universität wien

DIPLOMARBEIT

Titel der Diplomarbeit

Overcoming resistance against ErbB inhibitory drugs by simultaneously targeting downstream effector kinases in breast cancer cells

angestrebter akademischer Grad

Magistra der Naturwissenschaften (Mag. rer.nat)

Verfasserin / Verfasser:	Victoria Saferding
Matrikel-Nummer:	0300895
Studienrichtung /Studienzweig (lt. Studienblatt):	Biologie / Genetik und Mikrobiologie
Betreuerin / Betreuer:	Univ.-Prof. Dr Thomas Decker

Wien, im Oktober 2010

"In der Wissenschaft gleichen wir alle nur den Kindern, die am Rande des Wissens hier und da einen Kiesel aufheben, während sich der weite Ozean des Unbekannten vor unseren Augen erstreckt." (Isaac Newton)

Danksagung

Ich danke Univ. Prof. Dr. Thomas Decker, der mir die Durchführung meiner Diplomarbeit an der Medizinischen Universität Wien, Univ. Klinik für Innere Medizin I, Abteilung für Onkologie im AKH ermöglicht hat.
Mein besonderer Dank gilt Hr. Univ. Prof. Mag. Dr. Thomas W. Grunt für die Themenstellung sowie Finanzierung, aber vor allem für seine hervorragende Betreuung und Unterstützung. Weiters danke ich auch Hr. Univ. Prof. Dr. Peter Valent für seine Unterstützung.

Meiner Kollegin Renate Wagner danke ich ganz besonders für ihre großartige Hilfe, besonders am Beginn meiner Arbeit, für unzählige Ratschläge, große Geduld bei vielen Erklärungen und der Einschulung im Labor, sowie für ein wunderbares, lustiges und bestes Arbeitsklima das es gibt.

Außerdem möchte ich mich bei meinen Studienkolleginnen Caroline Brünner-Kubath und Katharina Tomek für ihre Unterstützung in manch verzweifelten Momenten und ihren Ermutigungen danken.
Meinem Freund Florian danke ich für die liebevolle Unterstützung und besonders für seine Geduld und seine Eigenschaft als guter Zuhörer während meines gesamten Studiums.
Weiters danke ich auch meinem Bruder Gabriel der mir bei unzähligen Computerfragen hilfreich zur Seite stand.
Mein größter Dank gilt allerdings meinen Eltern, Edeltraud und Franz, die mir mein Studium ermöglicht und mir immer mit Rat und Tat zur Seite gestanden haben.

Danke an alle, die an mich geglaubt haben und mich dazu ermutigt haben nicht aufzugeben und mich in diesem Abenteuer immer unterstützt haben.

Abstract

Breast cancer is the most common malignancy in women worldwide. Deregulations of members of the ErbB receptor family play an important role in breast cancer pathogenesis. Epidermal growth factor (EGF) binds to EGF receptor (EGFR) and activates a network of signalling pathways. The two major downstream signalling pathways of EGFR are the mitogen-activated protein kinase(MAPK)- and the phosphoinositide 3-kinase(PI3K)-transduction systems. Both pathways exhibit a high frequency of activating mutations and are good targets for cancer therapy.

Two irreversible ErbB inhibitors termed pelitinib and canertinib were tested in breast cancer cell lines. In some cell lines the drugs showed good growth inhibitory effects but there were still some cell lines, which were highly resistant to treatment with these drugs. In this study we concentrated on the two breast cancer cell lines SKBR3 and T47D, which proved to be resistant and sensitive to ErbB inhibition respectively. By treatment of SKBR3 and T47D cell lines with PI3K and MAPK pathway inhibitors we examined the significance of both pathways for growth of these cell lines and for the development of resistance against ErbB inhibitory drugs. We applied mTOR-(rapamycin), AKT-(Akti-1,2) and PI3K/mTOR-(NVP-BEZ 235) inhibitors to examine the PI3K/AKT pathway and MEK1,2 (AZD6244, U0126 and AS703026) inhibitors to silence the MAPK pathway. Results showed that in vitro growth of both cell lines was highly sensitive to inhibition by PI3K/AKT pathway inhibitors, especially to NVP-BEZ 235, but resistant to MAPK pathway inhibition. This finding let us suggest that the PI3K/AKT pathway plays a more important role in growth of these cell lines and could be a good target to overcome resistance against ErbB inhibition. Our assumption could be confirmed by a combined therapy using canertinib or pelitinib together with PI3K/AKT pathway inhibitors, which leads to a strong decrease of the ErbB drug resistance. Of note, this therapy showed especially good growth inhibitory effects by combination of ErbB inhibitors with NVP-BEZ 235, the dual PI3K and mTOR inhibitor. On the other hand, our data reveal that

inhibition of the MAPK pathway did not have any growth inhibitory effect. In contrast, the data indicate that an ErbB independent activation of the PI3K/AKT pathway is a very important determinant, for resistance against the ErbB inhibitors pelitnib and canertinib.

Zusammenfassung

Brustkrebs ist die häufigste Krebserkrankung bei Frauen weltweit. Die ErbB Rezeptor Familie spielt eine wichtige Rolle in der Entstehung dieser Erkrankung. Sie zeigt sehr oft eine Deregulierung in Krebserkrankungen. Der epidermale Wachstumsfaktor (EGF) bindet am EGF Rezeptor (EGFR) und aktiviert ein Netzwerk von Signalwegen. Die zwei bedeutendsten nachgeschalteten Signalwege von EGFR sind der mitogen aktivierte Proteinkinase (MAPK)- und der Phosphoinositide 3-kinase (PI3K)- Weg. Beide Signalwege weisen eine hohe Frequenz an Mutationen auf und sind daher gute Ziele für die Krebstherapie.

Der Effekt von zwei irreversiblen ErbB Inhibitoren genannt Pelitinib und Canertinib wurde in Brustkrebszelllinien getestet. In einigen Zellen zeigten sie gute wachstumsinhibierende Effekte, jedoch gibt es immer noch einige, die resistent auf eine Behandlung mit diesen Substanzen reagieren. In unserer Studie haben wir uns auf zwei bestimmte Brustkrebszelllinien, SKBR3 und T47D, konzentriert, die resistent und sensitiv auf ErbB Inhibierung reagieren. Durch Behandlung von SKBR3 und T47D Zelllinien mit PI3K- und MAPK-Signalweginhibitoren wollten wir die Bedeutung beider Signalwege für diese Zelllinien untersuchen und darüber hinaus die Mechanismen der Resistenz definieren. Wir haben mTOR-(Rapamycin), AKT-(Akti-1,2) und PI3K/mTOR-(NVP-BEZ 235) Inhibitoren angewandt um den PI3K/AKT Signalweg und MEK1,2 (AZD6244, U0126 und AS703026) Inhibitoren um den MAPK Signalweg zu bestimmen. Die Ergebnisse zeigten, dass beide Zelllinien sehr gut auf eine Inhibierung mit PI3K/AKT Signalweginhibitoren, besonders mit NVP-BEZ 235, aber in sehr schlechtem Ausmaß auf eine MAPK Signalweginhibierung ansprechen. Diese Erkenntnisse ließen uns vermuten dass der PI3K/AKT Signalweg eine wichtigere Rolle in diesen Zelllinien spielt und eine sehr gute Zielstruktur wäre, um die Resistenz gegen ErbB Inhibierung zu überwinden. Unsere Vermutung konnte durch eine kombinierte Therapie von Canertinib beziehungsweise Pelitinib mit PI3K/AKT Signalweginhibitoren bestätigt werden. Diese führte zu einer starken Senkung der Resistenz

gegenüber den ErbB Inhibitoren. Des weiteren zeigte diese Therapie besonders gute wachstumsinhibierende Effekte in der Kombination von ErbB Inhibitoren mit NVP-BEZ 235, einem zweifachen PI3K und mTOR Inhibitor.

Wir konnten zeigen, dass die Inhibierung des MAPK Signalweges keinen wachstumsinhibierenden Effekt hatte, des weiteren ließen unsere Daten erkennen, dass eine ErbB unabhängige Aktivierung des PI3K/AKT Signalweges ein sehr wichtiger Faktor für die Entwicklung von Resistenz gegenüber den ErbB Inhibitoren Pelitinib und Canertinib darstellt.

Table of contents

1.Introduction

1.1 Cancer

1.1.1 What is cancer

Cancer is characterized as a disease with uncontrolled expansion and invasion of cells. These cells display deregulted cell proliferation and suppression of apoptosis (Evan et al., 2001).

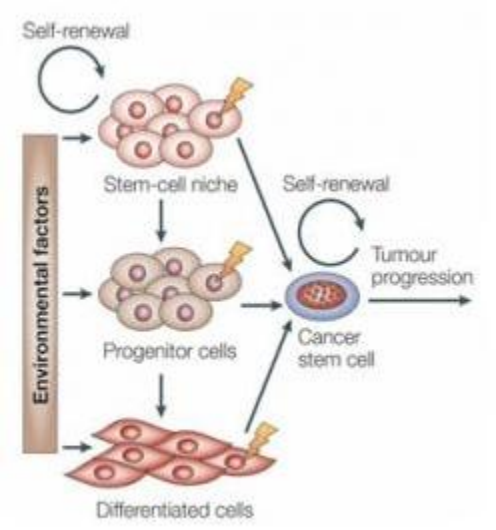


Figure 1: *Mutations in stem cells or progenitor cells might give rise to cancer stem cells (Bjerkvig et al., 2005).*

The progress which leads to a transformation of normal cells to malignancy requires mutations which arise as a consequence of damage to the genome. Cancer stem cell might appear after mutations in specific stem cells or early stem cell progenitors. Moreover it is also possible that cancer stem cells can be derived from differentiated cells. There might be numerous factors in the host microenvironment that trigger the initial steps of tumor formation (Figure 1). Mutational damage can be the result of endogenous processes such as errors in replication of DNA, through the intrinsic chemical instability of certain DNA bases or from the attack by free radicals generated during metabolism.

DNA damage can also be a result from interactions with exogenous agents such as ionizing radiation, UV radiation and chemical carcinogens.
Cells have evolved some mechanisms to repair such damage, but for different reasons errors occur and permanent changes in the genome are introduced.
The evolution of a normal cell to a malignant one, involves processes by which genes involved in normal homeostatic mechanisms that control proliferation and cell death suffer mutational damage. This can result in the activation of genes, which stimulate proliferation or protection against cell death. These genes are called oncogenes. In contrast, altered genes that in their wild type-form would inhibit proliferation and called tumor suppressor genes. When a cancer cell overcomes normal controls on cell brith and cell death, this cell has two challenges, it must overcome replicative senescence and become immortal. In addition, this cell must obtain adequate supplies of nutrients and oxygen, to maintain this high rate of proliferation (Bjerkvig et al., 2005; Bertram et al., 2000).

1.2 Hallmarks of cancer

There are a lot of different types of cancer and subtypes of tumors found in specific organs. In general there are six essential alterations in cell physiology, which cause malignant growth (Figure 2).

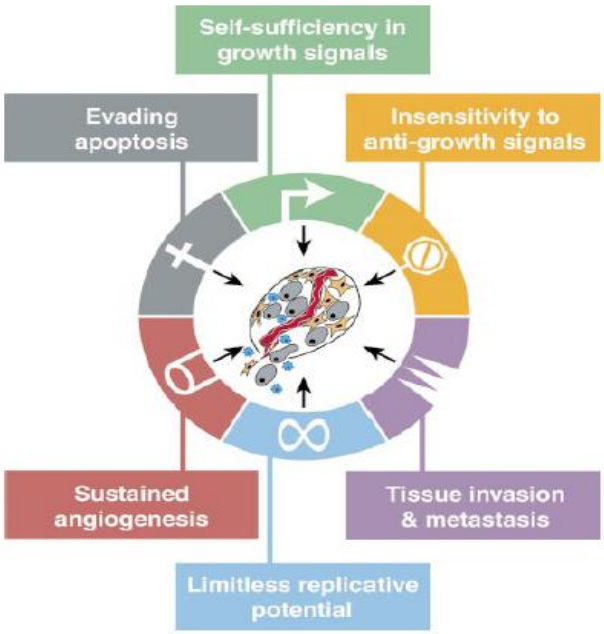


Figure 2: *Hallmarks of cancer, suggestion that most cancers have acquired the same set of function capabilities during their development (Hanahan et al.,2000).*

These six alterations are self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis, tissue invasion and metastasis. This multiplicity of defenses may explain why cancer is relatively rare during an average human lifetime (Hanahan et al.,2000).

1.2.1 Self-sufficiency in growth signals

Normal cells require mitogenic growth signals, before they can move to an active proliferative state. These signals are transmitted into the cell by transmembrane receptors that bind specific signalling molecules. No normal type of cell is able to proliferate in the absence of such stimulatory signals. However tumor cells show a strongly reduced dependence on exogenous growth stimulation. A possible reason could be that they generate many of their own growth signals and thereby reducing their dependence on stimulation from

their normal tissue microenvironment. This mechanism disrupts a critically important homeostatic function that normally ensures a proper behavior of different cell types. To stimulate tumor cell growth, cancer cells possess uncontrolled overexpression or activation of cell surface receptors, which transduce stimulating signals into the cell. For example the epidermal growth factor receptor EGFR/ErbB1 or more frequently (~20%), or the HER2/neu/ErbB2 receptor is overexpressed in mammary carcinomas (Hanahan et al., 2000).

1.2.2 Insensitivity to antigrowth signals

Normal tissues have mutiple antiproliferative signals to maintain cellular quiescence and tissue homeostasis. These signals include soluble growth inhibitors and immobilized inhibitors embedded in the extracellular matrix and on the surfaces of nearby cells.

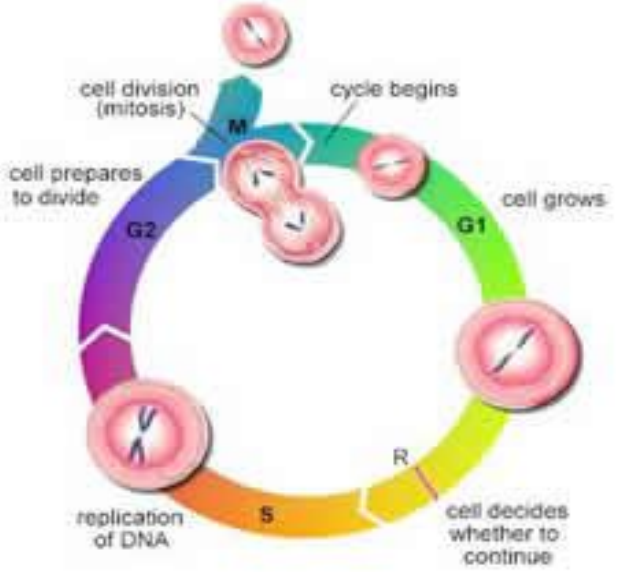


Figure 3: *Phases of the cell cycle* (1).

Antigrowth signals can block proliferation by two distinct mechanisms. Cells can be forced out of the active proliferative cycle into the quiescent (G0) state from which they may reemerge on some future occasion when extracellular signals permit (Figure 3). Second mechanism is that cells may be induced to permanently relinquish their proliferative potential by being induced to enter into postmitotic states, which are normally associated with the acquisition of specific differentiation traits. Cancer cells have to avoid these antigrowth signals, for an unlimited proliferation.
Therefore often mutations are found in components which are responsible for the regulation of proliferation. For example retinoblastoma protein (pRb), which controls the expression of different genes, is important for the cell cycle progression from G1 to S phase (Hanahan et al., 2000).

1.2.3 Evading apoptosis

The ability of tumor cells to expand is determined by the rate of cell proliferation and also by the rate of cell death. A resistance against apoptosis is a hallmark of most and maybe all types of cancer. To circumvent apoptosis tumor cells intervene antiapoptotic survivalsignals and regulate tumor suppressors. Oncogenes which are expressed in a high level help to turn normal cells into cancer cells. Such genes for example RAS and c-Myc as well as tumor suppressors like p53 and PTEN are an important part of this alteration (Hanahan et al., 2000).

1.2.4 Limitless replicative potential

Furthermore cancer cells require unlimited replicative potential. For this feature telomere play an essential role. Telomeres are the end of chromosomes and consist of several thousand repeating sequences and have a protective function. Telomere maintenance is evident in all types of malignant cells.

Therefore, they upregulate the expression of telomerase enzyme, which adds hexanucleotide repeats onto the ends of telomeric DNA or they use a rare mechanism called alternative lengthening of telomeres (ALT). In both mechanisms telomeres are maintained at a length above a critical threshold and this in turn permits unlimited multiplication of descendant cells.
These two mechanisms seem to be strongly suppressed in most normal human cells, to deny them unlimited replicative potential (Hanahan et al., 2000).

1.2.5 Sustained angiogenesis

The oxygen and nutrients supplied by the vasculature are crucial for cell function and survival. Therefore, it is necessary that all cells in a tissue are provided with a capilary blood vessel. When a tissue is formed, the growth of new blood vessels, this process which is called angiogenesis is very carefully regulated. Tumor cells, which rapidly divide, often lack angiogenic ability. So they have to develop angiogenic ability to get a good supply with nutrients, which is necessary for growth. Counterbalancing positive and negative signals encourage or block angiogenesis. These signals are for example vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF). Moreover integrins and adhesion molecules, which mediate cell-matrix and cell-cell association, also play critical roles. Tumors activate the angiogenic switch by changing the balance between angiogenesis inducers and countervailing inhibitors. One common strategy for shifting the balance involves altered gene transcription (Hanahan et al., 2000).

1.2.6 Tissue invasion and metastasis

The capability for invasion and metastasis enable cancer cells to escape the primary tumor mass and colonize new terrain in the body where no limiting of nutrients and space is present. Such distant settlements of tumor cells and

metastases are the cause of 90% of human cancer deaths. Invasion and metastasis are complex processes, which involves changes in the physical coupling of cells to their microenvironment and activation of extracellular proteases. Several different proteins which are involved in the tethering process of cells to their surroundings in tissue are altered. These proteins include cell-cell adhesion molecules (CAM). The most observed alteration in cell to environment interactions in cancer involves E-cadherin, a homotypic cell to cell interaction molecule, which is ubiquitously expressed on epithelial cells. A coupling between adjacent cells by E-cadherin bridges results in the transmission of antigrowth and other signals, via cytoplasmic contacts with β-catenin to intracellular signalling circuits.
The second parameter of the invasive and metastatic capability involves extracellular proteases. Docking of active proteases on the cell surface can facilitate invasion by cancer cells into nearby stroma, across blood vessels walls and through normal epithelial cell layers (Hanahan et al., 2000).

1.3 Distribution of cancer

1.3.1 The geographical distribution and incidence rates of breast cancer

Breast cancer is the most common malignancy in women worldwide and is the leading cause of cancer related mortality. 45% of the more than one million new cases of breast cancer diagnosed each year, and more than 55% of breast-cancer–related deaths, occur in low- and middle income countries. There is a higher prevalence of breast cancer in industrialised than in non industrialised countries.

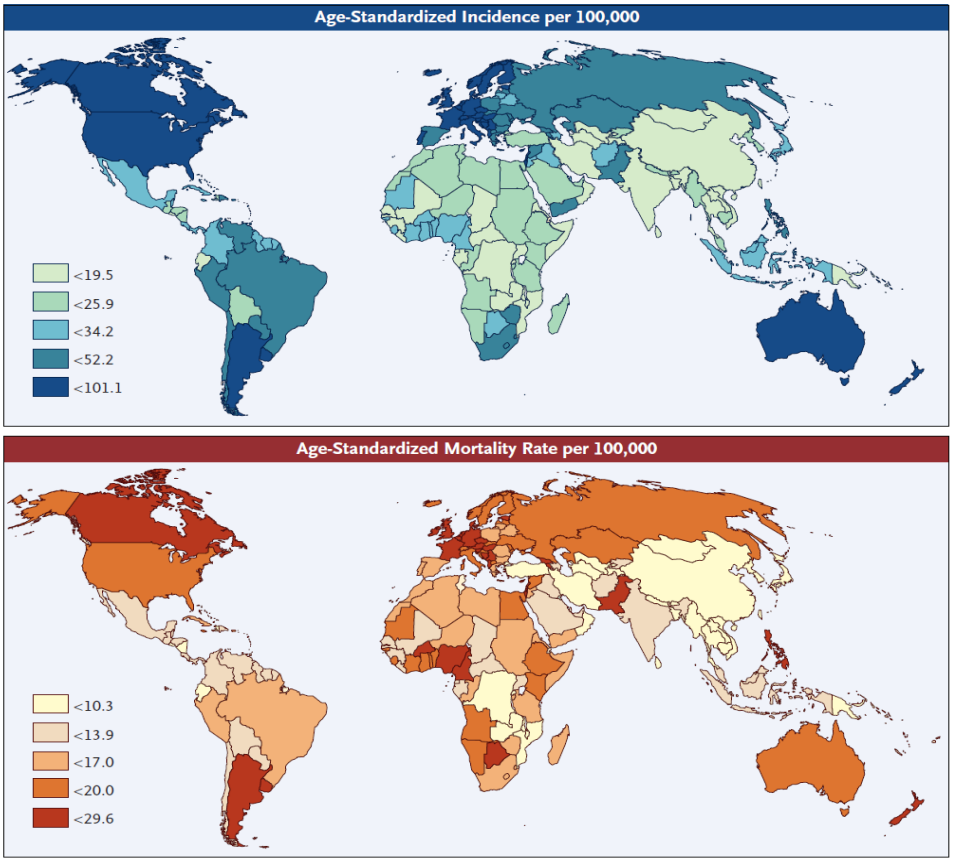


Figure 4: *Incidence and mortalitiy rate of breast cancer in the world (Porter et al., 2008).*

East Asian women still have the lowest rates of breast cancer (Figure 4). Japan, Singapore and Korea, countries with the most developed registries, documented increasing rates. They have doubled or tripled in the past 40 years. In India there are similar trends observed, with increased concentrations in urban areas.

The incidence of breast cancer in African countries is lower than overall rates in North America or Western Europe, as well as lower than rates among black women living in these Western countries.

The most reason for the global increase in breast cancer is the socioeconomic improvement that increase life expectancy and allow women reproductive

control as well as the adoption of less desirable habits. This is called Westernization and can strongly contribute to an increase in breast cancer risk (Porter et al., 2008). Breast cancer is a postmenopausal disease, with more than three-quarters of tumors hormone responsive.

This hormone dependency interacts with environmental and genetic factors to determine incidence and progression of the disease. Age and female sex are major risk factors for breast cancer, with rapidly rising incidence rates, between the age of 35 and 39 years. The age of increase slows around the age of 50 years, corresponding to the average age of menopause. Figure 5 shows two separate rate curves for oestrogen receptor positive and negative tumors. The incidence of oestrogen receptor negative tumors increases rapidly until age 50 years and then flattens or decreases. But the incidence of oestrogen receptor positive tumors is similar up to the age of 50 years, but then continues to climb at a slower pace. Oestrogen receptor negative tumors occur earlier in life and oestrogen receptor positive tumors are more common in older women (Benson et al., 2009).

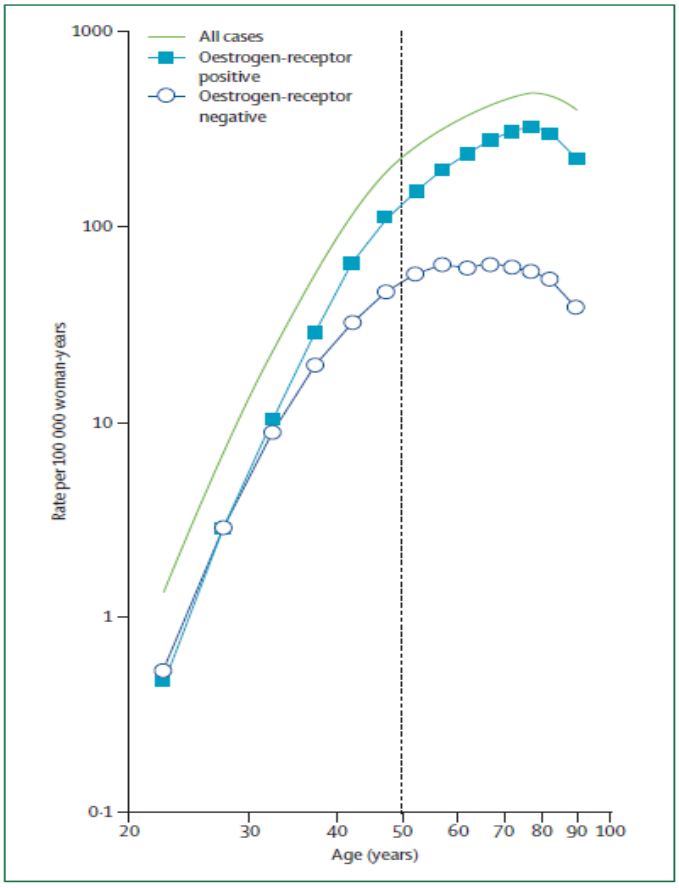


Figure 5: *Age specific incidence rates in female patients suffer on breast cancer (Benson et al., 2009).*

1.4 Risk factors for breast cancer

One of the strongest risk factors for breast cancer is increasing age. Early age at menarche, late age at menopause, nulliparty and late age at first birth increase the risk for breast cancer. But breastfeeding is associated with lower risk. Obesity in Western countries is associated with breast cancer development. Moreover, there are some gene mutations identified, which also contribute to breast cancer. These mutations are tumor suppressor genes, for example BRAC1, BRAC2 and PTEN (Hankinson et al., 2004).
A germ line mutation in the BRCA1 or BRCA2 gene is the most detectable cause of a heritable risk of breast cancer. 40-50% of families with multiple cases of female breast cancer are linked to these genes (Robson et al., 2007). An association between alcohol consumption and breast cancer was observed in epidemiologic studies. Alcohol affects plasma oestrogenes, which also have an influence of breast cancer risk. Alcohol consumption is positively associated with ER positive and PR positive breast cancers, but not with ER negative and PR negative cancers. This leads to the suggestion that alcohol may act in part through the estrogen pathway, to affect breast cancer risk. It may also affect breast cancer risk by acting as a co carcinogen. Alcohol increases the permeability of cell membranes to carcinogenes, being mutagenic through acetaldehyde, inhibiting detoxification of carcinogenes, activation of procarcinogenes, inducing oxidative stress and affecting folate metabolism (Zhang et al., 2007). Moreover it was found, that oestrogen and progesterone play a very important role in breast cancer development. Studies revealed that hormone replacement therapy (HAT) leads to an increased risk of breast cancer in healthy women (Holmberg et al., 2008).

1.5 Diagnosis of breast cancer

Patients with breast cancer have to be scanned precisely. Different factors have to be evaluated clinically. A mammographic screening leads to the detection of malignancy. Moreover, screening mammography in postmenopausal women reduces breast cancer mortality by about 20%. Complementary to mammography, breast ultrasound is made, it increases diagnostic accuracy. Tissue diagnosis is also essential and can be obtained with fine needle aspiration cytology. Percutaneous needle biopsy techniques can now provide a definitive diagnosis for benign and malignant diseases. To clarify the extent of a lesion and to establish, whether satellite foci are present in patients, magnet resonance imaging (MRI) is used (Benson et al., 2009).

1.6 Treatment strategies in breast cancer

The primary treatment of localized breast cancer is either breast conserving surgery and radiation therapy or mastectomy with or without breast reconstruction. Systemic adjuvant therapies, which eradicate microscopic deposits of cancer cells that may have spread or metastasized from the primary breast cancer, show an increasing chance for long term survival in breast cancer patients. Such systemic adjuvant therapies include chemotherapy, with anticancer drugs and hormone therapy.
In addition radiotherapy in some cases as a local adjuvant treatment to destroy breast cancer cells, which remain in the chest wall or in regional lymph nodes after mastectomy. Breast cancer must be determined precisely in tumor size, histological type, age of the patient and biomarkers like estrogen receptor status and progesterone receptor status, to find the best treatment option for breast cancer patients (Eifel et al., 2001). Breast conservation surgery is a procedure, which is preferred for women with early stage breast cancer. Moreover widespread mammographic screening has contributed to a stage shift for newly

diagnosed disease, with an average tumour size of less than 2cm. Interestingly, long term follow up of breast conservation trials show increased rates of local relapse when radiotherapy is omitted. Therefore breast conservation surgery is combined with whole breast irradiation. With new partial breast irradiation techniques called intraoperative radiotherapy and MammoSite intraoperative radiotherapy, high dose of radiation can be applicated as one fraction at the time of surgery. This technique allows a precise application of radiation to the targeted area, to eliminate tumor foci around the surgical bed (Benson et al., 2009).

1.7 ErbB receptors

1.7.1 ErbB receptor family

The ErbB receptor family plays a very important role in the pathogenesis of breast cancer (Karamouzis et al., 2006). Moreover, when epidermal growth factor-like ligands bind to the ErbB family receptors, a network of signalling pathways is activated, which influence cell division, apoptosis, motility and adhesion (Yarden et al., 2006). The ErbB receptor family consists of four different types of receptors. EGFR/ErbB1/Her1, ErbB2/Her2/neu, ErbB3/Her3 and ErbB4/Her4. All these receptors have intrinsic tyrosine kinase activity except Her3. Her2 does not bind any ligand, but it is a preferred partner for heterodimerisation. Because Her2-containing heterodimers show higher membrane stability and decreased degradation rates than heterodimers containing other partners, they are more mitogenic than others. In particular, the Her2/Her3 heterodimer is the most potent growth-stimulating ErbB signaling complex (Karamouzis et al., 2006).

1.7.2 Ligands of the ErbB receptor familiy

ErbB receptors are activated by a number of ligands that constitute the EGF family of peptide growth factors. These peptides are produced as membrane-anchored precursors and are processed and released by proteolysis.
There exist a large number of ErbB specific ligands (Figure 6), and all of them contain an EGF like domain, which is sufficient for binding specificity.

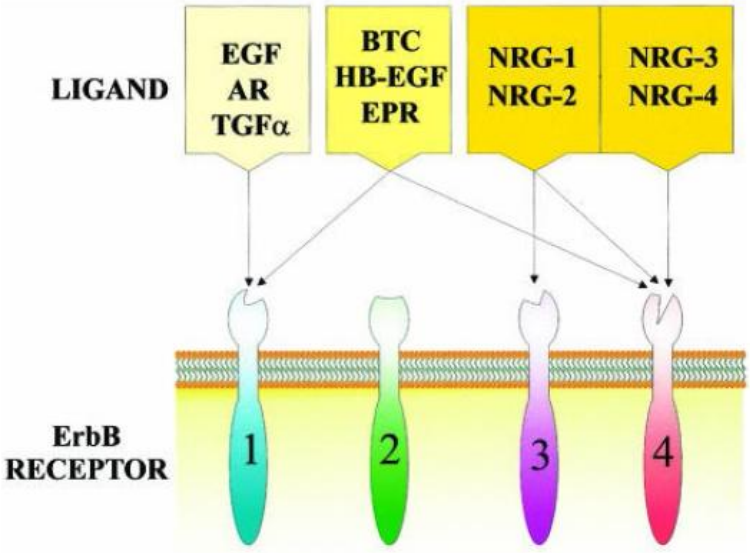


Figure 6: *Binding specficities of the EGF related peptide growth factors (Olayloye et al., 2000).*

These ligands included EGF, amphiregulin (AR), and transforming growth factor-α (TGFα), which bind specifically to ErbB1. Moreover, there are betacellulin (BTC), heparin binding EGF (HB-EGF) and epiregulin (EPR). EPR exhibits dual specificity, it binds both ErbB1 and ErbB4. Neuregulins (NRG) comprise the third ligand family.
NRG-1 and NRG-2 bind to ErbB3 and ErbB4 whereas NRG-3 and NRG-4 only bind to ErbB4 but not to ErbB3. The binding of ligands to these receptors leads to the formation of homo- and heterodimers. This stimulates the intrinsic

intracellular tyrosine kinase activity of the receptors and triggers autophosphorylation of specific tyrosine residues in the cytoplasmic of the receptor protein domain. These phosphorylated residues serve as docking sites for signalling molecules, which are involved in the regulation of intracellular signalling cascades.

For ErbB2 no direct ligand has been discovered so far. Therefore, the suggestion is that its primary function is as a coreceptor. Moreover, ErbB2 is the most preferred heterodimerization partner (Figure 7) for all other ErbB familiy members (Olayloye et al., 2000).

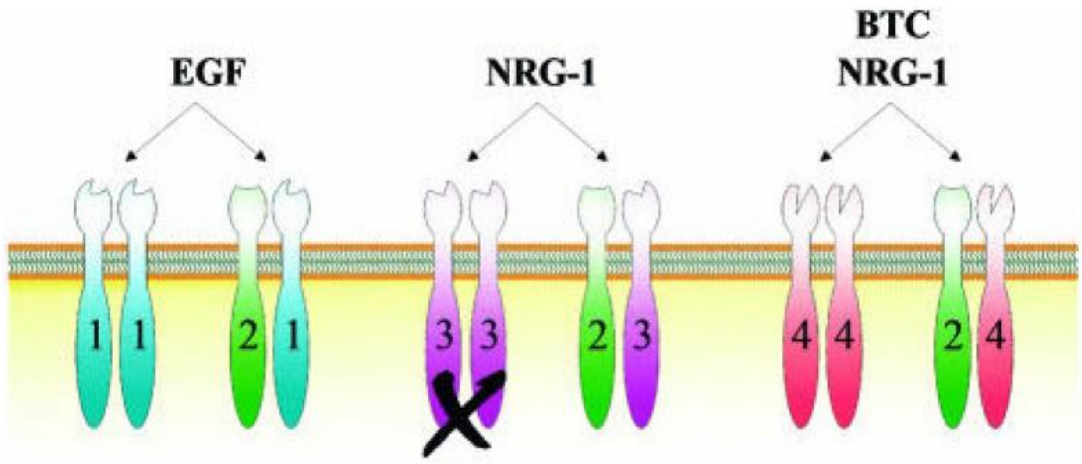


Figure 7: *ErbB2 serves as a preferred dimerization partner for the other ErbB receptors (Olayloye et al., 2000).*

Furthermore ErbB3 homodimers are not able to signal, because ErbB3 harbours a catalytically deficient kinase domain. Therefore, ErbB3 signalling relies on the formation of signalling competent heterodimers with other ErbB family members (Karamouzis et al., 2006). ErbB2 and ErbB3 heterodimers are the most potent ErbB signalling complexes. In cancer EGFR and ErbB2 are often overexpressed and are associated with a poor clinical prognosis (Olayloye et al., 2000).

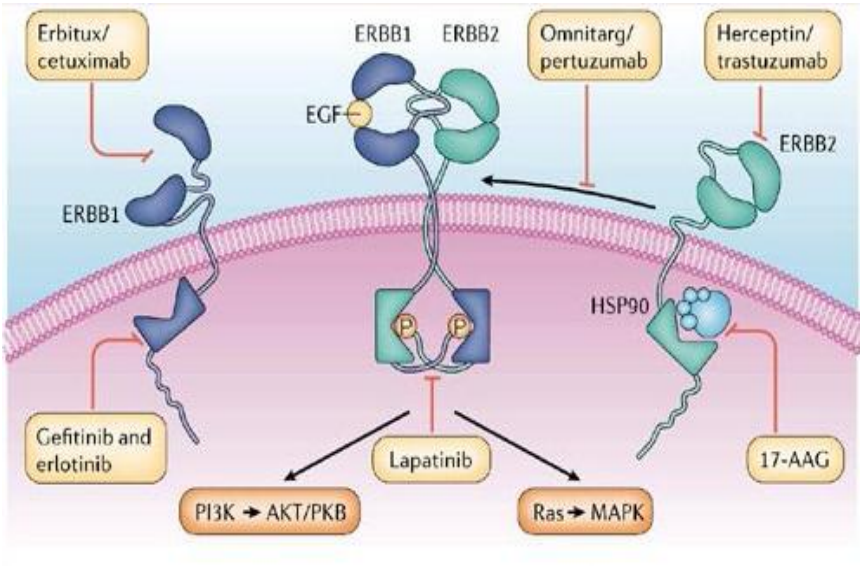
1.7.3 Downstream signalling of ErbB receptors

Regulation of the intracellular signalling pathways, which become activated by ErbB receptors is very complex. The ligand-receptor composition on a cell, the availability of intracellular docking molecules and, moreover, negative effectors, which downregulate the signal are some key parameters that determine the final outcome of ErbB activation.

The binding of ligand influences, which tyrosines on a particular ErbB protein become phosphorylated, which in turn determines what type of signalling molecules is to be enrolled in the downstream events. Binding of cytoplasmic mediators, including adaptor proteins or enzymes, occur through their Src-homology 2 (SH2) - or phosphotyrosin-binding (PTB)-domains, whereas the recruitment specificity is determined by the amino acids surrounding the autophosphorylation site on the receptor itself. The Shc- or Grb2- activated mitogen–activated protein kinase (MAPK) pathway is a common downstream target of many receptors. In the same way, the phosphatidylinositol-3-kinase (PI3K) pathway becomes activated by most ErbBs. However, the signalling extent depends upon the receptor, because for example ErbB3 couples particularly well to this pathway because of its multiple p85 (subunit of PI3K pathway) docking sites, whereas ErbB4 directly recruits p85. There are other cytoplasmic docking proteins, which are reruited by specific ErbB receptors and less exploited by others. These include for example the GTPase activation proteins (GAP), the protein tyrosine phosphatise SHP-1 and the intracellular tyrosine kinase Src. Moreover, the duration of signalling is another key factor in the defining of the biological output. The stimulation of cytoplasmic pathways transduces the signal to the nucleus, where a lot of transcription factors undergo activity changes, this leads to the initiation of many transcription programs. There are numerous transcription factors downstream of ErbBs, which have been described. They include c-Jun, c-Fos and c-Myc, NF-κB, zinc-finger transcription factors and signal transducer and activator of transcription

(Stat). Therefore, the ErbB signalling network controls a wide array of biological outcomes, including cell proliferation, differentiation, survival, adhesion and migration (Holbro et al., 2004).

1.7.4 Targeting of ErbB receptors in cancer



ErbB receptors, especially EGFR and ErbB2 play a very important role in the development and malignancy of human cancer (Olayloye et al., 2000).

Figure 8: *Different targeting strategies of ErbB receptors in cancer cells (Citri et al., 2006).*

For treatment, monoclonal antibodies, tyrosine kinase inhibitors, heat shock protein- 90 (HSP90) inhibitors and drug combination are used.
Monoclonal antibodies act by recruiting cytotoxic lymphocytes. Moreover, they have direct effects on signalling in the target tumor cell itself. Antibodies, which are nowadays in use, are trastuzumab, which targets ErbB2 and Cetuximab, which targets ErbB1 (Figure 8) Tyrosine kinase inhibitors are small molecules which act by blocking the nucleotide binding site of ErbB receptors. Two ErbB1 inhibitors, erlotinib and gefitinib, are in use for the treatment of non small lung cancer. But a much more effective strategy is the use of dual inhibitors like lapatinib, which targets both ErbB1 and ErbB2. ErbB2 strongly depends on the

HSP90 chaperone complex to maintain its stability. This could therefore be a good possibility to target ErbB2. Therefore, the clinical efficacy of HSP90 inhibitors like 17-N-allylamino-17-demethoxygeldanamycin are under evaluation. However, cellular dynamics and genetic heterogeneity underlie tumor resistance to these drugs. Therefore, combination treatment strategies are being developed, that aim to prevent tumor recurrence. Combination of anti-ErbB drugs with anti cancer chemotherapy and radiotherapy appears to be a promising way to overcome drug resistance (Citri et al., 2006).

1.7.5 Overexpression of EGFR

Overexpression of EGFR is associated with malignancy and with enhanced ability of certain tumors to invade normal tissues and to metastasize (Korc et al., 1992). EGFR is overexpressed in a variety of human cancers. This overexpression is correlated with a loss of estrogen responsiveness and poorer prognosis. EGFR is involved in later stages of human breast cancer and may play an important role in the metastatic process (Biscardi et al., 2000).
By the use of EGFR-targeting small-molecule tyrosine kinase inhibitor gefitinib (ZD1839, Iressa), the growth of cell lines that express high levels of EGFR could be inhibited. ZD1839 promotes the formation of inactive EGFR/ErbB2 and EGFR/ErbB3 heterodimers (Anido et al., 2003). Moreover, gefitinib, which is a quinazoline tyrosine kinase inhibitor, shows good growth inhibitory effects in EGFR and Her2 overexpressing breast cancer, which was associated with the dephosphorylation of EGFR and Her2 and a down-regulation of Akt activity (Moasser et al., 2001). Erlotinib is another EGFR receptor tyrosine kinase inhibitor. It inactivates EGFR downstream signalling (Schaefer et al., 2007).

1.7.6 Overexpression of ErbB2

Ligand-independent activation and prolonged membrane stability of overexpressed ErbB2 in combination with its preferred role as a dimerization partner is responsible for its high oncogenic potential. Moreover, the level of ErbB2 in human cancer cells with membrane overexpression is much higher than in normal adult tissues. Therefore, ErbB2 is a good target for cancer therapy. A monoclonal antibody (clone 4D5) targets the extracellular domain of ErbB2. The humainized version of this antibody (trastuzumab, herceptin) is used to treat ErbB2-positive metastatic breast cancer. This treatment results in a rapid reduction of ErbB2 phosphorylation. But not all ErbB2 overexpressing cells show downstream effects to Herceptin treatment. Phase II and III trials in metastatic disease showed that trastuzumab has relevant clinical activity against ErbB2 positive breast cancer (Valabrega et al., 2007; Olayioye et al., 2000). But lots of patients, who originally responded to trastuzumab therapy, become resistant within one year of treatment. Trastzuzmab inhibits ErbB2, therefore EGFR/ErbB3 heterodimers are formed, which signal through the MAPK and PI3K/AKT signalling pathway. Hence, breast cancer cells become resistant against trastuzumab. Therefore, combination of different inhibitors could be a good therapeutic strategy (Natha et al., 2006).
ErbB2 overexpression stimulates the MAPK and PI3K/AKT pathway and therefore promotes cell cycle progression. The combination of ErbB2 overexpression and activation of ErbB downstream signalling pathways often leads to multidrug resistance in breast cancer cell lines (Knuefermann et al., 2003).

1.7.7 Overexpression of ErbB3

In the ErbB family ErbB3 is inimitable, because of its catalytically deficient kinase domain and the high propensity to self associate in the absence of

ligand. The ErbB3 gene is located on chromosome 12q13. Its signalling relies on the formation of heterodimers with other ErbB members. ErbB3 is overexpressed in many cancers, like breast, lung, pancreas and stomach. Furthermore, this overexpression is documented in 20-30% of invasive and in approximately one third of in situ breast carcinomas. Moreover, its overexpression in cancer is associated with poor prognostic factors. The most mitogenic dimerization partners of ErbB receptors are ErbB2/ErbB3 heterodimers. They are constitutively active in breast cancer cells with ErbB2 overexpression. Drug resistance in a lot of ErbB2 overexpressing cancers rely on an increasing level of ErbB3 and/or EGFR. The EGFR/ErbB3 heterodimer signals through RAS-MAPK signalling pathway. PI3K activation in response to the binding of ligands is mostly mediated by ErbB3, because this receptor has multiple binding sites for the p85 regulatory subunit of the PI3K (Karamouzis et al., 2006).

1.7.8 Overexpression of ErbB4

The ErbB4 gene is located on chromosome 2q33.3-34 and encodes a transmembrane glycoprotein, which is very important in the development and differentiation of various tissues, especially the cardiovascular, the neural system and mammary glands, as well as in different pathological processes, including malignancy. However, ErbB4 is not frequently overexpressed in breast carcinomas, compared to other ErbB members. Its overexpression is associated with increased estrogen receptor and cell proliferation rates. Cell line experiments show that ErbB2 positive cancer cells transfected to overexpress ErbB4 lead to a reduction in proliferation and an increase in apoptosis (Karamouzis et al., 2006). Therefore, ErbB4 expression can serve as a marker of survival in breast cancer. Moreover, it was described that ErbB4 overexpression can be associated with cell growth and cell progression, which leads to the suggestion that there are differences in breast cancer subtypes,

according to ER+ and ER- tumors. Furthermore, breast cancer overexpressing ErbB4 in ER+ tumors have a good prognosis (Sundvall et al., 2008). Mutations of ErbB4 have been found in many cancers including breast cancer. Mutated ErbB4 is most frequently seen in melanoma, where it appears to be the only ErbB receptor which is altered by mutation. Moreover, mutant ErbB4 is one of the driving forces for the development of melanoma. It should therefore be targeted by novel pan-ErbB inhibitors (Rudloff et al., 2010).

1.8 Downstream signalling pathways of ErbB

1.8.1 Mitogen activated protein kinase (MAPK) signalling pathway

The phosphorylation by the receptor tyrosine kinase of specific tyrosine residues on Shc and IRS-1 generates binding sites, which are necessary for the recruitment of the Grb2/Sos complex to the plasma membrane, where Sos mediates Ras activation by stimulating Ras to exchange GDP for GTP (Figure 9). The activation of Ras leads to the recruitment of Raf, a MAP kinase kinase kinase, to the membrane. There it is subsequently activated. Raf phosphorylates a MEK which phosphorylates the MAP kinases (ERK1 and ERK2) on critical tyrosine and threonine residues. The activation of MAP kinase leads to the phosphorylation of both cytoplasmic and nuclear targets, including the ribosomal subunit protein kinase $p90^{RSK}$ and transcription factors Elk-1, cMyc, c-Jun and c-Fos, which are all important in mediating cell cycle progression and proliferation. Moreover, the Ras/MAP kinase pathway is involved in the regulation of cell differentiation and cell survival signalling (Hermanto et al., 2000).

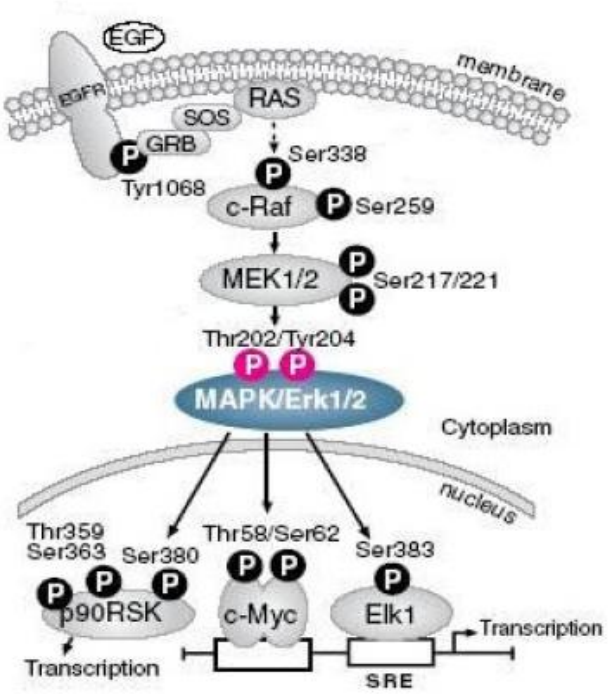


Figure 9: *The mitogen activated protein kinase signalling (MAPK) pathway (2).*

1.8.2 The phosphatidylinositol 3 kinase (PI3K)/AKT signalling pathway

After activation of the ErbB receptor family, the PI3K signalling pathway activates the protein kinase Akt and therefore promotes proliferation, growth, glycogen synthesis, and nitric oxide synthesis is associated with tumorigenesis. The initial activation of the phosphatidylinositol 3 kinase pathway occurs at the cell membrane (Figure 10), where the signal for pathway activation is propagated through PI3K. Activation of PI3K can occur through tyrosine kinase growth factor receptors like ErbB family receptors. When PI3K is activated, the p85 regulatory subunit of the PI3K binds to the phosphorylated tyrosine residues and recruits the second catalytic subunit p110 of the PI3K. After this activation, PI3K transforms phosphatidylinositol 4,5 bisphosphate [PI(4,5)P2] also called PIP2 into phosphatidylinositol 3,4,5 triphosphate [PI(3,4,5)P3] also called PIP3. Thereafter, PI(3,4,5)P3 binds to the pleckstrin homology (PH)

domains of the 3'-phosphoinositide dependent kinase 1 (PDK-1) and the serine/threonine kinase Akt, which cause both proteins to be translocated to the cell membrane, where they are subsequently activated. PTEN (phosphatide and tensin homolog on chromosome ten)-a tumor suppressor- antagonizes PI3K by dephosphorylating PI(3,4,5)P3, to prevent translocation and activation of Akt and PDK-1. Akt is available in three structurally similar isoforms, Akt1, 2 and 3. The activation of Akt1 at the cell membrane occurs through two crucial phosphorylations. First, phosphorylation takes place at Thr308 in the catalytic domain, by PDK-1. The second one occurs at Ser473, in the hydrophobic motif, mediated by different kinases for example PDK-1, integrin linked kinase, Akt itself or mammalian target of rapamycin (mTOR). Phosphorylation of Akt2 and 3 takes place by the same mechanism (Testa et al., 2001; Nicholson et al., 2002; LoPiccolo et al., 2007). Moreover, the PI3K/AKT pathway is very important for normal cellular homeostasis and cancer growth, because it has a central role as a cross-over point between growth, differentiation, apoptotic pathways and metabolic systems like tumorigenic lipogenesis (Grunt et al., 2009).

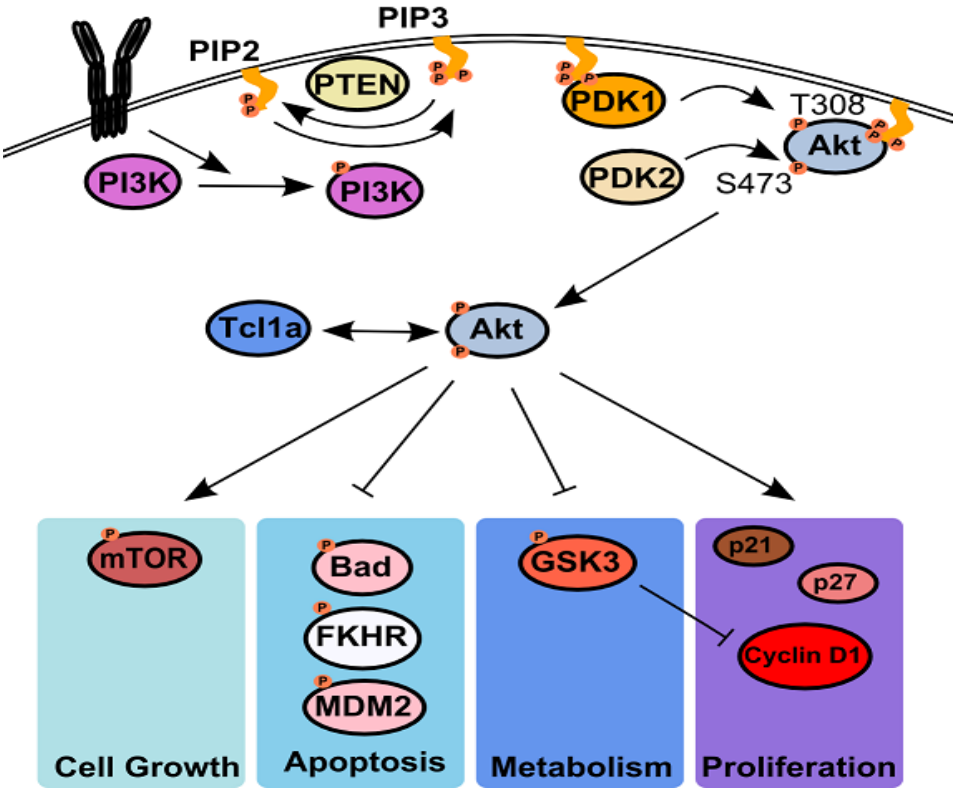


Figure 10: *The phosphatidylinositol 3 kinase (PI3K)/AKT signalling pathway (3).*

1.8.3 Targeting MAPK and PI3K in cancer

The protein kinase MEK occupies a crucial signalling node downstream of RAS and RAF and directly upstream of ERK. Analyses of MEK function shows, that it is necessary for the transforming and proliferative effects of the MAPK pathway. Therefore, the suggestion was to block its function with specific enzyme inhibitors such as with PD0325901 and AZD6244. These molecules act through a noncompetitive allosteric mechanism of inhibition, by the binding to a site adjacent to the ATP-binding site and locking the kinase in a closed and catalytically inactive conformation. Furthermore, the phosphatidylinositol 3 kinase (PI3K) pathway, which signals through AKT represents a key mechanism of transducing prolifertive and antiapoptotic signals. Activating

mutations in the catalytic subunit of p-110α (PIK3CA) occur in 25-30% of breast tumors. Moreover, inactivation of the tumor suppressor protein PTEN, which acts as an upstream inhibitor of PI3K, has been observed in a significant proportion of breast cancer patients. Treatment of breast cancer cell lines with a MEK inhibitor can lead to a feedback upregulation of the PI3K signalling pathway (Figure 11).

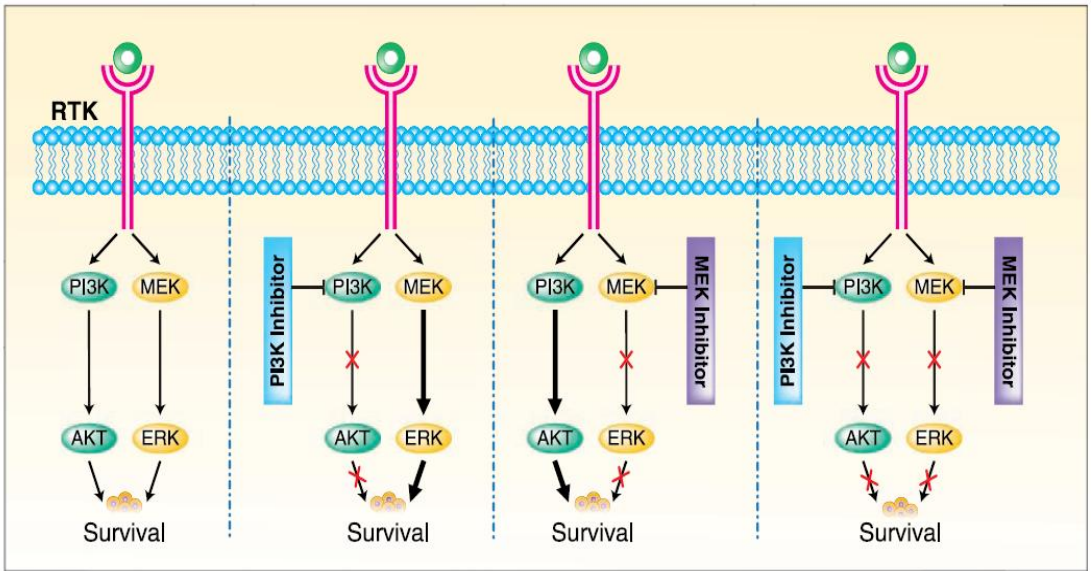


Figure 11: *Different treatment strategies of receptor tyrosine kinases (Rexer et al., 2009).*

Moreover the inhibition of pathways downstream of PI3K results in an upregulation of MEK. Therefore, in many cancer cells, in which PI3K and MEK are hyper active, the combined inhibition is necessary to affect tumor cell survival (Hoeflich et al., 2009; Rexer et al., 2009).

2 Material and Methods

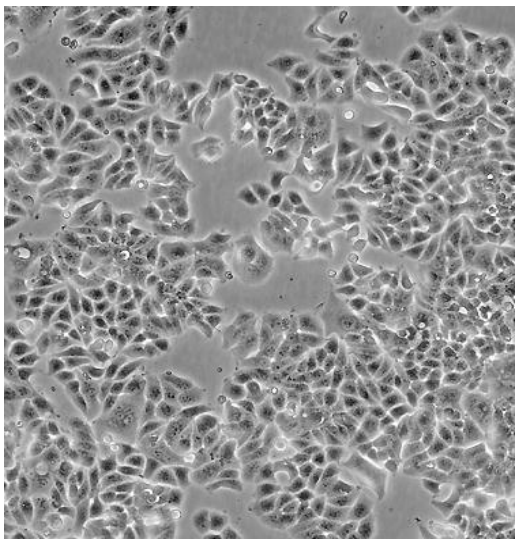
2.1 Cell culture and cell lines

2.1.1 Cell lines

Table 1: *Overview of the used cell lines, their growth medium, specification and source (Hoeflich et al., 2009).*

Cell line	Growth medium	Specification	Source
T47D	D-MEM	H1047R PIK3CA mutation, PTEN WT, low pAkt(S473) level	ATCC
SKBR3	D-MEM	EGFR protein expression, PTEN WT, moderate pAkt(S473) level	ATCC
MCF7	D-MEM	EGFR protein expression, E545K PIK3CA mutation, PTEN WT, low pAkt(S473) level	ATCC
BT474	α-MEM	K111N PIK3CA mutation, PTEN WT, moderate pAkt(S473) level	ATCC
MDA-MB 231	α-MEM	EGFR protein expression, KRAS mutation, PTEN WT, low pAkt(S473) level	ATCC

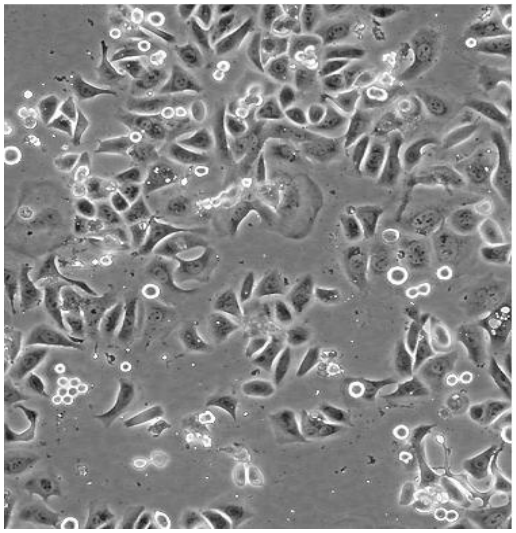
T47D



The T47D ductal carcinoma cell line (Figure 12) was isolated from a human mammary tumor. T47D was cultivated in D-MEM with 10% heat inactivated fetal calf serum (FCS-HI) and 100 U/ml penicillin, 100 µg/ml streptomycin and 2mM L-glutamine (PSG). The cells were passaged once a week, at a splitting ratio of 1:12.

Figure 12: *Morphology of T47D cell line (4).*

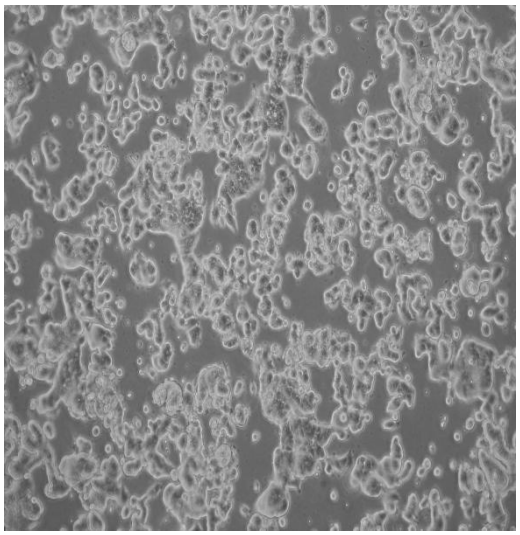
SKBR3



The adenocarcinoma cell line SKBR3 (Figure 13) was islolated from a human mammary tumor.
This cell line was maintained in D-MEM supplemented with 10% FCS-HI and PSG. The cells were subcultured once a week, at a ratio of 1:8.

Figure 13: *Morphology of SKBR3 cell line (5).*

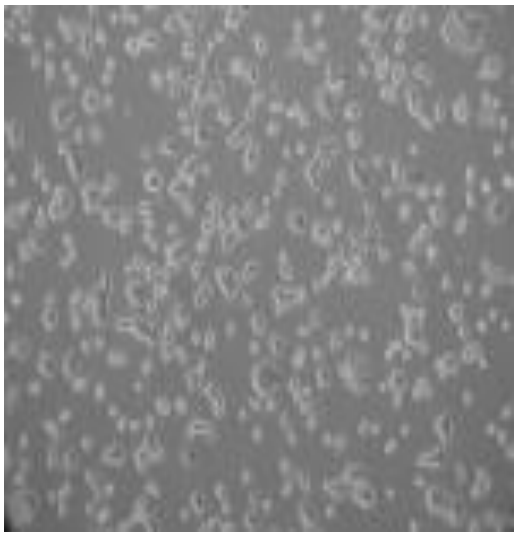
MCF7



This adenocarcinoma cell line (Figure 14) descends from a human mammary tumor. The MCF7 cell line was cultivated in D-MEM with 10% FCS-HI and PSG.
The cells were passaged once a week, at a splitting ratio of 1:12.

Figure 14: *Morphology of MCF7 cell line (6).*

BT474

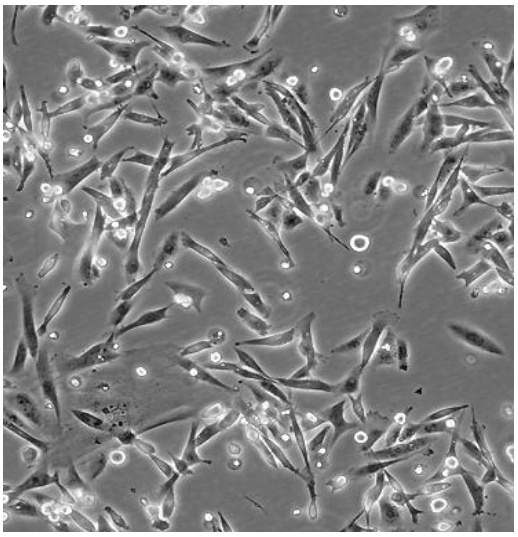


The BT474 ductal carcinoma cell line (Figure 15) was isolated from a human mammary tumor.

This cell line was maintained in α-MEM with 10% FCS-HI and PSG. BT474 cell line was subcultured once a week, at a ratio of 1:3.

Figure 15: *Morphology of BT474 cell line (7).*

MDA-MB 231



The MDA-MB 231 adenocarcinoma cell line (Figure 16) descends from a human mammary tumor.

This cell line was cultivated in α-MEM with 10% FCS-HI and PSG. MDA-MB 231 cells were subcultured once a week, at a splitting ratio of 1:12.

Figure 16: *Morphology of MDA-MB 231 cell line (8).*

2.1.2 Materials for cell culture

Table 2: *List of used materials for cell culture and their source.*

Material	Source
T25 tissue culture flasks 50ml/25cm^2	Falcon 35/3108
T75 tissue culture flasks 250ml/75cm^2	Falcon 35/3111
60x15mm tissue culture dishes	Corning 8430166
96-well plates with flat bottom	TPP 92696
Cryotubes	NUNC 368632
Alpha MEM w/nucleosides	GIBCO 22571-020
D-MEM high glucose 4500mg/l	GIBCO 41965-039
Penicillin/Streptomycin	GIBCO 15140-148
L-Glutamin 200mM	GIBCO 25030-032
Fetal calf serum (FCS)	GIBCO 10270-106
Trypsin-EDTA 0,05%	GIBCO 25300-054
Dulbecco's sulphate buffered saline (D-PBS)	GIBCO 14190
Dimethyl-sulfoxide DMSO	Sigma D2650

2.1.3 Cell culture

All cell lines (Figure 12 –16) were maintained at 37°C in 5 % CO_2 atmosphere with 95 % humidity. They were cultured in two T25 tissue culture flasks per cell line, in 7ml medium containing 10% Fetal calf serum (FCS) with 100U/ml Penicillin, 100µg/ml Streptomycin and 2mM L-Glutamine (PSG). The cells were passaged at a confluence of approximately 90-95%. For the passage, the culture medium was removed. Then the cells were washed carefully with D-PBS and incubated with Trypsin-EDTA for 5 minutes at 37°C to detach them. These cells were resuspended with respective medium to obtain a single cell suspension and plated into two new T25 culture tissue flasks at a splitting ratio of 1:3 to 1:12. The cell lines were passaged once a week and the confluence was controlled with a light microscope.

2.1.4 Storage of the cells

The cells were only used until passage 30 for the experiments, to prevent mutations or other long term effects. For that reason cells were expanded and stored (at a density of $2x10^6$ cells/ml) in liquid nitrogen. To freeze the cells, they were washed carefully with PBS and incubated with Trypsin-EDTA for 5 minutes at 37°C to detach them. Then the cells were resuspended in 10ml respective culture medium with 10% FCS and PSG and centrifuged at 1000rpm for 5 minutes. The cell pellet was again resuspended in 2ml culture medium with 15% FCS and PSG. Then the cells were counted in a counting chamber under the light microscope. After the counting, the cell suspension was adjusted to $4x10^6$ cells/ml with culture medium containing 15% FCS and PSG. Then this suspension is diluted 1:2 with culture medium containing 20% DMSO, so the final DMSO concentration is 10%. Before the cells were frozen in liquid nitrogen, they were slowly frozen in cryotubes. First in an isolated styrofoam box at -80°C for 48 hours and then the cells were stored in liquid nitrogen.
For experiments, cells were thawed from liquid nitrogen. The cryotube with cells was transferred from liquid nitrogen to ice, and then to lukewarm water. Subsequently, the cell suspension was transferred into a 50ml falcon tube, which contained 20ml precooled culture medium (respective for the cell line) and centrifuged at 1000rpm for 5 minutes at 4°C.
The cell pellet was resuspended in 14 ml culture medium and seeded into two T25 culture tissue flasks, 7ml each. The cells were cultured for 3 passages before they were used in experiments.

2.2 Used drugs

Pelitinib:

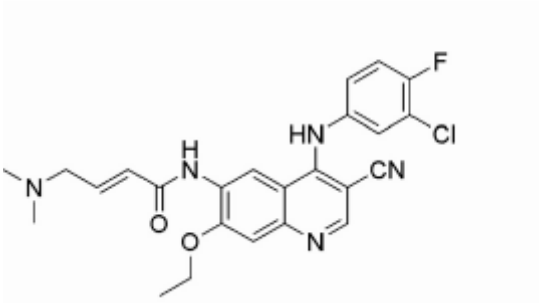


Figure 17: *Molecular structure of pelitinib (Wissner et al., 2008).*

Pelitinib or EKB-569 (Figure 17) provided from Wyeth (22 88 32-2) is an irreversible tyrosine kinase inhibitor. It was dissolved under sterile conditions in DMSO and stored at -80°C.

This inhibitor targets the epidermal growth factor (EGF or ErbB1, Her1) and the epidermal growth factor receptor 2 (EGFR2/Her2 or ErbB-2). It is a 4-anilino-3-cyano quinoline derivate. This drug inhibits the function of the target enzymes by forming a covalent interaction with a conserved cysteine residue, which is located in the kinase domain of these proteins. Pelitinib is also used in clinical trials to treat colon and lung cancer (Wissner et al., 2008).

Canertinib:

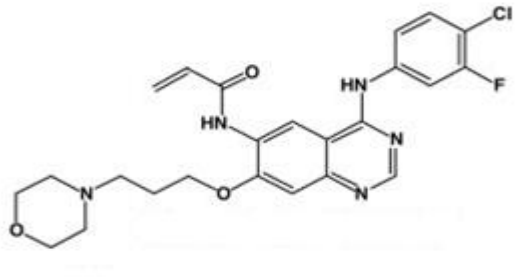


Figure 18: *Molecular structure of Canertinib (Arora et al., 2005).*

Canertinib or CI1033 (Figure 18) provided from Pfizer (0183 805-00028) is an irreversible pan-reactive ErbB inhibitor. This drug was dissolved under sterile conditions in DMSO and stored at -80°C.
Canertinib produces rapid irreversible inhibition of all members of the EGFR family. It inhibits EGFR kinase activity and has antitumor activity in EGFR-and ErbB2-dependent preclinical models. It is also active against ErbB4, but has no effect on other tyrosine kinases (Arora et al., 2005).

Rapamycin:

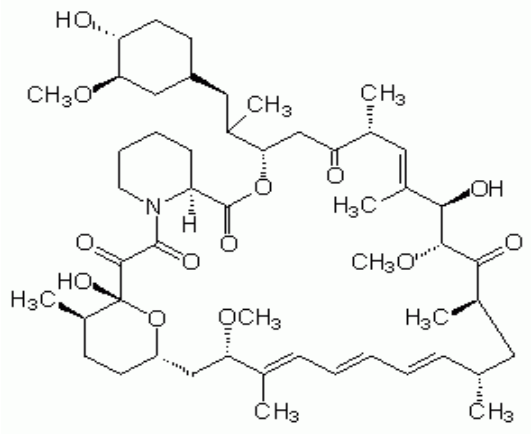


Figure 19: *Molecular structure of Rapamycin (9).*

Rapamycin (Calbiochem; 553210; Figure 19) inhibits the serine-threonine kinase mammalian target of rapamycin (mTOR). The inhibition of the mTOR kinase leads to dephosphorylation of its two major downstream signaling components, p70 S6 kinase (S6K1) and 4E-BP1 (Mondesire et al., 2004). Rapamycin was dissolved in DMSO under sterile conditions and stored at -80°C.

Akti-1,2:

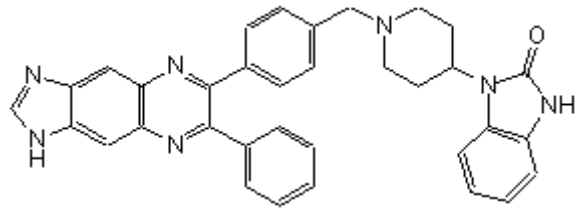


Figure 20: *Molecular structure of Akti-1,2 (10).*

Akti-1, 2 (Akt Inhibitor VIII; Calbiochem; 124018; Figure 20) is a reversible quinoxaline compound that selectively inhibits Akt1/Akt2. The inhibition appears to be pleckstrin homology (PH) domain-dependent. It does not exhibit any inhibitory effect against PH domain-lacking Akts (10).
This drug was dissolved under sterile conditions in DMSO and stored at -80°C.

NVP-BEZ 235:

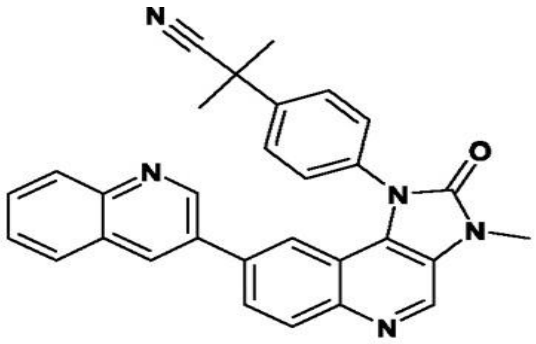


Figure 21*:* *Molecular structure of NVP-BEZ 235 (Maria et al., 2008).*

NVP-BEZ 235 (Chemietek; 915019-65-7; Figure 21) is a dual PI3K and mTOR Inhibitor. NVP-BEZ 235 is an imidazo[4,5-*c*]quinoline derivative that inhibits PI3K and mTOR kinase activity by binding to the ATP-binding cleft of these

enzymes. In human tumor cells this drug was able to block the dysfunctional activation of the PI3K pathway, including G1 arrest (Maria et al., 2008).
This drug was dissolved under sterile conditions in DMSO and stored at -80°C.

AZD6244:

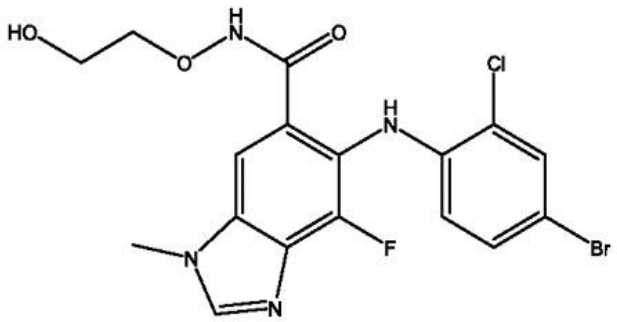


Figure 22: *Molecular structure of AZD6244 (Yeh et al., 2007).*

AZD6244 or ARRY-142886 (Chemietek; CT-A6244; Figure 22) is a potent and selective MEK1/2 inhibitor and moreover it potently inhibits the basal ERK1/2 phosphorylation.
This drug was dissolved under sterile conditions in DMSO and stored at -80°C (Yeh et al., 2007).

U0126:

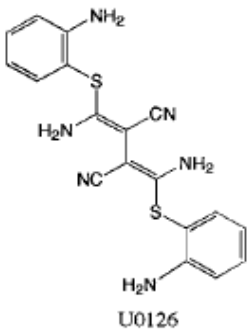


Figure 23: *Molecular structure of U0126 (Favata et al., 1998).*

U0126 (Cell Signaling; NEB9903; Figure 23) was dissolved under sterile conditions in DMSO and stored at -80°C. It directly and selectively inhibits mitogen activated protein kinase kinases MEK1 and MEK2 (Favata et al., 1998).

AS703026:

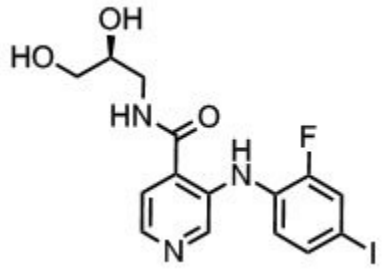


Figure 24: *Molecular structure of AS703026 (11).*

AS703026 (kind gift from Merck Serono; Figure 24) is a small- molecule inhibitor of MEK1 and MEK2. It selectively binds to MEK1/2 and prevents the activation of MEK1/2 dependent effector proteins and transcription factors, which may lead to the inhibition of growth factor mediated cell signalling and cell proliferation.
This drug was dissolved under sterile conditions in DMSO and stored at -20°C (11).

2.3 Cell proliferation assay (EZ4U)

2.3.1 Background

This assay is a colorimetric assay used to measure cell proliferation. It is based on the activity of mitochondrial dehydrogenase enzymes in cells (Ahmadian et al., 2009).

3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) salt is reduced to colored formazan (Figure 25) by active mitochondria in viable cells (Liu Y et al., 1997).

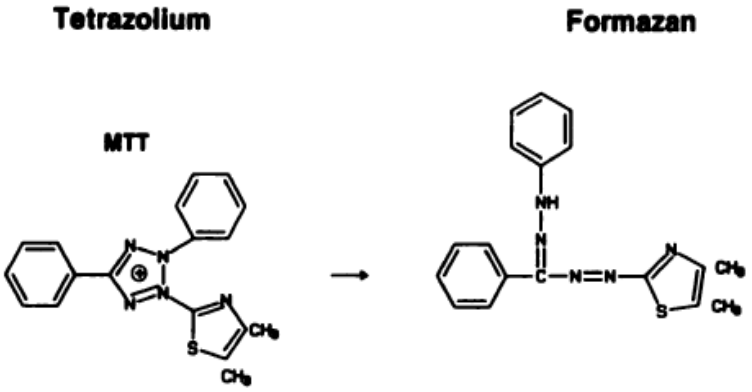


Figure 25: *Reduction of 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) to formazan by mitochondrial reductase (Scudiero et al., 1988).*

2.3.2 Performance of EZ4U assay

Cells were plated in 96-well plates, at a density of 1,5 x 10^5 cells per well, in 100µl medium respective for the cell line, containing 10% FCS and PSG. After 24 hours, the attachment was controlled microscopically. Then 100µl D-MEM or α-MEM without FCS and containing PSG as well as different concentrations of the above mentioned inhibitors was given to the attached cells. Controls were treated with solvent (DMSO) only. Blank wells contained only medium with 5% FCS and are without cells.

Three wells were analyzed for each treatment condition. After 72 hours of incubation at 37°C and light protected, the non-radioactive cell proliferation and cytotoxicity assay termed EZ4U (Biomedica BI-5000, Vienna, Austria) was performed. Intact mitochondria of viable cells reduce uncoloured tetrazolium salt

into intensely coloured formazan derivates. This chemical reaction is detected as a colour change of the medium. First, one vial EZ4U substrate was dissolved in 2,5 ml activator and prewarmed to 37°C for 10 minutes. Then 20 µl/well of the EZ4U reagent was added. After two hours of incubation at 37°C, the absorbance was measured at 490nm against a 655nm reference wave length, on the microplate reader spectrophotometer (Bio-Rad Labortories Model 680). Dose response curves and IC_{50} values were calculated with SigmaPlot and Microsoft Excel.

2.4 Protein analysis (Western blotting)

2.4.1 Background

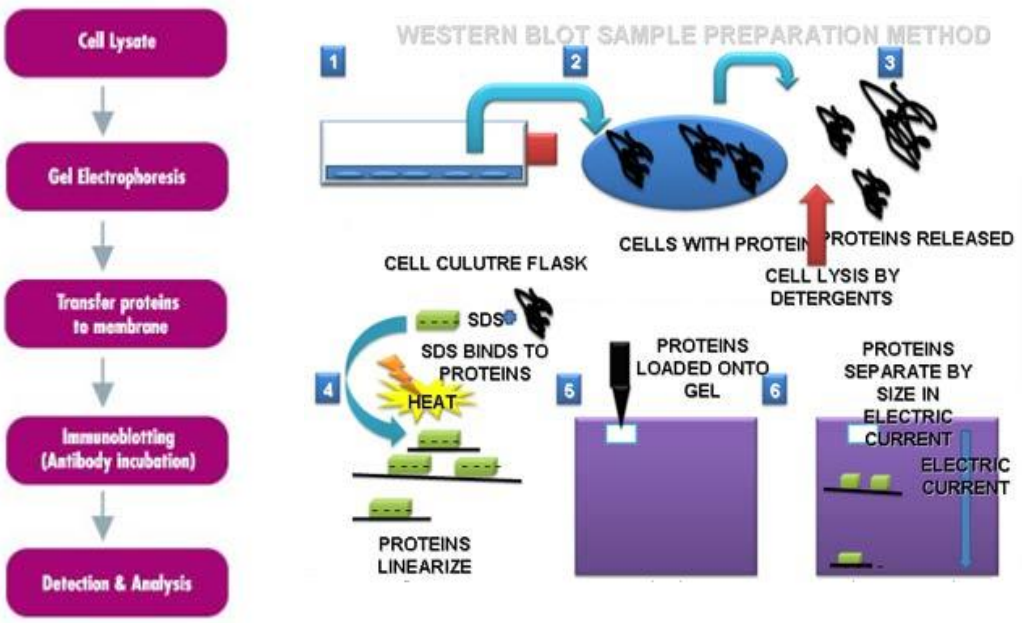


Figure 26: *Workflow of the Western Blot (12; 13).*

Western blotting (Figure 26) is an analytical technique, which is used to identify proteins. This assay is based on the ability of these proteins to bind to a specific

antibody. With this method the size of the interesting proteins can be determined, by comparison to the sizes of a mixture of prelabelled marker proteins (kDa). Moreover, it gives an information of protein expression, by the comparison of an untreated sample (control). Therefore, sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) is performed. This method separates proteins in a polyacrylamide gel matrix according to their size, in the presence of an electric current. Low percentage (8-10%) gels separate larger proteins, whereas higher percentage (15-20%) gels separate smaller proteins. SDS is given to the protein sample. It binds to proteins and neutralizes the electrical charge. This is necessary to separate them by size and not by charge. To reduce disulphide bridges in proteins, which is necessary linearize them for a separation by size, all protein samples were boiled in mercaptoethanol-containing loading buffer at 95°C. To separate proteins, two different polyacrylamide gels were used. One is called stacking gel and the other running or separating gel. The stacking gel with a pH of 6,8 is used to pack proteins together, after loading. The running gel has a pH of 8,8 and is used at the bottom to separate proteins. Polyacrylamide gels are formed by the polymerization of two compounds, acylamide and N,N-methylenebis-acrylamide (Bis). Bis serves as a cross-linking agent in these gels. Polymerisation is initiated by the addition of ammonium persulfate together with tetramethylethylenediamine (TEMED). After electrophoresis, separated proteins were transferred in an electrical field from the gel to a membrane. Therefore, a wet electrotransfer was used during which the negatively charged proteins traveled towards the positively charged electrode and were bound by the membrane. Then the membrane with the bound proteins was blocked with bovine serum albumin (BSA), to prevent non-specific background. This is followed by washing steps. Then the membrane is incubated with the first antibody solution, directed against a specific protein. Then secondary antibody, which is directed against a species-specific region of the first antibody, is used. Normally this secondary antibody is linked to horseradish peroxidase, a reporter

enzyme, which cleaves an added substrate. This results in a detectable light signal (enhanced cehmiluminescence), (14;15).

2.4.2 Protein extractions

$5x10^5$ cells were plated in 60x15mm tissue culture dishes. Cells were cultured in 7ml respective growth medium per well, supplemented with 5% FCS, 100U/ml penicillin, 100µg/ml streptomycin and 2mM L-glutamin. They were allowed to adhere over night. Then the treatment with the respective drug (see used drugs 2.) started. Moreover, a DMSO solvent control culture was included. Cells were exposed for 6 hours at 37°C and protected from light, because drugs are light sensitive. After the incubated time period, cells were checked under the light microscope to determine their degree of confluence and apoptosis.

2.4.2.1 RIPA Lysis

All of the following steps were done on ice. Before lysis, culture media was removed and cells were washed twice with cold PBS. Then the remaining PBS was carefully removed from the dish and 80µl freshly prepared $RIPA^+$ lysis buffer (Table 3 and 4 ingredients of RIPA and $RIPA^+$) was added to the dish. Thereafter, cells were left on ice for 5 min and collected by scraping with a sterile scraper. Then, these lysates of the cells were transferred into an Eppendorf tube and vortexed for several times. After vortexing, the lysates were centrifuged at 12.500rpm at 4°C for 30min. The pellet was discarded and the supernatant was saved at -20°C for the protein assay.

Table 3: *Ingredients of RIPA Lysis Buffer; it was adjusted to pH 7,4, filled up with water to 95ml and stored at 4°C.*

Reagent	MW	Stock Concentration	Volume	Final Concentration
Na Cl	58.44	5M	3ml	150mM
Tris pH 7,4	121.14	1M	5ml	50mM
DOC (Na-deoxycholate)	414.6	10%	5ml	0,5%
EGTA	380.4	50mM	4ml	2mM
EDTA pH 7,4	372.2	50mM	10ml	5mM
NaF	41.99	500mM	6ml	30mM
β-Glycero-phosphate pH7,2	216	400mM	10ml	40mM
Tetrasodium-pyrophosphate	446.06	100mM	10ml	10mM
Benzamidine	156.6	30mM	10ml	3mM
Nonidet P-40		pure	1ml	1%

Table 4: *Ripa^{+} Lysis Buffer; it has to be prepared freshly before use.*

Reagent	Volume
RIPA	1.90ml
200mM Na-Orthovanadate	20µl
25xComplete stock solution (Protease Inhibitor Cocktail)	80µl

2.4.2.2 Protein Quantification

The determination of the protein concentration of each sample was done with the BioRad Protein Assay Kit II (Bio-Rad 500-0112). This assay was accomplished in a 96 well plate. First, a 10mg/ml bovine serum albumine (BSA) stock solution for protein standard was produced. Therefore, 10mg BSA was

dissolved in 1ml Aqua bidest., then this protein standard was serially diluted 1:2 with $RIPA^+$ to get 5, 2.5, 1.25, 0.625, 0.315, 0.15625 µg/µl protein standards. $RIPA^+$ allone was used as a blank control. This assay was also done in triplicates. Then, 5µl of each standard was set up in a 96 well plate in increased concentrations or 5µl of the sample. All following steps were done under light protection. Reagent A' of this Protein Assay Kit II was freshly prepared by adding 1ml Reagent A (BioRad 5000113) to 20µl Reagent S (BioRad 5000115). Thereafter, 20µl of the Reagent A' and 200µl Reagent B (BioRad 5000114) of this kit was added to each well, which contains samples or standards. After incubation for 20min the absorbtion was measured at 655/450nm with a microplate reader spectrophotometer (Bio-Rad Labortories Model 680). After this measurement, the protein concentration for each sample and standard curve was calculated using Microsoft Excel Software. Then the protein samples were stored in a 1x sample buffer at -20°C, at a final concentration of 1µg/µl. Therefore, the samples were diluted with three parts RIPA+ to a concentration of 1.33µg/µl and then one part 4x sample buffer (Table 5).

Table 5: *Ingredients of 4x Sample Buffer (Beta-mercaptoethanol was added just before use).*

Reagent	MW	Stock	Volume	Final Concentration
Glycerol	92.09	pure	5ml	50%
Tris-HCl, pH 6.8	121.14	1M	1.25ml	125mM
SDS		20%	2ml	4%
Bromophenol blue		1%	1.25ml	0.125%
Beta-mercaptoethanol		pure	0.5ml	5%

2.4.3 SDS- Polyacrylamide Gel Electrophoresis (PAGE)

The sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) is used to separate the proteins according to their size by the use of a 10% separating gel and a 4% stacking gel (Table 6, 7 and 8). SDS is used to denature proteins. This detergent wraps around the polypeptide backbone, so the intrinsic charges of the protein becomes negligible compared to the negative charges contributed by SDS. After SDS treatment proteins become a rod like structure possessing a uniform charge density. This allows separating proteins according to their size by migration from the cathode to the anode (Ornstein L. et al 1964 and Davis B.J. et al 1964). The separating gel was carefully overlayed with Aqua bidest to avoid any air bubbles and was allowed to polymerize for 30-45min. After this time, Aqua bidest was poured off and remaining water was drained with a small filter paper. Then the 4% stacking gel was pipette up to the very top of the plates. The comb, which is used to form 10 slots in the gel, was slightly inserted. The stacking gel required 20min for polymerization. The plates with the polymerized gels were inserted into the gel holder cassette (Bio-Rad 1703931) and then into the electrophoresis chamber. A 1x running buffer containing 25mM Tris, 192mM glycine and 0,1% SDS in Aqua bidest was added to the chamber. Each protein sample was then boiled for 10min at 95°C for denaturation. Afterwards, 30µg protein per slot was loaded and 2µl of the molecular weight marker (Magic Mark XP from Invitrogen LC5602) were loaded into an extra slot. Then electrophoresis was conducted with constantly 90V. It is important to stop electrophoresis before proteins run off the gel. But proteins are mostly colourless. Therefore, bromophenol blue, which was added to the proteins with the 4x sample buffer (see 2.4.2.2.), is used as a marker dye. It is a small molecule, which is negatively charged and moves towards the anode. It moves ahead of the most proteins, because it is very small and when it reaches the anode at the end of the gel, electrophoresis is stopped.

Table 6: *Ingredients of 30% Acrylamide/Bis solution.*

Reagent	Volume	Source
40% Acrylamide	11,1ml	Bio-Rad 1610140
2% Bis Solution	3ml	Bio-Rad 1610142
Aqua bidest.	0,9ml	
Total volume of 30% A/B	15ml	

Table 7: *Ingredients of the 10% seperating gel.*

Reagent	Volume	Source
30% A/B (Table 6)	6.7ml	
Aqua bidest.	8ml	
1.5M Tris 8.8	5ml	Bio-Rad 1610798
10% SDS	200µl	Bio-Rad 1610418
10% APS	100µl	Bio-Rad 1610700
Temed pur	10µl	Bio-Rad 1610800

Table 8: *Ingredients of the 4% stacking gel.*

Reagent	Volume	Source
30% A/B (Table 6)	1.673ml	
Aqua bidest.	7.46ml	
0.5M Tris pH 6.8	3.15ml	Bio-Rad 1610799
10% SDS	125µl	Bio-Rad 1610418
10% APS	125µl	Bio-Rad 1610700
Temed pur	12.5µl	Bio-Rad 1610800

2.4.4 Electrotransfer

After the proteins were separated according to their molecular weight, they were transferred to a polyvinylidene difluoride (PVDF) membrane (NEF 153196). Blotting rely upon current and a transfer buffer solution, which drive proteins

onto the PVDF membrane. To blot the proteins from the gel to the membrane an electrical field was used. Negatively charged proteins migrate from the cathode to the anode. It is necessary that the membrane is located between the gel and the anode. Proteins bind to the membrane because of hydrophobic interaction (Burnette W.N. et al 1981). Thereafter, the membrane was marked and shortly soaked in pure methanol. Then a blotting sandwich was made (Figure 27). The used filter papers and scotch brites were soaked in a precooled transfer buffer, which contains 150mM glycine, 50mM Tris pH8.3, 0.05% sodium dodecylsulfate (SDS) and 20% methanol. To remove air bubbles from the sandwich, a pipet was rolled over it. Then the blotting sandwich was placed into the blotting chamber. This chamber was filled with the precooled transfer buffer. One small stirring rod was placed into this chamber. The electrotransfer was performed over night at constant current of 290mA, at 4°C.

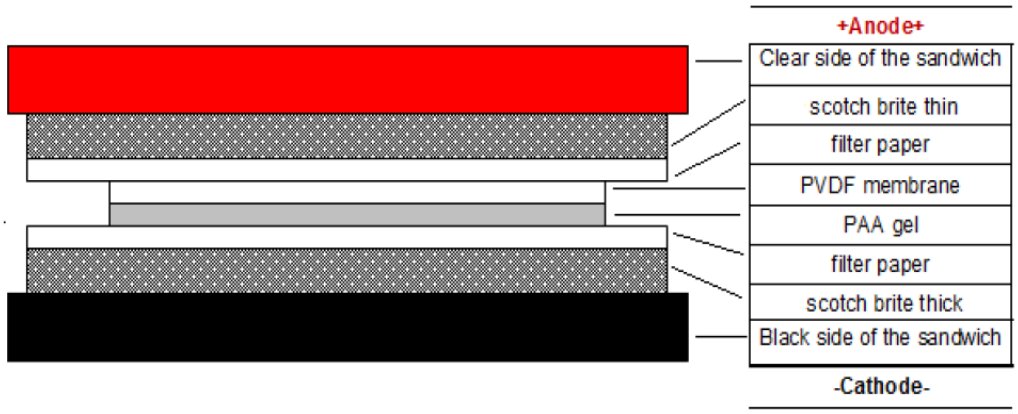


Figure 27: *Assembly of the blotting sandwich.*

2.4.5 Immunostaining of the blotted membranes

After the electrotransfer, the blotting sandwich was disassembled. The PVDF membrane was cut exactly into the size of the gel. Then the non-specific protein binding sites on the membrane had to be blocked. Therefore, a blocking

solution was made, which contains 4% bovine serum albumin (BSA, Fluka 05480) in 1xTris buffered saline (TBS, Bio-Rad 1706435). This TBS contains 0.1M Tris pH 7.5, 0.5M NaCl and Aqua bidest, supplemented with 0,1% Tween20 (Bio-Rad 1706531), TBS together with Tween is abbreviated as TBS-T. 4% heat incativated fetal calf serum (Gibco 10270-106) was added to TBS-T and BSA, to achieve the blocking solution. The cut membrane was incubated for one hour in blocking solution, at room temperature, during shaking.
Then the blocking solution was removed and the membrane was washed three times with TBS-T for 5min each. Afterwards the first antibody solution, which consisted of 1xTBS-T with 1% blocking solution, 0,05% Na-azide (for longer storage of the solution) and the respective antibody (Table 9), was added to the membrane. Incubation with the first antibody was over night at 4°C (Figure 28). After this incubation the first anibody solution was removed and the membrane was washed, twice for 5min and twice for 10min with TBS-T. Then the secondary antibody solution, which consisted of 1xTBS-T with 1% blocking solution and in which a HRP conjugated antibody (Table 10) was diluted, was added to the membrane for one hour shaking at room temperature. Thereafter, the secondary antibody solution was removed and the membrane was washed four times with TBS-T (twice for 5min and twice for 10min) and four times with TBS (twice for 5min and twice for 10min). Primary antibodies against α/β-Tubulin or Actin were used as loading control.

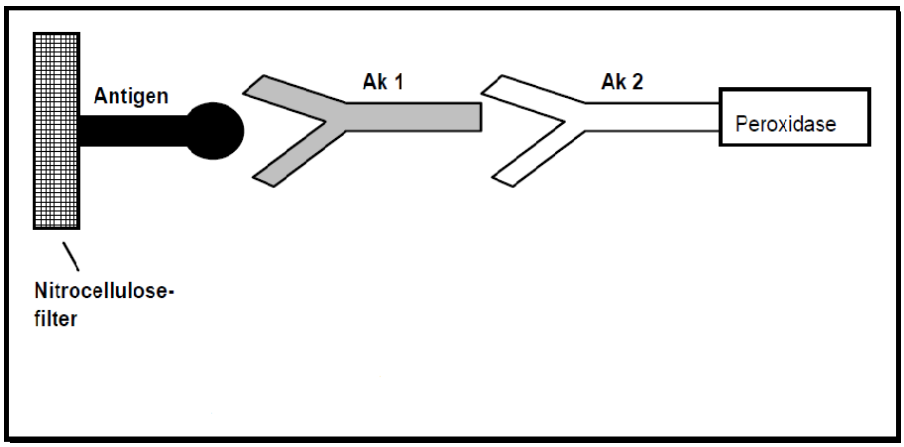


Figure 28: *Detection of proteins by the use of two different antibodies. Peroxidase, which is linked to the sencondary antibody, reacts with ECL-reagent, so that the antigen becomes visible (Hawkes et al., 1982).*

Then for enhanced chemiluminescence (ECL), the membrane was incubated with a mixture of 2ml detection solution A and 2ml detection solution B (Pierce 32106) for 5 minutes. This solution reacts with the horseradish peroxidase, which was conjugated to the secondary antibody. Horseradish peroxidise catalyses the oxidation of luminol to 3-aminophthalate, which is accompanied by emission of light (Veitch N.C. et al, 2004). This reaction occurs at the place where the specific antigen is located on the PVDF membrane. Detection occurs by the exposure of the membrane to Hyperfilm ECL high performance chemiluminescence film from Amersham (RPN3103K).

Table 9: *List of primary antibodies used for the detection of proteins of interest.*

Antibody	Species	Dilution	Source
AKT1,2,3	Rabbit	1:1000	Cell Signalling 9272, 60kD,polyclonal Antibody
pAKT(Ser473)	Rabbit	1:1000	Cell Signalling 9271, 60kD, polyclonal Antibody
pAKT(Thr308)	Rabbit	1:1000	Cell Signalling 9275, 60kD, polyclonal Antibody
ERK1,2	Rabbit	1:3000	Upstate 06-182 P44/42 MAP Kinase, polyclonal Antibody
pERK1,2 (Thr202/Tyr204)	Rabbit	1:1000	Cell Signalling 9101 Phospho-p44/42 MAP Kinase polyclonal Antibody
MEK1,2	Rabbit	1:1000	Cell Signalling 9122 45kD, polyclonal Antibody
pMEK1,2 (Ser217/221)	Rabbit	1:1000	Cell Singalling 9121, 45kD, polyclonal Antibody
S6	Rabbit	1:1000	Cell Signalling 2217 32kD, monoclonal Antibody
pS6(Ser240/244)	Rabbit	1:2000	Cell Signalling 2215 32kD, polyclonal Antibody
α/β Tubulin	Rabbit	1:1000	Cell Signalling 2148 55kD, polyclonal Antibody
β Actin	Goat	1:1000	Santa Cruz Biotechnology sc-1616, 43kD, polyclonal Antibody

Table 10: *List of secondary antibodies.*

Antibody	Dilution	Source
Donkey-anti-rabbit	1:15000	Promega V7591, horseradish peroxidase (HRP) linked
Donkey-anti-goat	1:15000	Santa Cruz Biotechnology sc2020 horseradish peroxidase (HRP) linked

2.4.6 Stripping of the membranes

After the detection of the proteins of interest, the membrane can be stripped, to detach all antibodies from the proteins in order to make the membrane suitable for another round of incubation with antibodies. For that reason a 1x stripping solution (Table 11) was prepared. Twenty ml of this stripping solution and 200µl β-Mercaptoethanol were added to the blot. Stripping was accomplished at 50°C in a water-bath for 20min, with constant shaking. Afterwards the membrane was washed with TBS-T, twice for 5min and twice for 10min. Then the blot was blocked with blocking solution containing 4% FCS for one hour and further processed for immunostaining in the regular way as described above (2.4.5.).

Table 11: *Ingredients of the stripping solution.*

Reagent	Quantity
SDS	10g
1M Tris pH 6.7	31.3ml
Aqua bidest.	filled up to 500ml

2.5 Statistical analysis

2.5.1 Analysis of variance (ANOVA)

Analysis of variance is a general method for statistical estimation of different means between more than two groups. Variance analysis is used to test, if variance between two groups is greater than variance inside these groups. Therefore, it could be analysed if these groups differ significantly or not. In the two factorial variance analysis two factors are considered (16). Anova was performed using Microsoft Excel 2007.

$Y_{ijk} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + e_{ijk}$ $e_{ijk} \sim N(0,\sigma^2)$ i=1,....,I, j=1,....,J, k=1,....,K

μ.....global expectancy
α_i.....effect of i-factor
β_j.....effect of j-factor
$(\alpha\beta)_{ij}$....effect of interaction
e_{ijk}.....random difference of the mean pro factor combination

2.5.2 Scheffé-Test

This analysis is done, only when anova yields significance. It compares all possible subgroups of means (17). Scheffé-Test was performed using Microsoft Excel 2007.

$$F_S = \frac{(\mu_i - \mu_j)^2}{s_{in}^{(k-1)} \cdot \left(\frac{1}{n_i} + \frac{1}{n_j}\right)}$$

μ_i.....mean i
μ_j.....mean j
$s_{in.}$.....standard deviation

F..... F-distribution

n.....sum of single values of all groups

k.....sum of all means

2.5.3 Student's t-Test

This statistical test assess whether the means of two groups statistically differ from each other, used to compare the means of two groups. The top part of the ratio is the difference between the two means. The bottom part is a measure of the variability of the groups (Paul D. Lewis, R for Medicine and Biology). Student's t-Test was performed using Microsoft Excel 2007.

$$t = \frac{\bar{X}_1 - \bar{X}_2}{S_{X_1X_2} \cdot \sqrt{\frac{2}{n}}}$$ and $$S_{X_1X_2} = \sqrt{\frac{S_{X_1}^2 + S_{X_2}^2}{2}}$$

S_{X1X2} .....grand standard deviation

1.....group one

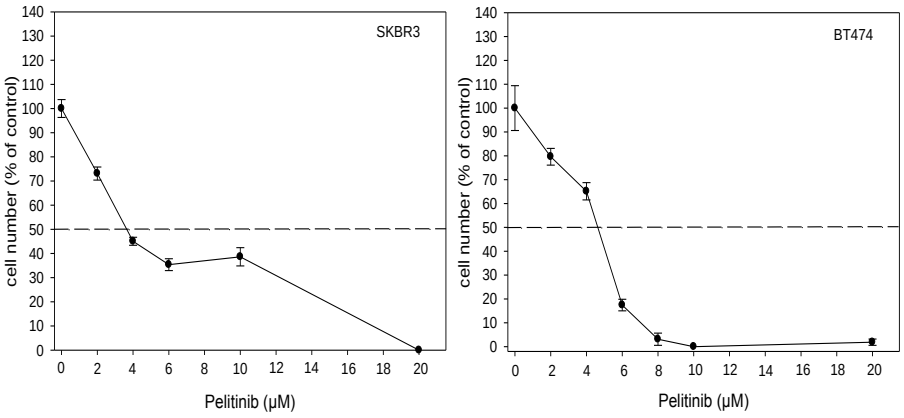
2.....group two

n.....number of participants

3 Results

3.1 Comparison of pelitinib sensitivity and resistance in different breast cancer cell lines

In breast cancer, the epidermal growth factor (EGF) receptor (EGFR) or ErbB family is very important for its pathogenesis (Olayioye et al., 2000). Previous experiments showed that the two breast cancer cell lines T47D and SKBR3 respond in different ways to EGFR/ErbB-2 receptor tyrosine kinase inhibitor pelitinib. T47D was resistant, whereas SKBR3 was sensitive against this inhibitor (Brünner-Kubath et al., in press). To define further breast cancer cell lines that respond either resistant or sensitive against pelitinib, MCF7, BT474 and MDA-MB 231 were tested with in vitro growth assays in addition to the two established cell lines. Cells were treated for 72 hours with 0-20µM pelitinib (Figure 29) and IC_{50} values were determined (Figure 30).



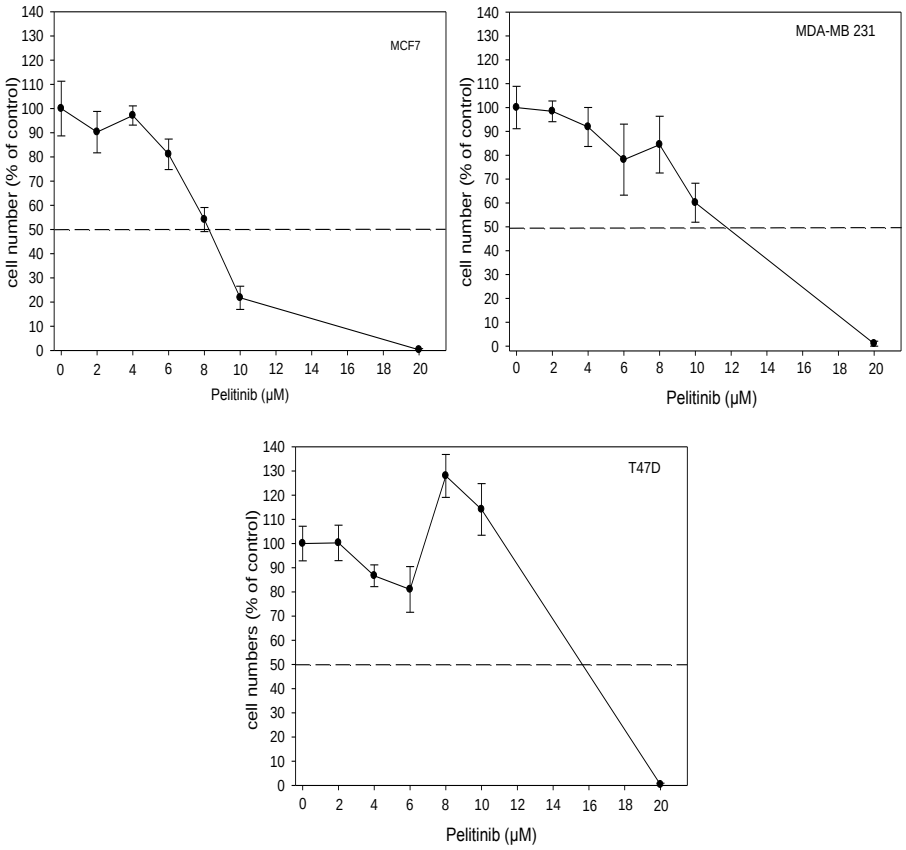
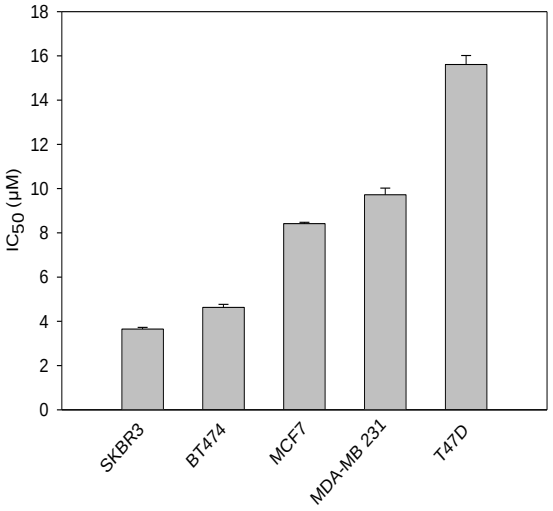


Figure 29: ***Growth curves from a panel of 5 different breast cancer cell lines treated with pelitinib.*** *SKBR3, BT474, MCF7, MDA-MB 231 and T47D cell lines were exposed to 0-20µM pelitinib for 72 hours. Cell proliferation was analysed by EZ4U. Means ± SD, n≥3.*

The findings indicate that MDA-MB-231, MCF7 and T47D cells exhibit high IC_{50} values (Figure 30 and Table 12). Especially T47D, with an IC_{50} value of 15,61µM was very resistant. In contrast, SKBR3 and BT474 cells showed much lower IC_{50} values, which indicate that these two cell lines are more sensitive against the inhibitor. Moreover, this outcome supports earlier findings of resistance and sensitivity in T47D and SKBR3, respectively. SKBR3 reveal an IC_{50} of 3,65µM and BT474 an IC_{50} of 4,63µM, which are the lowest IC_{50} values in the tested panel of cell lines. However, the other cell lines MDA-MB 231 and

MCF7 reveal higher IC_{50} values ranging from 8,41µM to 9,72µM, but they could not reach such a high IC_{50} value than T47D cells.



Cell line	IC_{50}
SKBR3	3,65µM
BT474	4,63µM
MCF7	8,41µM
MDA-MB 231	9,72µM
T47D	15,61µM

Figure 30 and Table 12: ***IC_{50} value of 5 different cell lines exposed to pelitinib.***

Effects on cell growth were determined by EZ4U of 72 h inhibitor treatment.

3.2 ErbB downstream signalling profiles from a panel of breast cancer cell lines exposed to pelitinib

After the findings we achieved in growth assays done with pelitinib, Western blot experiments using the three cell lines BT474, MCF7 and MDA-MB 231 in the presence or absence of pelitinib were performed in order to find out whether these cell lines reveal downstream Akt and Erk activation profiles that are analogous to the previously characterized patterns obtained from pelitinib sensitive SKBR3 and pelitinib resistant T47D cells (Brünner-Kubath et al., in press).

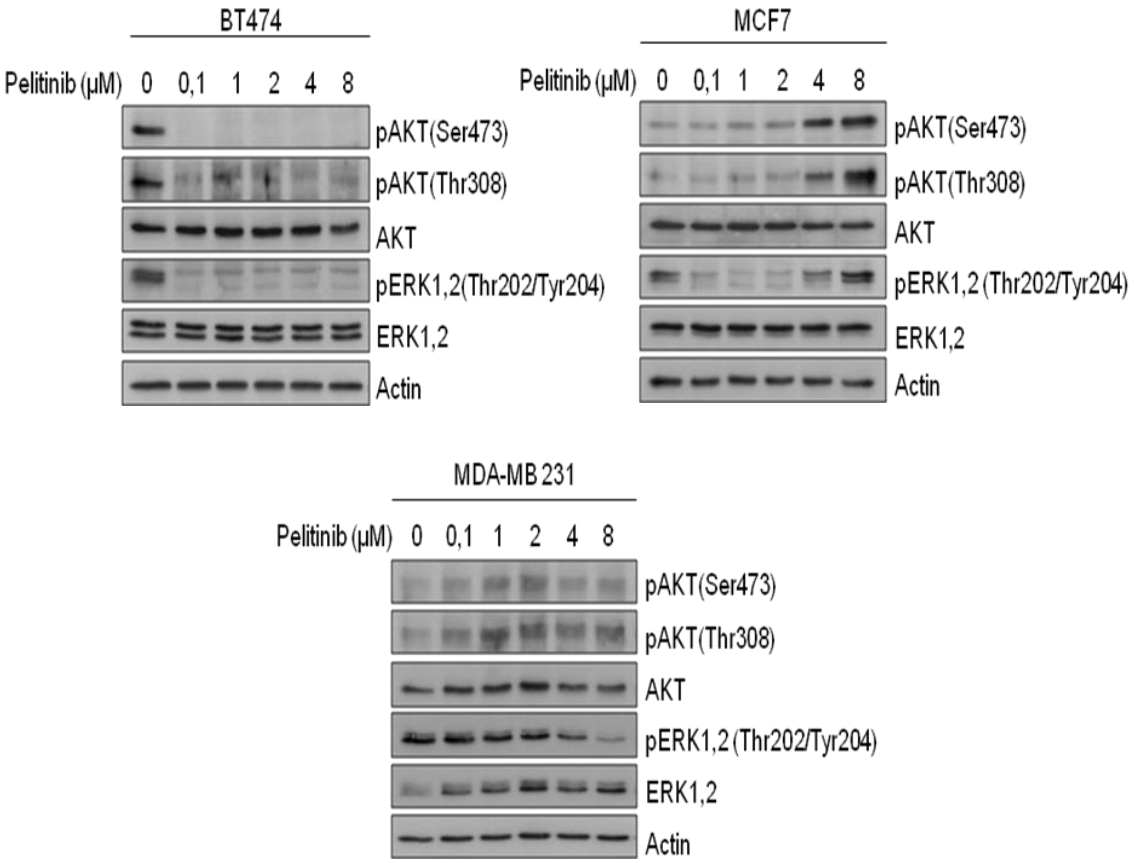


Figure 31: ***Breast cancer cell lines BT474, MCF7 and MDA-MB 231 exposed to phospho-AKT (Ser473), phospho-AKT (Thr308), AKT, phospho-ERK1,2 (Thr202/Tyr204) and ERK1,2 antibodies.*** *Cells were grown in 60mm dishes containing medium with 5% FCS for 24h. The cultures were treated with 0,1µM, 1µM, 2µM, 4µM and 8µM pelitinib as well as only with the solvent (DMSO) without pelitinib for 6h. Cell lysates were collected as described in material and methods. β-Actin was used as a loading control.*

This panel of cell lines were cultivated as described in Material and Methods, exposed for 6h to different concentrations of pelitinib and afterwards results were detected using Western blotting analysis (Figure 31).

These findings indicate that BT474 cell lines respond sensitive to the used drug. They show a reduction of pAKT (Ser473) and pAKT (Thr308) already at a

concentration of 0,1µM inhibitor. Likewise, the pERK1,2 (Thr202/Tyr204) level is very low at this concentration, but it does not completely disappear until the highest concentration of the drug. This result goes along with the finding obtained in growth assay analysis, where BT474 seems to be sensitive against pelitinib.
Interestingly, MCF7 shows a very low level of pAKT (Ser473) and pAKT (Thr308), in the untreated control. However, treatment with pelitinib, leads to an enhanced pAKT level, especially at concentrations of 4µM and 8µM inhibitor. Furthermore, the pERK1,2 (Thr202/Tyr204) level accumulates in this cell line. These findings correspond to growth assay analysis done with pelitinib, in which MCF7 proved to be resistant to this drug.

MDA-MB 231 cells exhibit low concentrations of pAkt (Ser473) and pAkt (Thr308) already in the untreated control and no further reduction by exposure to pelitinib. In contrast, this cell line reveals a high level of pErk1/2 (Thr202/Tyr204), which is reduced only at high concentration of the inhibitor.

3.2.1 Investigation of the importance of the MAPK pathway in MDA-MB 231 cells

Due to the results achieved from Western blotting analysis of MDA-MB 231 cells, we suggested that the MAPK pathway, compared to the PI3K system, may be more important in this cell line. These cells show high pERK1,2 (Thr202/204) level, but very low pAKT concentrations in the untreated control. Therefore, a growth assay analysis with the two MEK inhibitors U0126 and AZD6244 was done (Figure 32). To determine if decreased growth of this cell line will occur in response to inhibition of MEK. But a treatment of these cells with the inhibitor U0126 revealed no inhibition. The IC_{50} value could not be reached.

Exposure to the second inhibitor AZD6244 showed a high IC_{50} value of 14,15µM. According to these results, both MEK inhibitors do not significantly affect in vitro growth of this cell line.

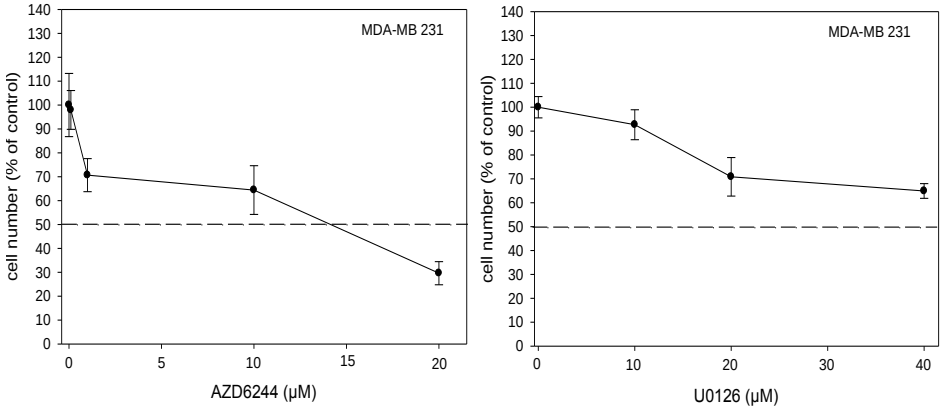


Figure 32: ***Treatment of MDA-MB 231 with U0126 and AZD6244.***
Cells were exposed to 0-20µM AZD6244 and 0-40µM U0126 for 72h. Proliferation was analysed by EZ4U. Means ± SD, n≥3.

3.3 Determination of the fundamental ErbB downstream signalling pathways in SKBR3 and T47D

3.3.1 Inhibition of the phosphatidylinositol-3-kinase pathway

The mammalian target of rapamycin (mTOR) belongs to the phosphatidylinositol kinase-related kinase family and plays central role in regulation of protein synthesis, cell growth and proliferation control. In response to mitogen stimulation, mTOR regulates translation through two distinct pathways, the ribosomal p70S6 kinase and the eukaryotic translation initiation factor 4E (eIF4E) inhibitor binding protein 1 (4EBP1) (Sun SY. et al., 2005).

Hence, we wanted to examine how the pelitinib sensitive and resistant cells SKBR3 and T47D respond to inhibition of mTOR, to determine whether the PI3K pathway is important or not for these two cell lines. Therefore, a growth assay with rapamycin, a selective mTOR inhibitor, was performed (Figure 33).

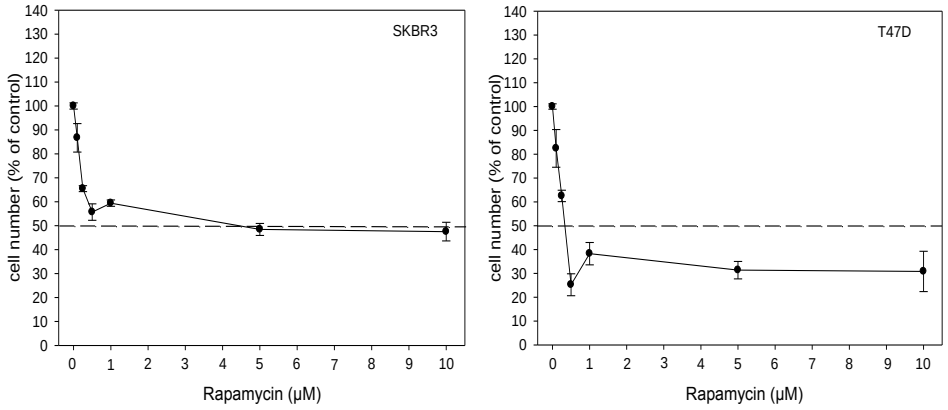


Figure 33: ***The growth effects of rapamycin in SKBR3 and T47D cells.***

Cell lines were exposed to 0-10µM rapamycin for 72h. Proliferation was analysed by EZ4U. Means ± SD, n≥3.

Rapamycin shows good inhibitory effects on T47D cells compared to SKBR3 cells. The IC_{50} value in rapamycin treated T47D cells lies at 0,33µM. SKBR3 cells do not respond as good as T47D cell line on mTOR inhibition. The IC_{50} value for SKBR3 is 4,09µM. These results indicate that rapamycin exerts much better growth inhibitory effects on T47D when compared to SKBR3 cells. Rapamycin only inhibits mTOR, therefore another inhibitor called NVP-BEZ 235, which acts as a dual mTOR and PI3K inhibitor, was used to treat SKBR3 and T47D cells (Figure 34). Several isoforms of the PI3K family are implicated in pathologic processes and diseases. It facilitates the phosphorylation of Akt, which is a central effector of this pathway (Serra et al., 2008).

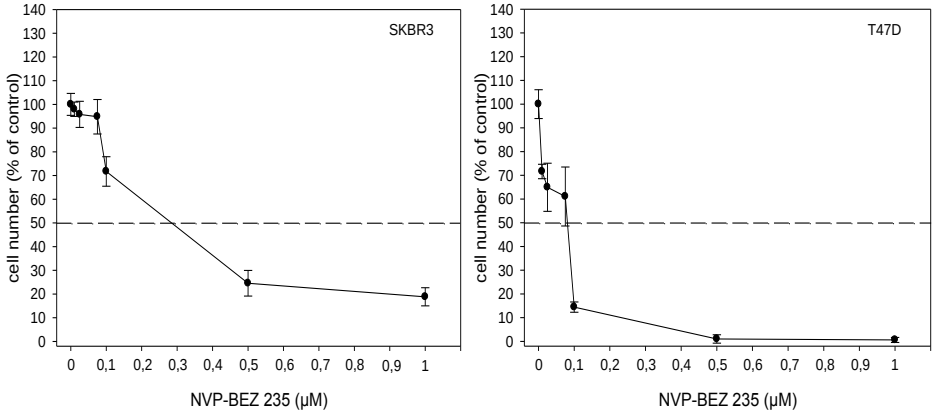


Figure 34: ***The growth effects of NVP-BEZ 235 on T47D and SKBR3 cells.***

Cells were exposed to 0-1µM NVP-BEZ 235 for 72h. Effects on growth were determined by EZ4U. Means ± SD, n≥3.

Results from growth assay performed with NVP-BEZ 235 show, that the dual inhibitor acts strongly on both cell lines. T47D as well as SKBR3 reveal low IC_{50} values. However, T47D cells show a stronger response to the inhibitor than SKBR3 cells, with an IC_{50} value of 0,08µM, which is very low, whereas the IC_{50} value of NVP-BEZ 235 against SKBR3 cells with 0,29µM is nearly four times higher. Previous studies showed that NVP-BEZ 235 had different effects dependent on the used concentration. The inhibition of mTOR predominates at low concentrations, but dual mTOR/PI3K blockade occur at higher drug concentrations. Hence, we next used a direct Akt inhibitor called Akti-1,2 to treat these two cell lines (Figure 35). Akt is a central effector of the PI3K/Akt pathway. It promotes for example protein synthesis and cell growth by alleviating suppression of mTOR (Serra et al., 2008).

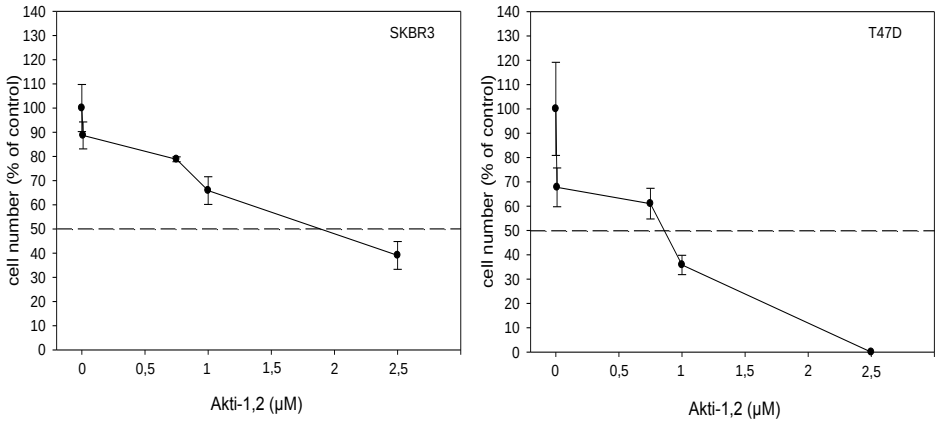


***Figure 35:* The growth effects of Akti-1,2 on SKBR3 and T47D cells.**

The two cell lines were exposed to 0-2,5µM Akti-1,2 for 72h. Cell growth was analysed by EZ4U. Means ± SD, n≥3.

Akti-1,2 drug exposure shows good inhibitory effects on T47D (IC_{50} 0,86µM), but is less efficient in SKBR3 (IC_{50} 1,9µM). Akti-1,2 was less potent than NVP-BEZ 235 or rapamycin. These results indicates that dual inhibition of both mTOR and PI3K by NVP-BEZ 235 shows much more inhibitory effect than single inhibition of mTOR by rapamycin or of Akt by Akti-1,2, in these two cell lines.

3.3.2 Inhibition of the mitogen–activated protein kinase pathway

MEK is part of the mitogen-activated protein kinase pathway. It activates ERK1/2 by phosphorylation. Activated ERK1/2 phosphorylates a wide range of protein substrates including transcriptional and apoptosis regulators (Whyte J. et al., 2009). To determine if MEK1/2 inhibitors are also able to inhibit growth of breast cancer cells, growth assays with the three MEK1/2 inhibitors AZD6244, U0126 and AS703026 were performed (Figure 36-38).

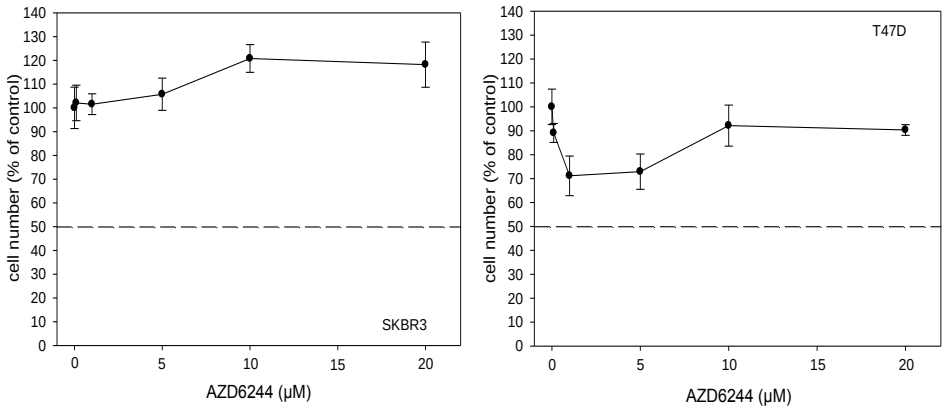


Figure 36: ***The growth effects of AZD6244 on SKBR3 and T47D cells.***

Cells were exposed to 0-20µM of the inhibitor for 72h. Effects on cell growth were determined by EZ4U. Means ± SD, n≥3.

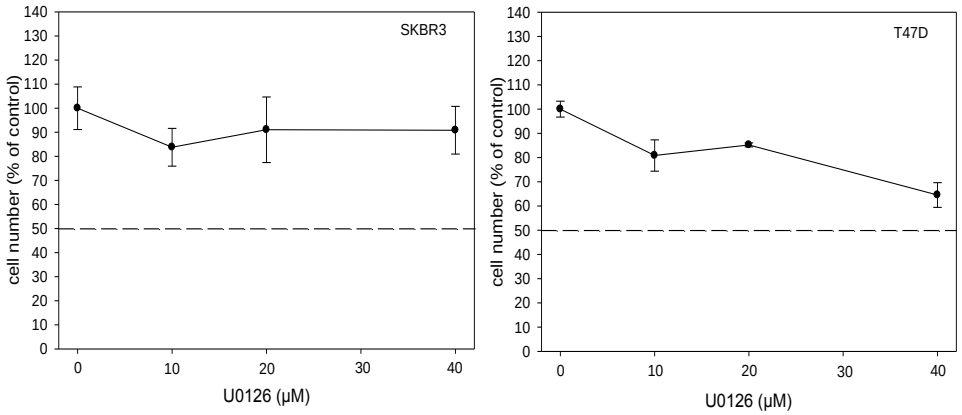


Figure 37: ***The growth effects of U0126 on SKBR3 and T47D cells.***

Cells were treated with 0-40µM U0126 for 72h. Proliferation was analysed by EZ4U. Means ± SD, n≥3.

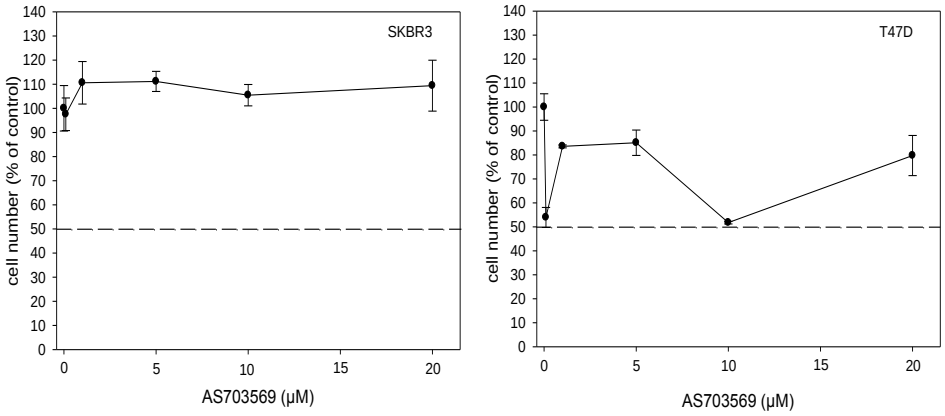


Figure 38: ***The growth effects of AS703026 on SKBR3 and T47D cells .***

The two cell lines were exposed to 0-20µM inhibitor. Effects on cell growth were determined 72h after treatment by EZ4U. Means ± SD, n≥3.

Treatment of SKBR3 and T47D cells with these three MEK inhibitors revealed no significant inhibition of growth in these cell lines, because IC_{50} value of both cell lines lies over 20µM with AZD6244, U0126 and AS703026.

Together, these findings in SKBR3 and T47D cells treated with PI3K or MAPK inhibitors showed that both cell lines respond much more sensitive to inhibition of PI3K- than of MAPK pathways (Figure 39).

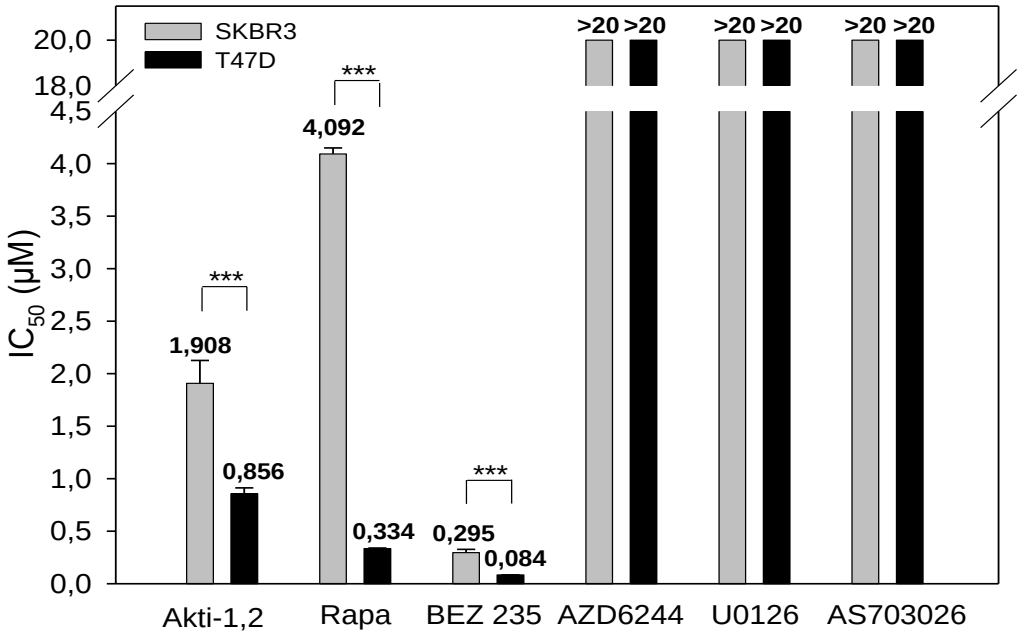


***Figure39:* IC50 values of SKBR3 and T47D treated with different inhibitors.**
*Cells were exposed to PI3K pathway inhibitors (Akti-1,2, rapamycin or NVP-BEZ 235) or to MAPK pathway inhibitors (AZD6244, U0126 or AS703026). IC_{50} values were determined after 72h of drug exposure. Statistical significance between SKBR3 (grey) and T47D (black) was determined by two-tailed Student´s t-Test, *** indicates significant difference at p<0,001.*

3.4 Effects of PI3K pathway inhibitors on AKT, ERK and S6 in the two cell lines SKBR3 and T47D

3.4.1 Influence of mTOR inhibition in the pelitinib sensitive and resistant cell lines

To determine the downstream signalling effects of mTOR inhibition by rapamycin, Western blot analysis in the presence and absence of this drug in

both cell lines was done. Growth assay analysis of SKBR3 treated with rapamycin showed low growth inhibitory effects. Treatment of T47D in contrast worked very good and showed potent growth inhibitory effects. Therefore, it was necessary for us to determine how this inhibitor affects the downstream signalling pathway in these cell lines. SKBR3 and T47D were exposed to 0,1-10µM rapamycin for 6h (Figure 40 and 41). Antibodies against AKT, ERK and S6 or their phosphorylated forms were used. β-Actin was utilized as a control, for equal loading and efficient protein transfer on PVDF membranes.

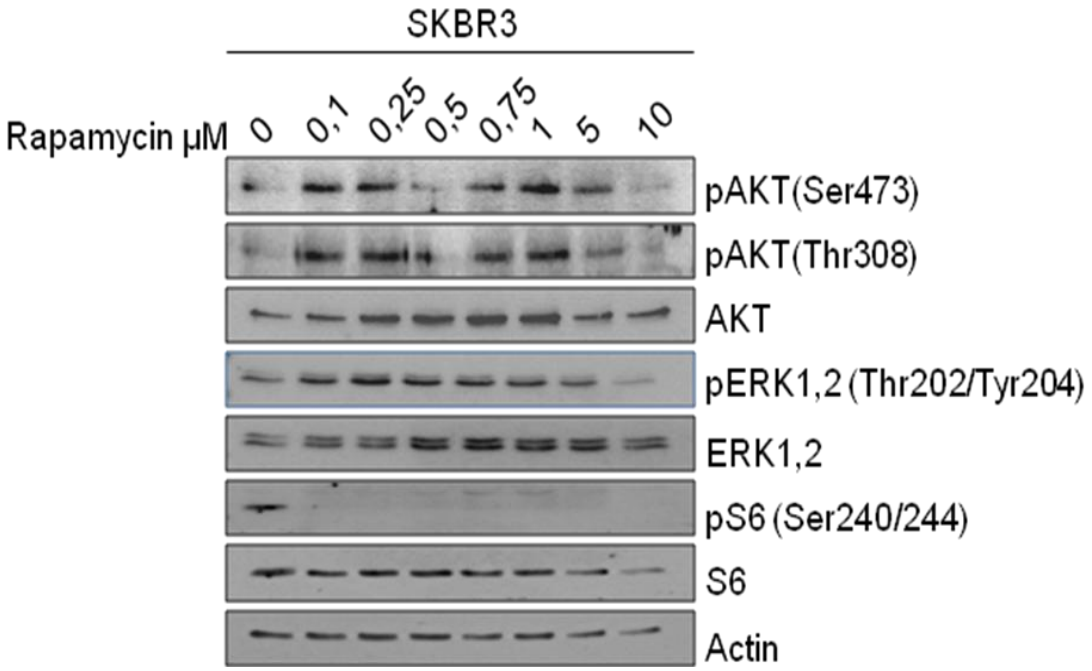


Figure 40: ***Separated total protein from SKBR3 cells treated with rapamycin was labelled with phospho-AKT (Ser473), phospho-AKT (Thr308), AKT, phospho-ERK1,2 (Thr202/Tyr204), ERK1,2, S6 and phospho-S6(Ser240/244) antibodies.***
Cells were allowed to attach over night in 5% FCS in 60mm dishes. Cultures were treated with 0,1µM, 0,25µM, 0,5µM, 0,75µM, 1µM, 5µM and 10µM rapamycin as well as only with the solvent (DMSO) without the inhibitor. Cell lysates were collected after 6h and β-actin was used as a loading control.

This result shows that pAKT (Ser473) and equally pAKT (Thr308) level decreases only in high concentrations of rapamycin. Likewise, pERK1,2 (Thr202/Tyr204) level is reduced in high concentration of the drug. In contrast, pS6 (Ser240/244) is reduced at very low drug levels. These findings go along with growth assays done with rapamycin in this cell line, where SKBR3 responds with low growth inhibition to this drug. Moreover as expected, this result shows that rapamycin inhibits mTOR, because the level of pS6 (Ser240/244), which is located downstream of mTOR, decreases in low drug concentrations.

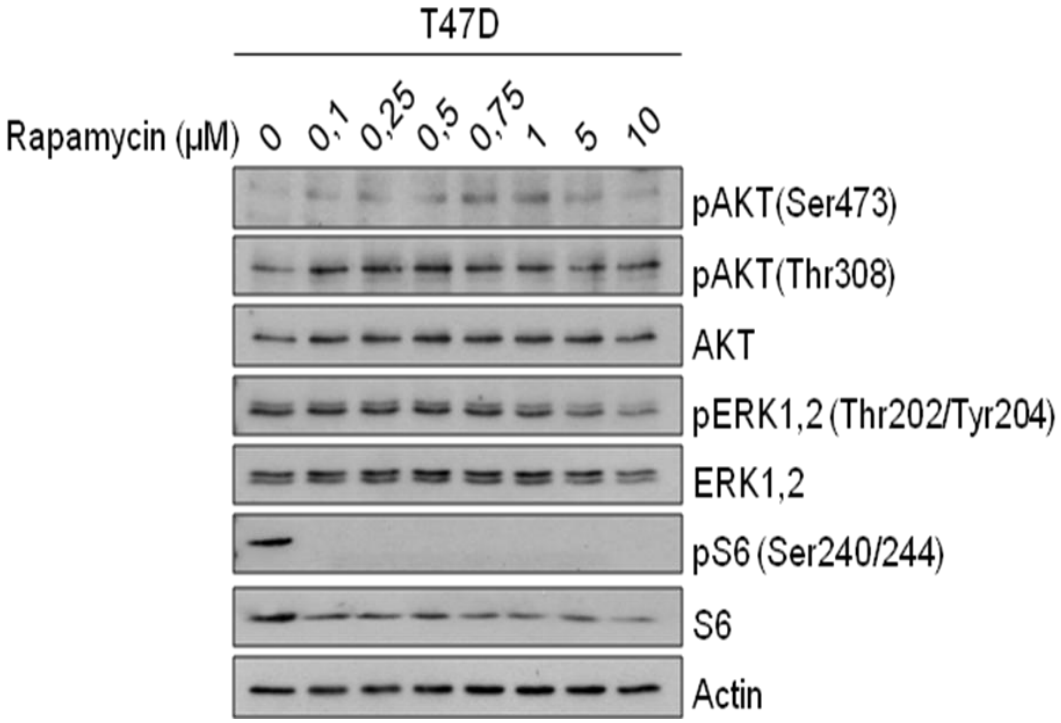


Figure 41: ***Separated total protein from T47D cells treated with rapamycin was labelled with phospho-AKT (Ser473), phospho-AKT (Thr308), AKT, phospho-ERK1,2 (Thr202/Tyr204), ERK1,2, S6 and phospho-S6 (Ser240/244) antibodies.*** *Cells were grown in 60mm dishes containing medium with 5% FCS for 24h. Then cells were exposed to 0,1µM, 0,25µM, 0,5µM, 0,75µM, 1µM, 5µM and 10µM rapamycin as well as only with the solvent (DMSO) without the inhibitor. Cell lysates were collected after 6h and β-actin was used as a loading control.*

Treatment of T47D cells with rapamycin leads to an increasing level of pAKT (Thr308) and also of pAKT (Ser473) but less stronger. Moreover, ERK1,2 phosphorylation level is minor decreased in high drug concentrations. The pS6 (Ser240/244) level is reduced already at a inhibitor concentration of 0,1µM. This cell line is sensitive against growth inhibition by rapamycin, which goes along with the results achieved in Western blotting, because pAKT (Ser473) is very low as well as pS6 (Ser240/244) level.

3.4.2 Dual mTOR and PI3K inhibition in SKBR3 and T47D cell lines

Inhibition with rapamycin in the pelitinib resistant cell line T47D showed some good effects, to overcome the resistance, but rapamycin inhibits only mTOR. To examine how dual inhibition of mTOR and PI3K influences downstream signalling in SKBR3 and T47D cell lines, Western blotting with NVP-BEZ 235 was done. Both cell lines were treated with 0-1µM inhibitor for 6h. Thereafter, Western blotting was done (Figure 42 and 43) and β-actin was used as a loading control. Exposure of SKBR3 cell lines to NVP-BEZ 235 revealed a dose-dependent increasing level of pAKT (Thr308) up to 0,075µM which then strongly decreased at higher inhibitor concentrations. pAKT (Ser473) decreases already at lower inhibitor concentrations. The level of pS6 (Ser240/244) is reduced already at the lowest drug concentration used. Phosphorylated S6 is located downstream of mTOR and decreases already at very low concentrations of NVP-BEZ 235. Phosphorylation of AKT increases at low, but then decreases at higher concentrations of NVP-BEZ 235. This finding goes along with the results achieved from Serra et al., 2008, which indicate that this dual inhibitor blocks mTOR at very low concentrations and both mTOR and PI3K in high concentrations and is due to a negative regulatory feed-back loop from mTOR to PI3K. The level of pERK1,2 (Thr202/Tyr204) increased upon NVP-BEZ 235-mediated inhibition of mTOR and PI3K.

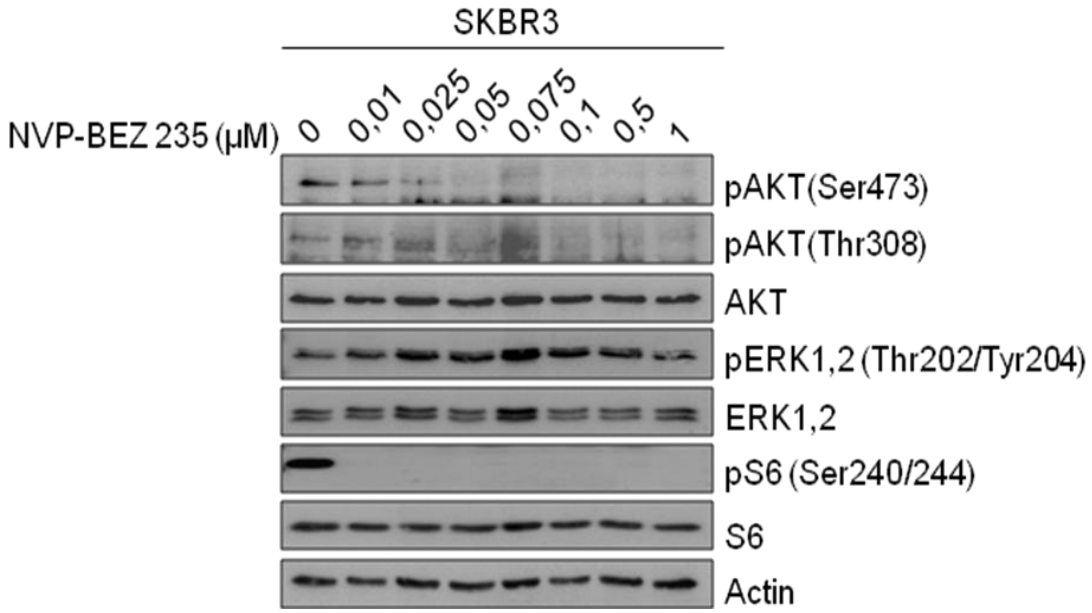


Figure 42: ***SKBR3 cells treated with the dual mTOR and PI3K inhibitor NVP-BEZ 235 and labelled with phospho-AKT (Ser473), phospho-AKT (Thr308), AKT, phospho-ERK1,2 (Thr202/Tyr204), ERK1,2, S6 and phspho-S6 (Ser240/244) antibodies.***

Cells, which were grown in 60mm dishes containing medium with 5% FCS, were treated for 6h with 0,001µM, 0,025µM, 0,05µM, 0,075µM 0,1µM, 0,5µM and 1µM NVP-BEZ 235 or with the solvent (DMSO) only. Cell lysates were collected as described in Material and Methods. β-actin was used as a loading control.

Treatment of the pelitinib resistant cell line T47D with NVP-BEZ 235 showed that the pAKT (Ser473) level is strongly reduced at a concentration of 0,05µM. However, the pAkt (Thr308) level is increased at low inhibitor concentrations; only at high drug levels a reduction is seen. This might be caused by the differential affinity of the inhibitor for its two target kinases, which is significantly higher for mTOR than for PI3K. Thus mTOR becomes inhibited at very low drug concentrations already, whereas PI3K inhibition occurs only at much higher doses of NVP-BEZ 235 (Serra et al., 2008). The phosphorylation level of S6 is very intense in the untreated control, but pS6 level strongly decreases after

0,01µM NVP-BEZ 235 treatment and nearly disappears at higher concentrations. However, even at the highest concentration it could not be totally wiped out. At low concentrations of NVP-BEZ 235, an increase of pErk1/2 (Thr202/204) level was observed, but it decreased at higher concentrations of the dual inhibitor.

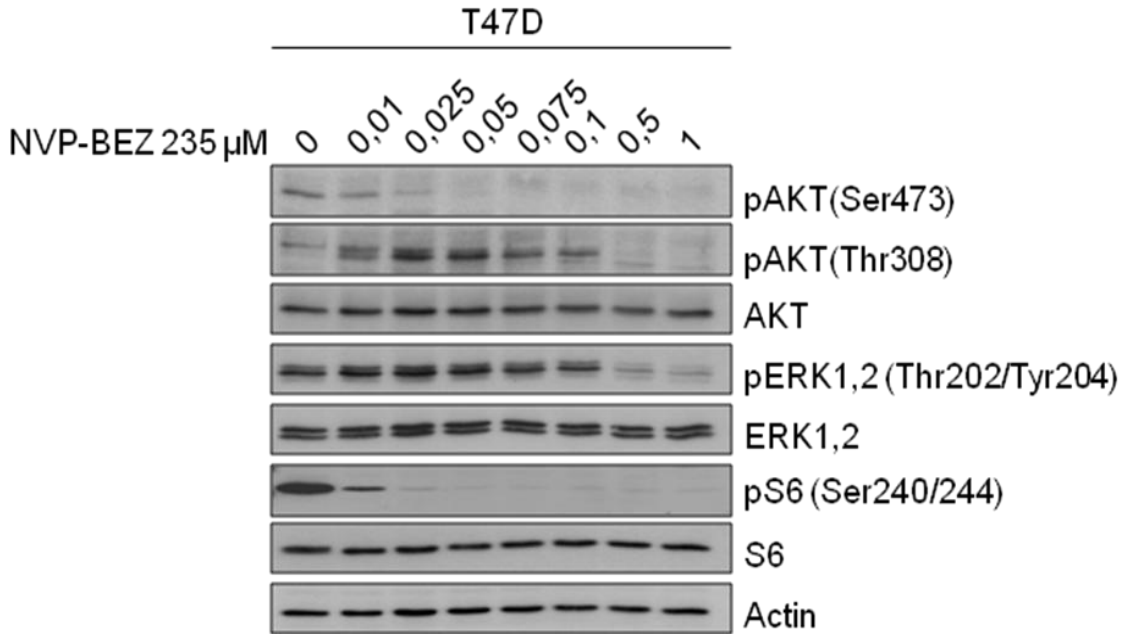


Figure 43: ***Pelitinib resistant cell line T47D exposed to NVP-BEZ 235, a dual mTOR and PI3K inhibitor, labelled with phospho-AKT (Ser473), phospho-AKT (Thr308), AKT, phospho-ERK1,2 (Thr202/Tyr204), ERK1,2, S6 and phspho-S6 (Ser240/244) antibodies.*** *Cells were grown in 60mm dishes containing medium with 5% FCS for 24h. Afterwards, cells were treated for 6h with 0,001µM, 0,025µM, 0,05µM, 0,075µM 0,1µM, 0,5µM and 1µM NVP-BEZ 235 or well as with the solvent (DMSO) only. Cell lysates were collected as described in Material and Methods. β-actin was used as a loading control.*

3.4.3 Determination of signalling effects through inhibition of AKT in T47D and SKBR3 cells

Inhibition of these two cell lines with the mTOR/PI3K inhibitor NVP-BEZ 235 reduces downstream pS6 more efficiently than AKT, which acts more upstream in the PI3K cascade. Thus, we wanted to determine how direct inhibition of AKT influences the signalling cascade in pelitinib resistant T47D and sensitive SKBR3 cells. Cells were cultivated for one day in medium containing 5% FCS, afterwards both cell lines were exposed to 0-5µM Akti-1,2 for 6h, followed by Western blot analysis (Figure 44 and 45). β-actin was used as a loading control.

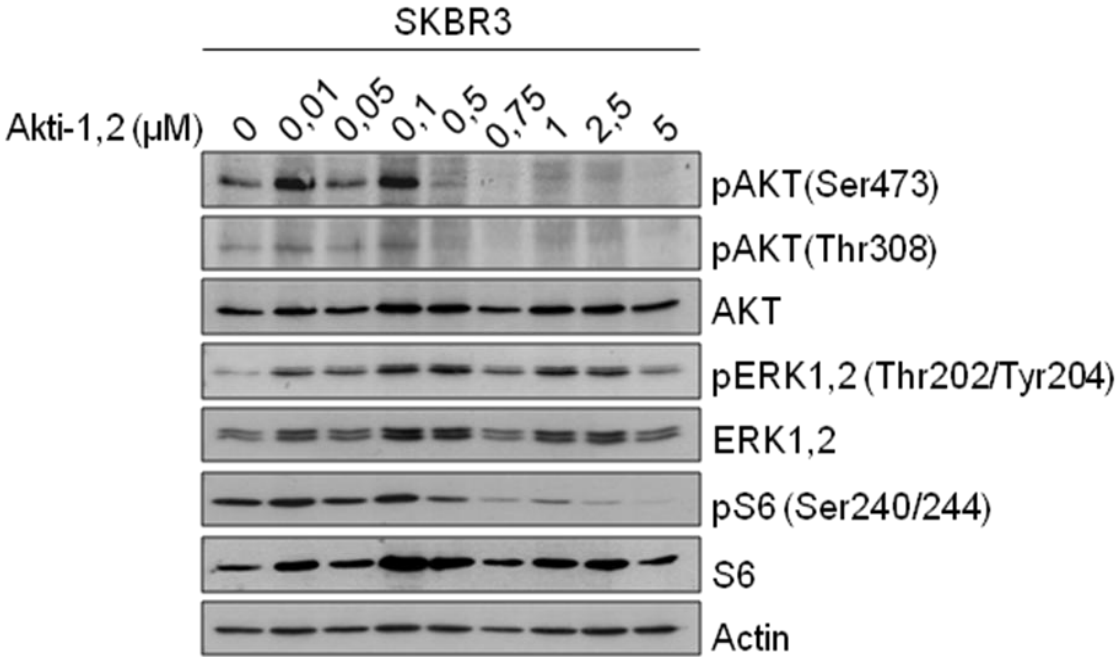


Figure 44: ***Breast cancer cell line SKBR3 exposed to Akti-1,2 was labelled with phospho-AKT (Ser473), phospho-AKT (Thr308), AKT, phospho-ERK1,2 (Thr202/Tyr204), ERK1,2, S6 and phspho-S6 (Ser240/244) antibodies.***

Cells were grown in 60mm dishes containing medium with 5% FCS for 24h. The cultures were treated for 6h with 0,01µM, 0,05µM, 0,1µM, 0,75µM 0,5µM, 1µM, 2,5µM and 5µM Akti-1,2 with the solvent (DMSO) only. Cell lysates were collected as described in Material and Methods. β-actin was used as a loading control.

The level of pAKT (Ser473) is strongly decreasing at an inhibitor concentration of >0,1µM. Level of pAKT (Thr308) compared to pAKT (Ser473) is very low in the untreated control and decreases also at a concentration of >0,1µM Akti-1,2. Interestingly, the pERK1,2 (Thr202/Tyr204) level, which is very low in untreated SKBR3 cells, is enhanced by drug exposure. This may suggest that inhibition of AKT by Akti-1,2 lifts an inhibitory cross talk loop from the PI3K to the MAPK system. Moreover, the phosphorylation of S6 is reduced at inhibitor concentrations of >0,5µM.

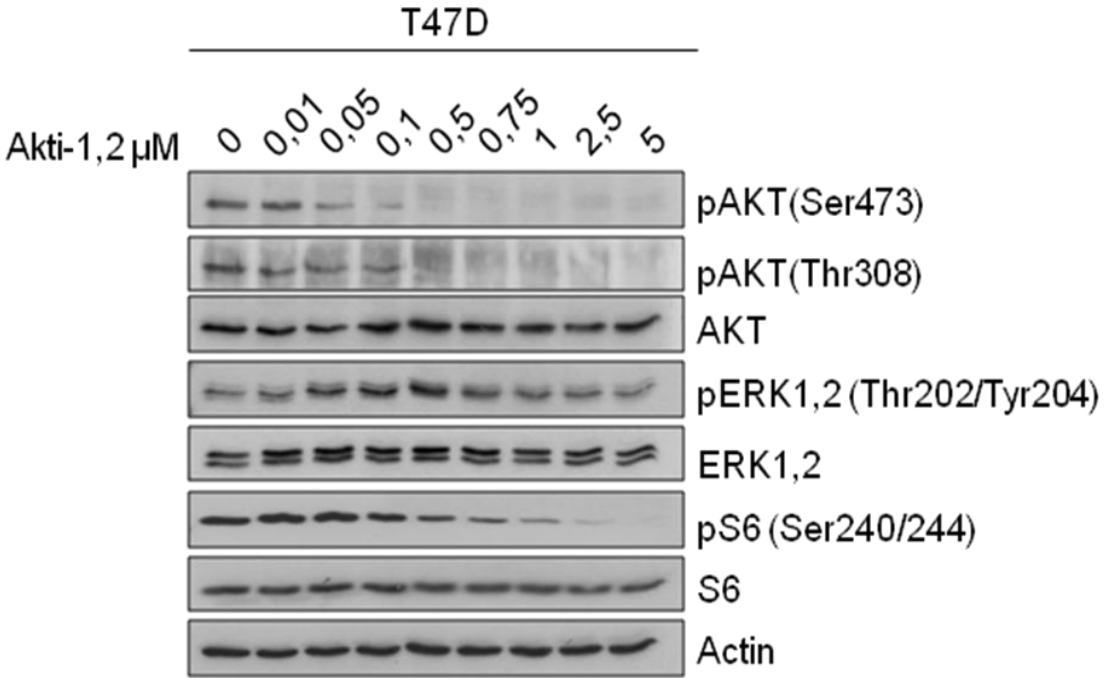


Figure 45: ***Breast cancer cell line T47D exposed to Akti-1,2 was labelled with phospho-AKT (Ser473), phospho-AKT (Thr308), AKT, phospho-ERK1,2 (Thr202/Tyr204), ERK1,2, S6 and phspho-S6 (Ser240/244) antibodies.***

Cells were allowed to attach over night in 5% FCS in 60mm dishes. Cultures were treated for 6h with 0,01µM, 0,05µM, 0,1µM, 0,5µM, 0,75µM, 1µM 2,5µM and 5µM Akti-1,2 as well as only with the solvent (DMSO) only. β-actin was used as a loading control.

Results from inhibition of T47D cells with Akti-1,2 show that the level of AKT (Thr308) and AKT (Ser473) and S6 (Ser240/244) phosphorylation decreases at ≥0,5µM drug concentrations. Equally to SKBR3 inhibition with this drug, the pERK1,2 (Thr202/Tyr204) level is increased with low Akti-1,2 dose and only decreases at high inhibitor concentrations. Western blotting analysis with Akti-1,2 revealed that these two cell lines respond nearly in the same way to the inhibition of AKT. Compared to the other PI3K inhibitors, Akti-1,2 does not work as good as rapamyicn or NVP-BEZ 235 in reducing the pS6 level. This result goes along with growth assay analysis, where AKT inhibition alone showed not the same good growth inhibitory effects when compared to the other two PI3K pathway inhibitors in T47D cell lines.

3.5 Effects of MAPK pathway inhibitors on MEK and ERK in the two cell lines SKBR3 and T47D

Growth assay analysis with MEK1,2 inhibitors revealed, that they do not have any growth inhibitory effect on SKBR3 or T47D cells. This result leads to the suggestion that the MAPK pathway plays a minor role in the growth of these cell lines. In order to determine, if the used MEK1,2 inhibitors really lead to inhibition of this pathway. Western blot analysis of total proteins from SKBR3 and T47D cells exposed for 6h to the MEK1,2 inhibitors AZD6244, U0126 or AS703026 was performed.

3.5.1 Influence of AZD6244 on MAPK pathway signalling in the two pelitinib sensitive and resistant cell lines

Previous growth analysis using AZD6244 showed no significant growth inhibitory effects on SKBR3 and T47D, with an IC_{50} value over 20µM. In order to analyze the inhibitory effect on the MAPK pathway signalling, Western blotting in both cell lines treated with AZD6244 was done. Cells were cultured as

described in Material and Methods. SKBR3 and T47D were exposed to 0-20µM AZD6244 for 6h (Figure 46 and 47). Antibodies against MEK and ERK or their phosphorylated forms were used. α/β- Tubulin was used as a control for equal loading and efficient protein transfer to PVDF membranes.

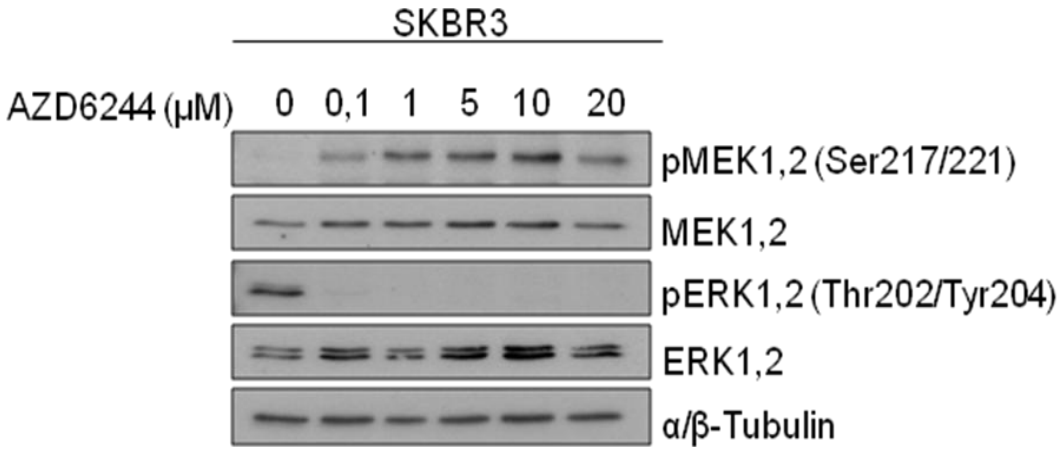


Figure 46: ***Total protein from SKBR3 cells treated with AZD6244 were labelled with phospho-MEK1,2 (Ser217/221), MEK, phospho-ERK1,2 (Thr202/Tyr204) and ERK1,2 antibodies.***

Cells were allowed to attach over night in 5% FCS in 60mm dishes. Cultures were treated for 6h with 0,1µM, 1µM, 5µM, 10µM and 20µM AZD6244 or with the solvent (DMSO) only. α/β-Tubulin was used as a loading control.

Results from Western blotting analysis of SKBR3 cells treated with the MEK1,2 inhibitor AZD6244 interestingly showed an increasing level of MEK1,2 phosphorylation also in the highest drug concentration, compared to a treatment with the solvent only. Moreover, the level of pERK1,2 (Thr202/Tyr204) is reduced strongly at a drug concentration of 0,1µM. This result shows that the inhibitor AZD6244 acts downstream of MEK1,2. Because an inhibition is only seen in the posphorylation of ERK1,2 which is located downstream of MEK1,2.

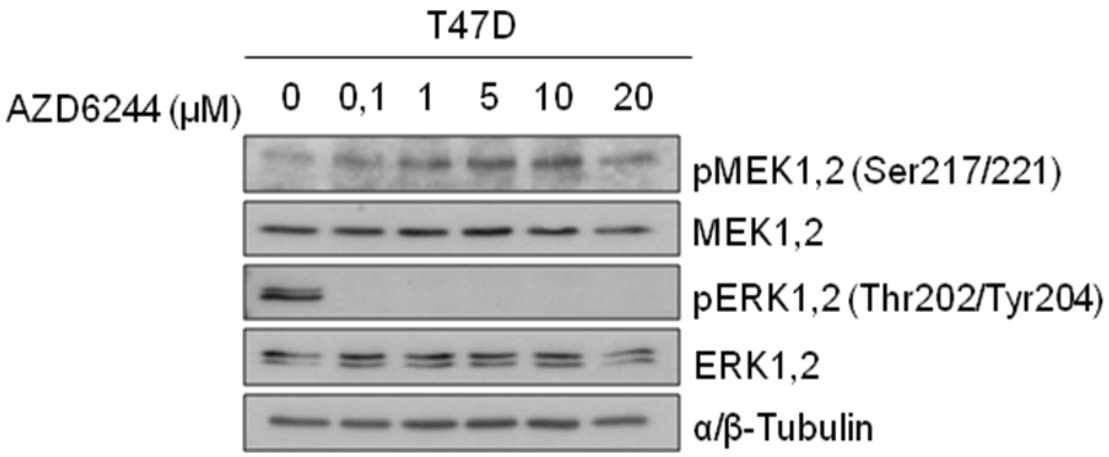


Figure 47: ***Total protein from T47D cells treated with AZD6244 were labelled with phospho-MEK1,2 (Ser217/221), MEK, phospho-ERK1,2 (Thr202/Tyr204) and ERK1,2 antibodies.***

Cells were allowed to attach over night with 5% FCS in 60mm dishes. Cultures were treated for 6h with 0,1µM, 1µM, 5µM, 10µM and 20µM AZD6244 or with the solvent (DMSO)only. α/β-Tubulin was used as a loading control.

Western blotting analysis of T47D cells treated with AZD6244 showed nearly the same results like as compared to SKBR3 cells. Inhibitor exposure leads to an increasing level of pMEK1,2 (Ser217/221), but not with the same intensity than in SKBR3 cells. Furthermore, ERK1,2 phosphorylation is decreased at the lowest inhibitor concentration already. Therefore, AZD6244 acts also downstream of MEK1,2 in this cell line.

3.5.2 Influence of U0126 on MAPK pathway signalling in the two pelitinib sensitive and resistant cell lines

U0126 did not induce growth inhibition in pelitinib resistant T47D and sensitive SKBR3 cell lines. Analysis of the inhibitory effects of U0126 on the MAPK pathway, was done by Western blotting. Cells were cultivated for one day in medium containing 5% FCS. Afterwards, both cell lines were exposed to

0-80µM U0126 for 6h. α/β- Tubulin was used as a loading control (Figure 48 and 49).

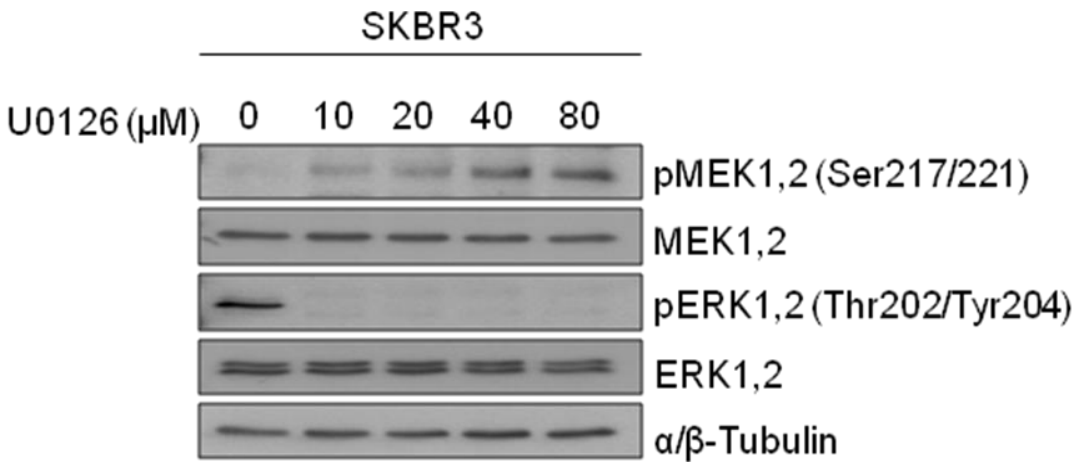


Figure 48: ***Breast cancer cell line SKBR3 exposed to U0126 was labelled with phospho-MEK1,2 (Ser217/221), MEK, phospho-ERK1,2 (Thr202/Tyr204) and ERK1,2 antibodies.*** *Cells were grown in 60mm dishes containing medium with 5% FCS for 24h. The cultures were treated for 6h with 10µM, 20µM, 40µM and 80µM U0126 or with the solvent (DMSO) only. Cell lysates were collected as described in Material and Methods. α/β-Tubulin was used as a loading control.*

Western blot analysis of SKBR3 cells exposed to the MEK1,2 inhibitor U0126, shows a dose-dependent increasing level of pMEK1,2 (Ser217/221). In contrast, phosphorylation of ERK1,2 decreases rapidly already at the lowest U0126 concentration. This result show that U0126 acts in the same way as AZD6244, namely downstream of MEK1,2.

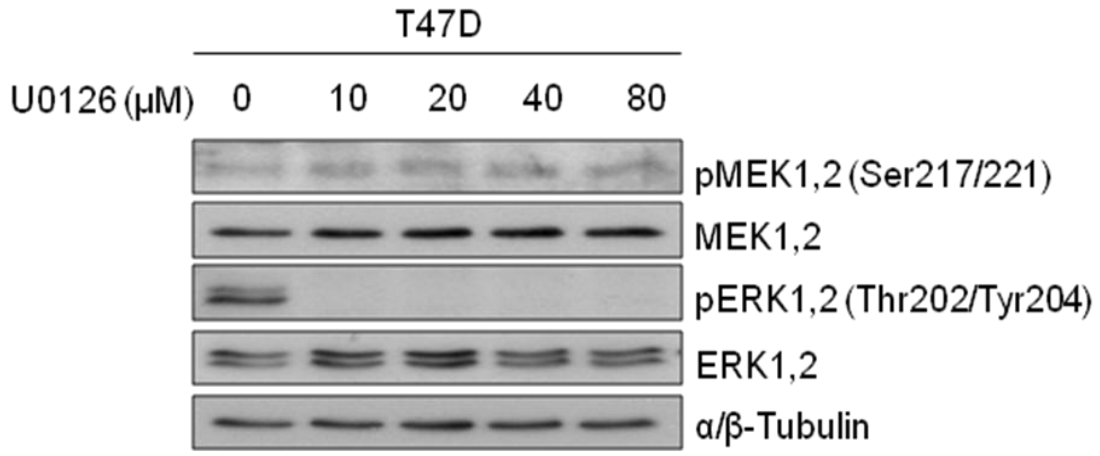


Figure 49: ***Total protein from T47D cells treated with U0126 was labelled with phospho-MEK1,2 (Ser217/221), MEK, phospho-ERK1,2 (Thr202/Tyr204) and ERK1,2 antibodies.*** *Cells were grown in 60mm dishes containing medium with 5% FCS for 24h. Then cells were exposed for 6h to 10µM, 20µM, 40µM and 80µM U0126 or to solvent (DMSO) only. α/β-Tubulin was used as a loading control.*

T47D cells show a very low level of pMEK1,2 (Ser217/221), which was only very slightly elevated by U0126. In contrast, a strong reduction of pERK1,2 (Thr202/Tyr204) can be seen already at the lowest dose of U0126.

3.5.3 Influence of AS703026 on MAPK pathway signalling in the two pelitinib sensitive and resistant cell lines

The third used MEK1,2 inhibitor, termed AS703026, did also not show any growth inhibitory effects on SKBR3 and T47D cells. To check the function of this drug, Western blotting in both cell lines was performed. Cells were cultured as described in Material and Methods. SKBR3 and T47D were exposed to 0-20µM AS703026 for 6h (Figure 50 and 51). Antibodies against MEK and ERK or their phosphorylated forms were used. α/β- Tubulin served as a control for equal loading and efficient transfer of the proteins onto the PVDF-membranes.

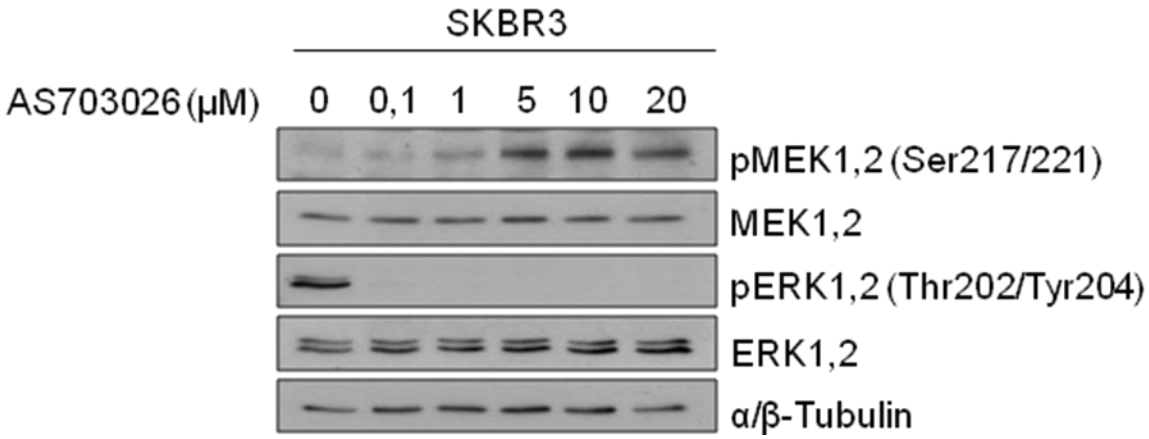


Figure 50: ***SKBR3 cell line exposed to AS703026 and labelled with phospho-MEK1,2 (Ser217/221), MEK, phospho-ERK1,2 (Thr202/Tyr204) and ERK1,2 antibodies.***

Cells were grown in 60mm dishes containing medium with 5% FCS for 24h. The cultures were treated for 6h with 0,1µM, 1µM, 5µM 10µM and 20µM AS703026 or with the solvent (DMSO) only. Cell lysates were collected as described in Material and Methods. α/β-Tubulin was used as a loading control.

Our results indicate that inhibition of MEK1,2 with AS703026 leads to a dose-dependent increase of pMEK1,2 (Ser217/221). In the absence of the drug the level of pMEK1,2 is very low in SKBR3 cells. On the other hand, AS703026 leads to a strong inhibition of phosporylated ERK1,2, already at the lowest inhibitor concentration. These findings indicate, that AS703026 works in the same way, compared to the other used MEK1,2 inhibitors. It shows inhibitory effects downstream of MEK1,2.

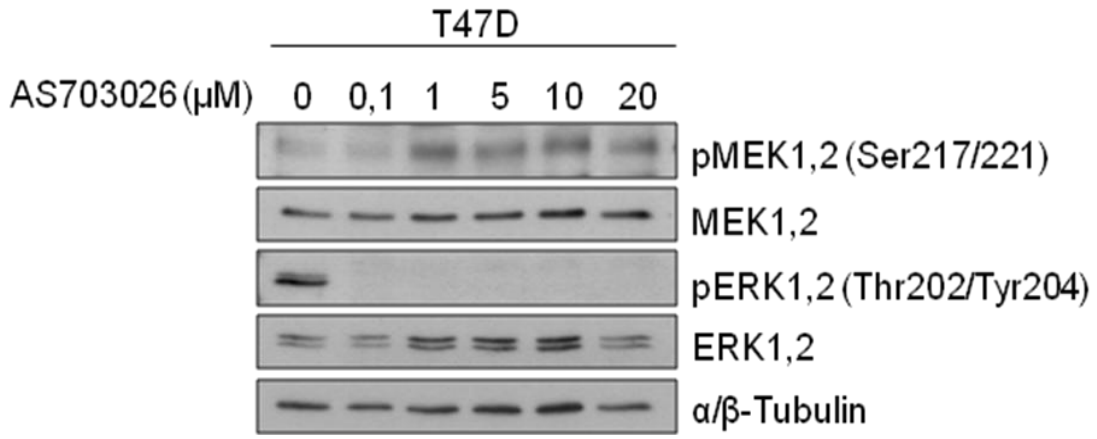


Figure 51: ***T47D exposed to AS703026 and labelled with phospho-MEK1,2 (Ser217/221), MEK, phospho-ERK1,2 (Thr202/Tyr204) and ERK1,2 antibodies.*** *Cells were grown in 60mm dishes containing medium with 5% FCS for 24h. The cultures were treated for 6h with 0,1µM, 1µM, 5µM, 10µM and 20µM inhibitor or the solvent (DMSO) only. Cell lysates were collected as described in Material and Methods. α/β-Tubulin was used as a loading control.*

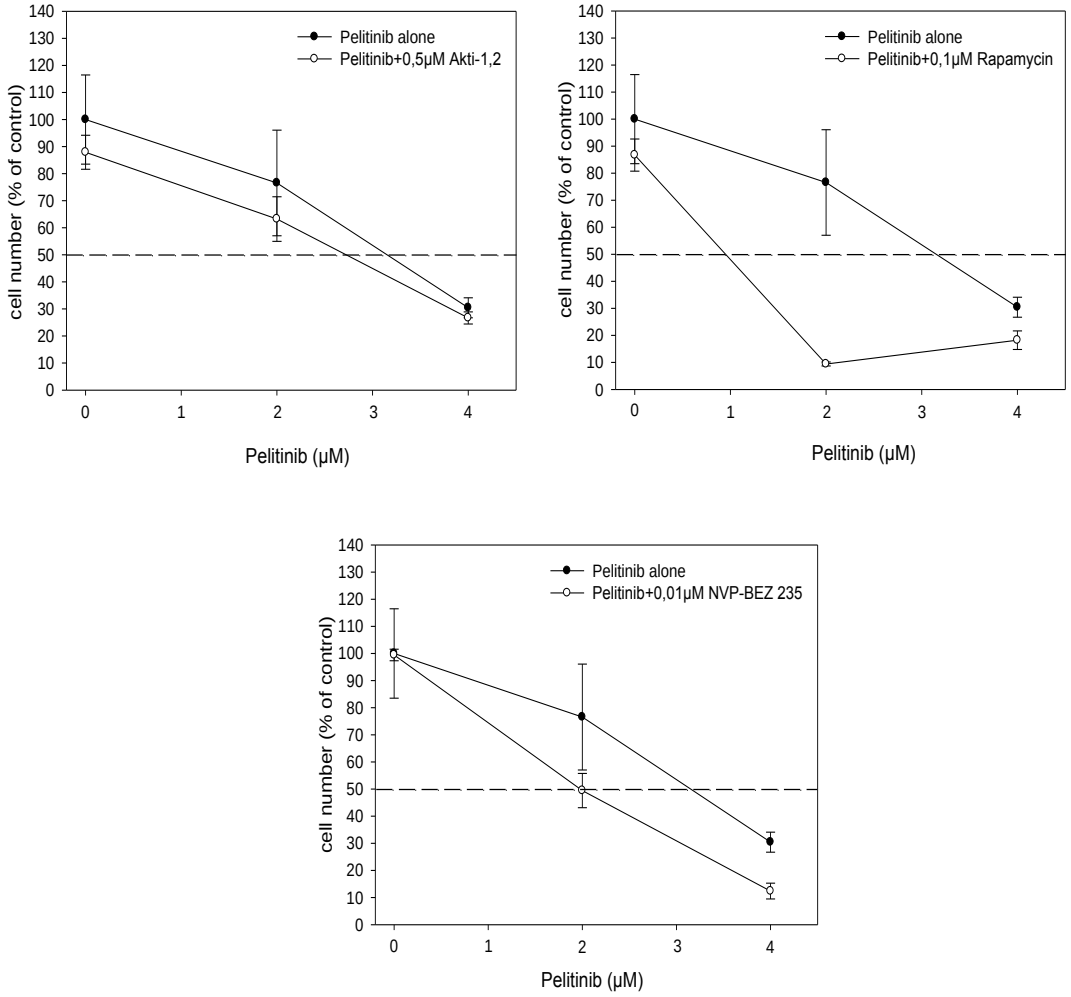
Findings achieved by treatment of T47D with AS703026 revealed that the level of pMEK1,2 (Ser217/221) strongly increases at drug concentrations ≥ 1µM. In contrast, the level of phosphorylated ERK1,2 strongly decreases at inhibitor concentration as low as 0,1µM. Thus, the results obtained with AS703026 are similar to those obtained with the two other MEK1,2 inhibitors. In general, all three MEK1,2 inhibitors, which did not show any growth inhibitory effects in either cell line, act downstream of MEK1,2 by inhibiting phosphorylation of ERK1,2. This reveals that these drugs are potent inhibitors of the MAPK cascade but growth of SKBR3 and T47D breast cancer cells appears to be largely independent of MAPK activity.

3.6 Overcoming the resistance of T47D against pelitinib

Treatment of SKBR3 and T47D with PI3K inhibitors showed some good inhibitory effects. Especially in the pelitinib resistant cell line T47D, single treatment with PI3K inhibitors was very effective. To see how the growth in both cell lines responds to a combined therapy with ErbB and PI3K or MAPK inhibitors, combinational growth assays with SKBR3 and T47D cell lines were performed.

3.6.1 Combined treatment of SKBR3 or T47D with pelitinib and PI3K inhibitors

Single treatment of SKBR3 cells with PI3K inhibitors showed lower growth inhibitory effects than in T47D cells, especially with rapamycin. To assess the effects of combined therapy with pelitinib and the three PI3K inhibitors Akti-1,2, rapamycin and NVP-BEZ 235, combined treatment and growth assay analysis was done. SKBR3 cells were treated for 72h with various concentrations of gefitinib combined with one concentration of PI3K pathway inhibitors. As expected growth assay analysis showed that SKBR3 cells are sensitive against pelitinib alone. Combinational treatment with Akti-1,2 does not significantly elevate the antiproliferative response (Figure 52). However, combinational exposure to pelitinib plus rapamycin or plus NVP-BEZ 235 revealed improved inhibitory effects. Figure 53 showed the calculated significance of each combination. Combined treatment of SKBR3 cells with 2µM pelitinib and 0,1µM rapamycin showed high significance, compared to treatment with pelitinib alone. But the significance of combinational exposure of 4µM pelitinib with 0,1µM rapamycin revealed lower significance. Moreover exposure of this cell line to 4µM pelitinib and 0,01µM NVP-BEZ 235 showed high significance compared to single treatment with this ErbB inhibitor.



<u>Figure 52:</u> Growth curves of pelitinib sensitive cell line SKBR3 treated with pelitinib alone or in combination with PI3K inhibitors.

This cell line was exposed to 0-4µM pelitinib, 0,5µM Akti-1,2, 0,1µM rapamycin or 0,01µM NVP-BEZ 235 alone or in combination. Cell growth was analysed by EZ4U. Means ± SD, n≥3.

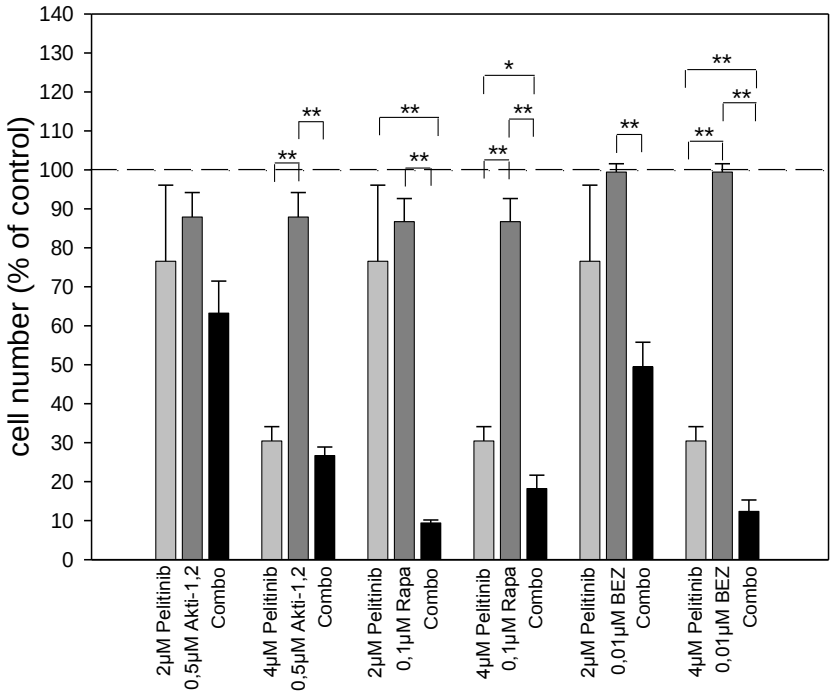


Figure 53: ***Combinational treatment of SKBR3 with pelitinib plus PI3K inhibitors.*** *Bars indicate cell numbers (% of control), treatment with pelitinib alone light grey, with PI3K inhibitor dark grey and combinational therapy black. Significance was calculated using ANOVA followed by Scheffé-Test. * indicates significant difference at $p < 0.05$ and ** at $p < 0.01$. Means ± SD, $n \geq 3$.*

Single treatment of T47D cells with PI3K inhibitors showed very good growth inhibitory effects, especially with the dual mTOR and PI3K inhibitor NVP-BEZ 235. To assess the effect of both ErbB inhibitor pelitinib and the three PI3K inhibitors together, combined therapy with these inhibitors was done.

Combined growth assay analysis showed that T47D cells are resistant to pelitinib alone (Figure 54). However, combined exposure to pelitinib and PI3K inhibitors lead to better growth inhibitory effects. Especially exposure to 2µM pelitinib combined with 0,1µM rapamycin was very effective. Similarly, treatment of pelitinib with Akti-1,2 and NVP-BEZ 235 revealed significant reduction of the cell number, compared to an exposure to the ErbB inhibitor alone (Figure 55).

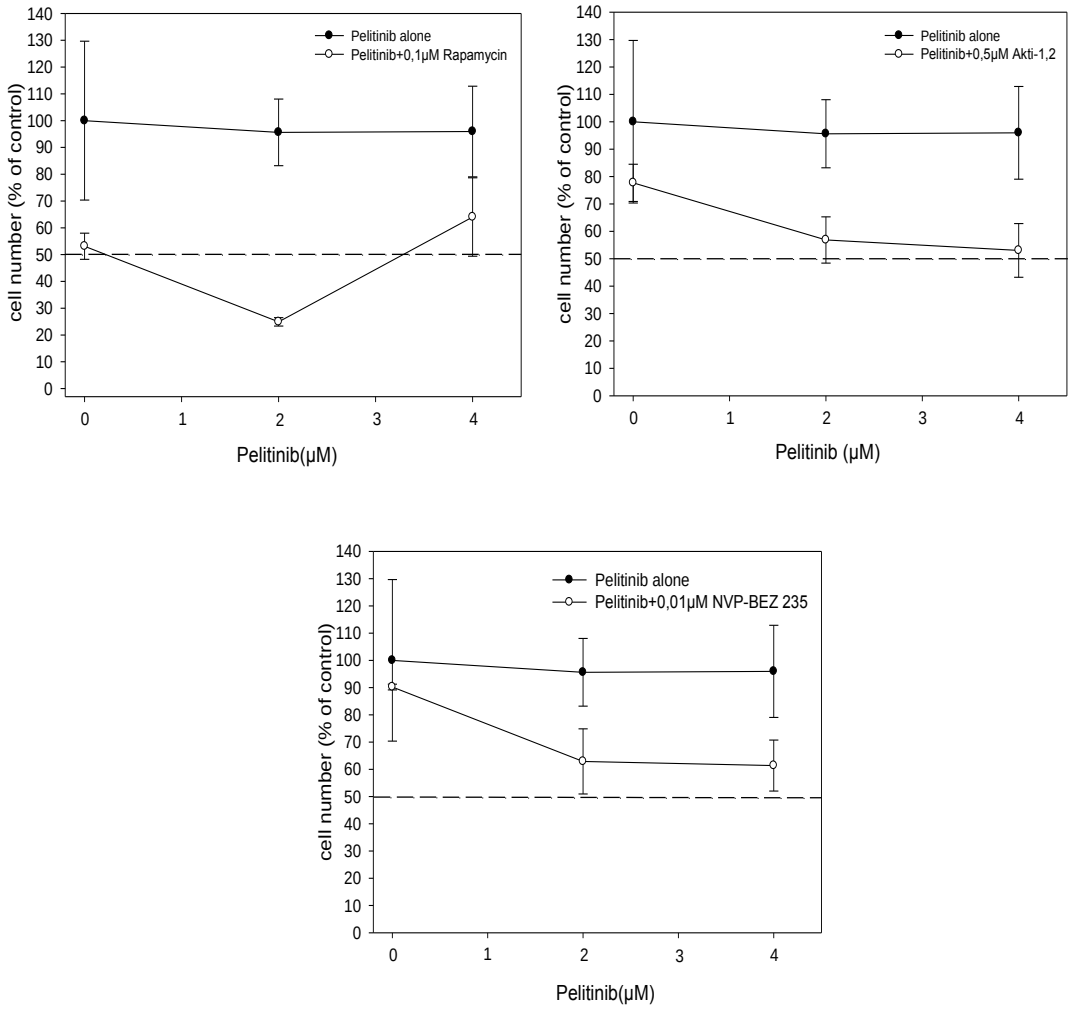


***Figure 54:* Growth curves of pelitinib resistant cell line T47D treated with pelitinib alone or in combination with PI3K inhibitors.**

This cell line was exposed to 0-4µM pelitinib, 0,5µM Akti-1,2, 0,1µM rapamycin or 0,01µM NVP-BEZ 235 alone or in combination. Cell growth was analysed by EZ4U. Means ± SD, n≥3.

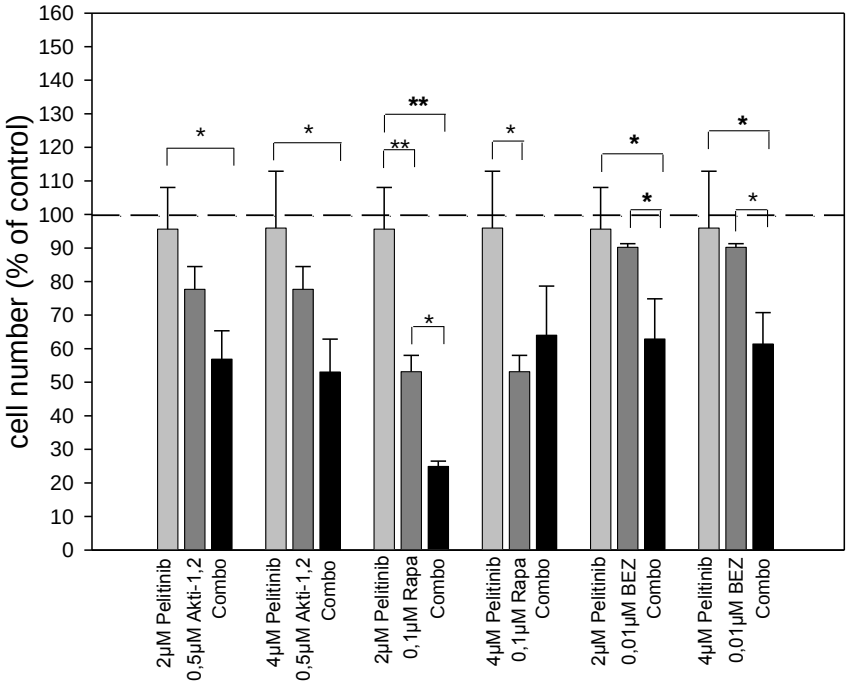


***Figure 55:* Combinational treatment of pelitinib and PI3K inhibitors in T47D.**
*Bars indicate cell numbers (% of control), treatment with pelitinib alone light grey, with the respective PI3K inhibitor dark grey and combinational therapy black. Significance was calculated using ANOVA followed by Scheffé-Test. * indicates significant difference at p < 0.05 and ** at p < 0.01. Means ± SD, n≥3.*

3.6.2 Combined treatment of SKBR3 and T47D with canertinib and PI3K inhibitors

Furthermore, a second ErbB inhibitor called canertinib was used in combination with PI3K inhibitors, to determine how SKBR3 and T47D cell lines respond to another ErbB inhibitor and, moreover, because canertinib is in clinical development. Therefore, the same combinational therapies using canertinib and PI3K inhibitors were done. Growth assay analysis of SKBR3 cells with canertinib alone showed as expected a very good growth inhibitory effect, even better than single treatment with pelitinib. Cotreatment with Akti-1,2 did not lead to a significant increase of the sensitivity of SKBR3 cells (Figure 56 and 57).

Likewise, combined treatment of SKBR3 with canertinib and NVP-BEZ 235 did not show a significant decrease of the cell number compared to single treatment with canertinib. This result shows that a combination of pelitinib and NVP-BEZ 235 (Figure 52 and 53) worked better than with canertinib. However, cotreatment with 2µM or 4µM canertinib and 0,1µM rapamycin lead to a significantly stronger growth inhibitory effect as opposed to canertinib alone, which conforms findings achieved when pelitinib was combined with rapamycin.

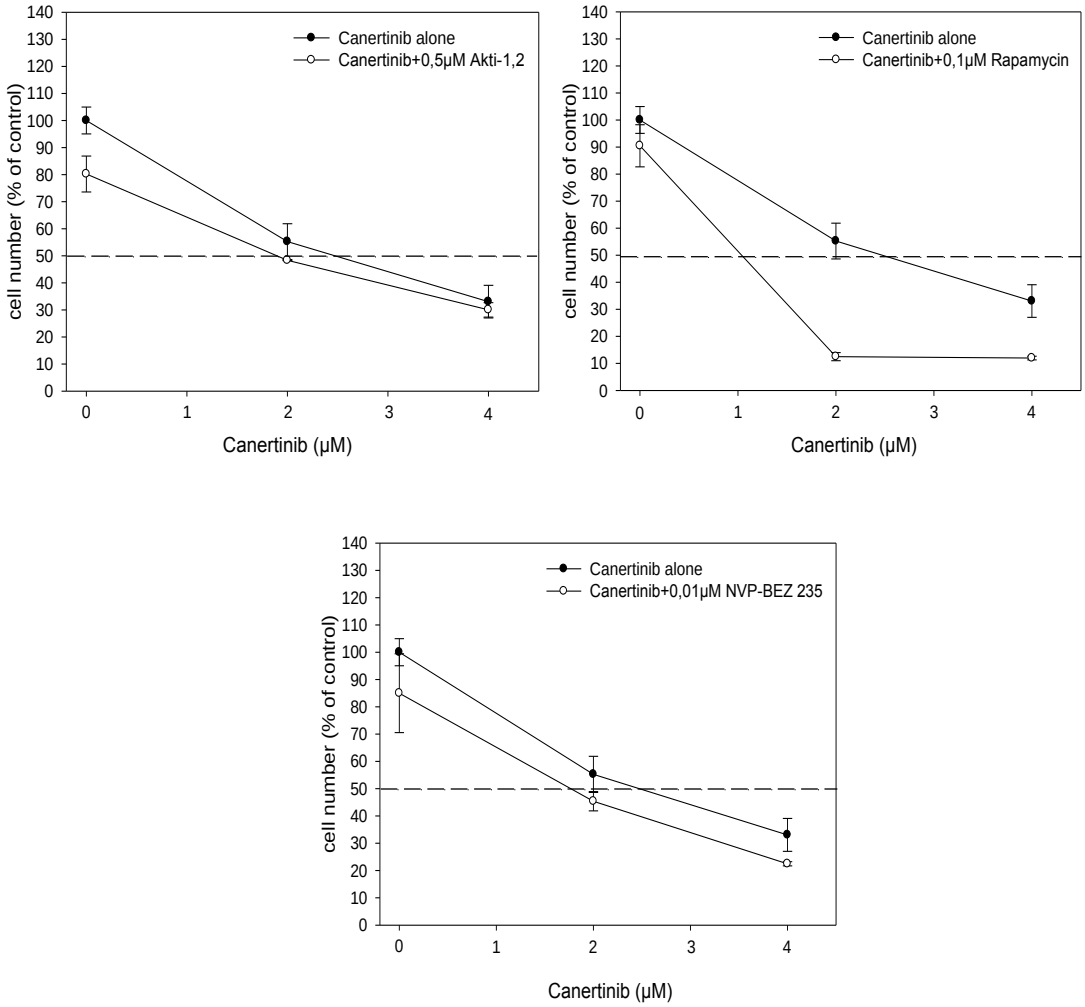


Figure 56: ***Growth curves of SKBR3 treated with canertinib alone or in combination with PI3K inhibitors.***

This cell line was exposed to 0-4µM canertinib, 0,5µM Akti-1,2, 0,1µM rapamycin or 0,01µM NVP-BEZ 235 alone or in combination. Cell growth was analysed by EZ4U. Means ± SD, n≥3.

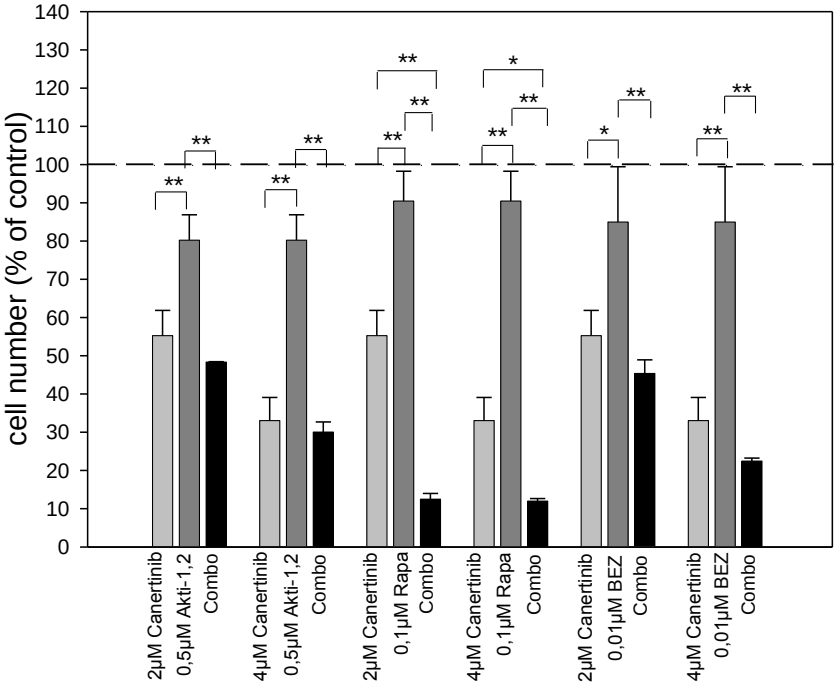


Figure 57: ***Combinational treatment of canertinib and PI3K inhibitors in SKBR3.***
*Bars indicate cell numbers (% of control), treatment with canertinib alone light grey, with the respective PI3K inhibitor dark grey and combinational therapy black. Significance was calculated using ANOVA followed by Scheffé-Test. * indicates significant difference at $p < 0.05$ and ** at $p < 0.01$. Means ± SD, $n \geq 3$.*

In growth assay analysis (Figure 58) pelitinib resistant T47D cells were cross-resistant to canertinib alone. Combinational therapy using this ErbB inhibitor together with PI3K inhibitors lead to highly significant growth inhibition versus single canertinib treatment (Figure 59). The effects for canertinib were even more pronounced than that seen after pelitinib/PI3K inhibitor cotreatment in this cell line.

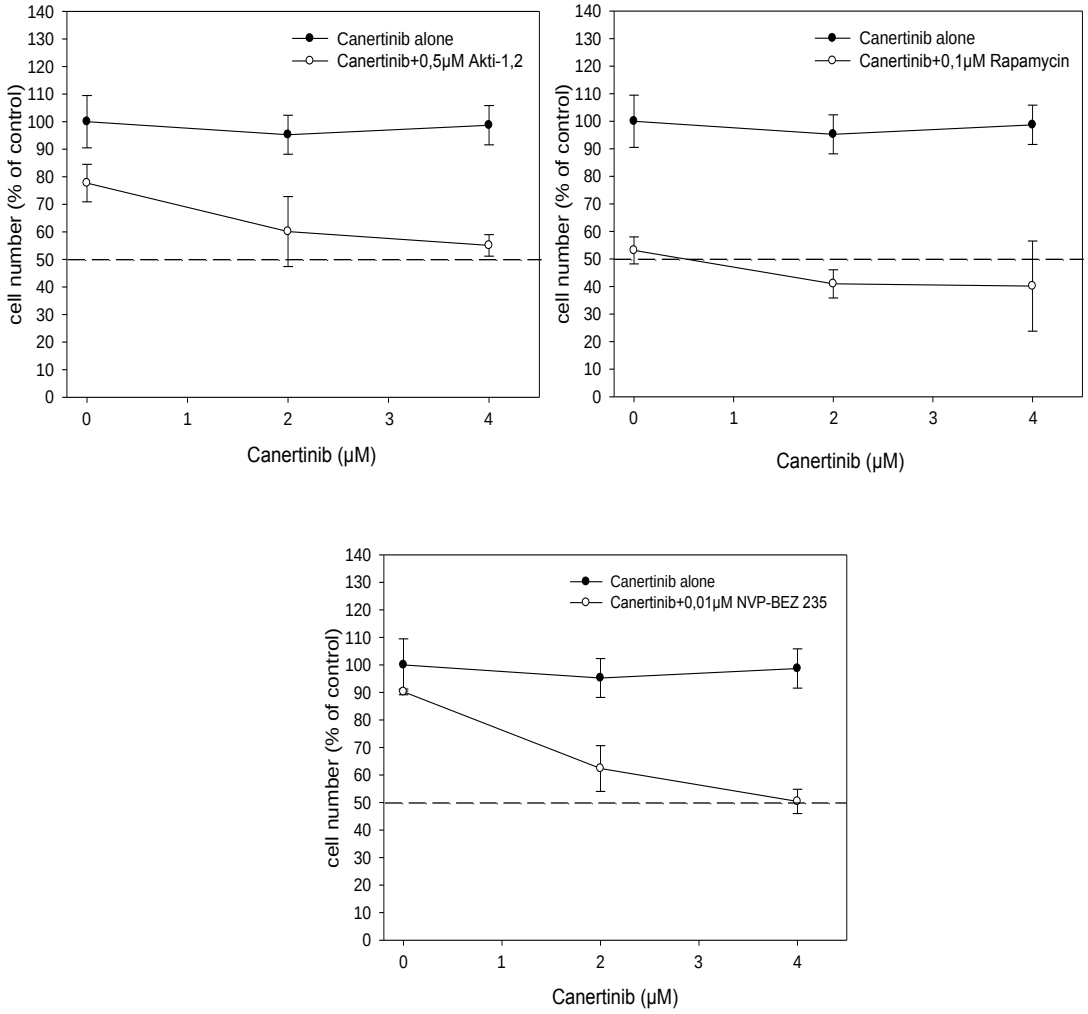


Figure 58: ***Growth curves of T47D treated with canertinib alone and in combination with PI3K inhibitors.***

This cell line was exposed to 0-4µM canertinib, 0,5µM Akti-1,2, 0,1µM rapamycin or 0,01µM NVP-BEZ 235 alone or in combination. Cell growth was analysed by EZ4U. Means ± SD, n≥3.

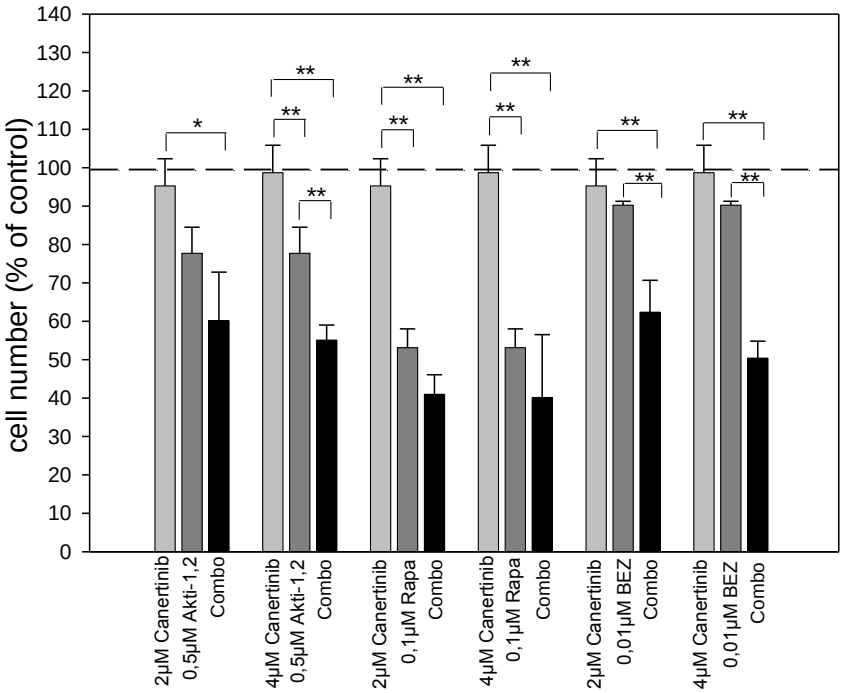


Figure 59: ***Combinational treatment of canertinib and PI3K inhibitors in T47D.*** *Bars indicate cell numbers (% of control), treatment with canertinib alone light grey, with the respective PI3K inhibitor dark grey and combinational therapy black. Significance was calculated using ANOVA followed by Scheffé-Test. * indicates significant difference at p < 0.05 and ** at p < 0.01.*

Cotreatment of ErbB inhibitor resistant T47D cells with both ErbB drugs and with the three PI3K inhibitors, respectively, significantly reduced the growth and therefore the resistance of the cells against pelitinib and canertinib. In contrast, the ErbB inhibitor sensitive cell line SKBR3 did not show such high growth inhibitory effects in combinational therapy.

3.6.3 Cotreatment of pelitinib and MAPK inhibitors

There were no growth inhibitory effects obtained in previous growth analysis done with MAPK inhibitors in SKBR3 and T47D cells. Nevertheless, we wondered, if there is any improvement, when MAPK- and ErbB drugs are

combined. Therefore, growth assay analysis with pelitinib combined with AZD6244 or U0126 combined was done.

Cotreatment of SKBR3 with pelitinib and with AZD6244 or with U0126 failed to show improved growth inhibitory effects (Figure 60 and 61). Therefore, as expected, neither MAPK inhibitor could improve the effect caused by pelitinib. Similar analyses of treatment in T47D cells, also failed increase growth inhibition by pelitinib (Figure 62 and 63).

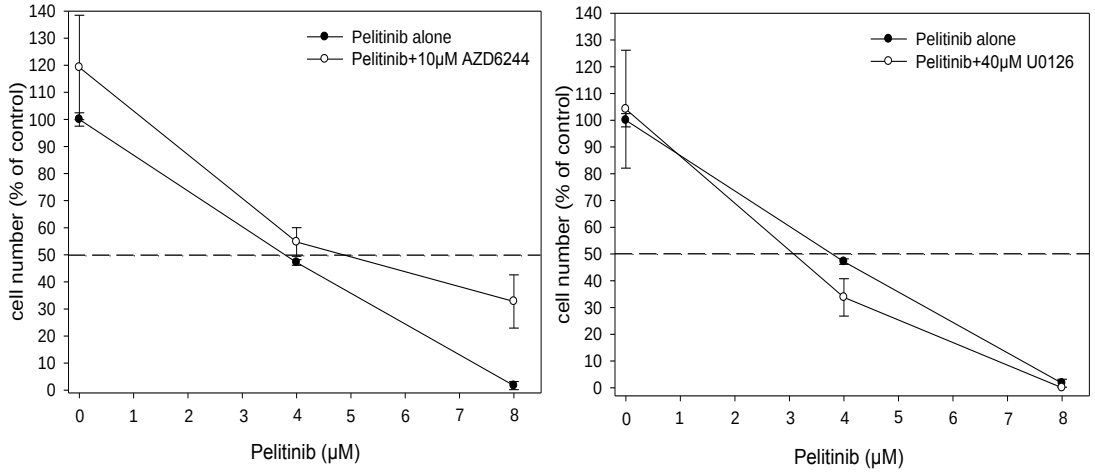


Figure 60: ***Growth curves of SKBR3 treated with pelitinib alone and in combination with two MAPK inhibitors.***

This cell line was exposed to 0-8μM pelitinib, 10μM AZD6244 or 40μM U0126 alone or in combination. Cell growth was analysed by EZ4U. Means ± SD, n≥3.

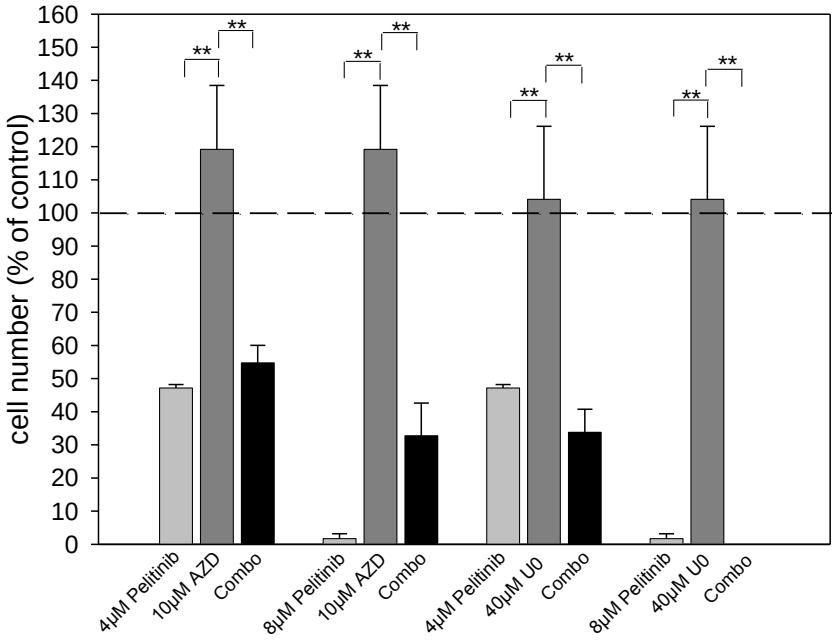


***Figure 61:* Combinational treatment of pelitinib and MAPK inhibitors in SKBR3.**

*Bars indicate cell numbers (% of control), treatment with pelitinib alone light grey, with the respective MAPK inhibitor dark grey and combinational therapy black. Significance was calculated using ANOVA followed by Scheffé-Test. **indicates significant difference at p < 0.01.*

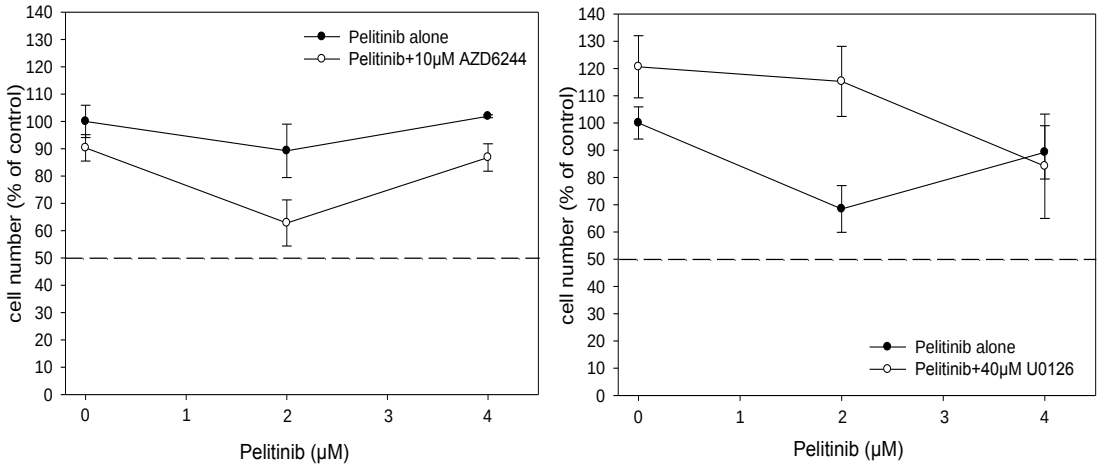


***Figure 62:* Growth curves of T47D treated with pelitinib alone and in combination with two MAPK inhibitors.**

This cell line was exposed to 0-4µM pelitinib, 10µM AZD6244 and 40µM U0126 alone or in combination. Cell growth was analysed by EZ4U. Means ± SD, n≥3.

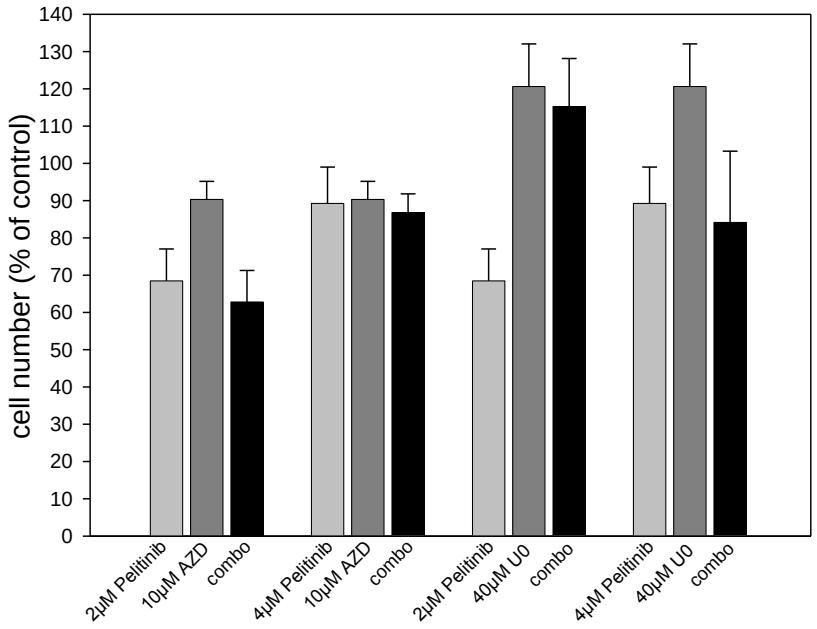


Figure 63: ***Combinational treatment of pelitinib and MAPK inhibitors in T47D cells.***

Bars indicate cell numbers (% of control), treatment with pelitinib alone light grey, with the respective MAPK inhibitor dark grey and combinational therapy black. (Significance was not calculated, because data set was not relevant) Means ± SD, n≥3.

3.6.4 Cotreatment of SKBR3 and T47D cells with canertinib and MAPK inhibitors

To determine how a combined treatment of SKBR3 and T47D with canertinib and MAPK inhibitors affects the growth of these cell lines, growth assays with these drugs were performed. As expected, this cotreatment did not show any increased growth inhibitory effects, neither with AZD6244 nor with U0126 (Figure 64 and 65) in the SKBR3 cell line. Moreover, such a combinational treatment revealed the same result in T47D cell line (Figure 66 and 67).

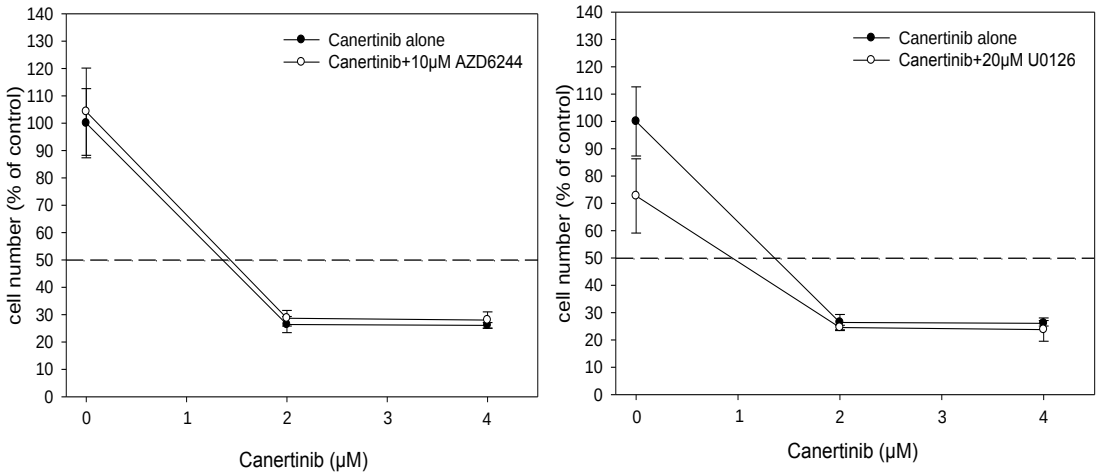


Figure 64: ***Growth curves of SKBR3 treated with canertinib alone and in combination with two MAPK inhibitors.***

This cell line was exposed to 0-4µM canertinib, 10µM AZD6244 or 20µM U0126 alone or in combination. Cell growth was analysed by EZ4U. Means ± SD, n≥3.

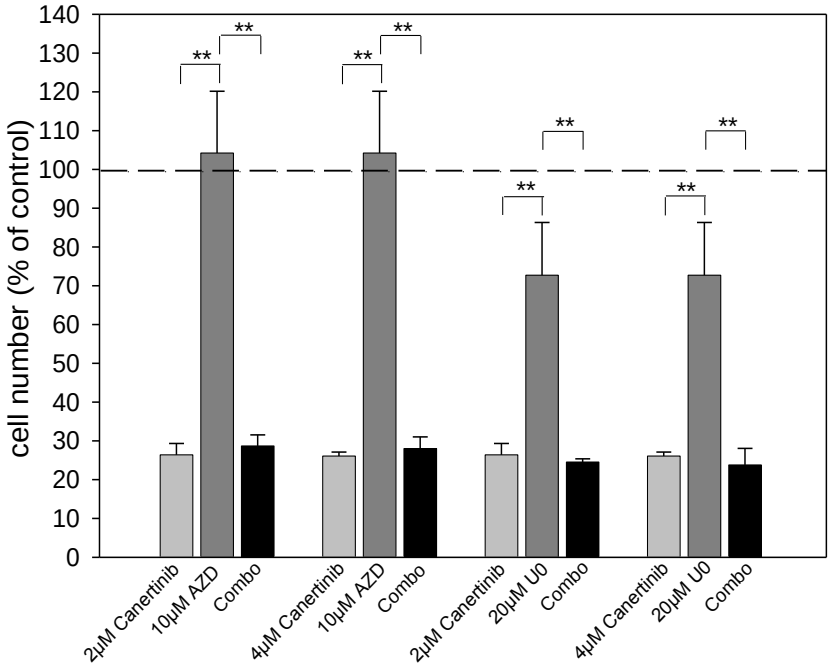


Figure 65: ***Combinational treatment of SKBR3 cells with canertinib and MAPK inhibitors.***

Bars indicate cell numbers (% of control), treatment with canertinib alone light grey,

*with the respective MAPK inhibitor dark grey and combinational therapy black. Significance was calculated using ANOVA followed by Scheffé-Test. ** indicates significant difference at p < 0.01. Means ± SD, n≥3.*

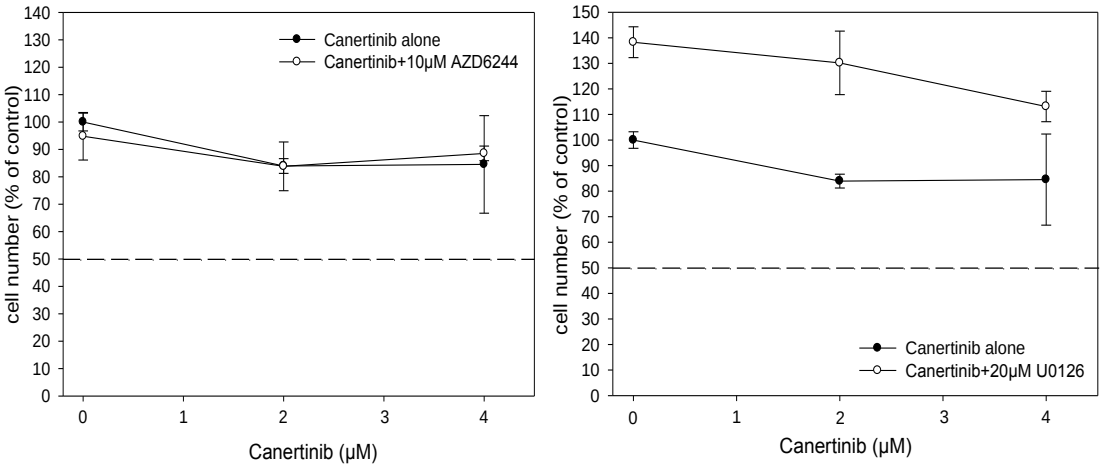


***Figure 66:* Growth curves of T47D treated with canertinib alone and in combination with two MAPK inhibitors.**

This cell line was exposed to 0-4µM canertinib, 10µM AZD6244 and 20µM U0126 alone or in combination. Cell growth was analysed by EZ4U. Means ± SD, n≥3.

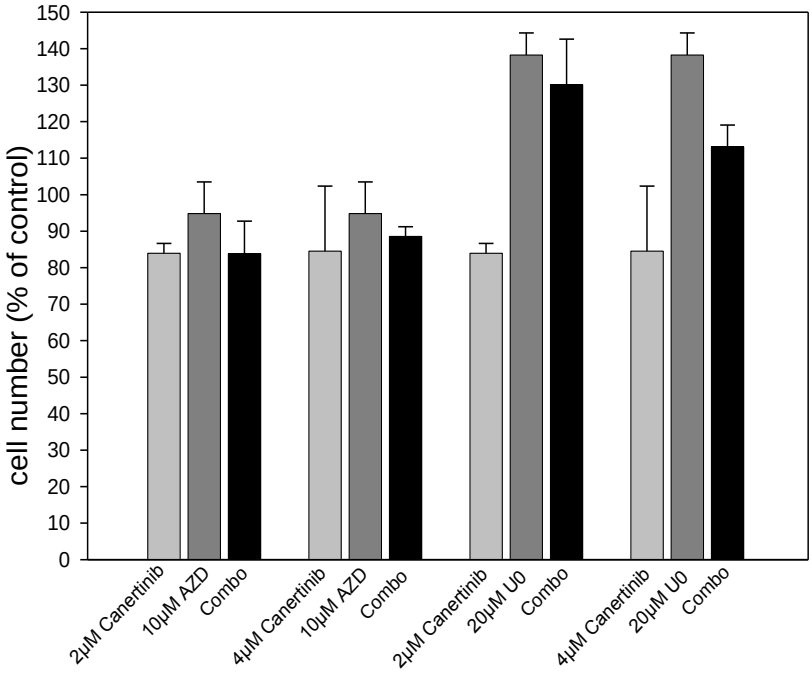


Figure 67: ***Combinational treatment of T47D cells with canertinib and MAPK inhibitors.***

Bars indicate cell numbers (% of control), treatment with canertinib alone light grey, with the respective MAPK inhibitor dark grey and combinational therapy black. (Significance was not calculated, because dataset was not relevant). Means ± SD, n≥3.

4. Discussion

Treatment of breast cancer includes surgery, radiotherapy and application of cytotoxic or cytostatic inhibitors of tumor growth. Unfortunately, however, the death rate of breast cancer has not significantly decreased in recent years. This appears to be the result of rising incidence or resistance to therapy. Therefore, development of new drugs is very important (Dickson et al., 2006). A hallmark of breast cancer is its great heterogenity. Moreover, the ErbB receptor family plays a crucial role in the development of this form of cancer. This transmembrane receptor family, which bears tyrosine kinase activity, consists of four homologous members (EGFR/ErbB1/Her1, ErbB2/Her2/neu, ErbB3/Her3 and ErbB4/Her4). EGFR is deregulated in a number of human malignancies. ErbB2 shows overexpression in 20-30% of breast cancer and correlates with an increased resistance to hormone-based therapy. Overexpression of EGFR is often associated with poor survival and chemoresistance. Therefore, EGFR has become an important target for anticancer drug development. (Karamouzis et al., 2006; Hermanto et al., 2000 and Laheru et al., 2008). The monoclonal antibody trastuzumab (Herceptin) is being used to treat patients with ErbB2 overexpressing breast cancer. It provides good clinical benefits in such patients, but patients with advanced disease, who initially responded to this antibody develop resistance against this drug (Eichhorn P. et al., 2008). Moreover, long-term treatment using reversible EGFR inhibitors such as gefitinib and erlotinib also causes resistance. Pelitinib (EKB-569) reveals retained inhibitory activity against cancers that became resistant against erlotinib and gefitinib, because it is an irreversibly acting inhibitor, which binds to the target proteins EGFR and ErbB2 by covalent interaction (Wissner et al., 2008).

In cancer a high frequency of mutations in the downstream signalling pathways of ErbB, the RAS-RAF-mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) kinase (MEK)-ERK and phosphoinositide 3-kinase (PI3K)-PTEN-AKT signaling pathway, occur. They play central roles in

the signal transduction networks, by promoting tumor initiation and tumor progression. Mutations of RAS, RAF, PI3KCA, AKT and PTEN and, moreover, amplification of AKT can be found in many human malignancies. Targeting pathways downstream, could lead to greater therapeutic efficacy than upstream inhibition, because there exists multiple upstream inputs (Mirzoeva et al., 2009).Therefore, these pathways provide a number of promising molecular targets for cancer therapy. Hence, a new generation of PI3K inhibitors is emerging, which hopefully can overcome the problems of ErbB drug resistance. For example, NVP-BEZ 235, a dual mTOR and PI3K inhibitor, or Akti-1,2, a selective AKT1 and AKT2 inhibitor, show good inhibitory effects in breast cancers, which are depending on the PI3K pathway (Serra et al., 2008; She et al., 2008). Moreover, there are also mitogen activated protein kinase pathway inhibitors in use. Oncogenic mutations in RAS lead to deregulation of several effector pathways that control cell proliferation and survivial. Deregulation of MAPK pathway often occurs through KRAS or BRAF mutations. These findings validate this pathway as a therapeutic target in breast cancer, but treatment with MEK inhibitors have shown only limited antitumor activity. A possible reason for that could be that the PI3K pathway activation strongly influences the sensitivity of RAS mutant cells to MEK inhibitors. Activating mutations in PIK3CA reduce the sensitivity of MEK inhibition whereas PTEN mutations cause complete resistance, which could be avoided by combined blockade of the MAPK and the PI3K pathway (Wee et al., 2009 and Mirzoeva et al., 2009).

4.1. Responsiveness of different breast cancer cell lines to ErbB inhibition

In a previous study (Brünner-Kubath et al., in press) we have shown that the in vitro growth of SKBR3 breast cancer cells can be blocked by exposure to the irreversible EGFR/ErbB2 inhbitor pelitinib, whereas T47D cells were found to be resistant to this drug. To examine how the growth of other breast cancer cell

lines responds to a treatment with pelitinib, a panel of cancer cells including T47D and SKBR3 was treated with this drug. Concordant to the results achieved in previous assays, T47D cells displayed distinct higher resistance than SKBR3 cells. Moreover, we found additional pelitinib resistant (MDA-MB 231 and MCF7) and sensitive (BT474) breast cancer cell lines. The two pelitinib sensitive cell lines reveal a high ErbB2 expression level which is not displayed by the other tested cell lines and therefore a possible reason for a higher sensitivity (Brünner-Kubath et al., in press). On the other hand although ErbB2 is overexpressed in 20-30% of invasive breast carcinomas, the monoclonal antibody trastuzumab which specifically targets the extracellular domain of HER2 reveal only limited efficiency because of primary resistance or acquired resistance. Moreover, ErbB1, -3 or -4 do not correlate with sensitivity against pelitinib of these cell lines (Brünner-Kubath et al., in press; Eichhorn P. et al, 2008). Downstream signalling effects of pelitinib revealed that the pelitinib resistant cell line MCF7 responds with increasing levels of pAKT and pERK1/2. Maybe this cell line responds in the same way as T47D cells to the inhibitor, because both exhibit a mutation in the catalytic subunit p110α of the PI3K (PIK3CA). PI3K signals through AKT and other downstream effectors and represents a key mechanism of transducing proliferative and antiapoptotic signals. This pathway is activated in many breast cancer cell lines and, moreover, activating mutations in the catalytic subunit of p110-α occur in 25-30% of breast tumors, alike in our tested cell lines T47D which harbour a H1047R and MCF7 revealing a E545K mutation. Conversely, BT474 cells respond very sensitive to pelitinib, although they harbour a K111N mutation in PIK3CA. However, BT474 cells also show an ErbB2/Her2 amplification, whereas T47D express low levels of ErbB2 (Hoeflich et al., 2009 and She et al., 2008). Moreover, BT474 is the only tested cell line harbouring both, PIK3CA mutation and overexpression of ErbB2. She et al.,(2008) found out that activation of the PI3K/AKT pathway through ErbB2 signalling plays an important role in mediation the transformed phenotype in breast cancer with ErbB2

overexpression. Interestingly, MDA-MB-231 cells, which do not have a PIK3CA mutation, show extremely low levels of phosphorylated AKT in the untreated control. Therefore, we hypothesize that the MAPK pathway could be more important in this cell line, because they reveal a high ERK1/2 phoshporylation level in the untreated control and, moreover, they harbour a KRAS mutation (Hoeflich et al., 2009). But growth analysis with MEK1/2 inhibitors revealed no significant response to the growth inhibitory effects of these drugs. Therefore, our hypothesis could not be confirmed.

4.2. Fundamental ErbB downstream signalling pathway of SKBR3 and T47D

4.2.1. Examination of the growth inhibitory effects of PI3K and MAPK pathway inhibitors

Previous findings from Brünner-Kubath et.al (in press) showed that pelitinib resistance is associated with drug-refractory phosphorylation (activation) of AKT, whereas the phosphorylation of the MAPKs Erk1/2 does not correlate with pelitinib resistance or sensitivity. Both, the PI3K and the MAPK systems are major ErbB downstream signalling pathways. Treatment of SKBR3 and T47D with an mTOR inhibitor (rapamycin) exerted a higher growth inhibitory effect on T47D relative to SKBR3 cells. This may be caused by an activating mutation in the catalytic p110α subunit of PI3K (PIK3CA) in T47D, whereas SKBR3 cells express wild type PIK3CA (Crowder et al., 2009; She et al., 2008). mTOR signals through two distinct complexes. The first one is mTORC1, also called RAPTOR, which is composed of regulatory- associated protein of mTOR (RAPTOR), mLST8 also known as GβL and the proline-rich AKT substrate (PRAS). RAPTOR positively regulates mTOR, but PRAS acts as an inhibitor of MTR kinase activity in a phosphorylation-dependent manner. The PI3K and MAPK signalling pathways regulate the mTORC1 function through tuberous

sclerosis complex 2 (TSC2). The second one is mTORC2, also called RICTOR, which is composed of mLST8, rapamycin-insensitive companion of mTOR (RICTOR) and SIN1 or RICTOR. RICTOR leads to the full activation of the AKT kinase through phosphorylation of Ser(473). Notably, rapamycin inhibits only mTORC1, but not mTORC2 (Carracedo et al., 2008). Therefore a dual inhibitor of mTOR and PI3K called NVP-BEZ 235 was used. Antiproliferative effects of NVP-BEZ 235, which blocks mTORC1, mTORC2 and PI3K (Serra et al., 2008), were analysed in growth asssays. This inhibitor acted as a strong growth inhibitor in both cell lines, especially in T47D cells. These results go along with the finding of Brachman et al., (2009), who showed that a treatment of cell lines with PIK3CA mutation or ErbB2 amplification with NVP-BEZ 235 leads to induced cell death. In contrast, the drug is inefficient in inhibiting growth of cells, which harbour KRAS mutations or loss of PTEN function. Our two cell lines show PIK3CA mutation (T47D) or Her2 amplification (SKBR3), but neither KRAS nor PTEN loss (Hoeflich et al., 2009 and She et al., 2008).

Growth assay analysis with a special AKT inhibitor called Akti-1,2 show good inhibitory effects in both cell lines, but in T47D it was less efficient than NVP-BEZ 235 or rapamycin. This result goes along with the finding of Vasudevan et al. (2009), where they uncovered an AKT-independent signal, which is active in many cancer cell lines possessing a PIK3CA mutation, so that AKT inhibition does not have any growth inhibitory effect. Moreover, they suggested that an inhibition of PI3K or downstream effectors could be more effective than an inhibition of AKT. Usage of theses different PI3K inhibitors showed that the PI3K pathway must be important for both cell lines, because they respond sensitive to their exposure. Treatment of these two cell lines with MEK1,2 inhibitors showed no growth inhibitory effects, neither on SKBR3 nor on T47D cells. Mirzoeva et al. (2009) demonstrated that MEK1,2 inhibitors exhibit only limited antitumor activity in luminal breast cancer cell lines like T47D and SKBR3. Moreover, they suggested that luminal breast cancer cell lines depend

more on the PI3K than on the MAPK-pathway, which is supported by the preferential occurrence of PI3K mutations in this subtype of breast cancer cell lines. Furthermore, they found that the PI3K pathway becomes activated in response to MEK inhibition, through a negative MEK- EGFR- PI3K feedback loop, which therefore limits the efficiency of the MEK1,2 inhibitors.

4.2.2. Examination of the downstream signalling effects of PI3K and MAPK pathway inhibitors

Our results achieved in growth assay analysis with SKBR3 and T47D indicate that the PI3K pathway seems to play more important role in growth regulation of both cell lines than the MAPK pathway. We then wanted to examine not only the response of growth inhibition, but we also wanted to get a more precise information on how these inhibitors exactly do affect the two ErbB downstream signalling pathways. As expected the mTOR inhibitor rapamycin caused an immediate reduction of the S6 phosphorylation level in SKBR3 and T47D cells, because S6 is located downstream of mTOR. But this inhibitor showed minor inhibitory effects of the PI3K and MAPK pathway, except at high drug concentrations. This finding goes along with a study from Carracedo et al. (2008), who found that there exists an mTORC1-MAPK feedback loop. Normally, mTORC1 activation leads to PI3K and MAPK inhibition through a negative feedback loop stemming from S6K1 (p70 ribosomal protein S6 kinase 1). Consequently, rapamycin-mediated inhibition of mTORC1 results in hyperactivation of PI3K, and also activates the MAPK pathway. This finding explains the increasing level of phosphorylated AKT and ERK1,2 seen in our Western blotting analysis after rapamycin treatment. It is known that selective inhibition of mTOR can abrogate a negative feedback loop, which usually keeps phosphorylation of AKT in check, thereby causing a paradoxic upregulation of pAKT (Thr308) levels (Mirzoeva et al., 2009).

The dual PI3K and mTOR inhibitor NVP-BEZ 235 inhibits class 1 PI3K catalytic activity by competing at its ATP-binding site and, compared to rapamycin, which inhibits only mTORC1, this drug shows inhibitory effects on both, mTORC1 and mTORC2 (Serra et al., 2008; Carracedo et al., 2008). Our findings interestingly showed that low concentrations of NVP-BEZ 235 inhibited only pAKT (Ser473), but not pAKT (Thr308), in T47D cell lines. This result could be explained by a differentially phosphorylation of them. AKT (Ser473) becomes directly phosphorylated by mTORC2, but in contrast, AKT (Thr308) becomes phosphorylated through PI3K, in which PDK1 is an essential link (Qian Yang et al., 2006). Moreover, NVP-BEZ-235 shows a higher affinity to mTOR than to PI3K (Serra et al., 2008). Therefore mTOR becomes inhibited at low drug concentrations, but not PI3K. Low drug concentrations inhibit mTORC2, which lead to an inhibition of the phosphorylation of AKT (Ser473). But, the blockade of mTORC1, through NVP-BEZ 235, decreases mTOR-dependent negative feedback-regulation of PI3K and therefore of PDK1, thus stimulates the phosphorylation of AKT (Thr308) (Huang et al., 2009). On the other hand, high inhibitor concentrations result in downregulation of both, AKT (Ser473) and AKT (Thr308) phosphorylation, because NVP-BEZ 235 inhibits both mTOR and PI3K. Constitutive phosphorylation of S6 ribosomal protein is quite high in control cells and was decreased by the drug at the lowest inhibitor concentration in SKBR3 and at a slightly higher drug concentration in T47D cells. This is compatible with the notion that mTOR inhibition occurs at lower drug concentrations, whereas both mTOR and PI3K become inhibited at higher concentrations of NVP-BEZ 235, because S6 ribosomal protein is located downstream of mTOR (Serra et al.,2008). Moreover, NVP-BEZ 235 leads to an increase of the phosphorylation level of ERK1,2. This finding goes along with results we achieved with rapamycin, where we suggested that there exists, a negative mTORC1-MAPK feedback loop. Inhibition of mTORC1 through NVP-BEZ 235 and rapamycin results in an activation of the MAPK pathway, because mTORC1 normally leads to MAPK inhibition through a negative feedback loop

based from S6K1. But inhibition of mTOR leads to an increase of this pathway (Mirzoeva et al., 2009). Direct inhibition of AKT through Akti-1,2 in SKBR3 and T47D cells is not as efficient as the other pathway inhibitors used (rapamycin and NVP-BEZ 235). Previous studies showed that breast cancer cell lines, which possess a PIK3CA mutation, exhibit only minimal AKT activation. Therefore, a diminished reliance on AKT for anchorage independent growth was found. These cells seem to use a signalling pathway that is independent of AKT. Therefore, inhibition with Akti-1,2 does not lead to a significant inhibitory effect at low drug concentrations (Vasudevan et al., 2009).

Our growth assay analysis done before showed no growth inhibitory effect of MEK1,2 inhibitors in SKBR3 and T47D cells. We therefore wanted to examine if these inhibitors actually do inhibit the MAPK cascade in these cells and performed Western blot analyses in cells exposed to the MEK1,2 drugs AZD6244, U0126 or AS703026. These inhibitors showed good inhibition of phosphorylated ERK1,2 but no significant inhibitory effects on MEK1,2 phosphorylation. This finding suggests that these three inhibitors act downstream of MEK by inhibiting phosphorylated ERK1,2. Moreover, these results together with these obtained from growth assays indicate that MEK1,2 inhibitors are not adequate for inhibition of growth of these cell lines. Furthermore, these results showed that the MAPK pathway does not seem to be important for growth regulation in both cell lines and, therefore, inhibition of only this pathway is not enough to overcome the resistance of T47D cells against pelitinib. Furthermore Hoeflich et al., (2009) underline the notion that MEK inhibition in luminal breast cancer cell lines (SKBR3 and T47D) does not have any significant growth inhibitory effect, despite the fact that they also achieved a strong reduction of the pERK1,2 level in luminal breast cancer cells.
All our data achieved in growth assay and Western blotting analysis with PI3K and MAPK pathway inhibitors suggested that the PI3K pathway plays a more important role in growth regulation of SKBR3 and T47D cells. Therefore,

inhibition of this pathway could be a good approach to overcome the resistance of T47D cells against the ErbB inhibitor pelitinib.

4.3. Combined therapy in SKBR3 and T47D cell lines

Here we demonstrated that treatment of SKBR3 and T47D with PI3K inhibitors leads to good growth inhibitory effects, especially in the pelitinib resistant cell line T47D. Therefore, we wondered which effect a cotreatment using the ErbB inhibitors pelitinib or canertinib together with the three different PI3K inhibitors may exert on the growth of SKBR3 and T47D cells. Canertinib as a second ErbB inhibitor was used to see how these cell lines respond to another drug than pelitinib and, moreover, because it is in clinical trials. Combinational therapy of SKBR3 cell lines with PI3K inhibitors and pelitinib showed a good elevation of the cellular sensitivity against pelitinib. Treatment with pelitinib together with rapamycin or with NVP-BEZ 235 showed high significant growth inhibition compared to pelitinib single treatment. Single treatment with the ErbB inhibitor canertinib leads to very good growth inhibition, but only treatment with rapamycin showed significantly elevated effect.

Cotreatment of T47D cells with pelitinib and rapamycin revealed a highly significant growth inhibition compared to exposure to pelitinib alone. Therapy with this ErbB inhibitor and Akti-1,2 or with NVP-BEZ 235 revealed a significant growth reduction. As expected, single therapy of T47D cells with canertinib was inefficient. However, elevated sensitivity was observed by combined exposure of the cells to canertinib and Akti-1,2, rapamycin or NVP-BEZ 235. These observations revealed that rapamycin, Akti-1,2 and NVP-BEZ 235 increased the antiproliferative effects of pelitinib and canertinib in ErbB resistant T47D cells. This effects seems to be dependent on inhibition of mTOR, because rapamycin, which inhibits mTOR shows growth inhibitory effects like other drugs inhibiting PI3K and mTOR (NVP-BEZ 235) or Akt (Akti-1,2). Treatment with ErbB and PI3K inhibitors is very effective and, moreover, able to overcome the resistance

of T47D against the two ErbB inhibitors. In contrast cotreatment with ErbB inhibitors and MEK1,2 inhibitors does not improve the growth inhibitory effect. Hoeflich et al., (2009) found that basal like breast cancer cell lines, which lack expression of ErbB2/Her2, respond sensitive to the inhibitory effects of MEK inhibition, in contrast to luminal breast cancer cell lines. They also found that the tumor suppressor PTEN is a negative predictive factor for response to MEK inhibition. Moreover, they showed that the combination of MEK and PI3K inhibitors lead to enhanced efficacy. Our tested cell lines are of luminal origin and showed that the combined therapy with ErbB2 and PI3K inhibitors yield strong growth inhibitory effects and, moreover, efficiently overcomes cellular resistance against ErbB-inhibitory drugs.

5 References

Ahmadian S, Barar J, Saei AA, Fakhree MA, Omidi Y. Cellular toxicity of nanogenomedicine in MCF-7 cell line: MTT assay. J Vis Exp (2009) 10:1191

Anido J, Matar P, Albanell J, Guzman M, Rojo F, Arribas J, Averbuch S, Baselga J. ZD1839, a specific epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor, induces the formation of inactive EGFR/HER2 and EGFR/HER3 heterodimers and prevents heregulin signaling in HER2-overexpressing breast cancer cells. Clin Cancer Res (2003) 9:1274-83

Aurora A, Scholar EM. Role of tyrosine kinase inhibitors in cancer therapy. J Pharmacol Exp Ther (2005) 315:971-9

Benson JR, Jatoi I, Keisch M, Esteva FJ, Makris A, Jordan VC. Early breast cancer. Lancet (2009) 373:1463-79

Bertram JS. The molecular biology of cancer. Mol Aspects Med. Review (2000) 21 (6):167-223

Biscardi JS, Ishizawar RC, Silva CM, Parsons SJ. Tyrosine kinase signalling in breast cancer: epidermal growth factor receptor and c-Src interactions in breast cancer. Breast Cancer Res (2000) 2(3):203-10

Bjerkvig R, Tysnes BB, Aboody KS, Najbauer J, Terzis AJ. The origin of the cancer stem cell: current controversies and new insights. Nat Rev Caner (2005) 5(11):899-904

Brachmann SM, Hofmann I, Schnell C, Fritsch C, Wee S, Lane H, Wang S, Garcia-Echeverria C, Maira SM. Specific apoptosis induction by the dual PI3K/mTor inhibitor NVP-BEZ235 in HER2 amplified and PIK3CA mutant breast cancer cells. Proc Natl Acad Sci U S A (2009) 106(52):22299-304

Brünner-Kubath C, Shabbir W, Saferding V, Wagner R, Singer CF, Valent P, Berger W, Marian B, Zielinski CC, Grusch M, Grunt TW. The PI3 Kinase/mTOR Blocker NVP-BEZ235 Overrides Resistance against Irreversible ErbB Inhibitors in Cancer Cells. Breast Cancer Res Treatm (2010) in press

Burnette WN. Western blotting: electrophoretic transfer of proteins from sodium dodecyl sulfate -polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. Anal. Biochem (1981) Bd. 112 [195–203]

Carracedo A, Ma L, Teruya-Feldstein J, Rojo F, Salmena L, Alimonti A, Egia A, Sasaki AT, Thomas G, Kozma SC, Papa A, Nardella C, Cantley LC, Baselga J, Pandolfi PP. Inhibition of mTORC1 leads to MAPK pathway activation through a PI3K-dependent feedback loop in human cancer. J Clin Invest (2008) 118(9):3065-74

Citri A, Yarden Y. EGF-ERBB signalling: towards the systems level. Nat Rev Mol Cell Biol (2006) 7: 505 516

Crowder RJ, Phommaly C, Tao Y, Hoog J, Luo J, Perou CM, Parker JS, Miller MA, Huntsman DG, Lin L, Snider J, Davies SR, Olson JA Jr, Watson MA, Saporita A, Weber JD, Ellis MJ. PIK3CA and PIK3CB inhibition produce synthetic lethality when combined with estrogen deprivation in estrogen receptor-positive breast cancer. Cancer Res (2009) 69(9):3955-62

Davis BJ. "Disc Electrophoresis. 2, Method and application to human serum proteins". Ann. New York Acad. Sci (1964) 121: 404–427

Dickson RB, Johnson MD, Maemura M, Low J. Anti-invasion drugs Breast Cancer Res Treat (1996) 38(1):121-32

Eichhorn PJ, Gili M, Scaltriti M, Serra V, Guzman M, Nijkamp W, Beijersbergen RL, Valero V, Seoane J, Bernards R, Baselga J. Phosphatidylinositol 3-kinase hyperactivation results in lapatinib resistance that is reversed by the mTOR/phosphatidylinositol 3-kinase inhibitor NVP-BEZ235. Cancer Res (2008) 68(22):9221-30

Eifel P. National Institutes of Health Consensus Development Conference Statement: adjuvant therapy for breast cancer. J Natl Cancer Inst (2001) 93(13):979-89

Evan GI, Vousden KH. Proliferation, cell cycle and apoptosis in cancer. Nature Review (2001) 411(6835):342-8

Favata MF, Horiuchi KY, Manos EJ, Daulerio AJ, Stradley DA, Feeser WS, Van Dyk DE, Pitts WJ, Earl RA, Hobbs F, Copeland RA, Magolda RL, Scherle PA, Trzaskos JM. Identification of a novel inhibitor of mitogen-activated protein kinase kinase. J Biol Chem (1998) 73(29):18623-32

Grunt TW, Wagner R, Grusch M, Berger W, Singer CF, Marian B, Zielinski CC, Lupu R. Interaction between fatty acid synthase- and ErbBsystems in ovarian cancer cells. Biochem Biophys Res Commun (2009) 385(3):454-9

Hanahan D, Weinberg RA. The hallmarks of cancer. Cell Review (2000) 100(1):57-70

Hankinson SE, Colditz GA, Willett WC. Towards an integrated model for breast cancer etiology: the lifelong interplay of genes, lifestyle, and hormones. Breast Cancer Res (2004) 6(5):213-8

Hawkes R. A dot-immunobinding assay for monoclonal and other antibodies. Anal Biochem (1982) 119(1):142-7

Hermanto U, Uong CS, Wang L. Inhibition of Mitogen-activated Protein Kinase Kinase Selectively Inhibits Cell Proliferation in Human Breast Cancer Cells Displaying Enhanced Insulin-like Growth Factor I-mediated Mitogen-activated Protein Kinase Activation. Cell Growth and Differentiation (2000) 11(12):655-64

Hoeflich KP, O'Brien C, Boyd Z, Cavet G, Guerrero S, Jung K, Januario T, Savage H, Punnoose E, Truong T, Zhou W, Berry L, Murray L, Amler L, Belvin M, Friedman LS, Lackner MR. In vivo antitumor activity of MEK and phosphatidylinositol 3-kinase inhibitors in basal-like breast cancer models. Clin Cancer Res (2009) 15(14):4649-64

Holbro T, Hynes NE. ErbB receptors: directing key signaling networks throughout life. Annu Rev Pharmacol Toxcol (2004) 44:195-217

Holmberg L. Increased risk of recurrence after hormone replacement therapy in breast cancer survivors. J Natl Cancer Inst (2008) 100(7):475-82

Huang J, Wu S, Wu CL, Manning BD. Signaling events downstream of mammalian target of rapamycin complex 2 are attenuated in cells and tumors deficient for the tuberous sclerosis complex tumor suppressors. Cancer Res (2009) 69(15):6107-14

Karamouzis MV, Badra FA, Papavassiliou AG. Breast cancer: the upgraded role of HER-3 and HER-4. Int J Biochem Cell Biol (2007) 39(5):851-6

Knuefermann C, Lu Y, Liu B, Jin W, Liang K, Wu L, Schmidt M, Mills GB, Mendelsohn J, Fan Z. HER2/PI-3K/Akt activation leads to a multidrug resistance in human breast adenocarcinoma cells. Oncogene (2003) 22(21):3205-12

Korc M, Chandrasekar B, Yamanaka Y, Friess H, Buchier M, Beger HG. Overexpression of the epidermal growth factor receptor in human pancreatic cancer is associated with concomitant increases in the levels of epidermal growth factor and transforming growth factor alpha. J Clin Invest (1992) 90(4):1352-60

Laheru D, Croghan G, Bukowski R, Rudek M, Messersmith W, Erlichman C, Pelley R, Jimeno A, Donehower R, Boni J, Abbas R, Martins P, Zacharchuk C, Hidalgo M. A phase I study of EKB-569 in combination with capecitabine in patients with advanced colorectal cancer. Clin Cancer Res (2008) 14(17):5602-9

Liu Y, Peterson DA, Kimura H, Schubert D. Mechanism of cellular 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction. J Neurochem (1997) 69(2):581-93

LoPiccolo J, Granville CA, Gills JJ, Dennis PA. Targeting Akt in cancer therapy. Anticancer Drugs Review (2007) 18(8):861-74

Maira SM, Stauffer F, Brueggen J, Furet P, Schnell C, Fritsch C, Brachmann S, Chène P, De Pover A, Schoemaker K, Fabbro D, Gabriel D, Simonen M, Murphy L, Finan P, Sellers W, García-Echeverría C. Identification and characterization of NVP-BEZ235, a new orally available dual

phosphatidylinositol 3-kinase/mammalian target of rapamycin inhibitor with potent in vivo antitumor activity. Mol Cancer Ther (2008) 7:1851-63

Mirzoeva OK, Das D, Heiser LM, Bhattacharya S, Siwak D, Gendelman R, Bayani N, Wang NJ, Neve RM, Guan Y, Hu Z, Knight Z, Feiler HS, Gascard P, Parvin B, Spellman PT, Shokat KM, Wyrobek AJ, Bissell MJ, McCormick F, Kuo WL, Mills GB, Gray JW, Korn WM. Basal subtype and MAPK/ERK kinase (MEK)-phosphoinositide 3-kinase feedback signaling determine susceptibility of breast cancer cells to MEK inhibition. Cancer Res (2009) 69(2):565-72

Moasser MM, Basso A, Averbuch SD, Rosen N. The tyrosine kinase inhibitor ZD1839 (Iressa) inhibits HER2-driven signaling and suppresses the growth of HER2-overexpressing tumor cells. Cancer Res (2001) 61:7184 7188

Mondesire WH, Jian W, Zhang H, Ensor J, Hung MC, Mills GB, Meric-Bernstam F. Targeting mammalian target of rapamycin synergistically enhances chemotherapy-induced cytotoxicity in breast cancer cells Clin Cancer Res (2004) 10:7031-42

Nahta R, Yuan LX, Zhang B, Kobayashi R, Esteva FJ. Insulin-like growth factor-I receptor/human epidermal growth factor receptor 2 heterodimerization contributes to trastuzumab resistance of breast cancer cells. Cancer Res (2008) 68(22):956

Nicholson KM, Anderson NG. The protein kinase B/AKT signalling pathway in human malignancy. Cell Signal (2002) 14(5):381-395

Olayioye MA, Neve RM, Lane HA, Hynes NE. The ErbB signaling network: receptor heterodimerization in development and cancer. EMBO J Review (2000) 19(13):3159-67

Ornstein L. "DISC ELECTROPHORESIS. I. BACKGROUND AND THEORY." Ann N Y Acad Sci (1964) 121: 321–349

Paul D. Lewis.R for Medicine and Biology (Jones and Bartlett Series in Biomedical Informatics) ISBN 978-0-7637-5808-0 (2010)

Porter PL. "Westernizing" women's risks? Breast cancer in lower-income countries. N Engl J Med (2008) 358(3):213-6

Renshaw JS, Patnaik A, Gordon M, Beeram M, Fischer D, Gianella-Borradori A, Lin C, Mendelson D. A phase I two arm trial of AS703569 (R763), an orally available aurora kinase inhibitor, in subjects with solid tumors: preliminary results. Journal of Clinical Oncology (2007) 14130

Rexer BN, Ghosh R, Arteaga CL. Inhibition of PI3K and MEK: it is all about combinations and biomarkers. Clin Cancer Res (2009) 15(14):4518-20
Robson M, Offit K. Clinical practice. Management of an inherited predisposition to breast cancer. N Engl J Med (2007) 357(2):154-62

Rudloff U, Samuels Y. A growing family: Adding mutated Erbb4 as a novel cancer target. Cell Cycle (2010) 9(8)

Schaefer G, Shao L, Totpal K, Akita RW. Erlotinib directly inhibits HER2 kinase activation and downstream signaling events in intact cells lacking epidermal growth factor receptor expression. Cancer Res (2007) 67(3):1228-38

Scudiero DA, Shoemaker RH, Paull KD, Monks A, Tierney S, Nofziger TH, Currens MJ, Seniff D, Boyd MR. Evaluation of a soluble tetrazolium/formazan assay for cell growth and drug sensitivity in culture using human and other tumor cell lines. Cancer Res (1988) 48(17):4827-33

Serra V, Markman B, Scaltriti M, Eichhorn PJ, Valero V, Guzman M, Botero ML, Llonch E, Atzori F, Di Cosimo S, Maira M, Garcia-Echeverria C, Parra JL, Arribas J, Baselga J. NVP-BEZ235, a dual PI3K/mTOR inhibitor, prevents PI3K signaling and inhibits the growth of cancer cells with activating PI3K mutations. Cancer Res (2008) 68(19):8022-30

She QB, Chandarlapaty S, Ye Q, Lobo J, Haskell KM, Leander KR, DeFeo-Jones D, Huber HE, Rosen N. Breast tumor cells with PI3K mutation or HER2 amplification are selectively addicted to Akt signaling. PLoS One (2008) 3(8):3065

Sun SY, Rosenberg LM, Wang X, Zhou Z, Yue P, Fu H, Khuri FR. Activation of Akt and eIF4E survival pathways by rapamycin-mediated mammalian target of rapamycin inhibition. Cancer Res (2005) 65(16):7052-8

Sundvall M, Iljin K, Kilpinen S, Sara H, Kallioniemi O-P, Elenius K. Role of ErbB4 in Breast Cancer. J Mammary Gland Biol Neoplasia (2008) 13:259 268

Testa JR, Bellacosa A. AKT plays a central role in tumourigenesis. Proc Natl Acad Sci USA (2001) 98:10983-10985

Valabrega G, Montemurro F, Aglietta M. Trastuzumab: mechanism of action, resistance and future perspectives in HER2-overexpressing breast cancer. Ann Oncol (2007) 18(6):977-84

Vasudevan KM, Barbie DA, Davies MA, Rabinovsky R, McNear CJ, Kim JJ, Hennessy BT, Tseng H, Pochanard P, Kim SY, Dunn IF, Schinzel AC, Sandy P, Hoersch S, Sheng Q, Gupta PB, Boehm JS, Reiling JH, Silver S, Lu Y, Stemke-Hale K, Dutta B, Joy C, Sahin AA, Gonzalez-Angulo AM, Lluch A, Rameh LE, Jacks T, Root DE, Lander ES, Mills GB, Hahn WC, Sellers WR, Garraway LA. AKT-independent signaling downstream of oncogenic PIK3CA mutations in human cancer. Cancer Cell (2009) 16(1):21-32

Veitch NC. "Horseradish peroxidase: a modern view of a classic enzyme". Phytochemistry (2004) 65 (3): 249–259

Whyte J, Bergin O, Bianchi A, McNally S, Martin F. Key signalling nodes in mammary gland development and cancer. Mitogen-activated protein kinase signalling in experimental models of breast cancer progression and in mammary gland development. Breast Cancer Res (2009) 11(5):209

Wee S, Jagani Z, Xiang KX, Loo A, Dorsch M, Yao YM, Sellers WR, Lengauer C, Stegmeier F. PI3K pathway activation mediates resistance to MEK inhibitors in KRAS mutant cancers. Cancer Res (2009) 69(10):4286-93

Wissner A, Mansour TS. The development of HKI-272 and related compounds for the treatment of cancer. Review Arch Pharm (2008) 341:465-77
Yang Q, Inoki K, Ikenoue T, Guan KL. Identification of Sin1 as an essential TORC2 component required for complex formation and kinase activity. Genes Dev (2006) 20(20):2820-32

Yarden Y, Sliwkowski MX. Untangling the ErbB signaling network. Nat Rev Mol Cell Biol (2001) 2(2):127 37

Yeh TC, Marsh V, Bernat BA, Ballard J, Colwell H, Evans RJ, Parry J, Smith D, Brandhuber BJ, Gross S, Marlow A, Hurley B, Lyssikatos J, Lee PA, Winkler JD, Koch K, Wallace E. Biological characterization of ARRY-142886 (AZD6244), a potent, highly selective mitogen-activated protein kinase kinase 1/2 inhibitor. Clin Cancer Res (2007) 13(5):1576-83

Zhang SM, Lee IM, Manson JE, Cook NR, Willett WC, Buring JE. Alcohol consumption and breast cancer risk in the Women's Health Study. Am J Epidemiol (2007) 165(6):667-76

References to websites as of October 2010:

(1) http://kirschner.med.harvard.edu/files/html/research.shtml
(2) http://www.cell signalling.com
(3) http://knol.google.com/k/protein-kinase-b#
(4) http://www.lgcstandards-atcc.org/Attachments/1975.jpg
(5) http://www.lgcstandards-atcc.org/Attachments/1983.jpg
(6) http://icbp.lbl.gov/breastcancer/viewline.php?id=40
(7) http://icbp.lbl.gov/breastcancer/viewline.php?id=6
(8) https://www.atcc.org/Attachments/1982.jpg
(9) http://www.merck-chemicals.de/life-science-research/rapamycin/EMD_BIO-553210/p_BI6b.s1LTrAAAAEWx2EfVhTm
(10) http://www.merck-chemicals.com/life-science-research/akt-inhibitor-viii- isozyme-selective-akti-1-2/EMD_BIO-124018/p_1P.b.s1Ls8oAAAEWxmEfVhTm
(11) http://activebiochem.com/Product/AS-703026.html
(12) http://www.millipore.com/immunodetection/id3/westernblottingprotocols
(13) http://www.molecularstation.com/protein/western-blot/#gelinfo

(14) http://www.molecularstation.com/protein/western-blot/

(15) http://www.abcam.com/ps/pdf/protocols/WB-beginner.pdf

(16) http://www.wiso.uni-koeln.de/statistik_lernmaterial/Kurs-Neue-Statistik/content/MOD_91773/modul_91773.pdf

(17) http://www.reiter1.com/Glossar/Post_Hoc_Test.htm#F-Verteilung

6 Curriculum vitae

General information

Name	Victoria Saferding
Date of birth	02.09.1985
Place of birth	St.Pölten
Adress	Eitzendorf 15, 3123 Obritzberg
Citizenship	Austria

Education

1999-2003	BORG St.Pölten, with natural scientific focus
2003-2004	Medical study at the Universitiy of Vienna
2004-2010	Study of biology with the branch of “Microbiology and Genetics” at the University of Vienna

Laboratory course

Experience by working in the laboratory of the “Austrian Research Institute for Chemistry and Technology (OFI)” in the department of medical devices in August 2007, from September 2007 till March 2009 in a minor employment.

Diploma thesis at the Division of Oncology, Department of Medicine I, Medical University of Vienna. Supervision by Univ.-Prof. Dr Thomas Decker and Univ. Prof. Mag. Dr. Thomas W. Grunt.

Publications

Brünner-Kubath C, Shabbir W, Saferding V, Wagner R, Singer CF, Valent P, Berger W, Marian B, Zielinski CC, Grusch M, Grunt TW. The PI3 Kinase/mTOR Blocker NVP-BEZ235 Overrides Resistance against Irreversible ErbB Inhibitors in Cancer Cells. Breast Cancer Res Treatm (2010) in press

Abstracts

Brünner-Kubath C, Shabbir W, Saferding V, Wagner R, Singer CF, Valent P, Berger W, Marian B, Zielinski CC, Grusch M, Grunt TW. Characterization of signaling pathways related to resistance of breast cancer cells against irreversible EGFR/HER/ErbB kinase inhibitors. AACR Translational Cancer Medicine Conference 2010, Amsterdam, The Netherlands, 21-24 March 2010.

Brünner-Kubath C, Shabbir W, Saferding V, Wagner R, Singer CF, Valent P, Berger W, Marian B, Zielinski CC, Grusch M, Grunt TW. Downstream signaling pathways determine resistance of cancer cells against novel irreversible ErbB-targeting drugs. EACR 21, Oslo, Norway, 26 - 29 June 2010. Eur. J. Cancer Suppl. 8, Abstr# 206, 2010.

Grunt TW, Brünner-Kubath C, Shabbir W, Saferding V, Wagner R, Singer CF, Valent P, Berger W, Zielinski CC, Grusch M. The Dual PI3K/mTOR Blocker NVP-BEZ235 Sensitizes Cancer Cells Against Irreversible ErbB Inhibitors. 22nd EORTC-NCI-AACR Symposium on “Molecular Targets and Cancer Therapeutics”, Berlin, Germany, 16 - 19 November 2010, Eur. J. Cancer, Submitted