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Abstract

Background: The development of epithelial ovarian cancer is, in most cases, not detected at an early stage because of its lack of symptoms or even asymptomatic progress. It is a serious diagnosis with a rather dismal prognosis. This led to the interest of finding new drug targets or prognostic markers. In the case of ovarian cancer the pH-regulating enzyme carbonic anhydrase IX (CAIX) is of special interest. It is responsible for a change of pH in tumors, where an acidic environment is developed, which simplifies the proliferation and invasiveness of the tumor tissue.

Methods: The CAIX gene expression was detected with qPCR in ovarian cancer cell lines when treated with cisplatin. Furthermore, via Western Blot the CAIX protein levels were analyzed in cell lines, where hypoxia-like conditions were evoked. Chemosensitivity assays were done to determine the cell survival rate after treating them with cisplatin and in combination with a CAIX inhibitor. In order to detect the soluble form of CAIX (sCAIX) dependent on inducing hypoxia-like conditions again, ELISA analysis was done.

Results: Under hypoxic conditions the membrane-bound form of CAIX was decreased and the levels of sCAIX were increased. This leads to the assumption that a change of the hypoxic conditions evokes a shedding of CAIX, from the membrane bound to the soluble form. Also an antagonistic interaction could be observed concerning the treatment with carboplatin and the CAIX inhibitor.

Furthermore, the treatment with cisplatin led to a down regulation of CA9 gene expression. It can also be derived that certain cell lines from clear cell carcinomas have a high constitutive expression of CAIX.

Conclusion: The expression of CA9 mRNA as well as the CAIX protein expression were significant in the ovarian cancer cell lines. This might give new possibilities in using CAIX as an identification marker of ovarian cancer or help evaluating the progress of ovarian cancer treatment.

1 Introduction

1.1 The ovaries

The paired ovaries are part of the female reproductive system, which is completed by the paired fallopian tubes, the uterus, the vagina and the external genitals. They are developed, like male testes, from the indifferent gonads, and produce the female sex hormones, estrogen and progesterone, and provide oocytes. The ovaries are located intraperitoneal at the pelvic wall on both sides of the uterus and are oval-shaped. The menstrual cycle influences the ovary's size due to changing processes (Mayerhofer, Vogt, & Zanner, 2005).

1.2 Ovarian cancer

1.2.1 Epidemiology

In Austria, 646 women were diagnosed with ovarian cancer in 2011. This constitutes an uncorrected incidence rate of 8.6 per 100,000 inhabitants. The prevalence was 6,315 women, who were suffering from this type of cancer, while 486 women deceased as a consequence in that year. This leads to a mortality rate of 5.3 per 100,000 inhabitants (Statistik Austria, 2011). Different numbers are published by the International Agency for Research on Cancer (IARC) (2012) giving an incidence rate of 14.8 and a mortality rate of 11.7 per 100,000 inhabitants. Considering both data sources, incidence and mortality in Austria are above the world average, since the IARC numbers the worldwide incidence rate at 6.8 and mortality at 4.3 per 100,000 inhabitants for ovarian cancer. According to this source ovarian cancer is the eighth most common cancer in women.

As a general trend the morbidity has been decreasing in the past three decades. For 2011 it can be said that one out of 111 women (0.9%) will develop ovarian cancer in her life (Statistik Austria, 2011).

1.2.2 Etiology

According to Schorge et al. (2010) the etiology of epithelial ovarian cancer (EOC) is still not fully understood. The origin of the tumor tissue is unclear as well as the exact development. Landen, Birrer, and Sood (2008) propose that in

90% of the cases EOC develops from a single multidysfunctional cell, while 10% of the cases are linked to genetic alterations. The authors discuss four hypotheses trying to explain the physiological susceptibility. These hypotheses are: (1) incessant ovulation, (2) gonadotropin stimulation, (3) hormonal stimulation, and (4) inflammation.

The incessant ovulation hypothesis focuses on damaged ovarian surface epithelium (OSE) through ovulation, while the repairing processes by cells lead to the accumulation of mutations. This means a decreased number of ovulations minimize the chance of mutations and malignancies. Thus it may be concluded that the risk of developing EOC is proportional to the number of menstrual cycles, depending on the deliveries, lactation and use of oral contraceptive pills (OCPs). This hypothesis was first mentioned by Fathalla (1971), as well as Purdie et al. (2003).

The gonadotropin stimulation describes the effects of follicle-stimulating hormone (FSH) and luteinizing hormone (LH), which lead to cell growth, division and mutations. This implies that infertility and polycystic ovarian syndrome (PCOS) increase the risk of developing EOC, while progesterone-only OCPs decrease the risk. Also the expression of FSH increases the expression of many oncogenes and favors the growth of preclinical lesions. This is also stated by Whittemore, Harris, and Itnyre (1992).

Following, the hormonal stimulation causes carcinogenesis by high rates of androgens in the tumor microenvironment, e.g. through PCOS, acne or hirsutism, leading to a higher risk of developing EOC due to activation of androgen receptors on the OSE cells initiating proliferation. Additionally, androgens are excessively present in inclusion cysts. This is also mentioned by Edmondson, Monaghan, & Davies (2002). In opposite to androgens, progestin decreases the possibility of EOC and is supposed to lead to OSE apoptosis.

At last, inflammation can be caused by the ovulation damaged OSE and can evoke cytokine release and tissue reconstruction. This stresses the OSE cells, which also leads to mutations. This is in line with findings that patients with a highly regular use of nonsteroidal anti-inflammatory drugs (NSAIDs), which generally reduce inflammations, have a reduced risk of EOC. Indeed, exposure

to talc or asbestos, which promote inflammations, lead to a higher risk of EOC; also described by Ness and Cottreau (1999).

These four hypotheses explain how the processes of developing EOC can work, but there are many other factors that may have an impact as well.

As mentioned before, in addition to the 90% of tumors evoking from physiological susceptibilities, 10% of tumors emerge from genetic predisposition. From the latter, 90% of cases are associated with mutations in the breast cancer antigen 1 or 2 (BRCA1 or 2) gene and the rest is suspected to evolve from other, partially random, mutations (Landen et al., 2008; Schorge et al., 2010).

The hereditary component is an important factor because the cumulative risk of having an ovarian tumor, when a mutation in the BRCA1 gene is detectable, is 30-40% and a mutation in the BRCA2 gene results in a risk of 10-15%. BRCA1 and 2 are proteins with major functions in regulating the accurate processing of DNA, especially detecting double strand breaks. They control the completeness of the genome and cell cycle by promoting DNA repair mechanisms. They also regulate part of the mitosis and cell division steps (O'Donovan & Livingston, 2010).

Furthermore, the Lynch syndrome or hereditary nonpolyposis colon cancer (HNPCC), a hereditary germline mismatch repair (MMR) gene mutation, is associated with a high risk of colorectal cancer, and 10% of the genetically predisposed cases of EOC (Ketabi et al., 2011). This results in a general risk of 1-2% of having ovarian cancer, when the Lynch syndrome is present. In that case it is also noticeable that the median age of the diagnosis was 48 years, compared to 61 years as a the age of diagnosis considering all ovarian cancers (Landen et al., 2008).

Another aspect is the influence of lifestyle factors such as eating habits, obesity, or smoking. For example Kushi et al. (1999) suggest that the consumption of green leafy vegetables is associated with a risk reduction of ovarian cancer. More recent studies show that for instance a high cholesterol intake enhances the risk of ovarian cancer. Additionally as mentioned before, higher consumption of cruciferous vegetables, and vegetables in general, decreased the risk. In their review the authors show that current studies on the

effect of dairy products on ovarian cancer induction produce ambivalent results (Pan, Ugnat, Mao, Wen, & Johnson, 2004).

Regarding the role of obesity and a high body mass index (BMI), Webb (2013) found that a 5-unit increase in BMI increases the risk of suffering from certain ovarian cancer subtypes. She also describes a poorer prognosis for women suffering from obesity. Furthermore, according to McLemore, Miaskowski, Aouizerat, Chen, and Dodd (2009) obesity leads to a higher production of androgens, which enhances the conversion into estrogens. At the same time the binding capacity for sex hormones is decreased, which leads to a higher concentration of free and active estradiol. The negative effect of androgens on the risk level of developing EOC is hypothesized by Landen et al. (2008) in the hormonal stimulation hypothesis (3), which was mentioned at the beginning of this section.

McLemore et al. (2009) also mention the influence of smoking on ovarian cancer. They detected nicotine metabolites in ovarian follicular cells. This was also proposed in other studies, which show that smoking increases the risk of ovarian cancer (Modugno, Ness, & Cottreau, 2002; Pan, Ugnat, Mao, Wen, Johnson, et al., 2004).

1.2.3 Pathogenesis and tumor classification

Ovarian cancers are highly heterogeneous tumors, differing in morphology, clinical appearances, genetic variations and development; that is why ovarian cancer can be treated more accurately by better diagnoses of subtypes, stages and grade of the tumor. Because a general screening method does not exist yet and ovarian carcinomas do not show any typical symptoms, or are even asymptomatic until an advanced stage, most tumors (~75%) are detected late (at advanced stage III/IV) (Karst & Drapkin, 2009).

The only detection methods so far are transvaginal sonography (TVS) and biomarker screenings, especially of cancer antigen 125 (CA125). But in both cases a sufficient sensitivity is not given, which makes both methods inefficient as early detection methods (Karst & Drapkin, 2009).

To identify the type of the tumor, immunohistochemistry and a detection of certain biomarkers are used. For instance, the proliferation marker Ki-67 and CA125 are expressed differently in varying kinds of tumors (Köbel et al., 2008).

1.2.3.1 High-grade serous carcinoma (HGSC)

In 75% of the cases women diagnosed with EOC have this aggressive kind of ovarian cancer. HGSC is also linked to BRCA1/2 mutations in 10% of the cases (Kessler, Fotopoulou, & Meyer, 2013).

The histopathological appearance of the cells is characterized by various alterations. A multilayered epithelium with fenestrated cells and slit-like interstitial spaces, as well as giant mononuclear cells with immense nuclei can be seen. The latter is the criterion for differing between HGSC and low-grade serous carcinoma (LGSC). Another characteristic of HGSC is a high mitotic rate (Gilks & Prat, 2009).

There is a high detection rate of the mutated tumor suppressor gene p53, of which 95% of mutation in BRCA genes are seen in over 20% of the cases and estrogen receptors (ER) in over 65% of the cases (Prat, 2012).

The development of HGSC, as hypothesized by Kessler et al. (2013) is linked to toxic substances that occur after ovulation and lead to an inflammation at the epithelium of the fallopian tube. This inflammation is the origin of cellular changes and the development of the malignancy. A contrary position suggests that p53 loss and genetic instability are the decisive factors of the tumor (Prat, 2012).

Another possibility for the origin of HSGCs is the serous tubal intraepithelial carcinoma (STIC), which is a precursor lesion in the fallopian tube mucosa. However, it is not detectable in all HGSC cases (Erickson, Conner, & Landen, 2013). Furthermore, STIC and HGSC have the same p53 mutations and express the same oncogenes (Kessler et al., 2013).

HGSC is a carcinoma with a high chemosensitivity initially, which relapses in form of a chemoresistent tumor, resulting in a poor prognosis (Prat, 2012).

1.2.3.2 Low-grade serous carcinoma (LGSC)

Only 5% of all cases of ovarian carcinoma are assigned to LGSC. It differentiates from HGSC by showing more consistent nuclei and having a lower mitotic rate. It was assumed that LGSC develops into HGSC, but the two tumors differ in their pathways and phenotypes. In early stages LGSC has a promising prognosis, but in advanced stages its prognosis is comparable to the dismal HGSC prognosis (Gilks & Prat, 2009; Jones et al., 2012).

The origin of LGSC is a noninvasive serous borderline tumor (with or without a micropapillary structure), which shows invasive growth and is then classified as LGSC. The characteristic molecular abnormalities are BRAF or KRAS mutations, respectively in 38% and 19% of LGSC. Interestingly, only one of these mutations occurs in a tumor. Furthermore, LGSC is not associated with BRCA germline mutations (Prat, 2012; Singer et al., 2003).

Generally, LGSC affects younger women more often and is less aggressive than HGSC. It shows a high rate of multiple recurrences requiring several operations. Overall, the ten year survival rate is less than 50% (Jones et al., 2012).

1.2.3.3 Mucinous carcinoma (MC)

Of all ovarian carcinomas only 3-4% belong to the mucinous type, but if also metastases are counted, it results in a total of 10-15%. Characteristically these tumors are primarily unilateral and are typically of big volume (>13cm) at the time of diagnosis. In most cases, metastases appear bilateral and are significantly smaller (Gilks & Prat, 2009).

There are four different appearances: benign, borderline, invasive, and non-invasive (intraepithelial carcinoma) MCs; they can all occur simultaneously in one tumor and have to be determined after histopathology. Usually the tumor is limited to the ovary, not entering the microenvironment. In latest studies, the MC is separated into the expansile type and the infiltrative type. The first type is also considered as noninvasive, whereas the infiltrative type is characterized by stromal invasion through glands, cell clusters or individual cells. The prognosis is favorable, but even better for the expansile type.

As precursor lesions, cystadenoma or borderline tumors, are proposed. The phenotype is also associated with Kirsten rat sarcoma viral oncogene homolog (KRAS) mutations, the lack of ER and the transcription factor Wilms tumor suppressor 1 (WT1). Another characteristic is the expression of human epidermal growth factor receptor 2 (HER2) in 15-20% of the cases.

In patients with MC, a platinum-based chemotherapy shows a lower response rate, while a Trastuzumab therapy (a monoclonal antibody binding to HER2) reduces tumor proliferation more effectively (Gilks & Prat, 2009; Köbel et al., 2008; Prat, 2012).

1.2.3.4 Endometrioid carcinoma (EC)

This subtype is present in approximately 10% of all cases of ovarian cancer and is usually diagnosed in a quite early stage (I/II) in peri- or postmenopausal women (McMeekin, Burger, Manetta, DiSaia, & Berman, 1995; Prat, 2012). Almost half of the patients also show an ipsilateral or pelvic endometriosis, while 15-20% are associated with endometrium carcinomas. The neoplasms occur in 28 % on both ovaries. Tumors are of spherical structure or are tubular glands, which are lined by stratified epithelium without mucin. Generally, it is hard to differentiate between EC and HGSC, especially in high-grade EC cases. Characteristic in most cases is the squamous differentiation of the tissue, and besides that, also clear cells can occur (Gilks & Prat, 2009; Köbel et al., 2008; McMeekin et al., 1995; Prat, 2012; Schwartz et al., 2002).

Most mutations occur in the AT-rich interactive domain 1A gene (ARID1A), the tumor suppressor gene phosphatase, and tensin homologue gene (PTEN). These ARID1A mutations can be present in EC, but also in clear cell carcinomas (CCC). It is part of the transcriptional suppressor and enhancer SWItch/Sucrose NonFermentable (SWI-SNF-A) complex, a chromatin remodeling complex. The mutations of PTEN lead to an enhanced inhibition of apoptosis, which depends on the PI3K-AKT pathway.

Furthermore, the cell-cell adhesion gene β -catenin (CTNNB1) mutation is important, whereby cellular β -catenin is resistant to Adenomatous Polypsis Coli (APC)-mediated downregulation. β -catenin is seen in the nuclei of the carcinoma cells in most of the patients.

EC has a high chemosensitivity and the prognosis is favorable (Gilks & Prat, 2009; Prat, 2012).

1.2.3.5 Clear cell carcinoma (CCC)

In another 10% of the cases, when women are diagnosed with ovarian carcinoma, they have CCC. It is diagnosed by a clear appearance of the cytoplasm, and the architectural and cytological appearance, which is characterized by few stratified epithelium and complex papillae, as well as the papillae extending to the hyaline basement membrane and hyaline bodies. Another distinction from HGSC is the lower mitotic rate (Komiyama et al., 1999; Prat, 2012; Sugiyama et al., 2000).

Considering the genetic abnormalities in almost half of the cases, ARID1A mutations occur especially in those tumors deriving from endometriosis (Wiegand et al., 2010). Over 90% of the carcinomas are positive for the transcription factor hepatocyte nuclear factor-1beta (HNF-1beta), which triggers various pathogenic processes, such as upregulation of CCC specific genes: dipeptidyl peptidase IV, osteopontin, angiotensin converting enzyme 2 etc. Furthermore, in 95% of the tumors, ER and WT1 are absent (Kato, Sasou, & Motoyama, 2005; Köbel et al., 2009).

Interestingly, most patients are diagnosed with early stage carcinomas (I/II), often in combination with endometriosis. This leads to a more favorable prognosis. In general, the chemosensitivity is relatively low. Only cases with high proliferation rates show better response to platinum-based chemotherapy (Itamochi et al., 2002; Komiyama et al., 1999; Silva & Young, 2007).

1.2.3.6 Brenner/Transitional carcinoma

This is an extremely rare type of carcinoma, which can occur as transitional cell carcinomas, with or without Brenner components. The ones without Brenner component cannot clearly be discriminated from HGSC, which leads to the assumption that it is a variation of HGSC. It also expresses the transcription factor WT1 (Gilks & Prat, 2009; Kommoss et al., 2005; Ledermann et al., 2013).

1.2.3.7 Mixed epithelial tumors

Sometimes tumors cannot be clearly classified because they show different structural and cytological components. Usually these tumors are identified as HGSC in combination with other types, e.g. HGSC/EC, HGSC/CCC etc. These types of tumors are treated like HGSC, for a higher recovery rate (Prat, 2012).

1.2.4 Diagnosis

Patients developing ovarian carcinomas do not have clear symptoms or even no symptoms at all at an early stage. Therefore, more than 75% of the cases are detected at an advanced stage (III/IV), which leads to a more unfavorable prognosis of these tumors (Karst & Drapkin, 2009).

Symptoms that could be observed in patients at all stages are pelvic and abdominal pain, increased abdominal size, bloating, urinary frequency, vaginal bleeding, constipation, diarrhea, and fatigue. Later, the usually increased abdominal size leads to an increased abdominal girth, bloating, nausea, anorexia, dyspepsia, and early satiety. Additionally, depression can be associated with ovarian cancer, especially in patients of younger age. Furthermore, a correlation between frequency, severity, and duration of symptoms could be seen in patients suffering from these listed symptoms daily when compared to the control group, which shows these unspecific symptoms only two to three times a month (Goff et al., 2007; Ledermann et al., 2013).

Diagnosis is performed through measurement of serum CA125 to confirm the malignancy. However, only in 80% of all ovarian cancers CA125 is positive, whereas it is detected in almost all HGSCs, and only in 60% of MC and CCC tumors (Bast et al., 2005; K. H. Lu et al., 2004). A recent study shows that the combination of CA125 and a symptom index can identify 80% of all early stage cancers (Andersen et al., 2008).

As screening method, transvaginal sonography (TVS) is performed to determine the volume of the ovaries as normal or abnormal. This means that tumors can only be detected if they enlarge the ovary, or if TVS is conducted annually, and alterations in the volume can be identified (van Nagell Jr. et al., 2000).

Other possibilities for further staging of the tumors, are computed tomography (CT) or magnetic resonance (MR) imaging techniques which can also determine metastases and exact size of the tumors (Kurtz et al., 1999).

1.2.5 Staging of the tumors

Table 1: *Staging of ovarian cancer through FIGO and AJCC.*

FIGO	Stage characteristics	TNM
	Primary tumor cannot be assessed	TX
0	No evidence of primary tumor	T0
I	Tumor confined to the ovaries	T1
IA	Tumor limited to 1 ovary (capsule intact); no tumor on ovarian surface; no malignant cells in the ascites or peritoneal washings	T1a
IB	Tumor limited to both ovaries (capsule intact); no tumor on ovarian surface; no malignant cells in the ascites or peritoneal washings	T1b
IC	Tumor limited to 1 or both ovaries, with any of the following:	
IC1	Surgical spill	T1c1
IC2	Capsule ruptured before surgery or tumor on ovarian surface	T1c2
IC3	Malignant cells in the ascites or peritoneal washings	T1c3
II	Tumor involves 1 or both ovaries with pelvic extension (below pelvic brim) or primary peritoneal cancer	T2
IIA	Extension and/or implants on uterus and/or fallopian tubes and/or ovaries	T2a
IIB	Extension to other pelvic intraperitoneal tissues	T2b
III	Tumor involves 1 or both ovaries or fallopian tubes, or primary peritoneal cancer, with cytologically or histologically confirmed spread to the peritoneum outside the pelvis and/metastasis to the retroperitoneal lymph nodes	T3 and/or N1
IIIA1	Positive retroperitoneal lymph nodes only (cytologically or histologically proven)	T1/T2 N1
IIIA1i	Metastasis up to 10 mm in greatest dimension	T1/T2 N1a
IIIA1ii	Metastasis more than 10mm in greatest dimension	T1/T2 N1b
IIIA2	Microscopic extrapelvic (above the pelvic brim) peritoneal involvement with or without positive retroperitoneal lymph nodes	T3a2
IIIB	Macroscopic peritoneal metastasis beyond the pelvis up to 2cm in greatest dimension, with or without metastasis to the retroperitoneal lymph nodes	T3b
IIIC	Macroscopic peritoneal metastasis beyond the pelvis more than 2cm in greatest dimension, with or without metastasis to the retroperitoneal lymph nodes (includes extension of tumor to capsule of liver and spleen without parenchymal involvement of either organ)	T3c
IV	Distant metastasis excluding peritoneal metastases	M1
IVA	Pleural effusion with positive cytology	M1a
IVB	Parenchymal metastases and metastases to extra-abdominal organs (including inguinal lymph nodes and lymph nodes outside of the abdominal cavity)	M1b

Note. Adapted from Prat (2014) and Union for International Cancer Control (2014).

A consistent grading system is necessary in order to achieve a homogeneous characterization of tumors. This is important for the therapy and prognosis for the patients.

The most common staging classification system was established by the Fédération Internationale de Gynécologie et d'Obstétrique (FIGO) and the American Joint Committee on Cancer System (AJCC), which is labeled as TNM. The classification is made by tumor and its general characteristics, the nodes and the metastases (AJCC, 2014; Benedet, Bender, Jones, Ngan, & Pecorelli, 2000; Prat, 2014). Table 1 summarizes both classification systems.

Through light microscopy the tissue can be differentiated and then sub-classified by grading, which is only applied to EOC, not non-epithelial. The grading is proportional to the prognosis and is shown in table 2 (Benedet et al., 2000; Prat, 2014).

Table 2: *Grading of ovarian cancer.*

Gx	Grade cannot be assessed
G1	Well differentiated
G2	Moderately differentiated
G3	Poorly differentiated

Note. Adapted from Benedet et al. (2000).

1.2.6 Prognosis

While ovarian cancer is the eighth most common cancer in women, it is the most deadly of all gynecological malignancies. Several aspects can be taken into consideration when determining the survival rate and prognosis. The most important prognostic factors are: i) the FIGO stage of the tumor and ii) the amount of residual disease after surgery. Other predictive factors are the age of the patient, type and grade of the tumor, serum CA125 levels, presence and amount of ascites, and performance status (Clark, Stewart, Altman, Gabra, & Smyth, 2001; Tingulstad, Skjeldestad, Halvorsen, & Hagen, 2003).

For patients discovered with a stage I carcinoma the five year survival rate is more than 90%, whereas the five year survival rate for stage III/IV patients is only 30%, while approximately 75% of the patients are diagnosed with stage III/IV tumors (Jemal et al., 2008).

Regarding the residual tumor after surgery, the numbers show significant differences. When no visible residuals are left, the average surviving time is more than eight years for patients with any FIGO stage. For any residual tumor after surgery, the survival time for patients with a tumor 1-10mm is around 36 months, and with a tumor >10mm about 29 months (du Bois et al., 2009).

Remarkably, the number of ovarian cancer related deaths above the age of 75 is three times higher than in women at the age 45 or younger (Tingulstad et al., 2003).

A prognosis can also be made based on the histological type of the tumor as mentioned before. In general the MC and the EC have more favorable prognoses, whereas the HGSC has a rather dismal prognosis. LGSC and CCC have intermediate prognoses compared to the other subtypes (Prat, 2012).

1.2.7 Therapy

The first step of therapy is a laparotomy with comprehensive surgical staging. Through this the number of occult metastases can be determined and these tumors can also be resected. This is important to make a valid prognosis and to decide whether chemotherapy is needed or not. Surgical staging itself is characterized by an exploratory laparotomy, peritoneal washings for cytology, total abdominal hysterectomy, bilateral salpingo-oophorectomy, omentectomy, bilateral pelvic and paraaortic lymphadenectomy, and peritoneal biopsies (Garcia-Soto, Boren, Wingo, Heffernan, & Miller, 2012; Ledermann et al., 2013). This is particularly important for advanced stage carcinomas, where a complete resection of the tumor favors the overall survival rate (Vergote et al., 2010).

At an early stage, an adjuvant platinum-based chemotherapy should be given after surgery, if there are no metastases beyond the pelvis. But it can be omitted, when the tumor is staged I (FIGO) and could be removed entirely (Winter-Roach, Kitchener, & Dickinson, 2009).

Advanced staged carcinomas are always treated with the standard chemotherapy consisting of carboplatin (area under the curve (AUC) 6-5) and paclitaxel (175-185mg/m²). Therefore, the dose of the carboplatin is calculated from the formula established by Calvert. It includes the glomerular filtration rate (GFR) in the following equation: carboplatin (mg) = AUC x (GFR + 25).

Commonly, paclitaxel is administered intravenously over 3 hours, followed by intravenously administered carboplatin for 30-60 minutes. This scheme is repeated every three weeks for six cycles. This treatment is equally effective as the combination of cisplatin and paclitaxel, but it is less toxic and associated with a higher tolerability (du Bois et al., 2003; Ledermann et al., 2013; Ozols et al., 2003).

As an alternative for patients who are intolerant to paclitaxel, the combinations of carboplatin and pegylated liposomal doxorubicin (Caelyx; PLD) are given (Pignata et al., 2011; Vasey et al., 2004). Furthermore, bevacizumab, a monoclonal antibody that inhibits the vascular endothelial growth factor, was tested in recent studies in combination with chemotherapeutics, and it can be administered to advanced staged patients. It benefits the overall survival time and progression-free survival, but it is also associated with severe hypertension (Burger et al., 2011; Perren et al., 2011).

Around 70% of the patients experience a relapse within the first three years after platinum-based chemotherapy. These recurrences can be separated into platinum-resistant and platinum-sensitive cases. The patients with the platinum-resistant tumor have a dismal prognosis. Therefore, a palliative treatment should be administered in order to improve the quality of life and decrease symptoms. In this case paclitaxel, topotecan, PLD, and gemcitabine show acceptable effects if administered once a week or three times weekly. When the relapsed tumor is platinum-sensitive, platinum is used in different combinations and, additionally, trabectedin can be used (Fung-Kee-Fung et al., 2007; Ledermann et al., 2013).

In order to evaluate the efficacy of the treatment, or as follow-up after treatment, progressive serial elevation of CA125 is performed (Rustin et al., 2011). Physical examinations (breast, pelvic, rectal) should be done the first year after treatment every three months, then every four to six months until the fifth year after treatment and then annually (Benedet et al., 2000).

1.3 Regular and tumor cell metabolism

The regular cell metabolism is based on the production of energy via glycolysis and oxidative phosphorylation. Thereby, glucose is phosphorylated to glucose-6-phosphate, which is converted into pyruvate and results in an allocation of two molecules of adenosine triphosphate (ATP), protons (H^+), and two molecules of nicotinamide adenine dinucleotide phosphates (NADH). If oxygen is available in the cells, the mitochondria oxidize pyruvate to form HCO_3 and 36 molecules ATP are produced. These processes are part of the respiratory chain, where electrons are transferred through different kind of enzymes, the so called complexes I-IV, ubiquinone and cytochrome c. This electron transfer into the intermembrane space is accompanied by the efflux of H^+ , which build up an electrochemical gradient. The ATP synthase produces the ATP by transporting the protons. If there is no oxygen available, pyruvate is further reduced into lactate, which is transported out of the cells and nicotinamide adenine dinucleotide (NAD^+) is regenerated. Via this pathway only two molecules of ATP are produced per glucose molecule. This inefficient pathway for energy is downregulated under normal conditions, but it is upregulated in the absence of oxygen (Fang, Gillies, & Gatenby, 2008; Gatenby & Gillies, 2004; Rich, 2003). In contrast to normal cells, which generate energy in order to maintain cell homeostasis, tumor cells need to provide more energy for their proliferation. In these cells, control mechanisms are removed, which allows tumor cells to generate energy through glycolysis even if oxygen is available. This was first mentioned by Otto Warburg and is consequently called the Warburg effect (Cairns, Harris, & Mak, 2011; Warburg, 1956).

Furthermore, proliferated tumors obtain hypoxic conditions, which means lower concentrations of oxygen can be observed in the tumor tissue. Two different explanations are presented in the literature. First, due to a lower vascularity or, second, due to a lower blood flow through the vessels (Cairns et al., 2011).

Another significant difference between regular cells and tumor cells lies in the pH gradient of the intercellular pH (pH_i) and the extracellular pH (pH_e). In normal cells the pH_i is ~ 7.2 and the pH_e is ~ 7.4 . However, a higher rate of glycolysis

leads to an increased production of protons and a higher efflux of protons into the extracellular microenvironment of the tumor cells. This leads to an extracellular acidification, which results in a shift of pH_i to ≥ 7.4 and a pH_e of ~ 6.7 to 7.1 in tumors. Clinical consequences of hypoxia and acidosis are a higher radioresistance and drug resistance of tumor cells. The extracellular acidification leads to enhanced metastases, invasion of the microenvironment, higher mutation rates, and tumor progression (Gatenby & Gillies, 2004; Webb, Chimenti, Jacobson, & Barber, 2011).

1.4 Tumor growth influencing factor

1.4.1 Hypoxia-inducible factor-1 (HIF-1)

The transcription factor hypoxia-inducible factor-1 is a heterodimer, which is responsible for the alternate gene expression in cells with present hypoxia. It consists of two subunits, HIF- α , which is expressed depending on the availability of oxygen, and HIF- β , which is constitutively expressed and independent of oxygen levels. HIF- β is identical to the aryl hydrocarbon nuclear translocator (ARNT), a partner of the arylhydrocarbon receptor (Bertout, Patel, & Simon, 2008).

HIF- α contains an oxygen-dependent degradation domain (ODD), which regulates the oxygen-dependent expression of the protein (Huang, Gu, Schau, & Bunn, 1998). Under normoxic conditions, the HIF-prolyl hydroxylase domain (PHD) hydroxylates HIF- α , which is recognized by the von Hippel-Lindau tumor suppressor protein (pVHL) and mediates the polyubiquitination of the subunit (Cockman et al., 2000). The ubiquitinated substrates are identified and hydrolysed by the 26S proteasome. This results in a degraded HIF- α protein and an inactivated transcription factor HIF-1 (Kallio, Wilson, O'Brien, Makino, & Poellinger, 1999). Furthermore, HIF-1 α is stabilized by the endproducts of the aerobic glycolysis, pyruvate, and lactate. This suggests that malignant transformation is produced via the Warburg effect (H. Lu, Forbes, & Verma, 2002).

It is known that HIF-1 activates the expression of more than 100 genes, which contain a hypoxia responsive element (HRE), such as the vascular endothelial cell growth factor (VEGF), glucose transporters 1 and 3 (GLUT1/3),

glyceraldehyde phosphate dehydrogenase (GAPDH), lactate dehydrogenase (LDH), carbonic anhydrases 9 and 12 (CAIX/XII), monocarboxylate transporter 4 (MCT4), pyruvate dehydrogenase kinase (PDK), and Na^+/H^+ exchanger 1 (NHE1) (Ke & Costa, 2006; Kroemer & Pouyssegur, 2008; Maxwell, Pugh, & Ratcliffe, 2001; Semenza, Neifelt, Chi, & Antonarakis, 1991).

In HIF-1 and many other proteins, e.g., tumor suppressor p53, phosphatidylinositol 3-kinase (PI3K)/Akt/mTor signaling pathway, proto-oncogene KRAS, and myc mutations were found, which favor the tumor growth (DeBerardinis, 2008).

1.5 pH Regulation and transport proteins

Differing from the pH_i of ~ 7.2 and a pH_e of ~ 7.4 in normal tissue, tumor cells have a pH_i of ≥ 7.4 and the pH_e of tumors range from ~ 6.7 to 7.1 .

The pH_e of the normal cells depend on the levels of carbon dioxide (CO_2), and its constant partial pressure (pCO_2), as well as the concentration of bicarbonate (HCO_3^-), which is regulated by the lungs and kidneys. The pH_i is controlled by the efflux of protons, CO_2 and lactic acid ($\text{C}_3\text{H}_6\text{O}_3$) (De Brabander, Geuens, Nuydens, Willebrords, & Mey, 1982; Roos & Boron, 1981; Swietach, Vaughan-Jones, & Harris, 2007).

In the tumor tissue changes take place and a different pH gradient develops. This happens on a molecular basis through weak bases, e.g., HCO_3^- , that are transported into the cells. At the same time, weak acids, such as CO_2 , carbonic acid (H_2CO_3), and $\text{C}_3\text{H}_6\text{O}_3$ are exported from the cell; H^+ are exchanged for other cations (Neri & Supuran, 2011).

Many different transporters are part of this pH regulating system in tumor cells because it maintains the survival and proliferation of the tumor cells under altered conditions such as hypoxia. The Na^+/H^+ -Exchanger generates an influx of Na^+ , accompanied by an export of H^+ and it can be activated by growth factors. From the co-transporter family of the monocarboxylate transporters (MCT) the isoforms 1 to 4 export lactic acid together with H^+ . Furthermore, there is carbonic anhydrase IX (CAIX) or CA XII, an ectoenzyme bound to the outer membrane of the cell, converting CO_2 , which diffuses across the plasma

membrane to the extracellular space, into carbonic acid. Closing this circle, the Na^+ -dependent and Na^+ -independent chloride (Cl^-)/ HCO_3^- exchangers shift bicarbonate back into the cell and alkalizes the intracellular space (Pouysségur, Dayan, & Mazure, 2006).

As mentioned before, the consequence of an acidosis favors the development and proliferation of the tumor. The radioresistance is increased, as well as the resistance towards certain medication, more invasion takes place and metastases are developed, leading to apoptosis (Gatenby & Gillies, 2004).

1.5.1 Carbonic anhydrase IX

The CAIX is a metalloenzyme with a zinc (Zn^{2+}) ion in the active binding site consisting of 459 amino acids. It has the N-terminal structure of proteoglycans, a transmembrane domain, and a C-terminus inside of the cell. It belongs to the gene family of α -carbonic anhydrases (α -CA). These are found in vertebrates, bacteria, algae, and plants. There are 16 isozymes that differ in their catalytic activity, localization, and tissue distribution, which are separated into the cytosolic forms (CAI, CAII, CAIII, CAVII, and CA XIII), the membrane bound ones (CAIV, CAIX, CAXII, CAXIV, and CAXV), the mitochondrial forms (CAVA and CAVB), and the secreted form (CAVI) (Neri & Supuran, 2011; J. Pastorek et al., 1994; Supuran, 2008; “UniProt,” 2014).

In CAIX the extracellular domain is responsible for the catalytic activity, whereas the N-terminus, with its proteoglycans, is suggested to be important for cell adhesion. Furthermore, parts of the C-terminus can be phosphorylated and therefore it can be involved in signaling pathways, dimerization or bicarbonate transporter interaction. This leads to the conclusion that CAIX is important for pH regulation as well as intercellular communication (Dorai, Sawczuk, Pastorek, Wiernik, & Dutcher, 2005; Sterling, Reithmeier, & Casey, 2001; Švastová et al., 2003).

As previously mentioned, CAs are very efficient in the reversible transformation of CO_2 to HCO_3^- and protons ($\text{CO}_2 + \text{H}_2\text{O} \leftrightarrow \text{HCO}_3^- + \text{H}^+$). These acids play a part in contributing to the buffer system of the cells. Usually CAIX is expressed in gastrointestinal tract, small intestine and gallbladder, and it also has the highest efficiency in transferring protons among all CAs. But it is

overexpressed in tumor tissue, together with CAXII, which is induced by HIF-1. Therefore it is considered to be involved in tumor progression and therapeutic response. Additionally, CAs are involved in bone resorption and bone calcification, electrolyte secretion, and respiration (Neri & Supuran, 2011; Pastorekova et al., 1997; Saarnio et al., 1998; Wingo, Tu, Laipis, & Silverman, 2001).

The CAIX was first discovered in HeLa cells and called MN protein. It was localized via immunofluorescence on the outside of the cell, but also nuclear localization was detected (Opavský et al., 1996; Pastoreková, Závadová, Košťál, Babušíková, & Závada, 1992).

1.5.2 Soluble form of carbonic anhydrase IX

In addition to the cell-associated CAIX there is another isoform, namely the soluble CAIX (sCAIX). It was first detected in the serum and urine of renal clear cell carcinoma patients. It is shown that the soluble version is shed by the tumor cells in form of a shortened protein with a molecular mass of 50/54kDa, compared to regular 'full' protein with 54/58kDa. It could be shown by monoclonal antibodies binding experiments that the proteoglycan-like domain and the CA domain as the extracellular part of the molecule are being separated from rest to build the new soluble CAIX (Závada, Závadová, Zatořovičová, Hyršl, & Kawaciuk, 2003).

In ovarian cancer, serum levels of CAIX were measured and were found to be in the range of 30 to 1687pg/ml. However, no significant prognostic relevance was found for the serum levels (Woelber et al., 2010).

2 Aims of the study

Ovarian cancer is the eighth most common cancer in women, but it is also the deadliest among all gynecological malignancies. Since there are no reliable prognostic methods for an early detection of these tumors, most cases are discovered at advanced stage (FIGO stage III or IV), which also leads to poor prognosis for the patients. Despite radical surgery and chemotherapy, which can lead to a remission, most patients suffer from recurrences and cancer-related death within five years.

Ovarian cancer was found to be a heterogeneous disease. This fact complicates diagnosing and choosing a proper therapy. The carcinomas are classified histologically after surgery into high grade or low grade serous carcinomas, clear cell, endometrioid, and mucinous carcinomas. Depending on classification and stage, a therapy regimen containing carboplatin and paclitaxel is selected.

It is known that most tumors rapidly proliferate and expand through the acidic microenvironment generated by tumor cells. Through the extensive tumor cell proliferation, hypoxia is developed within the tumor. There is a lack of oxygen caused by insufficient vascularization, leading to a rapid adjustment in the cells. The oxygen-sensitive transcription factor HIF-1 is induced and several genes necessary to provide survival at low oxygen levels are upregulated, including the genes for glycolysis. This assures a sufficient ATP supply without the oxidative phosphorylation. For example glucose transporter GLUT increases the influx of glucose, which is provided for the glycolysis. These processes generate a high amount of protons that are exported from the cell by different transmembrane proteins, including NHE-1 or MCT 1-4, in exchange or via a co-transport with other ions.

Additionally, CO_2 diffuses through the membrane to the extracellular space and is converted by the ectoenzyme CAIX together with H_2O into HCO_3^- and H^+ . This accumulation of H^+ in the extracellular space leads to an acidosis. The result is a shift of the regular pH_i of ~ 7.2 and pH_e of ~ 7.4 , to a more basic pH_i in tumor cells of ≥ 7.4 and a more acidic pH_e of ~ 6.7 to 7.1 . This favors the development and proliferation of the tumor in many ways.

In addition to the intracellular CA isoform a soluble form of the CAIX (s-CAIX) was discovered, constituting a shortened version of the regular CAIX, which is shed by tumor cells. It can be detected in the serum, ascites, and urine, depending on the origin and position of the tumor and its metastases. This form is not well investigated in ovarian cancer, yet.

Therefore, aim of this study was to examine the presence of CAIX and sCAIX in ovarian cancer cell lines. Moreover, the research question concerned the concentrations of proteins when cells were treated with chemotherapeutic cisplatin. Also the effect of deferoxamine (DFX), which is known to induce HIF-1 and simulate hypoxia, was tested. Studies were done using qPCR and Western Blot analysis, as well as ELISA and chemosensitivity assay to analyze gene and protein expression as well as to study the efficiency of the treatments.

The understanding of pH regulators in general and their behavior under common chemotherapy might give new opportunities for a better treatment of ovarian cancer by developing new therapeutics.

3 Materials and methods

3.1 Solutions and buffers

The solutions and buffers listed here were used throughout all working processes and are in line with Klameth (2013), who used the same materials and methods.

10x Phosphate buffered saline (PBS):

2.0g	potassium dihydrogen phosphate (KH_2PO_4) ^a
80.0g	sodium chloride (NaCl) ^a
2.0g	potassium chloride (KCl) ^a
14.4g	disodium hydrogen phosphate (Na_2HPO_4) ^a
ad 1.0l	double distilled water (ddH_2O)

The pH was adjusted to 7.4.

Radio-immunoprecipitation (RIPA) buffer:

0.1%	sodium dodecyl sulfate (SDS) ^a
25mM	TRIS ^a
150mM	NaCl
1.0%	sodium deoxycholate ^a
1.0%	Nonidet P40 substitute (NP 40) (Sigma-Aldrich, St. Louis, MO, USA)

10x Laemmli buffer:

10.0g	SDS ^a
30.0g	TRIS ^a
144.0g	Glycine ^a
ad 1.0l	ddH_2O

4x Sample buffer:

3.0ml	87% Glycerine ^a
5.0ml	0.5M TRIS pH 6.8
0.8g	SDS ^a
0.5ml	0.1% bromphenol blue ^a
20.0%	β-mercaptoethanol (Sigma-Aldrich, St. Louis, MO, USA) → freshly added before each use

10x TAE buffer:

96.8g	TRIS ^a
54.5g	sodium acetate ^a
7.6g	ethylenediaminetetraacetic acid (EDTA) ^a
ad 2.0l	ddH ₂ O

3.2 Cell culture

The following list of chemicals and appliances is in line with Klameth (2013).

- 25cm² and 75cm² cell culture flasks (TPP, Trasadingen, CH)
- Pipettes 10ml and 25ml (Biozym Scientific GmbH, Oldendorf, GER)
- RPMI-1640 medium with L-glutamine and sodium bicarbonate R8758 (Sigma-Aldrich, St. Louis, MO, USA)
- Calcium and magnesium free 1x PBS
- Safe-seal tips (Biozym Scientific GmbH, Oldendorf, GER)
- Pasteur pipettes (Sigma-Aldrich, St. Louis, MO, USA)
- 15ml centrifugation tubes (TPP, Trasadingen, CH)
- Hettich Rotante centrifuge (Hettich Bäch, CH)
- Lamina flow (Thermo Fisher Scientific, Asheville, US)
- Heraeus cytoperm 2 (Thermo Fisher Scientific, Asheville, US)
- Olympus CK2 inverted microscope with phase contrast (Spach Optics, Rochester, NY)
- Fetal Bovine Serum FBS superior (Biochrom AG Biotechnologie, Berlin, GER)

^a Products were purchased from Merck, Darmstadt, GER.

- 10x Trypsin - EDTA solution Nr. 59418 (Sigma-Aldrich, St. Louis, MO, USA)
- Ethanol (EtOH) (AnalaR NORMAPUR, West Sussex, UK)
- Penicillin - Streptomycin P4083 (Sigma-Aldrich, St. Louis, MO, USA)
- Cisplatin and etoposide (Sigma-Aldrich, St. Louis, MO, USA)
- CAIX inhibitor C207A (obtained from Prof. Claudiu T. Supuran, Firenze, ITA)

3.2.1 Cell lines

All of the following cell lines were obtained from the ATCC (Wesel, Germany)

- ES2
- PA1
- PE01
- A2780
- A2780 ADR^b
- OV90
- COV318
- CaOV4
- COV362

3.2.2 Medium and cultivation conditions

The adherent cells were cultivated in cell culture flasks in RPMI-1640 basic medium with an addition of 10% PBS, 50µg/ml streptomycin, and 100µl/ml neomycin as well as approximately 50units penicillin/ml. To determine the cell growth and confluency a phase contrast microscope was used.

The supernatant medium of the cells was discarded and the cells were washed once with 1x PBS. Then 10x Trypsin was diluted with 1x PBS to 1x Trypsin (0.5%) and added to the cells, which were incubated for 4 min at 37°C. When the cells where detached from the bottom and rounded, the medium was transferred into a 15ml flask and centrifuged for 3 min at 450g. Afterwards the supernatant was discarded and medium was added again, so the cells could be

^b Adriamycin resistant variant of A2780 (Adriamycin = Doxorubicin)

split. From 1/3 till 1/6 of the cells were moved to new culture flasks and incubated at 37°C and 5% CO₂.

All steps were done in a sterile Lamina Airflow and the reagents were used at room temperature.

3.2.3 Treatment with chemotherapeutics and modulators

The cells were treated with one of the following agents in different concentrations:

Carboplatin and Cisplatin:

- both platinum based chemotherapeutic drugs, counting to the alkylating like drugs
- interact with the DNA and they are DNA-repair interfering

C207A:

- [4-(2,4,6-trimethylpyridinium-N-methylcarboxamido)-benzensulfonamide perchlorate]
- Compound 39
- inhibits the zinc binding site in the catalytic domain of CAIX

DFX:

- Deferoxamine is an iron chelator, which is able to induce hypoxia in cells
- promotes the expression of hypoxia-inducible factor 1 α (HIF-1 α)

Three days after the treatment the cells were harvested at 37°C and 5% CO₂ and the listed methods were performed.

3.3 RNA isolation

The following list of chemicals and appliances is in line with Klameth (2013).

- 25cm² cell culture flask (Nunc, Roskilde, DK)
- 6-well plate (TPP, Trasadingen, CH)
- Calcium and magnesium free 1x PBS
- Cell scrapers (TPP, Trasadingen, CH)

- peqGold Trifast (PEQLAB Biotechnologie GmbH, Erlangen, GER)
- Gibco distilled water, RNase/DNase free (Life Technologies, Carlsbad, CA)
- Ethanol 70% (EtOH) (AnalaR NORMAPUR, West Sussex, UK)
- Isopropanol (Merck, Darmstadt, GER)
- Chloroform (Merck, Darmstadt, GER)
- Centrifuge 5415R (Eppendorf AG, Hamburg, GER)
- Thermomixer comfort (Eppendorf AG, Hamburg, GER)
- Nanodrop Spectrophotometer ND-100 (Thermo Fisher Scientific, Asheville, US)
- Reaction tubes 1.5ml (Biozym Scientific GmbH, Oldendorf, GER)
- Safe-seal tips (Biozym Scientific GmbH, Oldendorf, GER)

The RNA isolation was done under sterile working conditions.

Cells were grown in a 25cm² cell culture flask or a 6-well plate until a confluency of 90-95% was achieved. In order to collect the cells the medium was removed and the adherent cells were washed twice with 1x PBS and lysed with 1ml Trifast, an amount that is for about 5-10*10⁶ cells. Trifast consists of phenol and guanidine-isothiocyanate and is able to lyse the cells and stabilize RNA, so the cells are incubated for 5 min at room temperature to complete the lysis. The content was transferred into reaction tubes.

For each ml of Trifast in the tubes, 200µl Chloroform were added to dissolve the RNA. The tubes were shaken by hand for about twenty times and incubated at room temperature for 7 min. Then they were centrifuged in an Eppendorf centrifuge at 12,000g for 15 min at 4°C to divide the RNA into the phases. The lower red phase contains Trifast and Chloroform, the cloudy interphase contains the genomic DNA and proteins, while the upper phase contains the wanted RNA. This upper RNA phase was carefully pipetted into fresh tubes each containing 500µl Isopropanol. Again the tubes were shaken by hand for about twenty times and put on ice for 10 min. After another round of centrifugation at 12,000g for 10 min at 4°C, a pellet of RNA should be seen at the bottom of the tube. The Isopropanol was carefully pipetted and discarded and the pellet was then washed twice with 1ml Ethanol and after each time

centrifuged at 12,000g for 10 min at 4°C. After that the supernatant Ethanol was removed entirely and the open tubes were put on the Thermomixer comfort at 55°C for a few minutes while constantly controlling if the pellet has dried.

Subsequently the pellet was dissolved in RNase/DNase free water and depending on the size of the pellet between 20 and 80µl were added. The aim was to attain a concentration of 1µl/ml. Therefore it was better to add a little less than acquired so it was still dilutable afterwards. For a consistent distribution the water and the RNA were pipetted thoroughly. In order to dissolve all of the RNA the tubes were put on the Thermomixer at 1,400rpm for 10 min at 55°C. After these steps the RNA needed to be kept on ice constantly or stored at -80°C.

For determining the concentration of the RNA solution the Nano Drop Spectrophotometer was used and measured against 1µl of RNase/DNase free water as the blank value. Thereupon the RNA quantification was controlled via gel electrophoresis.

3.4 Agarose gel electrophoresis

The following list of chemicals and appliances is in line with Klameth (2013).

- 1x TAE buffer
- peqGold Universal Agarose (PEQLAB Biotechnologie GMBH, Erlangen, GER)
- Amersham Pharmacia Biotech Electrophoresis EPS 301 Power Supply (Analytical Instruments, LLC, Golden Valley, MN)
- 6x loading dye (Fermentas GmbH, St. Leon-Rot, GER)
- Ethidium bromide (Merck, Darmstadt, GER)
- Horizontal submarine agarose gel unit HE 133 (Hoefer, Holliston, MA)
- E.A.S.Y Win 32 Transilluminator (Herolab, Wiesloch, GER)

In order to part the RNA fragments by size, from 100bp to 25kbp, the gel electrophoresis was performed using a 1.5% Agarose gel, to check the RNA isolation.

The gel was prepared by mixing 450mg Agarose and 30ml TAE buffer, heated in a microwave at highest level until it started to boil and the Agarose

was dissolved. Then it was cooled until lukewarm, 1.5µl ethidium bromide were added and mixed well. The solution was then poured into the electrophoresis chamber and immediately the combs were put in to create the slots for the samples. The gel was then kept at room temperature for 10 min and in the refrigerator for 15 min to harden.

The results from the Nano Drop were used to calculate the needed amount of RNA and RNase/DNase free water, so that there was a total of 10µl with a concentration of 500ng RNA for each sample. Furthermore, 2µl of loading dye were added to each sample and mixed gently.

The gel was put into the submarine unit, which was filled with 1x TAE buffer and 10µl of the samples were pipetted into the slots. Then the lid was closed and the electrophoresis ran at 100 Volt, 400 mA for 25 min. Due to negatively loaded phosphate backbone of the RNA the electro-osmotic flow is towards the anode.

After the process the RNA was visualized through the intercalating ethidium bromide with the double stranded nucleotides in the Transilluminator via UV light.

3.5 Reverse transcription

The following list of chemicals and appliances is in line with Klameth (2013).

- Gibco distilled water, RNase/DNase free (Life Technologies, Carlsbad, CA)
- High Capacity cDNA Reverse Transcription Kit (Life Technologies, Carlsbad, CA)
- Biometra personal cycler (Gatt-Koller, Innsbruck, AT)
- PCR-Softstrips 0.2ml (Biozym Scientific GmbH, Oldendorf, GER)
- Safe-seal tips (Biozym Scientific GmbH, Oldendorf, GER)

A master-mix was required to perform the reverse transcription. Therefore 1µg of RNA was pipetted in each sample with a calculated amount of RNase/DNase free water, so there was a total of 10µl in the tubes. Then the master-mix was produced, with each sample containing:

2.0µl	10x reverse transcription buffer
2.0µl	10x random primer
0.8µl	25x dNTPs
1.0µl	reverse transcriptase
4.2µl	RNase/DNase free water

Thus, 10µl of this master-mix were added to the RNA and water mixture in each tube and mixed thoroughly. After that they were put on the personal cycler and underwent the following program: 10 min at 25°C to activate the enzymes, 120 min at 37°C for the reverse transcription, 5 min at 85°C to deactivate the enzymes. From there on the samples were kept at 4°C. Now the samples contained the transcribed cDNA, which was required to perform the quantitative real time polymerase chain reaction (qPCR). Therefore, the cDNA was diluted adding 80µl of RNase/DNase free water leading to a final concentration of 10ng/µl. The samples were now stored at -20°C.

3.6 Quantitative real time PCR (qPCR)

The following list of chemicals and appliances is in line with Klameth (2013).

- Gibco distilled water, RNase/DNase free (Life Technologies, Carlsbad, CA)
- 2x TaqMan Gene Expression Master Mix (Life Technologies, Carlsbad, CA)
- TaqMan primer/probes (all purchased from Life Technologies, Carlsbad, CA):
 - 13363
 - ES2
 - COV318
 - OV56
 - PE01
- MicroAmp Fast Optical 96-well Reaction Plate with Barcode (0.1ml) (Life Technologies, Carlsbad, CA)
- MicroAmp Clear Adhesive Film (Life Technologies, Carlsbad, CA)

- 7900HT-Fast Real Time PCR System with SDS 2.4 Software (Life Technologies, Carlsbad, CA)
- geNorm Reference (house keeping) gene selection kit (PrimerDesign, Southampton, UK)
- Safe-seal tips (Biozym Scientific GmbH, Oldendorf, GER)
- Microsoft Excel 2003 (Microsoft Corporation, Redmond, USA)
- GraphPad Prism 5 (Graphpad, La Jolla, CA)

The qPCR is a method to amplify a specific DNA of a gene, which is quantified via detection of the fluorescence, added to the sample.

Therefore, a 96-well plate was used and filled with 2µl of cDNA and another master-mix was produced with each sample containing:

7.0µl	RNase/DNase free water
1.0µl	Primer
10.0µl	2x TaqMan

Summed up, a total of 18µl were added to the cDNA in each well. After that, an adhesive film was applied on the 96-well plate and it was centrifuged at 200g for 1min. Then the plate was transferred to the Real Time PCR system machine, which ran a program at 50°C for 2 min, 95°C for 10 min, 95°C for 15 sec and 60°C for 1 min. The last two steps were repeated forty times.

The resulting cycle threshold (C_T) values given by the Real Time PCR software SDS were transferred into Excel in order for the fold changes to be calculated.

The PCR is based on the method of specifically varying the temperature, so that the DNA is first denaturated, then the primer anneals and the elongation takes place. After each cycle the DNA is amplified. To quantify the amplification TaqMan was used in this case, which works via the fluorescence resonance energy transfer (FRET). Therefore, TaqMan is added to the master-mix consisting of a specific nucleic acid sequence, complementary to the sample DNA, with a reporter on one side and a quencher on the other. During the annealing phase TaqMan binds to the sample DNA and the Taq polymerase, that also has a 5'→3' exonuclease activity, splits the TaqMan. As a

consequence the reporter is separated from the quencher and its fluorescence signal can be detected constantly during the cycles of the PCR. Thus, the amplification of the DNA can be quantified via the fluorescence signal.

In this case we used TaqMan with the reporter FAM, 6-carboxyfluorescein, and the quencher TAMRA, 6-carboxy-tetramethyl-rhodamine.

3.7 Protein isolation

The following list of chemicals and appliances is in line with Klameth (2013).

- Calcium and magnesium free 1x PBS
- 25cm² cell flask (Nunc, Roskilde, DK)
- Cell scrapers (TPP, Trasadingen, CH)
- Radio-Immunoprecipitation Assay (RIPA) buffer
- Protease inhibitor for use with mammalian cell and tissue extracts (Sigma-Aldrich, St. Louis, MO, USA)
- Insulin syringe 30G needle (Insumed, Artsana, Grandate, IT)
- Thermomixer comfort (Eppendorf AG, Hamburg, GER)
- Centrifuge 5415R (Eppendorf AG, Hamburg, GER)
- Reaction tubes 1.5ml (Biozym Scientific GmbH, Oldendorf, GER)
- 15ml centrifugation tubes (TPP, Trasadingen, CH)

Cells were grown in a 25cm² cell culture flask or a 6-well plate until a confluency of 90-95% was achieved. In order to collect the cells the medium was removed and the adherent cells were washed twice with cold 1x PBS and lysed with a varying amount of 100-250µl RIPA buffer depending on the confluency in the reservoir. Additionally, 1µl of protease inhibitor had to be added for each 100µl of buffer in order to prevent proteolysis. The content was transferred into reaction tubes and mechanically homogenized insulin syringes.

The samples were then incubated in the Thermomixer at 1,400rpm for 30 min at 4°C and afterwards centrifuged at 10,000g for 10 min at 4°C. The supernatant phase was pipetted into new tubes and stored at -20°C from then on.

3.8 Bicinchoninic acid (BCA) assay for protein quantification

The following list of chemicals and appliances is in line with Klameth (2013).

- Reaction tubes 1.5ml (Biozym Scientific GmbH, Oldendorf, GER)
- 15ml centrifugation tubes (TPP, Trasadingen, CH)
- 96-well plate (Nunc, Roskilde, DK)
- BCA Protein Assay Reagent A (Thermo Fisher Scientific, Asheville, US)
- BCA Protein Assay Reagent B (Thermo Fisher Scientific, Asheville, US)
- Biozym Ritips professional 10ml (Biozym Scientific GmbH, Oldendorf, GER)
- Infinite M200 Pro (Tecan Group Ltd. Männedorf, CH)
- BSA – Albumin (PAN Biotech, Aidenbach, GER)
- Heraeus cytoperm 2 (Thermo Fisher Scientific, Asheville, US)

For the BCA assay a serial dilution from 3.0µg/µl to 0.05µg/µl was pipetted in duplicate into the 96-well plate for the standard curve. The solution of the proteins was diluted 1:5 with ddH₂O and 25µl of this dilution were applied in duplicate onto the 96-well plate. Following the BCA reagents A and B were mixed 50:1 from which 200µl were added into each single well.

The plate was incubated at 37°C for 30 min and then the absorbance was measured in the Infinite M200 Pro at a wavelength of 562nm.

The BCA reagents are reduced from Cu²⁺ ions to Cu⁺ by the proteins, and the Cu⁺ forms a dye with the BCA, while the amount is equivalent to the amount of proteins in the sample. Thus the absorbed wavelength at 562nm measures the quantity of proteins in the sample.

3.9 Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Blot

The following list of chemicals and appliances is in line with Klameth (2013).

- 1x Laemmli buffer
- Calcium and magnesium free 1x PBS
- 4x sample buffer

- Mini-Protean TGX Gels, 4-15% (Bio-Rad Laboratories Inc., California, US)
- Mini Protean Tetra Cell (Bio-Rad Laboratories Inc., California, US)
- Trans-Blot Turbo Transfer Pack Mini format, 0.2µm PVDF (Bio-Rad Laboratories Inc., California, US)
- Trans-Blot Turbo Transfer System (Bio-Rad Laboratories Inc., California, US)
- Thermomixer comfort (Eppendorf AG, Hamburg, GER)
- Skim milk powder (Maresi, Vienna, AT)
- Tween 20 for electrophoresis, (Sigma-Aldrich, St. Louis, MO, USA)
- PageRuler Prestained Protein Ladder (Fermentas GmbH, St. Leon-Rot, GER)
- Rabbit anti carbonic anhydrase IX antibody 1:2000 (Novus Biologicals, Littelton, US)
- Rabbit anti GAPDH polyclonal antibody 1:3000 (Proteintech, Chicago, US)
- Goat anti rabbit IgG-HRP 1:5000 (Santa Cruz Biotechnology, Santa Cruz, US)
- Roto-Shake Genie (Scientific Industries, Bohemia, NY)
- Immun-Star Western C Kit (Bio-Rad Laboratories Inc., California, US)
- VersaDoc 4000MP Imaging System (Bio-Rad Laboratories Inc., California, US)
- Software Imagelab and Quantity One (Bio-Rad Laboratories Inc., California, US)

First the SDS-PAGE was performed and therefore the samples had to be prepared by pipetting 15µg of protein into the tube; an amount calculated from the results of the BCA assay. This was added up to 18.15µl with ddH₂O. Also 4.84µl of 4x sample buffer and 1.21µl of β-mercaptoethanol were pipetted into each tube, which resulted in a total of 22µl in each tube. After that the samples were mixed thoroughly and incubated in the Thermomixer at 70°C for 8 min.

Following, the gel electrophoresis was set up and 22µl of each sample were applied in the gel pockets as well as the marker; PageRuler in these cases. Then the gel ran at constant 350V for approximately 10 min. Afterwards the gel was removed, carefully washed with ddH₂O and stacked in the following way from the top to the bottom: cassette electrode (cathode), ion reservoir stack, gel, blotting membrane, ion reservoir stack, and cassette electrode (anode). The transfer was made in the Trans-Blot Turbo Transfer system at 1.3A and 25V for 7 min, where the proteins migrate from the gel onto the membrane, which is out of polyvinyliden-fluorid. After that the membrane was put immediately into a mixture of PBS-0.05% Tween-20 (PBST) and 3% non-fat instant milk, the blocking solution, for 30 min and shaken on the Roto-Shake Genie at room temperature. During this process all unspecific binding sites were connected to the membrane. After the 30 min the medium was discarded.

The first antibody was diluted in its specific ratio (e.g. 1:4000) in a mixture of 1:1 of blocking solution and PBST and put onto the membrane in a small plastic dish for 60 min on the Rotor Shake Genie. Subsequently the membrane was washed with PBST three times in a row for 5 min. Then the second antibody was diluted 1:8000 in a 1:1 mixture of blocking solution and PBST. This second antibody was conjugated with an anti-rabbit horseradish peroxidase (HRP) IgG. These steps were performed for the specific detection of the proteins. After the second antibody the membrane was washed again with PBST twice and the third time only with 1x PBS for 5 min each time.

In order to achieve a chemiluminescence signal the Peroxide Solution and the Enhancer Solution from the Immun-Star WesternC Kit were mixed 1:1 and diluted with ddH₂O 1:5 and added to the membrane and incubated for 2 min. So the substrate was then converted by the HRP and the intensity of the bands could be detected with the VersaDoc Imaging System and its Imagelab and Quantity One Software.

To verify the results, housekeeping genes had to be detected and in this case glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used, which had a size of 37.5kDa and could be differentiated from the bands of CAIX with 50-55kDa.

3.10 Chemosensitivity Assay

The following list of chemicals and appliances is in line with Klameth (2013).

- 96-well microtiter plate (Greiner, Kremsmuenster, Austria)
- Carboplatin and cisplatin (Sigma-Aldrich, St. Louis, MO, USA)
- Heraeus cytoperm 2 (Thermo Fisher Scientific, Asheville, US)
- CAIX inhibitor C207A (obtained from Prof. Claudiu T. Supuran, Firenze, ITA)
- EZ4U (Biomedica, Vienna, Austria)
 - Substrate - lyophilised
 - Activator solution - ready for use
- Infinite M200 Pro (Tecan Group Ltd. Männedorf, CH)
- Safe-seal tips (Biozym Scientific GmbH, Oldendorf, GER)

In order to evaluate the response of tumor cells to chemotherapy, the chemosensitivity assay can be applied. Therefore, 1×10^4 cells were sowed in 100 μ l of medium in each well of the 96-well plate. Then, carboplatin was dissolved in physiologic saline and added in additional 100 μ l of medium. This was added to the first row of the plate, where the cells were applied in triplicates, and the medium was mixed thoroughly. Next, 100 μ l of the resultant medium were pipetted into the following row, mixed again and 100 μ l of the mixture transferred into the next row and so forth. This emerges in a bisecting dilution in each row, starting with 10 μ g/ml in the first row. Also the inhibitor was added in a concentration of 50.0 μ M. The samples were then incubated for four days at 37°C and 5% CO₂.

After that the substrate, tetrazolium, of one vial was dissolved in 2.5ml of activator solution. 20 μ l of the solution were added to each well and the plate was incubated again for four days at 37°C and 5% CO₂. During this step the mitochondria of the living cells reduced the substrate, which led to a coloration through formazan derivates. In opposite to that, Mitochondria of dead cells were inactivated and could not transform any of the substrate.

Subsequently the absorbance was measured at 450nm, so that the percentage of the surviving cells after treatment could be determined by using

an empty well as blank and a well with non-treated cells as 100% of proliferation.

3.11 Enzyme Linked Immunosorbent Assay (ELISA)

The following list of chemicals and appliances is in line with Klameth (2013).

- Human Carbonic Anhydrase IX Quantikine ELISA Kit (R&D Systems, Inc., Minneapolis, MN, USA)
- Safe-seal tips (Biozym Scientific GmbH, Oldendorf, GER)
- Pipettes 10ml and 25ml (Biozym Scientific GmbH, Oldendorf, GER)
- 15ml centrifugation tubes (TPP, Trasadingen, CH)
- Hettich Rotante centrifuge (Hettich Bäch, CH)
- Microplate shaker (Scientific Industries, Bohemia, New York, USA)
- Infinite M200 Pro (Tecan Group Ltd. Männedorf, CH)

In this case the sandwich ELISA was performed to quantify the amount of the protein in the samples and therefore, the wells of the 96-well plate are pre-coated with a monoclonal antibody whereon the 50µl of Assay Diluent and 100µl of the sample fluid were added. The plate was incubated for 120 min at room temperature on a shaker. During this step all present CAIX bound to the antibody. Then the fluid was discarded and the plate was washed with Wash Buffer four times to remove any unbound substances. Then 200µl of CAIX Conjugate were added, a polyclonal antibody against CAIX and conjugated to HRP, which bound to the antigen; this was incubated for 120 min at room temperature, also on a shaker. This was washed again for four times and 200µl of Substrate Solution were pipetted into each well and incubated for 30 min in the dark. This solution consisted out of two mixed reagents, A and B, and stabilized the hydrogen peroxide and the chromogen, which is responsible for the development of the blue color in the samples, that is proportional to the amount of CAIX bound in the first step. In the end 50µl of sulfuric acid were added to each well to stop the reaction and the color turned from blue to yellow. The absorbance could then be measured at a wavelength of 450nm.

There was also a serial dilution added to the plate, so a standard curve could be compiled and the concentrations of soluble CAIX calculated.

4 Results

4.1 Gene expression of CA9 and Western Blot analysis

All qPCR experiments were run in duplicate. All given values represent the mean of both experimental runs.

4.1.1 Gene expression of all cell lines selected

Figure 1 shows the absolute Ct of the CA9 mRNA expression, for the protein CAIX, for all control groups of the cell lines that were investigated.

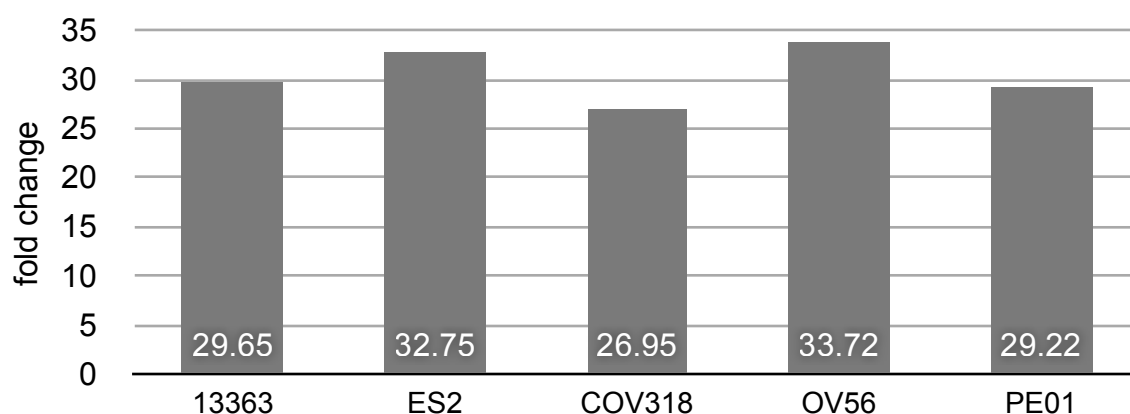


Figure 1: *qPCR analysis of CA9 in all cell lines (13363, ES2, COV318, OV56, PE01)*

The following figures show the treatment effect of cisplatin on the different cell lines.

4.1.2 Cisplatin in concentration of 0.625µg/ml and its influence on CA9

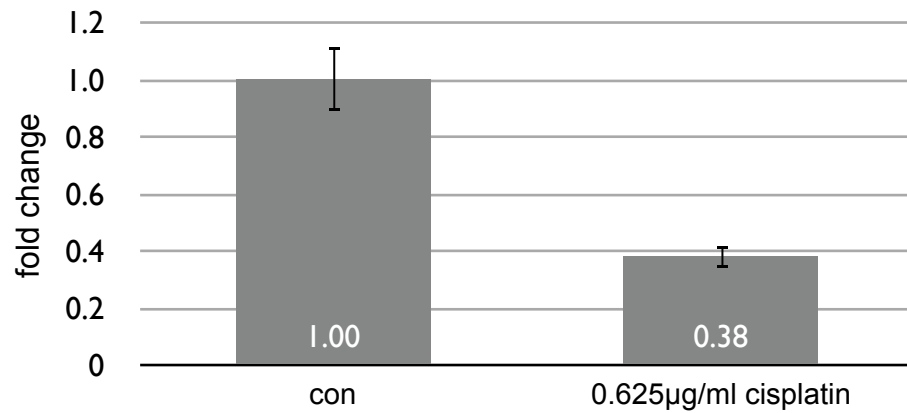


Figure 2: *qPCR analysis of cell line 13363 treated with cisplatin*

Con shows the CA9 mRNA expression of the untreated control group in this figure, as in the following qPCR figures of this section.

When the cells were treated with 0.625µl/ml cisplatin the expression was decreased to almost one third of the control group.

4.1.3 Cisplatin in concentration of 0.5µg/ml and 1.0µg/ml and its influence on CA9

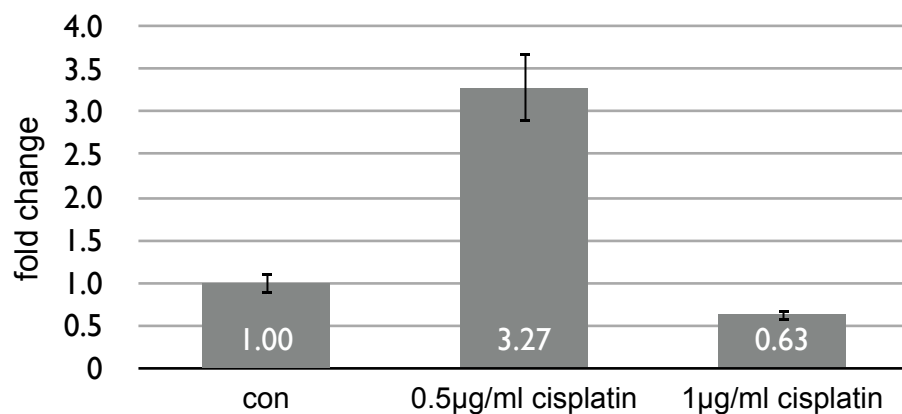


Figure 3: *qPCR analysis of cell line ES2 treated with cisplatin*

When the cells were treated with 0.5µg/ml cisplatin an outlier appeared with an over threefold expression of CA9 mRNA.

When the cells were treated with 1.0µg/ml cisplatin the expression was decreased to about two third of the control group.

4.1.4 Cisplatin in concentration of 1.25µg/ml and its influence on CA9

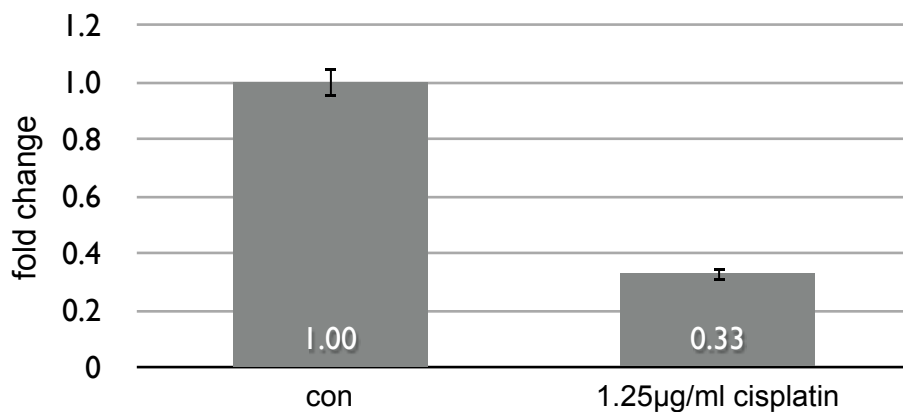


Figure 4: qPCR analysis of cell line COV318 treated with cisplatin

When the cells were treated with 1.25µg/ml cisplatin CA9 mRNA expression was decreased to one third of the control group.

4.1.5 Cisplatin in concentration of 0.6µg/ml and 1.25µg/ml and its influence on CA9

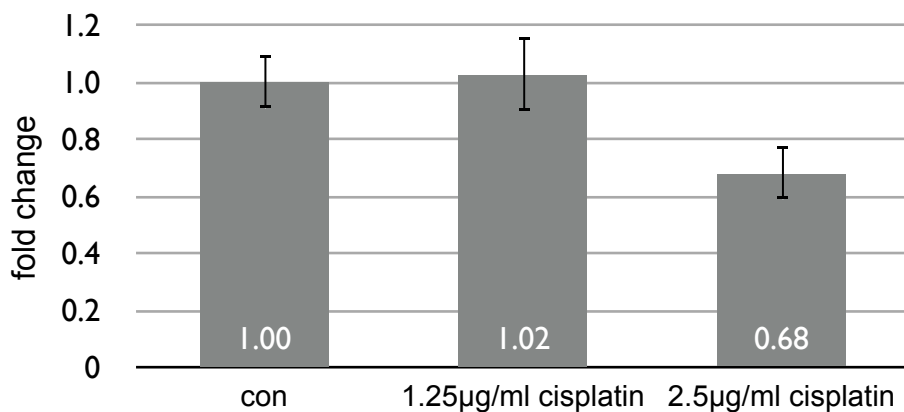


Figure 5: qPCR analysis of cell line OV56 treated with cisplatin

When the cells were treated with 0.6 μ g/ml cisplatin no effect on CA9 mRNA expression was observed.

When the cells were treated with 1.25 μ g/ml cisplatin the expression was decreased to two third of the control group.

4.1.6 Cisplatin in concentration of 1.25 μ g/ml and 2.5 μ g/ml and its influence on CA9

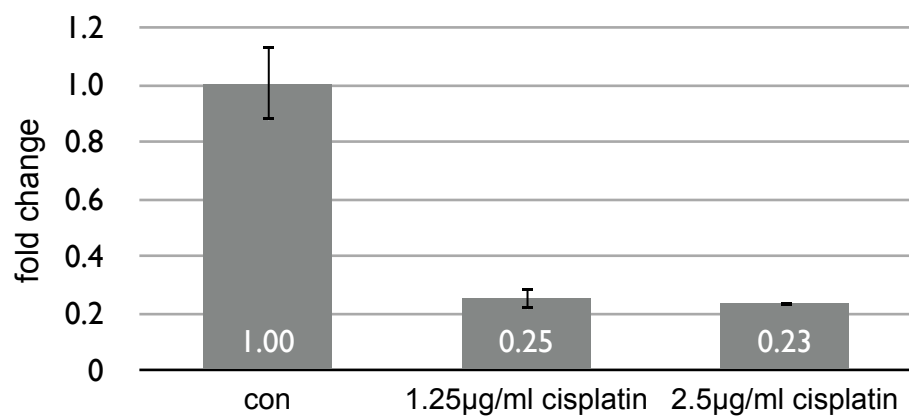


Figure 6: *qPCR analysis of cell line PE01 treated with cisplatin*

When the cells were treated with 1.25 μ g/ml cisplatin CA9 mRNA expression was decreased to one fourth of the control group.

When the cells were treated with 2.5 μ g/ml cisplatin CA9 mRNA expression was decreased to a little less than one fourth.

4.1.7 Western Blot of CAIX

In the following cell line Western Blots were done to detect the presence of CAIX. In this Western Blot and all of the following GAPDH was used as the housekeeping control.

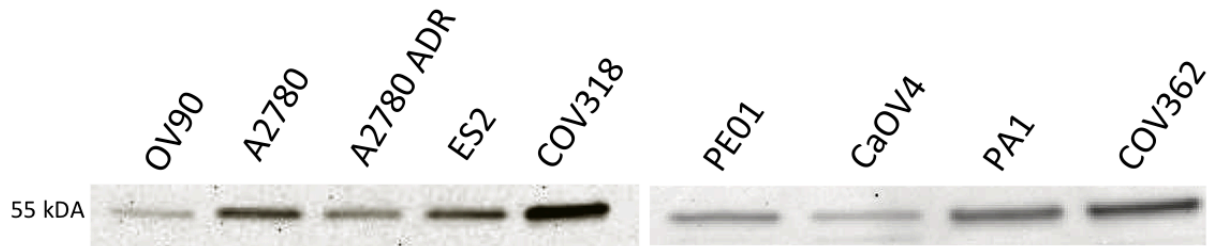


Figure 7: Western Blot of CAIX in various cell lines

This Western Blot shows a detection of CAIX in all cell lines. The strongest band can be seen at COV318. Also distinct bands can be seen at A2780, ES2, PA1, and COV362. Less obvious, but still very clear bands can be seen at OV90, PE01, and CaOV4.

4.1.8 Western Blot of DFX treated CAIX in OV90, CaOV4 and PA1

After treating the cells with two different concentrations of DFX, 50 μ M, and 100 μ M, Western Blots were done to show the effect of the simulated hypoxia onto the different cell lines.

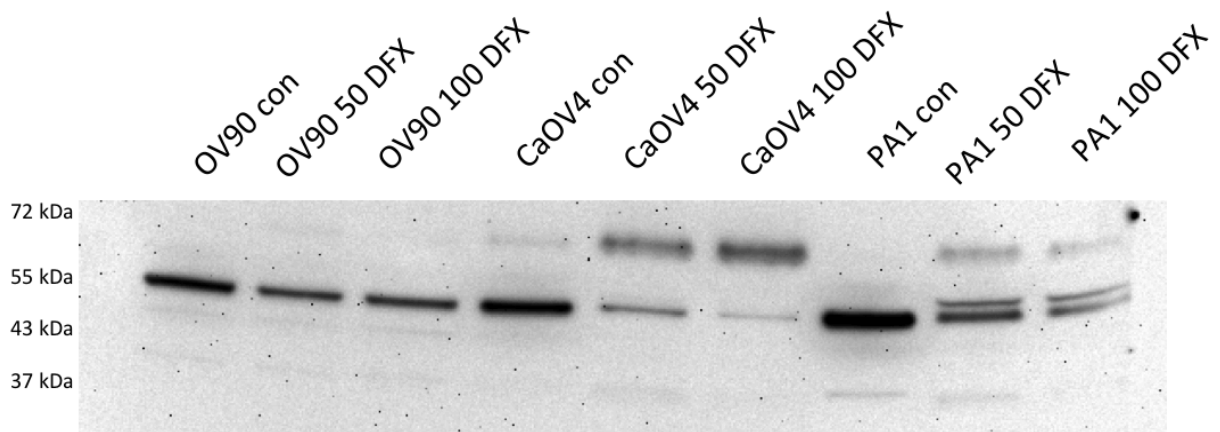


Figure 8: Western Blot of CAIX in DFX treated OV90, CaOV4 and PA1

Observing cell line OV90, there is a decreased quantity of CAIX in the DFX treated groups compared to the control group.

In cell line CaOV4 an obvious decreased quantity of protein levels compared to the control group can be seen, but another visible band at 72 kDa, which is more visible at the treated groups.

In cell line PA1 also a decreased quantity of protein can be seen and the band is split into a double-band in the treated groups and additionally light bands at 72 kDa, only visible in the treated groups.

4.1.9 Western Blot of DFX treated CAIX in ES2, A2780 and A2780 ADR

These cells were treated the same way, with two different concentrations of DFX, 50 μ M, and 100 μ M. Western Blots were done to determine the quantity of CAIX proteins.

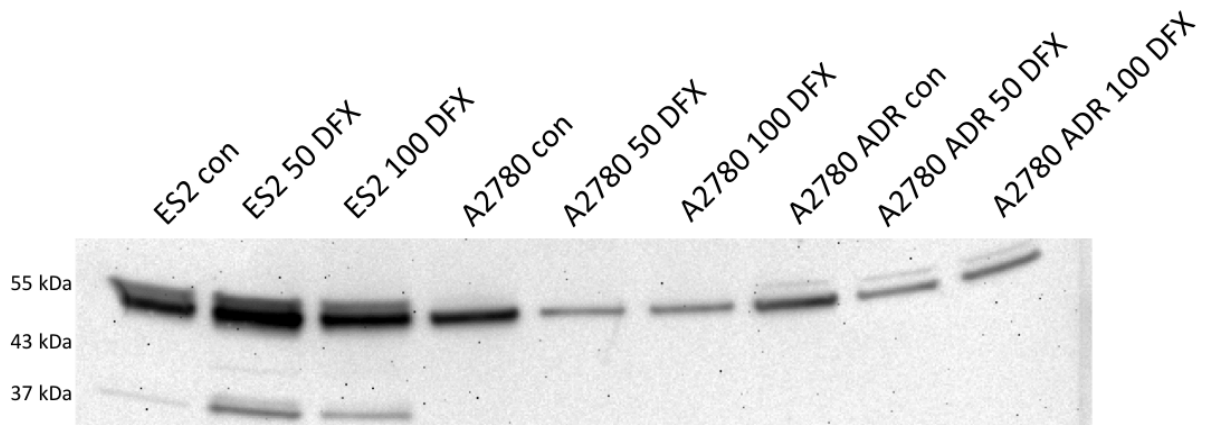


Figure 9: *Western Blot of CAIX in DFX treated ES2, A2780, A2780 ADR*

In cell line ES2 an obvious increase of CAIX can be seen in the DFX treated groups, and another additional signal underneath the CAIX band, which is more intense at the treated groups.

In cell line A2780 there is a decreased quantity of CAIX in the DFX treated groups.

In cell line A2780 ADR is another decrease in the protein level, but an additional very light band directly above the CAIX band can be observed.

4.2 Chemosensitivity assay

For this chemosensitivity assay 1×10^4 cells of each cell line were treated with $10 \mu\text{g/ml}$ Carboplatin, reducing the concentration nine times by half, and additionally with a constant concentration of $50 \mu\text{M}$ of the CAIX inhibitor C207A. The cells were kept at 37° and $5\% \text{CO}_2$ for four days, and analyzed with the EZ4U kit to determine the quantity of surviving cells.

4.2.1 MTT-Assay of OV90

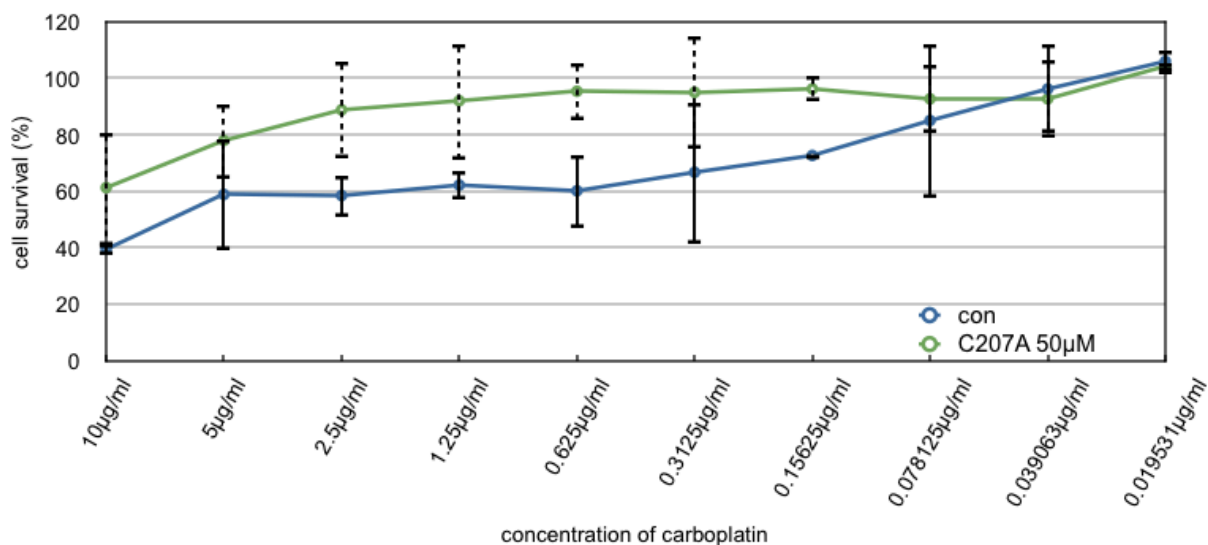


Figure 10: MTT-Assay of OV90 treated with carboplatin and C207A

The control group cells were treated a total of ten times with carboplatin. The first treatment started with a carboplatin concentration of $10 \mu\text{g/ml}$. For the subsequent treatment the carboplatin concentration was reduced by 50%, leading to a concentration of $0.01952 \mu\text{g/ml}$ in the tenth treatment. The C207A group was treated the same way, however in addition they were also treated with a constant concentration of $50 \mu\text{M}$ C207A, the CAIX inhibitor, in every round. This procedure was repeated for all cell lines, which are listed below.

For the cell line OV90 it can be seen that approximately half of the cells are still vivid when treated with $10 \mu\text{g/ml}$ carboplatin. Comparing the control group with the C207A treated group, it is obvious that the CAIX inhibitor decreases the efficacy of carboplatin until a very low concentration $0.039063 \mu\text{g/ml}$. At this

point the survival rates meet at around 95%, so the influence of the CAIX inhibitor is rescinded.

4.2.2 MTT-Assay of CaOV4

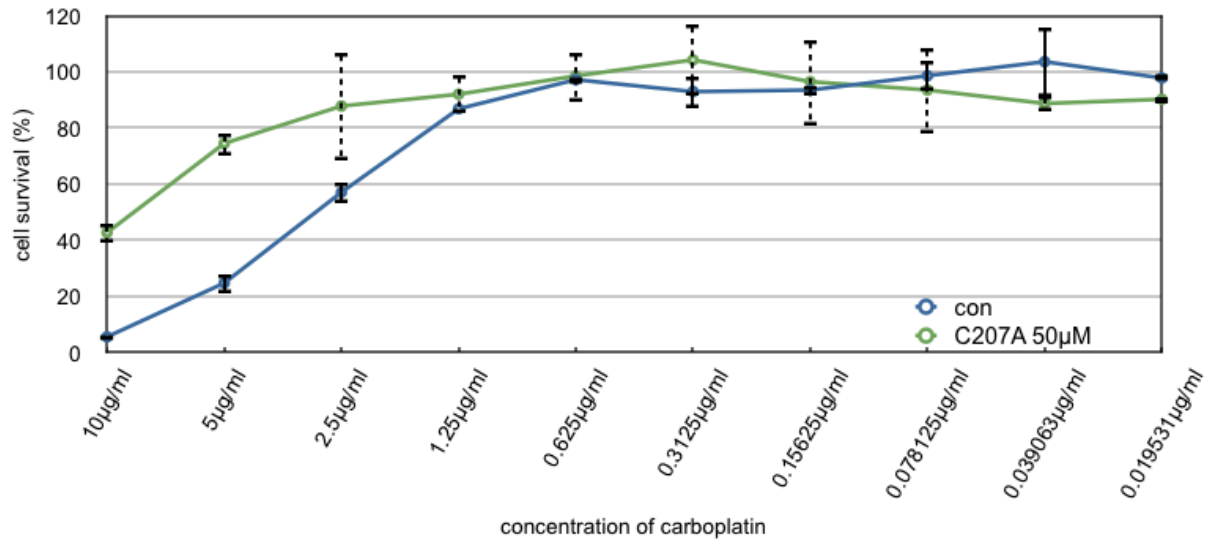


Figure 11: MTT-Assay of CaOV4 treated with carboplatin and C207A

The cell survival rate of the control group starts at 5% and of the C207A group at around 40%, which also shows a decrease in the efficacy of carboplatin, when treated with the CAIX inhibitor. At a concentration 1.25 µg/ml carboplatin, both conditions have a cell survival rate of around 90% and progress around this level, also for the lower concentrations.

4.2.3 MTT-Assay of PA1

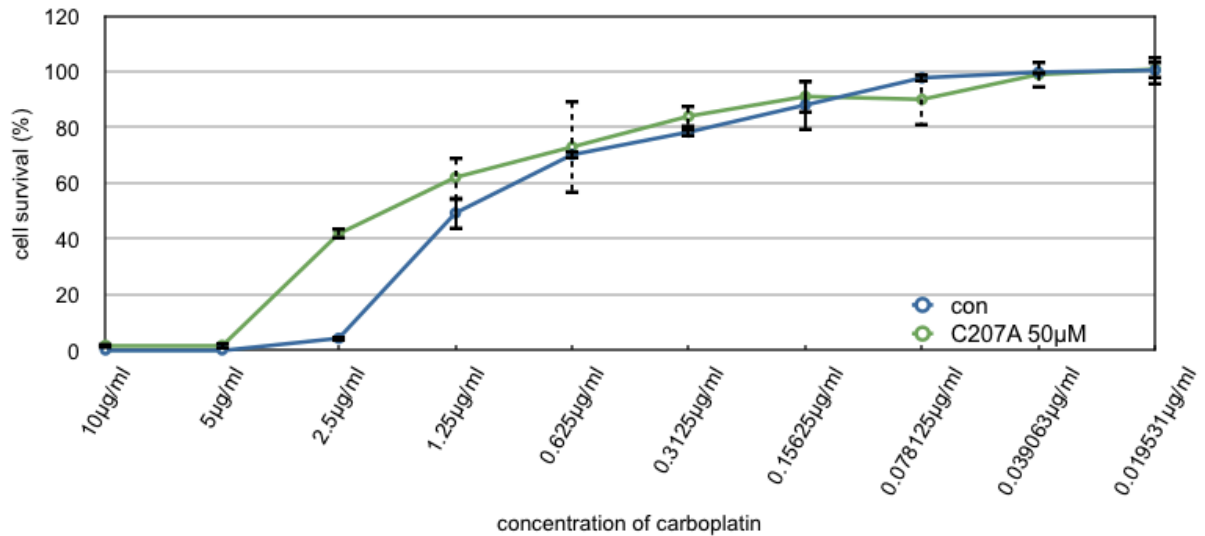


Figure 12: MTT-Assay of PA1 treated with carboplatin and C207A

Until a concentration of 5µg/ml carboplatin both conditions of the PA1 cell line do not have any surviving cells. In the control group even the 2.5µg/ml concentration of carboplatin barely has any surviving cells. The efficacy of carboplatin is decreased by the CAIX inhibitor again. But at a concentration of 0.625µg/ml carboplatin both treatment groups have a matching survival rate of around 70%; they also show a similar progress for the rest of the treatment.

4.2.4 MTT-Assay of ES2

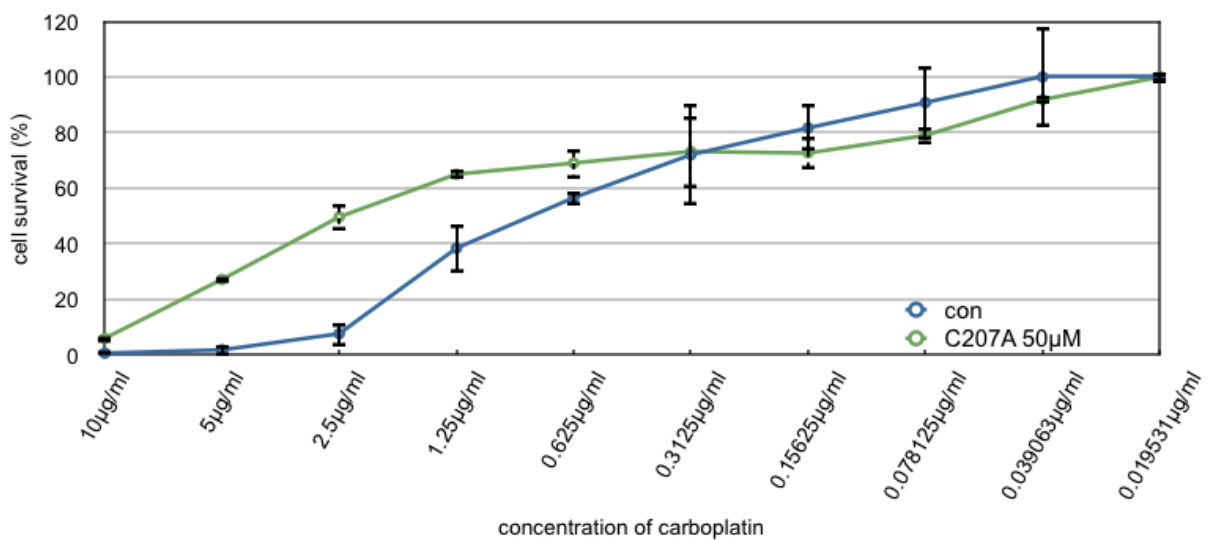


Figure 13: MTT-Assay of ES2 treated with carboplatin and C207A

For the cell line ES2 the starting survival rate is close to zero in both treatment groups. Until a concentration of 0.3125µg/ml carboplatin, its efficacy is decreased when the CAIX inhibitor is added. But for the lower concentrations the efficacy is inverted and the double treated group shows a lower survival rate.

4.2.5 MTT-Assay of A2780

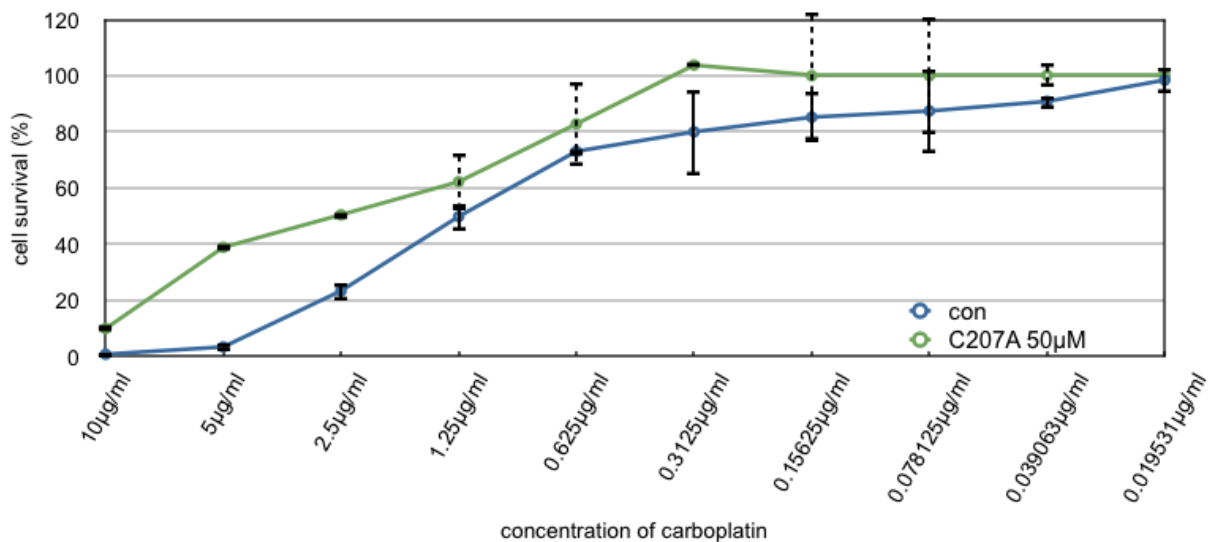


Figure 14: MTT-Assay of A2780 treated with carboplatin and C207A

The A2780 cells have no sign of vitality in the control group at the beginning of the treatment with 10µg/ml carboplatin. At a concentration of 0.625µg/ml carboplatin around 70% of the control group cells survive and reach almost 100% of cell survival at the last treatment. In this case, the C207A treated group shows a constant decrease in the efficacy of carboplatin, since more cells survive the treatment.

4.2.6 MTT-Assay of COV318

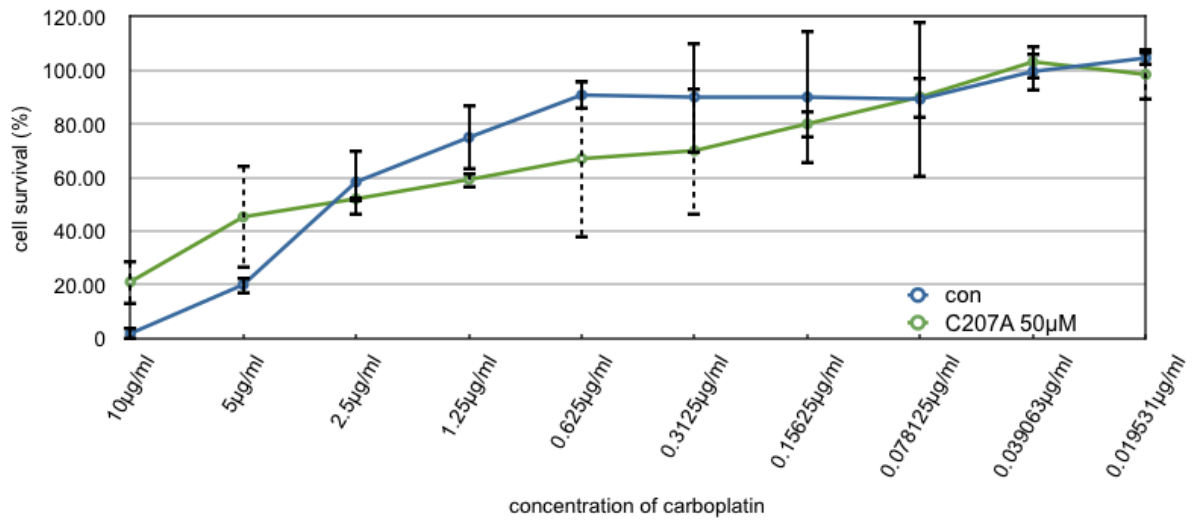


Figure 15: MTT-Assay of COV318 treated with carboplatin and C207A

The COV318 cell line also has a decrease in the efficacy of carboplatin at the beginning of the treatment with the highest dose, 10µg/ml carboplatin. At a concentration of 2.5µg/ml carboplatin and the following concentrations, the C207A treated group shows more vivid cells than the group only treated with carboplatin. Both groups reach the same level of 90% cell survival at 0.078125µg/ml carboplatin.

4.2.7 MTT-Assay of PE01

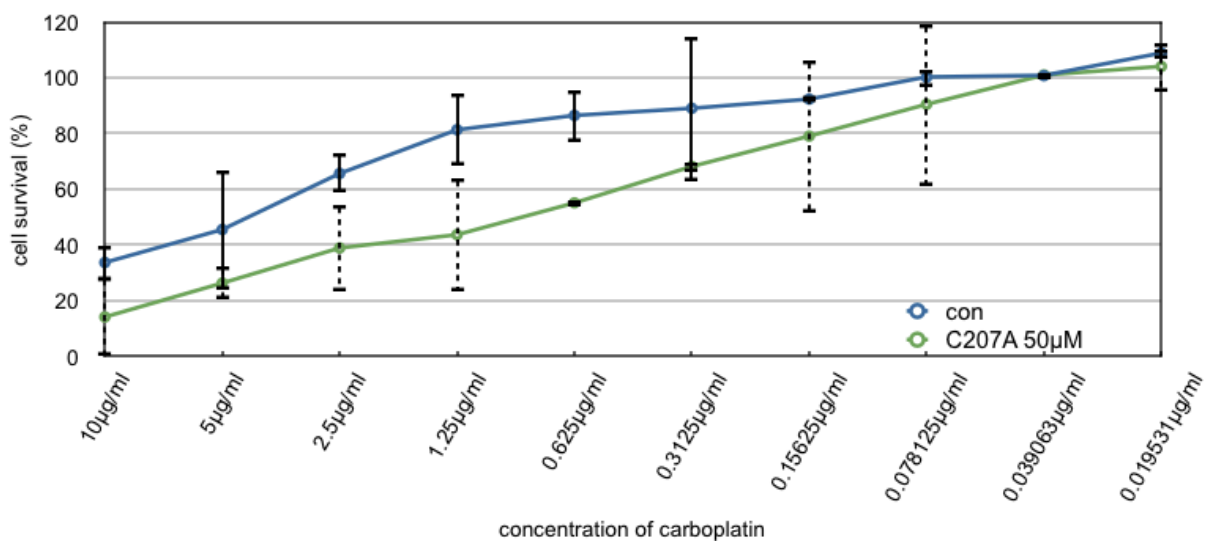


Figure 16: MTT-Assay of PE01 treated with carboplatin and C207A

In the PE01 cell line the converse effect can be seen. Almost throughout the whole progress of the treatment the control group has an obvious higher cell survival than the C207A treated group. When the concentration of 0.039µg/ml carboplatin is reached both groups have 100% surviving cells. This means that the efficacy of Carboplatin is increased when the CAIX inhibitor is added.

4.3 ELISA of Soluble CAIX in supernatant medium

The cell lines were centrifuged and the supernatant medium separated and sCAIX checked via ELISA. Here additionally, the DFX-treated (50µg and 100µg) cells were used.

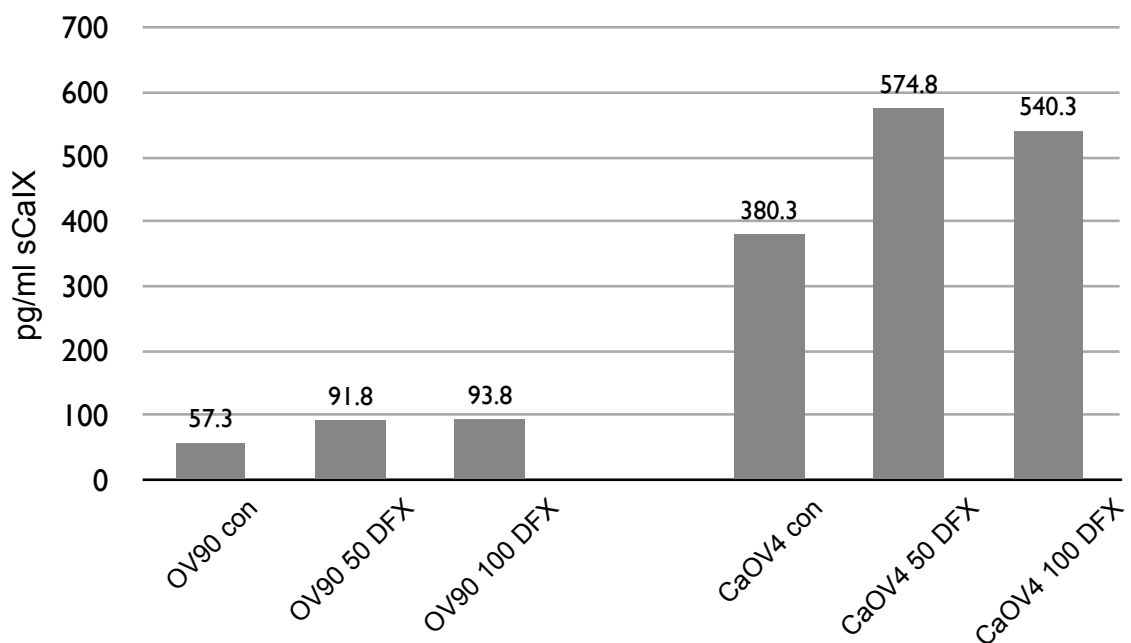


Figure 17: sCAIX ELISA of the supernatant of DFX treated cell lines OV90 and CaOV4

In cell line OV90 the concentration of sCAIX is increased by approximately 50% in both DFX treated groups, but overall rather low.

In cell line CaOV4 the initial concentration is 380.3pg/ml, whereas the 50µg DFX treated cell line also has a 50% increase and the higher treated cell line marginally less.

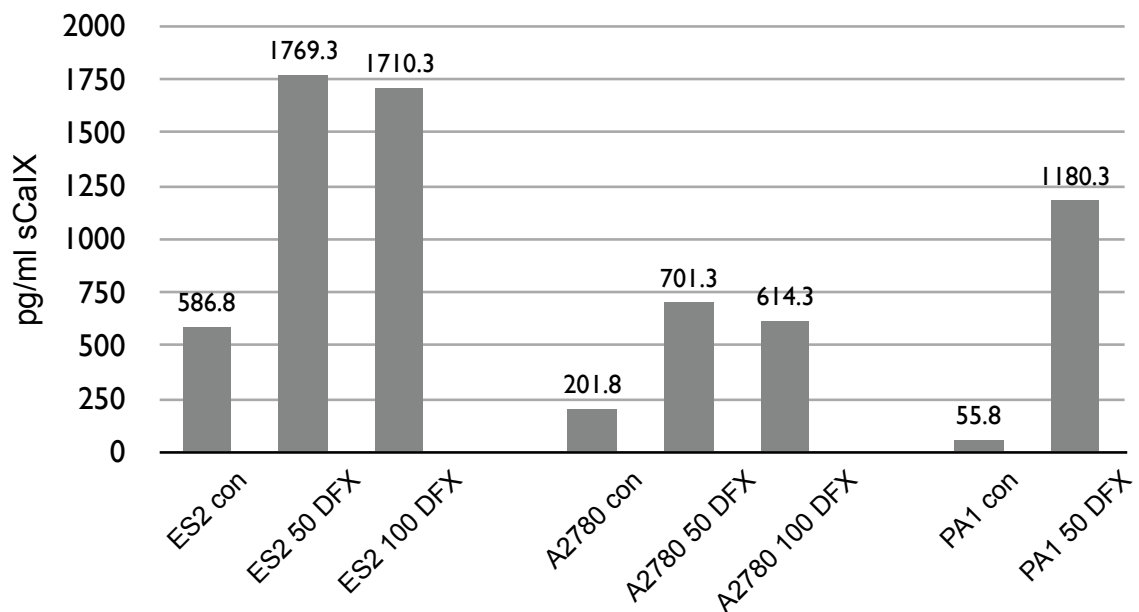


Figure 18: sCAIX ELISA of the supernatant of DFX treated cell lines ES2, A2780 and PA1

Cell line ES2 has the highest initial concentration and also a very high increase with almost a threefold increase in sCAIX concentration in the treated groups with only a marginal difference between 50µg and 100µg of DFX.

In cell line A2780 there is a medium initial concentration with a three and half times increase of concentration in the 50µg DFX treated group. The group treated with a higher concentration results in a little lower protein concentration.

Cell line PA1 starts with an extremely low concentration, whereas the 50µg treated group has an over 20-fold increase of protein concentration.

5 Discussion

The present study investigated the occurrence of the protein CAIX in ovarian cancer cell lines, the expression through its gene CA9, and the effects of certain treatments on CAIX expression.

The metalloenzyme CAIX is expressed under normal conditions in the stomach and gallbladder only, but also plays an important role in pH regulation of tumor cells such as in ovarian cancer, usually in their more aggressive kinds. The protein CAIX is bound to the extracellular membrane and converts carbon dioxide into protons and bicarbonate, whereby the protons provide the acidic conditions, which lead to a higher migration rate and invasiveness of the tumor. The protein is encoded in the gene CA9, which is induced by the transcription factor HIF-1, activated under hypoxic conditions (Greijer et al., 2005; Kim et al., 2012).

In this study first, the expression of the CA9 gene, which encodes for the corresponding protein was investigated in a few ovarian cancer cell lines via qPCR. All of them show a significant expression of the mRNA of the gene, also the cells from line 13363 (patient identification number), cultured from the ascites of an ovarian cancer patient.

Following the cell lines were each treated with an individual concentration of cisplatin, a common chemotherapeutic drug used in the treatment of ovarian cancer (Luvero, Milani, & Ledermann, 2014). It can be shown that the gene expression of CA9 is downregulated, with exception of ES2; these cells descend from a clear cell carcinoma, and not from high serous carcinoma like the other cell lines. This leads to the conjecture of a high constitutive expression of CAIX in ES2. All the other cells responded to the cisplatin treatment with a lower expression of the mRNA. This may be an additional advantage of cisplatin in the treatment of ovarian cancer.

Furthermore, Western Blots were performed to detect the CAIX protein, which is bound to the cell membrane, in the cell lines. All of them were positive and showed a clear visible band at 54 kDa. Then the cells were treated with different concentrations of DFX (50 μ M and 100 μ M), an iron chelator, which promotes the expression of HIF-1 and simulates hypoxia-like conditions in the

cells. Here, CAIX is decreased, except for cell line ES2; there the protein shows a higher expression. This controversial result will be discussed later.

The chemosensitivity was done treating the cells with carboplatin, another platinum based compound and often used in clinics to see the cell survival rate dependent on the different carboplatin concentrations. In addition to the control group, another cell arm was treated with carboplatin and C207A, a compound which is able to inhibit the active binding site of the CAIX protein and interacts with the pH regulation of the cells. The combination of carboplatin and C207A could show effects on the functionality of CAIX and the cell survival. In most cell lines the combination of both therapeutics leads to an antagonistic interaction. This means that the control group only treated with carboplatin has a lower survival rate. Consequently, this treatment is more effective because the cells should be eliminated. The additional treatment with C207A interacts with carboplatin and as a consequence a higher cell survival rate is the result. Exceptions are given for the cell lines PE01 and partially COV318; here the efficacy of both substances is higher than in the control group and leads to a lower cell survival rate.

This leads to the conclusion that a direct application of the combination of carboplatin and the CAIX inhibitor cannot be used as an effective treatment because of its mostly antagonistic effect on the cells. Though certain cells show a beneficial behavior, it would acquire a specific analysis to determine the characteristics of the cells and its suitability for this treatment.

Besides the membrane bound form of CAIX, a soluble form (sCAIX) can be detected – for instance – in the supernatant medium of the cell lines. Here the cells were treated with DFX, centrifuged and the supernatant medium was analyzed with ELISA to detect sCAIX. The results show that rising concentrations of DFX, which induces hypoxia like conditions lead to higher concentrations of sCAIX. The more hypoxic the condition in a cell is, the more CAIX proteins are shed and lead to rising concentrations of sCAIX. This explains why membrane bound CAIX is decreased under DFX treatment, which was mentioned earlier; the CAIX proteins are shed and converted into sCAIX.

As a conclusion it can be said that the gene CA9 and its mRNA, as well as the CAIX protein are significantly expressed in ovarian cancer cell lines. This

gives a new perspective in diagnosing and, maybe, treating ovarian cancer because it specifically occurs in these kind of neoplasms.

In summary, it can be stated that under hypoxia like conditions the membrane bound CAIX is decreased and sCAIX levels are increased. The treatment with cisplatin leads to a downregulation of CA9 mRNA. In combination the assumption is that lower CAIX levels are associated with higher CAIX shedding and antagonistic interaction because the combination of a CAIX inhibitor and carboplatin show antagonistic effects. An administration is impossible and cannot be seen as useful.

With respect to ovarian cancer treatment these results show a significant detection of CAIX under various conditions. This could lead to a development of using CAIX in its membrane bound form or soluble form as a (circulating) marker in processes of diagnosing or evaluating the progress of the treatment. This could be especially helpful since there is currently no reliable preventive checkup for ovarian cancer.

Points of criticism in this study could be that it would be helpful to differentiate between the various classes of ovarian cancer in detail. Also more cell lines could have been examined for making a clear statement.

Further studies have to be conducted to reinforce these results and to specify the use of CAIX as a marker for diagnosing ovarian cancer. It can also be researched what a high shedding of CAIX means; do high sCAIX levels affect the progress of healing or the development of metastasis?

In preliminary experiments we found that ascitic fluid from ovarian cancer patients, which is hypoxic, contains μg amounts of sCAIX, which in turn is expected to promote metastasis.

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German Abstract

Einleitung: Die Entstehung von Ovarialkarzinomen kann in den meisten Fällen nicht frühzeitig erkannt werden, weshalb Tumore oft erst im fortgeschrittenem Stadium diagnostiziert werden und zu einer äußerst ungünstigen Prognose führen. Außerdem verläuft die Erkrankung oft mit atypischen Symptomen oder sogar symptomlos, was die Diagnostik weiter erschwert. Daraus resultierte das Interesse neue Marker zu erforschen, die eine Entdeckung der Krankheit möglich machen. In diesem Zusammenhang wurde das Enzym Carboanhydrase IX genauer untersucht, welches an der pH Regulation von Tumorzellen beteiligt ist. Dieses sorgt für eine saure extrazelluläre Umgebung und ermöglicht so die Zellproliferation und Einwanderung in gesundes Gewebe.

Methoden: Die Expression des Gens CA9, sowie die Expression des CAIX Proteins wurde mittels qPCR in Ovarialkarzinom-Zelllinien festgestellt, welche mit Cisplatin behandelt wurden. Mittels Western Blot wurden in den Zelllinien die CAIX Protein Levels bestimmt, wobei die Zellen mit DFX behandelt wurden, was zu Hypoxie-ähnlichen Bedingungen in der Zelle führt. Außerdem wurden Chemsensitivitäts-Assays durchgeführt, um die Überlebensrate der Zellen zu bestimmen, nachdem diese mit Carboplatin und einem CAIX-Inhibitor behandelt wurden.

Die lösliche Form von CAIX (sCAIX) wurde mittels ELISA analysiert, wobei die Konzentration in Abhängigkeit von der Behandlung mit einer Substanz ermittelt wurde, welche Hypoxie-ähnliche Bedingungen erzeugt.

Ergebnisse: Unter den hypoxischen Bedingungen kam es zu einer Abnahme der membrangebundenen Form des CAIX Proteins, sowie zu einer Zunahme der sCAIX Proteine. Dies führt zu der Annahme, dass eine Veränderung der hypoxischen Bedingungen zu einer Freisetzung von CAIX, von der gebundenen in die lösliche Form, führt. Außerdem konnte ein antagonistischer Effekt bei der Behandlung mit Carboplatin und einem CAIX Inhibitor beobachtet werden.

Des Weiteren führte die Behandlung mit Cisplatin zu einer Down-Regulation der Genexpression von CA9. Außerdem kann von den Untersuchungen abgeleitet werden, dass eine bestimmte Form des Clear Cell Karzinoms eine sehr hohe konstitutive Expression von CAIX aufweist.

Schlussfolgerung: Die Expression der CA9 mRNA, sowie die CAIX Proteinexpression sind signifikant in Ovarialkarzinom-Zelllinien. Dies könnte neue Möglichkeiten darstellen, um CAIX als Identifikationsmarker von Ovarialkarzinomen zu verwenden, sowie um den Fortschritt einer Behandlung bewerten zu können.

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