *et al.*[9] have demonstrated that in a randomized phase III trial involving patients with advanced bladder cancer, the gemcitabine/cisplatin regimen showed an efficacy similar to MVAC but with a superior safety and tolerability profile. Based on the synergistic interaction between cisplatin and gemcitabine, the combination of the two drugs has been considered as a standard alternative to MVAC.[8,10]

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Cisplatin (*cis*-diamminedichloroplatinum, CDDP) is one of the most potent antitumor agents available due to its ability to induce DNA cross-links and severe lesions that lead to apoptosis, especially in highly replicating cells such as tumor cells.[11,12] Gemcitabine (2′,2′-difluorodeoxycytidine, dFdC) is a deoxycytidine analog with clinical activity against a variety of tumors.[13,14] This drug is phosphorylated into its active dFdCTP metabolite and can be incorporated into DNA, causing replication blockage.[15] It has been suggested that deoxycytidine kinase (the enzyme involved in the rate-limiting phosphorylation step to the monophosphate form) is a critical factor for gemcitabine cytotoxicity.[16]

Mutations in the *TP53* gene are frequently found in bladder cancer cells.[17] The major roles of *TP53* include the induction of a transient (cell cycle arrest) or permanent (senescence) blockage of cell proliferation and activation of cell death signaling pathways in response to genotoxic stress.[18] Attempts to correlate tumor chemoresistance with *TP53* status have shown that the therapeutic response depends on the type of *TP53* mutation and the treatment used; it is also highly cell/tissue specific and dependent on the experimental design used to evaluate cellular responses (proliferation, apoptosis and clonogenic survival),[19] often leading to apparently conflicting results. While several reports have shown that mutated *TP53* cancer cells are more resistant to cisplatin than are wild-type (wt) cancer cells,[20–22] *TP53* mutations are associated with increased susceptibility to chemotherapy-induced apoptosis.[23,24] In addition, it has been observed that gemcitabine treatments can cause lower cytotoxicity in cells with inactivated *TP53*[25] and increased apoptosis in *TP53*-mutated cells.[26] Conversely, some reports have demonstrated cellular responses that are independent of *TP53* status.[27,28]

Because cisplatin and gemcitabine are widely used as antineoplastic agents to treat urinary bladder cancer, the aim of this study was to investigate the effects of these two drugs on cellular responses by analyzing the apoptotic index and cell cycle distribution for urinary bladder transitional carcinoma cell lines with wt or mutated *TP53* (RT4, with wt *TP53*; 5637 and T24, with mutated *TP53*). Cytotoxicity, cell survival and clonogenic survival were also evaluated.

## Materials and methods

### Cell lines, culture conditions and chemicals

The established cell lines RT4, 5637 and T24 from human bladder TCC were obtained from the Cell Bank of the Federal University of Rio de Janeiro, Brazil. The 5637 cells harbor two *TP53* mutations, at codon 72 (Arg > Pro) and codon 280 (Arg > Thr).[29,30] T24 cells contain a *TP53* allele encoding an in-frame deletion of tyrosine 126.[30] Both cell lines were established from high-grade bladder tumors. No specific mutations were detected in RT4 cells, which had been established from a low-grade papillary bladder tumor.[30]

The RT4 and T24 cell lines were maintained in Dulbecco's modified Eagle's medium (Sigma-Aldrich, Inc, St Louis, MO, USA), and the 5637 cells were kept in Roswell Park Memorial Institute medium (Sigma-Aldrich). Both media were supplemented with 10% fetal bovine serum (Cultilab Ltd, Campinas, Brazil), 100 U/mL penicillin G (Sigma-Aldrich), 100 U/mL streptomycin (Sigma-Aldrich) and 1% kanamycin sulfate (Amresco, Branded Products Group, Solon, OH, USA); cells were cultured at 37°C in an atmosphere of 5% $CO_2$. The antineoplastic drugs gemcitabine (dFdC, Gemzar) and cisplatin (CDDP) were obtained from Eli Lilly Laboratory (Eli Lilly and Company, Indianapolis, IN, USA) and Sigma-Aldrich, respectively. Ultra-pure sterilized water was used for dilutions.

### Cytotoxicity and cell survival assays

The cytotoxicity and cell survival induced by cisplatin and gemcitabine were assessed using the XTT assay (Cell Proliferation Kit II, Roche Diagnostics, Mannheim, Germany). Briefly, cells were seeded into 12-well culture plates ($1.5 \times 10^4$ and $6 \times 10^4$ cells/well for cell survival and cytotoxicity assays, respectively). Twenty-four hours later, cells were treated with different concentrations of cisplatin (0.5, 1.0, 2.5, 5.0, 10 or 20 $\mu$mol/L), gemcitabine (0.78, 1.56, 3.12, 6.25, 12.5 or 25 $\mu$mol/L) or both drugs simultaneously (0.5 $\mu$mol/L cisplatin + 0.78 $\mu$mol/L gemcitabine, 0.5 $\mu$mol/L cisplatin + 1.56 $\mu$mol/L gemcitabine, 1.0 $\mu$mol/L cisplatin + 0.78 $\mu$mol/L gemcitabine or 1.0 $\mu$mol/L cisplatin + 1.56 $\mu$mol/L gemcitabine). Untreated cells were cultured in parallel as a negative control. After 24 h of incubation, cells were washed with Hank's solution (0.4 g KCl, 0.06 g $KH_2PO_4$, 0.04 g $Na_2HPO_4$, 0.35 g $NaHCO_3$, 1 g glucose and 8 g NaCl in 1000 mL $H_2O$), and complete fresh medium was added. Cells were sampled for cytotoxicity and cellular survival assays at 24 h and five days after treatment, respectively. A 50 $\mu$L aliquot of the XTT test solution (1 mL XTT labeling/20 $\mu$L electron-coupling reagent) was added to each well at the end of the experiment. After 90 min of incubation, the absorbance was measured at both 492 nm and a reference wavelength (690 nm).[31]

### Clonogenic survival assay

A clonogenic assay was used to evaluate the long-term effects of the drugs. To determine clonogenic ability following treatment, cells were plated at a density of $1 \times 10^6$ cells/25 $cm^3$ culture flask. After 24 h, different concentrations of cisplatin (0.5, 1.0, 2.5 or 5.0 $\mu$mol/L), gemcitabine (0.78, 1.56, 3.12 or 6.25 $\mu$mol/L) or a combination of both (0.5 $\mu$mol/L cisplatin + 0.78 $\mu$mol/L gemcitabine, 0.5 $\mu$mol/L cisplatin + 1.56 $\mu$mol/L gemcitabine, 1.0 $\mu$mol/L cisplatin + 0.78 $\mu$mol/L gemcitabine and 1.0 $\mu$mol/L cisplatin + 1.56 $\mu$mol/L gemcitabine) were added to the culture medium. After another 24 h incubation, cultures were rinsed with Hank's solution and trypsinized, and approximately 1000 cells were plated onto 25 $cm^3$ culture flasks and allowed to grow for 15 days to form colonies. Cells were stained with Giemsa, and colonies with 50 or more cells were counted.

### Analysis of cell cycle kinetics

To determine cell cycle distributions, $1 \times 10^6$ cells were plated into 25 $cm^3$ culture flasks, incubated for 24 h and treated with 1.0 $\mu$mol/L cisplatin, 1.56 $\mu$mol/L gemcitabine or a combination of both (1.0 $\mu$mol/L cisplatin + 1.56 $\mu$mol/L

gemcitabine) for 24 h. These concentrations were chosen because they caused lower clonogenic survival (0 [RT4], $0.28 \pm 0.49\%$ [5637] and $0.81 \pm 0.70\%$ [T24]). After treatment, the cells were washed with Hank's solution, and complete fresh medium was added. Cells were sampled at 0 (just after washing), 24, 48 and 72 h after treatment (recovery time). An aliquot of $5 \times 10^5$ cells was used to analyze cell cycle kinetics using a CycleTEST PLUS DNA Reagent Kit (Becton Dickinson, BD Sciences, San Jose, CA, USA). Instrument performance and data quality were checked using a DNA QC Particles Kit (Becton Dickinson). Cells were analyzed using a FACSCalibur flow cytometer (Becton Dickinson), and their distribution by cell cycle phase was calculated using the Modfit 2.0 cell cycle analysis software.

DNA ploidy was determined based on the DNA index (DI). DI was defined as the comparison between DNA content in tumor cells at the G0/G1 phase and in blood cells from safe individuals (reference cells): a DI value of 0.8–1.2 represents diploid cells, 1.20–1.30 represents peridiploid cells, 1.40–1.60 represents triploid cells and 1.80–2.20 represents tetraploid cells.

## Apoptosis assay

A quantitative assessment of apoptosis was performed using an annexin V assay kit (BD Biosciences). Briefly, cells were incubated and treated as described previously for the cell cycle analysis. Cells were sampled at 24, 48 and 72 h after the end of treatment. Approximately $1 \times 10^5$ cells were resuspended in binding buffer (10 mmol/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid/NaOH [pH 7.4], 140 mmol/L NaCl and 2.5 mmol/L $CaCl_2$) and stained with annexin V-fluorescein isothiocyanate and 7-amino-actinomycin D (7-AAD) at room temperature for 15 min in the dark. Cells were immediately analyzed with an FACSCalibur flow cytometer (Becton Dickinson) using CellQuest software. Data from 10,000 cells were collected in each data file. Cellular status was defined as follows: unstained cells were classified as 'live', cells stained only with annexin V were 'early apoptotic', cells stained with both annexin V and 7-AAD were 'late apoptotic' and cells stained only with 7-AAD were 'dead'.[32–35]

## Combination index

In the clonogenic survival assay, the combination index (CI) was used to analyze the interaction (antagonistic, additive and synergistic effects) between the two drugs. The CI for the fractions of dead cells was calculated using the following equation, described by Chou *et al.*:[36] $CI = d1/D1 + d2/D2 + \{(d1d2)/(D1D2)\}$, where $D1$ and $D2$ are the doses of drugs 1 and 2 (which by themselves result in a given fraction of dead cells) and $d1$ and $d2$ are the doses resulting in the same fraction of dead cells in combination. The CI values were interpreted as an antagonistic effect if $CI > 1.2$, an additive effect if $1.2 > CI > 0.8$ and a synergistic effect if $CI < 0.8$.

## Quantitative real-time PCR

Quantitative real-time polymerase chain reaction (PCR) was performed to investigate the relationship between *TP53* expression and *TP53* status in the three cell lines submitted to different treatments.

### cDNA synthesis

Differential expression of *TP53* in the three cell lines was assayed using real-time PCR with the TaqMan system (Applied Biosystems, Foster City, CA, USA). Total RNA (1 $\mu$g) from control (untreated) cell cultures and cultures treated with 1.0 $\mu$mol/L cisplatin, 1.56 $\mu$mol/L gemcitabine or a combination of both drugs was reverse transcribed using 6 $\mu$L random hexamer primers (10×), 6 $\mu$L reaction buffer (10×), 2.5 $\mu$L dNTPs (25×) and 3 $\mu$L MultiScribe (50 U/$\mu$L) (High Capacity, Applied Biosystems). After incubation at 25°C for 10 min and 37°C for two hours, cDNA was stored first at 4°C and then at −20°C.

### Quantitative real-time PCR

Each reaction tube contained 2 $\mu$L cDNA template, 5 $\mu$L Master Mix TaqMan 2X (Applied Biosystems) and 0.5 $\mu$L 20X primers/probe (Assays-on-Demand gene expression products, Applied Biosystems). $\beta$-Actin was used as a housekeeping gene. The PCR program consisted of the following steps: two minutes at 50°C, 10 min at 95°C and 40 cycles of 15 s at 95°C and one minute at 60°C. Fluorescence data were collected during each annealing/extension step. The reactions were carried out using an Applied Biosystems 7500 FAST Real-Time PCR System and SDS software, version 1.2.3 (Sequence Detection Systems 1.2.3, 7500 Real-Time PCR Systems, Applied Biosystems). For every PCR, a negative (no template) control was processed as a routine assay quality control. Assays were carried out in triplicate in two independent experiments.

### Standard curve and data analysis

To generate standard curves, mRNA that was derived from the RT4, 5637 and T24 cell lines (samples pooled together) and prepared by serial dilutions was used. The smallest RNA standard dilution was assigned a relative value of 100, and following the same reasoning, the other three points were assigned as 20, 4.0 and 0.8. The mRNA quantity in the samples was expressed as values relative to the standard curve.

## Statistical analysis

Statistical analyses were performed using SAS software, v.9.1.3 (Statistical Analysis System, SAS Institute, Cary, NC, USA). For cytotoxicity and cellular survival assays, data were compared using a factorial analysis adjusted to Tukey's test, considering the cell lines, time and concentration effects, time versus concentration and cell lines versus concentration. For clonogenic survival, the analysis was performed based on a Poisson distribution; apoptosis was analyzed using a delineation adjusted to Tukey's test. The cell cycle analysis was performed using a delineation adjusted in factorial based on a binomial distribution. Results from the quantitative real-time PCR experiments were analyzed by analysis of variance and Bonferroni's multiple comparison test. $P < 0.05$ was considered significant.

## Results

### Cytotoxicity and survival assays

Significant toxicity was detected in RT4 (wt *TP53*; low-grade tumor) cells 24 h after treatment with cisplatin (20 $\mu$mol/L) or gemcitabine (12.5 or 25 $\mu$mol/L) ($P < 0.05$). No effect was observed in 5637 or T24 cell lines.

After five days of treatment (survival assay), cisplatin significantly ($P < 0.05$) reduced cell survival in RT4 (1.0–20 $\mu$mol/L), 5637 (0.5–20 $\mu$mol/L) and T24 (5–20 $\mu$mol/L) cells. Similarly, significant effects were detected when gemcitabine was applied to RT4 (0.78–25 $\mu$mol/L), 5637 (0.78–25 $\mu$mol/L) and T24 (1.56–25 $\mu$mol/L) cell lines. Simultaneous treatments with cisplatin and gemcitabine also reduced survival in these three cell lines. In addition, statistically significant differences were detected between the *TP53*-mutated (T24 and 5637) and wt cell lines treated with cisplatin or the combined treatment, suggesting that the mutated cells had higher sensitivities than wt cells. By contrast, T24 cells were more resistant to gemcitabine than RT4 cells at 0.78, 1.56 and 5 $\mu$mol/L concentrations, and 5637 cells were more resistant to gemcitabine than RT4 cells at a 1.56 $\mu$mol/L concentration (Figures 1 and 2).

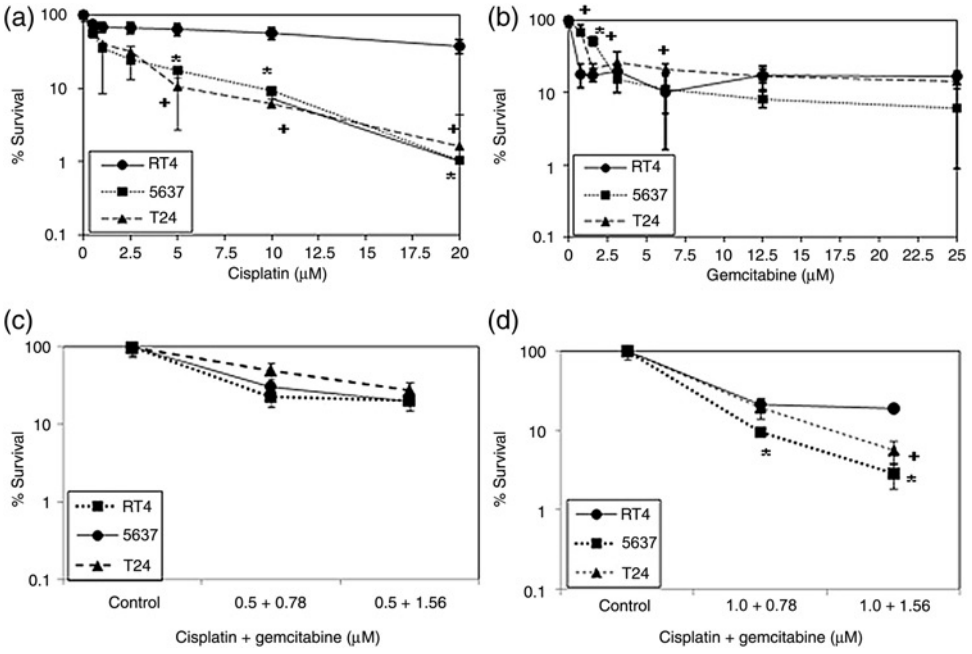


**Figure 1** Percent cell survival (logarithmic scale) for RT4, 5637 and T24 cell lines five days after treatment with cisplatin (a), gemcitabine (b) or both drugs simultaneously (c, d). $P < 0.05$ ($^{*}$5637 in relation to RT4; $^{+}$T24 in relation to RT4). Each point represents the mean value obtained in three independent experiments

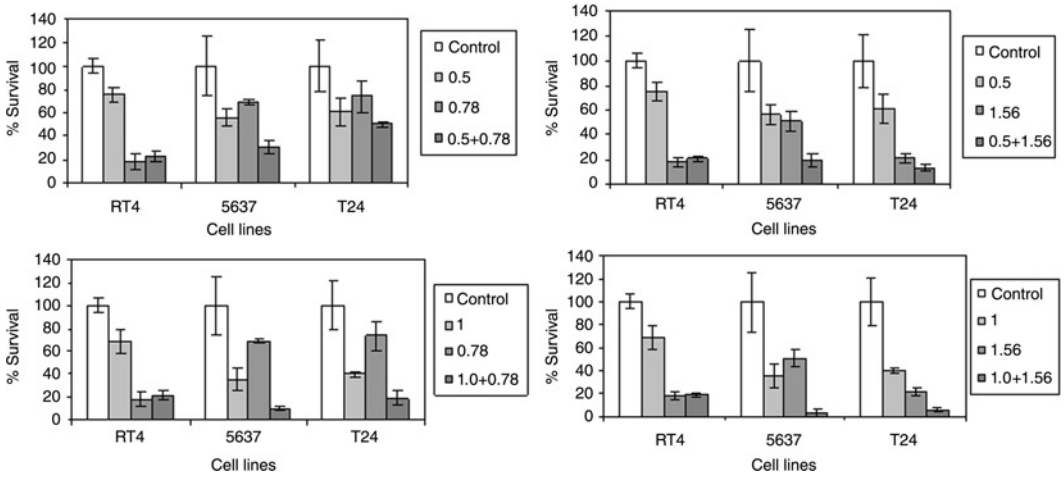


**Figure 2** Percentage of survival of RT4, 5637 and T24 cells five days after treatment with cisplatin (0.5 or 1.0 $\mu$mol/L), gemcitabine (0.58 or 1.56 $\mu$mol/L) or both drugs simultaneously

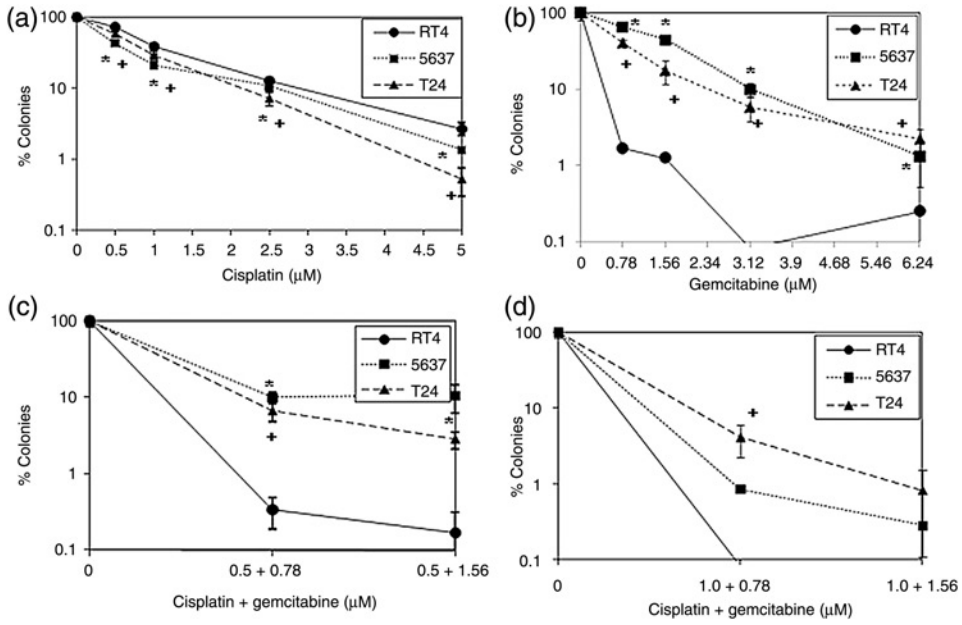


**Figure 3** Clonogenic survival profiles for RT4, 5637 and T24 cells treated with cisplatin (a) and gemcitabine (b). Data correspond to the percentage of colonies counted 15 d after treatment (logarithmic scale). $P < 0.05$ ($^{*}$5637; $^{+}$T24 in relation to RT4). Each point represents the mean value obtained in three independent experiments

### Clonogenic survival assay

Clonogenic survival assays with the three cell lines showed a significant increase in cell death after treatment with cisplatin, gemcitabine or a combination of both drugs. Moreover, RT4 cells (wt for *TP53*) were more resistant to cisplatin and more sensitive to gemcitabine than were mutated *TP53* cells. For the combined treatment, *TP53*-wt cells showed higher drug sensitivity, mainly at concentrations of 0.5 μmol/L cisplatin and 0.78 μmol/L gemcitabine (Figure 3). The CI showed a synergistic interaction between gemcitabine and cisplatin for all cell lines (CI values of 0.657, 0.603 and 0.633 for RT4, 5637 and T24 cells, respectively).

### Cell cycle kinetics and apoptosis

To determine the effects of the drugs on cell cycle kinetics, all cell lines were treated with 1 μmol/L gemcitabine, 0.5 μmol/L cisplatin or a combination of both. For cisplatin, most RT4 cells were at the S phase just after treatment, and T24 cells accumulated in the G2 phase (in controls, most cells were in the G1 and S phases (65.71 and 73.57%, respectively)). At 24 h, T24 cells accumulated in the G1 phase, while untreated cells (control) were more evenly distributed throughout the cell cycle. For 5637 cells, untreated cells were either at the G1 phase (24 h) or distributed throughout the cell cycle (48 h), while most cells treated with cisplatin were at the S and G2 phases 24 h after treatment and at the G1 phase after 48 h (data not shown). For gemcitabine and combined treatments, G1 cell cycle arrest was observed in RT4 (98.62% and 84.47%, respectively) and T24 (45.34% and 47.21%, respectively) cells immediately after treatment (T0) and in 5637 (77.6% and 68.56%, respectively) cells at 24 h (T1). This effect was sustained until 72 h of recovery time (data not shown). Except in control conditions (untreated cells), tetraploid cells were observed in the RT4 cell line after all treatment protocols (Figure 4).

Cisplatin induced apoptosis only in RT4 cells at 48 h (7.84%) and 72 h (10.68%) after treatment. Gemcitabine induced high levels of apoptosis in RT4 cells at 24 h (9.09%), 48 h (83.96%) and 72 h (82.16%), in 5637 cells at 48 h (7.24%) and 72 h (9.56%) and in T24 cells at 72 h (15.92%). Following treatment with both drugs, high levels of apoptosis were observed at 24, 48 and 72 h in RT4 (9.13%, 80.71% and 83.72%, respectively) and 5637 (17.74%, 11.30% and 10.72%, respectively) cells. The combined treatment induced apoptosis earlier (24 h) than gemcitabine treatment alone. For T24 cells, apoptosis was observed at 72 h (31.05%) (Figure 5). Therefore, cisplatin induced apoptosis only in *TP53*-wt cells; for the gemcitabine and combined treatments, apoptosis occurred independently of *TP53* status, although higher percentages were observed in *TP53*-wt cells.

#### *TP53 gene expression*

Our data showed *TP53* gene upregulation in RT4 cells 24 h after treatment with either cisplatin alone or with cisplatin and gemcitabine simultaneously; in 5637 cells, upregulation was detected just after treatment with the drug combination (0 h); in T24 cells, upregulation occurred just after (0 h) and 24 h after treatment with gemcitabine or with both drugs simultaneously. Downregulation of *TP53* was observed only in RT4 cells (0 h) treated with the combination of drugs (Figure 6).

## Discussion

Mutations in the *TP53* gene are thought to be one of the central events in urothelial carcinogenesis; they have been

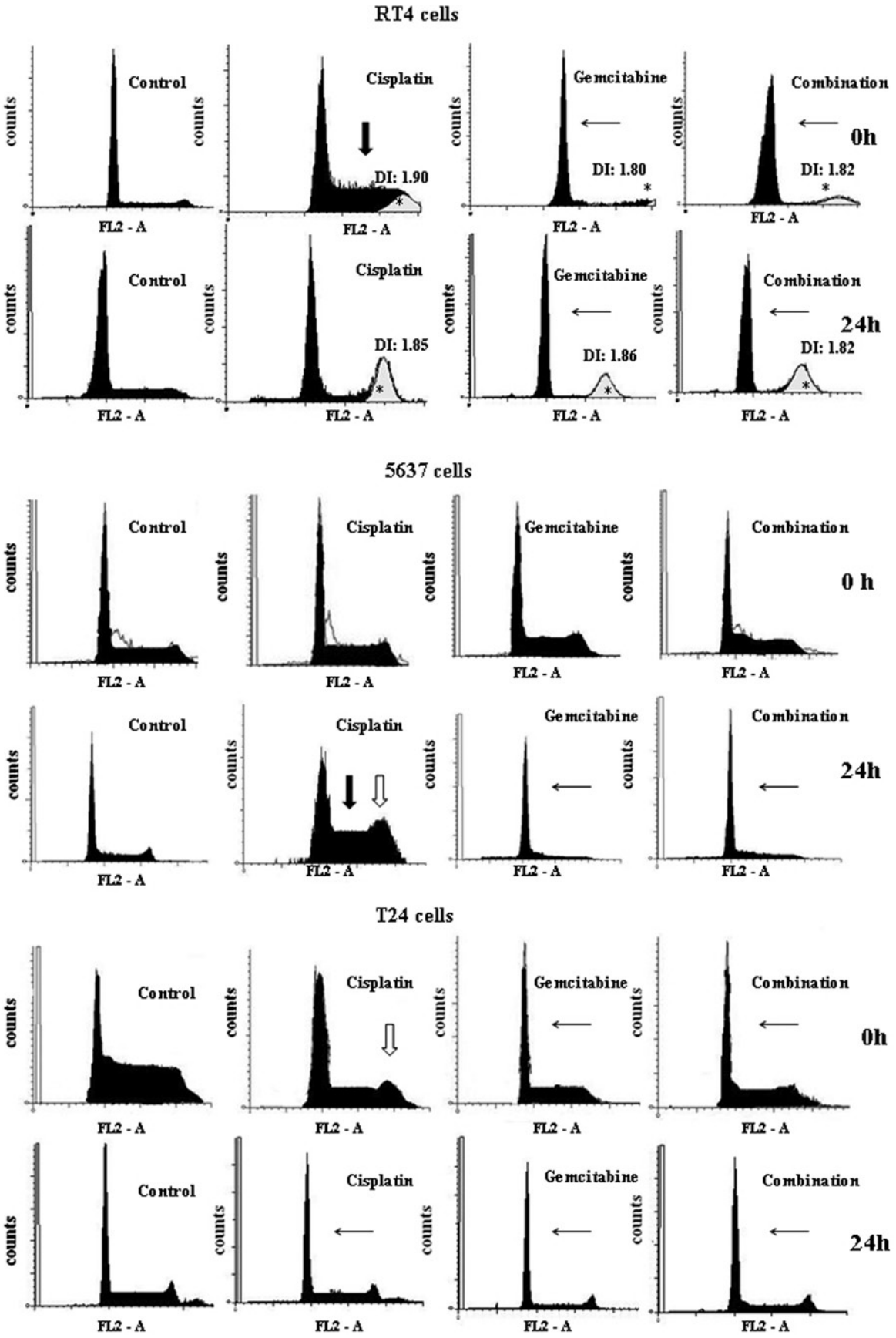


**Figure 4** Representative DNA histograms (flow cytometry) for RT4, 5637 and T24 cells treated with 1.0 $\mu$mol/L cisplatin, 1.56 $\mu$mol/L gemcitabine or both drugs simultaneously. Arrow indicates increased number of cells in $G_0/G_1$(←), S (⬇) and $G_2$/M (⇩) phases; *tetraploid cells. DI, DNA index

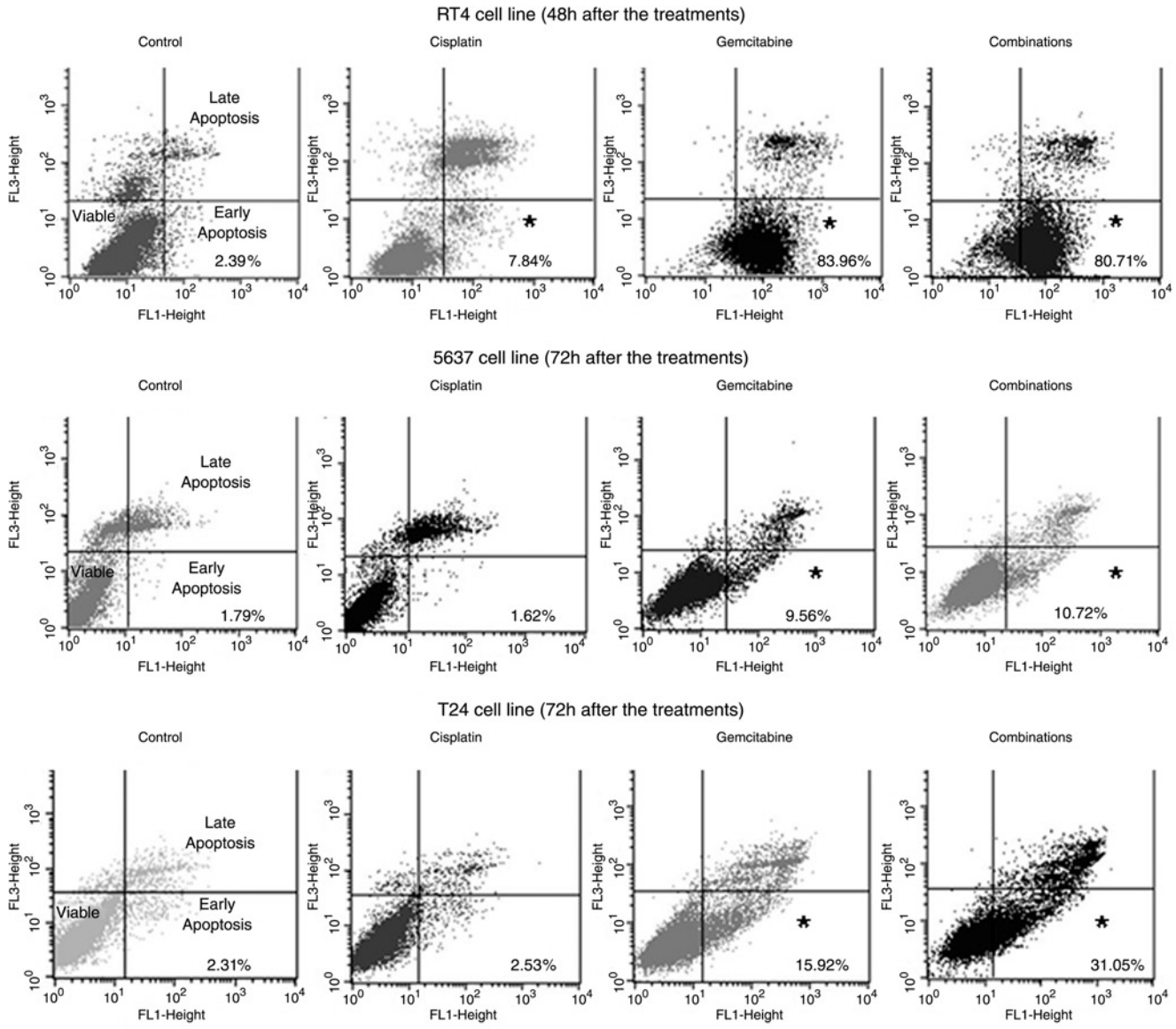


**Figure 5** Early apoptotic index (%) in RT4, 5637 and T24 cells treated with 1.0 $\mu$mol/L cisplatin, 1.56 $\mu$mol/L gemcitabine or both drugs simultaneously. FL1-H, annexin V-fluorescein isothiocyanate fluorescence; FL2-H, 7-amino-actinomycin D fluorescence. $^{*}P < 0.05$ (compared with respective controls)

associated with tumor progression and poor prognosis.[17] Acute DNA damage triggers a rapid *TP53* response that inhibits phase-specific cell cycle progression (G1–S). *TP53* also activates the DNA repair and redox systems, leading to cell survival.[37] Invasive tumors are characterized by loss-of-function mutations affecting prototypical tumor suppressor genes including *TP53*, but these alterations are absent or very rare in superficial, papillary, non-invasive tumors.[4]

The influence of *TP53* mutations on cellular responses to chemotherapy is poorly understood because it depends on a complex signaling cascade. Hypothetically, *TP53*-mutated cells should be more resistant to chemicals because of the role that *TP53* plays in apoptosis control. However, induction of apoptosis does not occur by a single *TP53*-dependent pathway. Conversely, recent findings have yielded somewhat unexpected insights concerning the preponderance of survival-promoting effects of wt *TP53* in cancer cells, a rather undesirable property from a therapeutic standpoint.[18] In bladder cancer, the interaction between the *TP53* status of a primary tumor and the response to chemotherapy is not well defined.[27] Most likely, the genetic background of each cell line must be taken into account. Here we analyze three human bladder cancer cell lines, RT4 (wt *TP53*), 5637 and T24 (both with mutated *TP53*), to investigate the relationship between *TP53* status and sensitivity to the antitumor drugs cisplatin and gemcitabine.

Cisplatin cytotoxicity at an early time point (24 h) was not observed in the *TP53*-wt cell line treated with 0.5–10 $\mu$mol/L cisplatin or in the two mutated cell lines at any concentration tested. However, additional results showed decreased cellular survival (five-day test) in cisplatin-treated cells. Results examining whether *TP53* mutations confer increased

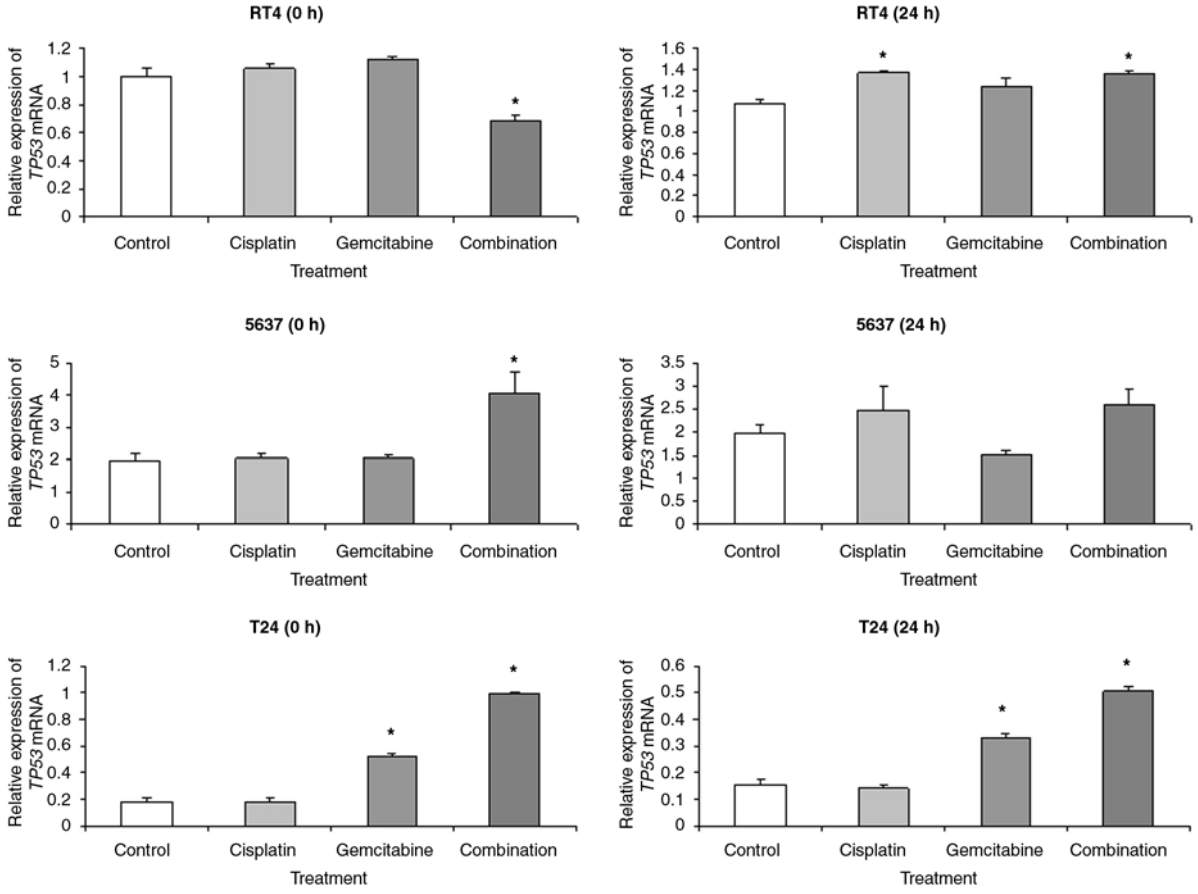


**Figure 6** Relative TP53 mRNA levels in RT4, 5637 and T24 cell lines after treatment with cisplatin (1.0 $\mu$mol/L), gemcitabine (1.56 $\mu$mol/L) or both drugs simultaneously (combined treatment) at zero hour (just after washing) and 24 h (recovery time). Values represent relative mRNA levels (quantitative realtime-polymerase chain reaction), normalized to the values of control cells (without treatment) with duplicate determinations. $\beta$-Actin was used as the endogenous RNA control. $^{*}P < 0.05$ in relation to the control

responsiveness or increased resistance to the effects of cisplatin-based systemic chemotherapy have been conflicting. Some studies have demonstrated increased resistance of *TP53*-mutant cancer cell lines to cisplatin compared with *TP53*-wt cell lines,[38,39] while others have shown increased sensitivity of *TP53*-mutant cells to cisplatin, with *TP53*-wt cells being more resistant.[24,40]

*TP53* is known to be activated by genotoxic drugs capable of inducing apoptosis, and it is widely considered to be a mediator of chemotherapy-induced cell death. In our study, cisplatin did not significantly increase apoptosis in *TP53*-mutant cells, although low percentages of cellular and clonogenic survival were detected. By contrast, a significant increase in the apoptotic index was observed in wt RT4 cells, which correlates with increased *TP53* expression. The results of Gallagher *et al.*[41] support the hypothesis that *TP53* mutations result in an inability to initiate apoptosis in response to DNA damage. Additionally, cisplatin-induced growth arrest in human cancer cells has characteristics of senescence rather than apoptosis.[42] According to Wang *et al.*,[42] cancer cells lacking *TP53* function can also be killed by cisplatin via a *TP53*-independent mechanism that is similar to replicative senescence.

Cisplatin has properties similar to alkylating agents and forms a variety of DNA adducts. Although not specific to a particular cell cycle phase,[43] cisplatin-induced DNA adducts may inhibit DNA replication, transcription and ultimately cell division.[44] It has been shown that in certain tumors, *TP53* protects cells from cisplatin toxicity due to induction of G1 arrest.[45,46] Nevertheless, Kannan *et al.*[21] have shown that silencing *TP53* in a testicular tumor cell line protects cells from cisplatin-mediated toxicity. Our data demonstrated a temporary S-phase arrest in *TP53*-wt cells immediately after treatment (0 h). However, a significant increase in *TP53* expression was observed only 24 h after cisplatin exposure. Regarding T24 and 5637 *TP53*-mutant cells, G1-phase arrest was detected 24 and 48 h after treatment, respectively, but no changes were detected in *TP53* gene expression. Therefore, cell cycle kinetics varied according to *TP53* status, but were independent of *TP53* expression.

Gemcitabine activity in relation to *TP53* status has been less thoroughly investigated in bladder cancer and other tumors. Studies have indicated apparently contradictory results about the effects of gemcitabine on cell cycle kinetics. We observed a G1-phase arrest in the three cell lines that was independent of *TP53* status. Using bromodeoxyuridine

labeling, Merlin *et al.*[47] observed that low concentrations of gemcitabine ($IC_{50}$ values) cause arrest in early *S* phase, while high concentrations induce a G1-phase arrest. In addition, it has been shown that the effects of gemcitabine are not related to *TP53* status in human epithelial lung A549 cells.[48] In the present study, no difference in *TP53* expression was detected between treated and untreated wt cells. Significant upregulation was observed in T24 cells at zero hour and 24 h after treatment, but only at zero hour in 5637 cells. Therefore, G1-phase arrest also does not seem to be related to *TP53* gene expression. T24 cells harbor *TP53* mutations in the N-terminal transactivation domain, which produce proteins that preserve *TP53* activities such as DNA binding.[49] In 5637 cells, the *TP53* gene has point mutations at codons 72 and 280 (core domain), which have previously been reported to be part of a mutational hot spot for bladder cancer. Mutations in the core domain affect the ability of p53 to bind DNA.[49]

Higher levels of apoptosis were found in gemcitabine-treated RT4 cells compared with the *TP53*-mutated cell lines. However, based on previous studies, this apoptosis effect is still unclear. It has been reported that in *TP53*-wt gemcitabine-treated cells, DNA damage leads to increased *TP53* expression, resulting in DNA repair.[50] Fencher *et al.*[28] showed that apoptosis is independent of *TP53* status in TCC cell lines. Our data demonstrate that gemcitabine-induced apoptosis seems to be dependent on *TP53* status because *TP53*-wt cells present an early apoptotic index of 82.16% at 72 h, while 5637 and T24 *TP53*-mutant cells showed apoptotic indices of 9.56% and 15.92%, respectively. Our data also demonstrate that apoptosis occurs independently of *TP53* expression because only one of the two *TP53*-mutant cell lines presented increased gene expression.

Aside from the low apoptotic index, we also detected low cellular and clonogenic survival rates in *TP53*-mutant cells. Tannock and Lee[51] suggested that activation of the apoptosis pathway occurs either as a primary event induced by therapy or as a secondary event following lethal cell damage. This distinction is not trivial because if apoptosis occurs as a primary event following chemotherapy, then the treatment effectiveness will depend on the cells' ability to activate the apoptotic pathway. By contrast, if apoptosis is a secondary event that occurs in cells that have sustained lethal DNA damage and merely controls the rate of cell lysis for those that have already lost reproductive potential, manipulation of the pathway would not influence the long-term effects of the treatment.[51] In our experiments, we first assayed the drugs' antiproliferative effects using cytotoxicity and survival assays. The clonogenic assay was then used to determine the antitumor activities of the drugs. The most relevant endpoint for measuring cell death in tumor cell lines is detected by a colony-forming assay; cells that are unable to produce colonies under optimal conditions are also unlikely to produce tumors.[51] We observed that in the clonogenic assay, *TP53*-mutant cells were more resistant to gemcitabine treatment than the *TP53*-wt cell line. The different results observed between the cellular and clonogenic survival assays could indicate that antineoplastic drugs cause a reduction in cellular reproductive capacity, suggesting that the drugs induce DNA lesions that interfere with clonogenic potential. However, the extent of these lesions seems to be insufficient for inducing the cell death pathways because viable cells were detected five days after treatment.

Acquired resistance after prolonged and repeated gemcitabine exposure might be a mechanism of cell resistance.[52] To overcome the low rates of cell killing, combined chemotherapy regimens such as methotrexate–vinblastine–cisplatin, MVAC and gemcitabine–cisplatin have been used. In the present study, the combination of cisplatin and gemcitabine led to a synergistic effect that was independent of *TP53* status and expression. With regard to the cell cycle, the effects of gemcitabine prevailed over those of cisplatin when the drugs were administered together, with G1-phase arrest observed in all three cell lines. Apoptosis was detected after 24 h in 5637 cells, and a high percentage of apoptosis was observed in T24 cells after 72 h of post-treatment recovery time. Despite the fact that the results of the apoptosis and cell cycle analyses were similar between gemcitabine and cisplatin/gemcitabine treatments, lower survival rates were observed in the survival and clonogenic assays for RT4, 5637 and T24 cells following the combined treatment. Other studies had previously reported that cisplatin treatment followed by gemcitabine treatment produced a synergistic effect in human ovarian and lung cancer cell lines and that simultaneous administration (or treatment in the reversed order) also results in high cytotoxicity.[53,54] In addition, there is evidence that genes other than *TP53* also participate in the regulation of DNA repair and apoptosis mechanisms.[55] *TP53* might function as a molecular node, but other target genes can also be altered and thus could contribute to the success of drug therapy.

In conclusion, our data show that treatment with gemcitabine alone or gemcitabine with cisplatin induces G1-phase arrest and triggers apoptosis in *TP53*-wt and *TP53*-mutant cell lines. Moreover, bladder carcinoma cells with wt *TP53* are more sensitive to apoptosis than *TP53*-mutant cells under cisplatin and/or gemcitabine treatment. Nevertheless, the combination of cisplatin and gemcitabine resulted in low cell survival that was independent of *TP53* status and expression. It should be emphasized that chemotherapy with these two compounds can be effective regardless of tumor-associated *TP53* mutations or tumor grade. Therefore, because high concentrations of cisplatin are very toxic to humans, the use of low concentrations of cisplatin and gemcitabine simultaneously might be clinically relevant and reduce secondary effects. Further studies should be conducted to compare the efficiencies of different chemotherapy protocols.

**Author contributions:** All authors reviewed the manuscript; GNS was responsible for the study design and data interpretation, performed most of the experiments and wrote the manuscript; JPCM and EAC conducted the cell cycle experiments; GASPJ and ETS-H assisted in data interpretation and critical reading of the manuscript; and DMFS contributed to the study design, interpretation of data and critical reading of the manuscript.

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