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DIPLOMARBEIT

Titel der Diplomarbeit

Molecular Mechanisms of Resistance/Sensitivity of Breast Cancer against the ErbB-Inhibitor Pelitinib (EKB-569)

angestrebter akademischer Grad

Magistra der Naturwissenschaften (Mag. rer.nat.)

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Wien, Juni 2009

This diploma thesis was performed at the

Medical University of Vienna
Department of Medicine I
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Danksagung

Ich danke Herrn 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 Dank gilt besonders Herrn Prof. Mag. Dr. Thomas Grunt für die Themenstellung und Finanzierung, für seine hervorragende Betreuung und seine Geduld bei meiner Diplomarbeit. Bedanken möchte ich mich auch bei Frau Prof. Dr. Brigitte Marian und Herrn Doz. Dr. Michael Grusch, sowie Herrn Prof. Dr. Walter Berger für ihre Unterstützung.

Meiner Kollegin Frau Renate Wagner möchte ich für ihre Hilfe, Geduld und Unterstützung im Labor danken, sowie meiner Kollegin Frau Katharina Tomek.

Ebenso danke ich meinem Freund Gerhard für seine große Geduld während meiner gesamten Ausbildung. Mein größter Dank gilt meinen Eltern, die mich immer unterstützt haben, mein Studium ermöglicht und mir bei allen Problemen mit Rat und Tat beigestanden haben. Vielen, vielen Dank ohne euch wäre das nicht möglich gewesen!!!

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1 Aims of the study

In our experiments, SKBR3 and T47D, two human breast cancer cells proved to be sensitive and resistant against a particular epidermal growth factor receptor (EGFR)/ErbB-2 receptor tyrosine kinase inhibitor pelitinib (EKB-569), respectively. The aim of the project is to delineate the major signaling pathways downstream of the ErbB family of receptor tyrosine kinases (RTKs) in these two human breast cancer cell lines. We examined the effects of pelitinib on the phosphatidylinositol-3 kinase (PI3K) pathway and on the mitogen-activated protein kinase (MAPK) downstream signaling cascade. Proliferation data of these cells exposed to different concentrations of pelitinib, rendered SKBR3 sensitive and T47D resistant. Interestingly, differences in AKT phosphorylation levels were observed. To establish a solid basis for further statements of inhibitor sensitivity/resistance in these human cancer cell lines AKT activation and its ability to render cells resistant was examined by transfection procedures of sensible SKBR3 with constitutive active myr-AKT1 plasmids.

2 Introduction

2.1 Breast cancer

2.1.1 The geographical distribution and mortality incidence of breast cancer

In industrialized countries breast cancer is one of the most common cancers of women and more than 1 million cases are diagnosed with a mortality incidence of 500 000 death per year in the whole world (Benson et al., 2009). In earlier times breast cancer was not theme in not-industrialized countries but nowadays the disease rate increases. Peggy Porter (2008) reported the `Westernization` as a higher risk to increase breast cancer rates in Japan, Korea, Singapore, China, India and Africa.

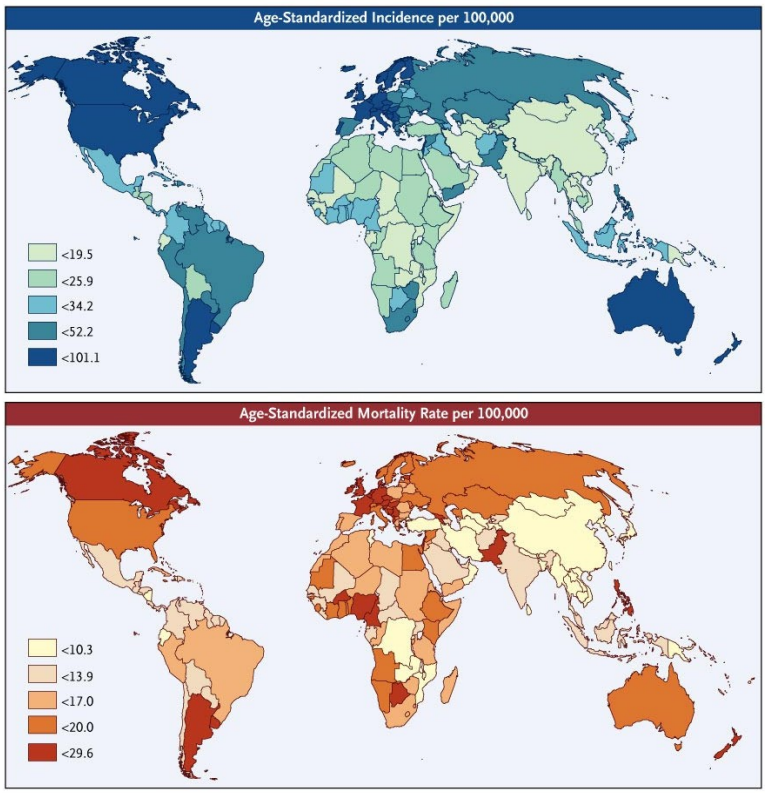


Fig.1: Incidence rates (top panel) and mortality rates (bottom panel) of breast cancer (Peggy Porter, 2008). *Data are from GLOBOCAN 2002, International Agency for Reasearch on Cancer.*

Anyway, East Asian woman represent lower incidence rates of breast cancer, about 21 per 100,000 compared to the United States, Europe and Australia with 101 per 100.000 the highest incidence rate, respectively. Moreover, Western Europe represents an incidence rate of breast cancer about 85 per 100,000 **(Fig.1)**. Interestingly, while African women still have lower incidence rates, 23 per 100,000, when compared to other countries except East Asia, local registries report in Africa two-fold higher incidence rates compared to the past 40 years. Nevertheless, the incidence rates of breast cancer increases worldwide, thus the mortality incidence continue to rise, with about 29,6 to 20 per 100,000 in the United States, Europe, Western Europe and Australia **(Fig.1)**. Although the mortality rates have increased the past years in Africa and Asia the mortality rates tend to be much lower, under 20 per 100,000, when compared to the rest of the world.

2.1.2 Breast cancer-risks and the modulating factors

Previous studies reported different risk factors which are associated with breast cancer development, such as genetic predispositions, hormones and reproductive patterns (Benson et al., 2009 and Hankinson et al., 2004). Risk factors such as decreasing age at menarche, late age at menopause, nullparity and reduced breast feeding have been known for many years. Moreover, different diet strategies and obesity in Western countries are associated with breast cancer development. Often genetic predispositions play crucial roles in human cancer development. Years ago, tumor suppressor genes have been identified, including BRAC1, BRAC2, and PTEN, to be associated with breast cancer-risks. It was reported that 5-10% of all breast cancer are hereditary and due to mutations within these two genes BRAC1 and BRAC2 (Robson et al., 2007).

As early as in the 1800s, it was recognized that estrogen plays an important role in the development and progression of human breast cancer. Many years later the estrogen receptor (ER) was identified and nowadays is used as an

important breast cancer phenotypic and prognostic parameter. Breast cancer risks due to hormone dependency, includes estrogen-receptor-positive tumors which are stimulated by estrogens and estrogen-receptor-negative tumors, which emanates from the former by possible lesions. Furthermore, hormone-replacement therapies (HRT) are methods for treatment of menopausal woman with diminished circulating estrogens and progesterone. Holmberg et al., (2008) reported HRTs as reasons which double the risks of recurrence in patients who have survived breast cancer.

Interestingly, alcohol consumption also correlates with breast cancer-risk in women through its effects on estrogens (Zhang et al., 2007). Moreover this paper has investigated the relation of alcohol intake and ER+PR+ tumors, whereas ER-PR- and ER+PR- tumors are not associated with alcohol consumption, like the Swedish Mammography Cohort study has revealed but in contrast to the Women`s Health study. These data suggest high association between alcohol consumption and breast cancer-risk mediated by the estrogen pathway.

2.1.3 Diagnosis of breast cancer

In patients with breast cancer, complete clinical evaluations of different factors have to be revealed, and for best therapeutic measures a rough estimate on local disease extent is made. Mammographic screening leads to the detection of malignancy and even reduces breast cancer-mortality in postmenopausal women. Furthermore, complementary to mammography (MMG), whole-breast ultrasonography (US) provides measurement of tumor size and increases diagnostic estimates of malignant foci. These both imaging technologies enable enhanced prevention of breast cancer development and further implicate exact coordination of patient treatment (Benson et al., 2009). For exact tissue diagnosis for benign or malignant diseases fine-needle aspiration cytology or core biopsy are used, whereas core biopsy enables higher specificity and sensitivity as compared to the former (Britton et al., 1999). To detect the extent

of a lesion of breast cancer in its accuracy, magnet resonance imaging (MRI) remains to be a diagnostic imaging method reported by Mameri et al., (2008).

2.1.4 Primary systemic and adjuvant-systemic therapies in breast cancer

Breast cancer is known to be a complex and heterogeneous disease and its biology is poorly understood. Primary treatment of breast cancer includes lumpectomy, radiotherapy and radical mastectomy, whereas adjuvant therapy is additional to provide long-term survival and avoid recurrence of the tumor. Ravdin et al., (2001) reported improvements of a computer program “Adjuvant” to decide about right adjuvant therapies, such as chemotherapy, hormone therapy or chemoendocrine therapy, dependent on tumor staging and characteristics. Therefore, systemic investigations characterize the existence of multiple subtypes of breast cancer, such as basal-like, ErbB-2 overexpressing, normal breast-like, luminal subtypes A,B,C using gene expression pattern profiles (Sorlie et al., 2001). The first target-based endocrine therapy was the treatment of luminal subtypes of estrogen receptor positive (ER+) breast cancer cells, with the selective estrogen receptor modulator (SERM) tamoxifen, the most effective systemic treatment since 100 years, responsible for reduction in mortality and local recurrence. Aromatase inhibitors and fulvestrant represent new developed drugs in treatment of post-menopausal breast cancer after disease progression following anti-estrogen therapies. Nevetheless, patients can respond poorly to endocrine therapies, irrespective of used endocrine agents, which can be associated with activated AKT a downstream mediator of ErbB-2 overexpressed in many ER+ breast carcinomas (Tokunaga et al., 2006).

2.2 ErbB tyrosine kinase receptors

2.2.1 ErbB tyrosine kinase receptors

The ErbB receptor network comprises a signaling transduction system which is involved in the process of malignant transformation during cancer development (Yarden et al.,2006). Darcy et al., (2000) reported involvement of all ErbB members in mammary gland development and expression of various cell-type specific patterns during growth, differentiation and apoptosis. This family consists of four homologous members: ErbB-1/HER-1/EGFR, ErbB-2/HER-2/Neu, ErbB-3/HER-3 and ErbB-4/HER-4. All family members have an extracellular ligand-binding domain, a short hydrophobic transmembrane domain and a cytoplasmic tyrosine kinase domain that plays a significant role in regulating cellular processes in cancer.

2.2.2 ErbB-specific ligands and ErbB-receptor heterodimerisation

ErbB receptors are activated by four categories of ErbB-specific ligands such as EGF-related peptide growth factors **(Fig.2)**. For epidermal growth factor-receptor (EGFR) the following ligands are known: amphiregulin (AR), betacellulin (BTC), epidermal growth factor (EGF), epiregulin (EPR), heparin-binding EGF-like ligand (HB-EGF), and transforming growth factor α (TGFα). EGFR binds EGF, AR and TGF-α, whereas BTC, HB-EGF and EPR can bind either to EGFR or to ErbB-4. The neuregulins (NRGs) or heregulins (HRGs) represent a large number of differently spliced proteins that bind to ErbB-3 and –4. NRG1 and NRG2 bind both ErbB-3 and ErbB-4 receptor, but NRG3 and NRG4 only bind to the ErbB-4 receptor.

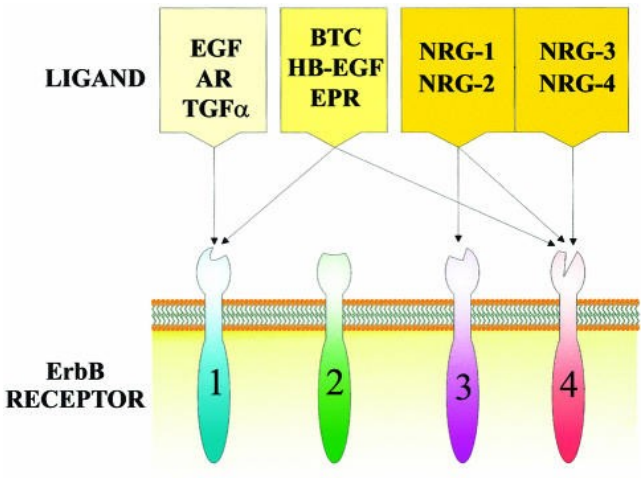


Fig.2: ErbB-specific ligands (Olayioye et al., 2000). *EGFR bind the following ligands, which are EGF, transforming growth factor α (TGFα) and amphiregulin (AR). Betacellulin (BTR), epiregulin (EPR) and heparin-binding EGF-like ligand (HB-EGF) bind both EGFR and ErbB-4. The neuregulins (NRGs) represent NRG1 and NRG2 that bind to ErbB-3 and –4, whereas NRG3 and NRG3 only bind ErbB-4.*

The receptors are phosphorylated upon ligand binding and form homo- or heterodimers. The nature of the ligand determines the ErbB dimers which are formed. However, it has to be mentioned that ErbB-2 does not have a cognate ligand implicating that it does not represent a true receptor. It rather acts as a dimer partner and co-receptor for the other ErbB receptors. ErbB-2 is the most preferred dimerisation partner for EGFR, ErbB-3 and ErbB-4 receptor **(Fig.3)**.

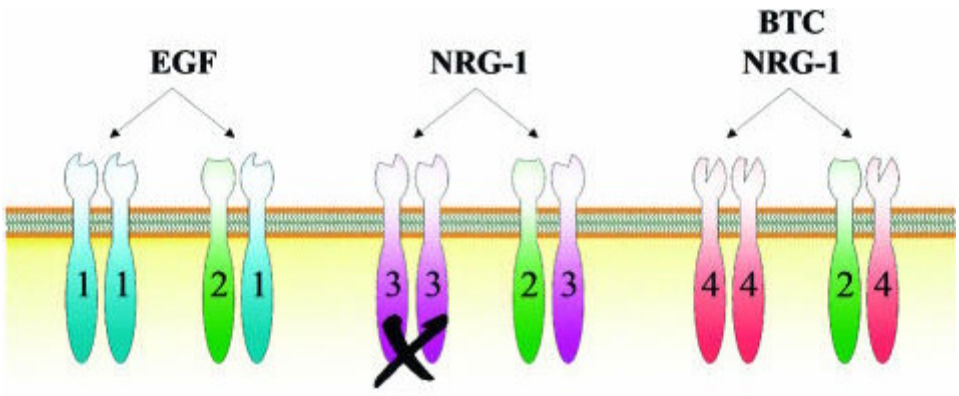


Fig.3: ErbB-receptor homo- heterodimerisation (Olayioye et al., 2000). *Formation of homo- and heterodimers, whereas ErbB-2 is the most preffered heterdimerisation partner for EGFR, ErbB-3 and ErbB-4. ErbB-3 exhibits a degenerated tyrosine kinase domain and cannot signal on its own.*

On the other hand, the tyrosine kinase domain of ErbB-3 is degenerated and thus functionally invalid. Consequently, ErbB-2 and ErbB-3 cannot signal on their own, but rather need a dimerisation partner for signaling. ErbB-2 is strongly activated after heterodimerisation with ligand-activated EGFR, ErbB-3 or ErbB-4, whereas ErbB-2 and ErbB-3 heterodimers tend to be the most favored ErbB constellation under the ErbB heterodimers. Anyway, EGFR and ErbB-2 are frequently overexpressed in cancer and correlate with a more aggressive clinical behavior and poor prognosis (Olayiode et al., 2008).

2.2.3 ErbB-specific downstream signaling and cross talk with other regulatory systems

After receptor dimerisation mediated by specific ligand binding, the cytoplasmic kinase domain of the receptor is activated and triggers autophosphorylation of the C-terminal tyrosine residues. These tyrosine residues provide docking sites for molecules with a src homology 2 (SH2) and a phosphotyrosine binding (PTB) domain, such as adapter proteins and different kinases. The most important signaling pathways are the phosphatidylinositol-3 kinase (PI3K)/AKT pathway, the mitogen-activated protein kinase (MAPK) signaling pathway as well as the signal transducer and activator of transcription (STAT), the phospho lipase C (PLC) and pospholipase D pathways (Marmor et al., 2004). The best characterized signaling cascades, which signal via the ErbBs are the PI3K/AKT and the MAPK pathways. Apart from these classical downstream pathways, ErbB receptors have increasingly be recognized as a family of receptors that cross talks with nuclear receptors such as the clinical important and relevant estrogen receptor (Grunt et al., 1995; Tang et al., 1996 and Saceda et al., 1996) and retinoid receptors (Flicker et al., 1997; Grunt et al., 1998; Offterdinger et al., 1998, 1999; Schneider et al.,1999; Grunt, 2003 and Grunt et al., 2005)

2.2.4 The phospatidylinositol-3 kinase (PI3K)/AKT signaling pathway

After ligand-induced activation of the ErbB receptor family, the PI3K-PDK1 (Vanhaesebroeck et al., 2000) signaling pathway activates protein kinase B/AKT and promotes proliferation survival, growth, glycogen synthesis, survival and nitric oxide (NO) synthesis **(Fig.4)**, and is associated with tumorigenesis (Testa et al., 2001 and Nicholson et al., 2002). First, the p85 regulatory subunit of the PI3K docks on the phosphorylated tyrosine residues and recruits the second catalytic subunit p110 of the PI3K. After the activation of PI3K, it transforms phosphatidylinositol 4,5 phosphate (PIP2) into phosphatidylinositol 3,4,5 phosphate (PIP3). After PIP2 is converted to PIP3 the phosphoinositol-dependent kinase-1 (PDK1) is recruited to the membrane via its pleckstrin homology (PH) domain. Furthermore PDK1 phosphorylates AKT at residues threonine 308 and serine 473, which is also recruited to the plasma membrane because of its PH domain.

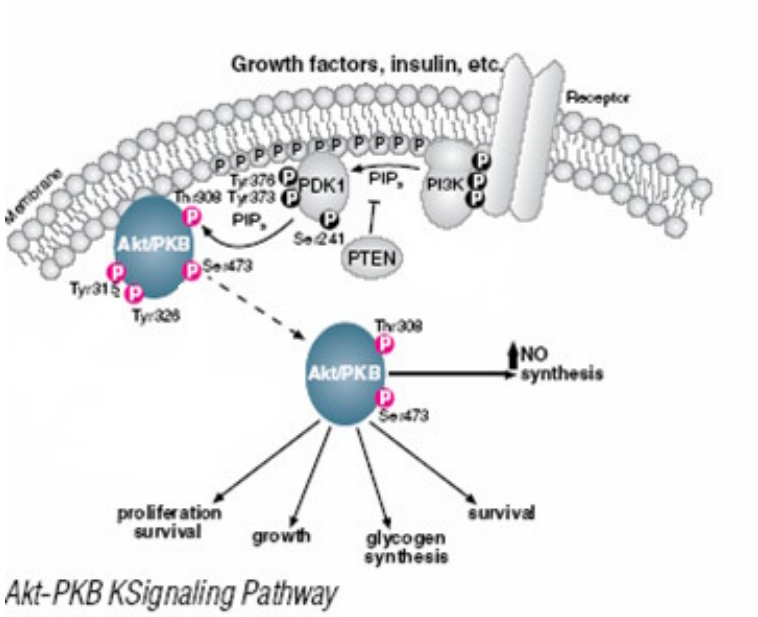


Fig.4: The phospatidylinositol-3 kinase (PI3K)/AKT signaling pathway (Cell Signaling Technology, http://www.cellsignal.com, slightly modified). *The mechanisms of AKT activation is mediated by phosphorylation of the PI3K through the ErbBs.*

In addition, the PI3K/AKT-pathway has major importance in normal cellular homeostasis and cancer growth due to its central role as a cross-over point between growth, differentiation and apoptotic pathways and major metabolic systems such as tumorgenic lipogenesis (Grunt et al., 2009).

2.2.5 The mitogen-activated protein kinase (MAPK) signaling pathway

The ErbB family members signal via the adaptor proteins Grb2 or Shc and activate the MAPK signaling pathway (Olayioye et al., 2000). Ras a small GTP-binding protein recruits after conformational change Raf-1 (c-Raf) to the membrane, where it is phosphorylated on serine/threonine and tyrosine residues. Raf1 activates the MEK-ERK signaling pathway and phoshorylates Myc and Elk two transcription factors, which control various biologic processes by modulating gene expression **(Fig.5)**. The RAF/MEK/ERK pathway therefore plays a crucial role in tumorgenesis and by regulating gene expression and preventing apoptosis (McCubrey et al., 2006).

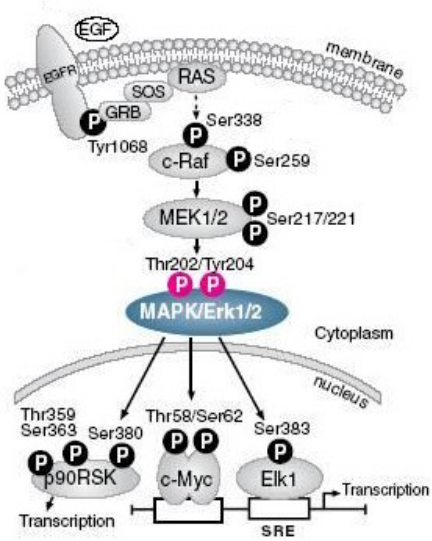


Fig.5: The mitogen-activated protein kinase (MAPK) signaling pathway (Cell Signaling Technology, http://www.cellsignal.com, slightly modified). *Gene expression triggerd by activation of Ras and Raf, which activate the MEK/ERK signaling pathway.*

2.2.6 Targeting ErbB receptors in cancer development

Signaling through the ErbB receptor family correlates with human cancer development and therefore serves as perfect target for different drugs (Citri et al., 2006). Nowadays, ErbB-neutralizing monoclonal antibodies, ErbB-specific tyrosine kinase inhibitors (TKIs) and inhibitors of heat-shock protein-90 (HSP90) are in clinical development **(Fig.6)**. For treatment of breast cancer the ErbB-2 antibody trastuzumab (herceptin) is in use and for colorectal cancer the monoclonal EGFR antibody cetuximab (erbitux) is clinically relevant, and pertuzumab (omnitarg) targets heterodimerisation of ErbB-2 as novel therapeutic agent. Monoclonal antibodies inhibit not only ErbB receptor signaling, but also recruit cytotoxic T-cells. Further achievements are small molecule TKIs, such as gefitinib (iressa) and erlotinib (tarceva), which inhibit the kinase domain of the EGFR receptor by blocking the ATP binding pocket. Gefitinib and erlotinib are two reversible small molecule TKI`s and are approved for non-small-cell lung cancer (NSCLC). More effective are dual-receptor TKIs such as lapatinib and pelitinib (EKB-569), a new generation of drugs targeting both EGFR and ErbB-2. Important for the stability of the ErbB-2 receptor is the HSP90 chaperone, which serves therefore as perfect target for new inhibitors such as 17-N-allylamino-17-demethoxygeldanamycin (17-AAG).

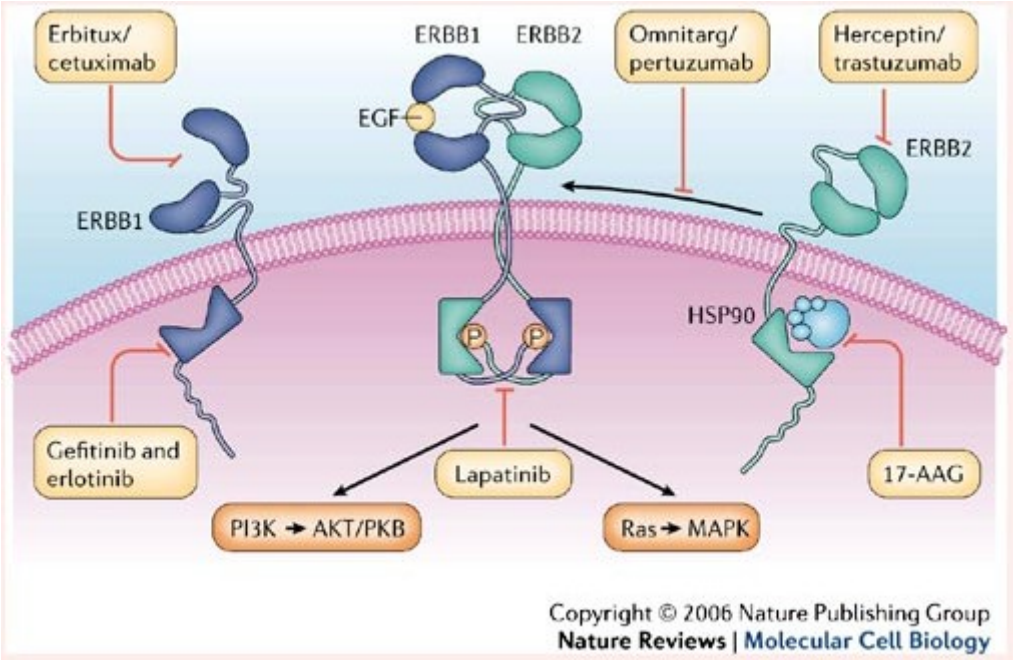


Fig.6: Targeting ErbB receptors (Citri et al., 2006). *Monoclonal antibodies cetuximab, pertuzumab and trastuzumab, as well as tyrosine kinase inhibitors (TKIs) gefitinib, erlotinib and lapatinib and heat-shock protein 90 (HSP90) inhibitors such as 17-AAG are in clinical use or in clinical development.*

2.2.7 EGFR overexpression and resistance mechanisms

Nicholson et al. (2001) reported EGFR expression to be a strong prognostic marker in head, neck, ovarian, cervical, bladder and esophagus cancer, whereas in non-small-cell lung cancer, EGFR expression acts as a weak prognostic factor. In the situation of breast, esophagus, colorectal, gastric and endometrial cancer EGFR is a modest prognostic factor. In breast cancer, EGFR expression correlates with decrease in patient survival and also acts as early prognostic factor suggesting EGFR triggers in cancer development more efficiently cell growth and cell progression. Elevated levels of EGFR in breast cancer are associated with poor prognosis and loss of sensitivity against endocrine therapies (Nicholson et al., 1994). In preclinical studies, breast cancer cells were treated with EGFR TKI gefitinib (iressa) and showed growth inhibition of breast cancer cells by dephosphorylation of EGFR, ErbB-2 and

ErbB-3 receptor resulting in down-regulation of AKT activity (Moasser et al., 2001 and Anido et al., 2003). Anyway, resistance mechanisms against the oral TKI gefitinib were shown in breast cancer cells that co-express EGFR, ErbB-2 and ErbB-3. Sergina, et al. 2007 reported the escape of EGFR-TKIs through ErbB-2-dependent activation of the ErbB-3/PI3K/AKT pathway. However, Normanno, et al. 2008 demonstrated development of resistance mechanisms to the drug in breast cancer cells, because of a persistent EGFR-independent activation of MAPK signaling pathway. Another EGFR receptor TKI is erlotinib (tarceva), an oral reversible inhibitor, which inactivates EGFR downstream signaling as well as the ErbB-2 receptor irrespective of the former (Schaefer et al., 2007). Anyway, cetuximab (erbitux), a chimeric human-mouse anti-EGFR monoclonal antibody undergoes testing in colorectal cancer, head & neck cancer and NSCLC as addition to chemotherapy (Stern et al., 2004).

2.2.8 ErbB-2 overexpression and resistance mechanisms

A crucial marker for clinical management of breast cancer is the ErbB-2/HER2/neu receptor, which belongs to the large family of ErbB receptor tyrosine kinases. ErbB-2 is overexpressed in 20-30% of breast tumors and serves as a perfect target for therapeutic agents. The first clinically approved drug targeting ErbB-2 in ErbB-2 positive cancer was trastuzumab (herceptin) (Ginestier et al., 2007 and Pal et al., 2007). Trastuzumab is a humanized monoclonal antibody with high specificity and affinity for the ErbB-2 extracellular domain. In metastatic breast cancer trastuzumab serves either as single therapeutic agent or is combined with chemotherapy (Stern et al., 2005). ErbB-2 overexpression and hyperactivation stimulate the MAPK and the PI3K/AKT pathway, and promote cell cycle progression. ErbB-2 overexpression combined with PI3K/AKT activation mediates multidrug resistance in breast cancer cell lines (Knuefermann et al., 2003). Most of the patients with metastatic breast cancer respond to trastuzumab but disease progression was seen within one year. Whilst trastuzumab inhibits ErbB-2 receptor signaling, EGFR forms

homodimers or EGFR/ErbB-3 heterdodimers and signals via the PI3K/AKT and the MAPK signaling pathway rendering breast cancer cells resistant against trastuzumab. Thus, combination therapies, herceptin with TKIs, such as gefitinib and pertuzumab a monoclonal antibody, will be new therapeutic strategies. As a new therapeutic agent, pertuzumab inhibits ErbB-2/EGFR and ErbB-2/ErbB-3 heterodimers (Natha et al., 2006). Other EGFR/ErbB2-dual targeting drugs tested in breast cancer include the small molecule tyrosine kinase inhibitors lapatinib and pelitinib (EKB-569). Nahta et al. (2007) reported induction of apoptosis in trastuzumab resistant breast cancer cells after treatment of lapatinib, whereas pelitinib a new irreversible inhibitor is referred to be in clinical trials in NSCLC and colon cancer (Wissner et al., 2008)

2.2.9 ErbB-3 overexpression and targeted therapies

Similar to ErbB-2, ErbB-3 overexpression correlates with aggressive clinical behavior of breast cancer (Sithanandam et al., 2008). Although ErbB-3 has a deficient kinase domain and ErbB-2 is lacking a specific ligand, the ErbB-2/ErbB-3 heterodimer yet constitutes the most active signaling complex and signifies clinically aggressive tumors. Moreover, resistance mechanisms against TKIs are often shown in breast cancer cells that co-express EGFR, ErbB-2 and ErbB-3. ErbB-3 upregulation in breast cancer cells is associated with resistance against different TKIs. Therefore, ErbB-3 overexpression provides EGFR-TKIs escape through the possibility of ErbB-3/PI3K/AKT signal pathway activation mediated by ErbB-2-dependent signaling (Sergina et al., 2007). Furthermore, downregulation of ErbB-3 by RNA interference abrogates secondary resistance to TKIs and induces apoptosis.

2.2.10 ErbB-4 overexpression

The fourth member of the ErbB family termed ErbB-4 exhibits different functions compared to the other ErbB receptors. ErbB-4 can signal as full lengths

membrane protein via the PI3K and MAPK signaling pathway, but can also translocate as soluble receptor to the nucleus after its processing. It has to be noted that all ErbB receptors have already been reported to be localized not only in the cell membranes, but also in the cell nuclei. Interestingly, full length EGFR (Lin et al., 2001), ErbB-2 (Dillon et al., 2008) and ErbB-3 (Offterdinger et al., 2002) have been found in the cell nuclei, where they can act as regulators of gene transcription. The biochemical mechanisms of nuclear entry and localization of these membrane proteins is still not fully elucidated. Not only the full-length and cleaved ErbB-4 plays essential roles, even spliced ErbB-4 isoforms must be considered in breast cancer patients. Therapeutic agents have been developed to target EGFR, ErbB-2 but relatively little is known about the biological significance of ErbB-4 in breast cancer. Experimental studies of ErbB-4 show that it is not overexpressed in breast cancer and when ErbB-2 overexpressing cells are forced to overexpress ErbB-4, it can be associated with a reduction of cell proliferation and increase of apoptosis. Therefore, ErbB4 expression is considered a marker of survival in breast cancer. Interestingly, it was described that ErbB-4 overexpression can also be associated with cell growth and cell progression, suggesting differences in breast cancer subtypes, according to ER+ and ER- tumors. Breast cancer cells, which overexpress ErbB-4 in ER+ tumors, are associated with favorable prognosis. (Sunvall et al., 2008).

3 Material and methods

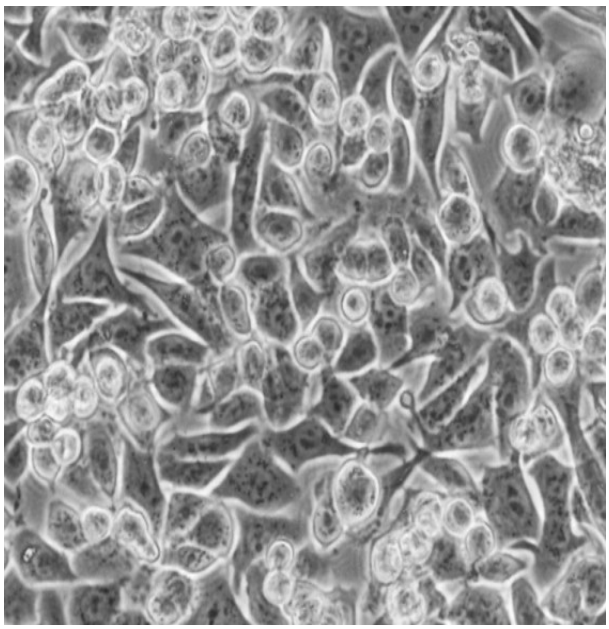
3.1 Cell culture and cell lines

3.1.1 List of used materials

T25 tissue culture flasks 50ml/25cm^2: Falcon, 35/3108
T75 tissue culture flasks 250ml/75cm^2: Falcon, 35/3111
60x15mm tissue culture dishes (petri dishes): Corning, 8430166
6-well plates: Corning Incorporated, 3506
96-well plates flat bottom: TPP 92696
0,05% Trypsin-EDTA: GIBCO, Cat.No. 25300-054
MEM alpha w/nucleosides. : GIBCO, Cat.No. 22571-020
D-MEM high glucose (4500mg/l): GIBCO, Cat.No. 41965-039
Fetal calf serum (FCS): S-America, GIBCO, Cat.No. 10270-106
Penicillin/Streptomycin: GIBCO, Cat.No. 15140-148
L-Glutamin 200mM: GIBCO, Cat.No. 25030-032
Dulbecco`s sulphate buffered saline (D-PBS): GIBCO, Cat.No. 14190
Recombinant epidermal growth factor (EGF): Sigma, E9644, 100µg/ml
Recombinant human heregulin ß1 (HRGß1): Thermo Fisher Scientific, RP-318-P1A,1nM

3.2 Cell lines

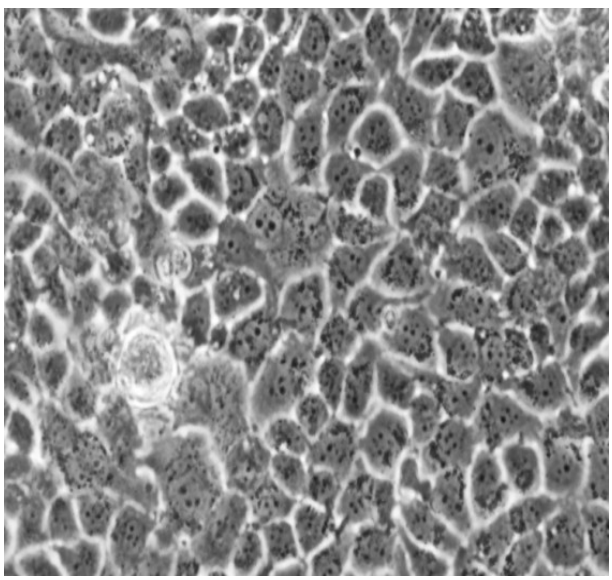
The SKBR3 cell line



The SKBR3 breast adenocarcinoma cell line descends from human mammary gland. This cell line was taken from a 43 years old female. SKBR3 is cultivated in D-MEM and supplemented with 10% heat inactivated fetal calf serum (FCS) and 1% 100U/ml penicillin, 100µg/ml streptomycin and 2mM L-glutamine (PSG). The cells were subcultured once a week at a ratio of 1:7.

Fig.7: Morphology of SKBR3

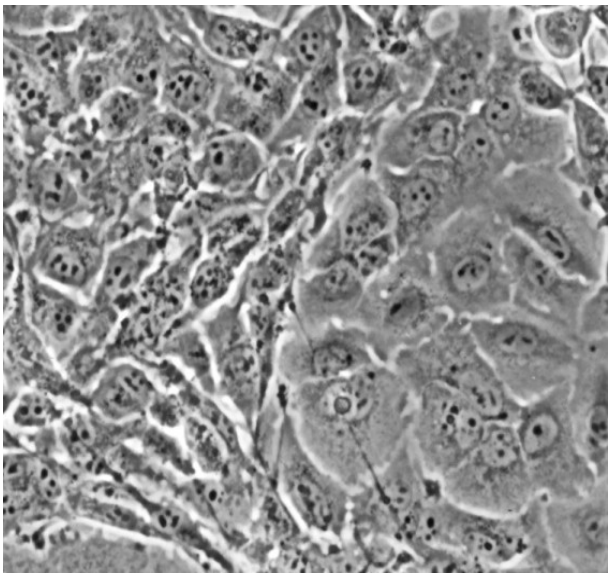
The T47D cell line



T47D represents a ductal carcinoma and was isolated from human mammary gland. This breast cancer cell line descends from a 54 years old female. T47D is maintained in D-MEM supplemented with 10% FCS and 1% PSG. The cells were subcultured once a week at a ratio of 1:10.

Fig.8: Morphology of T47D

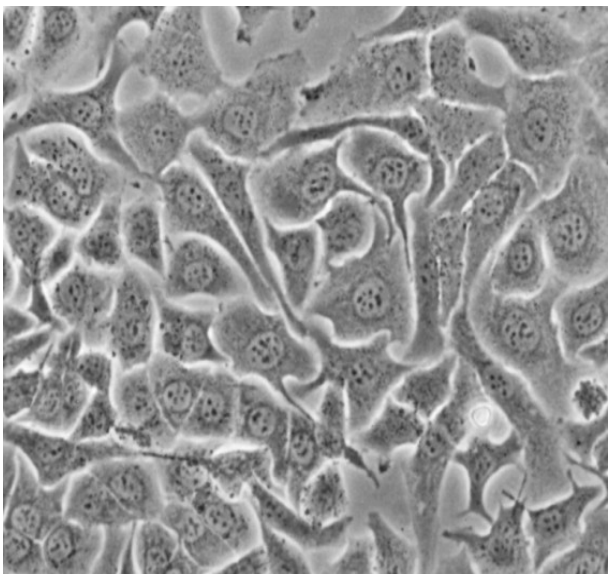
The CaOV3 (HTB-75) cell line



CaOV-3 (HTB-75) cells descend from a human ovarian adenocarcinoma. This cell line is cultivated in D-MEM supplemented with 10% FCS and 1% PSG. CaOV-3 (HTB-75) cells were subcultured once a week at a ratio of 1:12.

Fig.9: Morphology of CAOV-3 (HTB-75)

The SKOV3 cell line



SKOV3 cells were isolated from human ovarian adenocarcimoma. This cell line is maintained in α-MEM supplemented with 10% FCS and 1% PSG. The cells were subcultured once a week at a ratio of 1:13.

Fig.10: Morphology of SKOV3

3.3 Cell culture and passaging of cells

All cell lines **(Figs.7-10)** were cultured in two T 25 tissue culture flasks in 6 ml each of the respective medium containing 10% FCS with 1% PSG. Cells were passaged at a confluence of 95-100%. After removal of the culture medium cells were washed carefully with Dulbecco's phosphate buffered saline (D-PBS) and incubated for 5 min at 37°C, 5 % CO_2, 95 % humidity with trypsin ethylenediaminetetraacetic acid (EDTA) to detach the cells. These cells were resuspended in the respective medium and plated in two new T25 culture flasks at a ratio of 1:7 to 1:13. The cells were passaged once a week. In some cases cells were exposed to 100ng/ml EGF at various times and 3min to heregulin ß1 (HRGß1).

3.4 Used drug

Pelitinib (EKB-569), an irreversible tyrosine kinase inhibitor (TKI) was generously provided from Wyeth **(Fig.11)**. It targets both the epidermal growth factor (EGFR) and the human epidermal growth factor receptor-2 (EGFR2/HER2/ErbB-2) and inhibits the function of the kinase domain. As 4-anilino-3-cyano quinoline derivative it forms a covalent link with conserved cysteine residues, Cys-773 (or Cys-797) in EGFR and Cys-805 in ErbB-2.

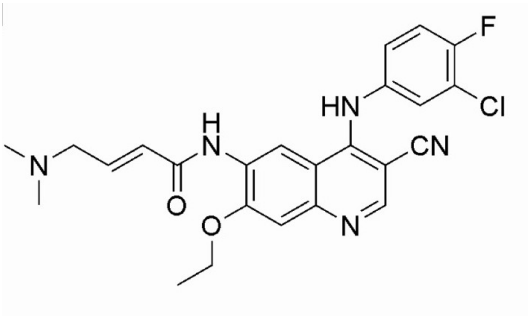


Fig.11: Structure of pelitinib (EKB-569), (Wissner et al., 2008)

EKB-569 currantly undergoes clinical trial for non-small cell lung (NSCLC) and colon cancer.

3.5 Proliferation assay

1,5x 10^3 cells per well were seeded in 96-well plates in culture medium containing 10% FCS with 1% PSG. These cells had to attach overnight and were controlled under the microscope. After 24 hours incubation the inhibitor pelitinib (EKB-569) was added at different concentrations in culture medium without FCS, to obtain a final concentration of 5% FCS per well. Three wells were analyzed per treatment condition. Blank wells were devoid of cells and consisted culture medium with 5% FCS. Controls were treated with the vehicle (DMSO) only.

After 72 hours of pelitinib treatment, a growth assay, called EZ4U (Biomedica, BI-5000) was performed. This assay is a non radioactive cell proliferation and cytotoxicity test. After adding the reagent to the 96-well plates the color of the medium changes because living cells with active mitochondria reduce a yellow colored tetrazolium compound to a soluble red formazan product. First the substrate of the EZ4U reagents had to be dissolved at 37°C by adding 2,5ml activator to the substrate. Then 20µl of the reagent was pipetted per well and after incubation of 2,5 hours the absorbance was measured at 450nm against a 655nm reference wavelength with a microplate reader spectrophotometer (Bio-Rad Laboratories, Model 680). IC50 values were calculated with Excel and dose-response curves were designed with SigmaPlot software.

3.6 Protein analysis

3.6.1 Protein extracts

$5x10^5$ cells were seeded in 60mm tissue culture petri dish before protein extraction was perfomed. Every step was done on ice. The respective culture media was removed and the cells were washed twice with cold PBS. Then the remaining PBS was removed and 80µl RIPA+ (prepared as described in (**Tab.1 and 2**) was added to one 60mm dish (1 confluent 60mm dish gives >150 µg protein).

Tab.1: RIPA lysis buffer

Reagent	MW	Stock	Storage	Vol	Final concentration
NaCl	58.44	5M	rt*	3ml	150mM
Tris pH 7.4	121.14	1M	rt	5ml	50mM
DOC (Na-deoxycholate)	414.6	10%	rt (keep dark)	5ml	0.5%
EGTA	380.4	50mM	rt	4ml	2mM
EDTA, pH 7.4	372.2	50mM	rt	10ml	5mM
NaF	41.99	500mM	rt	6ml	30mM
ß-Glycerophosph.pH7.2	216	400mM	4C	10ml	40mM
Tetrasodium pyrophosph.	446.06	100mM	rt	10ml	10mM
Benzamidine	156.6	30mM	4C	10ml	3mM
Nonidet P-40		pure	rt	1ml	1%

*rt…room temperature

After all components were mixed, pH was set to 7,4 and the volume was filled with Aqua bidest. up to 95,0 ml. The RIPA solution was stored at 4°C and shortly before use RIPA+ was prepared.

Tab.2: RIPA+ lysis buffer

RIPA+	2ml
RIPA	1,90ml
200mM Na-Orthovanadate	20µl
25 x Complete stock solution	80µl

After adding 80 µl RIPA+, cells were scraped with a sterile scrapper. Then the lysates were transferred to one Eppendorf tube, vortexed several times and spun for 30min at 12.500rpm in a microfuge at 4°C. The pellet was discarded and the supernatant was saved at -20°C for the protein assay.

3.6.2 Protein quantification

Determination of protein concentration in the samples was done with the BioRad Protein Assay Kit II (Cat# 500-0112). The Kit was performed in a 96-well plate. For measurements, first a 10mg/ml bovine serum albumine (BSA) stock solution was produced for protein standards by dissolving, for example 28.4mg BSA in 2.84ml Aqua bidest. This stock solution was serially diluted 1:2 with RIPA+ to obtain 5, 2.5, 1.25, 0.625, 0.3125, 0.15625 µgBSA/µl protein standards (Blank was RIPA+). Then these standards were set up in the 96 well plate.

Pipet after this concept:

-pipet 5µl RIPA+ in Blank wells

-pipet 5µl protein standards/well, triplicates

-pipet 5µl samples/well, triplicates

Reagent A' of the Protein Assay Kit II was prepared freshly every time by taking 1ml Reagent A + 20µl Reagent S. 20µl Reagent A'/well and 200µl Reagent B/well were added. Finally, the extinction was measured at 620/450 nm in the microplate reader (spectrophotometer Bio-Rad) after an incubation time of 20min. The standard curve and protein concentration were calculated using Excel. The samples were stored in 4x sample buffer **(Tab.3)** at a final concentration of 1µg/µl at -20°C, that means the protein sample had a concentration of 1,33 µg/µl before 4x sample buffer was added [e.g. 3 parts protein sample in RIPA+ (1,33µg/µl) + 1 part 4x sample buffer = 1 µg protein/µl].

Tab.3: 4x sample buffer

Reagent	MW	Stock	Storage	Volume	Final concentration
Glycerol	92.09	pure	rt	5 ml	50%
Tris-HCl, pH 6.8	121.14	1M	rt	1.25 ml	125mM
SDS		20%	rt	2,00 ml	4%
Bromophenol blue		1%	rt	1,25 ml	0,125%
Beta-mercaptoethanol (to be added just before use)		pure	rt	0.5ml	5%

*rt…room temperature

3.6.3 SDS- Polyacryl amide gel electrophoresis (PAGE)

The glass plates for PAGE were washed with Aqua bidest. and 70% ethanol (EtOH), the spacers only with Aqua bidest. and then assembled according to Bio-Rad instructions. Then the separating gel was prepared **(Tab.4a and 4b)**. A 7,5% gel was used, a middle solution depending on the size of proteins of interests.

Tab.4a: A/B Solution

Reagent	Volume
40% Acrylamid (Biorad #1610140)	11,1 ml
2% Bis Solution (Biorad # 1610142)	3 ml
Aqua bidest.	0,9 ml
30 % A/B	15 ml

Tab.4b: 7,5% separation gel

Reagent	7,5% gel
30% A/B	5 ml
Aqua dest.	9,7 ml
1,5M Tris 8,8	5 ml
10% SDS	200µl
10% APS	100µl
Temed pur	10µl

The cassette was filled ~¾ full with separating gel **(Tab.4b)** and carefully (slowly) overlayed with some Aqua bidest. to remove any air bubbles. The solution polymerized for 30 – 45 min. Aqua bidest. was discarded off the cassette and the comb was inserted. The 4% stacking gel solution **(Tab.4c)** was poured to the very top, the comb slightly drawn up and the gel polymerized for ~30min.

Tab.4c: 4% stacking gel

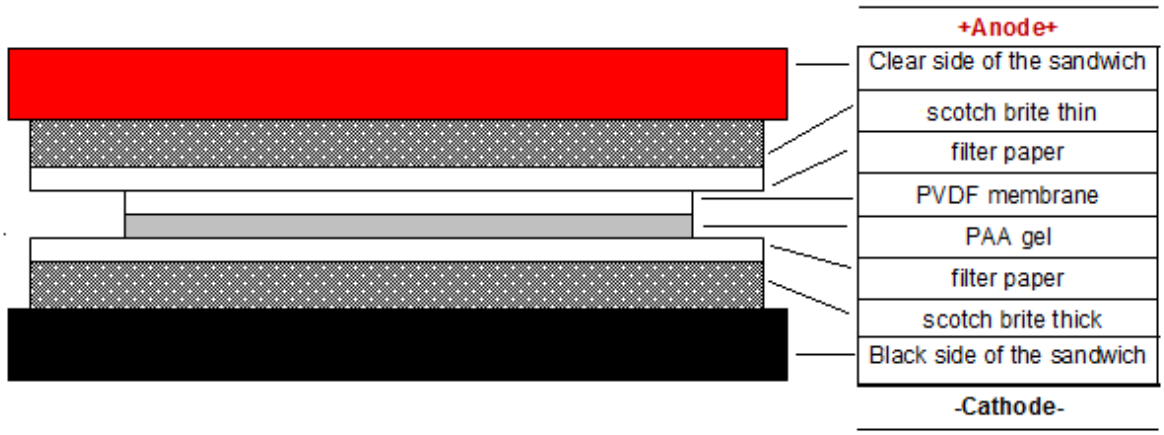
Reagent	4% gel
30% A/B	1,673ml
Aqua bidest.	7,47ml
0,5M Tris 6,8	3,15 ml
10% SDS	125µl
10% APS	125µl
Temed pur	12,5µl

The cassettes were inserted in the electrophoresis apparatus and 1x running buffer (25mM Tris, 192mM Glycine, 0,1% SDS in Aqua bidest.) was added. The protein samples were boiled for 10 min at 95°C before each use for denaturation and then put on ice. 20µl of the protein samples (20µg protein) and 2 µl of the molecular weight marker (Magic Mark XP from Invitrogen) were loaded on the gel. The gel was run at constant 100 Volt for approximately 2 hours. After electrophoresis, the gel was subjected to Western blotting

3.6.4 Western blotting

The separated proteins were transferred from the gel onto a Hybond-P polyvinylidene fluoride (PVDF) membrane. Before the transfer, the membrane had to be prewet in pure methanol. For identification cut marks in the rim of the PVDF (Polyscreen, NEF 1000, 153196) membranes corresponding to marks to be made in the rim of the gels. The blotting sandwich was assembled with the PVDF membrane and the gel in between as shown in **Fig.12**. A pipet fragment was used for rolling out some air bubbles that might have been formed during the assembly of the sandwich.

Fig.12: The blotting sandwich



*PAA…polyacrylamide

The sandwich was closed with the clamp, still submerged in transfer buffer (150mM Glycine, 50mM Tris pH8,3, 0,05% sodium dodecylsulfate (SDS), 20% Methanol) in the tray. The chamber was filled with precooled transfer buffer and the sandwich put into the sandwich holder (black side faces black electrode), which was already in the chamber. The rest of transfer buffer was filled into the transfer chamber and the stirring rod was put inside. The electrotransfer was performed at constant 290mA overnight at 4°C.

3.6.5 Immunostaining of the blot membranes

Specific binding of the first antibody to the proteins and detection of horseradish peroxidise (HRP) conjugated to the secondary antibody with enhanced chemiluminescense (ECL-Substrat,Pierce,#32106) led to the detection of the proteins.

The transfer apparatus and the blotting sandwich were disassembled and the blot membrane cut exactly to the size of the gel with a razor blade. First of all, the PVDF membrane was blocked in blocking solution (BS) with 4% FCS **(see Tab.5a)** for 1 h at room temperature.

Tab.5a: Immunostaining buffers part1

-10x Tris-buffered saline (TBS) (Biorad # 1706435):
•1x TBS: 50mM Tris pH7,5, 150mM NaCl
-TBS-T:
•1x TBS with 0,1% Tween20 (1ml Tween20 to 1000ml TBS)
-Blocking solution (BS):
•1x TBS-T with 4% bovine serum albumin (BSA) (4g BSA in 100ml 1x TBS-T)
-Blocking solution (BS) + 4% FCS:
•add 800 µl FCS to 20 ml BS

Tab.5b: Immunostaining buffers part2

-First antibody solution:
make 1% BSA in TBS-T: add 1 vol BS to 3 vol TBS-T
1x TBS-T with 1% BSA and 0,05% Na-azide (20ml TBS-T with 1% BSA + 100µl 10% Na-azide/blot)
-Secondary antibody solution:
•TBS-T with 1% BSA

The membrane was washed 3 times for 5min in TBS-T **(Tab.5a)** on the shaker and then incubated with the first antibody **(Tab.5b and 6)** at room temperature for 2 hours or overnight at 4°C. After incubation with the first antibody, the membrane was washed again two times for 5min and two times for 10min in TBS-T. In a second step the secondary antibody **(Tab.5b and 7)**, conjugated HRP, was diluted in TBS-T + 1% BSA just before use and the membrane was incubated for 1 hour at room temperature on the shaker. After that the membrane was washed 2 times for 5min, 2 times for 10min in TBST and 2 times for 5min, 2 times for 10min in TBS **(Tab.5a)**. For detection the membrane

was incubated with a mixture of 2ml Detection solution1 + 2ml Detection solution 2 (ECL-Substrate) which underwent a chemiluminescence reaction with the horseradish peroxidise (HRP) conjugated to the secondary antibody. The protein bands were visualised by exposing the membrane to a x-ray film (Hyperfilm ECL high performance chemiluminescence film, Amersham, RPN3103K).

Tab.6: First Antibodies

1st Antibody	Dilution	Feature	Company	Cat. No	Size of target protein
mTOR	1:1000	rabbit polyclonal IgG	Cell Signaling	#2972	287kDa
p-mTOR (Ser2448)	1:1000	rabbit polyclonal IgG	Cell Signaling	#2971	287kDa
EGFR	1:500	rabbit polyclonal IgG	Cell Signaling	#2232	170kDa
p-EGFR(Tyr 1068)	1:1000	rabbit polyclonal IgG,	Cell Signaling	#2234	170kDa
ErbB-2, NEU C-18	1:200 (=1µg/ml)	rabbit polyclonal IgG	Santa Cruz Biotechnology	sc-284	185kDa
pErbB-2, pNEU(Tyr1248)	1:200 (=1µg/ml)	rabbit polyclonal IgG	Santa Cruz Biotechnology	sc-12352R	185kDa
ErbB-3, C17	1:500 (=0,4µg/ml)	rabbit polyclonal IgG	Santa Cruz Biotechnology	sc-285	185kDa
Her3/ErbB-3 (1B2E)	1:1000	Rabbit monoclonalAB	Cell Signaling	#4754	185kDa

pErbB-3 (Tyr1289)	1:1000	rabbit polyclonal IgG	Cell Signaling	#4791S	185kDa
HER4/ErbB-4 (11B2)	1:1000	Rabbit monoclonal IgG	Cell Signaling	#4795	180kDa
AKT	1:1000	rabbit polyclonal IgG	Cell Signaling	#9272	60kDa
pAKT(Ser473)	1:1000	rabbit polyclonal IgG	Cell Signaling	#9271	60kDa
pAKT(Thr308)	1:1000	rabbit polyclonal IgG	Cell Signaling	#9275	60kDa
PTEN (138G6)	1:1000	Rabbit monoclonal AB	Cell Signaling	#9559	54kDa
Mek1/2	1:1000	Rabbit polyclonal IgG	Cell Signaling	#2122	45kDa
pGSK3ß (Ser9)	1:1000	rabbit polyclonal IgG	Cell Signaling	#9336	46kDa
ERK1/2	1:3000 (=0,1183µg/ml)	rabbit immunoaffinity purified polyclonal IgG	Upstate	#06-182	42/44kDa
pERK1/2 (pP44/42 MAPK)	1:1000	rabbit polyclonal IgG	Cell Signaling	#9101S	42/44kDa
p-S6Ribosomal Protein (Ser240/244)	1:2000	rabbit polyclonal IgG	Cell Signaling	#2215S	32kDa

Alpha/beta Tubulin	1:1000	rabbit polyclonal IgG	Cell Signaling	#2148	55kDa
Actin	1:200 (=1µg/ml) or 1:300 (=0,67µg/ml)	goat polyclonal IgG	Santa Cruz Biotechnology	sc-1616	43kDa

Tab.7: Secondary antibodies

2nd Antibody	Dilution	Feature	Company	Cat. No
Donkey-anti-rabbit	1:15000	horseradish peroxidase (HRP) linked	Promega	V795A
Donkey-anti goat	1:15000	horseradish peroxidase (HRP) linked	Santa Cruz Biotechnology	sc2020
Goat-anti-rabbit	1:2000	horseradish peroxidase (HRP) linked	Cell Signaling	#7074

3.6.6 Stripping of membranes

For the possibility to use the blots again and to avoid background signals the membranes were stripped with 20ml stripping solution **(Tab.8)** and 200µl ß-Mercaptoethanol per 20ml stripping solution. The blots were stripped for 20min at 50°C in a water-bath with frequent shaking. Before the subsequent procedure was followed in the regular way by blocking the blotting membranes in BS+4%FBS for 1 h, the blots were washed several times in TBS-T.

Tab.8: Stripping solution

Reagent	Quantity
SDS	10g
1M Tris pH6,7	31,3ml
	fill up with water to 500ml

3.7 Transformation of DNA into bacteria

3.7.1 Transformation of JM-109 (E-coli) competent cells

A human myc-ca-Mek1/pBlueskript KS plasmid on a filter paper was generously provided by Hiroshi Kiyama (Inst. of Japan, Namikawa et al., 2000) and for efficient transfection into eukaryotic cells, the myc-tagged constitutively active MEK1 fragment had to be excised and cloned in pcDNA3, which represents an ideal expression vector for eukaryotic cell lines.

A piece from the filter paper containing human myc-ca-MEK1/pBlueskript KS was cut off and dissolved in 100µl TE buffer, incubated for 2 hours at room temperature. Then 5µl of this solvent plasmid and pBlueskript control (kindly provided by Michael Grusch, Institute for Cancer Research) were added to 100µl competent cells (JM-109, E.coli) and stored on ice for 20min. After 1 min heat shock at 42°C 1ml SOC medium **(Tab.9)** was added to the cells. Two hours later cells were harvested by centrifugation for 3min at 2000g and the supernatant was discarded. 100µl were left back and the resulting culture was spread out on LB **(Tab.10)** plates (containing 50µg/ml ampicillin) and grown overnight at 37°C.

Tab.9: SOC-medium

Reagent	Quantity
tryptone	20g
yeast extract	5g
10mM NaCl	0,584g
2,5mM KCl	0,186g
10mM $MgCl_2$	0,952g
20mM glucose	3,603g
	adjust the pH to 7.0 with sodium hydroxide
	fill up with water to 1000ml
	autoclave at 121°C for 20min

Tab.10: LB-medium

Reagent	Quantity
tryptone	10g
yeast extract	5g
NaCl	10g
	adjust the pH of LB to 7.5 or 8 with sodium hydroxide
	fill up with water to 1000ml
	autoclave at 121°C for 20min

3.7.2 Plasmid isolation – Miniprep “Quick `n` Dirty” – Boiling lysis

A couple of eprouvettes containing 6ml LB-Broth (Sigma, L3022-1kg) with ampicillin (stock: 50mg/ml, Roche Art. Nr.10 835 242001, Lot 93271021) were inoculated with different colonies taken from the agar plate and incubated at 37°C overnight. The next day 1.5ml of the overnight culture were transferred to a 1.5ml microcentrifuge tube and harvested by centrifugation for 1min at 16000g. To increase the yield this step was repeated again. Then the medium is aspirated, the pellet was resuspended in 700µl STET-buffer (8% glucose, 5% Triton X100, 50mM EDTA and 50mM Tris pH8) and 13µl lysozyme (stock 50µg/ml, Sigma L-7651) was added. The tube was mixed 3 times and boiled at 100°C for 1-2min. The proteins were pelleted at 12000-16000g for 10 min and the pellet was discarded with an RNAse A (stock 10mg/ml, Sigma R-5503) covered toothpick. After adding 700µl isopropanol the plasmid DNA was precipitated for 20 min at -20°C and pelleted at 4°C with top speed for 15min.The supernatant was discarded and the pellet was washed with 500µl of 70% ethanol, and then centrifuged at room temperature with top speed for 5min. Then the ethanol was aspirated and the step was repeated again. After the pellet was dry it was resuspended in 20µl TE-buffer and stored at 4°C or -20°C.

3.7.3 Restriction assay for control

To control if the right colony was picked from the agar plate, a restriction assay was prepared in order to cut the plasmid in pieces. The use of restriction enzymes depends on the vector. Clone manager software was used to choose the right enzymes. For restriction analysis Aqua bidest. DNA and the 10xbuffer were prepared in a 1.5ml microcentrifuge tube and finally restriction enzymes were added **(Tab.11)**.

Tab.11: Restriction analysis

Probe	pBluscript 1	pBluscript 2
DNA	1µl	1µl
Restriction Enzyme I	1µl NotI	1µl XhoI
Activity	10U/µl, 1500U	10U/µl, 2000U
Company	Fermentas # ER 0592	Fermentas # ER 0691
Restriction Enzyme II	1µl KpnI	1µl BamHI
Activity	40U/µl, 10000U	10U/µl, 4000U
Company	Roche # 742953	Fermentas # ER 0051
10xBuffer	1µl 10x buffer BamHI	1µl 10x buffer BamHI
Company	Fermentas # B57	Fermentas # B57
Aqua bid.	6 µl	6µl
Sum	10µl	10µl

The mix was incubated for at least 2 hours at 37°C. Then a 1% agarose gel (Biozyme LE Agarose Art Nr 840004, 500g) was poured. To detect the bands, 10µl of the restriction mix were mixed with 2µl Vistra-Green (GE Healthcare) and loaded on the gel. Furthermore a marker solution consisting of 1µl Gene Ruler 1kb DNA ladder (Fermentas #SM1163) mixed with 9µl Aqua bidest. and 2µl Vistra-Green was loaded on the gel. For electroporetic seperation the gel was run for 1 hour at 110V in 0,5 x TBE buffer **(Tab.12)**. Data were analyzed using Fluor Imager 595 Scanner (Molecular Dynamics). The obtained MEK1 fragment had a size of 1181bp **(Fig.13)**.

Tab.12: 0,5 x TBE buffer

Reagent	Quantity
Tris	54g
Boric acid	27,5g
0,5ml EDTA/pH8,0	20ml
Aqua bidest.	fill up to 10l

3.7.4 Restriction assay for preparing vector DNA (pcDNA3) and insert DNA (MEK1) for ligation

After the control on the gel 19µl of the pBluescript (left from the miniprep) containing the MEK1 insert **(Fig.13)** and a pcDNA3 vector **(Fig.14)**, (kindly provided by Michael Grusch, Institute for Cancer Research) were prepared and cut with restriction enzymes **(Tab.13)** and religated.

Fig.13: pBlueskript KS – MEK1

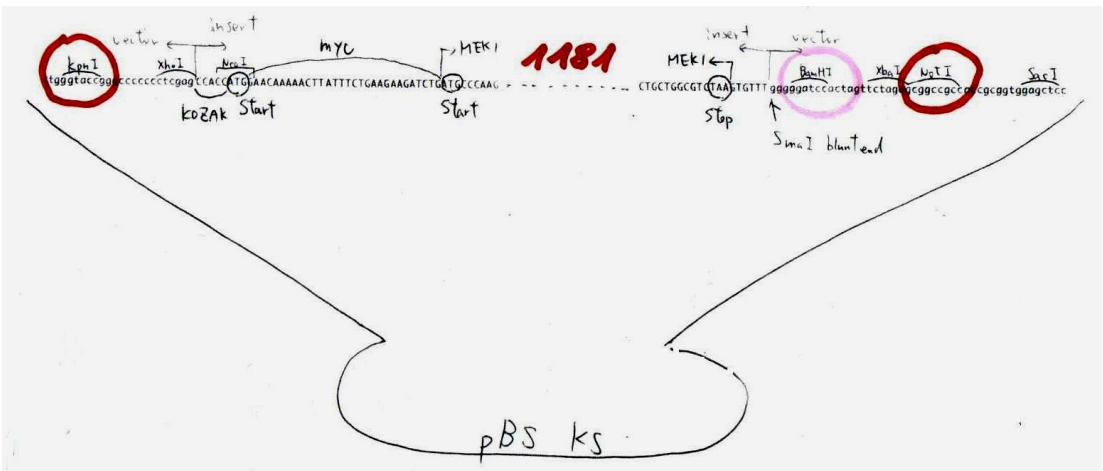
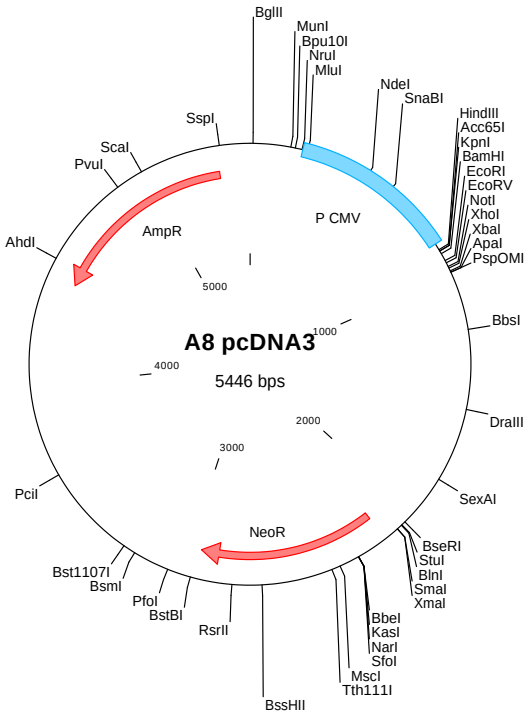


Fig.14: Restriction map of pCDNA3 (Institute of Cancer Research)



Tab.13: Restriction analysis

Probe	pBluscript MEK1	pcDNA3
DNA	19 µl	20µl
Restriction Enzyme I Activity Company	3µl KpnI 40U/µl, 10000U Roche # 742953	3µl KpnI 40U/µl, 10000U Roche # 742953
Restriction Enzyme II Activity Company	3µl BamHI 10U/µl, 4000U Fermentas # ER 0051	3µl BamHI 10U/µl, 4000U Fermentas # ER 0051
10xBuffer Company	6µl 10xbuffer BamHI Fermentas # ER 0051	6µl 10xbuffer BamHI Fermentas # ER 0051
Aqua bid.	29 µl	28µl
Sum	60µl	60µl

After incubation of the restriction mix the vector was dephosphorylated to avoid re-ligation by exposure to Calf Intestine Alkaline Phosphatase (CIAP), (Fermentas #Ef03U1).

Procedure:

pCDNA3
-pCDNA3 60µl + 1µl CIAP
-30min 37°C
-30min 56°C
+ 1µl CIAP
-30min 37°C
-30min 56°C

This reaction mix was incubated for 2 hours and then a 1% agarose gel was poured. For further experiments 60µl of the restriction mix MEK1 and 62µl of the dephosphorylated restriction mix pcDNA3 were mixed with 12µl Vistra-Green (GE Healthcare) and loaded on the gel. In addition a marker solution as mentioned above was loaded on the gel. For electroporetic seperation the gel was run for 1 hour at 110V in 0,5 x TBE buffer. Data were analyzed using the Fluor Imager 595 Scanner and the obtained bands of MEK1 (1181bp) and pcDNA3 (5446bp) were cut out of the gel and cleaned by using the Wizard SV Gel and PCR Clean-Up System (Promega Part #9F B072,Lot #259073, Exp. Date: JUL11).

3.7.5 Ligation of pCDNA3 (vector) with Mek1 (insert)

The gel purification was performed as described in the protocol and in addition 2µl of each isolated fragment was loaded on a 1% agarose gel with appropriate amount of Vistra-Green. Gel electrophoresis was performed at 110V for 2 hours. To figure out the concentration of vector DNA and insert DNA which were cut out of the gel, their quantities were calculated in relation to the 1000bp band of the marker. The vector DNA contained 271ng/µl and the insert DNA 178ng/µl. For the ligation assay the data were calculated using Image Quant 5.0

software and after calculation of the ratio between vector and insert the reagents were prepared for the ligation reaction **(Tab.14)**.

Tab.14: Ligation reaction

	Ligation 1 3x surplus of insert	Ligation 2 5x surplus of insert	Ligation 3 Negative Control
Vector-DNA	0,5µl	0,5µl	0,5µl
Insert-DNA	0,49µl	0,82µl	
10x T4 Ligase buffer Fermentas # B69	1µl	1µl	1µl
T4 DNA Ligase 5U/µl, 200U,Fermentas# EL0014	1µl	1µl	1µl
Aqua bid.	7µl	6,7µl	7,5µl
Sum	10µl	10µl	10µl

Then the ligation mix was incubated overnight at 16°C and 5µl DNA of ligation1 (3x surplus of insert), ligation2 (5x surplus of insert) and ligaton3 (negative control) were transformed in competent JM-109 (E.coli). on the next day, 12 eprouvettes containing 6ml LB-Broth supplemented with 50µg/ml ampicillin were inoculated with different colonies taken from the agar plate and incubated at 37°C overnight. 1ml LB-Broth from each eprouvette containing transformed bacteria was put into the freezer for later experiments. 24 hours later a Plasmid Miniprep like the “Quick `n` Dirty” – Boiling lysis protocol above was performed.

3.7.6 Restriction assay after Ligation

After plasmid isolation a restriction assay **(Tab.15)** was prepared to look if one of the 12 clones contained the right plasmid with MEK1 as insert with a size of 1181bp.

Tab.15: Restriction assay

Probe	Ligation 1-12
DNA	1µl
Restriction Enzyme I Activity Company	1µl KpnI 40U/µl, 10000U Roche # 742953
Restriction Enzyme II Activity Company	1µl BamHI 10U/µl, 4000U Fermentas # ER 0051
10xBuffer Company	1µl 10xbuffer BamHI Fermentas # ER 0051
Aqua bidest.	6 µl
Sum	10µl

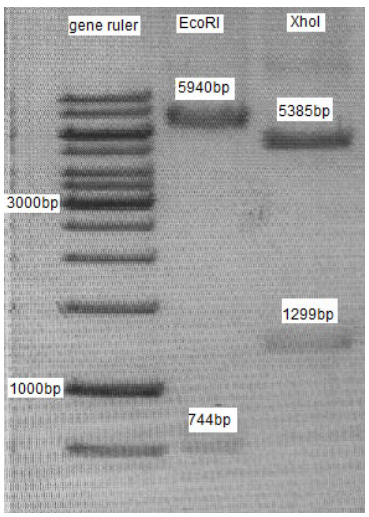
After the mix was incubated for 2 hours at 37°C, a 1% agarose gel was prepared and 10µl restriction mix supplemented with 2µl Vistra-Green and the marker solutuion was loaded. The gel was run for 1 hour at 110V and data were analysed using the Fluor Imager 595 Scanner. MEK1 fragment with 1181 bp size was expected. One clone exhibited the right size and to convince the correctness of this clone a further restriction assay was performed **(Tab.16)**.

Tab.16: Restriction assay

Probe	Right Clone	Right Clone
DNA	2µl	2µl
Restriction Enzyme I Activity Company	0,5µl EcoRI 10U/µl, 5000U Fermentas# ER 0271	0,5µl XhoI 10U/µl, 2000U Fermentas # ER 0691
10xBuffer Company	1µl 10x buffer EcoRI Fermentas # B12	1µl 10x buffer Red Fermentas # BR5
Aqua bid.	6,5 µl	6,5µl
Sum	10µl	10µl

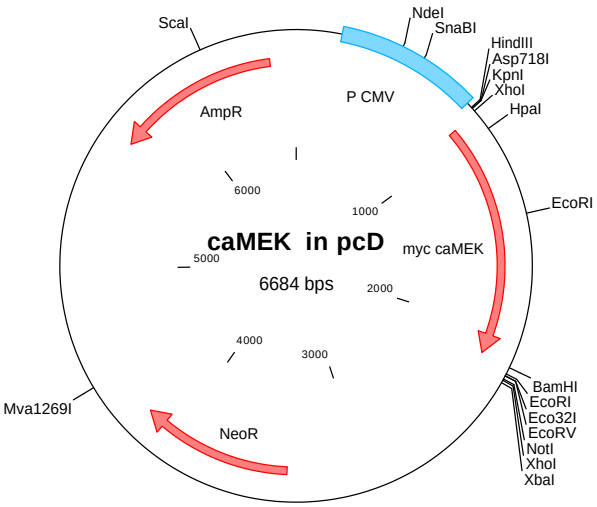
The restriction mix was incubated at 37°C for 2 hours and was then loaded on a 1% agarose gel and bands were analyzed expecting fragments after EcoRI digestion of 744bp and 5940bp and after XhoI digestion fragments of 1299bp and 5385bp **(Fig.15)**.

Fig.15: EcoRI and XhoI cut



By this transforming and cloning procedure we obtained a pcDNA3 plasmid containing the constitutively active MEK1 **(Fig.16)**.

Fig.16: Constitutively active Mek1 in pcDNA3



3.7.7 PureYield Plasmid Midiprep

After the correct clone has been obtained, 10µl from the remaining 1ml LB-Broth was inoculated into 100ml LB-Broth containing 50µl/ml ampicillin. As well as the correct clone, EGFP (Institute of Cancer Research) and dominant active murine AKT, which was a kind of gift from D. Efremov [(International Centre for Genetic Engineering & Biotechnology, Monterotondo Scalo, Italy), Longo et al., 2007] were inoculated into 100ml LB-Broth containing ampicillin. These cultures were incubated overnight at 37°C and isolated using PureYield Plasmid Midiprep System (Promega Cat # A2492). The DNA concentrations were calculated with Nanodrop ND-1000.

3.8 Transfection of DNA into eukaryontic cells

3.8.1 Transient transfection

One day before transfection, $8x10^5$ SKBR3 cells were plated in 2ml culture medium (D-MEM supplemented with 10% FCS and 1% PSG) in 6-well plates (Corning Incorporated, 3506). The cells should be 80% confluent before transfection. Next day, the attached cells were controlled under the microscope, the culture medium was removed and 2ml culture medium was added again. Further, the transfection sample was prepared (mix for 1 well) diluting plasmid DNA **(Tab.17)** in 400µl Opti-MEM I (Invitrogen, Cat.No. 31985-062), mixing, and adding 8µl Lipofectomine LTX reagent (Invitrogen, Cat.No. 15338-100) directly to the diluted DNA. This complex was incubated for 30min at room temperature. The Opti-MEM-DNA-LTX containing complex was added to the well with the cells and mixed gently. The cells were incubated at 37°C in a CO2 incubator for 24 hours. To check the transfection efficiency, a EGFP plasmid was used as control, which was expressed in the cell 24h after the transfection. Under the microscope, cells appeared green. The used filter block for the microscope I $2/_3$ emitted blue light. After microscopic control, the transfection complex was removed, and 2ml culture medium were again added. One day later, cells were trypsined with 300µl 0,05% Trypsin-EDTA, counted and seeded in 96-well plates for EZ4U growth assays or in 60mm petri dishes for Western blot analysis.

Tab.17: Plasmid DNA

Plasmide	Concentration	Source
pcDNA 3	647,9ng/µl	Institute of Cancer Research
pcDNA3 EGFP	207,7ng/µl	Instiute of Cancer Research
pcDNA3 MEK1	533,54ng/µl	Prepared by Cloning
pcDNA3 AKT	315,1ng/µl	Dimitar Efremov Longo et al., 2007

4 Results

4.1 EGFR/ErbB-2 receptor tyrosine kinase inhibitor pelitinib inhibits monolayer growth of SKBR3 but not of T47D breast cancer cells

Previous studies have shown that small-molecule tyrosine kinase inhibitors (TKIs), such as anti-EGFR and anti-ErbB2 drugs inhibit the growth of breast carcinoma cell lines (Moulder et al., 2001 and Erlichman et al., 2006). Whereas the majority of patients with breast cancer do not respond to TKIs, due to intrinsic or acquired resistance (Normanno et al., 2008).

SKBR3 and T47D human breast cancer cells proved to be sensitive and resistant against EGFR/ErbB-2 receptor TKI pelitinib (EKB-569), respectively. SKBR3 and T47D were cultured as described in Material and Methods. Sensitivity of SKBR3 and the resistance of T47D against pelitinib were demonstrated in invitro growth assays. Cells were treated for 72h with 0,1µM, 1µM, 2µM, 4µM, 6µM, 10µM and 15µM pelitinib. SKBR3 cells, a ErbB-2 overexpressing but estrogen receptor (ER) and progesterone receptor (PR) negative breast cancer cell line revealed a IC50 of 2,67µM **(Fig.17)**. Whereas T47D cells, which are positive for ER and PR but do not overexpress ErbB-2 demonstrated striking resistance against pelitinib with a four- to five-fold higher IC50 value (12,06µM) than SKBR3 **(Fig.17)**.

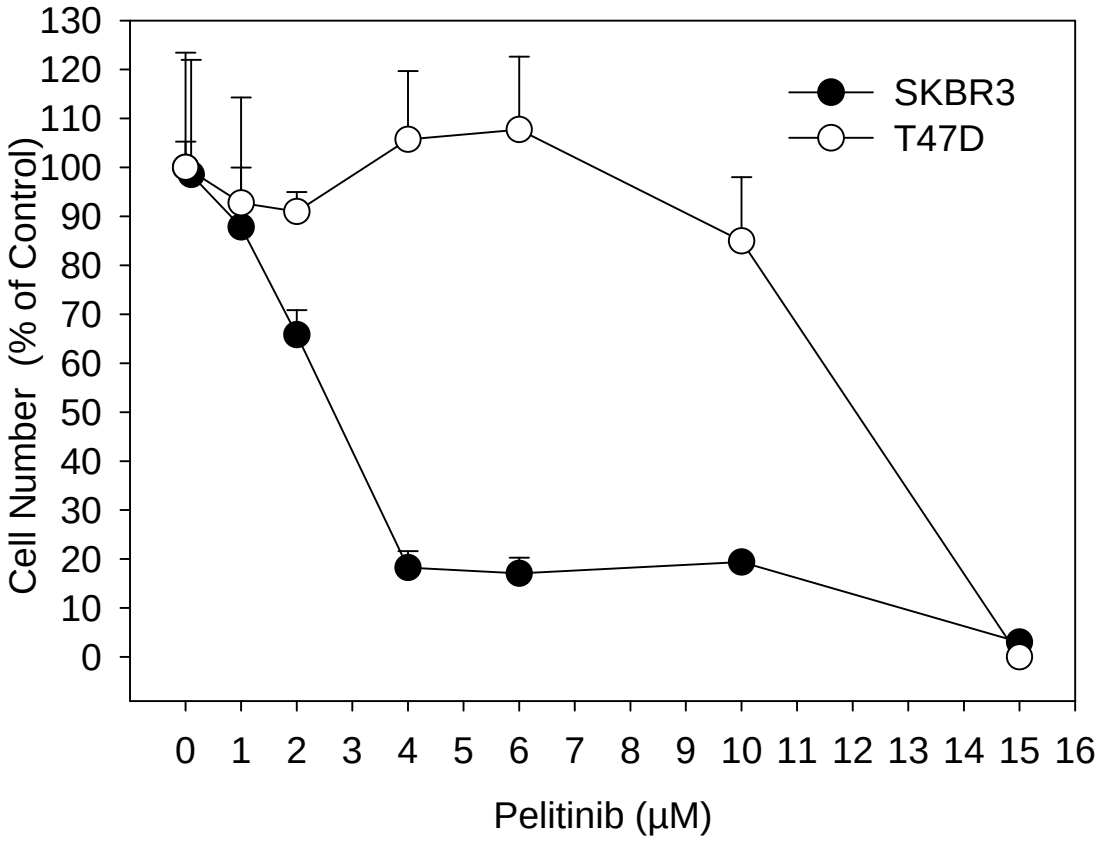


Fig.17: Breast cancer cell lines SKBR3 and T47D exposed to pelitinib*. Cells were grown in 96-well plates, allowed to attach o.n. and treated for 3 days with 0,1µM, 1µM, 2µM, 4µM, 6µM, 10µM and 15µM pelitinib. Before the absorbance was measured at 450nm against a 655nm reference wavelength with a microplate reader spectrophotometer, the EZ4U reagent was added.*

4.2 ErbB receptor expression profile and downstream signaling in SKBR3 and T47D breast cancer cells

To identify possible molecular parameters for sensitivity or resistance of SKBR3 and T47D cells against pelitinib the patterns of ErbB receptor expression and of the downstream signaling proteins were examined by Western blot analysis **(Fig.18)**. SKBR3 and T47D were plated in serum-free media. Twenty-four hours later protein extracts were prepared as described in Material and Methods.

The ErbB receptor signaling pathway and its downstream signaling components play an important role in cell survival, cell proliferation and cell differentiation. ErbB-2 is overexpressed in 20-30% of human breast carcinomas and correlate with an aggressive behaviour and poor clinical prognosis. SKBR3 cells overexpress EGFR. They also exhibit a high-copy-number amplicon in the centromere region of chromosome 17 containing the ErbB-2 onco-gene providing high ErbB-2 expression (Szöllösi et al., 1995 and Shadeo et al., 2006). We compared ErbB expression and phosphorylation in SKBR3 and T47D cells. In SKBR3 cells the ErbB-2 receptor appears to be constitutively phosphorylated whereas EGFR is not phosphorylated in the absence of growth factors or serum **(Fig. 18)**. SKBR3 show low levels of ErbB-3, which is however highly phosphorylated. In contrast, ErbB-4 protein and phosphorylation was not detectable in SKBR3 by Western blotting **(Fig.18)**.

T47D reveal constitutive expression of all four ErbB receptors. However, the levels of EGFR, ErbB-2 and ErbB-3 are lower than in SKBR3 **(Fig.18).** Moreover, in contrast to SKBR3 T47D exhibit ErbB-4 receptor expression. From these four ErbB receptors only ErbB-2 and ErbB-3 are constitutively phosphorylated **(Fig.18)**.

In summary, SKBR3 cells express relatively high levels of EGFR, ErbB-2 and ErbB-3, but no ErbB-4 whereas T47D express relatively low levels of all four ErbB receptors. In both cell lines, constitutive activation/phosphorylation is seen for ErbB-2 and ErbB-3, but not for EGFR and ErbB-4. Thus, while the principal building of ErbB baseline signal transduction appears quite similar in the nature

in both cell lines emanating from ErbB-2/ErbB-3 heterodimers, the intensity appears higher in SKBR3 than in T47D probably due to receptor overexpression in the former.

We next examined expression and baseline phosphorylation of AKT and ERK1/2, the crucial mediators of the PI3K and MAPK pathways, respectively **(Fig19)**, which represent the two major downstream signaling cascades of ErbB receptors. Both AKT and ERK1/2 are expressed at similar levels in both cell lines. However, despite relatively high intrinsic ErbB-2/ErbB-3 activity in SKBR3, pAKT and pERK1/2 levels are yet markedly lower in SKBR3 when compared to T47D cells indicating high constitutive activation of ErbB downstream pathways in T47D cells.

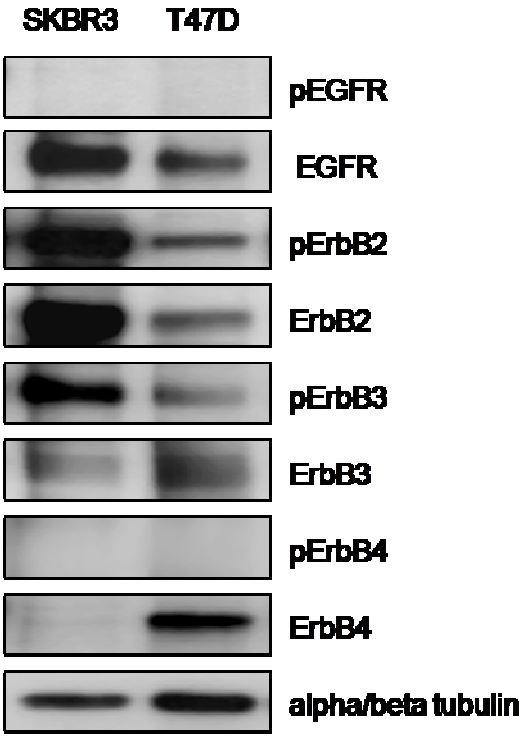


Fig.18: Total protein from SKBR3 and T47D cells were labeled with anti-EGFR, ErbB-2, ErbB-3, ErbB4 or anti-pEGFR, pErbB-2, pErbB-3, pErbB4 antibodies. *Cells were allowed to attach o.n. in 5% FCS and were grown in 60 mm dishes. Medium was changed to medium without FCS for 24 hours. Before sample preparation the cultures were exposed for 6 hours to DMSO. Alpha/beta tubulin was detected for loading control.*

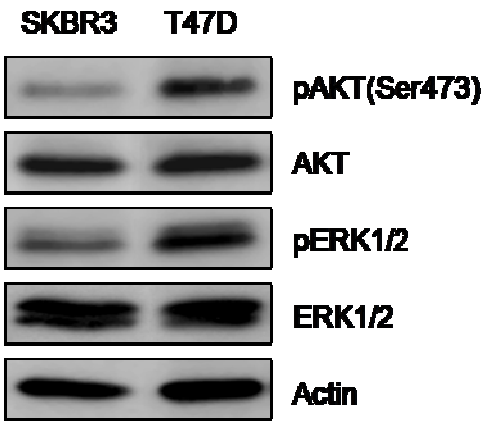


Fig.19: Total protein from SKBR3 and T47D cells were labeled with anti-AKT, ERK1/2 or anti-pAKT(Ser473) and pERK1/2 antibodies*. Cells were allowed to attach o.n. in 5% FCS and were grown in 60 mm dishes. Medium was changed to medium without FCS for 24 hours. Before sample preparation the cultures were exposed for 6 hours to DMSO. Actin was detected for loading control.*

4.3 Stimulation of ErbB receptors and of ErbB downstream pathways by EGF

ErbB receptors are activated by a repertoire of ErbB ligands which include EGF, amphiregulin (AR) and transforming growth factor-α (TGF-α), which bind to EGFR, and betacellulin (BTC), heparin-binding EGF (HB-EGF) and epiregulin (EPR) which interact with EGFR and ErbB-4. The third family of ligands is termed neuregulins or heregulins, which include HRG1 and HRG2 that bind to ErbB-3 and ErbB-4, and HRG3 and HRG4 which bind exclusively ErbB-4 (Olayioye et al., 2000).

Here we used EGF as a prototype ligand for stimulation of EGFR and the ErbB downstream pathways and compared signaling behavior in SKBR3 and T47D cells by using Western blotting analysis. Cells were cultured in normal growth medium with 5% FCS for one day and then depleted of serum for another day. SKBR3 and T47D cells were stimulated for 1, 2, 3, 10 or 20min with EGF. Antibodies against ErbB receptors or against their phosphoylated form were used. Actin was detected as a control for equal loading and efficient protein transfer on to the PVDF membranes. During the course of our studies we found that 2min of exposure to EGF is required to stimulate EGFR phosphorylation in SKBR3 **(Fig.20)**, whereas in T47D, 1min is already sufficient to stimulate EGFR **(Fig.21)**. Generally, in both cell lines EGF-mediated phosphorylation of EGFR and AKT is transient and returns near to baseline levels after 20min EHF stimulation, whereas ERK1/2 remains phosphorylated during the whole period of EGF exposure **(Fig.20 and 12)**. Furthermore, ErbB-2 just as ErbB-3 is constitutively active and is resistant to further stimulation by EGF. Interestingly, ErbB receptors respond to EGF stimulation quite similarly compared to SKBR3, irrespective of the fact that T47D express much less ErbB receptors than SKBR3 **(Fig.18)**. Moreover, in both cell lines 1min EGF stimulation is long enough to trigger the major ErbB controlled downstream signaling cascades, which is evidenced by increased levels of pAKT(Ser473), pAKT(Thr308) and pERK1/2 **(Fig.20 and 21)**.

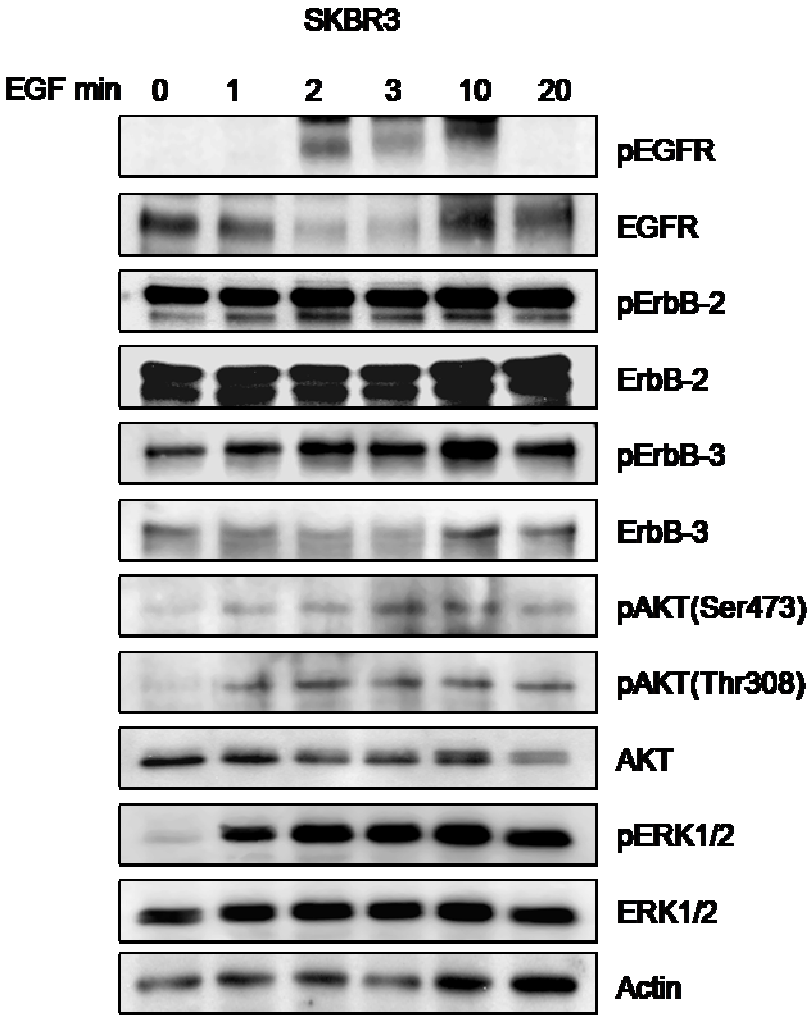


Fig.20: Total protein from SKBR3 cells were labeled with anti-EGFR, ErbB-2, ErbB-3, ErbB4, AKT, ERK1/2 or anti-pEGFR, pErbB-2, pErbB-3, pErbB4, pAKT (Ser473/Thr308) and pERK1/2 antibodies. *Cells were allowed to attach o.n. in 5% FCS and were grown in 60 mm dishes. Medium was changed to medium without FCS for 24 hours. Before sample preparation the cultures were exposed for 0,1, 2, 3, 10, 20min to recombinant human EGF(100ng/ml). Actin was detected for loading control*

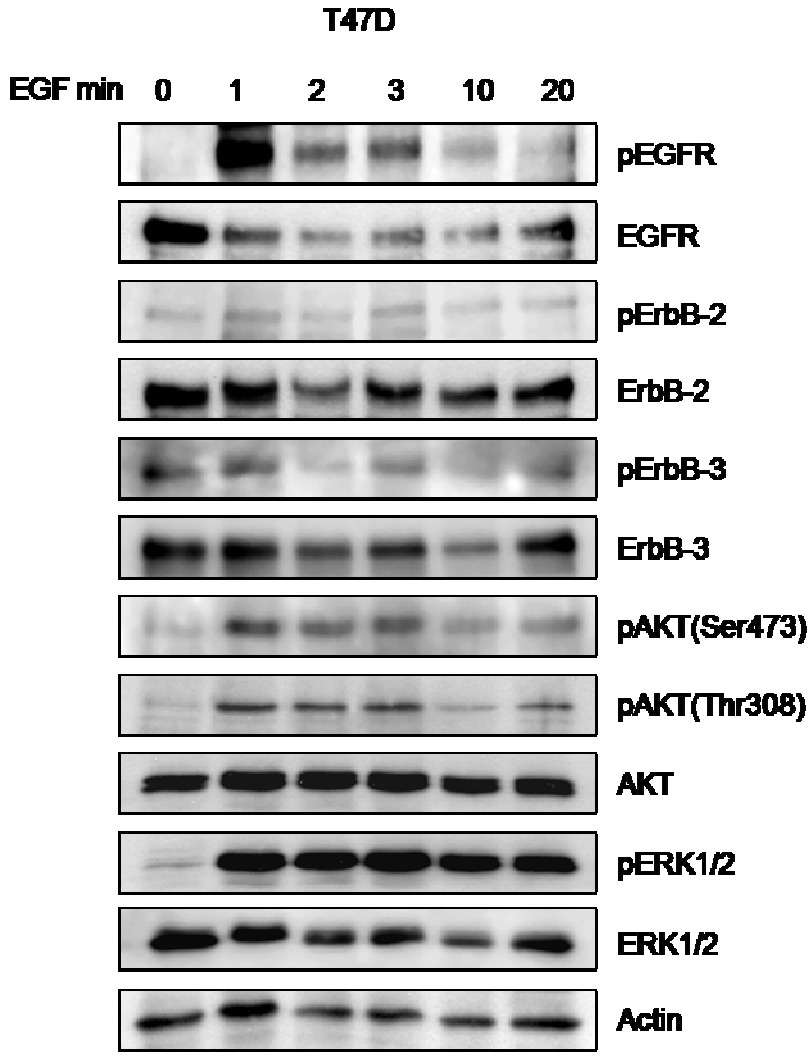


Fig.21: Total protein from T47D cells were labeled with anti-EGFR, ErbB-2, ErbB-3, ErbB4, AKT, ERK1/2 or anti-pEGFR, pErbB-2, pErbB-3, pErbB4, pAKT (Ser473/Thr308) and pERK1/2 antibodies*. Cells were allowed to attach o.n. in 5% FCS and were grown in 60 mm dishes. Medium was changed to medium without FCS for 24 hours. Before sample preparation the cultures were exposed for 0,1, 2, 3, 10, 20min to recombinant human EGF(100ng/ml). Actin was detected for loading control.*

4.4 The tyrosine kinase inhibitor pelitinib inhibits ErbB receptor signaling in SKBR3 and T47D cells stimulated with EGF and heregulin (HRG)

To test the function of the irreversible EGFR/ErbB-2-dual small-molecule RTKI pelitinib (Wissner et al., 2008) we cultivated SKBR3 and T47D cells as described in Material and Methods, depleted for one day from serum and then treated the cells for 6 hours with 0,1µM, 1µM, 2µM, 4µM or 8µM pelitinib. Finally, the cells were stimulated with 3min with EGF and heregulin ß1 (HRGß1). Results were then detected by western blotting analysis. Alpha/beta tubulin was used as loading control. First it was demonstrated that EGFR and the ErbB-3 are activated by EGF and HRGß1 stimulation in both SKBR3 and T47D cells **(Fig.22 and 23).** It has to be mentioned that ErbB-3 not only requires ligand for activation, but also has to form a heterodimer with one of the other ErbB receptors. For instance, cross talk between ErbB-3 and ErbB-2 triggers a very strong signaling cascade although each alone represents an invalid receptor. ErbB-2 lacks a cognate ligand and thus does not function as a receptor in the classical meaning domain, whereas ErbB-3 lacks functional tyrosine kinase. Nevertheless, these receptors complement each other and cooperate during signaling. In SKBR3 it is not possible to activate ErbB-2 because it is already maximally active even in the absence of ligands **(Fig.22)**. Interestingly, pretreatment for 6 hours with concentrations as low as 0,1µM of pelitinib depresses pEGFR,pErbB-2 and pErbB-3 levels already below those seen without ligands. ErbB-2 is constitutively active in both cell lines and requires relatively high concentrations of pelitinib for inhibition which was accompanied by reduced steady-state levels of ErbB-2 protein.

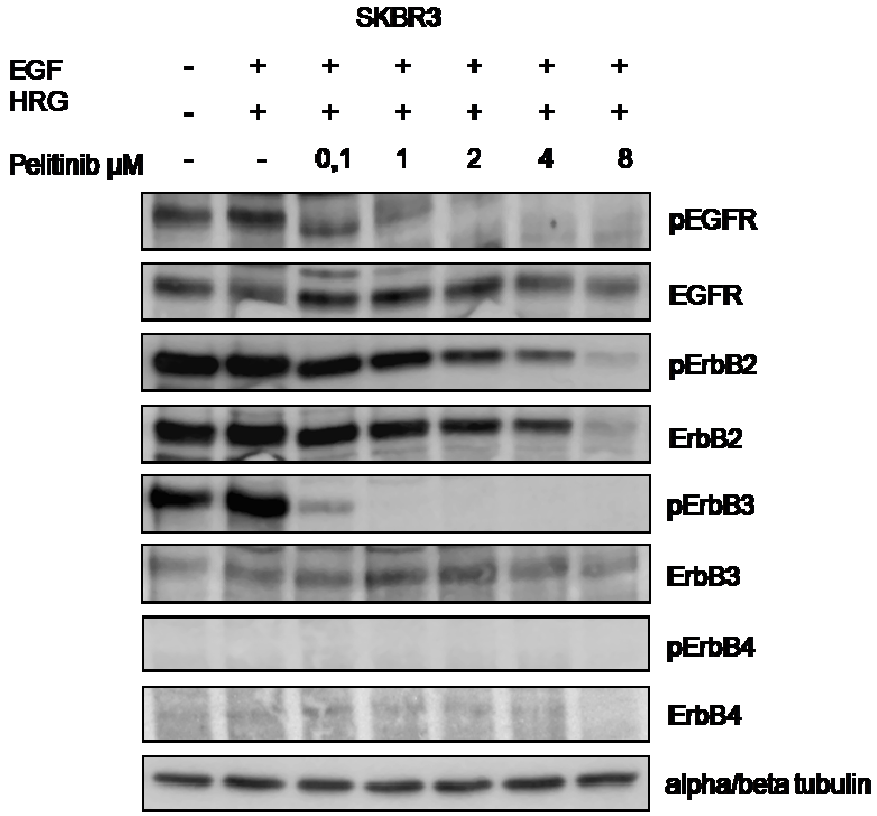


Fig.22: Breast cancer cell line SKBR3 is exposed to anti-EGFR, ErbB-2, ErbB-3, ErbB4, AKT, ERK1/2 or anti-pEGFR, pErbB-2, pErbB-3, pErbB4, pAKT (Ser473/Thr308) and pERK1/2 antibodies. *Cells were allowed to attach o.n. in 5% FCS and were grown in 60 mm dishes. Medium was changed to medium without FCS for 24 hours. The cultures were treated with 0,1µM, 1µM, 2µM, 4µM and 8µM pelitinib for 6 hours. Before sample preparation the cultures were exposed for 3min to recombinant human EGF(100ng/ml) and to heregulin ß1 (1nM). Alpha/beta tubulin was detected for loading control.*

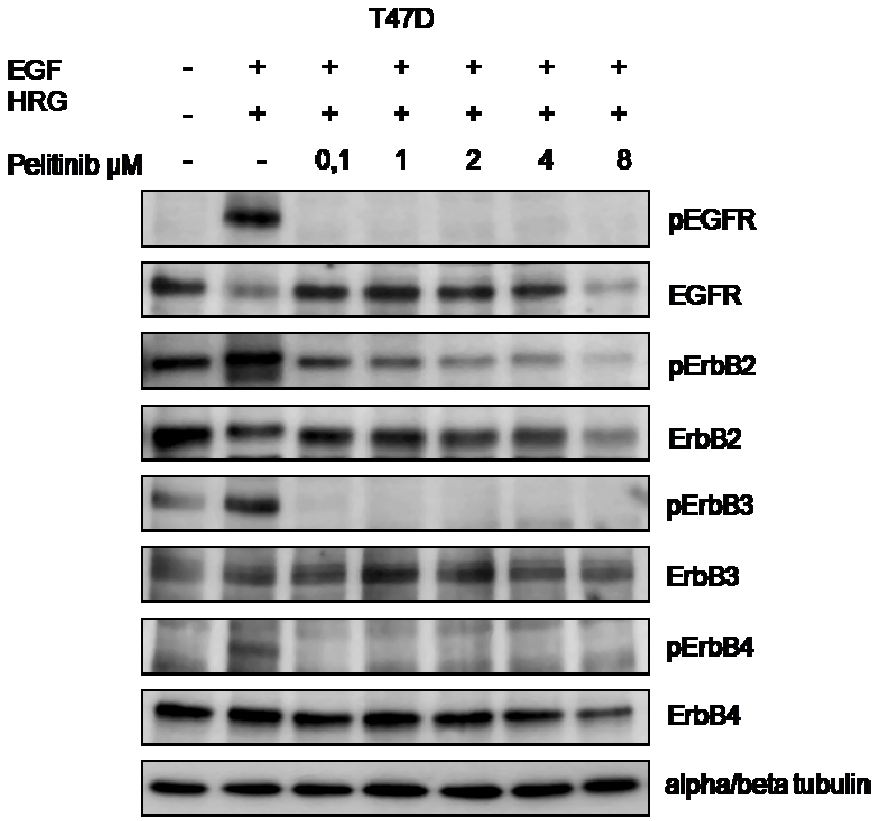


Fig.23: Breast cancer cell line T47D is exposed to anti-EGFR, ErbB-2, ErbB-3, ErbB4, AKT, ERK1/2 or anti-pEGFR, pErbB-2, pErbB-3, pErbB4, pAKT (Ser473/Thr308) and pERK1/2 antibodies. *Cells were allowed to attach o.n. in 5% FCS and were grown in 60 mm dishes. Medium was changed to medium without FCS for 24 hours. The cultures were treated with 0,1µM, 1µM, 2µM, 4µM and 8µM pelitinib for 6 hours. Before sample preparation the cultures were exposed for 3min to recombinant human EGF(100ng/ml) and to heregulin ß1 (1nM). Alpha/beta tubulin was detected for loading control.*

4.5 The effects of pelitinib on ErbB downstream signaling in SKBR3 and T47D cells by pelitinib

4.5.1 In the presence of serum supplementation

ErbB family reseptors signal via mitigen-activated protein kinase (MAPK) and phosphatidylinositol-3kinase (PI3K)/AKT cascades. AKT is a serine/threonine kinase also known as protein kinase B (PKB). Activated AKT plays a crucial role in progression of various breast carcinomas and can cause intrinsic or aquired drug resistance, including endocrine resistance (Tokunaga et al., 2006). In SKBR3 and T47D which proved to be sensitive and resistant against the ErbB TKI pelitinib, respectively, we demonstrated significant differences in downstream signaling. The cells were cultivated as described, cultivated for one day in medium containing 5% FCS and then treated for 6 hours with 0,1µM, 1µM, 2µM, 4µM and 8µM pelitinib following by Western blotting analyses. Actin was used as loading control. In SKBR3, which were growth inhibited by pelitinib **(Fig.17)**, the drug completely abrogated of AKT phosphorylation at serine 473 and threonine 308 at a concentration as low as 0,1µM (**Fig.24**). In contrast T47D, cells are not growth inhibited by pelitinib and do not show reduced pAKT (Ser473) and pAKT (Thr308) levels upon pelitinib treatment (**Fig.25**). Interestingly, pERK1/2 was inhibited by pelitinib in both cell lines irrespective of growth sensitivity or resistance to the drug.

Previous studies have demonstrated that constitutive activation of AKT could be caused by a deletion or mutation of PTEN, a protein and lipid phosphatase (Wu et al., 1998 and Dahia et al., 1999). Loss of PTEN, associated with ErbB overexpression means that PI3K-mediated signaling is hyperactive. Therefore, we examined the PTEN status in both cell lines and show that both. SKBR3 and T47D, maintain PTEN expression independent of pelitinib treatment **(Figs.24 and 25)**.

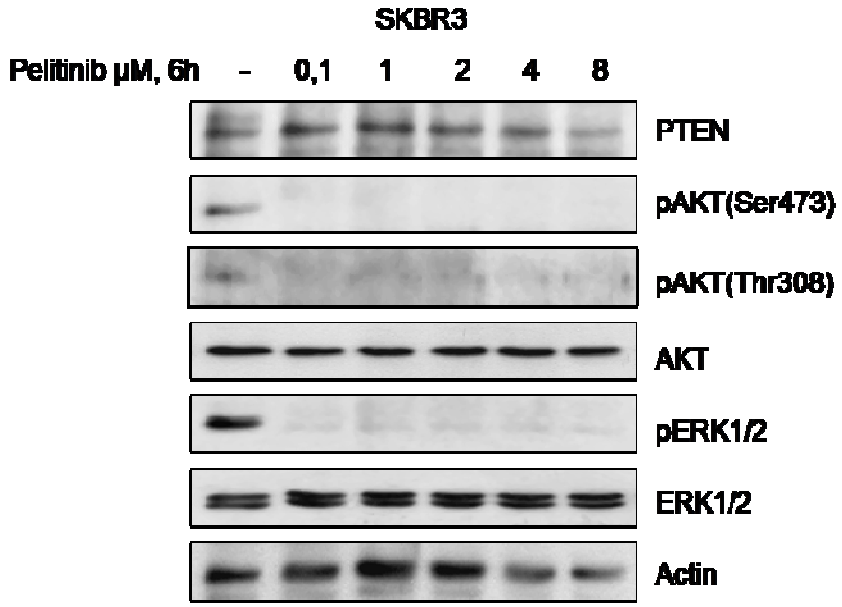


Fig.24: Total protein from SKBR3 cells were labeled with PTEN, anti-AKT, ERK1/2 or anti-pAKT (Ser473/Thr308) and pERK1/2 antibodies*. Cells were allowed to attach o.n. in 5% FCS and were grown in 60 mm dishes. Before sample preparation the cultures were treated with 0,1µM, 1µM, 2µM, 4µM and 8µM pelitinib for 6 hours. Actin was detected for loading control.*

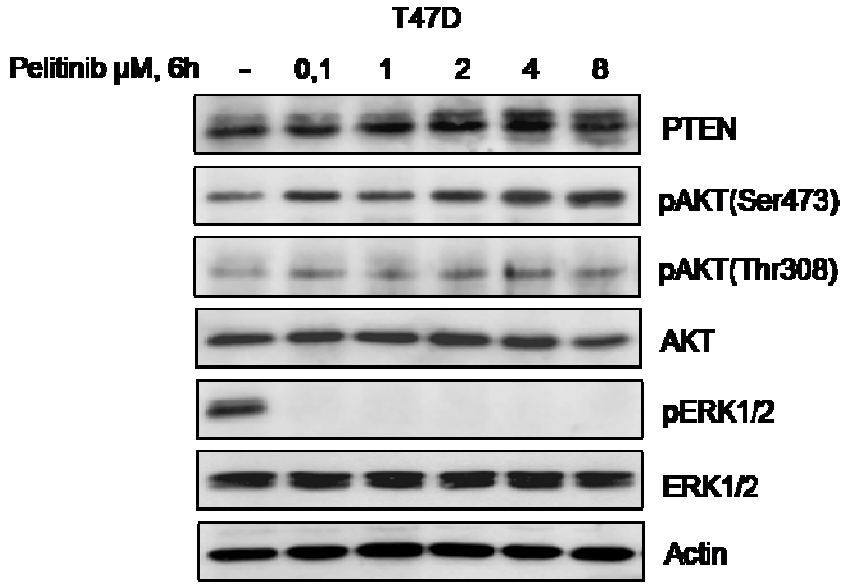


Fig.25: Total protein from T47D cells were labeled with anti-AKT, ERK1/2 or anti-pAKT (Ser473/Thr308) and pERK1/2 antibodies*. Cells were allowed to attach o.n. in 5% FCS and were grown in 60 mm dishes. The cultures were treated with 0,1µM, 1µM, 2µM, 4µM and 8µM pelitinib for 6 hours before sample preparation. Actin was detected for loading control.*

4.5.2 In the presence of serum supplementation and exogenous EGF

To examine where this inhibitor-resistant AKT phosphorylation in T47D comes from we raised AKT phosphorylation by exposure of the cells to EGF. SKBR3 and T47D were cultivated for one day in 5% FCS then pelitinib was added for another 6 hours followed by 20min of EGF stimulation, which significantly activated phosphorylation of AKT and ERK1/2 in both cell lines (data not shown), respectively. Treatment with pelitinib inhibits the EGF stimulus in SKBR3 and T47D. However, in T47D a marked baseline level of AKT phosphorylation triggered at serine 473 and threonine 308 persisted even at high doses of pelitinib stimulation, whereas in SKBR3 phosphorylation of both amino acids is completely inhibited by pelitinib even at the lowest concentration tested (0,1µM, **Figs.26 and 27**). Baseline phosphorylation of AKT at serine 473 and threonine 308 can be caused by activating mutations in AKT or in PI3K. Another possibility is that T47D produces non EGF-like autocrine factors (Nicholson et al., 2003) that cooperate with exogenous growth and survival factors present in the fetal calf serum thus maintaining high levels of AKT phosphorylation. Again phosphorylation of ERK1/2 is completely blocked by pelitinib pretreatment and can not be reactivated by EGF exposure in either cell line irrespective of pelitinib growth sensitivity or resistance **(Figs.26 and 27)**.

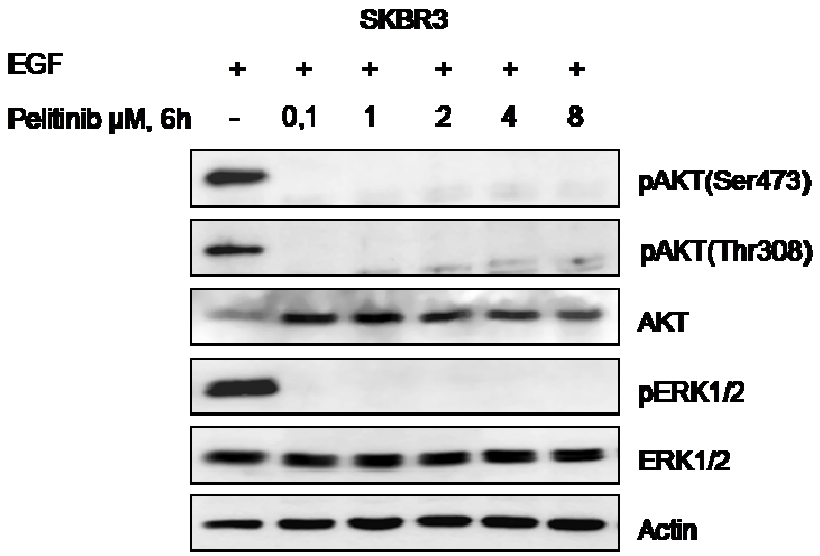


Fig.26: Total protein from SKBR3 cells were labeled with anti-AKT, ERK1/2 or anti-pAKT (Ser473/Thr308) and pERK1/2 antibodies*. Cells were allowed to attach o.n. in 5% FCS and were grown in 60 mm dishes. The cultures were treated with 0,1µM, 1µM, 2µM, 4µM and 8µM pelitinib for 6 hours. Before sample preparation the cultures were exposed for 20min to recombinant human EGF (100ng/ml). Actin was detected for loading control.*

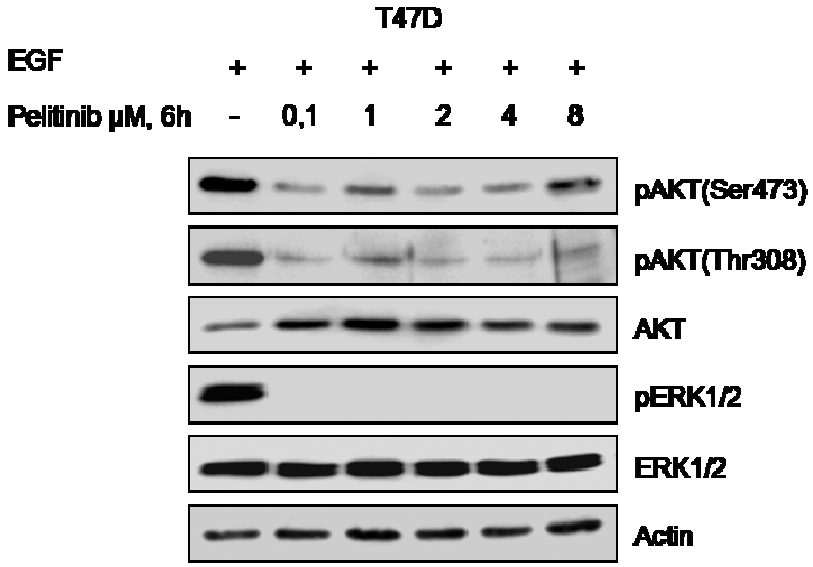


Fig.27: Total protein from T47D cells were labeled with anti-AKT, ERK1/2 or anti-pAKT (Ser473/Thr308) and pERK1/2 antibodies*. Cells were allowed to attach o.n. in 5% FCS and were grown in 60 mm dishes. The cultures were treated with 0,1µM, 1µM, 2µM, 4µM and 8µM pelitinib for 6 hours. Before sample preparation the cultures were exposed for 20min to recombinant human EGF (100ng/ml). Actin was detected for loading control.*

4.5.3 In the absence of serum supplementation

To eliminate possible effects of 5% FCS Western blot experiments were performed without serum. SKBR3 and T47D were cultivated as described in Material and Methods, depleted for one day of serum and then treated for 6 hours with 0,1µM, 1µM, 2µM, 4µM and 8µM pelitinib. Subsequently, both cell lines were stimulated for 20 min with EGF. Actin was used as loading control. As expected, EGF stimulates AKT and ERK1/2 phosphorylation in both cell lines. Pelitinib completely abrogates EGF-mediated phosphorylation of AKT in drug sensitive SKBR3 cells, whereas in drug-resistant T47D a low baseline phosphorylation of AKT persists even in the absence of serum **(Figs.28 and 29)**. This residual, ErbB-independent-AKT activity could come from autocrine production of growth and survival factors in T47D cells that lead signals into the PI3K/AKT pathway or from intrinsic activity of the PI3K, which harbors an activating point mutation in the p110 alpha catalytic subunit (PIK3CA H1047R) in T47D cells (She et al., 2008).

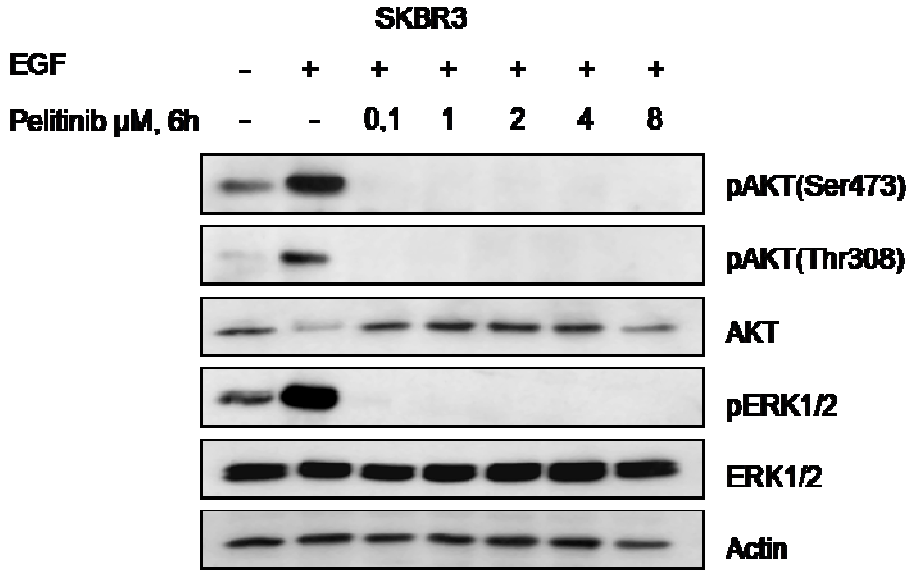


Fig.28: Total protein from SKBR3 cells were labeled with anti-AKT, ERK1/2 or anti-pAKT (Ser473/Thr308) and pERK1/2 antibodies*. Cells were allowed to attach o.n. in 5% FCS and were grown in 60 mm dishes. Medium was changed to medium without FCS for 24 hours. The cultures were treated with 0,1µM, 1µM, 2µM, 4µM and 8µM pelitinib for 6 hours. Before sample preparation the cultures were exposed for 20min to recombinant human EGF (100ng/ml). Actin was detected for loading control.*

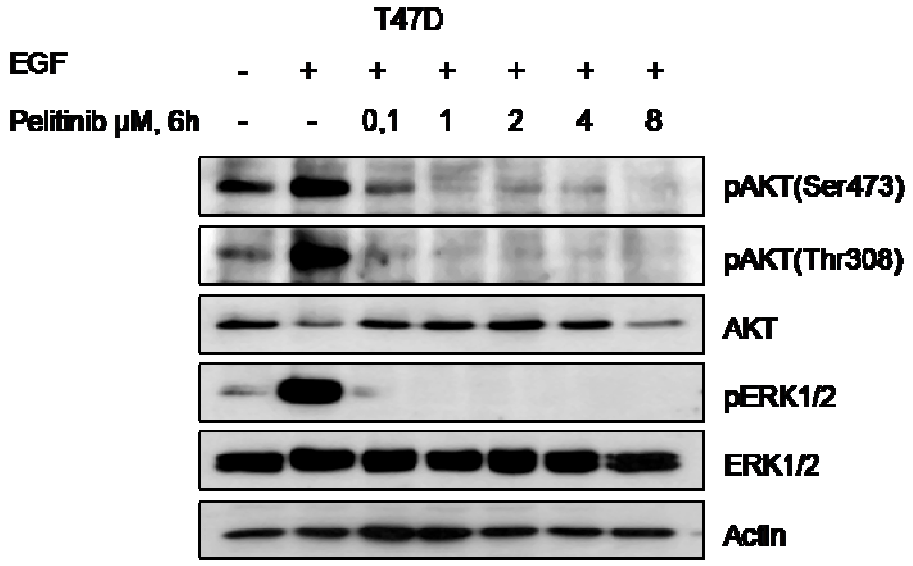


Fig.29: Total protein from T47D cells were labeled with anti-AKT, ERK1/2 or anti-pAKT (Ser473/Thr308) and pERK1/2 antibodies*. Cells were allowed to attach o.n. in 5% FCS and were grown in 60 mm dishes. Medium was changed to medium without FCS for 24 hours. The cultures were treated with 0,1µM, 1µM, 2µM, 4µM and 8µM pelitinib for 6 hours. Before sample preparation the cultures were exposed for 20min to recombinant human EGF (100ng/ml). Actin was detected for loading control.*

4.6 The effect of pelitinib on AKT and ERK1/2 activity in pelitinib sensitive and pelitinib resistant ovarian cancer cells

We next determined the growth inhibitory effect of pelitinib in a panel of ovarian cancer cell lines and found various degrees of sensitivity. For instance, SKOV3 cells revealed an IC50 of 7,3 ± 0,9µM, whereas CaOV3 are highly resistant to pelitinib showing an IC50 fo 11.5 ± 1,3µM (data not shown). In these two cell lines, the effect of pelitinib on AKT and ERK1/2 phosphorylation was also examined. The cells were cultivated in the absence ore presence of serum as described in Material and Methods, treated for 6 hours with pelitinib, then stimulated for 20min with EGF (100ng/ml), and subjected to Western blot analysis. **Fig. 31** demonstrates that AKT phosphorylation decreases even below constitutive levels seen in the absence of EGF and serum with increasing pelitinib concentration. In contrast, in CaOV3 cells, pelitinib concentrations as high as 8µM are not able to depress AKT phosphorylation below baseline levels seen in the absence of EGF and serum **(Fig.30)**. Unlike AKT, ERK1/2 phosphorylation is blocked by pelitinib below baseline levels in both cell lines, irrespective of their sensitivity against pelitinib in growth assays. These data indicates that the efficiency of pelitinib to silence AKT activity is a crucial parameter for growth inhibitory activity of the compound not only in breast but also in ovarian cancer cells.

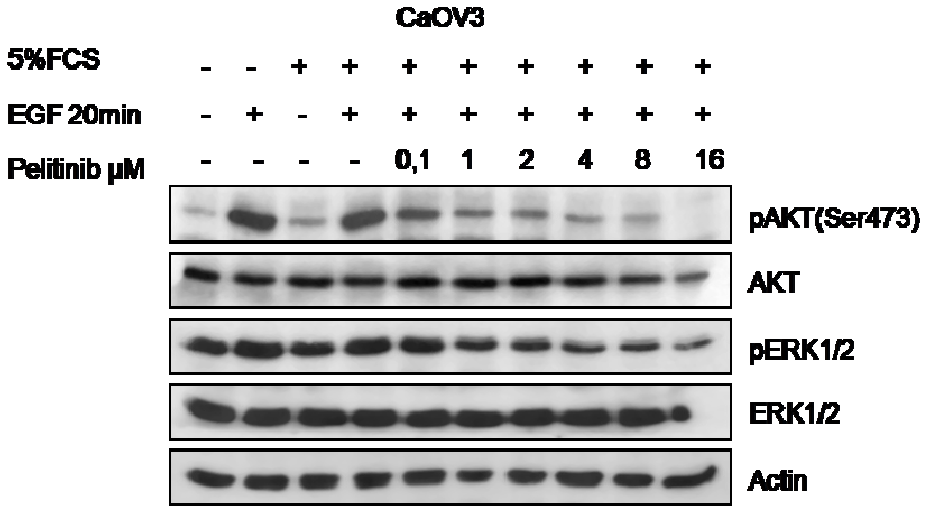


Fig.30: Total protein from CaOV3 cells were labeled with anti-AKT, ERK1/2 or anti-pAKT (Ser473) and pERK1/2 antibodies*. Cells were allowed to attach o.n. in 5% FCS and were grown in 60 mm dishes. Lane1 exhibits cells exposed to medium without FCS for 24 hours, the same with lane2 but exposed 20 min to recombinant human EGF (100ng/ml). The cell cultures in lane3 had medium supplemented with 5% FCS, the same with lane4 but also exposed to 20min EGF. The cultures in lane5-10 were treated with 0,1µM, 1µM, 2µM, 4µM, 8µM and 16µM pelitinib for 6 hours in medium with 5% FCS and exposed to 20min EGF. Actin was detected for loading control.*

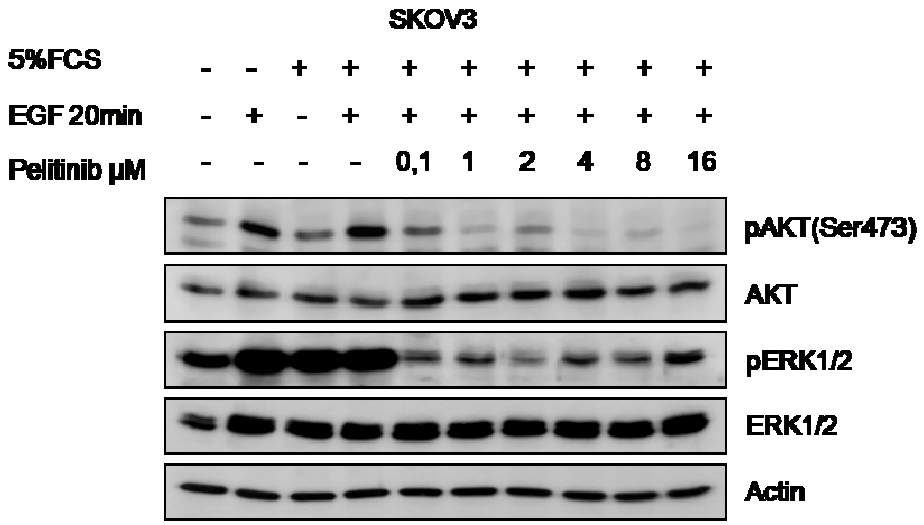


Fig.31: Total protein from SKOV3 cells were labeled with anti-AKT, ERK1/2 or anti-pAKT (Ser473) and pERK1/2 antibodies*. Cells were allowed to attach o.n. in 5% FCS and were grown in 60 mm dishes. Lane1 exhibits cells exposed to medium without FCS for 24 hours, the same with lane2 but exposed 20 min to recombinant human EGF (100ng/ml). The cell cultures in lane3 had medium supplemented with 5% FCS, the same with lane4 but also exposed to 20min EGF. The cultures in lane5-10 were treated with 0,1µM, 1µM, 2µM, 4µM, 8µM and 16µM pelitinib for 6 hours in medium with 5% FCS and exposed to 20min EGF. Actin was detected for loading control.*

4.7 The effects of constitutively active, exogenous MEK1 and AKT1 on pelitinib-mediated growth inhibition in SKBR3 breast cancer cells

ErbB receptors, especially ErbB-2, are overexpressed and hyperactivated in many breast cancer cells including SKBR3, and correlate with an aggressive clinical phenotype and unfavorable prognosis. However, EGFR, ErbB-2 and ErbB-3 hyperactivity **(Fig.18)** in SKBR3 is efficiently blocked by the dual EGFR/ErbB-2 targeting drug pelitinib. Correspondingly, pelitinib also inhibits invitro monolayer growth of SKBR3 cells **(Fig.17)**. This correlates with pelitinib dependent reduction of pAKT levels in SKBR3 cells. T47D cells, while showing pelitinib-mediated reduction of ErbB activity, do neither reveal down regulation of pAKT nor inhibition of invitro growth. In contrast, to that, both pelitinib-sensitive SKBR3 and pelitinib-resistant T47D reveal pelitinib-mediated blockade of ERK1/2 signaling. Moreover, a quite similar situation was found in ovarian cancer cells. In order to specifically examine the relative contribution of MAPK or PI3K/AKT signaling development of pelitinib- resistance, SKBR3 were transfected with either constitutively active MEK1 or constitutively active AKT1 and the effect of pelitinib on the growth of these transfectants was examined. **(Fig.32)**. AKT promotes survival of cancer cells and is often associated with resistance of chemo- (Clark et al., 2002 and LoPiccolo et al., 2007) radio- or hormone therapy (Tokunaga et al., 2006). On the other hand, the MAPK signaling pathway may play an essential role in developing resistance against tyrosine kinase inhibitors (Normanno et al., 2006 and 2008). Our data demonstrates that hyperactivation of the PI3K/AKT pathway by introduction of dominant active AKT1 markedly elevates the IC50 of SKBR3 cells to approximately 3,4µM, whereas hyperactive MAPK signaling due to exogenous MEK1 activity does not significantly change the IC50 for SKBR3 invitro growth (IC50 = 1,7µM) **(Fig.32)**. Thus AKT, but not MAPK signaling essentially contributes to pelitinib resistance.

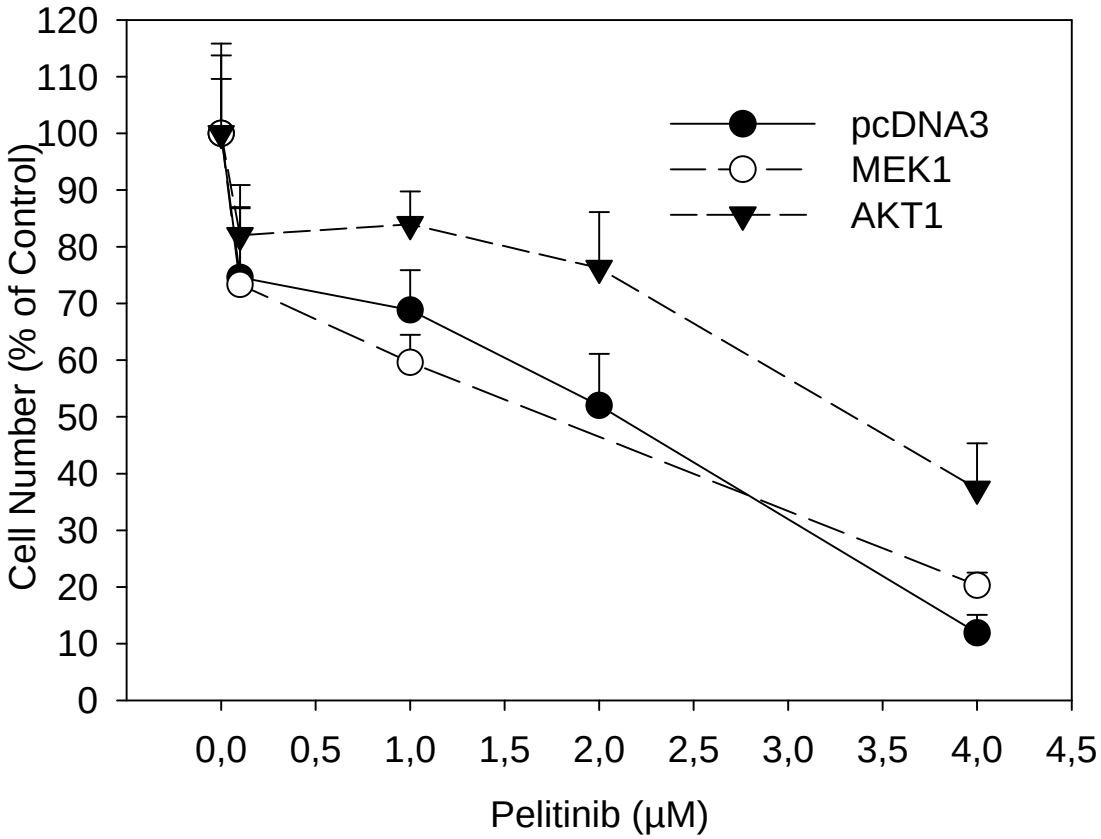


Fig.32: Transfected breast cancer cell lines SKBR3 exposed to pelitinib*. SKBR3 cells were transfected with pcDNA3, constitutively active AKT1 and constitutively active MEK1. Cells were grown in 96-well plates, allowed to attach o.n. and treated for 3 days with 0,1µM, 1µM, 2µM, 4µM pelitinib. Before the absorbance was measured at 450nm against a 655nm reference wavelength with a microplate reader spectrophotometer, the EZ4U reagent was added.*

4.8 The effects of constitutively active, exogenous MEK1 and AKT1 on the activation of downstream signaling cascades in SKBR3 breast cancer cells and their responsiveness to pelitinib

Transient overexpression and hyperactivation of MEK1, AKT1 and downstream signal mediators were checked by Western blot analysisSKBR3 were transfected as described and treated for 8 hours with 0,1µM pelitinib **(Fig.33)**. Alpha/beta tubulin was used as loading control. Using antibodies against MEK1/2 and AKT we could confirm effective MEK1 and AKT transfection. Activation of MEK1 downstream signaling was demonstrated with pERK1/2 antibody. ERK1/2 was phosphorylated in untreated wild type and all transfected cell lines. However, highest level of phosphorylation was seen in untreated MEK1 transfectants, which is resistant to pelitinib-mediated silencing **(Fig.33)**.
Furthermore, using a pAKT (Ser473) antibody, we demonstrated that pelitinib inhibits pAKT in untransfected, vector-transfected and MEK1 transfected, but not in AKT transfected, SKBR3 cells, which reveal strongly elevated AKT phosphorylation. These data suggest that the AKT pathway contributes to resistance of the cells against pelitinib. Moreover, AKT is known to phosphorylate glycogen synthase kinase (GSK)-3ß, thereby inhibiting it and enabling cell cycle progression by stabilizing cyclin D1 expression. Phospho-GSK3-ß is highest in untreated AKT transfectants and is reduced by pelitinib in wildtype and all transfected SKBR3 cell lines. However, pelitinib-mediated blockade of GSK3-ß phosphorylation is markedly less pronounced in SKBR3 expressing constitutively active AKT1 suggesting a lower efficacy of pelitinib to block cell cycle progression in cells transfected with AKT1 **(Fig.33)**.
Another downstream substrate of AKT is mTOR which phosphorylates S6 kinase. The S6 kinase activates the ribosomal protein S6 promoting increased protein translation. Unexpectedly, pelitinib inhibits mTOR phosphorylation in all cell lines tested, including AKT1 transfectants. In contrast, while pelitinib inhibits

phosphorylation of S6 in wildtype, vector transfected and MEK1 transfected SKBR3 cells, it can only partially block S6 phosphorylation in AKT1 transfected cells **(Fig.33).** This data demonstrates that forced expression of dominant active MEK1 and AKT1 effectively activate the MAPK and PI3K/AKT pathways in SKBR3 cells, respectively, and renders them partially resistant to pelitinib. Moreover, we have shown that PI3K/AKT, but not MAPK hyperactivation, markedly contributes to resistance of the cells against the growth- inhibitory effect of pelitinib.

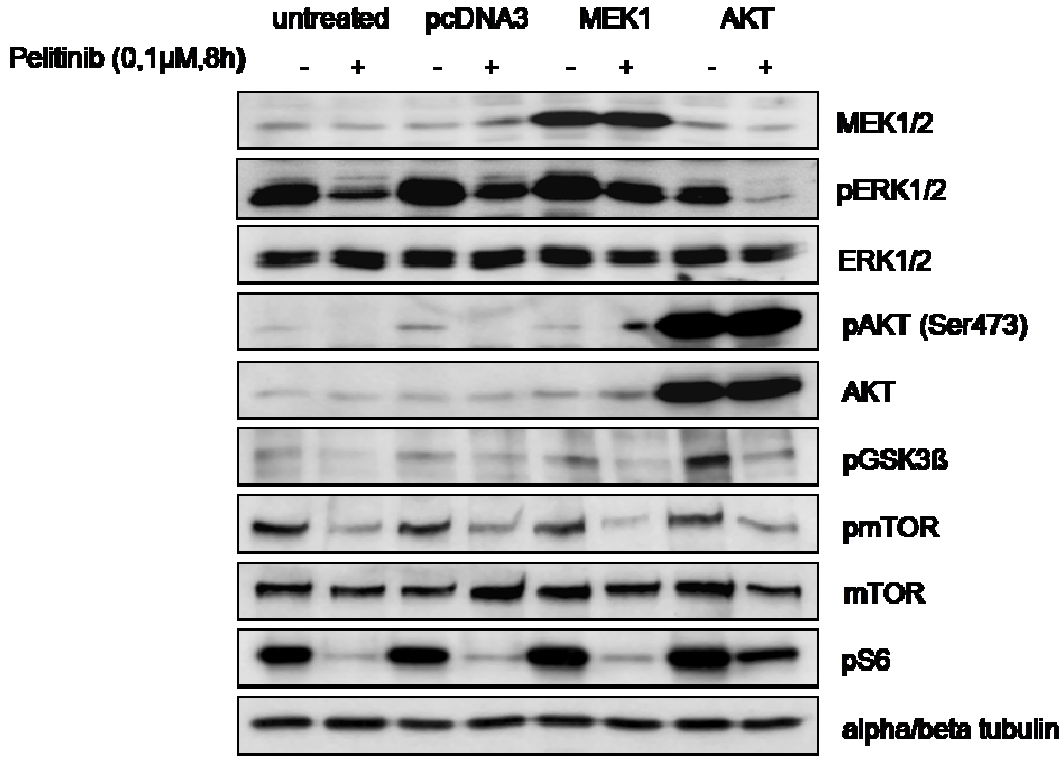


Fig.33: Transfected breast cancer cell lines SKBR3 exposed to pelitinib*. Cells were grown in 60mm dishes, allowed to attach o.n. and then transfected with pcDNA3, constitutively active AKT1 and constitutively active MEK1. After 24 hours culture was treated 8 hours with 0,1µM pelitinib or with DMSO.*

5 Discussion

ErbB receptors are associated with the development of aggressive solid tumors via the phosohoinositide 3-kinase (PI3K) and the mitogen-activated protein (MAP) kinase (MAPK) signaling pathways and are therefore important targets for anti-cancer therapies (Aifa et al., 2008). The family of ErbB receptor tyrosine kinases consists of four homologous members (ErbB-1/HER-1/EGFR, ErbB-2/HER-2/Neu, ErbB-3/HER-3 and ErbB-4/HER-4) whose signaling is mediated through ligand binding, which triggers enhanced network signaling (Monilola et al., 2000; Schessinger, 2000 and Yarden et al., 2001). EGFR and ErbB-2 tyrosine kinase receptors are often overexpressed in breast cancer, promote solid tumor growth and provide perfect targets for anti-cancer therapies (Nicholson et al., 2001 and Ginestier et al. 2007). Elevated levels of ErbB-2 are associated with aggressive clinical phenotypes and poor prognosis, therefore representing a perfect therapeutic target for trastuzumab, a recombinant humanized monoclonal anti-ErbB-2 antibody (Kumar Pal et al., 2007). Moreover, patients with ErbB-2 overexpression can benefit from therapy with trastuszumab, alone or in combination with other drugs, however not all patients respond to these therapies dependent on development of resistance against trastuzumab (Nahta et al., 2006). Furthermore, Sithanandam et al., (2008) reported ErbB-3 involvement to be responsible for escape from therapies targeting other ErbB`s and as a consequence the role of ErbB-3 in breast cancer development has to be revealed. ErbB-4 overexpression in breast cancer and the reports about biological and prognostic relevance have been contradictory compared to the other ErbB family members (Sundvall et al., 2008). These ErbB receptors activate signaling cascades, stimulate the PI3K and the MAPK and thereby control cell proliferation, differentiation, migration and tumor angiogenesis. Further signaling cascades are reviewed by Li et al., (2002) and concern the activation of signal transducers and activators of transcription (STAT) proteins by ErbB receptors. As a consequence, EGFR and ErbB-2 are the main molecular targets for cancer therapies because their

heterodimerization and “cross talk” longs for improved agents that collectively inhibit these two receptors (Reid et al., 2007).

5.1 ErbB receptor profile

In this thesis the effects of an irreversible dual EGFR/ErbB-2 inhibitor pelitinib (EKB-569) were assessed (Erlichman et al., 2006). Pelitinib is a small-molecule RTKI that competes with ATP and inhibit the tyrosine kinase domain (Wissner et al., 2008). This drug retains inhibitory effects against tumors which have become resistant against certain first generation RTKIs such as erlotinib (tarceva) and gefitinib (iressa), two selective inhibitors against the EGFR tyrosine kinase domain. We used two different breast cancer cell lines, SKBR3 and T47D, which proved to be sensitive or resistant to the dual EGFR/ErbB-2 inhibitor pelitinib, respectively. In the first step we performed growth assays to determine the antiproliferative efficacy of pelitinib. T47D exhibited an IC50 value that was 4,5 fold higher than that of SKBR3. Thus, pelitinib inhibited growth in SKBR3 cells, but not in T47D cells. We compared ErbB receptor expression levels in these two breast carcinoma cell lines. SKBR3 and T47D express EGFR, ErbB-2 and ErbB-3 whereas ErbB-4 was only found in T47D. SKBR3 cells showed higher levels of EGFR and ErbB-2 protein expression than T47D. However, phosphorylation of downstream signaling proteins like AKT and Erk1/2 is similar in both cell lines. To test the functionality of the EGFR signaling pathway, SKBR3 and T47D were stimulated with 100ng/ml EGF. Both cell lines are responsive to EGF challenge, resulting in the formation of active EGFR homodimers, or EGFR/ErbB-2 and EGFR/ErBB-3 heterodimers and subsequent cross-phosphorylation at their tyrosine residues (Citri et al., 2006) and downstream activation of PI3K/AKT and ERK1/2 signaling pathways.

5.2 The functionality of pelitinib (EKB-569)

Remarkable was the overexpression of EGFR and ErbB-2 in SKBR3 cells. A number of previous studies have successfully demonstrated inhibition of ErbB-2 and its downstream signaling using not only antibodies such as trastuzumab (Moasser et al., 2001 and Anido et al., 2003), but also EGFR TKIs such as erlotinib and gefitinib. Other studies, however, reported EGFR tyrosine kinase inhibitors such as AG1478 (Grunt et al., 2007a) or gefitinib (Grunt et al., 2007b) already weakly effective against breast cancer cells due to release of autocrine ligands, such as heregulin and betacellulin, that stimulate ErbB-3 receptor and ErbB-4 (Kong et al., 2008). ErbB-2 can escape EGFR TKI blockade by formation and activation of heterodimers containing autocrine-ligand activated ErbB-3 and ErbB-4 receptors. We first examined functionality of the whole ErbB system in SKBR3 and T47D cells, by maximally stimulating it with EGF and heregulin (Bazley et al., 2005). EGF directly binds EGFR, but not ErbB2-4, whereas heregulin binds ErbB-3 and ErbB4. ErbB-2 has no known ligand and is only activated by heterodimerisation wit other ErbB receptors. Thus, by combining EGF with heregulin, we could activate the whole ErbB system in both cell lines. We assessed pelitinib-mediated inhibition of phosphorylation of EGFR, ErbB-2 and ErbB-3 in sensitive SKBR3 as well as in resistant T47D cells. Interestingly, in SKBR3 MAPK and PI3K/AKT downstream signaling was completely blocked by pelitinib, whereas in T47D AKT phosphorylation remained active despite inhibition of upstream ErbB receptor activation by pelitinib. Our data indicates that pelitinib is not only a functional inhibitor of EGFR and ErbB-2, but also prevents further heterodimerisation with other ErbB members. Our results are not consistent with the possibility that pelitinib resistance may be caused by lateral escape from the drug-mediated blockade due to compensatory formation and activation of ErbB-3 and ErbB-4 homo- or heterodimers in pelitinib-resistant T47D cells. However, alterations in downstream signaling cascades or involvement of non-ErbB are still possible causes for the observed resistance of T47D cells.

5.3 The role of the PI3K/AKT signaling pathway in determing cellular resistance against pelitinib

Using Western bot analysis we were able to demonstrate differences in AKT (protein kinase B) signaling between SKBR3 and T47D exposed to pelitinib. Protein kinase B/AKT is activated by PI3K-PDK1 (Vanhaesebroeck et al., 2000) regulates cell survival and is therefore associated with tumorigenesis (Testa et al., 2001 and Nicholson et al., 2002). Moreover, PI3K/AKT pathway activation was revealed in breast cancer by Tokunaga et al. (2006), and is associated with endocrine resistance in metastatic breast cancer. T47D, representing the pelitinib resistant breast cancer cell line, exhibited high basal level of AKT phosphorylation, irrespective of pelitinib exposure. This situation is already seen in two different ovarian cancer cells such as SKOV3 and CaOV3, where differences in AKT signaling are already seen, and abrogates the possibility of involvement of AKT being a resistance factor against TKIs not only in breast cancer, but also in ovarian cancer.

Nicholson et al. (2003) described the role of AKT and its constitutive activation. In SKBR3 cells, it is associated with ErbB-2 receptor overexpression, whereas in T47D on released autocrine factors, which act through EGFR and ErbB-2 and induce the AKT signaling pathway. In the presence of serum, phosphorylation of AKT was completely non-responsive to pelitinib and persisted at high level in T47D. Nevertheless, EGF still was able to elevate AKT phosphorylation even higher. Interestingly, this portion of AKT phosphorylation was inhibited by pelitinib even in the pelitinib-resistant T47D. The residual AKT phosphorylation could be caused by serum factors, unrelated to the ErbB system, from autrocrine non-ErbB growth factors or from intrinsic upstream activation through PI3K. Serum growth factors were mostly not involved, because removal of serum from the culture did not alter the overall pattern of AKT phosphorylation after pelitinib exposure, indicating that residual AKT activation may be either due to autocrine non-ErbB growth factors or due to constitutive upstream signaling activity.

Regarding multidrug resistance in breast cancer cells, Knueferman et al. reported involvement of ErbB-2/PI3K/AKT activation by transforming MCF-7 cells with a dominant negative form of PI3K and AKT obtaining abrupt sensitivity against chemotherapeutics. According to our results, pelitinib sensitive SKBR3 remained resistant after transfection with the constitutive active myr-AKT1 plasmid suggesting involvement of AKT in achievement of the resistant phenotype. After performing Western blot analysis of transfected SKBR3 further supported this notion and revealed hyperactive AKT downstream signaling as demonstrated by elevated levels of pGSK3ß, a cell cycle regulator, and of phospho-S6 ribosomal protein, which is involved in protein translation.

It was found that 26% of breast cancer cell lines exhibit a mutation in the catalytic subunit p110α of the PI3K (PIK3CA), an early event in development of the disease (Saal et al., 2005). These authors report that PIK3CA mutations are associated with PTEN functionality, ErbB-2 overexpression and with tumors exhibiting estrogen and progesterone receptors. PTEN is frequently seen in estrogen and progesterone receptor negative breast cancer suggesting new possibilities of molecular targeting anti-cancer strategies. In these cases, the PI3K/AKT pathway plays a pivotal role. SKBR3 cells are estrogen and progesterone receptor negative and overexpress ErbB-2, whereas T47D cells are positive for both receptors and do not overexpress ErbB-2. Consistent with this, T47D in contrast to SKBR3, exhibited basal phosphorylation of AKT despite pelitinib treatment. Consequently, pelitinib inhibited growth of SKBR3, lacking a PIK3CA mutation, but not of T47D cells, which exhibit a PIK3CA H1047R mutation. These data suggest important roles of PIK3CA mutations and hormone receptor pathways. Moreover, She et al. (2008) underline the notion that ErbB-2 amplification or PI3K mutations activate the PI3K/AKT pathway in breast cancer. Apart from PIK3CA mutation it was reported that T47D also exhibits a functional p110ß (PIK3CB) and both isoforms must be inhibited to completely inactivate AKT (Crowder et al., 2009). The latter report also demonstrates the relevance of blocking the estrogen receptor in estrogen receptor-positive breast cancer cells and inhibiting both PI3K p110α and p110ß subunits. These studies implicate the importance of PI3K/AKT signaling and its

role in breast cancer development. Interestingly, another group reported that PIK3CA mutation is correlated with PTEN loss in estrogen receptor positive breast cancer cell lines with no ErbB-2 overexpression, which is not associated with pAKT signaling suggesting that PI3K signals via other pathways that do not involve AKT (Pèrez-Tenorio et al., 2007).

5.4 The role of MAPK signaling pathway in determining cellular resistance against pelitinib

Similar to the PI3K/PTEN/AKT signaling pathway, the RAF/MEK/ERK MAPK cascades can also generate tumor development and drug resistance (McCubrey et al., 2006). Moreover, Moelling et al. (2002) demonstrated crosstalk regulation between RAF and AKT in the breast carcinoma cell line MCF-7. When the RAF/MEK/ERK pathway is activated it correlates with cell differentiation and rapid cell growth. We have explored the role of MAPK signaling in pelitinib resistance by transfecting pelitinib sensitive SKBR3 with a constitutive active MEK1 plasmid. To examine possible resistance development dependent on MAPK signaling, growth assays and Western blot analysis were performed. As expected, constitutive MEK1 transfection did not render SKBR3 resistant against pelitinib suggesting that the relevant resistant mechanisms are located in the PI3K/AKT rather than in the MAPK pathway. In contrast, Magne et al., (2002) showed development of resistance in head and neck cancer against the EGFR-TKI gefitinib (iressa, ZD1839) to be dependent on MAPK signaling. Moreover, overcoming resistance in non-small cell lung cancer (NSCLC) was only achieved with EGFR-TKIs such as gefitinib associated with combinatory inhibition of PI3K/AKT or MAPK signaling pathways (Janmaat et al., 2003). Interestingly, in contrast to our data Normanno et al., (2006 and 2008) examined acquired resistance against gefitinib in long-term gefitinib-treated SKBR3 and associated loss of sensitivity with upregulation of the MAPK signaling pathway.

5.5 The role of IGFR signaling pathway

The insulin-like growth factors (IGFs) bind two ligands, IGF1 and IGF2, consist of two cell surface receptors, such as IGF-1R and IGF-2R which mediate cell proliferation and inhibition of apoptosis. Upon ligand binding, the kinase is activated and recruits signaling molecules such as Src homology 2 containing-domain (Shc) protein and insulin receptor substrates (IRS1-4) to the phosphorylated kinase domain and signals via the MAPK and the PI3K signaling pathway. The body-wide expression of IGF-1R and its contribution to growth makes the development of drugs targeting this receptor unavoidable. Moreover, IGF-1R expression in breast cancer is associated with hormone receptor expression (Hartog et al., (2007). Nowadays, IGF-1R monoclonal antibodies and TKIs are in early clinical and preclinical development. Furthermore, previous studies described involvement of IGF-1R in development of resistance mechanisms. Nahta et al. (2005) reported possible `cross talk` mechanisms between the ErbB-2 and the IGF receptors in SKBR3 breast cancer cells. Many ErbB-2 positive breast cancer patients receiving the monoclonal ErbB-2 neutralizing, growth inhibiting antibody trastuzumab (herceptin) developed resistance and experienced disease progression within several months. However, Nahta et al. (2007) reported in trastuzumab resistant SKBR3, that apoptosis can again be induced in these cells by treatment with lapatinib (GW572016) a small molecular inhibitor of EGFR and ErbB-2 and subsequent inhibition of IGF-1R. Consequently, in order to assess the role of IGF-1R signaling in pelitinib resistance, we treated SKBR3 and T47D cells with the IGF-1R tyrosine kinase inhibitor picropodophyllin (PPP) (data not shown). Interestingly, PPP had no inhibitory effects on downstream signaling, but instead activated AKT phosphorylation in T47D cells. This data suggests that IGF-1R signaling to AKT is not the cause for the observed pelitinib-resistant elevated basal level of constitutive AKT phosphorylation. Therefore, IGF-1R appears not to be involved in pelitinib resistance of T47D cells. These hypotheses must be scrutinized in continuative experiments.

6 Conclusion

Our results dealt with the relationship between ErbB RTK mediated signaling pathways and the development of resistance mechanisms against ErbB RTK`s. We compared two breast cancer cell lines, SKBR3, which proved to be sensitive and T47D, which is resistant against the dual EGFR/ErbB-2 inhibitor pelitinib. Functional ErbB signaling mediated through EGF and heregulin ligand binding was assessed in both cell lines. Moreover, the ability of EGF to bind its receptors increased the level of activated AKT and MAPK downstream signaling. In addition, treatment of SKBR3 and T47D with pelitinib decreased phosphorylation levels of EGFR, ErbB-2 and ErbB-3 in both cell lines. Interestingly, downstream signaling via AKT was only inhibited in SKBR3, but not in T47D. Furthermore, pelitinib completely inhibited MAPK activation in both cell lines. To reveal the mechanisms of pelitinib resistance in T47D we transfected pelitinib sensitive SKBR3 either with a constitutive active myr-AKT1 plasmid or with a constitutive active MEK1 plasmid, respectively. Interestingly, SKBR3 transfection with the constitutive active AKT1 but not with the constitutive MEK1 plasmid rendered SKBR3 resistant against pelitinib. These findings demonstrate the relevance of AKT signaling in contrast to MAPK signaling in development of resistance mechanisms against pelitinib and suggest that AKT should be cotargeted in order to overcome ErbB RTKI resistance in breast cancer cells.

7 Abstract

Breast cancer already occurs in women for a long time and ErbB receptor tyrosine kinases are associated with its pathogenesis. This family consists of four homologous members: ErbB-1/HER-1/EGFR, ErbB-2/HER-2/Neu, ErbB-3/HER-3 and ErbB-4/HER-4. In breast cancer, as well in ovarian cancer, EGFR and ErbB-2 receptors are overexpressed. They stimulate carcinogenesis and correlate with a more aggressive clinical behavior and poor prognosis. EGFR and ErbB-2 overexpression therefore serve as perfect targets for therapeutic agents.

Our studies focused on SKBR3 and T47D human breast cancer cells which proved to be sensitive and resistant against the particular Epidermal Growth Factor Receptor (EGFR)/ErbB-2 receptor tyrosine kinase inhibitor pelitinib (EKB-569, Wyeth). This novel irreversible ErbB inhibitor showed effects on the growth activity and on ErbB-triggered signaling. In contrast, the difference between sensitivity and resistance of the ovarian cancer cell lines did not vary as much.

The project delineated the effect of the drug on major signaling pathways downstream of the ErbB family of receptor tyrosine kinases (RTKs) in these two human breast cancer cell lines. The examined effects of pelitinib were drug-dependent inhibition of EGFR, of ErbB2 and of ERK1/2 phosphorylation in both pelitinib-sensitive and pelitinib-resistant cells. Interestingly, phosphorylation of AKT at Ser473 and Thr308 was only blocked in sensitive SKBR3, but not in resistant T47D, suggesting that inhibition of ERK1/2 downstream signaling is not sufficient for drug-dependent growth arrest. Moreover, transfection of a constitutively active AKT, but not of constitutively active MEK1, in SKBR3 induced resistance to pelitinib. This data suggests crucial roles of the PI3K/AKT signaling pathway but not of the Erk1/2 pathway in determining sensitivity/resistance of the cells against pelitinib.

8 Zusammenfassung

Brustkrebs ist eine potenziell tödliche Erkrankung bei Frauen und seine Pathogenität steht im Zusammenhang mit den ErbB Tyrosinkinase Rezeptoren. Diese Familie besteht aus vier gleichartigen Mitgliedern: ErbB-1/HER-1/EGFR, ErbB-2/HER-2/Neu, ErbB-3/HER-3 und ErbB-4/HER-4. In Brustkrebs, und auch in Eierstockkrebs sind die EGFR und ErbB-2 Rezeptoren überexpremiert. Diese Rezeptoren stimulieren die Krebsentstehung, sorgen für klinisch aggressives Verhalten und schlechte Prognosen. Die Überexpression von EGFR und ErbB-2 Rezeptoren macht sie deshalb zu perfekten Zielen für eine Therapie.

Unsere Versuche konzentrierten sich auf die zwei Brustkrebslinien SKBR3 und T47D, welche jeweils sensibel gegenüber dem EGFR/ErbB-2 Inhibitor Pelitinib (EKB-569, Wyeth) oder resistent dagegen sind. Dieser neue irreversible Inhibitor hat einen Effekt auf das Wachstum der Zellen und auf die ErbB getriggerte Signaltransduktion. Im Gegensatz dazu ist der Unterschied zwischen Sensibilität und Resistenz bei den Eierstockkrebszellen nicht so groß.

Dieses Projekt beschreibt die Effekte dieses Medikaments in den zwei Brustkerbs Zelllinien auf die Hauptsignalwege die unterhalb der ErbB Tyrosinkinase Rezeptoren (RTKs) liegen. Die untersuchten Effekte von Pelitinib beziehen sich auf die Inhibierung der Phosphorylierung von EGFR, ErbB-2 und ERK1/2 in den sensiblen und resistenten Zelllinien. Interessanterweise wird die Phosphorylierung von AKT nur in den sensiblen SKBR3 gehemmt aber nicht in den resistenten T47D, dies führt zu der Annahme, dass die Inhibierung vom ERK1/2 Signalweg nicht ausreicht für die Inhibierung des Zellwachstums. Die Transfection von SKBR3 mit dem konstitutiv aktiven AKT führt im Gegensatz zum konstitutiv aktiven MEK1 zu einer induzierten Resistenz gegenüber Pelitinib. Diese Daten zeigen uns die wichtige Rolle vom PI3K/AKT Signalweg, nicht vom ERK1/2 Signalweg, der entscheidend ist für die Sensibilität/Resistenz der Zelllinien gegenüber Pelitinib.

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10 Abbrevations

APS	ammonium persulfat
CIAP	calf intestine alkaline phosphatase
DMSO	dimethyl sulfoxide
DOC	2,5-dimethoxy-4-chloroamphetamine
ECL	enhanced chemiluminescence
EDTA	ethylene-diamine tetraacetic acid
EGTA	ethylene glycol tetraacetic acid
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ER	estrogen receptor
ErbB-2	epidermal growth factor 2
ErbB-3	epidermal growth factor 3
ErbB-4	epidermal growth factor 4
ERK	extracellular signal- regulated kinase
FCS	fetal calf serum
GFP	green fluorescence protein
GSK-3	glycogen synthase-3
HB-EGF	heparin-binding EGF
HRP	horseradish peroxidase
HRG	heregulin
IGF	insulin-like growth factor
IGF-1R	insulin-like growth factor-1 receptor
Jak	janus tyrosine kinases
MAPK	mitogen-activated protein kinase
MEK1/2 MAP	kinase ERK kinase (=MAPKK 1/2)
mTOR	mammalian target of rapamycin
NaCl	sodium chloride
NaF	sodium fluoride
NRGs	neuregulins
NSCLC	non small cell lung cancer

PAA	polyacrylamide
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PDK1	phosphoinositide dependent kinase
PH	pleckstrin homology domain
PI3K	phosphatitylinositol-3 kinase
PKP	protein kinase B
PPP	Picropodophyllin
PR	progesterone receptor
PTB	phosphotyrosine binding
PTEN	phosphatase and tensin homologue deleted from chromosome 10
PVDF	polyvinylidene fluoride
RTK	receptor tyrosine kinase
RTKI	receptor tyrosine kinase inhibitor
SDS	sodiumdodecylsulfat
SH	scr homology domain
STAT	signal transducer and activator of transcription
TBE t	ris borat EDTA buffer
TEMED	N,N,N´,N´,- tetramethylethylendiamine
TGFα	transforming growth factor α
Tris	tris(hydroxymethyl)-aminomethan

11 Curriculum Vitae

11.1 Personal Data

Name:	Caroline Brünner-Kubath
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11.2 Education

1990 – 1994:	Elementary school Götzendorf/Leitha
1994 – 2002:	High school, Don Bosco Gymnasium Unterwaltersdorf
Juni 2002:	Matura
10/2002-06/2003:	Student at the Medical University Vienna
10/2003:	Student at the University of Vienna (Molecular Microbiology & Genetics)
9/2005/2006/2007:	One month practical course at OMV Refining & Marketing GmbH
5/2008-5/2009:	Diploma Thesis, Medical University Vienna, Supervisior: Ao. Univ.-Prof. Mag. Dr. Thomas Grunt, Supervisior of the University of Vienna: Univ.-Prof. Dr. Thomas Decker

11.3 Scientific achievement

Poster:

Shabbir,W., Brünner-Kubath,C., Grusch,M., Berger,W., Marian,B., Singer,C.F., Wagner,R., Zielinski,C.C., Grunt,T.W.: Resistance against the irreversible dual EGFR/HER2 inhibitor pelitinib (EKB-569) correlates with hyperactive HER3/PI3K/AKT signaling. Proc. AACR, Denver, CO, 50, Abstr.# 2776, 2009.

Shabbir,W., Brünner-Kubath,C., Grusch,M., Berger,W., Marian,B., Wagner,R., Lötsch,D., Zielinski,C., Grunt,T.W.: The PI3K/AKT pathway determines EGFR/HER/ErbB drug efficacy in breast cancer cells. EHRLICH II, 2nd World Conference on Magic Bullets, Oct 3-5, 2008 Nurnberg, Germany, p. A113, 2008.

Shabbir,W., Brünner-Kubath,C., Grusch,M., Berger,W., Marian,B., Wagner,R., Lötsch,D., Zielinski,C., Grunt,T.W.: Phospho-AKT as a biomarker for EGFR/HER/ErbB drug efficacy in breast cancer cells. Keystone Symposium & 15th International Conference of the International Society of Differentiation on “Stem Cells, Cancer and Aging”, Singapore, September 29 - October 4, 2008.

Shabbir,W., Brünner-Kubath,C., Grusch,M., Berger,W., Marian,B., Wagner,R., Lötsch,D., Zielinski,C., Grunt,T.W.: The role of the ErbB3/PI3K/AKT pathway in determining breast cancer cell sensitivity against the irreversible dual EGFR/ErbB2 inhibitor EKB-569. 20th EORTC-NCI-AACR Symposium on "Molecular Targets and Cancer Therapeutics”, Geneva, Switzerland, 21 - 24 October 2008, Eur. J. Cancer, 6, Abstr.# 559, 2008.

12 Lebenslauf

12.1 Persönliche Daten

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12.2 Ausbildung

1990 – 1994:	Volksschule Götzendorf/Leitha
1994 – 2002:	Don Bosco Gymnasium Unterwaltersdorf
Juni 2002:	Matura
10/2002-06/2003:	Studium der Medizinischen Universität
10/2003:	Studium der Biologie, Studienzweig Mikrobiologie und Genetik
9/2005/2006/2007:	Ferialpraxis bei der OMV Refining & Marketing GmbH
5/2008-5/2009:	Diplomarbeit, Medizinische Universität, bei Ao. Univ.-Prof. Mag. Dr. Thomas Grunt, Betreuer der Universität Wien, Univ.-Prof. Dr. Thomas Decker

12.3 Wissenschaftliche Leistungen

Poster:

Shabbir,W., Brünner-Kubath,C., Grusch,M., Berger,W., Marian,B., Singer,C.F., Wagner,R., Zielinski,C.C., Grunt,T.W.: Resistance against the irreversible dual EGFR/HER2 inhibitor pelitinib (EKB-569) correlates with hyperactive HER3/PI3K/AKT signaling. Proc. AACR, Denver, CO, 50, Abstr.# 2776, 2009.

Shabbir,W., Brünner-Kubath,C., Grusch,M., Berger,W., Marian,B., Wagner,R., Lötsch,D., Zielinski,C., Grunt,T.W.: The PI3K/AKT pathway determines EGFR/HER/ErbB drug efficacy in breast cancer cells. EHRLICH II, 2nd World Conference on Magic Bullets, Oct 3-5, 2008 Nurnberg, Germany, p. A113, 2008.

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