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The addition of the novel heparan sulfate (HS) mimetic PG545 to standard chemotherapy shows promising efficacy in the treatment of ovarian cancer *in vitro* and *in vivo*.

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Für meine Familie

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1 List of abbreviations

BCA	bicinchoninic acid
bFGF	basic fibroblast growth factor
BM	basement membrane
BMI-1	B cell-specific Moloney murine leukemia virus integration site 1
CCR	complete clinical remission
CD-31	cluster of differentiation 31
CDDP	cisplatin
CI	combination index
CSC	cancer stem cell
DMSO	dimethyl sulfoxide
DPBS	Dulbecco's phosphate-buffered saline
ECM	extracellular matrix
EC	endothelial cell
ECL	enhanced chemiluminescence
ED	effective dose
FBS	fetal bovine serum
FGF	fibroblast growth factor
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GF	growth factor
HB-EGF	heparin-binding epithelial growth factor
HCl	Hydrogen chloride
H&E stain	haematoxylin and eosin stain
HPF	high power field
HRP	horseradish peroxidase
HS	heparan sulfate
HSPG	heparan sulfate proteoglycan
IC	inhibitory concentration
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MVD	micro vessel density

List of abbreviations

NBF	neutral buffered formalin
Oct-4	octamer-binding transcription factor 4
OS	overall survival
PARP	poly ADP ribose polymerase
PBS	phosphate buffered saline
PFS	progression-free survival
PI	proliferation index
PNPP	p-Nitrophenyl Phosphate
PVDF	polyvinylidene difluoride
TBS	Tris-Buffered Saline
TBST	Tris-Buffered Saline and Tween 20
TEMED	tetramethylethylenediamine
SDF-1	stromal cell-derived factor 1
SDS-PAGE	sodium dodecyl sulfate- PAGE
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor

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3 Introduction

3.1 Ovarian Cancer

With an estimated 22,440 new cases in the United States in 2017, ovarian cancer is the second most common gynecologic cancer (Cancer Facts & Figures 2017). "Epithelial ovarian cancer (EOC) is the fifth leading cause of cancer deaths among women in the United States" (Flesken-Nikitin, Hwang et al. 2013) and with 15,500 estimated deaths per year it is the most lethal gynecologic cancer (Siegel, Naishadham et al. 2012).

The highly lethal nature of this cancer stems from its asymptomatic growth, resulting in advanced, widespread disease in about 70% of women at diagnosis (Yoneda, Lendorf et al. 2012).

The current treatment for women with newly diagnosed advanced ovarian cancer is, surgical cytoreduction followed by platinum and taxane- based chemotherapy, which results in complete clinical remission (CCR) in up to 75% of cases (Mei, Chen et al. 2013). However, 75% of the responders will relapse within 18 to 28 months (Mei, Chen et al. 2013). The disease is no longer curable at recurrence due to the lack of effective therapies resulting in only 30% 5 year survival of advanced stages (Naora and Montell 2005).

" The average lifetime risk for women is about 1 in 70" (Cannistra 2004) and with a median age of 60, the disease predominantly affects postmenopausal women (Liu 2005). Most ovarian cancers result from sporadic mutations (Yoneda, Lendorf et al. 2012). Although only around 10 percent of ovarian cancer patients have an identifiable genetic predisposition, the most important risk factor of ovarian cancer is a family history of ovarian or breast cancer (Cannistra 2004). Moreover, there is a general agreement that nullparity, early menarche and late menopause are associated with an elevated risk of ovarian cancer and that oral contraceptive use, pregnancy, and lactation are associated with a reduced risk (Whittemore 1994, Cannistra 2004). These findings propose that repeated stimulation of the epithelium of the ovarian surface, as a result of a high number of lifetime

ovulations, may predispose the epithelium to malignant transformation (Cannistra 2004, Liu 2005).

Epithelial ovarian cancer, also ovarian carcinoma, is the most common type of ovarian cancer (Schildkraut, Iversen et al. 2013). Of the five main subtypes of ovarian carcinoma (high grade serous, endometrioid, clear cell, mucinous and undifferentiated), high- grade serous epithelial ovarian cancer is the most common (Schildkraut, Iversen et al. 2013) and most lethal (Kessler, Fotopoulou et al. 2013) and characterized by its chromosomal instability (Wang, Birkbak et al. 2012).

3.1.1 Metastasis in ovarian cancer

As the majority of patients present at an advanced stage, with widely metastatic spread within the peritoneal cavity, metastasis plays an important role in ovarian cancer. In contrast to hematogenously metastasizing tumors, ovarian cancer metastasizes either by direct extension to neighboring organs or when cancer cells detach from the primary tumor (Lengyel 2010). This process is facilitated by enzymes, like heparanase, that disconnect intercellular adhesions, loosen the ECM (extracellular matrix) and lead to shedding of single cells from the primary tumor (Vlodavsky and Friedmann 2001). Then, transported throughout the peritoneum by normal peritoneal fluid, exfoliated tumor cells spread within the abdominal cavity (Lengyel 2010). The dissemination of tumors cells within the peritoneal cavity is often associated with the production of ascites (Gavalas, Liontos et al. 2013). Carried by the peritoneal fluid, cancer cells have to overcome anoikis, an intrinsically programmed cell death (apoptosis) that occurs due to detachment of a cancer cell from the extracellular matrix (Kandala and Srivastava 2012). Resistance to anoikis is an essential feature of a metastasizing tumor cell and contributes to the formation of metastasis in other organs (Kandala and Srivastava 2012). After overcoming anoikis and circulating in the peritoneal fluid, cancer cells attach, preferentially on the abdominal peritoneum or omentum (Lengyel 2010). The viability of these cells and successful tumor formation on a metastatic site is further dependent upon the development of sufficient neovasculature (Jelovac and Armstrong 2011) through neoangiogenesis.

3.1.2 Angiogenesis in ovarian cancer

Angiogenesis is defined as the growth and recruitment of new blood vessels (Nadkarni, Geest et al. 2013). It is a complex and highly regulated process and a key factor for tumor progression and development of metastasis (Spannuth, Sood et al. 2008). Tumor growth beyond a few millimeters is dependent on angiogenesis, as the production of new blood vessels is necessary to supply the tumor with oxygen and nutrients (Teodoro, Evans et al. 2007).

Angiogenesis is a multi-step process (Martin and Schilder 2007), which is regulated by a balance between proangiogenic and antiangiogenic molecules such as cytokines and proangiogenic growth factors (i.e. VEGF, platelet-derived growth factor, fibroblast growth factor). In tumors this equilibrium is altered, balance leans "towards the greater expression of pro-angiogenic factors and angiogenesis occurs" (Gavalas, Liontos et al. 2013). Markers of angiogenesis such as VEGF and CD31 have been identified to have prognostic value in ovarian cancer patients (Nadkarni, Geest et al. 2013) and increased vascularity is associated with poor survival in patients with ovarian carcinoma (Alvarez, Krigman et al. 1999). Furthermore, with a strong expression in ovarian carcinoma, moderate expression in borderline tumors and a weak or no expression in normal ovarian tissue, VEGF expression is cancer specific (Schmitt and Matei 2012).

Angiogenesis also contributes to the metastatic process (Folkman 1990). Neovascularization permits the dissociation of cells from the primary tumor (Folkman 1990). It was also shown that VEGF overexpression may lead to peritoneal related neoangiogenesis and increased vascular permeability in the peritoneum, suggesting that angiogenesis is involved in the metastasis of tumor to the peritoneum (Gavalas, Liontos et al. 2013). A decrease of angiogenesis is associated with a decreased rate of metastasis (Folkman 1990). The understanding of the molecular mechanisms of tumor angiogenesis led to the identification of angiogenic targets and the development of anti-vascular agents.

3.1.3 Heparan sulfates and heparanase in ovarian cancer

Due to the inherent instability of the genome of high- grade serous ovarian cancer, the focus of current research has shifted from the tumor itself, to a new target: the tumor microenvironment (Vaughan, Coward et al. 2011). According to Paget's 'seed and soil' hypothesis, which is now widely accepted, a cancer's unique microenvironment and as part of it the extracellular matrix (ECM), present the 'soil' on which 'the seed' (the tumor cell) can grow, proliferate and metastasize (Talmadge and Fidler 2010).

A key component of the ECM is the ubiquitous macromolecule heparan sulfate proteoglycan (HSPG) (Sasisekharan, Shriver et al. 2002). The HS (heparan sulfate) chains of HSPGs, which are attached to core proteoglycans (Joyce, Freeman et al. 2005), are structurally heterogeneous and provide binding-sites for a diverse repertoire of heparin- binding proteins, including extracellular signaling molecules such as the growth factors (for example FGF, VEGF165 and HB-EGF), chemokines, morphogens and enzymes (Bernfield, Gotte et al. 1999). Moreover, HSPGs function as key co-receptors for these ligands and thus play an important role in modulating heparin-binding growth factor signaling (Blackhall, Merry et al. 2001). As HSPGs also bind to structural proteins of the extracellular matrix, they are part of cell-cell and cell-matrix adhesion complexes (Davies, Blackhall et al. 2004) and thereby contribute significantly to the integrity of the ECM. By mediating adhesion, migration and cellular responses to mitogenic and angiogenic growth factors, HSPGs regulate multiple pathways including those important in tumorigenesis such as tumor progression, angiogenesis, and metastasis (Sasisekharan, Shriver et al. 2002).

Regulation of HSPGs can occur through the enzyme heparanase, "which cleaves heparan sulfate (HS) and hence participates in degradation and remodeling of the extracellular matrix (ECM)" (Ilan, Elkin et al. 2006) and release of HS- bound biological mediators. It has been shown that ovarian carcinomas express higher levels of heparanase than normal ovaries and borderline tumors (Ginath, Menczer et al. 2001) and that heparanase is highly expressed in carcinomas with large

ascites and high grade (Kodama, Shinyo et al. 2003), suggesting an important role of this molecule in aggressive ovarian neoplams.

By cleaving HS chains, resulting in the degradation of the ECM and the loosening of intercellular connections, heparanase promotes cellular mobility and cell dissemination. Consequently, "the expression of heparanase correlates with the metastatic potential of tumor cells" (Vlodavsky and Friedmann 2001). In ovarian carcinoma, it has been shown that heparanase is frequently expressed in metastatic ovarian cancer, it is secreted into the peritoneal fluid and that increased heparanase expression correlates with poor overall survival (Davidson, Shafat et al. 2007).

By degrading HS in the sub-endothelial basement membrane of blood vessels, heparanase facilitates endothelial cell migration and sprouting, which is an early event in the angiogenic process. Moreover, the release of HS- bound angiogenic growth factors (bFGF, VEGF) through heparanase activity liberates pro-angiogenic molecules that may act cooperatively to promote neovascularization (Vlodavsky and Friedmann 2001). Therefore it is not surprising, that increased heparanase expression is associated with increased microvessel density (Vlodavsky, Abboud-Jarrous et al. 2006). Targeting heparanase thus presents a promising target to mediate anti-angiogenic and anti-metastatic effects.

3.2 Therapy of ovarian cancer

The standard therapy of ovarian cancer consists of primary cytoreductive surgery followed by platinum- based chemotherapy. The amount of residual tumor is the most important prognostic survival factor of ovarian cancer patients (Elattar, Bryant et al. 2011), the surgical treatment is aggressive and the goal of cytoreductive surgery is optimal debulking, which means no residual tumor < 1cm. More recently optimal debulking is considered as no residual tumor (R0). The surgical goal is a complete cytoreduction with no residual after surgery. Nevertheless, surgical cytoreduction is, due to the cancer's diffuse nature, rarely sufficient to render patients disease-free and adjuvant chemotherapy is required

to eradicate residual disease, except for women with organ-confined, low-grade disease (Naora and Montell 2005).

The current standard chemotherapy for advanced ovarian cancer is the intravenous administration of six cycles of carboplatin and paclitaxel. Platinum based compounds, such as carboplatin and cisplatin, are the most potent chemotherapeutics in ovarian cancer treatment (Cannistra 2004). They mediate their effect through the formation of intrastrand cross-links with DNA (Cannistra 2004). Taxanes, for example paclitaxel, exert their cytotoxic effects through binding to and stabilization of tubulin polymers (Cannistra 2004). As ovarian cancer is highly sensitive to chemotherapy, the majority of patients will achieve a full remission with initial treatment (Jelovac and Armstrong 2011). It is commonly acknowledged that a platinum and taxane-based chemotherapy improves both, progression-free survival (PFS) and overall- survival (OS) (Binaschi, Simonelli et al. 2011).

Despite an initial response in about 80% of patients, relapse occurs in most patients with advanced-stage disease, making ovarian cancer essentially incurable (Jelovac and Armstrong 2011). Although recurrent cancer is highly treatable, it has a very poor prognosis. Patients have to receive multiple lines of chemotherapy with unsatisfactory results with regard to PFS and OS, due to the occurrence of drug resistant cancer clones (Tomao, Papa et al. 2013). A large number of chemotherapeutics (liposomal doxorubicin, topotecan, gemcitabine, paclitaxel, oral etoposide and vinorelbine (Cannistra 2004)) have been tested and can be used as second-line therapeutics to treat (even platinum-resistant) recurrent cancer, but the second remission is in more than 95% of cases shorter than the first (Jelovac and Armstrong 2011) resulting in a median survival of less than two years from the time of recurrence (Gadducci, Cosio et al. 2005).

3.2.1 Maintenance therapy, recurrence and cancer stem cells (CSC)

As recurrence of chemo-resistant ovarian cancer after initial response to first-line chemotherapy is the major obstacle in the treatment of ovarian cancer, one

potential strategy that may improve patient outcome is maintenance therapy targeting residual subclinical disease. Strategies of maintenance therapy aiming to impede recurrence and to prolong progression-free survival (PFS) have been studied, but a statistically significant prolongation of PFS has only been achieved by prolonged administration of paclitaxel and the combination of bevacizumab and chemotherapy followed by a maintenance period of bevacizumab (12- 15 cycles) (Binaschi, Simonelli et al. 2011).

Clearly, disease recurrence is caused by persisting dormant and drug-resistant cells after front-line therapy (Binaschi, Simonelli et al. 2011). "Recent studies have identified and characterized a self-renewing subpopulation of cancer-initiating cells in ovarian cancers endowed with stem cell-like properties" (Siu, Wong et al. 2013). These cancer-initiating cells, also called cancer stem cells, have the ability to self-renew, differentiate and to form tumors (Zhan, Wang et al. 2013). Moreover, cancer stem cells have been reported to be involved in drug resistance and have been identified as key players in the development of recurrence. "Traditional chemotherapy can kill the majority of cancer cells, while failing to target cancer stem cells" (Zhan, Wang et al. 2013). These cells survive chemotherapy and enter a state of dormancy during which they survive in still undefined niches within the body before being stimulated to initiate re-growth (Binaschi, Simonelli et al. 2011). Recent studies, showing that cisplatin treatment of ovarian cancer cell lines *in vitro* leaves residual cells that are enriched in cancer stem cell-like traits, underline the importance of cancer-initiating cells in disease recurrence (Abubaker, Latifi et al. 2013). The identification of cancer-initiating cells "has opened up a novel field of research aimed at identifying additional innovative therapeutic approaches" (Binaschi, Simonelli et al. 2011).

3.2.2 Targeted therapies

Despite intensive research on chemotherapeutic drugs and the significant progress made in understanding the molecular pathogenesis and signaling pathways of epithelial ovarian cancer, this has not led to satisfactory improvement in outcomes or overall survival (Howlader et al. 2011; Jelovac et al. 2011).

As a result, efforts are still ongoing to identify novel anti- cancer agents. The exploration of the molecular pathways within normal and malignant cells has led to the development of drugs with distinct molecular targets. Their aim is on individual pathways that are important to tumor development including angiogenesis, invasive and metastatic potential (Martin and Schilder 2007). Those "targeted therapies" have the potential to improve long-term disease management and decrease unwanted toxic side effects (Martin and Schilder 2007).

In the treatment of ovarian cancer, the most promising results in the field of targeted therapy have come from targeting angiogenesis (Martin and Schilder 2007). Anti-angiogenic therapies have shown to slow primary tumor growth and resulted in promising and well-validated therapeutics (Gavalas, Liontos et al. 2013). Inhibitors of the VEGF/VEGFR angiogenesis pathways, such as the VEGF monoclonal antibody bevacizumab, have been approved for use in several cancers, including ovarian cancer. However, since they target an individual signaling pathway, they miss the development of metastasis and thus are not sufficient to prevent death from metastatic cancer. Animal models of cancer have even shown that the treatment with anti-angiogenic drugs might favor the spread of tumor cells and promote the formation of metastasis (Ebos, Lee et al. 2009) (Ostapoff, Awasthi et al. 2013) (Paez-Ribes, Allen et al. 2009). As a mode of resistance to anti-VEGF treatment, other growth factors have been found to remain functionally active. In a maintenance setting, anti-angiogenic therapeutics were unable to prevent recurrence and render patients disease free.

Thus a dual approach with inhibition of angiogenesis and modulation of the tumor environment and extracellular matrix to decrease the metastatic potential would be needed to effectively target the features of ovarian cancer.

3.3 The heparan sulfate mimetic PG545

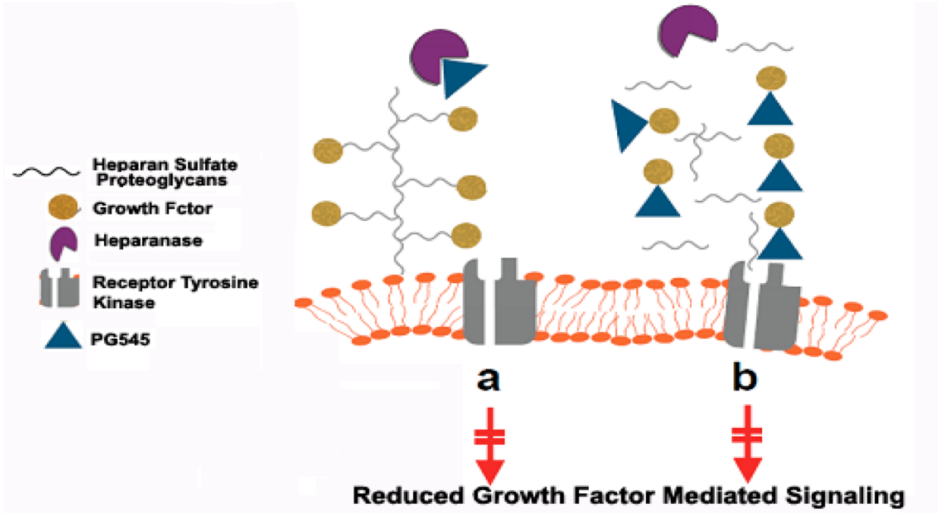


Figure 1: Scematic view of PG545 interacting with (a) Heparanase to prevent cleavage and release of (b) HS bound GF to prevent binding to receptor. Either process blocks intracellular signaling in tumor and endothelial cells.

Fig.1: The mechanism of action of PG545. Figure provided by Progen.

Aiming attention at HSPGs and heparanase in the development of potential cancer therapeutics led to the identification of sulfated oligosaccharides as structural mimetics of heparan sulfate with a dual mechanism of action: inhibition of "angiogenesis by sequestering angiogenic growth factors in the extracellular matrix (ECM), thus limiting subsequent binding to receptors" (Dredge, Hammond et al. 2011) and inhibition of metastasis by inhibiting heparanase, the only endglycosidase, which cleaves HS chains in the ECM.

The hypothesis was that HS mimetics, targeting heparanase and multiple growth factors in the tumor microenvironment, would function as inhibitors of multiple signaling pathways, resulting in inhibition of tumor growth, angiogenesis and metastatic spread. A heparan sulfate mimetic of the first generation, which had the important property of simultaneously inhibiting angiogenesis and heparanase, is PI-88 (Parish, Freeman et al. 1999, Joyce, Freeman et al. 2005, Ferro, Dredge et al. 2007). Seeking to improve upon the functionality of PI-88, the PG500 series was born and PG545 was identified as the leading compound for further investigation as a novel anti-cancer agent (Dredge, Hammond et al. 2010).

"PG545 is a fully sulfated tetrasaccharide functionalized with a cholestanyl aglycon designed at Progen Pharmaceuticals Ltd (Brisbane, QLD, Australia)" (Dredge, Hammond et al. 2011). Compared to PI-88, increased potency and lower anticoagulant property was achieved by adding a lipophilic moiety (aglycon) (Ferro, Liu et al. 2012). Testing for HS-binding site activity indicated low nanomolar binding of PG545 to HS- binding proteins, which are important in tumor angiogenesis such as FGF1, FGF2 and VEGF and potent inhibition of heparanase (Ki= 6 nM) (Dredge, Hammond et al. 2010). In cell-based assays, PG545 potently inhibited growth factor-induced endothelial cell proliferation, which is the first essential cellular step in angiogenesis (Dredge, Hammond et al. 2010). In an *ex vivo* rat aortic ring assay, PG545 had an over 80% inhibitory effect on angiogenesis (Dredge, Hammond et al. 2010).

In vivo, PG545 was shown to inhibit angiogenesis and solid tumor growth in murine mouse models of multiple types of cancer (Ferro, Liu et al. 2012). The anti-metastatic activity of PG545 was tested in three murine models of metastasis: a B16 melanoma model, a HT-29 colon carcinoma model and a Lewis Lung Carcinoma model, in all of which PG545 significantly inhibited metastatic spread, resulting in a reduced number of metastasis (Dredge, Hammond et al. 2011).

In ovarian cancer, it has been shown recently, that independent of the heparin-binding growth factor (HB-EGF, HGF, FGF-2, VEGF or SDF-1) used to stimulate SKOV3- ovarian cancer cells, the exposure to PG545 reduced cellular migration and invasive capacity. The theory, that PG545, by inhibiting heparanase and sequestering growth factors, attenuates downstream cell signaling, has been confirmed by the finding that treatment of SKOV3 and OV202 ovarian cancer cells with PG545 results in decreased activation of the EGF receptor and subsequent decreased phosphorylation of the downstream signaling proteins of the ERK/ MAPK pathway *in vitro*. Furthermore, PG545 showed significant anti-tumor activity with marked reduction in tumor burden in cisplatin-sensitive, cisplatin-resistant and immunocompetent ovarian tumor mouse models *in vivo* as both, a single agent and in combination with the standard chemotherapeutics cisplatin and paclitaxel. The anti-metastatic effect of PG545 in ovarian cancer has been studied *in vitro* and *in vivo.* PG545 was found to inhibit adhesion of ovarian cancer

cells to mesothelial cells *in vitro* and significantly inhibited bowel and peritoneal dissemination in a highly metastatic *in vivo* tumor model (data not published).
PG545 is currently being investigated in a Phase I clinical trial in advanced solid cancer patients (Winterhoff, Freyer et al. 2015).

4 Aim of the study

Despite significant progress in understanding the nature of ovarian cancer and considerable advances in cytotoxic therapy, the overall 5-year survival rate in patients suffering from ovarian cancer remains at 30%. This is mainly attributed to recurrence of the disease after initial response to surgical treatment and first-line chemotherapy. Currently there is no drug or drug combination available that effectively eradicates remaining cancer cells after surgery and thus prevents recurrence of the disease. The novel heparan-sulfate mimetic PG545 has shown promising results as an anti- cancer drug *in vitro* and *in vivo*. PG545 provides, next to formation of intrastrand cross-links with DNA by cisplatin and binding to tubulin polymers by taxanes, a third mechanism of action to attack ovarian cancer cells and thus seems to be a promising addition to conventional chemotherapy. Furthermore, due to the characteristic overexpression of VEGF (vascular endothelial growth factor), a growth factor promoting angiogenesis, in 90% of human ovarian tumors and the high metastatic potential of this disease, epithelial ovarian cancer provides an ideal target for PG545.

The principal aim of this study is to test if the addition of PG545 to standard chemotherapy with cisplatin and paclitaxel provides a more powerful anti-cancer regimen. We also seek to identify the most effective schedule of drug administration and examine if the addition of PG545 to standard chemotherapy is effective in preventing recurrence of ovarian cancer in a maintenance setting *in vivo*.

First, the effect of PG545 on A2780 and SKOV3 ovarian cancer cells as a single agent and in combination with the standard chemotherapeutics for ovarian cancer, cisplatin and paclitaxel, is investigated *in vitro*. As PG545 has previously shown anti-metastatic potential, the effect of PG545, alone and in combination with cisplatin, on ovarian cancer cells growing under detachment growth conditions is examined *in vitro*.

Next, the anti-tumor effect of PG545 is tested in an *in vivo* model for recurrent ovarian cancer. We investigate the efficacy of PG545 in combination with standard chemotherapy and as a maintenance agent and test, if the addition of PG545 to

standard chemotherapy has the potency to maintain the clinical complete response after initial chemotherapy, resulting in progression- free and overall survival benefits. Using immunohistochemistry and western blot analysis, we determine the effect of PG545 treatment on angiogenesis and CSC *in vivo.*

5 Material and Methods

5.1 Materials

5.1.1 Instruments

Microscope Leica DMIL	Leica, North Central Instruments, Plymouth, MN, USA
Camera Leica ICC50 HD	Leica, North Central Instruments, Plymouth, MN, USA
Centrifuge Legend X1R	Thermo Scientific, Waltham, MA, USA
Centrifuge IEC Centra CL2	Thermo Scientific, Waltham, MA, USA
Victor3 Multilabel Plate Reader	PerkinElmer, Waltham, MA, USA
Spectrophotometer, DU 530	Beckman Coulter, USA
Heater	Fisher Scientific, Manassas, VA, USA
PowerPac 200, Electrophoresis Power Supply	Bio-Rad Laboratories, Hercules, CA, USA
Hemacytometer Reichert Bright-Line	Hausser Scientific, Horsham, PA, USA

5.1.2 Cell lines

A2780	Dr. Thomas Hamilton, Fox Chase Cancer Center, Philadelphia, PA, USA
SKOV3	American Type Culture Collection, Manassas, VA, USA
A2780 luciferase expressing	Department of Experimental Pathology, Mayo Clinic, MN, USA

5.1.3 Cell culture reagents and plasticware

RPMI 1640, 1X (with L-Glutamine)	Cellgro, Mediatech Inc, Manassas, VA, USA
Mc Coy's 5A, 1X(with L-Glutamine)	Cellgro, Mediatech Inc, Manassas, VA, USA

HyClone Standard Fetal Bovine Serum	HyClone Laboratories, Thermo Scientific, Logan, Utah, USA
Penicillin/Streptomycin	Gibco by life technologies, Invitrogen, Grand Island, NY, USA
Novolin R Insulin	Novo Nordisk, Novo Allé, Danmark
DPBS, 1X(without Calcium and Magnesium)	Cellgro, Mediatech Inc, Manassas, VA, USA
0,25% Trypsin/EDTA (1X)	Gibco by life technologies, Invitrogen, CAN
Cell Culture Flask 150 cm2	Corning Life Sciences, Big Flats, NY, USA
Cell Culture Flask 75 cm2	BD Falcon, Franklin Lakes, NY, USA
6 Well Cell Culture Plates	Corning Life Sciences, Big Flats, NY, USA
96-Well Plates	Corning Life Sciences, Big Flats, NY, USA
6-Well ultra-low Attachment Plates	Fisher Scientific, Manassas, VA, USA
60 mm Tissue Culture Dish	BD Falcon, Franklin Lakes, NY, USA

5.1.4 Reagents and materials

Trypan Blue Solution 0,4%	Cellgro, Mediatech Inc, Manassas, VA, USA
MTT Reagent	Sigma, St. Louis, MO, USA
Coomassie Blue	Department of Experimental Pathology, Mayo Clinic, MN, USA
DMSO	Fisher Scientific, Manassas,VA, USA
BSA	Thermo Scientific, Rockford, IL, USA
BCA Reagent A+B	Thermo Scientific, Rockford, IL, USA
Protein Assay Dye Reagent Concentrate	Bio-Rad Laboratories, Hercules, CA, USA
ProtoGel Ultrapure	National Diagnostics, Atlanta, GA, USA
ProtoGel Resolving Buffer 4X	National Diagnostics, Atlanta, GA, USA
Ammonium Persulfate	Bio-Rad Laboratories, Hercules, CA, USA
TEMED (N,N,N',N'-Tetramethylethylenediamine)	Sigma Aldrich, St. Louis, MO, USA
ProtoGel Stacking Buffer	National Diagnostics, Atlanta, GA, USA
Isopropyl Alcohol	Mayo Clinic, Rochester, MN, USA
NuPage LDS Sample Buffer 4X	Invitrogen, Carlsbad, CA, USA
Criterion Empty Cassettes	Bio-Rad Laboratories, Hercules, CA, USA
Pageruler plus prestained	Fisher Scientific, Manassas, VA, USA
Running buffer	Bio-Rad Laboratories, Hercules, CA, USA
Blot Paper, extra thick	Bio-Rad Laboratories, Hercules, CA, USA
Nictrocellulose Membrane, 0,45 um	Bio-Rad Laboratories, Hercules, CA, USA

Immobilon-P Membrane, PVDF	EMD Millipore, Billerica, MA, USA
Methanol	Mayo Clinic, Rochester, MN, USA
Blotting Grade Blocker (Non-fat dry milk)	Bio-Rad Laboratories, Hercules, CA, USA
Amersham ECL Prime Western Blotting Detection Reagent	GE Healthcare, Little Chalfont Buckinghamshire , UK
Pierce ECL Western Blotting Substrate	Thermo Scientific, Rockford, IL, USA
HyBlot CL Autoradiography Film	Denville Scientific Inc., South Plainfield, NJ, USA
D- Luciferin Sodium Salt	Regis, Morton Grove, IL, USA
Formaldehyde	Fisher Scientific, Manassas, VA, USA
Forane (Isoflurane, USP)	Baxter Healthcare Corporation, Deerfield, IL, USA
Specimen containers	Kendall, Tyco Healthcare, Mansfield, MA, USA
Fisher Tissue Path Cassettes IV	Fisher Scientific, Manassas, VA, USA
Xylene	EMD Millipore, Billerica, MA, USA
Ethanol	Sigma Aldrich, St. Louis, MO, USA
Target Retrieval Solution	Dako, Carpinteria, CA, USA
Hydrogen peroxide solution 30% (w/w)	Sigma Aldrich, St. Louis, MO, USA
Grease Pen	Dako, Carpinteria, CA, USA
Protein Block	Dako, Carpinteria, CA, USA
Biotin Blocking system	Dako, Carpinteria, CA, USA
LSAB+ System-HRP	Dako, Carpinteria, CA, USA
Mayer's Hematoxylin	Dako, Carpinteria, CA, USA
Bluing Reagent	Lermer Laboratories, Pittsburgh, PA, USA
Shandon EZ-Mount	Thermo Electron Corporation, Pittsburgh, PA, USA
Premium Microscopic Slides	Fisher Scientific, Manassas, VA, USA

5.1.5 Solutions and buffer

Transfer Buffer	Mayo Clinic, Rochester, MN, USA
TBS-Tween-20 (20X)	Boston BioProducts, Ashland, MA, USA
Restore Western Blot Stripping Buffer	Thermo Scientific, Rockford, IL, USA

5.1.6 Antibodies

Bmi1, rabbit, ploYclonal	Novus, Littleton, CO, USA
Oct-4, rabbit, polyclonal	Cell Signaling Technology, Danvers, MA, USA
Nanog, rabbit, polyclonal	Cell Signaling Technology, Danvers, MA, USA

GAPDH, rabbit, monoclonal	Cell Signaling Technology, Danvers, MA, USA
CD31, mouse, monoclonal	PECAM, Santa Cruz Biotechnology, Dallas, TX, USA
Ki67, rabbit, monoclonal	Clone EP5, Epitomics, Burlingame, CA, USA

5.1.7 Drugs and chemotherapeutics

Cisplatin 50 mg	EMD Millipore, Billerica, MA, USA
Paclitaxel (30mg/5ml)	Novaplus
PG545	Progen Pharmaceuticals, Brisbane, QLD, Australia

5.1.8 Mouse model

50 six-week old, female, athymic nude mice were obtained from the National Cancer Institute (Frederick, MD).

5.2 Methods

5.2.1 Cell culture

Maintenance of cell lines

A2780 and A2780-luci cells were cultured in RPMI 1640 with L-Glutamine medium supplemented with 10% fetale bovine serum (FBS), 100 IU penicillin, 100 µg/ml streptomycin and 0.01mg/ml human insulin and SKOV-3 were cultured in McCoy's 5A with L-Glutamine medium supplemented with 10% FBS, 100 IU penicillin and 100 µg/ml streptomycin.

Cells were grown in 150cm^2 tissue culture flasks and maintained in humidified incubators at 37°C circulated with 5% CO2/95% air. Cells were passaged every 3 days.

5.2.2 In vitro proliferation assay

The anti-proliferative effect of PG545, cisplatin and paclitaxel and the combination of cisplatin and PG545 and paclitaxel and PG545 was assessed using the MTT colorimetric assay, first described by Mosmann in 1983 (Mosmann 1983).

Cisplatin was dissolved in PBS to a stock concentration of 1mM. Paclitaxel was made as a 1μM stock solution in PBS and PG545, stored as a 20μM solution in PBS, was further diluted in media to a 1mM stock solution.

A2780 and SKOV-3 cells in exponential growth were washed with phosphate-buffered saline (PBS), trypsinized with 0.25% trypsin/EDTA at 37°C, counted and seeded as a single cell suspension in 200 μl media at a concentration of $5x10^3$ cells/well in 96-well plates and allowed to attach over night at 37°C. Serial dilutions were made from the stock solutions in complete culture media and, to test the drugs as single agents, cells were exposed to various drug concentrations between 1-150 μM for cisplatin, 1-150 nM for paclitaxel and 1-200 μM for PG545 for 48h. To test drug combinations, cells were incubated with increasing concentrations of cisplatin (1-25 μM) or paclitaxel (1-75 nM) without or with the addition of PG545 (2.5 and 10 μM). After a 48h incubation at 37°C, 20 μl MTT reagent (5mg/ml) was added to each well and cells were incubated for another 2 to 4 hours at 37°C. To terminate the experiment, the culture media was replaced with 100 μl DMSO. The absorbance was read at 570 nM with Victor3 Multilabel Plate Reader. All proliferation assays were performed in triplicate.

The sort of interaction between cisplatin and PG545 was assessed by median effect analysis, first described by Chou and Talalay (Chou and Talalay 1984).

The experiment was conducted similarly to the proliferation assay, with the only difference in the drug treatment. A2780 cells were seeded as a single cell suspension in 200 μl media at a concentration of $5x10^3$ cells/well in 96-well plates and were allowed to attach over night at 37°C. The cells were then exposed to increasing concentrations of the chemotherapeutic for 48h. Dose-response curves were first determined for each drug alone and the IC50 value for cisplatin was calculated using the Calcusyn Software. Then the dose- response curves were determined with cisplatin and PG545 in combination at a fixed ratio equivalent to the ratio of their IC50 values. Calcusyn Software was used to calculate the combination indices at the different levels of cell kill (ED). Combination index values <1 indicate synergy, a value of 1 indicated additivity and values >1 indicate antagonism.

5.2.3 Anoikis assay

Anoikis assay was performed as previously described with few modifications (He, Ota et al. 2010).

A2780 cells were plated in duplicate at a density of $4x10^5$ cells/well in 2ml growth media/well in 6-well ultra-low attachment plates. PG545 or cisplatin was added in a volume of 1ml to achieve final concentrations of 1-50 µM for PG545 and 1-15 µM for cisplatin. Cells were treated in duplicate and incubated for 48 hours. After 48 hours, cells were spun down at 1500 rpm for 3 min. Cells were re-suspended in fresh drug-free or PG545 containing media (for PG545 only treatment) and plated as a single cell suspension at a volume of 3ml/dish on 60mm dishes. After an incubation of 8 to 12 hours at 37°C, 1ml MTT reagent was added and cells were incubated for another 1 to 4 hours. After aspiration of the media, 2ml DMSO was added. After transferring 50 µl of each DMSO-cell dilution from the 60 mm dishes in triplicate to 96-well plates, already containing 150 µl of fresh DMSO, plates were read at 500 nm with Victor3 Multilabel Plate Reader.

The same protocol was followed to study the combination of cisplatin and PG545. Cells were incubated with 2.5 µM cisplatin with and without 25 µM PG545 and 5µM cisplatin with and without 25 µM PG545.

To analyze the cleavage of PARP, after being plated as a single cell suspension culture, cells were lysed and analyzed by Western Blotting as described below.

5.2.4 Xenograft model

The animal experiments were conducted according to an Institutional Animal Care and Use Committee–approved protocol and institutional guidelines were followed. 50 six-week old, female, athymic nude mice were obtained from the National Cancer Institute.

Before injection, A2780 luciferase-expressing ovarian cancer cells were washed with PBS, counted and resuspended in PBS at 625000 cells/100 µl. On Day 0, 1.25 Mio cells/mouse were injected into the intraperitoneal cavity. Mice were distributed randomly into 5 treatment groups and treatment was administered as

shown in Fig. 7. The first group was a control group. The second group was a cisplatin/paclitaxel combination treated group receiving 4mg/kg cisplatin plus 16mg/kg paclitaxel on Day 3, 6, and 9 of the study. The third group received a combination of cisplatin (4mg/kg), paclitaxel (16mg/kg) and PG545 (20mg/kg) on Day 3, 6, and 9 followed by PG545 treatment twice weekly (20mg/kg). The fourth group was treated with cisplatin (4mg/kg) and paclitaxel (16mg/kg) on Day 3, 6, and 9 followed by PG545 treatment twice weekly (20mg/kg). The fifth group received PG545 (20mg/kg) twice- weekly. For drug preparation, cisplatin was dissolved firstly in 1ml DMSO and then diluted in phosphate buffered saline (PBS) pH 7.2. Paclitaxel and PG545 were diluted in phosphate buffered saline (PBS) pH 7.2. All drugs were administered i.p. (intra peritoneally).

Body weight was measured twice weekly and tumor growth of 5 mice/group was measured radiographically using the IVIS200 system twice weekly until the end of the study. All mice, surviving until the end of the study, were sacrificed on day 63. To examine the antitumor efficacy of each treatment regimen, all mice were autopsied at death.

5.2.5 Western blot analysis

Generation of cell lysates

Western plot analysis was performed using protein lysates of snap-frozen tumor samples of three mice/ treatment-group. Snap-frozen tumor samples were homogenized and lysed in a cold lysis buffer containing a protease-inhibitor cocktail (Roche, Indianapolis, IN) for 30 minutes on ice, centrifuged at 13,000 rpm for 30 minutes and supernatant was collected.

Lysis buffer AM1 (active motif):

20 mM	Hepes
0.1 mM	EDTA
400 mM	Sodium Chloride
10 mM	Sodium Fluoride
10 µM	Sodium Molybdate

1 mM	Sodium Metavanadate
10 mM	PNPP
10 mM	Beta- glycerophosphate
20%	glycerol
Solution pH 7.5	

Freshly added: Protease inhibitor cocktail

Determination of protein concentration

Protein concentration was estimated by the Bradford method (BioRad, Hercules, CA). The Bradford protein assay, a colorimetric protein assay, is based on the absorbance shift of Coomassie Brilliant Blue G-250 dye in response to various concentrations of protein. The Bradford reagent (5x) was diluted in a 1:5 ratio with deionized water. To estimate protein concentrations, 1000 µl of the diluted Bradford reagent was mixed with 1000 µl ddH2O and 1µl of protein sample and optical density of the reaction was measured at 595 nm with a spectrophotometer.

SDS- polyacrylamide gel electrophoresis

For sample preparation, protein samples with equal amounts of protein were resolved by SDS-PAGE (polyacrylamide gel electrophoresis) gel and electrotransferred onto PVDF (EMD Millipore, Billerica, MD) or nitrocellulose (BioRad, Hercules, CA) membranes.

For separation of proteins, SDS- PAGE was used and gels and solutions were freshly prepared. Gels from 7.5% -15% were prepared, depending on the size of the proteins to be separated and after polymerization were overlaid with a 4% stacking gel. Protein samples with equal amounts of protein were denatured by the addition of 4x nupage LDS sample buffer and heated for 3 minutes at 90 °C. Samples were then applied to the slots of a 4% stacking gel. The cell was filled up with running buffer and samples were run for approximately three hours at 60 V.

Resolving gel:

	7,5%	10%	12%	15%
30% Gel	5 ml	6.7 ml	6 ml	10 ml
ddH20	9.8 ml	8.0 ml	6.8 ml	4.8 ml
4x resolving buffer	5 ml	5 ml	5 ml	5 ml
10% APS	200 µl	200 µl	200 µl	150 µl
TEMED	20 µl	20 µl	20 µl	20 µl

Stacking gel:

	4%
30% Gel	1.3 ml
ddH2O	6.1 ml
Stacking buffer	2.5 ml
10% APS	100 µl
TEMED	10 µl

Sample buffer:

10%	Glycerol
141 mM	Tris base
106 mM	HCl
2%	LDS
0.51 mM	EDTA
0.22 mM	SERVA Blue G250
0.175 mM	Phenol red
pH 8.5	

Tris-glycine-SDS running buffer (1x):

25 mM	Tris HCl
192 mM	Glycine
0.1%	SDS
pH 8.3	

Western blotting

To transfer the proteins on a membrane, carbon paper and nicrocellulose or PVDF membrane were cut to the size of the separating gel and the stacking gel was removed. The membrane was activated by dipping it for one minute into methanol, and the transfer sandwich was set up in the gel holder cassette from bottom to top as followed: foam, carbon paper, gel, membrane, carbon paper, foam. The transfer was done with a voltage of 60V for 1 hour and 20 minutes. After the protein transfer, the membrane was blocked for one hour in blocking solution (5% non fat milk dissolved in TBST) and immunoblotted with the primary antibody (1:500) overnight at 4°C. Post incubation, the membrane was washed in TBST and probed with a HRP-conjugated secondary antibody (1:3000) in blocking buffer for 90 minutes at RT. After another washing step, the proteins of interest were visualized by enhanced chemiluminescence (ECL) (Amersham, Buckinghamshire, UK).

Transfer buffer: premixed

Washing buffer (TBST):

20 mM Tris base

140 mM NaCl

0.05% Tween 20

pH 7.6

5.2.6 Immunohistochemistry

After fixation in 10% neutral buffered formalin (NBF), tumor tissue samples were embedded in paraffin. Paraffin-embedded tissue blocks were cut and processed for H&E staining and immunohistochemistry. Immunohistochemistry evaluated microvessel density (MVD) by staining for CD31. H&E staining and immunohistochemistry were performed by the core facility at Mayo Clinic.

MVD was determined by counting the number of vessels lined by CD31- positive endothelial cells in 5 HPF of viable tumor tissue per specimen. Numbers of vessels were averaged and compared between the groups.

6 Results

6.1 In vitro

6.1.1 *PG545 decreases cell viability of A2780 and SKOV3 cells in a dose dependent manner*

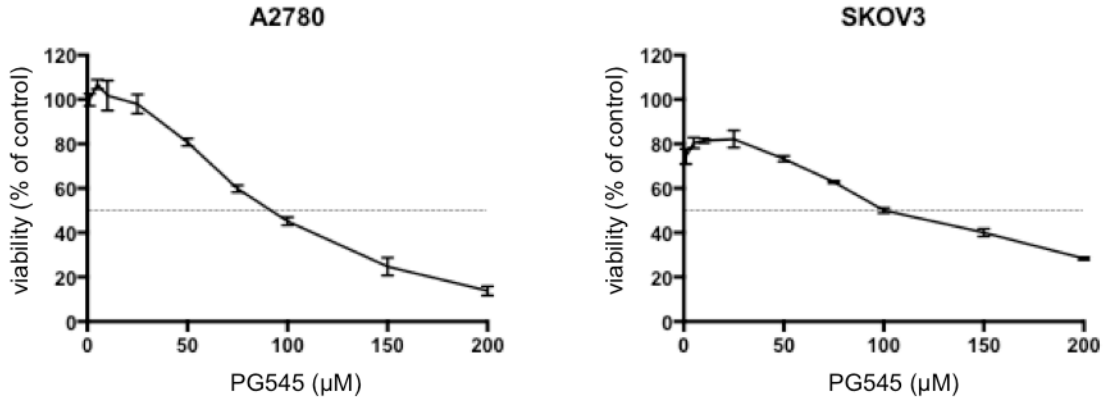


Fig. 2: Percent (%) cellular viability of ovarian cancer cell lines, A2780 and SKOV3, treated with increasing concentrations of PG545 for 48 hours.

First, we examined the inhibitory effect of PG545 on viability of human ovarian cancer cells *in vitro*. Cells were treated with increasing concentrations of PG545 and after 48 h viability of the cells was assessed using the colorimetric MTT assay. "The 3-(4,5-dimethylthiazol-2-yl)-2.5-diphenyltetrazolium bromide (MTT) assay measures the mitochondrial activity of viable cells by quantifying the conversion of the tetrazolium salt to its formazan product" (Aras, Hartnett et al. 2008). The inhibition of cell viability under PG545 treatment is shown as the percentage of viable treated cells compared to viable untreated cells (% of control). The graphs (Fig. 2) show that PG545 exhibited concentration-dependent growth inhibition in A2780 and SKOV3 cell lines. While low concentrations of PG545 showed no inhibitory effect on the proliferation of A2780 cells, a growth inhibition up to 20% was achieved with doses between 1-10 μM in SKOV3 cells. The concentration of PG545 to inhibit 50% cell growth (IC50) was estimated to be around 90 μM on A2780 and 100 μM on SKOV3 cells.

6.1.2 PG545 enhances cisplatin and paclitaxel induced cytotoxicity in vitro

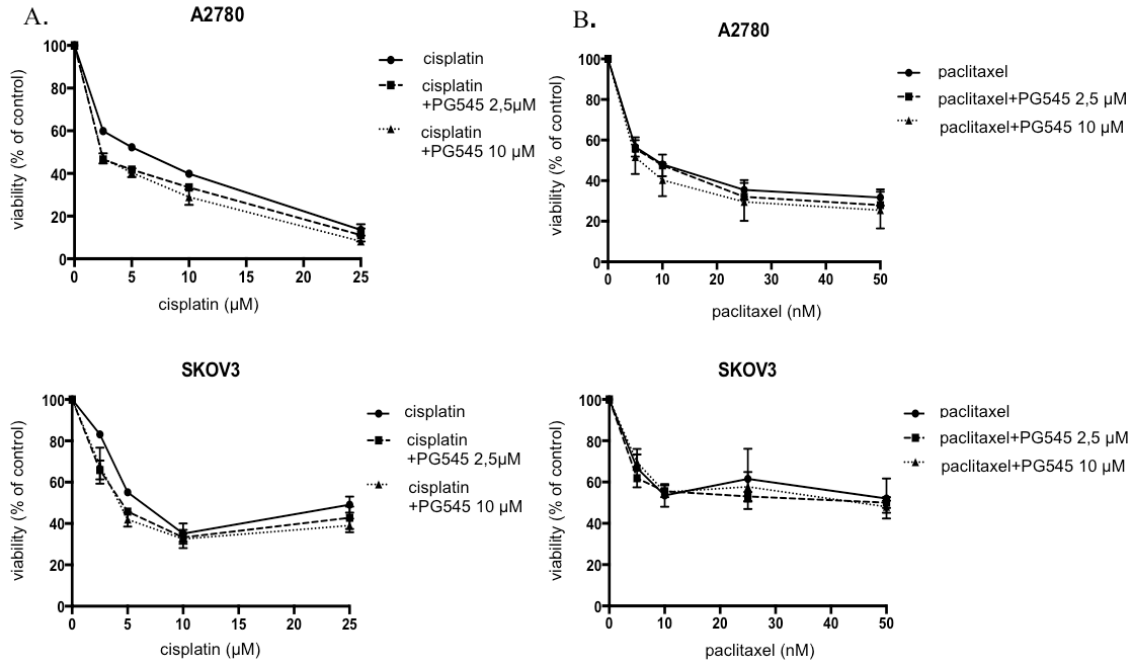


Fig. 3: Percent (%) cellular viability of ovarian cancer cell lines, A2780 and SKOV3 treated with cisplatin or paclitaxel +/- PG545.
A. Viability (%) of A2780 and SKOV3 cells treated with cisplatin alone, cisplatin + 2.5 μM PG545, or cisplatin + 10 μM PG545.
B. Viability (%) of A2780 and SKOV3 cells treated with paclitaxel alone, paclitaxel + 2.5 μM PG545, or paclitaxel + 10μM PG545.

As platinum compounds and taxanes are the key components of chemotherapy for ovarian cancer patients, we next examined whether PG545 can enhance cisplatin and paclitaxel induced cytotoxicity in A2780 and SKOV3 ovarian cancer cells.

Cells were exposed to increasing concentrations of cisplatin or paclitaxel with or without the addition of PG545 (2.5 μM and 10 μM) and cell viability was assessed by MTT assay after 48h. To describe the additive effect of PG545 on cisplatin and paclitaxel efficacy, we analyzed if the addition of PG545 can reduce the dose of these anti-cancer drugs needed to achieve 50% of growth inhibition (IC50).

Both cell lines, A2780 and SKOV3 were inhibited in proliferation by cisplatin and paclitaxel. In A2780 cells the IC50 for cisplatin was 6.1μM. In combination with PG545, less than halve the dose of cisplatin, 2.4 μM, was sufficient to achieve 50% growth inhibition. A greater additional cytotoxic effect of the higher dose of PG545

(10μM) compared to the lower dose (2.5μM) was only seen when the dose of cisplatin exceeded 5μM. When SKOV3 cells were treated with increasing concentrations of cisplatin, a cisplatin concentration of 4.4 μM was sufficient to achieve the IC50 when treated with both, 10 μM PG545 and cisplatin, instead of 6.4 μM when treated only with cisplatin. The higher dose of PG545 had more cytotoxic effect in comparison to the lower dose when cisplatin was above 5μM. When A2780 cells were treated with paclitaxel, the addition of 10 μM PG545 reduced the IC50- paclitaxel dose from 9.1 nM to 5.5 nM. Addition of PG545 to increasing doses of paclitaxel demonstrated an additional effect in paclitaxel doses above 5 nM with the higher PG545 dose of 10μM, while the lower dose enhanced the effect of paclitaxel only in doses above 10 nM. In SKOV3 cells, concentrations up to 50 nM of paclitaxel were not sufficient to achieve an IC50, but the adding of 10 μM PG545 led to an IC50 of 44.6 nM for paclitaxel. Both PG545 concentrations had a similar additional cytotoxic effect.

To further analyze the nature of the combination of PG545 and cisplatin, we used the combination index method as described by Chou and Talalay (Chou and Talalay 1984). Serial dilutions were prepared, based on the IC50 of each drug providing a 1:1 equipotent cisplatin: PG545 ratio to ensure that the contributing effects from each drug were equal. To determine the nature of the interaction between PG545 and cisplatin, we used median effect analysis, which offers a way to examine the degree of synergism and antagonism at different levels of cell kill. This analysis provides a combination index (CI), where a CI <1 indicates synergism and a CI>1 indicates antagonism (Chou 2006). The smaller the value <1, the greater is the degree of synergy and the greater the value >1, the greater is the antagonistic effect.

A synergistic effect of cisplatin and PG545 was observed, when the effective dose (ED) of the drug combination inhibited cell viability by 50% with a CI of 0.93. At a low level of cytotoxicity (ED20) no synergistic effect was seen. Synergy occurred when the level of cell kill exceeded 45% (ED45). A significant synergistic effect was seen only between ED70 and ED85. At the 90% level of cell kill, the combination of cisplatin and PG545 had a CI of 0.73, indicating synergy at levels of high cytotoxicity.

6.1.3 PG545 decreases cell survival and potentiates the effect of cisplatin in suspension culture

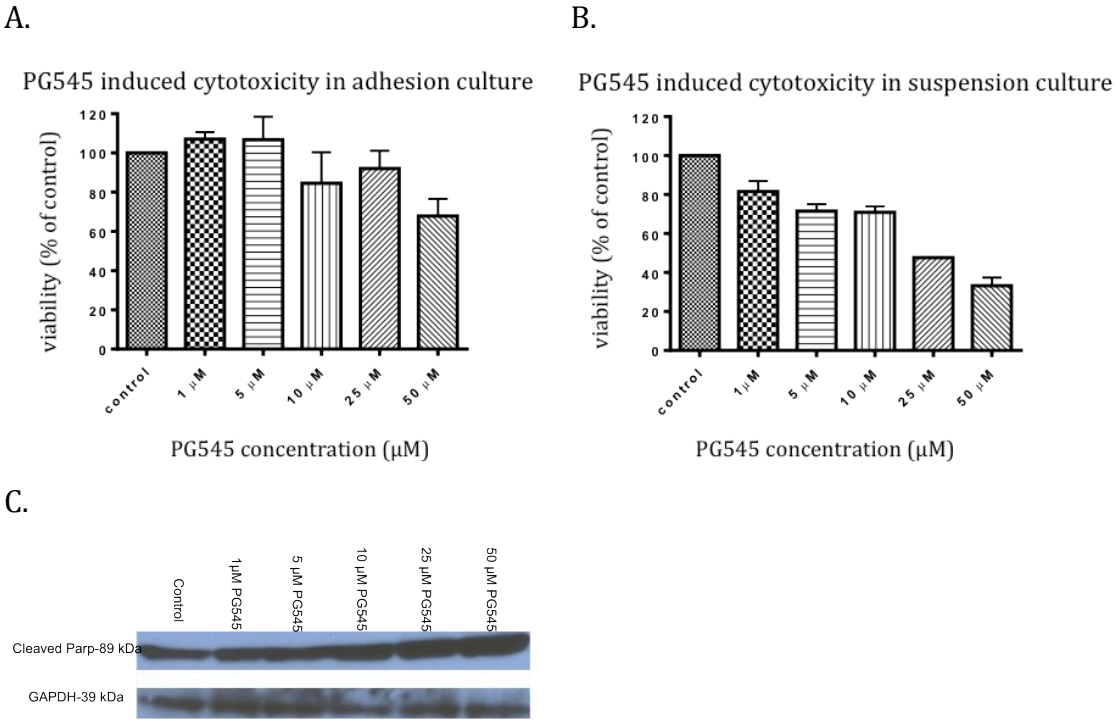
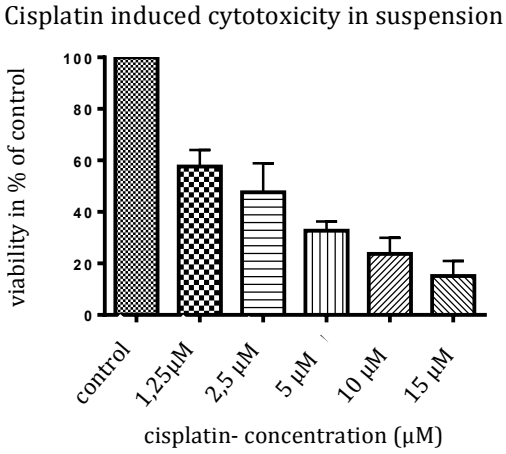


Fig. 4: Anoikis assay under PG545 treatment.
A. Percent (%) cell viability under PG545 treatment in adhesion culture.
B. Percent (%) cell viability under PG545 treatment in suspension culture.
C. Increased PARP-cleavage under PG545 treatment in suspension culture.

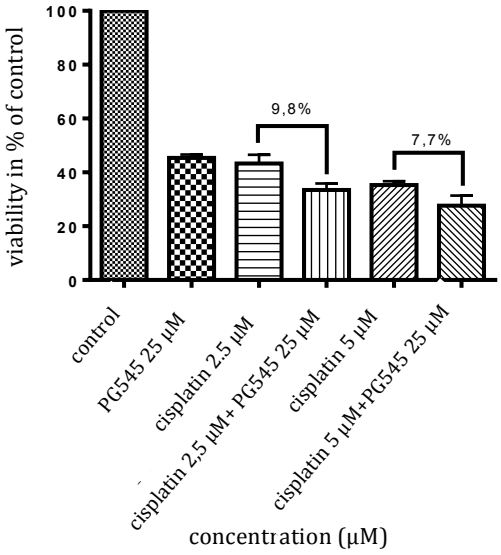
In this experiment, we examined the effect of PG545, cisplatin, and the combination of both drugs on cancer cells that have lost anchorage- dependence. Suspension culture was used to simulate extracellular matrix detachment growth. Recent studies indicated that malignant cells are able to survive the detachment from a primary tumor and thus acquire metastatic potential only after acquiring new features. One feature is overcoming anoikis, a type of apoptotic cell death, which is triggered by detachment of cells from the extracellular matrix (ECM)(Kandala and Srivastava 2012). In other words, cells surviving in detachment growth conditions have lost sensitivity to anoikis signals and become anoikis resistant. To determine if PG545 and cisplatin are able to reduce the number of cells surviving under detachment growth condition and affect floating tumor cells, A2780 cells were cultured as a suspension culture on ultra-low

attachment plates and exposed to increasing concentrations of PG545 and/or cisplatin for 48h. The cells were re-plated on adherent culture dishes, where only surviving, anoikis- resistant cells attach and grow (Kandala and Srivastava 2012). For comparison, the same experiment was conducted under adhesive growth conditions. The cell survival rate was determined by MTT assay and is shown as percentage of viability compared to the control. As shown in Fig. 4A and 4B, treatment with PG545 reduced cell survival under both, adhesive and non-adhesive growth conditions, in a concentration-dependent manner. Concentrations of 1μM PG545, which show no effect on anchorage- dependent cell proliferation, show a decrease of cell viability to 80.6%. By 5-50 μM PG545 treatment, cell viability was reduced by 32.3-70.5% in suspension culture. On attachment plates, concentrations up to 10 μM were not sufficient to decrease cellular viability. PG545 concentrations from 10-50 μM were able to reduce cell viability by 15.5-32.1%. Hence, the effect of PG545 on A2780 ovarian cancer cells is more pronounced, when cells are grown under suspension culture conditions, compared to attachment growth conditions.

Subsequently, we investigated if the decrease in cell viability under PG545 treatment is linked to an increase of suspended cells undergoing apoptosis. As the apoptotic process is associated with the cleavage of PARP, we analyzed if cell viability was associated with an increase in the cleavage of PARP. Cleaved PARP in the untreated control represents the cells that underwent apoptotic cell death as a result of the transfer from attachment- to detachment- growth conditions, called anoikis, hence- did not become anoikis resistant. Treatment with PG545 led to an increase of cleaved PARP in a concentration dependent manner. This result suggests that PG545 induced apoptosis in suspension culture, possibly by overcoming anoikis resistance, resulting in an increase of apoptotic cell death and decreased cell survival rates under PG545 treatment.



A.



B.

Fig. 5: Anoikis assay under cisplatin and combination treatment.
A. Percent (%) cell viability under cisplatin treatment in suspension culture.
B. Percent (%) cell viability under combination treatment in suspension culture.

For better evaluation and comparison, the same experiment was also conducted with cisplatin, the standard first-line agent in the treatment for ovarian cancer. The results in Fig. 5A show that cisplatin potently decreases cell viability and 2.5 µM cisplatin were sufficient to reduce the cell survival to under 50%. At 10 µM, cisplatin decreased viability to 23.8% and PG545 to 51.3%, and at a concentration of 15 µM cisplatin only 15% of cells survived the treatment. Fig. 5B shows the effect of the combination of cisplatin and PG545. For this experiment, cells were

treated with 2.5 µM or 5 µM cisplatin with and without 25 µM PG545 for 48h. The HS-mimetic was able to enhance the cytotoxic effect of cisplatin and to decrease survival at 2.5 µM cisplatin by an additional 9.8% and at 5 µM cisplatin by 7.7%.

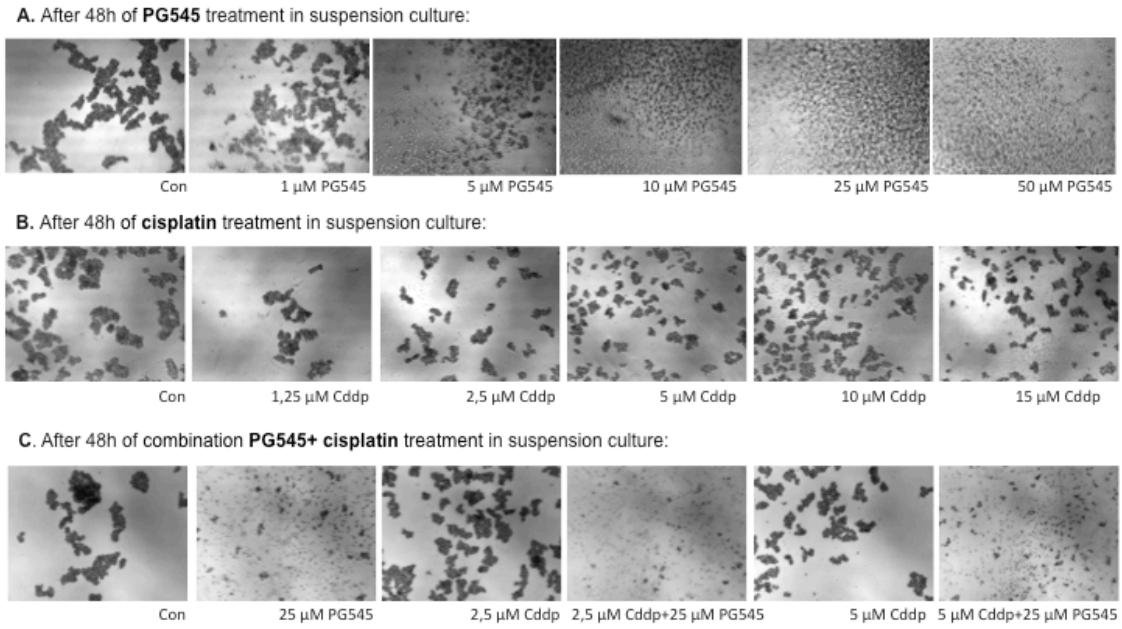


Fig. 6: Anoikis assay. Suspension culture photographs. Cddp=cisplatin

To get a better notion of how PG545 and cisplatin influence cell growth in the suspension culture, pictures were taken after 48h of drug exposure at a 50X magnification. Untreated cells, which had been grown for 48h in suspension culture, were able to form cell-cell adhesion mediated multicellular aggregates. Cells, which had been exposed to a 48h cisplatin treatment showed smaller aggregates, compared to the untreated control, but even under highly toxic concentrations of cisplatin, i.e. 15 µM under which only 15% of cells survived, the tumor cells maintained the ability to form floating multicellular clusters. Whereas cisplatin seemed not to be able to prevent the formation of cell aggregates, PG545 seemed to impede cell-cell adhesion and as a result, the formation of spheroid aggregates in a concentration-dependent manner. The higher the concentration of PG545 in the suspension, the more cells remained in suspension and did not adhere to each other. This observation was reinforced by photographs of the cisplatin and PG545 combination. Only in combination with PG545, but not by cisplatin treatment alone, were cells visibly detained from aggregating.

6.2 In vivo

6.2.1 PG545 as a combination and maintenance drug prolongs overall survival in an in vivo ovarian cancer model

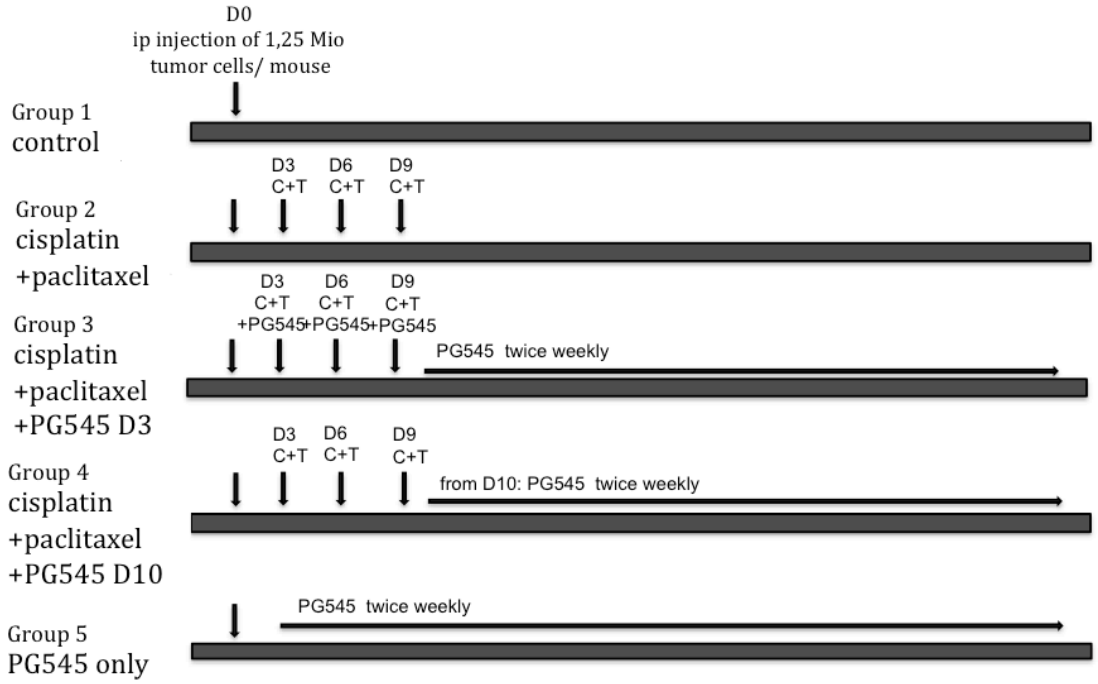


Fig. 7: Treatment schedule. C= cisplatin, T= taxol

To examine our hypothesis that patients might benefit from an addition of PG545 to the regular first-line chemotherapeutic treatment and from a maintenance treatment with PG545, we designed an *in vivo* study.

Mice receiving three cycles of platinum- and taxane- based chemotherapy were compared to mice additionally receiving PG545 as a maintenance agent, mice receiving a combination therapy of cisplatin, paclitaxel and PG545 followed by a PG54 maintenance therapy and mice treated with PG545 as a single agent. The treatment schedule is shown in Fig.7.

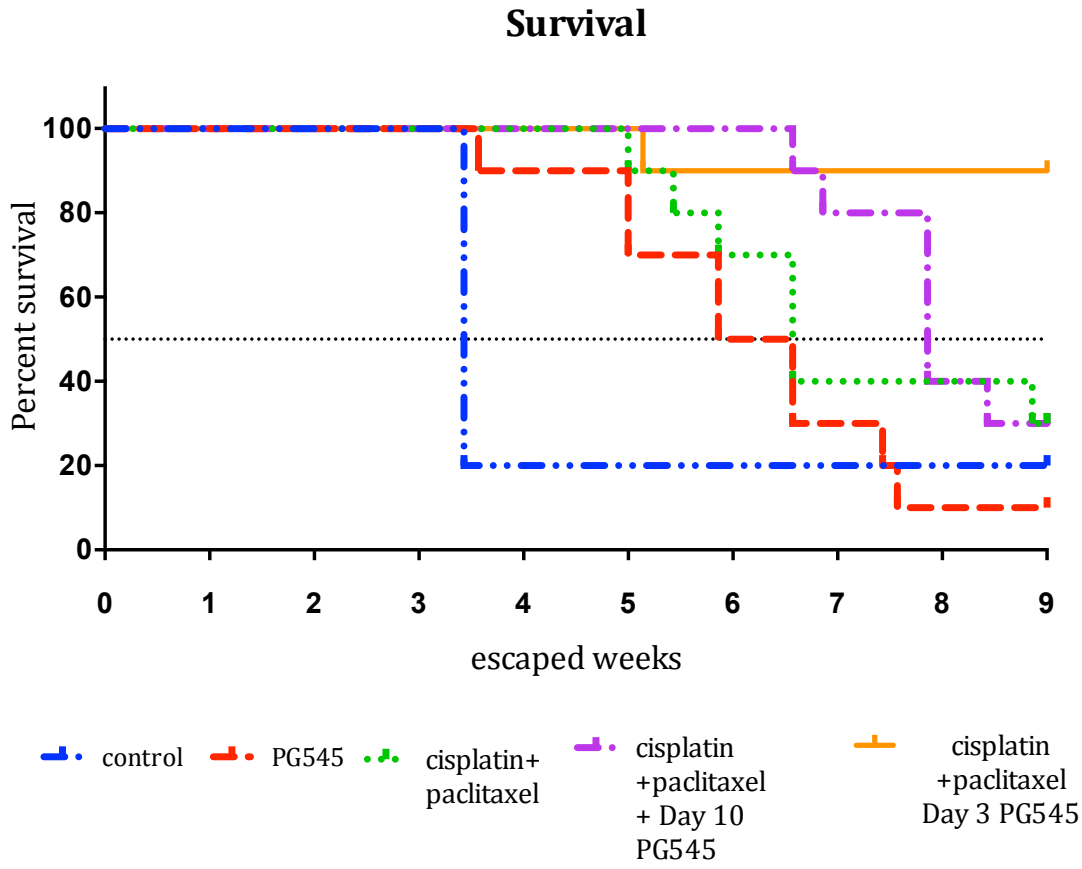


Fig. 8: Kaplan- Meyer survival analysis.

Untreated mice had a mean survival 3.5 weeks. Two mice survived until the end of the study and were tumor-free. Mice treated with cisplatin and paclitaxel showed a mean survival of 6.5 weeks. Mice, which received PG545 as a single agent, had a mean survival of about 6 weeks. The study thus showed that PG545, administered twice weekly as a single agent and treatment with 3 cycles of standard chemotherapeutics were able to significantly ($p<0.05$ and $p<0.01$, respectively) prolong survival compared to the control group.

PG545, given as a maintenance drug after cisplatin and paclitaxel front-line therapy, prolonged the mean survival to nearly 8 weeks.

The most powerful anti-cancer regime was the combination of PG545, cisplatin and paclitaxel for three cycles followed by a PG545 maintenance therapy. In this group, the median survival was not reached as 90% of mice survived nine weeks until the end of the study. The addition of PG545 to standard chemotherapy followed by PG545 maintenance treatment showed a significant ($p<0.05$) prolongation of survival compared to mice treated only with the traditional

chemotherapeutics cisplatin and paclitaxel. These results show that the addition of PG545 to standard chemotherapy followed by maintenance therapy with PG545 produces a significant overall-survival benefit in ovarian cancer-bearing mice.

6.2.2 *The addition of PG545 to regular chemotherapy inhibits recurrence of ovarian cancer and prolongs progression free survival*

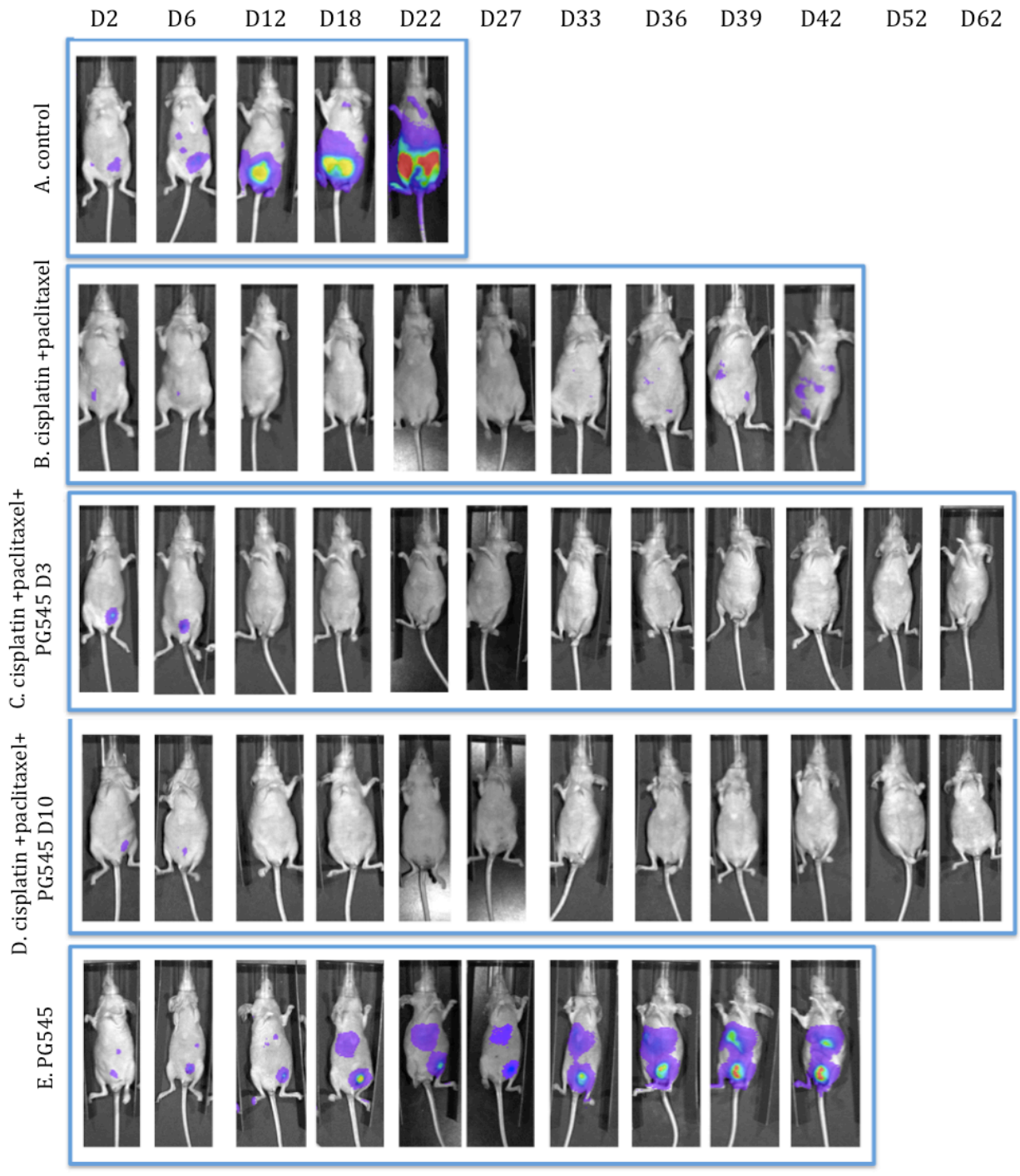


Fig. 9: Tumor growth development determined by measuring the fluorescence intensity in tumor using IVIS luminescence imaging system series 2000.

During the course of the study tumor growth development was determined by measuring the fluorescence intensity in tumor using IVIS luminescence imaging system series 2000 in 5 mice/group. Fig. 9 shows one representative mouse per group. The comparison of the combination of cisplatin and paclitaxel (B) and PG545 as a single agent (E) to control mice (A) shows that both treatment regimes we able to slow down tumor growth. While PG545 inhibits primary tumor growth, resulting in a slower growth progression and smaller tumors compared to control mice, the three cycles of cisplatin and paclitaxel chemotherapy were even able to decrease the tumor size and as a result, in some mice tumors were not detectable via imaging between day 12 and day 33, when they started to recur. One mouse from this group showed a progression of tumor growth despite chemotherapy. Maintenance therapy with PG545 after initial chemotherapy with cisplatin and paclitaxel (D) resulted in a complete remission of the primary tumors (with the exception of one mouse) and, in contrast to the cisplatin and paclitaxel treatment regimen, no signs of relapse were detected. The combination therapy with cisplatin, paclitaxel and PG545 followed by a PG545 maintenance therapy (C) resulted also in a complete remission of the disease and none of the mice showed signs of relapse through the end of the study. One mouse showed an increase of fluorescence intensity on day 62, which was too weak to be attributed to a tumor with certainty. It might correlate with the tiny tumor nodule which was found in one mouse. These results show that the addition of PG545 to standard chemotherapy not only results in a benefit in overall survival, but inhibits recurrence after initial chemotherapy resulting in a prolonged progression free survival.

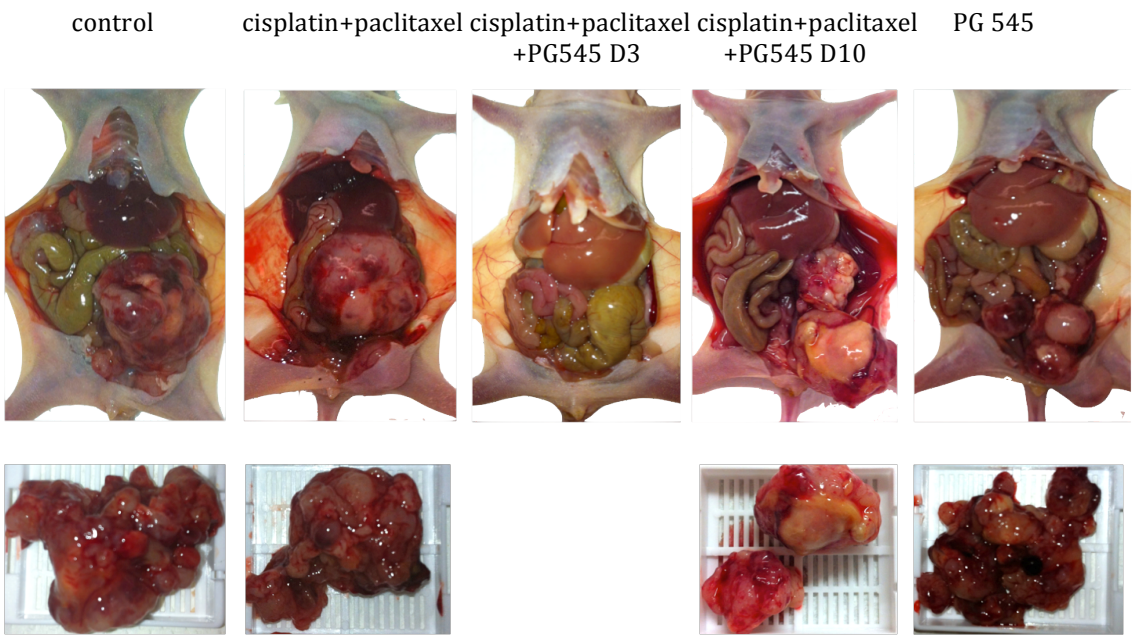


Fig. 10: One autopsied mouse/ group and the excised tumor.

To further examine the efficacy of each treatment regime, all mice were autopsied at death and the tumor was excised. Fig. 10 shows one representative mouse/group and the excised tumor. The amount of mice bearing tumor and the mean tumor weight at autopsy are indicated in Table 1.

Treatment	Mice with (recurrent) tumor	Tumor weight (g), mean of excised tumors	Abdominal circumference (cm), mean
Control	8/10	3,65	7,6
cisplatin+ paclitaxel	7/10	3,78	7,5
CP +PG545 D3	1/10	0,09^	7,1^
CP +PG545 D10	3/10	2,39	7,2
PG545	9/10	1,65^	7,1

Table 1: Effect of PG545 in *in vivo* mouse model; ^ significantly different from control group.

It has to be noted that the interpretability of tumor weight is limited as mice were sacrificed at different time points. In the control group, 8 out of 10 mice had a tumor with a mean tumor weight of 3.65 g. 9 out of 10 mice, which received PG545 as a single agent had a tumor, but with a mean tumor weight of 1.65 g, the mean tumor weight is only 45% of the weight of untreated mice. The significantly lower

tumor weight is, beside the inhibitory effect on tumor growth seen in the imaging and the survival benefit, another sign for the anti-cancer efficacy of PG545 as a single agent *in vivo*. Of mice treated with cisplatin and paclitaxel, 7 out of 10 autopsied mice bore tumors with a mean tumor weight of 3.78 g. With the addition of PG545 as a maintenance agent, only 3 out of 10 mice had tumor at the time of death. Compared to 7 mice bearing tumors after chemotherapy with the conventional drugs, the maintenance treatment with PG545 led not only to prolonged survival, but also to a higher rate of disease free mice.

The most promising effect was seen when PG545 was given directly in combination with platinum and taxane based chemotherapy followed by a continuing administration of PG545. In this group, only one mouse showed a very small tumor nodule on her bowl. In nine out of ten mice no sign of recurrent tumor was found. Biopsies were taken for microscopic analysis, but no microscopically detectable tumor lesions were discovered. These results indicate that the addition of PG545 to conventional chemotherapy significantly inhibits and delays the regrowth of recurrent ovarian cancer cells. Comparing this group to mice receiving PG545 as a maintenance agent shows that the initial addition of PG545 to first-line therapy leads to a lower rate of recurrent tumors and prolonged survival. This finding indicates that the addition of PG545 to first- line chemotherapy leads to an enhanced response to initial treatment and consequently that PG545 has an additive effect in combination with cisplatin and paclitaxel *in vivo*, which is consistent with our *in vitro* data. The enhanced response to initial treatment may reduce minimal residual disease and thus decrease the risk of recurrence.

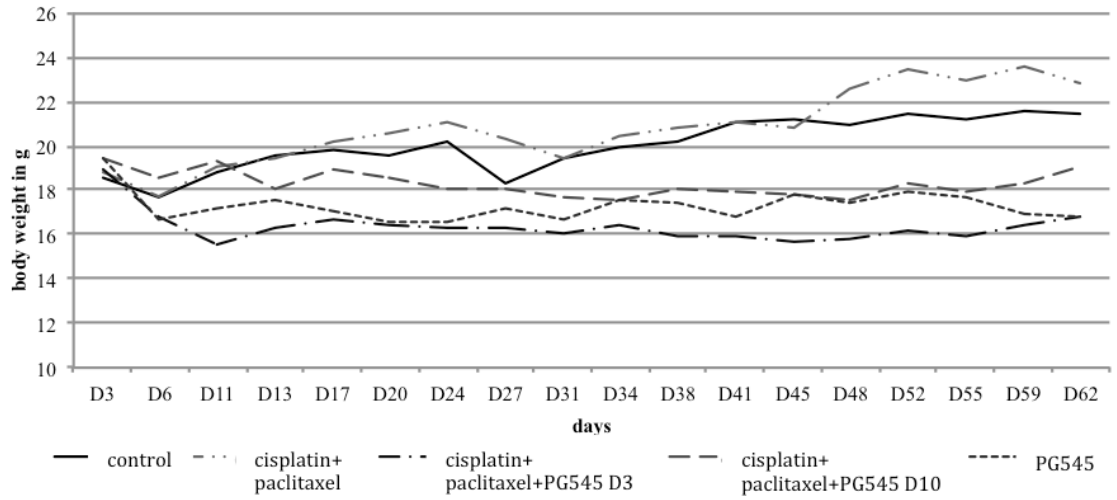


Fig. 11: Body weight development of tumor bearing mice during the course of the study

As previous studies indicated weight loss in tumor bearing mice associated with PG545 treatment, all mice were weighed twice weekly during the course of the study (Fig. 11). Additionally, abdominal circumference was measured during autopsy (Table 1). We observed that all mice treated with PG545 showed an initial weight loss. Despite this initial weight loss, mice did not keep on losing weight during the course of the study. One mouse receiving PG545 as a single agent had to be sacrificed due to low weight. The lower weight in the three treatment groups receiving PG545 treatment compared to the control and cisplatin and paclitaxel treated mice corresponds to the lower mean tumor weight by the time of autopsy and the lower abdominal circumference.

6.2.3 PG545 has effect on tumor necrosis and on angiogenesis in ovarian cancer in vivo

For further examination, representative portions of the excised tumors were paraffin- fixed and processed for histologic examination. Although the comparison of microscopic features of the excised tumor tissue is limited as mice were sacrificed at different time points, we analyzed the tumor tissue microscopically.
As there was only one very small tumor nodule of the cisplatin, paclitaxel and PG545 day 3 treated group, this group was not included in the analysis.

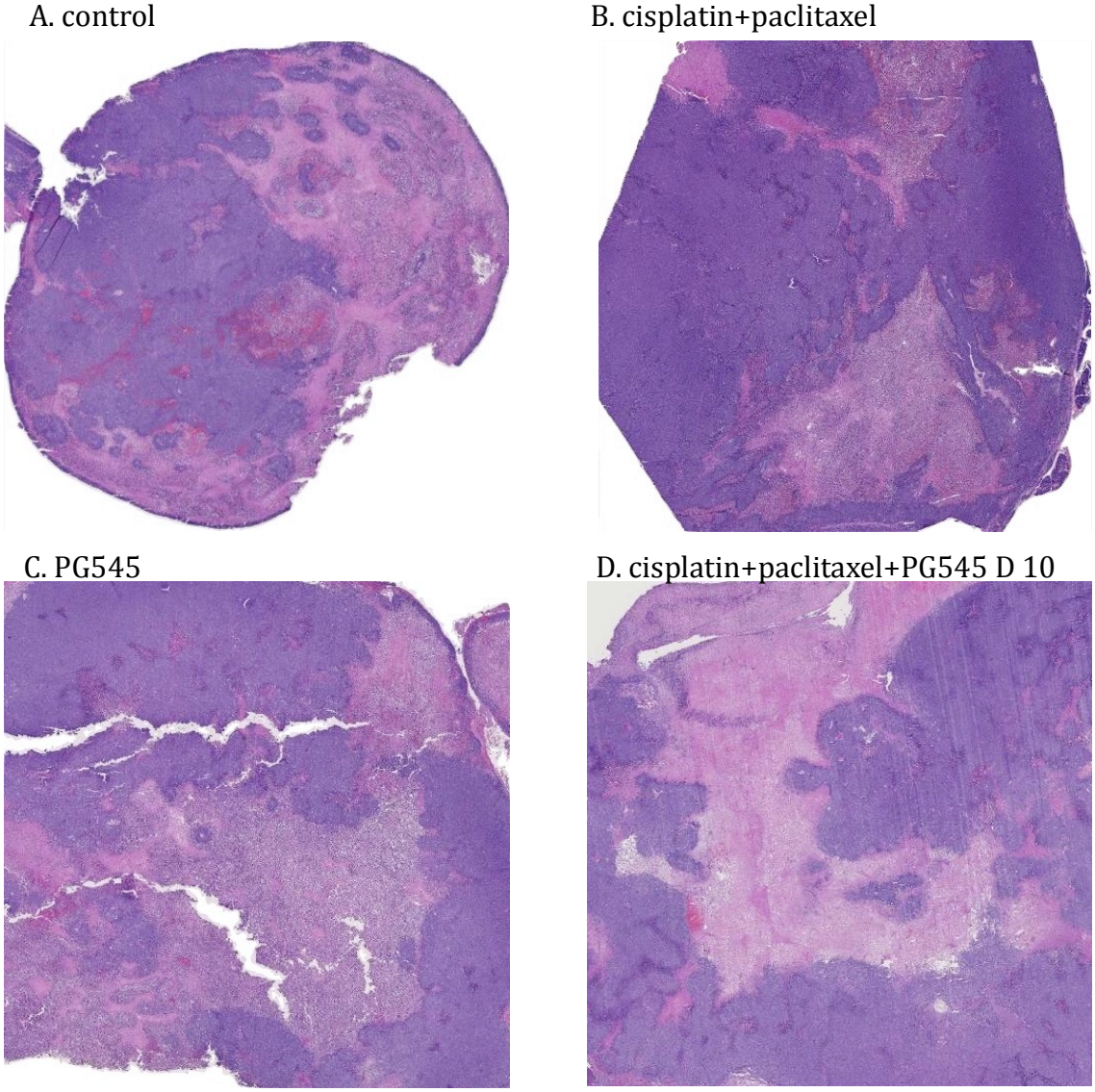


Fig. 12: H&E staining of representative A2780 xenografts in mice at the time of autopsy.

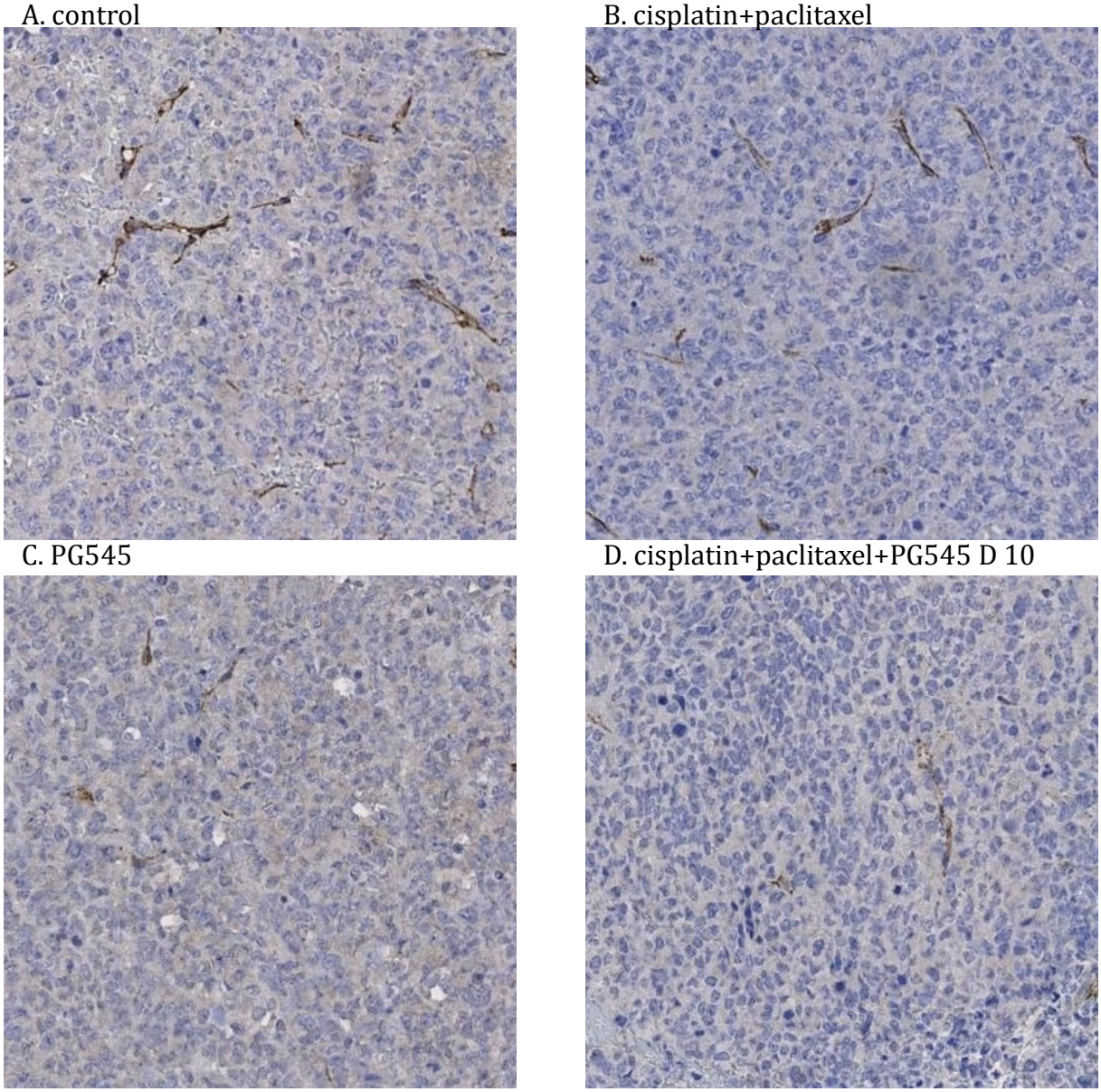


Fig. 13: Representative staining of CD31 of blood microvessels of A2780 xenografts in mice at time of autopsy.

First, to determine the viability of the tumor tissue, necrotic and viable tumor size was assessed from H&E stained sections of all excised tumors and the ratio of necrotic tumor size to total tumor size was calculated using Image J (Fig. 12). Necrosis (dead tissue) in cancerous tumors is typically the consequence of oxygen deficiency, occurring when tumors grow rapidly and outgrow their blood supply (Leek, Landers et al. 1999). As shown in Figure 12, xenografts derived from untreated mice showed the largest necrotic tumor portions. We attribute this

finding to the fact that tumor growth in untreated tumors was uninhibited by a chemotherapeutic agent, tumors grew rapidly and quickly outgrew their blood vessel supply.

Comparing xenografts derived from mice receiving chemotherapy, we found that mice treated with PG545 had a higher percentage of necrosis compared to xenografts derived from cisplatin and paclitaxel- treated mice. Consistent with this finding, maintenance treatment with PG545 resulted in an increase of necrotic tumor compared to tumor tissue derived from mice receiving only primary chemotherapy. As PG545 front-line- and maintenance- treated mice showed a similar and longer median survival (respectively) and a smaller tumor burden at time of death than the cisplatin and paclitaxel treated group, this effect can probably not be attributed to faster tumor growth with consequent outgrowth of blood vessel supply. Thus, these results indicate that the addition of PG545 in a maintenance setting may increase necrosis in growing ovarian tumors and reduce tumor tissue viability.

As necrosis is linked to insufficient blood vessel supply within a tumor, which can result from anti-angiogenic chemotherapeutics, we evaluate the anti- angiogenic efficacy of the treatment regimens by assessing microvessel density (MVD) in our xenografts (Fig.13).

Staining with the endothelial cell marker anti- CD31 showed reduced microvessel density in tumors derived from mice treated with PG545 as a single agent, as well as a maintenance agent compared to tumors derived from untreated and cisplatin and paclitaxel treated mice. PG545- treated tumors showed a reduction of microvessel density compared to untreated tumors and first-line chemotherapy treated tumors, which indicates the inhibition of angiogenesis in primary tumor growth by PG545. Tumors from mice receiving PG545 as a maintenance agent showed also a microvessel reduction compared to untreated mice and to mice receiving front-line chemotherapy. This finding shows that PG545 maintains its ability to inhibit angiogenesis in front-line chemotherapy pretreated tumors. Collectively, this data suggests that PG545 treatment inhibits angiogenesis in primary tumor growth as well as in re-growing tumors.

6.2.4 *Conventional chemotherapy increases the expression of cancer stem cell markers in ovarian tumors and maintenance with PG545 possibly affects CSC*

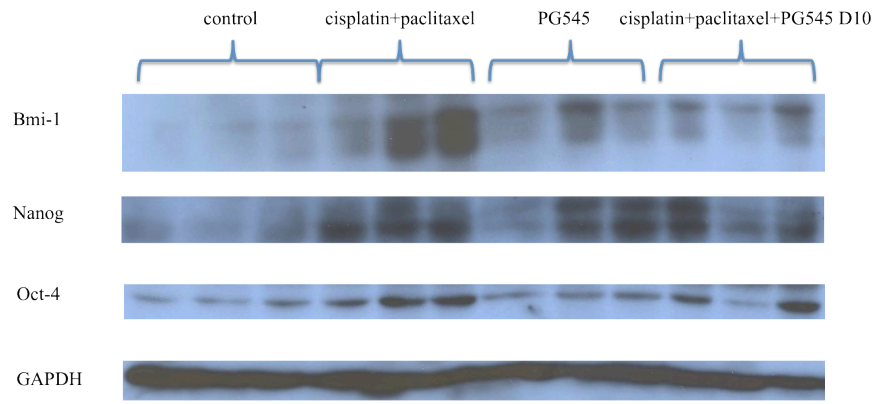


Fig. 14: Stem cell marker expression in tumor samples of untreated mice, cisplatin+ paclitaxel treated mice, PG545 treated mice and cisplatin+ paclitaxel+ PG545 day 10 treated mice.

Since we showed that the addition of PG545 to the regular chemotherapeutic regimen inhibits regrowth of ovarian cancer cells after response to first-line chemotherapy, resulting in a lower rate of relapse *in vivo*, we next analyzed the expression of cancer stem cell markers in our tumor samples. The expression of cancer stem cell specific markers indicates the existence of tumor cells with stem cell-like properties. This group of cells survives chemotherapy and is responsible for chemo-resistance and relapse after initial remission (Binaschi, Simonelli et al. 2011). The aim was to determine if PG545 has the potency to affect this cell population. We therefore determined the expression of characteristic cancer stem cell markers (Nanog, bmi-1 and oct-4) in samples of the excised tumors using western blot analysis. The expression of both, Oct-4 and nanog is essential for the maintenance of embryonic stem cell properties (Samardzija, Quinn et al. 2012). "Bmi-1, a member of the polycomb group (PcG) family, participates in the self-renewal and maintenance of CSCs" (Tomao, Papa et al. 2013). Moreover, it can enable cells to escape apoptosis (Zhan, Wang et al. 2013). Oct-4 is a member of the POU family of transcription factors and plays a crucial role in maintenance of self-renewal and pluripotency in embryonic stem cells. Nanog is another key

transcription factor essential for maintaining stem cell properties such as self-renewal and pluripotency (Siu, Wong et al. 2013).

The expression of the cancer stem cell markers bmi-1, nanog and oct-4 was determined in three tumor samples per treatment group. Due to the lack of tumor material of the cisplatin, paclitaxel and PG545 day 3 treated group, we were not able to include this group in the analysis.

Compared to mice receiving any kind of chemotherapy, the three samples of the control group showed the lowest expression levels of all three stem cell markers. Especially cisplatin and paclitaxel treated mice showed enhanced expression of all three stem cell markers, compared to the control. This finding is consistent with other publications that report an enhanced expression of cancer stem cell markers (including nanog and oct-4) in chemotherapy surviving ovarian cancer cells *in vitro* (Abubaker, Latifi et al. 2013).

For bmi-1 and oct-4 the highest expression levels were found in tumors from mice, treated only with the standard primary chemotherapy. Tumors from mice treated with PG545 alone or PG545 as a maintenance agent after first-line therapy tended to express lower levels of bmi-1 and oct-4 compared to the cisplatin and paclitaxel treated group, but slightly higher levels compared to the control. While no distinct difference was seen in the expression levels of bmi-1 in the PG545 treated group compared to the group, receiving PG545 as a maintenance agent, two samples of the maintenance group showed higher levels of oct-4 expression compared to the PG545 treated group. All three cisplatin+ paclitaxel group samples showed high expression levels for nanog. PG545 treated samples showed similar expression levels in two tumors and with addition of PG545 as a maintenance drug, only one tumor showed a high nanog expression level.

7 Discussion

Epithelial ovarian cancer is the leading cause of death from gynecologic malignancies and is referred to as the 'silent killer' (Binaschi, Simonelli et al. 2011). The lethality of ovarian cancer mainly originates from the inability to detect the disease at an early, organ- confined stage when the disease is most treatable, and the lack of effective therapies to cure advanced- stage disease (Naora and Montell 2005). Since early symptoms are unspecific and vague, "seventy percent of women present with advanced disease in which the tumor has spread to the peritoneal surfaces of the upper abdomen" (Cannistra 1993). The standard treatment, aggressive debulking surgery followed by six cycles of platinum-based chemotherapy, results in complete clinical remission in up to 75% of patients. However, 75% of the responders will relapse within a median time of 18 to 28 months (Mei, Chen et al. 2013). Currently, there are no chemotherapeutics that effectively prevent and inhibit the recurrence of ovarian cancer. Thus, efforts are still ongoing to identify new anti-cancer agents with the main focus on targeted therapy. One of these newly identified agents is PG545, a heparan-sulfate mimetic which uses a dual approach as an anti- cancer therapeutic, with both, anti-angiogenic and anti- metastatic potential. Due to its mechanism of action, PG545 is a promising agent to target some main features of ovarian cancer. Typical traits that present a target for PG545 are the characteristic overexpression of VEGF, a growth factor promoting angiogenesis (Binaschi, Simonelli et al. 2011) and the highly metastatic potential of this disease.

As the mechanism of action of PG545 make this drug a promising agent to target some main features of ovarian cancer, the combination of PG545 and current first-line therapy as a triple drug regimen might enhance the efficacy of initial chemotherapy resulting in a decrease or eradication of minimal residual disease. Additionally, as the mechanism of action of PG545 differs from that of standard chemotherapeutics cisplatin and paclitaxel it might even affect chemo-resistant disease and as it is able to interfere with fundamental steps in tumor cell re-growth, inhibit the growth of recurrent chemo-resistant cancer. Hence, in this study we sought to test the addition of PG545 to standard chemotherapy and

identified the most potent treatment regimen. Furthermore, we investigated in a maintenance setting, if one of the regimens is effective in maintaining the clinical complete response after initial chemotherapy resulting in progression- free and overall survival benefit.

First, *in vitro* analysis of PG545 was performed to assess the effect of PG545 as a single agent on A2780 and SKOV3 ovarian cancer cells. PG545 was found to decrease the viability of ovarian cancer cells in a dose-dependent manner. The concentration necessary to inhibit cell growth by 50% (IC50) was 90 μM in A2780 and 100 μM in SKOV3 cells.

Currently, the most active agent in the treatment of ovarian cancer is cisplatin, which often produces good initial therapeutic responses (Jekunen, Christen et al. 1994). Unfortunately, resistance to cisplatin develops regularly and leads to chemotherapeutic failure (Jekunen, Christen et al. 1994). As it is nonetheless a very important agent in the treatment of ovarian cancer, there is a strong interest in identifying agents that synergistically enhance cisplatin activity. To determine if PG545 has this ability, we tested this drug in combination with the commonly used chemotherapeutic agents cisplatin and paclitaxel. When tested in combination PG545 potentiated cisplatin- and paclitaxel- induced cytotoxicity in A2780 and SKOV3 *in vitro* and, in combination with PG545, lower concentrations of these traditional chemotherapeutics were sufficient to obtain the same anti- cancer effect. This *in vitro* finding supports the hypothesis that the combination of PG545 and current front-line chemotherapy to a three-drug regimen could enhance the efficacy of initial chemotherapy. Moreover this data is consistent with *in vivo* results, which showed that combination of PG545 and cisplatin or paclitaxel increased the anti-tumor effect in ovarian cancer bearing mice, compared to cisplatin or paclitaxel single agent therapy.

From a clinical perspective, a reduction of the cisplatin and paclitaxel dose is of great interest, as it may lead to a reduction of toxic side effects and allow patients to perform more treatment cycles with an enhanced quality of life.

As well as an inhibitory effect on primary tumor growth, PG545 has shown anti-metastatic potential in various *in vitro* and *in vivo* experiments. Metastasis in

ovarian cancer is a multi-stage process including (a) disconnection of intercellular adhesions and detachment of single cells from the primary tumor (b) single cells surviving the circulation in the peritoneal fluid (c) attachment to distant sites and (d) growth at metastatic sites (Lengyel 2010, Arvatz, Shafat et al. 2011). PG545 has shown to inhibit several of these steps. In initial studies, the heparan- sulfate mimetic was shown to potentially inhibit the enzyme heparanase (Ki= 6nM)(Dredge, Hammond et al. 2010), which is critical for the remodeling and loosening of extracellular matrix and whose activity is strongly involved in cell dissemination associated with tumor metastasis (a).

Recently, our laboratory has shown, that PG545 reduces the adhesion of ovarian cancer cells to mesothelial cells *in vitro* (data not published). *In vivo,* the mesothelium covers the abdominal cavity and inner organs and is thus the first surface encountered by tumor cells circulating in the peritoneal fluid. Inhibition of adhesion to these cells leads to a decreased attachment of tumor cells to distant sites, hence the inhibition of step (c). As angiogenesis is necessary for tumor growth at the metastatic site, PG545 may also influence step (d).

In this study we tested if PG545 has an effect on cancer cells that have lost anchorage-dependence. *In vivo*, tumor cells are in anchorage- independent growth condition between step (a) and (c), when they circulate as detached cancer cells in the peritoneal fluid (b). Following the detachment from the ECM, cells usually undergo an intrinsically programmed cell death (apoptosis) called anoikis (Kandala and Srivastava 2012). Thus, in order to survive under detachment growth conditions and to metastasize, a cancer cell has to acquire new features. One primary feature of metastasizing tumor cells is the loss of sensitivity to the anoikis signal, allowing them to survive under suspension conditions and to circulate in the peritoneal fluid (He, Ota et al. 2010). As ovarian cancer commonly metastasizes through the peritoneal fluid, studying cells in detachment-growth condition it is of great interest in this type of cancer. It was, for example, recently shown that treatment with diindolylmethane reduced anoikis resistance in various ovarian cancer cell lines (Kandala and Srivastava 2012).

In this study, we showed that PG545 and cisplatin were able to decrease survival rates of cells cultured in anchorage-independent growth condition in a concentration-dependent manner. The decrease of cell viability was associated

with an increase in the cleavage of the apoptosis marker PARP, indicating an increased number of suspended cells undergoing apoptosis. These results suggest that PG545 induces apoptosis in suspension culture, possibly by overcoming anoikis resistance, resulting in an increase of apoptotic cell death and decreased cell survival rates under PG545 treatment. Thus, PG545 might effectively inhibit step (b) of the metastasis process, the circulation of detached tumor cells in the peritoneal fluid. Furthermore, PG545 was able to potentiate the effect of cisplatin on A2780 ovarian cancer cells in suspension culture. It was also observed that PG545 treatment prevents the formation of cell-cell mediated multicellular aggregates in a concentration-dependent manner. Previous studies report that cell-cell adhesion mediated multicellular aggregate formation leads to activation of Erk, PI3K/Akt and other survival pathways and that this stimulation of intracellular survival signaling can sustain the evasion of anoikis in suspension culture (Zhong and Rescorla 2012).

Knowing that PG545 directly inhibits growth factor mediated signaling through blockage of heparin-binding growth factors (Winterhoff, Freyer et al. 2015) and that PG545 impedes cell-cell mediated multicellular aggregate formation; PG545 has two effects, that both lead to a decrease of growth factor mediated signaling and therefore deprive the cell of this mechanism to evade anoikis. Thus, it is possible that anoikis-resistant cells become anoikis-sensitive under PG545 treatment. The underlying principle of how PG545 inhibits cell-cell adhesion and if it affects anoikis resistance have yet to be elucidated and require further research.

The anti- metastatic properties of PG545 are not only important for metastasis but also with regard to disease recurrence after first- line chemotherapy, and thus of great interest for this study. Disease recurrence originates from minimal residual disease, either at the location of the primary tumor or on metastatic sites.

As PG545 is able to decrease the survival of floating tumor cells *in vitro*, it might be able to decrease the spread of cancer cells from minimal residual disease after cytoreduction and consequently the risk of recurrence on metastatic sites. As it has shown to enhance the efficacy of cisplatin and paclitaxel induced cytotoxicity, the three drug combination might be able target minimal residual disease more effectively.

There is currently a strong rational to target angiogenesis as an approach for novel anti- cancer strategies and in the search for maintenance agents that may impede disease recurrence (Mabuchi, Terai et al. 2008). Since it has been shown that tumors require vascular blood supply to grow beyond 1 to 2 mm in diameter (Folkman 1990), minimal residual disease remaining after cytoreductive surgery and complete clinical response to first-line chemotherapy should depend on angiogenesis for proliferation, invasion and metastasis (Mabuchi, Terai et al. 2008). Similar to our study, a Japanese study group reported in 2008 a survival benefit in mice treated with the angiogenesis inhibitor bevacizumab in a maintenance setting (Mabuchi, Terai et al. 2008). Used in maintenance therapy, bevacizumab significantly inhibited the regrowth of ovarian cancer and 50-60% of mice treated with cisplatin and bevacizumab, followed by a maintenance therapy with bevacizumab, survived until the end of the study without evidence of recurrence (Mabuchi, Terai et al. 2008).

Several clinical trials have studied the efficacy of adding bevacizumab to standard chemotherapy, both in first-line treatment (ICON7 and GO-0218 trial) and in patients with recurrent ovarian cancer (OCEANS and AURELIA trial) (Aravantinos and Pectasides 2014). It was shown that the addition of bevacizumab to standard chemotherapy in first-line treatment improved progression-free survival in ovarian cancer patients, with a greater benefit in terms of progression- free, as well as overall survival in women with a high risk of disease progression (Perren, Swart et al. 2011). The use of bevacizumab has also shown significant efficacy benefits in patients with recurrent ovarian cancer (Aravantinos and Pectasides 2014).

As recent studies have shown that anti-angiogenic approaches can promote metastasis in animal models of cancer (Ostapoff, Awasthi et al. 2013) and drug resistance was reported, the strategy of targeting angiogenesis has been questioned (Ostapoff, Awasthi et al. 2013) and future research is focusing on agents that inhibit metastasis as well as angiogenesis.

One aim of our study was to analyze the efficacy of a combination therapy to prevent or inhibit relapse of ovarian cancer in a maintenance setting. We therefore developed an *in vivo* recurrence mouse model in which mice, treated with three cycles of cisplatin and paclitaxel first-line chemotherapy, showed regrowth of the

disease after initial treatment response, visualized by IVIS luminescence imaging. We used this model to identify the most effective treatment schedule and test if a combination therapy followed by a maintenance therapy with PG545 is effective in eradicating or reducing the minimal residual disease burden.

To our knowledge, this is the first study showing that treatment with PG545, administered as a single agent, not only inhibits and decelerates primary tumor growth, but leads to prolonged overall survival in ovarian cancer bearing mice compared to an untreated control group ($p<0.05$). The significantly lower tumor weight at time of death, the inhibitory effect on tumor growth, seen in the imaging, and the survival benefit, emphasizes the anti-cancer efficacy of PG545 as a single agent *in vivo*. Three cycles of chemotherapy with cisplatin and paclitaxel also significantly prolonged the overall survival of mice ($p<0.01$). Although this treatment regime led to full remission in some mice, it was not sufficient to prevent disease recurrence.

The addition of PG545 as a maintenance agent, following initial chemotherapy, led to further survival benefit. Mice did not show relapse after initial remission in the imaging and a reduced number of mice were tumor-positive at the time of autopsy, compared to the cisplatin and paclitaxel- treated group. The direct addition of PG545 to standard chemotherapy showed a significant ($p<0.05$) survival benefit compared to conventional treatment. This anti-cancer regimen was able to sustain disease remission and impede recurrence of the disease and only one mouse was tumor-positive at time of death. The fact that the direct addition of PG545 to standard chemotherapy is more potent than the maintenance regimen mirrors our *in vitro* data, where PG545 has shown to potentiate the cytotoxic effect of cisplatin and paclitaxel and suggests that addition of PG545 to first-line chemotherapy might make it more effective. The enhanced response to initial treatment may reduce minimal residual disease and thus decrease the risk of recurrence.

Targeting angiogenesis is based on the knowledge that growth of new blood vessels is an important requirement for tumor growth beyond a few millimeters (Nadkarni, Geest et al. 2013) and thus a necessity for primary tumor growth as well as metastization. A retrospective study has shown that high vessel counts in tumors of ovarian cancer patients are associated with a worse prognosis for

disease-free survival (Hollingsworth, Kohn et al. 1995). Moreover, increased vascularity is associated with poor survival in patients with ovarian carcinoma (Alvarez, Krigman et al. 1999) and with a higher risk of recurrence (Nadkarni, Geest et al. 2013). One mechanism of action in PG545 is the sequestration of angiogenic growth factors in the extracellular matrix. PG545 inhibits angiogenesis and was previously found to inhibit angiogenesis potently in an ex vivo rat aortic ring assay (Dredge, Hammond et al. 2010) and in mouse models of several types of cancer (Ferro, Liu et al. 2012).

In this study, we used immunohistochemistry to analyze the microvessel density in the tumor specimens. It has to be noted, the interpretability of our result is limited as mice were sacrificed at different time points and as the group of mice receiving triple chemotherapy with cisplatin, paclitaxel and PG545 followed by a PG545 maintenance therapy was not included into the analysis as only one small tumor nodule was obtained.

We found that tumors derived from mice treated with PG545, as a first-line agent and as a maintenance agent, showed a higher mean percentage of necrotic area than tumors derived from cisplatin and paclitaxel-treated mice. Staining with the endothelial cell marker anti- CD31 showed reduced microvessel density in tumors derived from mice treated with PG545 as a single agent, as well as a maintenance agent, compared to tumors derived from untreated mice and mice treated with conventional primary chemotherapy. These findings indicate that PG545 inhibits angiogenesis in primary tumor growth as well as in relapsing tumors, after treatment with standard chemotherapy. Although the tumors of relapsing mice might be resistant to conventional chemotherapeutics, the decreased MVD is an indication that they respond to PG545 treatment and that the anti-angiogenic effect of PG545 is preserved.

The recurrence of successfully treated ovarian cancer has been associated with the existence and persistence of drug- resistant cancer stem cells (Binaschi, Simonelli et al. 2011). Cancer stem cells are a small proportion of cells (0.01% to 0.1%) in a tumor that somewhat mimics normal stem cell biology (Pasquier and Rafii 2013). Cancer stem cells have the capacity to self-renew, have unlimited division potential, are able to recapitulate the heterogeneity of tumors (Pasquier and Rafii

2013) and are highly tumorigenic (Cheng, Ramesh et al. 2010). It has been shown that cisplatin treatment induces transition from an epithelial to a mesenchymal morphology (epithelial mesenchymal transition), a process believed to correlate with a 'cancer stem cell- like' phenotype in residual cancer cells (Latifi, Abubaker et al. 2011). Moreover, chemotherapeutical treatment of ovarian cancer cell lines leads to increased expression levels of cancer stem cell markers (Abubaker, Latifi et al. 2013). We investigated, in this study, if chemotherapy leads to changes in the expression levels of the cancer stem cell markers Bmi-1, Oct-4 and nanog. The aim was to determine if PG545 has the potency to affect this cell population.

Bmi-1 plays a role in the self-renewal and maintenance of CSCs and, as an oncogene, can enable cells to escape apoptosis (Zhan, Wang et al. 2013). Oct-4 participates in maintenance of self-renewal and pluripotency in embryonic stem cells. While upregulation of Oct-4 preserves a pluripotent stem cell character, loss of Oct-4 promotes differentiation of stem cells (Samardzija, Quinn et al. 2012). Oct-4 was associated with tumorigenesis, when it was first shown that Oct-4 increases the malignant potential of embryonic stem cells in a dose-dependent manner (Gidekel, Pizov et al. 2003). Oct-4 expression was shown to significantly increase from normal ovarian surface epithelium to benign tumors to malign serous ovarian cancers, proposing the Oct-4 expression is associated with onset and progression of serous ovarian carcinoma (Zhang, Li et al. 2010). Elevated levels of Oct-4 in tumors have been associated with poor survival outcome and metastasis (Gidekel, Pizov et al. 2003). Nanog is another key transcription factor essential for maintaining stem cell properties such as self-renewal and pluripotency (Siu, Wong et al. 2013). Increased nanog expression has been associated with high-grade cancer, reduced chemosensitivity, pro-metastatic properties, and poor survival. Furthermore, nanog has been shown to be an independent prognostic factor for overall and disease-free survival of ovarian cancer patients (Siu, Wong et al. 2013).

Our western blot analysis showed that cisplatin and paclitaxel treatment led to enhanced expression of all three stem cell markers, compared to untreated tumors. This observation indicates that recurrent tumors, re-growing after first—line chemotherapy contain an increased percentage of tumor cells with cancer stem cell properties compared to untreated tumors. In accordance with our findings, it has been shown previously in a colorectal cancer *in vivo* mouse model, that

residual xenogeneic tumors after chemotherapy were enriched for cells with a cancer stem cell phenotype (Dylla, Beviglia et al. 2008). This finding was expected as the tumor bulk was hypothesized to be more sensitive to chemotherapeutic agents than cells with a cancer stem cell phenotype, resulting in a higher frequency of these cells in the residual tumors (Dylla, Beviglia et al. 2008). The enrichment of a cell population with cancer stem cell characteristics after chemotherapy has been reported in various malignancies, such as breast cancer (Calcagno, Salcido et al. 2010), hepatocellular carcinoma (Tan, Chen et al. 2009) and lung cancer. For ovarian cancer, it has been shown previously that chemotherapeutical treatment of ovarian cancer cell lines *in vitro* leads to increased expression levels of cancer stem cell markers (Abubaker, Latifi et al. 2013). Furthermore, it has been observed that patients that have relapsed following initial chemotherapy have a higher percentage of tumor cells with stem-cell like properties in their ascites than untreated ovarian cancer patients (Rizzo, Hersey et al. 2011). This is consistent with the selection for tumor cells with stem cell like properties during chemotherapy. Our finding indicates that compared to primary ovarian tumors, tumors re-growing after first-line chemotherapy are enriched in tumor cells with cancer stem cell characteristics. This might be a consequence of the selection for resistant and thus residual cancer stem cells by conventional chemotherapy, which initiate tumor recurrence.

Tumors from mice treated with PG545 expressed higher levels of bmi-1 and nanog and similar levels of oct-4 compared to the control. This finding, although less pronounced than in cisplatin/paclitaxel treated tumors, indicates that PG545 treatment leads to increased expression levels of cancer stem cell markers. Compared to cisplatin and paclitaxel treated tumors, PG545 treated tumors showed a tendency towards lower expression levels, especially for bmi-1 and oct-4. Hence, it is possible that PG545 exhibits an effect even on tumor cells with stem cell like properties. Another explanation would be that the proportion of CSC in relation to other tumor cells is lower in PG545 treated mice compared to cisplatin and paclitaxel treated mice.

As we have shown that PG545 maintenance leads to inhibition of regrowth after initial remission, we then determined if PG545 maintenance might have an effect on cancer- initiating cells that are most likely responsible for relapse. Comparing

tumors of mice treated with initial chemotherapy and of mice additionally receiving PG545 as a maintenance agent showed a tendency for PG545 maintenance treatment to lead to decreased expression levels of cancer stem cell markers. The fact that mice of both groups had the same first- line chemotherapy, which led to an enrichment of chemo-resistant cancer initiating cells and the finding that PG545 maintenance treated mice showed lower marker expression levels of the CSC markers indicates that PG545 may have the ability to affect cancer cells with stem cell like properties.

Intensive research on the microenvironment of cancer stem cells led to the identification of so called stem cell niches, which play a crucial role in the maintenance of stem cell characteristics (Tomao, Papa et al. 2014). "Recent evidence indicates that CSC live in a "vascular niche" that promotes their long-term growth and self- renewal" (Yi, Hao et al. 2013). The interaction between the 'vascular niche' and CSC is highly complex and bidirectional (Ribatti 2012). In brain tumors, it was shown that endothelial cells, as a critical component of the CSC particular niche, supply secreted factors that maintain CSC in a stem cell state and in turn, CSC secrete high levels of vascular endothelial growth factor (VEGF), promoting the formation of new blood vessels (Ribatti 2012). Indeed, the significantly increased VEGF expression of cancer stem cells, compared to normal tumor cells, makes them the most strongly angiogenic cells in the tumor (Ribatti 2012). This strong pro-angiogenic activity of CSC may also play a crucial role in the early stages of growth and re-growth after therapy (Ribatti 2012). These findings led to the idea that therapeutic targeting of the vascular could destroy the CSC niche microenvironment and to the attempt to target CSC via inhibition of angiogenesis (Calabrese, Poppleton et al. 2007). It was indeed shown that bevacizumab, a VEGF inhibitor, was able to reduce the stem- like cell fraction in brain tumors (Calabrese, Poppleton et al. 2007) (Ribatti 2012) .

In ovarian cancer, it was shown that metformin decreased CSC *in vitro* and *in vivo* and inhibited the growth of ovarian tumor spheroids (Shank, Yang et al. 2012). Metformin treatment was also associated with a decrease in microvascular density (Pasquier and Rafii 2013) (Shank, Yang et al. 2012). This led to the conclusion that the decline in CSC may lead to a decrease in blood vessel growth and that the anti-

angiogenic effect of metformin might be caused by its anti-CSC activity (Shank, Yang et al. 2012).

In this study it was not only observed that PG545 shows promising efficacy in a maintenance setting, but also that maintenance treatment with PG545 leads to a decrease in tumor vascularity and lower expression levels of cancer stem cell markers, compared to mice without maintenance treatment. Considering that CSC play a crucial role in not only tumor-development, but chemo-resistance and relapse after initial treatment (Tomao, Papa et al. 2014), further investigation of the interplay between CSC and the microvascular and how PG545 interferes with the two, might bring new insights on the mechanisms of how the addition of PG545 to initial standard chemotherapy effectively prevents disease recurrence and prolongs progression free and overall survival.

As angiogenesis has also been associated with ascites formation in ovarian cancer (Gavalas, Liontos et al. 2013), the effect of PG545 on malignant ascites formation has been assessed in an immunocompetent ID8- mouse model. Treatment with PG545 was found to significantly reduce ascites accumulation. Reduction of ascites accumulation has been also observed in ovarian cancer patients treated with bevacizumab (Winterhoff, Freyer et al. 2015). The formation of ascites is thought to be caused by increased vascular permeability resulting from VEGF expression (Masoumi Moghaddam, Amini et al. 2012). It was observed that plasma levels of VEGF, HB-EGF, FGF-2 and heparanase were elevated in mice undergoing PG545 treatment, whereas ascites VEGF level were reduced. The main factor of the VEGF level in ascites is the secretion of VEGF by tumour, stromal and immune cells. Thus it is likely that the reduced VEGF levels in ascites can be explained by the suppression of one of these cells (or a combination) under PG545 treatment.

Pharmacokinetic studies revealed that AUC is the relevant measure of exposure to associate with anti-cancer activity as twice–weekly low dose administration exhibited similar effects to high dosing once-weekly. The dosage to achieve therapeutic levels in patients was estimated to be 150 mg when applicated s.c. (Winterhoff, Freyer et al. 2015).

PG545 is currently being investigated in a Phase I clinical trial in patients with advanced, recurrent solid cancers. Four patients (with thyroid cancer, colon cancer, pancreatic cancer and melanoma) were treated with once weekly s.c. administration of 25 or 50 mg of PG545. The study was halted after two to eight injections due to unexpected local injection site reactions.

Monitoring of pharmacodynamic changes showed that VEGF plasma levels were elevated in patients during treatment with PG545, which is in accordance with the findings of the ID8 mouse model. Similar changes were observed in other target proteins like FGF-2, HB-EGF and heparanase.

Elevated VEGF plasma levels, as well as reduced VEGF ascites levels, as seen under PG545 treatment, have been previously reported for treatment with bevacizumab. The elevation of these target proteins may be the result of blocking the interaction of VEGF and heparanase with HS within the tumor and as a consequence liberation of the free ligands into the plasma. The rise of plasma VEGF levels under PG545 treatment, makes VEGF a potential biomarker for drug response (Winterhoff, Freyer et al. 2015).

In conclusion, this thesis provides preclinical data to support clinical testing of PG545 as a novel anti-cancer agent in patients with ovarian cancer.

First, it was shown that treatment with PG545 decreases cell viability of A2780 and SKOV3 ovarian cancer cells in a dose dependent manner *in vitro*. PG545 was also found to enhance cisplatin and paclitaxel induced cytotoxicity *in vitro* and to have a synergistic effect in combination with both drugs. Moreover, treatment with PG545 decreases cell survival and potentiates the effect of cisplatin in suspension culture.

Our *in vivo* study showed, that the addition of PG545 to standard chemotherapy and the use of PG545 as a maintenance agent prolongs overall survival in ovarian cancer bearing mice. Imaging showed that triple therapy with cisplatin, paclitaxel and PG545 followed by a PG545 maintenance regimen inhibited disease recurrence, led to sustained disease remission and prolonged progression free survival.

Immunohistochemistry of tumor specimens showed a reduced microvessel density under PG545, as a single, as well as a maintenance agent. CSC were found to be

elevated in tumors treated with standard chemotherapy compared to untreated mice. Maintenance therapy with PG545 led to a reduced expression of CSC markers compared to standard chemotherapy without a maintenance regimen.

8 Summary

Background: Despite ongoing efforts to improve treatment strategies, epithelial ovarian cancer remains the leading cause of death among gynecologic malignancies. In search for new treatment options, there is currently a particular focus on anti-angiogenic agents, such as bevacizumab, which have shown significant efficacy benefits in clinical trials. Nonetheless, efficacy remains limited due to resistance linked to alternative angiogenic pathways and metastasis. Therefore, we investigated PG545, a novel heparan-sulfate mimetic with anti-angiogenic and anti-metastatic properties using preclinical models of ovarian cancer.

Methods: We investigated the anti-cancer activity of PG545 *in vitro* and *in vivo* as a single agent and in combination with paclitaxel and cisplatin. Furthermore, we identified the most effective treatment schedule and investigated if the addition of PG545 to standard chemotherapy can prevent disease recurrence *in vivo*.

Results: The *in vitro* experiments of this study showed that treatment with PG545 decreases cell viability of A2780 and SKOV3 ovarian cancer cells, enhances cisplatin and paclitaxel induced cytotoxicity and has a synergistic effect in combination with both drugs. Most importantly, our *in vivo* study showed, that the addition of PG545 to standard chemotherapy, and the use of PG545 as a maintenance agent, let to sustained disease remission and prolonged progression free and overall survival in ovarian cancer bearing mice. Immunohistochemistry of tumor specimens showed a reduced microvessel density under PG545, as a single- as well as a maintenance agent. Maintenance therapy with PG545 led to a reduced expression of CSC markers compared to primary chemotherapy without a maintenance regimen.

Conclusion: In conclusion, this thesis provides further preclinical data to support clinical testing of PG545 as a novel anti-cancer agent in patients with ovarian cancer.

9 Zusammenfassung

Hintergrund: Trotz fortlaufenden Bemühungen die Therapie zu verbessern, ist das epitheliale Ovarialkarzinom weiterhin die häufigste Todesursache unter den gynäkologischen malignen Erkrankungen. Bei der Suche nach neuen zielgerichteten Therapeutika stehen aktuell anti-angiogene Substanzen, wie Bevacizumab, im Fokus. Diese haben in klinischen Studien bereits einen signifikanten Effektivitätsvorteil gezeigt. Dennoch ist die Effektivität des gezielten Angreifens eines einzelnen Signalwegs durch Bildung von Resistenzen über alternative Signalwege und Metastasierung limitiert. Daher haben wir in dieser Studie PG545, ein neues Heparansulfat- Mimetikum mit anti-angiogenen und anti-metastatischen Eigenschaften an präklinischen Modellen des Ovarialkarzinoms untersucht.

Methoden: In der hier vorgelegten Dissertation haben wir die chemotherapeutische Wirkung von PG545 als Einzelsubstanz sowie in Kombination mit Cisplatin und Paclitaxel *in vitro* und *in vivo* untersucht. Wir haben den effektivsten Behandlungsplan identifiziert und geprüft, ob die Hinzunahme von PG545 zur Standardchemotherapie Rezidive verhindern kann.

Ergebnisse: Die *in vitro* Versuche dieser Studie haben gezeigt, dass die Behandlung mit PG545 das Zellüberleben von A2780 und SKOV3 Ovarialkarzinomzellen reduziert, die durch Cisplatin und Paclitaxel induzierte Zytotoxizität verstärkt und synergistisch mit beiden Medikamenten wirkt. Als bedeutendstes Ergebnis dieser Arbeit haben die *in vivo* Studien gezeigt, dass die Ergänzung von PG545 zur Standardchemotherapie und der Einsatz als Erhaltungsmedikament zu einem Erhalt der Tumorremission führte und das progressionsfreie und Gesamtüberleben in Tumor-tragenden Mäusen verlängern konnte. Immunhistochemische Analysen des Tumorgewebes zeigten, dem anti-angiogenen Charakter des Medikaments entsprechend, eine reduzierte Mikrogefäßdichte unter PG545 Behandlung, als Einzel- sowie als Erhaltungsmedikament. Eine Erhaltungstherapie mit PG545 führte zu einer reduzierten Expression von

Krebsstammzell-spezifischen Markern verglichen mit Tumorgewebe, welches ausschließlich mit Erstlinien-Chemotherapie behandelt wurde.

Schlussfolgerung: Zusammenfassend bietet diese Arbeit weitere präklinische Daten, welche eine klinische Testung von PG545 als neues onkologisches Medikament bei Patientinnen mit fortgeschrittenem Ovarialkarzinom unterstützen.

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12 Curriculum vitae

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Eidesstattliche Versicherung

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Ich erkläre hiermit an Eides statt,

dass ich die vorliegende Dissertation mit dem Thema

The addition of the novel heparan sulfate (HS) mimetic PG545 to standard chemotherapy shows promising efficacy in the treatment of ovarian cancer in vitro and in vivo.

selbständig verfasst, mich außer der angegebenen keiner weiteren Hilfsmittel bedient und alle Erkenntnisse, die aus dem Schrifttum ganz oder annähernd übernommen sind, als solche kenntlich gemacht und nach ihrer Herkunft unter Bezeichnung der Fundstelle einzeln nachgewiesen habe.

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Muenchen, 07.06.2018

Ort, Datum

Luisa Freyer

Unterschrift Doktorandin/Doktorand