universität wien

MASTERARBEIT

Titel der Masterarbeit

„Modulation of regulators of cellular pH in Small Cell Lung Cancer cells by chemotherapeutic drugs“

verfasst von

Lukas Klameth, BSc

angestrebter akademischer Grad

Master of Science (MSc)

Wien, 2013

Studienkennzahl lt. Studienblatt:	A 066834
Studienrichtung lt. Studienblatt:	Masterstudium Molekulare Biologie
Betreuerin / Betreuer:	Ao. Univ.-Prof. Dipl.-Ing. Dr. Natale-Erwin Ivessa

Danksagung

Mein aufrichtigster Dank gilt Assoc. Prof. Gerhard Hamilton, Ph.D., der es mir ermöglicht hat, diese Masterarbeit zu verfassen und mir immer mit Rat und Tat zur Seite stand.

Ein besonderer Dank geht an Ao. Univ. Prof. Dr. Theresia Thalhammer, für die fabelhafte und harmonische Zusammenarbeit im Labor und die gute Betreuung vom Anfang bis zum Ende der Arbeit.

Bei Ao. Univ.-Prof. Dipl.-Ing. Dr. Natale-Erwin Ivessa bedanke ich mich für das rasche und sorgfältige Begutachten der Arbeit, sowie seinen Anregungen und Vorschlägen zur Verbesserung dieser Arbeit.

All meine Kenntnisse der praktischen Arbeit im Labor habe ich von Dr. Martin Svoboda, dem ich für seine Mühe und Hilfe sehr dankbar bin.

Allen Kollegen und Kolleginnen die im Laufe der Zeit im Labor waren und mit denen ich zusammengearbeitet habe, möchte ich für das angenehme und produktive Klima danken.

Zuletzt möchte ich noch jenen Personen danken, ohne die das Ganze nie möglich gewesen wäre: meine Verlobte und meine Familie. Danke für all die Nerven, Mühe und Freude die ihr während dieser Zeit mit mir geteilt habt.

Abstract

Background: Small Cell Lung cancer (SCLC) is characterized by an early relapse with a poor prognosis in second-line therapy. Targeted therapy approaches failed so far. A new treatment attempt can be to target pH-regulating proteins like carbonic anhydrases IX (CA IX) and XII (CA XII), sodium proton exchanger 1 (NHE1) and monocarboxylate transporters 1 (MCT1) and 4 (MCT4), because tumor cells shift their energy metabolism under hypoxic conditions from oxidative phosphorylation towards an increased rate of anaerobic glycolysis. Many solid tumors stay at this energy producing system even after re-oxygenation leading to an activation of several mechanisms to avoid intracellular acidification.

Aim: The aim of this study was to determine, whether these pH regulating proteins are expressed in SCLC cell lines (DMS-153, NCI-H417 and NCI-H526) and how currently used chemotherapeutic agents influence their gene expression patterns and protein quantities. Can CA IX and NHE1 inhibitors be combined with cisplatin and etoposide treatment and how do they influence the quantity of soluble CA IX?

Methods: SCLC cell lines were treated with cisplatin, etoposide or topotecan and the expression of the CA IX, CA XII, MCT1, MCT4 and NHE1 genes was determined by using qPCR. Protein levels of CA IX were analysed by using Western Blot and the effect of CA IX and NHE1 inhibitors was assessed in chemosensivity assays. The altered quantities of soluble CA IX in response to treatment were determined by ELISA analysis.

Results: The cells lines analysed react in their gene expression regulation in different ways to the treatments, even though they are all SCLC cells, partially depending on their degree of chemoresistance. It is clear that CA IX and NHE1 inhibitors (C207A, zoniporide) are acting antagonistic to cisplatin treatment and thus are increasing tumor cell survival. C207A increases the quantity of soluble CA IX in DMS-153 cells, while cisplatin increases it in NCI-H417 cells.

Conclusion: Some basic findings on pH regulation in SCLC – hitherto little investigated – were obtained in this study. Important pH regulating proteins are expressed in SCLC, and a basis was established for more detailed experiments also in other SCLC as well as in NSCLC cell lines. Taken together, the results may lead to an improved second-line therapy for SCLC patients including targeting of pH regulating proteins combining with chemotherapeutic agents with a special focus on CA IX.

Contents

2. Abbreviations

ACTH	adrenocorticotropic hormone
ADH	antidiuretic hormone
ARNT	aryl hydrocarbon receptor nuclear translocator
ATP	adenosine triphosphate
CA	carbonic anhydrase
CDE	cyclophosphamide/doxorubicin/etoposide
cDNA	complementary DNA
CO_2	carbon dioxide
CSC	cancer stem cell
C_t	Cycle threshold
ECM	extracellular matrix
EGF	epidermal growth factor
EtBr	ethidium bromide
$FADH_2$	flavine adenine dinucleotide
FAM	6-carboxyfluoresceine
FBS	fetal bovine serum
FHIT	fragile histidine triad protein
FRET	fluorescence resonance energy transfer
GLUT	glucose transporter
H_2O	water molecule
HCO_3^-	bicarbonate
HIF	hypoxia inducible factor
HRE	hypoxia response element
HRP	horseradish peroxidase
KRAS	Kirsten rat sarcoma viral oncogene homolog
LDH	lactate dehydrogenase
MCT	monocarboxylate transporter
mTOR	mammalian target of rapamycin
NADH	nicotinamide adenine dinucleotide
NBC	sodium driven bicarbonate co-transporter
NHE	sodium proton exchanger

NMR	nuclear magnetic resonance
NSCLC	non small cell lung cancer
ODDD	oxygen dependent degradation domain
PCR	polymerase chain reaction
PDH	pyruvate dehydrogenase
PDK	pyruvate dehydrogenase kinase
PHD	prolyl hydroxylase
pH_e	extracellular pH
pH_i	intracellular pH
PNEC	pulmonary neuroendocrine cells
PPP	pentose phosphate pathway
PVDF	poly-vinyli-dene-fluoride
qPCR	quantitative Real Time PCR
ROS	reactive oxygen species
RPMI	Roswell Park Memorial
SCLC	small cell lung cancer
SLC2A1	solute carrier family 2 (facilitated glucose transporter) member 1 = glucose transporter 1
SLC9A1	solute carrier family 9 (sodium/hydrogen exchanger) member 1 = sodium proton exchanger 1
SLC16A1	solute carrier family 16 (monocarboxylic acid transporter) member 1 = monocarboxylate transporter 1
SLC16A3	solute carrier family 16 (monocarboxylic acid transporter) member 1 = monocarboxylate transporter 4
TAMRA	6-carboxy-tetramethyl-rhodamine
TGF	transforming growth factor
VHL	von Hippel-Lindau protein

3. Introduction

3.1. Small Cell Lung Cancer (SCLC) – Clinical description

In 2010 approximately 4140 new cases from lung cancer (small cell lung cancer, SCLC, and non-small cell lung cancer, NSCLC, combined) and 3650 deaths were estimated in Austria (Statistik Austria). In 2012, 225,160 new cases and 160,340 deaths were recorded in the United States (National Cancer Institute at the National Institutes of Health, 2012). 80-90% of all cases are related to a long time exposure to tobacco smoke.

By the time of diagnosis, SCLC tends to be more disseminated than other lung cancer types. On the other hand, the response to chemotherapy and radiation treatment is also better than for other lung cancer types. Without chemotherapy and radiation therapy, the median survival of SCLC patients is just 2-4 months after the time of diagnosis. This makes this cancer type to the most aggressive one of any pulmonary tumors (Micke et al., 2001; Ettinger, 2011).

The majority of patients suffering from SCLC have distant metastases at the time of diagnosis. That is the main reason why surgery or radiation therapy of the primary tumor does not lead to a long time survival (Prasad et al., 1989; Gadgeel et al., 2012). The life span can be prolonged up to the 5-fold by the current chemotherapy regimens. Just 10% of all patients treated with chemotherapy remain disease-free for 2 years – most relapses occur during this time. Patients with these tumors have an overall survival rate of 5% - 10% after 5 years. Therefore, most of the patients will die from SCLC within this period (Johnson et al., 1990; Lassen et al., 1995; Fry et al., 1996; National Cancer Institute at the National Institutes of Health, 2012; U.S National Library of Medicine, 2013).

3.2. Small cell lung cancer– cell origin

SCLC develops, in most cases, from pulmonary neuroendocrine cells (PNEC) but also from alveolar type II cells. If other cell types or precursors exist which are able to develop to SCLC is not known (Swarts et al., 2012).

Neuroendocrine cells, in general, are able to release signal molecules (hormones) after a neuronal input signal. PNECs are localized throughout the whole respiratory tract. SCLC mainly develops from so called Feyrter cells which are PNECs localized in the bronchus. These cells produce ectopically antidiuretic hormone (ADH) and adrenocorticotropic hormone (ACTH) (Chon et al., 2006; Mitchell et al., 2007; Swarts et al., 2012).

Type II alveolar cells are found at the alveolar septal junction site and are responsible for the secretion of phospholipids to reduce surface tensions and to generate best circumstances for gas exchange (Ward and Nicholas, 1984).

These cells stay phenotypically normal while more and more genetic alterations take place including:

- epigenetically methylation of fragile histidine triad protein (FHIT) which results in loss of protein expression
- deletion of chromosome 3p, 5q, 13q and 22q
- loss of heterozygosity at 9p and 17p
- p53 mutations
- chromosomal instability

All these events occur in the majority of SCLC patients. Further changes, such as epithelial to mesenchymal transition, result in metastic SCLC phenotype (Swarts et al., 2012).

3.3. Cellular classification and staging of SCLC

It is very important, that an experienced pathologist graduates the pathological material before any SCLC treatment is initiated. The pathologist divides in following types (Hirsch et al., 1988, Travis et al., 1999; Kalemkerian, 2011):

- SCLC
- mixed small cell/large cell carcinoma
- combined small cell carcinoma (i.e. SCLC combined with neoplastic squamous and/or glandular components)

A broad spectrum of diseases is represented by neuroendocrine carcinomas of the lung with two extremes: on one hand, SCLC with neuroendocrine features with a poor prognosis and on the other hand, bronchial carcinoids with an excellent prognosis after surgery and excision of the tumor (Harpole et al., 1992, Detterbeck, 2010; National Cancer Institute at the National Institutes of Health, 2012). Well-differentiated neuroendocrine

carcinoma of the lung is an unusal entity between SCLC and bronchial carcinoids which is called malignant carcinoid. (Lequaglie et al., 1991).
A simple classification system from the Veterans Administration Lung Cancer Study with two different stages of SCLC, limited and extensive stage, is widely accepted and commonly used (Micke et. al, 2001).
At limited stage, which is found in approx. 30% of all patients at the time of diagnosis, the tumor is limited to the mediastinum, the supraclavicular nodes or to the hemithorax of origin. However, this definition term is not universally accepted and patients with massive pulmonary tumor and pleural effusion are either included or excluded from this stage depending on the institutional regulations in different countries. The limited stage tumors are treatable with tolerable radiation therapy and then the survival time is increased to 16-24 months (Murray et al., 1993; Johnson et al., 1996; Mountain, 1997, Califano et al., 2012).
Extensive stage is reached when the tumor already spread widely, and distant metastases are found. The latter is always seen in patients with extensive stage tumors. People with extensive stage tumors just have a median survival of 6 – 12 months with the currently applied therapy. A long time disease-free survival is rare (Mountain, 1997; Youlden et. al, 2008; William and Glisson, 2011). If patients have also an involvement of the liver or the central nervous system, the survival time is much shorter (Socha et al., 2012). A longer survival time is observed in patients with limited-stage disease at diagnosis, female gender and good performance status (Rawson and Peto, 1990; Wolf et al., 1991; Chute et al., 1997; William et al., 2011).

It is very important to distinguish between limited and extensive stage with distant metastases prior to the initiation of the chemotherapy. There are different methods used for determining the stage of SCLC (Ihde et al., 1997, Kalemkerian, 2011):

- CT scans of the chest and the abdomen
- bone marrow examination
- computed tomography (CT) or magnetic resonance imaging scans of the brain
- radionuclide bone scans

3.4. Treatment of small cell lung cancer

Most patients with SCLC will die because of the disease, even if they get the best available medicine and therapy (Califano, 2012). Clinical studies were performed to determine the best timing of thoracic radiation therapy for limited stage patients and the role of surgery combined with different schedules and doses of chemotherapeutic regimes. Surgery is just possible if patients are assigned to limited stage and have a single small tumor without any infiltration of the surrounding lymph nodes (Comis et al., 1998).

The survival rate of both stages is increased by the application of current chemotherapy regimens, but a complete cure occurs only in a minority of patients (Comis et al. 1998; Agra et al.; 2003; William, 2011). Randomized trials have shown that higher doses of chemotherapeutic drugs are not able to increase patients' survival time (Klasa et al., 1991; Arriagada et al., 1993; Elias et al., 1993; Ihde et al., 1994). A standard dose of cyclophosphamide, doxorubicin, vincristine/etoposide and cisplatin was compared to a high-dose cisplatin, vincristine, doxorubicin, and etoposide treatment in a multicenter study with the result that the more dose-intensive regimen produced a higher response rate of the tumor but the treatment related mortality increased as well. Therefore, increasing the dose does not necessarily result in a prolonged progression-free or overall survival (Murray et al., 1999; National Cancer Institute at the National Institutes of Health, 2012).

3.5. Treatment of SCLC patients with limited stage disease

The treatment of limited stage SCLC with combined regimes and moderately intensive doses of drugs are clearly more effective than single agent treatments and doses with only a minimal hematologic toxic effect. Current therapies are able to achieve an overall objective response of 65% to 90%. Complete response appears in rates of 45% to 75%. Hidden distant metastatic diseases occur very frequently in patients with limited stage. So it is inevitable to treat these patients additionally to the radiation therapy with chemotherapy. It is necessary to combine at least 2 different drugs to achieve the maximal possible effect (Pignon et al., 1992; Warde and Payne, 1992; Califano et al., 2012).

The effect of etoposide and cisplatin chemotherapy in combination with concurrent chest radiation therapy has been determined in several (single institutional as well as cooperative group) studies. This treatment was able to yield a median survival of 18 to 24 months throughout the studies as well as a 2 year survival of 40% - 50% with less than 3% mortality based on the treatment (Turrisi and Glover, 1990; McCracken et al.,

1990; Murray et al., 1993; Johnson et al., 1996; Takada et al., 2002). If patients have adequate and sufficient lung function as well as a tumor confined to the origin it may be an advantage to resect affected parts of the lung. However, this is applicable just in a minority of patients (Prasad et al., 1989; Smit et al., 1994; National Cancer Institute at the National Institutes of Health, 2012).

3.6. Treatment of SCLC patients with extended stage disease

To achieve best results for patients with extended stage, it is important to use a chemotherapy regimen which includes different drugs and at concentrations at which at least moderate toxic effects on the tumors can be achieved. Their application should allow an overall response rate of 70% - 85% and complete response rates of 20% to 30% (Schiller et al., 2001; Noda et al., 2002; Eckardt et al., 2006; William et al., 2011; Califano et al., 2012). There is no evidence available to define the most effective application time for any chemotherapy. It is not known if a therapy for more than 6 months may increase the life span (Bleehen et al., 1993; Sculier et al., 1996).

If patients with SCLC achieve a complete remission it may be an advantage to administer prophylactic cranial irradiation (PCI). This method is a preventative radiation therapy to the brain to kill cancer cells, which may have spread into the head but cannot be seen in scans. It is useful because there is a 60% risk for patients to develop central nervous system metastases in the next 2-3 years after the onset of treatment. The majority of these metastases occur in the brain only. In most cases, patients die after a secondary tumor in the central nervous system (Cull et al., 1994; Auperin et al., 1999; Socha et al., 2012). There is a median survival of 2-3 months for unresponsive patients with SCLC. In these patients, the tumor progresses despite of the standard chemotherapy. These patients should be advised to palliative therapy or they may participate into clinical trials for new drugs.

However, patients with a tumor which is primarily resistant to the first-line therapy as well as patients who have received multiple chemotherapy regimes are not likely to respond to second-line therapy. In contrast, patients with an initial response to the first-line treatment and a relapse after 6 months are more probable to respond to a second-line chemotherapy. Such second-line treatment options are:

- oral etoposide
- etoposide/cisplatin

- cyclophosphamide/doxorubicin/vincristine (CAV)
- lomustine/methotrexate
- paclitaxel
- topotecan

(Johnson et al., 1990; Evans et al., 1985; Chute et al., 1996; Arrdizoni et al., 1997; Califano et al., 2012)

There is no significant difference between the response rate of CAV and topotecan in a second-line treatment regimen. However, symptoms of common lung cancer were better controllable by topotecan and so it is the only FDA approved chemotherapeutic agent for this application (von Pawel et al., 1999).

3.7. Why SCLC is resistant to second-line chemotherapy – new approaches needed

SCLC is characterized by a rapid relapse which doesn't respond very well to current used second-line therapies. The increased number of treatment related deaths with higher concentrations of anticancer drugs in SCLC therapy led to a treatment with less toxicity without any improvement for patient survival in the last years. Responsible for the high chemoresistance are different factors (Knez et al., 2011):

- upregulation of multidrug resistance associated proteins
- alterations in topoisomerase II
- increased DNA repair via DNA excision repair protein ERCC1
- p53 mutations
- genomic instability
- presence of chemoresistant cancer stem cells (CSC)
- impaired induction of drug induced cell death

Many mechanism and pathways are still unknown in this cancer type. A new approach for a better therapy can be to target different proteins which are responsible for pH regulation. The cancer cell is confronted to an increased amount of acids and protons from an altered cell metabolism. These products must be extruded from the cell - otherwise many physiological reactions would stop. A novel anticancer strategy can be to target and inhibit or influence key players in tumor pH regulation e.g. carbonic anhydrase IX

(CA IX) and sodium proton exchanger 1 (NHE1 – SLC9A1). The main metabolism changes in cancer cells and the function of selected pH regulation proteins are explained in the following chapters.

3.8. Cell metabolism

Under normal physiological conditions, cells preferentially obtain their energy from glycolysis and oxidative phosphorylation. Glucose is phosphorylated to glucose–6–phosphate under the consumption of 1 adenosine triphosphate (ATP). Overall, one molecule glucose is converted into two molecules of pyruvate (pyruvic acid). The net result of the glycolysis is: 2 pyruvate, 2 ATP, 2 nicotinamide adenine dinucleotide phosphate (NADH), 2 H^+ and 2 water molecules (H_2O).
During the Krebs cycle, which takes place in the mitochondria, acetyl-CoA is oxidized producing 3 NADH and 1 flavine adenine dinucleotide ($FADH_2$). The electrons of these co-enzymes are removed and transported step by step via different sets of enzymes, which are organized in complexes I-IV and ubichinone as well as cytochrome c in the inner membrane – the so called electron transport chain. The transport of these electrons is coupled to a release and re-uptake of protons. This chain pumps protons from the mitochondrial matrix into the intermembrane space, which results in an electrochemical gradient. ATP is produced when the protons move back into the matrix space through the ATP-synthase. The electrons used are transferred to a terminal acceptor: oxygen, which is reduced to water. This process provides, in total, 38 moles of ATP per used mole of glucose (Rich, 2003; Gatenby and Gillies, 2004).

Growing tumors cannot provide sufficient oxygen supply through new blood vessels, particular in the center of large tumors, even if angiogenesis is highly increased. The lack of oxygen will lead to a hypoxic microenvironment, especially in the core of the tumor. There, cells will shift their energy metabolism from oxidative phosphorylation towards an anaerobic metabolism. Anaerobic glycolysis provides 2 moles of ATP per mole of glucose consumed, but also 2 protons are produced in every glycolysis cycle.
In the 1920s, Otto Warburg was the first who observed a high glucose uptake in tumor cells and the increased production of lactic acid. He postulated that this is the result of an anaerobic glycolysis. For some tumor cells, it has been demonstrated that even in the presence of oxygen, anaerobic glycolysis is the main metabolic pathway to produce energy. In 1930, Warburg stated that this change in energy metabolism results from a mitochondrial injury. This was considered the most important conversion, which occurs

during malignant transformation and points to the “origin of cancer cells” (Warburg, 1930; Gatenby and Gillies, 2004; Fang and Gillies and Gatenby 2008; Ebbesen et al. 2009).

However, this hypothesis of the origin of cancer cells is not generally accepted, because some tumors do not carry any defect in oxidative phosphorylation mechanisms (Chance B, 2005; Funes et al. 2007).

Nevertheless, altered metabolism with increased glucose uptake and conversion to lactate provides an advantage for tumor survival and growth. This may explain why tumor cells continue to produce energy by anaerobic glycolysis even under normoxic conditions.

- Different intermediates of the glycolytic pathway can be used by the tumor cells for anabolic reactions, e. g. pyruvate for triacylglyceride synthesis (Gatenby and Gillies, 2004)
- To regenerate NADPH through the pentose phosphate pathway (PPP), which serves as a protection against oxidative stress (Gatenby and Gillies, 2004; Langbein et al., 2006; Foldi et al., 2007)
- Tumor cells can survive fluctuating oxygen tension (Pouyssegur et al., 2006).
- Acids, like bicarbonic and lactic acid, which are produced in high amounts in cancer cells, can suppress anticancer immune effectors and favor tumor invasion (Koukourakis et al., 2006; Swietach et al., 2007; Fischer et al., 2007).

Hypoxic conditions are also related to an increased resistance of the tumor to anticancer drugs:

The distance of hypoxic cancer cells and the circulating blood system is too far for them to get exposed enough and to be affected by some anticancer agents (Durand, 1994; Tannock, 1998). Another reason is that just cancer cells that have lost p53 survive these harsh hypoxic conditions. This might also reduce the sensitivity to some treatments. Also the up-regulation of some hypoxia induced genes, such as P-glycoprotein (a membrane bound protein able to pump many drugs out of the cell), increases drug resistance (Comerford et al., 2002; Wartenberg et al., 2003). Some anticancer drugs (like bleomycin) also need the presence of oxygen to increase their cytotoxicity (Batchelder et al., 1996).

3.9. Hypoxia-inducible factor-1 - HIF-1

A big subset of oncogenes and tumor suppressor genes play a role in the metabolic switch of tumor cells, e.g. Akt, myc, Kirsten rat sarcoma viral oncogene homolog (KRAS), epidermal growth factor (EGF), mammalian target of rapamycin (mTOR), adenosine monophosphate (AMP) - activated protein kinase and especially hypoxia-inducible factor 1 (HIF-1) (Kroemer and Pouyssegur, 2008; DeBerardinis RJ. 2008; Levine and Puzio-Kutter, 2010).

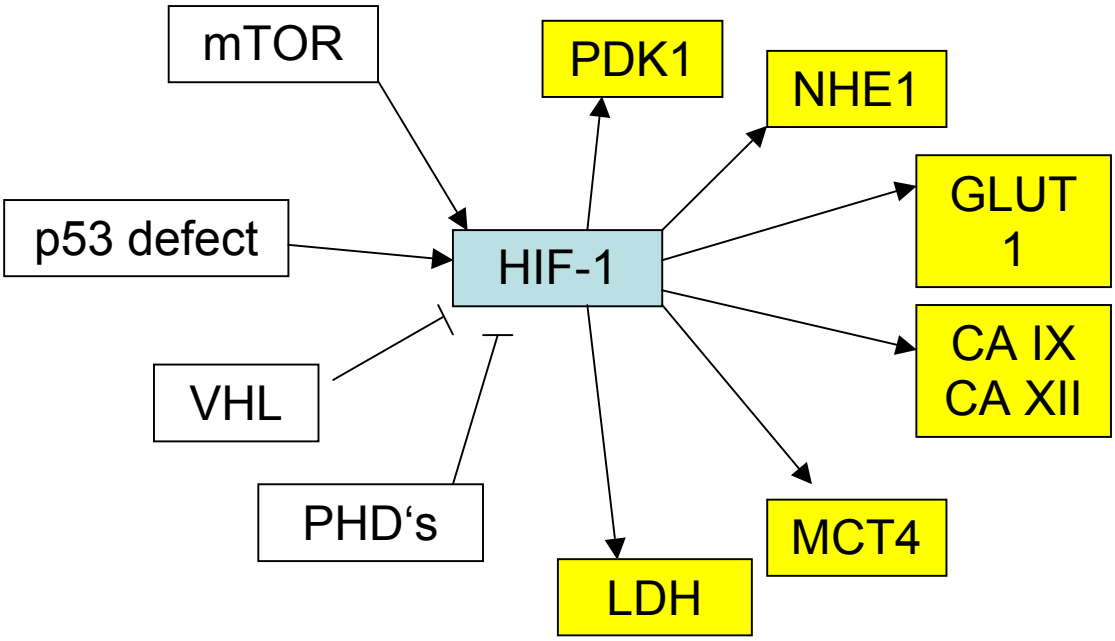

Figure 1 Hypoxia inducible factor 1 (HIF-1) is the main player in hypoxic situations
Many genes like lactate dehydrogenase (LDH), monocarboxylate transporter 4 (MCT4), carbonic anhydrase IX and XII, glucose transporter 1 (GLUT1), Na^+/H^+ exchanger 1 (NHE1) or pyruvate dehydrogenase kinase 1 (PDK1) have a hypoxia response element (HRE) in their promoter. HIF-1 is able to bind to these sequences and to induce gene expression. The van Hippel-Lindau protein (VHL) and HIF-prolyl hydroxylases (PHDs) are able to inhibit HIF1 by degradation of the HIF-1 alpha subunit. Mammalian target of rapamycin (mTOR) and p53 defects are able to induce HIF-1.

Hypoxia induced genes, such as genes coding for the glucose transporters GLUT1 and 3 and monocarboxylate transporters like MCT4 (SLC16A3), or genes coding for enzymes responsible for glycolytic activity, like lactate dehydrogenase (LDH) and pH regulation, like carbonic anhydrases, CA IX, CA XII, have a hypoxia response element (HRE) in their promoter. This element is recognized by the HIF-1 complex (Fig.1) (Caro, 2001; Maxwell et. al 2001). The HIF-1 complex consists of two subunits: HIF1β, also known as aryl hydrocarbon receptor nuclear translocator (ARNT), and HIF1α, building up a heterodimeric complex. The beta subunit is expressed constitutively, while the ex-

pression of the alpha subunit depends on the oxygen level in the tissue (Semenza, 2002). The level of HIF1α is undetectable in cells under normoxic conditions, because the oxygen dependent degradation domain ODDD, located in the center of the HIF1α protein, is hydroxylated by the HIF-prolyl hydroxylases (PHDs). The von Hippel-Lindau (VHL) protein recognizes this hydroxylated structure and then ligates ubiquitin to the alpha subunit of HIF1, which results in the degradation of the protein in the 26S proteasome (Huang et al., 1996; Salceda and Caro, 1997; Gleadle and Ratcliffe, 1998; Pugh and Ratcliffe, 2003; Mole et al., 2001; Kaelin, 2005).

HIF-1 is also responsible for the suppression of the Krebs cycle by pyruvate dehydrogenase kinase 1 (PDK1) induction. The activated PDK1 phosphorylates the E1 subunit of pyruvate dehydrogenase (PDH) and thus prevents the conversion of pyruvate to acetyl-CoA. This mechanism results in an inhibition of the Krebs cycle (Kim et al., 2006; Papandreou et al., 2006).

3.10. Lactate and monocarboxylate transporters (MCTs)

Increased glucose uptake for glycolysis is provided by the elevated expression of GLUT1 (SLC2A1). If the pyruvate formed accumulates in the cell, it would block glycolysis. Pyruvate subsequently converted into lactate, as up-regulation of lactate dehydrogenase 5 (LDH5) occurs simultaneously in cancer cells with altered cell metabolism (Semenza et al., 1994 and 1996).

Both products of glycolysis, pyruvate and lactate, are able to stabilize the HIF-1α subunit, even under normoxic conditions. This leads to an increased accumulation of this transcription factor in the cells.

Accumulation of lactate will also result in the saturation of the glycolysis process. The ensuing intracellular acidosis will dramatically affect energy metabolism. Koukourakis et al. suggested in 2005 a key role of the surrounding stroma for the lactic acid metabolism in colon cancer. Especially the tumor associated fibroblasts may have an important role in the uptake, via monocyrboxylate transporters (MCTs), and the metabolism of lactate extruded from the cancer cells (Koukourakis et al., 2005).

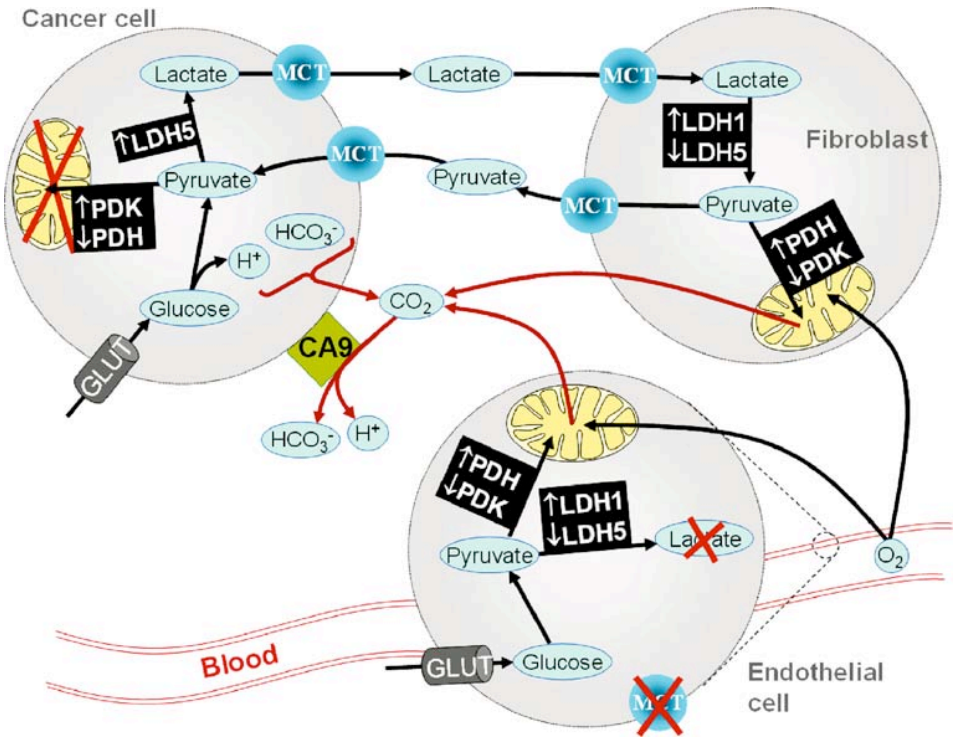

Figure 2. Model of a possible lactate metabolism system in colon cancer
The different expression patterns of involved enzymes, namley LDH, lactate dehydrogenase, PDH pyruvate dehydrogenase and PDK, pyruvate dehydrogenase kinase as well as the monocarboxylate transporters (MCT'S) suggests a key role of the tumor stroma, especially the tumor associated fibroblasts for lactate metabolism. Tumor generated lactate is transported via MCT's into the stroma and is taken up again via MCTs by the fibroblasts. This protects the tumor enviroment from lactate overload which otherwise would saturate glycolytic flux. The produced acid is trapped in the extracellular space via carbonix anhydrase IX (CA IX). Taken from Swietach et al. 2007.

The monocarboxylate transporter (MCT) family consists of 14 members. 6 members are already characterized and MCT1 (SLC16A1) and MCT4 (SLC16A3) are the only members so far, where the transport of lactate has been shown to be coupled to proton transport (Halestrap and Price, 1999; Grollman et al., 2000; Halestrap and Meredith, 2004).

The expression of MCT1 occurs in most cells, while MCT 4 is highly expressed in glycolytic cells, e.g., white muscle cells (Halestrap and Price, 1999; Juel and Halestrap, 1999; Halestrap and Meredith, 2004). MCT2, which is absent in most human tissues, is found in rats. There it is found in neurons and liver, both with a high capacity for lactic acid uptake (Halestrap and Price, 1999; Halestrap and Meredith, 2004).

Basigin (CD147) or embigin (gp70) are necessary for the expression of MCTs on the plasma membrane. Both are ancillary glycoproteins and are important for the activity of the transporter (Wilson et. al 2002 and 2009).

MCT1 mediates the export from lactate from colon cancer cells (Fig. 2). The surrounding tumor-associated fibroblasts import the lactate via MCT1 and MCT2. These cells have low LDH5 and high LDH1 levels and favor the conversion of lactate into pyruvate, the Krebs cycle and subsequently, the electron transport chain in the mitochondria. The glucose uptake in tumor-associated fibroblasts is decreased by a reduced expression of GLUT1. The tumor-associated endothelium shows low MCT1 expression, which indicates that these cells do not import tumor-derived lactate. Instead, they gain their energy from “normal” oxidative phosphorylation (Vaupel et al., 1989; Koukourakis et al., 2005 and 2006; Harris, 2005; Kim et al., 2006; Papandreou et al., 2006).
It is interesting to note that neither MCT1 nor MCT2 have a HRE in their promoter and they don’t respond to hypoxia via HIF-1. Only MCT4 is inducible by hypoxia (Ullah et al., 2006).

3.11. Lactate and the immune system, cell migration and radioresistance

3.11.1. Immune system

Tumors developed several mechanisms to avoid elimination by the immune system. Also lactate plays a role in inhibiting the immune system (Fig. 3). In the presence of high levels of extracellular lactate, monocytes will not differentiate to dendritic cells. Additionally, the release of cytokines from dendritic cells is blocked by lactate (Gottfried et al., 2006; Fischer et al., 2007; Singer et al., 2011).
Lactate-dependent immune inhibition is also able to block activated cytotoxic T-cell function. These cells are key players to prevent tumor growth. Activated T-cells mainly gain their energy from glycolysis and they are not able to suppress their own lactate production, even if the extracellular lactate concentration is high and despite the fact that in most cells, the rate of lactate uptake depends on the ratio between the concentration of intra- and extracellular lactate. Lactate transport also results in a decreased extracellular pH, because protons are transported simultaneously with lactate by the MCT. This reduces the function of cytotoxic T-cells (Fischer et al., 2007; Dietl et al., 2010; Michalek et al., 2011).

3.11.2. Cell migration

Lactate also favors cell migration (Fig. 3). Various cancer cell lines showed an increased capacity for movement depending on the concentrations of lactate in the medi-

um. Some data also showed that high lactate concentrations increased cell motility. However, the exact molecular mechanisms are not understood so far, although it seems that integrins play a role. Some data showed an influence of the transforming growth factor-β2 (TGF- β2) signaling pathway on the lactate-induced/increased cell motility (Baumann et al., 2009; Goetze et al., 2011).

3.11.3. Resistance against ionizing radiation

Lactate plays a role in the resistance to ionizing radiation, which induces an increased production of reactive oxygen species (ROS). These will damage DNA, RNA, proteins and lipids, and will lead to subsequent cell death. Through lactate, which acts as an antioxidant, cancer cells can get resistant to radiation therapy (Fig. 3) (Groussard et al., 2000; Sattler et al., 2009 and 2010).

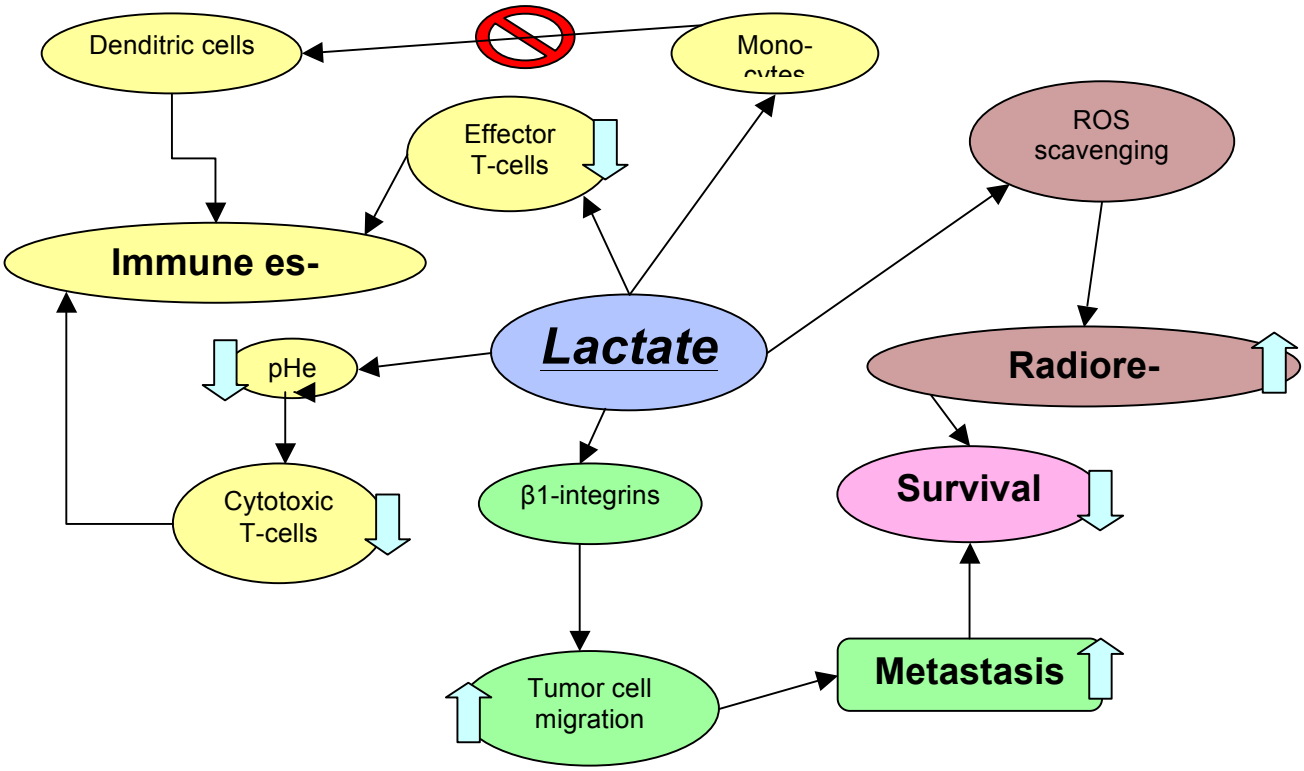

Figure 3 Positive effects of lactate for the cancer cell
High levels of lactate in the cancer cell, generated by the altered metabolism, play an important role in tumor progression and tumor survival. Lactate acts as antioxidant and protects the cell from damage caused by irradiation therapy. Lactate may also play a role in increased tumor cell migration and metastasis by interacting with β1-integrins. Monocytes are not able to differentiate to dendritic cells, when a high amount of lactate is present. Also effector T-cells are inhibited, because these cells gain their energy from glycolysis and are not able to extrude their lactate produced. High lactate levels decrease the extracellular pH and inhibit the function of cytotoxic T-cells.

3.12. Regulation of the pH in tumor cells: importance of carbonic anhydrase IX and Na^+/H^+ exchanger NHE1

The acid/base regulation in the human body is an essential part of life. The pH in the extracellular space (pH_e) is maintained at 7.4. This value depends on the carbon dioxide (CO_2) partial pressure and the bicarbonate (HCO_3^-) concentration in the blood. The blood carbon dioxide and bicarbonate concentrations are regulated mainly by the lung and the kidneys.

The carbonic buffer system is able to stabilize the pH_e immediately and to mediate a physiological adjustment in order to maintain normal cellular processes. The intracelllar pH (pH_i) has a pH value of approx. 7.2. Changes to a more acidic pH_i affects cell division, growth and other functions. It may even lead to apoptosis.

This difference between an alkaline pH_e and near neutral pH_i suggests an advantage on cell survival as well as on growth and development. Cellular respiration, glycolysis and the activity of the respiration chain lead to a production of protons, CO_2 and lactic acid, which decreases the pH_i. If these products are allowed to accumulate within the cell, the pH could fall to a hazardous level (Roos and Boron, 1981; De Brabander et al., 1982; Morita et al., 1992; Isfort et al., 1993;; McConkey et al., 1996).

Cells developed multiple membrane transport mechanisms, e.g. MCTs and Na^+/H^+ exchangers (NHEs), which are able to extrude these acids and protons from the cell in order to stabilize the pH_i at ~ 7.2. Warburg's observation of high glucose uptake and high lactic acid production in tumors suggested a low pH_i and pH_e in tumor tissues (Warburg, 1930).

It was determined by nuclear magnetic resonance (NMR) analysis in the 1980s, that pH_e ranges between 6.9 and 7.0 in tumors and is therefore quite acidic. Since the pH_i ranges between 7.0 and 7.4, this milieu provides a selective advantage for tumor development and growth. The surrounding healthy cells undergo apoptosis if exposed to this more acidic microenvironment for a longer time (Griffiths et al., 1981; Vaupel et al., 1990; Gatenby and Gillies, 2004). The acidic pH_e even provides an advantage for tumor metastasis, as shown with melanoma and sarcoma cells *in vitro* because degradation of the basement membrane and the extracellular matrix (ECM) is favored under these acidic conditions (Schlappack et al., 1991).

Mutated p53 also protects tumor cells from apoptosis in the low pH_e medium (Williams et al., 1999).

3.13. Carbon dioxide (CO_2)

Not only lactic acid plays a role in extracellular acidification. In an experiment by Newell et al., glycolysis-deficient Chinese hamster lung fibroblasts were transplanted into mice. These cells produced a pH_e of approx. 6.7, but only a small amount of lactic acid. Therefore, other factors may cause the pH shift (Newell et al., 1993).
In another experiment, LDH-deficient hamster ovary cells were transplanted into mice. These cells showed significant lower glucose usage and lactic acid output, but they also led to acidification of the extracellular microenvironment, in this case via interfering with the CO_2 content (Yamagata et al., 1998).
There are a few sources of CO_2 in cells:

- Krebs cycle
- pentose phosphate shunt
- titration of HCO_3^- with H^+ from metabolic activities

One molecule of CO_2 is produced per one carbon atom during the Krebs cycle. It is possible, however, that this source of CO_2 is perhaps negligible because of the metabolic alterations in tumor metabolism, which inhibit the Krebs cycle.
An alternative to glycolysis, the pentose phosphate shunt, may also be a source of CO_2. This mechanism regenerates NADH and produces pentoses. The CO_2 flux is increased during nucleotide synthesis, which is up-regulated in tumor development.
The third source of CO_2 is independent of glycolysis or Krebs cycle enzymes. It is due to the titration of HCO_3^- by H^+ producing CO_2 (Swietach and Vaughan-Jones and Harris, 2007).

3.14. H^+ transport out of the cell

CO_2 together with HCO_3^- works as an open buffer system, which ensures sufficient supply with HCO_3^- (Roos and Boron, 1981). To maintain an intracellular pH value of > 7 it is important to extrude H^+ ions, which are produced in high amounts because of the increased metabolic rate, from the cancer cells. The protons can be transported into intracellular vesicles or exported from the cells via V-type H^+-ATPases. Also monocarboxylate or Na^+-H^+ transporters like NHE1 are able to extrude H^+ from the intracellular space (Fig. 4).

A mechanism, which involves the enzyme carbonic anhydrase, is the intracellular reaction of H^+ with HCO_3^- forming CO_2. CO_2 is able to travel freely through the plasma membrane to the extracellular space, where it is then hydrated again and dissipates to H^+ and HCO_3^- via membrane-bound carbonic anhydrase IX. The H^+ ions are trapped at the outside of the cell leading to an acidification of the extracellular milieu and the HCO_3^- ions consumed in the cells can be replaced using sodium-driven bicarbonate co-transporter mechanisms (NBC), via the Na^+/HCO_3^-co-transporters (Fig. 4) (Meyerson et al. 1991; Swietach and Vaughan-Jones and Harris, 2007).

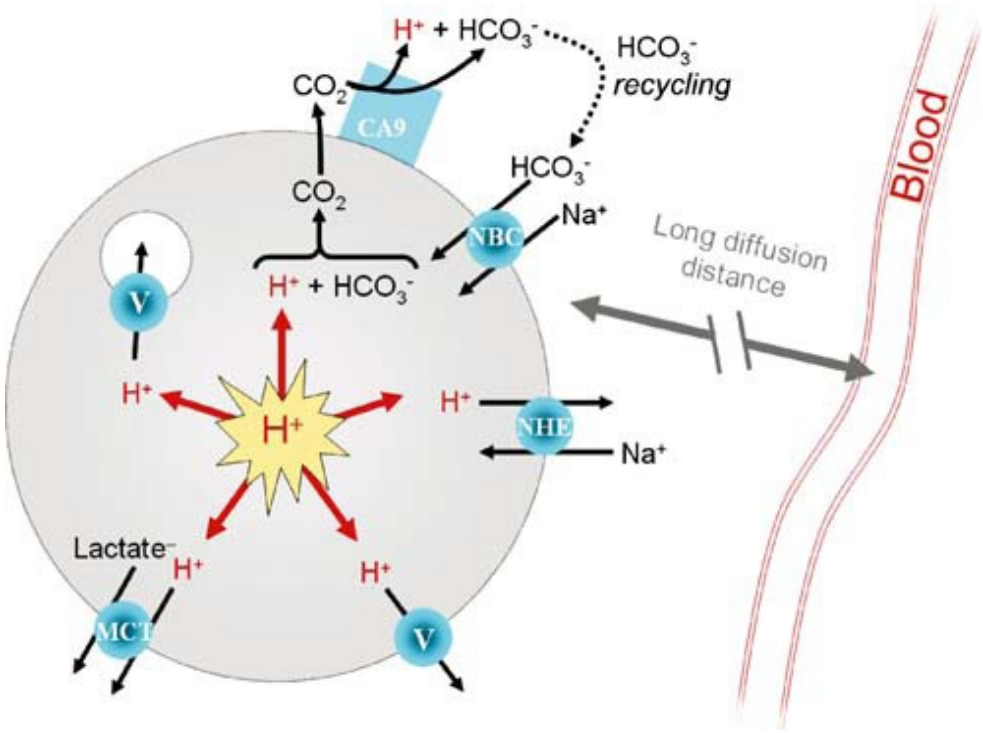

Figure 4 Overview how cancer cells extrude overproduced protons from the cell
High amount of protons (H^+) are produced due to the altered metabolism of cancer cells. These protons must be transported out of the cell to maintain an intracellular pH of > 7. Protons can be extruded by Na^+/H^+ exchangers (NHEs), monocarboxylate transporters (MCTs) or via V-type H^+ ATPases (V). Another possibility is the titration of H^+ with HCO_3^- forming CO_2, which can travel freely through the plasma membrane to the extracellular space, where carbonic anhydrase IX (CA9) reversibly hydrates CO_2 back to HCO_3^- and H^+. Taken from Swietach et al. 2007.

3.15. The role of carbonic anhydrase IX

Alpha carbonic anhydrases (CAs) are metalloenzymes, which are found in vertebrates. There are 16 different isotypes of carbonic anhydrases known, which differ in their localization within the cell and their catalytic activity:

- Cytosolic: CA I, CA II, CA III, CAVII, CA XIII
- Membrane bound: CA IV, CA IX, CA XII, CA XIV, CA XV
- Mitochondrial: CA V A and B
- Secreted: CA VI

The missing isotypes CA VIII, CA X and CA XI are so-called carbonic anhydrase-related proteins, because they lack the typical carbonic anhydrase activity.
CA IX is a 466 amino acid containing protein with a N-terminal proteoglycan structure, a hydrophobic signal peptide, a catalytic domain with a zinc-binding histidine residue, a hydrophobic transmembrane segment and a short intracellular C-terminal domain (Pastorek et al., 1994).
The physiological role of the intracellular part of the protein is still unclear, but some data showed that it contains sites that can be phosphorylated. This may play a role in signaling pathway/dimerization/interaction (Sterling and Reithmeier and Casey, 2001; Dorai et al., 2005). Also the role of the proteoglycan structure is still not clear, but it may influence cell adhesion (Svastova et al., 2003).
Carbonic anhydrase reversibly hydrates CO_2 to HCO_3^- and this contributes to the bicarbonate – carbonic acid buffer system. The carbon dioxide, which is produced during the Krebs cycle, is subsequently converted into bicarbonate by carbonic anhydrases. This is important, because the uncatalysed reaction would be to slow too remove the accumulating carbon dioxide in the cell.

There are several processes in which the carbonic anhydrases are involved:

- Bone respiration
- Bone calcification
- Electrolyte secretion
- Respiration
- and in plants: photosynthesis

(Scozzafava et. al, 2004 and 2006; Supuran, 2008, 2010 and 2010 (b)).

3.16. Carbonic anhydrases (CAs) in cancer

There are two isoforms of CA, which are highly overexpressed in many solid tumors: CA IX and CA XII. Both types are induced by HIF-1, but CA IX is the most frequently over-expressed gene in cancer cells in response to hypoxia. It is also the most active form for hydrating CO_2. CA IX is also the only isotype with a dimeric structure – all other carbonic anhydrases are monomeric enzymes. CA XII, in comparison with CA IX, shows a lower catalytic activity and it is expressed more diffuse in healthy tissues (Pastorek et al., 1994; Borsi et al., 1996; Svastová et al., 2004; Scheurer et al., 2004; Vullo et al., 2005; Hilvo, 2008; Innocenti et al., 2009; Supuran, 2008 and 2010).

CA IX was first detected in HeLa cell and was called MN protein. Immunofluorescence analysis showed localization on the cell membrane, but also nuclear localization was described (Pastorekova et al., 1992; Opavsky et al., 1996).

In many solid tumors, including lung, breast, cervix, brain, thyroid and vulvar cancer, high CA IX levels are suitable as diagnostic markers. There expression seems to correlate with a poor prognosis for cancer patients (Gieling and Williams, 2012).

Carbonic anhydrases are already well-characterized and a variety of different CA-inhibitors are available (e.g. sulfonamides, which bind to the catalytic zinc domain in the active site). This makes CA blockage to an attractive anticancer strategy (Supuran, 2008 and 2010).

3.17. Na^+/H^+ exchanger NHE1

The sodium proton exchanger (NHE) family consists of seven isoforms of integral membrane proteins and transports sodium into and protons out of the cell simultaneously. There are two structurally related domains found throughout the protein family with a 20%-70% identity (Orolowski and Grinstein, 1997; Fliegel, 2001):

- an approximately 500 amino acid long membrane domain with 12 trans-membrane helices
- an approximately 300 amino acid long C-terminal cytoplasmic tail

NHE1 was the first isoform of this exchanger family discovered first and is expressed ubiquitously in the plasma membrane of nearly all tissues. The most important function of NHE1 and the whole Na^+/H^+ exchanger family is to counteract a falling intracellular pH. The intracellular proton concentration plays a crucial role for NHE1 activity, as protons act as independent allosteric regulator for NHE1 by binding to an internal allosteric proton binding regulatory site at the C-terminal end of the protein (Aronson et al., 1982; Pouyssegur et al., 1984; Grinstein et al., 1989). In addition to the regulation of the pH, NHEs are able to regulate the cell volume and Na^+ flux after osmotic shrinkage (Grinstein et al., 1989; Shrode et al., 1996). NHE1 also acts as scaffolding protein by interacting with a variety of proteins including: calmodulin, 14-3-3 adaptor protein, calineurin homologous protein (CHP), and B-Raf. The latter is able to activate NHE1 (Li X et al., 2002 and 2006; Stock et al., 2012; Karki et al., 2011; Boedtkjer et al., 2012).
NHE1 can also be activated by extrinsic factors such as growth factors, hormones or ECM ligand receptors. These activations shift the pH dependence of the exchanger to a more alkaline range. By doing so, the activity of the exchanger is increased even at an alkaline pH (Fliegel, 2001).
The C-terminal domain of the protein contains serine and threonine residues, which are constitutively phosphorylated and which get further phosphorylated by extrinsic activation. This kind of regulation is complex and varies between different cell types (Meima et al., 2009). Rho kinase, Nck – interacting kinase and mitogen-activated protein kinase are known for NHE1 to activate phosphorylation, while in contrast protein kinase p38 inhibits the exchanger (Wang et al., 1997; Tominage et al., 1998; Kusuhara et al., 1998; Moor and Fliegel, 1999; Moor et al., 2001; Yan et al., 2001). Some other kinases, such as protein kinase C and D, are also regulators for NHE1 activity, but they do not phosphorylate the protein directly (Fliegel et al., 1992; Hanworth et al. 1999).

3.18. NHE1 and neoplastic transformation

NHE1 plays an important role in oncogene driven neoplastic transformation. Cancer cells have, based on their altered metabolism, a higher H^+ production, as already mentioned above. Tumor cells are able to increase the affinity of the C-terminal proton-binding site of NHE1 to mimic a low pH within the cell to increase enzyme activity even

under normoxic conditions (Reshkin et al., 2000). However, in two studies it has been suggested that dimerization of NHE1 may occur, which would also increase the enzyme activity, but not by increasing the affinity of the proton-binding site (Lacroix et al., 2004; Fuster et al., 2008).

This proton gradient switch in cancer cells is one of the very first steps in oncogene driven neoplastic transformation. It has been shown that in v-mos and ras-oncogene driven transformation an increased intracellular pH occurs very early and is based on the increased activity of NHE1 (Hagag et al., 1984; Doppler et al., 1987; Reshkin et al., 2000). The pH switch in tumor cells is an important driver change which has a high impact on other hallmarks of cancer, such as: anaerobic glycolysis even in the presence of oxygen, tumor growth in nude mice, increased growth rate, growth factor independence and substrate independent growth (Reshkin et al., 2000).

4. Aim of the diploma thesis

Small Cell Lung Cancer (SCLC) is untreated the most aggressive form of all pulmonary tumors. It is very complicated to treat, because this tumor type tends to be more disseminated at the time point of diagnosis and distant metastases occur very early in tumor development.

SCLC is commonly dived into two stages: limited and extensive stage. Just 30% of all patients are in the limited stage at the time point of diagnosis, which is characterized by a limitation of the tumor to one area. If the tumor spreads beyond the supraclavicular nodes or distant metastases are found, the patients are assigned to extensive stage. Both stages need a combinational chemotherapy with at least two different anticancer agents. Limited stage patients also receive chest radiation therapy. The median survival rate for patients without therapy lies between two and four months. Current available therapy regimes are able to prolong patient life span: limited stage patients have a median survival of 18-24 months, while patients with extensive stage survive only 6-12 months.

SCLC is characterized by a rapid relapse without a good response to a second-line therapy. Novel anticancer strategies are urgently needed. One new approach can be to target different key players for tumor pH regulation, because tumors are confronted with altered cell metabolism and shifted pH microenvironment based on hypoxia. Fast growing tumors can not provide sufficient oxygen supply for the core of the tumor, even if angiogenesis is increased. Oxidative phosphorylation, the energy producing system which is used by most cells of the body, is blocked, because the final electron acceptor, oxygen, is missing. A variety of genes are activated through Hypoxia Inducible Factor 1 (HIF-1) in response of hypoxia. This activation shifts the energy production towards a 200 times increased oxygen independent glycolysis. This system produces only 2 moles of ATP per mole of glucose used compared to 38 moles ATP per mole of glucose metabolized by oxidative phosphorylation. Many solid tumors stay at this energy producing system even after reoxygenation, providing a selective advantage for the tumor cell: glycolytic intermediates can be used for anabolic reactions and NADH regenerated through the pentose phosphate pathway serves as protection against oxidative stress. Furthermore, tumor cells survive fluctuating oxygen tensions, and acids produced during glycolysis protect the cancer cells against anticancer immune effects. Lactate, one of the overproduced acids, contributes to immune escape by inhibiting the differentiation of monocytes to dendritic cells and by inhibiting cytotoxic and effector T-cells. It also

interacts with β1-integrins and by doing so it increases tumor cell motility leading to early metastasis. Lactate works also as antioxidant and leads to increased radioresistance.
Accumulating pyruvate and lactate as well as protons, due to increased glycolysis, would lead to acidosis in the cell and eventually would stop all physiological reactions. HIF-1 is able to induce gene expression of important pH regulating proteins, such as carbonic anhydrase IX (CA IX), sodium proton exchanger 1 (NHE1) and monocarboxylate transporter 4 (MCT 4).
Protons react with bicarbonate forming carbon dioxide, which is able to travel through the plasma membrane. CA IX, a transmembrane protein with the catalytic domain at the extracellular space, reversibly hydrates carbon dioxide back to protons and bicarbonate. NHE1 is able to transport sodium into and protons out of the cell simultaneously. And MCTs transport monocarboxylated substances, like pyruvate and lactate, out of the cell together with protons. All these mechanisms are able to stabilize the intracellular pH at ~7 and to trap acids and protons at the outside of the cancer cell producing an acidic microenvironment in which healthy cells undergo apoptosis.

It was assessed, whether these important pH regulators are expressed in different cell lines representing SCLC (DMS-153, NCI-H526 and NCI-H417). The influence of currently used chemotherapy agents (etoposide, cisplatin and topotecan) as well as the influence of CA IX and NHE1 inhibitors on the gene expression of these key players was determined by performing qPCR. Protein levels with and without therapy were analyzed with Western Blot analysis. Also chemosensitivity assays and ELISAs were used to evaluate the efficacy of cisplatin in combination with CA IX and NHE1 inhibitors.
These experiments should give a first insight into the changes of pH regulation in SCLC with currently used chemotherapy regimes. The results of this study should be useful as basis for novel anticancer strategies.

5. Materials and Methods

5.1. Solutions and buffers

5.1.1. 10x PBS – phosphate buffered saline with pH = 7.4

potassium dihydrogen phosphate (KH_2PO_4)*	2g
sodium chloride (NaCl)*	80g
potassium chloride (KCl)*	2g
disodium hydrogen phosphate ($Na_2HPO_4.2H_2O$)*	14.4g

The pH value was adjusted to 7.4 after dissolving all chemicals in 1l ddH_2O

5.1.2. Radio-Immunoprecipitation Assay (RIPA) buffer

SDS*	0,1%
TRIS*	25mM
NaCl	150mM
sodium deoxycholate*	1%
Nonidet P40 substitute–NP-40 (SIGMA-ALDRICH, Buchs, CH)	1%

5.1.3. 10x Laemmli buffer

SDS*	10g
TRIS*	30g
Glycine*	144g
ddH_2O	ad 1l

5.1.4. 4x sample buffer

87% Glycerine*	3ml
0,5M TRIS pH = 6.8	5ml
SDS*	0,8g
0,1% bromophenol blue *	0,5ml

Freshly add 20% β-mercaptoethanol (SIGMA-ALDRICH, Buchs, CH) before each use.

5.1.5. 10x TAE buffer

TRIS*	96,8g
sodium acetate*	54,5g
EDTA*	7,6g
ddH_2O	ad 2l

*purchased from Merck (Darmstadt, GER)

5.2. Cell culture

- $25cm^2$ and $75cm^2$ 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, Buchs, CH)
- calcium and magnesium free 1x PBS
- Safe-seal tips (Biozym Scientific GmbH, Oldendorf, GER)
- Pasteur pipettes (SIGMA-ALDRICH, Buchs, CH)
- 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)
- 10x Trypsin - EDTA solution Nr. 59418(SIGMA-ALDRICH, Buchs, CH)
- Ethanol (EtOH) (AnalaR NORMAPUR, West Sussex, UK)
- Penicillin - Streptomycin P4083 (SIGMA-ALDRICH, Buchs, CH)
- Cisplatin and etoposide(SIGMA-ALDRICH, Buchs, CH)
- Toptecan (GlaxoSmithKline plc., London, UK)
- CA IX inhibitor C207A (obtained from Prof. Claudiu T. Supuran, Firenze, ITA)
- NHE1 inhibitor: Zoniporide (Tocris Bioscience, Bristol, UK)

5.2.1. Cell lines

SCLC cell lines were obtained from Dr. Nina Pedersen, Department of Radiation Biology, The Finsen Centre, National University Hospital, Copenhagen DK-2100, Denmark (Pedersen et al., 2003). The NSCLC cell line A549 was obtained from ATCC.

- GLC14, GLC16, and GLC19: These three cell lines were isolated from the tumor of a 55 year old woman suffering from SCLC.
    - GLC14: biopsy from a supraclavicular node at the time of diagnosis – tumor showed a complete response to 5 cycles of CDE therapy (cyclophosphamide/doxorubicin/etoposide)
    - GLC16: tumor (SCLC) recurrence with another 4 cycles of CDE therapy (stopped after the fourth cycle according to the wish of the patient) – biopsy of the orificium of the liungula segment. After radiation therapy the X-Ray was normal
    - GLC19: tumor (SCLC) recurrence - Biopsy of the upper lobe, the area which had been irradiated. Again 2 cycles of CDE therapy were given, but without any response of the tumor

- DMS-153: Cells were isolated during an autopsy from the liver of a 44 year old male. The cells were identified as SCLC. The patient was treated with cytoxan and methotrexate.

- NCI-H417: is an aggressive variant SCLC cell line which is derived from a female without prior treatment and this cell line is refractory to treatment.

- NCI-H526: This cell line was derived from a bone marrow metastasis from a patient prior therapy and was identified as SCLC. This cell line shows no chemo resistance.

- A549: A549 is a NSCLC cell line derived from a 58 year old Caucasian male. (ATCC)

5.2.2. Medium used and cell culture conditions

- GLC14/16/19, NCI-H417, NCI-H526 and DMS-153: RPMI-1640 (with L-glutamine and sodium bicarbonate) supplemented with 10% FBS, 50µg/ml streptomycin and 100µg/ml neomycin and ~50units penicillin/ml

- A549: RPMI-1640 (with L-glutamine and sodium bicarbonate) supplemented with 10% FBS

All reagents and media were warmed up to room temperature before using.
The sterile working bench was washed with 70% EtOH before starting. The cells were checked under a phase contrast microscope to determine cell density and confluency every second day.
The medium of the adherent cells was aspirated and the cells were washed once with 1x PBS. After removing of the PBS 1x trypsin (0.5%) was added. 10x trypsin was diluted with 1xPBS for this purpose. The cell flask was incubated for ~4 minutes at 37°C until all cells became rounded and started to detach. The cell suspension was transferred into a 15ml centrifugation tube and was centrifuged for 3 minutes at 450g. Afterwards, the supernatant was discarded and the cells were diluted in an appropriate amount of medium for splitting. 1/3 up to 1/6 of the cells were transferred into a new cell culture flask with 6ml (for a 25cm^2 flask) or 12ml (for a 75cm^2) medium. The cells were incubated at 37°C and 5% CO_2.
For maintaining suspension cells, a part of the cell-medium suspension was transferred into a new cell culture flask and fresh medium was added.
The medium of semi-adherent cells was transferred into a centrifuge tube and was retained. The residual adherent cells were washed once with 1x PBS and 1x trypsin (0.5%) was added. After 4 minutes incubation at 37°C the cells were added to the cell-medium suspension in the centrifuge tube, and then the samples were treated as described above for the adherent cells.

5.2.3. Treatment of the cells

The cells were treated with chemotherapeutic agents (cisplatin, etoposide, topotecan or in combination) or C207A or zoniporide and then further processed for RNA or protein isolation to determine the influence of the agents on gene expression and the protein level in the cells. These samples were also used for ELISA analysis.

- cisplatin crosslinks guanine and adenine within a DNA strand and between different DNA strands. This cross linking inhibits normal mitosis. Additionally, cisplatin inhibits DNA repair mechanisms and the activity of the telomerase, leading to apoptosis
- etoposide is able to form a complex with the DNA and DNA topoisomerase II. It triggers DNA strands to break up by inhibition of re-ligation.
- topotecan inhibits DNA replication by inhibition of DNA topoisomerase I. A double strand break occurs if the DNA polymerase reaches the complex of topotecan-DNA-DNA topoisomerase I.
- zoniporide dihydrochloride is a selective inhibitor for NHE1. It inhibits the NHE1 dependent $^{22}Na^{+}$ uptake *in vitro.* Formula: $C_{17}H_{16}N_6O.2HCl$
- C207A: is a positively charged sulfonamide which inhibits the zinc binding site in the catalytic domain of CA IX. Formula: $C_{16}H_{21}ClN_2O_6S$

The cells were treated once with the chemotherapeutic agent in different concentrations (0,6µg/ml – 1,25µg/ml – 2,5µg/ml – 5µg/ml). The samples were harvested after three days at 37°C and 5% CO_2 for RNA or protein isolation.

5.3. RNA isolation

- calcium and magnesium free 1x PBS
- 25cm² cell culture flask (Nunc, Roskilde, DK)
- cell scrapers (TPP, Trasadingen, CH)
- peqGold Trifast (PEQLAB Biotechnologie GmbH, Erlangen, GER)
- GIBCO distilled water, RNase/DNase free (Life Technologies, Carlsbad, CA)
- Ethanol (EtOH) (AnalaR NORMAPUR, West Sussex, UK)
- Isopropanol (Merck, Darmstadt, GER)
- Chloroform (Merck, Darmstadt, GER)

- Centrifuge 5415R (Eppendorf AG, Hamburg, GER)
- Hettich Rotante centrifuge (Hettich Bäch, CH)
- 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)

All steps were carried out on a sterile working bench.
Cells in a 25cm^2 culture flask were grown until 90%-95% confluency. For collecting the cells, the medium was removed. Adherent cells were washed twice with 1x PBS and harvested and lysed directly in the flask with 1ml Trifast (1ml Trifast for 5-10*10^6 cells). Soluble cells were centrifuged for 3 minutes at 400g in a Hettich centrifuge. The medium was discarded and the pellet was lysed in 1ml Trifast. Trifast, a phenol and guanidine-isothiocyanate mixture is able to lyse cells, inactivate RNases, and to stabilize the RNA in one step.
The cells lysate was incubated for 5 minutes at room temperature to allow completely lysis of the cells. Afterwards, 200µl chloroform per 1ml Trifast was added. The tubes were shaken manually for 15-20 times and the lysate was incubated again for 5 minutes at room temperature. To separate the RNA from proteins and genomic DNA, the samples were centrifuged in an Eppendorf centrifuge for 15 minutes at 12000g (4°C). After this step, the RNA is in the upper aqueous phase, while the Trifast and chloroform remains in the lower red phase. Genomic DNA and proteins are found in the interphase. The aqueous phase was transferred into a fresh tube and was centrifuged again for 15 minutes at 12000g at 4°C. If genomic DNA or proteins are still in the sample, they will precipitate and form a pellet.
For RNA precipitation, the aqueous phase was transferred in a fresh reaction tube containing 500µl isopropanol. After carefully mixing (repeated pipetting 10x), the samples were kept on ice for 10 minutes, followed by a centrifugation step (10 minutes at 12000g and 4°C). The RNA should be visible as a gel-like pellet in the reaction tube.
The RNA pellet was washed two times with 1ml of 70% EtOH by vortexing and centrifugation for 10 minutes at 12000g and 4°C. The EtOH supernatant was discarded and the pellet was dried for max. 3 minutes in the thermocycler.
The pellet was resolved in 20µl-80µl distilled water, depending on the size of the pellet, by pipetting for a few times. After resolving the pellet, the solution was incubated at 55°C for 5-10 minutes in the thermo-cycler. Thereafter, it was stored at -80°C until use.

To quantify the RNA concentration 1µl of the sample was measured in the Nanodrop using 1µl of distilled RNase/DNase free water as blank. After evaluation of the concentration the RNA, RNA integrity was controlled in a 1.5% agarose gel.

5.4. Agarose gel electrophoresis

- 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)

Agarose gel electrophoresis is suitable to separate DNA and also RNA fragments in a range of 100bp – 25kbp. For this application, the gel concentration varies between 0.5% – 3%, depending on the samples analysed.
Due to the high amount of phosphate in the DNA and RNA backbone, the fragments are negatively charged at a neutral pH and will migrate to the anode.
After RNA isolation, the RNA was checked routinely by agarose gel electrophoresis. By mixing 450mg of agarose and 30ml of TAE buffer, a 1.5% agarose gel was prepared. The mixture was heated in the microwave up to the boiling point until all of the agarose was dissolved. After cooling to approx. 50°C, 1.5µl ethidium bromide (EtBr) was added to a final concentration of 0,5µg/ml and the mixture was poured into the gel electrophoresis chamber. The slots for the samples were formed by combs. After 30 minutes, the gel became polymerized. EtBr intercalates into double stranded nucleotides, and they can be detected under UV light.
Depending on the amount of RNA available, 1µg or 500ng of RNA were used for gel electrophoresis. The RNA was mixed with RNase/DNase free water to give a volume of 10µl and 2µl 6x loading dye was added.
The gel was placed into the submarine unit and 1x TAE buffer was added until the whole gel was covered. 10µl of the sample mixture was applied to one slot of the gel.

After 25 minutes of electrophoresis (constant voltage 100V), the nucleotide bands were visualized under UV light in the Herolab Transilluminator. A sample gel is shown in Fig. 5.

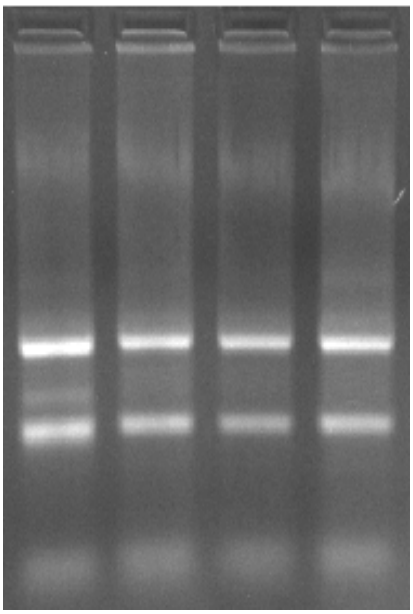

Figure 5 RNA quality assessments by electrophoresis
RNA was isolated from Small Cell Lung Cancer cell lines and was separated on a denaturing 1,5% agarose gel
Upper band: 28S rRNA
Lower band: 18S rRNA
Intact RNA is found in all samples and can be further used for reverse transcription

5.5. Reverse Transcription

- 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)

For real time polymerase chain reaction (qPCR) analysis, 1µg RNA was reverse transcribed into complementary DNA (cDNA) by using the high capacity cDNA reverse transcription kit (Life technologies). The amount of RNA required was diluted in RNase free water to give a total volume of 10µl. A Mastermix was prepared according to the following table:

Reverse Transcription (RT) Mastermix for one sample	
10x RT buffer	2µl
10x primer	2µl
10x dNTP's	0,8µl
reverse transcriptase	1µl
RNase free water	4,2µl
Total volume	10µl

10µl of the RT-Mastermix was added to each of the RNA samples, which were then incubated in the cycler. The following program was applied:

1. 25°C for 10 minutes for enzyme activation
2. 37°C for 120 minutes for the reverse transcription
3. 85°C for 5 minutes for deactivating the enzyme
4. 4°C unlimited

After the reverse transcription, the cDNA was diluted 1:5 by adding 80µl of distilled RNase-free water to obtain a final concentration of 10ng/µl. The cDNA was stored at -20°C.

5.6. Quantitative Real Time PCR - qPCR

- GIBCO distilled water, RNase/DNase free (life technologies, Carlsbad, CA)
- 2x TaqMan Gene Expression Master Mix*
- TaqMan primer/probes:*
    - CA9 – Hs00154208_m1
    - CA12 - Hs01080909_m1
    - SLC16A1/MCT1 - Hs00161826_m1
    - SLC16A3/MCT4 - Hs00358829_m1
    - SLC9A1/NHE1 - Hs01011912_g1
- MicroAmp Fast Optical 96-well Reaction Plate with Barcode (0.1ml)*
- MicroAmp Clear Adhesive Film*
- 7900HT-Fast Real Time PCR System with SDS 2.4 Software*
- geNorm Reference (house keeping) gene selection kit (PrimerDesign, Southhampton, UK)

- Safe-seal tips (Biozym Scientific GmbH, Oldendorf, GER)
- Microsoft Excel 2003 (Microsoft Corporation, Redmond, USA)
- GraphPad Prism 5 (Graphpad, La Jolla, CA)

*all purchased from Life technologies, Carlsbad, CA

5.6.1. Conventional PCR

Polymerase Chain Reaction is a standard method in molecular biology to amplify DNA *in vitro*. To perform the experiment, the sequences of the flanking regions of the gene of interest have to be known as the DNA-polymerase synthesizes the new strands in 5' to 3' direction starting from short complementary oligonucleotides, the primers, on both sides of the sequence of interest.
The DNA-polymerase, a thermo stable Taq-Polymerase, was originally isolated from the thermophilic bacteria *Thermus aquaticus* because the enzyme has to be stable at 95°C. This heating step is essential for the denaturation of the double stranded DNA. Afterwards the temperature is lowered to about 50°C, at which the primers bind to the DNA (annealing). Starting from that, the enzyme synthesizes the complementary new DNA strands (extension). This denaturation and annealing/elongation step is repeated 25-40 times depending on the starting amount of DNA. To evaluate the result of the conventional PCR, a gel electrophoresis analysis must be performed to analyze the product generated.

5.6.2. Real Time PCR

Real time Polymerase Chain Reaction is a sensitive and reproducible quantitative method to analyze gene expression in cells.
This method is able to detect the fluorescence signal generated during the formation of the PRC product.

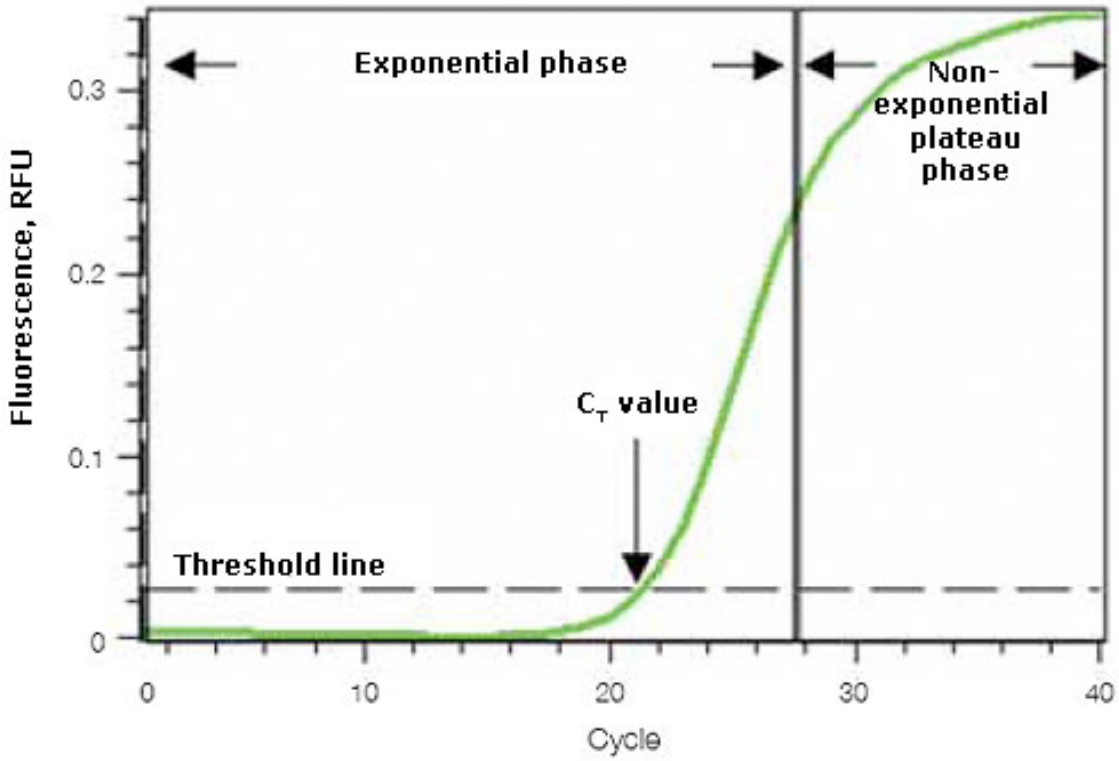

Figure 6 Schematic representation of qPCR data
This scheme shows the Ct value. It represents the cycle in which the fluorescence signal from the PCR product raises off from the background signal. Taken from Bio-Rad Laboratories Inc. Application Guide for Real Time PCR, 2006.

In the conventional PCR, the product formed is measured in the non-exponential plateau phase at the end of the reaction. The reaction is no longer in the exponential phase, but has already reached a plateau, because reaction compounds (dNTP's, primers and polymerases) are already consumed and also a product inhibition is possible. Real Time PCR is able to measure an increase of a fluorescence signal during the experiment, which is proportional to the produced DNA. The cycle number, when the signal gets intense enough to be higher than the background, is called Ct, Cycle threshold (see Fig. 6). The value of Ct depends on the amount of template, which is present at the beginning of the reaction. If a high amount of template is available, the fluorescence signal will start to increase earlier and the Ct value is accordingly low. The fewer templates are available, the higher is the Ct value.

Two commonly used fluorescence dye-based methods are available to create this fluorescence signal: SYBR Green and TaqMan method. SYBR Green is a DNA binding dye, which is able to bind unspecifically to double strand DNA. This increases the fluorescence signal up to 1000x fold.

In this master thesis the TaqMan system was used. This system is based on the fluorescence resonance energy transfer, FRET, method which is summarized in Fig. 7.

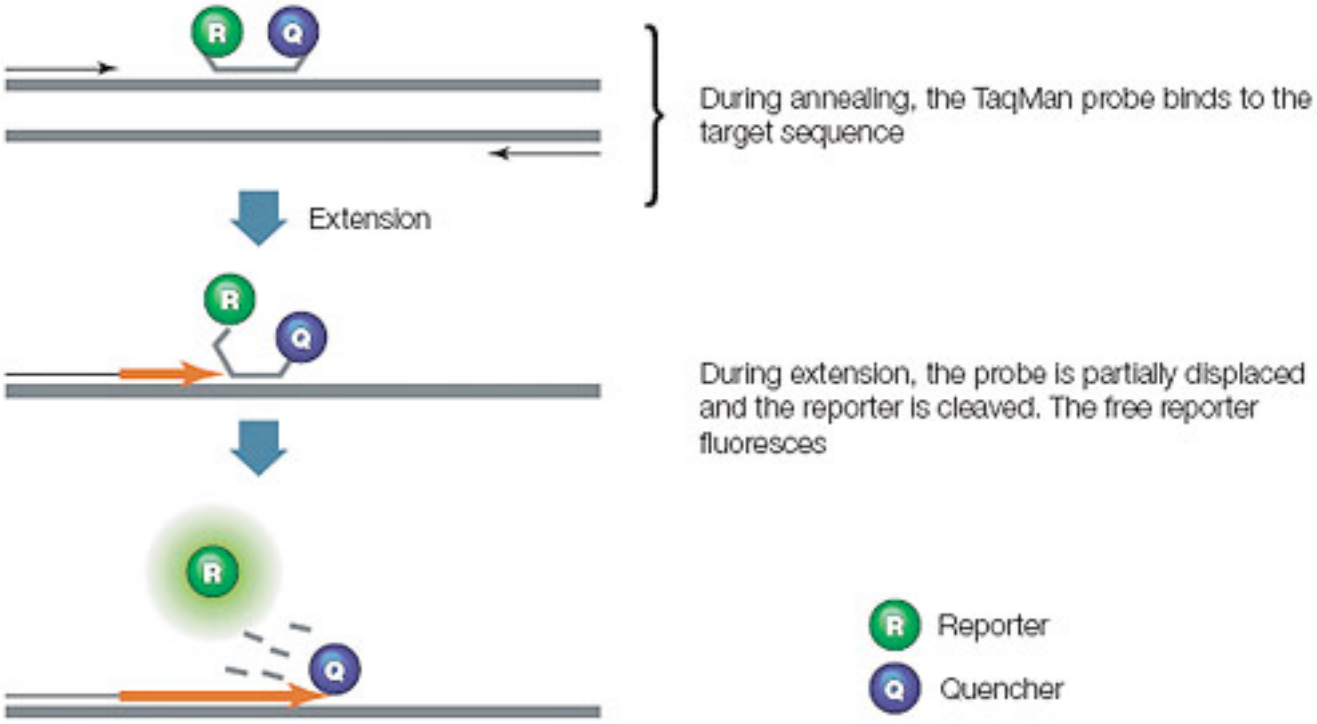

Figure 7 Summary of the TaqMan qPCR method.
A fluorescence labeled oligonucleotide binds to the cDNA. No fluorescence signal can raise through the vicinity of the high energy reporter (R) at the 5' end and a low energy quencher (Q) at the 3' end. After digestion through the DNA polymerase, the distance between reporter and quencher increases and the fluorescence intensity increases. Taken from Bio-Rad Laboratories Inc. Application Guide for Real Time PCR, 2006.

A probe consisting of fluorescence-dye labeled oligonucleotides, is added to the primer solution. The probe carries a high-energy fluorescent reporter at the 5'site and a low energy quencher on the 3'end. As long as the probe doesn't bind specifically to a template, the quencher suppresses the signal of the reporter, because of its vicinity. In the annealing phase of the PCR, the probe binds specifically to the template. Within the extension phase, the 5' → 3' exonuclease activity of the polymerase is able to cleave the reporter from the quencher. This will result in a fluorescence signal, which is proportional to the amount of amplified DNA and which can be detected in the Real Time PCR system (Fig. 7). The commonly used reporter in the TaqMan method is FAM (6-

carboxyfluoresceine), which is quenched by TAMRA (6-carboxy-tetramethyl-rhodamine).

5.6.3. Real-time TaqMan PCR

The first step of the qPCR is the preparation of a MasterMix for each gene of interest. It contains (for one well) the following:

TaqMan Primer/Probe	1µl
2x TaqMan Gene Expression MasterMix	10µl
RNase free H2O	7µl
Total	18µl

Afterwards, 2µl of the cDNA (containing 20ng DNA), was transferred to the optical 96-well plate. Samples were done in duplicates. 18µl of the MasterMix were added to each well and the plate was sealed with a clear adhesive film.
The following settings were used in the Real Time PCR system machine:

Duration	Temperature
2 minutes	50°C
10 minutes	95°C
15 seconds	95°C
1 minute	60°C

The last two steps were repeated 40 times.

The Ct values obtained from the Real Time PCR software, SDS, were transferred into a 2003 Excel sheet in order to calculate the fold changes, as described in Data analysis. The results were transferred into the GraphPad Prism software for further graphical depiction and statistical analysis. GraphPad Prism is a software, which allows good data organisation and understandable statistical calculation. Additionally, it is easily possible to represent the data in graphs.

5.6.4. qPCR - Data analysis

There are two different ways to analyze the qPCR data obtained: the absolute and the relative quantification. In the absolute quantification method, the data are related to a standard curve. It determines the starting copy number of the template in a sample.
In the relative quantification, a change in the gene expression (expressed as fold change of the target gene) in one group is set into relation to the gene expression levels in another group. Such groups could be the treated and untreated groups, respectively.

5.6.5. Relative quantification with the $2^{-(\Delta\Delta Ct)}$ method

To find a suitable house-keeping gene for the calculations, 12 housekeeping genes, were determined by using the geNorm reference gene selection kit. GAPDH (glyceraldehyde-3 phosphate dehydrogenase gene), YWHAZ (14-3-3 protein zeta/delta gene) and TOP1 (DNA topoisomerase 1 gene) showed the most stable expression.

GAPDH
GAPDH is an important enzyme during glycolysis. It catalyses the conversion of glycerine aldheyde – 3 – phosphate to 1,3-bisphosphoglycerate.

YWHAZ
The YWHAZ gene encodes for a 14-3-3 zeta/delta protein, which is able to bind to phosphoserine-containing proteins.

TOP1
The topoisomerase 1 protein is responsible for the alteration of the topological states of the DNA during transcription and replication. It catalyzes the transient break of one strand of the DNA and it is also rejoining the break.

The geometric mean of the Ct values from the housekeeping genes was calculated and subtracted from the Ct of the target resulting in the ΔCt value. Now the ΔCt of the treated samples was subtracted from the ΔCt of the untreated reference group, which is then called ΔΔCt. To receive the fold change (compared to the reference control group) the values were inserted in the formula 2^-(ΔΔCt).

5.7. Protein isolation

- 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, Buchs, CH)
- Insulin syringe 30G needle (Insumed, Artsana, Grandate, IT)
- Hettich Rotante centrifuge (Hettich Bäch, CH)
- 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)
- 96well 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)

Cells were seeded in 25cm² cell flasks and grown until ~95% confluency. After removing the growth medium, the adherent cells were washed twice with ice cold PBS. Afterwards, the cells were lysed directly with RIPA buffer. Depending on the cell number or pellet size, approximately 200µl-300µl RIPA buffer was used for cells in one 25cm² flask. Protease inhibitors must be added to the RIPA buffer before its application to prevent proteolysis (1µl per 1 million cells).
Solubilized cells were centrifuged for 3 minutes at 450g and the pellet was washed twice with ice cold PBS, before the solution was further treated with RIPA buffer.
Additionally, cells in the RIPA buffer were also homogenised mechanically by using a 30G insulin syringe. Afterwards the cells were incubated in the thermomixer at 4°C and the suspension was shaked at 1400 rpm for 30 minutes. The suspension was again centrifuged in the Eppendorf centrifuge for 10 minutes at 10000g and 4°C. The super-

natant which contains the soluble protein fraction was transferred into a new reaction tube and was stored at -20°C.

5.8. Protein quantification by the bicinchoninic acid assay (BCA)

For protein quantification, a modified Lowry method, the BCA (bicinchoninic acid) assay was performed. This method is able do detect protein concentrations in a range from 0.5µg/ml up to 1.5mg/ml. It is based on a colour change depending on the amount of protein.
25µl of the protein solution (if a high concentration of protein is expected, because of many cells or low amounts of RIPA buffer used, a dilution of 1:5 up to 1:10 was performed) was added to a 96-well plate in duplicate as well as the BSA standard curve ranging from 3µg/µl to 0,05µg/µl. The two reagents A and B of the BCA assay were mixed in a 50:1 ratio. 200µl of the mixture were added to each well following by incubation at 37°C for 30 minutes. This reagent mixture contains BCA, sodium carbonate, sodium bicarbonate, BCA detection reagent and sodium tartrate and is able to reduce the Cu^{2+} ions to Cu^{+} by the peptide bonds. In the next step, BCA chelates with Cu^{+} and changes the colour from green to purple depending on the Cu^{+} ions available in the protein solution. This purple solution absorbs light at a wavelength of 562nm, and the absorbance was measured in the M200 Pro from Tecan.

5.9. SDS – Page (Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis) and Western Blot

- 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, Buchs, CH)
- 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)

5.9.1. Sample preparation

Before electrophoresis, proteins must first be mixed with sample buffer (3:1) which contains sodium dodecyl sulphate, SDS, and β-mercaptoethanol.
SDS is a highly negative charged anionic detergent, which binds to the proteins and unfolds them. Approximately 1,4mg SDS binds per mg of protein, which results in a negative net charge of the proteins. Therefore, they migrate in the gel towards the anode. After mixing with sample buffer, the proteins were heated at 37°C for 30 minutes. Normally the heating step would be at 95°C for three minutes, but it is preferable to treat transmembrane proteins (like CA9) at lower temperatures. The hydrogen bonds in a protein structure will be cleaved in this heating step and the proteins will be linearized. Also disulfide bridges will be cleaved by β-mercaptoethanol.

5.9.2. SDS-PAGE, protein transfer to the membrane and detection

22µl of the samples and 6µl of the prestained PageRuler marker were applied to the precast gels from Bio-Rad Laboratories Inc. This gel allows a separation in 10 minutes at 350V.

After the run the gel was washed gently with ddH2O and then the gel box was opened. The transfer system was stacked like shown in Fig. 8 below using a Poly-Vinyli-Dene-Fluoride, PVDF, membrane.

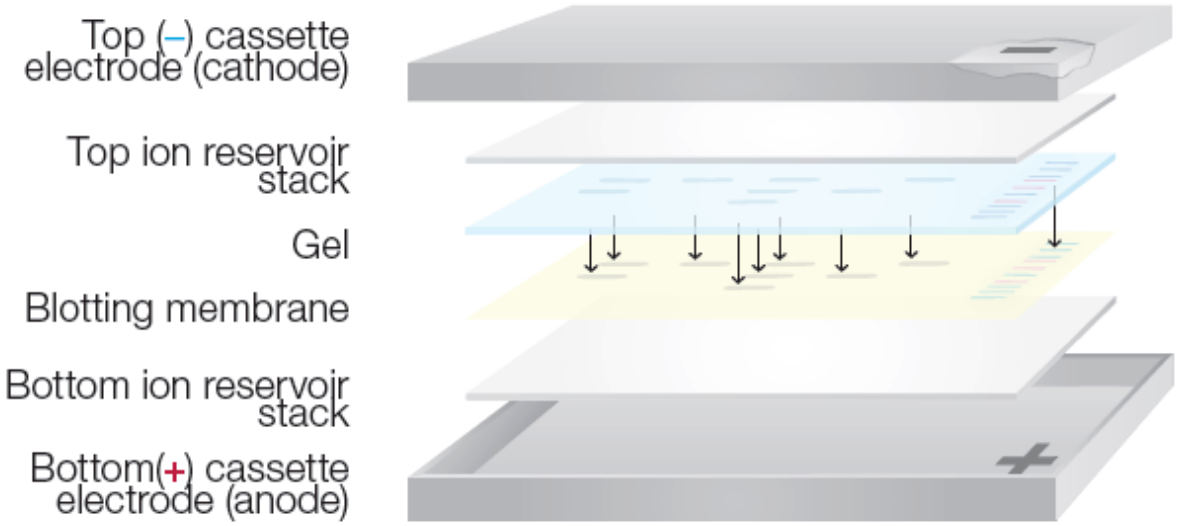

Figure 8 Assembly of the Western Blot sandwich
On the upper and bottom side are ion reservoir stacks. In-between is the SDS-gel (at the cathode site) and the PVDF, polyvinylidenefluoride, membrane (at the anode site).Taken from Bio-Rad Laboratories Inc. TransBlot Turbo instructions

The sandwich was put into the cassette of the Trans-Blot Turbo Transfer System and the “Mixed Molecular Weight” program with constant 1,3A and 25V for 7 minutes was chosen. Proteins migrate to the anode and, because of the hydrophobic interactions and hydrogen bonds, they are able to bind to the membrane. After the transfer, the membrane was put immediately in a ~3% non fatty instant milk PBS-0,05% Tween-20 solution for 30 minutes under constant shaking. Unspecific binding sites of the membrane will be blocked during this time –avoiding a high background signal.

For the specific detection of the proteins of interest, the antibodies were diluted in a 1:1 mixture of blocking solution and PBS-0,05% Tween-20.

The membrane was put into 7ml antibody solution into a plastic dish and was incubated for 1hour under constant shaking. Afterwards, the membrane was washed three times with PBS-0,05% Tween-20 at room temperature - each time for 5 minutes. During this time the secondary antibody, which is conjugated with horseradish peroxidase (HRP), an anti rabbit HRP IgG, was prepared in a 1:5000 dilution – again diluted with a 1:1 mix of blocking solution and PBS-0,05% Tween 20.

After the third washing step, the secondary antibody solution was added to the membrane and incubated for 45 minutes under constant shaking. Two washing steps with

PBS-0,05% Tween 20 at room temperature are following the incubation step with the secondary antibody. The third and last washing step was done with 1x PBS.
The detection solution was prepared by mixing the two substances of the immune star detection kit by 1:1. The membrane was incubated for about 2 minutes. After removing the solution, the membrane was transferred into a plastic wrap avoiding any air bubbles. The plastic wrap was put into the VersaDoc 4000MP and the membrane was exposed, depending on the intensity of the chemiluminescence signal, for 30 seconds up to 15 minutes.
A housekeeping gene must be evaluated on the membrane in order to allow comparison of different bands of proteins of interest, and to control the amount of protein loaded. Glyceraldehyde-3 phosphate dehydrogenase was chosen because of its size, which is 37,5kDa. CA IX has a size around 50-55kDa and can be separated from the housekeeping enzyme. Other housekeeping proteins, like alpha-tubulin (approx. 50kDa) would interfere with this signal.
The intensity of immunoreactive bands was measured with the Imagelab software and was then normalized to the housekeeping gene. The results were expressed as %-shift compared to the untreated control group.

5.10. Chemosensitivity assay

- 96-well microtiter plate (Greiner, Kremsmuenster, Austria)
- Cisplatin and etoposide (SIGMA-ALDRICH, Buchs, CH)
- Heraeus cytoperm 2 (Thermo Fisher Scientific, Asheville, US)
- CA IX inhibitor C207A (obtained from Prof. Claudiu T. Supuran, Firenze, ITA)
- NHE1 inhibitor: Zoniporide (RandD Systems, Minneapolis, MN, USA)
- 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)

One step toward personalized medicine is achieved by chemosensitivity assays. These laboratory tests are able to predict the efficacy of chemotherapies and the response of the tumor in different patients.

$1*10^4$ cells were seeded in 100µl medium into each well of a microtiter plate. Cisplatin, a commonly used cytostatic reagent, and the inhibitors used were added in another 100µl medium. Cisplatin (dissolved in physiologic saline) was diluted in twofold steps, starting with 40µg/ml or 20µg/ml, and was added in triplicates. The inhibitors used were added in concentrations of 2.5µM, 5µM and 10µM, and the samples were incubated for four days at 37°C and 5% CO_2.

After four days, the substrate of one vial from the EZ4U kit was dissolved in 2.5ml activator solution. This solution was warmed up to 37°C and is enough for one 96 well plate. 20µl substrate solution was added to each well and the cells were incubated for 4 hours at 37°C and 5% CO_2.

During this time, the mitochondria of living cells are able to reduce uncolored tetrazolium into intensely colored formazan derivates. Mitochondria of dead cells are inactivated within a few minutes after cell death.

The optical density was measured at 450nm after 4 hours using a microplate reader to determine the %-cell survival after treatment. An empty well was used as blank and non-treated cells with media alone were set to 100% proliferation.

5.11. Enzyme Linked ImmunoSorbent Assay - ELISA for soluble CA IX

- Human Carbonic Anhydrase IX Quantikine ELISA Kit (RandD Systems, 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)

Enzyme linked immunosorbent assay (ELISA) is an antibody based method for the detection of antigens in solutions or liquids. It is a diagnostic tool in medicine as well as a quality control tool in industrial productions. Several different types are used: direct, indirect/ sandwich ELISA

Basically, antigens in a sample are allowed to bind to a solid surface (like the bottom of a microtiter plate – direct ELISA) or to an antibody which is coupled to a solid phase (indirect ELISA). All other binding sites are blocked with a non-reacting protein, such as albumin. After some washing steps, which remove other unbound substances, a second antibody is added which binds also to the antigen but to another epitope. The second antibody is coupled to an enzyme. Again some washing steps are performed and a substrate is added to the sample. The enzymes catalyze, in most cases, a color change within the sample (other possibilities: fluorescence or electrochemical signals). The intensity of the color change depends on the amount of antigen available in the sample and the absorbance can be measured.

All reagents for the soluble carbonic anhydrase IX ELISA were diluted and dissolved as described in the kit manual.
The medium of the cells was transferred into a 15ml centrifuge tube and centrifuged for four minutes at 450g. The supernatant was used as sample.
50µl of the RD1-21 assay diluent were added to each well in duplicate. 100µl of the sample and the standard were added to the wells, and the plate was incubated for 2 hours at room temperature on a microplate shaker (500rpm). Afterwards, the solution was discarded and the wells were washed four times with 400µl wash buffer. 200µl of the carbonic anhydrase IX conjugate were added to each well, and the samples were incubated for another 2 hours at room temperature on the microplate shaker at 500rpm. The four washing steps were repeated after the incubation. 200µl of the substrate solution were added to each well and the samples were incubated for 30 minutes on the benchtop protected from light. Thereafter, 50µl of the stop solution were added to each well, and the color should change from blue to yellow depending on the amount of soluble CA IX in the sample. The intensity of the yellow color was determined at a wavelength of 450nm, and a well with medium alone was used as blank.

5.12. Immunofluorescence microscopy

- calcium and magnesium free 1x PBS
- H_2O and ddH_2O
- paraformaldehyde fixed cells
- Reaction tubes 1.5ml (Biozym Scientific GmbH, Oldendorf, GER)
- Dako Pen *
- Chem Mate Hematoxylin *
- Immuno-Fluor (MP Biomedicals, Inc., Ohio, US)
- Rabbit anti carbonic anhydrase IX antibody 1:1000 (Novus Biologicals, Littelton , US)
- Goat anti rabbit IgG – Alexa Fluor 488 (Molecular Probes, Eugene, OR)
- 4´,6-Diamidin-2´-phenylindoldihydrochlorid - DAPI (Roche Diagnostics GmbH, Bael, SUI)
- BSA – Albumin (PAN Biotech, Aidenbach, GER)
- Centrifuge 5415R (Eppendorf AG, Hamburg, GER)
- Slides (Thermo Scientific, Asheville, US)
- Cover Glass 24x50mm,ThicknessNo.1 (VWR International, Radnor, PA)
- Safe-seal tips (Biozym Scientific GmbH, Oldendorf, GER)
- AXIO Imager Zi (Zeiss, Jena, GER)

*purchased from DakoCytomation, Glostrup, Denmark

Immunofluorescence is a method to localize antigens in a cell or tissue by fluorescently labelled antibodies. Fluorophores which are conjugated to antibodies are able to absorb light or electromagnetic radiation and to emit it with a, in most cases, longer wavelength than the absorbed radiation. This shifted wavelength results in a different colour and can be detected with fluorescence microscopy.

There are two different ways to mark the antigens:

- direct immunofluorescence
- indirect immunofluorescence

In the direct method, the fluorophore is directly labelled to the first antibody and can be detected directly. In the method used for this thesis, the indirect immunofluorescence, a secondary antibody is needed. In a first step, an unlabelled antibody binds specifically to the antigen. In a following step, a secondary antibody, which is labelled with the fluorophore, detects the first antibody, building up an antigen - primary antibody – secondary antibody sandwich, which then can be further analysed.

If not mentioned otherwise, the centrifugation steps were carried out at 400g and 4°C for 7 minutes.
Cells already fixed with paraformaldehyde were washed twice with 1x PBS followed by a washing step with PBS/10% BSA. The cells were centrifuged after each washing step. Even though carbonic anhydrase IX is a transmembrane protein, it is necessary to permeabilize the cells, because the antibody binds to the cytosolic C-terminus of the protein. The cell pellet was resuspended in PBS/10% BSA and incubated for 30 minutes on ice. During the following centrifugation step the primary antibody was prepared by diluting it 1:1000 with PBS/10% BSA. The pellet was resuspended with the antibody dilution and incubated for 30 minutes on ice. It is important to mix the cells after 15 minutes to avoid aggregation of the cells on the bottom of the reaction tube. Excess antibody was removed by a 10 minute centrifugation step at 400g and 4°C. Two washing steps with PBS/10% BSA were performed to eliminate possible residues of the antibody solution.
All light sources were shut down while using the fluorophore-marked antibody. The Alexa 488 marked secondary anti rabbit IgG antibody was diluted 1:1000 with PBS/BSA 10%. The cells were incubated for 45 minutes on ice with the secondary antibody followed by a 10 minute centrifugation step at 400g and 4°C and two washing steps with PBS/10% BSA.
DAPI (4',6-diamidino-2-phenylindole) forms a fluorescent complex by attaching in the minor grove of A-T rich sequences [d(CGACGTCG)]2 of the DNA (Tanious 1992). Additionally, DAPI interacts with G-T rich sequences [d(CGAC**GT**CG)]2 by intercalation. After excitation, the nuclei are stained blue and visible under UV light.
A 1:1000 dilution of DAPI with PBS was prepared and the cell pellet was resuspended in this solution and incubated for 10 minutes on ice. The pellet was resuspended after the centrifugation in an appropriate amount of Immuno-Fluor and was applied to the slide. After covering the samples with a cover glass, they were dried for 4 hours at room temperature. The samples were analyzed under the Zeiss fluorescent microscope. Afterwards, the samples were put to 4°C for long time storing.

6. Results

6.1. Gene expression and Western Blot analysis of GLC14-16-19

All gene expression datasets show the mean fold change from two experiments as well as the standard error of the mean (SEM). * represents significant data:

- * - P<0,05
- ** - P<0,01
- *** - P<0,001

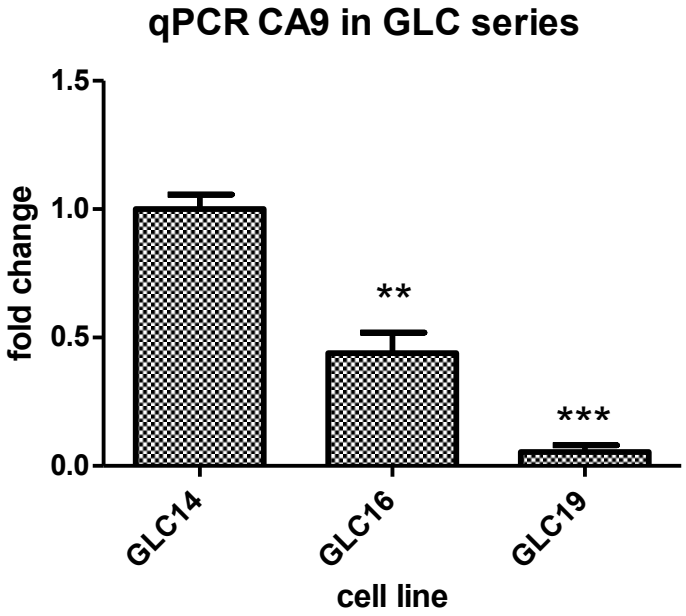

Figure 9 qPCR analysis of CA IX in the GLC14-16-19 cell lines
Gene expression analysis of three different SCLC cell lines derived from one patient at different stages of cancer treatment and development.
GLC14: prior treatment
GLC16: after tumor recurrence
GLC19: without any tumor response to treatment
CA IX expression decreases during therapy to 0,1fold in the therapy resistant tumor
*: P<0,05; **:P<0,01; ***:P<0,001

The gene expression of carbonic anhydrase IX decreases significantly during *in vivo* cyclophosphamide/doxorubicin/etoposide treatment (Fig. 9).

6.1.1. CA IX Western Blot – GLC series

Western Blot analysis shows, after normalisation, a 10% reduced level of CA IX protein in GLC16 cells but no difference in GLC19 cells compared to GLC14 cells (Fig. 10).

cell line	CA IX	GAPDH	**normalised CA IX**
GLC14	1	1	1
GLC16	0.383815	0.48411	0.899705
GLC19	0.73725	0.721099	1.016151

Figure 10 Western Blot and normalisation with GAPDH of GLC14-16-19 cells detecting CA IX protein levels
Left: GLC14 which is set to 100% protein quantity
Middle: GLC16 with a 10% reduced quantity of CA IX compared to GLC14
Right: GLC19 without any difference of protein level compared to GLC14

Immunofluorescence stainings do not appear to reveal differences in the quantity of protein present in the three different cell lines. The localization of CA IX in GLC-19 cells may move into a more cytoplasm localization.

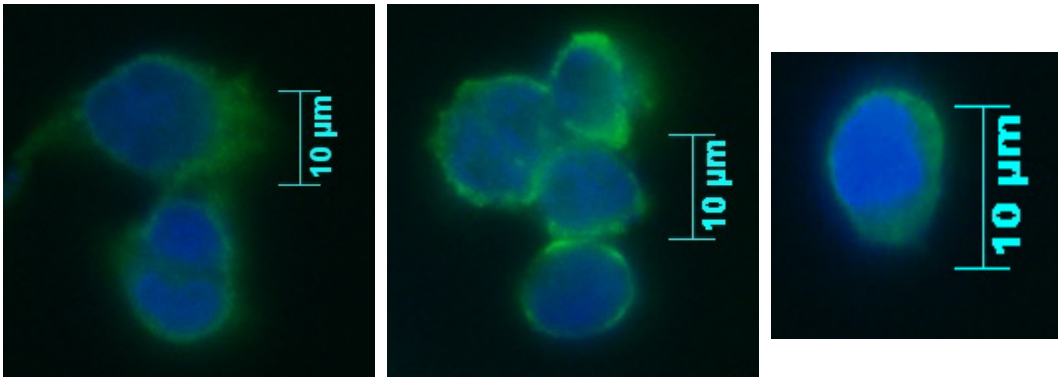

Figure 11 Immunofluorescence of GLC14-16-19 detecting CA IX
Green: carbonic anhydrase IX
Blue: nucleus
Left: GLC14 **Middle:** GLC16 **Right:** GLC19
No difference in intensity of fluorescence can be seen at first glance. The localisation of CA IX in GLC19 maybe has changed to a more cytoplasm site

Quantitative analysis by FACS is planned for the future to determine if there is a difference between the intensity of the fluorescence signal between the three different cell lines.

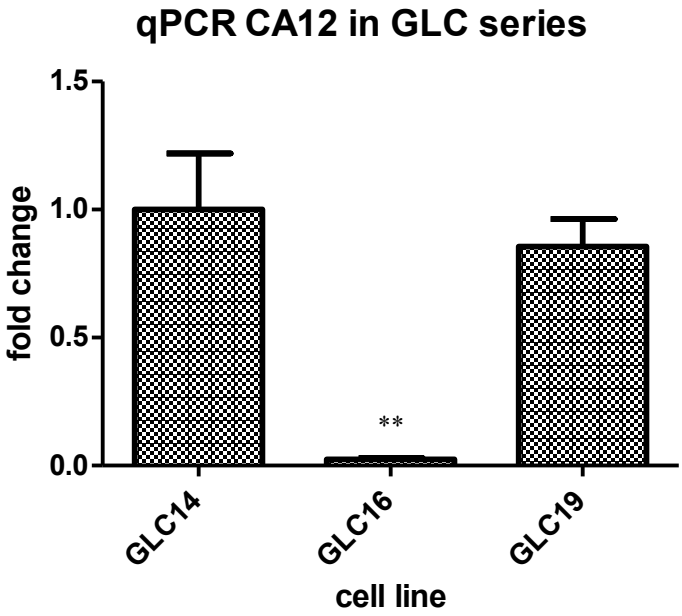

Figure 12 qPCR analysis of CA XII in GLC14-16-19
Gene expression analysis of three different SCLC cell lines derived from one patient at different stages of cancer treatment and development.
GLC14: prior treatment
GLC16: after tumor recurrence
GLC19: without any tumor response to treatment
CA XII expression decreases during cancer treatment (GLC16) to 0,02fold. No difference in gene expression is seen in the therapy resistant stage (GLC19)
*: P<0,05; **:P<0,01; ***:P<0,001

The gene expression of CA XII decreases in the GLC16 cells to 0,02 fold compared to GLC14, but stays at almost the same level in GLC19 as prior treatment (Fig. 12).

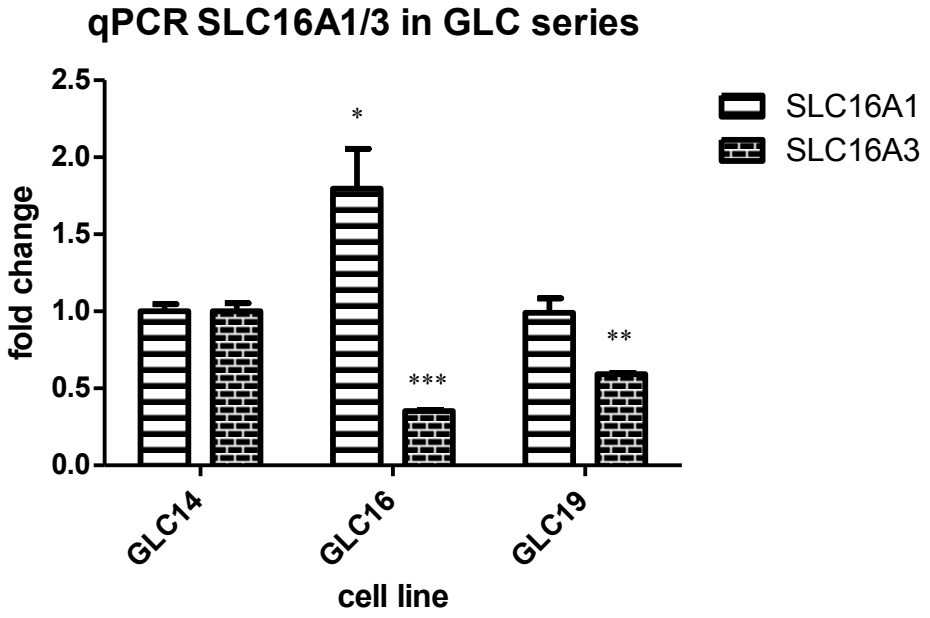

Figure 13 qPCR analysis of monocarboxylate transporters 1 and 4 in GLC14-16-19
Gene expression analysis of three different SCLC cell lines derived from one patient at different stages of cancer treatment and development.
GLC14: prior treatment
GLC16: after tumor recurrence
GLC19: without any tumor response to treatment
SLC16A1 (MCT1) gene expression increases 2fold in GLC16 cells and stays at the same level in GLC19 cells compared to GLC14 cells. SLC16A3 (MCT4) decreases significantly during cancer treatment to 0,45fold in GLC16 and 0,55fold in GLC19
*: P<0,05; **:P<0,01; ***:P<0,001

Different results were obtained in the gene expression analysis of monocarboxylate transporters 1 (SLC16A1) and 4 (SLC16A3). The expression of SLC16A1 nearly increased 2fold in GLC16 compared to GLC14, but falls back to the same level in GLC19 as prior treatment. SLC16A3 expression is decreased in GLC16 to 0,45fold and in GLC19 to 0,55fold compared to GLC14 (Fig. 13).

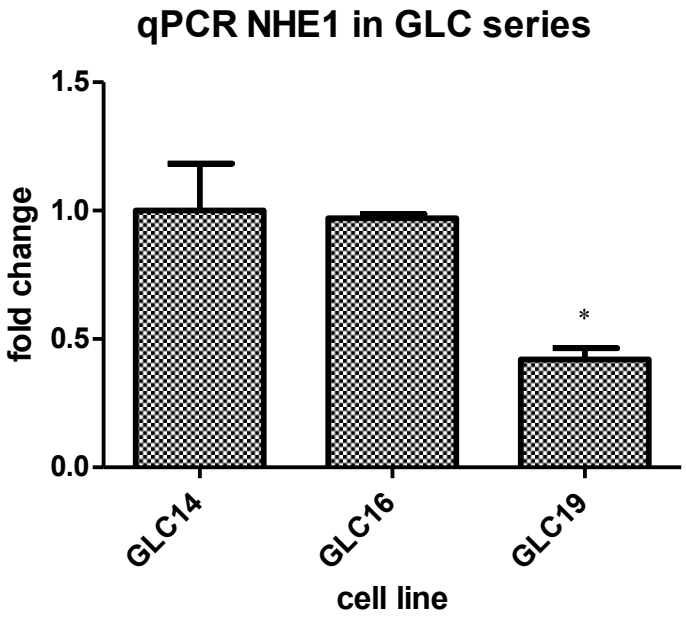

Figure 14 qPCR analysis of sodium proton exchanger NHE1 in GLC14-16-19
Gene expression analysis of three different cell lines derived from one patient at different stages of cancer treatment and development.
GLC14: prior treatment
GLC16: after tumor recurrence
GLC19: without any tumor response to treatment
Gene expression does not change after the first cycle of *in vivo* treatment (GLC16), but significantly decreases to 0,5fold in the therapy resistant tumor GLC19
*: P<0,05; **:P<0,01; ***:P<0,001

NHE1 expression does not change after the first turn of treatment in GLC16, but decreases significantly to 0,5fold after the second-line treatment in the therapy resistant tumor (GLC19) compared to prior treatment (GLC14) (Fig. 14).

6.2. Gene expression and Western Blot analysis of treated SCLC – cell lines

All three cell lines, DMS-153, NCI-H526 and NCI-H417 were treated once with defined concentrations of etoposide, topotecan or cisplatin ranging from 0,6µg/ml to 5µg/ml. The cells were harvested after three days at cell culture conditions, 37°C and 5% CO_2. RNA and proteins were isolated and used for further gene expression and protein analysis.

6.2.1. 0,6µg/ml and 1,25µg/ml cisplatin – influence on CA IX

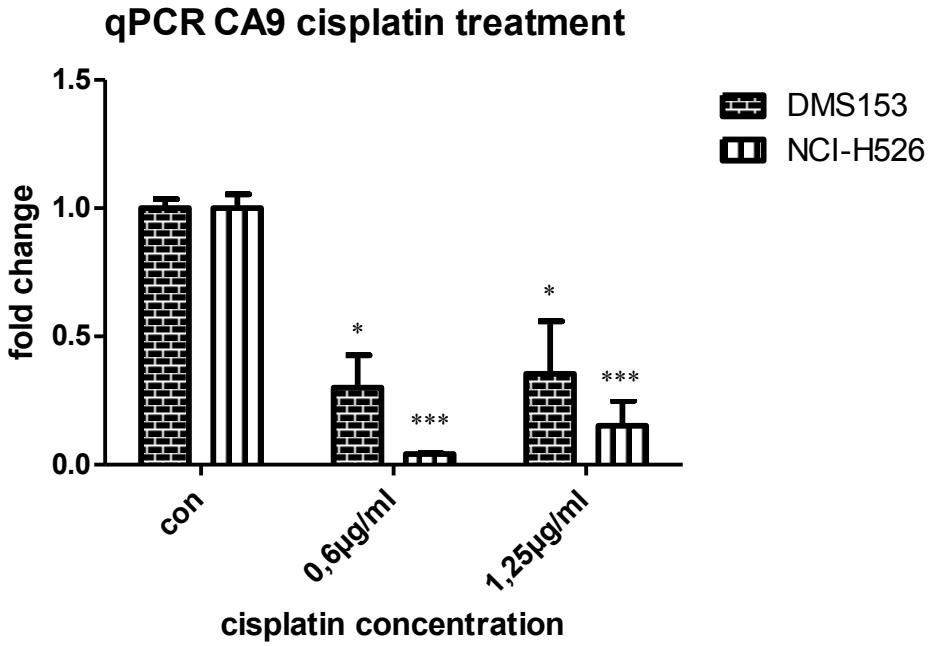

Figure 15 qPCR analysis of CA IX in DMS-153 and NCI-H526 SCLC cell lines in response to cisplatin treatment
con: untreated control group
0,6µg/ml: significantly decreases the gene expression of CA IX in both cell lines to 0,3 fold and 0,1 fold, respectively
1,25µg/ml: has the same effect as the lower concentration and decreases significantly the CA IX gene expression to 0,4 fold and 0,2 fold, respectively
*: P<0,05; **:P<0,01; ***:P<0,001

The CA IX gene expression in both cell lines, DMS-153 and NCI-H526, decreases already at a 0,6µg/ml cisplatin concentration to 0,3 and 0,1 fold and stays suppressed also at 1,25µg/ml (Fig. 15).

6.2.2. Western Blot – DMS-153 cells treated with cisplatin

Western Blot analysis below does not show a significant difference in protein levels in DMS-153 cells with and without treatment (Fig. 16).

cisplatin	CA IX	GAPDH	**normalised CA IX**
DMS153 con	1	1	***1***
DMS153 0.6 cis	1.08	0.97	***1.11***
DMS153 1.25 cis	0.96	1.04	***0.92***

Figure 16 Western Blot and normalisation with GAPDH of cisplatin treated DMS-153 cells detecting CA IX protein levels
Left: untreated control group which is set to 100% protein quantity
Middle: 0,6µg/ml cisplatin shows a 11% increased quantity of CA IX compared to the control
Right: 1,25µg/ml cisplatin shows a 8% decreased quantity of protein level compared to the control

6.2.3. Western Blot – NCI-H526 cells treated with cisplatin

The CA IX protein level in NCI-H526 cells with 1,25µg/ml cisplatin treatment decreases to about 70% of the control group (Fig. 17).

cisplatin	CA IX	GAPDH	**normalised CA IX**
NCI-H526 con	1	1	***1***
NCI-H526 0,6µg/ml	0.901278	0.772493	***1.128785***
NCI-H526 1,25µg/ml	0.6006	0.873871	***0.726729***

Figure 17 Western Blot and normalisation with GAPDH of cisplatin treated NCI-H526 cells detecting CA IX protein levels
Left: untreated control group which is set to 100% protein quantity
Middle: 0,6µg/ml cisplatin shows a 13% increased quantity of CA IX compared to the control
Right: 1,25µg/ml cisplatin shows a 30% decreased quantity of protein level compared to the control

6.2.4. 1,25µg/ml and 2,5µg/ml cisplatin – influence on CA IX

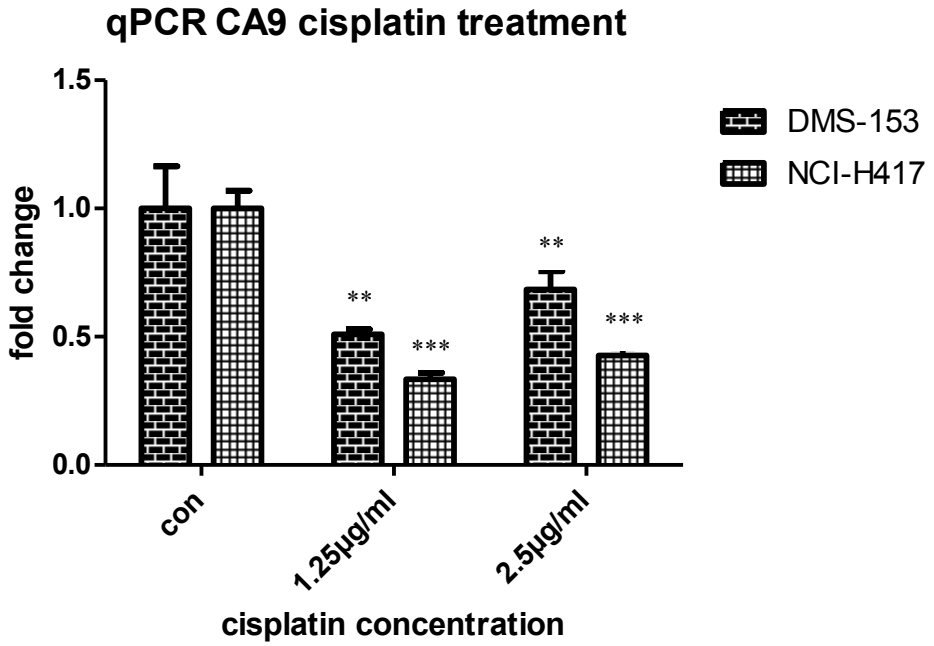

Figure 18 qPCR analysis of CA IX in DMS-153 and NCI-H417 SCLC cell lines in response to cisplatin treatment
con: untreated control group
1,25µg/ml: significantly decreases the gene expression of CA IX in both cell lines to 0,5fold with a stronger effect in NCI-H417 cells
2,5µg/ml: does not have the strong effect like with the lower concentration. CA IX gene expression decreases in DMS-153 and NCI-H417 cells to 0,6 fold and 0,35 fold, respectively
*: P<0,05; **:P<0,01; ***:P<0,001

CA IX gene expression in DMS-153 and NCI-H417 cell lines decreases with 1,25µg/ml cisplatin to 0,5fold compared to the control group – with a stronger effect in NCI-H417 cells in which the expression decreases to 0,35fold. A higher concentration of cisplatin, 2,5µg/ml, does not have the same strong effect as the lower concentration in DMS-153 cells where the gene expression is just reduced to 0,7fold. The expression in NCI-H417 cells with 2,5µg/ml cisplatin decreases to 0,5fold (Fig. 18).

6.2.5. Western Blot – NCI-H417 cells with cisplatin

Western Blot analysis of cisplatin treated NCI-H417 cells shows a quantity of CA IX protein decreased by about 13%-14% with both concentrations used (Fig. 19).

cisplatin	CA IX	GAPDH	**normalised CA IX**
NCI-H417 con	1	1	***1***
NCI-H417 1,25µg/ml	0.728774	0.851337	***0.877437***
NCI-H417 2,5µg/ml	0.785028	0.924322	***0.860706***

Figure 19 Western Blot and normalisation with GAPDH of cisplatin treated NCI-H417 cells detecting CA IX protein levels
Left: untreated control group which is set to 100% protein quantity
Middle: 1,25µg/ml cisplatin shows a 13% decreased quantity of CA IX compared to the control
Right: 2,5µg/ml cisplatin shows a 14% decreased quantity of protein level compared to the control
*: P<0,05; **:P<0,01; ***:P<0,001

6.2.6. Influence of 2,5µg/ml and 5µg/ml cisplatin on CA IX gene expression

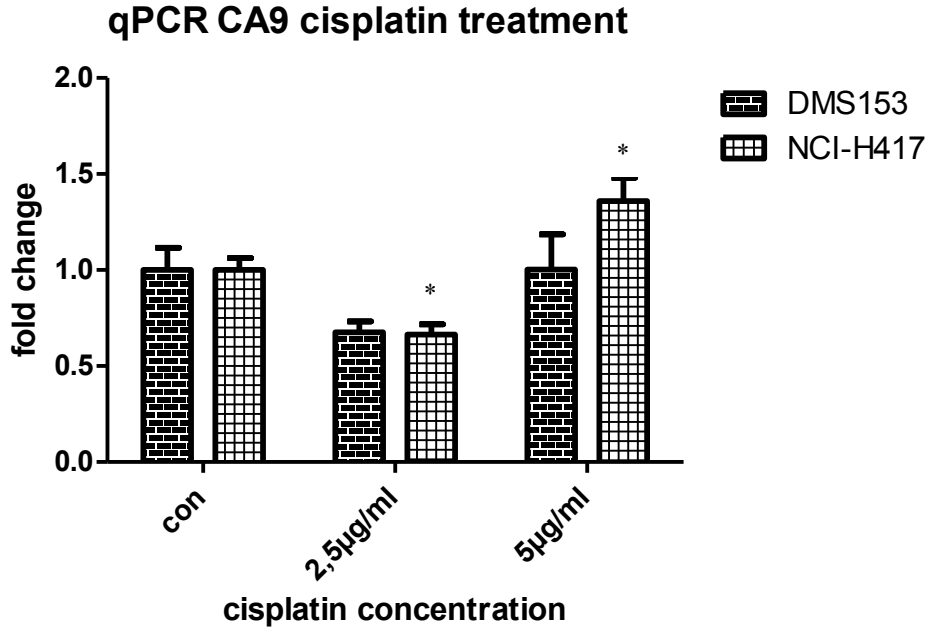

Figure 20 qPCR analysis of CA IX in DMS-153 and NCI-H417 SCLC cell lines in response to cisplatin treatment
con: untreated control group
2,5µg/ml: decreases CA IX gene expression in both cell lines to 0,6fold
5µg/ml: shows inconsistent results when compared to the lower concentration. No effect is seen in DMS-153 cells, while the gene expression slightly increases in NCI-H417
*: P<0,05; **:P<0,01; ***:P<0,001

Again, the expression of CA IX decreases slightly at 2.5µg/ml, but the higher concentration of cisplatin, 5µg/ml, has no effect in DMS-153 cells and even increases CA IX expression in NCI-H417 (Fig. 20).

6.2.7. Summary CA IX expression with cisplatin treatment

Low concentrations of cisplatin, 0,6µg/ml and 1,25µg/ml, have stronger effects in decreasing the gene expression of carbonic anhydrase IX, especially in NCI-H26 cells. Higher concentrations are not able to achieve the same effect - 5µg/ml cisplatin even increases, slightly, the gene expression of CA IX in NCI-H417 cells.

6.2.8. Influence of 0,6µg/ml and 1,25µg/ml cisplatin on CA XII gene expression

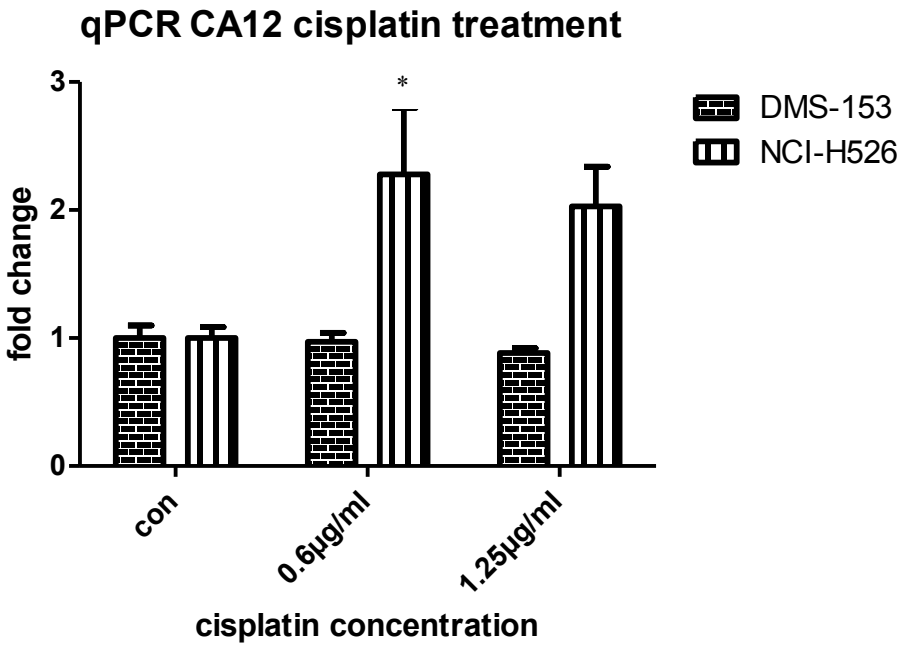

Figure 21 qPCR analysis of CA XII in DMS-153 and NCI-H526 SCLC cell lines in response to cisplatin treatment
con: untreated control group
0,6µg/ml: has no effect on the gene expression in DMS-153 cells. NCI-H526 cells have a 2,3fold increased CA XII expression in response to cisplatin
1,25µg/ml: has no effect on the gene expression in DMS-153 cells. CA XII gene expression is increased 2,1fold in NCI-H526 cells
*: P<0,05; **:P<0,01; ***:P<0,001

While the gene expression of CA XII in DMS-153 cells stays at the same level with cisplatin treatment, it increases in NCI-H526 cells up to 2,3fold in response to cisplatin (Fig. 21).

6.2.9. Influence of 2,5µg/ml and 5µg/ml cisplatin on CA XII gene expression

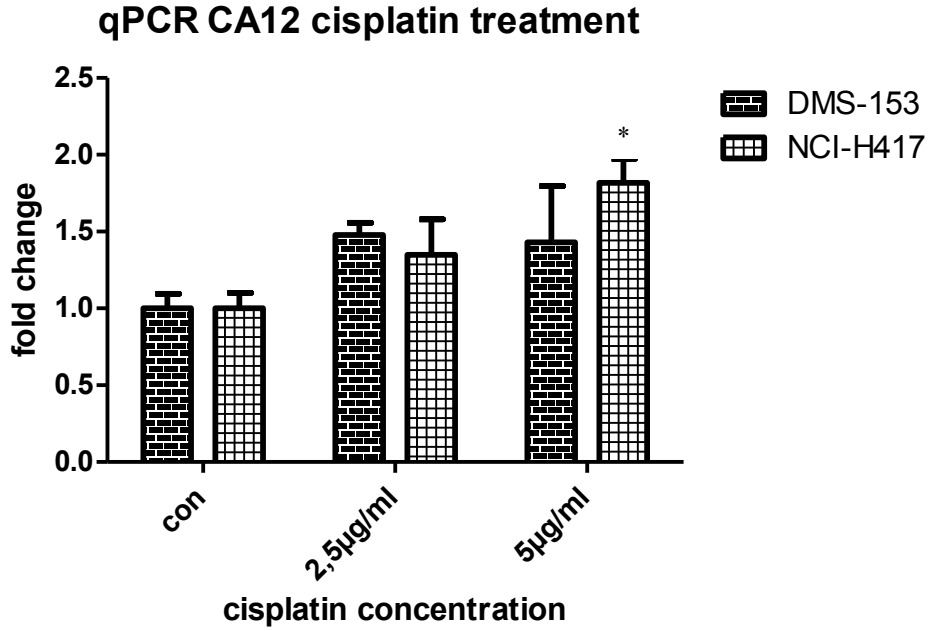

Figure 22 qPCR analysis of CA XII in DMS-153 and NCI-H417 SCLC cell lines in response to cisplatin treatment
con: untreated control group
2,5µg/ml: increases CA XII gene expression in both cell lines
5µg/ml: increases CA XII gene expression in DMS-153 and NCI-H417 cells with a significant increase to nearly 2fold in NCI-H417 cells
*: P<0,05; **:P<0,01; ***:P<0,001

A higher concentration of cisplatin increases the gene expression of CA XII in DMS-153 cells to 1,5fold. The same effect is seen in NCI-H417 cells with a significant 2fold increased gene expression in response to 5µg/ml cisplatin when compared to the control group (Fig. 22).

6.2.10. Summary CA XII expression with cisplatin treatment

Lower concentrations of cisplatin, 0,6µg/ml and 1,25µg/ml, have no effects on the gene expression of CA XII in DMS-153 cells while these concentrations are able to increase the gene expression 2fold in NCI-H526 cells.
Higher concentrations of cisplatin, 2,5µg and 5µg/ml, are able to increase the gene expression of carbonic anhydrase XII in DMS-153 and NCI-H417 cells to 1,5fold and 2fold, respectively.

6.2.11. Influence of 0,6µg/ml and 1,25µg/ml cisplatin on SLC16A1 gene expression

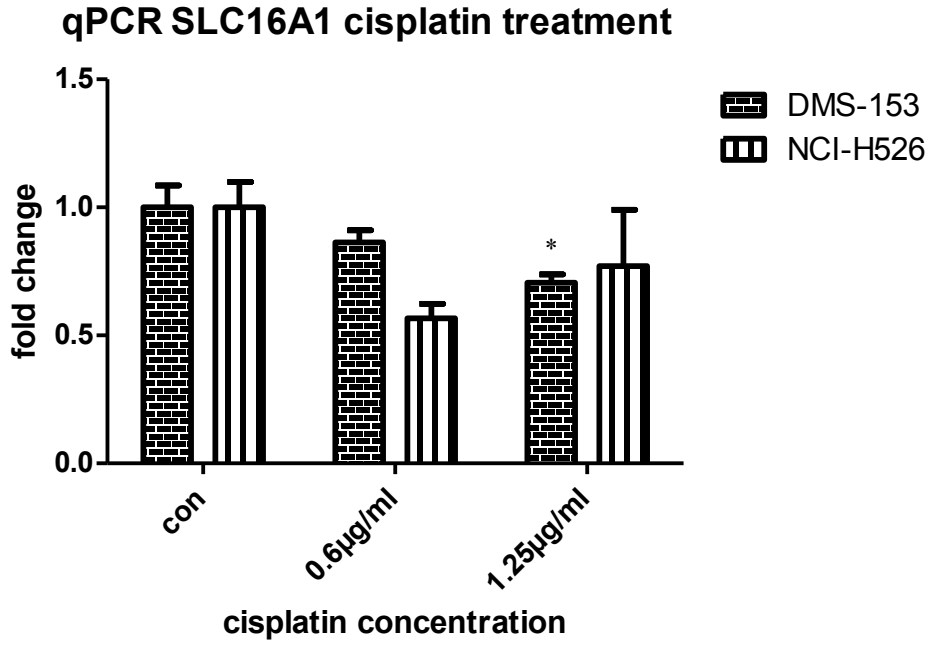

Figure 23 qPCR analysis of SLC16A1 (MCT1) in DMS-153 and NCI-H526 SCLC cell lines in response to cisplatin treatment
con: untreated control group
0,6µg/ml: reduces the SLC16A1 gene expression in NCI-H526 cells to 0,5fold and slightly decreases it in DMS-153 cells
1,25µg/ml: has a stronger effect on DMS-153 cells than the lower concentration and decreases gene expression to 0,8fold in both cell lines
*: P<0,05; **:P<0,01; ***:P<0,001

0,6µg/ml cisplatin is able to decrease the gene expression of SLC16A1 in NCI-H526 cells to nearly 0,5fold compared to the control group. The higher concentration does not achieve the same strong effect like the lower concentration and decreases the expression just to 0,8fold compared to the control group. These results are not significant in a first experiment.

In DMS-153 cells the gene expression does not change with 0,6µg/ml cisplatin and decreases with higher concentrations to about 0,75fold compared to the control group (Fig. 23).

6.2.12. Influence of 2,5µg/ml and 5µg/ml cisplatin on SLC16A1 gene expression

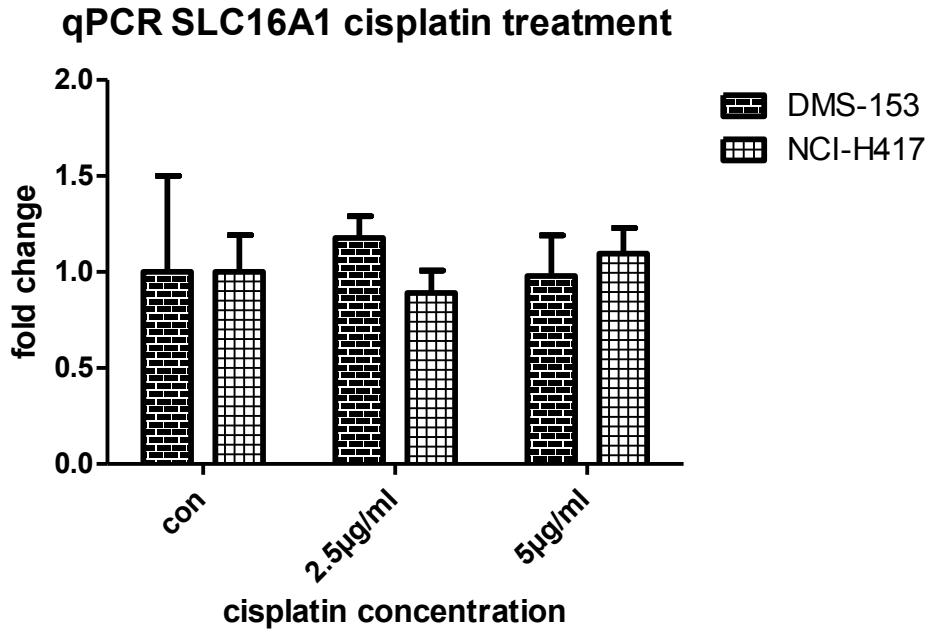

Figure 24 qPCR analysis of SLC16A1 (MCT1) in DMS-153 and NCI-H417 SCLC cell lines in response to cisplatin treatment
con: untreated control group
2,5µg/ml and 5µg/ml cisplatin do not show significant changes in SLC16A1 gene expression in both cell lines
*: P<0,05; **:P<0,01; ***:P<0,001

Higher concentrations of cisplatin, 2,5µg/ml and 5µg/ml, do not have any significant effect on the gene expression of SLC16A1 in DMS-153 and NCI-H417 cells (Fig. 24).

6.2.13. Summary SLC16A1 gene expression with cisplatin treatment

In NCI-H526 cells a reduction of gene expression is seen at low concentrations, 0,6µg/ml and 1,25µg/ml, of cisplatin. 1,25µg/ml cisplatin is able to reduce the SLC16A1 gene expression in DMS-153 to 0,75fold but higher concentrations of cisplatin, 2,5µg/ml and 5µg/ml, do not have significant effects on the gene expression in DMS-153 and NCI-H417 cells.

6.2.14. Influence of 0,6µg/ml and 1,25µg/ml cisplatin on SLC16A3 gene expression

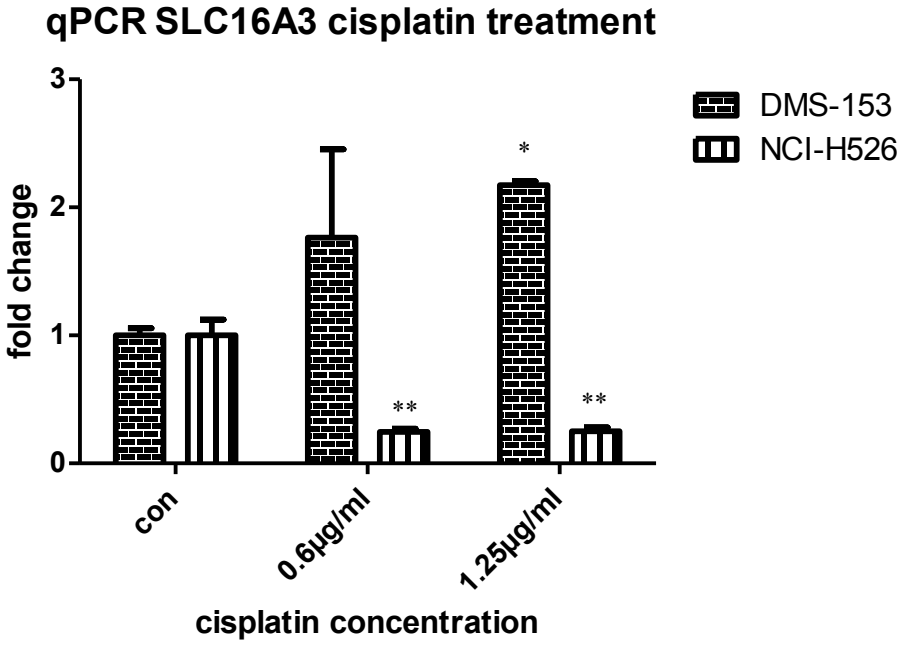

Figure 25 qPCR analysis of SLC16A3 (MCT4) in DMS-153 and NCI-H526 SCLC cell lines in response to cisplatin treatment
con: untreated control group
0,6µg/ml: achieves different results: MCT4 gene expression increases to 2fold in DMS-153 cells and decreases to 0,2fold in NCI-H526 cells
1,25µg/ml: significantly increases the SLC16A3 gene expression in DMS-153 cells to 2,2fold and decreases it to 0,2fold in NCI-H526 cells
*: P<0,05; **:P<0,01; ***:P<0,001

Already 0,6µg/ml cisplatin significantly decreases the gene expression of SLC16A3 in NCI-H526 cells to 0,2 fold compared to the control group and stays at the same level with 1,25µg/ml. An opposed effect is seen in DMS-153 cells in which the expression increases significantly with higher treatment concentrations of cisplatin to 2,2 fold (Fig. 25).

6.2.15. Influence of 2,5µg/ml and 5µg/ml cisplatin on SLC16A3 gene expression

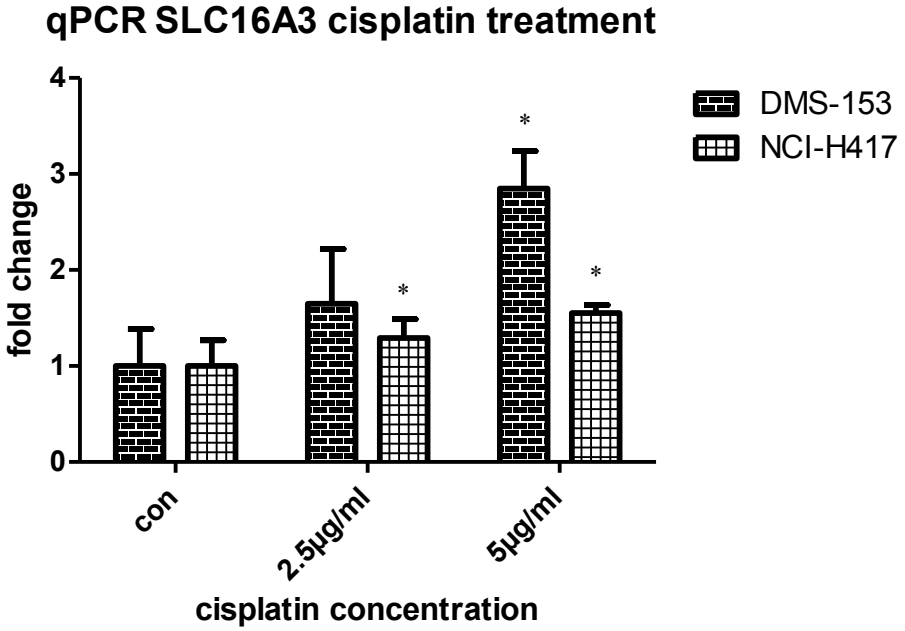

Figure 26 qPCR analysis of SLC16A3 (MCT4) in DMS-153 and NCI-H417 SCLC cell lines in response to cisplatin treatment
con: untreated control group
2,5µg/ml: increases the SLC16A3 gene expression in both cell lines with a stronger effect in DMS-153 cells
5µg/ml: increases the SLC16A3 gene expression in DMS-153 cells to 3fold and in NCI-H417 cells to 1,8fold
*: P<0,05; **:P<0,01; ***:P<0,001

The influence of 5µg/ml cisplatin on the gene expression of SLC16A3 in DMS-153 cells is higher than lower concentrations – it increases the gene expression to 3fold when compared to the control group. SLC16A3 expression in NCI-H417 cells increases the SLC16A3 gene expression significantly to 1,8fold (Fig. 26).

6.2.16. Summary SLC16A3 expression with cisplatin treatment

While the gene expression of SLC16A3 decreases to 0,1fold with 0,6µg/ml and 1,25µg/ml cisplatin in NCI-H526 cells, it increases in DMS-153 cells where it reaches with lower concentrations a 2fold increased expression. With 5µg/ml cisplatin it already increases to 3fold.
The SLC16A3 gene expression increases in NCI-H417 cells with cisplatin treatment to about 1,5fold compared to the untreated control group.

6.2.17. Influence of 0,6µg/ml and 1,25µg/ml cisplatin on NHE1 gene expression

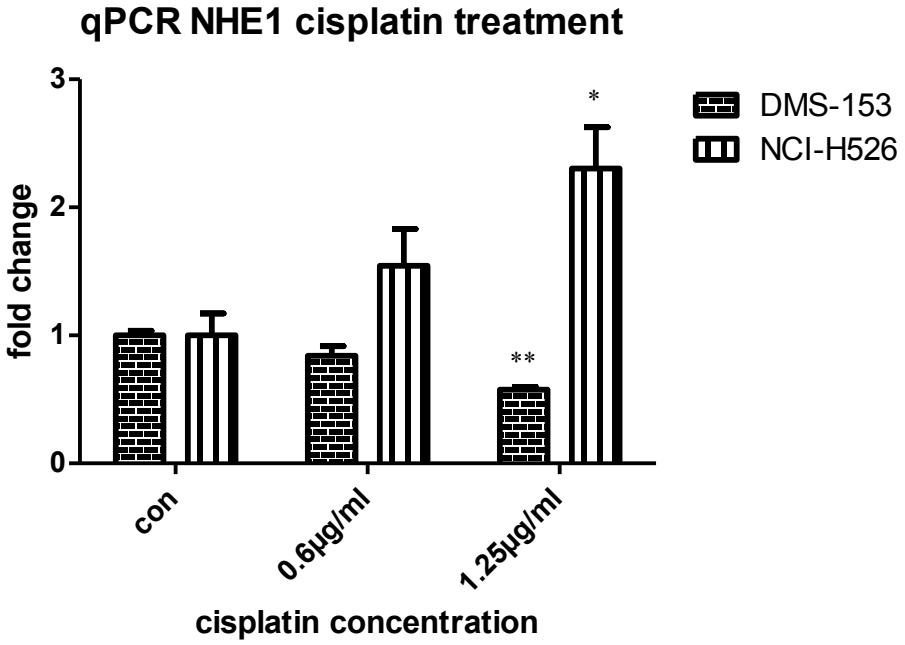

Figure 27 qPCR analysis of sodium proton exchanger NHE1 in DMS-153 and NCI-H526 SCLC cell lines in response to cisplatin treatment
con: untreated control group
0,6µg/ml: increases NHE1 gene expression in NCI-H526 cells to 1,5fold and has no effect in DMS-153 cells
1,25µg/ml: increases NHE1 gene expression in NCI-H526 cells to 2,5fold and decreases it in DMS-153 cells to 0,5fold
*: P<0,05; **:P<0,01; ***:P<0,001

Different results are observed at the gene expression analysis of the key regulator NHE1 in response to low cisplatin concentrations in DMS-153 and NCI-H526 cells. While the expression in DMS-153 cells decreases to 0,5fold, it increases in NCI-H526 cells to nearly 2,5fold when compared to the control group (Fig. 27).

6.2.18. Influence of 1,25µg/ml and 2,5µg/ml cisplatin on CA IX gene expression

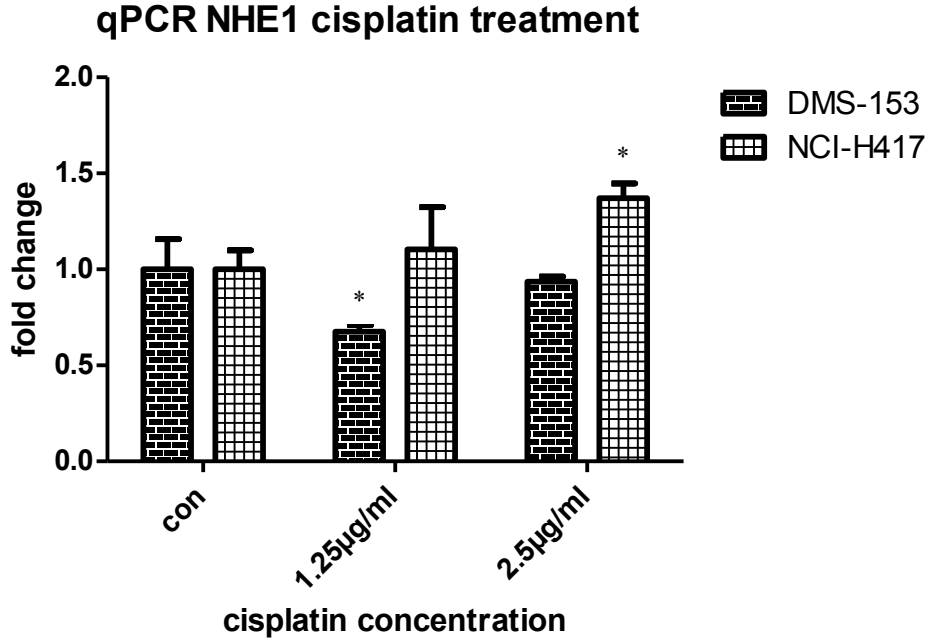

Figure 28 qPCR analysis of sodium proton exchanger NHE1 in DMS-153 and NCI-H417 SCLC cell lines in response to cisplatin treatment
con: untreated control group
1,25µg/ml: decreases NHE1 gene expression in DMS-153 cells to 0,6fold and has no effect in NCI-H417 cells
2,5µg/ml: increases NHE1 gene expression in NCI-H417 cells to 1,5fold and has no effect in DMS-153 cells
*: P<0,05; **:P<0,01; ***:P<0,001

While the gene expression of NHE1 decreases at 1,25µg/ml cisplatin in DMS-153 cells to 0,6fold, it stays almost at the same level like in the control group with 2,5µg/ml. The treatment slightly increases the gene expression in NCI-H417 cells to 1,5fold (Fig. 28).

6.2.19. Influence of 2,5µg/ml and 5µg/ml cisplatin on NHE1 gene expression

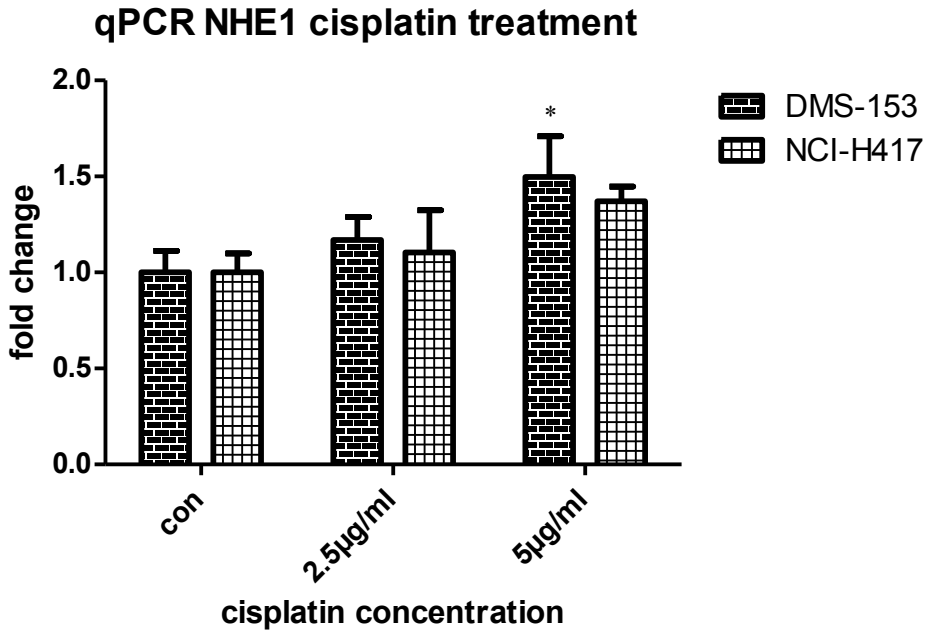

Figure 29 qPCR analysis of sodium proton exchanger NHE1 in DMS-153 and NCI-H417 SCLC cell lines in response to cisplatin treatment
con: untreated control group
2,5µg/ml: has no effect in NHE1 gene expression in both cell lines
5µg/ml: increases NHE1 gene expression in DMS-153 cells to 1,5fold and in NCI-H417 cells to 1,4fold
*: P<0,05; **:P<0,01; ***:P<0,001

5µg/ml cisplatin is able to increase the NHE1 gene expression significantly to 1,5fold when compared to the control group in DMS-153 cells. The slightly increase of the gene expression in NCI-H417 with 5µg/ml is not significant (Fig. 29).

6.2.20. Summary NHE1 expression with cisplatin treatment

0,6µg/ml and 1,25µg/ml cisplatin increases the NHE1 expression in NCI-H526 cells to nearly 2,5fold with the higher concentration. 1,25µg/ml cisplatin is able to decrease the gene expression of NHE1 in DMS-153 cells to almost 0,5fold while higher concentrations, 5µg/ml cisplatin, increase the gene expression to 1,5fold. The gene expression in NCI-H417 cells slightly increases with all used cisplatin concentrations without any significant data.

6.2.21. Etoposide – influence on CA IX gene expression

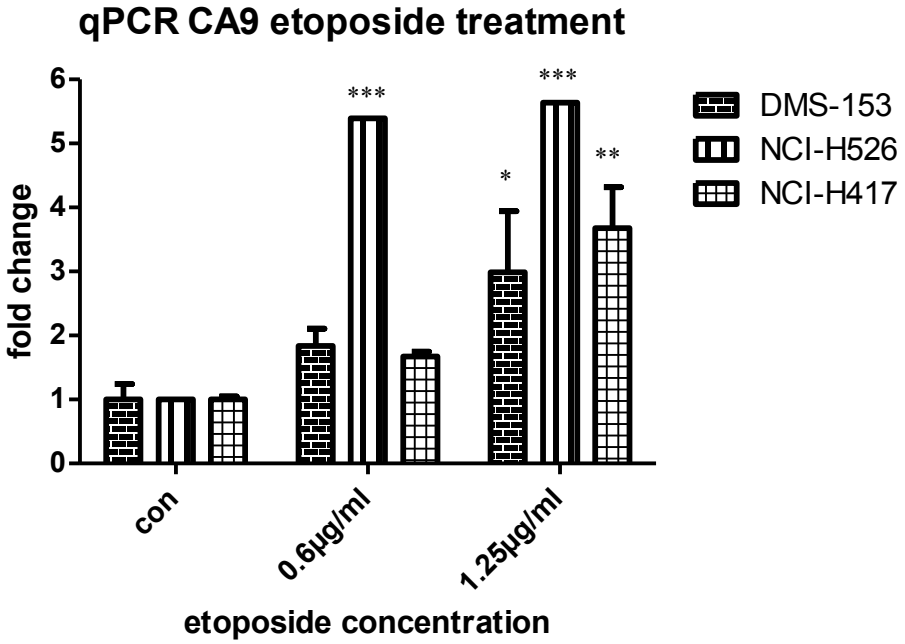

Figure 30 qPCR analysis of CA IX in DMS-153. NCI-H526 and NCI-H417 SCLC cell lines in response to etoposide treatment
con: untreated control group
0,6µg/ml: increases CA IX gene expression to 2fold in DMS-153 and NCI-H417 cells. In NCI-H526 cells it increases significantly to 5,2fold
1,25µg/ml: increases CA IX gene expression significantly on all three cell lines. In DMS-153 cells it increases to 3fold – in NCI-H417 cells to 3,5fold – in NCI-H526 cells to 6fold when compared to the control groups
*: P<0,05; **:P<0,01; ***:P<0,001

A significant increase of CA IX gene expression occurs in all three cell lines with etoposide treatment. In NCI-H526 cells it increased the strongest to 6fold when compared to the control group with 0,6µg/ml and 1,25µg/ml etoposide. CA IX expression in DMS-153 increases to 2fold with 0,6µg/ml etoposide. The higher concentration, 1,25µg/ml, achieves a 3fold increased gene expression. The same effect is seen in NCI-H417 cells where the expression almost increases 2fold at the lower concentration. 1,25µg/ml etoposide increases the gene expression to 3,5fold when compared to the control group (Fig. 30).

6.2.22. CA IX Western Blot – DMS-153 cells in response to etoposide

Western Blot analysis shows an increased CA IX protein quantity by about 20% in DMS-153 cells in response to 0,6µg/ml etoposide treatment. With 1,25µg/ml etoposide the protein level is increased by about 12% compared the control group (Fig. 31).

	CA IX	GAPDH	normalised CA IX
DMS153 con	1	1	**1**
DMS153 0.6 etopo	1.14	0.94	**1.2**
DMS153 1.25 etopo	1.09	0.97	**1.12**

Figure 31 Western Blot and normalisation with GAPDH of etoposide treated DMS-153 cells detecting CA IX protein levels
Left: untreated control group which is set to 100% protein quantity
Middle: 0,6µg/ml etoposide increases CA IX protein quantity by about 20%
Right: 1,25µg/ml etoposide treated DMS-153 cells have a 12% increased quantity of CA IX protein

6.2.23. Etoposide – influence on CA XII gene expression

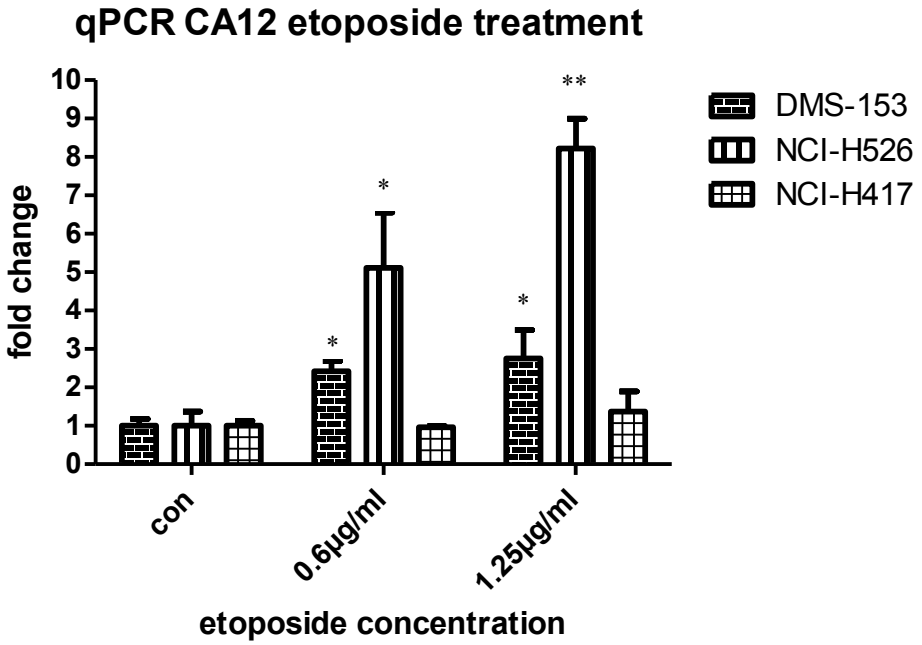

Figure 32 qPCR analysis of CA IX in DMS-153, NCI-H526 and NCI-H417 SCLC cell lines in response to etoposide treatment
con: untreated control group
0,6µg/ml: increases CA XII gene expression significantly in DMS-153 and NCI-H526 cells to 2,5fold and 5fold, respectively. In NCI-H417 cells no change in CA XII gene expression is detectable.
1,25µg/ml: increases CA XII gene expression significantly in DMS-153 and NCI-H526 cells to 3fold and 8fold, respectively. In NCI-H417 cells no change in CA XII gene expression is detectable
*: P<0,05; **:P<0,01; ***:P<0,001

While the gene expression of CA XII in NCI-H417 cells with etoposide treatment stays at the same level with and without therapy, it increases in DMS-153 up to 3fold and especially in NCI-H526 cells up to 8fold in response to 1,25µg/ml etoposide when compared to the untreated control group (Fig. 32).

6.2.24. Etoposide – influence on SLC16A1 gene expression

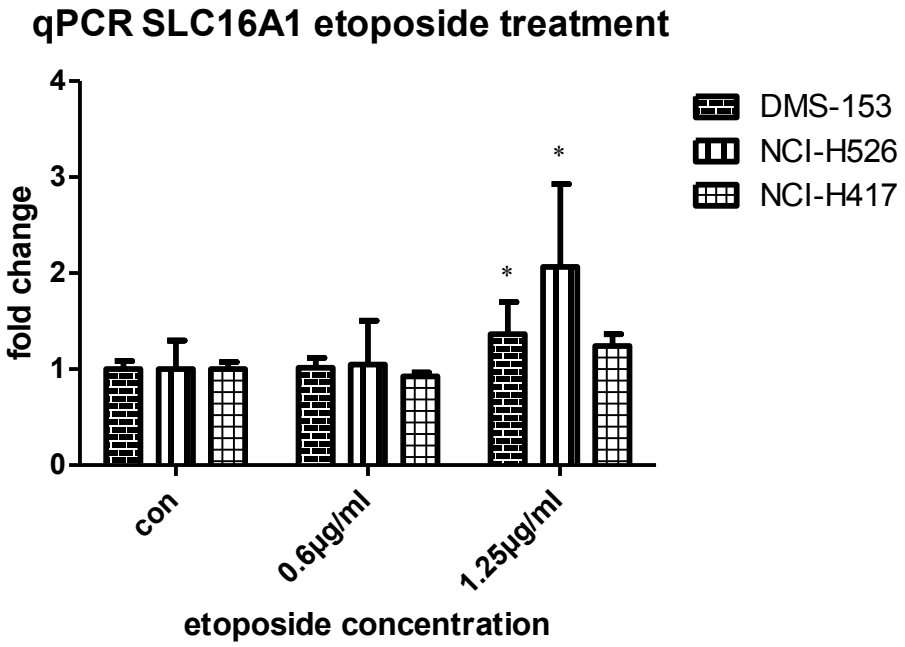

Figure 33 qPCR analysis of SLC16A1 (MCT1) in DMS-153, NCI-H526 and NCI-H417 SCLC cell lines in response to etoposide treatment
con: untreated control group
0,6µg/ml: no changes in MCT1 gene expression is visible in all three cell lines
1,25µg/ml: increases MCT1 gene expression significantly in DMS-153 and NCI-H526 cells to 1,3fold and 2fold, respectively. Gene expression does not change significantly in NCI-H417 cells
*: P<0,05; **:P<0,01; ***:P<0,001

A low concentration of etoposide, 0,6µg/ml, do not change the gene expression in all three tested cell lines. A higher concentration, 1,25µg/ml, increases gene expression in NCI-H526 cells to 2fold and slightly increases it in DMS-153 and NCI-H417 cells (Fig. 33).

6.2.25. Etoposide – influence on SLC16A3 gene expression

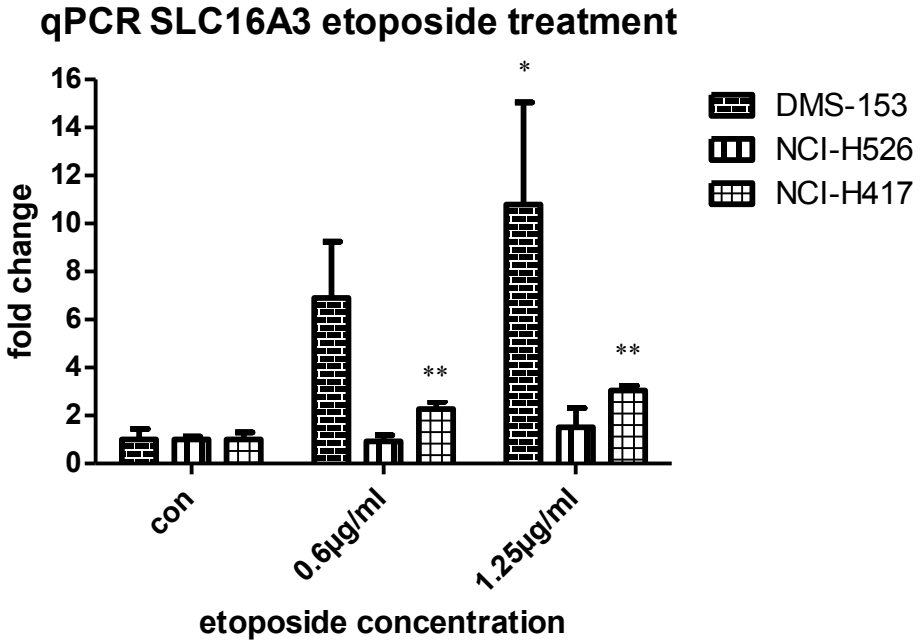

Figure 34 qPCR analysis of SLC16A3 (MCT4) in DMS-153, NCI-H526 and NCI-H417 SCLC cell lines in response to etoposide treatment
con: untreated control group
0,6µg/ml: increases MCT4 gene expression in DMS-153 cells and NCI-H417 cells to 7fold and 2fold, respectively. No change in MCT4 gene expression in visible in NCI-H526 cells
1,25µg/ml: increases MCT1 gene expression significantly in DMS-153 and NCI-H417 cells to 11fold and 3fold, respectively. No change in MCT4 gene expression in visible in NCI-H526 cells
*: P<0,05; **:P<0,01; ***:P<0,001

An increased expression of SLC16A3 is seen in DMS-153 cells treated with etoposide. The higher concentration of etoposide, 1,25µg/ml, results in a 11 fold increased expression of SLC16A3.

In NCI-H417 cells the gene expression increases to 2fold with 0,6µg/ml etoposide and 3fold in response to 1,25µg/ml, while in NCI-H526 cells the expression does not change significantly (Fig. 34).

6.2.26. Etoposide – influence on NHE1 gene expression

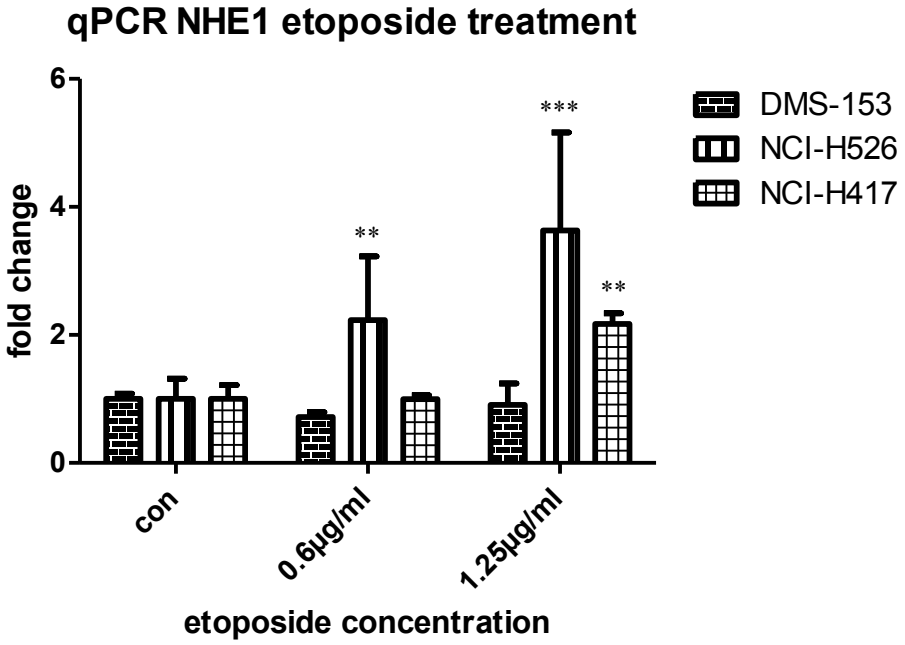

Figure 35 qPCR analysis of sodium proton exchanger NHE1 in DMS-153, NCI-H526 and NCI-H417 SCLC cell lines in response to etoposide treatment
con: untreated control group
0,6µg/ml: no change in NHE1 gene expression is seen in DMS-153 and NCI-H417 cells. In NCI-H526 cells it increases to 2fold
1,25µg/ml: no change in NHE1 gene expression in seen in DMS-153 cells while it increases in NCI-H526 and NCI-H417 cells to 4fold and 2fold, respectively
*: P<0,05; **:P<0,01; ***:P<0,001

With 1,25µg/ml etoposide the expression increases in NCI-H526 cells to about 4fold compared to the control group. Also the expression in NCI-H417 increases to 2fold at this concentration while in DMS-153 cells no difference is observed (Fig. 35).

6.2.27. Topotecan – influence on CA IX gene expression

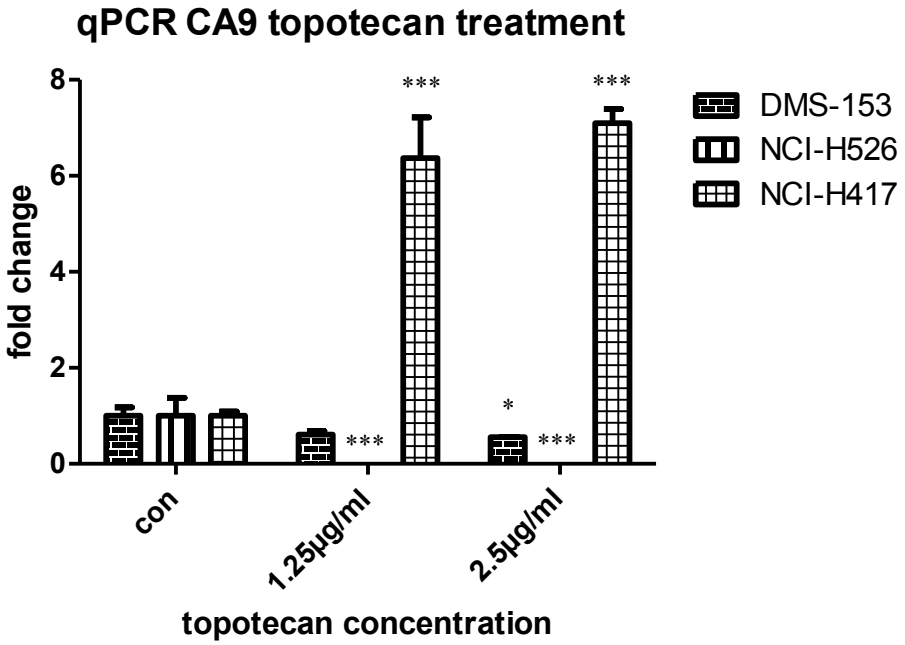

Figure 36 qPCR analysis of CA IX in DMS-153, NCI-H526 and NCI-H417 SCLC cell lines in response to topotecan (TPT) treatment
con: untreated control group
1,25µg/ml: no CA IX gene expression is detectable in NCI-H526 cells while it increases to 6,5fold in NCI-H417 cells in response to TPT treatment. CA IX gene expression decreases to 0,5fold in DMS-153 cells.
2.5µg/ml: no CA IX gene expression is detectable in NCI-H526 cells while it increases in NCI-H417 cells to 7fold in response to TPT treatment. CA IX gene expression decreases to 0,5fold in DMS-153 cells
*: P<0,05; **:P<0,01; ***:P<0,001

No CA IX gene expression can be detected in NCI-H526 cell in response to topotecan (TPT) treatment. Not even at the lowest concentration of 0,6µg/ml TPT. In NCI-H417 cells the gene expression increases to 7fold in response to TPT treatment while it decreases to 0,5fold in DMS-153 cells (Fig. 36).

6.2.28. Topotecan – influence on CA XII gene expression

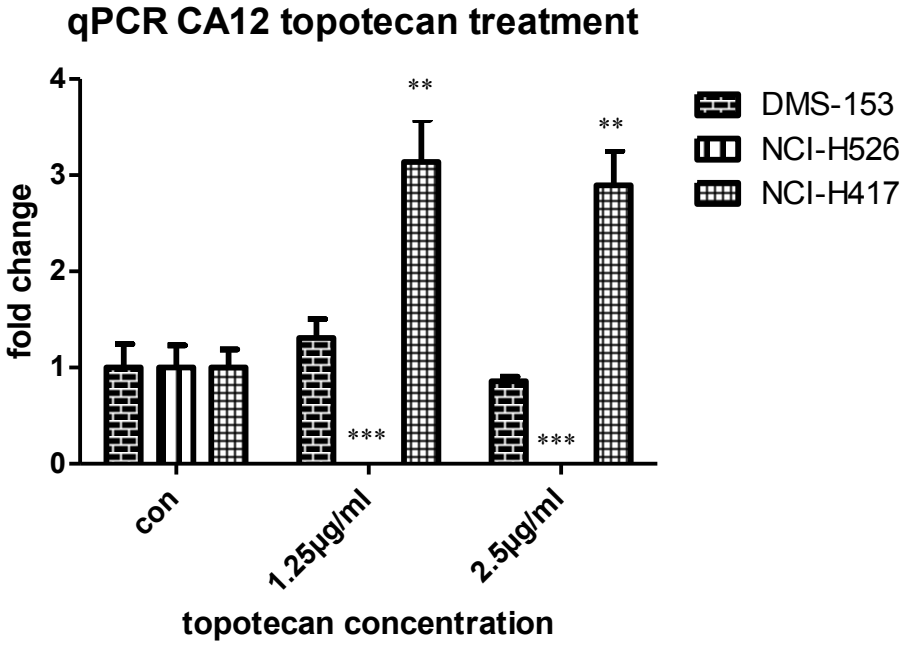

Figure 37 qPCR analysis of CA XII in DMS-153, NCI-H526 and NCI-H417 SCLC cell lines in response to topotecan (TPT) treatment
con: untreated control group
1,25µg/ml: no CA XII gene expression is detectable in NCI-H526 cells while it significantly increases to 3fold in NCI-H417 cells in response to TPT treatment. CA IX gene expression in DMS-153 cells stays at nearly the same level as in the control group
2.5µg/ml: no CA XII gene expression is detectable in NCI-H526 cells while it increases in NCI-H417 cells to 3fold in response to TPT treatment. CA IX gene expression stays in DMS-153 cells at the same level as in the control group
*: P<0,05; **:P<0,01; ***:P<0,001

Gene expression of CA XII increases to 3fold in NCI-H417 cells which were treated with 1,25µg/ml and 2,5µg/ml TPT compared to the untreated control group. The expression of CA XII in NCI-H526 cells is not measurable in response to TPT treatment. CA XII expression in DMS-153 cells stays with treatment at almost the same level as in the control group (Fig. 37).

6.2.29. Topotecan – influence on SLC16A1 gene expression

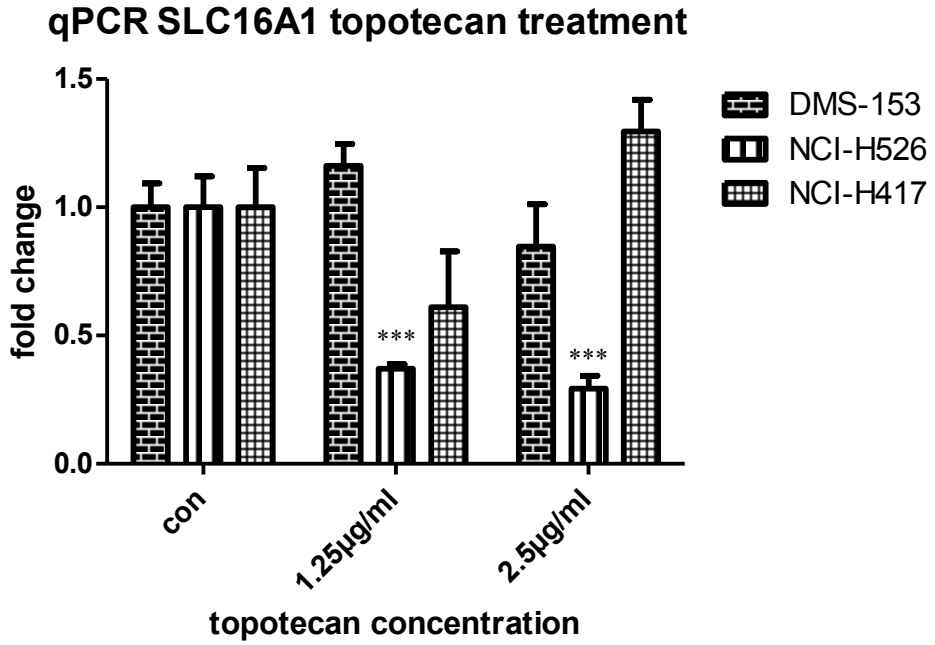

Figure 38 qPCR analysis of SLC16A1 (MCT1) in DMS-153, NCI-H526 and NCI-H417 SCLC cell lines in response to topotecan (TPT) treatment

con: untreated control group

1,25µg/ml: MCT1 gene expression stays at nearly the same as in DMS-153 cells when compared to the control group. Gene expression decreases in NCI-H526 and NCI-H417 cells to 0,4fold and 0,7fold, respectively.

2.5µg/ml: MCT1 gene expression stays at nearly the same as in DMS-153 cells when compared to the control group. Gene expression decreases in NCI-H526 to 0,4fold but increases in NCI-H417 cells to 1,3fold

*: P<0,05; **:P<0,01; ***:P<0,001

SLC16A1 gene expression decreases in NCI-H526 cells to 0,4fold when exposed to TPT treatment – at all different concentrations used.

In NCI-H417 cells the gene expression decreases in response to 1,25µg/ml TPT to nearly 0,5fold. With a higher concentration, 2,5µg/ml, SLC16A1 expression increases to 1,3fold in NCI-H417.

The gene expression in DMS-153 does not change significantly with TPT treatment (Fig. 38).

6.2.30. Topotecan – influence on SLC16A3 gene expression

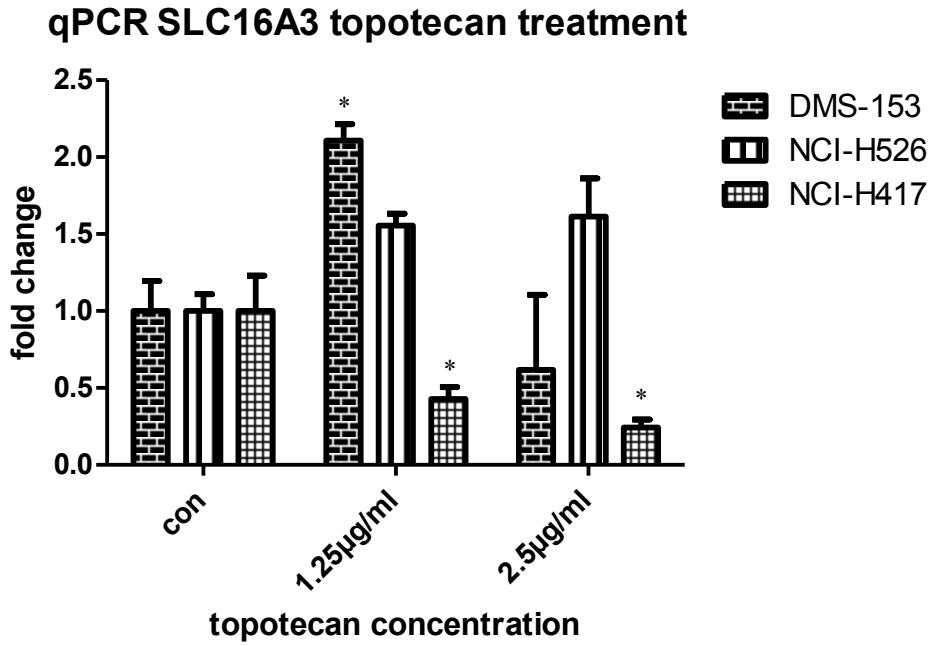

Figure 39 qPCR analysis of SLC16A3 (MCT4) in DMS-153, NCI-H526 and NCI-H417 SCLC cell lines in response to topotecan (TPT) treatment
con: untreated control group
1,25µg/ml: MCT4 gene expression increases in DMS-153 and NCI-H526 cells to 2,1fold and 1,5fold, respectively. Gene expression decreases in NCI-H417 cells to 0,5fold in response to TPT treatment
2.5µg/ml: MCT4 gene expression increases in NCI-H526 cells to 1,5fold while it is decreased in DMS-153 and NCI-H417 cells to 0,5fold and 0,25fold, respectively
*: P<0,05; **:P<0,01; ***:P<0,001

SLC16A3 gene expression increases in NCI-H526 cells in response to TPT treatment to 1,5fold – at any concentration used. In DMS-153 cells it increases 2fold with 1,25µg/ml but decreases to 0,5fold when exposed to 2,5µg/ml TPT.

The gene expression in NCI-H417 cells decreases at the low concentration to 0,5fold and keeps decreasing to 0,25fold at 2,5µg/ml topotecan when compared to the control (Fig. 39).

6.2.31. Topotecan – influence on NHE1 gene expression

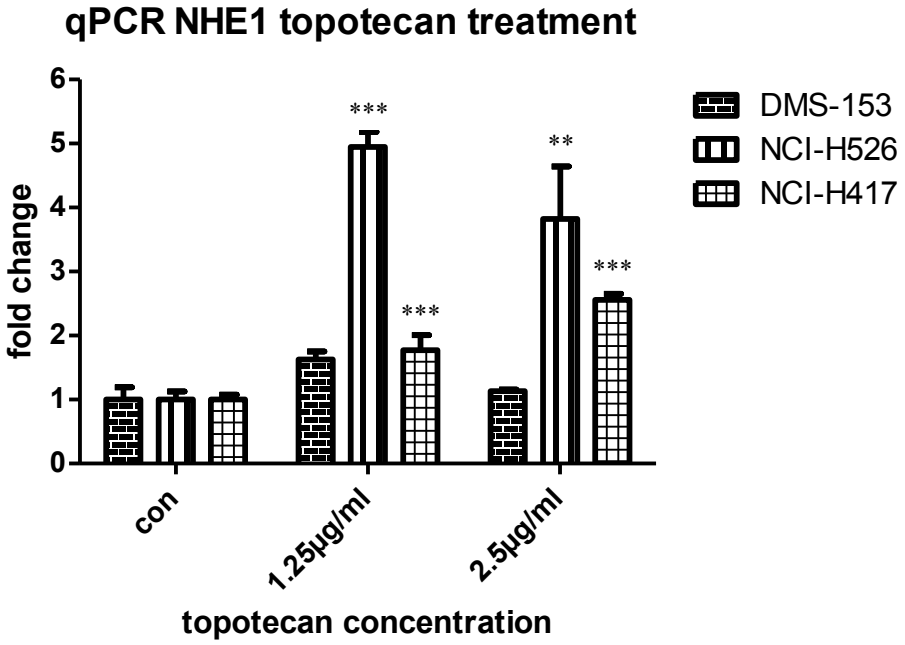

Figure 40 qPCR analysis of sodium proton exchanger NHE1 in DMS-153, NCI-H526 and NCI-H417 SCLC cell lines in response to topotecan (TPT) treatment
con: untreated control group
1,25µg/ml: NHE1 gene expression increases in DMS-153 and NCI-H417 cells to 2fold in both cell lines. NHE1 gene expression in NCI-H526 cells increases significantly to 5fold.
2,5µg/ml: NHE1 gene expression does not change in DMS-153 cells while it increases in NCI-H526 and NCI-H417 cells to 4fold and 2,8fold, respectively
*: P<0,05; **:P<0,01; ***:P<0,001

The treatment of NCI-H526 cells with topotecan increases the gene expression of NHE1 up to 5fold at 1,25µg/ml topotecan and 2,5µg/ml increases it to 4fold. In DMS-153 cells it increases 2fold at 1,25µg/ml but stays at the same level with a higher concentration, 2,5µg/ml, of topotecan when compared to the control group.
NHE1 gene expression increases 2fold in NCI-H417 cells treated with 1,25µg/ml topotecan and it is also increased to 2,8fold in 2,5µg/ml topotecan treated NCI-H417 cells (Fig. 40).

6.2.32. Effect on CA IX gene expression of combined treatment with cisplatin and etoposide

Cisplatin treatment decreases the gene expression of CA IX in the three used SCLC cell lines while etoposide increases it. Both substances are used as combinational treatment for cancer patients. Therefore, the gene expression of carbonic anhydrase IX was determined in DMS-153, NCI-H526 and NCI-H417 cells by using 0,6µg/ml cisplatin + 0,6µg/ml etoposide (Low) and 1,25µg/ml cisplatin + 1,25µg/ml etoposide (Hi).

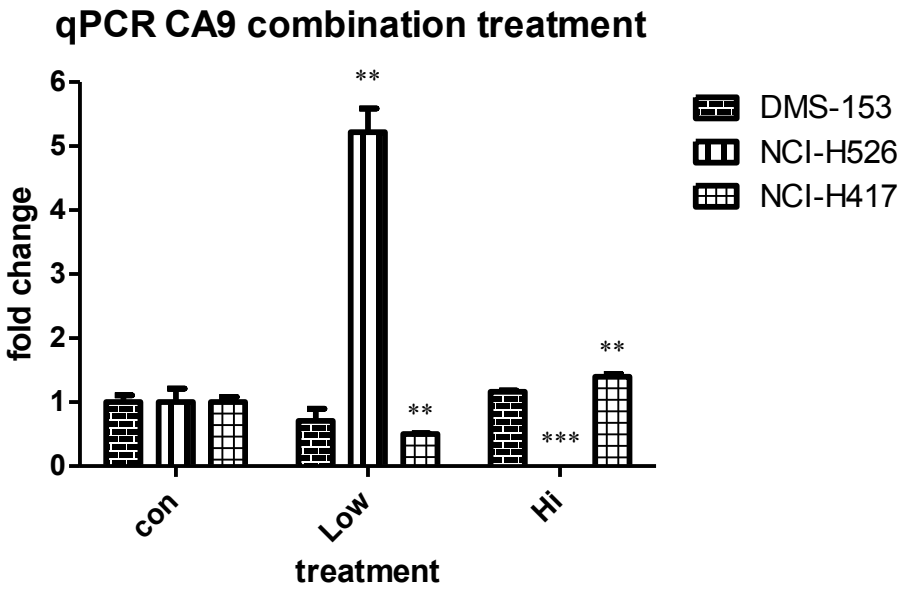

Figure 41 qPCR analysis of CA IX in DMS-153, NCI-H526 and NCI-H417 SCLC cell lines in response to combinational treatment with cisplatin and etoposide
con: untreated control group
Low - 0,6µg/ml cisplatin and etoposide: CA IX gene expression decreases in DMS-153 and NCI-H417 cells to 0,75and 0,5fold, respectively. Gene expression increases to 5fold in NCI-H526 cells
Hi – 1,25µg/ml cisplatin and etoposide: No CA IX expression is detectable in NCI-H526 cells. Gene expression increases in NCI-H417 cells to 1,5fold while no change in DMS-153 cells is visible
*: P<0,05; **:P<0,01; ***:P<0,001

While 0,6µg/ml cisplatin and etoposide increases the gene expression of CA IX in NCI-H526 cells to 5fold, the higher concentration, 1,25µg/ml of each substance results in a not measurable gene expression of CA IX.

The low treatment of DMS-153 and NCI-H417 cells decreases the CA IX expression to 0,75fold and to 0,5fold, respectively. But the Hi treatment does not change the gene expression significantly when compared to the control group in DMS-153 cells while in NCI-H417 cells a significant 1,5fold increase occurs (Fig. 41).

6.3. MTT-Assays

NCI-H417 cells were treated with defined concentrations of C207A (CA IX inhibitor) or zoniporide (NHE1 inhibitor) together with cisplatin. After four days at cell culture conditions, 37°C and 5% CO_2, the EZ4U kit was used to determine the number of living cells.

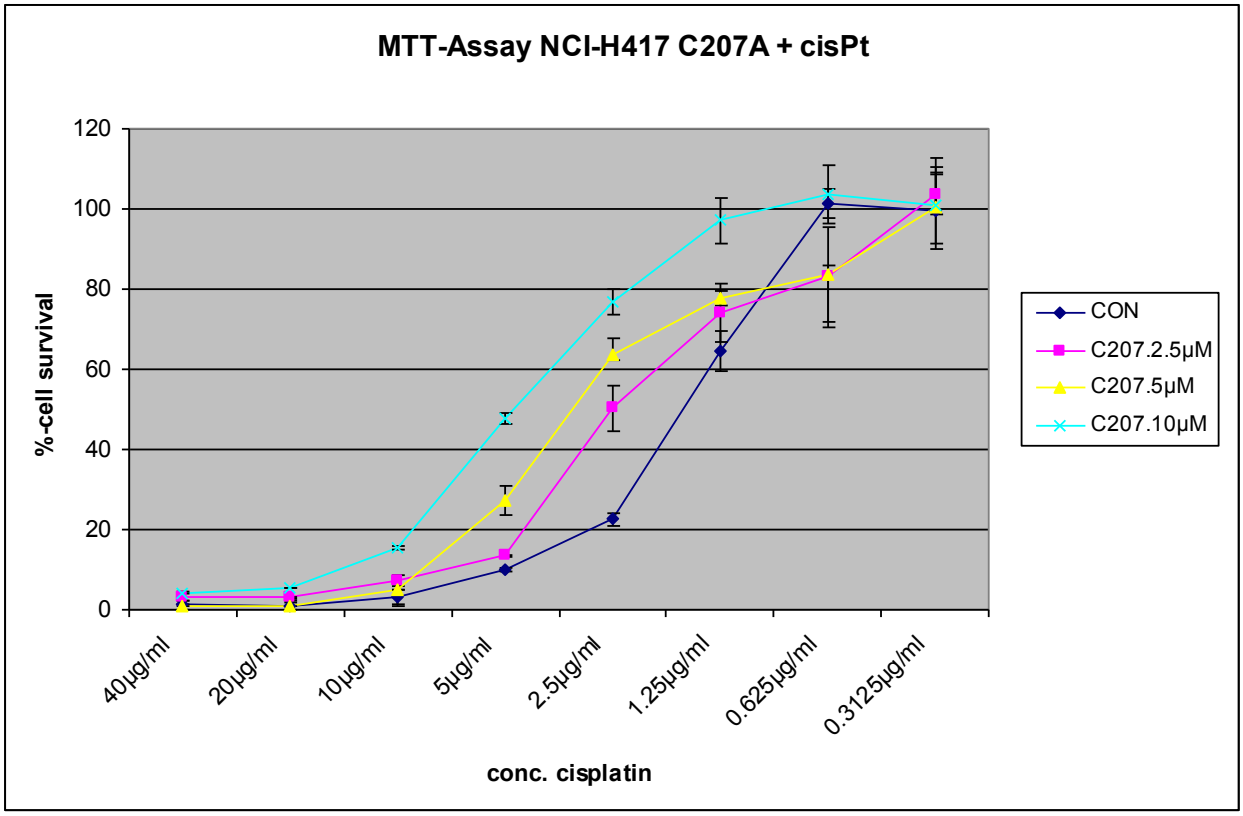

Figure 42 MTT-Assay of NCI-H417 cells treated with cisplatin and the CA IX inhibitor C207A
$1*10^4$ NCI-H417 cells were treated with either 2,5µM, 5µM or 10µM C207A (CA IX inhibitor) together with cisplatin, which was diluted in twofold steps starting with 40µg/ml. After four days at cell culture conditions, 37°C and 5% CO_2, the EZ4U kit was used to determine the number of living cells. C207A acts antagonistic to cisplatin treatment and increases tumor cell survival

It can be observed that the carbonic anhydrase inhibitor C207A decreases the efficacy of cisplatin. At a concentration of 10µg/ml cisplatin nearly all cells in the control group are dead while about 20% of the cells survive this treatment with the presence of 10µM C207A. This effect increases with lower concentrations of cisplatin: just 20% of the cells are able to survive with 2,5µg/ml cisplatin in the control group, while

- nearly 80% of the cells with 10µM C207A
- nearly 65% of the cells with 5µM C207A
- nearly 50% of the cells with 2,5µM C207A

survive this cisplatin concentration (Fig. 42).

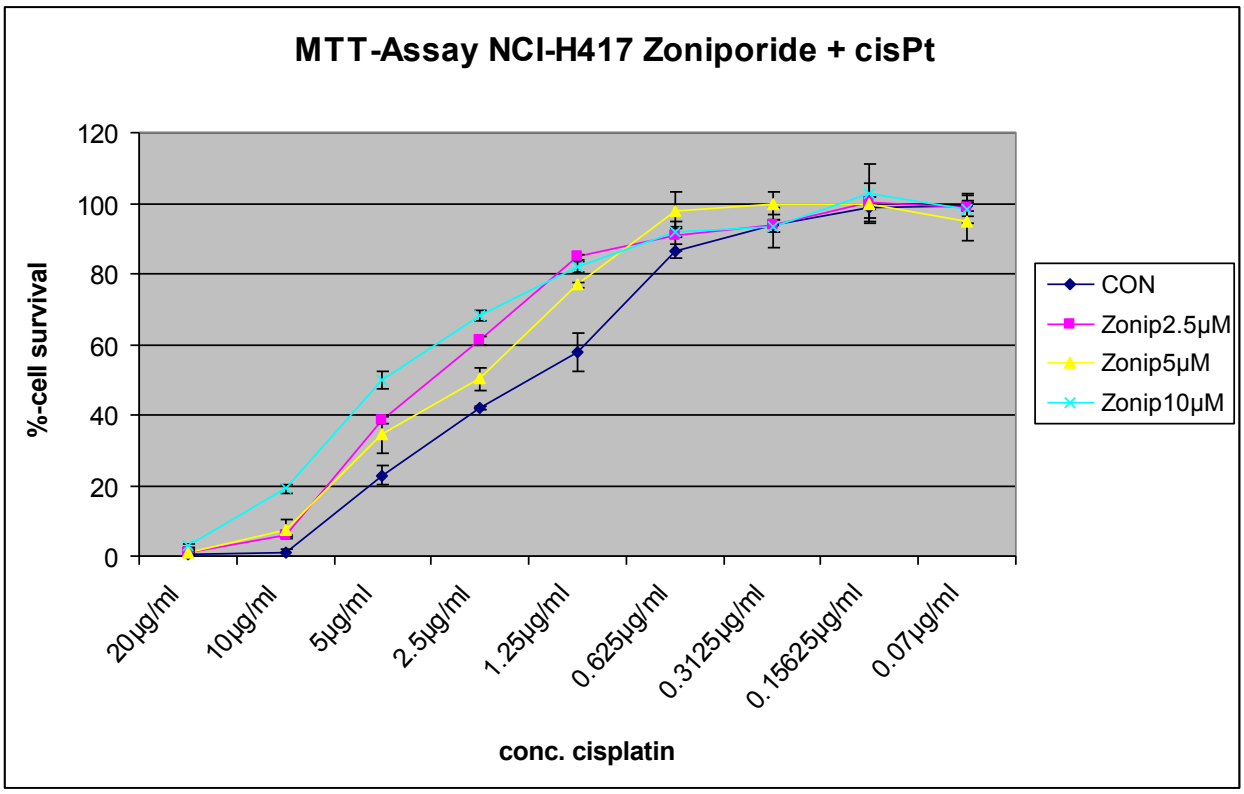

Figure 43 MTT-Assay of NCI-H417 cells treated with cisplatin and NHE1 inhibitor zoniporide
$1*10^4$ NCI-H417 cells were treated with either 2,5µM, 5µM or 10µM zoniporide (NHE1 inhibitor) together with cisplatin, which was diluted in twofold steps starting with 20µg/ml. After four days at cell culture conditions, 37°C and 5% CO_2, the EZ4U kit was used to determine the number of living cells. C207A acts antagonistic to cisplatin treatment and increases tumor cell survival. Zoniporide acts antagonistic to cisplatin treatment and increases tumor cell survival

Also zoniporide acts antagonistic to the cisplatin treatment. The presence of 10µM zoniporide leads to a 50% cell survival at 5µg/ml cisplatin while just 20% of the cells survive in the control group at this cisplatin concentration.

At 1,25µg/ml cisplatin nearly 60% of the cells live in the control group – but in the presence of zoniporide, at 2,5µMn 5µM and 10µM, around 80% of the cells are alive (Fig. 43).

6.4. Soluble CA IX-ELISA

The cell medium was centrifuged and separated from the cells. The supernatant was used as sample for ELISA analysis (for more details see Material and Methods)

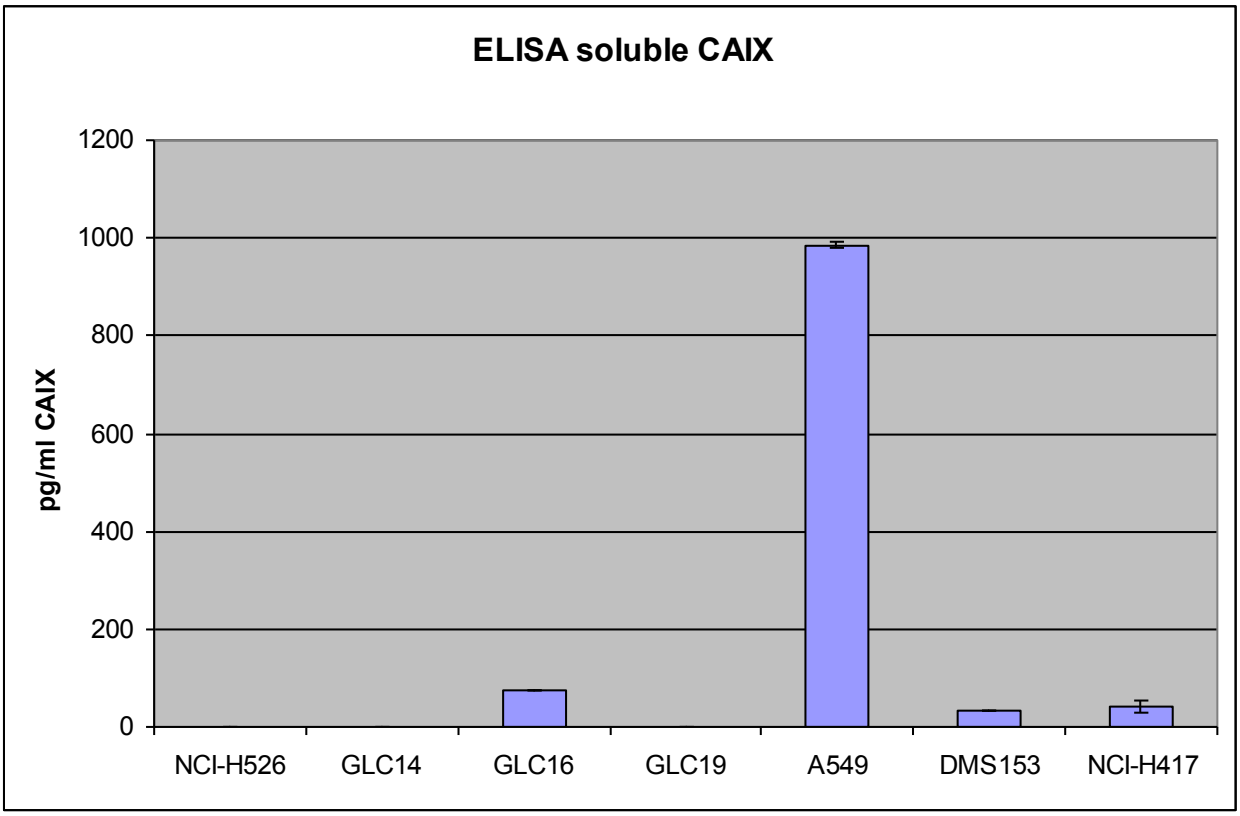

Figure 44 soluble CA IX – ELISA of different SCLC and one NSCLC cell line
No levels of soluble CA IX are detectable in NCI-H526, GLC14 and GLC19 cells. ~40pg/ml soluble CA IX is found in DMS-153 and NCI-H417 cells and ~70pg/ml in GLC16 cells. The only NSCLC in this experiment, A549, shows the highest level of nearly 1ng/ml soluble CA IX

The quantity of soluble CA IX in culture media of different SCLC cell lines and one NSCLC cell line was determined before starting to evaluate the influence of anticancer treatment on the quantity of soluble CA IX present in the media. Soluble CA IX is not detectable in NCI-H526, GLC 14 and 19 cells, while in GLC16 (~70pg/ml), DMS-153 (~40pg/ml) and NCI-H417 (~40pg/ml) cell lines small quantities of soluble CA IX are present. The non small cell lung cancer cell line A549 shows the highest quantity of nearly 1ng/ml soluble CA IX (Fig. 44).

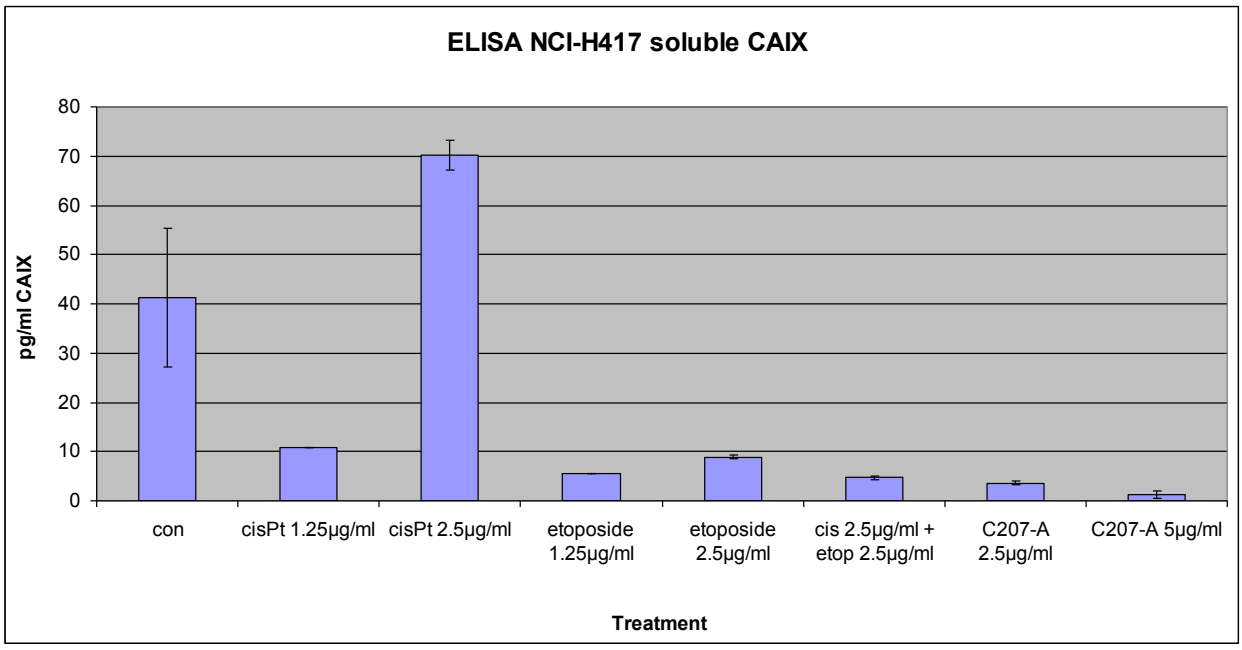

Figure 45 soluble CA IX – ELISA of NCI-H417 cells with cisplatin, etoposide, topotecan and CA IX inhibitor C207A treatment

Only 2,5µg/ml cisplatin increases the quantity of soluble CA IX from 40pg/ml to 70pg/ml. All other treatments decrease the quantity of soluble CA IX to around 10pg/ml or less.

All treatment approaches, with one exception, decreases the quantity of soluble CA IX in NCI-H417 cells in the culture medium. 40pg/ml are present in the control group and 1,25µg/ml cisplatin, etoposide, combination with cisplatin and etoposide, topotecan and C207A decreases the soluble CA IX level to 5-10pg/ml. Just the treatment with 2.5µg/ml cisplatin increases the soluble CA IX level to 70pg/ml (Fig. 45).

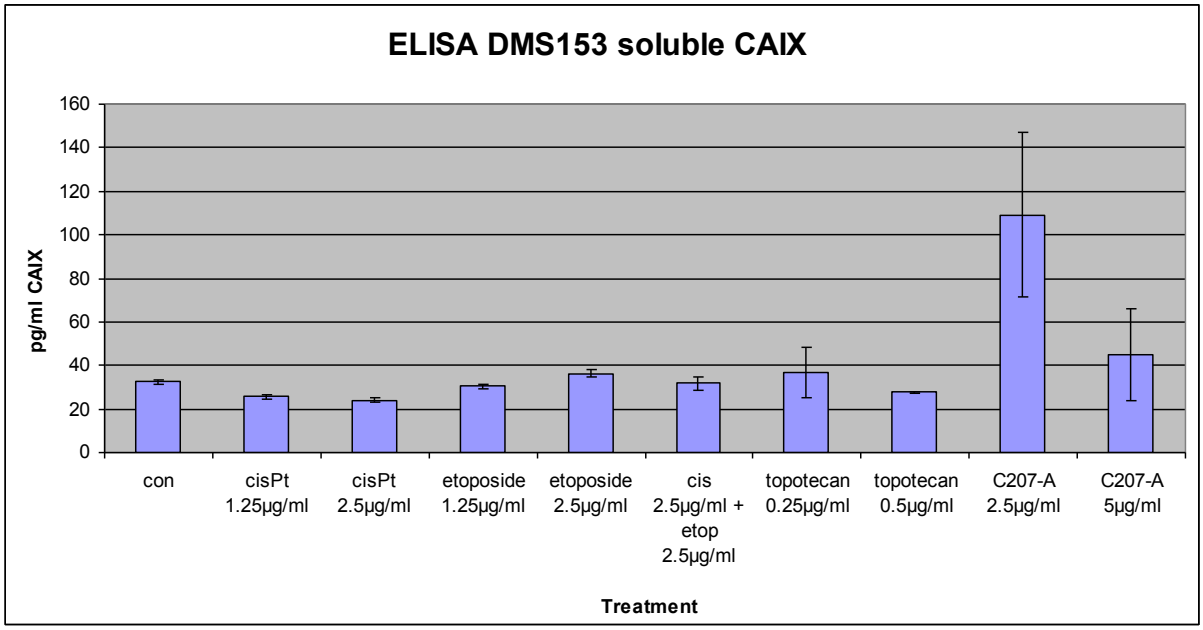

Figure 46 soluble CA IX – ELISA of DMS153 cells with cisplatin, etoposide, topotecan and CA IX inhibitor C207A treatment

Only 2,5µg/ml CA IX inhibitor C207A increases the level of soluble CA IX from 30pg/ml to 110pg/ml. All other treatments do not change the level of soluble CA IX

Treatment with cisplatin, etoposide, combination with cisplatin and etoposide and topotecan does not change the quantity of soluble CA IX significantly in DMS-153 cells compared to the control group with 30pg/ml. Only the treatment with 2,5µg/ml CA IX inhibitor C207A increases the level of soluble CA IX to 110pg/ml (Fig. 46).

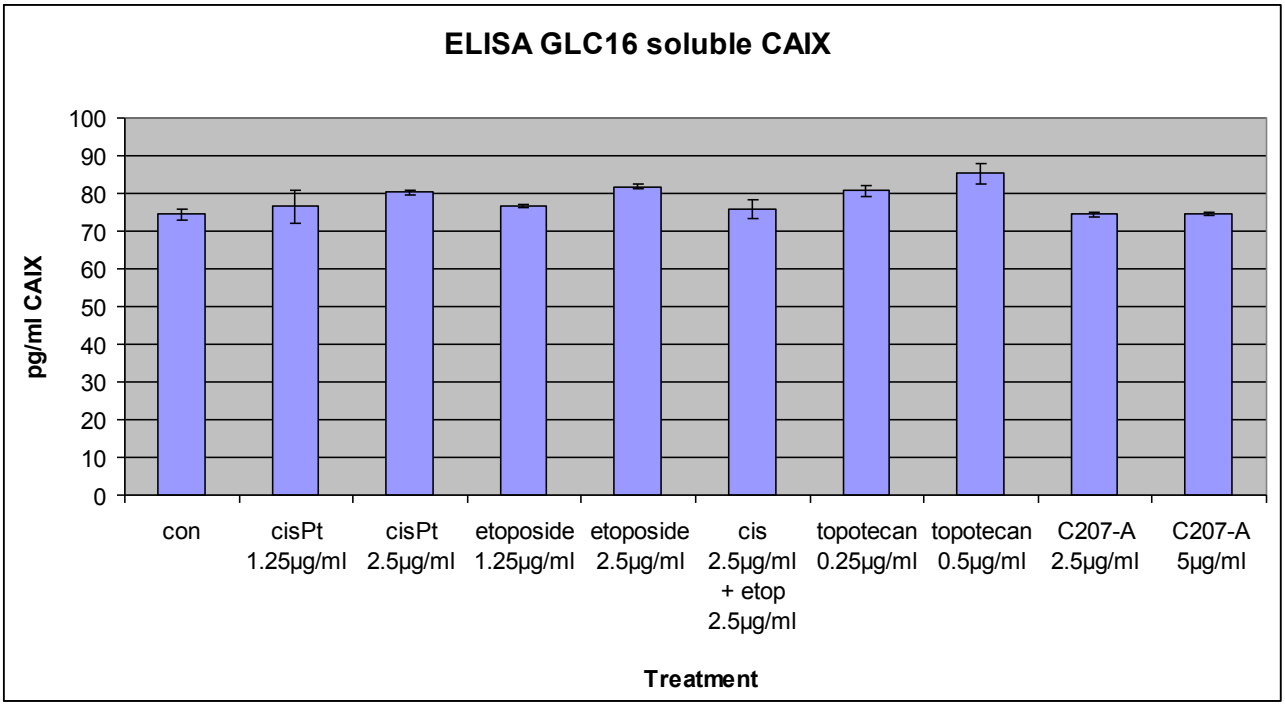

Figure 47 soluble CA IX – ELISA of GLC16 cells with cisplatin, etoposide, topotecan and CA IX inhibitor C207A treatment
No treatment achieves any change in the level of soluble CA IX compared to the control group

No significant changes in soluble CA IX levels are visible with any treatment approach in GLC16 cells. The quantity stays nearly at the same level as in the control group with 75pg/ml soluble CA IX (Fig. 47).

7. Discussion

Small cell lung cancer (SCLC) is untreated the most aggressive form of all pulmonary tumors with a median survival of 2-4 months and gets more and more important especially for women who have a rising incidence due to smoking. Lung cancer in general is already the leading cause of cancer deaths in the United States and the second most common cancer type among women (American Society of Clinical Oncology; Radzikowska et. al, 2002). Combinational treatment of platinum (cisplatin or carboplatin) with etoposide is the standard primary therapy approach (Okamoto et al., 2007). SCLC shows a good response to first-line therapy, but frequently a relapse occurs very early. Further treatments with topotecan are very toxic, because its target, topoisomerase 1, is an enzyme, which is expressed ubiquitously in all tissues. However, this treatment regime is not very effective, but it is till now the only FDA approved drug for SCLC second-line therapy (Perez-Soler, 1996; Eckardt et al., 2007). The reason for this early relapse and the high chemoresistance is still unclear, but cancer stem cells (CSCs) may play a role in these mechanisms (Abdullah and Chow, 2013).
Many studies were trying to improve second-line therapies:

- new chemotherapeutic drugs in multimodal chemotherapy
- targeting different signaling pathways: e.g. epidermal growth factor receptor tyrosine kinases, Src kinase, mTOR
- targeting angiogenesis: e.g. matrix metalloproteinases
- targeting apoptosis promotion: e.g. Bcl-2, 26S ubiquitin proteasome complex
- targeting multidrug resistance effectors

All these approaches failed in clinical trials for prolonging patients' survival so far.
(Peck et al., 2001; Shepherd et al., 2002; Rigas et al., 2003; Moore et al., 2006; Rudin et al., 2008; Molina et al., 2009; Tarhini et al., 2010; McDonald et al., 2012)

A new approach of targeted therapy can be the interaction with pH-regulating proteins in SCLC. Very little is known about pH regulation in this cancer type. The question during this master thesis was to determine to which extent these pH regulating key players are expressed in different representative SCLC cell lines (DMS-153, NCI-H526 and NCI-H417) are expressed. What is the influence of currently used chemotherapeutic agents, cisplatin, etoposide, topotecan, and a combination of cisplatin and etoposide, on the

gene expression levels of CA IX, CA XII, NHE1, SLC16A1 and SLC16A3, and how do the protein quantitites of the corresponding proteins change in response to these treatments? Are these drugs combinable with inhibitors of pH regulators?

Especially CA IX is in the focus for novel anticancer strategies, because this isoform of α-carbonic anhydrases is only present in a few healthy tissues (not in lung), but is overexpressed in many different malignant counterparts, including SCLC and NSCLC. High CA IX levels are correlated in many solid tumors, including lung, breast, cervix and thyroid, with a poorer prognosis for patients, because extracellular acidosis leads to an increased rate of metastasis and a higher immune escape, providing a selective advantage for tumor survival and a more aggressive phenotype. First of all, acidosis leads to an increased cell death of healthy cells through p53 induction, which increases the available volume for tumor growth. Cancer cells can elude this cell death by the upregulation of these important transporters and proteins already mentioned and/or by lacking p53 expression. Additionally, extracellular matrix degradation is increased by acidosis through activation of proteolytic enzymes like cathepsin B and matrix metalloproteases, which show an increased activation in a more acidic microenvironment favouring metastasis.

It is also interesting to note that no SLC16A1 and SLC16A3 gene expression is seen in healthy lung tissue, while these two transporters are found to be expressed in NSCLC without any data available for SCLC so far (Kallio et al., 2010; Kennedy and Dewhirst, 2010; Gieling and Williams, 2012; Dhup et al., 2012).

7.1. Selected GLC SCLC cell lines: GLC14, GLC16 and GLC19

The GLC-14-16-19 cell lines are ideally suited to observe the change in gene expression of the important master pH regulators in response to SCLC treatment, because these cell lines were obtained from one female patient during successive stages of SCLC treatment.

- GLC 14 was established at the first time point of diagnosis prior treatment – the tumor showed a complete response after 5 cycles of cyclophosphamide/doxorubicin/etoposide treatment (CDE)
- GLC 16 was established after relapse following chemotherapy – the patient was treated after the biopsy with 4 cycles CDE and radiation therapy with a normal X-ray after treatment

- GLC 19 was established after tumor recurrence after radiation therapy without any response of the tumor to a new CDE therapy

Then the therapy was aborted due the wish of the patient who died shortly after.

All three cell lines were compared to the tumor from which they were derived and showed a good match with respect to biochemical, immunohistological and morphological features (Pedersen et al., 2003). It is important to note that the tumors were treated with CDEs, but the standard therapy nowadays is based on platinum and etoposide combination treatment (Okamoto et al., 2007). This series of SCLC cell lines is very interesting, because a biopsy is normally taken just at the first time point of diagnosis prior treatment with a fine needle, leaving scarcely material for cell biology studies.

These cell lines are a very unique chance to investigate the influence of *in vivo* SCLC treatment to the gene expression of the important pH-regulators. The cell lines were analysed by qPCR, chemosensitivity assays and Western Blot.

CA IX gene expression dramatically drops during *in vivo* treatment (GLC 16 and GLC 19) to 0,1fold compared to prior treatment in GLC 14. CA XII gene expression shows the same effect after the first cycle of *in vivo* treatment with CDEs (GLC16), but stays at the same level in the therapy resistant tumor GLC19 compared to prior treatment.

Kallio et al. suggested that in breast cancer the gene expression of CA XII is perhaps not mainly regulated through HIF-1-induced pathways, but involves the interactions with estradiol and certain growth factors, while CA IX gene expression is mainly regulated by the HIF-1 pathway (Kallio et al., 2010). Alternative regulation mechanisms can be an explanation for the stabilisation of CA XII gene expression in GLC 19 cells, while CA IX gene expression drops continuously during therapy. The finding that the same quantity of CA IX protein in the *in vivo* CDE treated cell lines GLC 16 and GLC 19 is observed compared to GLC 14, but at the same time its gene expression is reduced, suggests also a hitherto unknown stabilisation mechanism of CA IX protein.

Moreover, the localisation of CA IX seems to change from the membrane site to a more cytoplasmic localisation in GLC 19 cells according to preliminary immunofluorescence tests. The exact localisation and the quantity of CA IX in these three cell lines will be determined in future immunofluorescence and confocal microscopy analysis.

The SLC16A1 and SLC16A4 genes are not expressed in healthy lung tissue, but these genes are upregulated in NSCLC cells, without available data for SCLC so far. SLC16A1 gene expression increases in GLC 16 to 2fold when compared to prior treatment, while SLC16A3 gene expression drops to 0,5fold. Significant high amounts of

lactate are found in patients with lung cancer with tumor recurrence, and lactate is a positive inducer for SLC16A1 gene expression, which might explain the increased gene expression of SLC16A1. SLC16A3 gene expression is linked to aerobic glycolysis in cancer cells. The treatments used may interact with this kind of cell metabolism and inactivate the need for this type of transporter (Kennedy and Dewhirst, 2010).

The reduced gene expression of SLC16A3 as well as NHE1 to 0,5fold in GLC 19 cells leads to the question: what happens to the intracellular alkalisation and the extracellular acidification? The reduced gene expression of those two important transporters is expected to result in a reduced extracellular acidification, which needs to be determined in future functional analysis.

7.2. SCLC cell lines – Influence of treatments on gene expression and protein quantity

7.2.1. Changes in carbonic anhydrase IX gene expression and protein quantity in response to treatment

CA IX gene expression decreases significantly with low concentrations of cisplatin, 0,6µg/ml – 1,25µg/ml – 2,5µg/ml, in all three cell lines. 1µg/ml cisplatin peak plasma concentration is the concentration which can be achieved in patients in response to cisplatin treatment (Urien et al., 2005). The strongest effects are seen with 0,6µg/ml and 1,25µg/ml cisplatin where the gene expression is reduced to <0,5fold, while 5µg/ml cisplatin already increases the gene expression of this carbonic anhydrase isoform in NCI-H417 cells. NCI-H526 cells, the chemonaive cell line, have the most effective reduction of CA IX gene expression in response to cisplatin. One explanation for the stronger effect of low cisplatin concentrations cells can be that higher concentrations of cisplatin already lead to an induction of cell death, while lower concentrations arrest the cells in the G2M phase during the cell cycle. An interesting point is that etoposide treatment increases CA IX gene expression in all three cell lines, with the strongest effect in NCI-H526 cells where it increases to 8fold. Both substances, cisplatin and etoposide, are used as combinational therapy for SCLC patients (Okamoto et al., 2007). In DMS-153 cells the combinational treatment has no effect on the CA IX gene expression, leading to the suggestion that the anticancer agents used abolish the effect of each other. 0,6µg/ml cisplatin + 0,6µg/ml etoposide increases the gene expression in NCI-H526

cells to 5fold, where the effect of etoposide can be seen, and decreases it in NCI-H417 cells to 0,75fold with a stronger effect of cisplatin. Converse results are seen when 1,25µg/ml cisplatin + 1,25µg/ml etoposide is used: in NCI-H526 cells no CA IX gene expression is detectable in qPCR analysis, what is maybe the effect of cisplatin, while it increases in NCI-H417 cells to 1,5fold, what may be the result of etoposide. More analysis needs to be performed to evaluate the influence of these substances on the gene expression of CA IX in patients and if this effect is useable for a combination treatment with CA IX inhibitors or antibodies.

Topotecan treatment shows controversial results: CA IX gene expression is not detectable in NCI-H526 cells in response to topotecan treatment. DMS-153 cells show a 0,5fold reduced gene expression, while NCI-H417 cells show a 7fold increased gene expression. These three cell lines analysed seems to have, even though they are all SCLC cell lines, different regulations of gene expression of CA IX in response to topotecan.

Western Blot analysis of CA IX protein levels in cisplatin treated DMS-153 cells shows the same effect like in *in vivo* CDE-treated GLC 16 and GLC 19 cells: the protein level does not change in response to cisplatin treatment, while the gene expression decreases. A just ~14% reduced quantity of CA IX protein is seen in NCI-H417 cells which were exposed to cisplatin. Only the chemosensitive cell line NCI-H526 has a 30% reduced quantity of CA IX protein when treated with cisplatin. It seems that more chemoresistant cell lines, DMS-153 and NCI-H417, have some regulation mechanisms, able to stabilize the protein within the cell, while the gene expression is decreased. The more chemosensitive cell line, NCI-H526, seems to lack these mechanisms.

7.2.2. Changes in carbonic anhydrase XII gene expression in response to treatment

Gene expression of CA XII is increased in all three SCLC cell lines analysed, which were treated with cisplatin again, with the strongest effect in chemosensitive NCI-H526 cells. This induction of gene expression leads to the suggestion that the reduced CA IX gene expression, in response to cisplatin, is compensated by an increased CA XII gene expression.

Etoposide has the same effect on CA XII gene expression like on CA IX expression and increases it in all cell lines except NCI-H417 cells, where it stays at the same level. Topotecan, however, increases the CA IX and CA XII gene expression in NCI-H417 cells and leads to a suppression of expression in NCI-H526 cells. No changes are observed in DMS-153 cells. Similar regulation adjustments as with cisplatin treatment are not seen in response to etoposide or topotecan treatment.

7.2.3. Changes in monocarboxylate transporter 1 gene expression in response to treatment

Cisplatin treatment does not affect the gene expression of SLC16A1 in chemoresistant cell lines DMS-153 and NCI-H417. Only in the chemosensitive cell line NCI-H526 a reduction of SLC16A1 gene expression to 0,5fold is seen with 0,6µg/ml cisplatin, but 1,25µg/ml has not such a strong effect and reduces the gene expression only to 0,8fold. Also lower concentrations of etoposide do not change the SLC16A1 gene expression in all cell lines, but higher concentrations are able to double the gene expression in chemosensitive NCI-H526 cells, while it stays at the same level in DMS-153 and NCI-H417 cells. Topotecan treatment also results in different gene expression patterns between the SCLC cell lines: DMS-153 cells do not change their SLC16A1 gene expression in response to topotecan at all, while it decreases to 0,4fold and 0,7fold in NCI-H526 and NCI-H417 cells, respectively, at 1,25µg/ml. This gene expression stays reduced in NCI-H526 cells with a higher concentration of topotecan, but this agent increases it in the chemoresistant NCI-H417 cells to 1,3fold.
It seems that different regulations of SLC16A1 gene expression occur between chemoresistant and chemosensitive cell lines in response to these treatments.

7.2.4. Changes in monocarboxylate transporter 4 gene expression in response to treatment

Gene expression data of SLC16A3 in response to anticancer treatment is more interesting compared to that of MCT1. Cisplatin treatment increases the SLC16A3 gene expression in DMS-153 (2,2fold) and NCI-H417 (1,8fold) cells, but reduces it in NCI-H526 cells (0,2fold).
The chemoresistant cell lines, DMS-153 and NCI-H417, increases their SLC16A3 gene expression in response to etoposide to 11fold and 3fold, respectively. However, no changes in gene expression are detectable in NCI-H526 cells.
The most resistant cell line, NCI-H417, shows a reduced SLC16A3 gene expression in response to topotecan treatment (0,25fold). DMS-153 cells increase their SLC16A3 gene expression at 1,25µg/ml topotecan (2,1fold), but with a higher concentration it falls back to 0,5fold. Only the chemosensitive cell line NCI-H526 shows a constant increased gene expression to 1,5fold with both concentrations of topotecan used.
All these gene expression profiles differ significantly, depending on the anticancer treatment used. Therefore, it is plausible to assume that different regulation mechanisms occur between these SCLC cell lines analysed.

7.2.5. Changes in sodium proton exchanger 1 gene expression in response to treatment

NHE1 gene expression decreases to 0,5fold in DMS-153 cells in response to 1,25µg/ml cisplatin. This concentration also leads to a decreased SLC16A1 expression (0,8fold), but to a 2,2fold increased SLC16A3 expression. The reduction of NHE1 as well as SLC16A1 gene expression may explain the increased SLC16A3 gene expression in DMS-153 cells, in which this transporter might resume the proton extrusion function.
Perhaps some similar effects occur in topotecan-treated NCI-H417 cells, in which SLC16A1 as well as SLC16A3 gene expression decreases to 0,7and 0,5fold, respectively, while NHE1 expression is increased to 2fold. NHE1 possibly resumes proton extrusion from the cell from the SLC16A1/3 transporters.

The changes of NHE1 gene expression together with SLC16A1/3 expression changes could result in different acidification of the tumor microenvironment, which needs to be determined in functional analysis in future experiments.

7.2.6. Chemosensitivity assays

The results of the chemosensitivity assay clearly show that C207A (CA IX inhibitor) and zoniporide (NHE1 inhibitor) act antagonistic to the cisplatin treatment in NCI-H417 cells and increase cancer cell survival. This data should have an important impact for novel combinational anticancer treatments of CA IX and NHE1 inhibitors together with cisplatin. This approach needs to be evaluated in different cell lines together with different types of inhibitors to determine, whether this increased cancer cell survival occurs also with other inhibitors and treatments.

7.2.7. ELISA of soluble CA IX

Soluble CA IX ELISA was performed, since it is known that a secreted soluble form of CA IX is present in different solid tumours. This form has an impact on treatment and targeting of CA IX on the tumor plasma membrane with e.g. antibodies. Most of the antibodies would bind to the circulating soluble CA IX if present at high quantities, and not to the membrane bound CA IX on cancer cells. This would result in a decreased effect of treatments targeting CA IX (Hyrsl et al., 2009; Woelber et al., 2010).
The first interesting point here is that just a low quantity of soluble CA IX is found in the SCLC cell lines analysed (30-70pg/ml), while the NSCLC cell line A549 has nearly 1ng/ml soluble CA IX.
2,5µg/ml cisplatin treatment leads to an increased quantity of soluble CA IX protein in NCI-H417 medium (70pg/ml), while all other treatments decreases the level to <10pg/ml. It will be interesting to determine, whether the reason for the increased quantity of soluble CA IX is that cisplatin leads to an increased shedding of CA IX from the membrane, therefore leading to an increased interference with treatments targerting CA IX or if the cell membrane is already ruptured and releases normal membrane bound CA IX.
C207A, however, results in an increased shedding of CA IX in DMS-153 cells and increases the quantity from 30pg/ml to >100pg/ml soluble CA IX, which would be interesting for CA IX targeting treatments.
Treatments in GLC16 cells do not increase the quantity of soluble CA IX in the medium and would not interfere with CA IX targeted therapy.

A high quantity of soluble CA IX is found in NSCLC, while SCLC cell lines do not show these high quantities, which would not impair targeted CA IX therapy in SCLC patients.

7.2.8. Summary and Outlook

The important pH regulating proteins, CA IX, CA XII, SLC16A1, SLC16A3 and NHE1 are expressed and present in the SCLC cell lines investigated. The gene expression regulation of these proteins differs from cell line to cell line analysed and the treatment used, but partially it seems to depend on the degree of chemoresistance. It is very interesting that cisplatin and etoposide treatments, both used as combinational SCLC primary treatment, result in different outcomes: in general, etoposide increases the gene expression, while cisplatin decreases or increases it.

CA IX and NHE1 inhibitors, C207A and zoniporide, respectively, are acting antagonistic to cisplatin treatment and increase tumor cell survival when cells are exposed to cisplatin. This data is important for further combinational treatment employing cisplatin together with CA IX and NHE 1 inhibitors.

ELISA analysis shows a low quantity of soluble CA IX protein in SCLC, while higher quantities are present in NSCLC. Targeted CA IX therapy does not interfere with soluble CA IX in SCLC treatment.

With this work, a foundation has been established for more detailed analysis in a broader spectrum of SCLC and NSCLC cell lines. In future experiments it should be possible to determine, whether the targeting of these important pH regulating proteins can lead to an improved combinational second-line therapy together with chemotherapeutic agents.

8. Deutsche Zusammenfassung

Kleinzelliger Lungenkrebs ist unbehandelt die aggressivste Form aller pulmonalen Tumorarten mit einer durchschnittlichen Lebenserwartung von 6-24 Monaten je nach Tumorstadium und Therapieerfolg. Die Behandlung dieser Tumorart ist sehr kompliziert, da sich der Tumor bereits sehr früh weit verbreitet und sich Metastasen bilden. Auch wenn der Tumor auf die Erstbehandlung, eine Kombinationstherapie aus Platinumsubstanzen und Etoposid, sehr gut anspricht, so kommt es in der Mehrheit der Patienten zu einem sehr frühen Rückfall, welcher nicht mehr gut durch eine weiterführende Therapie behandelbar ist. Das einzige FDA zugelassene Chemotherapeutikum für die weiterführende Behandlung ist Topotecan, ein Zytostatikum welches in der Lage ist die DNA-Topoisomerase I zu inhibieren. Dieses Enzym wird jedoch ubiquitär in fast jedem Gewebe exprimiert, wodurch die Behandlung mit Topotecan äußerst toxisch wird, bei jedoch nur geringem Behandlungserfolg.

Neue Alternativen für die Behandlung nach einem Rückfall werden für diese Tumorart dringend benötigt. Eine Möglichkeit hierfür bildet eventuell die Interaktion mit pH regulierenden Proteinen. Rasch wachsende Tumore sind nicht in der Lage, den Kern des Tumors mit ausreichend Sauerstoff zu versorgen, wodurch eine hypoxische Umgebung entsteht, in der normale Zellen absterben würden. Verschiedene Tumorarten sind jedoch in der Lage diesen Zelltod zu umgehen, indem sie, durch ein HIF-1-induziertes Signalnetzwerk, die Energiegewinnung von der sauerstoffabhängigen oxidativen Phosphorylierung zur 200fach erhöhten sauerstoffunabhängigen Glykolyse wechseln, welche nur 2 Mol ATP pro Mol Glukose erzeugt. Diese Umstellung geht jedoch mit der erhöhten Produktion von Säuren (besonders Laktat und Pyruvat) und Protonen einher, welche zu einer Ansäuerung des intrazellulären Bereichs führen würden und viele physiologischen Reaktionen inhibieren könnten.

Die Expression verschiedener pH regulierender Proteine wie Carboanhydrase IX (CA IX) und XII (CA XII), Monocarboxylat-Transporter 1 und 4 (MCT1, MCT4) sowie des Natrium-Protonen Austauschers NHE1 ist in diversen Tumorarten hinaufreguliert. All diese Proteine sind in der Lage, Protonen oder monocarboxylierte Substanzen aus der Krebszelle hinauszutransportieren um zu verhindern, dass es zur intrazellulären Ansäuerung kommt. Dadurch kommt es intrazellulär zu einer pH Verschiebung in den etwas mehr alkalinen Bereich, während es extrazellulär zu einer Ansäuerung kommt, in der gesunde Zellen absterben, während es für die Tumorzelle ein selektiver Wachstums- und Entwicklungsvorteil ist.

Ziel dieser Masterarbeit war es, einen ersten Einblick in diese pH Regulierung in verschiedenen kleinzelligem Lungenkrebs Zelllinien zu bekommen. Der Einfluss derzeit verwendeter Chemotherapeutika auf die Genexpression dieser pH regulierenden Proteine wurde ermittelt sowie deren Einfluss auf die Proteinmenge. Auch die Interaktion von Inhibitoren auf die Cisplatin und Etoposid Behandlung wurde mittels chemosensitiven Assays ermittelt sowie der Einfluss dieser Substanzen auf die Menge von löslichem CA IX im Medium.
Die untersuchten Zelllinien reagieren, obwohl sie alle kleinzelliger Lungenkrebs sind, verschieden auf die unterschiedlichen Behandlungen. Jedenfalls scheint es, dass mehr chemoresistente Zelllinien eine teilweise andere Expressions-Regulierung aufweisen als chemosensitive Zelllinien. Klar ersichtlich ist jedoch, dass die Verwendung von CA IX (C207A) und NHE1 (Zoniporid) Inhibitoren der Wirkung von Cisplatin in NCI-H417 Zellen entgegensteuert und somit das Tumorzell Überleben erhöht. Außerdem führt C207A zu einem Anstieg von löslichem CA IX in DMS-153 Zellen sowie der Einsatz von Cisplatin in NCI-H417 Zellen zu einer erhöhten Menge von löslichem CA IX. Diese erhöhte Menge könnte mit einer CA IX gerichteten Therapie interferieren.

Diese Arbeit hat gezeigt, dass die wichtigen pH Regulatoren in kleinzelligem Lungenkrebs exprimiert werden. Damit wurde die Grundlage für weiterführende Experimente in kleinzelligem sowie nicht-kleinzelligem Lungenkrebs gelegt. Die Ergebnisse könnten zu einem verbesserten Therapieansatz führen, in der Chemotherapie mit pH Regulator-Inhibitoren kombiniert werden.

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10. List of figures

Fig. 2 and 4: Taken from: Swietach Pawel, Vaughan-Jones Richard D. and Harris Adrian L., “Regulation of tumor pH and the role of carbonic anhydrase 9”, Cancer Metastasis Rev, 2007, 26: 299 – 310 with permission from Prof. Pawel Swietach

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11. Curriculum Vitae

ANGABEN ZUR PERSON — Klameth Lukas Michael Andreas

BERUFSERFAHRUNG

März 2012 – Heute

Masterpraktikum

Medizinische Universität Wien - Institut für Pathophysiologie und Allergieforschung, Waehringer Guertel 18-20, 1090 Wien (Österreich)

03. November 2011 – 30. November 2011

Wahlbeispiel der Universität Wien

MFPL, Dr. Bohrgasse 9, 1030 Wien (Österreich)

Untersuchung des Proteins Ribophorin 1 in Hamsterfibroblasten und Mutanten

22. August 2011 – 22. Oktober 2011

Wahlbeispiel der Universität Wien

Institut für Krebsforschung, Borschkegasse 8a, 1090 Wien (Österreich)

Erforschung der Auswirkung von bestimmten Genen auf die EMT (Epithelial to Mesenchymal Transition) in Leberkarzinomen

03. Mai 2010 – 31. Mai 2010

Ferialangestellter

Wirtschaftskammer Niederösterreich, Landsbergerstraße 1, 3100 St. Pölten (Österreich)

In der IT Abteilung: Aufsetzen von Laptops

13. Januar 2010 – 10. Juni 2010

Bachelorpraktikum

Hirnforschungszentrum Wien, Spitalgasse 4, 1090 Wien (Österreich)

Einfluss verschiedener Lösungsmittel auf [3H]EBOB binding; Test von "ROD-Substanzen", im Vergleich mit Benzodiazepinen

SCHUL- UND BERUFSBILDUNG

2010 – Heute

Student an der Universität Wien für das Masterstudium Molekulare Biologie

EQF Niveau 7

Universität Wien, Dr. Karl-Lueger-Ring 1, 1010 Wien (Österreich)

Molekulare Medizin
Epigenetik
Zellbiologie
Molekularbiologie

2007 – 2010

Bachelor of Science in Natural Sciences

EQF Niveau 6

FH Campus Wien, Viehmarktgasse 2A, 1030 Wien ((Österreich)

2006 – 2007

Zivildienst - Rettungssanitäter

Arbeiter Samariterbund Österreich, Hollergasse 2-6, 1150 Wien (Österreich)

1998 – 2006

Maturant

BGRG VIII (Bundesgymnasium und Bundesrealgymnasium), Albertgasse 18-22, 1080 Wien (Österreich)

1994 – 1998

Volksschule Langegasse (Volksschule), Lange Gasse 36, 1080 Wien (Österreich)

PERSÖNLICHE FÄHIGKEITEN

Muttersprache(n) Deutsch

Weitere Sprache(n)

	VERSTEHEN		SPRECHEN		SCHREIBEN
	Hören	Lesen	An Gesprächen teilnehmen	Zusammenhängendes Sprechen	
Englisch	C1	C1	C1	C1	C1

A1/A2: elementare Sprachverwendung - B1/B2: selbstständige Sprachverwendung - C1/C2: kompetente Sprachverwendung
Gemeinsamer Europäischer Referenzrahmen für Sprachen