Concomitant exposure of ovarian cancer cells to docetaxel, CPT-11 or SN-38 and adenovirus-mediated p53 gene therapy

Susanna Miettinen[a,b] and Timo Ylikomi[a,c]

Owing to its central role in multiple cellular functions, p53 is an attractive candidate for gene replacement therapy. We studied the role of adenovirus-mediated p53 gene (p53Ad) therapy on sensitivity of two ovarian cancer cell lines, OVCAR-3 ($p53^{mut}$) and SK-OV-3 ($p53^{wt}$), to docetaxel, CPT-11 and SN-38 exposures. Expressions of Bcl-XL, Bcl-XS, p53, Gadd45, c-fos, $p21^{waf1/cip1}$, Bax, Bcl-2 and Mcl-1 were measured after concomitant p53Ad and drug exposures. In SK-OV-3 cells containing a normal p53 gene, p53Ad alone or concomitantly with docetaxel, CPT-11 or SN-38 exposures did not have an effect on cell growth, cell cycle distribution or induction of apoptosis. In OVCAR-3 cells, p53 gene therapy inhibited the cell growth and sensitized cells to CPT-11/SN-38, but not to docetaxel. Growth inhibition and sensitization were results of G_2M cell cycle arrest and increased apoptosis. In SK-OV-3 cells, but not in OVCAR-3 cells, CPT-11/SN-38 exposures alone increased $p21^{waf1/cip1}$ expression. The p53Ad therapy induced strong $p21^{waf1/cip1}$ expression in both cell lines. In addition, the expression of Bax and expression ratios Bax/Bcl-2 and Bax/Bcl-XL increased in p53Ad-infected OVCAR-3 cells, but not in SK-OV-3 cells. These expression ratios were further increased in p53Ad + CPT-11/SN-38-exposed OVCAR-3 samples. These results support the combination of p53 gene therapy with topoisomerase I inhibitors SN-38/CPT-11 when tumour cells contain mutated p53. When p53 status is normal, p53 gene therapy is not effective alone or concomitantly with CPT-11/SN-38. Increased expression ratios of Bax/Bcl-2 and Bax/Bcl-XL might serve as positive markers for effective p53 gene therapy and concomitant topoisomerase I inhibitor therapy. *Anti-Cancer Drugs* **20:589–600 © 2009 Wolters Kluwer Health | Lippincott Williams & Wilkins.**

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Introduction

Ovarian cancer is the leading cause of death among patients bearing gynaecological malignancies [1]. The development of new treatment strategies is important towards improving the survival rate of ovarian cancer patients.

The role of tumour suppressor protein p53 in human cancer development has been extensively studied. Alterations in p53 gene are the most frequent genetic events in many cancers, and accordingly p53 abnormalities are found in 30–79% of malignant ovarian tumours [2,3]. In normal cells, p53 has a role in controlling cell cycle, apoptosis and DNA repair in response to various stress stimuli, such as DNA damage, hypoxia or oncogenic signals [4]. Mutations in p53 gene might lead to accumulation of further mutations, increased tumour growth and insensitivity to many chemotherapeutic drugs. In ovarian cancer, functionally null p53 represents an independent molecular predictor of compromised survival [5].

Upon cellular stress, such as DNA damage, p53 may induce cell cycle arrest or apoptosis. Induction of cell cycle arrest is mediated through transcriptional activation of p53 downstream effector genes, such as $p21^{waf1/cip1}$ and Gadd45. A cyclin-dependent kinase inhibitor $p21^{waf1/cip1}$ controls G_1/S and G_2M progression of the cell cycle [6,7]. Gadd45 is a genotoxic stress-responsive gene, which mediates growth suppression through the induction of cell cycle arrest in G_2M phase [8–10]. In addition to p53-dependent activation, expression of both $p21^{waf1/cip1}$ [11–13] and Gadd45 [14,15] may be induced by p53-independent mechanisms.

Activation of p53 and its downstream effectors may also cause the induction of apoptosis. The Bcl-2 gene family encodes a group of proteins that regulate programmed cell death [16]. Antiapoptotic members of this gene family, including Bcl-2, Bcl-XL and Mcl-1, promote cell survival, whereas proapoptotic members, such as Bax and Bcl-XS, promote cell death [17]. Although Bax is involved in p53-mediated apoptosis, Bax is not directly regulated by p53 [18,19].

Docetaxel, CPT-11 and its active metabolite SN-38 are anticancer drugs that induce G_2M cell cycle arrest and apoptosis [7,20–24]. CPT-11 is a semisynthetic derivative of a plant alkaloid, camptothecin, obtained from the

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Chinese tree *Camptotheca acuminata* [25,26]. Within cells, CPT-11 is converted to an active metabolite, SN-38 (7-ethyl-10-hydroxycamptothecin) [27]. CPT-11 and SN-38 interrupt DNA replication through the inhibition of topoisomerase I activity, thereby inducing single- and double-strand breaks into DNA [28]. Docetaxel is a semisynthetic taxane derivative from 10-deacetylbaccatin III, a compound extracted from the needles of the European yew *Taxus baccata* [29]. In cells, taxanes disrupt mitosis by enhancing microtubule polymerization and inhibiting depolymerization, thus inducing formation of abnormal and stable microtubule bundles [29].

Several studies have shown that growth of human cancer cell lines, including ovarian, can be suppressed by introducing the wild-type p53 gene into cancer cells [30–35]. In this report, we have studied the effect of adenovirus-mediated p53 gene therapy on growth and responsiveness of two ovarian cancer cell lines, OVCAR-3 ($p53^{mut}$) and SK-OV-3 ($p53^{wt}$), to docetaxel, CPT-11 and SN-38 exposures, and on expressions of cell survival regulators Bcl-XL, Bcl-XS, p53, Gadd45, c-fos, $p21^{waf1/cip1}$, Bax, Bcl-2 and Mcl-1.

Materials and methods

Cell culture

The human ovarian adenocarcinoma cell lines, OVCAR-3 and SK-OV-3 (ATCC, Manassas, Virginia, USA) were cultured in DMEM medium (Sigma Aldrich, St. Louis, Missouri, USA) supplemented with 10% foetal bovine serum, non-essential amino acids and antibiotics (100 IU/ml penicillin, 100 µg/ml streptomycin) at 37°C in a humidified 95% air/5% CO_2 incubator. For OVCAR-3 cells, the mutated p53 gene status is described by the manufacturer. SK-OV-3 cells have normal (wild-type) p53 gene [36].

Drug preparations

Docetaxel (Taxotere), SN-38 and CPT-11 (Irinotecan) were kindly provided by Sanofi Aventis (Bridgewater, New Jersey, USA). Docetaxel was dissolved in ethanol and both SN-38 and CPT-11 in dimethyl sulfoxide (Sigma Aldrich). Each drug was first diluted in ethanol and the final ethanol concentration in cell culture was 0.001%.

Adenovirus vectors

Adenoviral vector encoding a wild-type human p53 gene (Ad5 CMV–p53, p53Ad) was kindly provided by Sanofi Aventis. The empty adenoviral vector, Adeno-X-Null, and Adeno-X-LacZ adenovirus were obtained from BD Biosciences (Erembodegem, Belgium).

X-gal staining

X-gal staining was used to determine the infection rate of adenoviruses in SK-OV-3 and OVCAR-3 cell lines. Fifty thousand cells were plated on objective glass chambers (Lab-TekII Chamber Slide System; Nalge Nunc Inc., Illinois, USA). One day after plating, the old medium was removed and replaced with new one containing Adeno-X-LacZ viruses [multiplicity of infection (MOI) 0, 1, 5, 10, 50 and 100]. After 24-h infection, cells were washed twice with phosphate-buffered saline (PBS; pH 7.4) and fixed with 2% paraformaldehyde and 0.2% glutardialdehyde in PBS for 10 min and stained with 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-gal) solution (1 mg/ml X-gal, 4 mmol/l $K_3Fe(CN)_6$, 4 mmol/l $K_4Fe(CN)_6$, 2 mmol/l $MgCl_2$) for 2 h at 37°C. Stained cells were counted under a light microscope.

Immunocytochemistry

Immunocytochemistry was used to determine p53 expression after p53Ad infection in SK-OV-3 cell line. Fifty thousand cells were plated on objective glass chambers (Lab-TekII Chamber Slide System; Nalge Nunc Inc.). One day after plating, the old medium was removed and replaced with new one containing adenoviruses (MOI 0, 1, 5, 10, 50 and 100). After 24-h infection, cells were washed twice with PBS (pH 7.4) and fixed with 5% acetic acid in ethanol. Immunocytochemistry was performed using Histostain Plus Broad Spectrum kit (Zymed, San Francisco, California, USA). The p53 antibody (Novo Castra Laboratories, Newcastle upon Tyne, UK) was diluted 1 : 100 in sterile water, according to the manufacturer's instructions. OVCAR-3 cells were used as a positive control for p53 expression. Stained cells were counted under a light microscope.

Cell growth assay

When cell culture flasks were *ca.* 70% confluent, the cell growth assay was started and 2500 (SK-OV-3) or 10 000 (OVCAR-3) cells per well were plated on 96-well culture plates. One day after plating, the old medium was replaced by a medium containing adenoviruses (empty adenovirus vector or p53Ad). After a 24 h infection, the medium containing adenoviruses was removed. When the effect of adenoviral infection (MOI 0, 1, 5, 10, 50 or 100) on cell growth was determined, the medium containing adenovirus was replaced by the fresh one, and the samples were cultured for 5 days. When infections were combined with anticancer drugs the old medium was replaced by a medium containing docetaxel (0, 0.1, 0.5, 1, 2.5 and 5 nmol/l), CPT-11 (0, 1, 2.5, 5, 10, 25 µmol/l), SN-38 (0, 1, 2.5, 5, 10, 50 nmol/l) or vehicle and the cell growth samples were taken after a 5-day incubation period. The relative cell number was assessed using PreMix WST-1 Cell Proliferation Assay System (Takara Bio Inc., Shiga, Japan). The cell culture medium was removed, and PBS and PreMix WST-1 were added in the ratio of 10 : 1. The well plate was incubated for 4 h in + 37°C, and the relative cell number was measured in a microplate reader (Multiscan MS; Labsystems, Waltham, Massachusetts, USA) at 450 nm. At least eight

determinations were used to calculate the mean optical density in each MOI or drug concentration. The relative cell number in uninfected and untreated cells was set as 100%.

Cell cycle analysis

Cells were plated on T25 (Nalge Nunc Inc.) culture flasks (2×10^6 cells/flask). On the next day, the old medium was replaced by a medium containing p53Ad (MOI 10). After 24-h infection, the medium containing adenoviruses was removed and medium containing docetaxel (2.5 nmol/l), CTP-11 (10 μmol/l), SN-38 (20 nmol/l) or vehicle was added to cells. One half of the samples was exposed to drugs or vehicle alone without adenovirus infections. The cell cycle parameters were measured after 48 h of drug exposure. Trypsinized and floating cells were pooled, washed with PBS–EDTA, fixed with 70% (v/v) ethanol for 2 h at −20°C, and RNA was digested with RNAase (0.15 mg/ml). To assess DNA content, cells were stained with propidium iodide (Sigma Aldrich) and monitored with a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, New Jersey, USA). Cell cycle distribution was determined with ModFit LT (Verity Software House Inc., Topsham, Maine, USA).

Apoptosis measurements

The amounts of caspase-3 and caspase-7 were measured by using Caspase-Glo 3/7 Assay (Promega, Madison, Wisconsin, USA). Cells were plated on 96-well culture plates, 10 000 cells per well. On the next day the old medium was replaced by a medium containing adenoviruses (p53Ad or empty vector, MOI 10). After 24-h infection, the medium was removed and medium containing docetaxel (2.5 nmol/l), CTP-11 (10 μmol/l), SN-38 (20 nmol/l) or vehicle was added to cells. One half of the samples was exposed to drugs or vehicle alone without adenovirus infections. After 48 h incubation, Caspase-Glo 3/7 Assay reagent was added according to manufacturer's instructions. The luminescence was measured by using Multiscan MS (Labsystems). Eight parallel samples in each treatment were studied.

Ribonuclease protection assay

Cells were plated on T25 (Nalge Nunc Inc.) culture flasks (2×10^6 cells/flask). On the next day, the old medium was replaced by a medium containing adenoviruses (empty adenovirus vector or p53Ad, MOI 10). After 24-h infection, the medium containing adenoviruses was removed and medium containing docetaxel (2.5 nmol/l), CTP-11 (10 μmol/l), SN-38 (20 nmol/l) or vehicle was added to cells. After a 48-h incubation period, total RNA was extracted from cells using Trizol reagent (Invitrogen Life Technologies, Carlsbad, California, USA). Ribonuclease protection assay (RPA) was used to detect mRNAs of different cell cycle regulators. In-vitro transcription reaction kit and multiprobe template set were obtained from BD Bioscience Pharmingen (San Diego, California, USA). ^{32}P-labelled ([α-^{32}P]UTP, Amersham Bioscience, Pittsburgh, Pennsylvania, USA) RNA probes were synthesized in in-vitro transcription reaction using template set hStress-1, which generates probes for Bcl-XL, Bcl-XS, p53, Gadd45, c-fos, p21$^{waf1/cip1}$, Bax, Bcl-2, Mcl-1, glyceraldehydes-3-phosphate dehydrogenase (GAPDH) and L32. RPA (RPA III, Ambion, Austin, Texas, USA) was performed according to the manufacturer's instructions. In brief, ^{32}P-labelled RNA probes and 5 μg total RNA samples were hybridized. After an overnight hybridization, single-stranded RNA was digested and remaining double-stranded hybridization products of different lengths were separated by gel electrophoresis. An intensifying screen was exposed and scanned (Storm, Molecular Dynamics, Amersham Pharmacia Biotech). The intensities of Bcl-XL, p53, Gadd45, c-fos, p21$^{waf1/cip1}$, Bax and Mcl-1 bands were measured after 12-h exposure and the intensities of Bcl-XS and Bcl-2 after 24-h exposure. GAPDH and L32 bands were quantified after 6-h exposure. The results were obtained using computer program ImageQuant 5.1 (Molecular Dynamics, Amersham Bioscience). Intensity of GAPDH and L32 bands were used to equalize loadings between samples. Expression of studied gene is given as relative expression when compared with expression in vehicle-treated OVCAR-3 cells (set as 1).

Statistical analyses

All experiments except flow cytometry ($n = 3$) were repeated five times. The data are expressed as mean values with standard deviations. Statistical analyses of the cell growth data and apoptosis were carried out from the original data using Student's *t*-test. One-way analysis of variance followed by Bonferroni's post-hoc test (GraphPad Prism 3.03; GraphPad Software Inc., San Diego, California, USA) was carried out to study differences between gene expressions.

Results

Infection efficiency

Cell lines SK-OV-3 and OVCAR-3 were infected with adenoviruses (Adeno-X-LacZ or p53Ad) at MOI 0, 1, 5, 10, 50 or 100, and the infection efficiencies were studied using p53 immunocytochemistry and X-gal staining. As OVCAR-3 cells constantly express the mutated form of p53, immunocytochemistry was used to determine the infection rate only in SK-OV-3 cells. The p53Ad infection did not have an effect on p53 expression on immunocytochemical level in OVCAR-3 cells and the empty adenoviral vector, Adeno-X-Null did not have an effect on p53 expression in either of cell lines. We did not find a difference in infection rate between cells lines SK-OV-3 and OVCAR-3. Table 1 shows the summary of infection efficiencies in cell lines and Fig. 1a–f show the increase of p53 expression rate

Table 1 Efficiency of adenovirus infections in SK-OV-3 and OVCAR-3 cells

	SK-OV-3		OVCAR-3
MOI	p53 positive[a] cells (%)	LacZ positive cells (%)	LacZ positive cells (%)
0	0	0	0
1	12 ± 2	14 ± 3	17 ± 3
5	43 ± 3	48 ± 4	55 ± 4
10	78 ± 6	86 ± 4	91 ± 9
50	86 ± 12	100	100
100	92 ± 5	100	100

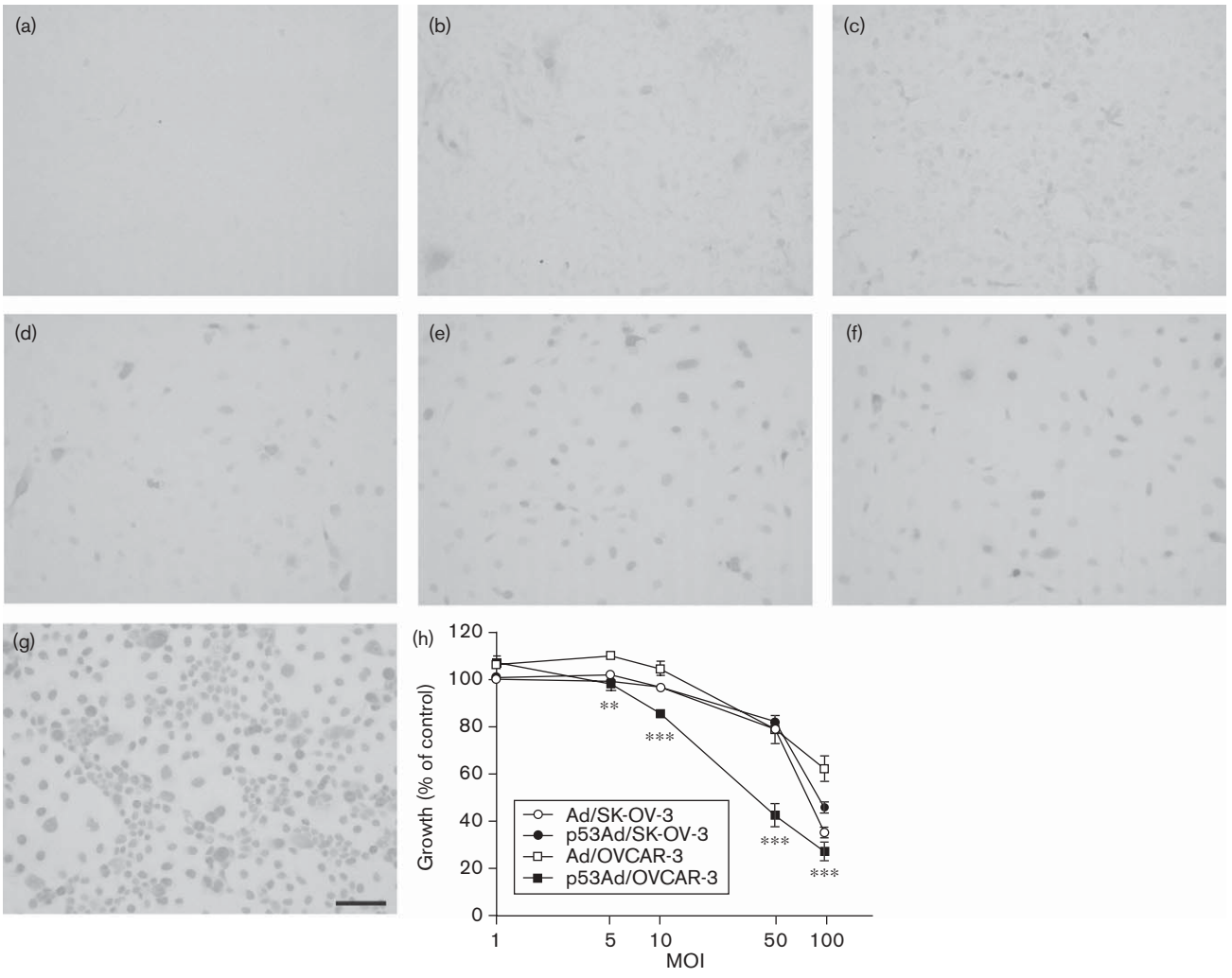
MOI, multiplicity of infection.
[a]Immunocytochemistry.

in SK-OV-3 cells after p53Ad MOI 0, 1, 5, 10, 50 or 100 infections. Figure 1g shows the constant expression of p53 in untreated OVCAR-3 cells.

Growth response to p53 gene therapy

Cell lines SK-OV-3 and OVCAR-3 were infected with adenoviruses (empty or p53Ad) at MOI 0, 1, 5, 10, 50 or 100. Figure 1h shows the growth inhibitory effect of adenovirus infections after a 5-day growth period. In SK-OV-3, the growth inhibition was as efficient in Adeno-X-Null as in p53Ad-infected samples. In OVCAR-3 cells, there was a statistically significant difference between Adeno-X-Null and p53Ad-infected samples at

Fig. 1



The expression of p53 in uninfected SK-OV-3 cells (a) and after adenovirus-mediated p53 gene (p53Ad) infections; multiplicity of infection (MOI) 1 (b), MOI 5 (c), MOI 10 (d), MOI 50 (e) and MOI 100 (f). The expression of p53 in uninfected OVCAR-3 cells is shown in (g). The scale bar in (h) is 80 μm. Effect of adenovirus infections on cell growth after a 5-day growth period (h). Cell lines SK-OV-3 and OVCAR-3 were infected with either empty (Ad) or p53Ad (p53) adenoviruses at MOI 0, 1, 5, 10, 50 or 100. The growth effect is indicated as percentage of the growth of uninfected control cells. The data represent the mean of five independent experiments ± SD. In OVCAR-3 cells, there was a statistically significant difference between empty adenovirus and p53Ad-infected samples at MOI 5 (**$P<0.001$), MOI 10 (***$P<0.0001$), MOI 50 (***$P<0.0001$) and MOI 100 (***$P<0.0001$).

MOI 5 ($P < 0.001$), MOI 10 ($P < 0.0001$), MOI 50 ($P < 0.0001$) and MOI 100 ($P < 0.0001$).

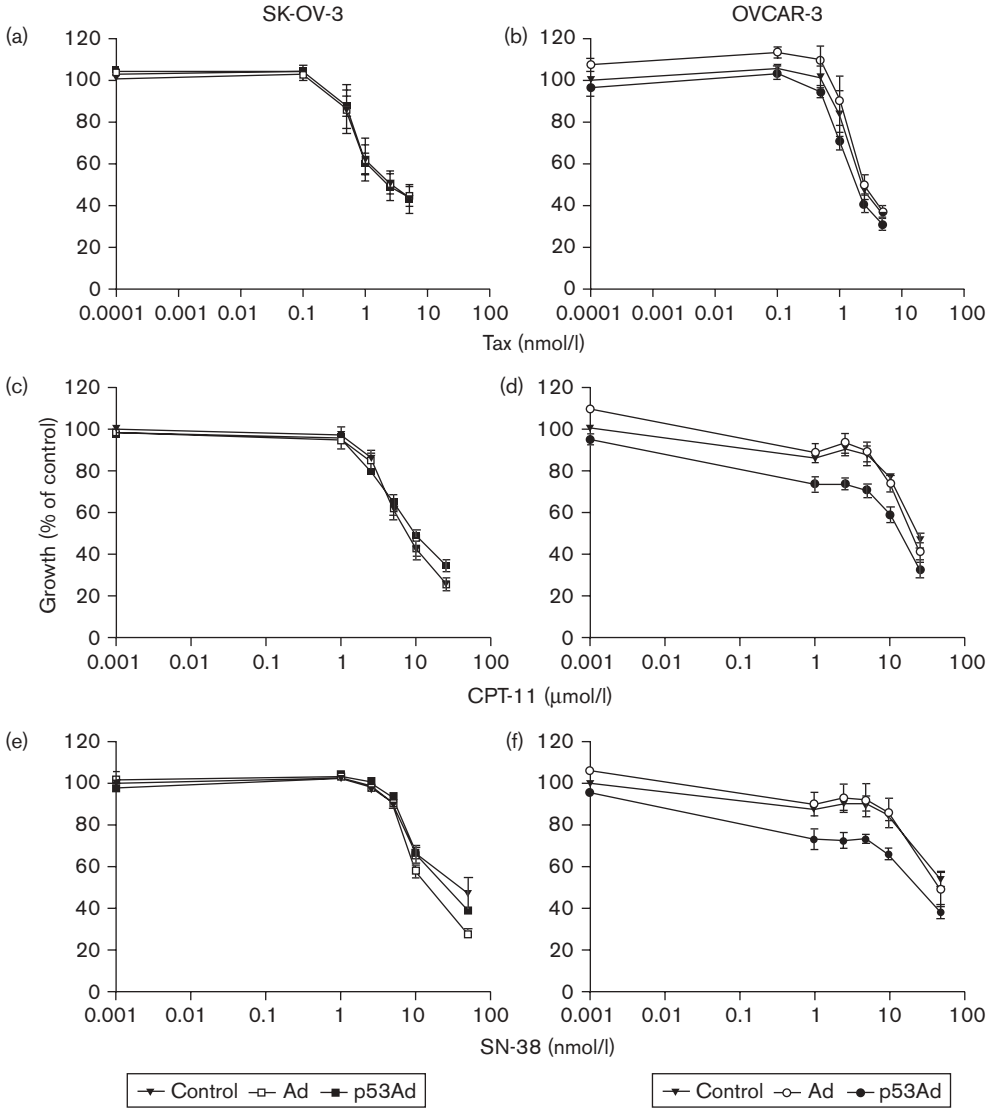
Growth response to concomitant docetaxel and p53 gene therapy

Figure 2a and b shows the effect of adenovirus infections on docetaxel-mediated growth inhibition. Infection of SK-OV-3 or OVCAR-3 cells with either p53Ad or empty adenovirus vector did not have significant effect on docetaxel-mediated growth inhibition.

Growth response to concomitant p53 gene therapy and CPT-11 or SN-38

Figure 2c and d shows the effect of adenovirus infections on CPT-11-mediated growth inhibition and Fig. 2e and f shows the effect on SN-38-mediated growth inhibition. Infection of SK-OV-3 cells with either Ad5 CMV–p53 or empty adenovirus vectors did not have significant effect on CPT-11 or SN-38-mediated growth inhibition. In OVCAR-3 cells, the differences between p53Ad and empty adenovirus-infected samples were statistically

Fig. 2



Effect of adenovirus infection on chemotherapeutic drug response. Cell lines SK-OV-3 and OVCAR-3 were infected with either empty (Ad) or p53Ad (p53) adenoviruses at multiplicity of infection 10. After adenoviral infections, cell lines were treated with docetaxel (0, 0.1, 0.5, 1, 2.5 and 5 nmol/l; a and b), CPT-11 (0, 1, 2.5, 5, 10, 25 μmol/l; c and d) or SN-38 (0, 1, 2.5, 5, 10, 50 nmol/l; e and f). Adenovirus-mediated growth effect was compared with uninfected control cells (control). Results were obtained after a 5-day treatment period. The data represent mean of five independent experiments ± SD. In OVCAR-3 cells, there were statistically significant differences between p53Ad-infected and uninfected samples when cells were exposed to CPT-11 ($P = 0.0008$) and SN-38 ($P < 0.0001$).

significant ($P = 0.0008$ for CPT-11 and $P < 0.0001$ for SN-38).

Expression of p53 and its downstream effectors

Expression of cell survival regulatory genes was measured on mRNA level using a RPA method. Figure 3 shows an example of one RPA analysis (12-h exposure) of cell lines SK-OV-3 and OVCAR-3 after indicated treatments.

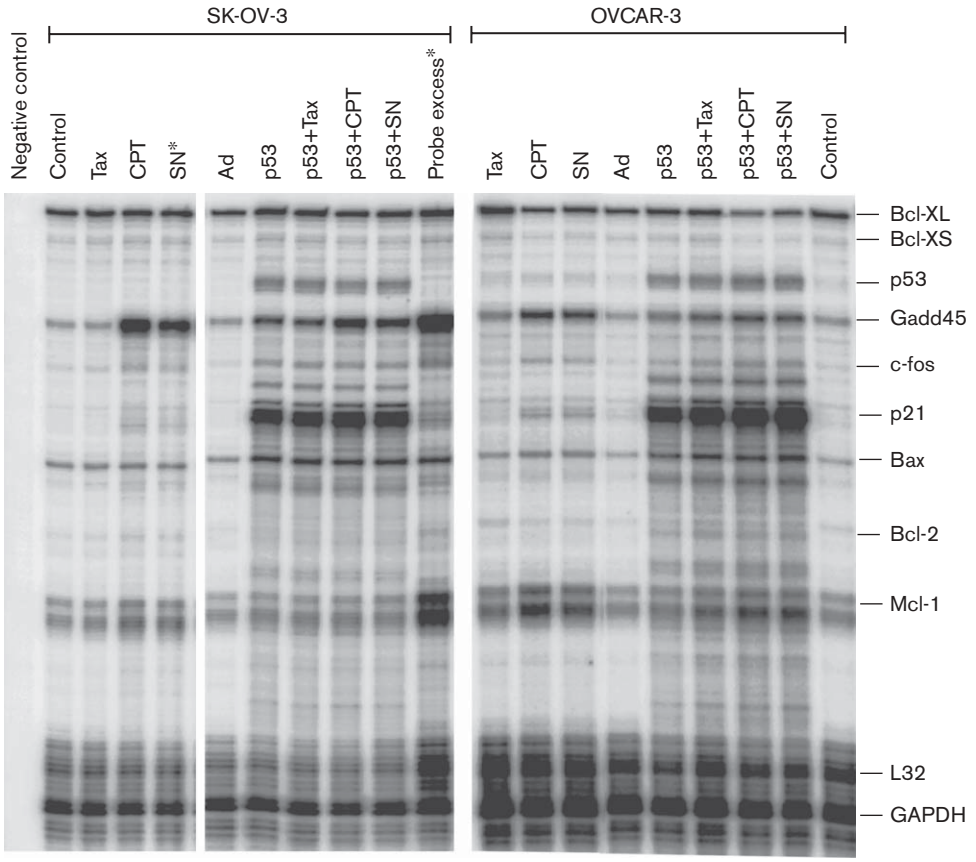
In OVCAR-3 cells, a low level of p53 was expressed in untreated control cells. The expression was not changed by docetaxel, CPT-11 or SN-38 exposures or by infection of empty adenovirus. In uninfected or Adeno-X-Null-infected SK-OV-3 cells, the expression of p53 was not detected. When both cell lines were infected by Ad5 CMV–p53 vector, an intense p53 band could be detected in the RPA analysis. After p53Ad infections, the expression of p53 was three times stronger in SK-OV-3 cells than in OVCAR-3 cells (Fig. 4a).

In SK-OV-3, but not in OVCAR-3 cells, the expression of Gadd45 was extensively upregulated by CPT-11 and SN-38 exposures (Fig. 4b). Infection of SK-OV-3 cells with p53Ad increased the expression of Gadd45 when compared with control sample, and the expression was further upregulated after CPT-11 and SN-38 treatments. These regulations in SK-OV-3 cells were statistically significant ($P < 0.05$). Docetaxel did not have an effect on Gadd45 expression either with or without p53Ad.

In SK-OV-3 cells, the $p21^{waf1/cip1}$ expression was upregulated by CPT-11 and SN-38 exposures without p53Ad infection ($P < 0.05$). In both cell lines, the expression of $p21^{waf1/cip1}$ was extensively upregulated by p53Ad infection ($P < 0.05$), but docetaxel, CPT-11 or SN-38 treatments did not further increase the expression (Fig. 4c).

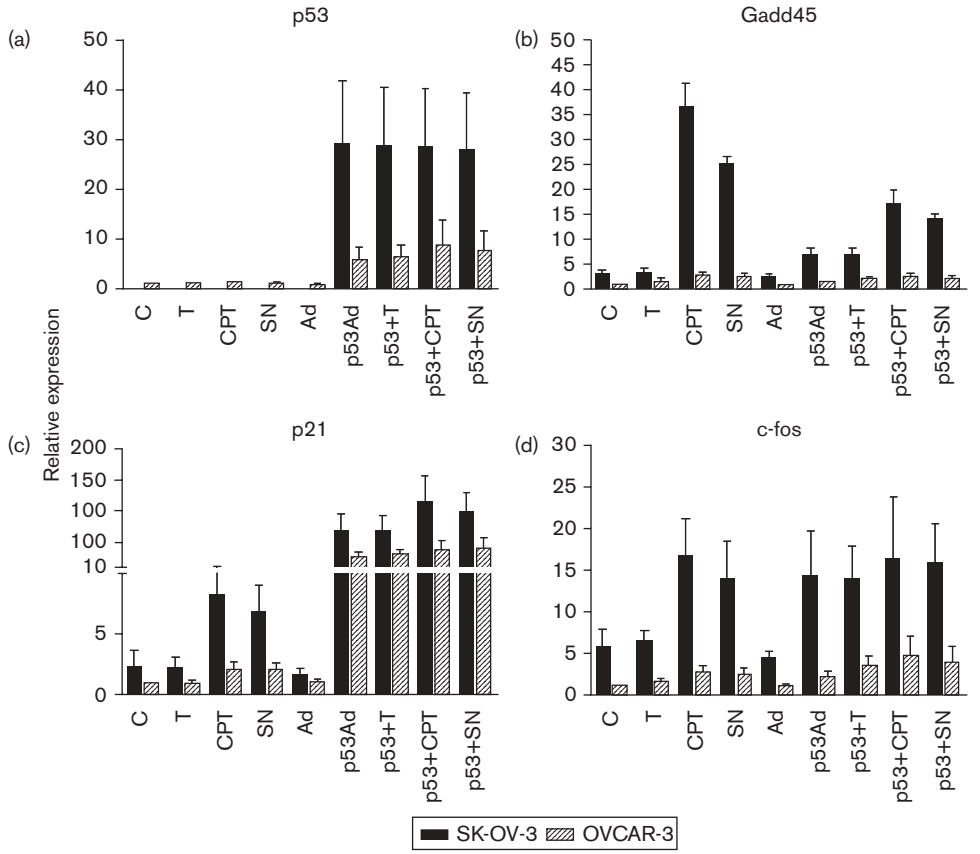
The expression of c-fos was slightly upregulated by SN-38 and CPT-11 exposures in both cell lines. In

Fig. 3



Ribonuclease protection assay (RPA) was used to detect mRNA expression levels of cell survival regulatory genes Bcl-XL, Bcl-XS, p53, Gadd45, c-fos, $p21^{waf1/cip1}$, Bax, Bcl-2 and Mcl-1 in ovarian cancer cell lines SK-OV-3 and OVCAR-3. Cells were treated with vehicle (Control), docetaxel (2.5 nmol/l, Tax), SN-38 (20 nmol/l, SN), CPT-11 (10 μmol/l, CPT), empty adenovirus (Ad), p53Ad (p53), docetaxel and p53Ad (p53 + Tax), CPT-11 and p53Ad (p53 + CPT) or SN-38 and p53Ad (p53 + SN). *For probe excess control three times more RNA is used when compared with SN sample to verify probe excess in RPA reactions. GAPDH, glyceraldehydes-3-phosphate dehydrogenase.

Fig. 4



The ribonuclease protection assay results of expression levels of cell cycle regulators p53 (a), Gadd45 (b), $p21^{waf1/cip1}$ (c) and c-fos (d) in ovarian cancer cell lines SK-OV-3 and OVCAR-3. Cells were treated with vehicle (C), docetaxel (2.5 nmol/l, T), SN-38 (20 nmol/l, SN), CPT-11 (10 μmol/l, CPT), empty adenovirus (Ad), p53Ad (p53), docetaxel and p53Ad (p53 + T), CPT-11 and p53Ad (p53 + CPT) or SN-38 and p53Ad (p53 + SN). The columns represent mean of five independent experiments ± SD.

SK-OV-3 cells, p53Ad upregulated the expression of c-fos, but in OVCAR-3 cells it did not have a clear effect on the expression (Fig. 4d). In SK-OV-3 cells, drug exposures did not increase p53Ad-mediated c-fos upregulation.

Expression of Bcl-2 family members

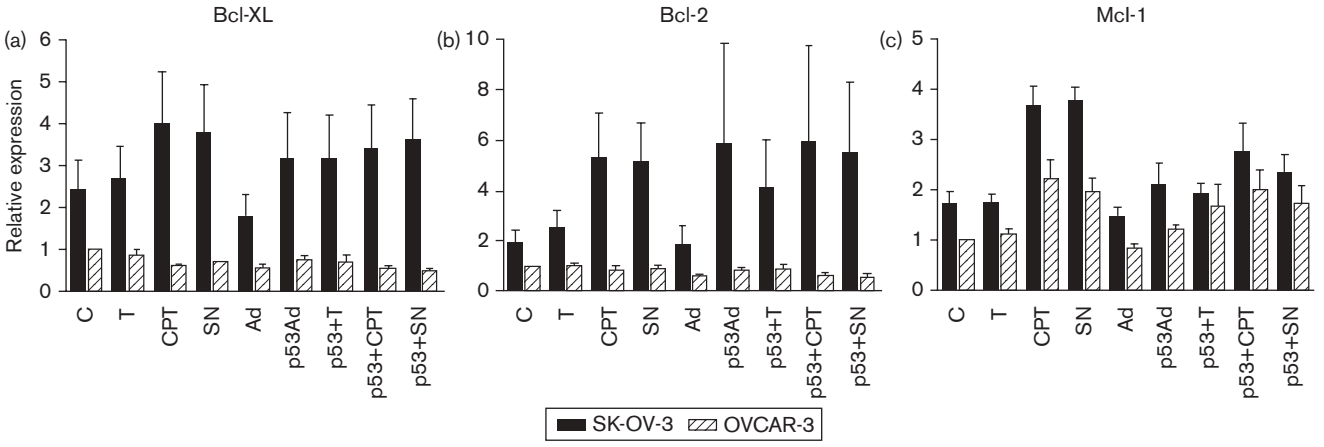
Figure 5a–c shows the expressions of antiapoptotic genes Bcl-XL, Bcl-2 and Mcl-1 genes. Drug exposures or p53Ad infection did not significantly regulate the expression of Bcl-XL in either cell lines (Fig. 5a). In SK-OV-3 cells, but not in OVCAR-3 cells, the expression of Bcl-2 was slightly increased after CPT-11 and SN-38 treatments and by p53Ad infection (Fig. 5b). The expression of Mcl-1 was slightly increased in SK-OV-3 and OVCAR-3 cells after CPT-11 and SN-38 exposures, but docetaxel and p53Ad exposures did not have a significant effect on Mcl-1 expression (Fig. 5c).

In SK-OV-3 cells, the expression of proapoptotic Bcl-XS was slightly enhanced by CPT-11 and SN-38 treatments and by p53Ad infection (Fig. 6a). The expression remained unchanged in OVCAR-3 cells. The other proapoptotic gene, Bax, was upregulated by CPT-11 and SN-38 treatments in OVCAR-3 cells and this was even more pronounced in p53Ad-infected cells (Fig. 6b). This difference was statistically significant when SN-38/CPT-11 and p53Ad + SN-38/CPT-11-treated samples were compared ($P < 0.05$). In SK-OV-3 cells, the expression of Bax was upregulated by p53Ad infection ($P < 0.05$), but drug exposures did not further increase the expression.

Bax/Bcl-2 and Bax/Bcl-XL expression ratio

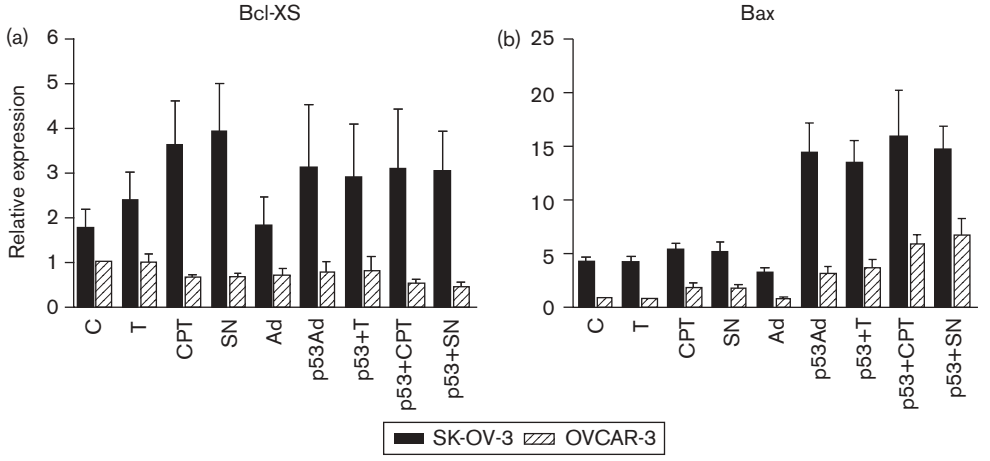
The balance between expressions of proapoptotic and antiapoptotic members of the Bcl-2 family is hypothesized to decide the cell fate after cellular stress. High expression ratios of Bax/Bcl-2 and Bax/Bcl-XL suggest increased apoptotic activity, whereas low ratios imply stronger resistant to apoptosis, improved cell survival and ultimately worse prognosis [37,38]. Both Bax/Bcl-2 and Bax/Bcl-XL expression ratios showed that cells lines SK-OV-3 and OVCAR-3 might respond differentially to p53Ad gene therapy (Fig. 7a and b). In SK-OV-3 cells, the expression ratios decreased in samples exposed to CPT-11 and SN-38. This was not observed in OVCAR-3

Fig. 5



The ribonuclease protection assay results of expression levels of antiapoptotic Bcl-XL (a), Bcl-2 (b) and Mcl-1 (c) in ovarian cancer cell lines SK-OV-3 and OVCAR-3. Cells were treated with vehicle (C), docetaxel (2.5 nmol/l, T), SN-38 (20 nmol/l, SN), CPT-11 (10 μmol/l, CPT), empty adenovirus (Ad), p53Ad (p53), docetaxel and p53Ad (p53 + T), CPT-11 and p53Ad (p53 + CPT) or SN-38 and p53Ad (p53 + SN). The columns represent the mean of five independent experiments ± SD.

Fig. 6



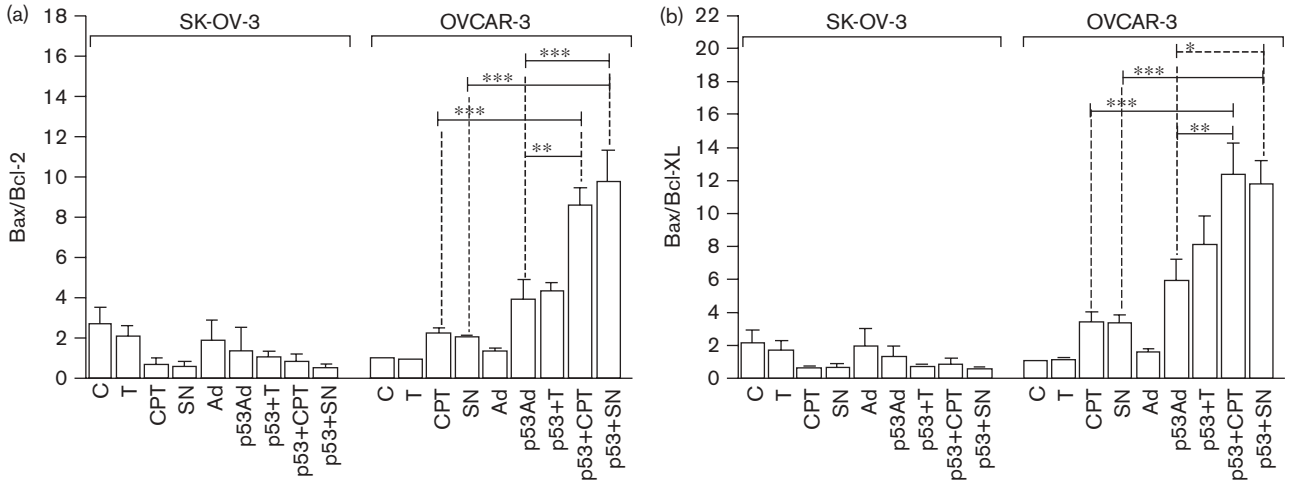
The ribonuclease protection assay results of expression levels of proapoptotic Bcl-XS (a) and Bax (b) in ovarian cancer cell lines SK-OV-3 and OVCAR-3. Cells were treated with vehicle (C), docetaxel (2.5 nmol/l, T), SN-38 (20 nmol/l, SN), CPT-11 (10 μmol/l, CPT), empty adenovirus (Ad), p53Ad (p53), docetaxel and p53Ad (p53 + T), CPT-11 and p53Ad (p53 + CPT) or SN-38 and p53Ad (p53 + SN). The columns represent the mean of five independent experiments ± SD.

cells, where the expression ratios were at least doubled after CPT-11 and SN-38 exposures. In OVCAR-3 cells, the p53Ad infection upregulated the expression ratios and these ratios were further increased after CPT-11 and SN-38 exposures. Differences between Bax/Bcl-2 and Bax/Bcl-XL expression ratios in SN-38/CPT-11 and p53Ad + SN-38/CPT-11 and p53Ad and p53Ad + SN-38/CPT-11-treated samples were statistically significant (Fig. 7a and b, respectively). In contrast to SN-38 and CPT-11, docetaxel did not have an effect on Bax/Bcl-2 or Bax/Bcl-XL expression ratios in either uninfected or infected samples.

Cell cycle distribution and induction of apoptosis

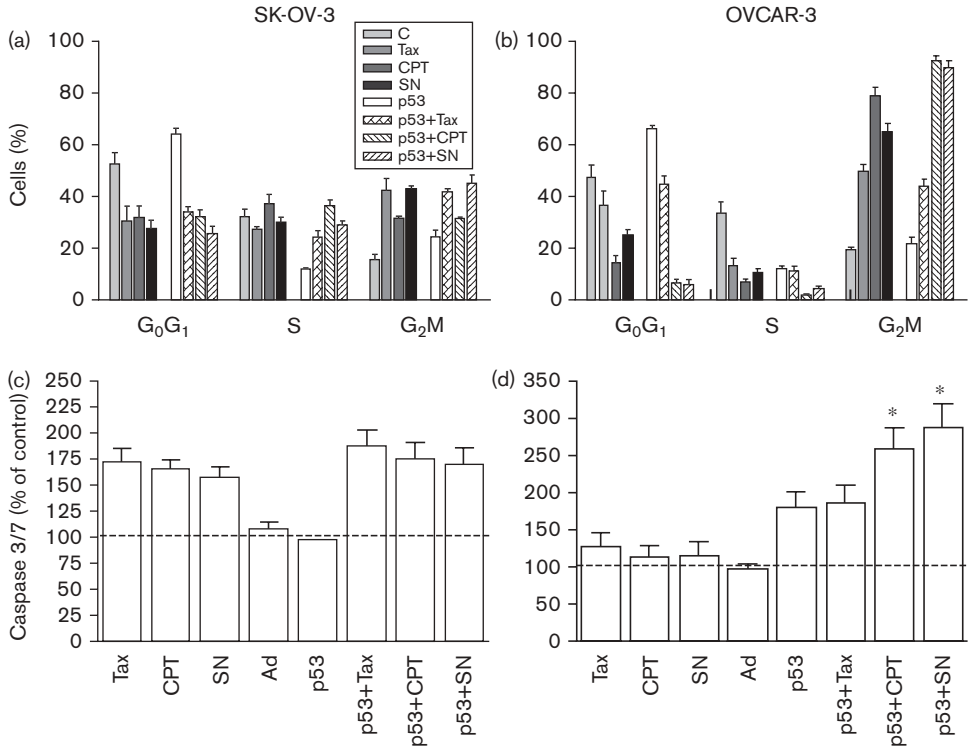
The proportions of cells in different cell cycle phases were analysed to explain differences in the growth response and gene expressions between SK-OV-3 (Fig. 8a) and OVCAR-3 (Fig. 8b) cell lines. In SK-OV-3 cell line (Fig. 8a), the cell number in the G_2M phase was increased after 48-h drug exposures and the cell counts

Fig. 7



Expression ratios of proapoptotic and antiapoptotic genes Bax/Bcl-2 (a) and Bax/Bcl-XL (b) were calculated based on the results of ribonuclease protection assay. Cells were treated with vehicle (C), docetaxel (2.5 nmol/l, T), SN-38 (20 nmol/l, SN), CPT-11 (10 μmol/l, CPT), empty adenovirus (Ad), p53Ad (p53), docetaxel and p53Ad (p53 + T), CPT-11 and p53Ad (p53 + CPT) or SN-38 and p53Ad (p53 + SN). High expression ratio indicates increased tendency of apoptosis and low ratio indicates resistance to apoptosis. In OVCAR-3 cells, the increases of Bax/Bcl-2 and Bax/Bcl-XL expression ratios were statistically significant when CPT-11/SN-38 or p53Ad-treated samples were compared with p53Ad + CPT-11/SN-38-treated samples (*$P<0.05$; **$P<0.01$; ***$P<0.001$).

Fig. 8



The cell cycle parameters (a and b) and caspase-3/7 activation (c and d) were measured after 48-h drug exposures in SK-OV-3 (a and c) and OVCAR-3 (b and d, respectively) cell lines. Cells were treated with vehicle (C), docetaxel (2.5 nmol/l, Tax), SN-38 (20 nmol/l, SN), CPT-11 (10 μmol/l, CPT), p53Ad (p53), docetaxel and p53Ad (p53 + Tax), CPT-11 and p53Ad (p53 + CPT) or SN-38 and p53Ad (p53 + SN). In caspase-3/7 experiment also, empty adenovirus vectors (Ad) were used. *Statistically significant difference ($P<0.05$) between p53Ad and p53 + CPT or p53 + SN-exposed samples.

in phases G_0G_1 and S were decreased when compared with control. Infection of cells with p53Ad and following drug exposures did not change the distribution of SK-OV-3 cells in different cell cycle phases, except that the cell count was slightly decreased in the S phase in p53Ad-alone-treated samples. In OVCAR-3 cell line (Fig. 8b), docetaxel, CPT-11 and SN-38 exposures increased cell number in the G_2M phase and cell counts in G_0G_1 and S were decreased. The p53Ad infection slightly increased cell counts in the G_0G_1, and in the S phase the cell counts were decreased. In contrast to SK-OV-3, in OVCAR-3 cells CPT-11 and SN-38 exposures concomitantly with the p53Ad infection decreased cell numbers in both G_0G_1 and S phases and cells accumulated in G_2M phase.

The apoptotic status of SK-OV-3 (Fig. 8c) and OVCAR-3 (Fig. 8d) cells after drug and p53Ad exposures was studied by measuring the induction of caspase-3 and caspase-7. The amount of caspase-3 and caspase-7 was increased in SK-OV-3 cells exposed to docetaxel, CPT-11 and SN-38. Infection with p53Ad did not increase the caspase activation, and concomitant drug plus p53Ad exposure did not further increase caspase activation when compared with uninfected samples exposed to drugs alone. In OVCAR-3 cells, drug exposures alone did not notably increase caspase activation. Infection with p53Ad increased the caspase activation and it was even more increased after concomitant CPT-11 or SN-38 and p53Ad exposures. This effect was statistically significant in both drug exposures ($P < 0.05$).

Discussion

Several studies have shown that growth of human cancer cell lines, including cervical, lung, colon and ovarian, can be suppressed by introducing the wild-type p53 gene into cancer cells [30–35]. In this study, we have used replication-deficient adenoviruses to deliver normal p53 gene to two human ovarian carcinoma cell lines. Cell line OVCAR-3 contains a point mutation in p53 gene, whereas cell line SK-OV-3 expresses normal p53 [36].

We found that OVCAR-3 and SK-OV-3 cell lines reacted differentially to adenovirus-mediated p53 gene therapy. In OVCAR-3 cells, p53 gene therapy significantly inhibited cell growth when compared with empty adenovirus-infected samples or uninfected control cells. In contrast, the growth of SK-OV-3 cells was inhibited after p53Ad infection, but infection with empty adenovirus produced equal growth inhibition. These results were not because of different infection efficiencies, as both cell lines were equally infected as shown by X-gal staining. SK-OV-3 cells, however, expressed three times more p53 mRNA than OVCAR-3 cells after p53Ad infection.

In OVCAR-3 cells, SN-38 and CPT-11 concomitantly with p53 therapy produced enhanced growth inhibition when compared with empty adenovirus infections and samples exposed to SN-38 and CPT-11 alone. This effect was not observed in SK-OV-3 cells. In fact, with high SN-38 concentrations, empty adenovirus was more effective growth inhibitor than p53Ad. These results indicate that in cells containing a normal p53 gene, p53Ad therapy does not provide additional benefit. In earlier studies, p53Ad has shown to facilitate the efficacy of DNA-damaging drugs, such as CPT-11 or SN-38, in both normal and neoplastic cells [33]. In our earlier study, the OVCAR-3 cell line was shown to be nearly resistant to SN-38 [20]. In this study, the induction of wild-type p53 gene facilitated the efficacy of both SN-38 and its prodrug CPT-11 in the OVCAR-3 cells. CPT-11 and SN-38 may, however, cause p53-independent cell growth inhibition. For example, in ovarian cancer cells, SN-38-induced G_2M arrest is independent of p53 function [23].

To study the mechanisms behind differential responses of cell lines to p53 gene therapy, we analysed expressions of several cell cycle and cell survival regulatory genes after drug and adenoviral infections. Gadd45 is a genotoxic stress-responsive gene, whose expression is rapidly induced by wide variation of DNA-damaging agents [8,9]. Gadd45 mediates growth suppression through the induction of cell cycle arrest in G_2M phase [10]. It is known to interact with $p21^{waf1/cip1}$ [39,40] and it is also involved in p53 stabilization in response to DNA damage [41]. $p21^{waf1/cip1}$ is a cyclin-dependent kinase inhibitor that controls G_1/S progression in cell cycle [6]. It may also have a role in G_2M progression, as SN-38 has been shown to induce cell cycle arrest in G_2M phase concomitantly with increased $p21^{waf1/cip1}$ expression levels [7]. Observed inductions of $p21^{waf1/cip1}$ and Gadd45 genes in ovarian cancer cells suggest that CPT-11 and SN-38 may induce cell cycle arrest in G_2M phase. Expressions of $p21^{waf1/cip1}$ and Gadd45 were both upregulated after SN-38 and CPT-11 exposures in SK-OV-3 and OVCAR-3 cell lines. P53Ad increased the expression of $p21^{waf1/cip1}$ even further in both cell lines. Infection of cell lines with p53Ad did not further increase Gadd45 upregulation. After infection of SK-OV-3 cells with p53Ad, the Gadd45 expression was even lower in response to SN-38 or CPT-11 treatments than in samples exposed to either drug alone. When cell cycle distribution was analysed, we noticed that in both cell lines exposed to p53Ad cells accumulated in G_0G_1 phase, but this effect was more efficient in OVCAR-3 cells. In bladder cancer cells, overexpression of wild-type p53 gene induces rapid G_1 and G_2M growth arrest associated with increased $p21^{waf1/cip1}$ expression. This growth arrest becomes irreversible and cells enter into senescence [42]. In our earlier study, we have shown that in ovarian cancer cells SN-38 cause cell accumulation in G_2M phase [20].

In OVCAR-3 cells, concomitant exposure to p53Ad and SN-38 or CPT-11 increased cell accumulation in G_2M even further. In SK-OV-3 cells, p53Ad used alone or concomitantly with SN-38 or CPT-11 did not increase the proportion of cells in G_2M.

Expressions and regulation of proapoptotic and antiapoptotic members of Bcl-2 family were also different in SK-OV-3 and OVCAR-3 cell lines. In SK-OV-3 cells, both proapoptotic and antiapoptotic genes were slightly upregulated by CPT-11, SN-38 and p53Ad exposures. In contrast, in OVCAR-3 cells only the expression of Bax was clearly upregulated by CPT-11, SN-38 and p53Ad exposures. Concomitant use of p53Ad with SN-38 or CPT-11 did further increase Bax expression in OVCAR-3 cells. Earlier, it has been shown that overexpression of Bax may increase sensitivity of head and neck squamous cancer cells to SN-38 [43], and it is upregulated concomitantly with p53, $p21^{waf1/cip1}$, Bcl-XL, cyclin A and cyclin E expressions by SN-38 [7,22].

The balance between expressions of proapoptotic and antiapoptotic members of the Bcl-2 family is hypothesized to decide the cell fate after cellular stress. High expression ratios of Bax/Bcl-2 and Bax/Bcl-XL suggest increased apoptotic activity, whereas low ratios imply stronger resistant to apoptosis, improved cell survival and ultimately worse prognosis [37,38]. In SK-OV-3 cells, the expression ratios decreased in CPT-11 and SN-38-treated samples. This was not observed in OVCAR-3 cells, where the expression ratios were at least doubled after CPT-11 and SN-38 treatments. In OVCAR-3, p53Ad infection upregulated the expression ratios and these ratios were further increased in samples exposed to p53Ad + CPT-11 or SN-38. In OVCAR-3 cells, p53Ad alone induced apoptotic cell death, as suggested by the increased expression ratios of both Bax/Bcl-2 and Bax/Bcl-XL, but this was not observed in SK-OV-3 cells.

The p53 expression status of ovarian cancer samples has been associated with improved sensitivity to taxane–platinum therapy [44], and adenoviral p53 therapy has sensitized ovarian cancer cells to paclitaxel [32]. In our study, infection of cells with p53Ad did not improve the efficacy of docetaxel in either cell lines. Our results show that in contrast to SN-38 and CPT-11, docetaxel does not regulate expression of cell cycle regulators on transcriptional levels or the regulation is minimal. Lack of gene regulation in docetaxel-treated samples might indicate that growth inhibition and induction of apoptosis is mediated through other mechanisms than activation of p53 pathway. The anticancer drug screen in the National Cancer Institute has revealed that p53 status may affect topoisomerase I inhibitor sensitivity, but sensitivity of cancer cells to antimitotic compounds, as taxol, is not dependent on p53 status [45]. Similar results have been found with paclitaxel sensitivity in gynaecological cancer cells including ovarian cancer [36]. Docetaxel sensitivity may be related to other mechanisms. Taxanes might induce phosphorylation of Bcl-2 protein, thereby decreasing the formation of Bcl-2/Bax heterodimers and allowing the proapoptotic action of Bax [24,46]. Earlier, it has been shown that overexpression of Bcl-2 could lead to increased resistance to treatment with another taxane, paclitacel [47], but there are also conflicting data showing Bcl-2 downregulation in association with paclitaxel resistance in ovarian cancer [48].

Although preclinical data show that p53 gene therapy might be a promising tool in improving the survival of ovarian cancer patients, clinical experiments have shown disappointing results and suggested that the selection criteria for patients who would benefit from the therapy need to be characterized more carefully [49]. Our study shows that Bax/Bcl-2 and Bax/Bcl-XL expression ratios might serve as indicators for efficacy of adenovirus-mediated p53 gene therapy and CPT-11 or SN-38 treatments. When p53 status of cancer cells is normal, p53 gene therapy is not effective alone or concomitantly with CPT-11 or SN-38 exposures.

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