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Targeting the Growth of Ovarian Cancer Cells

In vitro effect of vitamin D_3, anticancer drugs and p53 gene therapy

ACADEMIC DISSERTATION
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To My Father

ABSTRACT

Epithelial ovarian cancer has the highest mortality rates among gynaecological malignancies. Although the efficacy of ovarian cancer treatment has improved, survival remains low with a 5-year survival rate of only 50%. Thus, the demand for new treatment strategies is high. In the present study, we evaluated the efficacy of vitamin D_3 compounds; the anticancer drugs docetaxel, irinotecan, and SN-38; and p53 gene therapy to inhibit the growth of ovarian cancer cell lines.

Vitamin D_3 compounds regulate the growth of several different types of cancer cells. Our findings indicate that high concentrations of the active metabolite of vitamin D_3, calcitriol, and the vitamin D_3 analogue EB1089, inhibit ovarian cancer cell growth. On the other hand, low concentrations of calcitriol, pro-hormone $25(OH)_2D_3$, and probably also their metabolites produced by 24-hydroxylase stimulate growth.

Docetaxel and the active metabolite of irinotecan, SN-38, are potent inhibitors of ovarian cancer cell growth. When docetaxel and SN-38 are used concomitantly, however, SN-38 interferes with the growth-inhibiting actions of docetaxel by increasing the docetaxel efflux by p-glycoprotein.

Although both docetaxel and high concentrations of calcitriol inhibit ovarian cancer cell growth, their combinations do not necessarily have the same effect. The effect of concomitant treatment of ovarian cancer cells with calcitriol and docetaxel is dependent on the calcitriol-mediated cell cycle arrest at G_2M followed by the phosphorylation of cell death-regulating protein Bcl-2 and finally cell death. If calcitriol blocks the cell cycle at the G_1 phase, Bcl-2 phosphorylation does not occur and the antiproliferative actions of docetaxel are abolished.

The p53 gene is often mutated in ovarian cancer cells, leading to aberrant cell cycle control and increased resistance to some anticancer drugs. When irinotecan and SN-38 treatments are combined with adenovirus-mediated p53 gene therapy (p53Ad), the p53 gene status of cancer cells might be the determining factor for the response. In cells expressing mutated p53 protein, p53Ad therapy might increase the cytotoxicity of both irinotecan and SN-38. Additionally, p53Ad therapy itself inhibits growth of these cells. In cells expressing normal p53, p53Ad as a single therapy is not sufficient and concomitantly with irinotecan or SN-38 does not provide additional efficacy. Our findings suggest that the expression ratios of pro- and anti-apoptotic genes, such as Bax/Bcl-2 and Bax/Bcl-XL, might serve as indicators for the efficacy of p53Ad therapy and irinotecan or SN-38 exposure. In contrast to irinotecan and SN-38, the efficacy of docetaxel is not dependent on the p53 status and p53Ad does not increase the cytotoxicity of docetaxel.

Together, these results indicate that concomitant use of antiproliferative agents might abolish the efficacy of each individual agent. Furthermore, cells do not respond similarly to a given therapy. Cell-specific properties, such as the activity of metabolizing enzymes and drug efflux proteins, ability to induce cell cycle arrest, and expression of cell survival-regulating proteins, influence the efficacy of anticancer agents to inhibit cell growth.

TIIVISTELMÄ

Gynekologisista syövistä suurin kuolleisuus on epiteliaalisessa munasarjasyövässä. Vaikka hoidon tehokkuus on kasvanut, kuolleisuus on edelleen korkea; viiden vuoden elossaoloennuste on vain noin 50%. Siksi uusien hoitomuotojen kehittäminen ja testaaminen ovatkin ensiarvoisen tärkeitä. Tässä tutkimuksessa olemme testanneet D_3-vitamiiniyhdisteiden, solunsalpaajien doketakseli, irinotekaani ja SN-38 sekä p53-geeniterapian tehokkuutta munasarjasyöpäsolulinjojen kasvun hidastamisessa.

D_3-vitamiiniyhdisteiden tiedetään säätelevän syöpäsolujen kasvua. Tulostemme mukaan D_3-vitamiinin analogi EB1089 sekä korkea pitoisuus D_3-vitamiininin aktiivista muotoa, kalsitriolia, hillitsee munasarjasyöpäsolujen kasvua. Sen sijaan kalsitriolin esimuoto 25-hydroksi D_3, matalat kalsitriolipitoisuudet sekä 24-hydroxylaasi-enstyymin muodostamat aineenvaihduntatuotteet kiihdyttävät syöpäsolujen kasvua.

Doketakseli sekä irinotekaanin aktiivinen muoto SN-38 ovat tehokkaita munasarjasyöpäsolujen kasvun hidastajia. Yhdessä käytettyinä SN-38 kuitenkin estää doketakselin syöpäsolujen kasvua hidastavia vaikutuksia, esimerkiksi lisäämällä doketakselin p-glykoproteiinivälitteistä ulosvirtausta syöpäsoluista.

Vaikka doketakseli sekä suuret kalsitriolipitoisuudet hidastavatkin munasarjasyöpäsolujen kasvua, näiden aineiden yhdistelmät eivät välttämättä ole tehokkaita. Kalsitriolin ja doketakselin yhteisvaikutus on riippuvainen kalsitriolivälitteisestä solusyklin salpauksesta G_2M-vaiheeseen, sitä seuraavasta solukuolemaa säätelevän proteiinin, Bcl-2, fosforylaatiosta ja lopulta ohjelmoidusta solukuolemasta. Jos kalsitrioli salpaa solut solusyklin G_1-vaiheeseen, Bcl-2 ei fosforyloidu ja doketakselin syöpäsolujen kasvua hidastava vaikutus estyy.

P53 on munasarjasoluissa yleisesti muuntunut geeni, jonka mutaatioiden vaikutuksesta solujen jakaantumisen säätely ei toimi tarkoituksenmukaisesti ja solujen herkkyys useille solunsalpaajille laskee. Kun munasarjasyöpäsolut altistetaan samanaikaisesti irinotekaanille tai SN-38:lle sekä p53-geeniterapialle, solujen p53-status on ratkaiseva tekijä soluvasteessa. Jos solujen p53 on muuntunut, p53-geeniterapia edesauttaa irinotekaanin ja SN-38:n syöpäsolujen kasvua hidastavia vaikutuksia. Lisäksi pelkkä p53-geeniterapia ehkäisee sellaisten solujen kasvua, joissa on muuntunut p53-geeni. Jos p53 toimii soluissa normaalisti, p53-geeniterapiasta ei ole hyötyä yksinään eikä irinotekaaniin tai SN-38:aan yhdistettynä. Tutkimustulostemme mukaan solukuolemaa edistävien ja ehkäisevien geenien ilmenemisen suhde voisi toimia soluvasteen ennusmerkkinä; korkeat Bax/Bcl-2 ja Bax/Bcl-XL suhdeluvut viittaisivat hoidon tehokkuuteen, kun taas matalat suhdeluvut ennakoisivat hoidon tehottomuutta. Doketakselin tehokkuus munasarjasyöpäsoluissa ei ole riippuvainen p53:sta, eikä p53-geeniterapia lisää doketakselin tehokkuutta.

Vaikka kukin testaamistamme hoidoista yksinään osoittautuikin tehokkaaksi syöpäsolujen kasvun estäjäksi, hoitojen yhteisvaikutukset eivät aina olleet ennakoitavissa, vaan ne saattoivat häiritä tai jopa estää toistensa toimintaa. Kaikki syöpäsolutyypit eivät myöskään reagoi samalla tavalla annettuun käsittelyyn. Solulinjakohtaiset erot metaboliassa, lääkeaineiden kuljetuksessa sekä solusyklin ja solukuoleman säätelyssä vaikuttavat ratkaisevasti hoitojen tehokkuuteen.

CONTENTS

ABBREVIATIONS

$1,25(OH)_2D_3$	1,25-dihydroxycholecalciferol, calcitriol
$25(OH)D_3$	25-hydroxycholecalciferol
1αOHase	1α-hydroxylase
24OHase	24-hydroxylase
ABC	ATP-binding cassette
Ad	adenovirus vector
ARC	activator recruited cofactor
ATP	adenosine triphosphate
Bad	Bcl-2 antagonist of cell death
Bak	Bcl-2 antagonist/killer
Bax	Bcl-2-associated X protein
Bcl-2	B-cell CLL/lymphoma 2
Bcl-XL/XS	Bcl-like 1 (XL/XS)
Bcl-w	Bcl-like 2
BCRP	breast cancer resistant protein
BH	Bcl-2 homology
Bid	BH3 interacting domain death agonist
Bik	Bcl-2-interacting killer
Bim	Bcl-like 11
Bok	Bcl-2-related ovarian killer
BRAF	v-raf murine sarcoma viral oncogene homolog B1
BRCA1/2	breast cancer gene 1 or 2
CA 125	cancer antigen 125
CAR	coxsackie adenovirus receptor
CBP	CREB (cAMP responsive element binding protein) binding protein
cDNA	complementary DNA
CIN	chromosomal instability
CMV	cytomegalovirus
CPT-11	7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxycamptothecin, irinotecan
CSC	cancer stem cell
CTNNB1	catenin (cadherin-associated protein), beta 1
CYP	Cytochrome P450 enzyme
DBP	vitamin D_3 binding protein
DBD	DNA binding domain
DIABLO	Drosophila inhibitor of apoptosis protein-binding protein
DNA	deoxyribonucleic acid
Docetaxel	(2*R*,3*S*)-*N*-carboxy-3-phenylisoserine, *N*-tert-butyl ester, 13-ester with 5, 20-epoxy-1, 2, 4, 7, 10, 13-hexahydroxytax-11-en-9-one 4-acetate 2-benzoate, trihydrate
DRIP	vitamin D receptor interacting protein
EB1089	(1(S),3(R)-dihydroxy-20(R)-(5´-ethyl-5´-hydroxy-hepta-1´(E),3´(E)-dien-1´-yl)9,10-secopregna-5(Z),7(E),10,19-triene), calcitriol analogue
EC_{50}	effective concentration 50, concentration that inhibits cell growth by 50%
EGF	epidermal growth factor
ERp60	protein disulfide isomerase family A, member 3 (MARRS)
EOC	epithelial ovarian carcinoma
Fas	TNF (tumor necrosis factor)receptor superfamily member 6
FBS	foetal bovine serum

FIGO	International Federation of Gynaecology and Obstetrics
Gadd45	growth arrest and DNA-damage-inducible, alpha
HAT	histone acetyltransferase
HDAC	histone deacetylase
HIF-1	hypoxia-induced factor 1
IGF	insulin-like growth factor
LBD	ligand binding domain
Mcl-1	myeloid cell leukaemia 1
Mdm2/X	mouse double minute 2/4 homolog
MDR-1	multidrug resistance protein 1
MOI	multiplicity of infection
MPR	multidrug resistance-associated protein
mRNA	messenger RNA
NOXA	phorbol-12-myristate-13-acetate-induced protein 1
NCoR	nuclear receptor corepressor
$p21^{waf1/cip1}$	cyclin-dependent kinase inhibitor 1A/wild-type p53-activated fragment 1
p300	E1A binding protein p300
p53	tumor protein p53 (TP53)
PBGD	human porphobilinogen deaminase
PCR	polymerase chain reaction
PGP	p-glycoprotein
PGP-I	PGP-4008, p-glycoprotein inhibitor
PIK3CA	phosphoinositide-3-kinase, catalytic, alpha polypeptide
PTEN	phosphatase and tensin homolog
PTH	parathyroid hormone
PUMA	p53 up-regulated modulator of apoptosis
QPCR	quantitative PCR
RNA	ribonucleic acid
RPA	ribonuclease protection assay
RPLP0	human acidic ribosomal phosphoprotein P0
RXR	retinoid X receptor
RT-PCR	Reverse transcription PCR
SMAC	second mitochondria-derived activator of caspases
SMRT	silencing mediator for the retinoid and thyroid hormone receptor
SN-38	7-ethyl-10-hydroxycamptothecin
SRC	steroid receptor coactivators
t1/2	half-live
TERT	telomerase reverse transcriptase
TGFβ	transforming growth factor β
TIF2	transcription intermediary factor 2
TRAIL	tumor necrosis factor-related apoptosis-inducing ligand
TRAP	thyroid hormone receptor associated protein
VDR	vitamin D receptor
VDRE	vitamin D response element
VID400	24-hydroxylase inhibitor
Vitamin D	calciferol
Vitamin D_2	ergocalciferol
Vitamin D_3	cholecalciferol

LIST OF ORIGINAL COMMUNICATIONS

This thesis is based on the following articles, which are referred to in the text by their roman numerals.

I Miettinen S, Ahonen MH, Lou YR, Manninen T, Tuohimaa P, Syvälä H, Ylikomi T. Role of 24-hydroxylase in vitamin D_3 growth response of OVCAR-3 ovarian cancer cells. Int J Cancer. 2004 Jan 20;108(3):367-73.

II Miettinen S, Grènman S, Ylikomi T. Inhibition of P-glycoprotein-mediated docetaxel efflux sensitizes ovarian cancer cells to concomitant docetaxel and SN-38 exposure. Anticancer Drugs. 2009 Apr;20(4):267-76.

III Miettinen S, Yli-Kuha AN, Grènman S, Ylikomi T. In ovarian cancer cells antiproliferative effect induced by combination of calcitriol and docetaxel is mediated by increased Bcl-2 phosphorylation. Submitted.

IV Miettinen S and Ylikomi T. Concomitant exposure of ovarian cancer cells to docetaxel, CPT-11 or SN-38 and adenovirus-mediated p53 gene therapy. Anticancer Drugs. 2009 Aug;20(7):589-600.

INTRODUCTION

Ovarian cancer is one of the most common gynaecologic malignancies and the fifth most frequent cause of cancer death in women. Many different therapeutic strategies have been developed to treat ovarian cancer. These strategies are based on maximal cytoreduction in addition to cytotoxic and cytostatic methods to reduce tumour volume. Treatments that do not effectively destroy all the cancer cells may induce selective pressure on the cancer cell population, favouring mutations that aid in the survival of neoplastic cells. Once chemoresistance develops, a new treatment strategy must be used.

In these studies, we evaluated the efficacy of vitamin D_3 compounds; the anticancer drugs docetaxel, irinotecan, and SN-38; and p53 gene therapy to inhibit the growth of ovarian cancer cell lines. In addition, we identified the molecular mechanisms that affect the cellular responses to treatment with these agents administered as either a single therapy or concomitantly, such as hormone metabolism, induction of p-glycoprotein expression causing a multidrug resistant phenotype, and the differential regulation of pro- and anti-apoptotic genes. This thesis focuses on epithelial ovarian cancer because it is the predominant type of ovarian cancer and only epithelial ovarian cancer cell lines were used to conduct the studies included in the thesis.

Epidemiologic data suggest that sunlight and ultraviolet B irradiation are protective factors against ovarian cancer (Lefkowitz and Garland 1994; Al-Moundhri et al. 2003; Garland et al. 2006). Thus, decreased synthesis of vitamin D in the skin followed by vitamin D deficiency may contribute to the initiation and progression of ovarian cancer. Further, vitamin D inhibits the growth of several different cancer types in vivo and in vitro (Saunders et al. 1992; James et al. 1996; Ahonen et al. 2000a; Blutt et al. 2000; Villena-Heinsen et al. 2002; Kumagai et al. 2005; Zhang et al. 2005a). Because of its inhibitory effects on cell growth, vitamin D is an attractive molecule for anticancer drug development. Most studies have concentrated on more common neoplasms such as prostate, colon, and breast cancer, and the role of vitamin D in ovarian cancer is not clear. Even fewer studies have evaluated the effects of combinations of vitamin D compounds and anticancer drugs in ovarian cancer.

Docetaxel, irinotecan, and the active metabolite of irinotecan, SN-38, are anticancer drugs that inhibit cell growth by causing cell cycle arrest and cell death. Docetaxel disrupts cell division by enhancing microtubule polymerisation and inhibiting depolymerisation, thus inducing the formation of abnormal and stable microtubule bundles (Gelmon 1994). Irinotecan and SN-38 interrupt DNA replication via the inhibition of topoisomerase I activity, thereby inducing single and double-strand DNA breaks (Hsiang et al. 1985). Docetaxel, irinotecan, and SN-38 are not usually used as single agents in the treatment of ovarian cancer. Their potency against ovarian cancer has been demonstrated in vitro (McDonald and Brown 1998; O'Meara A and Sevin 1999; Markman et al. 2003; Rose et al. 2003), and these agents have been studied in several clinical trials (Polyzos et al. 2005; Clamp et al. 2006). The predominant chemotherapy used in the treatment of ovarian cancer is a combination of carboplatin and paclitaxel. In randomised clinical trials, however, a carboplatin-docetaxel combination has proven to be equally effective (Vasey et al. 2004). These findings suggest that the carboplatin-docetaxel combination may be an effective alternative treatment for ovarian cancer.

Alterations in the p53 gene are the most frequent genetic events detected in many cancers. In ovarian cancer, p53 abnormalities are detected in 30% to 80% of samples (Kohler et al. 1993; Kupryjanczyk et al. 1993; Lassus et al. 2003; Salani et al. 2008). In normal cells, p53 has a role in controlling cell proliferation and death in response to various stress stimuli such as DNA damage (Vogelstein et al. 2000). Mutations in the p53 gene might lead to the accumulation of additional mutations, tumour progression, and resistance to many chemotherapeutic drugs. In ovarian cancer, functionally null p53 represents an independent molecular predictor of decreased survival (Shahin et al. 2000). Several studies have demonstrated that the growth of human cancer cell lines, including ovarian cancer, are inhibited by introducing the wild-type p53 gene into cancer cells (Santoso et al. 1995; Blagosklonny and El-Deiry 1998; Gurnani et al. 1999; Wolf et al. 1999; Miyake et al. 2000; Quist et al. 2004), usually by the use of adenoviral vectors.

REVIEW OF THE LITERATURE

1. OVARIAN CANCER

The incidence of ovarian cancer is highest in developed areas such as Scandinavian countries and North America (Daly and Obrams 1998; Parkin et al. 2005). In Finland, ovarian cancer is the eighth most common cancer among women, accounting for 3.2% of all female malignancies. In 2007, the age-adjusted incidence rate of ovarian cancer was 8.3 per 100 000 women and 418 new ovarian cancers and 298 deaths were registered. In Finland, ovarian cancer is the fifth leading cause of cancer death in women (www.cancerregistry.fi).

Ovarian cancer has the worst prognosis among gynaecological neoplasms. Because the symptoms of ovarian cancer are usually vague, only fewer than 25% of ovarian cancers are found in the early stages and in most cases the disease has already spread beyond the ovaries at the time of diagnosis (Urban and Drescher 2008). Symptoms, such as gastrointestinal or urinary symptoms, unexplained weight loss or weight gain, pelvic and abdominal pain and swelling, fatigue, abnormal or postmenopausal bleeding, and pain during intercourse, usually occur only in advanced stages. Symptoms are present in 71% to 78% of ovarian cancers, and 7% of patients have no symptoms (Olson et al. 2001; Webb et al. 2004). Approximately 75% of women with ovarian cancer are not diagnosed until the disease is in an advanced stage (Urban and Drescher 2008). The 5-year survival rate for these women is only 18%, whereas the 5-year survival rate for patients with stage I disease approaches 90%; for stage II the rates are 67% to 74%, and for stage III the rates are 33% to 50% (Heintz et al. 2006).

Currently, there are no good screening tests for ovarian cancer. Cancer antigen 125 (CA 125) is a protein that is elevated in the serum in 80% of ovarian cancers (Bast et al. 2005). It is not specific enough to be used as a general screening tool, however, especially for pre-menopausal women, because serum CA 125 levels are also increased in patients with other cancers, hepatitis, pelvic inflammatory disease, endometriosis, early pregnancy, menstruation, and benign ovarian cysts. Therefore, the test is mainly used to monitor ovarian cancer treatment and to help detect early cancer recurrence. The test is approximately 50% sensitive for detecting early stage disease (Meyer and Rustin 2000; Colombo et al. 2006).

1.1 Risk and protective factors

The aetiology of ovarian cancer is poorly understood. Ovarian cancer generally develops after menopause. As with most cancers, a woman's chance for developing ovarian cancer increases with an increasing age. In Finland the highest risk is at age 70 to 79 (www.cancerregistry.fi). A common hypothesis of the genesis of ovarian cancer is incessant ovulation theory. It is based on the fact that the number of ovulations correlates with ovarian cancer incidence (Fathalla 1971; Daly and Obrams 1998). After ovulation, the mitotic activity required to repair the ovarian epithelium increases the likelihood of genetic abnormalities that can lead to malignant transformation (Murdoch 2003). Similarly, nulliparity (Daly and Obrams 1998; Rossing et al. 2004) and infertility drug use (Rossing et al. 1994) may lead to increased ovarian cancer risk. Conversely, multiparity, use of oral contraceptives, pregnancy, and lactation are associated with a reduced risk of ovarian cancer, because they degrease the number of ovulation cycles (Hartge et al. 1994; Vessey

and Painter 1995). Hormone replacement therapy increases the risk of ovarian cancer (Garg et al. 1998; Riman et al. 2002; Morch et al. 2009).

Hereditary ovarian cancer accounts for 5% to 10% of all cases (Sarantaus et al. 2001; Garber and Offit 2005). The most significant risk factor for ovarian cancer is an inherited mutation in one of two genes called breast cancer gene 1 and 2 (BRCA-1 and -2) (Scully et al. 1996; King et al. 2003). These genes were originally identified in families with multiple breast cancer cases, but they are also responsible for 5% to 10% of ovarian cancers. The lifetime risk of BRCA mutation carriers for developing ovarian cancer is 18% to 54% for BRCA-1 mutation carriers and 2% to 19% for BRCA-2 mutation carriers (Antoniou et al. 2003).

Another known genetic link involves an inherited syndrome called hereditary nonpolyposis colorectal cancer (HNPCC), also known as Lynch syndrome. Individuals in HNPCC families are at increased risk of colorectal cancer, as well as other extracolonic cancers including endometrial, gastric, hepatobiliary, urinary track and ovary (Kehoe and Kauff 2007). The risk of HNPCC associated ovarian cancer is 12% (Aarnio et al. 1999).

1.2 Classification of ovarian neoplasms

Ovarian cancer is a cancer that begins in the cells that constitute the ovaries, including surface epithelial cells, germ cells, and the sex cord-stromal cells. Because of their complicated histological structure, the ovaries produce multiple types of benign and malignant tumours (Soslow 2008). The most common ovarian neoplasm is epithelial cancer, which is traditionally thought to origin from epithelial cells covering the outer surface of the ovary. Most epithelial tumours are benign cystadenomas. Malignant epithelial tumours are carcinomas. Epithelial tumours are classified according to cell type and are considered benign, borderline, and malignant based on cellular proliferation, nuclear atypia, and stromal invasion (Soslow 2008). Epithelial ovarian carcinomas (EOC) account for 85% to 90% of all ovarian cancers, sex-cord stromal tumours account for approximately 6%, and germ cell tumours and other tumour types both account for approximately 1% of all ovarian cancers (2003).

1.1.1 Gradeing and stageing

EOCs are classified based on their grade and stage. The most commonly used grading systems are those proposed by the International Federation of Gynaecology and Obstetrics (FIGO), the World Health Organization (WHO), and the Gynaecologic Oncology Group (GOG) (Cho and Shih 2009). The grade of ovarian cancer is based on the degree of differentiation of the cancer cells. Poorly differentiated cancers are more common in the advanced stage and well-differentiated cancers are more common in the early stage (Cho and Shih 2009).

The FIGO stage of the tumour is ascertained during surgery (Heintz et al. 2006). In stage I tumours, cancer is limited to a single or both ovaries. In stage II tumours, the growth of the cancer involves one or both ovaries with extension into the pelvis. A tumour in stage III involves the growth of the cancer in one or both ovaries, and one or both of the following: (1) The cancer has spread beyond the pelvis to the lining of the abdomen or (2) the cancer has spread to the lymph nodes. The tumour is limited to the true pelvis but with histologically proven malignant extension to the small bowel or omentum. The most advanced stage of ovarian cancer is stage IV, where

the growth of the cancer involves one or both ovaries and distant metastases have occurred. Findings of ovarian cancer cells in the pleural fluid or a parenchymal liver metastasis are signs of stage IV cancer.

1.1.2 Histological classification

Epithelial ovarian tumours are further classified based on their histological type and these subtypes have distinct molecular pathogenesis (Shih and Kurman 2004; Heintz et al. 2006; Crum et al. 2007; Kobel et al. 2008; Kurman et al. 2008; Soslow 2008; Cho and Shih 2009; Kobel et al. 2009).

Serous carcinomas (Soslow 2008) are more common than all the other histological types together, accounting 80% to 85% of epithelial ovarian carcinomas (Heintz et al. 2006). As many as 95% of patients with FIGO stages III-IV EOC have serous carcinomas and FIGO stage I serous carcinomas are very uncommon. Serous carcinomas show a broad spectrum of histological appearances, perhaps due to the genetic heterogeneity of these tumours (Cho and Shih 2009), suggesting that some tumours diagnosed as serous carcinomas represent the transformation or progression of another tumour type. Serous tumour cells exhibit morphological features similar to the epithelial lining of the fallopian tube. Recent studies suggest that the distal fallopian tube might be the site of origin of at least some serous carcinomas that were previously thought to arise in the ovary or pelvic peritoneum (Crum et al. 2007; Kindelberger et al. 2007).

Endometrioid carcinomas (Soslow 2008) accounts for approximately 10% of all ovarian carcinomas and is the second most common EOC subtype. It is the most common tumour represented by FIGO I stage carcinomas, probably constituting at least half of such cases. Endometrioid carcinomas resemble their endometrial counterparts. In addition, they are usually associated with endometriosis, endometrioid borderline tumour, or a synchronous endometrial endometrioid neoplasm.

Clear cell carcinomas (Soslow 2008) are rare. Nevertheless, it is the third most common EOC in North America, accounting for approximately 5% of all ovarian tumours and its prevalence is even higher in Japan. Most clear cell carcinomas are FIGO stage I or II tumours. Low response rate to chemotherapeutic drugs is typical for clear cell carcinomas (Sugiyama et al. 2000; Ho et al. 2004).

Mucinous carcinomas (Soslow 2008) comprise less than 3% of all ovarian carcinomas. At the time of the diagnosis, approximately 50% to 65% of mucinous carcinomas are FIGO stage I tumours. These tumours have excellent prognosis, but advanced stages of mucinous carcinomas are associated with a very poor survival that surpasses the poor prognosis of women with advanced stage serous disease (Sherman et al. 2004). Mucinous tumour cells resemble epithelial cells of the intestinal (Heinzelmann-Schwarz et al. 2006) or endocervical origin (Moriya et al. 2003) and these types have distinct clinicopathological characters.

Transitional cell tumours (Soslow 2008) resemble urothelial carcinomas. **Mixed epithelial tumours** (Soslow 2008) are diagnosed when at least two histologically distinctive elements are present and each constitutes at least 10% of the tumour. Mixed epithelial tumours, transitional cell tumours, and **undifferentiated carcinomas** (Soslow 2008), lacking histological distinguishable features, are rare.

1.1.3 Proposed new classification

Emerging data support the idea that instead of representing one disease with variable characteristics, ovarian carcinoma constitutes several distinct disease entities (Shih and Kurman 2004; Crum et al. 2007; Kobel et al. 2008; Kurman et al. 2008; Cho and Shih 2009; Kobel et al. 2009). It is becoming evident that each major histological EOC type has its own genetic defects that affect specific signalling pathways. Additionally, within the most common histological types, the molecular pathogenesis of low-grade versus high-grade tumours appears to be different (Shih and Kurman 2004; Ouellet et al. 2005; Le Page et al. 2006; Kurman et al. 2008; Cho and Shih 2009). These characteristics are clinically relevant, although a specific therapy for each disease entity do not yet exist. For example low-grade serous, mucinous, and clear cell carcinomas are intrinsically resistant to standard chemotherapeutic agents (Shih and Kurman 2004; Kurman et al. 2008; Cho and Shih 2009). Recent studies have identified characteristic markers for different histological subtypes of EOC. Table 1 shows the proposed new classification of EOCs for type I and type II tumours (Shih and Kurman 2004; Kurman et al. 2008; Cho and Shih 2009).

Table 1. Common precursor lesions and molecular features of Type I and Type II EOCs (Modified from (Shih and Kurman 2004; Cho and Shih 2009)).

	Common precursors	Most frequent mutations	CIN[a]	Tumour characteristics
Type I tumours				
Low-grade serous	Serous borderline tumour	KRAS, BRAF	Low	
Low-grade endometrioid	Endometriosis	CTNNB1, PTEN, KRAS, LOH, microsatellite instability	Low	-Slow growth rate -Large tumour size -Confined to the ovary at diagnosis -Resistance to chemotherapeutic agents
Most clear cell	Endometriosis	PIK3CA, KRAS, microsatellite instability, TGFβ RII	Low	
Mucinous	Mucinous borderline tumours	KRAS	Low	
Type II tumours				
High-grade serous	Dysplasia in inclusion cysts /Fallopian tube/?	p53, amplification and overexpression of HER/neu and Akt2, inactivation of p16	High	-High grade -Spread disease at diagnosis
High-grade Endometrioid	?	p53	High	-Evolve rapidly -Highly aggressive
Clear cell	?	?	?	
Undifferentiated	?	?	?	
Carcinosarcoma	?	p53	?	

a, Low vs. high CIN (chromosomal instability) refers to comparison between low-grade and high-grade carcinomas within the same histological type.
Abbreviations: ?, Not yet identified; Akt2, thymoma viral proto-oncogene 2; BRAF, v-raf murine sarcoma viral oncogene homolog B1; CTNNB1, catenin (cadherin-associated protein), beta 1; HER/neu, Human Epidermal growth factor Receptor 2, ErbB-2; KRAS, v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog; LOH, loss of heterozygosity; PIK3CA, phosphoinositide-3-kinase, catalytic, alpha polypeptide; PTEN, phosphatase and tensin homolog; TGF, transforming growth factor.

1.3 Treatment and prognostic factors

Survival of patients with ovarian cancer has improved due to new cytostatic agents and improved surgical treatments. Treatment and prognosis depend on the type of ovarian cancer and how far it has spread at the time of diagnosis (Cho and Shih 2009). Most women are treated with surgical cytoreduction followed by

combination chemotherapy, usually a combination of carboplatin and paclitaxel or other platinum and taxane compounds (Ozols et al. 2003; Vasey et al. 2004). Combined docetaxel-carboplatin may be an alternative treatment for ovarian cancer (Pfisterer et al. 2004; Vasey et al. 2004).

Although the majority of patients, 70% to 80% have a complete clinical response to surgery and first-line chemotherapy, 50% to 70% of the patients will eventually relapse and die of their disease (Heintz et al. 2006). Known prognostic factors for ovarian cancer comprise age (Chan et al. 2006; Pectasides et al. 2007), FIGO stage (Heintz et al. 2006) and grade (Zanetta et al. 1998; Vergote et al. 2001), and residual tumour size (Griffiths 1975; Hoskins et al. 1994; Makar et al. 1995; Eltabbakh et al. 1998; Chi et al. 2001).

1.4 Stem cells in ovarian cancer

The classic hallmarks of cancer include growth factor independence, insensitivity to antiproliferative and apoptotic signals, limitless replication potential, sustained angiogenesis, and tissue invasion for metastasis (Hanahan and Weinberg 2000). Aggressive tumours selectively enhance these characteristics in progression to advanced state tumours. Two models explain cancer development and progression (Pan and Huang 2008). The first, termed the stochastic model, assumes that every cancerous cell has the capacity to extensively proliferate and regenerate a tumour. This model assumes that all cancer cells have an equal probability of regenerating a tumour. In contrast, the model of cancer stem cell (CSC) assumes that only a very small subset of cells within the tumour cell population actually has the capacity to initiate and sustain tumour growth. Stem cells are capable of dividing throughout life and creating highly specialised cells for cell renewal and tissue repair. On the other hand, cancer stem cells or tumour initiating cells are a subpopulation of tumourigenic cells that possess properties of stem cells. Thus, they are different from the bulk of cancer cells that are believed to be non-tumourigenic (Pan and Huang 2008). Emerging evidence has recently been provided to suggest that the capability to sustain tumour formation, growth, metastasis and resistance to chemotherapy in ovarian as well as other human malignancies, exclusively reside in CSCs (Clarke et al. 2006; Dalerba et al. 2007; Pan and Huang 2008). The stem cell-like phenotype of these cells and their limited number within the bulk of the tumour may lead to disease relapse although the primary lesion is eradicated by conventional therapies, and consequently their specific detection and targeting could be highly valuable for treatment of cancer.

A consensus of five defining criteria has been established to affirm the existence of CSCs: 1) self-renewal; 2) restriction to a small minority of the total tumour population; 3) reproducible tumour phenotype; 4) multipotent differentiation into non-tumourigenic cells; and 5) expression of distinctive cell surface markers, permitting consistent isolation (Clarke et al. 2006; Dalerba et al. 2007).

In ovarian cancer, Bapat and colleagues (Bapat et al. 2005) isolated two colonies of cells from ascites sample that could organise into anchorage-independent, spherical structures in culture, similar to those found in ascites (Burleson et al. 2004; Bapat et al. 2005). These clones were capable of forming xenografts in nude mice, with a histopathology similar to parental tumour, and expressed the stem cell factor receptor CD117 (Bapat et al. 2005). More recently, using primary human ovarian tumours, Zhang and co-workers isolated and characterized ovarian CSCs fully

capable of re-establishing their original tumour hierarchy in vivo (Zhang et al. 2008). In their study, ovarian CSCs organised in self-renewing, anchorage-independent spheres and were reproducible isolatable using antibodies against CD44 and CD117. Moreover, CSCs were also capable of intraperitoneal tumourigenesis and they could serially propagate tumours in animals (Zhang et al. 2008), fulfilling the accepted criteria for CSCs (Clarke et al. 2006; Dalerba et al. 2007).

2. ANTICANCER DRUGS

2.1 Docetaxel

Docetaxel ((2*R*,3*S*)-*N*-carboxy-3-phenylisoserine, *N*-tert-butyl ester, 13-ester with 5, 20-epoxy-1, 2, 4, 7, 10, 13-hexahydroxytax-11-en-9-one 4-acetate 2-benzoate, trihydrate) is a semisynthetic taxane derivative of 10-deacetylbaccatin III, a plant alkaloid extracted from the needles of the European yew *Taxus baccata* (Gelmon 1994). In the cells, taxanes disrupt mitosis by enhancing the polymerisation of the tubules into microtubules and inhibiting microtubule depolymerisation, thereby inducing the formation of abnormal and stable microtubule bundles and interfering with the function of mitotic spindles (Gelmon 1994). Because microtubule do not dissemble in the presence of taxanes, they accumulate inside the cell and cause initiation of apoptosis (Yvon et al. 1999). Two taxanes currently in clinical use are paclitaxel and docetaxel. Although their actions are quite similar, some differences exist. Docetaxel has greater affinity to β-tubulin, targeting centrosome organisation and acting on cells in three phases of the cell cycle, S/G_2/M. Paclitaxel is effective at the G_2M phase junction, whereas docetaxel is most effective against cells at the S phase and arrests cells at G_2M (Crown and O'Leary 2000). Additional differences include a greater uptake of docetaxel into the cells and slower efflux of docetaxel from the cells, leading to longer retention times and providing a possible explanation for the incomplete cross-resistance between these two taxanes (Hanauske et al. 1992; Riou et al. 1992).

Both taxanes have wide tissue distribution and are highly protein-bound, but they do not easily penetrate the central nervous system. Taxanes are extensively metabolised in the liver by the cytochrome P-450 enzymes and undergo biliary excretion as the main route of elimination (Gligorov and Lotz 2004). The side effects of docetaxel include reversible noncumulative neutropenia, fluid retention, cutaneous reactions, and hyperlacrimation, and appear to be schedule-dependent; that is, the side effects differ between administration every 3 three weeks or a weekly dosing schedule. Less acute toxicity emerges when docetaxel is administered weekly than when administered at 3-week intervals (Gligorov and Lotz 2004).

Conflicting explanations of the antitumour effects of docetaxel have been published, and its biochemical mechanisms of actions are not fully understood. Schimming and co-workers (Schimming et al. 1999) found that docetaxel potently induces mitotic arrest, but only weakly induces apoptosis. Neither event, however, is correlated with docetaxel's antitumour activity in a wide variety of cancer cells, including ovarian carcinoma (Kolfschoten et al. 2002). Instead of apoptosis, cells undergo lytic cell death. In breast cancer cell lines, docetaxel induces cell death via necrosis and a mitotic catastrophe (cell death occurring during metaphase) (Morse et al. 2005; Zamzami et al. 2005). This event is characteristic of cells with aberrant chromosome segregation after abnormal mitosis. Nuclear envelopes form around a single or

group of chromosomes, followed by the development of giant cells with multiple micronuclei that are morphologically distinguishable from apoptotic cells (Morse et al. 2005). Docetaxel induces apoptosis by inactivation of the anti-apoptotic function of Bcl-2 protein by phosphorylation. In hormone-refractory prostate cancer cells, docetaxel triggers apoptosis via a mitotic catastrophe (Fabbri et al. 2008). Cells are first transiently arrested at the G_2M phase, followed by the appearance of G_0G_1 hypo- and hyperdiploid cells, and finally Bcl-2 phosphorylation, activation of caspases 2 and 3, and apoptosis. In a wide variety of cancer cells, the mutational status of the p53 gene is not a determining factor for the antitumour activity of anti-mitotic drugs such as docetaxel (O'Connor et al. 1997; Schimming et al. 1999). In addition to cell cycle arrest and cell death, docetaxel possess antiangiogenic activity in vitro and in vivo (Sweeney et al. 2001).

2.1.1 Docetaxel and ovarian cancer

In ovarian cancer cells, docetaxel causes G_2M arrest, followed by the phosphorylation of Bcl-2, activation of caspase-3, and apoptosis. If docetaxel induces cell cycle arrest at the S phase, Bcl-2 phosphorylation, activation of the caspase cascade, and apoptosis do not occur, even though cell death is very efficient, suggesting that other cell death mechanisms besides apoptosis are involved (Kolfschoten et al. 2002). In ovarian cancer cells, similar to previous studies on breast cancer cells, a correlation between docetaxel sensitivity and an apoptotic response has not been established (Schimming et al. 1999; Kolfschoten et al. 2002). Furthermore, p53 status, induction of $p21^{waf1/cip1}$ expression, and Bcl-2 phosphorylation are not in correlation with cellular response to docetaxel (Kolfschoten et al. 2002).Recently, BRCA-1 status was associated with responsiveness of ovarian cancer cells to taxanes (De Ligio et al. 2009).

In vitro -studies suggest that docetaxel as a single agent (Kelland and Abel 1992; Riou et al. 1992; Engblom et al. 1997; Sato et al. 2004) or in combination with cisplatin (Engblom et al. 1999) would be more cytotoxic than paclitaxel or combined paclitaxel-cisplatin. Docetaxel as a single agent have been used in phase I and II clinical trials to treat recurrent or progressive ovarian cancer (Kaye et al. 1997; Fujiwara et al. 1999; Markman et al. 2003; Oishi et al. 2003; Rose et al. 2003; Mäenpää and Leminen 2009). In these trials the response rates have varied from 10% to 30% and the most severe side effect has been neutropenia. Single-agent docetaxel has proven to be effective against paclitaxel and platinum-resistant disease (Markman et al. 2003; Rose et al. 2003).

Combined docetaxel-carboplatin may be an alternative treatment for ovarian cancer. In a phase I/II study conducted by Pfisterer and co-workers, the response rate for docetaxel-carboplatin therapy was 70% (Pfisterer et al. 2004). Furthermore, in a randomized clinical trial, carboplatin-paclitaxel and carboplatin-docetaxel combinations have proven to be equally effective as first-line treatments for ovarian cancer (Vasey et al. 2004).

2.2 Irinotecan and SN-38

Camptothecin is an alkaloid obtained from the leaves, bark, and seeds of the Chinese tree *Camptotheca acuminata* (“Xi Shu” or tree of joy) (Lorence and Nessler 2004). Topotecan and irinotecan (CPT-11) are two camptothecin derivatives that are used in clinical practice (Pizzolato and Saltz 2003). Camptothecin derivatives act by

inhibiting topoisomerase I activity, thereby interrupting DNA replication (Hsiang et al. 1985; Hsiang and Liu 1988). DNA normally exists as a supercoiled double helix that unwinds during replication with single strands serving as templates for the synthesis of new strands. Torsional stress develops ahead of the replication fork, and transient cleavage of one or both strands of DNA is required to relieve the stress. Topoisomerases facilitate this process. Topoisomerase II induces transient double-stranded breaks, whereas topoisomerase I breaks one strand of the DNA helix. This action allows for rotation of the broken strand around the intact strand. Topoisomerase I then religates the broken strand to restore the integrity of the double-stranded DNA (Pizzolato and Saltz 2003). Camptothecin stabilises the cleavable complex between topoisomerase I and DNA breaks (Hsiang et al. 1985; Hsiang and Liu 1988). These stabilised breaks are reversible and non-lethal. When a DNA replication fork collides with the cleavable complex, however, single-strand breaks are converted to irreversible double-strand breaks, leading to cell cycle arrest and then senescence, apoptosis, or necrosis (Bhonde et al. 2006a). Because camptothecin primarily targets topoisomerase I, its action to induce cell death is dependent on active DNA replication and thus cells are most sensitive to camptothecin treatment during S-phase; cells at G_1 or G_2 phase are 100 to 1000 times less sensitive to camptothecin (Pizzolato and Saltz 2003). Camptothecin delays progression of the cell cycle at the G_2M or G_1 phase (Bhonde et al. 2006a).

Irinotecan (CPT-11, 7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxy-camptothecin) is a semisynthetic derivative of camptothecin. Irinotecan has an extremely complex pharmacological profile that depends on numerous enzymes involved in metabolic transformation and active protein transport, regulating intestinal absorption and hepato-biliary secretion mechanisms (Mathijssen et al. 2001).

The molecular structure of irinotecan includes a bulky side chain on the core structure, and the enzymatic cleavage of the side chain is required for its pharmacological activity. Irinotecan is converted to an active metabolite, SN-38 (7-ethyl-10-hydroxycamptothecin), by carboxylesterases found mainly in the liver, but neoplastic cells also possess carboxylesterase activity (Xu et al. 2002). Because of its prodrug nature, irinotecan has little intrinsic topoisomerase I inhibitory activity and *in vitro* SN-38 inhibits topoisomerase I activity 250 to 1000 times more effectively than irinotecan (Hsiang and Liu 1988; Jansen et al. 1997b). The relationship between carboxylesterase activity and cancer cell chemosensitivity has been demonstrated in vitro (van Ark-Otte et al. 1998) and in vivo (Kawato et al. 1991; Guichard et al. 1999). Carboxylesterase is also a potential target for gene therapy in cancer to increase the activity of irinotecan (Meck et al. 2001; Wierdl et al. 2001). Jonsson and co-workers reported that the activity of SN-38 and irinotecan do not correlate in all patient-derived tissues. Moreover, SN-38 is inactive in some gastrointestinal tumours in which irinotecan has strong activity (Jonsson et al. 2000). These findings suggest that irinotecan is not simply an inactive prodrug of SN-38, but possesses its own activities as well.

Both irinotecan and SN-38 are metabolised and converted to less active forms by hepatic CYP3A enzymes (Lokiec et al. 1995; Lokiec et al. 1996; Santos et al. 2000).

Anticancer drug screen in National Cancer Institute has revealed that p53 status may affect cells` sensitivity and response to irinotecan and SN-38 (O'Connor et al. 1997). In colon carcinoma cells expressing wild-type p53, SN-38 induces G_2M arrest,

followed by a long-term tetraploid G_1 arrest and senescence; whereas in p53-deficient cells, G_2M arrest is followed by apoptosis or necrosis (Bhonde et al. 2006b). Similar results have been obtained with colorectal cancer cells, where cell survival during SN-38 induced growth arrest is dependent on wild-type $p21^{waf1/cip1}$ and p53. Cells deficient in $p21^{waf1/cip1}$ and p53 expression enter into G_2M arrest and apoptosis (Hayward et al. 2003). The same study showed that downregulation of the heterodimerisation partner of Bax, Bcl-XL, may switch the response to SN-38 from senescence to apoptosis and enhance the general cytotoxicity of the drug. In human head and neck squamous cell carcinoma, Bax overexpression might indicate a better response to SN-38 (Guo et al. 2000). In colorectal and testicular cancer cells SN-38 can induce apoptosis via upregulating the expression of Bax (Ueno et al. 2002; Hayward et al. 2003).

In addition to topoisomerase I inhibition, camptothecin derivatives inhibit tumour angiogenesis (Nakashio et al. 2002). Furthermore, camptothecin derivatives inhibit the activity of hypoxia-inducible factor 1 (HIF-1), a master regulator of the ability of cancer cells to survive under oxygen deprivation (Rapisarda et al. 2002).

2.2.1 Irinotecan and SN-38 in ovarian cancer studies

In vitro studies indicate that ovarian cancer cells are sensitive to irinotecan and SN-38. Furthermore, these drugs do not share cross-resistance with other commonly used agents in ovarian cancer, such as cisplatin, carboplatin, and paclitaxel (O'Meara A and Sevin 1999). Sensitivity to irinotecan has also been demonstrated in a human ovarian cancer xenograft model in nude mice (Jansen et al. 1997a). The efficacy of irinotecan is dependent on topoisomerase I activity, which is enhanced in ovarian cancer cells by CAP-therapy consisting of cisplatin, doxorubicin, and cyclophosphamide (Kigawa et al. 1999). In ovarian cancer cells, the SN-38 and irinotecan-induced cell death is not dependent on p53 status (McDonald and Brown 1998).

Irinotecan became commercially available in Japan in 1994, where its approved indications are small-cell and non-small-cell lung cancers and cancers of the cervix and ovaries. In Europe, irinotecan is approved as a second-line treatment for colon cancer and in the USA for fluorouracil-refractory advanced colorectal cancer (Pizzolato and Saltz 2003). In phase II trials, irinotecan showed moderate efficacy and substantial toxicity in patients with metastatic platinum-resistant or platinum-refractory epithelial ovarian cancer (Bodurka et al. 2003). In these phase II trials, response rates of 14% to 30% were obtained with varying dosing schedules (reviewed in ref. (Bodurka et al. 2003)) and in combination with cisplatin, the response rate was approximately 40% (Sugiyama et al. 1998). The dose-limiting toxicities of irinotecan in clinical use are neutropenia, nausea and vomiting, anorexia, diarrhoea, and fatigue (Bodurka et al. 2003).

2.3 Concomitant use of taxanes and topoisomerase I inhibitors

The rationale for trials of docetaxel and irinotecan or SN-38 as a combination therapy is based on their different mechanisms of action and absence of cross-resistance (Jensen et al. 1997). Previous studies demonstrated that a combination of taxane and a topoisomerase I inhibitor have divergent growth effects on cancer cells (Chou et al. 1994; Kaufmann et al. 1996; Pei et al. 1997; Kano et al. 1998; Taron et al. 2000). Irinotecan produced additive cytotoxicity when combined with paclitaxel

in lung cancer cell lines (Pei et al. 1997). Topotecan and paclitaxel showed synergistic effects in a human teratocarcinoma cell line (Chou et al. 1994) and antagonistic effects in human lung cancer cells (Kaufmann et al. 1996). The growth effects of topoisomerase inhibitors and taxanes are schedule-dependent. Simultaneous administration might produce antagonistic growth effects, whereas additive or synergistic growth effects are observed with sequential administration (Kano et al. 1998; Taron et al. 2000). The combination of taxanes and topoisomerase I inhibitors has been tested as a treatment for non-small-cell lung cancer (Wachters et al. 2005), small-cell lung cancer (Stathopoulos et al. 2005a), gastric cancer (Park et al. 2006b; Sym et al. 2008), and breast cancer (Stathopoulos et al. 2005b) in phase I to III clinical studies with varying results.

Both irinotecan and docetaxel are effective in reducing ovarian cancer growth (McDonald and Brown 1998; O'Meara A and Sevin 1999; Markman et al. 2003; Rose et al. 2003). The feasibility of a docetaxel-irinotecan combination has been studied in ovarian cancer. Docetaxel-irinotecan treatment for recurrent disease produced a response rate of 63% in one study (Clamp et al. 2006). In another study the combination of docetaxel and irinotecan was moderately effective, with an overall response rate of 20%, for platinum-resistant, paclitaxel-pretreated patients (Polyzos et al. 2005). The addition of irinotecan to first-line carboplatin-docetaxel chemotherapy was also well tolerated, but the authors failed to show higher response rates for triple therapy when compared with carboplatin-docetaxel therapy alone (Clamp et al. 2006). The response rates for triple therapy vs. carboplatin-docetaxel therapy were 48% and 50%, respectively. Furthermore, the combination of weekly topotecan and docetaxel was shown to be effective and well tolerated in patients with platinum resistant ovarian cancer (Gupta et al. 2009), and addition of irinotecan with paclitaxel and carboplatin as a second-line therapy achieved 83% overall response rate (Escobar et al. 2004).

Because both irinotecan and docetaxel are metabolised by hepatic CYP3A4, competition might occur when these drugs are given sequentially, decreasing the clearance of docetaxel (Adjei et al. 2000). Irinotecan and paclitaxel produce a significant pharmacokinetic interaction that is characterised by increased plasma levels of both irinotecan and SN-38 (Mathijssen et al. 2001).

3. P-GLYCOPROTEIN AND RELATED PROTEINS IN DRUG RESISTANCE

Cancer cells may acquire resistance via multiple mechanisms. These mechanisms, however, can be divided into three major categories: 1), decreased uptake of water-soluble drugs such as cisplatin, which require transporters to enter cells; 2), various changes in cells that affect the capacity of cytotoxic drugs to kill cells, including alterations in cell cycle, increased repair of DNA damage, reduced apoptosis, and altered metabolism of drugs; and 3), increased energy-dependent drug efflux (Szakacs et al. 2006).

3.1 Multidrug resistance proteins

In tumour cells, a multidrug resistance (MDR) phenotype is commonly associated with an ATP-dependent decrease in cellular drug accumulations due to the

overexpression of certain ATP-binding cassette (ABC) transporter proteins located on the cell plasma membranes (reviewed in ref. (Leslie et al. 2005)). Thus, these proteins belong to the third category of resistance mechanisms. In addition to their roles in drug resistance, these proteins are expressed in normal tissues and are involved in protecting tissues from xenobiotic accumulation and resulting toxicity. In humans, this gene family contains at least 48 ABC genes that are organised into 7 subfamilies (A-G).

The first discovered protein of this family, and by far the most studied, is p-glycoprotein or MDR-1 encoded by the ABCB1 gene (Ambudkar et al. 1999). Isolation of a second distantly related ABC protein, the multidrug resistance-associated protein 1 (MRP1) encoded by ABCC1, led to discovery of eight more genes within the same ABC subfamily (Kool et al. 1997)(reviewed in ref. (Leslie et al. 2005)). A third drug transporter, also distantly related to p-glycoprotein and MRPs, is the breast cancer resistant protein (BCRP, encoded by ABCG2) (Doyle et al. 1998; Bates et al. 2001).

MRPs belong to the same ABC subfamily and share approximately 50% amino acid identity with each other. In contrast, p-glycoprotein and BRCP belong to separate subfamilies and share only approximately 20% identity with each other and with MRPs. Despite differences in amino acid sequence and domain organisation, there is considerable overlap in the spectrum of drugs to which these transporters confer resistance. Thus, p-glycoprotein confers high levels of resistance against bulky amphipathic natural product-type drugs such as taxanes, vinca alkaloids, and camptothecins (reviewed in ref. (Leslie et al. 2005)).

BCRP confers resistance to a narrower range of anticancer drugs than p-glycoprotein or MRPs. The spectrum includes anthracyclines, mitoxantrone, and topoisomerase I inhibitors such as camptothecin. It does not, however, confer resistance to the vinca alkaloids, paclitaxel, or cisplatin (reviewed in ref. (Leslie et al. 2005)).

The transport efficiency of p-glycoprotein differs between the camptothecin derivatives irinotecan and SN-38 and is higher with irinotecan than SN-38 (Hoki et al. 1997; Chu et al. 1999; Takeba et al. 2007), although the data are controversial and other transporting proteins, such as BCRP might be involved (Hoki et al. 1997; Chu et al. 1999; Maliepaard et al. 1999; Schellens et al. 2000; Mathijssen et al. 2003).

In EOC the p-glycoprotein overexpression is correlated with disease progression (Chen et al. 2009) and it may be an independent prognostic factor of survival in patients with serous ovarian carcinoma (Yakirevich et al. 2006). Furthermore, overexpression or polymorhism of p-glycoprotein in ovarian cancer cells or tissue samples is strongly associated with paclitaxel resistance (Kamazawa et al. 2002; Green et al. 2006; Hille et al. 2006; Johnatty et al. 2008). P-glycoprotein transfection studies, however, show that p-glycoprotein expression may by associated with enhanced sensitivity to the apoptotic effect of paclitaxel and reduced sensitivity to its G_2M arrest effect, but this effect is unrelated to the effect of p-glycoprotein on intracellular drug accumulation (Li and Au 2001). In ovarian cancer cell lines, paclitaxel and SN-38 can overcome cisplatin resistance by downregulating the MRP-mediated efflux system of cisplatin (Komuro et al. 2001). Although both SN-38 and paclitaxel upregulate the expression of p-glycoprotein, this upregulation is not involved in the sensitivity of ovarian cancer cells to these

drugs. Cisplatin alone upregulates the expression of MRP, but in cells exposed to cisplatin and SN-38 or paclitaxel, MPR expression is downregulated.

4. P53

4.1 Function of p53

Of the millions of patients diagnosed annually with cancer world wide, approximately half have tumours with p53 mutations. The p53 gene is a tumour suppressor gene that is responsible for orchestrating a complex system of responses to potentially cancer-causing DNA damage. The tumour suppressor gene p53 mediates cell cycle arrest and apoptosis as a response to stress stimuli or DNA damage (reviewed in ref. (Vousden 2000)). If DNA lesions cannot be repaired, cells die by apoptosis. Mutations in the p53 gene allow cells to divide despite DNA damage, favour the accumulation of further mutations, promote abnormal growth, and may reduce the efficiency of chemotherapeutic drugs (reviewed in ref. (Lowe et al. 2004)).

p53 is a DNA-binding phosphoprotein that normally exists as a homotetramer or a complex of tetramers. In several reports, p53 is described as a biochemically latent form that is rapidly degraded (t1/2 ~30 min). Alternative splicing, conformational change, phosphorylation, protein-protein interactions, and regulation of nuclear localisation regulate p53 activity post-transcriptionally and post-translationally. Based on various biochemical and cell culture studies, more than 36 different amino acids within p53 can be modified to regulate its activity (reviewed in ref. (Bode and Dong 2004; Kruse and Gu 2009)).

4.1.1 Activation of p53

The p53 gene is a transcription factor that regulates the activation of several genes responsible for cell cycle progression, apoptosis, senescence, DNA repair, autophagy, metabolism, and aging (Kruse and Gu 2009). The p53 activation comprises three key steps: (1) p53 stabilisation, (2) antirepression, and (3) promoter-specific activation (reviewed in ref. (Kruse and Gu 2009)). Stress-induced p53 stabilization (1) occurs through many different mechanisms, such as DNA damage, many of which act by affecting the ability of Mdm2 to ubiquitinate p53. In this phase acetylation of p53 is not required, and after DNA repair and feedback response, cells continue to divide. Antirepression (2) describes the release of p53 from the repression mediated by Mdm2 and MdmX. This step requires the acetylation of p53 at key lysine residues and facilitates the activation of specific subsets of p53 target genes, such as $p21^{waf1/cip1}$ and Gadd45 to induce cell cycle arrest and DNA repair. For the full activation (3) of specific promoters, p53 recruits and interacts with numerous cofactors. These act by modifying p53, the surrounding histones, or other transcription factors. Regulation of the activation of specific groups of p53 target genes may require exact combination of cofactors and posttranslational modifications. In this phase p53 targets pro-apoptotic genes, such as Bax, PUMA, and NOXA, and the induction of apoptosis is irreversible.

4.1.2 Regulation of cell cycle and DNA repair

p53 regulates the G_0G_1 and G_2M phases of the cell cycle following exposure of the cells to DNA-damaging agents. Because DNA damage can prevent the initiation and

progression of the cell cycle at multiple points, late G_1, S, G_2, checkpoint delays may allow time for DNA repair, thereby preventing replication or segregation of a damaged genome. The p53 gene triggers G_1 arrest through transcriptional activation of the cyclin-dependent kinase inhibitor $p21^{waf1/cip1}$ (Kruse and Gu 2009). The p53-induced proteins that contribute to maintain G_2M arrest include Gadd45 and $p21^{waf1/cip1}$ (Jin et al. 2000; Jin et al. 2003; Bhonde et al. 2006b). The latter is not required to trigger DNA damage-induced G_2M arrest, but is essential for maintaining G_2M arrest and for suppressing apoptosis while the DNA damage is being repaired (Bhonde et al. 2006b). The p53- $p21^{waf1/cip1}$ pathway preferentially leads to cell cycle arrest and the absence of $p21^{waf1/cip1}$ leads to apoptosis (Hayward et al. 2003).

Growth arrest after DNA damage constitutes a switch of cellular priorities from DNA replication to DNA repair (Kruse and Gu 2009). The p53 gene may act as a DNA damage recognition protein, inducing the activity of repair enzymes and recruiting repair proteins to DNA strand breaks and insertion/deletion mismatches. It can also reanneal DNA strand breaks. Moreover, the activity of nucleotide excision repair is partially regulated by p53 (Kruse and Gu 2009).

4.1.3 Apoptosis

If DNA lesions cannot be repaired, cells carrying wild-type p53 die by apoptosis. Apoptosis is a tightly regulated multi-step process, characterised by cell shrinkage, chromatin condensation, and nuclear and cell fragmentation (Cotter 2009).

Apoptosis is mediated by two converging cascades, referred to as the extrinsic and intrinsic pathways of apoptosis and p53 is involved in both (Kruse and Gu 2009). In the intrinsic apoptotic pathway, several p53-regulated genes, including bax, noxa, and puma, enhance the secretion of cytochrome *c* into the cytoplasm from the mitochondria (Fojo and Bates 2003). Furthermore, p53 downregulates the expression of anti-apoptotic protein Mcl-1 (Pietrzak and Puzianowska-Kuznicka 2008). In addition to the intrinsic pathway, p53 regulates a series of genes initiating the extrinsic apoptotic pathway, namely Fas ligand and TRAIL, resulting in activation of caspase-8 and -3 and apoptosis (Kruse and Gu 2009). Several lines of evidence suggest that the p53 protein interacts directly with the mitochondria and promote apoptosis (Kruse and Gu 2009). In response to DNA damage, mitochondrial p53 translocation triggers a rapid apoptotic response that occurs prior to p53 target gene activation (Bennett et al. 1998; Schuler et al. 2000). P53 can induce cytochrome c release directly by forming complexes with the pro-apoptotic proteins Bcl-2 and Bcl-XL (Mihara et al. 2003). During apoptosis, caspases cleave the p53 protein and regulate its activity (Sayan et al. 2006).

4.2 p53 in ovarian cancer

In cancer, p53 mutations are spread throughout the gene; they are mostly missense mutations leading to amino-acid substitutions in the wild-type protein. These mutations usually lead to the synthesis of a mutant protein that has increased cellular stability but impaired function (Bode and Dong 2004). The mutant p53 accumulates in the cells, reaching levels 10- to 100-fold higher than those of the wild-type protein (Runnebaum et al. 1996; Bode and Dong 2004).

Mutations in the p53 gene are detected in 30% to 80% of ovarian cancer patients (Kohler et al. 1993; Kupryjanczyk et al. 1993; Lassus et al. 2003; Salani et al.

2008). In several studies mutated p53 have been associated with a poor prognosis (Anttila et al. 1999a; Anttila et al. 1999b; Shahin et al. 2000; Skirnisdottir et al. 2002; Bali et al. 2004; Vartiainen et al. 2008; Schildkraut et al. 2009). The prevalence of p53 mutations is highly dependent on the histological subtype of the ovarian cancer. The mutation incidence is particularly high in high-grade serous carcinomas (Anttila et al. 1999a; Singer et al. 2005; Salani et al. 2008). The p53 expression status is used to classify serous ovarian carcinomas into two distinct subtypes: Patients with aberrant p53, i.e., excessive expression or completely negative p53 status, have a 5-year overall survival of 26%, whereas patients with normal p53 have a 5-year survival of 79% (Lassus et al. 2003). Based on results obtained in vitro, p53 status is particularly important in sensitivity of ovarian cancer cells to cisplatin (Brown et al. 1993; Righetti et al. 1996; Righetti et al. 1999; Clarke et al. 2004).

Accumulation of mutated p53 in malignant cells leads to the generation of anti-p53 autoantibodies in the ascites fluid of ovarian cancer patients. The prognostic significance of preoperative serum and ascitic p53 antibodies in advanced ovarian carcinoma was studied by Abendstein and co-workers, who reported that the detection of anti-p53 antibodies in ascites is a sign of shorter disease-free survival and worse overall survival (Abendstein et al. 2000).

Although p53 status is convincingly strong prognostic factor for survival in patients with ovarian cancer, in vivo mice study, where p53 mutant ovaries were transplanted into wild-type hosts, failed to show generation of ovarian cancers, suggesting that p53 mutation alone is not sufficient for ovarian cancer genesis (Chen et al. 2004).

5. P53 GENE THERAPY

Neoplastic cells often possess decreased chemosensitivity, which might be related to defects in the apoptotic cell death or cell cycle regulatory mechanisms in cancer cells (Fojo and Bates 2003). The frequent occurrence of p53 mutations in human cancers has led several investigators to evaluate its role as a therapeutic target. By restoring the normal function of p53, the goal is to turn off the malignant phenotype of a cancer cell, inhibit its growth, or enhance drug sensitivity. The first clinical trials aimed at restoring wild-type p53 function utilised retroviral vectors (Roth et al. 1996). Although the retroviral vector used in this study proved to be inefficient, the study provided the first proof of principle. Subsequent trials used adenoviral vectors to restore normal p53 function. The advantages of using adenovirus vectors are their ability to infect cells in all phases of the cell cycle, easily obtained high titers, and their capacity to accommodate large DNA inserts (Kamen and Henry 2004; Volpers and Kochanek 2004). Furthermore, adenoviral DNA does not integrate into the genome of a target cell and is not replicated during cell division, minimising the probability of cell transformation (Kamen and Henry 2004; Volpers and Kochanek 2004). The major disadvantages are the instability of transfection; the frequent occurrence of antibodies against adenoviruses in the general population; preferential infection of cells expressing the coxsackie adenovirus receptor (CAR) and $\alpha\upsilon$-integrin; and the lack of selectivity, which leads to the infection of normal cells, particularly hepatocytes, with high efficiency (Bergelson 1999; Fechner et al. 1999; Fojo and Bates 2003; Kamen and Henry 2004; Volpers and Kochanek 2004).

Despite their limitations, p53-adenovirus vectors (p53Ad) appear to be well tolerated. Preliminary results of clinical trials demonstrated antitumour activity of p53Ad gene therapy in patients with squamous cell carcinoma of the head and neck (Clayman et al. 1999), glioma (Lang et al. 2003) and non-small-cell lung cancer (NSCLC) (Swisher et al. 1999). In vitro the efficiency of p53Ad has been shown in lung cancer (Fujiwara et al. 1994), anaplastic thyroid (Blagosklonny et al. 1998), bladder (Miyake et al. 2000), and endometrial cancer cells (Ramondetta et al. 2000), among many other cancer cell types. In vitro studies have shown that docetaxel may enhance p53Ad transduction and increase the expression of exogenous p53 and apoptosis (Yoo et al. 2004).

Adenovirus infection may alter the pharmacokinetics and pharmacodynamics of anti-cancer agents and should be considered when designing therapeutic regimens for patients. For example, in rats infected with recombinant adenoviruses expressing the human p53 or lacZ gene, plasma clearance of docetaxel by hepatic CYP3A2, a rat homolog of human CYP3A4-metabolising chemotherapeutic agents, is significantly reduced (Wonganan et al. 2009).

5.1 P53 gene therapy in ovarian cancer

5.1.1 In vitro and in vivo studies

In ovarian cancer cell culture models, p53Ad gene therapy alone inhibits growth and promotes apoptosis, despite the endogenous p53 status of ovarian cancer cells (Santoso et al. 1995; Wolf et al. 1999). Also, concomitant treatment with adenovirus-mediated p53 gene therapy and cisplatin, doxorubicin, 5-fluorourasil, methotrexate, paclitaxel or etoposide inhibits ovarian cancer cell proliferation more efficiently than each of these agents alone (Nielsen et al. 1998; Gurnani et al. 1999; Ryu et al. 2009). The efficacy is further increased in combination with p53Ad, cisplatin, and paclitaxel (Gurnani et al. 1999). Furthermore, paclitaxel increases the number of ovarian cancer cells transduced by p53Ad in a dose-dependent manner (Nielsen et al. 1998). This effect requires lower concentrations of paclitaxel than is required for microtubule condensation.

In xenograft models, the effect of p53 gene therapy is controversial. In the p53 null ovarian cancer cell xenograft model, a dosing schedule of the p53Ad therapy that, by itself, had a relatively minimal effect on tumour burden, had a much greater effect on the tumour burden when combined with paclitaxel (Nielsen et al. 1998). In nude mice implanted intraperitoneally with human ovarian cancer cells bearing a mutated p53 gene, the response to p53Ad therapy showed a significant improvement in survival, with a survival time greater than that of untreated animals. No statistically significant survival advantage was observed, however, between p53Ad- and control Ad vector-treated mice (von Gruenigen et al. 1998). In another study, p53Ad treatment effectively suppressed the growth of peritoneal tumours and prolonged the survival of the treated group (Kim et al. 1999).

In addition to acting as gene delivery vectors, adenoviruses can be targeted to destroy tumour cells. In vivo data from an orthotopic murine model of peritoneally disseminated ovarian cancer suggest that an oncolytic adenovirus can be used to treat advanced ovarian cancer as a single agent or in combination with chemotherapeutic agents such as gemcitabine and epirubicin (Raki et al. 2008).

5.1.2 Clinical trials with p53Ad in ovarian cancer

Preclinical and preliminary/phase I/II clinical data (Buller et al. 2002a; Buller et al. 2002b; Wen et al. 2003; Wolf et al. 2004) indicated that intraperitoneal p53 gene therapy might be a promising tool for improving survival of ovarian cancer patients. The following phase III trial, however, was closed after the first interim analysis because an adequate therapeutic benefit was not reached (Zeimet and Marth 2003). Several reasons could have affected the outcome of this trial (reviewed in (Zeimet and Marth 2003)). P53 gene therapy can only restore full functionality of p53-regulated pathway, if none of the downstream genes are affected. In addition to p53 mutations, a malfunction of p53 downstream genes (e.g., c-fos, $p21^{waf1/cip1}$, Gadd45, Bax) might be involved in aberrant cell cycle and cell death control in cancer cells (Moorehead and Singh 2000; Qin and Ng 2001; Jin et al. 2003). For example, overexpression of a p53-regulated c-fos gene has been observed in ovarian cancer tissues from patients that failed cisplatin therapy and in ovarian cancer cells selected for cisplatin resistance (Scanlon et al. 1991; Elkeles et al. 1999). Moreover, defects in apoptosis cascades; epigenetic silencing of p53 target genes; and dominant negative effect of mutated p53 protein might have affected the response to p53 gene therapy (reviewed in (Zeimet and Marth 2003)). Additionally, in contrast to in vitro experiments, the number of cells transfected with the adenoviral vector is not very high in vivo. Too large residual tumour size; complex tumour structure with multiple cell types; lack of adenovirus receptors CAR and αυ-integrin on cancer cells; and antibodies against adenoviruses could have limited the number of cells transfected with the p53 transgene (Zeimet and Marth 2003).

6. BCL-2 FAMILY

The Bcl-2 gene family encodes a group of proteins that regulate apoptosis (Shimizu et al. 2004). Anti-apoptotic members of this gene family, including Bcl-2 (Haldar et al. 1997), Bcl-XL (Minn et al. 1996), and Mcl-1 (Michels et al. 2005), promote cell survival, whereas pro-apoptotic members like Bax (Yin et al. 1997), Bak (Degli Esposti and Dive 2003), and Bcl-XS (Minn et al. 1996; Fridman et al. 2001) promote cell death. The expression of these proteins plays a crucial role in oncogenesis as well as in the resistance to chemotherapy. Altered expressions is observed in many malignancies such as acute lymphocytic leukaemia and bladder, breast, prostate, colorectal, and ovarian cancers (Tsujimoto et al. 1984; Bargou et al. 1995; Tsujimoto 1996; Singh et al. 1998; Kassim et al. 1999; Yoshino et al. 2006). Bcl-2 was one of the first apoptosis-regulating proteins discovered (Tsujimoto et al. 1984; Tsujimoto 1996).

Proteins belonging to Bcl-2 family can be divided into three subfamilies (reviewed in references (Letai 2008; Yip and Reed 2008; Cotter 2009)). Proteins such as Bcl-2, Bcl-XL, Bcl-W, and Mcl-1 form the first subfamily and their structure contains four Bcl-2 homology (BH1-4) domains, which are required for their anti-apoptotic function. These domains drive interactions with other Bcl-2 family members, particularly at the level of the mitochondria, where they regulate the release of pro-apoptotic mediators such as SMAC (DIABLO). The second subfamily consists of pro-apoptotic members, such as Bax and Bak, which form pores or interact with pore-forming proteins at the level of the mitochondrial membrane, a function that is antagonised by the members of the first subfamily. These pro-apoptotic members

lack the BH4 domain. Members of the third subfamily provide a link between the terminal effector processes and the signalling network that informs the cell of its status regarding events such as growth factor stimulation, redox balance, DNA integrity, cell attachment, and microtubule function. These proteins have only a short BH3 domain, hence the name BH3-only proteins, and the subfamily consists of proteins such as Bim, Bid, and Bad (Bcl-2 antagonist of cell death). Their activity is regulated mainly by post-translational modifications. The BH3-only subfamily is further divided into activators (Bid and Bim) and sensitizers (Bad, Bik, Noxa). Activators stimulate the action of pro-apoptotic family members, whereas sensitizers restrain the activity of anti-apoptotic proteins of the Bcl-2 family.

Bax suppresses tumorigenesis and stimulate apoptosis. These actions are partially dependent on p53 (Yin et al. 1997). Both Bax and Bak are essential for caspase activation in intrinsic apoptosis (Ruiz-Vela et al. 2005). Bak, but not Bax, is required for Bcl-XS-induced apoptosis (Lindenboim et al. 2005). Bax forms homodimers as well as heterodimers with Bcl-2. As a homodimer, Bax induces apoptosis. When Bcl-2 is expressed in cells it forms a heterodimer with Bax and blocks apoptosis. Bax also heterodimerizes with other anti-apoptotic members of the Bcl-2 family, such as Bcl-XL and Mcl-1 (Sedlak et al. 1995). In addition to apoptosis, Bcl-2 family members are suggested to take part in regulation of a non-apoptotic programmed cell death, autophagy (Shimizu et al. 2004).

In hormone-refractory prostate cancer, overexpression of Bcl-2 is associated with aggressive phenotype of the disease, but the presence of Bcl-2 protein could indicate a survival benefit in patients scheduled for taxane therapy (Yoshino et al. 2006). In addition to its anti-apoptotic function, Bcl-2 may promote tumorigenesis by attenuating DNA repair by downregulating the mismatch repair activity (Youn et al. 2004).

6.1 Bcl-2 family proteins in normal ovary and in ovarian cancer

A high rate of follicular cell apoptosis continues in the ovaries during reproductive life. Mcl-1 has been identified as the main ovarian anti-apoptotic Bcl-2 family protein. The main pro-apoptotic proteins are Bok, Bim, and Bad (reviewed in (Hsu and Hsueh, 2000).

In a recent report, high Bcl-2 expression in primary ovarian tumours, but not in metastasis, was shown to correlate with worse overall and shorter progression-free survival (Elstrand et al. 2009). Also increased expression of Mcl-1 has been associated with poor prognosis (Shigemasa et al., 2002). In one study, expression of Bax without Bcl-2 expression correlated with poor clinical outcome (Marx et al. 1997). The results are controversial, however; in another study, reduced expression of Bax was associated with a shorter progression-free and overall survival (Schuyer et al. 2001). Furthermore, the balance between expression levels of anti- and pro-apoptotic members of Bcl-2 family might predict the likelihood of cancer cells to respond to chemotherapy and transition of cells from benign state to malignancy (Lohmann et al. 2000).

Overexpression of Bcl-2 alone (Kassim et al. 1999) or simultaneously with Bcl-XL (Yang et al. 2004) is linked to platinum resistance. Concomitant inhibition of Mcl-1 and Bcl-XL activity has recently been shown to induce apoptosis in ovarian cancer cells (Brotin et al. 2009). Moreover, concomitantly with cisplatin, downregulation

of these proteins caused a complete death of cancer cells thus avoiding subsequent in vitro recurrence of cancer cells.

Interestingly, elevated levels of Bcl-2 protein have been found from urine (Anderson et al. 2009), and serum (Camlica et al. 2008) of ovarian cancer patients, suggesting that detection of Bcl-2 from these samples together with CA 125 could serve as a tool for ovarian cancer diagnostics.

7. VITAMIN D

Vitamin D (calciferol) is involved in regulating calcium homeostasis in the human body. The lack of vitamin D_3 (cholecalciferol) in the diet or malfunction of its activating enzymes causes rickets and thus the compound is included in vitamins (Brown et al. 1999). Vitamin D regulates the growth and differentiation of several normal and neoplastic cells and tissue types (Issa et al. 1998; Brown et al. 1999; Omdahl et al. 2002). Moreover, vitamin D also exhibits immuno- and neuro-modulatory activity (Omdahl et al. 2002; Kalueff et al. 2006).

Vitamin D_3 is the naturally occurring form of vitamin D in vertebrates. Because vitamin D_3 is produced in the skin after exposure to sunlight, humans do not require dietary vitamin D when adequate sunlight is available. Adequate sunlight is not always available, however, and in these cases animal products constitute the major source of vitamin D_3. Saltwater fish and fish liver oils are good sources of vitamin D_3. Also eggs, meat, and butter contain small quantities of D_3. Vitamin D_2 (ergocalciferol) is the naturally occurring form of vitamin D in plants. Both D_3 and D_2 are used for human diet supplementation (Breslau and Zerwekh 1997; Horst and Reinhardt 1997). In Finland, milk is fortified with vitamin D_3. The requirement for vitamin D in healthy adults has not been precisely defined, but the estimates vary from 200 to 600 IU per day and might even exceed 2000 IU per day (Hollis 2005). Excessive amounts of vitamin D are not normally available from usual dietary sources. There is a wide margin of safety between the therapeutic and toxic doses of vitamin D intake. For example, abnormal increases in serum $25(OH)D_3$, causing hypercalcemia, are not observed up to a daily dose of more than 10000 to 20000 IU (Hollis 2005; Heaney 2008). The symptoms of vitamin D toxicity include hypercalcemia, hypercalciuria, anorexia, nausea, vomiting, thirst, polyuria, muscular weakness, joint pain, diffuse bone demineralization, and general disorientation. If toxicity continues, death will eventually occur (Todd et al. 1987; Blank et al. 1995; Vieth et al. 2002; Carroll and Schade 2003; Barrueto et al. 2005; Klontz and Acheson 2007).

7.1 Vitamin D metabolism

In vertebrates, vitamin D_3 is produced photochemically in the skin from 7-dehydro-cholesterol (provitamin D_3) by the actions of sunlight or ultraviolet light and heat. Vitamin D_3 has no known intrinsic biologic activity, but is considered to be a preprohormone. The compound is activated when it is first metabolized in the liver to 25-hydroxyvitamin D_3 [$25(OH)D_3$, prohormone] and then to $1,25(OH)_2D_3$ (calcitriol, active hormone) by the kidney or other target organs (Reviewed in (Wikvall 2001)). The normal range of serum $25(OH)D_3$ is between 25 to 137.5 nmol/l (Weaver and Fleet 2004). The normal range of serum calcitriol concentrations (0.05-0.15 nmol/l) is 500 to 1000 times lower than that for serum

$25(OH)D_3$ (Mehta and Mehta 2002). Both calcitriol and serum $25(OH)D_3$ exhibit seasonal variations (Hine and Roberts 1994). In the blood, vitamin D_3 metabolites bind to vitamin D_3 binding protein (DBP) and the major storage sites are fat and muscle tissue (Jones et al. 1998; Collins and Norman 2001).

7.1.1 25-Hydroxylase

The 25-hydroxylation of vitamin D_3 is the first step in vitamin D activation (Wikvall 2001), producing $25(OH)D_3$. Three cytochrome P450 enzymes, CYP27A1, CYP2R1, and CYP3A4, possess 25-hydroxylase activity (Cali and Russell 1991; Guo et al. 1993; Cheng et al. 2003; Gupta et al. 2004; Gupta et al. 2005). The liver is the major site of 25-hydroxylation. Extra-hepatic tissues, such as kidney, intestine, skin, and testis, however, also express these enzymes (Gascon-Barre et al. 2001; Schuessler et al. 2001; Cheng et al. 2003; Theodoropoulos et al. 2003). The production of $25(OH)D_3$ by 25-hydroxylases is not significantly regulated and production is mainly dependent on the substrate concentration. Thus, serum $25(OH)D_3$ concentrations are used as an indicator of the vitamin D status (Hollis 2005). Calcitriol and $25(OH)D_3$ downregulate the expression of CYP27A (Axen et al. 1995; Theodoropoulos et al. 2001), whereas growth hormone, insulin-like growth factor-1, and dexamethasone all enhance CYP27A expression (Araya et al. 2003). The regulation varies, however, depending on the cell and tissue type. CYP3A4 expression is upregulated by calcitriol due to a vitamin D response element (VDRE) on its promoter site (Thompson et al. 2002). CYP3A4 is a broad spectrum enzyme and, in addition to vitamin D, is involved in the metabolism of several anticancer drugs such as docetaxel and irinotecan (Engels et al. 2007).

7.1.2 1α-Hydroxylase (1αOHase)

The pro-hormone $25(OH)D_3$ is converted to an active $1,25(OH)_2D_3$ (calcitriol) form by the enzyme 1α-hydroxylase (1αOHase), encoded by the gene CYP27B1 (Fu et al. 1997; Monkawa et al. 1997; St-Arnaud et al. 1997). The main site of this hydroxylation is the kidney, where the enzyme is located in the inner mitochondrial membrane of the proximal and distal convoluted tubule cells. In addition to the kidney, 1αOHase expression is detected in several tissues, including skin, intestine, pancreas, adrenal medulla, brain, and placenta (Zehnder et al. 2001). Unlike 25-hydroxylases, renal 1αOHase is tightly controlled. It is downregulated by calcitriol, hypercalcemia, and hyperphosphatemia, and upregulated by parathyroid hormone (PTH), calcitonin, hypocalcemia, and hypophosphatemia (Horst and Reinhardt 1997; Bland et al. 1999; Omdahl et al. 2002; Zhang et al. 2002). Similar to 25-hydroxylases, however, 1αOHase regulation may be cell and tissue-specific, especially in extra-renal sites (Omdahl et al. 2002).

7.1.3 24-Hydroxylase (24OHase)

24-Hydroxylation by 24-hydroxylase (24OHase, CYP24A1) is the primary step in a metabolic pathway to inactive and degrade vitamin D metabolites. It is a mitochondrial enzyme that catalyses the hydroxylation of calcitriol ($1,25(OH)_2D_3$) to $1,24,25(OH)_3D_3$, while $25(OH)D_3$ is converted to $24,25(OH)_2D_3$. 24OHase might also catalyse further hydroxylation steps of vitamin D metabolism (Sakaki et al. 1999). 24OHase is co-expressed with the vitamin D receptor (VDR) and is directly regulated by calcitriol (Akeno et al. 1997; Horst and Reinhardt 1997). Different vitamin D analogues, retinoids, androgens, PTH, and ethanol, also regulate

24OHase expression (Allegretto et al. 1995; Roy et al. 1995; Armbrecht et al. 1998; Akutsu et al. 2001a; Zierold et al. 2001; Lou et al. 2005; Lou and Tuohimaa 2006; Shankar et al. 2008), but there are tissue-specific variations. 24OHase is a ubiquitous enzyme and may be present in every cell and tissue that contains the VDR. In addition to the kidney, where it was originally discovered, 24OHase is detected in the prostate, intestine, ovary, and many other organs expressing the VDR (Christopherson et al. 1986; Armbrecht and Boltz 1991; Lum et al. 1993; Skowronski et al. 1993; Alroy et al. 1995; Albertson et al. 2000; Harant et al. 2000).

7.2 Vitamin D Receptor

Most of the effects of calcitriol are mediated via a specific intracellular receptor, the VDR, which belongs to the nuclear receptor superfamily and contains transcription factors involved in many physiological functions, such as the control of homeostasis, reproduction, metabolism, cell differentiation, and embryonic development. Nuclear receptors bind to their response elements in DNA and regulate the transcription of specific genes in response to their cognate ligands. In addition to vitamin D, the superfamily comprises receptors for hydrophobic molecules, such as steroid hormones, the retinoic acid isoforms all-trans and 9-cis retinoic acid, thyroid hormones, fatty acids, leukotrienes, and prostaglandins. The superfamily also includes numerous orphan receptors for which ligands are still unknown or have only recently been identified (Renaud and Moras 2000; Chawla et al. 2001). There are currently 48 members in the nuclear receptor superfamily, which makes it the largest family of eukaryotic transcription factors known to date (Maglich et al. 2001). Similar to other nuclear receptors, the VDR is composed of five domains (A-E), of which the DNA binding domain (DBD, C) and the ligand binding domain (LBD, E) are highly conserved among nuclear receptors (Tsai and O'Malley 1994; Miyamoto et al. 1997; Jones et al. 1998; Kumar and Thompson 1999; Pike et al. 2003).

Calcitriol has the greatest binding affinity to the VDR. The binding affinity of $25(OH)D_3$ is 50-fold less, which partially explains its weaker activity when compared to calcitriol (Bouillon et al. 1995). In addition to vitamin D metabolites, some bile acid derivatives also have an affinity for the VDR (Adachi et al. 2005). The VDR is ubiquitously expressed in almost all tissues in the human body (Walters 1992; Pike et al. 2003).

7.2.1 VDR as a transcription factor

Most of the known biological effects of calcitriol occur via the direct transcriptional regulation of specific target genes. These effects are mediated through its interaction with the VDR, followed by binding to specific DNA sequences called VDREs in the promoter regions of target genes. Target genes usually have more than one VDRE (Carlberg et al. 2007; Turunen et al. 2007). Several genes are reported to be regulated by calcitriol, but only a small number of genes contain a VDRE. Examples of such genes are 24OHase, 1αOHase, cyclin C, $p21^{cip/waf}$, coactivator TIF2, and corepressor SMRT (Dunlop et al. 2004; Väisänen et al. 2005; Carlberg et al. 2007; Turunen et al. 2007).

To regulate gene expression, the VDR binds DNA predominantly as a heterodimer with a common partner, the retinoid X receptor (RXR), a nuclear receptor for 9-*cis* retinoic acid, in a calcitriol-dependent manner (Lemon et al. 1997). As with other

steroid receptors, ligand binding to the VDR drives conformational changes within the LBD, dissociating corepressor proteins, and creating a binding site for coactivators that enhance transcription (Rochel et al. 2000). A number of coactivators interact with liganded VDR, including steroid receptor coactivators (SRC-1, -2, and -3) , and the VDR-interacting protein (DRIP/ARC/TRAP) complex (Naar et al. 1999; Rachez et al. 1999). These coactivators link the liganded VDR to enzymes with histone acetyltransferase (HAT) activity, such as p300/CBP, that cause chromatin relaxation, which allows binding and assembly of the transcription machinery and, finally, the initiation of transcription (Chan and La Thangue 2001).

In the absence of a ligand, the VDR recruits corepressor proteins such as the nuclear receptor corepressor-1 (NCoR-1), a silencing mediator for the retinoid and thyroid hormone receptor (SMRT); and Alien and Hairless, which subsequently recruit histone deacetylases (HDACs) to repress the activity of target genes (Chen and Evans 1995; Hörlein et al. 1995; Dwivedi et al. 1998; Polly et al. 2000b; Potter et al. 2002; Lempiäinen et al. 2005). Corepressor SMRT is involved in the transcriptional repression of genes such as osteocalcin and 24OHase by the unliganded VDR because both SMRT and the VDR are recruited to VDREs in the absence of calcitriol (Kim et al. 2009). In some cases, such as in regulating the expression of the transcription factor Pit-1, the VDR represses transcription as a liganded homodimer without RXR (Seoane and Perez-Fernandez 2006), whereas in other cases the VDR-RXR heterodimer recruits corepressors NCoR1 and SMRT in a strictly VDR agonist-dependent manner (Herdick and Carlberg 2000; Sanchez-Martinez et al. 2008). The capacity of corepressors and coactivators to associate in several diverse complexes increases the complexity of their transcriptional regulatory roles and allows for temporal- and cell-specific modulation of the regulation process.

7.3 Non-genomic action of vitamin D

In contrast to genomic actions that generally take hours and days to occur, rapid non-genomic effects of calcitriol occur within minutes or even seconds; and their signal transduction is thought to involve the formation of second messengers such as cyclic nucleotides, diacylglycerol, inositol trisphosphate, and arachidonic acid (Nemere and Campbell 2000; Boyan et al. 2003; Schwartz et al. 2003). The non-genomic actions of calcitriol include rapid intestinal absorption of Ca^{2+}, secretion of insulin by pancreatic β-cells, opening of voltage-gated Ca^{2+} and Cl^- channels in osteoblasts, and the rapid migration of endothelial cells (reviewed in (Norman 2006)). Contradictory reports have been published concerning the mediators of these non-genomic actions. Increasing evidence suggests that several mechanisms might be involved. Ligand conformation might have a role in triggering the genomic and non-genomic responses (Norman et al. 1999). Calcitriol is flexible and can adopt different conformations for genomic and non-genomic actions. A new ligand-binding pocket was recently found in the VDR structure. This pocket is hypothesized to bind only the calcitriol conformation that mediates non-genomic actions (Mizwicki et al. 2004; Mizwicki et al. 2005). For some non-genomic actions, a functional VDR located on the cell plasma membrane might be required (Lieberherr et al. 1989; Erben et al. 2002), but other reports suggest that the actions might be mediated via a different receptor such as plasma membrane protein annexin II (Baran et al. 2000a; Baran et al. 2000b) or membrane-associated rapid

response steroid receptor (MARRS/ERp60) (Farach-Carson and Nemere 2003). The non-genomic actions of calcitriol in ovarian cancer have not been studied.

8. Vitamin D and cancer

Both epidemiologic and *in vitro* studies indicate that vitamin D may be an important factor in the development and progression of cancer (Garland et al. 1989; Bhalla et al. 1994; Tangrea et al. 1997; Ahonen et al. 2000a; Knight et al. 2007). Because of its inhibitory effects on growth, vitamin D is an attractive molecule for anticancer drug development studies.

8.1 Anticancer mechanisms of calcitriol

Experimental studies demonstrated that calcitriol inhibits the growth of many cancer cell lines. Calcitriol inhibits cancer cell growth through several mechanisms that affect both cell cycle control and apoptosis. Furthermore, it has both differentiating (Gocek and Studzinski 2009) and anti-angiogenic properties (Merke et al. 1989; Getzenberg et al. 1997; Iseki et al. 1999; Mantell et al. 2000; Nakagawa et al. 2005a; Nakagawa et al. 2005b; Chung et al. 2006).

Calcitriol induces cell cycle arrest at the G_1 and G_2M phases, but the mechanisms for G_1 and G_2M arrest overlap and are cancer cell-specific. Some cell cycle-regulating genes, such as Gadd45, cyclin C, and $p21^{waf1/cip1}$ contain VDRE and are direct transcriptional targets of calcitriol (Freedman 1999; Polly et al. 2000a; Jiang et al. 2003). In breast cancer cells, calcitriol-induced cell cycle arrest at G_2M phase does not require a functional p53 gene (Mathiasen et al. 1999). In prostate cancer cells, however, p53 is required for calcitriol-induced G_0 arrest, but not for G_1 accumulation or apoptosis (Polek et al. 2003). Moreover, in prostate cancer cells, calcitriol reduces c-myc expression followed by cell cycle arrest at G_1 (Rohan and Weigel 2009). In addition, calcitriol may have many indirect effects on the cell cycle due to cross-talk with other pathways, such as transforming growth factor β (TGFβ), insulin-like growth factor, (IGF) and epidermal growth factor (EGF) (Huynh et al. 1998; Tong et al. 1998; Tong et al. 1999; Yanagisawa et al. 1999).

Calcitriol modulates the expression of several Bcl-2 family proteins to regulate apoptosis. Calcitriol downregulates anti-apoptotic Bcl-2 and Bcl-XL in breast and prostate cancer cells and leukaemia cells (James et al. 1996; Blutt et al. 2000; Kumagai et al. 2005) and upregulates pro-apoptotic Bax and Bak expression in prostate and breast cancer cells and in colorectal adenoma and carcinoma cells (Diaz et al. 2000; Narvaez and Welsh 2001; Narvaez et al. 2001). In addition, calcitriol may trigger apoptosis directly by activating caspase effector molecules, although it is unclear whether calcitriol-induced apoptosis is caspase-dependent (Mathiasen et al. 1999). Interestingly, executioner caspase-3, which is activated during apoptosis, cleaves and inactivates the VDR, thereby controlling VDR activity as well as calcitriol-induced apoptosis (Malloy and Feldman 2009).

Most vitamin D studies have concentrated on the effects of calcitriol on cancer cells and consequently little is known about the effects of the pro-hormone $25(OH)D_3$, even though it may be more feasible for clinical use because of its less calcemic nature. In primary prostate epithelial cells, the pro-hormone is a more active growth inhibitor than calcitriol (Barreto et al. 2000). Moreover, in mouse mammary gland

organ culture, $25(OH)D_3$ acts as a chemopreventive agent against carcinogen-induced precancerous lesions (Peng et al. 2009). The effect could be stage-specific, because $25(OH)D_3$ mainly inhibits lesion formation. Interestingly, in primary prostate stromal cells, there seems to be a difference between the growth effects of $25(OH)D_3$ and calcitriol, favouring the pro-hormone as a more active antiproliferative agent (Lou et al. 2004).

8.1.1 Vitamin D analogue EB1089

Vitamin D analogues have been developed to separate the growth-regulating effects from the calcemic effects. Attention has been focused on analogues such as EB1089 that have modifications on their side chain structure (Hansen et al. 2001). EB1089 (1(S),3(R)-dihydroxy-20(R)-(5´-ethyl-5´-hydroxy-hepta-1´(E),3´ (E)-dien-1´-yl)9,10-secopregna-5(Z),7(E),10,19-triene) is a synthetic analogue with strong antiproliferative and differentiation-inducing effects on cancer cells. EB1089 has a unique side chain structure featuring 26,27 dimethyl groups, insertion of an extra carbon atom (24a) at C-24, and two double bonds at C-22, 23 and C-24, 24a (Hansen and Mäenpää 1997). EB1089 is not degraded by 24OHase, as in the case of the parent hormone, calcitriol (Shankar et al. 1997). Instead of 24-hydroxylation, EB1089 is slowly hydroxylated at the distal C-26 and C-26a positions, explaining its relative stability compared to other analogues and the parent hormone in vitro and in vivo (Shankar et al. 1997). EB1089 more potently inhibits tumour cell growth than calcitriol both *in vivo* and *in vitro* (James et al. 1994; Gorospe et al. 1997; VanWeelden et al. 1998; Prudencio et al. 2001). The mechanisms mediating its effects are similar to calcitriol, but the impact is more effective and lasts for a longer period of time (Hansen et al. 2001; Mäenpää et al. 2001). Antiproliferative actions are observed in several cancer types, including head and neck squamous cell carcinoma (Akutsu et al. 2001b; Prudencio et al. 2001), prostate cancer (Wang et al. 1997), and breast cancer (James et al. 1994; VanWeelden et al. 1998) cell lines. EB1089 has been tested in vivo in breast cancer cell xenografts in combination with paclitaxel with a 4-fold greater effect on reducing tumour size compared to controls (Koshizuka et al. 1999), and in vitro with adriamycin, where both apoptosis and cell cycle arrest were observed in association with reduced p21 expression and inhibition of mitogen activated protein kinase activity (Sundaram et al. 2000).

8.2 Clinical trials with calcitriol and its analogues

In in vivo pre-clinical models, the anticancer effects of calcitriol require concentrations that are considerably higher than the physiological range, typically in at least nanomolar concentrations. Such concentrations are not achieved with daily dosing due to dose-limiting effects such as hypercalcemia and hypercalciuria (Koeffler et al. 1985; Rustin et al. 1996; Gross et al. 1998). Recently developed high-dose formulations of calcitriol, however, such as DN-101, which are aimed for cancer treatment and not just as replacement therapy for calcitriol deficiency, appear to be well tolerated (Beer et al. 2007a). Calcitriol has been studied in clinical trials in several cancers, including prostate, colon, lung, breast, pancreatic, and liver cancer, but the results have been modest or disappointing (Peehl et al. 2003)(reviewed in ref (Deeb et al. 2007)), supplementary data). Clinical studies of vitamin D analogues have focused on continuous daily administration of EB1089 to patients with breast and colorectal cancer (Gulliford et al. 1998), hepatocellular carcinoma (Dalhoff et al. 2003), and pancreatic cancer (Evans et al. 2002). EB01089

did not show anticancer activity in these studies, and although potentially problematic hypercalcemia was noted, it was not dose-limiting.

8.3 Calcitriol and anticancer drugs

8.3.1 In vitro

In addition to the activity of calcitriol as a single agent, in vitro studies show that calcitriol and its analogues enhance the activity of a number of anticancer drugs and treatments, including dexamethasone (Yu et al. 1998; Bernardi et al. 2001), retinoids (Koga and Sutherland 1991; Guzey et al. 1998), tamoxifen (Abe-Hashimoto et al. 1993; Welsh 1994), radiation (Dunlap et al. 2003; Polar et al. 2003), docetaxel (Beer et al. 2001; Ting et al. 2007), paclitaxel (Wang et al. 2000; Hershberger et al. 2001), and platinum compounds (Light et al. 1997; Moffatt et al. 1999; Hershberger et al. 2002). Optimal potentiation is observed when calcitriol is administered before or simultaneously with the chemotherapeutic drug; administration after the anticancer drug does not provide enhancement (Light et al. 1997; Hershberger et al. 2001). The combination of docetaxel and calcitriol has been studied mainly on androgen-independent prostate cancer cells (Beer et al. 2001; Ting et al. 2007). Calcitriol enhances the efficacy of docetaxel by increasing the expression of Bax, followed by increased apoptosis. Furthermore, calcitriol may reduce docetaxel efflux by downregulating the expression of multidrug resistance-associated protein 1 (MRP-1). Anticancer drugs such as daunorubicin hydrochloride, etoposide, and vincristine sulphate may selectively increase the degradation of 24OHase mRNA, thereby prolonging the bioavailability of calcitriol (Tan et al. 2007). Moreover, these drugs increase the expression of VDR protein, but not VDR mRNA (Tan et al. 2007).

8.3.2 Clinical trials

In clinical trials, calcitriol has been studied in combination with paclitaxel, dexamethasone, and carboplatin, and the studies have mainly concentrated on prostate cancer (reviewed in ref (Trump et al. 2004; Deeb et al. 2007)). These combinations were usually well tolerated, but the trials failed to demonstrate efficacy exceeding that of the anticancer drug as a single agent or demonstrated only modest increases in efficacy. The most impressive findings regarding calcitriol actions in concert with anticancer drugs came from studies conducted by Beer and co-workers (Beer et al. 2001; Beer et al. 2007b). In a phase II study, calcitriol combined with docetaxel decreased prostate-specific antigen by 50% or more in 81% of patients. Furthermore, in androgen-independent prostate cancer, survival improved in patients receiving docetaxel (36 μg once a week) and calcitriol (DN-101, 45 μg 1 day before docetaxel) compared to patients receiving docetaxel in combination with a placebo. Because survival was not the primary end point of this phase II study, further confirmation of the results is required. Importantly, severe or life-threatening side effects, including thromboembolic complications, are reduced in patients receiving both calcitriol and docetaxel (Beer et al. 2006). A 1000 patient phase III study is currently in progress to further analyse these findings. In addition to prostate cancer, the combination of calcitriol and docetaxel is also being tested for the treatment of lung and pancreatic cancers (reviewed in ref (Deeb et al. 2007), supplementary data), but the results of these trials are not yet available.

An important aspect to be considered before calcitriol or its analogues are used in cancer therapy is the fact that calcitriol regulates the expression of CYP3A4, CYP2C9, and CYP2B6, which are involved in the metabolism of several anticancer drugs, including docetaxel and camptothecins (Schmiedlin-Ren et al. 1997; Drocourt et al. 2002). Furthermore, CYP3A4 is one of the enzymes involved in the 25-hydroxylation of vitamin D_3 in the liver and intestine (Gupta et al. 2004; Gupta et al. 2005).

8.4 24-Hydroxylase inhibitors

In many cancer cells, a relatively high concentration of calcitriol is required before growth inhibition is observed. This may be due to 24OHase, which is rapidly induced by calcitriol and inactivates the antiproliferative actions of calcitriol. To circumvent this, EB0189 and other calcitriol analogues that are resistant to 24OHase have been developed. Another option is to develop 24OHase inhibitors. Compounds such as ketoconazole (Reinhardt and Horst 1989; Peehl et al. 2001) and liarozole (Ly et al. 1999) can be used concomitantly with calcitriol to produce a synergistic growth inhibition of cancer cells. Although these compounds markedly inhibit 24OHase activity, they also inhibit a wide variety of other enzymes such as 1αOHase and CYP3A4, and thus are not specific enough. More selective 24OHase inhibitors such as VID400 allow for the local production of calcitriol while inhibiting the degradation of either endogenous vitamin D metabolites or supplementary vitamin D compounds (Schuster et al. 2001a; Schuster et al. 2001b). In human keratinocytes, VID400 stabilizes calcitriol levels and thereby strongly amplifies and prolongs the expression of 24OHase. In parallel, VID400 causes a 100-fold increase in the antiproliferative activity of calcitriol.

9. Vitamin D and ovaries

9.1 Vitamin D in normal ovaries

The function of vitamin D in normal ovaries has not been studied in detail. Vitamin D receptors and vitamin D metabolizing enzymes 1αOHase and 24OHase are detected in human ovaries, suggesting that human ovaries are target organs for vitamin D (Villena-Heinsen et al. 2002; Agic et al. 2007). In addition, the vitamin D metabolites calcitriol, $25(OH)D_3$, and $24,25(OH)_2D_3$ are present in the follicular fluid of human ovaries, although in lower concentrations than in the serum (Potashnik et al. 1992). Furthermore, calcitriol levels vary during the menstrual cycle (Gray et al. 1982). The calcitriol levels in women's serum are doubled on day 15 when compared to day 1 or 8 and oral contraceptives decrease the calcitriol concentrations. In addition, low serum levels of $25(OH)D_3$ in patients with polycystic ovarian syndrome suggest that calcitriol or other vitamin D metabolites are involved in controlling normal ovarian functions (Thys-Jacobs et al. 1999).

Studies in female rats demonstrated that a vitamin D deficiency markedly reduces fertility (Halloran and DeLuca 1980; Kwiecinksi et al. 1989). On the other hand, high doses of calcitriol, either directly or indirectly by subsequent hypercalcemia, might disturb the oestrus cycle and cause hyperfunctional changes in the corpus luteum (Horii et al. 1992). The VDR and calcitriol are necessary for full ovarian function through their direct effects on oestrogen biosynthesis and the regulation of

aromatase gene expression. VDR- and 1αOHase-null mice have impaired fertility; gonadal insufficiency, such as abnormal folliculogenesis; reduced aromatase gene expression; low aromatase activity; and elevated serum levels of luteinizing and follicle-stimulating hormones (Yoshizawa et al. 1997; Panda et al. 2001). Dietary supplementation with calcium partially normalizes fertility and the aromatase enzyme needed for oestrogen biosynthesis (Kinuta et al. 2000; Johnson and DeLuca 2001). Gene knockout studies revealed contradictory results, however, because female mice in a study using a different VDR knockout model were fertile (Li et al. 1997).

9.2 Vitamin D in ovarian cancer

9.2.1 Ovarian cancer aetiology and vitamin D

Epidemiologic data suggest that sunlight and ultraviolet B irradiation might be protective factors for ovarian cancer (Lefkowitz and Garland 1994; Al-Moundhri et al. 2003; Garland et al. 2006). Thus, decreased vitamin D synthesis in the skin followed by a vitamin D deficiency may contribute to the initiation and progression of ovarian cancer. Furthermore, an inverse association between dietary vitamin D and ovarian cancer was demonstrated in two studies (Bidoli et al. 2001; Salazar-Martinez et al. 2002), although contradictory reports have also been published (Kushi et al. 1999; Genkinger et al. 2006; Koralek et al. 2006). One reason for the contradictory results might be that the effect of dietary vitamin D intake is difficult to study because it strongly correlates with lactose intake, the decrease of which may also contribute to the risk for ovarian cancer (Genkinger et al. 2006; Larsson et al. 2006). Plasma concentrations of vitamin D metabolites can be measured more reliably, and a study of the relationship between vitamin D and ovarian cancer indicated that the overall risk of ovarian cancer is not associated with plasma calcitriol or 25(OH)D_3 levels (Tworoger et al. 2007). In overweight and obese women, however, 25(OH)D_3 levels are significantly inversely associated with an increased risk for ovarian cancer (Tworoger et al. 2007). Obesity is associated with a vitamin D insufficiency, likely due to the reduced bioavailability of vitamin D from cutaneous and dietary sources because of its deposition in the body fat (Wortsman et al. 2000), and with ovarian cancer, especially in women who have never used menopausal hormone replacement therapy (Leitzmann et al. 2009). Furthermore, women with adequate (>32 ng/ml) vs. inadequate (<32 ng/ml) 25(OH)D_3 levels have a 36% decreased risk of serous ovarian cancer (Tworoger et al. 2007).

9.2.2 VDR, vitamin D metabolism, and ovarian cancer

The vitamin D receptor is expressed in 43% to 83% of ovarian cancers (Saunders et al. 1992; Ahonen et al. 2000a; Villena-Heinsen et al. 2002). Furthermore, VDR expression is increased in ovarian tissue in patients with endometriosis and to an even greater extent in patients with ovarian cancer when compared to normal ovarian tissue, although this higher expression is not correlated with the cell proliferation index (Villena-Heinsen et al. 2002; Agic et al. 2007). Single nucleotide polymorphisms such as *FokI*, *Cdx-2*, *BsmI,* and *ApaI* in the VDR gene are associated with the risk of breast (Curran et al. 1999; Chen et al. 2005), colon (Park et al. 2006a), and prostate cancer (Taylor et al. 1996; Bodiwala et al. 2004; Valdivielso and Fernandez 2006; Li et al. 2007). These polymorphisms do not

change the amino acid sequence of the VDR, but may modulate transcription of the VDR gene or alter VDR transactivation and mRNA stability (reviewed in(Lurie et al. 2007)). Conflicting results have been published concerning ovarian cancer susceptibility and these single nucleotide polymorphisms; a positive association was found in two studies (Lurie et al. 2007; Tworoger et al. 2009), and no correlation was observed in one study (Clendenen et al. 2008).

The expression of 25-hydroxylase, 1αOHase, and 24OHase has been studied in both normal and neoplastic ovaries and some differences in expression have been observed. Elevated levels of 24OHase or of all these enzymes have been reported in some studies (Friedrich et al. 2003; Anderson et al. 2006), whereas in one study the authors detected no differences between normal and cancerous samples (Agic et al. 2007). Increased 1αOHase expression is observed in breast (Townsend et al. 2005) and prostate (Schwartz et al. 1998) cancers and during early colon tumour progression in well-to-moderately differentiated states, but decreased expression is observed in poorly differentiated colon carcinomas (Bareis et al. 2001; Cross et al. 2001; Tangpricha et al. 2001; Bises et al. 2004). 24OHase has been described as an oncogene because overexpression of this gene may provide cancer cells a growth advantage by allowing them to escape $1,25(OH)_2D_3$-mediated growth control (Albertson et al. 2000). Interestingly, in addition to breast (Kallioniemi et al. 1994; Tanner et al. 1995) and prostate (Wolter et al. 2002) cancers, amplification of the chromosomal region 20q12-q13, containing the 24OHase gene, has been reported in ovarian cancer (Iwabuchi et al. 1995; Tanner et al. 2000).

9.2.3 In vitro, in vivo, and clinical studies of calcitriol in ovarian cancer

The first observations of the growth inhibition induced by calcitriol in ovarian cancer cells were reported by Saunders and co-workers (Saunders et al. 1992; Saunders et al. 1993; Saunders et al. 1995). They also demonstrated that calcitriol downregulates the expression of the c-myc proto-oncogene (Saunders et al. 1993). Seven years earlier, however, Christopherson and colleagues showed that 10 nM calcitriol induced faster proliferation of ovarian cancer cells (Christopherson et al. 1986). Thereafter, several reports were published describing the antiproliferative effects of calcitriol on ovarian cancer cells (Ahonen et al. 2000b; Jiang et al. 2003; Jiang et al. 2004; Li et al. 2004). In an ovarian cancer xenograft model in nude mice, EB1089 suppressed growth of tumour xenografts without causing hypercalcemia by inhibiting cell proliferation and the induction of apoptosis (Zhang et al. 2005a). Other molecular mechanisms of calcitriol in ovarian cancer cells besides c-myc downregulation have been studied. The results of these studies imply that Gadd45 expression and transcriptional upregulation is essential for calcitriol-induced cell cycle arrest at G_2M (Jiang et al. 2003). Additionally, calcitriol increases $p27^{Kip1}$ protein stability by downregulating cyclin E to induce G_1S cell cycle arrest (Li et al. 2004). In ovarian cancer cells, calcitriol induces apoptosis through destabilizing telomerase reverse transcriptase (TERT) mRNA, followed by telomere erosion (Jiang et al. 2004). Interestingly, whereas several reports have described the pro-apoptotic effects of calcitriol, Zhang and co-workers (Zhang et al. 2005b) reported that calcitriol suppresses death receptor-mediated apoptosis (extrinsic apoptosis pathway) in ovarian cancer cells.

Few clinical ovarian cancer trials have been performed to study the effects of vitamin D compounds. One published trial evaluated the efficacy of calcitriol combined with isotretinoin after chemotherapy (Rustin et al. 1996). The

investigators treated 22 women with the combination for up to 74 weeks and the response was monitored using CA 125. The treatment did not improve the response rate except in one patient with borderline ovarian carcinoma.

AIMS OF THE STUDY

The aims of the present study were:

1. To evaluate the role of vitamin D_3 metabolites calcitriol and $25(OH)_2D_3$, and vitamin D analogue EB1089 in growth regulation of ovarian cancer cells (I).

2. To study the efficacy of chemotherapeutic drugs docetaxel and SN-38 used as single agents or concomitantly in ovarian cancer cells (II).

3. To evaluate the efficacy of combination of calcitriol and docetaxel in ovarian cancer cells (III).

4. To study the efficiency of adenovirus mediated p53 gene therapy with or without docetaxel, irinotecan or SN-38 in ovarian cancer cells (IV).

5. To detect molecular mechanisms affecting the responsiveness of ovarian cancer cells to vitamin D compounds, anticancer drugs docetaxel, irinotecan and SN-38, and adenovirus mediated p53 gene therapy (I-IV).

MATERIALS AND METHODS

1. Reagents, devices and computer programs (I-IV)

Cell lines UT-OC-1-5 were kindly provided by Dr. Seija Grènman (Department of Obstetrics and Gynaecology, Turku University Central Hospital, Turku Finland) and cell lines SK-OV-3 and OVCAR-3 were purchased from ATCC (Manassas, VA).

$1,25(OH)_2D_3$, $25(OH)D_3$, and EB1089 were kindly provided by Leo Pharmaceutical Products (Ballerup, Denmark) and VID400 by Dr. Anton Stuetz (Novartis Research Institute, Vienna, Austria). Chemotherapeutic drugs docetaxel ((Taxotere®)), SN-38 and Irinotecan (CPT-11, Campto®) and adenoviral vector encoding a wild type human p53 gene (Ad5CMV-p53, p53Ad) were generous gifts from Sanofi Aventis (Antony, France).

Adeno-X-Null and Adeno-X-LacZ adenoviruses, as well as FacScan flow cytometer were obtained from BD Biosciences (Erembodegem Belgium). Lab-Tek®II Chamber Slide System and cell culture flasks were purchased from Nalge Nunc Inc (IL, USA). PGP-4008, RPMI 1640, DMEM, DMEM/F12, propidium iodide, hoechst bisbenzamide 33258, P-glycoprotein (MDR) antibody and peroxidase-conjugated goat anti-mouse IgG were obtained from Sigma Aldrich (St. Louis, MO), Histostain Plus Broad Spectrum kit from Zymed (San Francisco, California, USA) and p53 antibody from Novo Castra Laboratories (Newcastle upon Tyne, UK). WST-1 PreMix Cell Proliferation Assay System was bought from Takara Bio Inc. (Shiga, Japan), Caspase-Glo™3/7 Assay from Promega (Madison, WI, USA), acetonitrile-C18 Sep-Pak from Waters (Ireland) and Trizol reagent from Invitrogen Life Technologies (Paisley, Scotland, UK). RobusT RT-PCR Kit was obtained from Finnzymes (Espoo, Finland), LightCycler-RNA Master SYBR Green I kit and LightCycler instrument from Roche Diagnostics (Basel, Switzerland). High Capacity cDNA Archive Kit, SYBR® Green PCR Master Mix and ABI Prism 7000 Sequence Detection System were purchased from Applied Biosystems (Foster City, CA). The *in vitro* transcription reaction kit and the hStress-1 probe template set were obtained from BD Bioscience Pharmingen (San Diego, CA, USA), RPA III from Ambion (Austin, TX, USA) and both BCA protein Assay Reagent and mPER reagent were obtained from Pierce (Rockford, IL, USA). Phospho-Bcl-2 antibody was purchased from Cell Signalling Technology (Danvers, MA, USA), peroxidase-conjugated goat anti-rabbit IgG from Cappel (West Chester, PA, USA) and both ECL reagent and [α-^{32}P]UTP from Amersham Biosciences (Little Chalfont, Buckinghamshire, UK). Victor 1420 multilabel counter was obtained from Wallac (Turku, Finland), Multiscan MS microplate reader from Labsystems (Waltham, MA, USA) and Konica SRX-101A from Konica Minolta (Wayne, NJ, USA). Storm label detection system and ImageQuant 5.1 were obtained from Molecular Dynamics (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, UK) and Prism 3.03 from GraphPad Software Inc. (San Diego, CA, USA).

2. Cell culture (I-IV)

Human ovarian adenocarcinoma cell lines (table 2), SK-OV-3, OVCAR-3, UT-OC-1, UT-OC-2, UT-OC-3, UT-OC-4 and UT-OC-5 were maintained in DMEM supplemented with 10% FBS, non-essential amino acids and antibiotics (p/s, 100 IU/ml penicillin, 100 μg/ml streptomycin). In the first study (I) the OVCAR-3 cells

were maintained in RPMI 1640 supplemented with 10% FBS (foetal bovine serum), 10 μg/ml insulin, 0.25% glucose and antibiotics (p/s). All cell lines were kept at 37°C in a humidified 95% air/5% CO_2 incubator and media were changed every third day. A new cell stock from liquid nitrogen storage was thawed and cultured for three passages before starting experiments. A passage number variation between each repeat was not more than three. Exclusion of mycoplasma contamination was verified by Hoechst 33258 staining.

Table 2. Characteristics of the epithelial ovarian cancer cell lines. Table modified from (Engblom 1999).

Cell line (Studies)	Histological type	Grade	Site of specimen	Previous therapy	p53 status
UT-OC-1 (II – III)	Mucinous cystadeno-carcinoma	3	Primary tumour	None	normal[a]
UT-OC-2 (II – III)	Endometrioid cystadeno-carcinoma	3	Primary tumour	Vincristine sulphate, Doxorubicin Cyclo-phosphamide Radiotherapy	normal[a]
UT-OC-3 (II – III)	Serous cystadeno-carcinoma	1	Primary tumour	None	normal[a]
UT-OC-4 (II – III)	Endometrioid cystadeno-carcinoma	2	Primary tumour	Radiotherapy	normal[a]
UT-OC-5 (II – IV)	Serous cystadeno-carcinoma	2	Omental metastasis	Radiotherapy	normal[a]
SK-OV-3 (II – III)	Epithelial carcinoma	Na	Primary tumour	None	normal[a]
OVCAR-3 (I – IV)	Poorly differentiated papillary adeno-carcinoma	Na	Ascites	Combination therapy with cyclo-phosphamide, adriamycin and cisplatin	mutated[b]

Na, Information not available
a, (Rantanen et al. 2002)
b, (O'Connor et al. 1997)

3. Adenovirus infections (IV)

Adenoviruses infect both dividing and resting cells. Their DNA does not integrate into the cell genome, leading to transient expression of a transgene (Fojo and Bates 2003). Adenovirus infections were performed using SK-OV-3 and OVCAR-3 cells and Ad5CMV-p53, Adeno-X-Null, and Adeno-X-LacZ viral vectors. All these viruses are replication-defective adenoviruses derived from human adenovirus serotype 5. Ad5CMV-p53 contains the wild-type human p53 gene and Adeno-X-LacZ contains the LacZ gene under a cytomegalovirus (CMV) promoter. Adeno-X-Null does not contain a transgene. To increase the infection efficacy, the infections were performed in reduced medium volumes (~50%) and the medium did not contain antibiotics or fetal bovine serum. The next day, the medium containing the viruses was changed and the medium volume was increased back to the initial volume. The infections were performed in a safety level 2 laboratory.

3.1 Infection efficacy

3.1.1 P53 immunocytochemistry and X-Gal staining

The expression of p53 protein in the SK-OV-3 cells after p53Ad infection was detected by immunocytochemistry. X-Gal staining was used to determine the infection rate in both SK-OV-3 and OVCAR3- cells. Because OVCAR-3 cells constitutively express p53 protein due to a p53 mutation, p53 immunocytochemistry could not be used to determine the infection efficacy in OVCAR-3 cells. For both assays, $5x10^4$ cells were plated into the wells of chamber slides. One day after plating, the medium was removed and replaced with fresh medium containing Adeno-X-LacZ or p53Ad viruses (multiplicity of infection [MOI] 0, 1, 5, 10, 50, and 100). For X-Gal staining, cells were fixed with 2% paraformaldehyde/0.2% glutardialdehyde for 10 min and stained with 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal) solution (1 mg/ml X-Gal, 4 mM $K_3Fe(CN)_6$, 4 mM $K_4Fe(CN)_6$, 2 mM $MgCl_2$) for 2 h at 37°C. For p53 immunocytochemistry, cells were fixed with 5% acetic acid in ethanol. Immunostaining was performed using a Histostain Plus Broad Spectrum kit. The p53 antibody was diluted 1:100 in sterile water. OVCAR-3 cells were used as a positive control for p53 expression. In both cases, stained cells were counted under a light microscope.

4. Flow cytometric analysis (II-IV)

For flow cytometric analysis, cells were detached with trypsin-EDTA. Detached cells and the culture medium including previously detached and floating cells were placed into tubes and centrifuged. The pellets were resuspended in 0.5 ml phosphate-buffered saline (PBS). The cell suspensions were stirred and 4.5 ml ice-cold 70% ethanol was added to each tube to fix the cells. After fixation, RNA was digested with 0.5 mg/ml RNase and cells were stained with 50 μg/ml propidium iodide. The cell suspensions were filtered and flow cytometric analyses were performed using a FacScan flow cytometer.

5. Cell growth assays (I-IV)

To study the effects of vitamin D_3 compounds, chemotherapeutic drugs, and adenovirus infection on cell growth, the cells were plated on 96-well culture plates according to the growth rate of each cell line ($1x10^3$-$1x10^4$ cells/well). One day after plating, the medium was changed and appropriate concentrations of hormones, drugs, or adenoviruses, or their combinations were added.

5.1 Crystal violet staining (I-II, IV)

Crystal violet staining was used to measure relative cell growth after exposure of cells to vitamin D_3 compounds [calcitriol (0.1-100 nM), 25(OH)D_3 (10-500 nM), and EB1089 (1 and 100 nM)], 24-hydroxylase inhibitor VID400 (100 nM), chemotherapeutic drugs [docetaxel (0.01-50 nM) and SN-38 (0.001-100 nM)], or their combinations (docetaxel+SN-38 and 10 nM calcitriol+0.3-0.5 nM docetaxel). When vitamin D_3 compounds or the combination of calcitriol and docetaxel were studied, relative cell growth was analysed after 1, 3, 5, 7, 9, and 11 days, or after 6, 9, and 12 days, respectively. Before concomitant exposure of cells to docetaxel and SN-38, the EC_{50} values were determined for both as single agents. When different dosing schedules were analysed, the first drug was added after cell attachment (24 h). After another 24 h, the second drug was added and cells were incubated an additional 4 days, thus the total incubation time of 5 days was the same as in the simultaneous or the single agent experiments. The effect of a selective p-glycoprotein inhibitor (PGP-4008, 250 nM) on cell growth was studied by adding the inhibitor simultaneously with docetaxel, SN-38, or both drugs, and the relative growth was analysed after 5 days incubation.

Relative cell numbers were quantified as previously described (Kueng et al. 1989). Cells were fixed on the bottom of the wells by adding 10 µl 11% glutardialdehyde solution in 0.1% phosphate buffer to 100 µl growth medium. The plates were shaken at 500 cycles/min for 15 min, washed three times with de-ionised water and air-dried. Fixed cells were stained with 0.1% crystal violet dissolved in de-ionised water. After 20 min incubation, excess dye was removed by washing vigorously with de-ionised water. The plate was air-dried and the bound dye was dissolved in 100 µl 10% acetic acid. The relative cell number was given as absorbance units by measuring the optical density (590 nm) in each well using a Victor 1420 Multilabel Counter.

5.2 WST-1 (IV)

A WST-1 cell proliferation assay was used to quantify relative cell number after exposing cells to adenoviral vectors (p53Ad and Adeno-X-Null) with or without additional exposure to docetaxel, irinotecan, or SN-38. For WST-1 analysis, $2x10^3$ (SK-OV-3) or $1x10^4$ (OVCAR-3) cells per well were plated on 96-well culture plates. One day after plating, the medium was replaced with fresh medium containing adenoviruses. After 24 h infection, the medium containing adenoviruses was removed. When the effect of adenoviral infection [multiplicity of infection (MOI) 0, 1, 5, 10, 50, or 100] on cell growth was determined, the medium containing adenoviruses was replaced with fresh medium and the samples were cultured for 5 days. When adenovirus infections were combined with anticancer drugs, the medium was replaced with medium containing docetaxel (0, 0.1, 0.5, 1,

2.5, and 5 nM), irinotecan (0, 1, 2.5, 5, 10, 25 μM), SN-38 (0, 1, 2.5, 5, 10, 50 nM), or vehicle, and the cell growth samples were taken after 5 days. The relative cell number was analysed using the WST-1 PreMix Cell Proliferation Assay. The assay is based on the cleavage of the tetrazolium salt WST-1 to a formazan-class dye by mitochondrial succinate-tetrazolium reductase in viable cells. As the cells proliferate, more WST-1 is converted to the formazan product. The quantity of formazan dye is directly related to the number of metabolically active cells, and can be quantified by colorimetric assay (Roehm et al. 1991). The cell culture medium was removed and phosphate-buffered saline and WST-1 were added at a ratio of 10:1. The cells were incubated for 4 h at 37^{o}C and the relative cell number was measured with Multiscan MS microplate reader at 450 nm. The mean relative cell number of uninfected and unexposed cells was set as 100%.

6. Detection of apoptosis

6.1 Hoechst bisbenzamide 33258 staining (II)

To detect apoptosis, cells ($5x10^4$-$1x10^5$ cells/well) were plated on a chamber slide. After cell attachment, cells were exposed to drug dilutions according to the EC_{50} values of each drug and cell line. The effect of PGP-4008 (250 nM) on apoptosis was studied in UT-OC-3, UT-OC-5, SK-OV-3, and OVCAR-3 cell lines. PGP-4008 was added to cells simultaneously with docetaxel, SN-38, or both drugs, and cells were incubated for 24 h. The cells were fixed with 4% paraformaldehyde and cell membranes were permeabilised with 0.5 % Triton X-100. The samples were stained with Hoechst 33258 stain. Apoptotic cells were counted under a fluorescence microscope (excitation 365 nm, emission 480 nm). Apoptotic cell detection was based on nuclear morphology, folding of the nuclear membrane, and nuclear fragmentation. At least 100 cells were counted from each treatment group and 5 different areas of the chamber slide were counted.

6.2 Detection of caspase-3 and -7 (III-IV)

During the induction of apoptosis a cascade of strictly controlled events take place. This leads to the activation of a series of cysteine proteases, called caspases. Caspase-3 and -7 are executioner caspases and their activation is induced both by extrinsic and intrinsic apoptotic pathways (Boatright and Salvesen 2003). The activity of caspase-3 and caspase-7 and thereby activation of the caspase cascade of apoptotic cell death was measured using a Caspase-Glo™3/7 Assay. OVCAR-3 ($1x10^4$ cells, III, IV), SK-OV-3 ($1x10^4$ cells, IV), and UT-OC-5 ($5x10^3$ cells, III) were plated on 96-well culture plates. One day after plating, the indicated concentrations of calcitriol, docetaxel, or their combinations (III) were added. To analyse apoptosis after adenovirus infections (IV), cells were infected with p53Ad or Adeno-X-Null empty vector (MOI 10). After 24 h infection, the medium was removed and the indicated concentrations of docetaxel, irinotecan, or SN-38 were added. Half of the samples without adenovirus infections were treated with drugs. In both cases, apoptosis was detected after 48 h incubation when the Caspase-Glo™3/7 Assay–reagent was added and the assay was performed according to the manufacturer's instructions. Luminescence was measured using a Multiscan MS.

7. Detection of vitamin D_3 metabolites (I)

OVCAR-3 cells ($1.5x10^6$ cells/flask) were plated on a T25 culture flask. The following day, cells were exposed to 500 nM $25(OH)D_3$. After 0, 3, or 24 h incubation, the medium was collected and the cell monolayer was extracted with 1 ml methanol for 15 min at room temperature. The methanol was collected into the same tube with the sample medium. The samples for the measurement of the produced metabolites, $24,25(OH)_2D_3$ and $1,25(OH)_2D_3$, were purified using the acetonitrile-C18 Sep-Pak (Turnbull et al. 1982), followed by separation of the metabolites by high-performance liquid chromatography. The concentration of $24,25(OH)_2D_3$ was quantified by a competitive protein binding assay (Parviainen et al. 1981) and that of $1,25(OH)_2D_3$ by a radioreceptor assay (Reinhardt et al. 1984).

8. RNA extraction (I-II)

Total RNA was isolated using Trizol reagent according to the manufacturer's instructions. The concentration of RNA in each sample was calculated from absorbance values at 260 nm. The integrity of the RNA samples was confirmed on gel electrophoresis. RNA samples were stored at -70°C.

9. Polymerase chain reactions (PCR) (I-II)

9.1 Reverse transcription PCR (RT-PCR) (I)

The expression of 1αOHase mRNA was detected using a reverse transcription polymerase chain reaction (RT-PCR). RT-PCR was performed using a RobusT RT-PCR Kit according to the manufacturer's instructions from 1 μg total RNA. The RT-PCR protocol was as follows: 30 min reverse transcription at 48°C and 2 min denaturation at 94°C, followed by denaturation at 94°C for 30 s, annealing at 54°C for 30 s, and extension at 72°C for 30 s for 30 cycles. After the 30 cycles, the final extension step was at 72°C for 7 min. Primers are shown in table 3.

9.2. Quantitative real time PCR (QPCR)(I-II)

Two different devices were used to analyse the relative expression of studied genes in the samples. In both cases, the relative expression of the studied genes was calculated according to the following equation (Pfaffl 2001):

$$\text{Ratio} = (E_{\text{target}})^{\Delta CP \text{ target (control-sample)}} / (E_{\text{ref}})^{\Delta CP \text{ ref (control-sample)}},$$

where E is PCR efficiency ($E=10^{[-1/slope]}$), ΔCP ref (control-sample) is the crossing point change in the reference gene (PBGD or RPLP0), and ΔCP target (control-sample) is the crossing point change in the target gene, 24OHase or MDR-1, when the vehicle and drug/hormone-treated cells are compared. Primers for QPCR are shown in table 3.

Table 3. Primer sequences for QPCR.

Gene (AC#)		Primer sequence	Position
1αOHase[a]	F	5'-GTCAAGGAAGTGCTAAGACTG-3'	1241-1261
(AB005038)	R	5'-TGTTAGGATCTGGGCCAAAG-3'	1524-1543
PBGD[b]	F	5'-AAGTGCGAGCCAAGGACCAG-3'	695-714
(X04808)	R	5'-TTACGAGCAGTGATGCCTACCAAC-3'	969-992
24OHase[b]	F	5'-TGATCCTGGAAGGGGAAGAC-3'	833-852
(L13286)	R	5'-CACGAGGCAGATACTTTCAAAC-3'	1023-1044
RPLP0[c]	F	5'-AATCTCCAGGGGCACCATT-3'	515-533
(NM_001002)	R	5'-CGCTGGCTCCCACTTTGT-3'	571-588
MDR-1[c]	F	5′CTCAGACAGGATGTGAGTTGGTTT-3′	2807-2830
(NM_000927)	R	5′-GCGAGCCTGGTAGTCAATGC-3′	2855-2874

F, Forward primer; R, Reverse primer; [a]Primer used in RT-PCR; [b]Primer used in Light Cycler; [c]Primer used in ABI Prism 7000; AC#, Gene bank accession number

9.2.1 Light Cycler (I)

The reactions for 24OHase mRNA were performed with the LightCycler instrument from 0.3 μg total RNA using LightCycler-RNA Master SYBR Green I kit. Human porphobilinogen deaminase (PBGD) mRNA was used as a reference gene. The RT-PCR protocol was as follows: 20 min reverse transcription at 61°C and 30 s denaturation at 95°C, followed by amplification of the products for 45 cycles (denaturation at 95°C for 1 s, annealing at 62°C for PBGD or 57°C for 24OHase for 7 s, and extension at 72°C for 12 s). Fluorescence was detected at the end of the extension step of each cycle. To verify the specific products, a melting curve analysis and gel electrophoresis were performed.

9.2.2 ABI Prism 7000 (II)

The reactions for MDR-1 mRNA were performed with an ABI Prism 7000 Sequence Detection System. RPLP0 mRNA was used as a reference gene. The reverse transcriptase reactions were performed using the High Capacity cDNA Archive Kit from 5 μg of total RNA. The program for reverse transcription was: transcription activation at 25°C for 30 min followed by reverse transcription at 37°C for 2 h and enzyme inactivation at 94°C for 5 min. The real-time PCR step was performed using SYBR® Green PCR Master Mix from 50 ng cDNA. The program for PCR was: activation of polymerase at 95°C for 10 min, followed by amplification for 45 cycles (denaturation at 95°C for 15 s and annealing/extension at 60°C for 1 min). After amplification, the specificity of the PCR products was verified by melting curve analysis.

10. Ribonuclease protection assay (IV)

A ribonuclease protection assay (RPA) was used to analyse the expression of stress response genes after p53Ad gene therapy and docetaxel, SN-38, and irinotecan drug

exposures. Cells were plated on T25 culture flasks (2 x 10^6 cells/flask). The next day, the medium was replaced with fresh medium containing adenoviruses (empty adenovirus vector or p53Ad, MOI 10). After 24 h infection, the medium containing the adenoviruses was removed and medium containing docetaxel (2.5 nM), CTP-11 (10 μM), SN-38 (20 nM), or vehicle was added. After 48 h, total RNA was extracted from the cells using Trizol reagent. P32-Labelled ([α-^{32}P]UTP) RNA-probes were synthesised in *in vitro* transcription reaction using template set hStress-1, which generates probes of specific lengths for Bcl-XL, Bcl-XS, p53, Gadd45, c-fos, p21$^{waf1/cip1}$, bax, Bcl-2, Mcl-1, GAPDH, and L32. RPA was performed according to the manufacturer's instructions. Briefly, ^{32}P-labelled RNA-probes and 5 μg total RNA samples were hybridised. After 16 to 17 h hybridisation, single-stranded RNA was digested and the remaining double-stranded hybridisation products of different lengths were separated by gel electrophoresis. An intensifying screen was exposed and scanned with a Storm scanner. 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 bands after 24 h exposure. The GAPDH and L32 bands were quantified after 6 h exposure. The ImageQuant 5.1 computer program was used to quantify the band intensities. The intensities of GAPDH and L32 bands were used to normalise the differences in sample loading. Expression of the studied gene is given as relative expression compared to the expression of the same gene in vehicle-treated OVCAR-3 cells.

11. Western blot analysis of Bcl-2 (II) and p-glycoprotein (III)

UT-OC-5 and OVCAR-3 cells exposed to 10 nM calcitriol, 0.5 nM docetaxel, or a combination of calcitriol and 0.5 nM docetaxel were used to analyse Bcl-2 protein phosphorylation. P-glycoprotein expression was evaluated in SK-OV-3, OVCAR-3, and UT-OC-1-5 cells after exposure to docetaxel, SN-38, or both according to the EC_{50} values of the drugs for each cell line. Proteins were extracted after 48 h exposure to mammalian Protein Extraction Reagent (mPER) and the protein concentrations were measured with BCA protein Assay Reagent. Equal amounts of protein from each sample were fractionated in 12% (phospho-Bcl-2) or 7.5% (p-glycoprotein) polyacrylamide gels and the protein samples were transferred to a nitrocellulose membrane using an electrophoresis transfer apparatus. The samples were blocked with 5% (phospho-Bcl-2) or 3% (p-glycoprotein) milk-TBS-Tween solution and the membranes were incubated at 4°C overnight with primary antibody (monoclonal p-glycoprotein [MDR] produced in mouse clone F4, or the phospho-Bcl-2 antibody) diluted in 1%-milk-TBS-Tween solution. Peroxidase-conjugated goat anti-rabbit IgG was used as the secondary antibody for phospho-Bcl-2 and peroxidase-conjugated goat anti-mouse IgG for p-glycoprotein. The membranes were incubated with secondary antibody for 1 h at room temperature. The membranes were washed. Proteins were detected by luminol containing the chemiluminescence reagent ECL and the bands were developed using a Konica SRX-101A. The amount of phosphorylated Bcl-2 was quantified with a densitometer.

12. Data analysis

12.1 Dose-response curves and isobolographs (II)

Dose-response curves to calculate the EC_{50} values for chemotherapeutic drugs docetaxel and SN-38 were created using GraphPad Prism 3.03. Data obtained from crystal violet staining was fitted to a sigmoid dose-response curve, equation: Y = Bottom + (Top-Bottom) / $(1+10^{(logEC50-X)*hillslope})$, where X is the logarithmic concentration and Y is the growth response. The dose-response curves had a sigmoid shape with a variable slope factor.

The dose-response interactions between docetaxel and SN-38 or irinotecan using the EC_{50} of the compounds were studied using the isobolographic method (Greco et al. 1995; Nelson and Kursar 1999).

12.2 Statistical analyses (I-IV)

All experiments were repeated 3 to 5 times, except QPCR for the detection of 24OHase, and Western blotting for p-glycoprotein and quantification of vitamin D_3 metabolites, which were repeated twice.

Statistical analyses were performed with a Prism 3.03 computer program. The statistical significance of the growth regulatory effects and differences in gene expression were analysed with either one-way or two-way analysis of variance followed by Bonferroni's correction for multiple comparisons or Student's *t*-test. A p-value of less than 0.05 was considered statistically significant.

RESULTS

1. Effects of $25(OH)D_3$, $1,25(OH)_2D_3$, and EB1089 on ovarian cancer cells

1.1 Regulation of cell growth by $25(OH)D_3$, $1,25(OH)_2D_3$, and EB1089

$25(OH)D_3$ increased the growth of OVCAR-3 cells in a concentration-dependent manner. $25(OH)D_3$ (10, 50, 100, 200, and 500 nM) stimulated cell growth by 32%, 41%, 39%, 35%, and 11%, respectively. All differences were statistically significant compared to the control ($P < 0.05$).

High concentrations of $1,25(OH)_2D_3$ significantly inhibited OVCAR-3 cell growth; 100 nM $1,25(OH)_2D_3$ decreased growth by 74% ($P < 0.001$) and 10 nM by 8% ($P < 0.0001$). Low concentrations of $1,25(OH)_2D_3$ had the opposite effect; 0.1 nM $1,25(OH)_2D_3$ increased growth by 14% ($P < 0.0001$).

EB1089 inhibited growth when 1 and 100 nM concentrations were used; 100 nM EB1089 inhibited growth by 84% and 1 nM by 73% ($P < 0.0001$). The growth inhibition of 1 nM EB1089 (73%) was almost equal to that of 100 nM $1,25(OH)_2D_3$ (74%).

1.2 Expression of 1α-hydroxylase and 24-hydroxylase

Our data indicate that the ovarian cancer cell lines OVCAR-3, SK-OV-3, and UT-OC-1-5 express 1αOHase. The expression was not regulated by 100 nM $1,25(OH)_2D_3$.

24OHase is expressed in OVCAR-3 cells and the expression of 24OHase is regulated by EB1089 and $1,25(OH)_2D_3$. After 6 h exposure, 24OHase mRNA expression was increased 650-fold with 100 nM $1,25(OH)_2D_3$ and 600-fold with 100 nM EB1089. After 24 h, the expression levels were further increased; $1,25(OH)_2D_3$ exposure increased the expression by 1100-fold and EB1089 by 1000-fold. The induction was notably weaker when 100 nM $25(OH)D_3$ was used; after 6 h, the expression was slightly increased (3-fold), but returned to the same level as in control samples or even slightly lower (0.5-fold) after 24 h exposure.

1.3 Metabolism of $25(OH)D_3$ in OVCAR-3 cells

To study whether 24OHase and 1αOHase are functional in OVCAR-3 cells, we evaluated the concentrations of metabolites generated from $25(OH)D_3$ by 24OHase and 1αOHase. In the first experiment, the amount of $24,25(OH)_2D_3$ was 4 times higher after 3 h incubation than it was when the experiment started (0 h). After 24 h, production was further increased (18-27-fold). When the 24OHase inhibitor VID400 was used, the production of $24,25(OH)_2D_3$ was reduced to one-third.

The basal level of $1,25(OH)_2D_3$ was 23 pM and the concentration increased to 37 pM and 33 pM after 3 h and 24 h incubations, respectively. VID400 did not affect the production of $1,25(OH)_2D_3$.

1.4 Effect of 24OHase inhibitor on the growth response to $1,25(OH)_2D_3$ and $25(OH)D_3$

Because the metabolic measurements showed that 24OHase effectively produced $24,25(OH)_2D_3$ from $25(OH)D_3$, we evaluated the effect of VID400 on the growth response of $1,25(OH)_2D_3$ and $25(OH)D_3$. $25(OH)D_3$ at 100 nM stimulated growth by 18%. The combination of 100 nM $25(OH)D_3$ with VID400, however, conversely inhibited growth by 14% ($P < 0.001$). In addition, we analysed the effect of VID400 on the growth response of $1,25(OH)_2D_3$. By itself, 1 nM $1,25(OH)_2D_3$ did not affect cell growth. In combination with VID400, however, it inhibited the growth by 27% ($P < 0.0001$). Moreover, 10 nM $1,25(OH)_2D_3$ inhibited growth by 26%, but in combination with VID400, growth was inhibited by 77%.

2. Action of anticancer drugs SN-38 and docetaxel

2.1 Effects on growth

2.1.1 Docetaxel and SN-38 as single agents

To assess the concentrations of docetaxel and SN-38 that inhibit cell growth by 50% (EC_{50}), ovarian cancer cell lines were exposed to docetaxel and SN-38 in a concentration range of 0.001 to 10 nM (docetaxel) and 0.001 to 100 nM (SN-38). Cell lines responded to docetaxel treatment rather similarly. The most sensitive cell line was SK-OV-3. The least sensitive cell line, UT-OC-2, required 2.6 times more docetaxel to achieve the EC_{50} than SK-OV-3 cells. In contrast, the response to SN-38 was completely different across cell lines. There was 19-fold difference in concentrations between the most sensitive (UT-OC-3) and the least sensitive (UT-OC-5 and OVCAR-3) cell lines. There was no correlation between sensitivity to docetaxel and SN-38.

2.1.2 Concomitant use of docetaxel with SN-38

The concomitant effects of docetaxel and SN-38 were studied by drawing dose-response curves and isobolographs. Simultaneous and sequential exposure of cells to docetaxel and SN-38 led to additive, subadditive, or antagonistic growth effects without schedule dependency. Figure 1 shows dose-response curves and figure 2 isobolographs after sequential drug exposures; SN-38 was given first before docetaxel. There was a strong tendency towards subadditive and antagonistic effects in every cell line. The effects were not supra-additive in any cell line studied. Concomitant use of the tested drugs was most effective in UT-OC-3 cells. Combinations were least effective in OVCAR-3 cells, where growth effects were strongly antagonistic. The antagonistic effects were partially concentration-dependent.

Both docetaxel and SN-38 used as single agents induced cell cycle arrest at G_2M and concomitant exposure did not increase or decrease the proportion of cells at G_2M.

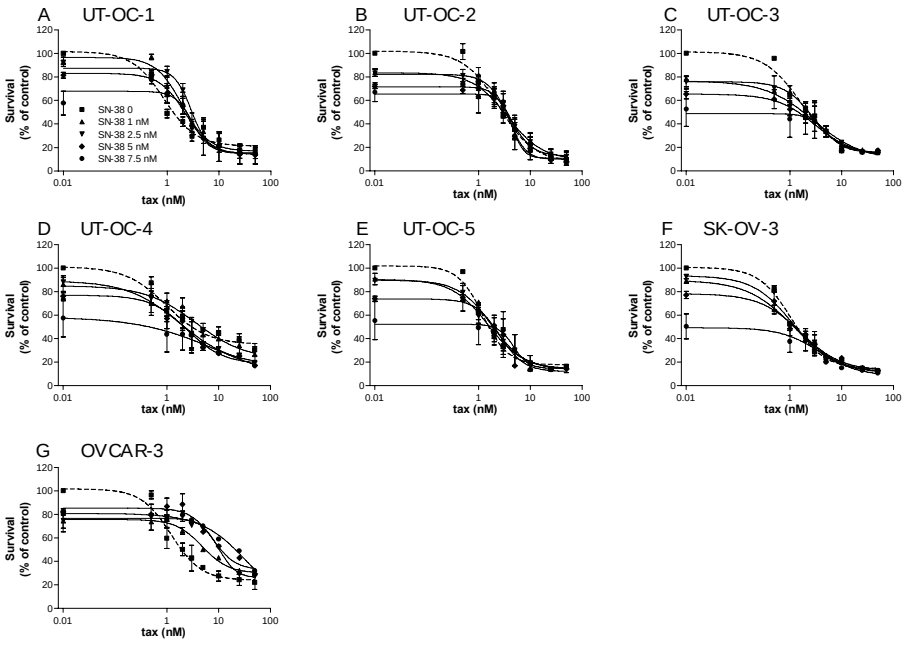

Figure 1. Dose-response curves for sequential SN-38 and docetaxel exposure. SN-38 was given first followed by docetaxel exposure. SN-38 concentrations are indicated in figure A.

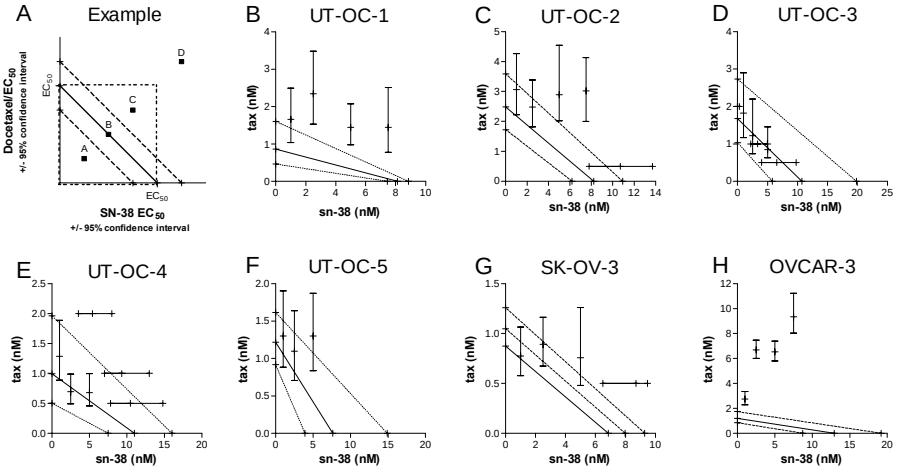

Figure 2. Isobolographs for sequential SN-38 and docetaxel exposure. SN-38 was given first followed by docetaxel exposure. The EC_{50} for docetaxel alone is plotted on the Y-axis and the EC_{50} for SN-38 on the X-axis. The straight solid line between the X- and Y-axis corresponds to isoeffective combinations (hypothetical combinations of docetaxel and SN-38 giving the same effect, 50% growth inhibition, as EC_{50} concentrations of either drug alone). The dashed lines represent 95% confidence intervals. In figure A characters a, b, c, and d indicate supra-additive, additive, sub-additive, and protective effects, respectively. If the combinatorial response 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 sub-additive; and if outside the rectangle (d), the effect is protective.

2.2 Effect of docetaxel and SN-38 on p-glycoprotein expression

To evaluate the role of p-glycoprotein on the growth response, we studied its expression in ovarian cancer cells and the regulation or its expression by docetaxel and SN-38. Docetaxel had only a modest effect on p-glycoprotein expression at both the mRNA and protein levels. P-glycoprotein was regulated by docetaxel only in UT-OC-1 cells. Both p-glycoprotein mRNA and protein expression, however, were regulated by SN-38 and the combination of SN-38 and docetaxel. The effect of SN-38 or SN-38+docetaxel was statistically significant in all cell lines except SK-OV-3.

2.3 Effect of p-glycoprotein inhibition on docetaxel- and SN-38 mediated apoptosis and cell growth inhibition

The effect of the selective p-glycoprotein inhibitor PGP-4008 on drug-induced apoptosis was studied using Hoechst bisbenzamide 33258 staining. In samples exposed to docetaxel, the amount of apoptosis was very high and this level was further increased by PGP-4008. This was not the case with SN-38; SN-38 was a notably weaker inducer of apoptosis than docetaxel, and PGP-4008 did not affect SN-38-induced apoptosis. The proportion of apoptotic cells was either not changed or only slightly elevated in docetaxel+SN-38 samples compared to SN-38 samples. When docetaxel, SN-38, and PGP-4008 were combined, the proportion of apoptotic cells was higher than that in samples exposed to docetaxel+SN-38. The effect of this combination, however, did not reach or only slightly exceeded the magnitude of apoptosis induced by docetaxel alone, and never exceeded the amount of apoptosis detected in samples exposed to docetaxel+PGP-4008.

Consistent with our apoptosis data, PGP-4008 enhanced docetaxel-induced growth inhibition. This effect was statistically significant ($p < 0.05$) in all cell lines. In UT-OC-3, UT-OC-4, and SK-OV-3 cells, the cell number was low in samples exposed to docetaxel+SN-38 and even lower in samples exposed to docetaxel+SN-38+PGP-4008. In UT-OC-1, UT-OC-2, UT-OC-5, and OVCAR-3 cells, the combination of docetaxel and SN-38 led to antagonistic growth effects. In UT-OC-5 and OVCAR-3, the growth effect was still antagonistic when cells were exposed to docetaxel+SN-38+PGP-4008. Growth inhibition, however, was significantly more effective than that in samples exposed to docetaxel+SN-38 ($p<0.05$).

3. Concomitant exposure of ovarian cancer cells to docetaxel and calcitriol

In this study we evaluated the concomitant effect of calcitriol and docetaxel on ovarian cancer cell growth, apoptosis, cell cycle distribution and Bcl-2 phosphorylation. Overview of results of publication III is shown in table 4.

Table 4. Results of publication III

Measurement (time point)/ exposure	OVCAR-3	UT-OC-5
Growth response to calcitriol (10 nM) (6, 9, and 12 days) and induction of apoptosis (48h)	Similar growth inhibition and induction of apoptosis	
Growth response to docetaxel (0.3, 0.4, and 0.5 nM) (6, 9, and 12 days) and induction of apoptosis (48h)	Similar growth inhibition and induction of apoptosis	
Growth response to calcitriol and docetaxel (6, 9, and 12 days) and induction of apoptosis (48h)	Additive growth inhibition and induction of apoptosis ($p<0.05$)	Calcitriol suppressed docetaxel-mediated growth inhibition ($p<0.01$) and induction of apoptosis ($p<0.05$)
Cell cycle (48h)		
docetaxel	G_2M arrest (both cell lines)	
calcitriol	G_2M	G_0G_1
docetaxel+calcitriol	Increased cell number at G_2M	No additional effect on cell cycle distribution
Bcl-2 phosphorylation (48h)		
docetaxel or calcitriol	Increased in both cell lines	
docetaxel+calcitriol	Increased ($p<0.05$)	Decreased ($p<0.05$)

4. Concomitant exposure of ovarian cancer cells to p53Ad and anticancer drugs docetaxel, irinotecan, and SN-38

In this study we evaluated the concomitant effect of p53Ad and docetaxel, SN-38 or irinotecan on ovarian cancer cell growth; apoptosis; cell cycle distribution; and expressions of several stress-responsive genes, such as $p21^{waf1/cip1}$, Gadd45, Bax, Bcl-2, and Bcl-XL. Overview of results of publication IV is shown in table 5.

Table 5. Results of publication IV.

Measurement (time point)/ exposure	OVCAR-3	SK-OV-3
Infections efficiency (Adeno-X-LacZ) (48h)	Similar infection efficiency	
Growth response to p53Ad (5 days)	Growth inhibition MOI 5 ($p < 0.001$), MOI 10, MOI 50 and MOI 100 ($p < 0.0001$)	No significant difference to empty vector
p53Ad + docetaxel	No effect on growth or gene regulation	
p53Ad + SN-38/irinotecan		
Growth (5 days)	Enhanced growth inhibition ($p = 0.0008$ for irinotecan, $p < 0.0001$ for SN-38)	Growth inhibition not enhanced by p53Ad
Cell cycle (48h)	-p53Ad increased cell counts at G_0G_1, and decreased at S -Irinotecan/SN-38 + p53Ad decreased cell numbers at G_0G_1 and S and increased cell number at the G_2M phase	-Cell number at G_2M increased and decreased at G_0G_1 and S -p53Ad + drug exposures did not affect the cell cycle distribution
Apoptosis (48h) (Activation of caspases 3 and 7)	p53Ad increased SN-38 and irinotecan- mediated caspase activation ($p<0.05$)	No additional effect on SN-38/irinotecan-mediated caspase activation
p53 (48h, RPA)	Strong expression in both cell lines after p53Ad, but no regulation with SN-38/irinotecan	
$p21^{waf1/cip1}$(48h, RPA)	Strongly induced by p53Ad, irinotecan/SN-38 exposures did not further increase the expression	
Gadd45 (48h, RPA)	No effect on expression	p53Ad increased expression and it was further enhanced by irinotecan and SN-38 exposures ($p<0.05$)
Bax (48h, RPA)	p53Ad upregulated Bax, and SN-38/irinotecan enhanced the expression ($p < 0.05$)	p53Ad upregulated Bax ($p<0.05$), but drug exposures did not further enhance the expression
Bax/Bcl-2 and Bax/Bcl-XL expression ratios (48h, RPA)	Increased expression ratios (SN-38/ irinotecan vs. p53Ad+SN-38/ irinotecan and p53Ad vs. p53Ad+SN-38/irinotecan ($p<0.05$))	Degreased expression ratios

DISCUSSION

1. Ovarian cancer cell response to vitamin D

In this study, we analyzed the growth-regulating effects of calcitriol, 25(OH)D_3, and EB1089 on OVCAR-3 ovarian cancer cells. High calcitriol concentrations (10-100 nM) inhibited OVCAR-3 cell growth, consistent with previous data on ovarian cancer cells (Saunders et al. 1992; Saunders et al. 1993; Saunders et al. 1995; Ahonen et al. 2000b; Jiang et al. 2003; Jiang et al. 2004; Li et al. 2004). The vitamin D analogue EB1089 also inhibited OVCAR-3 cell growth, but the EB1089 concentration required for growth inhibition was remarkably lower than that of calcitriol; equivalent growth inhibition was obtained with 1 nM EB1089 and 100 nM calcitriol in OVCAR-3 cells. In previous in vitro studies, similar results have been obtained, suggesting that EB1089 is 50 to 200-fold more potent for inhibiting growth than calcitriol (Blutt et al. 1997; Hansen et al. 2000; Zhang et al. 2005a). Earlier studies demonstrated that the calcitriol prohormone 25(OH)D_3 inhibits cell growth both in vitro (Chen et al. 1994; Barreto et al. 2000; Bae et al. 2001) and in vivo (Holt et al. 2002), but its effect on ovarian cancer cells had not been previously studied. Our findings indicate that 10 to 500 nM 25(OH)D_3 actually promotes cell proliferation, as does a low concentration of calcitriol (0.1 nM).

Because the metabolites of vitamin D compounds appear to have a significant effect on the cell growth response (Reinhardt and Horst 1989; Ly et al. 1999; Peehl et al. 2001), we analysed the expression of 24OHase and 1αOHase in ovarian cancer cells. We also evaluated 25(OH)D_3 hydroxylation by 24OHase and 1αOHase. Ovarian cancer cells expressed both 1αOHase and 24OHase, consistent with the data obtained from ovarian cancer tissue (Friedrich et al. 2003; Anderson et al. 2006; Agic et al. 2007). Both calcitriol and EB1089 strongly induced the expression of 24OHase, which is also consistent with a previous study (Christopherson et al. 1986). In contrast, however, 25(OH)D_3 had only a modest effect on 24OHase expression; a 3-fold upregulation after 6 h, followed by downregulation.

1αOHase metabolises 25(OH)D_3 to 1,25$(OH)_2D_3$ (calcitriol), whereas 24OHase converts 25(OH)D_3 to 24,25$(OH)_2D_3$ and also catalyses further hydroxylation reactions (Omdahl and May 1997). When the enzyme activities were analysed in OVCAR-3 cells, small amounts (only picomoles per litre) of calcitriol were produced from 500 nM 25(OH)D_3 by 1αOHase. In contrast, 24OHase produced relatively high concentrations (nanomoles per litre) of 24,25$(OH)_2D_3$. To further analyse the role of 24OHase in OVCAR-3 cell growth, we used a specific 24OHase inhibitor, VID400 (Schuster et al. 2001a; Schuster et al. 2001b), concomitantly with calcitriol and 25(OH)D_3. Inhibition of 24OHase enhanced the antiproliferative action of calcitriol. Moreover, in contrast to the growth stimulation effect of 100 nM 25(OH)D_3 alone, cell proliferation was inhibited by combining 100 nM 25(OH)D_3 with VID400.

High 24OHase enzyme activity together with the effect of the 24OHase inhibitor suggests that metabolites produced by 24OHase promote cell proliferation. 24OHase is considered to be a vitamin D metabolite-inactivating enzyme (Sakaki et al. 1999). Vitamin D metabolites might also have their own distinct effects on cell proliferation, but these effects are not yet clear. Previous studies showed that 1,24,25$(OH)_2D_3$ and a further oxidised 1,25$(OH)_2$-24-oxo-vitamin D_3 metabolite produced by 24OHase promoted cell proliferation, but the growth burst preceded

cell differentiation (Campbell et al. 1999; Rashid et al. 2001). In breast cancer, 24OHase is proposed to be an oncogene whose overexpression may provide a growth advantage to cancer cells, because these cells can escape vitamin D-mediated growth control (Albertson et al. 2000). The chromosomal region 20q12-q13 containing the 24OHase gene locus is amplified in 54% of ovarian cancers (Hahn et al. 1993; Tanner et al. 2000). The growth promoting role of 24OHase makes it an even more powerful oncogene, because it not only degrades the most antiproliferative form of vitamin D, calcitriol, but it also converts calcitriol to a metabolite that promotes cell growth. Differences in the metabolic pathways of EB1089 and calcitriol (Kissmeyer et al. 1997) might partially explain why EB1089, but not calcitriol, inhibits growth at a concentration as low as 1 nM. Instead, metabolites of EB1089 do not seem to act as effective regulators of gene expression (Quack et al. 1998) and consequently also do not likely act as growth regulators.

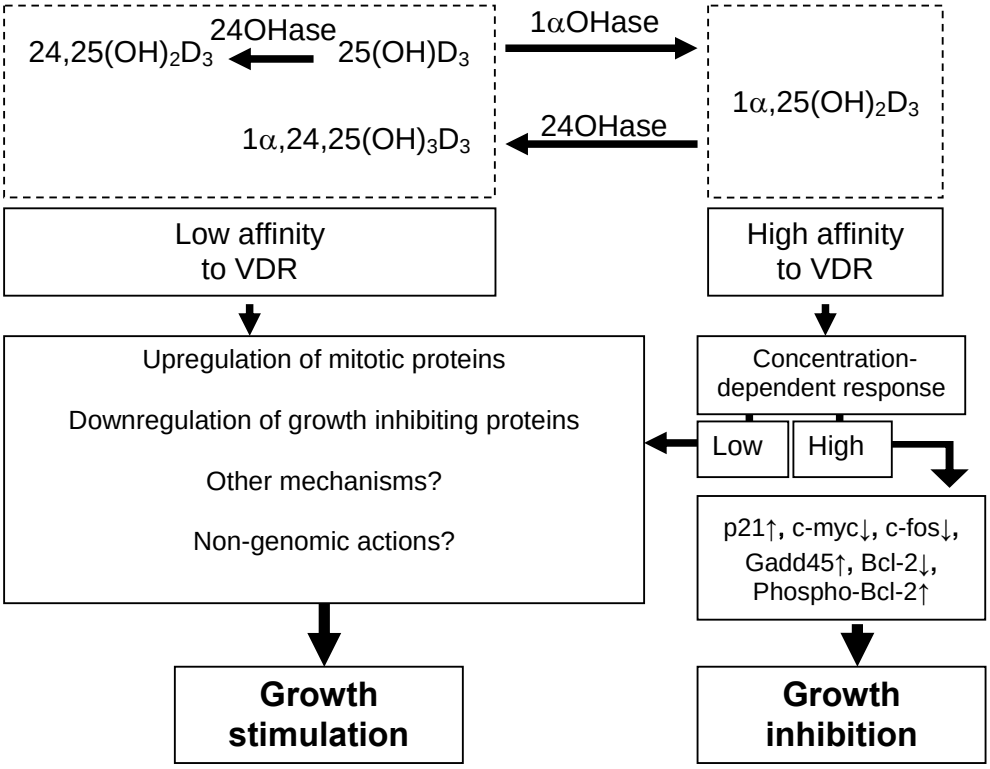

Figure 3. Hypothesis of vitamin D mediated growth effects in ovarian cancer.

Affinity of the VDR to its ligand might also be involved in the induction of vitamin D-responsive genes and the resulting growth regulation. Some growth modulating effects of $25(OH)D_3$, in addition to the 24OHase-metabolised products of calcitriol and $25(OH)D_3$, might be mediated via direct binding to the VDR, although the relative binding affinity of these products (e.g., $25(OH)D_3$ to VDR) is approximately 700-fold lower than that of calcitriol (Harant et al. 2000; Collins and Norman 2001). Hypothetically, different vitamin D metabolites might induce specific conformational changes in the VDR-RXR heterodimer, leading to distinct combinations of cofactors that further favour the induction of genes that promote cell proliferation or the repression of genes that induce growth inhibition. Instead, high concentrations of calcitriol lead to the activation of genes known to inhibit cancer cell growth, such as p21 or Gadd45 (Freedman 1999; Polly et al. 2000a;

Jiang et al. 2003). Figure 3 shows hypothesis of vitamin D mediated growth effects in ovarian cancer.

EB1089 mediates the stabilisation of VDR-RXR heterodimers on specific vitamin D response elements (ER9) at lower concentrations than it does on DR3-type response elements (Quack et al. 1998; Quack and Carlberg 2000), suggesting that ligand concentration is important in selecting the vitamin D target genes that are to be activated. Similarly, the calcitriol-induced mitogenic effects observed in this study might be explained by the activation of different vitamin D target genes after low-concentration calcitriol exposure. Moreover, the non-genomic actions of vitamin D compounds might play a role in this response, although this has not yet been studied in ovarian cancers. Furthermore, calcitriol suppressed the intrinsic apoptotic pathway induced by TRAIL and Fas in ovarian cancer cells (Zhang et al. 2005b), which is an important aspect to consider as several anticancer drugs cause cell death via an extrinsic pathway and, based on this result, calcitriol might prevent their action on cancer cells. Mitogenic effects have been reported with low concentrations of calcitriol in several cell culture models (Munker et al. 1986; Gniadecki 1996; Love-Schimenti et al. 1996; Gross et al. 1997; Rots et al. 1999; Rashid et al. 2001), including one report in ovarian cancer cells (Christopherson et al. 1986). Interestingly, the concentration used in the preceding study was rather high (10 nM) and is known to inhibit the growth of several ovarian cancer cell lines, including OVCAR-5 and UT-OC-5 used in this thesis, underscoring the cell-specific responses to vitamin D compounds.

2. Anticancer drug effects on ovarian cancer cells

In the present study, we evaluated the effects of docetaxel and SN-38, both alone and in combination, on cell proliferation, apoptosis, and p-glycoprotein expression in seven ovarian cancer cell lines UT-OC-1-5, OVAR-3 and SK-OV-3.

The efficacies of docetaxel and SN-38 to induce growth inhibition in ovarian cancer cells did not correlate with each other, possibly because of the different mechanisms of action; docetaxel induces microtubule polymerisation (Gelmon 1994) whereas SN-38 inhibits topoisomerase I activity (Hsiang et al. 1985; Hsiang and Liu 1988). Previous studies have indicated that ovarian cancer cells do not develop a cross-resistance between paclitaxel, a drug related to docetaxel, and irinotecan or SN-38 (O'Meara A and Sevin 1999).

The EC_{50} values for docetaxel in UT-OC-1-5 and SK-OV-3 cell lines were previously determined (Engblom et al. 1997). Although the present study used a different method (clonogenic assay vs. crystal violet staining), the present results are consistent with those obtained previously; the UT-OC-2 cell line was the most resistant to docetaxel exposure in both studies. Furthermore, the concentration scale for the EC_{50} values was very similar between both experiments, indicating that the two methods produce comparable results.

In lung cancer cell lines, concomitant exposure of cells to irinotecan and paclitaxel produces additive cytotoxicity (Pei et al. 1997). The combination of paclitaxel and topotecan induces a synergistic effect in a human teratocarcinoma cell line (Chou et al. 1994), whereas an antagonistic effect is observed in human lung cancer cells (Kaufmann et al. 1996). Additionally, in squamous larynx carcinoma, breast adenocarcinoma, and non-small cell lung cancer cells, sequential exposure to

topotecan and docetaxel induces a synergistic effect when docetaxel is given first, but the effect is antagonistic when topotecan is applied first (Taron et al. 2000). Concurrent administration of irinotecan and paclitaxel to human breast, lung, colon, and ovarian cancer cell lines induces antagonistic effects, whereas additive or synergistic effects are observed if the drugs are administered sequentially (Kano et al. 1998). In the present study, both simultaneous and sequential administration of SN-38 and docetaxel to cells induced additive, sub-additive, or clearly antagonistic effects, which were partially concentration-dependent and the results seemed more dependent on the cell line studied than on the dosing schedule. Both docetaxel and SN-38 induced cell cycle arrest at the G_2M phase, but after concomitant exposure, the number of cells at G_2M was not increased compared to the levels observed with either drug alone.

P-glycoprotein mediated drug efflux is the best described mechanism of resistance to antitubulin agents such as docetaxel (Dumontet and Sikic 1999). Therefore, we used a specific p-glycoprotein inhibitor (PGP-4008) to reduce the p-glycoprotein mediated drug efflux (Smith et al. 2000) and to further study its role in the observed antagonistic growth effects. The expression of p-glycoprotein was upregulated by SN-38, but the p-glycoprotein inhibitor did not improve the efficacy of SN-38 to inhibit growth. Docetaxel induced the expression of p-glycoprotein in some cell lines, but the effect was cell line-specific. Nevertheless, the p-glycoprotein inhibitor sensitised cells to both docetaxel and the combination of docetaxel and SN-38, as observed by an increase in apoptotic activity and growth inhibition. It is noteworthy that adding the p-glycoprotein inhibitor to docetaxel-SN-38 combinations did not exceed the efficacy of docetaxel combined with the p-glycoprotein inhibitor to induce apoptosis in any cell line or even cell growth inhibition in some cells, suggesting that p-glycoprotein induction is not the only mechanism involved in the growth-impairing effects of docetaxel and SN-38. Because cells are most sensitive to topoisomerase I inhibitors, such as irinotecan and SN-38 during S phase of the cell cycle (Pizzolato and Saltz 2003), agents that arrests cells at S phase might produce synergistic growth effects concomitantly with topoisomerase I inhibitors.

Our present results suggest (figure 4) that the SN-38 as a single agent or concomitantly with docetaxel upregulates p-glycoprotein expression, but docetaxel is transported more efficiently than SN-38, thus SN-38 may even enhance docetaxel efflux and induce drug resistance. In addition, our results suggest that p-glycoprotein expression might be a marker for the concomitant effects of docetaxel and SN-38. Furthermore, the use of novel taxanes that reduce p-glycoprotein mediated drug efflux (Ferlini et al. 2000) might be preferable for concomitant drug administration, such as docetaxel and SN-38.

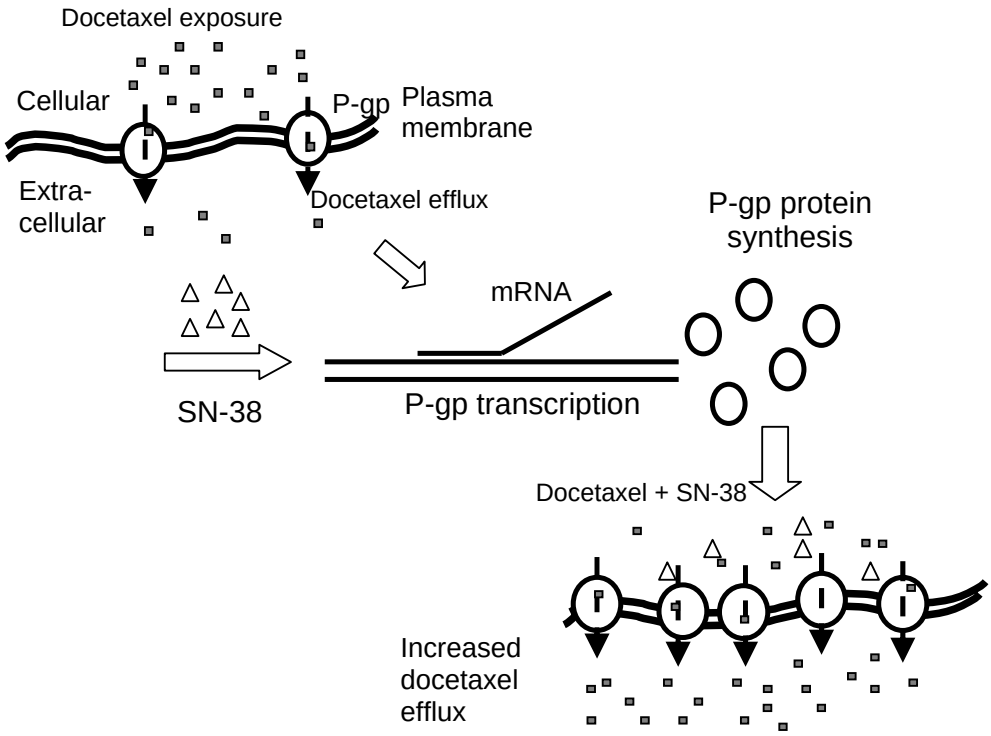

Figure 4. Overview of the results of study II. P-gp, p-glycoprotein.

3. Concomitant exposure to docetaxel and calcitriol

In this study, we assessed the concomitant growth effects of calcitriol and docetaxel in UT-OC-5 and OVCAR-3 cell lines. Calcitriol-mediated growth inhibition is established in OVCAR-3 cells (Saunders et al. 1992; Saunders et al. 1993; Saunders et al. 1995; Ahonen et al. 2000b; Jiang et al. 2003; Jiang et al. 2004; Li et al. 2004), but UT-OC-5 cells have not been used in vitamin D studies except to assess the expression of 1αOHase in study I of this thesis. Based on the results that OVCAR-3 and UT-OC-5 cells responded rather similarly to docetaxel exposure in study II, we selected both cell lines for this study.

The rationale for studying the combination of calcitriol and docetaxel in ovarian cancer cells was provided by data obtained with androgen-independent prostate cancer cells (Ting et al. 2007), where pre-treatment of cells with calcitriol enhanced the antiproliferative effect of docetaxel. In addition, the calcitriol-docetaxel combination has already been tested in clinical phase I/II trials for the treatment of prostate cancer (Beer et al. 2001; Beer et al. 2007b). In the clinical trials, prostate-specific antigen decreased by 50% or more in most of the patients. Furthermore, survival improved in the patient group receiving docetaxel and calcitriol compared to patients receiving docetaxel as a single agent. In ovarian cancer, the effects of vitamin D compounds are not as well studied and currently there are no reports of combinations of vitamin D and taxane.

In UT-OC-5 and OVCAR-3 cells, calcitriol and docetaxel inhibited cell growth when administered as single agents. When used simultaneously, they inhibited

OVCAR-3 cell growth to a similar extent. In UT-OC-5 cells, however, calcitriol and docetaxel induced a dose-dependent antagonistic effect. The antiproliferative effects of taxanes and calcitriol are mediated through the regulation of the cell cycle and the induction of apoptosis. Docetaxel arrests cells at G_2M (Crown and O'Leary 2000; Gligorov and Lotz 2004), whereas calcitriol induces arrest at either G_2M (Jiang et al. 2003) or G_1 (Li et al. 2004). Docetaxel induced G_2M arrest in both cell lines studied, supporting previously published data (Kolfschoten et al. 2002) and consistent with results obtained from studies II and IV. Calcitriol induced cell cycle arrest at the G_2M phase in OVCAR-3 cells and at G_1 in UT-OC-5 cells. When cells were concomitantly exposed to docetaxel and calcitriol, the number of cells in G_2M was further increased in OVCAR-3 cells, but not in UT-OC-5 cells.

Both calcitriol (Hershberger et al. 2002) and docetaxel (Fabbri et al. 2008) may trigger apoptosis by inducing caspase-3. Our results show that docetaxel and calcitriol increase the activity of caspases 3 and 7 in both UT-OC-5 and OVCAR-3 cells. The concomitant exposure of cells to docetaxel and calcitriol induced cell line-specific caspase activation; while caspase activation was further increased in OVCAR-3 cells, caspase activation was lower in UT-OC-5 cells than when exposed to docetaxel alone.

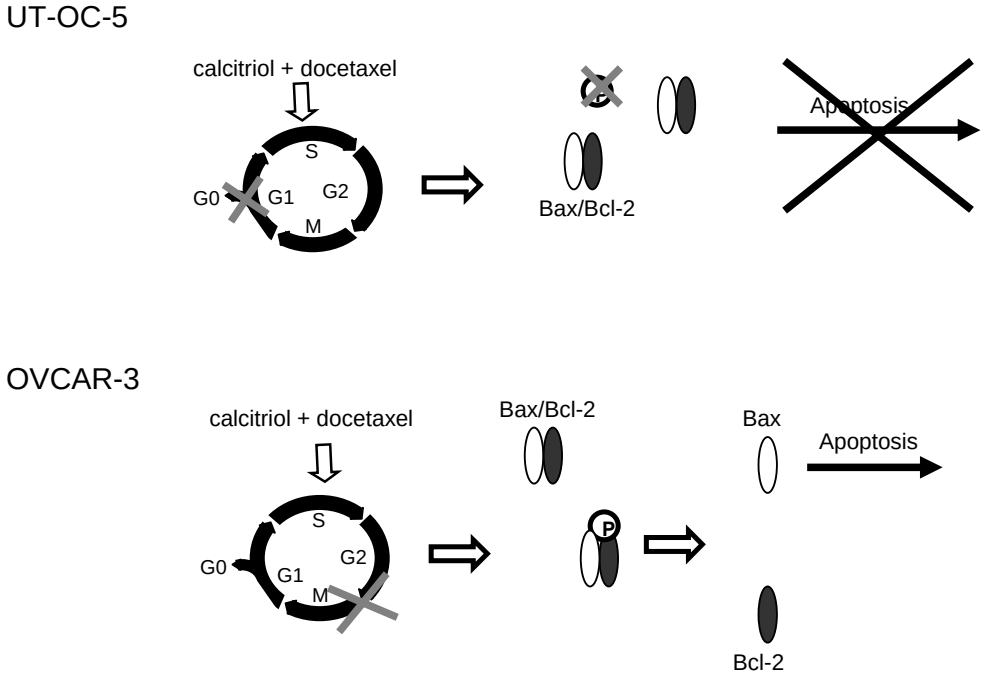

Figure 5. Cell line-dependent regulation of response to concomitant docetaxel calcitriol exposure. P, phosphorylation. A cross indicates cell cycle arrest, lack of phosphorylation and inhibition of apoptosis.

Docetaxel and calcitriol influence cancer cell survival by regulating the expression or phosphorylation status of Bcl-2 (Stein 1999; Guzey et al. 2002; Gligorov and Lotz 2004). In breast cancer cell lines, calcitriol enhances the ability of paclitaxel to phosphorylate Bcl-2, eventually leading to increased apoptosis (Wang et al. 2000).

In OVCAR-3 cells, the amount of phosphorylated Bcl-2 was higher in cells treated with the combination of calcitriol and docetaxel than in those treated with calcitriol or docetaxel alone. In contrast, the combination of calcitriol and docetaxel induced a smaller increase in Bcl-2 phosphorylation in UT-OC-5 cells than did docetaxel alone.

Docetaxel induces Bcl-2 phosphorylation during the G_2M phase of the cell cycle. This may lead to the dissociation of Bcl-2/Bax heterodimers and increased induction of apoptosis by Bax (Haldar et al. 1996; Kolfschoten et al. 2002). In the present study (figure 5), calcitriol differentially regulated cell cycle progression in OVCAR-3 and UT-OC-5 cells, causing G_2M arrest in OVCAR-3 cells and G_1 phase arrest in UT-OC-5 cells. In OVCAR-3 cells, the increased accumulation of cells at G_2M led to an increase in Bcl-2 phosphorylation and ultimately to an increase in apoptosis. In UT-OC-5 cells, however, the calcitriol-induced G_1 arrest decreased the apoptotic activity of docetaxel.

4. P53 gene therapy and anticancer drugs

In this study, we used a replication-deficient adenovirus to deliver a normal p53 gene to two human ovarian carcinoma cell lines. The OVCAR-3 cell line contains a point mutation in the p53 gene, while the SK-OV-3 cell line expresses normal p53 (O'Connor et al. 1997; Rantanen et al. 2002). The aim of this study was to assess the efficacy of p53Ad to inhibit cell growth and to enhance the antiproliferative effects of docetaxel, irinotecan, and SN-38.

In OVCAR-3 cells, the p53Ad gene reduced cell proliferation. This is consistent with several studies showing that human cancer cell line growth, including ovarian cancer cells, can be suppressed by introducing the wild-type p53 gene into cancer cells (Santoso et al. 1995; Blagosklonny and El-Deiry 1998; Gurnani et al. 1999; Wolf et al. 1999; Miyake et al. 2000; Quist et al. 2004). In contrast to previous results suggesting that ovarian cancer cell growth is inhibited by p53Ad regardless of the p53 status (Santoso et al. 1995; Wolf et al. 1999), however, p53Ad infection did not reduce SK-OV-3 cell growth more than did infection with empty adenovirus vectors. Furthermore, in OVCAR-3, but not in SK-OV-3 cells, SN-38 and irinotecan enhanced p53Ad-induced growth inhibition, implying that p53Ad does not provide additional antiproliferative effect in cells expressing functional p53.

In study II, the OVCAR-3 cell line was nearly resistant to SN-38. In the present study, p53Ad enhanced the efficacy of both SN-38 and irinotecan in OVCAR-3 cells. This result is consistent with previous findings that p53Ad increases the efficacy of DNA-damaging drugs such as irinotecan and SN-38 (Blagosklonny and El-Deiry 1998). The results of this study as well as the results of study II show that both irinotecan and SN-38 induce cell cycle arrest at the G_2M phase in OVCAR-3 and SK-OV-3 cells, regardless of their p53 status. Similar results were obtained in another ovarian cancer study showing that G_2M arrest is induced by SN-38 without p53 activation (McDonald and Brown 1998). The growth arrest was accompanied by the induction of $p21^{waf1/cip1}$ and Gadd45, which are both active at the G_2M phase of the cell cycle (Jin et al. 2000; Hayward et al. 2003). Gadd45 is a stress-responsive gene, whose expression is induced by various DNA-damaging agents and that stabilises p53 in response to DNA damage (Hollander et al. 1993; Zhan et al. 1994; Jin et al. 2003). $p21^{waf1/cip1}$ is a cyclin-dependent kinase inhibitor that controls G_1S

progression of the cell cycle (Rich et al. 2000). SN-38 increases the number of cells at G_2M, however, concomitantly with an increase in $p21^{waf1/cip1}$ expression (Hayward et al. 2003).

Although p53Ad did not inhibit SK-OV-3 cell growth, in both cell lines p53Ad induced G_0G_1 arrest together with increased expression of $p21^{waf1/cip1}$, emphasizing that p53 also has a role in G_1 arrest (Rich et al. 2000). At the same time, the number of cells at S phase decreased. In bladder cancer cells, overexpression of wild-type p53 induces rapid G_1 and G_2M arrest associated with an increase in $p21^{waf1/cip1}$ expression. The growth arrest is irreversible and the cells enter senescence (Sugrue et al. 1997). In our study, G_2M arrest was not obtained with p53Ad in either cell line. In OVCAR-3 cells, however, concomitant exposure with p53Ad and SN-38 or irinotecan increased the number of cells at G_2M, but the expression of $p21^{waf1/cip1}$ or Gadd45 was not further upregulated. In fact, after infection of SK-OV-3 cells with p53Ad, Gadd45 expression decreased even further in response to SN-38 or irinotecan treatment compared to samples exposed to either drug alone.

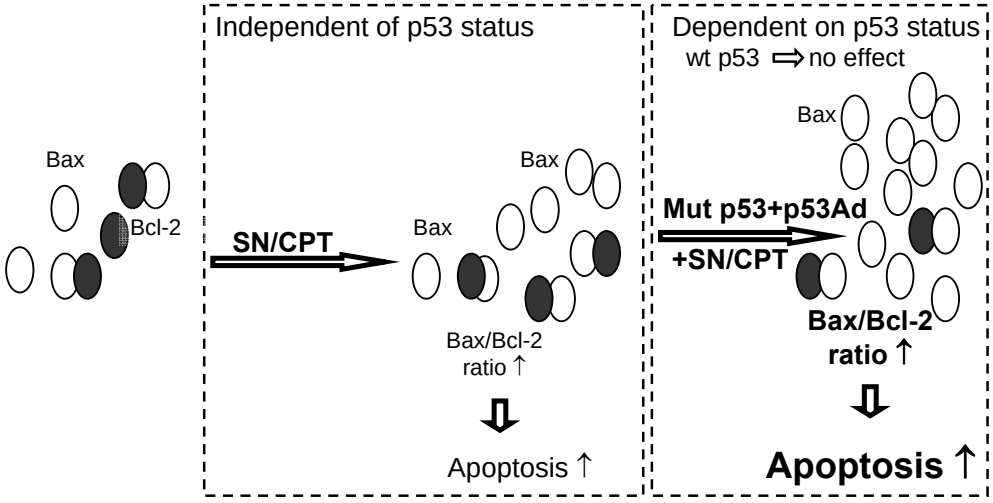

Figure 6. Response of ovarian cancer cells to concomitant p53Ad gene therapy and SN-38 (SN) /irinotecan (CPT) exposure is dependent on p53 status. Wt, wild-type.

The expression ratios of pro- and anti-apoptotic Bcl-2 family members may determine cell fate following exposure to a stressful stimulus: High expression ratios of Bax/Bcl-2 and Bax/Bcl-XL are thought to increase apoptotic activity, whereas low ratios imply stronger resistance to apoptosis and improved cell survival (Lohmann et al. 2000; Bokelmann and Mahlknecht 2008). The expression and regulation of these proteins were different in SK-OV-3 and OVCAR-3 cells, suggesting that the response might be dependent on p53 status (figure 6). In SK-OV-3 cells (wild-type p53), the transcription of both pro- and anti-apoptotic genes was slightly upregulated by irinotecan, SN-38, and p53Ad. In contrast, in OVCAR-3 cells (mutated p53) only the expression of pro-apoptotic Bax was notably upregulated. p53Ad together with SN-38 or irinotecan increased Bax expression in OVCAR-3 cells even further. In SK-OV-3 cells, the expression ratios of Bax/Bcl-2 and Bax/Bcl-XL decreased in samples exposed to irinotecan and SN-38. In OVCAR-3 cells, however, the expression ratios increased after irinotecan and SN-38 administration, and the expression ratios were even further amplified in samples

exposed to p53Ad and irinotecan 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, and apoptosis was enhanced even further by simultaneous irinotecan or SN-38 administration.

Previous studies suggested that the antiproliferative effects induced by anti-mitotic compounds such as taxanes are not dependent on functional p53 (O'Connor et al. 1997). Similar results have been reported regarding paclitaxel sensitivity of ovarian cancer cells (Rantanen et al. 2002). On the other hand, p53Ad therapy has also been reported to sensitise ovarian cancer cells to paclitaxel (Quist et al. 2004). Our data suggest that docetaxel sensitivity is not dependent on p53 status and p53Ad does not improve its efficacy. Docetaxel did not regulate expression of the studied genes, whether it was used with or without p53Ad. The lack of gene regulation might indicate that growth inhibition and induction of apoptosis are mediated through mechanisms other than activation of the p53 pathway, such as Bcl-2 phosphorylation, dissociation of Bax/Bcl-2 heterodimers and induction of apoptosis by Bax. In the study by Kolfschoten and co-workers, however, the expression levels of p53, p21, Bax, and Bcl-2 proteins as well as Bcl-2 phosphorylation were regulated by docetaxel in ovarian cancer cell lines, including OVCAR-3, indicating that docetaxel might regulate these genes at the protein level without affecting mRNA expression (Kolfschoten et al. 2002).

5. Future perspectives

Despite strong evidence supporting the antitumor activity of calcitriol and its analogues in vitro, it is still unclear how these results can be translated into clinical application, and which patients might benefit from vitamin D-based therapies. Similarly, promising results of p53 gene therapy in vitro and in vivo are not easily translated into effective therapeutic regimen in humans. Instead the results have been rather disappointing.

In this study, the histological type as well as grade of the original tumour was known for five of the cell lines, but this information was missing from two cell lines. In light of the proposed classification of epithelial ovarian cancer (Shih and Kurman 2004; Kurman et al. 2008; Cho and Shih 2009), it would have been interesting to compare the differences in response in histological types, but the number of cell lines in each histological category was too low to draw conclusions. The effects of hormones and growth factors, such as vitamin D and various drugs as single agents as well as in hormone-drug or drug-drug combinations on different histological subtypes of ovarian cancer might be an important new aspect to be considered in the treatment of ovarian cancer.

It is noteworthy that the vast majority of in vitro ovarian cancer studies and in vivo xenograft models have been conducted with only a few ovarian cancer cell lines, such as SK-OV-3 and OVCAR-3. The heterogeneity of ovarian cancers in addition to individual variations makes it difficult to draw conclusions about the efficacy of various treatments using such a small number of cell lines. Furthermore, all studies discussed in this thesis were conducted using in vitro monolayer cell cultures. The advantage of these cell culture systems is that the number of replicates and experiments can easily be increased to reach statistically significant and reproducible results. In addition, the costs of cell culture experiments are extremely

low when compared to in vivo or clinical trials. In the living body, however, the obtained effect is due to the sum of the various effects produced by several different cell types, such as epithelial and mesenchymal cells, vasculature, and organs metabolising the agent of interest. Fresh tissue culture models and in vivo studies would strengthen our ability to draw conclusions about the clinical relevance of these studies.

It is becoming evident that not only the epithelial cells but also the stromal compartment of tumours are chancing during the development and progression of ovarian cancer (Tuhkanen et al. 2004; Tuhkanen et al. 2006). Furthermore, ovarian cancer cells are not self-sustaining entities, but interact with the microenvironment. Among the cells found in this environment are macrophages, endothelial cells, lymphocytes, fibroblasts, and pericytes that interact with tumour cells through producing hormones, cytokines, and growth factors. Tumours are thought to develop stroma from several sources. Data in the literature currently support four origins: 1) the recruitment of resident tissue stem cells; 2) epithelial to mesenchymal transition (EMT) of the tumour parenchyma; 3) fibroblast recruitment into the tumour stroma; and 4) recruitment of bone marrow-derived cells from the circulation (reviewed in (Spaeth et al. 2009)). A fascinating new area of cancer research is cancer stem cells. Ascites-derived stromal cells that are similar to bone marrow-derived or adipose tissue-derived mesenchymal stem cells have shown to promote tumourigenicity and angiogenesis in ovarian cancer (Pasquet et al. 2009). Furthermore, the generation of cancer stem cells from immortalized human mammary epithelia has recently been shown to be triggered by EMT that involves the change in cell morphology, the loss of cell adhesion and acquisition of migratory and invasive properties (Mani et al. 2008; Morel et al. 2008; Sarrio et al. 2008). Interestingly, in ovarian cancer cells, EMT regulating factors Snail and Slug are mediators of radio- and chemoresistance by antagonizing p53-mediated apoptosis and acquiring a stem cell-like phenotype (Kurrey et al. 2009). The expression of Snail in borderline and malignant epithelial ovarian tumours is also associated with tumour progression (Tuhkanen et al. 2009). Epithelium-stroma interactions, EMT and ovarian cancer stem cells are interesting research topics and might be in a major role when new treatment strategies for ovarian cancer are developed.

SUMMARY AND CONCLUSIONS

The main disadvantage of using anticancer drugs is the wide capacity of cancer cells to acquire resistance to chemotherapeutic agents, and thus the development of new treatment strategies is required. The molecular events in cancer cells might provide clues to better strengthen the fight against cancer. In these studies, we evaluated the efficacy of vitamin D_3 compounds; the anticancer drugs docetaxel, irinotecan, and SN-38; and p53 gene therapy to inhibit growth of ovarian cancer cell lines. Moreover, we identified the molecular mechanisms that affect the cellular responses to these agents, either as single or combined therapies.

In ovarian cancer, high calcitriol concentrations may be required to produce inhibitory growth effects. Small amounts may stimulate the growth of ovarian cancer cells, as do low concentrations of $25(OH)D_3$ and calcitriol in the OVCAR-3 cell line. Because of this growth stimulation effect, the roles of vitamin D_3 and its metabolising enzymes 24OHase and 1αOHase in the development and progression of ovarian cancer must be characterised in more detail.

Our results indicate that docetaxel and SN-38 are potent antiproliferative agents in ovarian cancer cells. The efficacy of these drugs, however, especially that of SN-38, varied widely among different cell lines. When these drugs are used concomitantly, antagonistic growth effects are sometimes observed. Antagonistic effects are partially mediated by an SN-38 induced increase in p-glycoprotein expression, leading to an increase in the docetaxel efflux. Antagonistic effects must be considered when planning clinical studies of combinations of docetaxel and SN-38.

The response of ovarian cancer cells to simultaneous exposure of calcitriol and docetaxel is highly dependent on the cell line. The response appears to depend on whether calcitriol induces cell cycle arrest at G_1 or G_2M. Arrest at G_2M allows for the phosphorylation of Bcl-2 protein, leading to increased apoptosis. With G_1 arrest, however, Bcl-2 phosphorylation does not occur and the consequence is an antagonistic effect of docetaxel and calcitriol, which weakens or prevents the inhibition of growth.

Although preclinical data indicate that p53 gene therapy might be a promising tool for improving survival of ovarian cancer patients, the results of clinical experiments have been disappointing and suggest that the selection criteria for patients who might benefit from the therapy must be characterised more carefully. Our studies indicate that the efficacy of docetaxel is not dependent on p53 status. In contrast, the efficacy of irinotecan and its active metabolite SN-38 might be p53-dependent. In cancer cells containing a mutated p53 gene, the efficacy of irinotecan and SN-38 is improved by introducing a functional wild-type p53 gene into the cancer cells. When the p53 status of cancer cells is normal, however, p53 gene therapy is not effective alone or in combination with irinotecan or SN-38. Moreover, Bax/Bcl-2 and Bax/Bcl-XL expression ratios might serve as indicators for the efficacy of adenovirus-mediated p53 gene therapy and irinotecan or SN-38 administration.

Our results indicate that concomitant treatment with combinations of theoretically antiproliferative agents that as single agents inhibit the growth of ovarian cancer cells might abolish the individual actions of each agent. Furthermore, cells do not necessarily respond similarly to a given therapy. Instead, cell-specific properties, such as the activity of metabolising enzymes and drug efflux proteins, ease of

inducing cell cycle arrest, and expression of cell-survival regulating proteins, influence the therapeutic efficacy of these agents.

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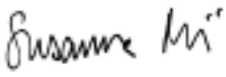

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ORIGINAL COMMUNICATIONS

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ROLE OF 24-HYDROXYLASE IN VITAMIN D_3 GROWTH RESPONSE OF OVCAR-3 OVARIAN CANCER CELLS

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Vitamin D and its analogues are potent regulators of cell growth and differentiation both *in vivo* and *in vitro*. We studied the effects of 25-hydroxyvitamin D_3 [25(OH)D_3], 1,25-dihydroxyvitamin D_3 [1,25(OH)$_2D_3$] and vitamin D analogue, EB 1089, on the growth of a human ovarian cancer cell line, OVCAR-3. We also studied the expression of vitamin D metabolising enzymes 24-hydroxylase (24OHase) and 1α-hydroxylase (1αOHase). Our results showed that high concentrations (10 and 100 nM) of 1,25(OH)$_2D_3$ inhibited a cell proliferation, whereas low concentration (0.1 nM) stimulated growth of the OVCAR-3 cells. In the concentration range of 10–500 nM a prohormone, 25(OH)D_3, stimulated growth. An amount of 1 nM EB 1089 and 100 nM 1,25(OH)$_2D_3$ inhibited growth with an equal magnitude. The expression of 24OHase was strongly induced by 1,25(OH)$_2D_3$ and EB 1089 in OVCAR-3 cells, and analysis of vitamin D metabolites showed the functionality of 24OHase. An inhibition of 24OHase activity with a novel 24OHase inhibitor enhanced growth-inhibiting effects of 1,25(OH)$_2D_3$ and suppressed the growth stimulation of 100 nM 25(OH)D_3. We also report the expression of a vitamin D activating enzyme, 1αOHase, in 7 ovarian cancer cell lines. The production of 1,25(OH)$_2D_3$ in OVCAR-3 cells was low, possibly due to an extensive activity of 24OHase or a low 1αOHase activity. These results suggest that in ovarian cancer cells vitamin D metabolizing enzymes might play a key role in modulating the growth response to vitamin D. The possible mitogenic effects of vitamin D should be considered when evaluating treatment of ovarian cancer with vitamin D.

Key words: *vitamin D; EB 1089; 1α-hydroxylase; 24-hydroxylase; ovary; cell growth*

Both epidemiologic and *in vitro* studies suggest that vitamin D may be an important factor in the development and progression of cancer.[1–4] Geographic data suggest that sunlight might be a protective factor for ovarian cancer.[5,6] Since the major source of vitamin D is through sunlight-induced synthesis in the skin, it has been hypothesized that vitamin D may mediate the protective effects observed. In addition, a case-control study in Mexico reports inverse association between vitamin D content of the diet and a risk of ovarian cancer.[7]

The action of vitamin D is mediated via its nuclear receptor, vitamin D receptor (VDR). Two different studies have reported that 43–50% of ovarian cancers express VDR.[8,9] In cell culture studies, vitamin D has been shown to inhibit the growth of various cancer cell lines.[4] We and others have shown that high 1,25-dihydroxyvitamin D_3 [1,25(OH)$_2D_3$] concentrations inhibit the growth of a human ovarian cancer cell line, OVCAR-3, which expresses VDR.[8–10]

Because of its inhibitory growth effects, vitamin D is an attractive molecule in the anticancer drug development. The clinical use of 1,25(OH)$_2D_3$ in the treatment of cancer patients is limited due to its hypercalcemic side effects. Because of its less calcemic nature, a pro-hormone 25(OH)D_3 could be used in the cancer therapy in higher concentrations than 1,25(OH)$_2D_3$. Vitamin D analogues have been developed to separate the growth-regulating effects from the calcemic effects. EB 1089 is one of these analogues generated by altering the side-chain structure of the parent hormone.[11]

The effect of vitamin D is modulated by vitamin D metabolising enzymes, 24-hydroxylase (24OHase) and 1α-hydroxylase (1αOHase) expressed predominantly in kidney. The pro-hormone 25-hydroxyvitamin D_3 [25(OH)D_3] is converted to an active 1,25(OH)$_2D_3$ form by an enzyme 1αOHase. In addition to kidney, the expression of 1αOHase has been shown in skin, intestine, pancreas, adrenal medulla, brain and placenta.[12] The enzymatic activity of 1αOHase has also been detected in both normal and neoplastic prostate and in lung and colon cancer cells.[13–17] Some studies suggest that the expression of 1αOHase might change during the cancer development and progression.[14–16] Knockout studies in mice have shown the important role of 1αOHase in normal ovarian development, since smaller ovaries and impaired folliculogenesis are observed in 1αOHase null mutant mice.[18]

24OHase is a mitochondrial enzyme that catalyses the hydroxylation of 1,25(OH)$_2D_3$ to 1,24,25(OH)$_3D_3$ while 25(OH)D_3 is converted to 24,25(OH)$_2D_3$. 24OHase might also catalyse further hydroxylation steps of vitamin D metabolism.[19,63] These metabolites have been considered as inactivation products, which do not have a clear biologic function, but some studies have shown that vitamin D metabolites may also have specific effects in target cells.[20,21] Besides kidney, 24OHase enzyme has been detected in prostate, intestine, ovary and many other organs expressing vitamin D receptor.[22–28]

Although high concentrations of 1,25(OH)$_2D_3$ have been shown to be a potent growth inhibitor of many neoplastic cell lines, mitogenic effects have been reported with low concentrations of 1,25(OH)$_2D_3$ in several cell culture models.[21,29–33] In this study, we report the concentration-dependent growth modulation with 1,25(OH)$_2D_3$, 25(OH)D_3 and EB 1089 in the ovarian cancer cell line, OVCAR-3. The addition of 24OHase inhibitor (VID400) enhances growth-inhibiting effects of 1,25(OH)$_2D_3$ and converts growth-promoting effects of 25(OH)D_3 to inhibiting effects. We also show the expression of 1αOHase in ovarian cancer cell lines and the induction of 24OHase mRNA by vitamin D compounds in OVCAR-3 cells.

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MATERIAL AND METHODS

Cell culture

The human ovarian adenocarcinoma cell line, OVCAR-3 (ATCC, Manassas, VA) was maintained, as recommended by the supplier, in RPMI 1640 medium (Sigma Aldrich, St. Louis, MO) supplemented with 10% FBS, 10 μg/ml insulin, 0.25% glucose and antibiotics (100 IU/ml penicillin, 100 μg/ml streptomycin). Human ovarian adenocarcinoma cell lines UT-OC-1, UT-OC-2, UT-OC-3, UT-OC-4, UT-OC-5[34] and SK-OV-3 and a human keratinocyte cell line HaCaT were grown in DMEM (Sigma Aldrich) with 10% FBS and antibiotics (100 IU/ml penicillin, 100 μg/ml streptomycin), and monkey kidney COS cells were maintained in DMEM/F12 (Sigma Aldrich) with 5% FBS. All cell lines were kept at 37°C in a humidified 95% air/5% CO_2 incubator.

Cell growth assay

When OVCAR-3 cells were on the logarithmic growth phase (70% confluent) the growth assay was started. For cell growth assay, 2,000 cell/200 μl/well were plated on 96-well culture plates. One day after plating, the medium (RPMI 1640, [Sigma Aldrich] supplemented with 10% FBS, 10 μg/ml insulin, 0.25% glucose and antibiotics) was changed and indicated concentrations of $1,25(OH)_2D_3$, $25(OH)D_3$, EB 1089 (kindly provided by Leo Pharmaceutical Products, Ballerup, Denmark), VID400 (specific 24OHase inhibitor, kindly provided by Dr. Anton Stuetz, Novartis Research Institute, Vienna, Austria) or combination of VID400 and $1,25(OH)_2D_3$ or $25(OH)D_3$ were added (day 0). Ethanol was used as a vehicle, and it was also included in the control. The medium containing ethanol vehicle and/or hormones were changed to a fresh one every third day. Cell growth samples were taken 0, 1, 3, 5, 7, 9 and 11 days after the treatment. Preliminary studies showed that during this period cells were at a logarithmic growth phase.

Relative cell numbers were quantified as described previously.[35] Cells were fixed on the bottom of the wells by addition of 10 μl of 11% glutaraldehyde solution in 0.1% phosphate buffer to 100 μl of medium. The plate was shaken 500 cycles/min for 15 min, washed 3 times by submersion in de-ionised water and air-dried. Fixed cells were stained with 0.1% solution of crystal violet dissolved in de-ionised water. After 20 min incubation, excess dye was removed by carefully washing with de-ionised water. The plate was air-dried prior to a bound dye solubilisation in 100 μl of 10% acetic acid. Relative cell number was given as absorbance units by measuring the optical density (590 nm) from each well using Victor 1420 multilabel counter (Wallac, Turku, Finland). Six determinations were used to calculate the mean optical density ± SD in each concentration at each time point. The absorbance value of day 0 (an overnight culture of 2,000 cells/well) was set as 0 by subtracting it from each value obtained from adjacent time-point measurements (days 1–11), and based on these values growth curves were created. Experiments were repeated 3–5 times. Day 11 was used to compare the effect of hormone treatments and 24OHase inhibitor. Statistical analyses were done using Student's *t*-test.

Detection of 24- and 1α-hydroxylase mRNAs

When cell culture bottles were grown to 70% confluence, the old medium was removed and replaced with medium containing 100 nM $1,25(OH)_2D_3$, $25(OH)D_3$ or EB 1089. Ethanol was used as a vehicle, and it was also added to the control cells. For RNA extraction, the cells were collected 4, 6 and 24 hr after the treatment with vitamin D compound or vehicle. RNA extractions were done with TRIZOL reagent (GIBCO Invitrogen Corporation, Paisley, UK). The integrity of RNA samples was confirmed on gel electrophoresis.

The expression of 24- and 1αOHase messenger RNA was detected using a reverse transcription-polymerase chain reaction (RT-PCR). To perform the RT-PCR, specific oligonucleotide primers were synthesised by Amersham Bioscience (Amersham, UK) (Table I). The reactions for 24-hydroxylase were performed in the LightCycler instrument (Roche Diagnostics, Basel, Switzerland) from 300 ng total RNA. PBGD (human porphobilinogen deaminase) mRNA was used as an external control. A master mix of the following components was prepared in a 20 μl volume: 0.5 μM PBGD primers or 0.3 μM 24OHase primers and 3.5mM Mn^{2+} for PBGD or 3.25 mM Mn^{2+} for 24OHase. Nucleotides, *Tth* DNA polymerase (DNA polymerase and reverse transcriptase activity), SYBR Green I and reaction buffer were included in the LightCycler-RNA Master SYBR Green I kit (Roche Diagnostics). For preparing the standard curve, total RNA from HaCaT cells, which express 24-hydroxylase mRNA,[28] was amplified in the same run as samples. The RT-PCR protocol was as follows: 20 min reverse transcription at 61°C and 30 sec denaturation at 95°C followed by 45 cycles with a 95°C denaturation for 1 sec, 62°C for PBGD or 57°C for 24OHase annealing for 7 sec and 72°C extension for 12 sec. Detection of fluorescent product was performed at the end of the extension step of each cycle. To verify the specific products, melting curve analysis and gel electrophoresis were done. The data were quantified by the Fit Points method with LightCycler Data Analysis software. The amplification efficiency and the relative expression ratio of 24OHase were calculated according to Pfaffl.[36] Hormone treatments and RT-PCR were done twice.

A normal RT-PCR was used for the detection of 1αOHase mRNA. RT-PCR (RobusT RT-PCR Kit, Finnzymes, Espoo, Finland) was performed according to the manufacturer's instructions from 1 μg total RNA. A negative control reaction (reactions without reverse transcriptase enzyme) was done from each sample. The RT-PCR protocol was as follows: 30 min reverse transcription at 48°C and 2 min denaturation step at 94°C followed by 30 cycles with 94°C denaturation for 30 sec, 54°C annealing for 30 sec and 72°C extension for 30 sec. The final extension after cycles was at 72°C for 7 min. Total RNA (0.5 μg) from monkey kidney COS cells transfected with human 1αOHase cDNA (kindly donated by Dr. S. Kato, Institute of Molecular and Cellular Biosciences, University of Tokyo, Japan) was used as a positive control. A transfection was done according to the manufacturer's instructions with 1αOHase ORF cDNA in pcDNA3 mammalian expression vector by a lipofection (Lipofectamine, Life Technologies). A functional control reaction (MS2 RNA and primers for amplification of 1100 bp sequence) was included in the kit, and it was carried out with the same run as other samples. After gel electrophoresis, RT-PCR products were extracted from the gel and sequences were verified by hybridisation with ^{32}P-labelled RNA-probe made from 1αOHase cDNA.

TABLE I – OLIGONUCLEOTIDE PRIMER SEQUENCES FOR RT-PCR

Gene (accession no.)	Base pairs	Oligos	Sequence	Product size (bp)
1αOHase	1241–1261	F	5′-GTCAAGGAAGTGCTAAGACTG-3′	303
(AB005038)	1524–1543	R	5′-TGTTAGGATCTGGGCCAAAG-3′	
24OHase	833–852	F	5′-TGATCCTGGAAGGGGAAGAC-3′	212
(L13286)	1023–1044	R	5′-CACGAGGCAGATACTTTCAAAC-3′	
PBGD	695–714	F	5′-AAGTGCGAGCCAAGGACCAG-3′	298
(X04808)	969–992	R	5′-TTACGAGCAGTGATGCCTACCAAC-3′	

F, forward primer; R, reverse primer.

Metabolic analysis of 25(OH)D_3

OVCAR-3 cells (1.5×10^6 cell/flask) were plated on T25 culture flasks. One day after plating, cells were treated with 500 nM 25(OH)D_3 in RPMI 1640 medium supplemented with 10% FBS, 10 μg/ml insulin, 0.25% glucose and antibiotics (100 IU/ml penicillin, 100 μg/ml streptomycin). After 0, 3 or 24 hr, the medium was collected and the cell monolayer was extracted with 1 ml methanol. After 15 min incubation at room temperature, the methanol was transformed into the same tube than the sample medium. The samples for the measurement of the 25(OH)D_3 metabolites were purified using the acetonitrile-C18 Sep-Pak (Waters Corporation, Milford, MA) procedure[37] followed by separation of the metabolites by a high-performance liquid chromatography. The concentrations of 24,25$(OH)_2D_3$ were quantified by a competitive protein binding assay[38] and 1,25$(OH)_2D_3$ by a radioreceptor assay.[39] The second measurement was done following the same procedure except dextran charcoal-treated FBS was used instead of FBS, 24OHase inhibitor (200 nM VID400) was used with the 500 nM 25(OH)D_3 treatment, and the samples were collected only after 0 and 24 hr.

RESULTS

Regulation of OVCAR-3 cell growth by different vitamin D compounds

Figure 1*a* illustrates the concentration-dependent stimulation of the cell growth with 25(OH)D_3 in OVCAR-3 cell line. An amount of 10 nM 25(OH)D_3 treatment stimulated growth by 32%, 50 nM stimulated growth by 41%, 100 nM by 39%, 200 nM by 35% and 500 nM 25(OH)D_3 by 11% when compared to the control (Fig. 1*a*). All differences were statistically significant when compared to the control ($p < 0.05$).

When high concentrations were used, 1,25$(OH)_2D_3$ inhibited growth of the OVCAR-3 cell line (Fig. 1*b*). An amount of 100 nM 1,25$(OH)_2D_3$ inhibited growth by 74% ($p < 0.001$) and 10 nM by 8% ($p < 0.0001$) when compared to the control. An amount of 0.1 nM 1,25$(OH)_2D_3$ stimulated growth by 14% ($p < 0.0001$), whereas 1 nM 1,25$(OH)_2D_3$ did not have an effect on the cell growth.

EB 1089 inhibited growth when 1 and 100 nM concentrations were used (Fig. 1*c*). When compared to the control, 100 nM EB 1089 inhibited growth by 84% and 1 nM by 73% ($p < 0.0001$). At 1 nM concentration, EB 1089 was as potent a growth inhibitor as 100 nM EB 1089. The growth inhibition was almost equal to 100 nM 1,25$(OH)_2D_3$ and 1 nM EB 1089 (74% *vs.* 73% of the control).

Expression of 1α-hydroxylase and 24-hydroxylase

To test whether enzymes 1α-hydroxylase and 24-hydroxylase might be involved in the metabolism of vitamin D compounds in the OVCAR-3 cell line, we studied the expression of these enzymes at mRNA level. We also studied whether the expression of 24OHase mRNA could be modulated by 25(OH)D_3, 1,25$(OH)_2D_3$ or EB 1089.

Our data indicate that the OVCAR-3 cell line expresses 1αOHase (Fig. 2). A single 303 bp band can be seen in 1αOhase-transfected COS sample (lane 3) and in both ethanol-treated control (lanes 7 and 8) and 100 nM 1,25$(OH)_2D_3$-treated (lanes 9 and 10) OVCAR-3 samples. A hybridisation with P^{32}-labelled probe showed that the 1αOHase sequence is amplified in RT-PCR. 1αOHase mRNA was also expressed in 6 other ovarian cancer cell lines (UT-OC-1–5 and SK-OV-3; data not shown).

Also 24OHase is expressed in OVCAR-3 cells and the expression of 24OHase is regulated by EB 1089 and 1,25$(OH)_2D_3$ almost equally. After 6 hr treatment, the expression of 24OHase mRNA (Fig. 3) was induced 650-fold with 100 nM 1,25$(OH)_2D_3$ and 600-fold with 100 nM EB 1089. After 24 hr, the expression levels were further increased. When compared to the control, 1,25$(OH)_2D_3$ treatment induced the expression by 1,100-fold and

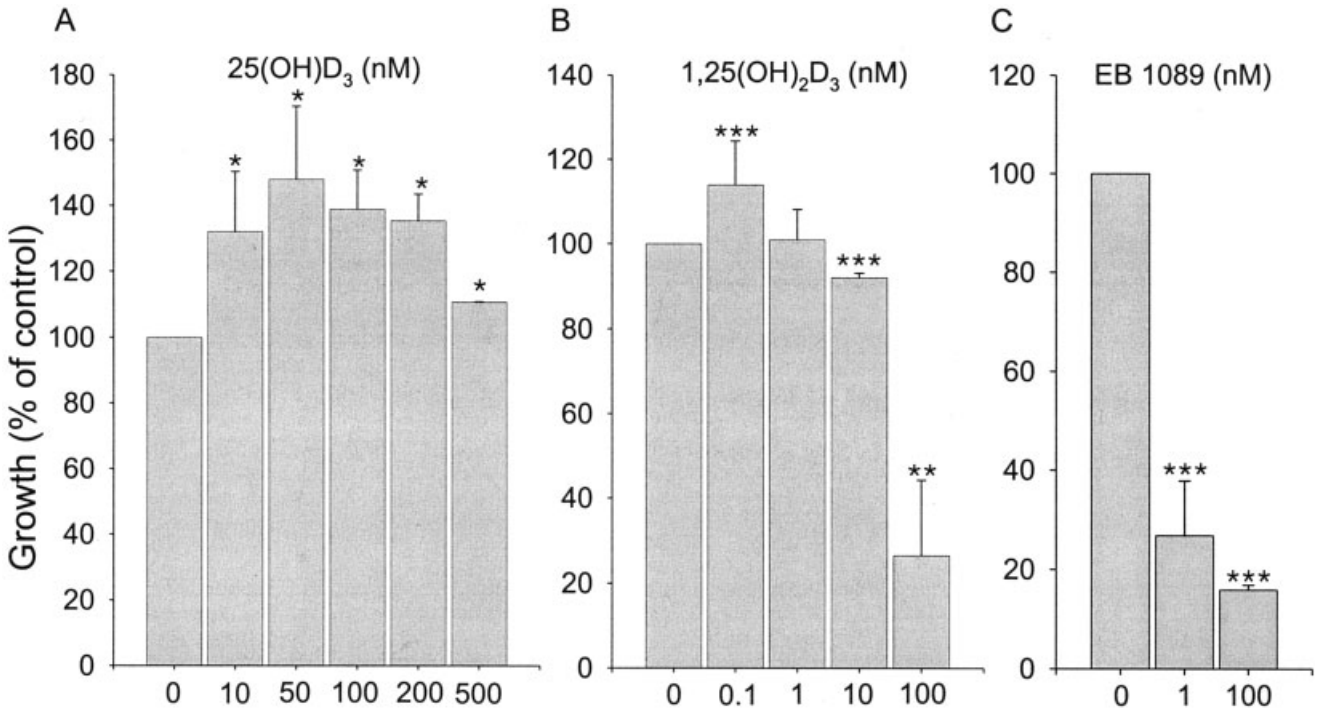

FIGURE 1 – Regulation of the cell growth by vitamin D compounds. The effect of 25(OH)D_3 *(a),* 1,25-$(OH)_2D_3$ *(b)* and EB 1098 *(c)* on the growth of OVCAR-3 cells. Cells were treated with indicated hormone concentrations for 11 days. Growth medium and hormones were changed to a fresh one every third day. After the treatment period, cells were fixed, stained with crystal violet, and the optical density (590 nm) was determined. The cell growth is presented as a percentage of ethanol-treated cells. The values represent the mean of 3–5 separate experiments ± SD. (*$p < 0.05$, **$p < 0.001$, ***$p < 0.0001$, Student's *t*-test).

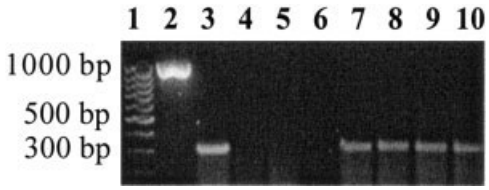

FIGURE 2 – The expression of 1αOHase in OVCAR-3 cells. A RT-PCR was used for the detection of 1αOHase mRNA from OVCAR-3 cells. A 303 bp band can be seen in the 1αOhase-transfected COS sample (lane 3) and in both ethanol-treated (lanes 7 and 8) and 100 nM 1,25-$(OH)_2D_3$-treated (lanes 9 and 10) OVCAR-3 samples. In lane 4, there is a negative control for the ethanol-treated sample, and lane 5 represents a negative control for the 1,25-$(OH)_2D_3$-treated sample. Lane 1 is a 100 bp marker, lane 2 is a RT-PCR functional control (1100 bp), and lane 6 is empty.

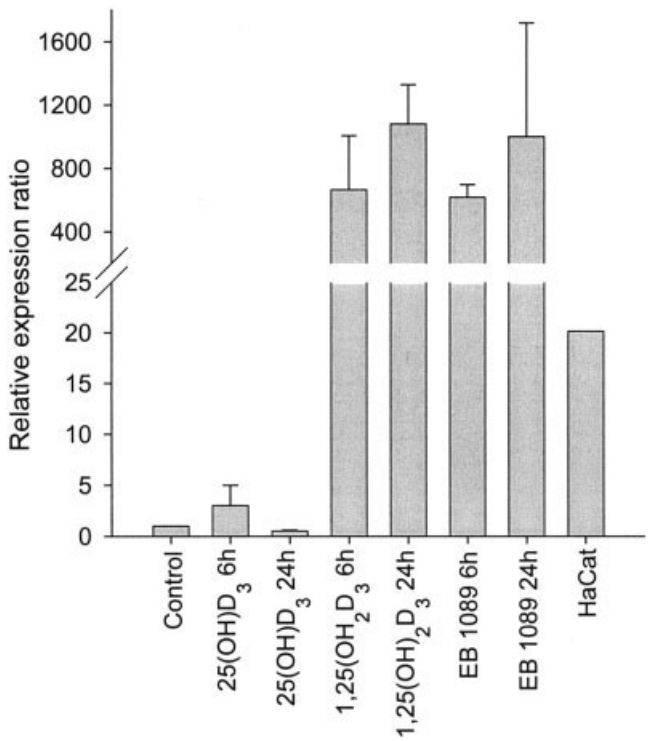

FIGURE 3 – The relative expression ratios of 24OHase mRNA in OVCAR-3 cells after 6 or 24 hr treatment with 100 nM 1,25-$(OH)_2D_3$, 25$(OH)D_3$, EB 1098 or ethanol (vehicle). A quantitative RT-PCR was done using 0.3 μg total RNA. The human keratinocyte cell line, HaCaT, was used as an expression control of 24OHase. The values represent the mean of 2 independent experiments ± SD.

EB 1089 by 1,000-fold. After 6 hr treatment, the expression in 25$(OH)D_3$- (100 nM) treated cells was slightly increased (3-fold) but returned to a basal level or even slightly downregulated (0.5 fold) after 24 hr treatment. The human keratinocyte cell line, HaCaT, was used as a control for the expression of 24Ohase, and our data indicate that the basal expression level is 20 times higher in HaCaT than in OVCAR-3 cells.

Metabolism of 25(OH)D_3 in OVCAR-3 cells

Since the both enzymes, 24OHase and 1αOHase, are expressed in the OVCAR-3 cell line, we studied the functionality of these enzymes. Analysis of metabolites generated from 25$(OH)D_3$ are shown in Table II. In the first experiment, the amount of 24,25$(OH)_2D_3$ was 4 times higher after 3 hr incubation than it was

TABLE II – METABOLITES OF 25$(OH)D_3$

	24,25$(OH)_2D_3$ (nM)		1,25$(OH)_2D_3$ (pM)	
	I	II[1]	I	II[1]
500 nM 25$(OH)D_3$, 0 hr	6	1	23	<20
500 nM 25$(OH)D_3$, 3 hr	24	ns	37	ns
500 nM 25$(OH)D_3$, 24 hr	112	27	33	28
500 nM 25$(OH)D_3$ + 200 nM VID400, 24 hr	ns	8	ns	27

[1]Cells were grown in RPMI 1640 supplemented with 10% dextran charcoal-treated FBS instead of FBS. ns, not studied.

when the experiment started (0 hr). After 24 hr, the production was further increased (18-fold). The basal level of 1,25$(OH)_2D_3$ was 23 pM, and after 3 hr incubation, the concentration was increased to 37 pM. After 24 hr, the concentration was almost equal or slightly decreased (33 pM).

In the second experiment, we supplemented RPMI 1460 medium with dextran charcoal-treated FBS instead of normal FBS. In this experiment, the concentration of 24,25$(OH)_2D_3$ was increased 27 times after 24 hr. When 24OHase inhibitor was used, the production reduced to one-third when compared to 500 nM 25$(OH)D_3$ treatment alone. At the beginning of the experiment (0 hr), the concentration of 1,25$(OH)_2D_3$ was undetectable, but after 24 hr we could detect 28 pM concentration of 1,25$(OH)_2D_3$. 24OHase inhibitor did not have an effect on production of 1,25$(OH)_2D_3$.

Effect of 24OHase inhibitor on growth response of 1,25(OH)$_2D_3$ and 25(OH)D_3

Because the metabolic measurements showed an extensive production of 24,25$(OH)_2D_3$ and an enzymatic activity of 24OHase, we decided to test the effect of 24OHase inhibitor, VID400, on the growth response of 1,25$(OH)_2D_3$ and 25$(OH)D_3$. As shown in Figure 4*a* and *b*, 200 nM VID400 alone had a growth-inhibitory effect on cells. The inhibition was 8% ($p < 0.05$) when compared to the control. In these experiments, 100 nM 25$(OH)D_3$ stimulated growth by 18% (Fig. 4*a*), but the difference was not statistically significant when compared to the control. When 100 nM 25$(OH)D_3$ was combined with 200 nM VID400, the stimulatory growth effect was converted to an inhibitory (14%, $p < 0.001$ when compared to the control).

We also studied the effect of 24OHase inhibitor on the growth response of 1,25$(OH)_2D_3$ (Fig. 4*b*). In these experiments, 1 nM 1,25$(OH)_2D_3$ alone did not have an effect on the cell growth. However, when it was combined with 200 nM VID400, it inhibited the growth by 27% ($p < 0.0001$) when compared to the control. An amount of 10 nM 1,25$(OH)_2D_3$ alone inhibited the growth by 26%, but a combination of 10 nM 1,25$(OH)_2D_3$ and 200 nM VID400 inhibited growth by 77%.

DISCUSSION

The growth inhibition of OVCAR-3 cells by 100 nM 1,25$(OH)_2D_3$ observed in our study is in an agreement with previous results on OVCAR-3 cells[8–10] and many other cancer cell lines.[4,40,41] Results of cell culture studies showing a growth inhibition are usually obtained with high 1,25$(OH)_2D_3$ concentrations (10–100 nM). However, there are also reports concerning the stimulation of cell proliferation with low 1,25$(OH)_2D_3$ concentrations. *In vitro* studies suggest that in normal tissues 1,25$(OH)_2D_3$ might have a role in maintaining the balance between proliferating and differentiating cell populations.[31–33] There are also reports that low concentrations of 1,25$(OH)_2D_3$ may stimulate the growth of neoplastic cell lines. Mitogenic effects of 1,25$(OH)_2D_3$ have been reported with low 1,25$(OH)_2D_3$ concentrations in prostate[41] and other cancer cell lines,[29,30] as well as in the ovarian cancer cell line in our study.

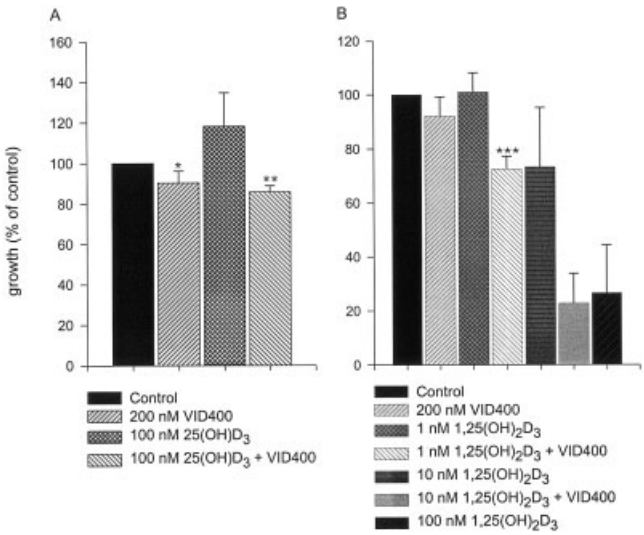

FIGURE 4 – The effect of 24OHase inhibitor on the cell-growth response to $25(OH)D_3$ *(a)* and $1,25\text{-}(OH)_2D_3$ *(b).* Cells were treated with indicated hormone concentrations or combinations of hormone and 24OHase inhibitor (VID400) for 11 days. The growth medium and hormones were changed to a fresh one every third day. After the treatment period, cells were fixed, stained with crystal violet, and the optical density (590 nm) was determined. The cell growth is presented as a percentage of ethanol-treated cells. The values represent the mean of 3 separate experiments ± SD. (*$p < 0.05$, **$p < 0.001$, ***$p < 0.0001$, Student's *t*-test).

Vitamin D analogue, EB 1089, has been shown to be more potent in the tumour cell growth inhibition than $1,25(OH)_2D_3$ in both *in vivo* and *in vitro* experiments.[42–45] In our study, EB 1089 was a potent inhibitor of OVCAR-3 cell growth already at 1 nM concentration. Studies on other cancer cell types have suggested that EB 1089 may be 50–200 times more potent than $1,25(OH)_2D_3$ in regulation of cell growth,[46] which is in line with our result showing similar growth inhibition with 1 nM EB 1089 and 100 nM $1,25(OH)_2D_3$ in OVCAR-3 cells. Similar results have been observed with prostate cancer cell line LNCaP, where 100 nM $1,25(OH)_2D_3$ was as potent a growth inhibitor as 1 nM EB 1089.[47] EB 1089 has been tested in phase I trials in breast and colorectal cancer patients.[48] Because it could be used in lower concentrations than the parent hormone to achieve a growth inhibition, calcemic and toxic effects observed with $1,25(OH)_2D_3$ treatment might be avoided.

The relatively high concentrations of $1,25(OH)_2D_3$ required to obtain an inhibitory growth response may be due to an enzyme 24OHase. The ability of $1,25(OH)_2D_3$ to induce 24OHase through a vitamin D receptor-dependent process is well known and used as a marker of $1,25(OH)_2D_3$ action.[49–51] Previously, induction of specific activity of 24OHase by $1,25(OH)_2D_3$ has been shown in ovarian cancer cells.[25] 24OHase was highly inducible by $1,25(OH)_2D_3$ and EB 1089 (1,100- and 1,000-fold, respectively) in OVCAR-3 cells. 24OHase has been considered as an inactivating enzyme of $1,25(OH)_2D_3$. Metabolites generated by 24OHase pathway may still have their own distinct effects on cell growth, but they are poorly known. $1,24,25(OH)_2D_3$ and a further oxidized product, $1,25(OH)_2$-24-oxo-vitamin D_3, might have growth-promoting effects.[21,52] Recently a selective inhibitor of 24OHase, VID400, has been developed.[53] Our results show that inhibition of 24OHase enhances the growth inhibition of $1,25(OH)_2D_3$. In breast cancer, 24OHase has been described as a candidate oncogene, whose overexpression may give a growth advantage to cancer cells, since these cells may escape from the vitamin D-mediated growth control.[24] The chromosomal region 20q12-q13 is amplified in 54% of ovarian cancers,[54] and it is the same region in which the gene locus of 24OHase is mapped.[55] The growth-promoting role of 24OHase makes it an even more powerful oncogene, since it does not only degrade the most growth-inhibitory form of vitamin D, $1,25(OH)_2D_3$, but also converts it to a growth-stimulatory metabolite. The strong induction of 24OHase by $1,25(OH)_2D_3$, possible because of a genomic amplification, might be a reason why calcitriol therapy in ovarian cancer has not been successful.[56]

The induction of 24OHase by EB 1089 has been shown previously in rat kidney and intestine[26] and in human head and neck squamous cell carcinoma cells.[57] Although in our study on OVCAR-3 cells EB 1089 upregulated 24OHase mRNA levels by 1,000-fold, the metabolism of EB 1089 may not involve 24OHase. Because of its side-chain double-bond structure, it is rather hydroxylated in distal C26 and C26a sites.[58] Differences in metabolic pathways between EB 1089 and $1,25(OH)_2D_3$ might explain why EB 1089 inhibited growth already at a 1 nM concentration, whereas 1 nM $1,25(OH)_2D_3$ did not have any effect on the cell growth.

$25(OH)D_3$ has been considered as a pro-hormone. Previous studies have shown an inhibition of cell proliferation by $25(OH)D_3$ in cultured prostate cells[15,59,60] and in colon tissue *in vivo.*[61] However, our results indicate that in the range of 10 nM to 500 nM, $25(OH)D_3$ promotes ovarian cancer cell growth. The effect of $25(OH)D_3$ on cell growth might be cell and tissue-type specific. Some growth-modulating effects of $25(OH)D_3$ might be mediated via a direct binding to VDR, although the relative binding affinity of $25(OH)D_3$ to VDR is about 700-fold lower than that of $1,25(OH)_2D_3$.[62] Besides the direct binding to VDR, both of the vitamin D metabolising enzymes, 1αOHase and 24OHase, might regulate the cellular responses to $25(OH)D_3$. Many cell types are reported to express 1αOHase and might be able to convert $25(OH)D_3$ to an active metabolite $1,25(OH)_2D_3$.[12–17] Our finding that 1αOHase mRNA is expressed in the human ovarian cancer cell lines suggests that $1,25(OH)_2D_3$ could be generated locally. In the early phase of the human colorectal cancer genesis, the expression of 1αOHase and VDR mRNA are upregulated, whereas in poorly differentiated late-stage carcinomas, only low levels of the respective mRNAs can be detected.[14] In prostate cancer cells, the activity of 1αOHase was reported to be 10- to 20-fold lower than in normal prostate cells, leading to a reduced antiproliferative action of $25(OH)D_3$.[15] As shown in our study, the activity of $25(OH)D_3$ 1αOHase in these cells may be so low that only minimal amounts of $1,25(OH)_2D_3$ is produced, and low $1,25(OH)_2D_3$ concentrations was shown to be growth stimulatory in our study.

24OHase converts $25(OH)D_3$ to $24,25(OH)_2D_3$ and also catalyses further hydroxylation reactions.[63] The role of these products in the regulation of the cancer cell growth has not been studied extensively, but $24,25(OH)_2D_3$ is believed to contribute to the bone formation and the fracture healing.[20] Although we could detect only a slight induction of 24OHase mRNA after 6 hr treatment and even a downregulation after 24 hr, the metabolite analysis showed the extensive production of 24-hydroxylated product, $24,25(OH)_2D_3$. In our cell growth studies, the inhibition of 24OHase activity converted the growth stimulation to the growth inhibition, which suggests that the mitogenic effects of $25(OH)D_3$ might be mediated partially through 24-hydroxylated products.

We conclude that in ovarian cancer, high vitamin D concentrations may be required to acquire a beneficial inhibitory growth effect. Small amounts of vitamin D may stimulate the growth of ovarian cancer cells as does low concentrations of $25(OH)D_3$ and $1,25(OH)_2D_3$ in the OVCAR-3 cell line. Because of the growth stimulation, the role of vitamin D and its metabolising enzymes, 24OHase and 1αOHase, needs to be established in more detail in ovarian cancer development and progression.

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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

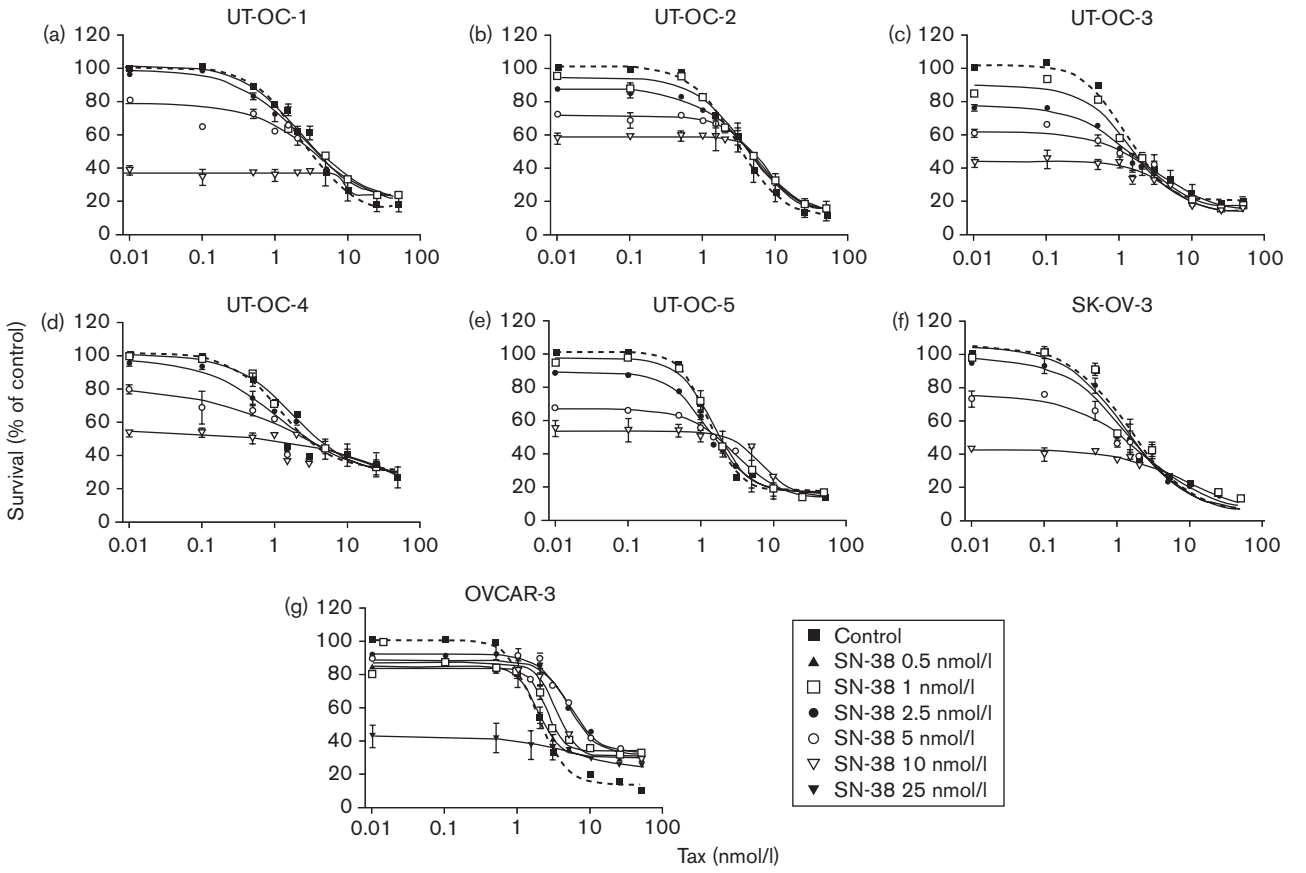
The cells (3.0×10^5) were plated on T25 culture flasks (Nalge Nunc International). After cell adhesion (24 h), the growth medium was replaced with media containing drug concentrations depending on the EC_{50} values of each drug and the cell line studied. After the 48-h treatment period, RNA was extracted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

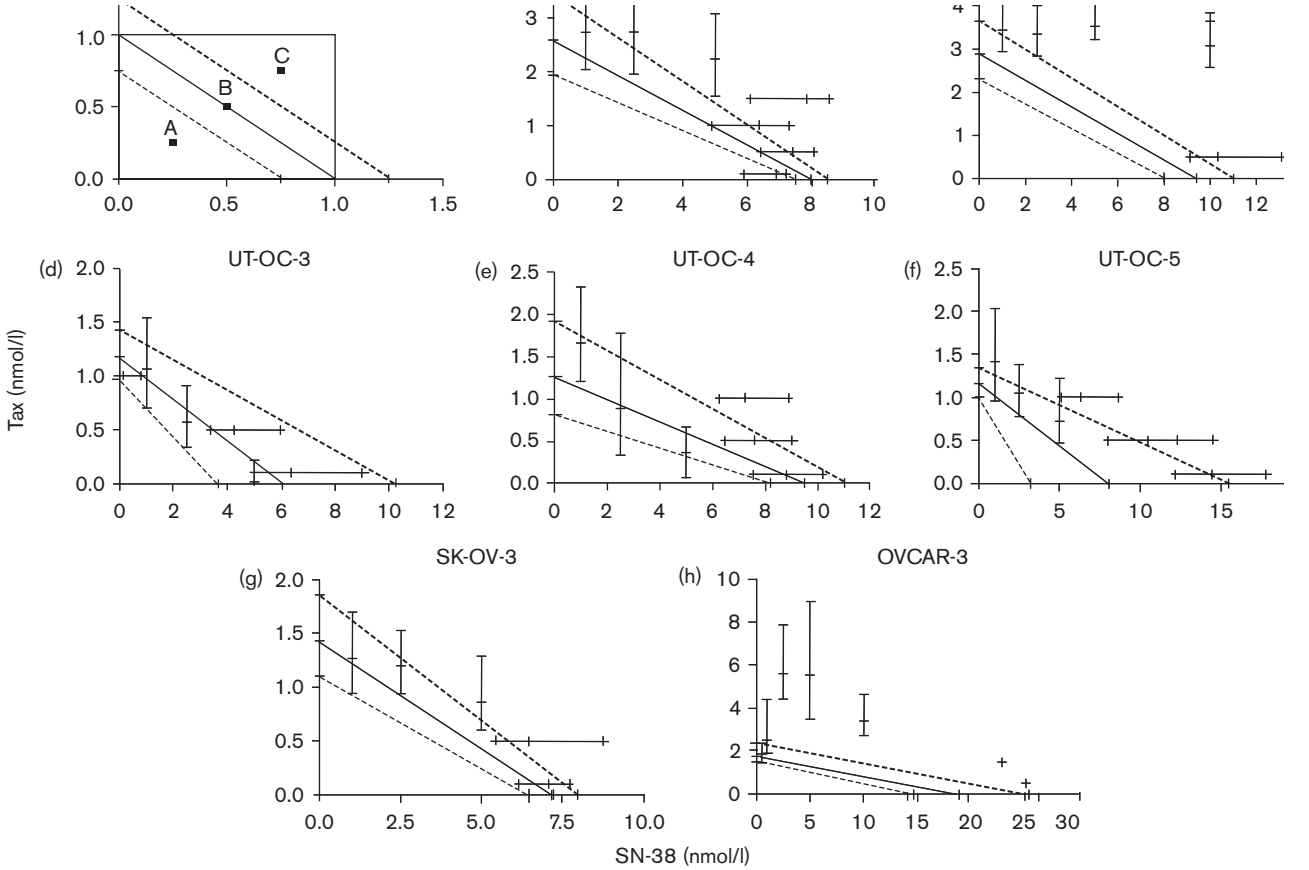
Quantitative real time-PCR (Fig. 4a–g) and western blotting (Fig. 4h) were used to study the effect of docetaxel and SN-38 on P-gp expression. On mRNA and protein levels, docetaxel had a moderate or no effect on P-gp expression. The regulation of P-gp mRNA expression by docetaxel was statistically significant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

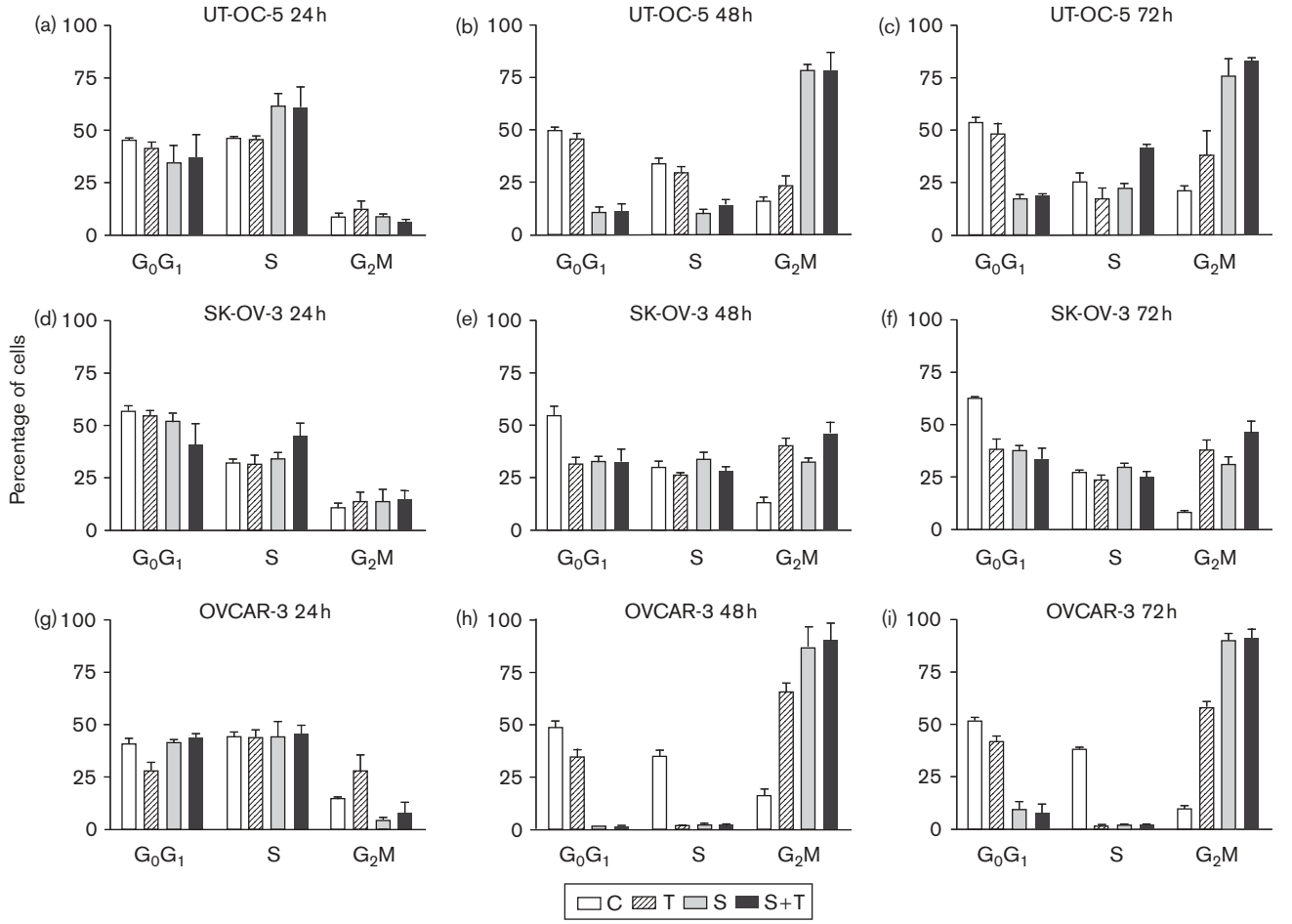
and SN-38 + docetaxel exposures increased P-gp expression on mRNA and protein levels (Fig. 4a–h). The effect of SN-38 and SN-38 + docetaxel exposures on P-gp mRNA expression was statistically significant in UT-OC-1 ($P < 0.001$, both exposures), UT-OC-2 ($P < 0.001$), UT-OC-4 ($P < 0.01$ and $P < 0.001$, 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.

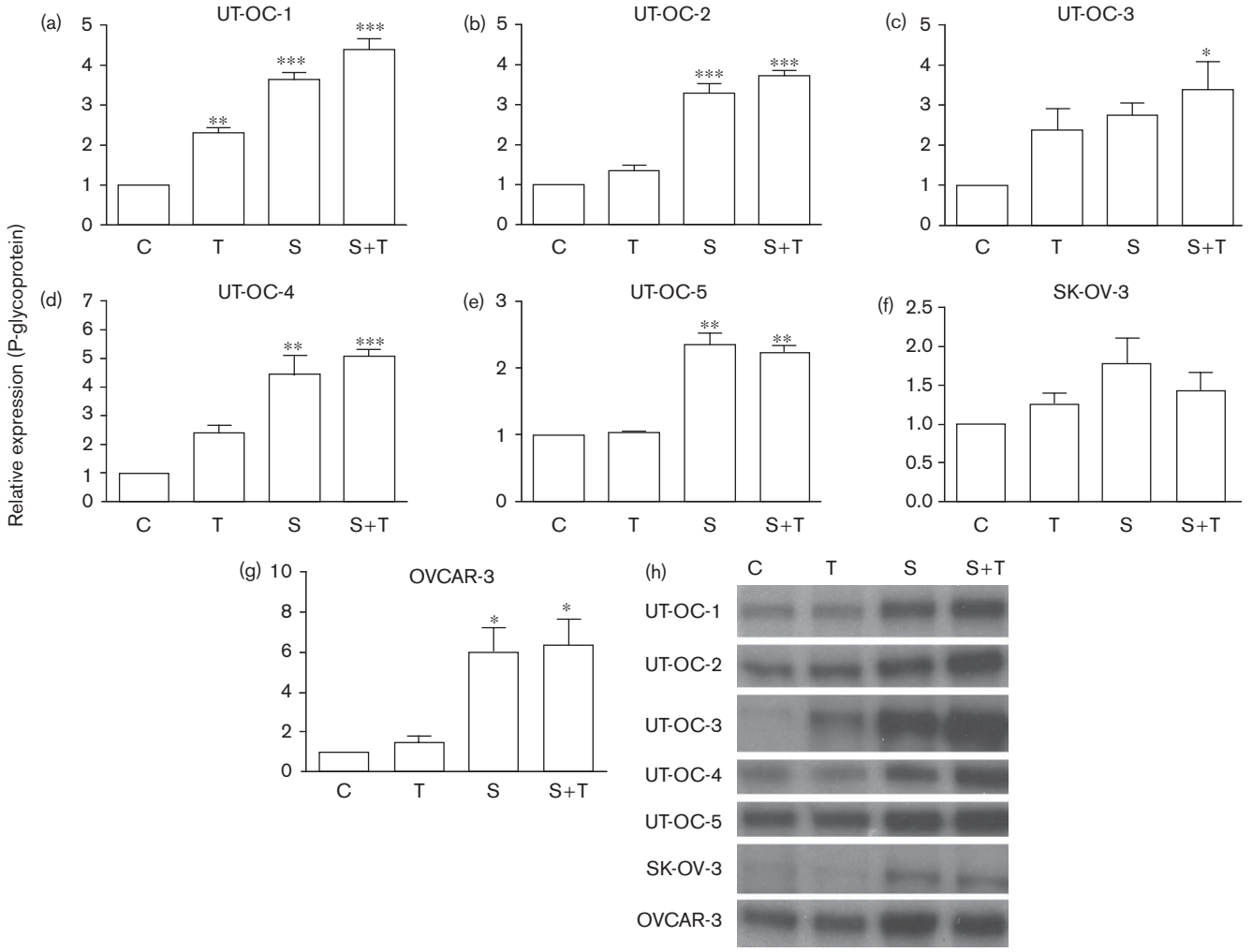
treatment of samples with docetaxel + SN-38 + PGP-4008 than after the treatment with only docetaxel + SN-38. The differences between docetaxel + SN-38 and docetaxel + SN-38 + PGP-4008 were statistically significant as shown in Fig. 5a.

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



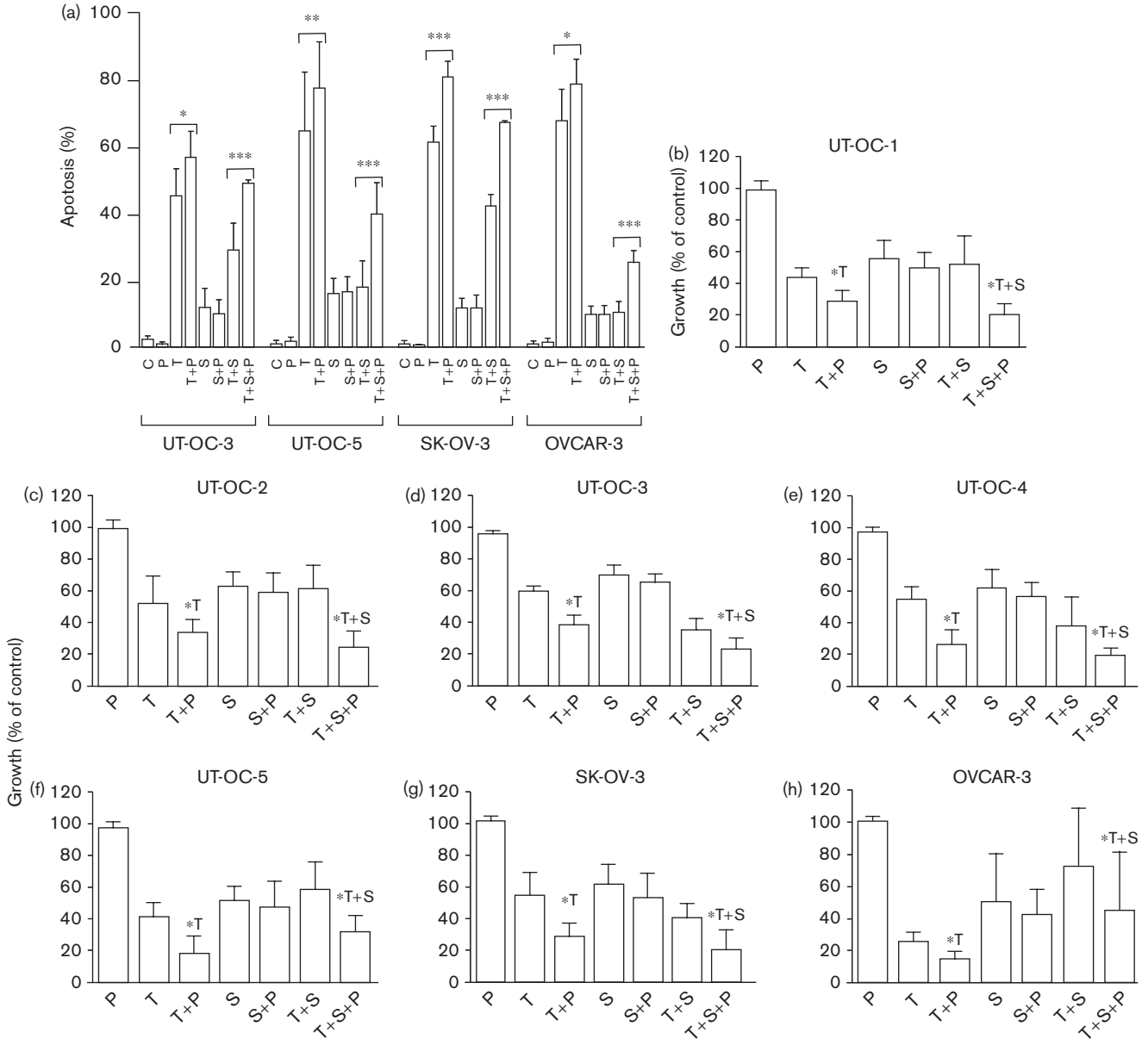
Quantitative real time (RT)-PCR (a–g) and western blotting (h) were used to detect regulation of P-glycoprotein (MDR-1) expression in cells after exposure to docetaxel and SN-38. Cells were exposed to cell culture medium without drugs (C), with docetaxel (T), SN-38 (S) or docetaxel and SN-38 (S + T) 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.

Acknowledgements

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In ovarian cancer cells antiproliferative effect induced by combination of calcitriol and docetaxel is mediated by increased Bcl-2 phosphorylation

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Abstract

In ovarian cancer cells the effect of calcitriol and docetaxel has been studied as single agents, but the combination of these compounds has not been evaluated. We compared the effects of docetaxel, calcitriol, and their combination on two ovarian carcinoma cell lines, OVCAR-3 and UT-OC-5. As single agents both calcitriol and docetaxel inhibited the proliferation of both cell lines. In OVCAR-3, calcitriol enhanced docetaxel-induced growth inhibition. In UT-OC-5, however, calcitriol decreased antiproliferative activity of docetaxel. In OVCAR-3, but not in UT-OC-5, the combination of docetaxel and calcitriol increased the number of cells at the G_2M phase of the cell cycle. In addition, the level of caspases 3 and 7 was increased in OVCAR-3 cells, which indicates increased activation of apoptosis. In both cell lines docetaxel and calcitriol used as single agents increased phosphorylation of the anti-apoptotic protein. The combination of calcitriol and docetaxel, however, had an opposite effect; phosphorylation of Bcl-2 was increased in OVCAR-3, and decreased in UT-OC-5. Our data indicate that the improved antiproliferative effect induced by the combination of calcitriol and docetaxel is mediated by G_2M cell cycle arrest and increased Bcl-2 phosphorylation. The growth inhibitory effect of simultaneous use these agents can be either additive or subadditive, and testing the effect on cell cycle or on Bcl-2 phosphorylation could be used as markers of effect achieved with the combination of calcitriol and docetaxel.

Key words: vitamin D, calcitriol, docetaxel, apoptosis, cell cycle, Bcl-2, ovarian cancer

Introduction

Ovarian cancer is a malignant neoplasm which is typically diagnosed in advanced stage. Primary therapy includes surgery [1] and chemotherapy, usually with a combination of carboplatin and paclitaxel [2-4] or docetaxel [5]. Although most patients respond to primary chemotherapy [1], 50-75% of the patients will eventually relapse [6]. Therefore, there is a demand for the development and testing of new treatment strategies.

Currently combinations of chemotherapeutic and biological agents are widely studied. Previous data indicate that calcitriol has growth inhibitory effect on several cancer cell types [7-11]. In high concentrations, the active form of vitamin D_3, calcitriol ($1,25(OH)_2D_3$), inhibits cancer cell proliferation and induces apoptosis [7-11]. The effects of calcitriol are mediated by the vitamin D receptor, VDR, which is a ligand-regulated transcription factor and a member of the steroid/thyroid hormone receptor superfamily [12]. In ovarian cancer cells calcitriol is known to regulate the expression of genes that are involved in apoptosis and cell growth [13]. Jiang et al. (2003) demonstrated that in ovarian cancer cells calcitriol induces expression of GADD45 in a p53-independent manner, followed by cell cycle arrest at the G_2M phase [14]. Increased stabilization of p27 by calcitriol in ovarian cancer cells, in turn, is suggested to arrest the cell cycle at G_0G_1 [15]. In addition, several other antiproliferative mechanisms including decrease of intracellular levels of anti-apoptotic Bcl-2-family proteins [16] have been reported [16-19].

Taxanes paclitaxel and docetaxel disrupt mitosis [20]. Both drugs bind to the β-tubulin subunit, thereby preventing depolymerisation of the microtubules and eventually cell division. In addition, taxanes regulate the cell cycle by

blocking cells from entering the G_2M phase [20, 21]. In ovarian cancer cells docetaxel regulates the expression of cell cycle and apoptosis related genes, such as p21/WAF1, Bax and Bcl-2, but growth inhibition is not necessarily dependent on regulation of these genes [22]. Also other cell death mechanisms besides apoptosis, such as mitotic catastrophe [23] and necrosis [24] may be involved in docetaxel induced cell growth inhibition.

The Bcl-2 gene family include proteins that regulate apoptosis [25]. Anti-apoptotic members of this gene family, including Bcl-2, promote cell survival, while pro-apoptotic members, such as Bax, promote cell death [26]. The balance between expressions of pro- and anti-apoptotic members of the Bcl-2 family is hypothesized to decide the cell fate after cellular stress, since heterodimerisation of Bcl-2 with Bax is critical in preventing Bax-mediated apoptosis [27, 28]. Phosphorylation of Bcl-2 results in decreased binding of Bcl-2 to Bax followed by Bax-mediated apoptosis. Bcl-2 phosphorylation occurs at the G_2M phase of the cell cycle [22, 29, 30]. Docetaxel accumulates cells at the G_2M phase and increases Bcl-2 phosphorylation, thus decreasing the anti-apoptotic function of the protein [22, 31, 32].

While in ovarian cancer cells the effect of calcitriol and docetaxel as single agents has been studied previously there are no reports on the effect of combination of docetaxel and calcitriol. In the present study, we compared the effects of docetaxel, calcitriol and their combination on growth, cell cycle regulation, apoptosis and phosphorylation of Bcl-2 protein in two ovarian carcinoma cell lines, OVCAR-3 and UT-OC-5.

Materials and methods

Cell culture

The experiments were performed using two ovarian cancer cell lines. OVCAR-3 (ATCC, Manassas, VA) has been established from Ascites of a patient treated for poorly differentiated papillary carcinoma. The non-commercial cell line UT-OC-5 have been established and characterized from epithelial ovarian carcinoma by our group [33-38]. It has been derived from an omental metastasis caused by moderately differentiated ovarian cystadenocarcinomas. Cell lines were maintained in Dulbecco's modified Eagles medium (DMEM, Sigma Aldrich, St. Louis, MO) supplemented with 10% fetal 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.

Drug preparations

Docetaxel (Taxotere®) was provided by Sanofi Aventis (Bridgewater, NJ) and dissolved in ethanol. Calcitriol was provided by Leo Pharmaceutical Products (Ballerup, Denmark) and it was dissolved in isopropanol in 4mM concentration by the manufacturer. Both drugs were first diluted in ethanol and then in growth medium. The final alcohol concentration for each experiment was 0.001%.

Cell growth assay

The cell growth assay was started when OVCAR-3 and UT-OC-5 cells were in logarithmic growth phase. 3000 cells/200 μl (OVCAR-3) and 1000 cells/200 μl (UT-OC-5) were plated on 96-well culture plates depending on the growth rate of the cell line. One day after plating, the medium was changed and fresh medium containing 10 nM calcitriol (Leo Pharmaceutical Products) or 0.3, 0.4 or 0.5 nM docetaxel (Sanofi Aventis) or the combination of calcitriol and each concentration of docetaxel was added in the wells. Ethanol, which was used as a vehicle, was included in the medium added on the control cells. The medium containing docetaxel, calcitriol or ethanol was changed every third day and samples were taken on day 0 and on days 6, 9 and 12 after starting the exposure. Four to eight parallel wells were used for each concentration in each time point. Relative cell numbers were quantified as described by Kueng et al [39]. Briefly, cells were fixed by adding 10 μl of 11% glutaraldehyde solution in phosphate buffer. Fixed cells were stained with 0,1 % solution of crystal violet and after the plates were air-dried, the bound dye was dissolved in 100 μl of 10% acetid acid. Relative cell number was determined as absorbance units (590 nm) using Victor 1420 multilabel counter (Wallac, Turku, Finland). Five independent experiments were done and statistical analyses were performed using Student's *t*-test.

Flow cytometer analysis

UT-OC-5 and OVCAR-3 cells were cultured in T25 flasks (Nalge Nunc International, Naperville, IL) until flasks had reached 70 % confluence. In cell culture media following concentrations of different compounds were added: 1) 10 nM calcitriol, 2) 0.5 nM docetaxel, 3) 5.0 nM docetaxel, 4) 10 nM calcitriol and 0.5 nM docetaxel and 5) 10 nM calcitriol and 5.0 nM

docetaxel. Ethanol, which was used as a vehicle, was included in the medium added on the control cells. Culture medium was removed 48 hours later and the cells were washed with phosphate buffered saline (PBS). The cells were detached with trypsin-EDTA in PBS and transferred to centrifuge tubes. Also previously removed culture medium containing detached floating cells and PBS that was used for washing was added to tubes and the tubes were centrifuged 800 G for 5 minutes. The pellets were resuspended with 0.5 ml PBS. The cell suspensions were stirred and 4.5 ml cold (-20°C) 70 % ethanol was added to each tube to fix cells. After two hours incubation the cell suspensions were centrifuged 800 G for 5 minutes and washed with 5 ml PBS. PBS was removed and 1 ml fresh PBS containing 0.5 mg/ml RNase (Sigma Aldrich) and 50 µg/ml propidium iodide (Sigma Aldrich) per $1x10^6$ cells was added. The tubes were protected from light and incubated at room temperature for 30 minutes. The cell suspensions were filtered and flow cytometric analyses were performed using FacScan (Becton Dickinson, Franklin Lakes, NJ, USA). Three independent experiments were done.

Apoptosis measurements

Induction of apoptosis was evaluated by measuring expression of caspases 3 and 7 in cells exposed to docetaxel, calcitriol or the combination. One day before starting the exposure 10 000 cells (OVCAR-3) and 5000 cells (UT-OC-5) were plated on a 96-well culture plates. On the following day 10 nM concentration of calcitriol, 0.5 nM and 5 nM concentration of docetaxel or the combination of 10 nM calcitriol and 0.5 nM and 5.0 nM docetaxel were added in fresh culture medium. Ethanol, which was used as a vehicle, was included in the medium added on the control samples. Eight parallel wells were used for each concentration. After 48 hours incubation Caspase-Glo™3/7 Assay –reagent (Promega, Madison, WI, USA) was added according to manufacturer's instructions. The luminescence was measured by using Multiscan MS (Labsystems, Waltham, MA, USA). Five independent experiments were done and statistical analyses were performed using Student's *t*-test.

Western blot analysis

Western blot analysis was used to detect phosphorylation of Bcl-2 protein by calcitriol, docetaxel and their combination. UT-OC-5 and OVCAR-3 cells were cultured in T25 flasks (Nalge Nunc International, Naperville, IL) until flasks had reached 70 percent confluence. Cells were lysed after 48 hours exposure to calcitriol (10 nM), docetaxel (0.5 nM) or combination of calcitriol and docetaxel in mPER reagent (Pierce, Rockford, IL, USA). Protein concentrations in each sample were measured by using BCA protein Assay Reagent (Pierce). Equal amount of proteins from each sample was loaded on 12 % polyacrylamide gel. After being resolved by electrophoresis, proteins were transferred to a nitrocellulose membrane via electroblotting. The membranes were blocked with 5% milk-TBS-Tween solution and incubated overnight at 4°C with phospho-Bcl-2 antibody (Cell Signalling Technology, Danvers, MA, USA) diluted in 1%-milk-TBS-Tween solution, followed by 1 hour incubation at room temperature with goat anti-rabbit IgG secondary antibody conjugated with peroxidase (Cappel, West Chester, PA, USA). The presence of phosphorylated Bcl-2 proteins was detected by using chemiluminescence reagent ECL (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK). A film was exposed and developed by using Konica SRX-101A (Konica Minolta, Wayne, NJ, USA) and phosphorylated Bcl-2 protein bands were quantified with a densitometer. Five independent experiences were done and statistical differences between samples were analyzed using two-way ANOVA followed by Bonferroni's post-hoc test (GraphPad Prism 3.03 software, San Diego, CA, USA).

Results

Growth-inhibitory effect of calcitriol, docetaxel, and their combination

Calcitriol (10 nM) and three concentrations of docetaxel (0.3, 0.4, and 0.5 nM) inhibited the growth of OVCAR-3 and UT-OC-5 cells. The growth-inhibiting effect of docetaxel was concentration-dependent in both cell lines. The effect of the combination of calcitriol and docetaxel, however, was cell line dependent (Figure 1). In OVCAR-3 cells calcitriol enhanced the growth inhibitory effect of docetaxel at each docetaxel concentration studied ($p<0.05$). In UT-OC-5 cells, combining calcitriol with docetaxel significantly decreased the growth inhibition compared to cells exposed to docetaxel alone ($p<0.01$).

Cell cycle regulation

The proportions of cells at different cell cycle phases were analyzed to examine differences in drug-induced cell cycle regulation in the OVCAR-3 and UT-OC-5 cell lines (Figure 2). After 48

hours incubation, the proportion of cells at the S phase in both cell lines was equivalent after exposure to different drugs. With a higher concentration of docetaxel, the proportion of cells at the G_2M phase notably increased and there was a corresponding decrease in the proportion of cells at the G_0G_1 phase in both cell lines. In OVCAR-3 cells, calcitriol alone increased the proportion of cells at the G_2M. In UT-OC-5 cells, similar G_2M cell cycle arrest was not detected and only the proportion of cells at the G_0G_1 was slightly increased. In OVCAR-3 cells, but not in UT-OC-5 cells, the addition of both calcitriol and docetaxel increased the proportion of cells at the G_2M. The exposure of UT-OC-5 cells to combination of calcitriol and docetaxel did not further increase the cell accumulation at the G_0G_1.

Activation of caspases 3 and 7

The apoptotic status of UT-OC-5 and OVCAR-3 cells was studied by measuring the activation of caspase 3 and caspase 7 levels in cells treated with calcitriol, docetaxel, or their combination (Figure 3). Simultaneous exposure to these agents increased the acitvity of caspases 3 and 7 in OVCAR-3 cells. At higher concentrations of docetaxel the relative activity of caspases 3 and 7 was significantly greater in cells exposed to a combination of calcitriol and docetaxel than in those exposed to docetaxel alone ($p<0.05$). This was not seen when lower 0.3 and 0.4 nM concentrations of docetaxel were used. Both docetaxel and calcitriol exposures increased the activity of caspases 3 and 7 in UT-OC-5 cells. The caspase activity was significantly lower when docetaxel was combined with calcitriol than when exposed to each drug alone ($p<0.05$).

Effect of calcitriol and docetaxel on Bcl-2 phosphorylation

We also studied phosphorylation of Bcl-2 protein in OVCAR-3 and UT-OC-5 cells exposed to calcitriol, docetaxel, and their combination (Figure 4). Docetaxel and calcitriol used as single agents increased the level of the phosphorylated form of Bcl-2 in both cell lines. In OVCAR-3, the expression of phosphorylated Bcl-2 was significantly stronger when the cells were exposed to the combination of calcitriol and docetaxel. In UT-OC-5 cells, calcitriol and docetaxel used as single agents induced a greater increase in the phosphorylation of Bcl-2 than the simultaneous use of these agents. In both cell lines, the results were statistically significant ($p<0.05$) when docetaxel or calcitriol-treated samples were compared to the samples exposed to the combination.

Discussion

The survival of ovarian cancer patients is low and consequently new treatment strategies are needed. Currently, a lot of research interest is focused on the growth inhibitory effect of biological compounds as well as to combining them with chemotherapeutic agents. Calcitriol in combination with docetaxel is already being used in clinical trials to treat prostate cancer [40-43]. The results suggest that this combination is well tolerated and effective, but more studies are needed to confirm the efficacy. We and others have demonstrated that calcitriol [7, 18, 44-46] and docetaxel [35, 47-49] inhibit proliferation of ovarian cancer cells. However, the combination of docetaxel and calcitriol has not been previously studied. In the present study, we compared the effects of docetaxel, calcitriol and their combination on growth, cell cycle regulation, apoptosis and phosphorylation of Bcl-2 protein in two ovarian carcinoma cell lines, OVCAR-3 and UT-OC-5.

According to our present data the efficacy of concomitant exposure of ovarian cancer cells to docetaxel and calcitriol is cell line-dependent. Exposure of cells to calcitriol either protected cells from docetaxel mediated growth inhibition or enhanced the antiproliferative activity of docetaxel. In vitro studies have shown that calcitriol and its analogues enhance the activity of a number of anticancer drugs and treatments, including dexamethasone [50, 51], retinoids [52, 53], tamoxifen [54, 55], radiation [56, 57], docetaxel [41, 58], paclitaxel [59, 60], and platinum compounds [61-63]. Synergism is observed when calcitriol is administered before or simultaneously with the chemotherapeutic drug; administration after the anticancer drug does not increase the efficacy [60, 63]. In androgen-independent prostate cancer cells calcitriol enhances the efficacy of docetaxel by increasing the expression of Bax, followed by increased apoptosis [41, 58].

Both docetaxel and calcitriol regulate the cell cycle of cancer cells. Docetaxel blocks cells from entering the G_2M phase [20, 21, 47] and calcitriol arrests cells at the G_2M [14] or G_0G_1 phase [15]. Docetaxel-induced cell cycle arrest at the G_2M phase was observed in both cell lines studied. Instead, the effect of calcitriol was different in the

two cell lines. In OVCAR-3 it blocked cells from exiting the G_2M phase in OVCAR-3 whereas in the UT-OC-5 calcitriol arrested cells at the G_0G_1. When both docetaxel and calcitriol were administered, the amount of cells at the G_2M was further increased in OVCAR-3 cells but not in UT-OC-5 cells.

Pretreatment of cells with calcitriol may sensitize cells to decetaxel mediated cell death by increasing apoptosis [59]. Caspases 3 and 7 are executioner caspases that are activated in apoptosis [64]. Both calcitriol [61] and docetaxel [65] may induce apoptosis by the induction of caspase 3. The results of the present study indicate that docetaxel alone increases the activity of caspases 3 and 7 in relation to control cells in both UT-OC-5 and OVCAR-3 cells. Calcitriol also had a modest increasing effect on the activity of caspases 3 and 7. Concomitant calcitriol and docetaxel exposure, however, had a different effect on activity of caspases depending on the cell line. In OVCAR-3 cells, the combination of calcitriol and docetaxel increased the caspase concentration more than docetaxel alone. In UT-OC-5 cells, the caspase amount was lower when docetaxel was used in combination with calcitriol.

Our data showed that concomitant calcitriol and docetaxel exposure had different effects on cell cycle and the activation of apoptosis in OVCAR-3 and UT-OC-5 cells. Therefore we studied the expression of phosphorylated Bcl-2 protein in cells exposed to docetaxel, calcitriol, or their combination. Proteins of the Bcl-2 gene family regulate apoptosis [25]. The pro-apoptotic proteins stimulate the release of cytochrome *c* from the mitochondria, which results in the activation of caspase-3 and apoptosis. Heterodimerisation of Bcl-2 with Bax prevents Bax-mediated apoptosis [27, 28] while phosphorylation of Bcl-2 results in decreased binding of Bcl-2 to Bax and enhancement of apoptotic cell death. This Bcl-2 phosphorylation occurs at the G_2M phase of the cell cycle [22, 29, 30]. Our present data and previous studies show that docetaxel accumulates cells at the G_2M phase and increases Bcl-2 phosphorylation, thus decreasing the anti-apoptotic function of the protein [22, 31, 32]. If cells are arrested at the S phase, neither Bcl-2 phosphorylation nor apoptosis occur [22]. In OVCAR-3 cells, both docetaxel and calcitriol induced the accumulation of cells in the G_2M phase and this effect was further increased by combination of these compounds. This in turn led to the increased phosphorylation of Bcl-2 and ultimately to efficient apoptosis. In UT-OC-5 cells, however, calcitriol induced G_0G_1 arrest and decreased the pro-apoptotic effect of docetaxel. Interestingly, data obtained with breast cancer cells indicated that calcitriol alone did not phosphorylate Bcl-2, but preincubation of cells with calcitriol potentiated the ability of paclitaxel to phosphorylate Bcl-2, leading to increased apoptosis [59]. These results support our results suggesting that the growth inhibitory and pro-apoptotic effects of simultaneous exposure to calcitriol and chemotherapeutic drugs are clearly dependent on the cell type.

In the present study, there was a clear relationship between the Bcl-2 phosphorylation and the efficacy of simultaneous exposure to docetaxel and calcitriol. The amount of phosphorylated Bcl-2 and ultimately induction of apoptosis was higher in OVCAR-3 cells treated with the combination of calcitriol and docetaxel than in those treated with calcitriol or docetaxel alone. In UT-OC-5 cells, however, the combination of calcitriol and docetaxel did not induce Bcl-2 phosphorylation at all and also docetaxel induced growth inhibition was weaker. The induction of apoptosis has not been reported to be associated with the duration or degree of Bcl-2 phorphorylation, since cell death may occur without Bcl-2 phosphorylation [22, 66].

The results of the present study indicate that the improved antiproliferative effect induced by the combination of calcitriol and docetaxel is mediated by G_2M cell cycle arrest and increased Bcl-2 phosphorylation. The growth inhibitory effect of simultaneous use these agents can be either additive or subadditive, and testing the effect on cell cycle or on Bcl-2 phosphorylation could be used as markers of effect achieved with the combination of calcitriol and docetaxel.

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Figures

Figure 1.

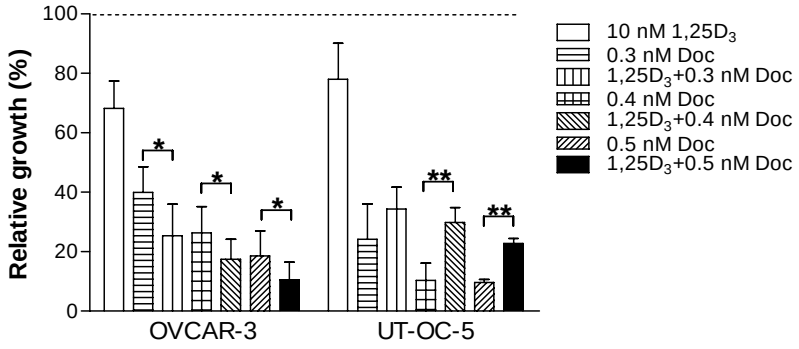

Figure 1. Relative growth (day 9) of OVCAR-3 and UT-OC-5 cells after 10 nM $1,25(OH)_2D_3$ and 0.3 - 0.5 nM docetaxel (Doc) exposures compared to control (horizontal line, set as 100%). Data represents mean of five independent experiments.* $p<0.05$, **$p<0.01$.

Figure 2.

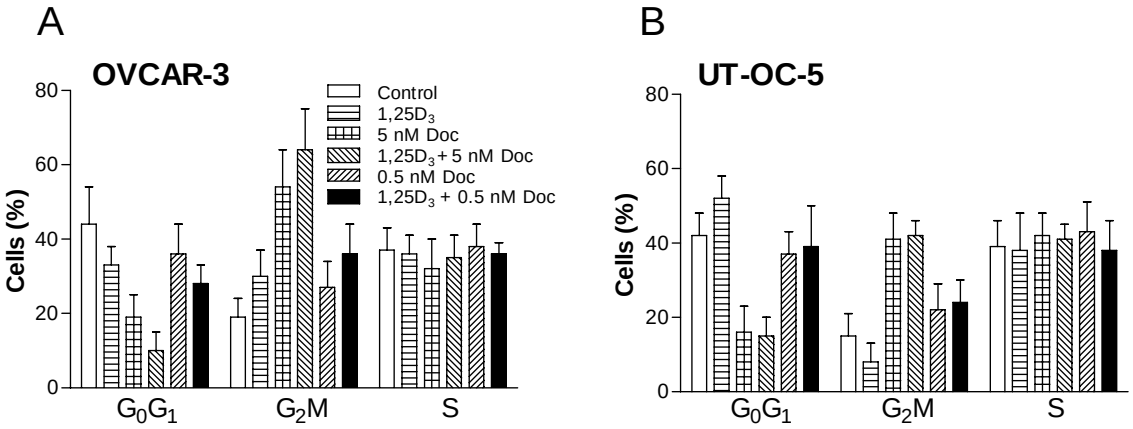

Figure 2. Cell cycle distribution of OVCAR-3 (A) and UT-OC-5 (B) cells after 48h $1,25(OH)_2D_3$ (10 nM) and docetaxel (Doc) exposures. Data represents mean of three independent experiments Exposures for both cells lines are indicated in figure A.

Figure 3.

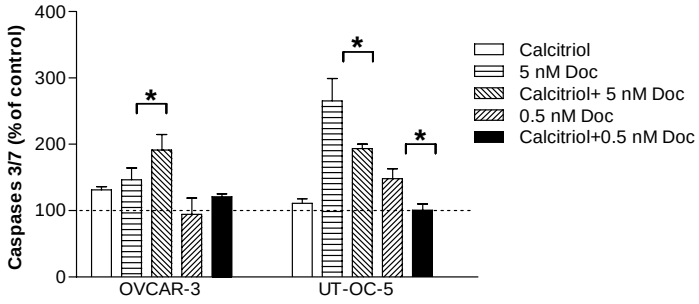

Figure 3. The relative amount of caspases 3 and 7 in OVCAR-3 and UT-OC-5 cells after 48h exposure to 10 nM $1,25(OH)_2D_3$, docetaxel (Doc) or both. The horizontal line represents control sample (set as 100). Data represents mean of five independent experiments.

Figure 4.

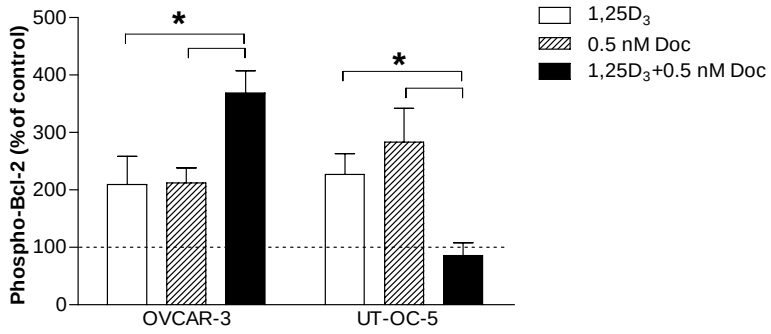

Figure 4. The amount of phosphorylated Bcl-2 in OVCAR-3 and UT-OC-5 cells after 48h exposure to 10 nM $1,25(OH)_2D_3$, docetaxel (Doc) or both. The horizontal line represents control sample (set as 100). Data represents mean of five independent experiments. * $p<0.05$.

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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Keywords: CPT-11, docetaxel, gene therapy, ovarian cancer, p53, SN-38

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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

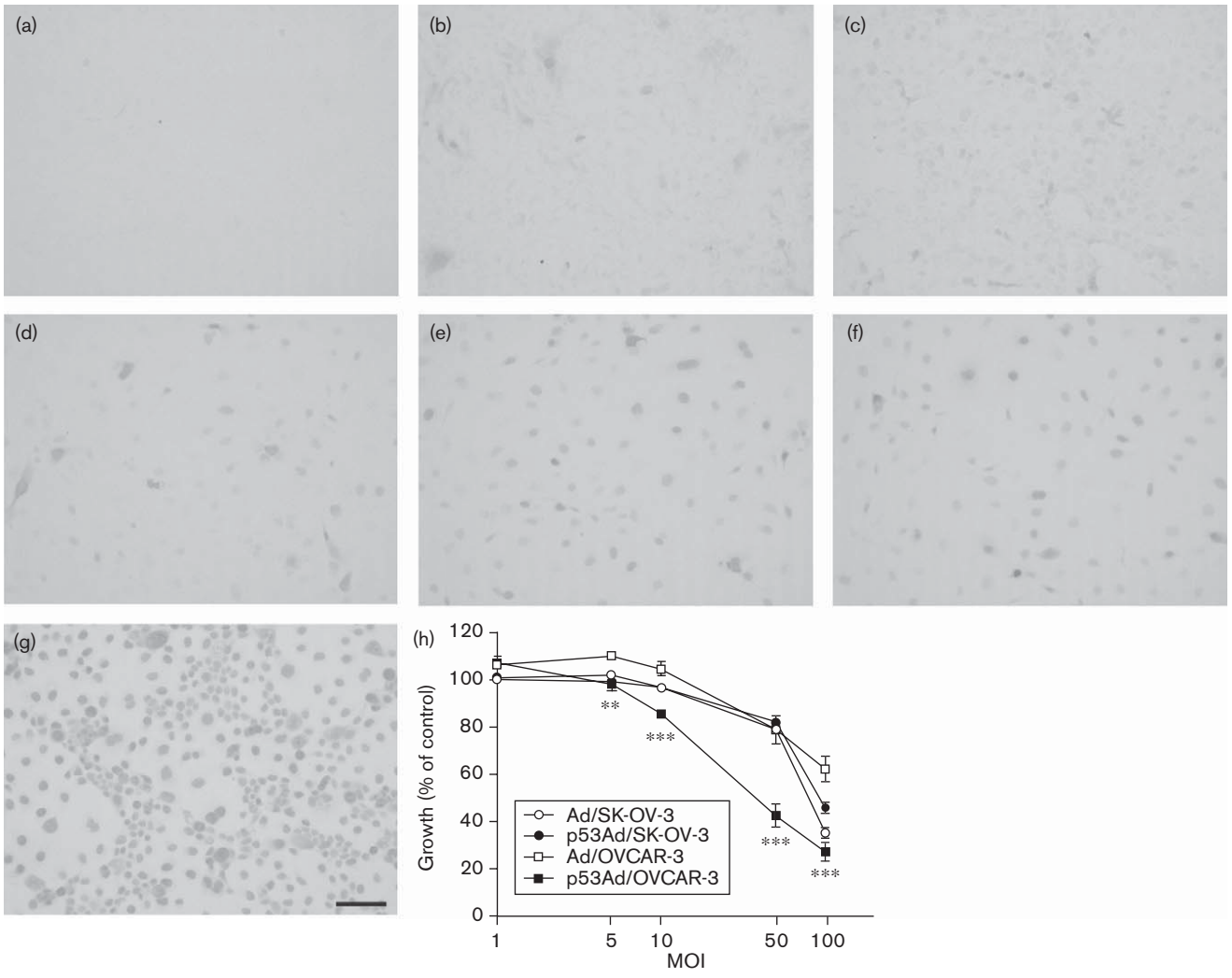
MOI, multiplicity of infection.
[a]Immunocytochemistry.

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 ($P < 0.0001$).

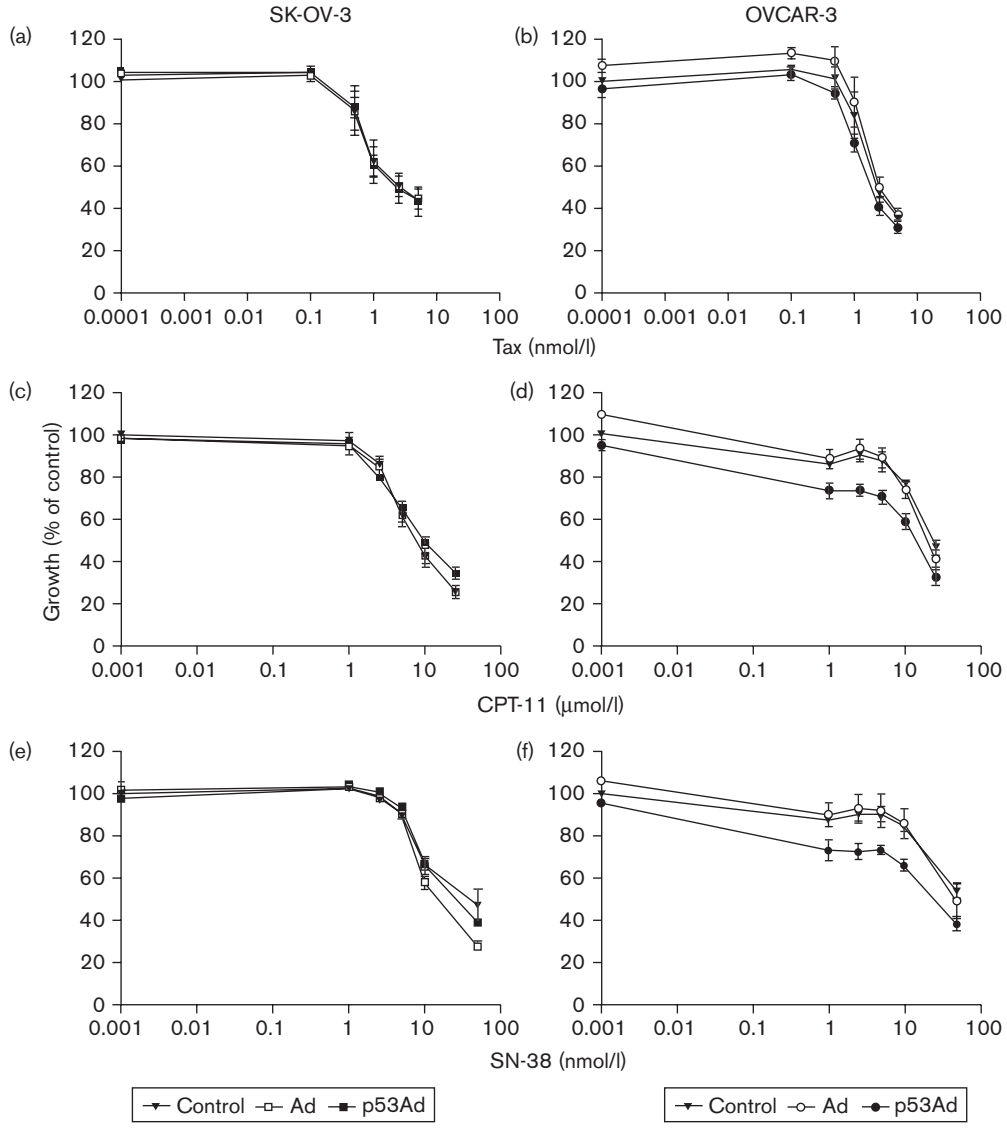
Growth response to concomitant docetaxel and p53 gene therapy

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.

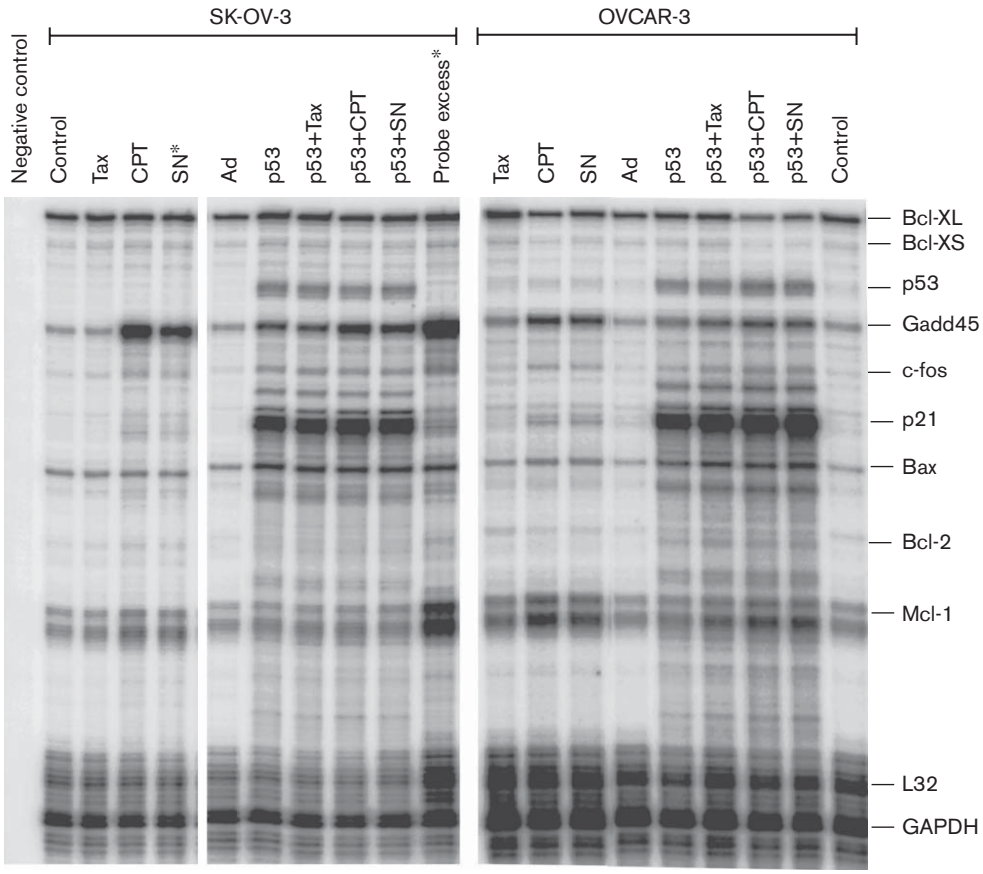
In OVCAR-3 cells, a low level of p53 was expressed in untreated control cells. The expression was not changed by docetaxel, CPT-11 or SN-38 exposures or by infection of empty adenovirus. In uninfected or Adeno-X-Null-infected SK-OV-3 cells, the expression of p53 was not detected. When both cell lines 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).

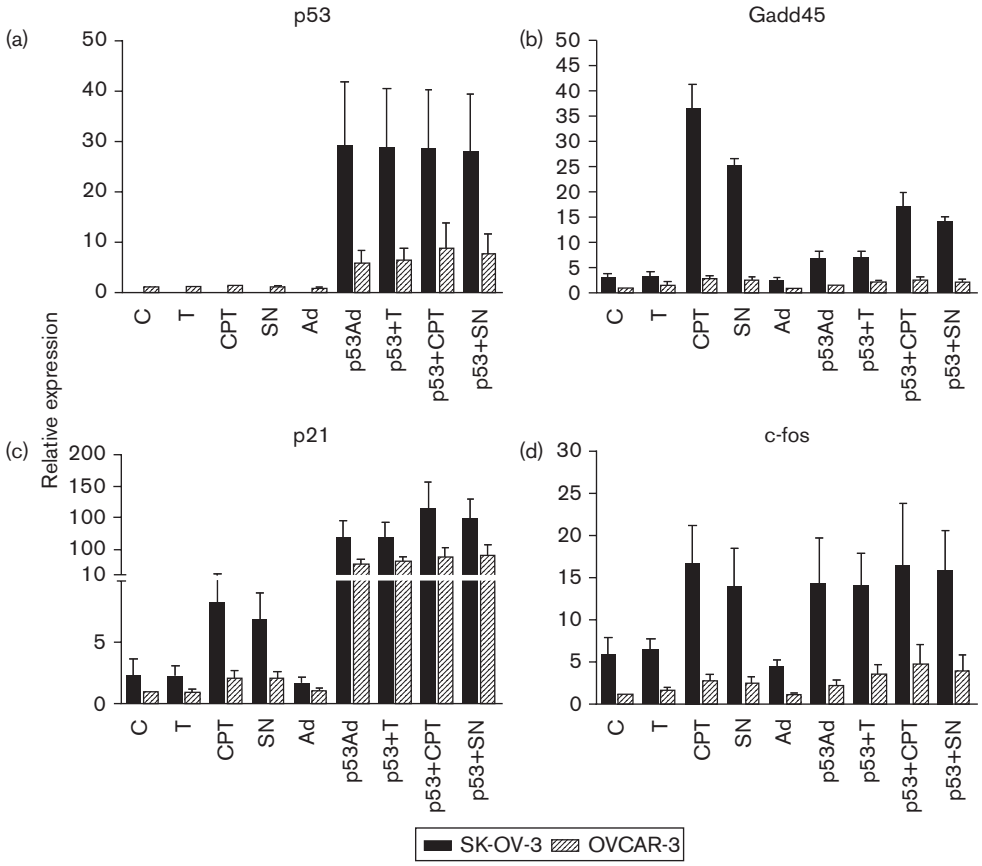
The expression of c-fos was slightly upregulated by SN-38 and CPT-11 exposures in both cell lines. In

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

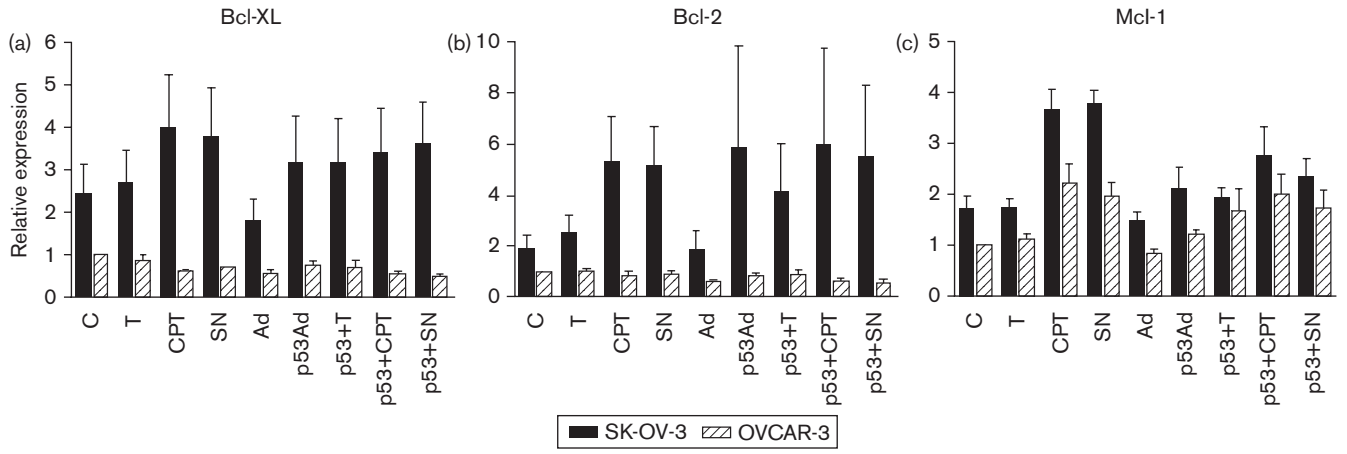
Figure 5a–c shows the expressions of antiapoptotic genes Bcl-XL, Bcl-2 and Mcl-1 genes. Drug exposures or p53Ad infection did not significantly regulate the expression of Bcl-XL in either cell lines (Fig. 5a). In SK-OV-3 cells, but not in OVCAR-3 cells, the expression of Bcl-2 was slightly increased 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

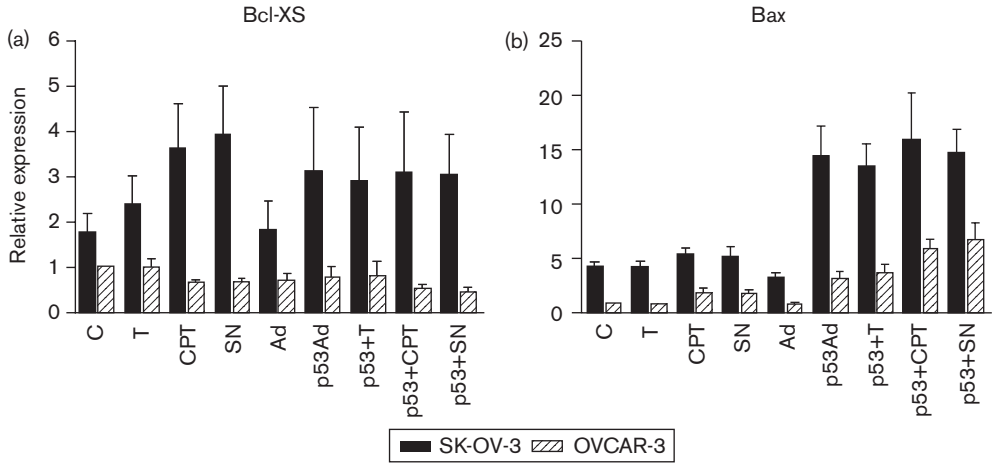
The balance between expressions of proapoptotic and antiapoptotic members of the Bcl-2 family is hypothesized to decide the cell fate after cellular stress. High expression ratios of Bax/Bcl-2 and Bax/Bcl-XL suggest increased apoptotic activity, whereas low ratios imply stronger resistant to apoptosis, 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



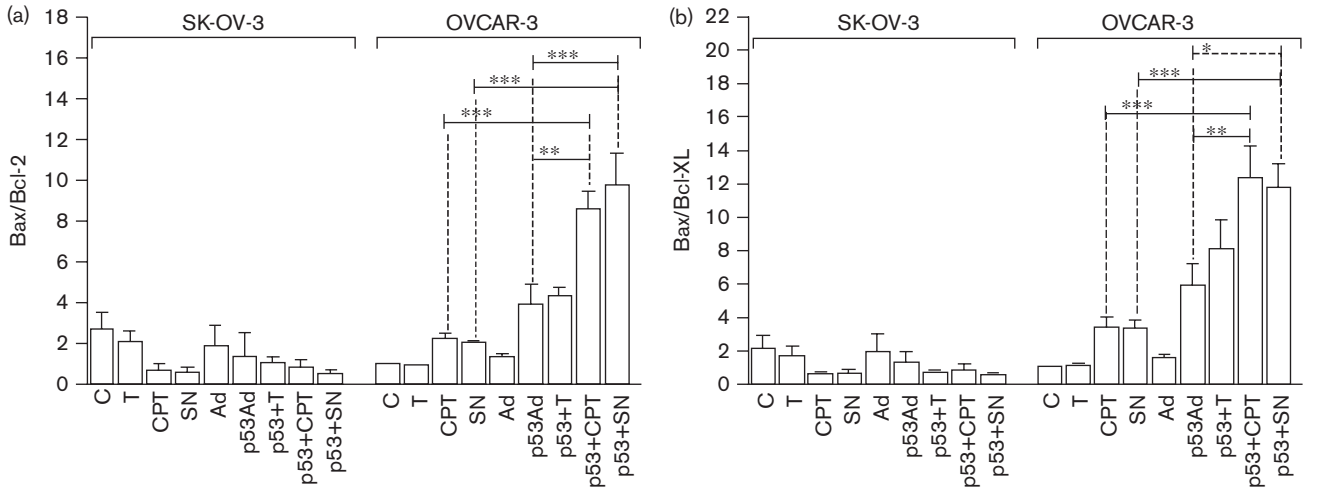
The ribonuclease protection assay results of expression levels of proapoptotic Bcl-XS (a) and Bax (b) in ovarian cancer cell lines SK-OV-3 and OVCAR-3. Cells were treated with vehicle (C), docetaxel (2.5 nmol/l, T), SN-38 (20 nmol/l, SN), CPT-11 (10 μmol/l, CPT), empty adenovirus (Ad), p53Ad (p53), docetaxel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

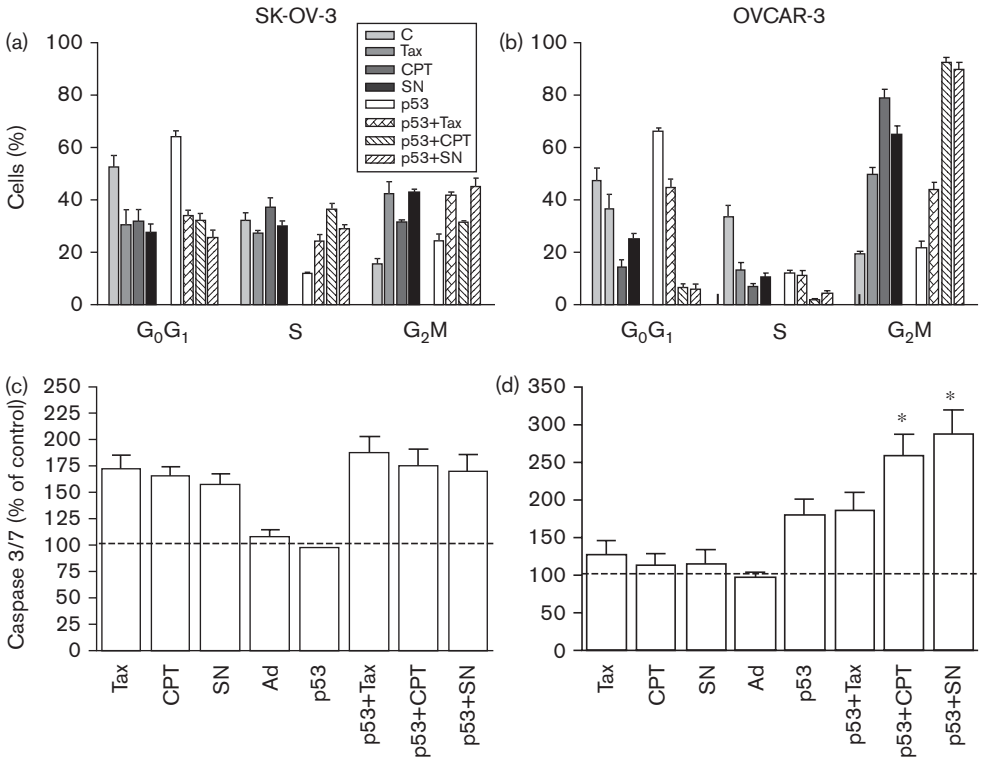
The proportions of cells in different cell cycle phases were analysed to explain differences in the growth response and gene expressions between SK-OV-3 (Fig. 8a) and OVCAR-3 (Fig. 8b) cell lines. In SK-OV-3 cell line (Fig. 8a), the cell number in the G_2M phase was increased after 48-h drug exposures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