1. Summary

Ovarian cancer is one of the highest fatality tumors among the gynecologic malignancies. Current treatment based on chemotherapy complemented with radiotherapy, the most frequent agents used in chemotherapy are paclitaxel, irinotecan and cisplatin. Most common genetic alterations in human cancers as well as in ovarian cancer are mutations or overexpression of the tumor suppressor p53. These mutations or overexpression were detected frequently in advanced stages of ovarian cancer. p53 is also an important factor responsible for drug resistance in ovarian cancer, and its level is a useful marker for cancer recurrence and for prognosis assessment. For these reasons, a strategy to treat ovarian cancer must include the knowledge of p53 status and the other cell cycle regulators. Another cell cycle regulator, namely phosphatase cdc25C has an important role in promoting G_2/M transition. Furthermore, the cytostatica which are used for the treatment of ovarian cancers target the G_2/M transition and therefore cdc25C was also analyzed in the present study. cdc25C is one member of cdc25 phosphatase family which contains two other members, cdc25C and cdc25B, which both have been shown to act as putative oncogenes and are overproduced in a variety of cancer cells whereas so far cdc25C was not shown to function as an oncogene and it is not overexpressed in tumors. Therefore I analyzed the sensitivity of the freshly established ovarian cancer cell lines OV-MZ-32, OvBH-1 and OvCBM for paclitaxel, irinotecan and cisplatin and the mechanism in which these drugs act in these cells. In addition I used gemcitabine which was already used to treat other types of cancers.

I found that ovarian cancer cells were insensitive towards cisplatin and irinotecan, whereas gemcitabine and paclitaxel induced apoptosis. There was a weak synergistic effect when both drugs were used together. Treatment of the ovarian cancer cells had no clear influence on the expression of p53 and of cdc25C. Although p53 expressed in OvBH-1 cells does not have a mutation and the deletion in p53 of OV-MZ-32 cells should not affect the wild type function of p53, it turned out that p53 did not behave like wild type p53. p53 from these ovarian cancer cell lines had only a limited transactivation function

for BAX and for WAF1 indicating that some wild type function of p53 got lost. The cell cycle checkpoint functions of cdc25C are not only regulated by the expression but also by its sub-cellular localization and its enzymatic activity. An altered sub-cellular localization of cdc25C was not found upon cytostatica treatment of the ovarian cancer cells but there was a considerable decrease in the phosphatase activity after treatment of OV-MZ-32 cells with gemcitabine. Since the cdc25C phosphatase activity is necessary for the cell cycle progression the present data indicate that the gemcitabine treatment of at least these cells acts as an inhibitor of the G_2/M transition of the cell cycle.

In all three cell lines the phosphorylation of cdc25C at serine 216 decreased after the treatment with a combination of gemcitabine and paclitaxel, stronger inhibition of the phosphorylation of serine 216 was observed by using the Chk1 inhibitor.

1.1. Zusammenfassung

Ovarialkrebs gehört zu den bösartigen gynäkologischen Tumoren mit der höchsten Sterblichkeitsrate. Derzeit basiert die Behandlung auf einer Kombination aus Chemotherapie und Strahlentherapie, wobei in der Chemotherapie hauptsächlich die Agenzien Paclitaxel, Irinotecan und Cisplatin zum Einsatz kommen. Die häufigsten genetischen Veränderungen bei menschlichem Krebs, so auch bei Ovarialkrebs, sind Mutationen oder Überexpression des Tumorsuppressors p53. Diese wurden regelmäßig in fortgeschrittenen Stadien des Eierstockkrebses gefunden. p53 ist auch ein wichtiger Faktor, der für Arzneimittelresistenz des Ovarialkrebses verantwortlich ist und dessen Menge einen nützlichen Marker für ein Rezidiv sowie die Festlegung einer Prognose darstellt.

Aus diesen Gründen muss eine Strategie zur Behandlung von Ovarialkrebs das Wissen über den Status von p53 und anderer Zellzyklusregulatoren beinhalten.

Ein weiterer Zellzyklusregulator, die Proteinphosphatase cdc25C, spielt eine wichtige Rolle am G_2/M-Übergang. Zudem zielen die Cytostatika, die zur Behandlung von Ovarialkrebs eingesetzt werden, auf den G_2/M-Übergang ab, weshalb auch cdc25C in der vorliegenden Studie untersucht wurde.

cdc25C gehört zur cdc25-Proteinfamilie. Diese umfasst neben cdc25C noch zwei weitere Proteinphosphatasen, cdc25A und cdc25B, welche nachgewiesenermaßen als mögliche Onkogene agieren und in einer Vielzahl von Krebszellen überexprimiert werden. Für cdc25C dagegen konnten bisher weder eine Funktion als Onkogen noch eine Überexpression in Tumoren gezeigt werden. Daher untersuchte ich die Sensibilität der neu etablierten ovarialen Krebszelllinien OV-MZ-32, OvBH-1 und OvCBM gegenüber Paclitaxel, Irinotecan und Cisplatin sowie den Wirkmechanismus dieser Medikamente in den Zellen. Zusätzlich verwendete ich Gemcitabin, welches bereits zur Behandlung anderer Krebsarten eingesetzt wurde.

Ich habe herausgefunden, dass die ovarialen Krebszelllinien insensitiv gegenüber Cisplatin und Irinotecan waren, wohingegen Gemcitabin und Paclitaxel Apoptose induzierten. Bei der gemeinsamen Verwendung beider

Medikamente konnte ein schwacher synergistischer Effekt nachgewiesen werden. Die Behandlung der ovarialen Krebszelllinien hatte keinen eindeutigen Einfluss auf die Expression von p53 und cdc25C.
Das in OvBH-1-Zellen exprimierte p53 beinhaltet keine Mutation, während das p53 aus OV-MZ-32-Zellen eine Deletion enthält, die jedoch die p53-Wildtyp-Funktionen nicht beeinträchtigen sollte. Dennoch stellte sich heraus, dass das p53 aus beiden ovarialen Krebszelllinien sich nicht wie die Wildtyp-Form verhält. Es hatte nur eine begrenzte Transaktivierungsfunktion für BAX und WAF-1, was darauf hindeutet, dass einige Wildtyp-Funktionen von p53 verloren gegangen sind.
Die Zellzyklus-Checkpoint-Funktionen von cdc25C werden nicht nur über die Expression des Proteins, sondern auch über seine subzelluläre Lokalisation sowie seine enzymatische Aktivität reguliert. Eine veränderte subzelluläre Lokalisation von cdc25C wurde bei der Behandlung mit Cytostatika nicht festgestellt, jedoch nahm die Phosphataseaktivität von cdc25C nach der Behandlung von OV-MZ-32-Zellen mit Gemcitabin beträchtlich ab. Da die cdc25C-Phosphataseaktivität notwendig für das Voranschreiten des Zellzyklus ist, deuten die vorliegenden Daten an, dass Gemcitabin-Behandlung zumindest in diesen Zellen zur Inhibition des G_2/M-Überganges führt. In allen drei Zelllinien nahm die Phosphorylierung an Serin 216 nach Kombinationsbehandlung mit Gemcitabin und Paclitaxel ab. Eine stärkere Inhibition dieser Phosphorylierung wurde nach Verwendung des Chk1-Inhibitors beobachtet.

2. Introduction

2.1. Ovarian Cancer

Of all the gynecologic cancers, ovarian malignancies represent the greatest clinical challenge, because they are usually asymptomatic until they have metastasized. Therefore patients usually present with an advanced disease (Bell *et al*., 1988; Novak, 1996). In more than two- third of the cases ovarian cancer has the highest fatality-to-case ratio of all the gynecologic malignancies (often the fourth place) (Clark *et al*., 2001; Mobus *et al*., 1992; Saga *et al*., 2002). The incidence for ovarian cancer increases with age, and the risk of its development through the women life is 1/70 (Moss and Kaye, 2002; Parker *et al*., 1996; Schuijer and Berns, 2003). Ovarian cancer is just a generic term for various types of ovarian cancers as presented in table 1.

Table 1: Classification of ovarian cancer types with the percentage of incidence (Novak, 1996).

1- epithelial ovarian cancers 90%		
serous tumors 75%	mucinous tumors 20%	endometrial tumors 2%
clear cell tumors 1%	brenner tumors 1%	undifferentiated cell tumors 1%.
2- nonepithelial ovarian cancers 10%		
germ–cell malignancy	Immature teratomas	sex cord tumors
metastatic carcinomas	Sarcomas	uncommon ovarian cancers

Approximately 90% of ovarian cancers are derived from tissues that come from the coelomic epithelium or "mesothelium" (Piek *et al*., 2006; Wright *et al*., 2005). More than 80% of epithelial ovarian cancers are found in postmenopausal women. The peak incidence of this disease occurs at 62 years whereas before the age of 45, these cancers are relatively uncommon (Novak, 1996).

To detect ovarian cancer in early stages the tumor marker CA 125 was used (Einhorn *et al*., 1992). Regarding the sensitivity of the test, CA 125 can detect 50% of patients with stage I disease and 60% of patients if those with stage II

disease are included (Zurawski, Jr. *et al*., 1990). However, the specificity of the test is low because the CA 125 level tends to be elevated in common benign conditions. The diagnosis of an ovarian cancer requires an exploratory laparotomy. Before the planned surgery, the patient should undergo routine hematologic biochemical assessments, an x-ray of the chest and an assessment of the urinary tract with an intravenous pyelography. In general patients with ovarian cancer have a poor prognosis (Campbell *et al*., 1989; Clark *et al*., 2001; Higgins *et al*., 1989; Kurjak *et al*., 1991; Schuijer and Berns, 2003).

Although radiotherapy of patients with ovarian cancer has some success, it is not sufficient alone for the management of ovarian cancer, and it is considered as complementary therapy to chemotherapy (Cardenes and Randall, 2000; Suzuki *et al*., 2000). Therefore, the first choice treatment of ovarian cancer patients is chemotherapy. Generally, patients with ovarian cancer show significant differences in chemosensitivity, even if the tumors share identical clinicopathological features. Either single-agent or multi-agent chemotherapy can be used to treat patients; the most frequently used single-agent chemotherapy has been melphalan given orally. Primary treatment with cisplatin gave response rates up to 70% (Gershenson *et al*., 1990). Recently, paclitaxel has become part of the standard primary therapy for advanced ovarian cancer. Because cisplatin, carboplatin, irinotecan and paclitaxel (Taxol) are active single agents against epithelial ovarian cancer, they may be preferable to melphalan for patients with low-stage disease (Du Bois and Pfisterer, 2005; McGuire *et al*., 1996; Wright *et al*., 2005). Cisplatin, carboplatin, and paclitaxel seem to be more active than alkylating agents (Du Bois and Pfisterer, 2005; Harries and Kaye, 2001; Ozols, 2000). Moreover, cisplatin, carboplatin and paclitaxel produce sufficiently high response rates to justify their routine use in primary therapy. Single-agent drugs, orally administered, are sometimes used for second-line chemotherapy because of their relative ease of administration and low toxicity. Second-line responses to paclitaxel, hexamethylmelamine, carboplatin and cisplatin have been observed in 10-36% of patients who have responded previously to cisplatin (Novak, 1996).

Numerous combination chemotherapeutic regimens have been tested in the treatment of advanced epithelial ovarian cancer to decrease the drug resistance and the side effects (Hirasawa *et al*., 2004; Young *et al*., 1990). Combination chemotherapy has been shown to be superior to single-agent therapy for most patients with advanced epithelial ovarian cancer (No authors listed, 1991). The cisplatin-based combination regimen seemed superior. Because of the toxicity of hexamethylmelamine, particularly the depression that some patients experience with the drug, many physicians omitted that agent (Alberts *et al*., 1992). Paclitaxel is an active agent in first-line therapeutic strategies because the overall response rates in phase II trials of 36% in previously treated patients. This is a higher rate than what was seen for cisplatin when it was first tested (McGuire *et al*., 1996). Chemotherapeutic recommendation in advanced ovarian cancer is a combination chemotherapy with cisplatin and paclitaxel which seems to be a more effective treatment. The cisplatin analog, carboplatin, is active as a second-line agent in patients who have responded to prior cisplatin treatment, and response rates for these patients have been 20-30%.

Among new agents tested as a medication for ovarian cancers is gemcitabine (Gem), that is a nucleotide 2',2'-difluorodeoxycytidine (deoxycytidine analogue), with two fluoride atoms at carbon 2 of the carbohydrate moiety. Gem is a hydrophobic molecule, transported into the cell by trans-nucleoside proteins related with cell membrane (Bookman, 2005; Heinemann, 2003; Plunkett *et al*., 1996). Gem diffusion is rapid within body fluids. Directly after entering into the cell, it is phosphorylated by deoxycytidine kinase to generate an active form: Gemcitabine di- and tri-phosphate. Studies have shown that Gem has a good tolerance, moderate toxicity and moderate side effects (Heinemann, 2003; Herzog, 2004; Nagourney *et al*., 2003; Yardley, 2005). Gem is metabolized by deamination in the plasma leading to formation of inactive uridine difluordexocycidine. The unmetabolized Gem is filtrated mainly from the kidney (Lawrence *et al*., 2001).

When Gem was used as second line therapy for epithelial ovarian cancer patients in stages III and IV, for those treated previously with cisplatin and having resistance to cisplatin, their response to Gem was about 20% (Barton-Burke, 1999; Shapiro *et al*., 1996; von Minckwitz *et al*., 1999). However, when used alone, Gem showed modest, albeit significant, activity in patients with ovarian cancer. The response rate is usually greater when it is used in combination with other agents (Mutch, 2003; Poveda, 2005).

Although some studies indicated the presence of synergic action between cisplatin and gemcitabine in the treatment of many cell lines of lung, breast and pancreas established specially from recurrent patients (Herzog, 2004; Kino *et al*., 2005; Nagourney *et al*., 2003; Theodossiou *et al*., 1998). On the other hand, there are many studies, which used gemcitabine together with paclitaxel for the first line treatment of lung, breast and bladder cancers where the response of this treatment was in general good (Heinemann, 2003; Yardley, 2005) although paclitaxel has decreased the uptake of Gem leading to its accumulation and to a decrease of active gemcitabine receptors (Shord *et al*., 2005). Gemcitabine and paclitaxel induce apoptosis leading to DNA fragmentation and lead cells to transformation into apoptotic bodies (Kaufmann *et al*., 1993). Based on the above mentioned data the role of Gem as a drug for ovarian cancer treatment is not yet clear at all.

The mode of action of Gem is different from the other drugs used in the therapy of ovarian cancer i.e. Gem is a nucleotide analog which causes DNA damage. Paclitaxel binds microtubules and causes kinetic suppression (stabilization) of microtubules dynamics, and inhibits γ- tubulin, leading to cell cycle arrest at M phase (Dumontet and Sikic, 1999). Cisplatin leads to formation of DNA interstrand and intrastrand cross-links followed by DNA strand breakdown. Cisplatin activates caspases and thereby apoptosis (Perez, 1998). Irinotocan inhibits topoisomerase II leading to DNA double-strand breaks (Gershenson, 2002).

In general, cancer is the result of an activation of an oncogene or an inactivation of a growth suppressor gene. Genetic alterations are also known

for ovarian cancer. A gene locus on chromosome 17 q, the BRCA1 gene, has been associated with breast and ovarian cancer (Lynch *et al*., 1991; Miki *et al*., 1994). The BRCA1 gene has been cloned, which suggests the prospect of screening of women at risk. The prevalence of BRCA1 mutations is about one in every 800-1000 women. Individuals who have a mutation in the BRCA1 have a cumulative lifetime risk of 85-90% developing breast cancer and 50% risk of ovarian cancer (Easton *et al*., 1995; Feki and Irminger-Finger, 2004; Piek *et al*., 2006; Zweemer *et al*., 1999). Recent studies have shown that BRCA1 and p53, a tumor suppressor, physically associate and that BRCA1 enhances p53-dependent gene expression, whereas mutant forms of BRCA1 show reduced p53-mediated transcriptional activation (Bar *et al*., 2002). The accumulation of p53 is an important event in hereditary ovarian cancer, and it is frequent in BRCA1-independent ovarian cancer (Zweemer *et al*., 1999).

It is widely accepted that the pathway leading to development of a tumor is a multistep process including the accumulation of genetic alterations of many oncogene and tumor suppressor genes (table 2).

Table 2: Putative oncogenes and tumor suppressor genes investigated in ovarian cancer.

gene	chromosome location	function	% altered	spectrum of alterations
oncogenes				
c-FMS	5q33.3-q34	receptor-like tyrosine kinase	57-100%	overexpresssion
CMYC	8Q24	transcription factor	30%	amplification, overexpression
K-RAS	12p12	signal transduction	4-30%	simple (codon 12.13 and codon 61)
HER-2/neu	17q21-q22	receptor-like tyrosine kinase	8-40%	amplification, overexpression
AKT2	19q13.1-q13.2	serine- threonine protein kinase	10-15%	amplification, overexpression
tumor suppressor genes				
FHIT	3p14.2	unknown	4-8%	altered transcripts
APC	5q21	binds α-and β-catenin: involved in adhesion	rare	multiple mutations
CDKN2/MTSI	9p21	cyclin-dependent kinase inhibitor	rare	multiple mutations
PTEN	10q23.3	phosphatase	rare	multiple mutations
WT1	11p13	transcription factor	none	mutations
ATM	11q22-q23	protein kinase	none	mutations
$p27^{KIP1}$	12p13	cyclin-dependent kinase inhibitor	30-50%	loss of expression
TEL	12p13	transcription factor	none	mutations

RB1	13q14	cell cycle regulator	rare	multiple mutations and loss of expression
				multiple mutations and overexpression
p53	17p13.1	cell cycle regulator; DNA	50%	
		repair and apoptosis		loss of expression mutation
OVCA1&2	17p13.3	unknown	?	mutations
NF1	17q11.2	down-regulates the active	none	
		form of RAS		mutations
NM23	17q21.3	nucleoside diphosphate	rare	enhanced expression
		kinase	70%	multiple mutations
BRCA1	17q21	transcription factor	rare	

Many studies showed that the most common genetic alterations in human cancers are mutations in tumor suppressor gene p53, being also present in approximately 50% of advanced stage ovarian carcinomas (Clark *et al*., 2001; Schuijer and Berns, 2003). On the long arm of chromosome 17, loss of 17q 12-q 21 has frequently been observed in ovarian cancers (p53 gene, maps to 17p 13.1) (Schuijer and Berns, 2003). Neither p53 mutations nor its overexpression have been described in benign epithelial ovarian tumors. The frequency of p53 mutations in border line tumors, which constitute about 10% of ovarian cancers, is less than 5%. p53 mutations were detected much more frequently in stages III and IV of ovarian cancer in comparison with stages I and II (Buttitta *et al*., 1997; Perego *et al*., 1996; Schuijer and Berns, 2003). Generally, in human cancers, and especially in ovarian cancers, p53 has point mutations or extended deletions that result in loss of its tumor suppressor function (Bar *et al*., 2001; Bar *et al*., 2002). Many studies performed on ovarian cancer cell lines, showed that cell lines, which express wild type p53 were sensitive to anticancer drugs and undergo p53-dependent apoptosis, whereas cell lines which lack functional p53 show drug resistance. Thus, p53 alterations might be one of the most important factors responsible for multidrug resistance in ovarian cancers (Bar *et al*., 2001; Debernardis *et al*., 1997; Hirasawa *et al*., 2004). On the other side, the presence of p53 mutations did not correlate with the response to primary therapy, but p53 is a direct determinant of chemotherapy response in ovarian cancers (Brown *et al*., 1993; Oggionni *et al*., 2005; Ortega *et al*., 1996). Therefore, drug resistance rises generally as a result of many causes, the most important one is the p53 status (Bar *et al*., 2001; Schuijer and Berns, 2003). Therefore,

cancers with non-functional p53 are less sensitive to chemotherapy, with low response to the treatment, and they are more aggressive than other cancers, in which p53 is functional (Bar *et al*., 2001; Oggionni *et al*., 2005; Perego *et al*., 1996). Other results have demonstrated that the presence of non-functional p53 is the main reason to delay apoptosis induced by cisplatin in cisplatin resistant cells (Lechpammer *et al*., 2004; Ortega *et al*., 1996; Sorenson *et al*., 1990). Many studies indicated a strong relationship between p53 accumulation and the response to cisplatin. On the other hand, p53 overexpression is correlated with a weak response to cisplatin (Bar *et al*., 2001; Niedner *et al*., 2001), and the resistance to cisplatin is in parallel with decreased ability of tumor cells to go into apoptosis induced by cisplatin (Fajac *et al*., 1996; Niedner *et al*., 2001; Perego *et al*., 1996). In addition there is a correlation between p53 levels and the degree of cancer recurrent after surgery (Shvarts *et al*., 2005). Therefore, many studies have considered that the p53 level is a useful marker for cancer recurrence and for prognosis assessment (Bar *et al*., 2001; Schuijer and Berns, 2003; Shvarts *et al*., 2005). Similar to cisplatin, lacking functional p53 makes the patients sensitive to paclitaxel because of delayed cell cycle arrest dependent on p53 (Wang *et al*., 2000).

The frequency of mutations in the p53 gene is 50%-80% in invasive serous carcinoma, whereas p53 mutations are not frequently found in serous borderline ovarian tumors. These mutations are found usually in exons 5, 6, 9 and 11 (No authors listed, 1993). The frequency of overexpression of p53 gene is low in low malignant tumors (Ceccaroni *et al*., 2004; No authors listed, 1993; Singer *et al*., 2005), whereas the frequency of p53 mutations is high in advanced ovarian cancers (Leitao *et al*., 2004; Singer *et al*., 2005). If a p53 mutation is accompanied with a mutation in BRCA1 gene, the status of cancer is bad (No authors listed, 1993). On the other hand, mutations in BRCA1 and BRCA2 are responsible for 95% of all inherited ovarian cancers (Zweemer *et al*., 1999). The spectrum of p53 mutations is different in ovarian cancers by origin and by exposure to oncogenic agents (Dansonka-Mieszkowska *et al*., 2006). However, there is a report showing that the p53 mutation status and mutation type (null vs missense) did not influence response to therapy or

overall survival (Galic *et al*., 2007). From all these data it is clear that a strategy to treat ovarian cancers must include the knowledge of the p53 status as well as other cell cycle checkpoint regulators.

2.2. Molecular biology of the cell cycle

The cell cycle represents a series of tightly integrated events that allow the cell to grow and proliferate, leading to the production of two daughter cells that are accurate copies of the parental cells. Successful cellular reproduction requires a cell to integrate the unfavorable events that occur once or a few times per cell cycle with the continuous processes of cell cycle (Pollard, 2002; Schwartz and Shah, 2005).

The cell cycle is divided into different phases (Fig. 1). The G_1 phase (first gap phase) is the interval between mitosis and DNA replication. The S phase is the synthesis phase, in which DNA is replicated. The G_2 phase is the interval between the completion of DNA replication and mitosis. During the G_2 phase, cells "proofread" the DNA and prepare for mitosis. If unreplicated or damaged DNA is detected, a protein kinase cascade, known as the G_2 DNA damage checkpoint, is triggered. This cascade ultimately leads to the inactivation of cyclin-dependent kinases required for entry into mitosis. The resultant increase in the length of the G_2 phase is called G_2 delay. Defects in enzymes in this checkpoint pathway can lead to cancer (Dash and El-Deiry, 2005; Graves *et al*., 2000; Suganuma *et al*., 1999). During M phase (or mitosis) and subsequent cytokinesis, chromosomes and cytoplasm are partitioned into two daughter cells.

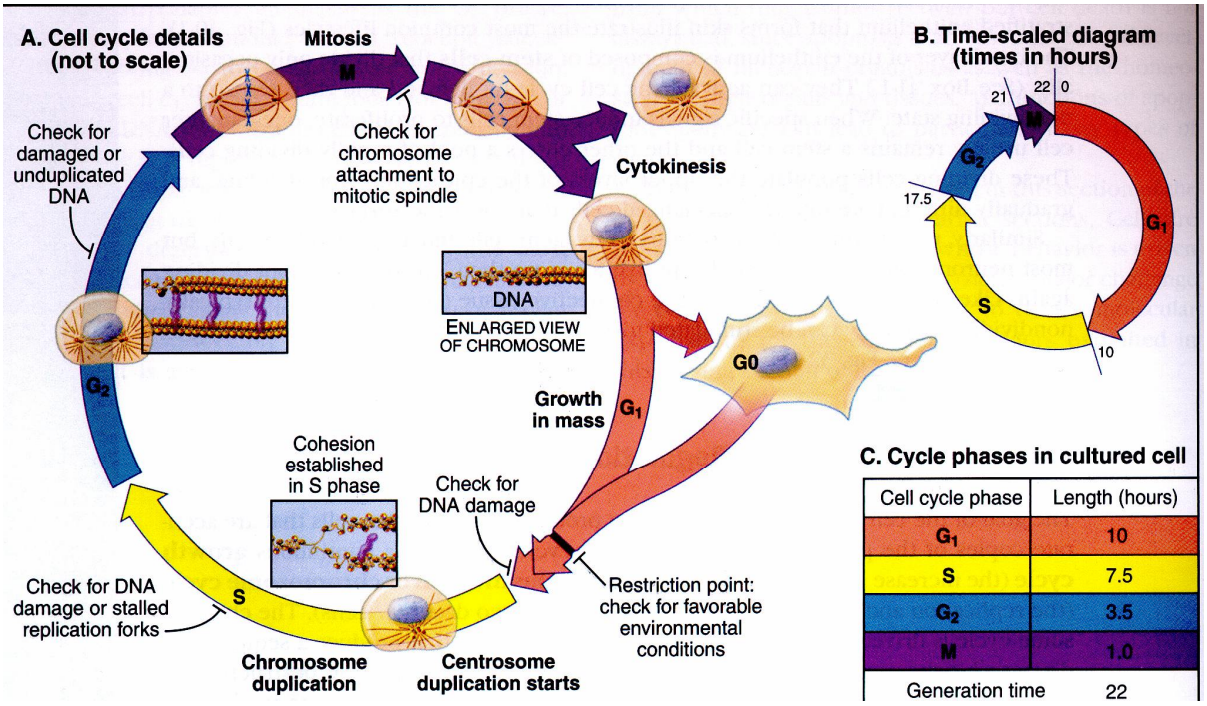


Figure 1: The cell cycle phases (Pollard, 2002).

Biochemical pathways termed checkpoints control transitions between cell cycle stages (Graves *et al.*, 2000). Four checkpoints are particularly well characterized. These DNA damage checkpoints monitor the integrity of DNA (Manke *et al.*, 2005; Xie *et al.*, 2005). Cells with damaged or partly replicated DNA arrest the cell cycle in late G_1 or G_2 phase, so that the damage can either be repaired or the cell can undergo programmed cell death by apoptosis (Arellano and Moreno, 1997; Motoyama and Naka, 2004). Transitions between cell cycle phases are triggered by a network of protein kinases and phosphatases that are tied to the discontinuous events of the chromosome cycle by the cyclic accumulation, modification and destruction of several key components. The cell cycle is regulated by different cyclin dependent protein kinases (Golsteyn, 2005; Xie *et al.*, 2005). Human beings have more than 10 distinct cyclin dependent kinases. To be active, these enzymes must associate with a regulatory subunit called cyclin. That is the reason why they have been termed cyclin dependent kinases (CDKs) (Schwartz and Shah, 2005). One of these cyclin dependent kinases is cdk1/ cyclin B1. Cyclin B1 binding also causes the reorientation of two residues

namely threonine 14 and tyrosine 15 in cdk1 so that they become accessible to protein kinases involved in regulation of CDK activity (Castedo *et al*., 2002; Pollard, 2002; Powers *et al*., 2004). Despite these changes, the CDK-cyclin complex has only partial catalytic activity. Complete activation requires the action of a kinase, called CAK (CDK-activating kinase), which phosphorylates threonine 160 in the T loop. This has a profound effect, stimulating the catalytic activity by up to 300-fold (Pollard, 2002).

At least two mechanisms inactivate CDKs and slow or stop the cell cycle. During the G_2 phase, cdk1 activity is controlled by the phosphorylation of two amino acid residues (threonine 14 and tyrosine 15) in the loop of the ATP-binding site by the protein kinases Myt1 and Wee1, respectively, leading to cell cycle arrest (Castedo *et al*., 2002; Rief *et al*., 2000). The product of the cdc2 (cell division cycle) gene is a protein kinase of 34,000 Da originally called $p34^{cdc2}$. $p34^{cdc2}$ now termed cdk1, seems to function primarily in the regulation of the G_2/M transition in animal cells. A second family member, cdk2, is involved in the regulation of the G_1/S transition and G_2/M transition. Kinases acting on cdk1 are found both in the nucleus and cytoplasm. Wee1 and CAK are both located in the nucleus. CAK phosphorylates cdk1 on threonine 161, triggering a refolding of the active site cleft and rendering the enzyme able to bind to its substrates (Hoffmann, 2000).

Once chromatids are separated, the cell must progress to a state with low levels of CDK activity so that nuclear envelope reassembly, spindle disassembly and cytokinesis can occur. Thus, exit from mitosis requires CDK inactivation. This occurs through the action of the ubiquitin-mediated proteolytic machinery that targets, among other key proteins, A and B-type cyclins (Pollard, 2002). cdk1 is the physiological substrate of the cdc25C phosphatase, which is responsible for the dephosphorylation of threonine 14 and tyrosine 15 and thereby triggering the final activation of the cdk1/cyclin B complex (Kristjansdottir and Rudolph, 2004).

cdc25 represents a class of well- studied cysteine phosphatases (Rudolph, 2007). Three closely related proteins termed cdc25A, cdc25B and cdc25C

constitute these cell regulating phosphatases. cdc25A regulates the G_1/S transition whereas cdc25B and cdc25C are mainly involved in G_2/M progression (Chen *et al*., 2001; Ferguson *et al*., 2005; Galaktionov *et al*., 1995; St Clair *et al*., 2004). The human cdc25s are between 423 and 566 amino acids long. The cdc25 proteins share approximately 40 to 60% amino acids identity with the highest homology in the C-terminal catalytic domain (Cans *et al*., 1999; Hirasawa *et al*., 2004). The N-terminal regulatory domain has low sequence homology (20-25% identity) and contains sites of phosphorylation, sequestration by 14-3-3 proteins, and ubiquitination (Eckstein, 2000; Kristjansdottir and Rudolph, 2004). The catalytic domain is able to dephosphorylate proteins substrates, where the physiological substrates of the cdc25 phosphatases are the cdk/cyclins. The three human cdc25 phosphatases A, B and C are responsible for dephosphorylation of threonine 14 and tyrosine 15 of cdk1 thereby triggering the final activation of the cdk/cyclin complexes (Barth *et al*., 1996; Chen *et al*., 2006; Rudolph, 2007).

cdc25C is relatively inactive during interphase for two reasons. First, its phosphorylation on the residue serine 216 creates a binding site for a protein called 14-3-3 (Peng *et al*., 1997; Roshak *et al*., 2000). This protein seems to inhibit nuclear import of cdc25C, causing the protein to accumulate in the cytoplasm (Hutchins *et al*., 2000; Kino *et al*., 2005). Second, to be fully activated cdc25C requires phosphorylation of its amino-terminal region. This is triggered by a protein called Polo like kinase (Plk1), and then completed by its substrate, cdk1/cyclin B1, creating a powerful positive feedback amplification loop that provides the burst of CDK activity triggering entry into mitosis (Xie *et al*., 2005).

Polo like kinases are involved in a variety of mitotic events, including formation of a bipolar spindle, cytokinesis and passage through certain cell checkpoints (Chen *et al*., 2006; Xie *et al*., 2005). cdc25B is activated much earlier in the cell cycle than cdc25C, with an activity first detected in late S phase and peaking during G_2 phase. Thus, this phosphatase is well positioned to initiate the G_2/M transition. cdc25B activity is limited in S and G_2

phases of the cell cycle. Its concentration is kept low, in part owing to its short half-life of less than 30 minutes (Millar *et al*., 1991). Overexpression of cdc25B during S or G_2 phase is sufficient to push cells prematurely into mitosis (Cans *et al*., 1999; Pollard, 2002). cdc25A is a nuclear enzyme, whereas cdc25B and cdc25C shuttle in and out of the nucleus throughout interphase, and this activity is dependent (at least in part) on their interactions with 14-3-3 proteins (Eckstein, 2000; Ferguson *et al*., 2005; Kristjansdottir and Rudolph, 2004). In order to shuttle between cytoplasm and nucleus cdc25B and cdc25C both have nuclear import and nuclear export signals (Ferguson *et al*., 2005; Noll *et al*., 2006).

Early in prophase phosphorylation of cyclin B1 inactivates its nuclear import signal allowing cdk1/cyclin B1 to accumulate rapidly in the nucleus (Noll *et al*., 2006). cdc25C also stops shuttling at the G_2/M transition, probably as a result of phosphorylation, apparently by Polo like kinase (Xie *et al*., 2005). There is an ample evidence that the cdk1/cyclin B1 that accumulates in the nucleus is already active, but it may be that restriction of cdk1/cyclin B1 complex to the nucleus together with cdc25C significantly increases their local concentration and may contribute to the final burst of cdk1/cyclin B1 activation (Pollard, 2002).

As shown earlier in our group cdc25C is also phosphorylated by another kinase, namely protein kinase CK2. CK2 is a protein kinase formerly known as casein kinase 2, which phosphorylates many proteins in the cell resulting in either gain or loss of functions. CK2 phosphorylates cdc25C at threonine 236 residue located close to the NLS of cdc25C. cdc25C phosphorylated at threonine 236 has lost binding to α/β importins leading to delayed import of cdc25C to the nucleus. Thus, the phosphorylation of cdc25C at threonine 236 by CK2 is an additional signal for the retention of cdc25C in the cytoplasm (Schwindling *et al*., 2004).

The restriction point in the G_1 phase is sensitive to the size and physiological state of the cell and to its interactions with the surrounding extra cellular

matrix. Cells that do not receive appropriate growth stimuli from their environment arrest at this point in the G_1 phase and may commit suicide by apoptosis. Exposure of cells to agents that damage DNA, including certain chemicals or ionizing radiation, halts the cell cycle temporarily in the G_2 phase. This G_2 delay gives cells the opportunity to repair damaged DNA before entering mitosis (Manke *et al.*, 2005; Ongkeko *et al.*, 1995). The G_2 checkpoint is also responsible for monitoring the completion of DNA replication. Absence of a proper checkpoint response causes premature entry to G_2/M transition, leading to cancer (Hoffmann, 2000; Kristjansdottir and Rudolph, 2004). The G_2 checkpoint involves three sorts of components: sensors, kinases and effectors. When sensors detect damage to DNA they become activated. In turn, they activate specialized protein kinases that transmit this information to a series of effector molecules. Effectors then either directly or indirectly block cell cycle progression. Having detected DNA damage, the various sensors transmit this information to a family of very large protein kinases (>2000 amino acids) that resemble the lipid kinase phosphatidylinositol -3. Rather than phosphorylating phosphatidylinositol, these G_2 checkpoint kinases phosphorylate proteins that stop the G_2/M transition. The best known of these G_2 checkpoint kinases is ATM, encoded by the gene defective in the human inherited disorder ataxia-telangiectasia. Ataxia-telangiectasia is complex, characterized by (among other things) premature aging, sensitivity to ionizing radiation and an elevated risk of cancer. Evidence that ATM is important for checkpoint control comes from the observation that caffeine, a molecule that can override cell cycle checkpoints in cultured cells, is a relatively specific inhibitor of ATM. Another member of the inositol 1,4,5-triphosphate (IP-3) kinase family, ATR (ataxia-telangiectasia and Rad3-related), is also involved in G_2 checkpoint control but may also have other roles, as it is essential for cellular life (Motoyama and Naka, 2004; Powers *et al.*, 2004; Roshak *et al.*, 2000).

When ATM and ATR are activated by DNA damage, they phosphorylate at least two important substrates: the famous tumor suppressor protein p53 and a protein kinase called Chk1 (checkpoint kinase 1) (Krause *et al.*, 2001). Chk1 is activated by phosphorylation and then it, in turn, phosphorylates cdc25C on

serine 216 (Niida *et al*., 2007). This inhibits the activity of cdc25C by producing a binding site for a member of the 14-3-3 group of adapter proteins (Chen *et al*., 1999; Roshak *et al*., 2000). These proteins bind sites on target proteins containing serine flanked by several other characteristic amino acids, but only when the critical serine is phosphorylated. When DNA is damaged by UV, two critical effectors, cdc25C and p53 are activated by checkpoint kinases ATM/ATR and Chk1/Chk2. The activation of cdc25C and p53 affects at least three stages of cell cycle: G_1/S transition, progress to S and G_2/M boundary (Chen *et al*., 2006; Taylor and Stark, 2001). This is an example of the general mechanism whereby phosphorylation regulates interactions between proteins in response to physiological signals. As a result of 14-3-3-binding, cdc25C is sequestered in the cytoplasm. This may also contribute to blocking the G_2/M transition (van Hemert *et al*., 2001; Wilker and Yaffe, 2004). Rad24 is one of the 14-3-3 proteins; it also controls the cdc25 phosphatases in the cell. When Rad24 is deleted, cdc25 accumulated in the nucleus. Rad24 enhances nuclear export of cdc25 in response to DNA damage (Chen *et al*., 1999; Masters and Fu, 2001). Some drugs used in chemotherapy of tumor patients abrogates a DNA damage-induced G_2 cell cycle arrest leading to premature activation of cdc25C and subsequently to «mitotic catastrophe» (Vogel *et al*., 2007).

p53 is another major target of ATM/ATR in response to DNA damage. Although p53 is not required to arrest the cell cycle in G_2 phase in response to DNA damage (Taylor and Stark, 2001), but it is absolutely required to prolong this cell cycle arrest. p53 regulates expression of proteins important for the G_2 checkpoint. One is p21, which inhibits cdk1/cyclin A 100-fold better than it inhibits cdk1/cyclin B1. p21 is an effector gene of p53, and the alterations in p21 mainly leads cells to arrest in G_1 phase of the cell cycle (Agarwal *et al*., 2006; Dash and El-Deiry, 2005; O'Connor, 1997; Wang *et al*., 1999b). In addition p21 expression provides an effective way of blocking the initiation of prophase by cdk1/cyclin A. p21 also participates in the G_1 DNA damage checkpoint (Cans *et al*., 1999; Dash and El-Deiry, 2005). Alterations in p21 or p53 lead to an abrogation of the G_1 checkpoint which may result in malignancies (Amikura *et al*., 2006).

A second target of p53 in the G_2 checkpoint is a member of the 14-3-3 protein family, namely 14-3-3σ. 14-3-3σ binds cdk1/cyclin B1 and interferes with its ability to shuttle between the nucleus and cytoplasm. As a result cdk1/cyclin B1 remains in the cytoplasm (Manke *et al*., 2005; van Hemert *et al*., 2001). The 14-3-3σ cdk1/cyclin B1 complex also contains the Wee1 inhibitory kinase, apparently providing a further level of assurance that cyclin B1-associated cdk1 kinase remains inactive. Disruption of the gene for 14-3-3σ is fatal for cells if they sustain DNA damage. Instead of activating their G_2 checkpoint, they enter an aberrant state with characteristics of both mitosis and apoptosis, and then die (Hermeking and Benzinger, 2006; Laronga *et al*., 2000; Masters and Fu, 2001).

The ability to block the G_2/M transition gives cells time to repair DNA damage. It is equally important, that cells must be able to turn off this checkpoint when the damage is repaired. How this is done is still a mystery. Limited evidence suggests that more active factors may also contribute to termination of G_2 checkpoint arrest. The Polo like protein kinase phosphorylates cdc25C, which has been previously phosphorylated on serine 216 by Chk1 and is held in the cytoplasm in a complex with 14-3-3 protein. Phosphorylation of cdc25C by the polo-like kinase disrupts the binding of 14-3-3 protein. This appears to directly promote activation of the cdc25C, and also enables shuttling into the nucleus. Overriding checkpoint arrests may be a major function of Polo like kinases (LeGac *et al*., 2006; Margolis *et al*., 2006; Mils *et al*., 2000).

A series of reports have correlated levels of cdc25 mRNA or protein with disease (Eckstein, 2000). The cdc25 phosphatases are overexpressed in numerous rapidly dividing cancer cells and this overexperession seems to correlate with many malignancies (Rudolph, 2007). Over half of the 15 different cancers studied showed overexpression of either cdc25A or cdc25B, but there was no significant overexpression of cdc25C (Eckstein, 2000; Kristjansdottir and Rudolph, 2004). The cdc25C gene is a target of transcriptional down regulation by p53 and this repression can be shown to contribute to p53- dependent cell cycle arrest. On the other hand, overexpression of cdc25C phosphatase results in suppression of a p53-

induced growth arrest (Bureik *et al*., 2000). Two independent mechanisms have been identified. One involves the direct binding of p53 to a site in the cdc25C promoter, and the second involves a CDE/CHR element in the cdc25C promoter. Both mediate p53 dependent repression at levels of p53 comparable to those produced by DNA damage. Repression of cdc25C by p53 represents an additional mechanisms for p53 dependent cell cycle arrest in response to DNA damage (St Clair *et al*., 2004; St Clair and Manfredi, 2006). In addition p53 interacts directly with cdc25C within its C-terminal region from amino acid 287 to 340 (Rief *et al*., 2000; St Clair *et al*., 2004).

Several cellular responses can be provoked by p53, including cell cycle arrest, senescence, differentiation and apoptosis (Vousden and Lu, 2002). In addition a variety of cells undergo programmed cell death. The path for accidental cell death is called **necrosis.** Accidental cell death occurs when cells receive a structural or chemical insult from which they cannot recover. Examples include ischemia, extreme temperatures and physical trauma. In contrast, cells that die by programmed cell death commit suicide actively as the result of activation of a dedicated intracellular program for programmed cell death, the most commonly described pathway is **apoptosis** (Earnshaw *et al*., 1999; Pollard, 2002). These can be separated into at least seven distinct classes, which are: 1- Harmful cells, 2- Developmentally defective cells, 3- Excess cells, 4- Unnecessary cells, 5- Obsolete cells, 6- Virus-infected cells, 7- Chemotherapeutic killing of cells. There are two principle ways of apoptosis which finally lead to the fragmentation of DNA by decay of the cell into apoptotic bodies which are finally removed by phagocytes: the receptor – mediated extrinsic pathway and the mitochondrial intrinsic pathway (Fig. 2). One of the late events in the apoptosis pathways is the cleavage of the poly-ADP- ribosyl- polymerase (PARP) (Kaufmann *et al*., 1993). Irreparable damage leads the cell into apoptosis by influencing the balance between pro and anti-apoptotic proteins (Haupt *et al*., 2003).

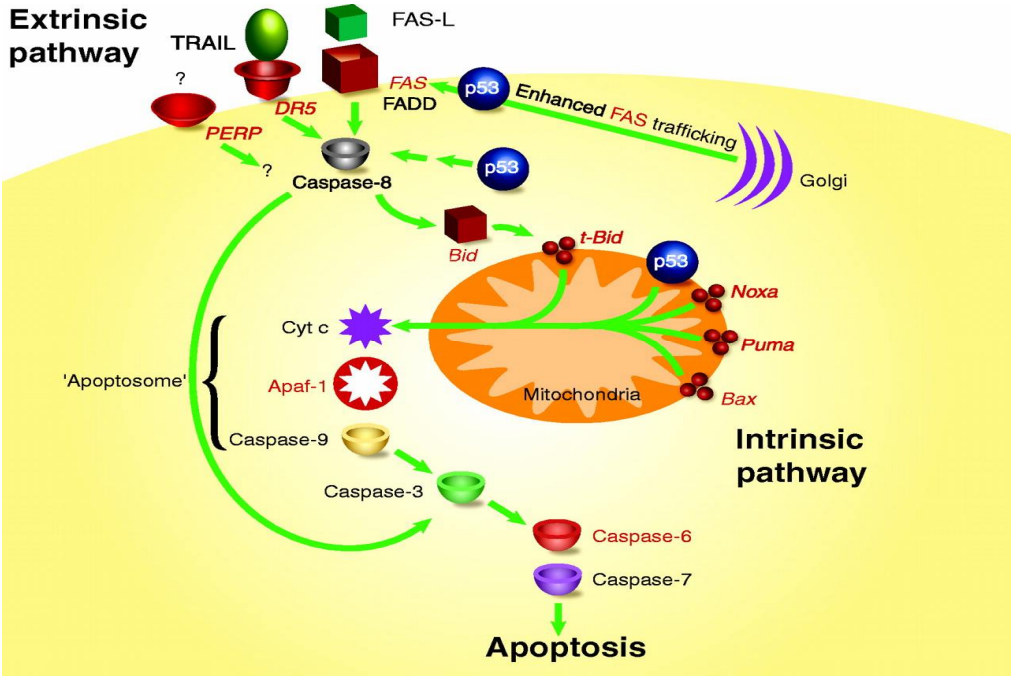


Figure 2: Apoptotic pathways (Haupt *et al*., 2003).

A direct connection between p53 and apoptosis was first noticed when the cloned p53 gene was introduced into a number of different cell types. In most cells, overexpression of p53 causes a cell cycle arrest at the G_1/S boundary. However, overexpression of cloned p53 in certain cancer-derived cell lines causes the cells to undergo apoptosis. Under some circumstances, p53 also contributes to the repair of genotoxic damage, potentially allowing the release of the rehabilitated cell back into the proliferating pool. In most cases, however, introduction of p53 leads to an irreversible inhibition of cell growth, most decisively by activating apoptosis (Schuler and Green, 2001; Vousden and Lu, 2002). Manipulation of the apoptotic functions of p53 constitutes an attractive target for the cancer therapy (Haupt *et al*., 2003).

p53 is a transcription factor that directly activates the expression of genes that contain p53-binding sites within their regulatory regions. p53 can independently regulate apoptosis and cell cycle arrest, through effecting on cell cycle arrest genes. Low levels of p53 can arrest cell cycle, whereas high levels of p53 induce apoptosis. In intact cells, the activity of p53 can be

further controlled by regulation of the sub-cellular localization of components of the p53-response pathway. p53 is actively transported into and out of the nucleus, and can be localized to distinct structures in both the nucleus, cytoplasm and mitochondria (Inoue *et al*., 2005; Pollard, 2002; Vousden and Lu, 2002).

p53 is a phosphoprotein, and the function of p53 can be regulated by phosphorylation and dephosphorylation. Protein serine/threonine phosphatase-1 dephosphorylates p53 at serine 15 and serine 37 leading to modulate its transcription and apoptotic activity negatively. Phosphorylation of human p53 on serine 20 and serine 15 is mediated by the Chk1/2 in response to UV radiation. This phosphorylation leads to the stabilization and activation of p53 (Chen *et al*., 2006; Li *et al*., 2006)

Two related proteins namely MDM2 and MDMX (MDM4) have crucial roles in regulating p53 activity to allow normal cell growth and development. MDM2 has been shown to function as ubiquitin ligase that targets p53 for degradation. The activation and stabilization of p53 is generally associated with inhibition of this function of MDM2 (Ferrone *et al*., 2006; Vousden and Lu, 2002). DNA damage causes dissociation of p53 from MDM2 (Chehab *et al*., 1999). The overexpression of p53 causes G_2 arrest attributed in part to the loss of cdk2 activity. Overexpression of p53 may also interfere with the accumulation of cdk1/ cyclin B1 in the nucleus, required for cells to enter mitosis (Taylor *et al*., 1999). Both wild-type p53 and mutant p53 naturally occurring could migrate into nucleus by essentially similar mechanisms (Shaulsky *et al*., 1990).

Some studies indicated that the ionized radiation (IR) leads to a dephosphorylation of p53 at serine 376 causing binding of p53 with 14-3-3 proteins without affecting the DNA binding activity of p53. This binding with 14-3-3 proteins activates p53 in response to IR (Stavridi *et al*., 2001).

In conclusion, there are many unclear points in ovarian cancer treatment which should be addressed in the present thesis:

1- What are the most effective drugs in ovarian cancer, and in which concentrations?

2- How do these drugs act, and is apoptosis involved?

3- What is the role of p53 in ovarian cancer cells treatment?

4- Do these drugs affect the behavior of cdc25C in ovarian cancer cells?

3. Materials and methods

3.1. Materials

3.1.1. Cell lines

OV-MZ-32	serous human ovarian adenocarcinoma cell line, these cells carry a 13-bp deletion from codon 314 to codon 318 of the p53 gene	Dr.I.B. Runnebaum, Freiburg
OvBH-1	temperature-sensitive ovarian clear carcinoma cell line, with a wild type p53	Prof. Dr. A. Harlozińska-Szmyrka, Dr. J. Bar; Breslau
OvCBM		Prof. Dr. A. Harlozyńska-Szmyrka, Dr. J. Bar, Breslau

3.1.2. Chemicals and laboratory materials

15 ml/ 50 ml Tubes	Greiner Bio-one GmbH, Frickenhausen
94 mm/ 60 mm Culture dishes	Greiner Bio-one GmbH, Frickenhausen
6-well plates (Cell culture)	Greiner Bio-one GmbH, Frickenhausen
96 Microwell plates (Cell culture)	Greiner Bio-one GmbH, Frickenhausen
Fluoro Nunc™ 96 Microwell plates (Phosphataseassay)	Nunc, Wiesbaden
Acrylamide-Stock solution: Rotiphorese® Gel 30	Carl Roth GmbH, Karlsruhe
Agarose (Type I, Low EEO)	Carl Roth GmbH, Karlsruhe
Ammonium persulfate (APS)	Amersham Biosciences, Freiburg
Bovine serum albumin (BSA)	PAA laboratories GmbH, Pasching
Bradford Protein Assay Kit	Bio-Rad, München
Bromophenol blue	Merck/VWR, Darmstadt
Cis-platinum(II)diamine dichloride	Sigma, München

Complete™-Protease inhibitor	Roche, Mannheim
CM-H_2DCFDA	Molecular Probes, Leiden, Niederlande
4´,6-Diamidino-2-phenylindol (DAPI)	Roche, Mannheim
Dimethyl sulfoxide (DMSO)	Merck KgaA, Darmstadt
Dithiothreitol (DTT)	Sigma, München
Dry milk powder, non fat	J.M Galber Saliter GmbH& Co.KG, Obergünzburg
Dulbecco's modified Eagle's medium (DMEM)	Invitrogen, Karlsruhe
Effectene™	Qiagen, Heidelberg
EGTA	Calbiochem/ Merk Biosciences GmbH, Schwalbach
Ethylene diamine tetraacetic acid disodium salt (EDTA)	Sigma, München
Ethidium bromide	Boehringer Ingelheim, Heidelberg
Filter paper 3MM	Schleicher+Schüll, Dassel
Fetal calf serum (FCS)	Biochrom KG, Berlin
Fluoresceine disulfate (FDP)	MolecularProbes, Leiden
Formaldehyde 37%	Carl Roth GmbH & Co, Karlsruhe
Glycerine	Fluka, Neu-Ulm
Glycine	Carl Roth GmbH & Co, Karlsruhe
Gemcitabine hydrochloride (Gemzar)	Lilly S.A., France
Hydrogen peroxide 30% (H_2O_2)	Merck KgaA, Darmstadt
Irinotecan hydrochloride(Campto)	Aventis Pharma Ltd.Essex, UK
Luciferase Assay Reagent	Promega, Manheim
Lumi-Light Western Blotting Substrate	Roche, Mannheim
3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazoliumbromid (MTT)	Sigma, München
Nonidet P40 (NP40)	Fluka, Neu-Ulm
Nucleobond® AX-Plasmid Purification KIT	Macherey-Nagel, Düren
Paclitaxel	Sigma, München
Penicillin/Streptomycin	Biochrom KG, Berlin

Prestained protein molecular weight maker	Fermentas, St. Leon-Rot
Propidium iodide	Sigma, München
Protein-A-sepharose^{TM} CL-4B	Amersham Biosiences, Freiburg
PVDF-Membrane	Roche, Mannheim
N,N,N´,N´-Tetramethylendiamine (TEMED)	Amersham Biosiences, Freiburg
SB218078 (Chk1 inhibitor)	Calbiochem/ Merk Biosciences GmbH, Schwalbach
Sepharose^{TM} CL-4B	Amersham Biosiences, Freiburg
Triton X-100	Serva, Heidelberg
Trypan blue	Serva, Heidelberg
Trypsin	Seromed Biochrom KG, Berlin
Tween 20	Serva, Heidelberg
X-ray films Agfa Cronex 5	Mortsel, Belgien

3.1.3. Buffers and solutions

APS-solution	10% (w/v) Ammonium persulfat
Blocking buffer	1 x PBS, pH 7.4 0.1% (v/v) Tween 20 5% (w/v) dry milk
Electrophoresis buffer	25 mM Tris-HCl, pH 8.8 192 mM glycine 3.5 mM SDS
Extraction buffer (1)(RIPA)	50 mM Tris-HCl, pH 8.0 150 mM NaCl 0.5% Na-Deoxycholat 1% Triton x-100

	0.1% SDS
Extraction buffer (2)	25 mM Tris-HCl, pH 10.5 1 mM EDTA 0.5 mM NaCl 10 mM β-MSH 0.5% Triton x-100
Gel solution A (Rotiphorese Gel 30)	4.2 M Acrylamid 52 mM N,N´-Methylenbisacrylamid
Gel solution B (Resolving gel buffer)	14 mM SDS 1.5 M Tris-HCl, pH 6.8
Gel solution C (Stacking gel buffer)	14 mM SDS 495 mM Tris-HCl, pH 6.8
Lysis buffer (1)	10 mM MES, pH 6.2 10 mM NaCl 1.5 mM $MgCl_2$ 1 mM EGTA 5 mM DTT 10% glycerol 1% NP40
Lysis buffer (2)	50 mM Tris-HCl, pH 7.4 250 mM NaCl 5 mM EDTA 100 mM $MgCl_2$ 50 mM NaF 1 mM DTT 0.1% Triton x-100 4%(v/v) Complete™

Lysis buffer (3)	25 mM Tris, pH 7.8 (using H_3PO_4) 2 mM EDTA 2 mM DTT 10% glycirol 1% Triton x-100
Mounting-Medium	5% (v/v) Polyvinylalcohol 23/140 10% (v/v) glycine in PBS
MTT-substrate solution	5 mg MTT/1 ml PBS
Neutralization Buffer	100 mM Tris-HCl, pH 7.0 10 mM β-MSH 0.5% Triton x-100
PBS	137 mM NaCl 2.7 mM KCl 8 mM Na_2HPO_4 1.5 mM KH_2PO_4, pH 7.3
Phosphatase assay-buffer	50 mM Tris-HCl, pH 8.0 1 mM DTT 20 µM FDP
Sample overlaying buffer (2.5x) (Laemmli, 1970)	130 mM Tris-HCl, pH 6.8 20% (v/v) glycerol 10% (w/v) β-mercaptoethanol 4% (w/v) SDS 0.02% (w/v) Bromophenol blue

Solubilization Solution	10% (w/v) SDS 0.01 M HCl
Transfer buffer	20 mM Tris-HCl, pH 8.3 150 mM glycine
Trypsin EDTA	0.25%(w/v)Trypsin 0.1%(w/v) EDTA in PBS, pH 7.1
Washing buffer (1)	1 × PBS, pH 7.4 0.1% (v/v) Tween 20
Washing buffer (2)	1 ×PBS, pH 7.4 0.1%(v/v) Tween 20 1%(w/v) dry milk
Washing buffer (3)	10 mM MES, pH 6.2 10 mM NaCl 1.5 mM $MgCl_2$ 1 mM EGTA 5 mM DTT 10% glycerol

3.1.4. Apparatus and instruments

AGFA, Curix ×60	AGFA, Belgium
Bio-Rad Microplate Reader 3550-UV	Bio-Rad, München
Digital camera AVT Horn 3CCD, Axiocam Arm	Zeiss, Oberkochen

Electrophoresis Chamber Mighty Small™ SE250	Serva (Hoefer)
Eppendorf Mastercycler gradient	Eppendorf, Hamburg
Eppendorf 5414C table centrifuge	Eppendorf, Hamburg
FACScan Flow cytometer	Becton Dickinson, Franklin Lakes (USA)
Fluorescence microscope Axioscope	Zeiss, Oberkochen
GENios Spectra Fluor plus Microplate Reader	Tecan Deutschland, Crailsheim
Incubator 6220 with CO_2	Heraeus, Hanau
Neubauer-counting chamber	WTW, Weilheim
pH-Meter: pH537	Carl Roth GmbH & Co, Karlsruhe
Photoanalysis system Axio Vision 2.0	Zeiss, Oberkochen
Power Supply	Amersham Biosiences, Freiburg
Reflecting microscope Axiovert 100	Zeiss, Oberkochen
Sigma 4k10 centrifuge	Sigma, Osterode
Sterile bench	Heraeus, Hanau
Ultra centrifuge	Beckman,
Ultrasonic bath Transonic 460	Elma GmbH, Singen
Ultraspec 2100 pro simple beam photometer	Amersham Biosiences, Freiburg
Stratalinker® UV crosslinker	Stratagene, Amsterdam Netherland
Varioklav® 400 Dampfsterilisator	H + P Labortechnik, Oberschleißheim

3.1.5. Antibodies

3.1.5.1. Primary antibodies

cdc25C-specific antibodies:

- C-20 (sc-327) Rabbit polyclonal antibody. The epitope is locatedin the last 20 C-terminal amino acids (454-473) of cdc25C (Santa Cruz Biotechnology, Heidelberg)
- C-2-2 Mouse monoclonal antibody. The epitope is between

	the amino acids 455-469 of cdc25C (PharMingen)
➢ H-6	Mouse monoclonal antibody, raised against amino acids 1-160 of cdc25C of human origin (Santa Cruz Biotechnology, Heidelberg)
➢ cdc25C-NT	Rabbit polyclonal antibody, raised against amino acids 134-210 of cdc25C of human origin (AG Montenarh, Dr. Nastainczyk, Homburg)
➢ phospho-cdc25C (Ser216)	Rabbit monoclonal antibody, detects endogenous level of cdc25C only whenphosphorylated on serine 216 (Cell Signaling Technology, USA)

p53-specific antibodies:

➢ DO-1	Mouse monoclonal antibody. It binds to the N-terminus between amino acids 11-25 of p53. (Santa Cruz Biotechnology, Heidelberg)
➢ p53 polyclonal	Rabbit polyclonal antibody (AG Montenarh, Dr. Nastainczyk, Homburg).
PARP-1(Ab-2)	Mouse monoclonal antibody. The epitope is located in the C-terminus of PARP DNA-binding domain (Oncogene Research Products, San Diego, USA)
GAPDH(FL-335)	Rabbit polyclonal antibody. It binds to the amino acids between 1-335 from GAPDH of human origin (Santa Cruz Biotechnology, Heidelberg)
Nucleolin	Rabbit polyclonal antibody binds to amino acids between 696-707 of human nucleolin (AG Montenarh, Dr. Nastainczyk, Homburg)

HSP90	Rabbit polyclonal antibody binds to amino acids between 610-723 of human HSP90 (Santa Cruz Biotechnology, Heidelberg)

3.1.5.2. Secondary antibodies

Western-blot	Horseradish peroxidase conjugated goat anti-mouse antibody (Dianova, Hamburg) Horseradish peroxidase conjugated goat anti-rabbit antibody (Dianova, Hamburg)
Immunofluorescence	AlexaFluor® 594 goat anti-rabbit IgG AlexaFluor® 488 goat anti-rabbit IgG AlexaFluor® 546 goat anti-mouse IgG AlexaFluor® 488 goat anti-mouse IgG (Molecular Probes, Leiden, Niederlande)

3.1.6. Enzymes

RNAse	Roche Diagnostics, Mannheim

3.2. Methods

3.2.1. Cell biology methods

3.2.1.1. Cell culture

Cells of all three cell lines were grown in 94-mm-culture dishes (Greiner) in DMEM medium (Invitrogen), and supplemented with 10% FCS in an incubator with 37°C and 5% CO_2 in moist atmosphere.

3.2.1.2. Cells splitting

The medium of the cells was sucked off the dish, 1 ml trypsin/EDTA [0.25% (w/v) Trypsin, 0.1% (w/v) EDTA] was added, and again sucked off the dish. The culture dish was incubated for 1 minute in the incubator for detachment of cells, new dishes were prepared with 5 ml medium and the cells were added.

3.2.1.3. Freezing of cells

Cells were stored in liquid nitrogen for conservation for long time.

After trypsinizing cells, fresh medium was added and the suspension was filled into a 15 ml tube, centrifuged for 7 min at 4°C and 250xg. The sediment was resuspended in 0.5 ml culture medium with 40% FCS, and then 0.5 ml medium with 20% DMSO were added drop wise. Cell suspension was incubated in cryo-atmosphere for two hours, and then transferred to liquid nitrogen.

3.2.1.4. Thawing of cells

Frozen cell suspension was placed for about 1 min in a water bath with 37°C, then the suspension was directly transferred to a sterile tube, 10-fold volume culture medium was added drop by drop with shaking. Cells were centrifuged for 5 min at 4°C and 250xg, and then the sediment was resuspended in culture medium with 10% FCS and transferred to a cell culture tube.

3.2.1.5. Counting of the cells

Cells were trypsinized and harvested with PBS. An aliquot from this suspension was mixed with the same volume of trypan blue. Only dead cells are stained with trypan blue. So we could count the living cells which are not colored by using a Neubauer-counting chamber.

3.2.1.6. Treatment of the cells with different drugs

Gemcitabine hydrochloride, irinotecan hydrochloride and cis-platinum (II)-diamine dichloride were dissolved in water. Paclitaxel was dissolved in dimethyl sulfoxide (DMSO). Twenty-four hours after seeding the cells, drugs were applied in concentrations of 1 nM, 10 nM, 100 nM, 1 µM and 10 µM, and then cells were incubated for seventy-two hours in 37°C and 5% CO_2.

3.2.1.7. MTT assay

Cell proliferation and viability was determined using a colorimetric MTT-based assay. This assay is based on the cleavage of the yellow tetrazolium salt MTT to purple formazan crystals by metabolically active cells. This reaction needs the pyridine nucleotide cofactors NADH and NADPH. The formazan crystals are solubilized and the resulting colored solution is quantified using a scanning multiwell spectrophotometer (ELISA reader):

5×10^3 (OV-MZ-32) or 3×10^3 (OvBH-1 and OvCBM) cells per well were grown in 96-well plate in a final volume of 100 µl culture medium. After twenty four hours cells were treated with drugs. Then, 10 µl of the MTT labeling reagent were added (the final concentration is 0.5 mg/ml). The enzymatic reaction needs at least four hours at 37°C in a humidified atmosphere to be complete. The formation of purple crystals was monitored in a light microscope. Hundred µl of solublization solution was added overnight in 37°C to dissolve the crystals. The spectrophotometrical absorbance of the blue dye was determined in a 96-well plate in an ELISA reader at 595 nm. The intensity of the absorbance correlates with the number of metabolic active cells.

3.2.1.8. Cells harvesting

After removal of the medium, cells were washed twice with cold PBS (pH 7.4), then fresh cold PBS was added and cells were scraped of the dish with a rubber. Cell suspension was transferred into 15 ml or 50 ml tube and kept on ice. Then the suspension was centrifuged for 7 min at 4°C and 250xg. The supernatant was discarded, and then proteins were extracted from the sediment.

3.2.1.9. Cytofluorimetry

The three cell lines (OV-MZ-32, OvBH-1, OvCBM) were treated with drugs as described above. Then cells were washed twice with cold phosphate buffered saline (PBS) and harvested by trypsinizing. Cells were centrifuged (250xg, 4°C, 10 min), and then resuspended in 200 µl PBS. Two ml cold 70% ethanol were used to fix cells and left at least 30 min at -20°C, then centrifuged again (250xg, 4°C, 10 min) and resuspended in 800 µl PBS. Hundred µl RNase (1 mg/ml) and 100 µl propidium iodide (400 µg/ml) were added, and cells were incubated at 37°C for 30 min. Cell cycle analysis of the suspension was performed using Becton-Dickinson FACScan II cytofluorimeter.

3.2.1.10. Measurement of intracellular reactive oxygen concentration

Intracellular concentration of reactive oxygen species was determined by staining the cells with the redox-sensitive dye CM-H_2DCFDA. Initially, CM-H_2DCFDA enters the cell where its acetate moiety is cleaved by intracellular esterases to C-H_2DCF. C-H_2DCF is then oxidized by reactive oxygen species using the intracellular peroxidases to yield the fluorescent C-DCF. OV-MZ-32, OvBH-1 or OvCBM cells were seeded in 6-well plates for 24 hours, then treated with a combination of 100 nM gemcitabine and 100 nM paclitaxel. Following treatment, cells were washed once with DMEM medium without FCS, harvested by trypsinizing and exposed to 5 µM CM-H_2DCFDA, and incubated for 30 min at 37°C. Subsequently, cells were washed once with DMEM medium and resuspended in 500 µl DMEM medium. Flow cytometric analysis was performed using Becton-Dickinson FACScan II cytofluorimeter.

3.2.1.11. Immunofluorescence

After seeding cells in 60-mm-dishes (Greiner), they were treated with drugs, and then washed for 3 x 10 min with PBS, fixed with 3.7% formaldehyde for 10 min at room temperature, washed again, permeabilized with 5% Triton-X100 for 10 min and washed again. For blocking bovine serum albumin 1% was added for 10 min. Diluted primary antibody (50 µl) were added and incubated for one hour at room temperature or half an hour at 37°C, cells were washed 3x 10 min with PBS and a secondary antibody (50 µl) was added and incubated for 30 min at room temperature. After washing with PBS 3x 10 min (50 µl) 4´, 6-Diamidino-2-phenylindol (DAPI) (0.1 µg/ml) was added to stain the chromatin in the nucleus. After 10 min at 37°C incubation, cells were washed three times with PBS embedded in mounting medium and monitored using an «Axioscope» microscope (Zeiss).

3.2.1.12. Transfection of cells

Transfection of cells was performed using Effectene® Transfection Reagent (Qiagen). The Effectene Reagent is used in conjunction with the “Enhancer” and the DNA-condensation buffer (Buffer EC). 3.5×10^5 (OV-MZ-32) or 2×10^5 (OvBH-1 or OvCBM) cells per well were grown in 6-well plates (Greiner). After twenty four hours 0.4 µg DNA of MDM2- or WAF1- or BAX- were added to 3.2 µl “Enhancer” mixed and incubated 2-5 minutes at room temperature to condensate DNA by interaction with the “Enhancer”. Then, 10 µl Effectene Transfection Reagent was added, incubated 10 minutes at room temperature to produce condensed Effectene-DNA complexes. Cells were washed with PBS and 1.6 ml fresh medium was added.

The Effectene-DNA complex was mixed with 0.6 ml culture medium and directly added to cells and incubated twenty- four hours at 37°C, 5% CO_2.

As a control a transfection mixture without DNA was used.

3.2.2. Protein methods

3.2.2.1. Protein extraction

Cells were extracted with RIPA buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.5% Na-Deoxycholat, 1% Triton x-100, 0.1% SDS) with complete™-protease inhibitor cocktail (1:25(v/v)) was added to the sediment and resuspended, tubes were left on ice about 15-20 min for extraction proteins, with shaking every 5 min, then sonicated 3 x 1 min at 4°C. Then the suspension was centrifuged for 30 min at 4°C and 16 000xg. The soluble proteins are in the supernatant.

3.2.2.2. Cell fractionation

Cells were harvested and sedimented, 2 ml lysis buffer (1) (10 mM MES, 10 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 5 mM DTT, pH 6.2, 10% (v/v) glycerol, 1% (v/v) NP40) with complete™- protease inhibitor cocktail (1:25 (v/v)) were added, mixed and sonicated for 1 min at 4°C, then the suspension was centrifuged for 7 min at 4°C and 1580xg, supernatant contains the cytoplasmic proteins. The sediment was washed with 2 ml washing buffer (3) (10 mM MES, 10 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 5 mM DTT, pH 6.2, 10% (v/v) glycerol) centrifuged for 7 min at 4°C and 1580xg, 1 ml extraction buffer (2) (25 mM Tris-HCl, 1 mM EDTA, 0.5 mM NaCl, 10 mM β-MSH, pH 10.5, 0.5% (v/v) Triton-X100) was added to the sediment, mixed, left on ice for 40 min for extraction proteins, with shaking every 7-8 min, then neutralization buffer (100 mM Tris-HCl, 10 mM β-MSH, pH 7.0, 0.5% (v/v) TritonX-100) (1:4 (v/v)) was added, centrifuged for 30 min at 4°C and 100 000xg. The soluble proteins of the nucleus are in the supernatant.

3.2.2.3. Determination of protein concentration according to Bradford

Measuring protein concentration in the extraction was performed using the Bio-Rad method which is based on Bradford reagent. One µl cell extract was pipetted to 800 µl distilled water and 200 µl Bradford reagent, mixed and incubated for 5 min at room temperature, then the absorbance was measured

using a spectrophotometer at 595 nm wavelength against extraction buffer as a control. Then, the protein concentration was calculated according to the bovine IgG-standard curve in µg/µl.

3.2.2.4. SDS-polyacrylamide gel electrophorese

SDS-polyacrylamide gel was cast in a vertical stand, in which 4-5 gels could be prepared. Glass plates, alumina plates and spacers were placed in the gel casting stand in a sandwich module, making spaces by spacers to fill the gel in.

The resolving gel was prepared and filled in till ¾ the volume of the spaces and 200 µl Isopropanol were added in each space to avoid air bubbles, after it was polymerized the stacking gel was prepared and filled in the spaces, combs were placed to make columns to fill the protein extract in. After gels were polymerized they were saved in moist atmosphere in a refrigerator.

For both gels we used an acrylamid solution (solution A) with 30% (w/v) acrylamide and 0.8% (w/v) N, N´-Methylenbisacrylamide [Carl Roth GmbH (Rotiphorese® Gel 30)], solution B for resolving gel and solution C for stacking gel. For polymerization of stacking gel 0.1% (w/v) APS and 0.2% (v/v) TEMED were used, and for polymerization of resolving gel 0.05% (w/v) APS and 0.06% (v/v) TEMED were used. Table (3) summarizes the solutions volumes used in preparing gels.

resolving gel	7.5%	10%	12.5%	15%	20%
solution A (ml)	9	12	15	18	24
solution B (ml)	9	9	9	9	9
distilled water (ml)	18	15	12	9	3
APS (µl)	200	200	200	200	200
TEMED (µl)	20	20	20	20	20

stacking gel	4.3%
solution A (ml)	2.2
solution C (ml)	3.8
distilled water (ml)	9

APS	(µl)	100
TEMED	(µl)	40

Table 3: solutions volumes used for preparing polyacrylamide gels.

Two times concentrated sample overlaying buffer (Laemmli, 1970) was added to the cell extract in equal volumes, samples were incubated in a water bath at 95°C for 5 min for denaturation. Then the cell extracts were loaded in the wells of the SDS-polyacrylamide gel in electrophoresis buffer (25 mM Tris-HCl, pH 8.8, 192 mM glycine, 3.5 mM SDS), prestained molecular weight marker (Fermentas) was loaded. Proteins were transferred to a PVDF-membrane (Roche) by a tank blotting with transfer buffer (20 mM Tris-HCl, pH 8.3, 150 mM glycine).

3.2.2.5. Western Blot

Membranes were blocked in blocking buffer [1x PBS, pH 7.4, 0.1% (v/v) Tween 20, 5% (w/v) dry milk] for one hour at room temperature. The membrane was incubated with the primary antibody diluted with washing buffer (2) [1x PBS, pH 7.4, 0.1%(v/v) Tween20, 1% (w/v) dry milk] for one hour at room temperature or overnight at 4°C. Then the membrane was washed with washing buffer (2) three times before incubating with the horseradish peroxidase-conjugated secondary antibody diluted with washing buffer (2) for one hour at room temperature. Then the membrane was washed with washing buffer (1) [1x PBS, pH 7.4, 0.1 %(v/v) Tween20] three times and signals were developed and visualized by the Lumilight system (Roche).

Antibodies:

primary antibodies:	**dilution**
C-20	1: 1000
C-2-2	1:500
cdc25C NT	1:1000
H-6	1:500
DO-1	1: 1000

p53 polyclonal	1: 1000
PARP-1 (Ab-2)	1: 100
GAPDH	1: 1000
Phospho-cdc25C (Ser216)	1: 1000
Hsp90	1: 1000
Nucleolin	1: 1000

secondary antibodies	**Dilution**
Goat anti-mouse POD	1:10000
Goat anti-rabbit POD	1:30000

3.2.2.6. Immunoprecipitation

After treating cells with drugs, cells were extracted using lysis buffer (2) (50 mM Tris-HCl, pH 7.4, 250 mM NaCl, 5 mM EDTA, 100 mM $MgCl_2$, 50 mM NaF, 1 mM DTT, 0.1% Triton x-100, 4%(v/v) complete™- protease inhibitor cocktail). 50% sepharose mix was prepared by mixing protein A sepharose with sepharose CL-4B [1:3(v/v)]. The sepharose mix was washed 3 times with cold PBS and divided into three equal volumes in 3 reaction tubes (first preprecipitation, second preprecipitation, immunoprecipitation). The preprecipitations were made to remove unspecific protein binding. Two to three mg protein extract were transferred to the first preprecipitation tubes and 10% BSA was put in second preprecipitation and immunoprecipitation tubes. All three tubes were incubated on a shaker at 4°C for two hours. After centrifugation the supernatant from the first preprecipitation was added to the sediment of second preprecipitation, and the antibody was added to the sediment of the immunoprecipitation tube. These two tubes were incubated again for two hours on a shaker at 4°C. After centrifugation the supernatant from the second preprecipitation, which contains the protein, was added to the sediment of immunoprecipitation which contains the antibody. The tube was incubated on a shaker at 4°C overnight. The precipitated proteins were collected by centrifugation; they could be used in the experiments which need purified protein like measuring enzyme activity.

3.2.2.7. Phosphatase activity assay

After protein precipitation, 20 µM fluoresceine disulfate (FDP) and 110 µl phosphatase assay buffer (50 mM Tris-HCl, pH 8.0, 1 mM DTT) were added to 40 µl of precipitated protein. FDP will convert to FMP by effect of phosphatase, and then fluoresceine was measured by GeNios spectra fluor plus at 595 nm wavelength, to know the phoshatase activity every 3 min during 2 hours in the immune precipitated protein (cdc25C) before and after treating cells with cytostatica.

3.2.2.8. Luciferase assay

Twenty- four hours after transfection cells were exposed to ultraviolet light (254 nm, 40 J/m2). After sixteen hours the induction of Luciferase activity was measured using the Luciferase Assay System from Promega. Cells were washed with PBS, a cell extract was obtained by adding 200 µl of lysis buffer (3) (25 mM Tris, pH 7.8 using H_3PO_4, 2 mM EDTA, 2 mM DTT, 10% Glycerol, 1% Triton x-100) to each well of a 6-well-plate and incubated for 15 min at room temperature, then the cell lysate was scraped of and moved to reaction tube and centrifuged for 1 min 13000xg. Fifty µl luciferase assay reagent was pipetted in each well of 96-well plate and 10 µl cell lysate were added. Luciferase activity was measured in a luminometer. Extract of CHOAA8Tetoff cells incubated without tetracycline was obtained from A. Hessenauer and used as a positive control.

4. Results

4.1. Growth inhibition in ovarian cancer cells

The current treatment for ovarian cancer includes a combination of surgery and chemotherapy. In the present thesis we wanted to analyze cell cycle checkpoint proteins in ovarian cancer cells after treatment with cytostatica. For this type of analysis we used the freshly established ovarian cancer cells OvBH-1 and OvCBM (kindly provided from Prof. Dr. A. Harlozińska and Dr. Julia Bar, Wrocław University, Poland) and an established cell line OV-MZ-32 (kindly provided by Prof. Dr. I. Runnebaum, Freiburg). With the freshly established cell lines we wanted to be as close as possible to the *in vivo* situation. As ovarian carcinomas show differences in chemosensitivity, we treated the ovarian cancer cells with drugs which are usually applied for the treatment of ovarian carcinoma such as cisplatin, paclitaxel, irinotecan and gemcitabine.

4.1.1. Viability of ovarian carcinoma cells after chemotherapy treatment

To check the effects of chemotherapeutic drugs on ovarian cell lines, and the most effective concentrations of these drugs, we analyzed the sensitivity of the three ovarian cancer cell lines after treatment with the drugs. Twenty-four hours after seeding, cells were treated with cisplatin, irinotecan, paclitaxel or gemcitabine in concentrations ranging from 1 nM to 10 µM. After seventy-two hours treatment the viability of cells was controlled with an MTT assay, and the percentages of surviving cells were calculated in comparison with untreated cells as a control (Fig. 3).

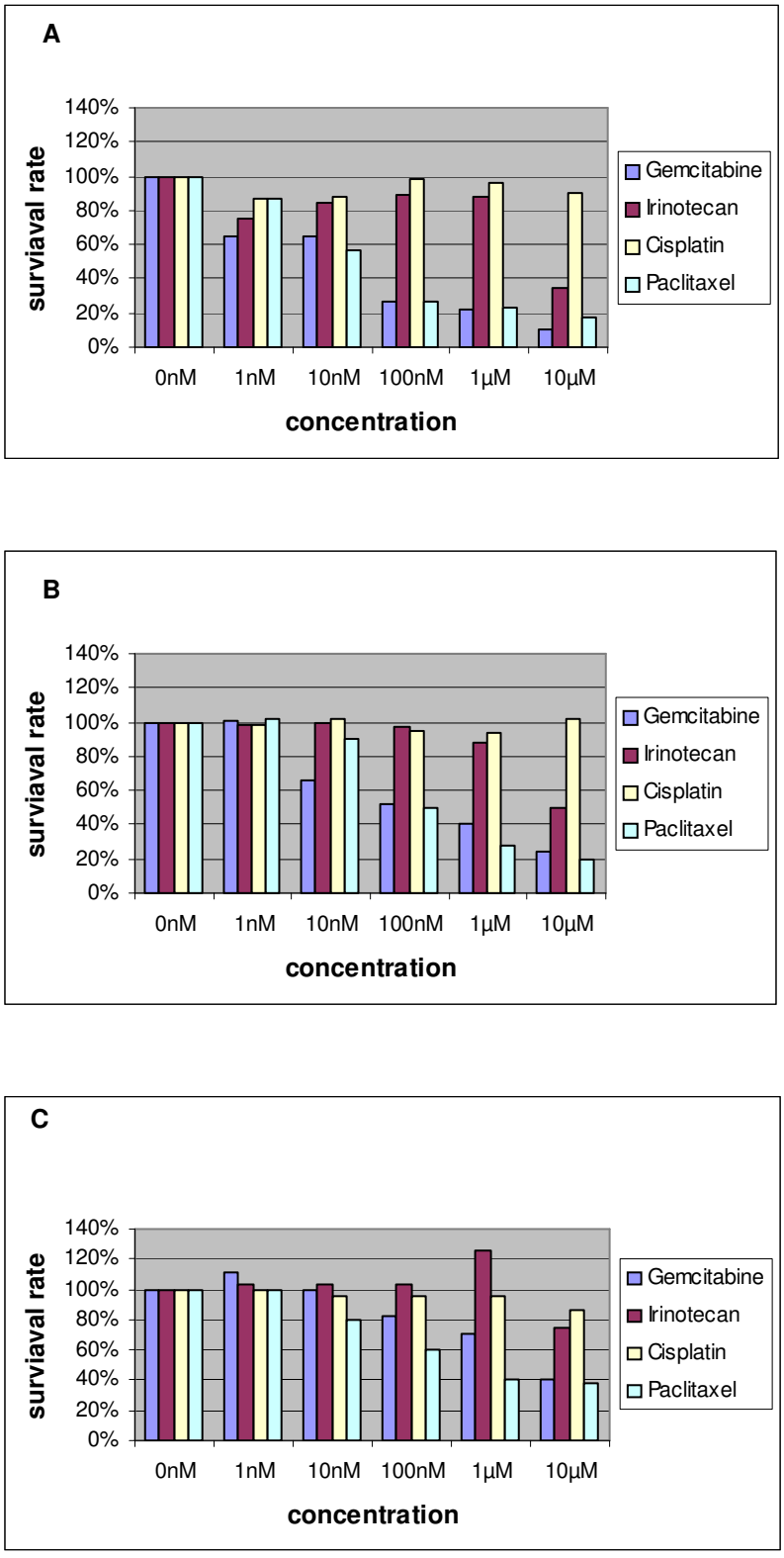
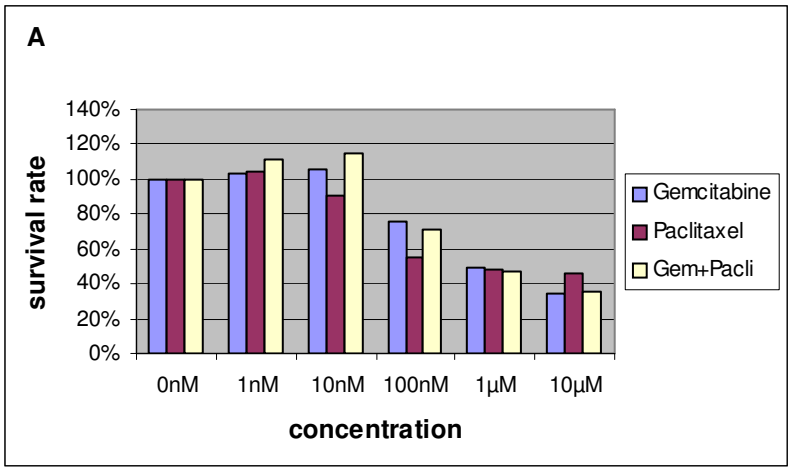
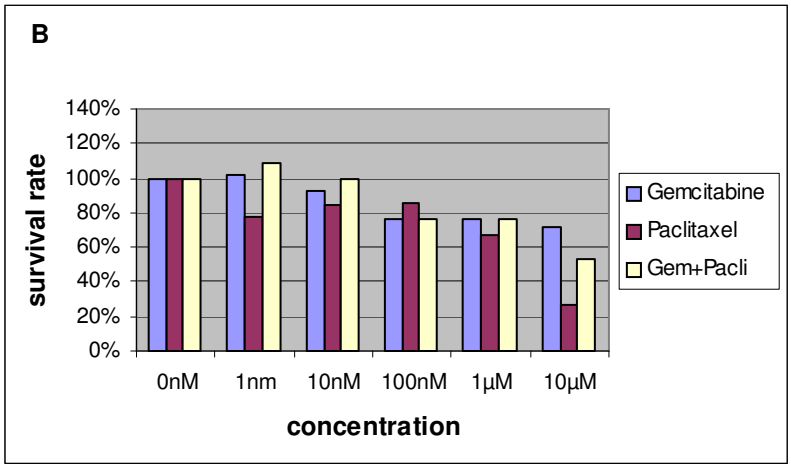


Figure 3: Survival of ovarian cancer cell lines after cytostatica treatment. Cells were treated with the indicated concentrations of cisplatin, irinotecan, paclitaxel or gemcitabine for 72 hours, and then the rate of living cells was determined with an MTT assay. Untreated cells were set to 100%. (A) OV-MZ-32; (B) OvBH-1; (C) OvCBM.

Treatment with cisplatin showed no effect on the growth of all three cell lines. The same result can be observed when using irinotecan, except at the highest concentration of 10 µM; the viability of the cells was reduced to a survival rate of 35% for OV-MZ-32; 50% for OvBH1; and 75% for OvCBM when compared to untreated cells. Gemcitabine was effective at 100 nM concentration for OV-MZ-32, which showed a reduction of 75% in viability; the same concentration was effective for OvBH-1 resulting in reduction of 50% in viability; whereas gemcitabine had only a slight effect on OvCBM cells at a concentration of 100 nM. It was more effective at a concentration of 1 and 10 µM, which caused a reduction in viability by about 35 or 50% respectively. Paclitaxel was already effective at a concentration of 100 nM which caused a reduction of 75% of viability of OV-MZ-32 cells; the same effect can be observed for OvBH-1 which showed a reduction of 50% of viability; OvCBM was affected also at 100 nM concentration causing reduction of 40% of viability. These results show that all three ovarian cancer cell lines were insensitive towards chemotherapy with cisplatin and irinotecan, however, the viability of cells was considerably reduced by using gemcitabine and paclitaxel starting at a concentration of 100 nM.

To analyze if there is a synergistic effect of gemcitabine and paclitaxel, the viability assay was performed after 48 hours using a combination of both drugs (Fig. 4).





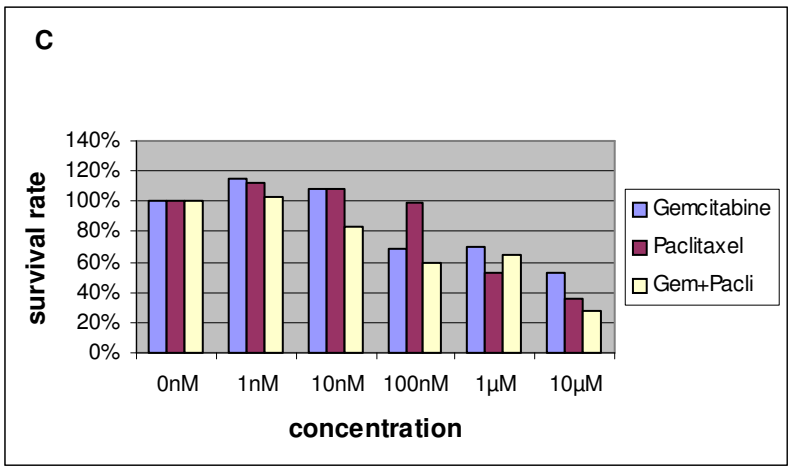


Figure 4: Survival of ovarian cancer cells after treatment with a combination of gemcitabine and paclitaxel. Cells were exposed to the indicated concentrations of gemcitabine, paclitaxel, or a combination of both. After 48 hours treatment the rate of surviving cells was determined with an MTT assay. Untreated cells were set to 100%. (A) OV-MZ-32; (B) OvBH-1; (C) OvCBM.

As shown in Fig. 4 the effect of gemcitabine or paclitaxel or the combination of both after 48 hours on OV-MZ-32 cells started at high concentrations (1 µM); and for OvBH-1 cells only paclitaxel showed an effect at 10 µM concentration; the effect of drugs on OvCBM cells started also at a concentration of 1 µM. One explanation for these results might be that 48 hours treatment is too short to get clear effects on the viability of cells. Therefore the next step was treatment of ovarian cancer cells for 72 hours (Fig. 5).

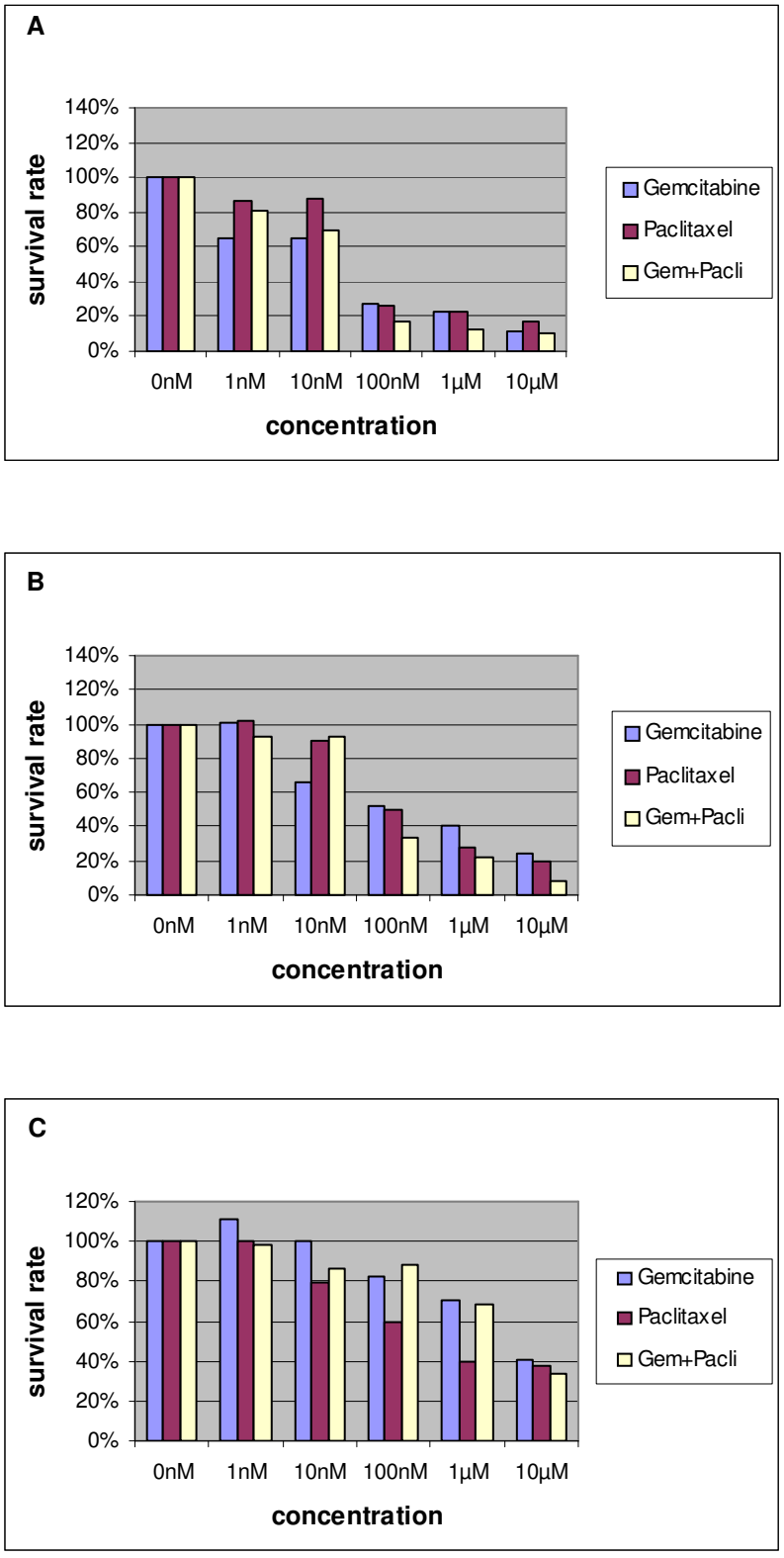


Figure 5: Survival of ovarian cancer cells after treatment with gemcitabine and paclitaxel. Cells were exposed to the indicated concentrations of gemcitabine, paclitaxel, or a combination of both. After 72 hours treatment the rate of surviving cells was determined with

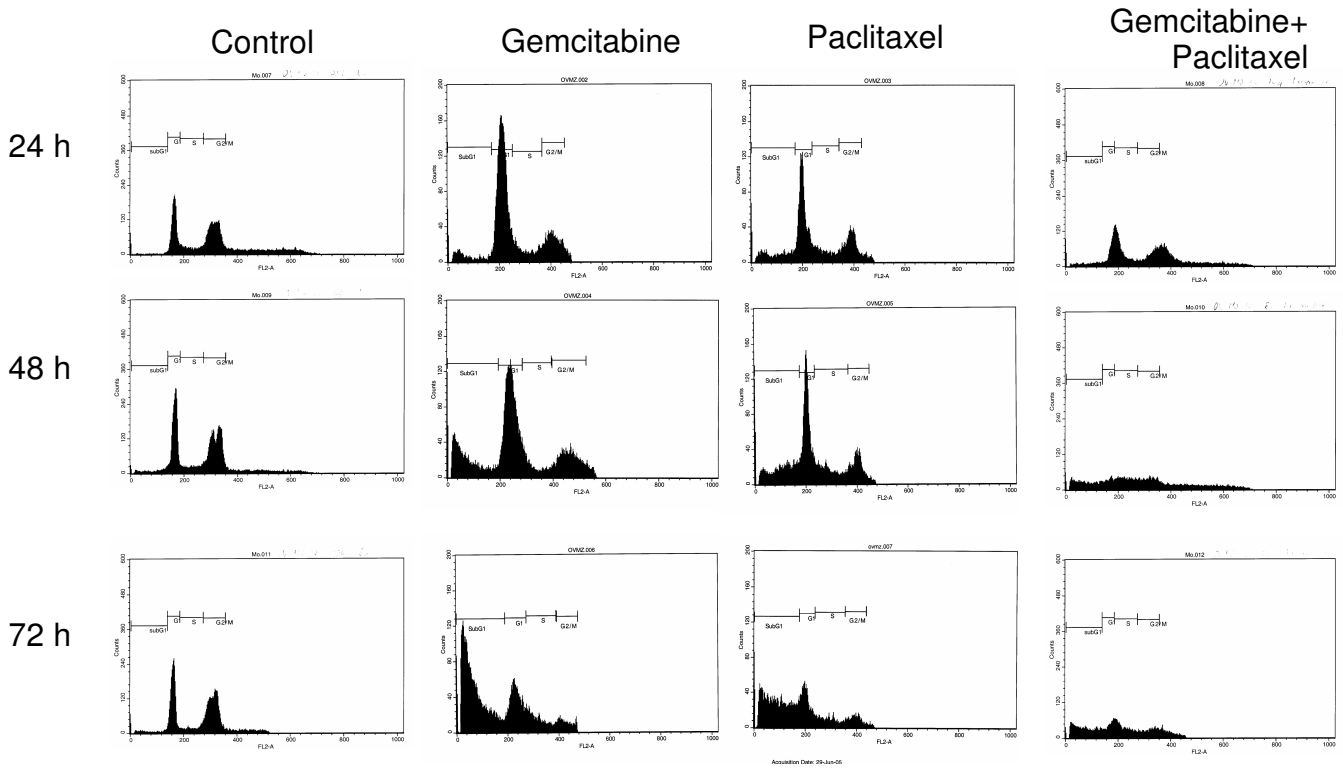
an MTT assay. The untreated cells were set to 100%. (A) OV-MZ-32; (B) OvBH-1; (C) OvCBM.

As shown in Fig. 5 the combination of both gemcitabine and paclitaxel had some but not a significant effect on the reduction of the survival of all three cell lines compared with gemcitabine or paclitaxel alone. Furthermore, viability is somewhat reduced upon long treatment of cells although the effect was limited.

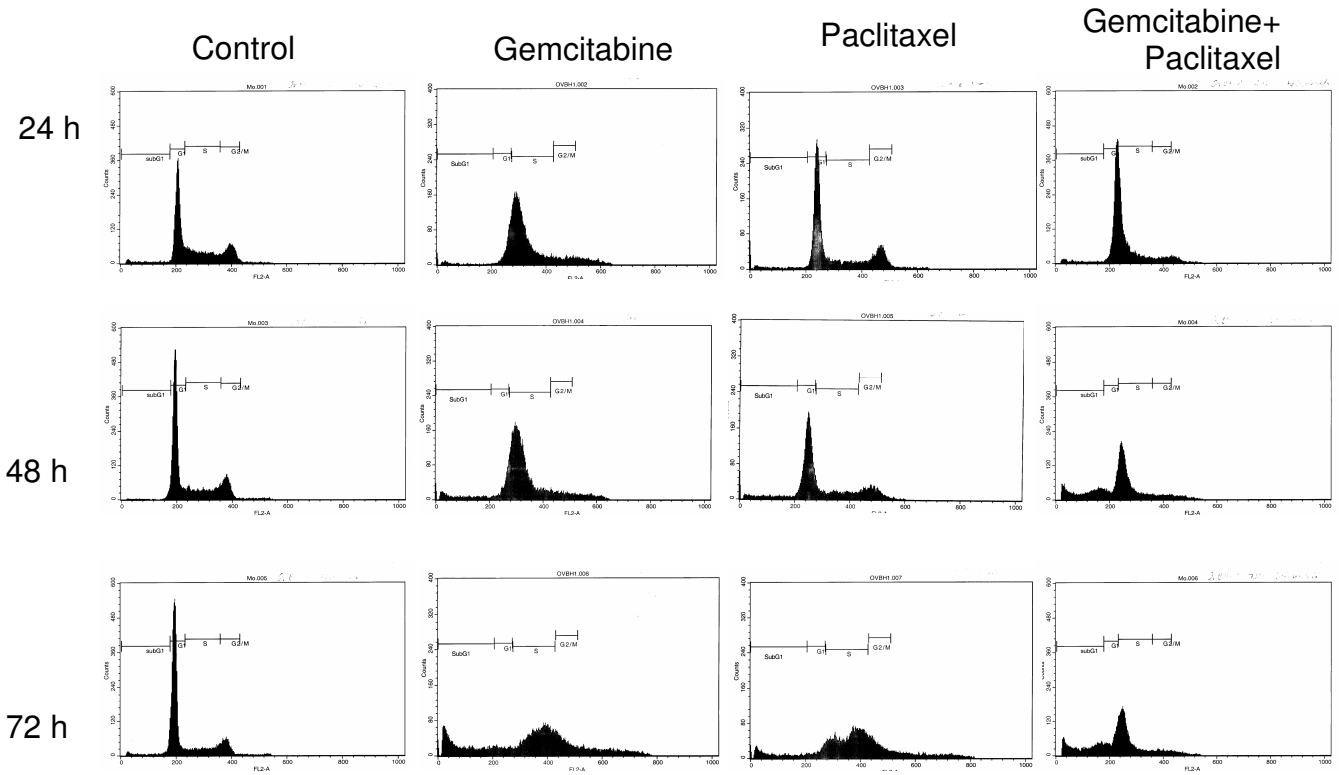
4.1.2. Apoptosis induction in ovarian cancer cells after chemotherapy treatment

As a considerable reduction in metabolically active cells was observed after treatment with gemcitabine or paclitaxel, we ask now if these drugs induce programmed cell death (apoptosis) in ovarian cancer cells. Two different assays were performed to detect apoptosis in treated cells: a cytofluorimetric analysis and the analysis of poly-ADP- ribosyl polymerase cleavage (PARP).
For the cytofluorimetric analysis, 24 hours after seeding, cells were treated with 100 nM gemcitabine, 100 nM paclitaxel or a combination of 100 nM gemcitabine/100 nM paclitaxel for 24, 48, or 72 hours. Then cells were harvested, fixed with ethanol and the DNA stained with propidium iodide. Cell cycle analysis was performed using a Becton-Dickinson FACscan II cytofluorimetrer (Fig. 6).

OV-MZ-32



OvBH-1



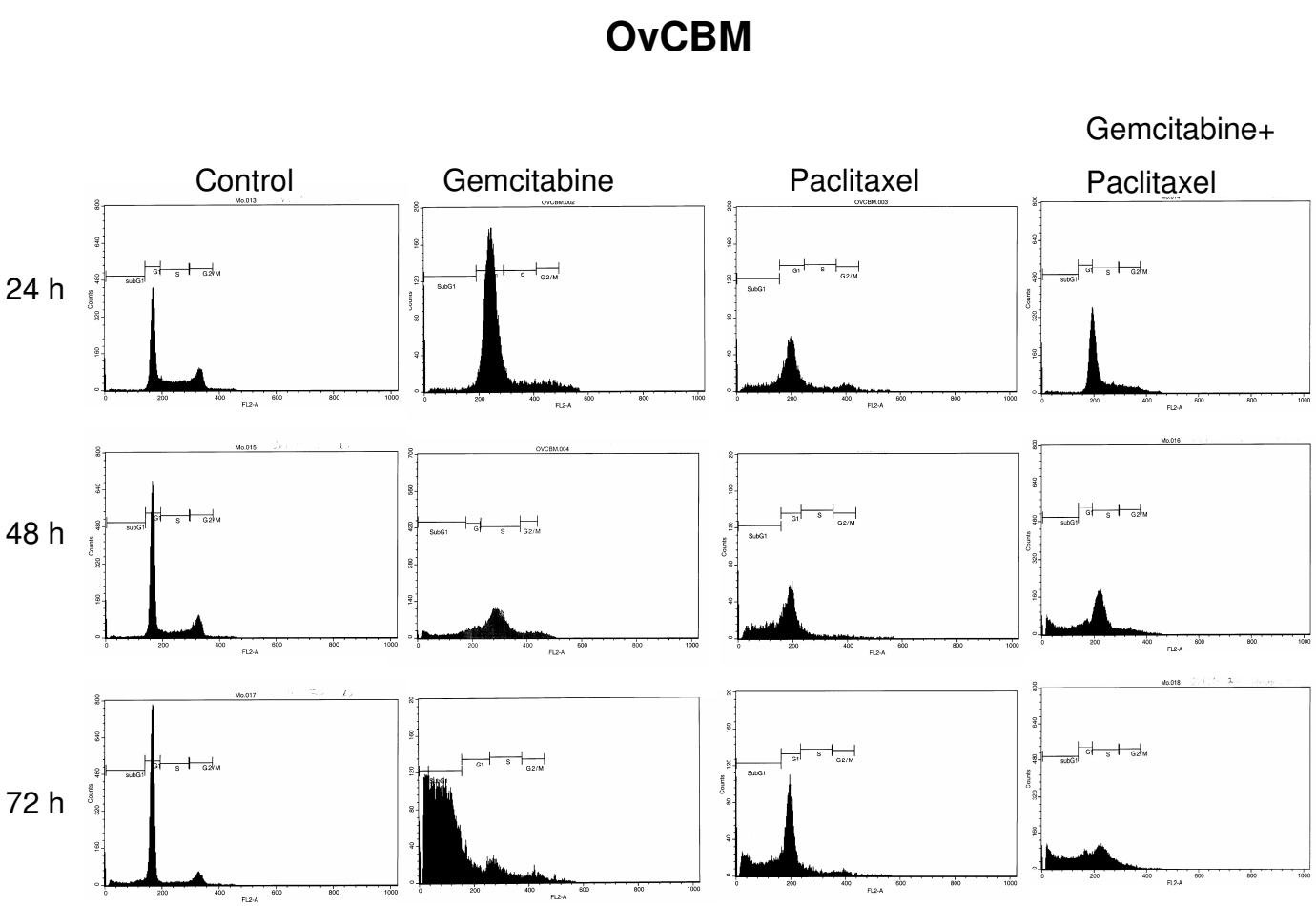


Figure 6: Cytofluorimetric assay of ovarian cancer cells after treatment with cytostatica. Cells were treated with 100 nM gemcitabine, 100 nM paclitaxel or a combination of 100 nM gemcitabine/100 nM paclitaxel for 24, 48 or 72 hours. Cell cycle analysis was performed using the FACScan II cytofluorimeter.

After treating OV-MZ-32 cells with gemcitabine or paclitaxel, the sub-G_1 population increased from 3.1% in control cells to 22.84 or 26.40% in cells treated for 48 hours with gemcitabine or paclitaxel, respectively, whereas it increased to 59.83 or 56.29% in cells treated for 72 hours. The increase after treating cells with a combination of both drugs was up to 19.92 or 31.90% in cells treated for 48 and 72 hours. No dramatic increase was observed in the S-phase cells after treating with gemcitabine or paclitaxel alone, whereas it increased after treating cells with a combination of both drugs from 10% to about 22-27%. The amount of G_1- and G_2- cells decreased in treated cells.

Table 4 summarizes the data for OV-MZ-32 cell line treated with both drugs separately or in combination.

OV-MZ-32	Sub-G_1	G_1	S	G_2/M
Control	3.1	30.53	10	42.95
Gem 24 h	7.03	61.77	11.90	17.98
Gem 48 h	22.84	48.06	9.16	18.58
Gem 72 h	59.83	23.17	11.72	5.47
Pacli 24 h	15.35	45.68	14.68	22.30
Pacli 48 h	26.40	38.40	18.91	16.12
Pacli 72 h	56.29	23.27	11.44	8.77
Gem/Pacli 24 h	5.29	15.24	27.41	18.62
Gem/Pacli 48 h	19.92	10.35	22.55	19.97
Gem/Pacli 72 h	31.90	15.95	25.09	15.98

Table 4: Percentage values of treated and untreated OV-MZ-32 cells in different cell cycle phases. Gem, gemcitabine 100 nM; Pacli, paclitaxel 100 nM.

After treating OvBH-1 cells 72 hours with gemcitabine or paclitaxel alone, sub-G_1 population increased from 4.89% in untreated cells to 23.60% in cells treated with gemcitabine and to 10.27% in cells treated with paclitaxel, whereas there was no increase after 24 or 48 hours. After treating cells with a combination of gemcitabine and paclitaxel the increase could be observed after 48 hours (26.11) % and remained the same after 72 hours (27.65%). I observed an increase in the S-phase cells from about 23.66% up to 42.13, 53.12 and 44.18% in cells treated with gemcitabine, paclitaxel, or a combination of both, respectively. I also observed that the amount of cells in G_1- and G_2- phases decreased. The data for OvBH-1 treated with gemcitabine, paclitaxel, or both are summarized in table 5.

OvBH-1	Sub-G_1	G_1	S	G_2/M
Control	4.89	55.25	23.66	15.58
Gem 24 h	1.62	13.35	69.77	7.44
Gem 48 h	6.82	9.00	70.24	7.20
Gem 72 h	23.60	6.31	42.13	13.57
Pacli 24 h	4.10	57.78	18.10	18.51
Pacli 48 h	8.04	53.34	20.28	14.77
Pacli 72 h	10.27	6.19	53.12	16.19
Gem/Pacli 24 h	3.55	40.72	47.81	6.29
Gem/Pacli 48 h	26.11	14.07	50.34	6.07
Gem/Pacli 72 h	27.95	18.43	44.18	6.51

Table 5: Percentage values of treated and untreated OvBH-1 cells in different cell cycle phases. Gem, gemcitabine 100 nM; Pacli, paclitaxel 100 nM.

Treating OvCBM cells for 72 hours with cytostatica resulted in an increase in sub-G_1 population from about 4.27% in untreated cells to 33.14, 25.19 and 36.61% in cells treated with gemcitabine, paclitaxel or a combination of both, respectively, but the increase was detected earlier in cells treated with a combination of the two drugs, it reached 22.92% after 48 hours. The percentage of cells in S-phase increased from 14.68% in control cells to 61.57 and 52.05% in cells treated for 48 hours with gemcitabine or a combination of the drugs, respectively, but in cells treated with paclitaxel there was only a slight increase. In all treated cells there was a decrease in the amount of G_1- and G_2- cells. Table 6 summarizes the data for OvCBM treated with gemcitabine, paclitaxel and the combination of both.

OvCBM	Sub-G_1	G_1	S	G_2/M
Control	4.27	62.49	14.68	17.25
Gem 24 h	4.70	50.49	25.77	19.12
Gem 48 h	14.81	12.55	61.57	7.49
Gem 72 h	33.14	51.23	7.16	7.18
Pacli 24 h	6.05	45.76	28.15	20.50
Pacli 48 h	8.84	61.79	16.39	12.00
Pacli 72 h	25.19	62.54	8.11	4.26
Gem/Pacli 24 h	1.99	28.74	55.78	11.92
Gem/Pacli 48 h	22.92	15.42	52.05	8.39
Gem/Pacli 72 h	36.61	18.80	38.08	6.17

Table 6: Percentage values of treated and untreated OvCBM cells in different cell cycle phases. Gem, gemcitabine 100 nM; Pacli, paclitaxel 100 nM.

The sub-G_1 population representing cells with fragmented DNA increased in all three cell lines after treatment. Thus, we assumed that treating ovarian cancer cells with gemcitabine or paclitaxel or the combination of both might lead cells to apoptosis. The combination of drugs had no clear visible synergistic effect.

To test the assumption a Western blot analysis was performed to check the cleavage of PARP. PARP is a 116-kDa protein, which is processed by

caspase 3 into fragments of about 89-and 26-kDa. In Western blot analysis the antibody used recognizes the full-length protein and the 89-kDa fragment. Thus we could follow-up the apoptosis induction in response to cytostatica treatment. After seeding cells for 24 hours, cells were treated with 100 nM gemcitabine, 100 nM paclitaxel or a combination of both for 72 hours. After harvesting cells, 150 µg protein were loaded on a 10% SDS polyacrylamide gel and analyzed by Western blot with the PARP-specific antibody (Ab-2). Signals were visualized with POD-conjugated secondary anti-mouse antibody and the Lumilight system. Fig. 7 shows the results.

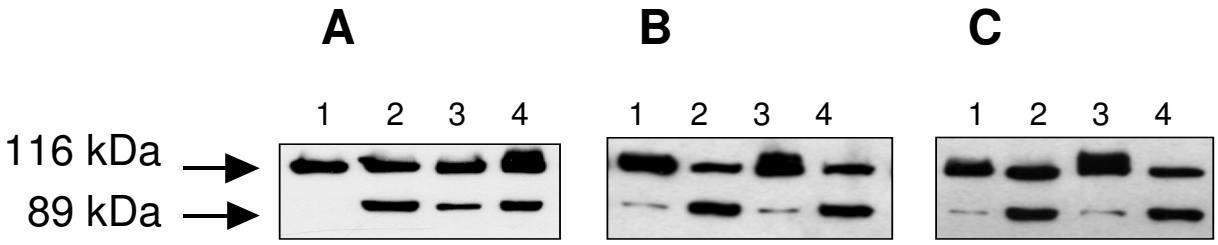


Figure 7: PARP cleavage in ovarian cancer cell lines after cytostatica treatment. Cells were treated for 72 hours with 100 nM gemcitabine (lane 2), 100 nM paclitaxel (lane 3), or combination of both (lane 4); untreated cells served as a control (lane 1). Cells were extracted and 150 µg proteins were analyzed on a 10% SDS polyacrylamide gel followed by Western blot. The two proteins of PARP (116 and 89 kDa) were detected with the PARP-specific antibody (Ab-2), and visualized with a POD-conjugated secondary anti-mouse antibody. (A) OV-MZ-32; (B) OvBH-1; (C) OvCBM.

Fig. 7 shows that in all ovarian cancer cell lines we detected the full length PARP protein before and after treatment. After treatment with gemcitabine in addition we detected the 89-kDa cleavage product of PARP. The same result was obtained for cells treated with paclitaxel and the combination of both drugs. PARP cleavage was reduced after treating the cells with paclitaxel in comparison to gemcitabine and the combination of gemcitabine and paclitaxel. Thus, from these results we conclude that treating ovarian cancer cells with gemcitabine or paclitaxel results in programmed cell death. The combination of both drugs has no co-operative effect.

4.1.3. Generation of oxygen radical species after cytostatica treatment

It is known from a variety of cytostatica that they generate reactive oxygen species (ROS). Cisplatin can lead to ROS generation and p53 transcriptional activity in many cell types (Bragado *et al.*, 2007). Gemcitabine induced apoptosis and led to elevated levels of ROS in pancreatic adenocarcinoma cells (Maehara *et al.*, 2004). In order to analyze whether the combination of gemcitabine and paclitaxel generated ROS the cells were treated with 100 nM gemcitabine and 100 nM paclitaxel for 30, 60, 120, 240 and 360 min, then cells were harvested and exposed to 5 µM CM-H_2DCFDA and the intracellular concentration of ROS was measured using a Becton-Dickinson FACscan II cytofluorimeter (Fig. 8).

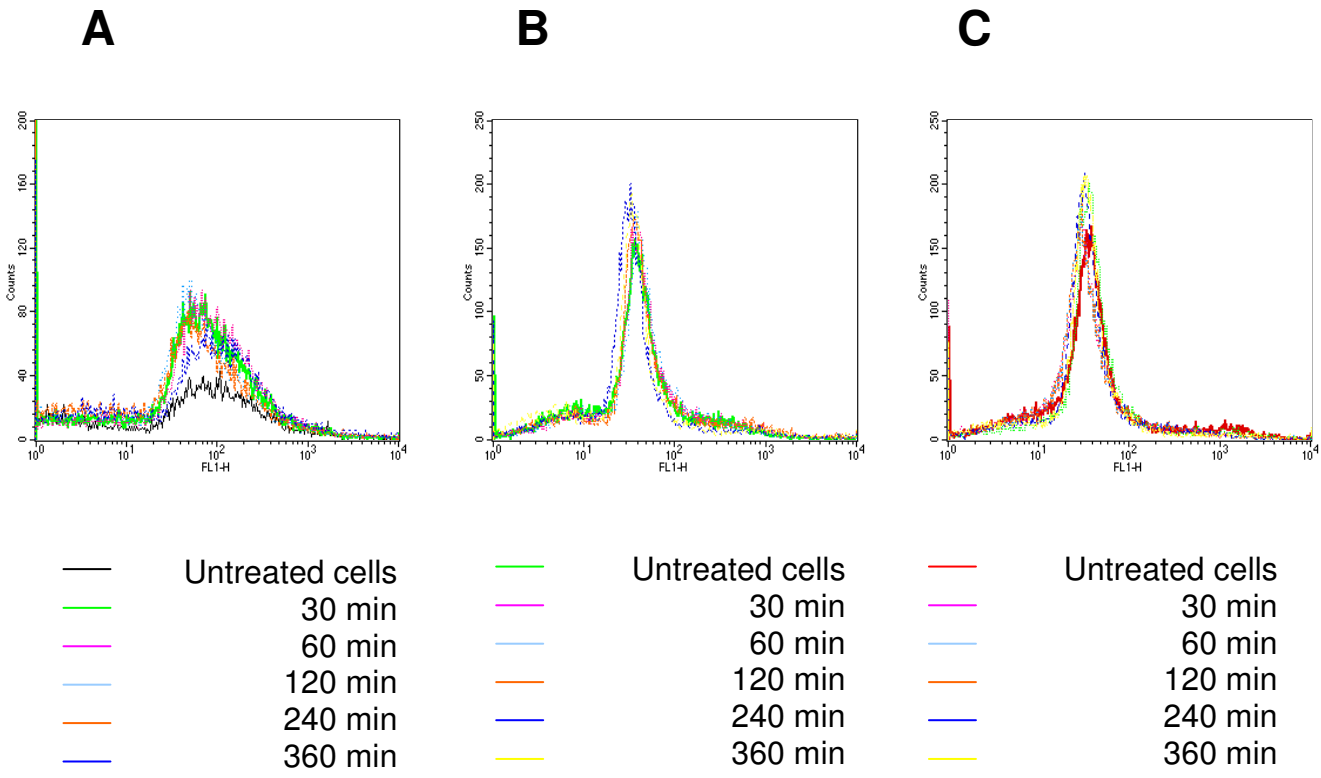


Figure 8: Measurement of intracellular concentration of ROS. Cells were treated with a combination of 100 nM gemcitabine and 100 nM paclitaxel for 30, 60, 120, 240 and 360 min. ROS was measured using the FACScan II cytofluorimeter. (A) OV-MZ-32; (B) OvBH-1; (C) OvCBM

As shown in Fig. 8, treating ovarian cancer cells with a combination of gemcitabine and paclitaxel did not increase the level of ROS in these cells, because no rightward shift in log fluorescence was detected by cytofluorimeter.

4.2. The role of p53 in the apoptosis induction in ovarian cancer cells

4.2.1. p53 expression in ovarian cancer cells

As all three ovarian cancer cell lines are undergoing apoptosis after cytostatica treatment, and as p53 is one of the key players leading to apoptosis, we tried to explore the role of p53 in causing apoptosis after cytostatica treatment. For this purpose we performed a Western blot analysis to detect the p53 expression before and after treatment. Twenty-four hours after seeding, cells were treated with 100 nM gemcitabine, 100 nM paclitaxel, 100 nM irinotecan or 100 nM cisplatin for 72 hours. Then cells were harvested, 20 µg of total protein from OV-MZ-32, 10 µg of total protein from OvBH-1 or 5 µg of total protein from OvCBM were loaded on a 10% SDS polyacrylamide gel and analyzed by Western blot with the p53 specific antibody (DO-1), signals were visualized with a POD-conjugated secondary anti-mouse antibody and the Lumilight system. The GAPDH-specific antibody was used as loading control. The results are shown in Fig. 9.

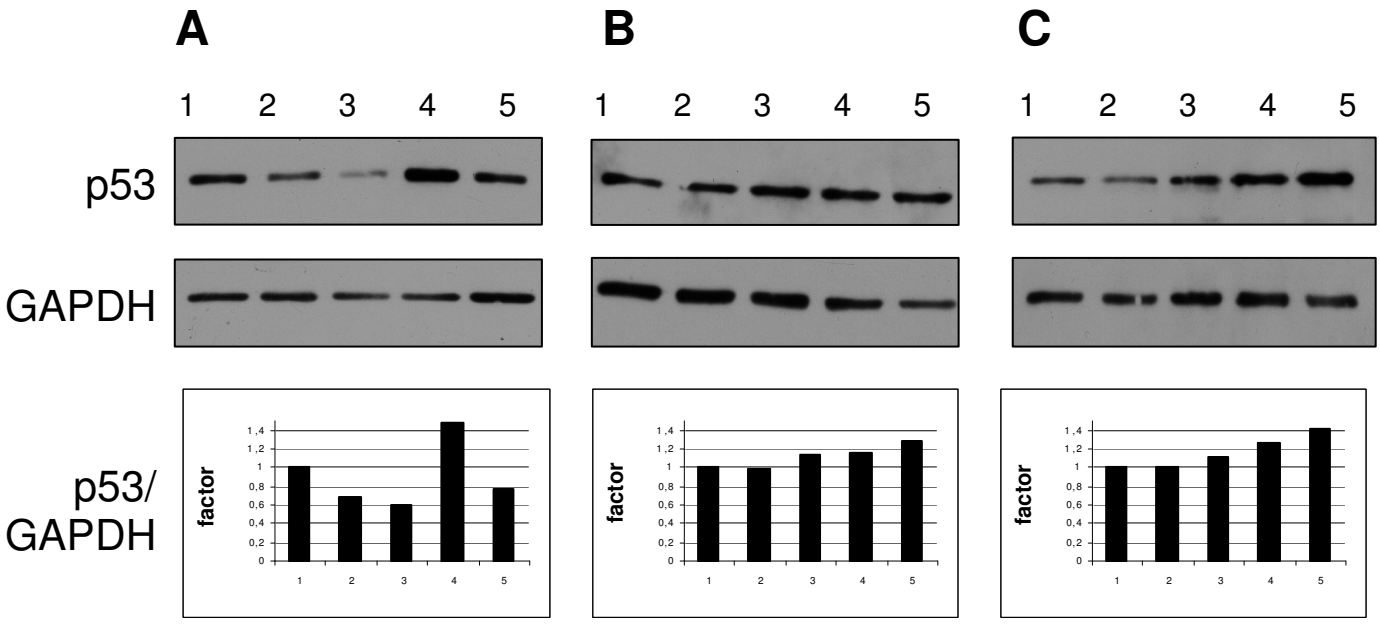


Figure 9: Expression of p53 in ovarian cancer cells after cytostatica treatment. Cells were treated with 100 nM gemcitabine (lane 2), 100 nM paclitaxel (lane 3), 100 nM irinotecan (lane 4) or 100 nM cisplatin (lane 5) for 72 hours; untreated cells served as control (lane 1). Cells were extracted and proteins were analyzed on a 10% SDS polyacrylamide gel followed by Western blot. p53 was detected with the DO-1 antibody and visualized with a POD-conjugated secondary anti-mouse antibody. A GAPDH-specific antibody was used as loading control. (A) OV-MZ-32; (B) OvBH-1; (C) OvCBM. The ratio of p53/GAPDH was calculated to detect changes of the p53 amount in cells before and after treatment.

As shown in Fig. 9 the expression of p53 increased only after treating OV-MZ-32 cells with irinotecan, whereas it decreased when cells were treated with other drugs, but when this experiment was repeated many times the p53 expression varied considerably. After treating OvBH-1 cells with gemcitabine the p53 expression did not change, whereas it increased about 17, 19 and 28% after treating cells with paclitaxel, irinotecan or cisplatin, respectively. The same result was observed for OvCBM cells where the expression of p53 remained the same when cells were treated with gemcitabine whereas it increased 10, 26 and 42% when cells were treated with paclitaxel, irinotecan or cisplatin, respectively. For these results it was impossible to assess a clear statement about the effect of the drugs on the p53 expression. The variability seems to be within experimental errors.

As we found in the MTT assay that the most effective drugs in these ovarian cancer cells were gemcitabine and paclitaxel, we performed a Western blot analysis after treating cells with these two drugs separately or in combination. Cells were seeded for 24 hours, and then treated for 72 hours with 100 nM gemcitabine, 100 nM paclitaxel or combination of both. Cells were harvested, and 30 µg of total protein from cell extracts were loaded on a 10% SDS polyacrylamide gel and analyzed by Western blot with the p53 specific antibody (DO-1), signals were visualized with a POD-conjugated secondary anti-mouse antibody and the Lumilight system. The GAPDH-specific antibody was used as loading control. The results of these experiments are shown in Fig. 10.

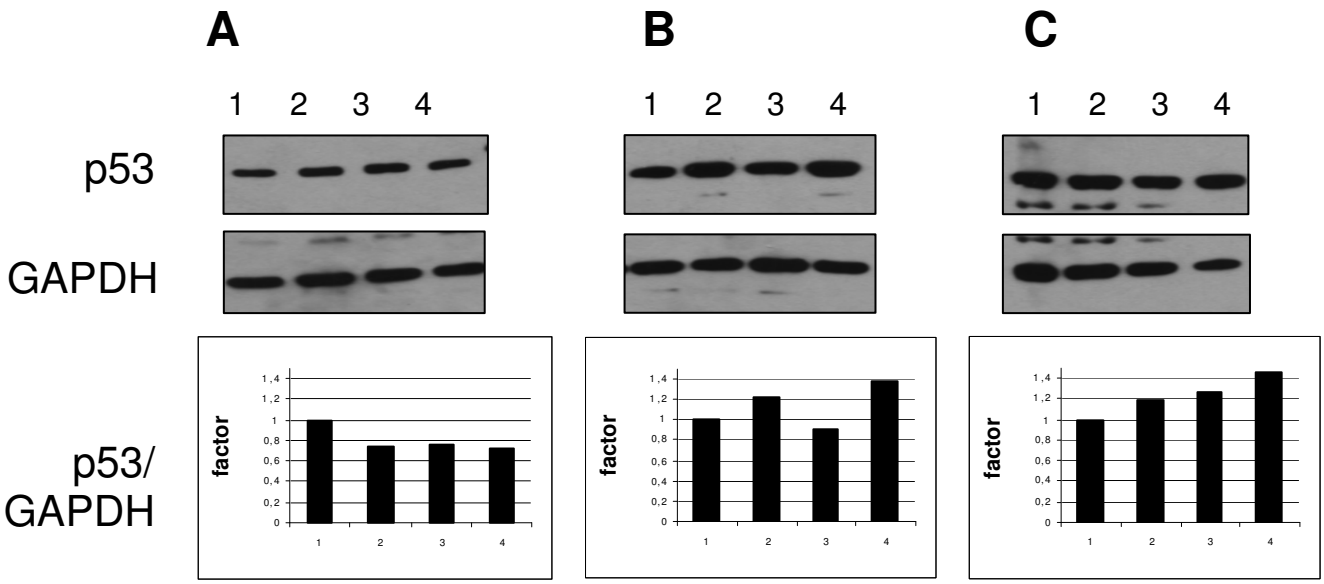


Figure 10: Expression of p53 in ovarian cancer cells after treatment with cytostatica. Cells were treated with 100 nM gemcitabine (lane 2), 100 nM paclitaxel (lane 3) or a combination of both (lane 4) for 72 hours; untreated cells served as control (lane 1). Cells were extracted and 30 µg of total proteins were analyzed on a 10% SDS polyacrylamide gel followed by Western blot. p53 was detected with DO-1 antibody and visualized with a POD-conjugated secondary anti-mouse antibody. A GAPDH-specific antibody was used as loading control. (A) OV-MZ-32; (B) OvBH-1; (C) OvCBM. The ratio of p53/GAPDH was calculated to detect changes of the p53 amount in cells before and after treatment.

As shown in Fig. 10, p53 expression in OV-MZ-32 cells was decreased about 24, 22 and 25% after treating cells for 72 hours with gemcitabine, paclitaxel or a combination of both, respectively. For OvBH-1 cells the expression of p53 was decreased 8% after treating cells with paclitaxel, whereas it increased about 25 and 40% when cells were treated with gemcitabine or a combination of both drugs. Also it increased in OvCBM cells about 20, 30 and 50% when cells were treated with gemcitabine, paclitaxel or a combination of both, respectively.

As DO-1 antibody detects the N-terminus between the amino acids (11-25) of p53 this region of p53 is heavily phosphorylated by different protein kinases

and it might be that DO-1 recognizes only a subclass of p53 generated by posttranslational modifications. Therefore the experiment described before we repeated but p53 was now detected with a polyclonal antibody which should detect all forms of p53 present in a cell. After seeding cells for 24 hours, cells were treated with 100 nM gemcitabine, 100 nM paclitaxel or a combination of both, then cells were harvested, 20 µg of total protein from OV-MZ-32, 10 µg of total protein from OvBH-1 or 5 µg of total protein from OvCBM were loaded on 10% SDS polyacrylamide gels. Proteins were analyzed by Western blot with the p53 polyclonal antibody, signals were visualized with a POD-conjugated secondary anti-rabbit antibody and the Lumilight system. The GAPDH-specific antibody was used as loading control (Fig.11).

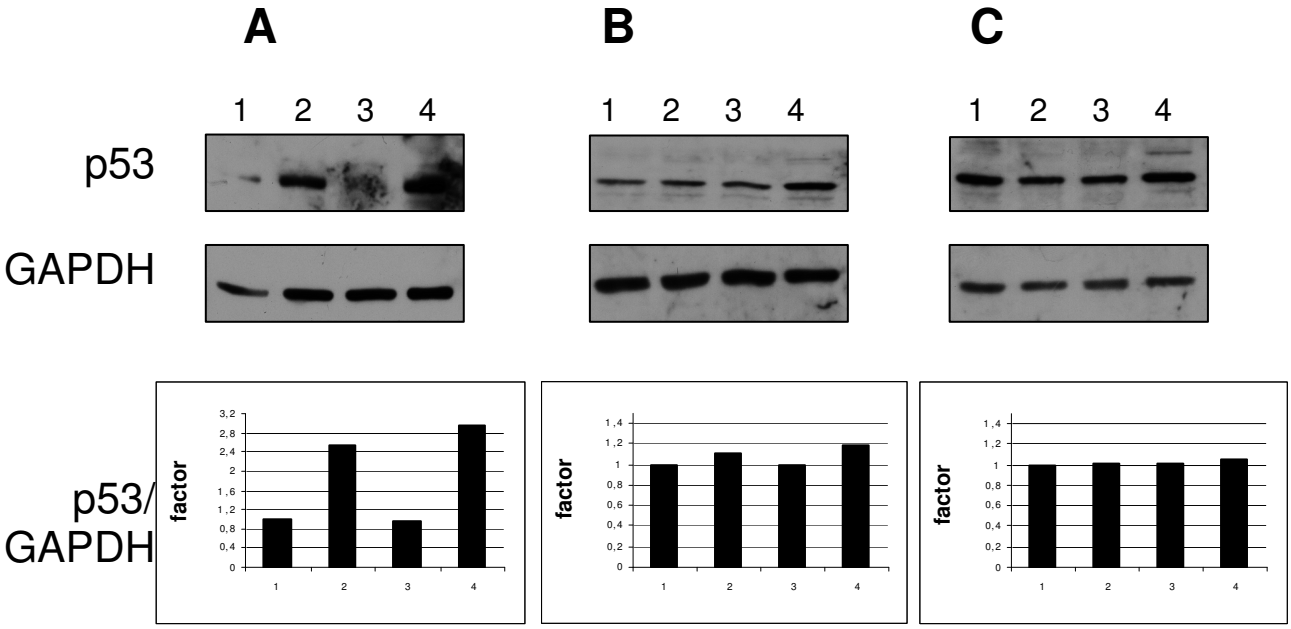


Figure 11: p53 expression in ovarian cancer cells after cytostatica treatment. Cells were treated with 100 nM gemcitabine (lane 2), 100 nM paclitaxel (lane 3), or a combination of both (lane 4) for 72 hours; untreated cells served as control (lane 1). Cells were extracted and proteins were analyzed on a 10% SDS polyacrylamide gel followed by Western blot. p53 was detected with the p53 polyclonal antibody and visualized with a POD-conjugated secondary anti-rabbit antibody. A GAPDH-specific antibody was used as loading control. (A) OV-MZ-32; (B) OvBH-1; (C) OvCBM. The ratio of p53/GAPDH was calculated to detect changes of the p53 amount in cells before and after treatment.

As shown in Fig. 11 the expression of p53 in OV-MZ-32 cells remained the same when cells were treated with paclitaxel and it increased about two or three folds after treating cells with gemcitabine or a combination of both drugs, respectively. In OvBH-1 cells it increased 12 and 20% after treatment of cells with gemcitabine or a combination of gemcitabine and paclitaxel, it remained the same after treatment with paclitaxel. In OvCBM cells the p53 expression did not change when cells were treated with drugs

So far the experiments about alterations in the p53 expression were performed after 72 hours, more extensive alterations might occur earlier therefore I looked for an induction of p53 after different earlier times.
Twenty-four hours after seeding, cells were treated with a combination of 100 nM gemcitabine and 100 nM paclitaxel. Then cells were harvested after 2, 4, 16 or 24 hours. Thirty µg of total proteins from the cell extracts were loaded on a 10% SDS polyacrylamide gel and analyzed by Western blot with the p53 specific antibody DO-1, signals were visualized with a POD-conjugated secondary anti-mouse antibody and the Lumilight system. The GAPDH-specific antibody was used as loading control (Fig. 12).

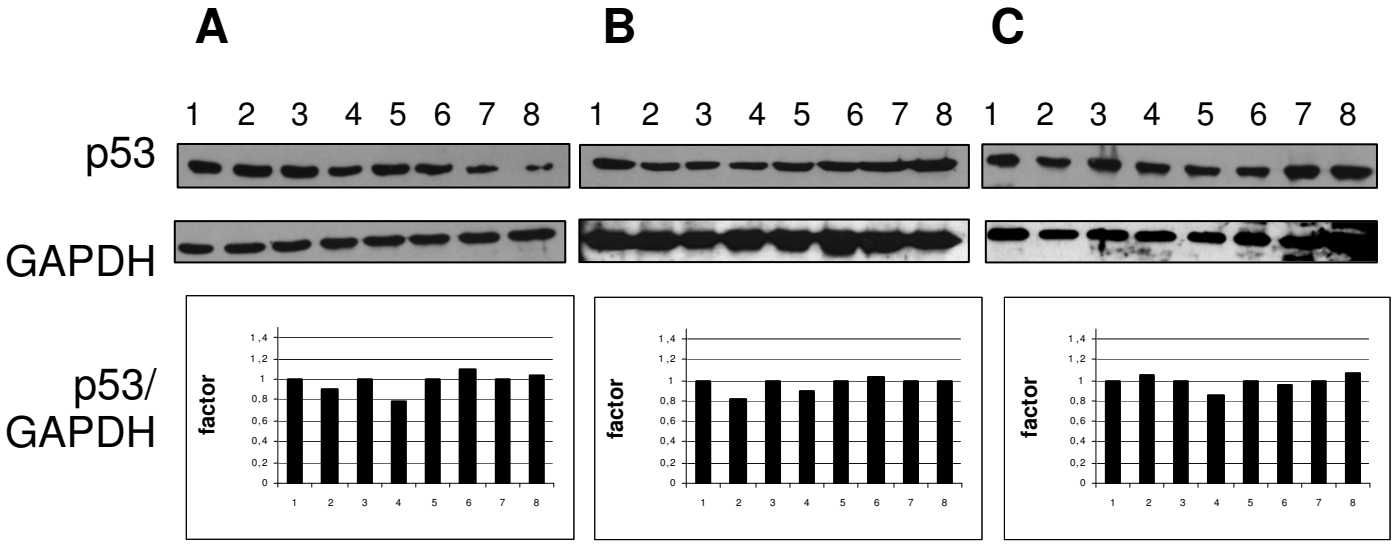


Figure 12: Expression of p53 in ovarian cancer cells after different time points of treatment. Cells were treated with a combination of 100 nM gemcitabine and 100 nM paclitaxel for 2

hours (lane 2), 4 hours (lane 4), 16 hours (lane 6) or 24 hours (lane 8); untreated cells served as control (lanes 1, 3, 5, and 7). Cells were extracted and 30 µg of total proteins were analyzed on a 10% SDS polyacrylamide gel followed by Western blot. p53 was detected with DO-1 antibody and visualized with a POD-conjugated secondary anti-mouse antibody. A GAPDH-specific antibody was used as loading control. (A) OV-MZ-32; (B) OvBH-1; (C) OvCBM. The ratio of p53/GAPDH was calculated to detect changes of the p53 amount in cells before and after treatment.

As shown in Fig. 12, after treating OV-MZ-32 cells with a combination of gemcitabine and paclitaxel the p53 expression was decreased 8% after 2 hours of treatment, and this decrease reached to about 20% after 4 hours, and then it increased again to become the same as it is in the control after 24 hours treatment. Also in OvBH-1 cells the expression of p53 decreased about 15% after 2 hours and then it increased again to the same value of the control after 24 hours. The expression of p53 in OvCBM cells remained almost the same after treating cells with a combination of gemcitabine and paclitaxel. Thus, we could say that the expression of p53 during 24 hours of treatment varied in the same range without any clear trend.

In order to exclude again that I might have detected only a particular subset of p53 also this experiment was repeated with a polyclonal antibody.
After seeding cells for 24 hours, cells were treated with a combination of 100 nM gemcitabine and 100 nM paclitaxel. Then cells were harvested after 2, 4, 16 or 24 hours. Thirty µg of total proteins from the cell extracts were loaded on a 10% SDS polyacrylamide gel and analyzed by Western blot with the p53 polyclonal antibody, signals were visualized with a POD-conjugated secondary anti-rabbit antibody and the Lumilight system. The GAPDH-specific antibody was used as loading control (Fig. 13).

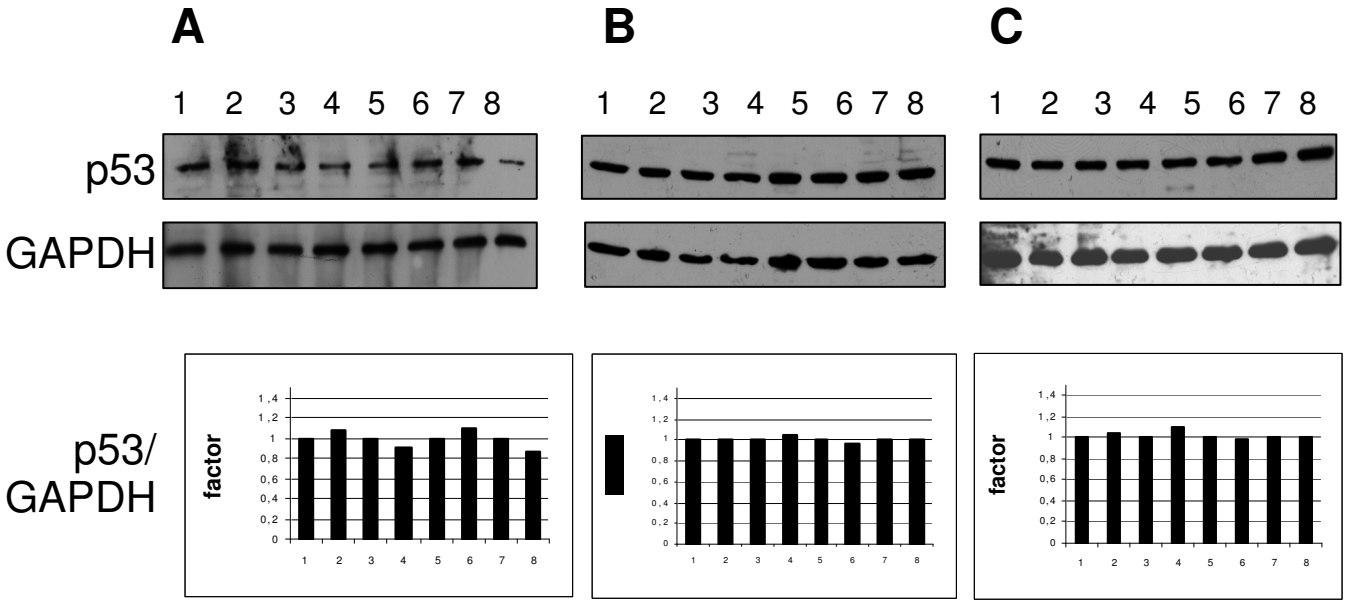


Figure 13: Expression of p53 in ovarian cancer cells after different time points of treatment. Cells were treated with a combination of 100 nM gemcitabine and 100 nM paclitaxel after 2 hours (lane 2), 4 hours (lane 4), 16 hours (lane 6) or 24 hours (lane 8); untreated cells served as control (lanes 1, 3, 5, and 7). Cells were extracted and 30 µg of total proteins were analyzed on a 10% SDS polyacrylamide gel followed by Western blot. p53 was detected with the p53 polyclonal antibody and visualized with a POD-conjugated secondary anti-rabbit antibody. A GAPDH-specific antibody was used as loading control. (A) OV-MZ-32; (B) OvBH-1; (C) OvCBM. The ratio of p53/GAPDH was calculated to detect changes of the p53 amount in cells before and after treatment.

As shown in Fig. 13, the expression of p53 in OvBH-1 cells detected with the polyclonal anti p53- antibody was almost the same during 24 hours treatment with a combination of gemcitabine and paclitaxel. In OvCBM cells the p53 expression increased about 11% after 4 hours of treatment then decreased again to the control value. In general the variations within the amount of p53 after treatment with a combination of gemcitabine and paclitaxel are within experimental errors.

To know if there are much more earlier effects on p53 expression after cytostatica treatment, we treated cells for shorter times with a combination of gemcitabine and paclitaxel. Twenty-four hours after seeding, cells were treated with a combination of 100 nM gemcitabine and 100 nM paclitaxel for

15, 30, 45, 60, 120, 240 and 360 min, then cells were harvested, and 10 µg total protein from cell extract of OV-MZ-32, 5 µg total protein from cell extract of OvBH-1 or 5 µg total protein from cell extract of OvCBM were loaded on 10% SDS polyacrylamide gels and analyzed by Western blot with the p53 specific antibody DO-1, signals were visualized with a POD-conjugated secondary anti-mouse antibody and the Lumilight system. The GAPDH-specific antibody was used as loading control (Fig. 14).

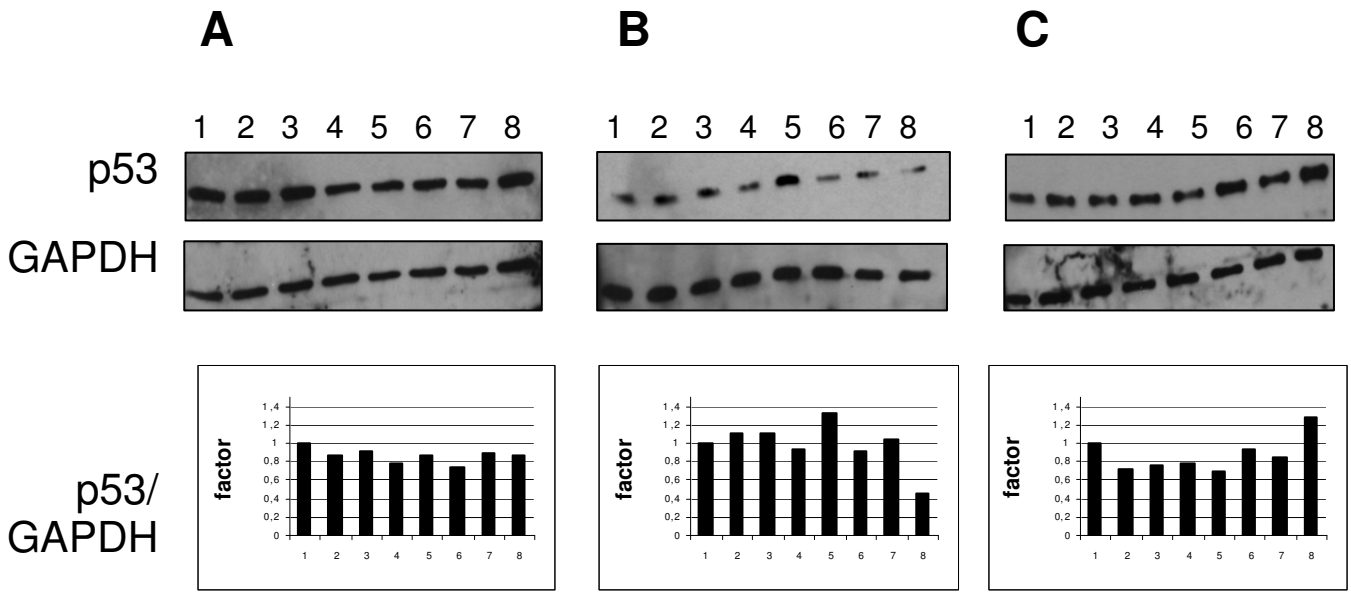


Figure 14: p53 expression in ovarian cancer cells after different time points of treatment. Cells were treated with a combination of 100 nM gemcitabine and 100 nM paclitaxel for 15 min (lane 2), 30 min (lane 3), 45 min (lane 4), 60 min (lane 5), 120 min (lane 6), 240 min (lane 7) and 360 min (lane 8); untreated cells served as control (lane 1). Cells were extracted and proteins were analyzed on a 10% SDS polyacrylamide gel followed by Western blot. p53 was detected with DO-1 antibody and visualized with a POD-conjugated secondary anti-mouse antibody. GAPDH was analyzed as loading control. (A) OV-MZ-32; (B) OvBH-1; (C) OvCBM. The ratio of p53/GAPDH was calculated to detect changes of the p53 amount in cells before and after treatment.

As shown in Fig. 14, the amount of p53 varied from time point to time point but there is no clear trend, thus, we conclude that the expression is in the same range within normal experimental errors.

We observe from Figs. 12 through 14 that the p53 expression was different when different antibodies were used, and that indicates to the presence of particular subset of p53 in these cells. Furthermore, these data indicate that there seems to be an alteration in the total amount of p53. These data suggest that a particular subclass of p53 might change upon treatment with these cytostatica. Since these observations go beyond of the scope of the present thesis this path was not followed any further.

4.2.2. Transcription factor activity of p53 in ovarian cancer cells

The results shown so far demonstrated that p53 does not clearly respond to cytostatica treatment, although we got some indications that a particular subclass of p53 might show an altered expression. OvBH-1 cells were known to express wild type p53. However it was already shown that there seems to be a particular subclass of p53 presents in these cells (Bar *et al*., 2002). OV-MZ-32 cells express a deletion mutant around the cdk1 phosphorylation site (Wu *et al*., 2000). The status of p53 gene in OvCBM cells is not known. For that we decided to analyze the main function of p53 which is transcriptional activator in the ovarian cancer cells used in this study. For that reason a Luciferase assay using reporter constructs of BAX, MDM2 and WAF1 was performed. BAX is a pro-apoptotic gene (Schuler and Green, 2005), MDM2 is one of the important regulator of the activity and stability of p53 (Vousden and Lu, 2002) and WAF1 is the cyclin-dependent kinase inhibitor (Dash and El-Deiry, 2005).

3.5×10^5 (OV-MZ-32) or 2×10^5 (OvBH-1 or OvCBM) cells per well were seeded for 24 hours on 6 well plates, then the reporter constructs for WAF1, MDM2 or BAX were transfected in the cells. Twenty-four hours after transfection cells were exposed to UV (254 nm, 40 Jol/m2) which is known to induce the transcription factor activity, 16 hours after irradiation cells were harvested, 10 µl cell extract was added to 50 µl Luciferase assay reagent, and the Luciferase activity was measured (Figs. 15-17).

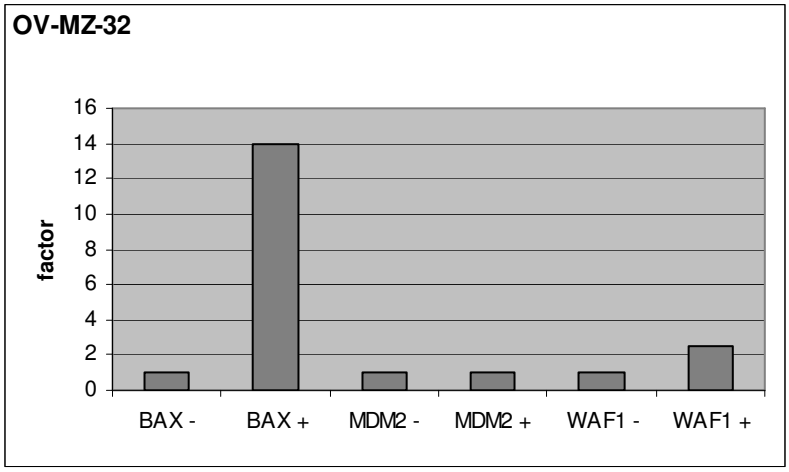


Figure 15: Luciferase assay in OV-MZ-32 cells. Cells were transfected with the reporter constructs for the WAF1, MDM2 or BAX genes and irradiated with UV. Sixteen hours after transfection Luciferase activity was measured. (-) non irradiated cells; (+) irradiated cells.

As shown in Fig. 15, p53 was acting as a transcription factor in OV-MZ-32 cells only on the BAX promoter.

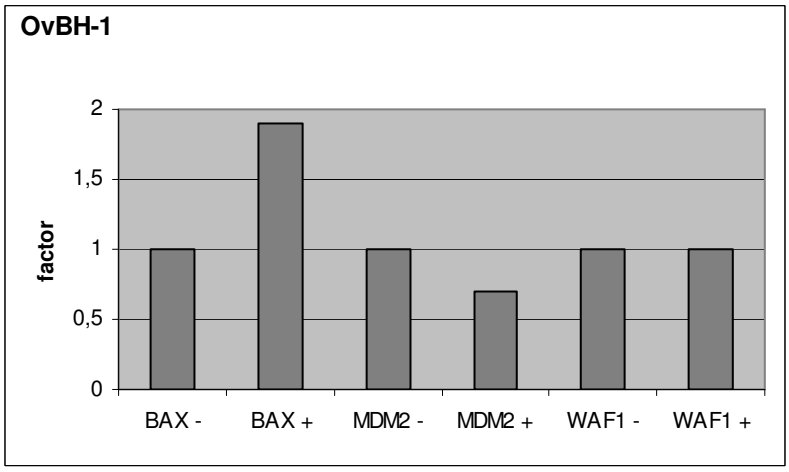


Figure 16: Luciferase assay in OvBH-1 cells. Cells were transfected with the reporter constructs for the WAF1, MDM2 or BAX genes and irradiated with UV. Sixteen hours after transfection Luciferase activity was measured. (-) non irradiated cells; (+) irradiated cells.

As shown in Fig. 16 also in OvBH-1 cells p53 acts weakly as a transcription factor only on the BAX promoter. The same experiment was also performed with OvCBM cells

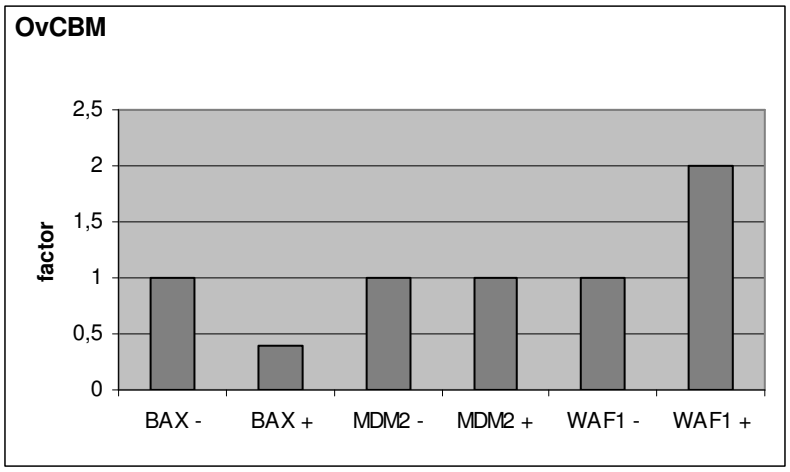


Figure 17: Luciferase assay in OvCBM cells. Cells were transfected with the reporter constructs for the WAF1, MDM2 or BAX gene and irradiated with UV. Sixteen hours after transfection Luciferase activity was measured. (-) non irradiated cells; (+) irradiated cells.

As shown in Fig. 17, in OvCBM cells and different from what was shown for the other two cell lines, p53 acts weakly as transcription factor on the WAF1 promoter only but not on the BAX and MDM2 promoter. Thus, these results showed that p53 did not function as wild type p53, instead it seems to be selectively active just for particular genes in these three cell lines. However, it is very interesting to notice that p53 activates the BAX promoter because BAX is an apoptosis promoting factor.

4.3. The effect of cytostatica treatment on cdc25C in ovarian cancer cells

cdc25C is one of the three members of phosphatases which are implicated in the cell cycle regulation. It activates the M-phase specific kinase, cdk1/cyclin B1. Furthermore the cdc25C gene has been shown to be a novel target for transcriptional down regulation by p53 (St Clair and Manfredi, 2006). In response to DNA damage p53 represses the promoter of cdc25C to maintain

the stability of G_2/M checkpoint, thereby preventing tumor formation and progression (Schwindling *et al*., 2004; St Clair and Manfredi, 2006). We tried to know the effects of cytostatica treatment on cdc25C.

4.3.1. The cdc25C expression in ovarian cancer cells before and after treatment

Western blot analysis was performed to show the expression of cdc25C before and after treatment. Cells were seeded for 24 hours, and then treated with 100 nM gemcitabine, 100 nM paclitaxel or a combination of both for 72 hours. After harvesting cells 75 µg total proteins from cell extracts were loaded on a 12.5% SDS polyacrylamide gel and analyzed by Western blot with the cdc25C specific antibody C-2-2. Signals were visualized with a POD-conjugated secondary anti-mouse antibody and the Lumilight system. The GAPDH-specific antibody was used as loading control (Fig. 18)

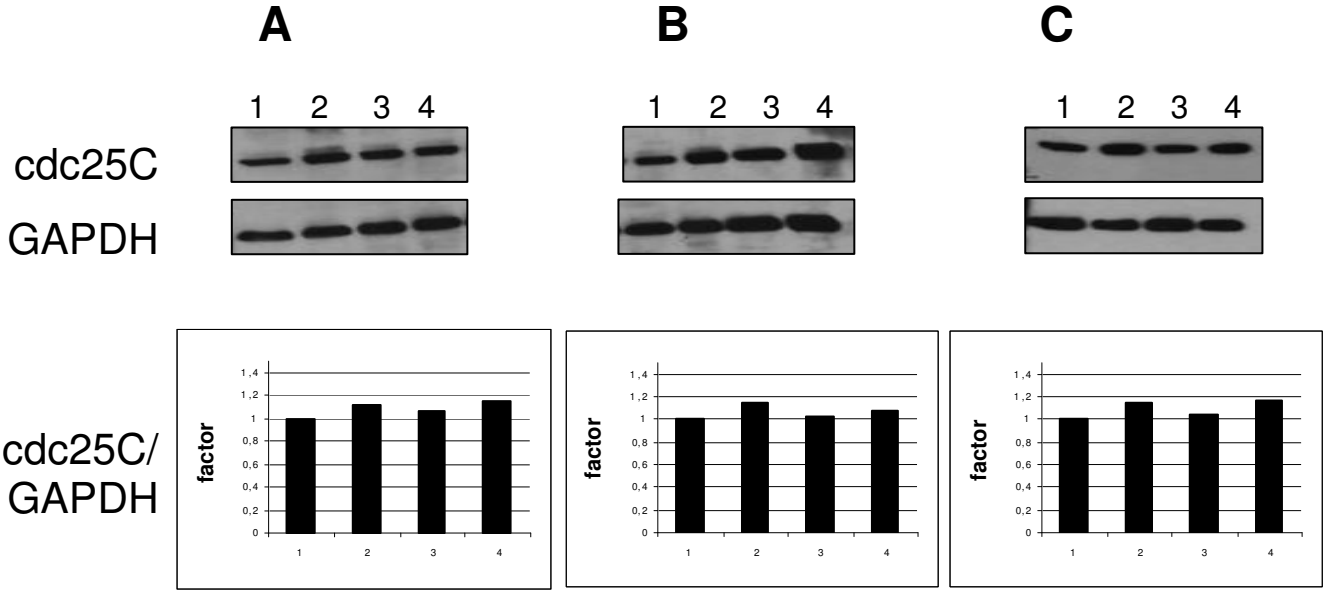


Figure18: Expression of cdc25C in ovarian cancer cells after cytostatica treatment. Cells were treated with 100 nM gemcitabine (lane 2), 100 nM paclitaxel (lane 3) or a combination of both (lane 4) for 72 hours; untreated cells served as control (lane 1). Cells were extracted (75) µg and proteins were analyzed on a 12.5% SDS polyacrylamide gel followed by Western blot. cdc25C was detected with C-2-2 antibody and visualized with a POD-conjugated secondary anti-mouse antibody. GAPDH was used as loading control. (A) OV-MZ-32; (B) OvBH-1; (C)

OvCBM. The ratio of cdc25C/GAPDH was calculated to detect changes of the cdc25C amount in cells before and after treatment.

As shown in Fig. 18, cdc25C expression in OV-MZ-32 cells increased about 12, 8 and 16% after treating cells for 72 hours with gemcitabine, paclitaxel or a combination of both, respectively. In OvBH-1 cells the expression of cdc25C remained almost the same after treating cells with paclitaxel, whereas it increased about 16 and 8% after treating cells with gemcitabine or a combination of both drugs. After treating with gemcitabine or a combination of both drugs it increased in OvCBM cells by about 17%, and it remained almost the same when cells were treated with paclitaxel.

The C-2-2 antibody detects the amino acids between 455-469 of cdc25C. In order to exclude that this antibody recognizes only a subset of cdc25C I repeated the experiment but instead of C-2-2 I used a cdc25C N-terminal antibody. Twenty-four hours after seeding, cells were treated with 100 nM gemcitabine, 100 nM paclitaxel or a combination of both drugs for 72 hours. Cells were harvested and 100 µg of total proteins from a cell extract were loaded on a 12.5% SDS polyacrylamide gel and analyzed by Western blot with the N-terminal polyclonal antibody against cdc25C. Signals were visualized with a POD-conjugated secondary anti-rabbit antibody and the Lumilight system. The GAPDH-specific antibody was used as loading control (Fig. 19)

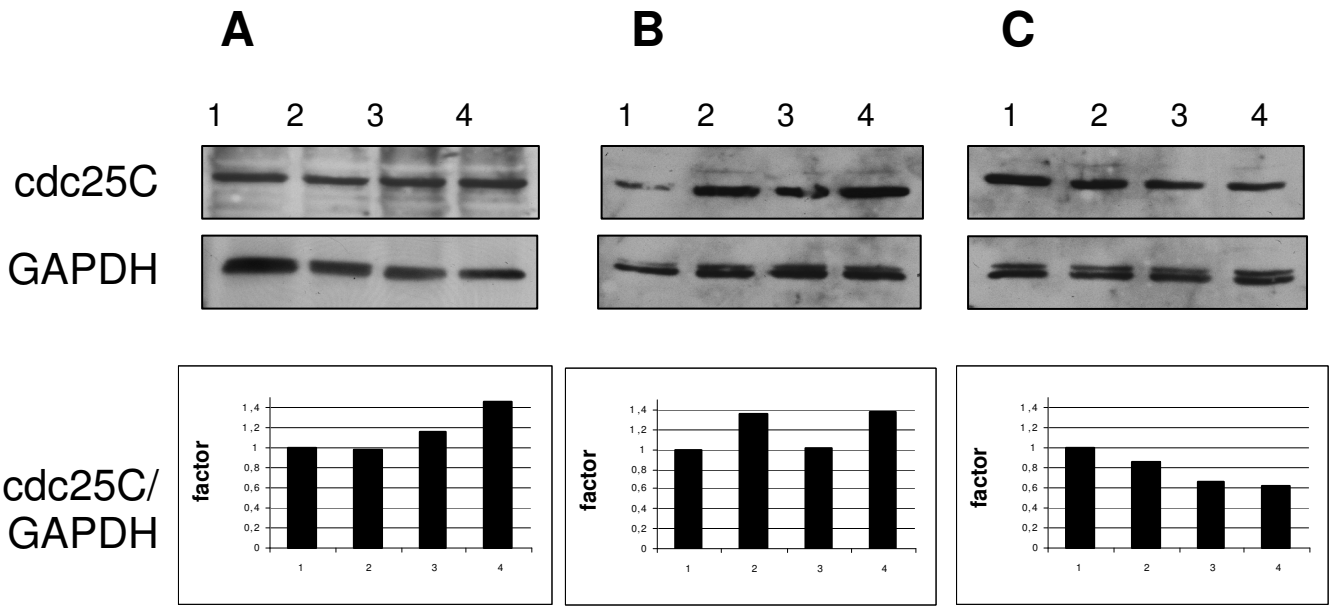


Figure 19: Expression of cdc25C in ovarian cancer cells after cytostatica treatment. Cells were treated with 100 nM gemcitabine (lane 2), 100 nM paclitaxel (lane 3) or a combination of both drugs (lane 4) for 72 hours; untreated cells served as control (lane 1). Cells were extracted (100 µg) and proteins were analyzed on a 12.5% SDS polyacrylamide gel followed by Western blot. cdc25C was detected with an N-terminal polyclonal antibody and visualized with a POD-conjugated secondary anti-rabbit antibody. GAPDH was used as loading control. (A) OV-MZ-32; (B) OvBH-1; (C) OvCBM. The ratio of cdc25C/GAPDH was calculated to detect changes of the cdc25C amount in cells before and after treatment.

The expression of cdc25C detected with the N-terminal polyclonal antibody in OV-MZ-32 cells increased about 18 and 45% after treating cells for 24 hours with paclitaxel or a combination of both drugs, respectively, whereas it remained the same when cells were treated with gemcitabine. The amount was the same in OvBH-1 cells after treating with paclitaxel and increased about 38% when cells were treated with gemcitabine or a combination of both drugs. It decreased about 18, 32 and 38% in OvCBM cells treated with gemcitabine, paclitaxel or a combination of both drugs, respectively

In order to exclude that I might have missed an earlier effect for the drugs on the expression of cdc25C in ovarian cancer cells, I repeated the experiment but analyzed the amount of cdc25C after treatment with the combination of 100 nM gemcitabine and 100 nM paclitaxel for 15, 30, 45, 60, 120, 240 and 360 min. Cells were harvested and 100 µg total proteins from a cell extract were loaded on a 12.5% SDS polyacrylamide gel and analyzed by Western blot with the N-terminal polyclonal antibody against cdc25C. Signals were visualized with a POD-conjugated secondary anti-rabbit antibody and the Lumilight system. The GAPDH-specific antibody was used as loading control (Fig. 20).

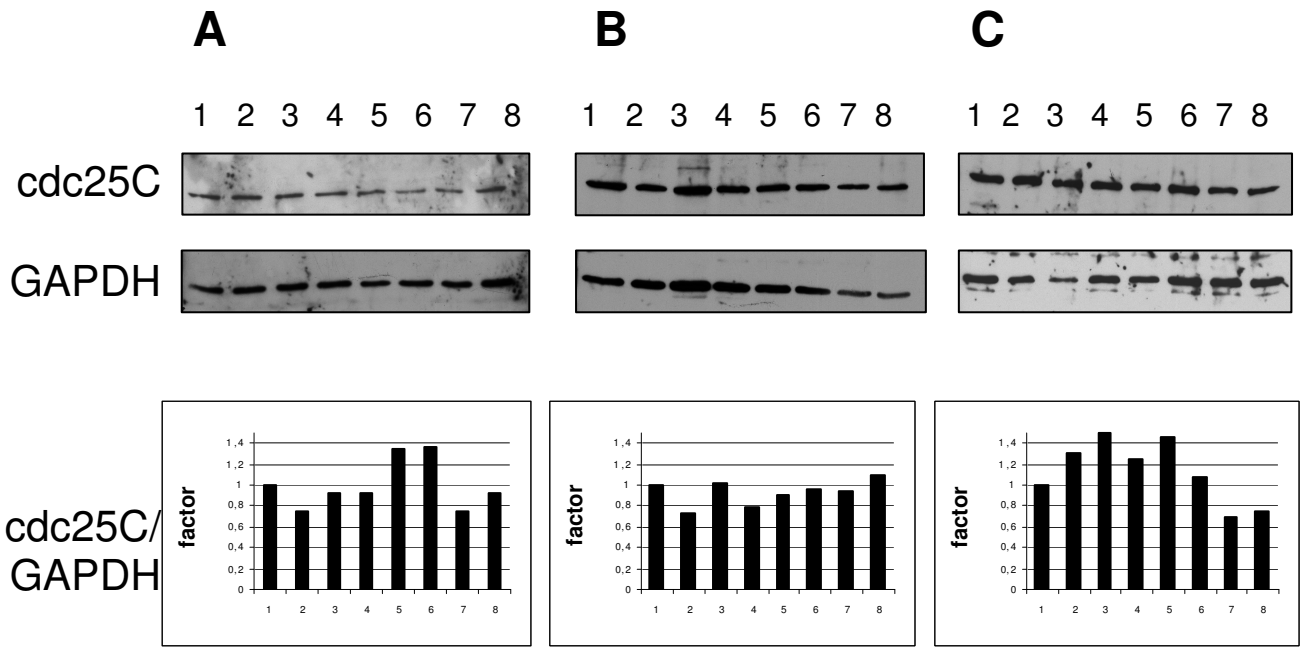


Figure 20: cdc25C expression in ovarian cancer cells after different time points of treatment. Cells were treated with a combination of 100 nM gemcitabine and 100 nM paclitaxel for 15 min (lane 2), 30 min (lane 3), 45 min (lane 4), 60 min (lane 5), 120 min (lane 6), 240 min (lane 7) and 360 min (lane 8); untreated cells served as control (lane 1). Cells were extracted (30 µg) and proteins were analyzed on a 12.5% SDS polyacrylamide gel followed by Western blot. cdc25C was detected with an N-terminal polyclonal antibody and visualized with a POD-conjugated secondary anti-rabbit antibody. GAPDH was used as loading control. (A) OV-MZ-32; (B) OvBH-1; (C) OvCBM. The ratio of cdc25C/GAPDH was calculated to detect changes of the cdc25C amount in cells before and after treatment.

As shown in Fig. 20, the amount of cdc25C varied from time point to time point but there is no clear trend. Thus, we conclude that the expression is in the same range within normal experimental errors.

It is known that treating cells with H_2O_2 increases the p53 expression (Chen *et al.*, 2000) and decreases the expression of cdc25C (Savitsky and Finkel, 2002). In order to analyze by another approach whether p53 and cdc25C in ovarian cancer cells behave as published before I checked the effect of H_2O_2 on p53 and cdc25C in ovarian cancer cells used in the present study. Twenty-four hours after seeding, cells were treated with 1 mM H_2O_2 for 15, 30, 45, 60, 120 and 180 min, then cells were harvested, and 40 µg of total protein from OV-MZ-32, 5 µg of total protein from OvBH-1 or 15 µg of total protein from

OvCBM were loaded on a 10% SDS polyacrylamide gel, and analyzed by Western blot with the p53 specific antibody DO-1. Signals were visualized with a POD-conjugated secondary anti-mouse antibody and the Lumilight system. The GAPDH-specific antibody was used as loading control (Fig. 21).

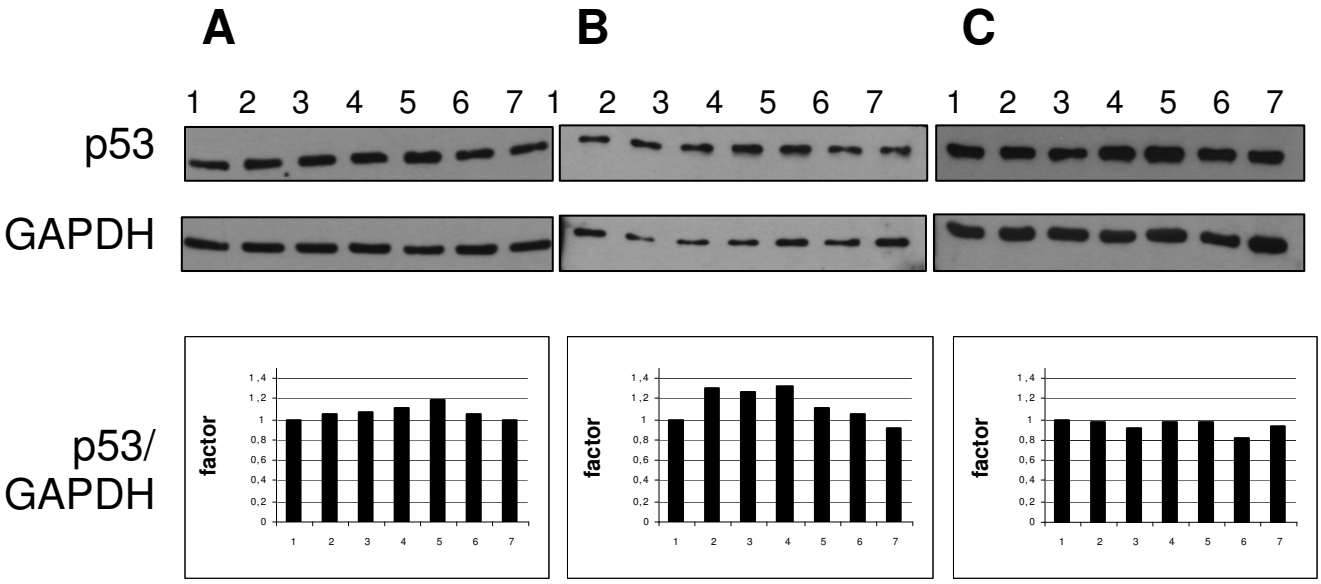


Figure 21: p53 expression in ovarian cancer cells after H_2O_2 treatment. Cells were treated with 1 mM H_2O_2 for 15 min (lane 2), 30 min (lane 3), 45 min (lane 4), 60 min (lane 5), 120 min (lane 6) or 180 min (lane 7); untreated cells served as control (lane 1). Cells were extracted and proteins were analyzed on a 10% SDS polyacrylamide gel followed by Western blot. p53 was detected with DO-1 antibody and visualized with a POD-conjugated secondary anti-mouse antibody. GAPDH was used as loading control. (A) OV-MZ-32; (B) OvBH-1; (C) OvCBM. The ratio of p53/GAPDH was calculated to detect changes of the p53 amount in cells before and after treatment.

As shown in Fig. 21, when OV-MZ-32 cells were treated with H_2O_2 the p53 expression was increased about 19% after 60 min, then it started decreasing to reach the same value as the control after 180 min. Also the p53 expression in OvBH-1 cells increased 15% after 60 min with H_2O_2, and then it decreased again to almost the same value of the control after 180 min. After H_2O_2 treatment of OvCBM cells, the p53 expression remained almost the same during 180 min, with a decrease about 16% after treatment for 120 min.
Thus, we have to conclude that H_2O_2 treatment had no significant effect on p53 expression.

The experiments were repeated exactly as for the analysis of p53 but now we analyzed the expression of cdc25C. The cell extracts were loaded on a 12.5% SDS polyacrylamide gel, and cdc25C was analyzed by Western blot with the N-terminal antibody against cdc25C. Signals were visualized with a POD-conjugated secondary anti-rabbit antibody and the Lumilight system. The GAPDH-specific antibody was used as loading control (Fig. 22).

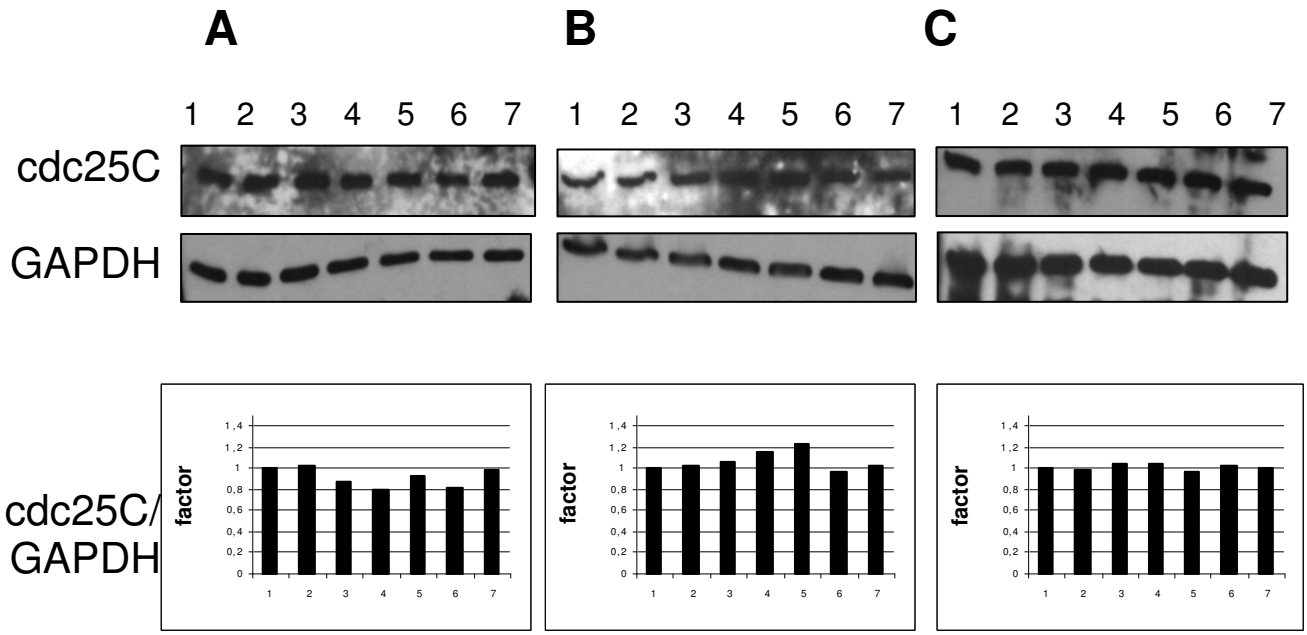


Figure 22: Effect of H_2O_2 on cdc25C expression in ovarian cancer cells. Cells were treated with 1 mM H_2O_2 for 15 min (lane 2), 30 min (lane 3), 45 min (lane 4), 60 min (lane 5), 120 min (lane 6) or 180 min (lane 7); untreated cells served as control (lane 1). Cells were extracted and proteins were analyzed on a 12.5% SDS polyacrylamide gel followed by Western blot. cdc25C was detected with an N-terminal polyclonal antibody and visualized with a POD-conjugated secondary anti-rabbit antibody. GAPDH was used as loading control. (A) OV-MZ-32; (B) OvBH-1; (C), OvCBM. The ratio of cdc25C/GAPDH was calculated to detect changes of the cdc25C amount in cells before and after treatment.

As shown in Fig. 22, the cdc25C expression in OV-MZ-32 cells was decreased about 18% during 120 min of treatment with H_2O_2, then it increased again to the same value of the control after 180 min. Whereas it increased about 25% in OvBH-1 cells during 60 min of treatment, then it decreased to the control value after 120 min. In OvCBM cells the expression

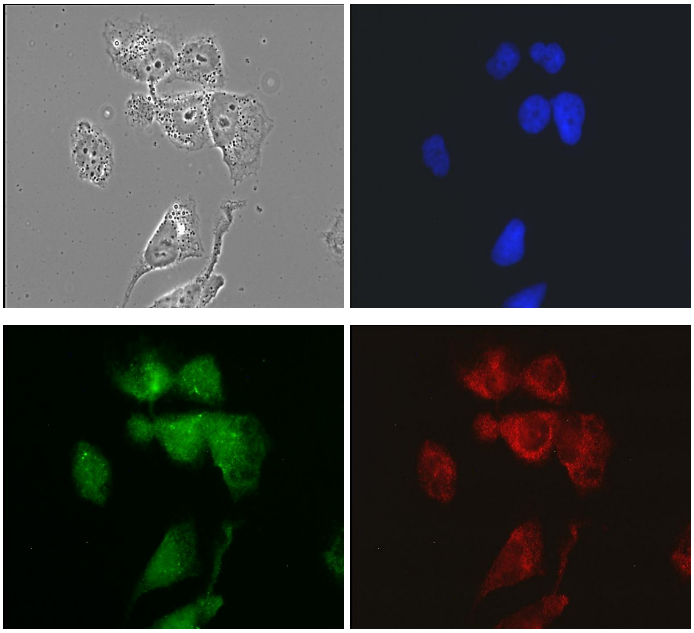
of cdc25C kept almost the same during 180 min of treatment. We conclude from the last two experiments that the variations in the amount of p53 as well as cdc25C after different times of treatment with H_2O_2 seem to be in the range of the experimental variations. Furthermore, these results support our previous findings that the p53 and cdc25C expression in the ovarian cancer cell lines used in the present study is different from published results in other cell lines.

4.3.2. Sub-cellular localization of p53 and cdc25C in ovarian cancer cells after cytostatica treatment

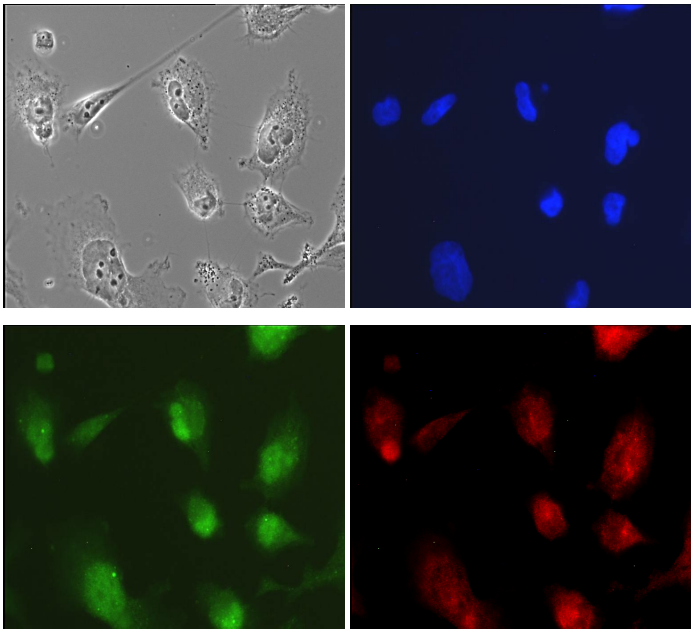
Since the only known substrate of cdc25C, which is cdk1, is located in the cell nucleus at G_2/M transition, the sub-cellular localization of cdc25C is an important mechanism for the regulation of its activity. A nuclear localization of cdc25C before G_2/M transition leads to mitotic catastrophe (Vogel *et al.*, 2007). After performing the experiments showing the expression of p53 and cdc25C in ovarian cancer cells after cytostatica treatment, the next step is to know if this treatment would change the sub-cellular localization of the cdc25C and p53. For this type of analysis we used the technique of immunofluorescence. Cells were seeded for 24 hours, then cells were treated with 100 nM gemcitabine, 100 nM paclitaxel or a combination of both for 24 hours. The cdc25C specific antibody (C-20) and the p53 specific antibody (DO-1) were added and then, AlexaFluor® 594 Goat anti-rabbit IgG, or AlexaFluor® 488 Goat anti-mouse IgG were added. The nucleus was stained with DAPI. Signals were analyzed under a fluorescence microscope (Axioscope Zeiss) (Fig. 23).

OV-MZ-32

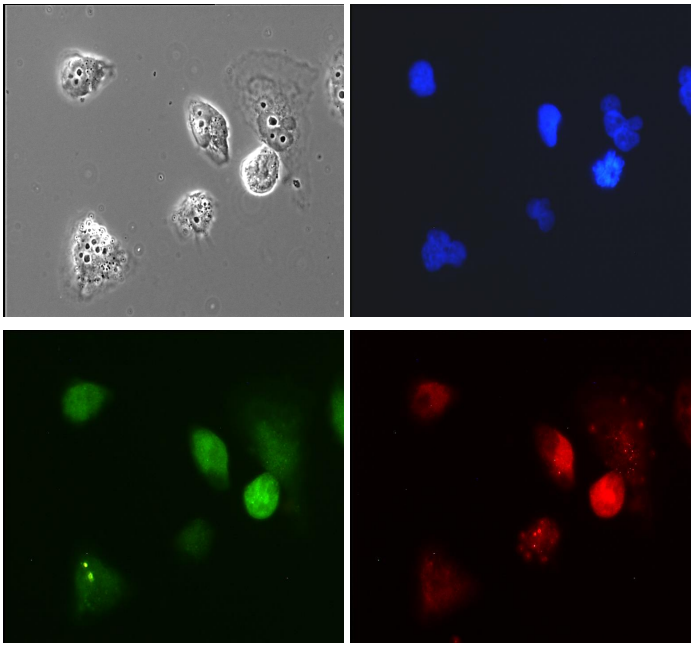
control



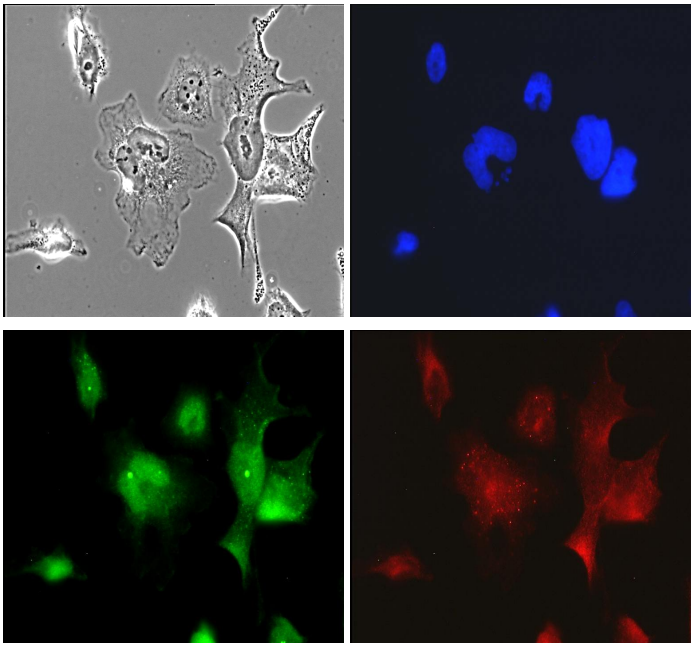
gemcitabine



paclitaxel

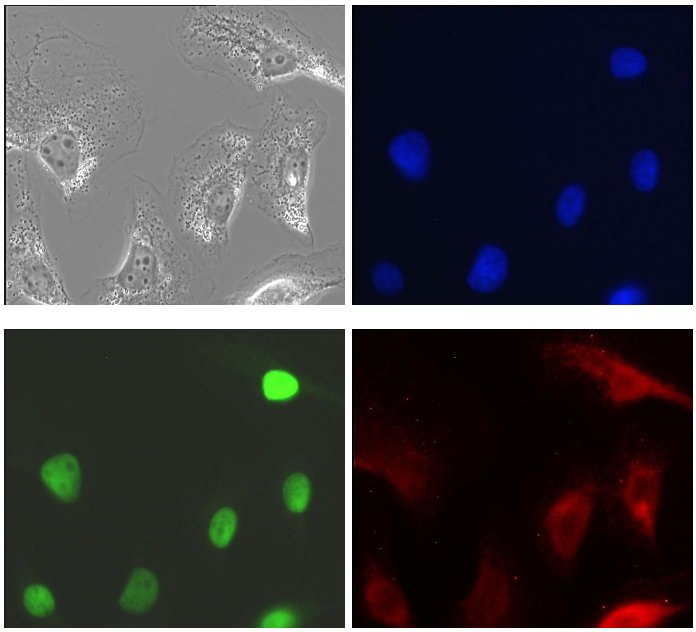


gemcitabine+paclitaxel

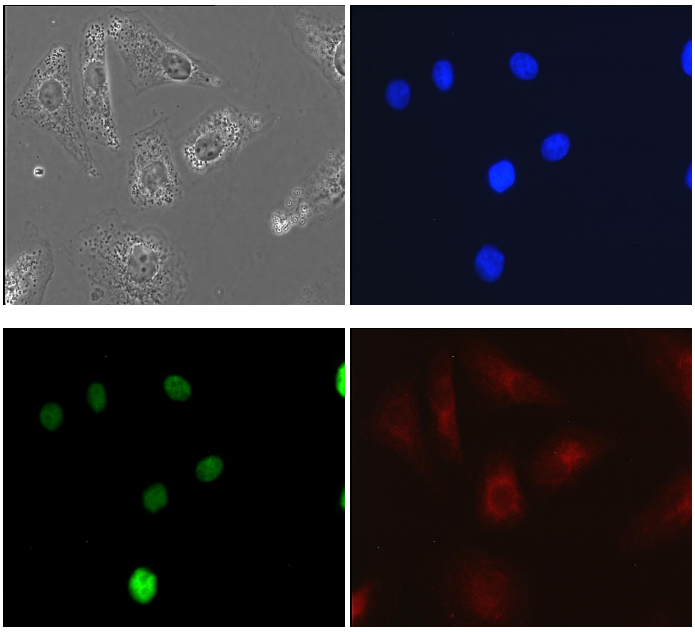


OvBH-1

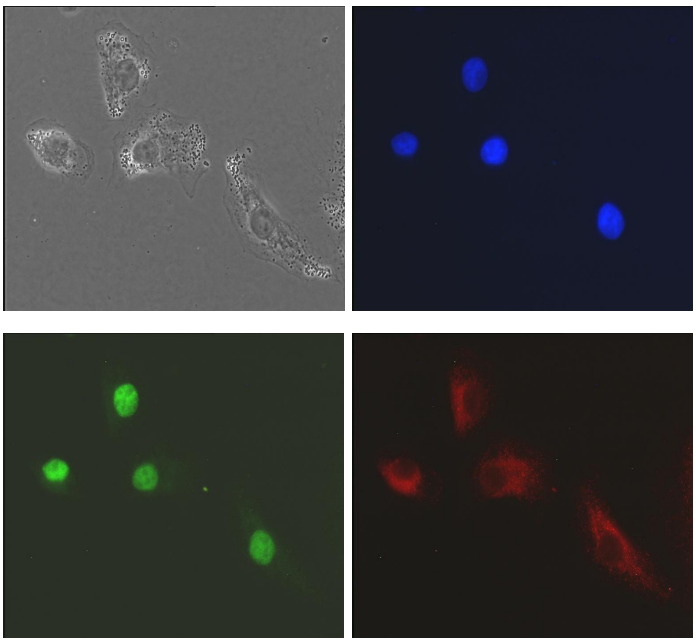
control



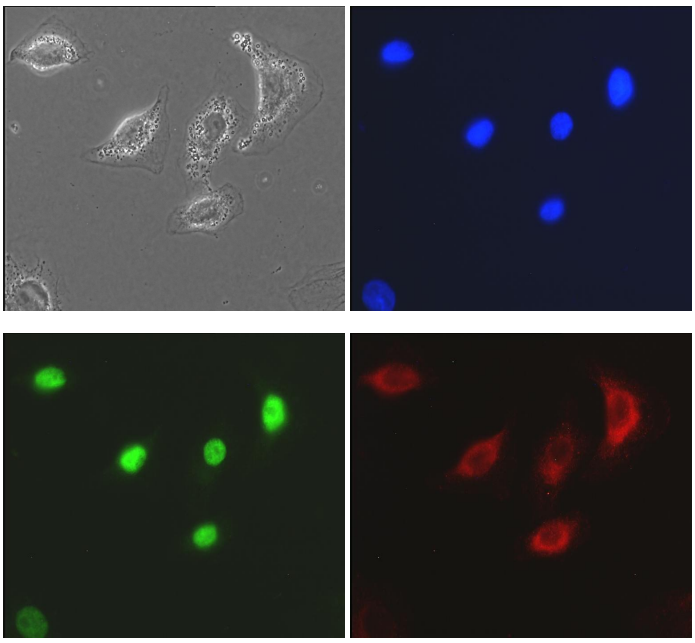
gemcitabine



paclitaxel



gemcitabine+paclitaxel



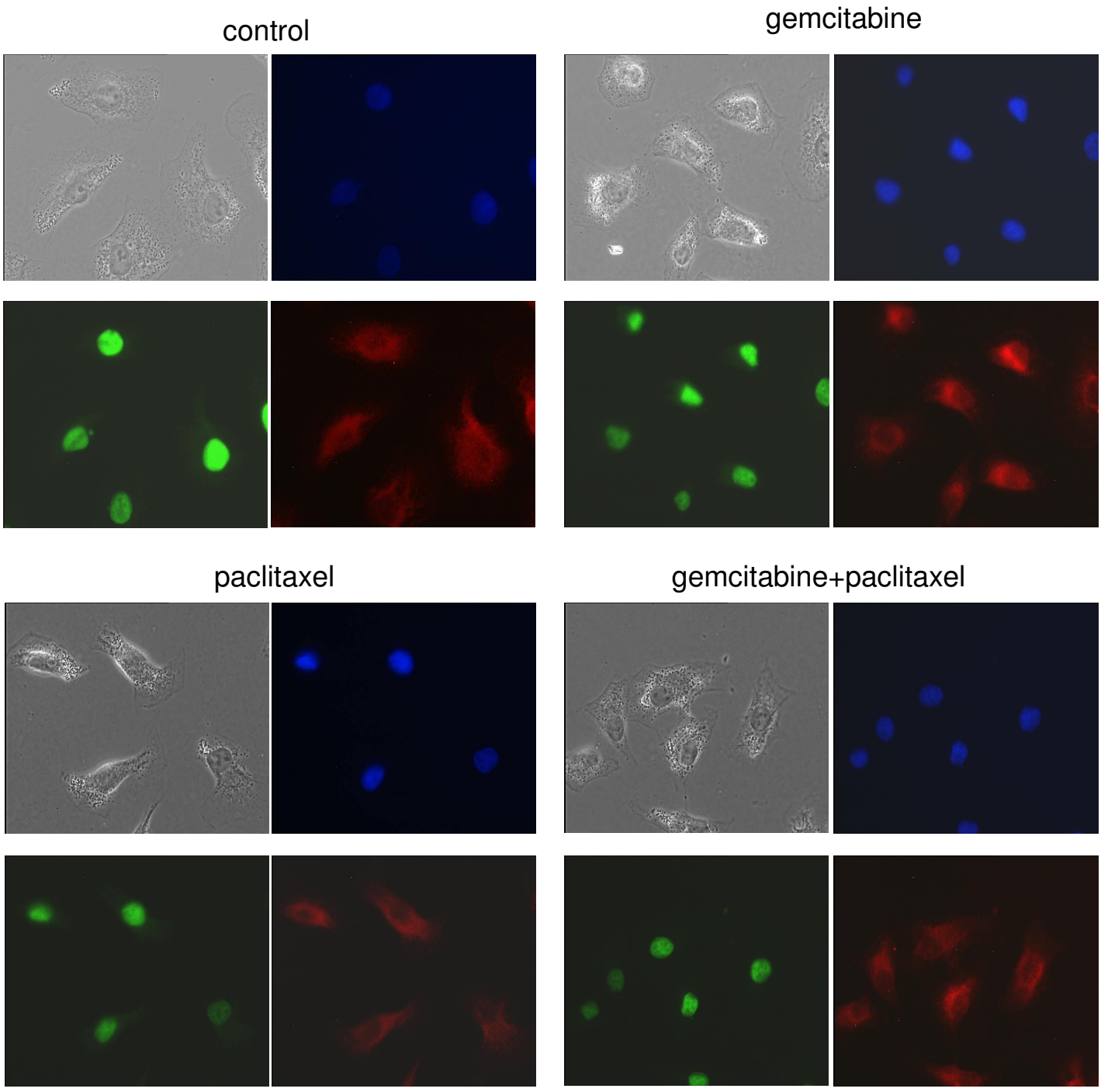


Figure 23: Immunofluorescense assay. Cells were treated for 24 hours with 100 nM gemcitabine, 100 nM paclitaxel or a combination of both. The sub-cellular localization of the cdc25C (red), and p53 (green), was controlled under a fluorescence microscope (Axioscope, Zeiss). The nucleus was stained with DAPI (blue).

As shown in Fig. 23, treating ovarian cancer cells with 100 nM gemcitabine, 100 nM paclitaxel or a combination of both did not affect the sub-localization of cdc25C or p53, as cdc25C was still in the cytoplasm and p53 in the nucleus.

In order to control these results by biochemical subfractionation, I extracted the nucleus and the cytoplasm separately, to assess the amount of cdc25C in each one. After seeding cells for 24 hours, they were treated with 100 nM paclitaxel, 100 nM gemcitabine or a combination of both drugs for 24 hours. After separation of the cytoplasm and the nucleus, 75 µg extract each were loaded on a 12.5% SDS polyacrylamide gel. cdc25C was analyzed by Western blot with the specific antibody H-6. Signals were visualized with a POD-conjugated secondary anti-mouse antibody and the Lumilight system. A HSP90 antibody or nucleolin antibodies were used to explore the purity of the cytosol and the nuclear fraction (Fig. 24).

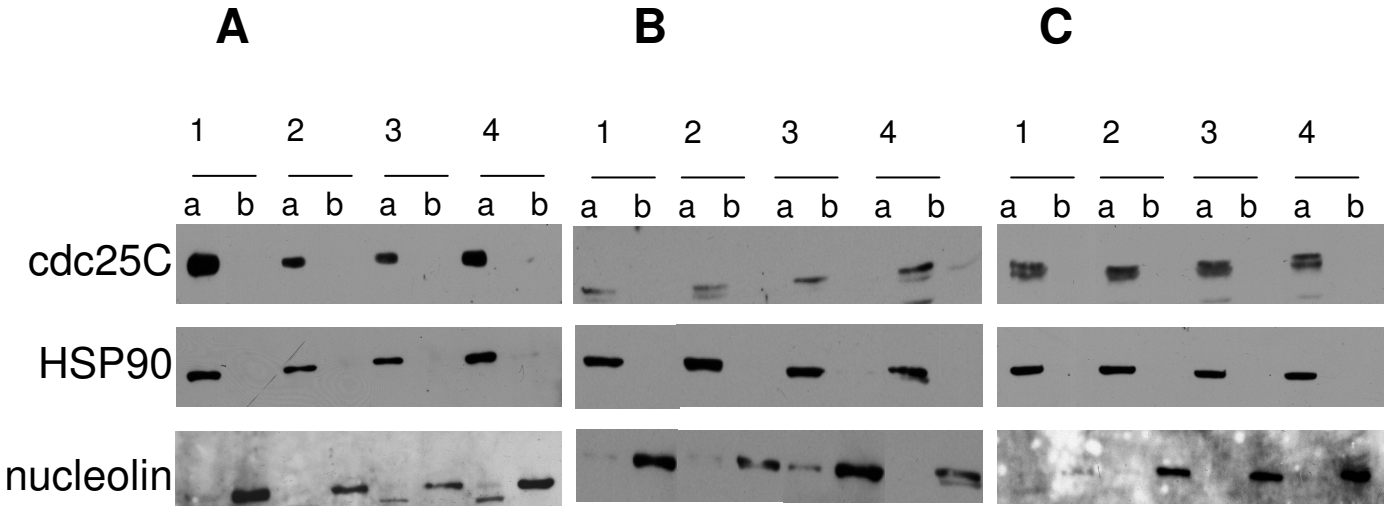


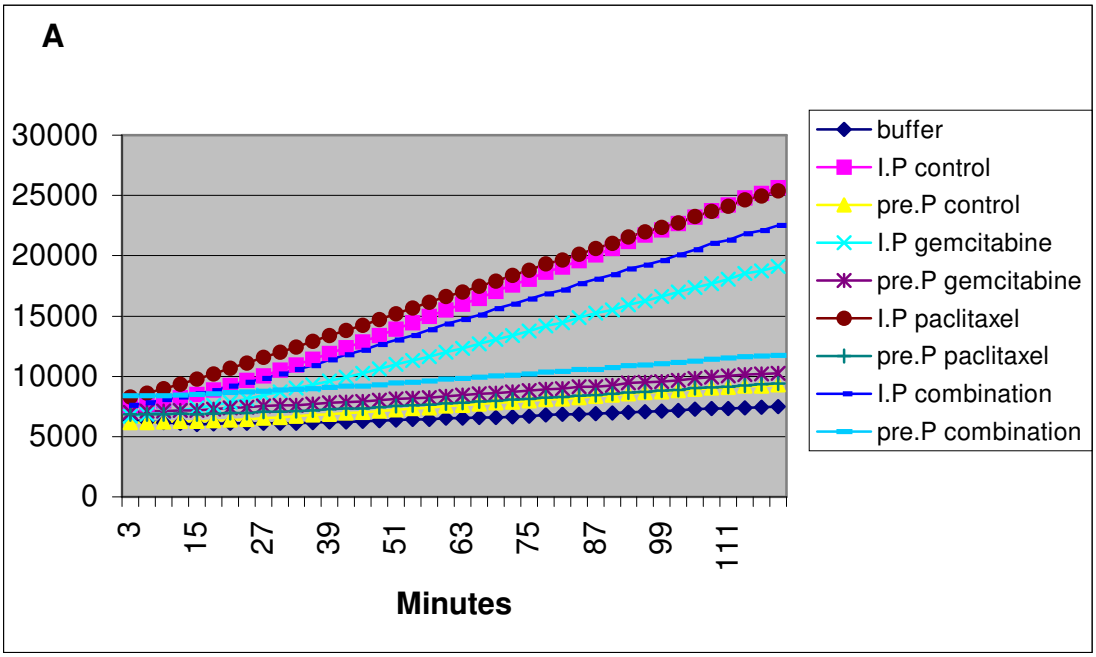
Figure 24: cdc25C expression in the cytoplasm (a) and nucleus (b) of ovarian cancer cells. Cells were treated for 24 hours with 100 nM paclitaxel (lane 1), 100 nM gemcitabine (lane 2) or a combination of both (lane 3); untreated cells served as control (lane 4). After separating of the cytoplasm and the nucleus, 75 µg extract each were loaded on a 12.5% SDS polyacrylamide gel. cdc25C was analyzed by Western blot with the specific antibody H-6.

HSP 90 and nucleoli antibodies both were used to demonstrate the purity of the cytosol or nuclear fraction. (A) OV-MZ-32; (B) OvBH-1; (C) OvCBM.

As shown in Fig. 24, the amount of cdc25C protein in the cytoplasm or nucleus did not change after treating ovarian cancer cells with drugs for 24 hours. The control experiments with HSP 90 and nucleolin antibodies showed that both cytosolic and nuclear fractions were pure.

4.3.3. cdc25C phosphatase activity in ovarian cancer cells

The enzymatic activity of cdc25C is low during interphase, and it increases sharply at the G_2/M transition point, changing of this activity is one of the mechanisms how cdc25C regulates cell cycle progression. Therefore we wanted to know the effects of the drugs on the phosphatase activity of the cdc25C. Twenty-four hours after seeding, cells were treated with 100 nM gemcitabine, 100 nM paclitaxel or a combination of both for 24 hours. Cells were harvested and 2-3 mg of total protein from the cell extract were used to precipitate the cdc25C protein, using the N-terminal antibody. The N-terminal antibody was used because its epitope on the polypeptide chain of cdc25C is far away from the active site. The immune precipitate was dissolved in phosphatase assay buffer (50 mM Tris-HCl, pH 8.0, 1 mM DTT, 20 µM FDP), 20 µM fluoresceine disulfate (FDP) were added and the phosphatase activity of cdc25C was measured by GeNios spectra fluor plus (Fig. 25).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I.P gemcitabine
pre.P gemcitabine
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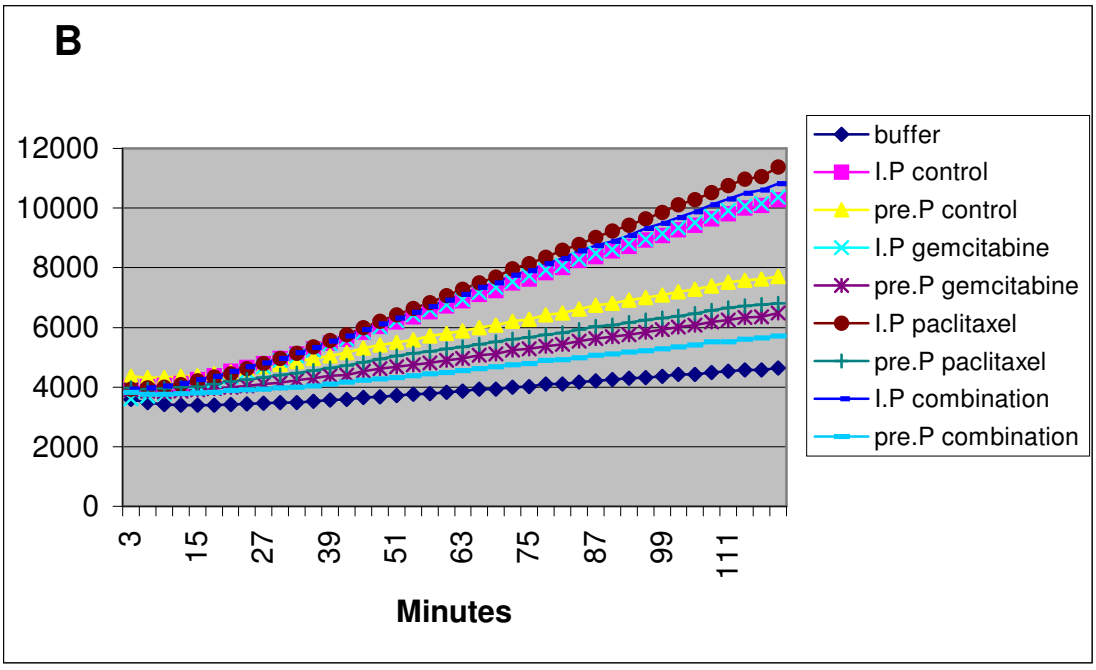
pre.P paclitaxel
I.P combination
pre.P combination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I.P combination
pre.P combination

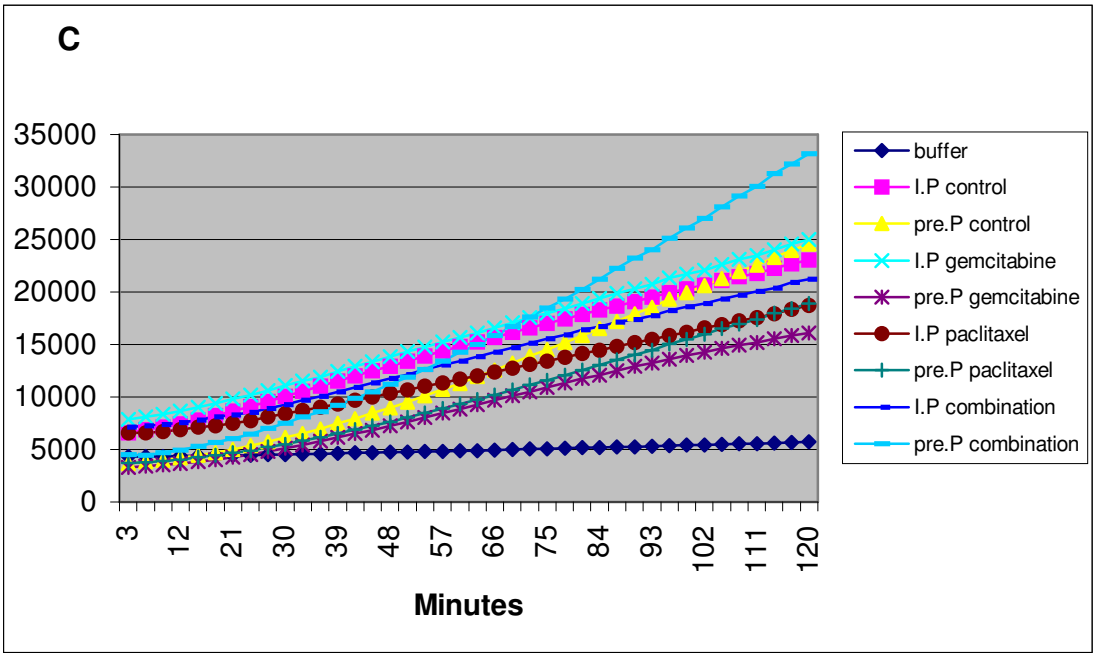
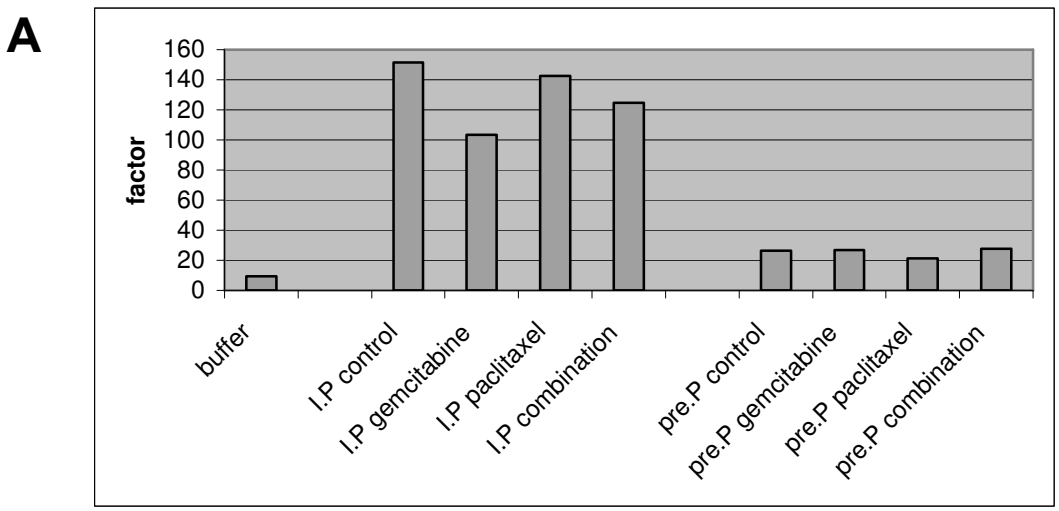
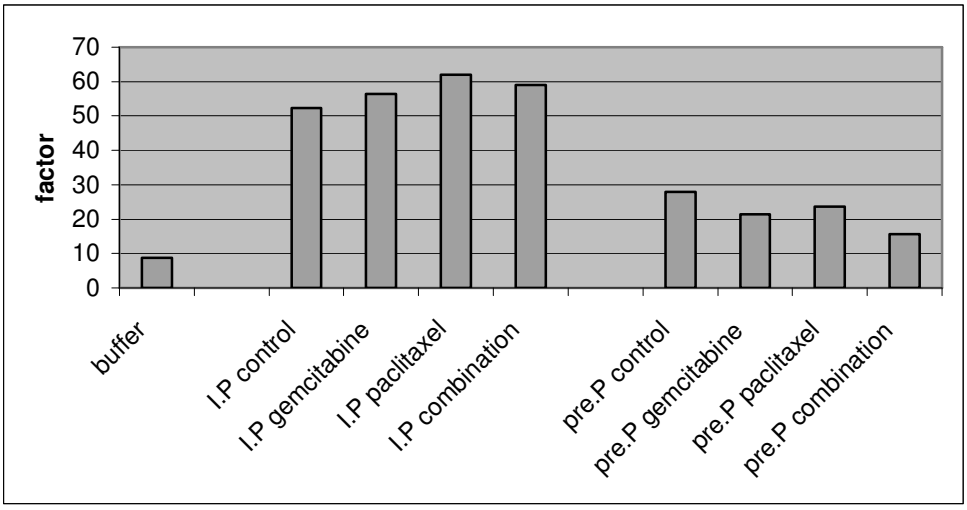


Figure 25: Phosphatase activity of cdc25C in ovarian cancer cells. Cells were treated with 100 nM gemcitabine, 100 nM paclitaxel or a combination of both for 24 hours. cdc25C protein was precipitated using the N-terminal antibody. Phosphatase activity of cdc25C was measured with FDP as substrate, the activity was also measured in the pre-precipitation samples and used as a control. (A) OV-MZ-32; (B) OvBH-1; (C), OvCBM.

Then I calculated the trend of the activity changes per min of every sample as it shown in Fig. 26.



B



C

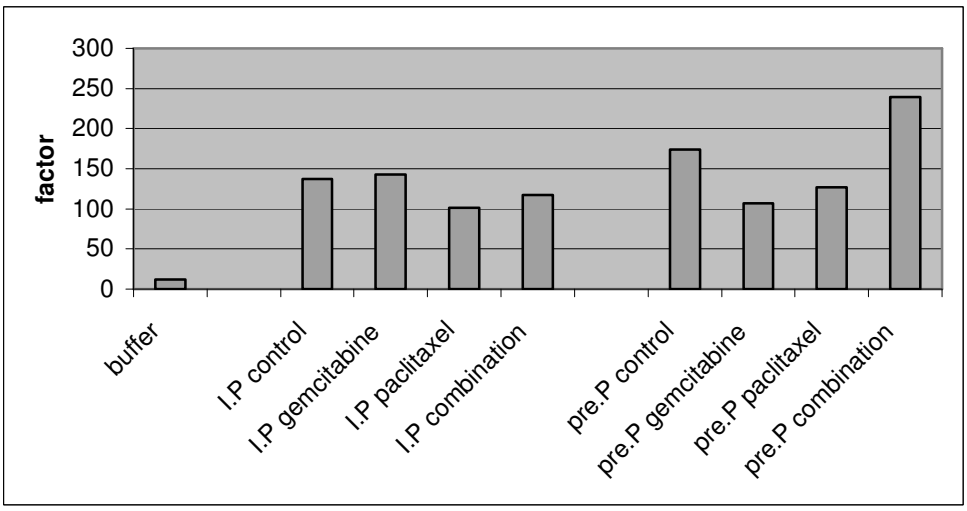


Figure 26: Phosphatase activity.

As shown in Figs. 25 and 26, the activity of cdc25C in the immune precipitate samples was higher than in the pre-precipitate samples which demonstrate that the assay is indeed exactly. Treating OV-MZ-32 cells for 24 hours with 100 nM gemcitabine decreased the phosphatase activity of cdc25C during 120 min of measuring, whereas paclitaxel had no effect, the combination of 100 nM gemcitabine and 100 nM paclitaxel had no additional effect compared to gemcitabine alone. Treating OvBH-1 cells with 100 nM gemcitabine, 100 nM paclitaxel or a combination of both for 24 hours had no effect on cdc25C phosphatase activity. The phosphatase activity of cdc25C in OvCBM cells

increased after treating cells for 24 hours with 100 nM gemcitabine, whereas a decreased activity was observed after treating cells with 100 nM paclitaxel. Also the combination of both drugs decreased the activity, but the effect was weaker than with paclitaxel alone.

In addition we performed Western blot analysis on the immunoprecipitation and pre-precipitation samples using the cdc25C specific antibody H-6, to check the cdc25C protein amounts (Fig. 27).

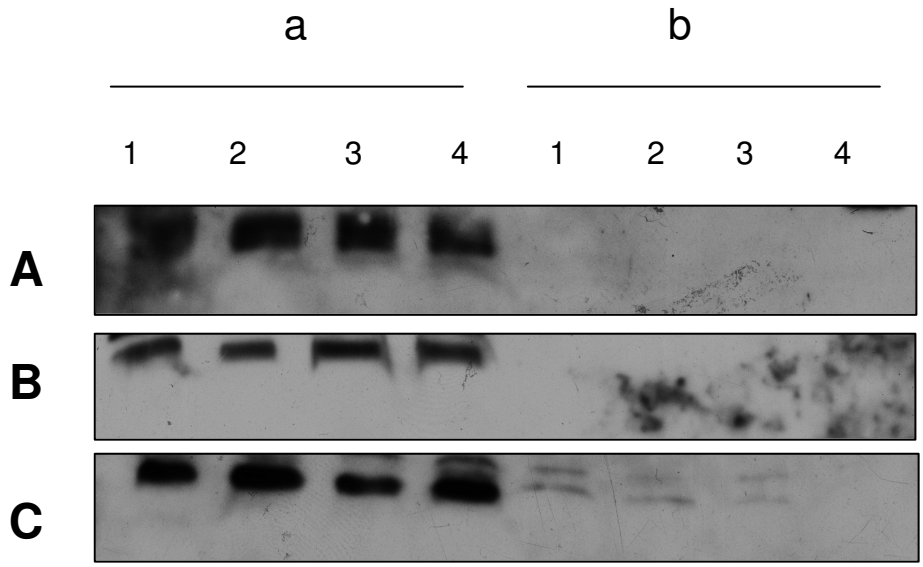


Figure 27: Western blot analysis on the immune precipitation (a) and pre-precipitation (b) of cdc25C as a control for the phosphatase activity. Cells were treated with 100 nM gemcitabine (lane 2), 100 nM paclitaxel (lane 3), a combination of both (lane 4); untreated cells (lane 1) then cdc25C was precipitated with an N-terminal antibody, the amount of cdc25C protein was controlled by Western blot analysis using the cdc25C specific antibody (H-6). (A) OV-MZ-32; (B) OvBH-1; (C) OvCBM.

As shown in Fig. 27, the amounts of the cdc25C protein was nearly equal in the immune precipitate samples, and there were no proteins in the pre-precipitate samples.

4.3.4. Phosphorylation of cdc25C

When ATM is activated by DNA damage it phosphorylates the protein kinase Chk1 which in turn phosphorylates the cdc25C on the residue serine 216 creating a binding site for the 14-3-3 protein. This binding leads to an accumulation of cdc25C in the cytoplasm and subsequently a decrease in its activity (Dalal *et al*., 1999). To analyze the effect of the cytostatica on this phosphorylation in the ovarian cancer cells a Western blot analysis was performed using serine 216 phosphorylation specific antibody.

Cells were seeded for 24 hours, then treated (Chk1 inhibitor) for 4 hours or with a combination of 100 nM gemcitabine and 100 nM paclitaxel for 24 hours. Part of dishes which were treated with the combination of drugs were treated with 2.5 µM SB218078 (Chk1 inhibitor) 4 hours before harvesting. After harvesting cells 100 µg total proteins from cell extracts were loaded on a 12.5% SDS polyacrylamide gel and analyzed by Western blot with the cdc25C phospho-specific antibody (Ser 216). Signals were visualized with a POD-conjugated secondary anti-rabbit antibody and the Lumilight system. The GAPDH-specific antibody was used as loading control (Fig. 28).

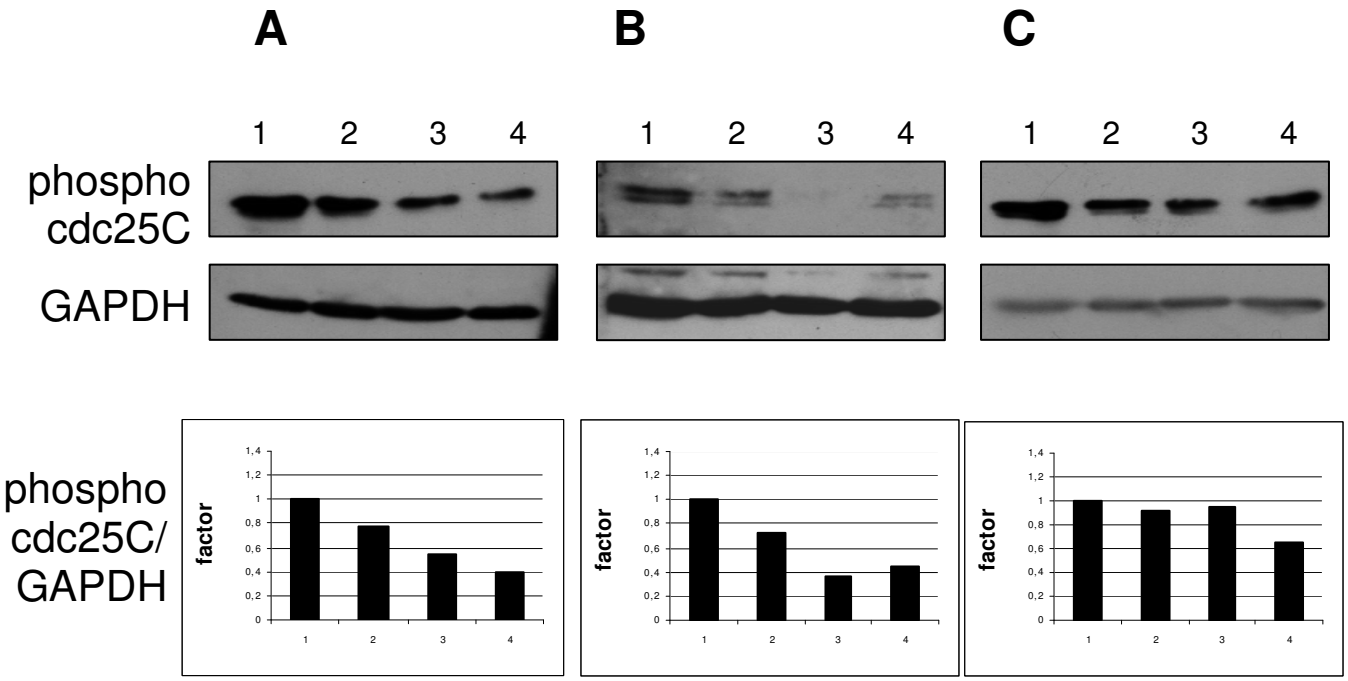


Figure 28: Phosphorylation cdc25C on serine 216. Cells were treated with a combination of 100 nM gemcitabine and 100 nM paclitaxel for 24 hours (lane 2); or 2.5 µM Chk1 inhibitor for 4 hours (lane 3), or a combination of drugs for 24 hours and Chk1 inhibitor for 4 hours (lane 4); untreated cells served as control (lane 1). Cells were extracted (100) µg and proteins were analyzed on a 12.5% SDS polyacrylamide gel followed by Western blot. cdc25C was detected with the phospho-specific antibody (Ser 216) and visualized with a POD-conjugated secondary anti-rabbit antibody. GAPDH was used as loading control. (A) OV-MZ-32; (B) OvBH-1; (C) OvCBM. The ratio of phosphor cdc25C/GAPDH was calculated to detect changes of the cdc25C amount in cells before and after treatment.

To control this experiment the Western blot analysis was performed on the same extract using the C-2-2 antibody (Fig. 29).

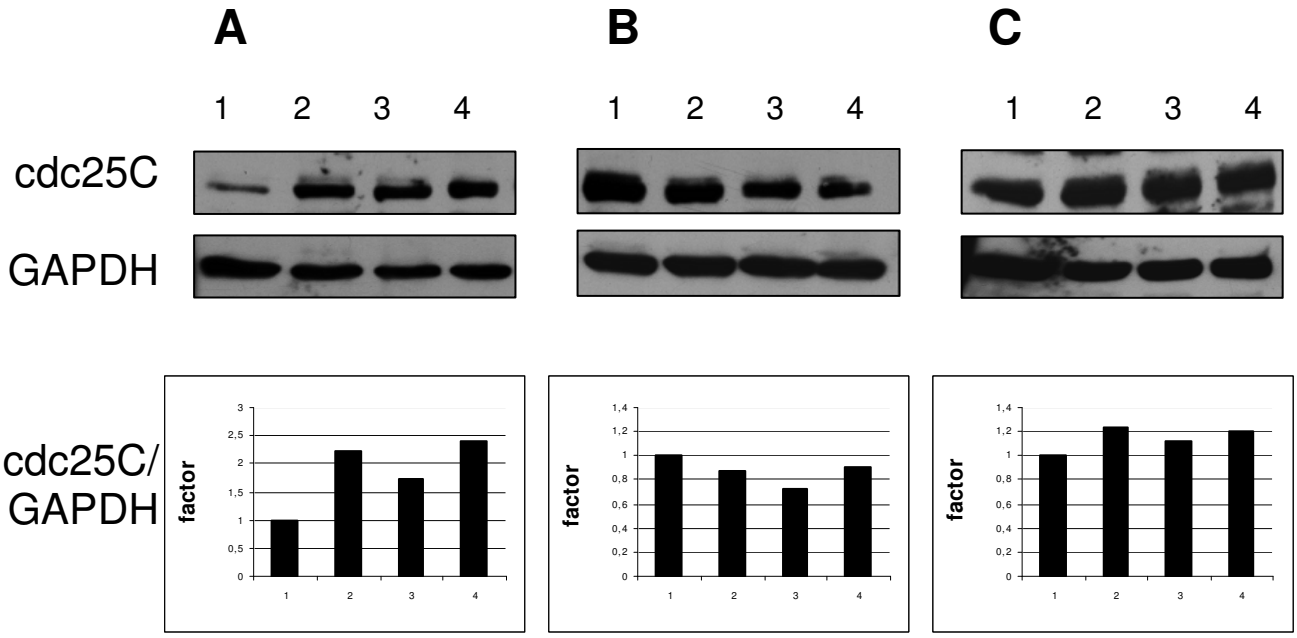


Figure 29: cdc25C expression. Cells were treated with a combination of 100 nM gemcitabine and 100 nM paclitaxel for 24 hours (lane 2), or 2.5 µM Chk1 inhibitor for 4 hours (lane 3), or a combination of drugs for 24 hours and Chk1 inhibitor for 4 hours (lane4); untreated cells served as control (lane 1). Cells were extracted (100) µg and proteins were analyzed on a 12.5% SDS polyacrylamide gel followed by Western blot. cdc25C was detected with C-2-2 antibody and visualized with a POD-conjugated secondary anti-mouse antibody. GAPDH was used as loading control. (A) OV-MZ-32; (B) OvBH-1; (C) OvCBM. The ratio of cdc25C/GAPDH was calculated to detect changes of the cdc25C amount in cells before and after treatment.

Then the ratio of the phospho- specific/total amount of cdc25C was calculated as shown in Fig. 30.

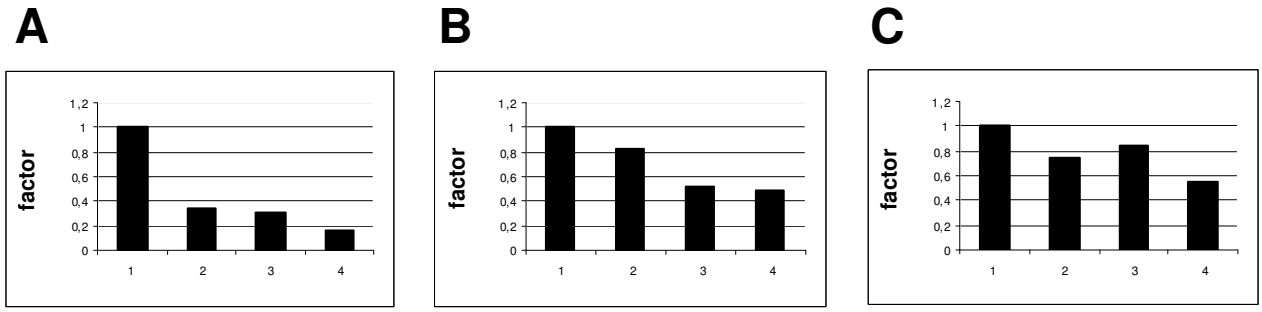


Figure 30: the ratio of phospho specific cdc25C/total amount of cdc25C

As shown in Figs. 28 -30, the phosphorylation of cdc25C on serine 216 was inhibited when all three cell lines were treated with the combination of paclitaxel and gemcitabine. The inhibition of the phosphorylation of cdc25C at serine 216 was stronger inhibited by the Chk1 inhibitor than with the combination of the two drugs in OvBH-1 cells but not in OvCBM cells, in OV-MZ-32 the Chk1 inhibitor had the same effect as the combination of drugs, in all cell lines when the combination of drugs was complemented with the Chk1 inhibitor we observed more inhibition effect than the using only the combination of drugs.

5. Discussion

In order to get an insight into molecular processes which are connected with cancer, animal models and especially established cell cultures from a particular type of cancer with defined properties are very helpful. Thus, different cell lines were established from a variety of different tumors including ovarian carcinomas. These cell lines were used to study alterations in comparison to normal cells, to study signaling cascades as well as the response to treatment with x-rays or chemotherapy. To study the molecular basis of alterations in the checkpoint control proteins such as p53 and cdc25C after chemotherapy we also used established ovarian cancer cell lines. Moreover, it is impossible to perform such experiments with tissue samples, the only way to perform such types of analysis is to use established cell lines with defined properties.

Most ovarian cancers are either ovarian epithelial carcinomas (cancer that begins in the cells on the surface of the ovary), or malignant germ cell tumor (cancer that begins in the egg cells). The majority of ovarian carcinoma cell lines had been established from ascites fluids of patients with serous ovarian carcinomas (Yaginuma and Westphal, 1992). Since cell lines may lose many of tumor-specific properties of the original tumor during prolonged *in vitro* culture (Mobus *et al*., 1992), I used freshly established ovarian cancer cell lines which are OV-MZ-32, OvBH-1 and OvCBM. OV-MZ-32 is a serous human ovarian adeno-carcinoma cell line; these cells carry a 13-bp deletion from codon 314 to codon 318 of the p53 gene (Wu *et al*., 2000). This deletion affects the cdk1 phosphorylation site at position 315 which is known to regulate the sequence specific DNA binding of p53 and conformational changes in the p53 protein (Wang and Prives, 1995). Introduction of wild-type p53 into these tumor cells resulted in inhibition of cell growth and induction of apoptosis. Subsequent chemotherapy with cisplatin, taxol, doxorubicin or mitomycin increased the growth inhibition significantly (Wu *et al*., 2000). OvBH-1 is a human ovarian carcinoma cell line derived from the ascitic fluid of

an untreated patient with ovarian clear-cell adenocarcinoma. These cells are large in size with indistinct cell borders, the cytoplasm contains small vacuoles with central or atopic localization of the nuclei. They are temperature-sensitive, growing normally at 37°C and showing a retarded growth when shifted to 31°C. Shifting cells to 31°C showed a G_2/M arrest. This arrest is WAF1 independent as these cells are negative for WAF1. In addition these cells are also negative for the MDM2 protein. DNA sequencing revealed that the p53 gene was wild type in these cells (Bar *et al*., 2002; Bar and Harlozinska, 2000). Complex rearrangement involving chromosome 3, 15 and 20 were found by karyotyping of these cells. FISH analysis with a p53 probe indicated a deletion of this region in two out of three copies of chromosome 17 (Schlade-Bartusiak *et al*., 2006). As these two cell lines present the most common ovarian cancers, I used them in my work. In addition I used OvCBM, a cell line which was freshly established from a patient with ovarian carcinoma which was obtained from Dr. Julia Bar, Wroclaw, Poland. Detailed informations about this cell line are currently collected. A new cell line named TAYA was established from the ascites of patients with clear cell adenocarcinoma of the ovary. These cells had a low sensitivity to anti cancer drugs such as cisplatin, paclitaxel, irinotecan and gemcitabine, whereas they were highly sensitive to radiotherapy (Saga *et al*., 2002). In our laboratory a study has been done by S. Kartarius on the same cells which I used in the present study. She found that these cell lines were resistant to γ-irradiation with an ID50 between 4 and 8 Gy. For that reason in my study I focused only on the effect of chemical drugs on these cells.

Most patients with ovarian cancer undergo surgery in addition to another form of treatment e.g. chemotherapy and/or radiotherapy. Debulking surgery is especially important in ovarian cancer because aggressive removal of cancerous tissue is associated with improved survival. Radiotherapy is not a common treatment for ovarian cancer because many women are diagnosed with late-stage cancers that have spread widely within the abdominal cavity. That means that results with radiotherapy on cell lines is not directly transferable to the treatment of women. The initial chemotherapy is now in

transition with most patients receiving primary therapy with drugs that contain platinum and taxane compounds e.g. cisplatin, carboplatin and paclitaxel. However, other drugs such as melphelan or doxorubicin also show first line activity in ovarian cancer. The dose, timing and the choice of therapies are determined by the stage of ovarian cancer or the health status of the patient. In many cases the patients are resistant to cisplatin or they develop a resistance after therapy. Because cisplatin, paclitaxel (Du Bois and Pfisterer, 2005; Ozols, 2000) and sometimes irinotecan (Brown *et al*., 1993; Gershenson, 2002) were used for the therapy of ovarian cancer I also used these drugs in the beginning of the present study. I also studied the effects of gemcitabine which has emerged as an important new agent in several tumor types including pancreas, non small-cell lung, bladder and breast cancer (Burris, III *et al*., 1997; Kaye, 1998).

Cisplatin leads to the formation of DNA interstrand and intrastrand cross-links and to an accumulation of p53 after DNA strand breaks. Cisplatin activate caspases, and thereby apoptosis (Perez, 1998). In tubular cells, a primary target for cisplatin is the genomic DNA. In response to DNA damage p53 is induced and implicated in subsequent DNA repair and cell death by apoptosis. Cisplatin used in 20 µM concentration led to apoptosis in approximately 70% of cells. Early during cisplatin treatment, p53 was up-regulated and therefore p53 activation might be an early signal for apoptosis during cisplatin treatment (Jiang *et al*., 2004). Some tumors which showed drug resistance require high concentration of cisplatin to induce apoptosis. Moreover the level of DNA damage stimulated by cisplatin plays a critical role in drug sensitivity (Ortega *et al*., 1996). It is clear from the results presented here that the ovarian carcinoma cell lines analyzed showed a resistance for cisplatin treatment as treating cells with cisplatin had no effect on the viability of all three cell lines even at high concentrations. In agreement with published results I also saw an increase in the expression of p53 after treatment of cells with 10 nM cisplatin which is (Jiang *et al*., 2004).

Topotecan is a water soluble camptothecin derivative that causes DNA damage by stabilizing the DNA-topoisomerase-I-cleavable complex

preventing re-ligation of broken DNA strands. This stabilization results in irreversible arrest of the replication fork and formation of lethal double-strand DNA breaks (Oggionni *et al*., 2005). Irinotecan is a camptothecin derivative. It inhibits topoisomerase II leading to double-strand break. Irinotecan is a corner stone drug in the management of metastatic colorectal cancer and in preclinical studies for antitumor drugs in ovarian cancer. Patients with recurrent or refractory diseases showed a good response to irinotecan (Gershenson, 2002). In my study, irinotecan had a cell growth inhibitory effect on ovarian cancer cells only at high concentrations.

Paclitaxel interferes with the normal function of the microtubule growth by hyper-stabilization of their structure. This destroys the ability of the cell to use its cytoskeleton in a flexible manner. Its binding to tubulin leads to cell cycle arrest at M phase (Jordan *et al*., 1993). Paclitaxel induces apoptosis in dose dependent manner leading to cell death specially in those cells with mutant p53 (Dumontet and Sikic, 1999; Henley *et al*., 2007; Wang *et al*., 1999a). Furthermore, it induces p53 and up regulates the expression of caspase 1, 2, 3, 4, 6, 9 and 10 leading to apoptosis in ovarian cancer cells (Sugimura *et al*., 2004; Wang *et al*., 2000).

p53 seems to be a critical factor in many paclitaxel treated tumors including ovarian cancer. Ovarian carcinomas with mutant p53 were described to be more responsive to a paclitaxel-based chemotherapy than p53 wild-type expressing tumors. Wild-type p53 carrying cells go into G_1 arrest and mutant p53 cells go into G_2 arrest (Lavarino *et al*., 1997; Wang *et al*., 2000). A study on ovarian cancer cells indicated that paclitaxel may have the ability to activate apoptosis in the absence of functional wild-type p53, as the authors found differences in activating downstream p53 genes in cells expressing wild-type p53 in contrast with those expressed mutant p53 (Debernardis *et al*., 1997). Paclitaxel induced apoptosis in p53-dependent manner in glioma cells and in renal cell carcinoma (Borbe *et al*., 1999; Reinecke *et al*., 2005). Paclitaxel is a highly effective chemotherapeutic agent against adeno carcinomas and squamous cell carcinomas of esophagus were it also induced apoptosis (Faried *et al*., 2006). In the present study I could show that

paclitaxel is very effective in the treatment of ovarian carcinoma cells, reducing the viability of cells at a moderate concentration of 100 nM. It turned out that this concentration was sufficient to reduce viability after a treatment for 72 hours. At earlier time point higher concentrations of paclitaxel were required.

Gemcitabine is a nucleotide 2',2'-difluorodeoxycytidine (deoxycytidine analogue). It has improved the survival of patients with many different tumors including non-small cell lung cancer, pancreatic cancer and breast cancer (Burris, III *et al*., 1997). Cytotoxity of gemcitabine is related to the cellular accumulation of gemcitabine tri-phosphate which blocks DNA replication inducing S-phase arrest in tumor cells (Kaye, 1998). It activates the S phase checkpoint of the cell cycle and the ATR/Chk1 pathway. Cells lacking ATR or Chk1 are more sensitive to gemcitabine. It was further shown that this sensitivity to gemcitabine is independent of the p53 status. Depletion of ATM, which is another DNA- damage induced protein kinase, makes the cells sensitive to gemcitabine and to ionic irradiation. In studies on lung cancer cells treated only with gemcitabine, it was shown to induce apoptosis via the activation of caspase 9 proteins (Karnitz *et al*., 2005). Mechanistic studies have demonstrated that there are at least two major pathways through which gemcitabine acts: (1) direct inhibition of DNA synthesis by formation of the tri-phosphate form, and (2) inhibition of ribonucleotide reductase, which results in the depletion of deoxynucleoside tri-phosphate necessary for DNA synthesis (Huang *et al*., 1991). The gemcitabine tri-phosphate is integrated with dCTP (deoxy-cyticine tri-phosphate) in order to incorporate into DNA as "faulty" base leading to inhibition of DNA synthesis. The di-phosphate form inhibits nucleoside reductase responsible for dCTP production, leading to decreased level of dCTP. Furthermore, it inhibits DNA polymerase and leads to decreased metabolic liquidation of gemcitabine, e.g. prolonged retention of the phosphorylated active form of gemcitabine in cancer cells (Huang and Plunkett, 1995; Lawrence *et al*., 2001; Plunkett *et al*., 1996). Many findings may lead to suggest gemcitabine as first line treatment in breast cancer (Nagourney *et al*., 2003; Yardley, 2005), leukemia (Karnitz *et al*., 2005) and in treatment of many types of solid tumors (Cartee *et al*., 1998; Plunkett *et al*.,

1996). In addition, it was shown that gemcitabine makes some solid tumors more sensitive to irradiation (Lawrence *et al*., 2001). The major finding of the first part of the present study was that gemcitabine is very efficient in its activity in inducing growth arrest and apoptosis in all these ovarian cancer cell lines tested (Touma *et al*., 2006).

Apoptosis was first shown to be a mechanism of paclitaxel-induced cytotoxity in human lymphoid leukemia cells, gastric carcinoma cells, ovarian carcinoma cells, head and neck tumors, prostate tumors, adenocortical carcinoma cells and human glioma cell lines (Wang *et al*., 2000). Disruption of microtubule structure by antimicrotubule drugs (paclitaxel) results in the induction of the tumor suppressor gene p53 and it is thought that this elevation leads to apoptosis (Wang *et al*., 1999a). G_2/M arrest occurs prior to paclitaxel-induced apoptosis and might be mediated by the mitochondrial apoptosis pathway at least in human esophageal squamous cell carcinoma cells (Faried *et al*., 2006). Some studies showed that gemcitabine also induces apoptosis in treated cancer cells by inducing fragmentation of DNA and inhibiting of DNA repair. Gemcitabine also induces dramatic morphological and biochemical alterations in cancer cells (Bookman, 2005; Cartee *et al*., 1998; Huang and Plunkett, 1995). However, as shown in the present study it does not cause alteration in the morphology of the ovarian cancer cells. Cytofluorimetric analysis demonstrated that treating ovarian cancer cells with gemcitabine or paclitaxel leads to an increase in sub-G_1 population which represents cells with fragmented DNA which is typical for apoptotic cells. Treating ovarian cancer cells with gemcitabine resulted in PARP cleavage to 89-kDa and 26-kDa fragments as shown by Western blot analysis. These both experiments mean that gemcitabine or paclitaxel treatment induces apoptosis in ovarian cancer cells. Thus, my data are in line with others in other tumors which also demonstrated the induction of apoptosis after gemcitabine treatment.

A gemcitabine-based combination therapy for pancreatic cancer provided better results than gemcitabine alone (Tsai *et al*., 2003). In breast and non-small cell lung cancers, paclitaxel increased the accumulation of dFdCTP, incorporation of gemcitabine into RNA and apoptosis (Kroep *et al*., 1999;

Kroep *et al*., 2000). The combining of gemcitabine and paclitaxel in chemotherapy for metastatic breast cancer was well tolerated and was not associated with any adverse impact on quality of life compared with paclitaxel alone (Yardley, 2005). There are results of some recent studies indicating the use of gemcitabine in combination with platinum as a first line therapy in early and advanced ovarian cancer (Poveda, 2005). Coexposure of glioma cells to taxol and either doxorubicin or camptothecin resulted in antagonistic effects rather than additive or synergistic cytotoxity (Borbe *et al*., 1999). In human leukemia cells paclitaxel substantially reduced the uptake and accumulation of gemcitabine and the formation of its metabolites (Shord *et al*., 2005). In a study on human lung and pancreas adenocarcinoma cells when gemcitabine and paclitaxel were incubated together, gemcitabine antagonized the cell killing produced by paclitaxel (Theodossiou *et al*., 1998). In this study the combination of gemcitabine and paclitaxel for treating ovarian cancer cells has no synergistic effect compared to the use of each drug alone. On the other hand there was also no inhibitory effect of paclitaxel on gemcitabine. We conclude that gemcitabine function as an efficient antitumor agent which for ovarian cancer is sufficient to reduce the viability of cancer cells and to induce apoptosis. Thus, our results show that gemcitabine can be used as a single agent for the treatment of ovarian cancer.

One mechanism of inducing apoptosis after cytostatica treatment is by generation reactive oxygen species (ROS). It was shown in a study performed on pancreatic adenocarcinoma cell lines that treating these cells with gemcitabine induced apoptosis which was associated with induction of ROS (Maehara *et al*., 2004). In contrast, in the present study the treatment of ovarian cancer cells with a combination of gemcitabine and paclitaxel did not increased the intracellular concentration of ROS. p53 plays an essential role in the cisplatin induced ROS production, its transcriptional activity is required for apoptosis and plays an important role in ROS generation (Bragado *et al*., 2007). In this study treating ovarian cancer cells with H_2O_2 caused little variations in the expression of p53 and cdc25C without a general trend.

In order to optimize cancer therapy the knowledge of signaling pathways which are affected by the drugs is absolutely necessary. The molecular mechanism by which these drugs caused apoptosis has not been fully defined yet. From many studies in ovarian tumors it is clear that p53 is a potential candidate gene and protein which responds to cytostatica treatment. Therefore, I studied the role of p53 function in cytotoxic effects of gemcitabine and paclitaxel in ovarian cancer cell lines. p53 is a transcription factor that is activated upon cellular stress and DNA damage. p53 restricts cellular growth by inducing senescence, cell cycle arrest at the G_1/S boundary and at the G_2/M boundary. If DNA damage can not be repaired p53 induces cell death by apoptosis (Haupt *et al*., 2003). Another reason for focusing on p53 expression is the observation that p53 has an impact on the efficiency an anticancer therapy.

The p53 gene is altered in more than 50% of human cancers (mutation or overexpression). For ovarian cancers an overexpression of p53 in 69% and mutations in 57% of studied ovarian cancer cases were observed. Patients with overexpression and mutation in p53 together, have survival rate less than patients only with mutations or overexpression of p53 (Amikura *et al*., 2006; de Graeff *et al*., 2006; Wen *et al*., 1999). Mutations in p53 lead to a loss of its tumor suppression function (Bar *et al*., 2002) and this loss of p53 function is responsible for an increased aggressiveness of cancer, while tumor chemoresistance is dependent upon the expression of mutant p53 protein. The ability of p53 to induce apoptosis in cancer cells is believed to be essential for cancer therapy (Bossi and Sacchi, 2007). Although alterations in the p53 gene in ovarian carcinoma cell lines are rarely described (Brown *et al*., 1993; Fajac *et al*., 1996; Yaginuma and Westphal, 1992), a significant correlation between p53 accumulation and p53 alteration and poor response to a cisplatin-based chemotherapy in ovarian cancers was already shown (Bar *et al*., 2001). The better outcome in relapsed patients with wild-type p53 suggests that the presence of a functional wild-type p53 confers stability of the drug-sensitive phenotype (Oggionni *et al*., 2005). A strong correlation has emerged between p53 alteration and response to chemotherapy. Epithelial ovarian tumors showing p53 aberrations are significantly less sensitive to

chemotherapy and more aggressive than those with functional p53 (Buttitta *et al*., 1997). Aberrant p53 overexpression was significantly associated with progression free survival in ovarian cancer patients (de Graeff *et al*., 2006). Transfection of mutant p53 gene into chemotherapy resistant cells (containing a mutation at codon 143 changing val to ala) resulted in obvious increased cisplatin sensitivity, suggesting that p53 is the direct determination for cisplatin resistance in these cells (Brown *et al*., 1993).

In OV-MZ-32 cells the p53 gene carries a 13-bp deletion from codon 314 to codon 318. In the original paper the authors stated that the p53 protein is undetectable in these cells (Wu *et al*., 2000). In contrast in the present study I could detect p53 protein in all three ovarian cancer cell lines using the specific monoclonal antibody DO-1 or a polyclonal antibody. The p53 gene in OvBH-1 cells has been shown to be wild type. The DO-1 antibody was also used in a previous study where this antibody recognized p53 in OvBH-1 cells. In immunoperoxidase staining as well as the immunofluorescence staining only a weak staining was observed with another p53 specific antibody namely PAb421 (Bar *et al*., 2002). In a Western blot analysis PAb421 did not react with p53 from OvBH-1 cells. It has been shown that p53 is sometimes phosphorylated at serine 20 which abolishes the recognition by DO-1 (Stephen *et al*., 1995; Vojtesek *et al*., 1992). Since p53 in OvBH-1 cells reacts with DO-1 one has to conclude that p53 in these cells is not phosphorylated at serine 20. In another study it was shown by immunohistochemical analysis of tissue specimen from ovarian cancer that p53 usually reacted with DO-7 antibody which recognizes the same epitope as DO-1 (Schuijer and Berns, 2003). Monoclonal antibody PAb1620 recognizes a conformation-sensitive epitope of wild-type p53 (Gannon *et al*., 1990; Milner *et al*., 1987). The fact that PAb1620 does not recognize p53 in OvBH-1 cells although it is a wild type by DNA sequencing indicated that p53 is in at least partial denatured state. Thus, one can understand why p53 does not behave like wild type in OvBH-1 cells. This finding was supported by the transactivation assays where p53 from OvBH-1 cells was unable to transactivate the WAF1 gene and the MDM2 gene. The fact that p53 does not transactivate the MDM2 gene might also explain why we did not find a rapid degradation of p53 because MDM2 is

known to be implicated in the degradation of p53 (Vousden and Lu, 2002). So far nothing is known about the status of the p53 gene in OvCBM cells. This is the only cell line where I found an induction of the p53 expression after cytostatica treatment which might indicate that these cells harbor wild-type p53. However, the transactivation assay showed that p53 from OvCBM was unable to transactivate BAX or MDM2 indicating that it does not behave completely like wild type p53.

A distinct decrease of p53 expression has been occasionally recognized after chemotherapy for ovarian cancers (Hirasawa *et al*., 2004). When cells were treated for different times with cytostatica, the p53 expression varied around the control value with no significant change. In all these observations about the expression of p53 after treating cells with cytotoxic drugs for different times, there is no general trend for p53 expression. However, from the published results as well as from the present study it is clear that the ovarian cancer cells express a subset of p53 which is immunologically distinct and which has a very limited transactivation function.

The role of p53 as a transcription factor appears to be essential, at least in response to some carcinogenic drugs. Inhibition of p53 transcriptional activity blocked cisplatin-induced apoptosis in human colorectal carcinoma cells expressing wild type p53 (Bragado *et al*., 2007). p53 is able also to promote apoptosis through transcription- independent apoptotic mechanism. This activity of p53 has been demonstrated in transformed cells rather than in normal cells, and this activity requires the cooperation with other apoptotic factors. The transcription-dependent and independent apoptotic functions of p53 appear to complement each other (Haupt *et al*., 2003). It was already shown that the p53-independent mitochondrial pathway plays a critical role in paclitaxel-induced apoptosis in ovarian cancer cells (Sugimura *et al*., 2004). In human non small cell lung cancer cells gemcitabine induced apoptosis independently of p53 (Chang *et al*., 2004). Cytotoxity induced by silvestrol (which has similar potency as paclitaxel against cultured human cancer cells) in human prostate cancer cells is associated with a block in the cell cycle at the G_2/M checkpoint and alterations in the expression of genes regulating apoptosis and cell cycle in a manner independent of p53, e.g. it caused a

decrease in p53 protein within 30 min of exposure with no p53 detectable after 6 hours (Mi *et al*., 2006). This finding is in agreement with the present finding where I found an induction of apoptosis after gemcitabine treatment of ovarian cancer cells. Moreover, I also did not detect an alteration in the expressing of p53.

Loss of normal p53 function has been demonstrated to sensitize patients to paclitaxel. Loss of the p53-dependent G_1 arrest promotes progression of cells to the G_2/M phase. The p53-upregulated WAF1 is known to facilitate the exit from mitotic arrest. In the absence of WAF1 paclitaxel-treated cells stay in the mitotic arrest state and die through apoptosis (Wang *et al*., 2000). The present results show that treating cells with gemcitabine and paclitaxel led to decrease in G_1- and G_2- phases and an increase was observed in the sub-G_1 population indicating to apoptosis. These data further show that wild-type function of p53 is not necessary for the induction of apoptosis. Moreover, it was shown earlier, that at least OvBH-1 cells do not express WAF1 (Bar *et al*., 2002) and thus we have to conclude that WAF1 at least in these cells is not required for apoptosis.

I further showed in the present study that BAX which is a proapoptotic factor is up-regulated by p53 whereas WAF1 is not induced at least in OV-MZ-32 and OvBH-1. Up-regulation of BAX may explain apoptosis induction after cytostatica treatment of these cells. In OvCBM cells p53 up-regulated WAF1 expression but not BAX. Therefore, we suggest that the apoptosis induced in these cells is WAF1 dependent, which is in agreement with a study on human colon cancer cell lines in which chemotherapy induced apoptosis in WAF1 dependent pathway but independently on p53 expression (Chen *et al*., 2004). In all three cell lines the p53 has no transcriptional activity on MDM2. It is known that MDM2 targets p53 for ubiquitin-mediated degradation. As it is not transactivated by p53 in these cells means that this pathway of p53 degradation is impaired in these cells. This result is in agreement with earlier findings about the MDM2 protein expression in these cells (Bar *et al*., 2002) and might explain the presence of high levels of p53 protein.

The cdc25C gene has been shown to be a novel target for transcriptional down regulation by p53, it targets the cdc25C gene for repression in two independent mechanisms. First, an element in the cdc25C promoter consisting of a binding site for p53 plus an adjacent 8 base pairs confers p53-dependent repression. Second, a minimal promoter containing a previous characterized CDE/CHR element is also repressed by p53 (St Clair *et al*., 2004; St Clair and Manfredi, 2006). As shown here only in OvCBM cells I observed an up-regulation of p53 after cytostatica treatment which goes along with a down-regulation of cdc25C. In the two other cell lines there were no gross alterations in the p53 expression and also no alterations in the cdc25C indicating that the repression function of p53 is only active in OvCBM cells.

Beside cdc25C in eukaryotic cells there exist two other cdc25 family members, namely cdc25A and cdc25B. While cdc25A plays a major role at G_1/S transition, cdc25B and C are required for entry into mitosis. The cdc25 phosphatases also play a key role in integrating the specific signals of checkpoint control in response to DNA damage at each of the stages of the cell cycle (Kristjansdottir and Rudolph, 2004). The cdc25 phosphatase family is involved in a variety of cancers and thus poses both a challenge and an opportunity for new therapy. Cdc25A and B have been implicated in cell transformation and tumorigenesis, checkpoint control and apoptosis (Eckstein, 2000). Many studies showed overexpression of either cdc25A or cdc25B in tumor cells but not of cdc25C. The overexpression of cdc25A or cdc25B in breast cancer correlates with clinical outcome, as in the case for ovarian and colorectal cancers (Kristjansdottir and Rudolph, 2004). Cdc25A and B act as putative oncogenes and are overproduced in a variety of cancer cells including small lung carcinomas, non-Hodgkin lymphomas, head and neck cancers and breast cancer. Interestingly, the cdc25C protein levels do not seem to be elevated in cancer cells (Hoffmann, 2000). In contrast to cdc25A and B the cdc25C phosphatase appears to be an enigma. Given its role in promoting the G_2/M transition and its role in S phase in human cells, it was expected to play an important role in cancer progression (Kristjansdottir and Rudolph, 2004). Furthermore, paclitaxel and gemcitabine are thought to be effective at G_2/M transition. For that I studied the behavior of cdc25C in the

ovarian cancer cells after treatment with cytostatica. When detected with C-2-2 antibody the cdc25C expression in all three cell lines increased in cells treated with gemcitabine or a combination of gemcitabine and paclitaxel, the same was observed for only OvBH-1 cells when cdc25C was detected with the N-terminal antibody, whereas it increased in OV-MZ-32 cells treated with gemcitabine or paclitaxel or a combination of both, and it decreased in OvCBM cells. These differences in cdc25C expression when different antibodies were used might indicate the presence of at least immunologically defined subset of cdc25C. Moreover, a general down-regulation of the total cdc25C in the ovarian carcinoma cells seems not to be the main mechanism how the cell cycle or/and entry into apoptosis is regulated after cytostatica treatment.

In addition to the transrepression of cdc25C by p53 there are other mechanisms known how cdc25C can regulate cell cycle progression. One mechanism is achieved by the Chk1 protein kinase which phosphorylates serine 216 of cdc25C in response to DNA damage. This phosphorylation creates a binding site for the 14-3-3 protein leading to cytoplasmic localization of cdc25C. Since the only substrate of cdc25C, which is cdk1, is located in the nucleus at G_2/M a translocation of cdc25C to the cytoplasm means inactivation of cdc25C activity (Eckstein, 2000). Recently it turned out that in addition to the phosphorylation at serine 216, cdc25C is also phosphorylated by protein kinase CK2 at serine 236. Both phosphorylation sites do not influence the enzymatic activity of cdc25C, but serine 236 phosphorylation is also implicated in the regulation of the sub-cellular localization. Inhibition of the serine 216 phosphorylation leads to increased localization of cdc25C into the nucleus. The elevated level of cdc25C results in a premature death of cell (Schwindling *et al.*, 2004).

Also cdc25C proteins that do not contain a binding site for 14-3-3 proteins showed a pancellular localization and an increased ability to induce premature chromosome condensation (Dalal *et al.*, 1999). As shown in the present study after treatment of cells with gemcitabine or paclitaxel or a combination of both there was no change in the sub-cellular localization of cdc25C or p53. This

observation is in agreement with finding that there was no premature cell death after cytostatica treatment of ovarian carcinoma cells. Instead the apoptotic pathway is induced.

Treatment of mitotic HeLa cells with TPA caused a rapid decrease of the specific enzyme activity of cdc25C due to dephosphorylation of the enzyme (Barth *et al*., 1996). Treatment of ovarian cancer cells with gemcitabine and paclitaxel led to reduction in the phosphorylation of serine 216 of cdc25C. A reduction in the serine 216 phosphorylation was observed with the Chk1 inhibitor. Chk1 is known to phosphorylate cdc25C at serine 216. This might indicate that an additional phosphorylation site might be affected by the cytostatica. Since we observed no additional reduction in the phosphorylation of cdc25C by the use of gemcitabine and paclitaxel together with the Chk1 inhibitor might indicate that the same phosphorylation seems to be affected.

Since it was known that a lower phosphorylated cdc25C also has a lower enzymatic activity, in the present study also the phosphatase activity of cdc25C was analyzed. The treatment of OV-MZ-32 cells with paclitaxel had no effect on the cdc25C phosphatase activity, but when cells were treated with gemcitabine or a combination of both drugs the cdc25C phosphatase activity decrease considerably. The phosphatase activity of cdc25C in OvBH-1 cells did not change after treatment. In OvCBM cells the cdc25C phosphatase activity increased after treatment of cells with gemcitabine, and it decreased when cells were treated with paclitaxel or a combination of both drugs.

From these results it might well be that the level of phosphorylation of cdc25C has an influence on the phosphatase activity of cdc25C. However, there is clearly more than one mechanism regulating the phosphatase activity of cdc25C. So far little is known about the regulation of the activity of cdc25C. It was recently found that p53 binds to cdc25C and this binding leads to a down-regulation of cdc25C (Ruppenthal *et al*., 2007). Another possibility for down-regulation of cdc25C is the rapid degradation which is observed in the M-phase of the cell cycle. Since the amount of cdc25C seems to be the same after cytostatica treatment degradation seems to be not responsible for the

down-regulation of the phosphatase activity of cdc25C observed in the present study.
Moreover, since p53 was found in the nucleus and cdc25C in the cytoplasm makes a downregulation of cdc25C by p53 highly unlikely.

In summary the present work has shown that Gemcitabine is an efficient drug for the treatment of ovarian cancer. Gemcitabine and paditaxel treatment of ovarian cancer cells leads to apoptosis. Apoptosis induction does not go along with an induction of p53 protein expression. Although at least OvBH-1 expressed wild type p53 it turned out that p53 in all three cell lines tested have only a restricted transcription factor activity indiated that at least some of the p53 function got lost in the tumor cells which is in agreement with earlier findings of our group. Furthermore the expression as well as its sub- cellular localization of the check- point control protein phosphatase cdc25C is not affected by the cytostatica treatment of the ovarian cancer cell lines studied here there is a reduction in the phosphorylation of cdc25C. Since a proper phosphorylation of cdc25C is necessary for the full activity of cdc25C one might speculated that the cytostatica treatment affected the activity of cdc25C via its phosphorylation.

6. References

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Curriculum Vitae (C.V)

Name:	Rania Elias Touma
Nationality:	Syrian
Personal information:	born in 09/07/1974, married with one child.
Education:	1- MSc in medical laboratory 2001. 2- Diploma of medical laboratory.1997-2000 3- Medical school (M.D degree) Damascus University 1997
Current position:	instructor, Faculty of Medicine, Damascus University, 1997 to current date.
Extra training:	1- Hematology, Clinical Chemistry, Microbiolog immunology and virology. Central Laboratory of the University Hospital of Damascus from 1997 to 2003. 2- Computing 2000-2001. 3- Workshop on Molecular Biology: Technique and applications– Faculty of medicine– Damascus University in collaboration with University of Saarland(medical Faculty)– Sep.2003.
Publication:	Growth inhibition and apoptosis induction in ovarian cancer cells. International Journal of Oncology. 2006

Eidesstattliche Versicherung

Hiermit versichere ich an Eides statt, dass ich die vorligende Arbeit selbständing und ohne Benutzung anderer als der angegebenen Hilfsmittel angefertig habe. Die aus anderen Quellen direkt übernommenen Daten und Konzepte sind unter Angabe der Quelle gekennzeichnen.

Die Arbeit wurde bisher weder im inland noch im Ausland in gleicher oder ähnlicher Form in einem anderen Verfahren zur Erlangung des Doktorgrades einer anderen Prüfungsbehörde vorgelegt.

Homburg, den 06.07.2007

Rania Touma