Inhibition of P-glycoprotein-mediated docetaxel efflux sensitizes ovarian cancer cells to concomitant docetaxel and SN-38 exposure

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The first-line treatment of ovarian cancer is based on cytoreductive surgery and the use of anticancer drugs. The main disadvantage in the usage of anticancer drugs is the wide capacity of cancer cells to acquire a resistance to chemotherapeutic agents and therefore new treatment strategies have to be developed and tested. In this study, the responses of seven ovarian carcinoma cell lines to docetaxel and a camptothecin derivative, SN-38, were evaluated. We further studied the expression of P-glycoprotein (P-gp), the best described mechanism of drug resistance, in these cells and the effect of treatment with a specific P-gp inhibitor (PGP-4008). Simultaneous treatment with docetaxel and SN-38 (docetaxel + SN-38) had an antagonistic growth effect that was not dependent on the administration schedule. Both drugs alone or in combination induced G_2M cell cycle arrest. Docetaxel was a more potent inducer of apoptosis than SN-38, but simultaneous treatment with docetaxel + SN-38 decreased the proportion of apoptotic cells to the same level observed after exposure to SN-38 alone. SN-38 increased P-gp expression in all cell lines. PGP-4008 enhanced docetaxel-mediated growth inhibition and apoptosis, but it did not have an effect when used simultaneously with SN-38. When cells were treated with docetaxel, SN-38, and PGP-4008 simultaneously, the growth was inhibited more efficiently and the proportion of apoptotic cells was higher than that without PGP-4008. Thus, treatment of ovarian cancer cells with docetaxel + SN-38 may have antagonistic effects. The simultaneous administration of a P-gp inhibitor may prevent docetaxel efflux, thereby sensitizing cells to docetaxel and other chemotherapeutic agents. *Anti-Cancer Drugs* 20:267–276 © 2009 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Introduction

Ovarian cancer is the sixth most common cancer among women, accounting for 4% of all female malignancies [1]. Although adequate staging, maximal cytoreduction, and combination chemotherapy have improved the outcome, ovarian cancer remains the most common cause of death from gynecologic cancers in developed countries because of the advanced stage at the time of diagnosis and the high relapse rate after an initial response to first-line treatment [2]. The development of new chemotherapeutic agents and methods to avoid developing drug resistance is therefore important toward improving the survival rate of ovarian cancer patients.

The camptothecin derivative SN-38 inhibits topoisomerase I activity, thereby interrupting DNA replication [3]. Topoisomerase I inhibitors induce cell cycle arrest in the G_2 phase and cells are most sensitive to SN-38 exposure during the S-phase [4–7]. Docetaxel disrupts mitosis by enhancing tubule polymerization into microtubules and by inhibiting microtubule depolymerization, resulting in the formation of abnormal and stable microtubule bundles [8]. Docetaxel induces G_2/M arrest, which is then followed by apoptosis [9].

The multi-drug resistance (MDR) phenotype in tumors is caused by the overexpression of P-glycoprotein (P-gp), also known as MDR-1, which is located in the plasma membrane of cancer cells [10]. This transport protein has a high number of substrates, including taxanes [11], and it is currently the best described mechanism of resistance to antitubulin agents such as docetaxel [12]. P-gp might also be involved in SN-38 transport, although the data are controversial and other transport proteins might be involved [13–17].

Docetaxel and SN-38 have different mechanisms of action and no cross-resistance [18]. Therefore, the combination of these drugs is clinically interesting. Single-agent therapy with docetaxel and irinotecan (CPT-11, a prodrug of SN-38) induces responses in clinical trials on ovarian cancer [7,19–21], and the combination of docetaxel and irinotecan has been used to treat ovarian carcinoma [22]. In previous studies, the combination of

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a taxane and a topoisomerase inhibitor have had divergent responses both *in vitro* [23–27] and in clinical settings [28,29].

In a randomized clinical trial, carboplatin–paclitaxel and carboplatin–docetaxel combinations were equally effective as first-line treatments for ovarian cancer [30], whereas in-vitro studies docetaxel is more cytotoxic than paclitaxel on a molar basis [31,32]. The development of platinum resistance is a major problem in clinical practice and therefore other taxane-based combinations must be evaluated. The purpose of this study was to evaluate the effects of combination treatment with docetaxel and SN-38 (docetaxel + SN-38) in ovarian cancer cells.

Materials and methods

Cell culture

The human ovarian adenocarcinoma cell lines, SK-OV-3, OVCAR-3 (ATCC, Manassas, Virginia, USA), UT-OC-1, UT-OC-2, UT-OC-3, UT-OC-4, and UT-OC-5 were maintained in Dulbecco's modified Eagles medium (Sigma-Aldrich, St Louis, Missouri, USA) supplemented with 10% fetal bovine serum, non-essential amino acids, and antibiotics (100 IU/ml penicillin, 100 μg/ml streptomycin). The noncommercial cell lines UT-OC-1, UT-OC-2, UT-OC-3, UT-OC-4, and UT-OC-5 have been established and characterized from epithelial ovarian carcinomas by our group [33–38]. All cell lines were maintained at 37°C in a humidified 95% air/5% CO_2 incubator.

Drug preparations

Docetaxel and SN-38 were provided by Sanofi Aventis (Bridgewater, New Jersey, USA). A selective P-gp inhibitor, PGP-4008 [11] was obtained from Sigma-Aldrich. Docetaxel and PGP-4008 were dissolved in ethanol and SN-38 in dimethylsulfoxide (Sigma-Aldrich). Drugs were first diluted in ethanol and then in growth medium. The final ethanol concentration was 0.001%. Final dilutions of docetaxel were 0.01–50 nmol/l and those of SN-38 were 0.001–100 nmol/l.

Cell growth assay

Before exposure of cells to docetaxel + SN-38, the half maximal effective concentration (EC_{50}) values were determined for each drug alone. For cell growth assay, $2.5 \times 10^3 - 1.0 \times 10^4$ cells/well (depending on the cell line growth rate) were plated on 96-well plates in Dulbecco's modified Eagles medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum, and 1% non-essential amino acids and antibiotics. Cells were allowed to adhere for 24 h before adding docetaxel, SN-38, or both. The vehicles, ethanol or dimethylsulfoxide, were added to control cells. Cell growth samples were harvested on day 5. When sequential exposures were studied, the first drug was added after cell adhesion (24 h). After another 24 h, the second drug was added and the cells were incubated for an additional 4 days. The P-gp inhibitor PGP-4008 (250 nmol/l) was added to the cells simultaneously with docetaxel, SN-38, or both drugs, and cells were incubated for 5 days. Different docetaxel and SN-38 exposure schedules were not studied with P-gp inhibitor.

Relative cell numbers were quantified as described previously [39]. Briefly, cells were fixed with glutaraldehyde on the bottom of 96-well plates and stained with crystal violet. Bound crystal violet dye was dissolved in acetic acid and optical density (590 nm) was measured from each well using a Victor 1420 multilabel counter (Wallac, Turku, Finland). The mean optical density ± SD for each concentration was calculated from 6 to 10 determinations, and dose–response curves were drawn based on these values.

Cell cycle analysis

The cell cycle parameters were measured after 24, 48, and 72 h of drug exposure. Trypsinized and floating cells were pooled, washed with phosphate-buffered saline (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). Cell cycle distribution was determined with ModFit LT (Verity Software House Inc., Topsham, Maine, USA).

Detection of nuclear morphology

Apoptotic cells were counted by fluorescence microscopy after Hoechst bisbenzamide 33258 staining (Sigma-Aldrich). Cells ($5.0 \times 10^4 - 1.0 \times 10^5$ cells/well) were plated on a chamber slide (Lab-Tek chamber slide, Nalge Nunc International, Naperville, Illinois, USA). After cell adhesion (24 h), the growth medium was replaced with medium containing drug dilutions according to the EC_{50} values of each drug and cell line. PGP-4008 (250 nmol/l) was added to the cells simultaneously with the other drugs. Cells were grown for 48 h, medium was removed, and cells were washed with PBS and fixed using 4% paraformaldehyde. After washing with PBS, cells were treated with 0.5% Triton X-100/PBS. The samples were washed and stained

Table 1 The EC_{50} values (95% confidence intervals) for docetaxel and SN-38

Cell line	Docetaxel (nmol/l)	SN-38 (nmol/l)
UT-OC-1	1.93 (1.38–2.69)	6.99 (6.11–8.00)
UT-OC-2	2.50 (1.94–3.23)	7.48 (6.07–9.23)
UT-OC-3	1.10 (0.90–1.35)	2.49 (1.91–3.23)
UT-OC-4	1.10 (0.84–1.43)	13.51 (11.60–15.73)
UT-OC-5	1.59 (1.33–1.89)	46.87 (38.54–57.00)
SK-OV-3	0.97 (0.88–1.08)	11.08 (9.65–12.71)
OVCAR-3	1.35 (1.24–1.46)	43.54 (37.43–50.65)

EC_{50}, half maximal effective concentration.

with Hoechst 33258. The apoptotic cells were counted using a fluorescence microscope (excitation 365 nm, emission 480 nm). The detection of apoptotic cells was based on nuclear morphology, folding of the nuclear membrane, and fragmentation of the nucleus. At least 100 cells were counted from each treatment group and the counting was repeated five times.

Quantitative real-time PCR

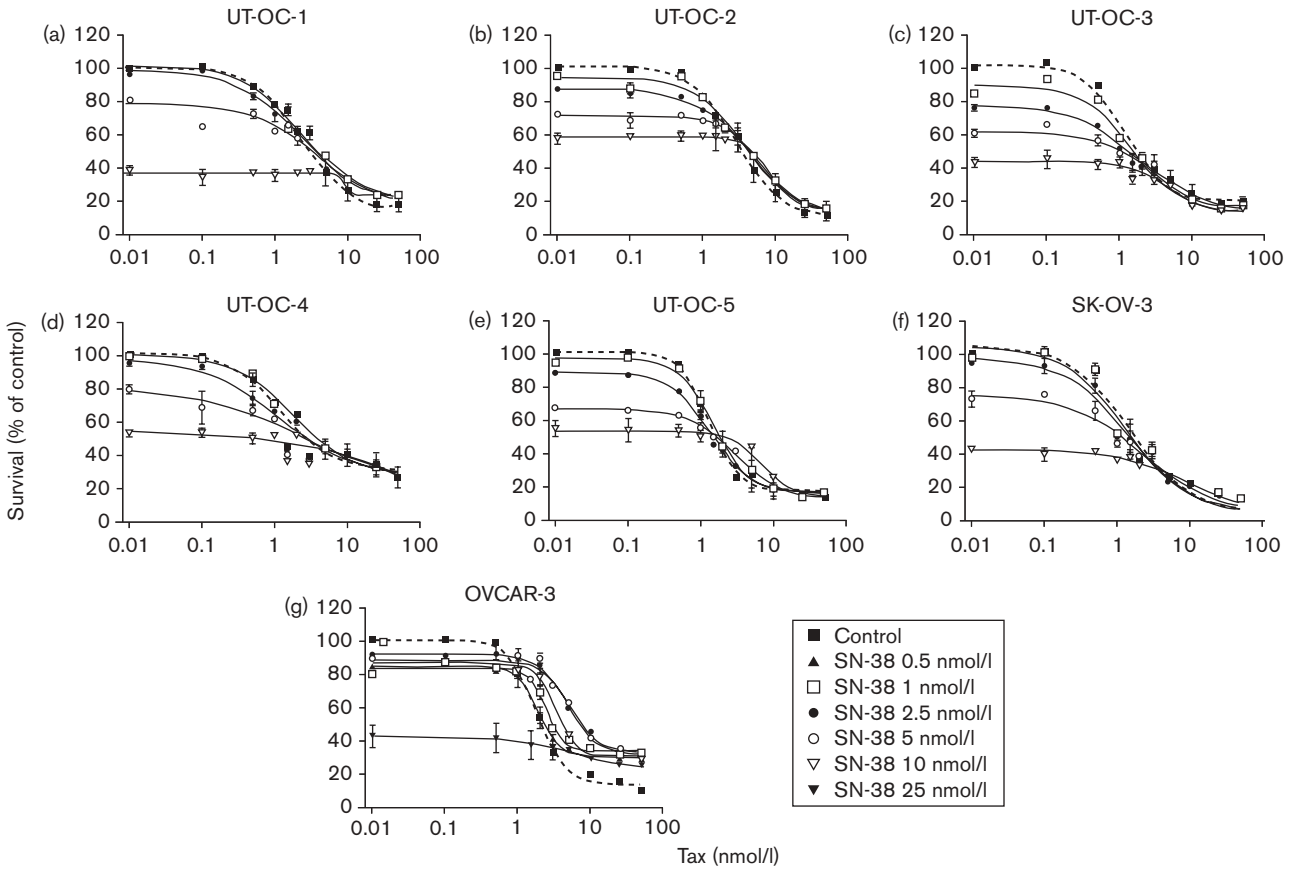
The cells (3.0×10^5) were plated on T25 culture flasks (Nalge Nunc International). After cell adhesion (24 h), the growth medium was replaced with media containing drug concentrations depending on the EC_{50} values of each drug and the cell line studied. After the 48-h treatment period, RNA was extracted with TRIzol reagent (Invitrogen Life Technologies, Paisley, Scotland, UK).

Quantitative real-time PCR was used to quantify P-gp (MDR-1) expression. The reverse transcriptase reaction was performed using the High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, California, USA) and the real-time PCR step was performed using SYBR Green PCR Master Mix and ABI Prism 7000 Sequence Detection System (Applied Biosystems). Primers were synthesized by TAG Copenhagen A/S (Copenhagen, Denmark). For amplification of the RPLP0 (reference gene, acidic ribosomal phosphoprotein, NM_001002), the forward primer was 5′-AATCTCCAGGGGCACCAT T-3′ and the reverse primer was 5′-CGCTGGCTCCCA CTTTGT-3′. For MDR-1 (NM_000927) amplification, the forward primer was 5′-CTCAGACAGGATGTGA GTTGGTTT-3′ and the reverse primer was 5′-GC GAGCCTGGTAGTCAATGC-3′. Relative quantification of the target gene (MDR-1) in comparison with the reference gene (RPLP0) was calculated as described previously [40].

Western blot analysis

For western blotting, cells were cultured in T25 flasks (Nalge Nunc International) until 70% confluent. After

Fig. 1



Dose–response curves after a concomitant exposure of cells [(a) UT-OC-1; (b) UT-OC-2; (c) UT-OC-3; (d) UT-OC-4; (e) UT-OC-5; (f) SK-OV-3; (g) OVCAR-3] to docetaxel (Tax) and SN-38. Cells were fixed with glutaraldehyde on the bottom of 96-well plates and stained with crystal violet. Bound crystal violet dye was dissolved in acetic acid and optical density (590 nm) was measured from each well. The mean optical density for each concentration was calculated from 6 to 10 determinations, and dose–response curves were drawn based on these values. Experiments were repeated three to five times and each data point represents mean ± SD of these experiments.

cell adhesion (24 h), the growth medium was replaced with media containing drug concentrations depending on the EC_{50} values of each drug and the cell line studied. After the 48-h treatment period, proteins were extracted with mPER reagent (Pierce, Rockford, Illinois, USA). Protein concentrations in each sample were measured with BCA protein Assay Reagent (Pierce). Equal amounts of protein from each sample was fractionated in 7.5% polyacrylamide gel. The protein samples were transferred to nitrocellulose membrane with electrophoresis transfer apparatus. The western blotting was carried out as described previously [41]. The samples were blocked with 3% milk–TBS–Tween solution and the membranes were incubated at 4°C overnight with monoclonal P-gp (MDR) antibody (Clone F4, does not recognize MDR-3) (Sigma-Aldrich) diluted in 1% milk–TBS–Tween solution. Peroxidase-conjugated goat anti-mouse IgG (Sigma-Aldrich) was used as secondary antibody. The membranes were incubated with secondary antibody 1 h at room temperature. The membranes were washed. Proteins (170–180 kDa) were detected by luminol-containing chemiluminescence reagent ECL (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK) and the bands were developed by using Konica SRX-101A (Konica Minolta, Wayne, New Jersey, USA).

Data analysis

The EC_{50} curves were generated using GraphPad Prism 3.03 (GraphPad Software Inc., San Diego, California, USA). Data were fitted to a sigmoid dose–response curve, equation: Y=Bottom + (Top–Bottom)/(1 + 10^[(logEC_{50}–X) × Hillslope)], where X is the logarithm of concentration and Y is the response. The dose–response interactions between docetaxel and SN-38 at the point of EC_{50} were evaluated using the isobolographic method [42,43]. All experiments were repeated three to five times and western blotting was carried out twice. Statistical analyses were done using GraphPad Prism 5 (GraphPad Software Inc.). Growth regulatory effects of different drug exposures and apoptosis data were compared using two-way analysis of variance followed by Bonferroni's post-hoc test. Statistical differences between P-gp expressions in treatment groups were analyzed using one-way analysis of variance followed by Bonferroni's post-hoc test. A P value of less than 0.05 was considered statistically significant.

Results

Concomitant docetaxel and SN-38 exposure produced subadditive or antagonistic growth effects without schedule dependency

Before exposure of the cells to docetaxel + SN-38, the EC_{50} values were determined for each drug alone. There was no correlation between sensitivity to docetaxel and SN-38. Table 1 summarizes the EC_{50} values of docetaxel and SN-38 in the cell lines.

To study the effect of simultaneous drug treatment, dose–response curves were drawn for individual cell lines. Concomitant (Fig. 1) or sequential exposure of cells to docetaxel and SN-38 resulted in additive, subadditive, or antagonistic effects that were not schedule dependent. The antagonistic effects were concentration dependent. Table 2 summarizes the effect of concomitant and sequential docetaxel and SN-38 exposure. There was a strong tendency toward a subadditive or antagonistic effect in every cell line. Growth regulation was evaluated using an isobolographic method and Fig. 2 shows isobolographs of all cell lines after concomitant treatment with docetaxel + SN-38.

Both docetaxel and SN-38 induced G_2M cell cycle arrest (Fig. 3). Simultaneous drug exposure did not increase or decrease the proportion of cells in the G_2M phase.

P-glycoprotein expression was regulated by SN-38

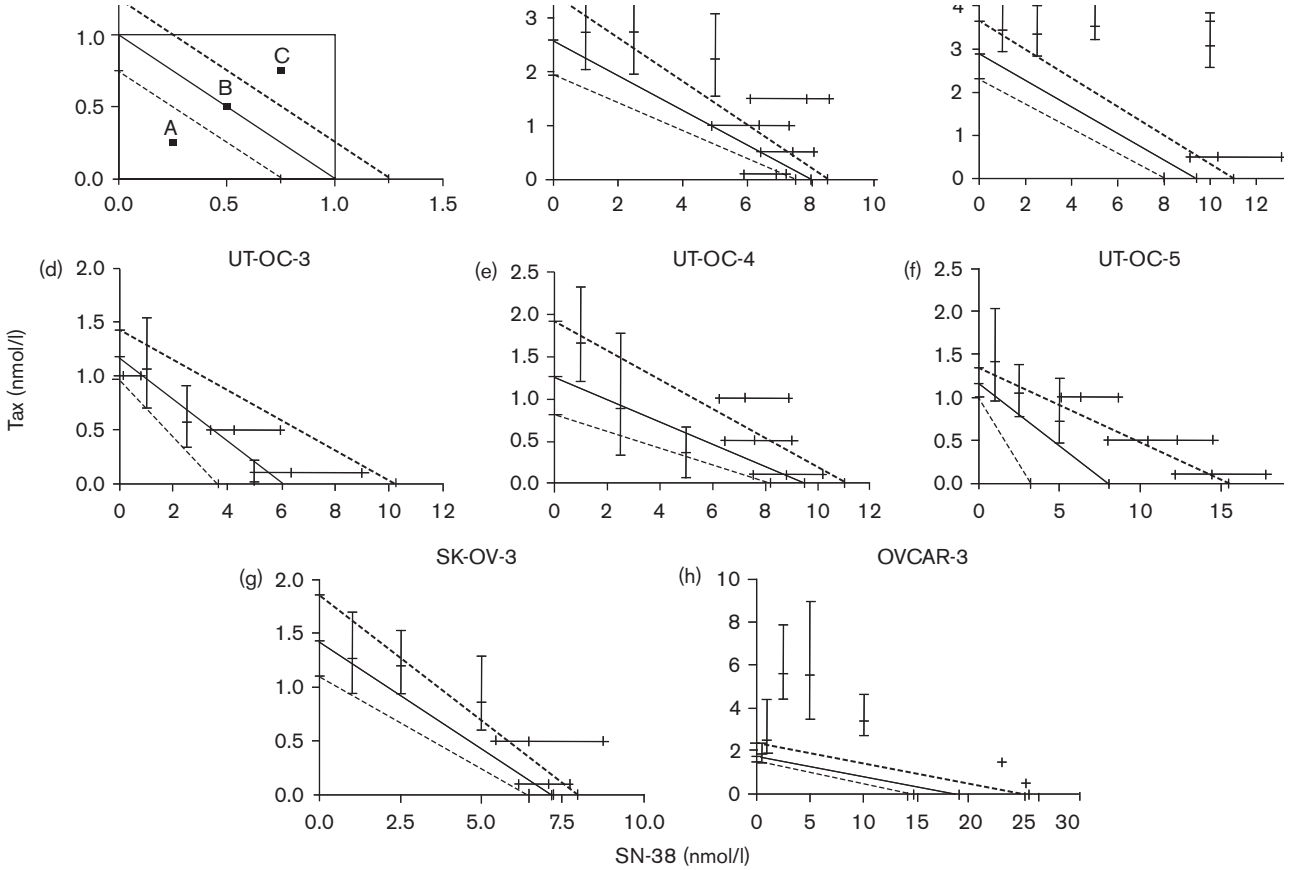
Quantitative real time-PCR (Fig. 4a–g) and western blotting (Fig. 4h) were used to study the effect of docetaxel and SN-38 on P-gp expression. On mRNA and protein levels, docetaxel had a moderate or no effect on P-gp expression. The regulation of P-gp mRNA expression by docetaxel was statistically significant ($P < 0.01$) only in UT-OC-1 cells (Fig. 4a). In contrast, both SN-38

Table 2 Effects of concomitant exposure of cells to docetaxel and SN-38

			Dosing schedule		
Cell line	Docetaxel (nmol/l)	SN-38 (nmol/l)	Tax/SN	SN[a] Tax[b]	Tax[a] SN[b]
UT-OC-1	0.01–50	1	P	P	P
		2.5	P	P	A
		5	SA	SA	P
		7.5	–	SA	A
UT-OC-2	0.01–50	1	P	P	P
		2.5	P	P	P
		5	P	P	P
		7.5	–	P	SA
		10	P	–	–
UT-OC-3	0.01–50	1	A	P	P
		2.5	A	A	P
		5	A	A	SA
UT-OC-4	0.01–50	1	P	P	P
		2.5	A	A	P
		5	A	A	P
		7.5	–	–	A
UT-OC-5	0.01–50	1	P	P	P
		2.5	A	A	P
		5	A	P	P
		7.5	–	–	A
SK-OV-3	0.01–50	1	A	A	A
		2.5	A	A	A
		5	SA	SA	SA
		7.5	–	–	SA
OVCAR-3	0.01–50	1	P	P	P
		2.5	P	P	P
		5	P	P	P
		7.5	–	P	P
		10	P	–	–

–, no data; A, additive growth effect; P, antagonistic growth effect; SA, subadditive growth effect; SN, SN-38; Tax, docetaxel; Tax/SN, simultaneous drug dosage.
[a]Drug given first.
[b]Drug given second.

Fig. 2



(a) A hypothetical isobolograph of docetaxel (Tax) and SN-38. The half maximal effective concentration (EC_{50}) value for docetaxel alone is plotted on *y*-axis and the EC_{50} value for SN-38 on *x*-axis. The straight solid line between the *x*-axis and *y*-axis represents isoeffective combinations (combinations of Tax and SN-38 giving the same effect, 50% growth inhibition, as either drug alone). The dashed lines represent 95% confidence intervals. Characters A, B, C, and D represent supra-additive, additive, subadditive, and antagonistic effects, respectively. If the response to the combination is plotted under the lower dashed line (A), the effect is supra-additive, if between the dashed lines (B), the effect is additive, if above the upper dashed line, but inside the rectangle (C), the effect is subadditive and if outside the rectangle (D), the effect is antagonistic. (b–h) Show the isobolographs of simultaneous exposure of indicated cell lines to docetaxel and SN-38.

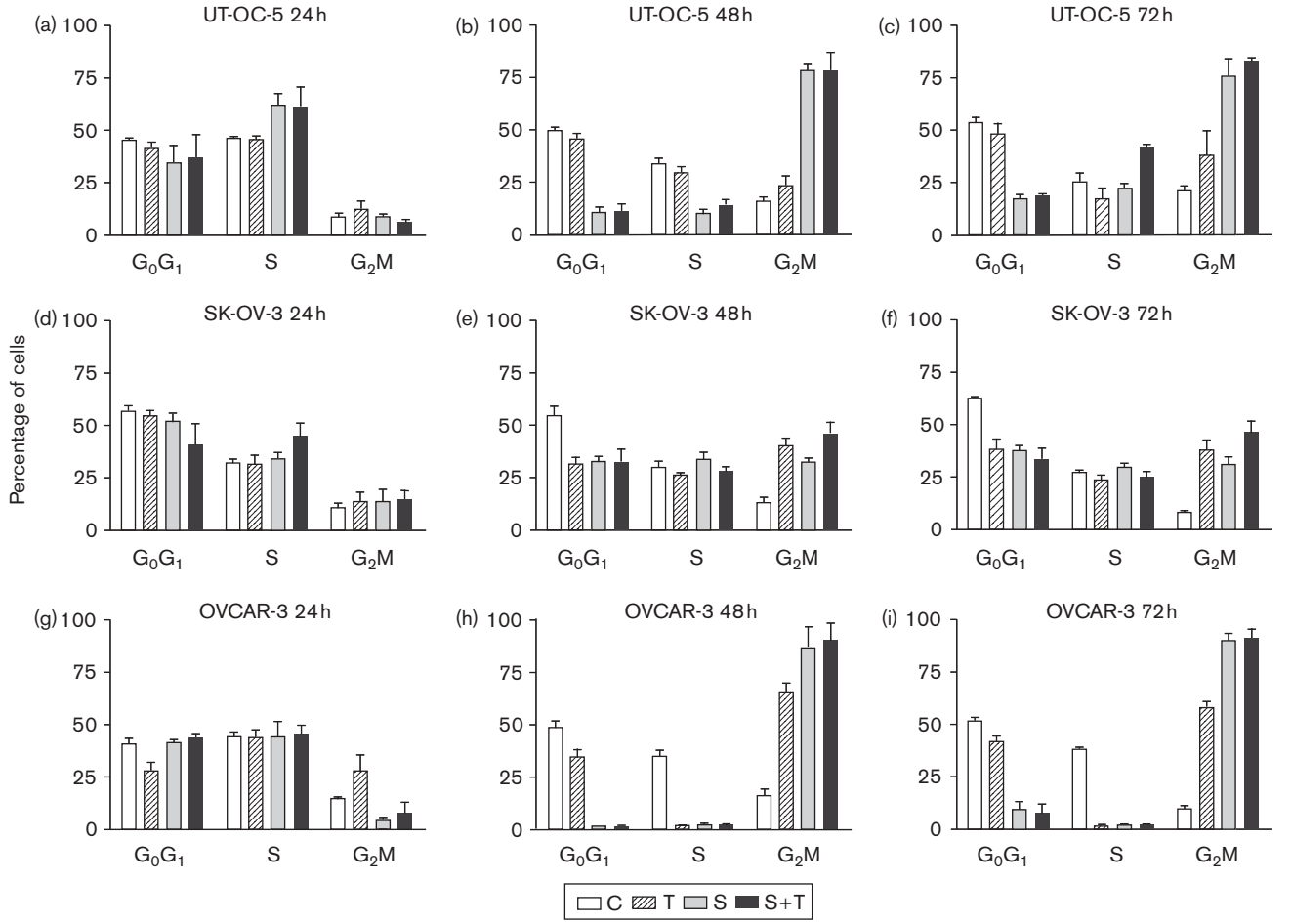
and SN-38 + docetaxel exposures increased P-gp expression on mRNA and protein levels (Fig. 4a–h). The effect of SN-38 and SN-38 + docetaxel exposures on P-gp mRNA expression was statistically significant in UT-OC-1 ($P < 0.001$, both exposures), UT-OC-2 ($P < 0.001$), UT-OC-4 ($P < 0.01$ and $P < 0.001$, respectively), UT-OC-5 ($P < 0.01$), and OVCAR-3 ($P < 0.05$) cells. In UT-OC-3 cells, only the SN-38 + docetaxel treatment led to a statistically significant ($P < 0.05$) regulation and in SK-OV-3 cells the differences between treatment groups were not statistically significant. The expression of P-gp on protein level was analyzed twice and the results were similar in both experiments.

Concomitant exposure to docetaxel, SN-38, and the P-glycoprotein inhibitor increased growth inhibition and apoptosis

As our present data suggested that the docetaxel + SN-38 induce an antagonistic effect in ovarian cancer cells, and SN-38 upregulates P-gp expression, we evaluated how inhibition of P-gp-mediated drug efflux affects cell growth and apoptosis.

Apoptosis was studied using the UT-OC-3, UT-OC-5, SK-OV-3, and OVCAR-3 cell lines. The proportion of apoptotic cells after exposure to docetaxel, SN-38, or both with PGP-4008 are shown in Fig. 5a. The percentage of apoptotic cells was low in samples treated with PGP-4008. In docetaxel-treated samples, the amount of apoptosis was very high and the percentage of apoptotic cells was increased even further when docetaxel was combined with PGP-4008. The differences between docetaxel and docetaxel + PGP-4008 were statistically significant as shown in Fig. 5a. When compared with the control or with PGP-4008-treated samples, the proportion of apoptotic cells was greater in samples treated with SN-38, SN-38 + PGP-4008, and docetaxel + SN-38. The percentage of apoptotic cells was higher after the

Fig. 3



The cell cycle parameters were measured after 24, 48, and 72-h drug exposures in the indicated cell lines [UT-OC-5 at (a) 24 h, (b) 48 h, (c) 72 h; SK-OV-3 at (d) 24 h, (e) 48 h, (f) 72 h; OVCAR-3 at (g) 24 h, (h) 48 h, (i) 72 h]. Cells were exposed to cell culture medium without drugs (C), with docetaxel (T), SN-38 (S) or docetaxel and SN-38 (S + T). Symbols for each treatment are indicated in Fig. 3a.

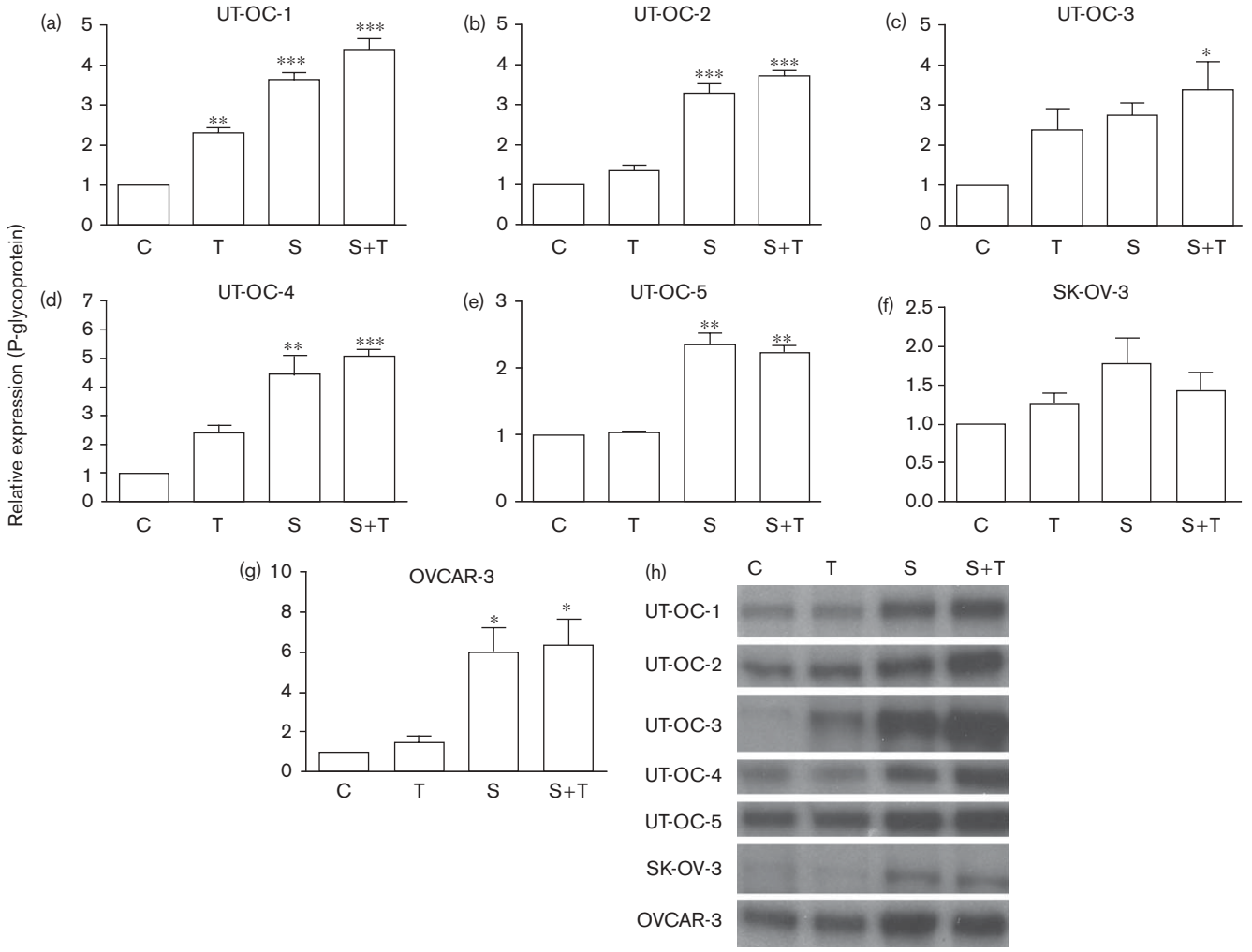
treatment of samples with docetaxel + SN-38 + PGP-4008 than after the treatment with only docetaxel + SN-38. The differences between docetaxel + SN-38 and docetaxel + SN-38 + PGP-4008 were statistically significant as shown in Fig. 5a.

The growth of the cell lines exposed to the indicated combinations of drugs with PGP-4008 is shown in Fig. 5b–h. Consistent with the apoptosis data, the number of viable cells was significantly decreased in samples treated with both docetaxel + PGP-4008 than when treated with docetaxel alone ($P < 0.05$, all cell lines). Although the number of cells was decreased in the UT-OC-3, UT-OC-4 and SK-OV-3 cells treated with docetaxel + SN-38, the number of cells was even lower in the cells treated with docetaxel + SN-38 + PGP-4008 ($P < 0.05$, all cell lines). This finding suggests that inhibition of P-gp function improves the growth inhibitory effect of combined docetaxel + SN-38. In the UT-OC-1, UT-OC-2, UT-OC-5, and OVCAR-3 cells, docetaxel + SN-38 had antagonistic effects. The effect was still antagonistic in the UT-OC-5 and OVCAR-3 cells when PGP-4008 was added, but the inhibition of growth was significantly stronger than that in cells treated with docetaxel + SN-38 ($P < 0.05$, both cell lines). Both the apoptosis and cell growth results suggest that inhibition of P-gp-mediated drug efflux sensitizes cells to the combined effects of docetaxel + SN-38.

Discussion

Long-term survival of patients with ovarian cancer is poor because the majority of cases are diagnosed at a late stage [2]. Cancer cells often acquire chemoresistance, and new treatment strategies and markers for the response to chemotherapy are crucial. In this study, we studied the effect of docetaxel and SN-38 alone and in combination on cell growth, apoptosis, and P-gp expression.

Fig. 4



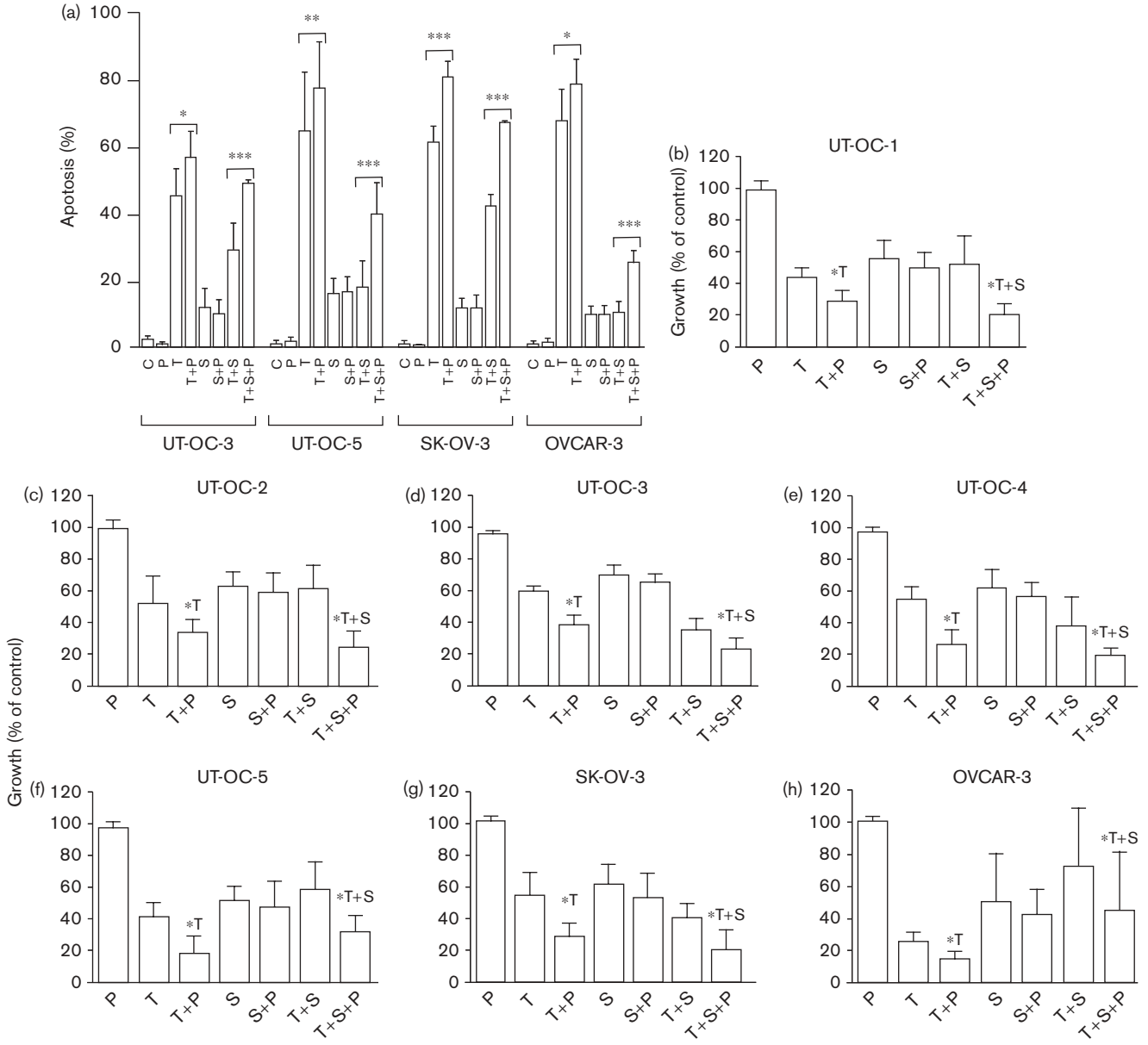
Quantitative real time (RT)-PCR (a–g) and western blotting (h) were used to detect regulation of P-glycoprotein (MDR-1) expression in cells after exposure to docetaxel and SN-38. Cells were exposed to cell culture medium without drugs (C), with docetaxel (T), SN-38 (S) or docetaxel and SN-38 (S + T) according to their half maximal effective concentration (EC_{50}) values. Quantitative RT-PCR experiments were repeated three times and western blotting was carried out twice. In a–g, the columns represent the mean of three independent experiments ± SD. *$P<0.05$, **$P<0.01$, and ***$P<0.001$ represent statistically significant differences between C and T, S or S + T treatments.

Sensitivities of ovarian cancer cells to docetaxel and SN-38 were not correlated, probably because of the different mechanism of action. The EC_{50} values for docetaxel in the UT-OC-1, UT-OC-2, UT-OC-3, UT-OC-4, and UT-OC-5 and SK-OV-3 cell lines were previously determined [35]. Although a different method was used to determine the EC_{50} values in this study, the results were consistent with those obtained previously; the UT-OC-2 cell line was the most resistant to docetaxel exposure in both studies.

The cells were treated with each drug according to the drug EC_{50} values, but docetaxel induced apoptosis more effectively than SN-38, which suggests that apoptosis is the main mechanism of growth inhibition and cell death in cells exposed to docetaxel. Other cell death mechanisms might also have a role after SN-38 exposure and greater levels of cell apoptosis might be observed over a longer period of time. SN-38 might also induce cell cycle arrest with or without subsequent apoptosis [6]. A correlation between taxane-induced growth inhibition and apoptosis has not been detected [9,44]. In this study, the cell lines were exposed to docetaxel and SN-38 according to the EC_{50} values of drugs and the level of apoptosis after docetaxel exposure varied from 35 to 70% in the individual cell lines.

Concomitant exposure of ovarian cancer cell lines to SN-38 and docetaxel produced additive, subadditive, or clearly antagonistic effects, which were partially concentration

Fig. 5



Effect of P-glycoprotein inhibitor (PGP-4008) on apoptosis and cell growth after concomitant exposures to indicated drugs. Cells were exposed to cell culture medium without drugs (C), with PGP-4008 (P), docetaxel (T), docetaxel and PGP-4008 (T + P), SN-38 (S), SN-38 and PGP-4008 (S + P), docetaxel and SN-38 (T + S) or docetaxel, SN-38 and PGP-4008 (T + S + P). (a) Proportion of apoptotic cells after combinations of docetaxel, SN-38 or both with PGP-4008 in indicated cell lines. The columns represent mean of five independent experiments ± SD. *$P<0.05$, **$P<0.01$, and ***$P<0.001$. (b–h) Cell growth data (day 5) of the indicated combinations of drugs with PGP-4008. The growth assay was repeated three times and the values represent the mean of three independent experiments ± SD. The growth of control cells (C) was set as 100%. *T represents statistically significant difference ($P<0.05$) between T and T + P treatments and *T + S represents statistically significant difference ($P<0.05$) between T + S and T + S + P treatments. In panel (a), *$P<0.05$, **$P<0.01$, and ***$P<0.001$.

dependent. In-vitro studies on paclitaxel combinations have produced varying results. In lung cancer cell lines, CPT-11 produces additive cytotoxicity when combined with paclitaxel [23]. The combination of paclitaxel and topotecan causes a synergistic effect in a human teratocarcinoma cell line [24] and an antagonistic effect in human lung cancer cells [25]. The variations in the responses might be dependent on the duration and concentration of the drug exposure as well as on the biological characteristics of the cells.

The growth effects of topoisomerase inhibitors and taxanes might be schedule dependent. Simultaneous exposure of human breast, lung, colon, and ovarian cancer

cell lines to irinotecan and paclitaxel produces antagonistic effects, whereas additive or synergistic effects are observed after sequential exposures [26]. In squamous larynx carcinoma, breast adenocarcinoma, and non-small cell lung cancer cells, the sequential exposure to topotecan and docetaxel produces synergistic effect when docetaxel is given first, but the effect is antagonistic when the reverse schedule of administration is applied [27]. Here, the effect was dependent on the cell line studied, but not on the schedule. In addition to variations in experimental settings, pharmacological differences of these drugs might cause the conflicting results between studies.

Our data indicate that both docetaxel and SN-38 induce cell cycle arrest in the G_2/M phase, but concomitant treatment does not have an additive effect on cell accumulation in the G_2/M phase and consequently docetaxel + SN-38-induced changes in the cell cycle could not account for the data on growth inhibition and apoptosis. P-gp-mediated drug efflux is the best described mechanism of resistance to antitubulin agents such as docetaxel [12]. Therefore, we evaluated the role of P-gp in the growth regulation of ovarian cancer. We used a specific P-gp inhibitor (PGP-4008) to reduce the P-gp-mediated drug efflux [11]. SN-38 upregulated the expression of P-gp on mRNA and protein levels; however, P-gp inhibitor did not have an effect on SN-38-mediated growth inhibition. Docetaxel did not have a consistent effect on P-gp expression, but P-gp inhibitor clearly sensitized cells to both docetaxel and combination of docetaxel and SN-38. Our data suggest that P-gp inhibitor may prevent docetaxel efflux and sensitize cells to concomitant treatments. Treatment of cancer cells with docetaxel + SN-38 + PGP-4008 increased the number of apoptotic cells toward those levels obtained with docetaxel treatment alone and cell growth was inhibited more efficiently when compared with cells treated with docetaxel + SN-38. These findings indicate that docetaxel-induced growth inhibition and apoptosis is inhibited, at least partially, by the action of SN-38.

Docetaxel, paclitaxel, and SN-38 have been shown to upregulate P-gp expression [45,46], indicating that these agents might be P-gp substrates. Contradictory data suggest that SN-38 might be exported through another transporter protein, cMOAT (MRP-2) (reviewed in Ref. [47]), which is not inhibited by P-gp inhibitors [11]. If both docetaxel and SN-38 or SN-38 alone increase P-gp expression in cells, but docetaxel is transported more efficiently, as our data suggest, SN-38 may even enhance docetaxel efflux, thereby inducing drug resistance. The observed effect might be dependent both on the drug concentration and the cancer cell line. The expression of P-gp in the cells might be one marker for the growth regulatory effects of docetaxel and SN-38.

Our results indicate that possible antagonistic effects must be considered when clinical studies of combinations of docetaxel and SN-38 are planned. The use of novel taxanes that have been developed to reduce P-gp-mediated drug efflux [48] might be preferable for concomitant treatments.

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