

Combination of bladder cancer-specific oncolytic adenovirus gene therapy with cisplatin on bladder cancer in vitro

Li Wang · Yunxin Zhang · Jinxia Zhao · Erlong Xiao ·
Jianzhong Lu · Shengjun Fu · Zhiping Wang

Received: 16 April 2014 / Accepted: 14 July 2014 / Published online: 2 August 2014
© International Society of Oncology and BioMarkers (ISOBM) 2014

Abstract Bladder cancer-specific oncolytic adenovirus Ad/PSCAE/UPII/E1A, carrying E1A gene regulated by human Uroplakin II (UPII) promoter and prostate stem cell antigen enhancer (PSCAE), could kill bladder tumor cells preferentially. The aim of this study was to examine the effects of Ad/PSCAE/UPII/E1A combined with cisplatin on human bladder cancer cells and to identify the underlying mechanisms. The combined effects of Ad/PSCAE/UPII/E1A and cisplatin on EJ, 5637, and BIU-87 bladder cancer cells were evaluated by MTT cell proliferation assay. Cell apoptosis was detected by flow cytometry with fluorescein isothiocyanate-conjugated annexin V (annexin V-FITC) and propidium iodide staining. The activation of the caspase pathway and the expression of Bcl-2 family proteins were determined by western blot assay. Ad/PSCAE/UPII/E1A adenovirus vector could infect bladder cancer cell lines selectively and induce growth inhibition effectively. Of note, the combination treatment of cisplatin and Ad/PSCAE/UPII/E1A could inhibit the proliferation of bladder cancer cells significantly compared with the “alone” treatment. Furthermore, Ad/PSCAE/UPII/E1A plus cisplatin combined treatment resulted in enhanced apoptosis in bladder cancer cells. The enhanced antitumor effects in vitro elicited by Ad/PSCAE/UPII/E1A plus cisplatin were closely related to

the increased Fas expression and cleavage of caspase-8 and Bid and decrease in the ratio of anti- to pro-apoptotic proteins followed by activation of caspase-9 and caspase-3, which may contribute to the activation of extrinsic and intrinsic apoptotic pathways. Our results indicate that the combination of Ad/PSCAE/UPII/E1A with cisplatin exerts a synergistic antitumor effect on human bladder cancer cells and is a potential combined treatment strategy for bladder cancer.

Keywords Bladder cancer · Gene therapy · Adenovirus · Cisplatin · Apoptosis

Introduction

Transitional cell carcinoma (TCC) of the bladder is a common cause of death of genitourinary tumours. Treatments are wide-ranging and include surgical resection, chemotherapy, radiotherapy, and immunotherapy. Meanwhile, gene therapeutic approaches to bladder cancer have shown research promise [1–4]. However, the low specificity and efficacy of gene therapy is an obstacle yet to be overcome.

Conditionally replicating adenovirus (CRAD) has been developed to reduce the side effects and obtain more beneficial efficacy [5, 6]. CRAD enables the gene therapy to induce tumor-specific cell death and amplify the oncolytic activity by replicating and spreading the surrounding cells [7–9]. The oncolytic process of the adenovirus life cycle can engender cancer cell death in a directed fashion when the gene E1A/B, a key regulator for viral replication, is modified to have a tumor-specific promoter [10, 11]. In the search for a tumor-specific adenovirus for the treatment of bladder cancer, we have constructed a bladder cancer-specific oncolytic adenovirus, Ad/PSCAE/UPII/E1A, by inserting human UPII promoter and PSCAE upstream of the E1A gene to regulate E1A gene expression, which has shown potent antitumor effects in bladder cancer cells in our previous studies [12].

Li Wang and Yunxin Zhang contributed equally to this work and are joint first authors.

L. Wang
School of Life Sciences, Lanzhou University,
Lanzhou 730000, Gansu, China

L. Wang · Y. Zhang · J. Zhao · E. Xiao · J. Lu · S. Fu · Z. Wang (✉)
Department of Urology, Institute of Urology, Key Laboratory of
Urological Diseases in Gansu Province, The Second Hospital of
Lanzhou University, Cuiyingmen No. 82, Linxia Road,
Lanzhou 730030, Gansu, China
e-mail: erywzp@lzu.edu.cn

L. Wang
Department of Pharmacology, School of Basic Medical Sciences,
Lanzhou University, Lanzhou 730000, Gansu, China

Chemotherapy is one of the most conventional therapeutic strategies for human cancers. Conventional chemotherapy agents include cisplatin, 5-fluorouracil, and adriamycin. Cisplatin, which is also named *cis*-diamminedichloroplatinum (CDDP), is deemed to be the “penicillin of cancer drugs” due to its universal, early, and effective treatment for a variety of cancers [13]. In the clinic, cisplatin is often used as part of an attractive chemotherapy regimen and is broadly used for the treatment of various human malignant tumors, including bladder cancer. However, severe side effects and drug resistance are the major clinical hurdles associated with cisplatin-based chemotherapy [14, 15]. The dose that is necessary to overcome even a small increase in cellular resistance can lead to severe cytotoxicity in normal cells. Therefore, in order to reduce drug dosage, minimize toxic side effects, and enhance therapeutic efficacy, novel therapeutic modalities are urgently needed to achieve the successful application of cisplatin in cancer therapy.

In this study, we constructed a bladder cancer-specific oncolytic adenovirus (Ad/PSCAE/UPII/E1A) that carries E1A gene under the control of human UPII promoter and PSCAE. Previous report has demonstrated that this bladder cancer-specific oncolytic adenovirus can kill bladder tumor cells preferentially [12]. In the present investigation, we evaluated whether the combination treatment of Ad/PSCAE/UPII/E1A plus cisplatin could perform robust synergistic killing in bladder cancer cells. Next, we studied the underlying molecular mechanisms of enhanced cytotoxicity induced by the combination therapy with attention to the alteration of Bcl-2 family proteins because pro- and anti-apoptotic Bcl-2 family proteins dictate the ultimate sensitivity or resistance of cancer cells to various apoptotic stimuli [16].

Materials and methods

Construction of recombinant adenoviruses

Two previously described recombinant adenovirus vectors [12] were used in this study: Ad/PSCAE/UPII/E1A, which carries the E1A gene under the control of UPII promoter and PSCAE, and Ad/PSCAE/UPII/Luc, which encodes a luciferase gene and was used as a control. All adenovirus handling, cotransfection, amplification, purification, and quantification have been described in detail in our previous studies [12]. The recombinant adenoviruses were amplified in HEK293 cells and purified by cesium chloride density gradient centrifugation. The virus titer was quantified using the standard TCID₅₀ (50 % tissue culture infective dose) assay. The MOI (multiplicity of infection, ratio of infectious virus particles to cells) was calculated from viral particle numbers.

Cell lines and cell culture

All cell lines were purchased from the American Type Culture Collection (Manassas, VA, USA). Human bladder cancer cell lines (5637, EJ, and BIU-87) were cultured in RPMI 1640 medium (Hyclone Laboratories, Logan, UT, USA), whereas human embryonic kidney cell line (HEK293) and normal human urinary cell line (SV-HUC-1) were grown in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Grand Island, NY, USA). The medium was supplemented with 10 % (vol/vol) heat-inactivated fetal bovine serum (Hyclone Laboratories), penicillin (final concentration 100 IU/ml), and streptomycin (final concentration 100 µg/ml). All cell lines were incubated at 37 °C in a humidified incubator containing 5 % carbon dioxide.

Infectivity of adenovirus

Bladder cancer cells were infected with Ad/PSCAE/UPII/E1A or Ad/PSCAE/UPII/Luc at a MOI of 10 for 24 h. The expression of adenoviral E1A in infected cells was tested using western blot analysis with anti-Ad5 E1A mouse monoclonal antibody (Abcam, Cambridge, UK).

Cell viability assay

The cytotoxic effects of recombinant adenoviruses or cisplatin on bladder cancer cells were first assessed using the MTT assay. Briefly, bladder cancer cells and human normal urinary cells SV-HUC-1 were seeded in 96-well plates (5×10^3 cells/well) and incubated for 24 h at 37 °C. Cells were then exposed to increasing concentrations of adenovirus vectors or cisplatin (Sigma, Ronkonkoma, NY, USA). After 72 h of incubation at 37 °C, 20 µl of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT; Sigma) in PBS (5 mg/ml) was added to each well, followed by an additional 4 h of incubation. Then, the supernatant was removed, and 150 µl dimethylsulfoxide (DMSO; Sigma) was subsequently added to each well and mixed thoroughly. The optical density (OD) was measured at 490 nm by a microplate reader (EXL-800, Bio-Tek Instruments, Inc., Winooski, USA). For combination therapy with adenoviruses and cisplatin, cells were seeded and infected with Ad/PSCAE/UPII/E1A or Ad/PSCAE/UPII/Luc at a MOI of 10. At 24 h postinfection, cells were then treated with cisplatin (1 µg/ml) for the indicated time points of 24, 48, and 72 h. At the indicated times posttreatment, the MTT assay was carried out. Each assay was repeated three times, each time in triplicate. Cell viability was expressed as the absorbance of the experimental group ($A_{\text{exp group}}$) compared with the absorbance of the control group (A_{control}) and was calculated as follows: $(A_{\text{exp group}}/A_{\text{control}}) \times 100$. The interaction between Ad/PSCAE/UPII/E1A and cisplatin was assessed by calculating the combination index (CI) using the following

equation as described previously [17, 18]: $CI = \text{FuAFuB}/\text{Fu(A+B)}$, where FuA represents the fraction unaffected by Ad/PSCAE/UPII/E1A alone compared with the untreated control group, FuB represents the fraction unaffected by cisplatin alone, and Fu(A+B) represents the fraction unaffected by Ad/PSCAE/UPII/E1A plus cisplatin. A value of $CI > 1$ denotes a synergistic effect between Ad/PSCAE/UPII/E1A and cisplatin, and $CI < 1$ denotes a less than additive effect.

Cell apoptosis analysis by flow cytometry

Ad/PSCAE/UPII/E1A or/and cisplatin-induced apoptosis in 5637 bladder cancer cells was measured by staining with fluorescein isothiocyanate-conjugated annexin V (annexin V-FITC) and propidium iodide (PI) using the annexin V-FITC Apoptosis Detection Kit I (BD PharMingen, San Diego, CA, USA). Briefly, cells were treated with Ad/PSCAE/UPII/Luc (10 MOI), Ad/PSCAE/UPII/E1A (10 MOI) or cisplatin (1 $\mu\text{g}/\text{ml}$) alone, Ad/PSCAE/UPII/Luc (10 MOI) plus cisplatin (1 $\mu\text{g}/\text{ml}$), or Ad/PSCAE/UPII/E1A (10 MOI) plus cisplatin (1 $\mu\text{g}/\text{ml}$). The medium containing PBS without cisplatin, Ad/PSCAE/UPII/E1A, Ad/PSCAE/UPII/Luc, cisplatin plus Ad/PSCAE/UPII/E1A, or cisplatin plus Ad/PSCAE/UPII/Luc was used as a cell control (PBS control). After 48 h, the cells were harvested, trypsinized, and washed twice with cold PBS. Then, the cells (1×10^5) were resuspended in 100 μl of $1 \times$ binding buffer and incubated with 5 μl of annexin V-FITC and 5 μl of PI at room temperature. After 15 min of incubation in the dark, 400 μl of $1 \times$ binding buffer was added, and the apoptotic cells were analyzed by flow cytometry, using a FACScan flow cytometer (BD Biosciences, San Jose, CA, USA). Apoptotic cells were defined as the population that was propidium iodide negative (indicating an intact plasma membrane) and annexin V-FITC positive [19, 20].

Western blot analysis

To elucidate the molecular mechanisms involved in Ad/PSCAE/UPII/E1A plus cisplatin-induced enhancement of growth inhibition and apoptosis in bladder cancer cells, the expression of apoptosis-related proteins in different treatments of 5637 cells was assessed by western blot analysis. Cells were treated with cisplatin (1 $\mu\text{g}/\text{ml}$), Ad/PSCAE/UPII/E1A (10 MOI), or Ad/PSCAE/UPII/E1A (10 MOI) plus cisplatin (1 $\mu\text{g}/\text{ml}$). The medium containing PBS without cisplatin, Ad/PSCAE/UPII/E1A, or cisplatin plus Ad/PSCAE/UPII/E1A was used as a cell control (PBS control). After 48 h of treatment, the 5637 cells were harvested and washed with cold PBS. Protein extraction was carried out using RIPA (Beyotime Biotechnology, Jiangsu, China) on ice. Protein concentrations were determined using a BCA Protein Assay Kit (Beyotime Biotechnology) according to the manufacturer's recommendations. Equal amounts (30 μg) of protein

from each sample were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto PVDF membranes (Millipore, Bioprocess Technology Center, Billerica, MA, USA). The membranes were blocked for 2 h at room temperature in Tris-buffered saline-Tween (TBST) containing 5 % nonfat dry milk. After washing three times with TBST, the membranes were incubated with the suitably diluted first antibodies overnight at 4 °C. Membranes were then washed with TBST again and incubated with appropriate HRP-conjugated secondary antibodies for 2 h at room temperature. The membranes underwent additional washing, and the immunoreactive bands were detected using the chemiluminescence detection kit (Beyotime Biotechnology) according to the manufacturer's protocols.

The primary antibodies specific for Fas, Bcl-2, Bcl-XL, Bax, and Bak were purchased from Abcam (Cambridge, UK). The antibodies specific for cleaved form of Bid, caspase-3, caspase-8, caspase-9, poly (ADP-ribose) polymerase (PARP), and β -actin were purchased from Cell Signaling Technology Inc. (Hertfordshire, UK). Horseradish peroxidase-conjugated anti-mouse IgG and anti-rat IgG were obtained from Beijing Zhong Shan-Golden Bridge Biological Technology Co., Ltd (Beijing, China).

Statistical analysis

The results were analyzed using SPSS 13.0 statistical software (SPSS Inc., Chicago, IL, USA) and were expressed as mean \pm SD. The significance of the difference between groups was evaluated by analysis of variance (ANOVA) and Student's *t* test. A value of $P < 0.05$ was considered as statistically significant.

Results

Infectivity of Ad/PSCAE/UPII/E1A

To assess the infectivity of oncolytic adenovirus Ad/PSCAE/UPII/E1A, bladder cancer cell lines EJ, 5637, and BIU-87 were treated with Ad/PSCAE/UPII/Luc (10 MOI), Ad/PSCAE/UPII/E1A (10 MOI), or PBS for 24 h, and western blot analysis was performed to detect the expression of adenovirus E1A protein. As shown in Fig. 1, a strong expression of E1A was found in the Ad/PSCAE/UPII/E1A-infected bladder cancer cells, but not in the Ad/PSCAE/UPII/Luc-infected and uninfected bladder cancer cells, indicating that Ad/PSCAE/UPII/E1A could efficiently infect bladder cancer cells and thereby replicate there at higher levels (Fig. 1).

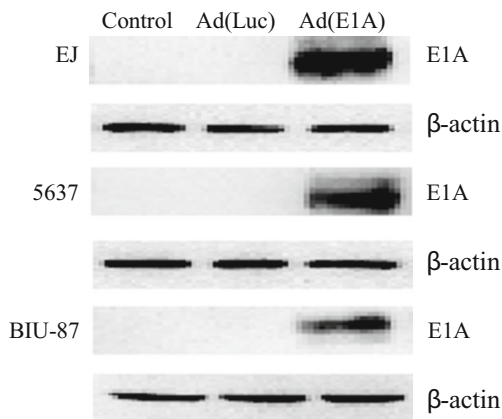


Fig. 1 The expression of E1A in EJ, 5637, and BIU-87 bladder cancer cells 24 h after infection of Ad/PSCAE/UPII/E1A or Ad/PSCAE/UPII/Luc. The cells were treated with Ad/PSCAE/UPII/Luc (10 MOI), Ad/PSCAE/UPII/E1A (10 MOI), or PBS for 24 h, and then the expression of E1A in bladder cancer cells were assessed by western blot analysis. Data shown are representative of three independent experiments. β -Actin was used as a loading control. *Ad(Luc)* Ad/PSCAE/UPII/Luc, *Ad(E1A)* Ad/PSCAE/UPII/E1A

Ad/PSCAE/UPII/E1A or cisplatin alone inhibits bladder cancer cell growth in vitro

The sensitivity of bladder cancer cells to cisplatin was first assessed by MTT assay. Cells were subjected to cisplatin at concentrations ranging from 0.5 to 8 $\mu\text{g/ml}$. As shown in Fig. 2, treatment of EJ, 5637, and BIU-87 cells with cisplatin resulted in a concentration-dependent reduction of cell viability, where a concentration of 1 $\mu\text{g/ml}$ reduced their surviving fraction to about 85 %.

We then tested the cytotoxic effects of oncolytic adenoviruses alone on the bladder cancer cells. EJ, 5637, and BIU-87 cells were infected with Ad/PSCAE/UPII/E1A or Ad/PSCAE/UPII/Luc at MOI ranging from 1 to 40. Results showed that Ad/PSCAE/UPII/E1A induced dose-dependent cell death in all tested bladder cancer cell lines (Fig. 3), whereas Ad/PSCAE/UPII/Luc did not cause detectable cytotoxic effects.

The combination of Ad/PSCAE/UPII/E1A and cisplatin synergistically suppresses bladder cancer cells growth in vitro

Next, we investigated the potential enhanced cytotoxic effect of Ad/PSCAE/UPII/E1A when used in combination with cisplatin. For combination therapies, EJ, 5637, and BIU-87 cells were first infected with Ad/PSCAE/UPII/E1A or Ad/PSCAE/UPII/Luc at a MOI of 10 for 24 h and then treated with 1 $\mu\text{g/ml}$ cisplatin. Cell survival was examined daily for 3 days using MTT assay. As shown in Fig. 4, Ad/PSCAE/UPII/E1A (10 MOI) plus cisplatin (1 $\mu\text{g/ml}$) combination treatment synergistically inhibited EJ, 5637, and BIU-87

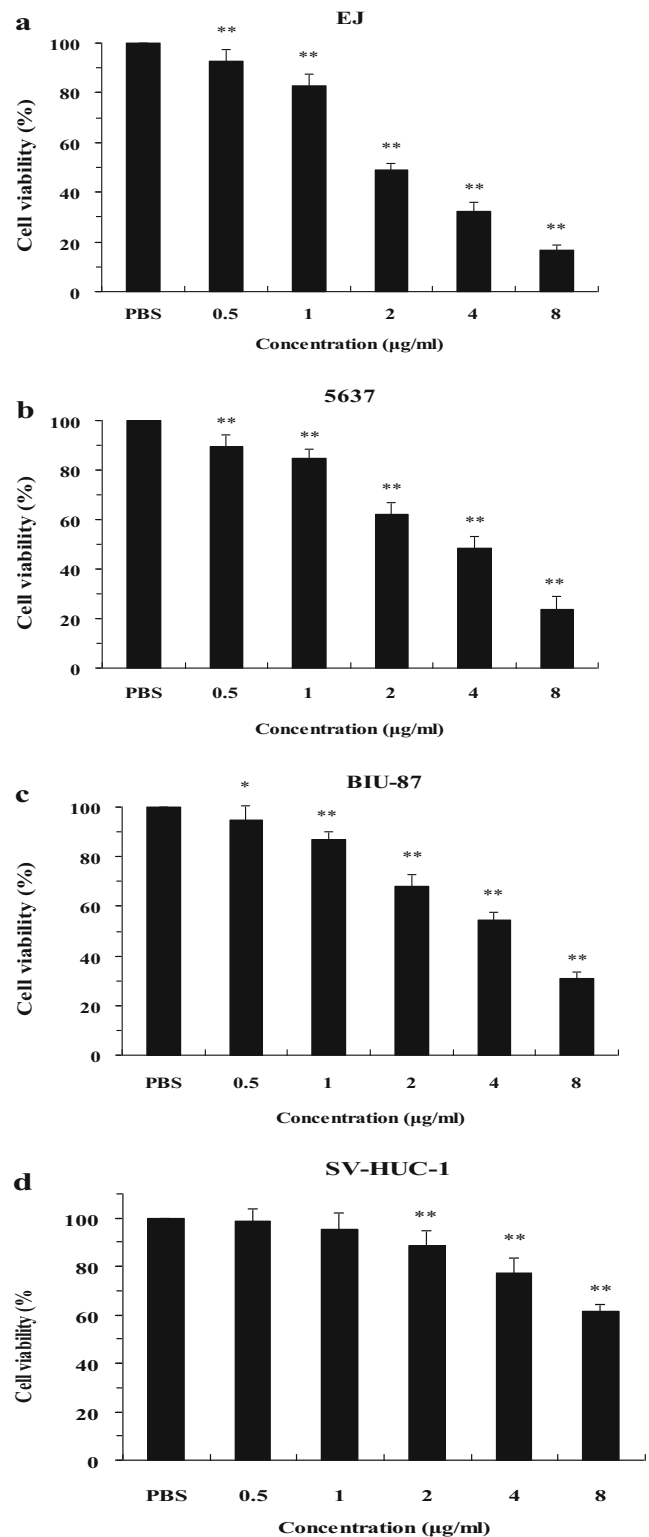


Fig. 2 The cytotoxic effects of cisplatin on EJ (a), 5637 (b), and BIU-87 (c) bladder cancer cells and SV-HUC-1 (d) normal urinary cells assessed by using the MTT assay. The data shown and bars represent the means \pm SD of three independent experiments. * $P < 0.05$, ** $P < 0.01$ compared with PBS group

bladder cancer cells growth in a time-dependent manner, compared with single Ad/PSCAE/UPII/E1A- and cisplatin-

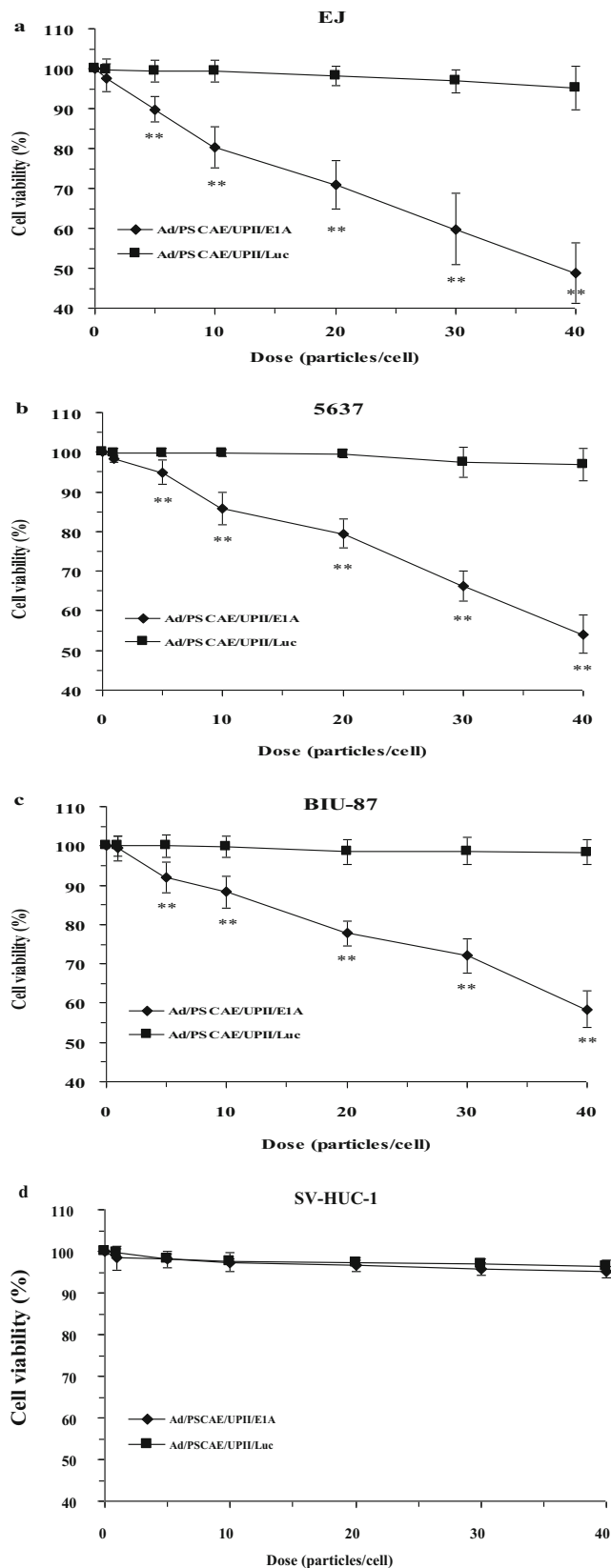


Fig. 3 The cytotoxic effects of Ad/PSCAE/UPII/E1A and Ad/PSCAE/UPII/Luc on EJ (a), 5637 (b), and BIU-87 (c) bladder cancer cells and SV-HUC-1 (d) normal urinary cells assessed by using the MTT assay. Each data *point* represents the mean of three independent experiments with standard deviation. ** $P < 0.01$ compared with Ad/PSCAE/UPII/Luc group

treated groups ($P < 0.01$; $CI > 1$). In particular, when used alone, cisplatin at 1 $\mu\text{g/ml}$ induced 17.1 % EJ cell death on day 3; Ad/PSCAE/UPII/E1A at 10 MOI induced 26.5 % cell death. In marked contrast, when 1 $\mu\text{g/ml}$ cisplatin was combined with 10 MOI Ad/PSCAE/UPII/E1A, cell death increased to 71.8 % (Fig. 4a).

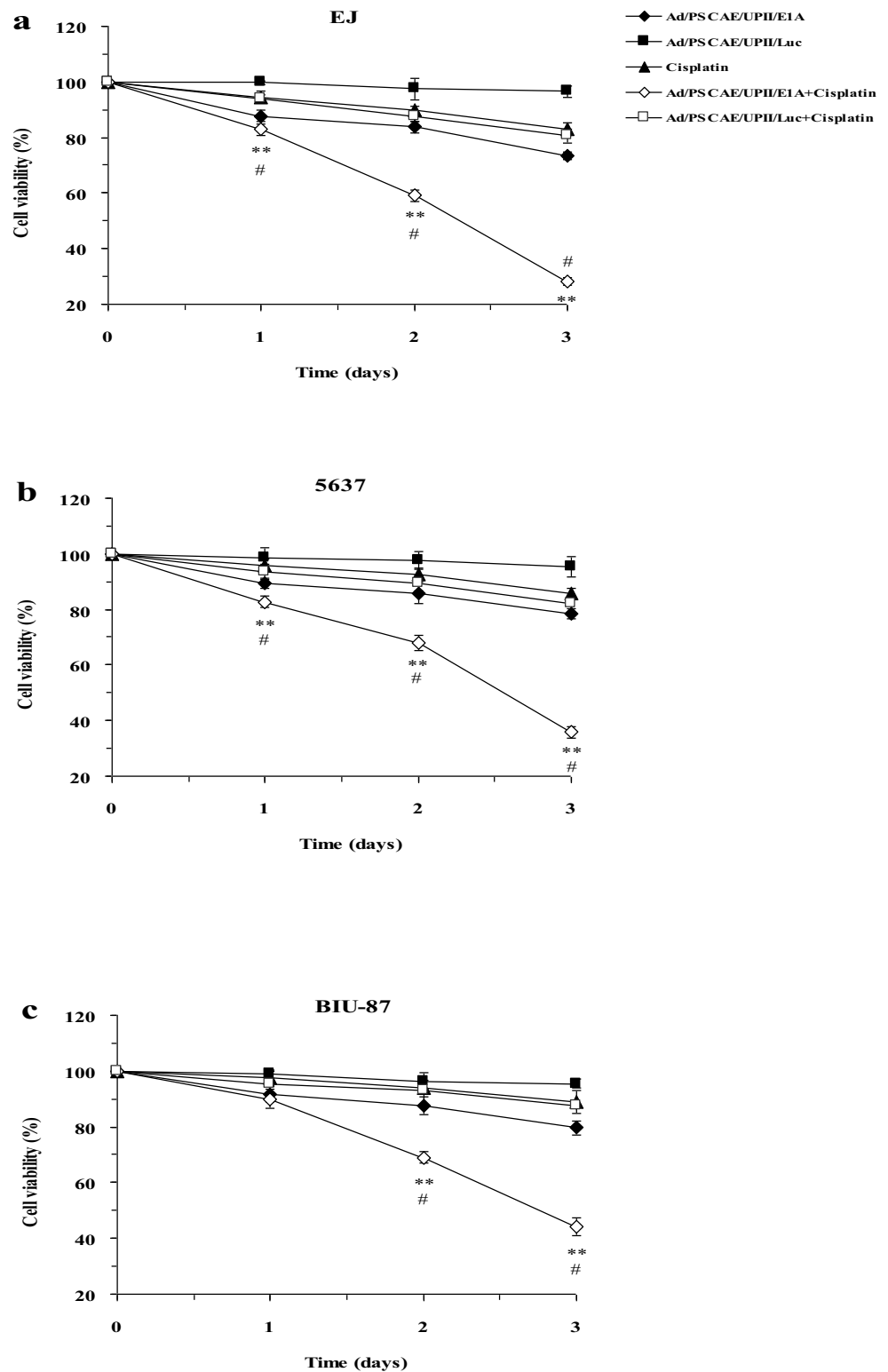
The effects of Ad/PSCAE/UPII/E1A or/and cisplatin on human normal urinary cell SV-HUC-1

As a tumor-selective replication vector, Ad/PSCAE/UPII/E1A should spare normal cells theoretically. We examined the effects of Ad/PSCAE/UPII/E1A or/and cisplatin on human normal urinary cell line SV-HUC-1. As shown in Fig. 2d, treatment of SV-HUC-1 cells with cisplatin at 2, 4, and 8 $\mu\text{g/ml}$ elicited a marked growth inhibition (11.3, 22.5, and 38.8 % reduction, respectively). In contrast, Ad/PSCAE/UPII/E1A and Ad/PSCAE/UPII/Luc at 1–40 MOI did not result in obvious cytotoxicity toward SV-HUC-1 cells (Fig. 3d, 0.4 to 5 % reduction). Furthermore, combined use of cisplatin and Ad/PSCAE/UPII/E1A had only a slightly greater cytotoxic effect compared with cisplatin alone (Fig. 5). These data suggest that Ad/PSCAE/UPII/E1A exerts selective killing activity against bladder cancer cells and is an ideal bladder cancer-specific oncolytic adenovirus for combination therapy of bladder cancer.

The combination of Ad/PSCAE/UPII/E1A and cisplatin enhances apoptosis in 5637 bladder cancer cells

To study a possible mechanism that contributes to the enhanced cytotoxicity induced by the combination of Ad/PSCAE/UPII/E1A and cisplatin, we investigated whether this combination treatment results in increased apoptosis in 5637 bladder cancer cells. Cell apoptosis was analyzed by flow cytometry. As shown in Fig. 6, the lower right quadrant (LR, PI-negative, and annexin V-FITC-positive cells) of each panel represents the early apoptotic cells. As shown in Fig. 6a–e, the early apoptotic cell fractions were 0.13, 1.75, 7.18, 8.58, and 9.25 % (lower right quadrant, LR) in 5637 bladder cancer cells treated with PBS, Ad/PSCAE/UPII/Luc, Ad/PSCAE/UPII/E1A, cisplatin, and Ad/PSCAE/UPII/Luc plus cisplatin, respectively. However, the early apoptotic cell fraction increased impressively to 33.73 % (Fig. 6f, lower right quadrant, LR) in the Ad/PSCAE/UPII/E1A plus cisplatin-treated cells. This means

Fig. 4 Synergistic induction of bladder cancer cells death by combination of Ad/PSCAE/UPII/E1A with cisplatin. Bladder cancer cell lines EJ (a), 5637 (b), and BIU-87 (c) were treated with Ad/PSCAE/UPII/Luc (10 MOI), Ad/PSCAE/UPII/E1A (10 MOI), cisplatin (1 μ g/ml), Ad/PSCAE/UPII/Luc (10 MOI) + cisplatin (1 μ g/ml), or Ad/PSCAE/UPII/E1A (10 MOI) + cisplatin (1 μ g/ml) for the indicated time periods (0–3 days), respectively. Cell viability was determined by the MTT assay. Data are presented as means \pm SD of three independent experiments. Asterisk significantly different from the treatment with Ad/PSCAE/UPII/Luc alone, Ad/PSCAE/UPII/E1A alone, cisplatin alone, and Ad/PSCAE/UPII/Luc plus cisplatin (** P <0.01). Number sign CI in the Ad/PSCAE/UPII/E1A plus cisplatin combination treatment >1

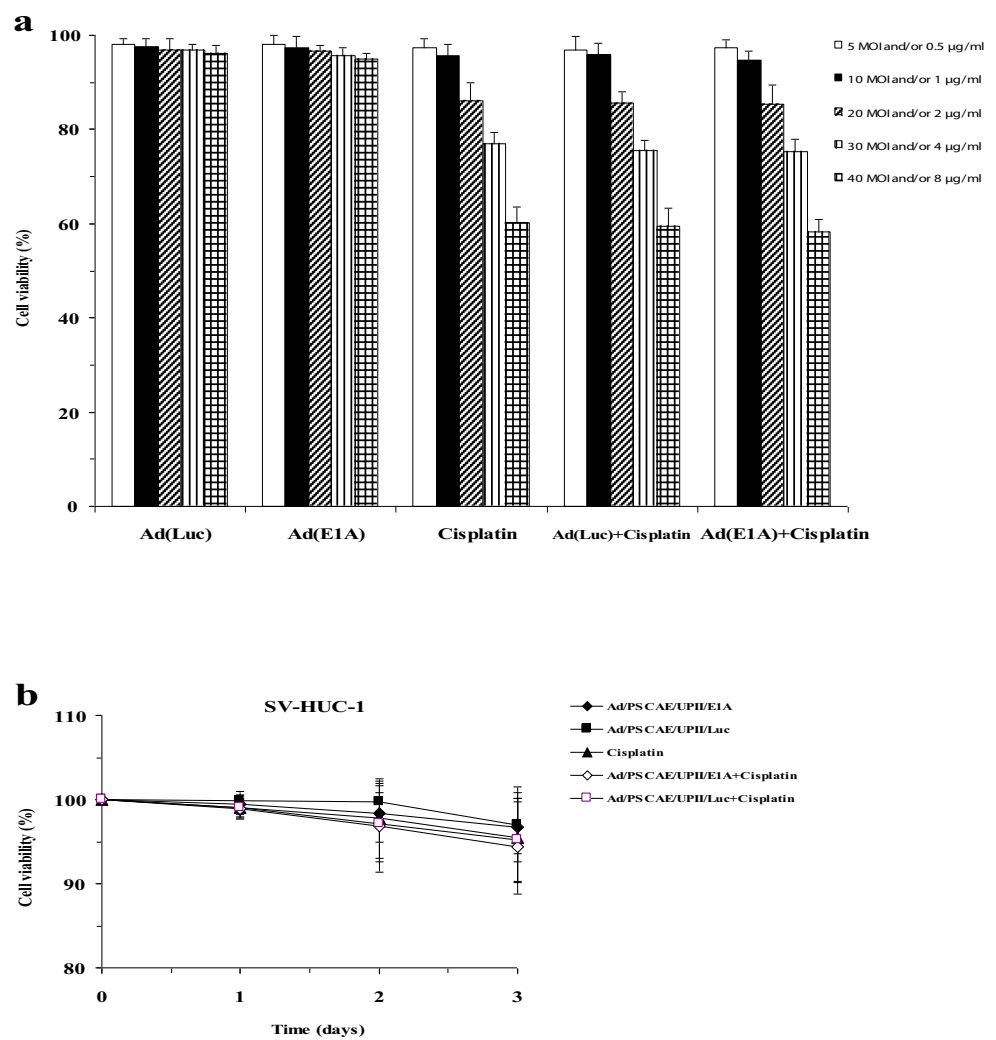


that, compared with Ad/PSCAE/UPII/E1A or cisplatin alone, their cooperation induces more 5637 cells apoptosis, which closely correlates with the Ad/PSCAE/UPII/E1A plus cisplatin-mediated in vitro synergistic growth inhibition of 5637 bladder cancer cells.

Ad/PSCAE/UPII/E1A plus cisplatin cooperatively regulates extrinsic and intrinsic apoptotic pathways

To further address the underlying molecular mechanism by which Ad/PSCAE/UPII/E1A plus cisplatin combination

Fig. 5 The cytotoxicity to human normal urinary cells SV-HUC-1 when Ad/PSCAE/UPII/Luc, Ad/PSCAE/UPII/E1A, and cisplatin were used alone or in combination. **(a)** SV-HUC-1 cells were treated with Ad/PSCAE/UPII/Luc, Ad/PSCAE/UPII/E1A, and cisplatin alone or two agents together at the dosage indicated for 72 h. Cell viability was assessed by the MTT assay. **(b)** SV-HUC-1 cells were treated with Ad/PSCAE/UPII/Luc (10 MOI), Ad/PSCAE/UPII/E1A (10 MOI), cisplatin (1 $\mu\text{g}/\text{ml}$), Ad/PSCAE/UPII/Luc (10 MOI) + cisplatin (1 $\mu\text{g}/\text{ml}$), or Ad/PSCAE/UPII/E1A (10 MOI) + cisplatin (1 $\mu\text{g}/\text{ml}$) for the indicated time periods (0–3 days). Cell viability was determined by the MTT assay. Data are presented as means \pm SD of three independent experiments. *Ad(Luc)* Ad/PSCAE/UPII/Luc, *Ad(E1A)* Ad/PSCAE/UPII/E1A



treatment results in enhanced antitumor effect, the expression of apoptosis-related proteins in 5637 bladder cancer cells with different treatments was determined by western blot analysis. As observed in Fig. 7, the expression of Fas, Bax, and Bak in Ad/PSCAE/UPII/E1A, cisplatin, and Ad/PSCAE/UPII/E1A plus cisplatin groups was significantly increased, whereas the expression of Bcl-2 and Bcl-XL was decreased, compared with the PBS control group. The significant activation of caspase-8, Bid, caspase-9, caspase-3, and PARP was also found in Ad/PSCAE/UPII/E1A, cisplatin, and Ad/PSCAE/UPII/E1A plus cisplatin groups, but not in the PBS control group. Moreover, Ad/PSCAE/UPII/E1A plus cisplatin combined treatment elicited an additive effect on the altered expression of apoptosis-related proteins involved in the activation of extrinsic and intrinsic apoptotic pathways, indicating that Ad/PSCAE/UPII/E1A plus cisplatin synergistically suppresses 5637 bladder cancer cell growth and induces apoptosis closely related to the cooperative regulation of extrinsic and intrinsic apoptotic pathways.

Discussion

Virotherapy is a cancer-specific strategy in which viruses are engineered to preferentially kill tumor cells through targeted alterations in the cancer such as p53 mutation, viral deletion, tumor-specific receptors, or tissue-specific transcriptional control, thus representing a promising approach for the treatment of human malignancies refractory to conventional therapies [21, 22]. Recently, many efforts have been made to realize tumor-specific adenoviral replication using a variety of gene promoters, including the prostate-specific antigen, osteocalcin, MUC1, midkine, L-plastin, and E2F-1 genes [23–28]. We were prompted by these studies to use the human UPII promoter and established a bladder cancer-specific oncolytic adenovirus vector (Ad/PSCAE/UPII/E1A), which contains the human UPII gene promoter and PSCAE upstream of the E1A gene in adenovirus type 5 genome. Ad/PSCAE/UPII/E1A can express E1A gene selectively in bladder cancer

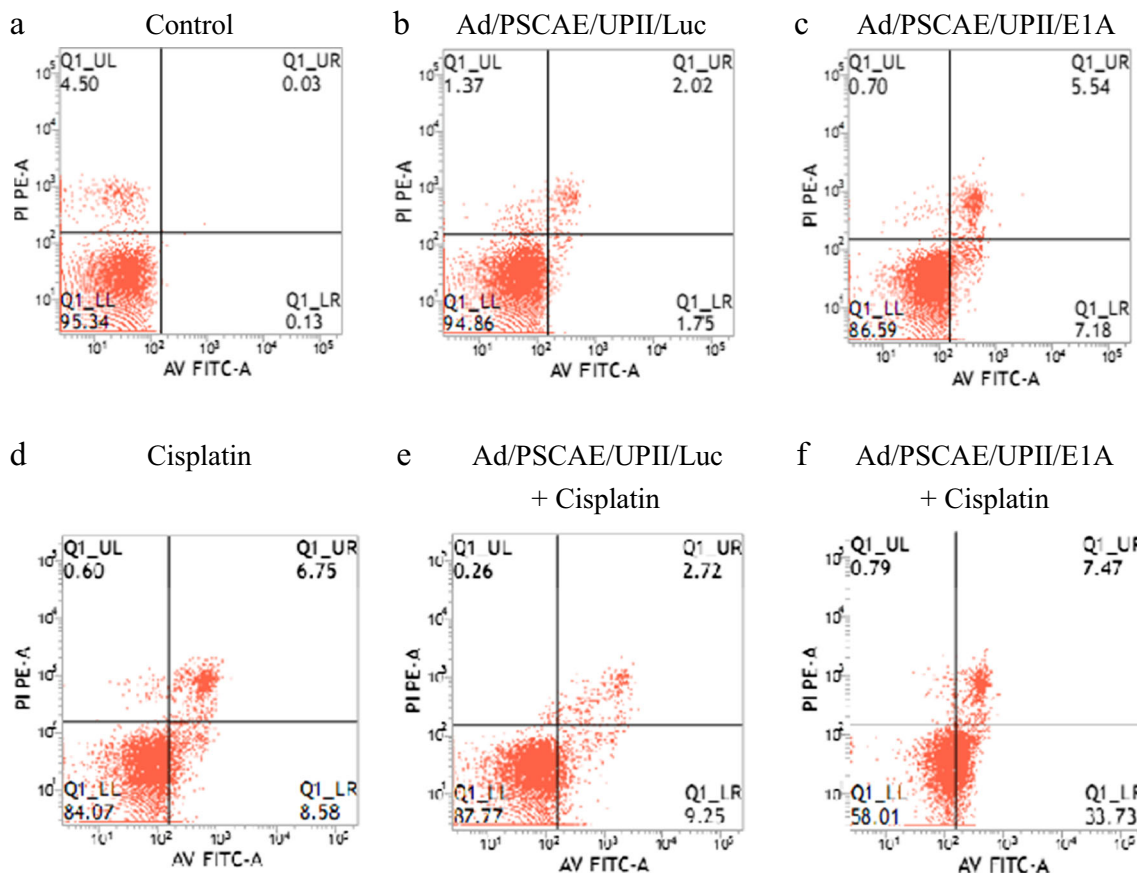


Fig. 6 Effects of Ad/PSCAE/UPII/E1A and cisplatin on 5637 bladder cancer cell apoptosis. Cells were treated with PBS (a), 10 MOI of Ad/PSCAE/UPII/Luc (b), 10 MOI of Ad/PSCAE/UPII/E1A (c), 1 μ g/ml of cisplatin (d), 10 MOI of Ad/PSCAE/UPII/Luc plus 1 μ g/ml of cisplatin (e), or 10 MOI of Ad/PSCAE/UPII/E1A plus 1 μ g/ml of cisplatin (f). At

48 h later, the cells were harvested and stained with annexin V-FITC and PI, which was immediately followed by flow cytometry analysis for apoptosis. The annexin V single-positive cells (*lower right quadrant, LR*) in the total cell population represent early apoptotic cells

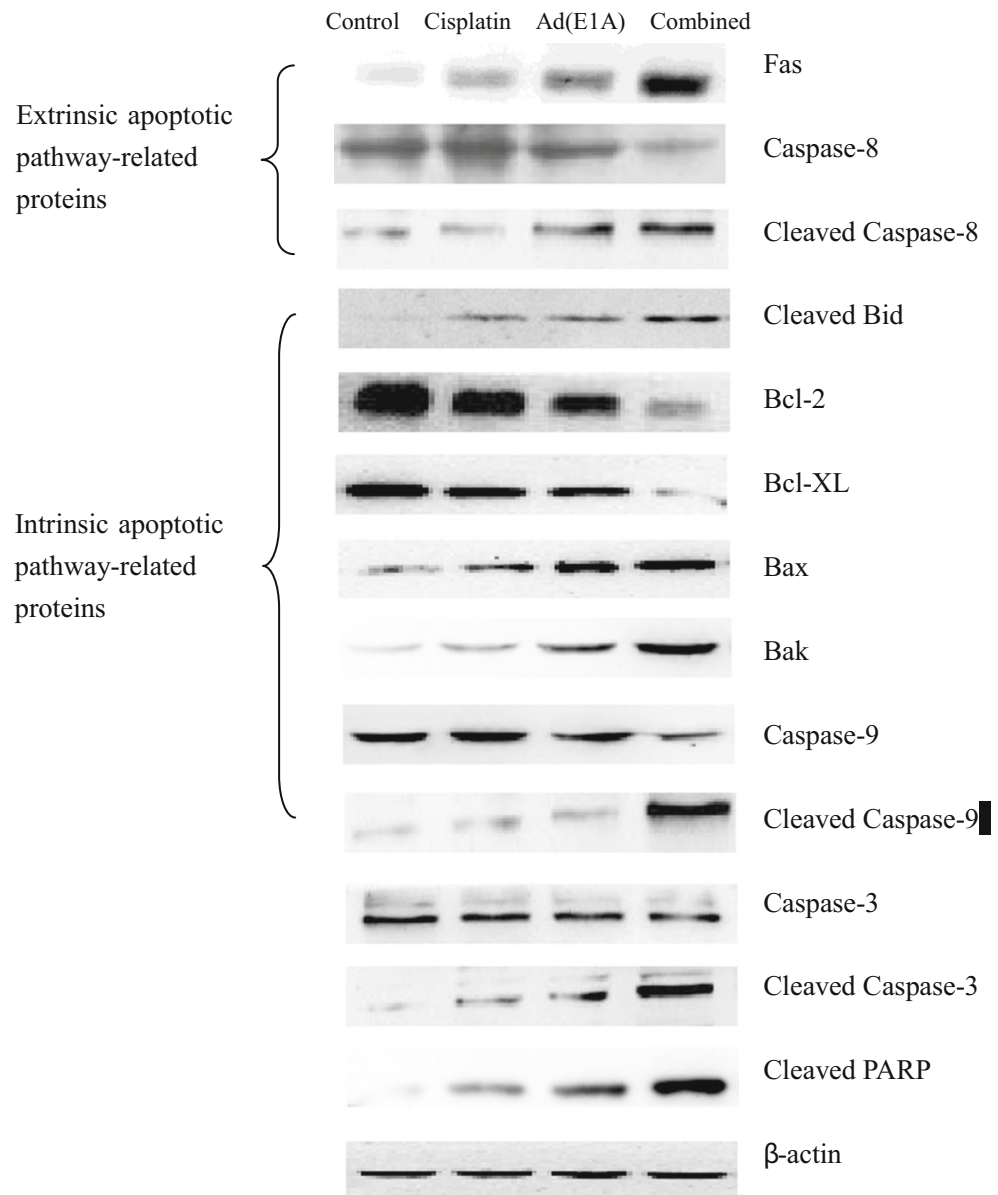
cells and thereby replicate there efficiently. This virus system does not require any specific transgenes to deliver because vigorous viral replication itself induces cell death as a result of viral toxicity. In this study, we used human bladder cancer cell lines and demonstrated that Ad/PSCAE/UPII/E1A, a new bladder cancer-specific oncolytic adenovirus, could efficiently infect bladder cancer cell lines and lead to remarkable killing of cancer cells in vitro without damage to human normal urinary cell line SV-HUC-1. These results suggest that Ad/PSCAE/UPII/E1A is a promising targeted bladder cancer-specific oncolytic adenoviral vector.

Cisplatin is a well-known chemotherapeutic agent that is broadly used for the treatment of ovarian, testicular, head and neck, bladder, lung, cervix, esophagus, stomach, endometrium, and other neoplasms [13, 29, 30]. The severe side effects encountered with cisplatin therapy include neurotoxicity and nephrotoxicity; drug resistance in response to cisplatin treatment is also a major hurdle in the clinical use of this drug [31]. Most cases of bladder cancer initially respond well to cisplatin-based

combination chemotherapy. Unfortunately, the initial response is not durable, and tumors in most patients become resistant to the agents with the progression of the disease [32, 33]. The dose escalation necessary to overcome even a small increase in cellular resistance to cisplatin can cause severe cytotoxicity to normal cells [34]. So, new approaches are urgently needed to achieve a high therapeutic response rate to cisplatin rather than the conventional approaches of increasing the drug dose or combining several kinds of chemotherapeutic drugs.

To enhance therapeutic effect, reduce side effects, and eliminate drug resistance, several conjugated strategies such as the combination of oncolytic adenoviruses with conventional chemotherapy or radiotherapy have been applied successfully in cancer therapy. Some clinical trials have demonstrated that oncolytic adenoviruses therapy combined with cisplatin yield greater antitumor effects in the patients with esophagus or head and neck cancer, without an increase in toxicity; recent studies have shown that the combination of cisplatin with oncolytic adenoviruses results in a remarkable synergistic cytotoxicity in

Fig. 7 Ad/PSCAE/UPII/E1A plus cisplatin cooperatively regulates extrinsic and intrinsic apoptotic pathways. The 5637 bladder cancer cells were treated with Ad/PSCAE/UPII/E1A (10 MOI) alone, cisplatin (1 μ g/ml) alone, Ad/PSCAE/UPII/E1A (10 MOI) plus cisplatin (1 μ g/ml), or PBS for 48 h, and then the expression of apoptosis-related proteins in 5637 bladder cancer cells was assessed by western blot analysis. Data shown are representative of three independent experiments. β -Actin was used as a loading control. *Ad(E1A)* Ad/PSCAE/UPII/E1A



human nasopharyngeal, colorectal, hepatocellular, lung, ovarian, and cervical cancer cells [30, 35–37]. In this study, we were interested in addressing the therapeutic effect of chemo-virus therapy by combining Ad/PSCAE/UPII/E1A with cisplatin on human bladder cancer cells in vitro and its potential mechanisms.

Before initiating in vitro combination therapy, we first assessed the cytotoxic effects of cisplatin or Ad/PSCAE/UPII/E1A alone on 5637, EJ, and BIU-87 human bladder cancer cells and SV-HUC-1 normal human urinary cells. Our results confirm the efficacy of Ad/PSCAE/UPII/E1A and cisplatin as monotherapy in the treatment of human bladder carcinoma. The results showed that Ad/PSCAE/UPII/E1A could significantly inhibit bladder cancer cell growth in a dose-dependent manner. However, Ad/

PSCAE/UPII/E1A had only a minimal effect on SV-HUC-1 human normal urinary cells, indicating that bladder cancer-specific oncolytic adenovirus Ad/PSCAE/UPII/E1A exerts selective tumor-killing activity in bladder cancer cells. Therapeutic effects of Ad/PSCAE/UPII/E1A varied between cell lines, most likely not only due to the differences in CAR expression with resulting variation in transduction efficiency but also due to the differences in growth rate and cell metabolism. In addition, as expected, cisplatin induced concentration-dependent growth inhibition in 5637, EJ, and BIU-87 bladder cancer cells or SV-HUC-1 normal urinary cells. The higher concentrations of cisplatin (2–8 μ g/ml) showed significant growth-suppressive effects on SV-HUC-1 normal urinary cells, whereas 1 μ g/ml of cisplatin only induced slight

cytotoxicity. To minimize the toxicity to normal cells and produce only modest antitumor activity when used as monotherapy, while leaving a window for observation of additive or synergistic effects of Ad/PSCAE/UPII/E1A plus cisplatin combination treatment, we selected the relatively lower doses of 1 $\mu\text{g/ml}$ of cisplatin and 10 MOI of Ad/PSCAE/UPII/E1A in the *in vitro* combination therapy. Moreover, the sequence and timing of combination treatments are of critical importance and should be considered in mind in practical use [38]. Difficulty in theoretically predicting the effect of combination therapy arises from the fact that the appropriate sequence of drugs may lead to synergism, while the reverse sequence using the same drugs may result in antagonism [39–41]. The phase of the cell cycle may influence the efficiency of adenovirus infection. Previous studies have shown that the expression of both CAR and α -v-integrins increases during the M phase [42]. Paclitaxel, which induces G2-phase/M-phase cell cycle arrest [43], was found to have a positive influence on adenovirus transduction in lung carcinoma cells [44]. Negative influence was observed when ovarian cancer cells were pre-treated with cytotoxic chemotherapy and then treated with tk-GCV gene therapy, while no interference was observed when gene therapy was followed by chemotherapy [45]. Therefore, administering chemotherapeutic agents prior to or during the virotherapy may negatively affect the outcomes of oncolytic adenoviruses therapy. Cisplatin, acting as a cytotoxic agent, interacts with DNA to produce intrastrand crosslinking through platination of DNA, leading to cell death [46]. The effects on crosslinking are not cell cycle specific but most pronounced during the S phase [47, 48]. In order to minimize the potential negative influence of chemotherapy on virotherapy and obtain the combination benefit, we performed virotherapy prior to chemotherapy and allowed for 24 h of undisturbed viruses infection and replication. Our *in vitro* data showed that Ad/PSCAE/UPII/E1A plus cisplatin combination treatment selectively exerted a synergistic cytotoxic effect in bladder cancer cells, but not in SV-HUC-1 normal urinary cells. These results suggest that Ad/PSCAE/UPII/E1A in combination with standard chemotherapeutic agent cisplatin may lead to enhanced chemosensitivity and probably greater tumor killing. The results also suggest that using a combination of Ad/PSCAE/UPII/E1A and cisplatin may offer a means of reducing the therapeutic doses of cisplatin and thus limiting its unwanted side effects in clinical.

Since classical apoptosis has been presumed as the mechanism of adenovirus-induced cell death [49], we then investigated the role of apoptosis in cytotoxicity resulting from the combination treatment. Our data showed that treatment of 5637 bladder cancer cells with Ad/PSCAE/UPII/E1A plus cisplatin resulted in more pronounced enhancement of

apoptosis. To further clarify the underlying mechanisms involved in Ad/PSCAE/UPII/E1A plus cisplatin-induced enhanced antitumor activity, the expression of apoptosis-related molecules such as Fas, caspase-8, Bid, Bax, Bak, Bcl-2, Bcl-XL, caspase-9, caspase-3, and PARP in different treatments of 5637 bladder cancer cells was assessed by western blot analysis. Fas as a critical apoptotic marker has been shown to regulate FasL–Fas extrinsic apoptotic pathway. Associated with the increase in Fas expression is the cleavage of caspase-8, downstream target of Fas, which has a key role in the extrinsic apoptotic pathway [50]. Moreover, caspase-8-mediated cleavage of Bid, which translocates to the mitochondria and activates Bax and Bak, also leads to the activation of intrinsic apoptotic pathway [51–53]. As we have known, apoptosis is regulated in part by the Bcl-2 protein family which consist of proapoptotic (Bak, Bax) and antiapoptotic (Bcl-2, Bcl-XL, Mcl-1, etc.) proteins [54]. The ratio of anti- to proapoptotic proteins constitutes a rheostat that sets the threshold of susceptibility to apoptosis for the intrinsic apoptotic pathway that promotes the pore formation in mitochondrial outer membrane, leading to the lesion of mitochondrial integrity and the release of cytochrome c followed by the activation of caspase-9 and caspase-3 and the cleavage of downstream substrate PARP [55, 56]. Our western blot results showed that Ad/PSCAE/UPII/E1A plus cisplatin combination therapy elicited an increased upregulation of Fas, cleaved caspase-8, Bid, Bax, Bak, cleaved caspase-9, caspase-3 and cleaved PARP, and downregulation of Bcl-2 and Bcl-XL in 5637 bladder cancer cells, leading to the cooperative activation of extrinsic and intrinsic apoptotic pathways, which may closely account for the Ad/PSCAE/UPII/E1A plus cisplatin-mediated synergistic growth inhibition and apoptosis in bladder cancer cells.

In summary, the present study demonstrated that Ad/PSCAE/UPII/E1A, a new bladder cancer-specific oncolytic adenovirus, could infect and kill bladder cancer cells effectively and selectively. We showed that Ad/PSCAE/UPII/E1A plus cisplatin combined treatment resulted in synergistic growth inhibition and enhanced apoptosis in bladder cancer cell lines, but without overlapping toxicities in normal cells. The enhanced antitumor effect *in vitro* elicited by Ad/PSCAE/UPII/E1A plus cisplatin was tightly associated with the increased Fas expression and cleavage of caspase-8 and Bid and decrease in the ratio of anti- to pro-apoptotic proteins followed by activation of caspase-9 and caspase-3, which may contribute to the activation of extrinsic and intrinsic apoptotic pathways. These results indicate that Ad/PSCAE/UPII/E1A plus cisplatin is a potential combined treatment strategy for bladder cancer.

Acknowledgments This work was supported by the National High-tech R&D Program of China (863 Program) (Grant No. 2008AA02Z421) and National Natural Science Foundation of China (Grant No. 30772177/H1619).

Conflicts of interest None

References

- Inoue K, Perrotte P, Wood CG, Slaton JW, Sweeney P, Dinney CP. Gene therapy of human bladder cancer with adenovirus-mediated antisense basic fibroblast growth factor. *Clin Cancer Res.* 2000;6:4422–31.
- Pan CX, Koeneman KS. A novel tumor-specific gene therapy for bladder cancer. *Med Hypotheses.* 1999;53:130–5.
- Kleinerman DI, Dinney CP, Zhang WW, Lin SH, Van NT, Hsieh JT. Suppression of human bladder cancer growth by increased expression of C-CAM1 gene in an orthotopic model. *Cancer Res.* 1996;56:3431–5.
- Burke JM, Lamm DL, Meng MV, Nemunaitis JJ, Stephenson JJ, Arseneau JC, et al. A first in human phase I study of CG0070, a GM-CSF expressing oncolytic adenovirus, for the treatment of nonmuscle invasive bladder cancer. *J Urol.* 2012;188:2391–7.
- Kim D. Clinical research results with dl1520 (Onyx-015), a replication-selective adenovirus for the treatment of cancer: what have we learned? *Gene Ther.* 2001;8:89–98.
- Ganly I, Kim D, Eckhardt G, Rodriguez GI, Soutar DS, Otto R, et al. A phase I study of Onyx-015, an E1B attenuated adenovirus, administered intratumorally to patients with recurrent head and neck cancer. *Clin Cancer Res.* 2000;6:798–806.
- Biederer C, Ries S, Brandts CH, McCormick F. Replication-selective viruses for cancer therapy. *J Mol Med (Berl).* 2002;80:163–75.
- Ring CJ. Cytolytic viruses as potential anti-cancer agents. *J Gen Virol.* 2002;83:491–502.
- Heise C, Kim DH. Replication-selective adenoviruses as oncolytic agents. *J Clin Invest.* 2000;105:847–51.
- Howe JA, Demers GW, Johnson DE, Neugebauer SE, Perry ST, Vaillancourt MT, et al. Evaluation of E1-mutant adenoviruses as conditionally replicating agents for cancer therapy. *Mol Ther.* 2000;2:485–95.
- Hallenbeck PL, Chang YN, Hay C, Golightly D, Stewart D, Lin J, et al. A novel tumor-specific replication-restricted adenoviral vector for gene therapy of hepatocellular carcinoma. *Hum Gene Ther.* 1999;10:1721–33.
- Zhai ZX, Wang ZP, Fu SJ, Lu J, Wang F, Li R, et al. Antitumor effects of bladder cancer-specific adenovirus carrying E1A-androgen receptor in bladder cancer. *Gene Ther.* 2012;19:1065–74.
- Kelland L. The resurgence of platinum-based cancer chemotherapy. *Nat Rev Cancer.* 2007;7:573–84.
- Morton RP, Rugman F, Dorman EB, Stoney PJ, Wilson JA, McCormick M, et al. Cisplatin and bleomycin for advanced or recurrent squamous cell carcinoma of the head and neck: a randomized factorial phase III controlled trial. *Cancer Chemother Pharmacol.* 1985;15:283–9.
- Williams SD, Birch R, Einhorn LH, Irwin L, Greco FA, Loehrer PJ. Treatment of disseminated germ-cell tumors with cisplatin, bleomycin and either vinblastine or etoposide. *N Engl J Med.* 1987;316:1435–40.
- Reed JC. Mechanisms of apoptosis. *Am J Pathol.* 2000;157:1415–30.
- Dings RP, Yokoyama Y, Ramakrishnan S, Griffioen AW, Mayo KH. The designed angiostatic peptide anginex synergistically improves chemotherapy and antiangiogenesis therapy with angiostatin. *Cancer Res.* 2003;63:382–5.
- Xie Y, Sheng W, Miao J, Xiang J, Yang J. Enhanced antitumor activity by combining an adenovirus harboring ING4 with cisplatin for hepatocarcinoma cells. *Cancer Gene Ther.* 2011;18:176–88.
- Fadok VA, Voelker DR, Campbell PA, Cohen JJ, Bratton DL, Henson PM. Exposure of phosphatidyl serine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages. *J Immunol.* 1992;148:2207–16.
- Zamai L, Falcieri E, Marhefka G, Vitale M. Supravital exposure to propidium iodide identifies apoptotic cells in the absence of nucleosomal DNA fragmentation. *Cytometry.* 1996;23:303–11.
- Chu RL, Post DE, Khuri FR, Van Meir EG. Use of replicating oncolytic adenoviruses in combination therapy for cancer. *Clin Cancer Res.* 2004;10:5299–312.
- Kim D, Martuza RL, Zwiebel J. Replication-selective virotherapy for cancer: biological principles, risk management and future directions. *Nat Med.* 2001;7:781–7.
- Rodriguez R, Schuur ER, Lim HY, Henderson GA, Simons JW, Henderson DR. Prostate attenuated replication competent adenovirus (ARCA) CN706: a selective cytotoxic for prostate-specific antigen-positive prostate cancer cells. *Cancer Res.* 1997;57:2559–63.
- Matsubara S, Wada Y, Gardner TA, Egawa M, Park MS, Hsieh CL, et al. A conditional replication-competent adenoviral vector, Ad-OC-E1a, to cotarget prostate cancer and bone stroma in an experimental model of androgen-independent prostate cancer bone metastasis. *Cancer Res.* 2001;61:6012–9.
- Kurihara T, Brough DE, Kovacs I, Kufe DW. Selectivity of a replication-competent adenovirus for human breast carcinoma cells expressing the MUC1 antigen. *J Clin Invest.* 2000;106:763–71.
- Adachi Y, Reynolds PN, Yamamoto M, Wang M, Takayama K, Matsubara S, et al. A midkine promoter-based conditionally replicative adenovirus for treatment of pediatric solid tumors and bone marrow tumor purging. *Cancer Res.* 2001;61:7882–8.
- Peng XY, Won JH, Rutherford T, Fujii T, Zelterman D, Pizzorno G, et al. The use of the L-plastin promoter for adenoviral-mediated, tumor-specific gene expression in ovarian and bladder cancer cell lines. *Cancer Res.* 2001;61:4405–13.
- Tsukuda K, Wiewrodt R, Molnar-Kimber K, Jovanovic VP, Amin KM. An E2F-responsive replication-selective adenovirus targeted to the defective cell cycle in cancer cells: potent antitumoral efficacy but no toxicity to normal cell. *Cancer Res.* 2002;62:3438–47.
- Go RS, Adjei AA. Review of the comparative pharmacology and clinical activity of cisplatin and carboplatin. *J Clin Oncol.* 1999;17:409–22.
- Pan QW, Liu BS, Liu J, Cai R, Wang Y, Qian C. Synergistic induction of tumor cell death by combining cisplatin with an oncolytic adenovirus carrying TRAIL. *Mol Cell Biochem.* 2007;304:315–23.
- Deng XY, Kim M, Vandier D, Jung YJ, Rikiyama T, Sgagias MK, et al. Recombinant adenovirus-mediated p14^{ARF} overexpression sensitizes human breast cancer cells to cisplatin. *Biochem Biophys Res Commun.* 2002;296:792–8.
- Sternberg CN, Yagota A, Scher HI, Watson RC, Ahmed T, Weiselberg LR, et al. Preliminary results of M-VAC (methotrexate, vinblastin, doxorubicin and cisplatin) for transitional carcinoma of the urothelium. *J Urol.* 1985;133:403–7.
- Sternberg CN, Yagota A, Scher HI, Watson RC, Geller N, Herr HW, et al. Methotrexate, doxorubicin and cisplatin for advanced transitional cell carcinoma of the urothelium: efficacy and patterns of response and relapse. *Cancer.* 1989;64:2448–53.
- Arafa e-SA, Zhu Q, Barakat BM, Wani G, Zhao Q, El-Mahdy MA, et al. Tangeretin sensitizes cisplatin-resistant human ovarian cancer cells through downregulation of phosphoinositide 3-kinase/Akt signaling pathway. *Cancer Res.* 2009;69:8910–7.
- Cheong SC, Wang Y, Meng JH, Hill R, Sweeney K, Kim D, et al. E1A-expressing adenoviral E3B mutants act synergistically with chemotherapeutics in immunocompetent tumor models. *Cancer Gene Ther.* 2008;15:40–50.
- Takakura M, Nakamura M, Kyo S, Hashimoto M, Mori N, Ikoma T, et al. Intraperitoneal administration of telomerase-specific oncolytic adenovirus sensitizes ovarian cancer cells to cisplatin and affects survival in a xenograft model with peritoneal dissemination. *Cancer Gene Ther.* 2010;17:11–9.
- Wu YM, Zhang KJ, Yue XT, Wang YQ, Yang Y, Li GC, et al. Enhancement of tumor cell death by combining cisplatin with an oncolytic adenovirus carrying MDA-7/IL-24. *Acta Pharmacol Sin.* 2009;30:467–77.
- Freund CTF, Tong XW, Rowley D, Engehausen D, Frolov A, Kieback DG, et al. Combination of adenovirus-mediated thymidine

- kinase gene therapy with cytotoxic chemotherapy in bladder cancer in vitro. *Urol Oncol.* 2003;21:197–205.
39. Parker RJ, Dabholkar MD, Lee KB, Bostick-Bruton F, Reed E. Taxol effect on cisplatin sensitivity and cisplatin cellular accumulation in human ovarian cancer cells. *J Natl Cancer Inst Monogr.* 1993;15:83–8.
 40. Milross CG, Peters LJ, Hunter NR, Mason KA, Milas L. Sequence-dependent antitumor activity of paclitaxel (taxol) and cisplatin in vivo. *Int J Cancer.* 1995;62:599–604.
 41. Smorenburg CH, Sparreboom A, Bontenbal M, Verweij J. Combination chemotherapy of the taxanes and antimetabolites: its use and limitations. *Eur J Cancer.* 2001;37:2310–23.
 42. Seidman MA, Hogan SM, Wendland RL, Worgall S, Crystal RG, Leopold PL. Variation in adenovirus receptor expression and adenovirus vector-mediated transgene expression at defined stages of the cell cycle. *Mol Ther.* 2001;4:13–21.
 43. Rowinsky EK, Donehower RC. Paclitaxel (taxol). *N Engl J Med.* 1995;332:1004–14.
 44. Seidman MA. Enhanced transgene delivery by adenovirus vectors in the presence of paclitaxel: potential mechanistic synergy between pharmaceutical and genetic therapies. In: *American Society of Gene Therapy 2nd Annual Meeting 1999.* Washington: American Society of Gene Therapy; 1999.
 45. Tong X, Shine DH, Agoulnik I, Freund CT, Hasenburger A, Aguilar-Cordova E, et al. Adenovirus mediated thymidine kinase gene therapy may enhance sensitivity of ovarian cancer cells to chemotherapeutic agents. *Anticancer Res.* 1998;18:3421–6.
 46. Cohen SM, Lippard SJ. Cisplatin: from DNA damage to cancer chemotherapy. *Prog Nucleic Acid Res Mol Biol.* 2001;67:93–130.
 47. Calvert H, Judson I, van der Vijgh WJ. Platinum complexes in cancer medicine: pharmacokinetics and pharmacodynamics in relation to toxicity and therapeutic activity. *Cancer Surv.* 1993;17:189–217.
 48. Reedijk J, Lohman PH. Cisplatin: synthesis, antitumor activity and mechanism of action. *Pharm Weekbl Sci.* 1985;7:173–80.
 49. Baird SK, Aerts JL, Eddaoudi A, Lockley M, Lemoine NR, McNeish IA. Oncolytic adenoviral mutants induce a novel mode of programmed cell death in ovarian cancer. *Oncogene.* 2008;27:3081–90.
 50. Nagata S. Fas ligand-induced apoptosis. *Annu Rev Genet.* 1999;33:29–55.
 51. Li H, Zhu H, Xu CJ, Yuan J. Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. *Cell.* 1998;94:491–501.
 52. Luo X, Budihardjo I, Zou H, Slaughter C, Wang X. Bid, a Bcl2 interacting protein, mediates cytochrome c release from mitochondria in response to activation of cell surface death receptors. *Cell.* 1998;94:481–90.
 53. Kuwana T, Bouchier-Hayes L, Chipuk JE, Bonzon C, Sullivan BA, Green DR, et al. BH3 domains of BH3-only proteins differentially regulate Bax-mediated mitochondrial membrane permeabilization both directly and indirectly. *Mol Cell.* 2005;17:525–35.
 54. Green DR, Kroemer G. The pathophysiology of mitochondrial cell death. *Science.* 2004;305:626–9.
 55. Oltvai ZN, Millman CL, Korsmeyer SJ. Bcl-2 heterodimerizes in vivo with a conserved homolog, Bax, that accelerates programmed cell death. *Cell.* 1993;74:609–19.
 56. Chao DT, Korsmeyer SJ. BCL-2 family: regulators of cell death. *Annu Rev Immunol.* 1998;16:395–419.