Gemcitabine-induced apoptosis in 5637 cell line: an in-vitro model for high-risk superficial bladder cancer

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Recent data suggest that new treatment options for superficial bladder cancer are necessary, owing to the high recurrence rate after conventional treatment, especially in T1G3 and Bacillus Calmette-Guerin-refractory patients. Phase I and II studies have demonstrated that gemcitabine may represent a candidate for intravesical therapy in superficial bladder cancer. Despite clinical trials, the in-vitro cytotoxic and proapoptotic effects of gemcitabine have been poorly investigated. In the present study, we investigated how gemcitabine affects apoptosis in bladder cancer cell line 5637, which has the same molecular features of high-risk superficial bladder cancer. Apoptosis was evaluated by DNA fragmentation, flow cytometry and caspase activation. *bcl-2*, *bcl-X*, *bax*, *survivin* and *fas* gene expression were also evaluated by reverse-transcriptase polymerase chain reaction. Nuclear factor-kappa B activation was assessed by immunofluorescence. Gemcitabine induced apoptosis in 5637 cells in a time-dependent manner, with activation of caspase-3, -8 and -9. Expression of *bcl-2*, *bax*, *survivin* and *bcl-X* was not affected by treatment, whereas *fas* strongly increased after 24 h of treatment. After treatment, we failed to find any nuclear localization of nuclear factor-kappa B. As gemcitabine-induced apoptosis involves *fas* upregulation, these results may encourage the investigation of intravesical gemcitabine in *fas*-negative bladder tumors. Furthermore, as nuclear factor-kappa B activation by cisplatin, doxorubicin and adriamycin may result in enhanced proliferation, migration, immortality and inhibition of apoptosis, the observation that gemcitabine does not activate nuclear factor-kappa B may have implications in intravesical therapy of high-risk superficial bladder cancer. *Anti-Cancer Drugs* 18:179–185 © 2007 Lippincott Williams & Wilkins.

Anti-Cancer Drugs 2007, **18**:179–185

Keywords: apoptosis, gemcitabine, nuclear factor-kappa B, superficial bladder cancer

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Sponsorship: This work was partially supported by A.R.Ger.On onlus, and the Italian Ministry for University and Research (MIUR).

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Received 12 June 2006 **Revised form accepted** 15 September 2006

Introduction

An increasing body of evidence suggests that new therapeutic options for superficial bladder cancer (SBC) are necessary, owing to the high recurrence rate after conventional treatment, especially in T1G3 and in Bacillus Calmette-Guerin (BCG)-refractory patients. The recurrence rate in intermediate-risk SBC after treatment with mitomycin C ranges between 20 and 45% according to different studies [1,2], and, although BCG is considered the most effective conservative treatment, its efficacy in T1G3 patients is controversial [3]. Furthermore, in BCG-refractory patients with superficial disease, cystectomy or alternative intravesical therapy are the only alternative approaches. High-risk SBC has a characteristic molecular profile, and high *bcl-2* and *bcl-X* expression has been described to identify a subset of patients with shorter relapse-free time [4]. In these patients at high risk to relapse, new therapeutic approaches are urgently needed. Clinical studies have demonstrated that intravesical gemcitabine has an excellent safety profile and minimal toxicity at concentrations up to 40 mg/ml [5], concluding that it may represent a candidate for intravesical therapy in SBC. Despite clinical trials, in-vitro effects of gemcitabine have been poorly investigated and mainly depend on the cell type. Most studies have been performed on non-small-cell lung cancer, in which gemcitabine activates the mitochondrial pathway without activation of initiator caspases. In some instances, $bcl\text{-}X_L$ overexpression induced resistance to gemcitabine [6]. In the pancreatic cell line, COLO357 gemcitabine increases CD95 expression and induces activation of caspase-8 [7], whereas in breast cancer, it induces apoptosis through downregulation of *bcl-2* and $bcl\text{-}X_L$ [8]. Although gemcitabine was

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described to induce apoptosis in bladder cancer cells [9], no data are available concerning the apoptotic pathway involved in gemcitabine-treated cells. Previous studies have shown that gemcitabine stimulates nuclear factor-kappa B (NF-κB) activation in pancreatic and non-small-cell lung cancer cell lines [10]. Once activated, NF-κB translocates to the nucleus and activates the transcription of multidrug resistance (MDR)-related and apoptosis-inhibiting genes [11]. Nevertheless, recent studies indicate that NF-κB may also induce apoptosis in some cell types through the activation of the genes encoding Fas and Fas ligand [12,13]. In this article, we describe how gemcitabine affects apoptosis and we investigated whether gemcitabine treatment may affect the NF-κB pathway in the bladder cancer cell line 5637. The choice of this in-vitro model was suggested by the observation that the 5637 cell line has molecular features (*bcl-2* and *bcl-X* positive expression, mutated p53) that are commonly observed in high-risk superficial bladder tumors.

Methods

Cell culture

Human bladder transitional cell carcinoma cell line 5637 was grown in RPMI 1640 (Euroclone, Life Science Division, GB, Pero, Italy) supplemented with 10% fetal bovine serum (Euroclone) in 5% CO_2 at 37°C. The cell line was obtained from the Bank of Biological Material Interlab Cell Line Collection (University of Genova, Genova, Italy).

Gemcitabine was kindly provided by Eli-Lilly Research Laboratories (Florence, Italy). Rabbit polyclonal antibody to NF-κB p65 was purchased from Santa Cruz Biotechnology (Santa Cruz, California, USA).

Cell viability assay

Cytotoxicity was tested by the trypan blue dye exclusion. Cells grown exponentially were suspended in a concentration of 250 000/ml and 24 h later the drug was added (1, 10, 50, 100 and 1000 μmol/l). After different incubation times (5,10, 20, 24, 48 and 72 h), cell viability was determined and expressed as a percentage of control cell growth.

Clonogenicity test

Cells were seeded 1×10^3 cells per well, adhered for 24 h, pulse treated for 0, 24, 48 and 72 h with the drug, and maintained drug-free 5–10 generation times in a complete medium. Proliferation was evaluated by crystal violet staining.

The cell culture medium was removed and surviving cells stained with 0.5% crystal violet in 20% methanol for 20 min at room temperature. The colonies were scored with an inverted microscope. In a different experiment, three surviving clones, named A, B and C, were allowed to grow in a complete medium, and then the drug was added again for 24, 48 and 72 h.

DNA fragmentation assay

DNA fragmentation into nucleosomal bands was detected by agarose gel electrophoresis.

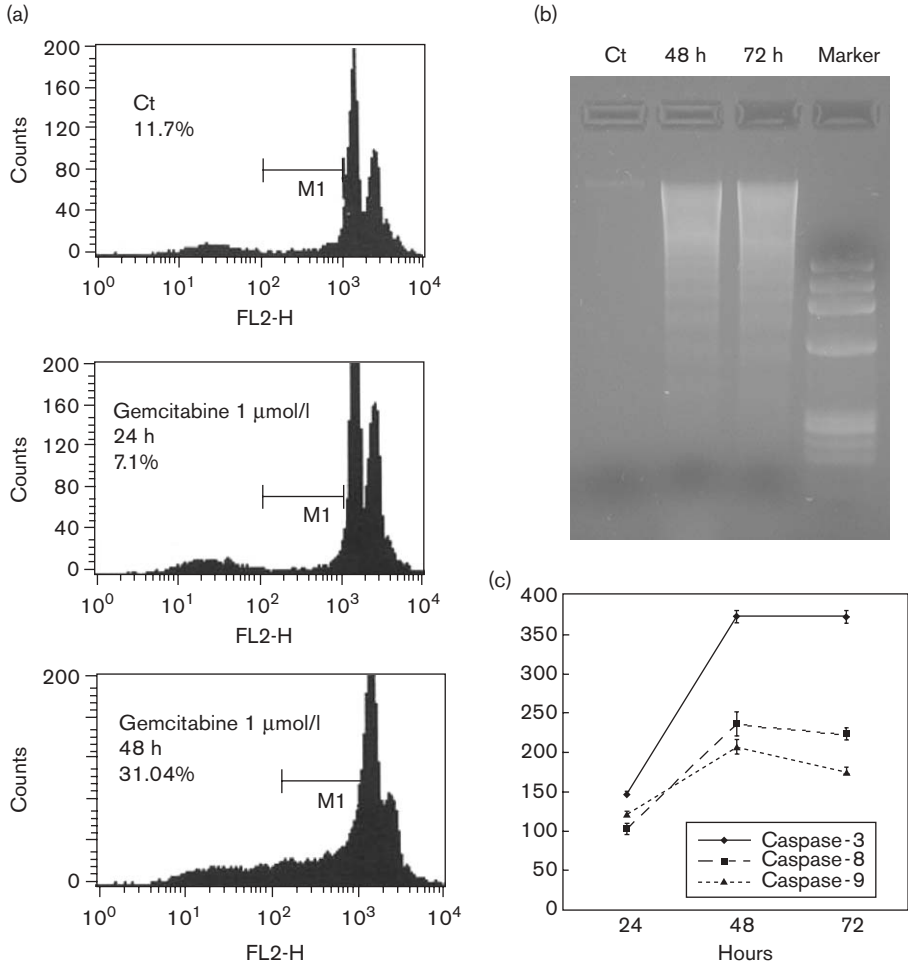
Briefly, 5×10^6 cells were lyzed with 0.5 ml of hypotonic lysis buffer (10 mmol/l Tris–HCl, 1 mmol/l ethylenediaminetetraacetic acid, 0.2%Triton X-100) for 60 min on ice. Cells lysated were centrifugated at 12 000 rpm for 10 min. Supernatant was incubated with DNase-free RNase A (ICN Biomed, Aurora, Ohio, USA) (5 μg/ml) for 1 h at 50°C, and proteinase K (200 μg/ml) (Sigma-Aldrich,

Table 1 Primer sequences and amplification conditions

Gene		Sequences 5′→3′	Annealing temperature (°C)	Size (bp)
gadph	upstream	AGATGTTCCAATATGATTCC	60	161
	downstream	TGGACTCCACGACGTACTCAG		
bcl-2	upstream	GTGGAGGAGCTCTTCAGGGA	60	304
	downstream	AGGCACCCAGGGTGATGCAA		
bax	upstream	GGCCCACCAGCTCTGAGCAGA	62	479
	downstream	GCCACGTGGGCGTCCCAAAGT		
bcl-X_L	upstream	TTGGACAATGGACTGGTTGA	58	780
bcl-X_S	downstream	GTAGAGTGGATGGTCAGTG		591
fas	upstream	TTCGGAGGATTGCTCAACA	60	501
	downstream	GGTGAGTGTGCATTCCTTG		
survivin	upstream	CAGATTTGAATCGCGGGACCC	60	206
	downstream	CCAAGTCTGGCTCGTTCTCAG		
mrp-4	upstream	GGATCCAAGAACTGATGAGTTAAT	65	358
	downstream	TCACAGTGCTGTCTCGAAAATAG		
mrp-1	upstream	CTGACAAGCTAGACCATGAATGT	60	353
	downstream	TCACACCAAGCCGGCGTCTTT		
mdr-1	upstream	TCCTCAGTCAAGTTCAGAGTCTTCA	62	193
	downstream	TAGCAAGGCAGTCAGTTACAGTCC		
$p27^{kip}$	upstream	AGGAGAGCCAGGATGTCAGC	60	235
	downstream	ACCGGCATTTGGGGAGCCGT		
p21	upstream	CTCAGAGGAGGCGCCATGTCA	66	255
	downstream	CGTGGGAAGGTAGAGCTTGGGC		

MDR, multidrug resistance; MRP, multidrug resistance protein.

Fig. 1



Apoptosis in gemcitabine-treated 5637 cell line evaluated by flow cytometry (a), DNA fragmentation (b) and caspase activation (c). (a) Cytometric analysis of apoptosis in gemcitabine-treated cells. DNA content was evaluated by staining cells as indicated in Methods and by analyzing the red fluorescence emission. Apoptotic cells were estimated as the region at the left of G_1/G_0 peak, as the sub-G_1 peak. Gemcitabine increased the proportion of apoptotic rate in 5637 cells; the percentage of events accumulated is indicated in each panel. (b) DNA fragmentation was detectable after 48 and 72 h of incubation with 1 μmol/l gemcitabine. (c) Gemcitabine induced apoptosis in 5637 cells through activation of the caspase pathaway. Activation of caspase-3, -8 and -9 was evaluated after 24, 48 and 72 h of incubation with 1 μmol/l gemcitabine. The results represent the mean on three independent experiments. Ct, control.

Milano, Italy) was then added and incubated for an additional hour at 65°C. The DNA laddering, extracted with phenol/chloroform followed by precipitation in chloroform, was separated on a 1.5% agarose gel electrophoresis and visualized by ethidium bromide staining.

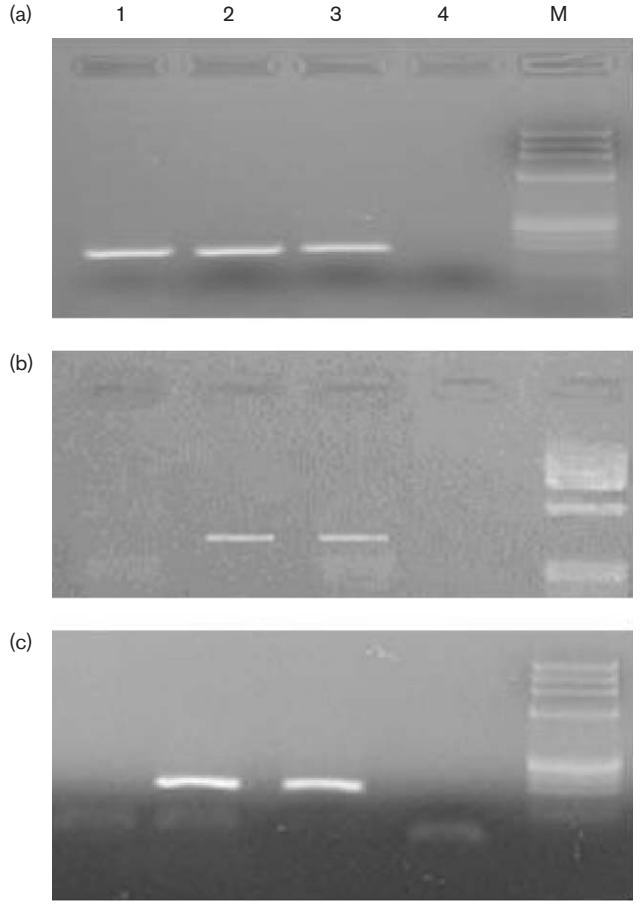
Flow cytometry analysis of apoptotic cells

Flow cytometry was used to detect quantitatively the apoptotic rate and the distribution of cell cycle. After 0, 24, 48 and 72 h of incubation with gemcitabine 1 μmol/l, cells were harvested, washed twice with ice-cold phosphate-buffered saline (PBS), and then 1.5×10^6 cells were fixed in 2 ml of 70% ethanol and then incubated for 1 h at 4°C. The cellular pellet was dissolved in 0.5 ml of solution containing propidium iodide 5 μg/ml and RNase A 1 mg/ml in PBS. The stained cells were incubated at room temperature for 30 min in the dark. The DNA content of the cells was analyzed by FACSCalibur flow cytometry using the CellQuest analysis program. The DNA content in the sub-G_1 population was considered to represent apoptotic cells.

Enzyme-linked immunosorbent assay for detection of caspase-3, -8 and -9

The cleavage activity of Ile-Glu-Thr-Asp conjugated to *p*-nitroanilide (IETD-*p*NA), Leu-Glu-His-Asp conjugated to *p*-nitroanilide (LEDH-*p*NA) and Asp-Glu-Val-Asp conjugated to *p*-nitroanilide (DEVD-*p*NA) was measured by using respectively FLICE/Caspase-8, Caspase-9/Mch6 or a Caspase-3/CPP32 colorimetric assay kit (Bio vision,

Fig. 2



Reverse transcription-polymerase chain reaction analysis for GADPH (a), Fas (b) and MRP-4 (c) expression in control and gemcitabine-treated 5637 cells. Lane 1: control 5637; lane 2: 5637 treated with 1 μmol/l gemcitabine 48 h; lane 3: positive control; lane 4: negative control.

Mountain View, California, USA) at 4, 48 and 72 h. About 3×10^6 cells were pelleted, washed twice in PBS and resuspended in 50 μl chilled lysis buffer. The formations of *p*NA were measured by an ELISA Microtiter Reader (Corning, Corning, New York, USA) at 405 nm before samples were incubated at 37°C for 2 h with appropriate substrates. Percentage increase in caspase activities was determined by comparing those results of the indicated time point with controls.

Reverse transcription-polymerase chain reaction

One microgram of total RNA extracted from the frozen cells was reverse-transcribed in a final volume of 20 μl containing 20 mmol/l Tris–HCl pH 8.3, 50 mmol/l KCl, 2.5 mmol/l $MgCl_2$, 100 pmol random examer and 50 units of MuLV reverse transcriptase (Life Technologies, Paisleyork, UK), according to the manufacturer's guidelines. Then, 4 μl of cDNA was amplified in polymerase chain reaction buffer containing 25 pmol each of upstream and 1.25 units of Platinum Taq polymerase (Life Technologies) in a final volume of 50 μl. The primers used were *bcl-2*, *bax*, *bcl-X*, *survivin* and *fas* for the apoptotic pathway; *p21* and *p27* involved in cell cycle progression; *mdr-1*, multidrug resistance protein (*mrp)-1* and *mrp-4* involved in the MDR profile. Amplifications were performed on a Techne Progene amplificatory (Techne Progene, Cambridge, UK). The conditions of amplification for each gene and the amplified product size are summarized in Table 1.

Immunofluorescence

Cells were grown on Labteck chamber slides (Nunc, Naperville, Illinois, USA) and treated with gemcitabine 1 μmol/l for 24, 48 and 72 h. After treatment, cells were washed with PBS and fixed with absolute methanol for 5 min at −20°C. Cells were then incubated for 1 h with rabbit polyclonal antibody to p65 (Santa Cruz Biotechnology), rinsed three times with PBS and then incubated with fluorescein isothiocyanate-conjugated anti-rabbit IgG (Sigma, Milano, Italy) for 1 h. Cells were then rinsed three times with PBS and mounted with Prolong antifade reagent, and the fluorescence was analyzed by an Olympus BX52 (Hamburg, Germany) fluorescence microscope. The images were acquired and elaborated with IAS 2000 software (Delta Sistemi, Rome, Italy).

Results

Detection of apoptosis

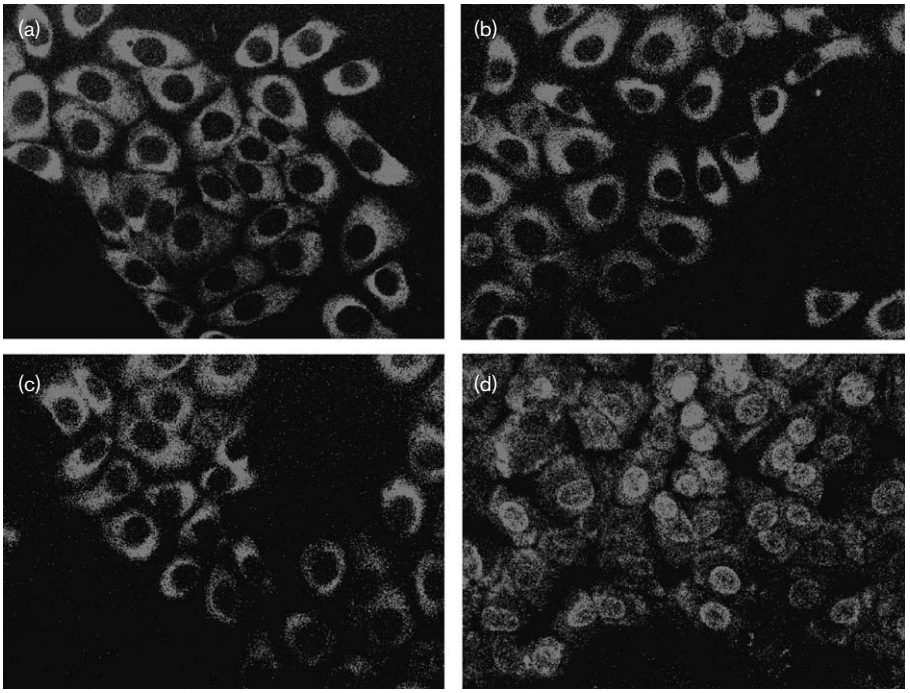
We first determined whether gemcitabine was able to affect the viability of the 5637 cell line using trypan blue dye exclusion. After testing different drug concentrations (1–1000 μmol/l), we decided to use 1 μmol/l because at this concentration the cells showed a viability of 95% that decreased to 72% after 48 h and 70% at 72 h. At this concentration, the drug was able to reduce colony formation significantly.

Flow cytometry assay showed that gemcitabine caused a significant increase in apoptotic rate of 5637 cells in a time-dependent manner (Fig. 1a). A consistent DNA fragmentation appeared after 48 and 72 h of treatment (Fig. 1b). A remarkable activation of caspase-8, -9 and -3 was detected from cells treated with 1 μmol/l gemcitabine at different time points (Fig. 1c). Caspase activation was observed after 24 h of treatment and persisted for at least 48 h.

Effects of gemcitabine on apoptotic genes profile and multidrug resistance

In the treated 5637 cell line, we analyzed the mRNA expression of several genes involved in cell cycle regulation, apoptosis and MDR. The analysis of genes involved in cell cycle progression did not demonstrate any significant increase in the expression of *p21* and *p27* after gemcitabine treatment. mRNA expression of apoptotic genes *bcl-2*, *bax*, *survivin* and *bcl-X* was not affected by

Fig. 3



Immunofluorescent detection of p65 nuclear factor-κB in untreated (a), 24 h (b) and 48 h (c) gemcitabine-treated and 48 h (d) doxorubicin-treated 5637 cell line.

treatment (data not shown). Caspase-8 being activated in treated cells, we analyzed *fas* mRNA expression in control and in gemcitabine-treated cells after 24, 48 and 72 h. *fas* resulted expressed after 24 h of treatment, persisting for 48 and 72 h, suggesting that gemcitabine-induced apoptosis in the 5637 cell line may pass through a death receptor pathway (Fig. 2a).

Among genes involved in MDR, only *mrp-4* was clearly induced by treatment. *mrp-4* mRNA expression started increasing after 9 h of treatment with gemcitabine 1 μmol/l, with a peak at 72 h (Fig. 2b). *mdr* and *mrp-1* gene expression was not affected by gemcitabine treatment.

Analogous results were obtained in clones A, B and C, in which both *fas* and *mrp-4* were found strongly expressed after treatment (data not shown).

Effects of gemcitabine on nuclear factor-kappa B activation

NF-κB was basally detected in the cytoplasm of 5637 cell line in 100% of cells; after 24 and 48 h of treatment with 1 μmol/l gemcitabine, we failed to find any nuclear localization, as shown in Fig. 3. As positive control for NF-κB translocation, we used doxorubicin-treated 5637 cells, as shown in Fig. 3(d). In clones A, B and C, we failed to find any nuclear translocation of NF-κB after retreatment with gemcitabine (data not shown).

Discussion

Few data are available concerning the modalities of gemcitabine-induced apoptosis in bladder cancer, although the use of intravesical gemcitabine entered phase II clinical trials. The objective of this study was to evaluate the mechanisms of gemcitabine-induced apoptosis in the 5637 bladder cancer cell line. Although chemotherapy-induced cell death was generally thought to depend on a pathway headed by caspase-9 activation, recent studies have focused on the role of caspase-8. [14,15]. Nevertheless, the role of caspase-8 in gemcitabine-induced apoptosis is still controversial and a better understanding of the molecular mechanism of gemcitabine is necessary to select patients for intravesical treatment. In the 5637 cell line, we observed an antiproliferative and apoptotic response after treatment; apoptosis was induced with activation of caspase-8, -3 and -9 through *fas* overexpression. As loss of *fas* has been described in many cancer cells, it is conceivable that this effect of gemcitabine may be one of the mechanisms by which this drug controls tumour progression. Our results also demonstrate that gemcitabine-induced apoptosis in the 5637 cell line does not pass through the down-regulation of *bcl-X*, *bcl-2* and *survivin*, these genes being unaffected by treatment.

We also speculated that Fas overexpression induced by gemcitabine could be dependent on NF-κB activation, as several reports have described a NF-κB-dependent, Fas-mediated apoptosis in cancer cell lines treated with gemcitabine and other antineoplastic agents [16]. Anyway, we failed to find any activation of NF-κB after 24 and 48 h of treatment, suggesting that gemcitabine-induced Fas activation and apoptosis are independent of NF-κB.

In our opinion, the observation that gemcitabine does not activate NF-κB in a bladder cancer cell line has crucial implications in cancer therapy. In fact, the nuclear translocation of NF-κB induced by doxorubicin, mitomycin C, cisplatin and other chemotherapeutic drugs may represent an undesirable effect, as NF-κB activation, through its target genes, may result in enhanced proliferation, migration, immortality and inhibition of apoptosis.

Thus, the activation of NF-κB increases resistance to apoptosis ordinarily induced by chemotherapy or radiation therapy. As some of these NF-κB-activating drugs are commonly used in intravesical treatment of SBC (e.g. doxorubicin), it should be taken into account that NF-κB-activation by these drugs may lead to the paradoxical effect of suppression of apoptosis and activation of drug resistance, through the induction, for example, of MDR family genes. Thus, NF-κB activation by the chemotherapeutic agent itself may partially account for the failure of intravesical chemotherapeutic approach, which is emerging from clinical studies. On the contrary, intravesical administration of gemcitabine, owing to its inability to activate NF-κB, should be expected to associate with a less incidence of therapy failure. In our hands, for example, no induction of *mdr* and *mrp-1*, which are NF-κB target genes, was observed after gemcitabine treatment.

Our results are in contrast with those from Delinger *et al.* [17] who found that gemcitabine induces NF-κB activation in lung cancer cell lines. Nevertheless, we could explain this discrepancy speculating that NF-κB activation by gemcitabine may be mainly dependent on the cell type.

A variety of chemotherapeutic agents have been shown to upregulate *fas* expression in cancer cell lines, and, specifically, in bladder cancer cells, adryamicin and mitomycin C are described to upregulate *fas*, thus restoring Fas-mediated cytotoxicity [18]. As downregulation of *Fas* occurs in advanced-stage bladder carcinomas [19], where it was found to be correlated with tumor stage, its upregulation by gemcitabine may represent one of the mechanisms by which the drug controls neoplastic progression, as previously described by Pace *et al.* [20] in lung cancer cell lines.

Although gemcitabine seems to share the ability to induce *fas* expression and apoptosis with other commonly used chemotherapy agents, the observation that it does not activate NF-κB may suggest its use in *fas*-negative superficial bladder tumors, although further studies are now in progress to clarify if this pathway of apoptosis induction observed in the 5637 cell line may be shared by other bladder cancer cell lines. Nevertheless, it is important to underline that the choice of the 5637 cell line was not casual, but suggested by the observation that it has the same molecular profile that we found in high-risk superficial bladder tumors [4]. Thus, the observation that gemcitabine induces apoptosis in a cell line that, from a molecular point of view, resembles high-risk SBC (5637 cell line is *bcl-2–bcl-X–survivin* expressing, with mutated p53), may suggest its efficacy in 'high-risk' SBC patients.

A further observation is that gemcitabine strongly induces *mrp-4* expression after 9 h of treatment. MRP-4 belongs to a family of ABC transporters, which was shown to confer resistance against nucleotide-based antiviral drugs as well as methotrexate. Gemcitabine-treated cells overexpress *mrp-4*, but still undergo apoptosis, thus suggesting that this ABC transporter should not be involved in gemcitabine resistance, as previously suggested by Reid *et al.* [21].

Nevertheless, the significance of this preliminary observation requires further studies.

Conclusion

In this paper, we evaluated the proapoptotic effects of gemcitabine in the 5637 cell line, derived from transitional cell carcinoma, which shares some important molecular features with 'high-risk' SBC .

Our results, which demonstrate that gemcitabine induces apoptosis through Fas upregulation without activation of NF-κB, may encourage clinical trials of intravesical gemcitabine for the treatment of SBC at high risk of relapse. To confirm our hypothesis, further studies are now in progress exploring the proapoptotic effects of gemcitabine in other transitional cell carcinoma-derived cell lines.

References

1 Bohle A, Jocham D, Bock PR. Intravesical Bacillus Calmette-Guerin versus mitomycin C for superficial bladder cancer. A formal meta-analysis of comparative studies on recurrence and toxicity. *J Urol* 2003; **169**:90–95.
2 Lamm DL. Intravesical therapy for superficial bladder cancer: slow but steady progress. *J Clin Oncol* 2003; **21**:4259–4260.
3 Shahin O, Thalmann GN, Rentsch C, Mazzucchelli L, Studer UE. A retrospective analysis of 153 patients treated with or without intravesical bacillus Calmette-Guerin for primary stage T1 grade 3 bladder cancer: recurrence, progression and survival. *J Urol* 2003; **169**:96–100.
4 Gazzaniga P, Gradilone A, Giuliani L, Gandini O, Silvestri I, Nofroni I, *et al.* Expression and prognostic significance of LIVIN, SURVIVIN and other apoptosis-related genes in the progression of superficial bladder cancer. *Ann Oncol* 2003; **14**:85–90.

5 Gontero P, Marini L, Frea B. Intravesical gemcitabine for superficial bladder cancer: rationale for a new treatment option. *BJU Int* 2005; **96**:970–976.
6 Kurdow R, Schniewind B, Zoefelt S, Boenicke L, Boehle AS, Dohrmann P, Kalthoff H. Apoptosis by gemcitabine in non-small cell lung cancer cell line KNS62 is induced downstream of caspase 8 and is profoundly blocked by Bcl-x_L over-expression. *Langenbeck Arch Surg* 2005; **390**:243–248.
7 Christgen M, Schniewind B, Jueschke A, Ungefroren H, Kalthoff H. Gemcitabine-mediated apoptosis is associated with increased CD95 surface expression but is not inhibited by DN-FADD in Colo357 pancreatic cancer cells. *Cancer Lett* 2005; **227**:193–200.
8 Galmarini CM, Clarke ML, Falette N, Puisieux A, Mackey JR, Dumontet C. Expression of a non-functional p53 affects the sensitivity of cancer cells to gemcitabine. *Int J Cancer* 2002; **97**:439–445.
9 Perabo FG, Lindner H, Schmidt D, Huebner D, Blatter J, Fimmers R, *et al.* Preclinical evaluation of gemcitabine/paclitaxel-interactions in human bladder cancer lines. *Anticancer Res* 2003; **23**:4805–4814.
10 El-Rayes BF, Ali S, Sarkar FH, Philip PA. Cyclooxygenase-2-dependent and -independent effects of celecoxib in pancreatic cancer cell lines. *Mol Cancer Ther* 2004; **3**:1421–1426.
11 Karin M, Greten FR. NF-kappaB: linking inflammation and immunity to cancer development and progression. *Nat Rev Immunol* 2005; **5**:749–759.
12 Jin F, Liu X, Zhou Z, Yue P, Lotan R, Khuri FR, *et al.* Activation of nuclear factor-kappaB contributes to induction of death receptors and apoptosis by the synthetic retinoid CD437 in DU145 human prostate cancer cells. *Cancer Res* 2005; **65**:6354–6363.
13 Guo LL, Xiao S, Guo Y. Activation of transcription factors NF-kappaB and AP-1 and their relations with apoptosis associated-proteins in hepatocellular carcinoma. *World J Gastroenterol* 2005; **11**:3860–3865.
14 Kalivendi SV, Konorev EA, Cunningham S, Vanamala SK, Kaji EH, Joseph J, Kalyanaraman B. Doxorubicin activates nuclear factor of activated T-lymphocytes and Fas ligand transcription: role of mitochondrial reactive oxygen species and calcium. *Biochem J* 2005; **15**:527–539.
15 Ferreira CG, Span SW, Peters GJ, Kruyt FA, Giaccone G. Chemotherapy triggers apoptosis in a caspase-8-dependent and mitochondria-controlled manner in the non-small cell lung cancer cell line NCI-H460. *Cancer Res* 2000; **60**:7133–7141.
16 Nakanishi C, Toi M. Nuclear factor-kappaB inhibitors as sensitizers to anticancer drugs. *Nat Rev Cancer* 2005; **5**:297–309.
17 Delinger CE, Rundall BK, Keller MD, Jones DR. Proteasome inhibition sensitises non small cell lung cancer to gemcitabine induced apoptosis. *Ann Thorac Surg* 2004; **78**:1207–1214.
18 Mizutani Y, Okada Y, Yoshida O, Fukumoto M, Bonavida B. Doxorubicin sensitizes human bladder carcinoma cells to Fas-mediated cytotoxicity. *Cancer* 1997; **79**:1180–1189.
19 Yamana K, Bilim V, Hara N, Kasahara T, Itoi T, Maruyama R. Prognostic impact of FAS/CD95/APO-1 in urothelial cancers: decreased expression of Fas is associated with disease progression. *Br J Cancer* 2005; **93**:544–551.
20 Pace E, Melis M, Siena L, Bucchieri F, Vignola AM, Profita M, *et al.* Effects of gemcitabine on cell proliferation and apoptosis in non-small-cell lung cancer (NSCLC) cell lines. *Cancer Chemother Pharmacol* 2000; **46**: 467–476.
21 Reid G, Wielinga P, Zelcer N, van der Heijden I, Kuil A, de Haas M, *et al.* Characterization of the transport of nucleoside analog drugs by the human multidrug resistance proteins MRP-4 and MRP-5. *Mol Pharmacol* 2003; **63**:1094–1103.