universität wien

DIPLOMARBEIT

Titel der Diplomarbeit

„Experimental targeting of basal-like breast cancer *in vitro*"

Verfasserin

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angestrebter akademischer Grad

Magistra der Naturwissenschaften (Mag.rer.nat.)

Wien, 2012

Studienkennzahl lt. Studienblatt:	A 490
Studienrichtung lt. Studienblatt:	Diplomstudium Molekulare Biologie
Betreuer:	ao. Univ.-Prof. Mag. Dr. Johann Rotheneder

Acknowledgement

First of all thanks are addressed to my supervisor ao. Univ.Prof. Dr. Robert Mader, who made my diploma work at the clinical divison for oncology possible.

I want to sincerely thank him for his support and competent guidance during my work. Further I want to thank him for being a mentor, who gave me the possibility to work independently but always had a helping hand whenever I needed one.

Special thanks are addressed to my colleagues Maria Kalipciyan and Birgit Forstner. I want to thank them for their patience, all the explanations and their help. Above all I want to thank them for the familiar atmosphere and the funny hours during my practical work.

Next my gratitude goes out to Dr. Zsuzsanna Bagó-Horváth (Department of Clinical Pathology) for making enormous amounts of photos of the cell lines.

I also want to thank ao. Univ.-Prof. Mag. Dr. Johann Rotheneder for serving as supervisor of the University of Vienna.

Further thanks are addressed to my family, my boyfriend and my friends. I want to thank them for their support, their encouragement and for always believing in me.

Contents

1 Introduction

1.1 Breast Cancer

Breast cancer is a disease of the mammary tissue. Luminal epithelial cells and basal or myoepithelial cells are the two major groups of differentiated cells which play an important role in this disease (Yamaguchi et al. 2009). Although many facts are known in respect of survival prognosis, a lack of information concerning the biology of this disease exists. Often breast cancer origins from a mutation in genes responsible for differentiation, cell growth or cell death. For better understanding of breast cancer genetic alterations are investigated constantly (Sørlie et al. 2001).

In addition to pathological and clinical classification, breast cancer subtypes can be distinguished based upon gene expression data. The following subtypes are known: human epidermal growth factor receptor 2 (Her2/ ErbB2) -positive, normal breast-like, luminal (luminal A, luminal B and luminal C) and basal-like (basal A and basal B) type (Yamaguchi et al. 2009; Sørlie et al. 2001). About 15% to 25% of all breast cancer cells belong to the ErbB2-postive group. These cells exhibit either an amplification of the corresponding gene or a high production of the protein. It is also possible that both of these abnormalities can be found in these cancer cells. This aggressive subtype is not easy to treat and has a poor survival prognosis (Piccart-Gebhart et al. 2005). Often there is a mutation in or a loss of the TP53 gene, which is responsible for an aggressive tumor and mostly leads to an early death. Sørlie and co-workers could show that 71% of the used ErbB2-positive cells had a mutation in the p53 gene (Sørlie et al. 2001). Like the ErbB2-positive breast cancer group, the normal-breast like cancer type is very aggressive with poor survival prognosis too. The normal-breast like cancer cells have many characteristics in common with the basal-like cancer cells. This breast cancer subtype mostly is negative for the hormone receptors of estrogen and progesterone and for the Her2 receptor. Furthermore Sieuwerts and co-workers could show that the epithelial - mesenchymal transition markers TWIST1 and vimentin are highly produced in these cancer cells (Sieuwerts et al. 2009). In contrast to the normal-breast like cancer cells, almost all cells of the three luminal cancer subtypes are positive for the estrogen receptor α (Sotiriou et al. 2003). Furthermore the luminal breast cancers are negative for the basal cytokeratins CK5, CK6, CK14, CK15, CK17, but they are positive for CK7, CK8, CK18 and CK19

(Laakso et al. 2006). The regulator protein GATA-3 plays an important role in luminal cell differentiation and can be used as predictive factor in luminal cancer cells (Kouros-Mehr et al. 2008). Expression properties of the subtype luminal A are for example GATA-3, ER-α, the tyrosine kinase c-kit, insulin-like growth factor-binding protein-3, cyclic AMP dependent transcription factor (ATF)-3, trefoil factor 3 and hepatocyte growth factor (Sotiriou et al. 2003; Sørlie et al. 2001). Sørlie and co-workers could show that only 13% of the used luminal A breast cancer cells have a mutation in the TP53 gene. This seems to be a reason why this subtype is the one with the best survival prognosis compared to the others (Sørlie et al. 2001). Luminal B breast cancer cells do not have an overexpression of Her2, but they show a much higher proliferation leading to the worst survival prognosis of the three luminal subtypes (Sotiriou et al. 2003; Sørlie et al. 2001). Further characteristics of this subtype are for example the expression of tumor necrosis factor receptor-associated factor 3, Breast Cancer Antigen (BRCA) 1-associated protein 1 (BAP1), RAD21 and lower levels of vascular cell adhesion molecule 1 and ATF-3. The survival rate of the last subtype, luminal C, is somewhere in between luminal A and luminal B (Sotiriou et al. 2003). The fourth group of breast cancer is basal-like breast cancer, the focus of my diploma thesis.

1.1.1 Basal-like breast cancer (BLBC)

About 15% of all breast cancers are the basal-like breast cancer subtype (Bauer et al. 2007). This group of breast cancer is predominantly negative for the hormone receptors of estrogen and progesterone and for the receptor of Her2 (= triple negative). Some of the cancer cells with basal-like phenotype can be positive for one of the receptors (Cleator et al. 2007; Rakha et al. 2009). Although the triple-negative cancer cells and the BLBC are very similar, only 85% of the triple negatives are basal-like (Bauer et al. 2007). The basal-like breast cancer cannot be treated with Her2 neutralizing antibodies or anti-hormone therapy because of the lacking receptors (Cleator et al. 2007). These myoepithelial cells from the outer layer of the mammary duct are positive for the basal cytokeratins CK5, CK6, CK14 and CK17. Some of the cells are also positive for luminal cytokeratins like CK8 and CK18. Further characteristics of this high proliferating and thus very aggressive cancer type are for example high expression of vimentin, epidermal growth factor receptor

(EGFR), laminin, c-kit, nestin, P-cadherin and osteonectin. Caveolins 1 and 2, Ki-67, Sox2 and moesin are also produced in this high grad tumors. An overexpression of p53 is very common in basal-like cancer cells (Cleator et al. 2007; Rakha et al. 2009; Laakso et al. 2006). Studies have shown that about 82% of this cancer cells have a mutation within this protein or the corresponding gene (Sørlie et al. 2001; Cleator et al. 2007). Pathological investigations of BLBC pointed out that this cancer is adenoid cystic or metaplastic (typical or atypical medullary), high mitotic and has frequent apoptotic cells. In addition, central necrosis, vesicular chromatin, rarely stromal content and a high nuclear-cytoplasmic ratio could be discovered via these investigations (Cleator et al. 2007; Rakha et al. 2009). This basal-like group can be differentiated into two subtypes. The first subunit is characterized by high amounts of CDC2, topoisomerase IIα, matrix metalloproteinase 7, mitotic MAD2L1 and proliferation cell nuclear antigen. The characteristics of the second subgroup of the basal-like breast cancer are overproduction of caveolin 1 and 2, activating transcription factor 3, fos B, c-fos, c-jun, transforming growth factor β receptor II and hepatocyte growth factor (Cleator et al. 2007, Sotiriou et al. 2003). Genetic analyses showed that often no locus at 5q11 could be found in case of BLBC. This seems to be one reason why this cancer type counts to the aggressive ones. On this chromosome arm tumor suppressor genes and DNA-repair genes are normally found. It is known that the basal-like subtype shares many properties with BRCA1 mutated breast cancer cells. The mutation or the loss of this gene responsible for homologous recombination often leads to incorrect repair and thus to the onset of cancer. The similarities of these two poor survival prognosticated cancer types are the high proliferation rate, the expression of the basal cytokeratins 5 and 6 and the EGFR. Further neither of them expresses the hormone receptor of estrogen (Cleator et al. 2007). Basal-like cancer cells seem to share characteristics with stem cells, such as expression of the stem cell regulatory genes CD133, Bmi1 and SLUG mRNA (Cleator et al. 2007, Storci et al. 2008). Visceral metastases of BLBC are often found in brain and lung and not in bone or lymph nodes, which is very often the case in other breast cancer types. It seems that the metastasis formation in basal-like breast cancer occurs in a different way compared to other breast cancer types (Rakha et al. 2009).

1.2 Receptors

1.2.1 Estrogen receptor (ER)

The dimeric estrogen receptor, which is activated by binding 17β-estradiol, plays an important role in about 40% - 70% of breast cancer cells. These cells, which are dependent on estradiol, can be treated with anti-estrogens. The two different isoforms of this receptor ER-α and ER-β are known. The first one is the more important one in respect of breast cancer. Breast cancers which are negative for this receptor are difficult to treat. Often an overexpression of EGFR or Her2/neu can be seen in ER negative cancer cells (Boerner et al. 2005).

1.2.2 Progesterone receptor (PR)

This receptor belongs to the steroid hormone family of nuclear receptors and it can be distinguished between PR-α and PR-β. This receptor is activated by binding a steroid, for example progesterone. PR-α seems to be the receptor which plays an important role in breast cancer. Although a lot of this steroid is known, the entire role of progesterone in breast cancer is not defined yet (Richer et al. 2002).

1.2.3 Human epidermal growth factor receptor (Her2/neu)

Her2/neu (ErbB2) is a member of the ErbB-like oncogene family. Therefore it is related to the epidermal growth factor receptor and the receptor tyrosine kinases. Herceptin (Trastuzumab) is a monoclonal antibody used for treatment of those 20% - 30% high-grade invasive breast cancer cells, which are overexpressing this receptor. The amount of this receptor plays an important role for survival and relapse time (Lakhani et al. 2002).

1.2.4 Epidermal growth factor receptor (EGFR)

The epidermal growth factor receptor (ErbB1) belongs to the ErbB family of receptors. This receptor as wells as the receptor Her2/neu mentioned above, are activated by dimerization (homo- or heterodimerization) after the ligand, for example EGF, has bound to the receptor. This receptor is likely to be expressed in a high amount in many breast cancer types. The consequence of this fact is more activation

of the Ras-Raf-MAPK pathway and the PI3K-Akt-mTOR pathway, leading to more proliferation of these cells (Anido et al. 2003).

1.2.5 Further important proteins

1.2.5.1 p53

In about 30% of breast cancer cells this nuclear tumor suppressor is mutated, leading to a high activation of proliferation (Moll et al. 1992). The corresponding gene of the tetrameric protein is located on chromosome 17. This tumor suppressor often plays a role in the poor survival or relapse time of patients with breast cancer. Often no apoptosis can be induced with a mutated form of p53 (Bergh et al. 1995).

1.2.5.2 Ki-67

This protein is related to cellular proliferation and is also used as a proliferation marker in pathological diagnosis. The corresponding gene is located on the chromosome 10. It can be distinguished between two isoforms of this marker. Ki-67, which can be phosphorylated on one serine and one threonine, plays an important role in cell division. Furthermore a lot of this protein is present during mitosis. Cancers with a high amount of this proliferation marker have poor prognosis for survival. Breast cancer cells with mutated p53 express a higher amount of Ki-67, representing a higher proliferation rate. It seems that chemotherapy can work more efficient if there is a higher amount of Ki-67 in early breast cancer (Urruticoechea et al. 2005).

1.2.5.3 Cytokeratin (CK) 5/6

Cytokeratin 5/6 is a basal cytokeratin marker. It seems that this basal marker is associated with worse survival prognosis compared to luminal cytokeratin markers. CK5/6 often is found in invasive breast cancer (Abd El-Rehim et al. 2004). CK5/6 serves as positive marker for basal-like breast cancer phenotype (Cheang et al. 2008).

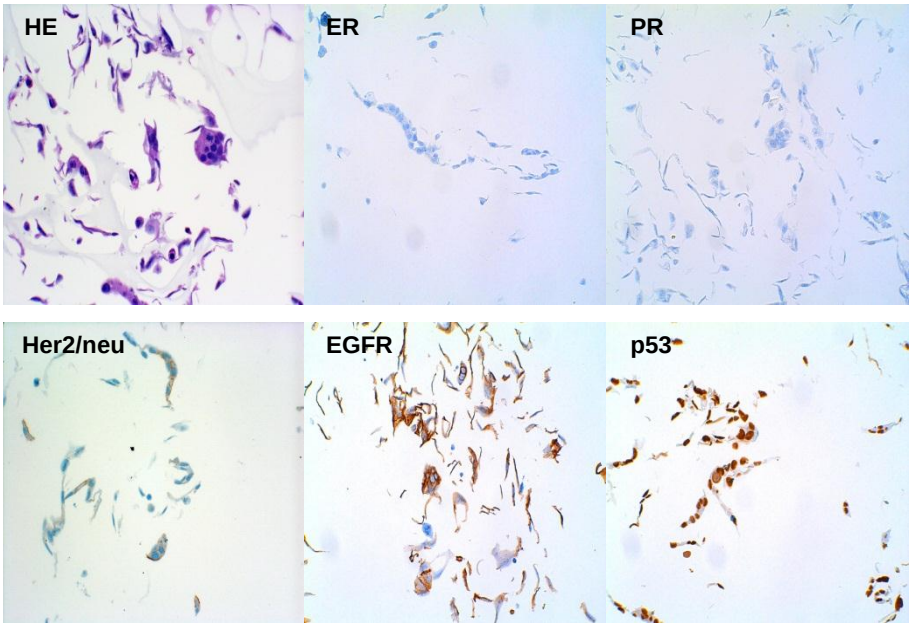
1.3 Cell lines

1.3.1 Basal-like breast cancer cells

Three different cell lines of this breast cancer subtype were used for the experiments (listed below).

1.3.1.1 HCC-38 (CRL-2314)

HCC-38 are adherent cells of the duct in the mammary gland. This cell line expresses Epithelial Glycoprotein 2 (EGP2) and cytokeratin 19. No receptors for the hormones of estrogen and progesterone are expressed. Further this cell line is negative for Her2/neu and positive for the protein p53 (http://www.lgcstandards-atcc.org). The gene cluster of HCC-38 is basal B (Neve et al. 2006). To verify these data, pictures of the ER, PR, Her2/neu, EGFR, CK5/6, p53, Ki67 and HE were taken. The photos illustrated that HCC-38 are negative for ER, PR, Her2/neu and are positive for EGFR, p53, Ki-67 and CK5/6 (see Figure1).



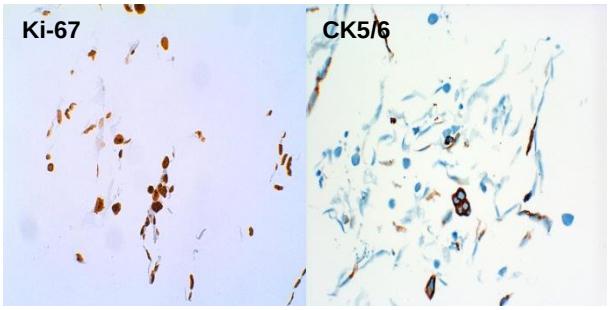
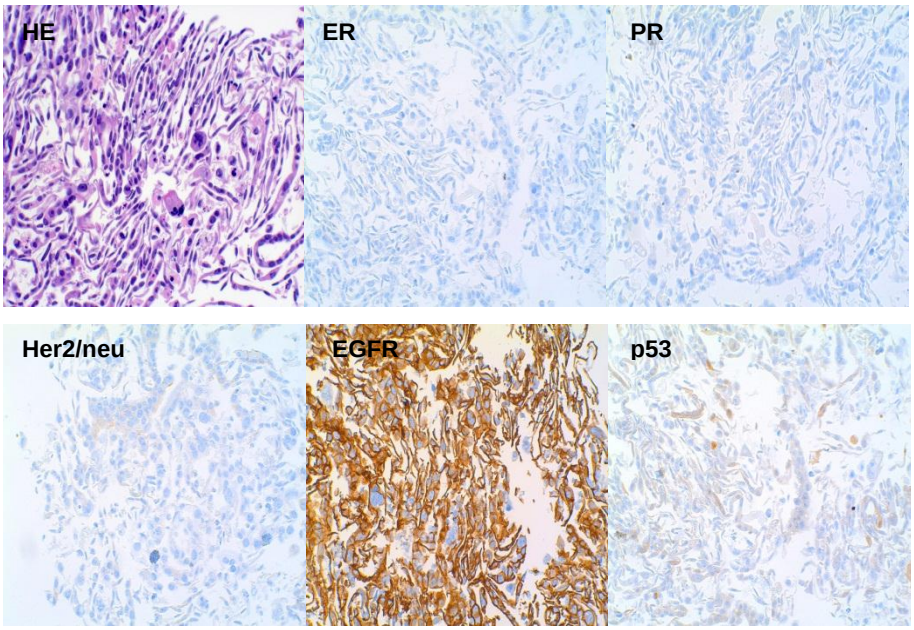


Figure 1: Photos of receptors and proteins of HCC-38. Paraffin blocks were made with HCC-38 followed by staining for HE, ER, PR, Her2/neu, EGFR, p53, Ki-67 and CK5/6. Legend: HE – Hematoxylin and Eosin; ER – estrogen receptor; PR – progesterone receptor, Her2/neu – human epidermal growth factor; EGFR – epidermal growth factor receptor; CK5/6 – cytokeratin 5/6. The pictures are kindly provided by Dr. Zsuzsanna Bagó-Horváth.

1.3.1.2 HCC-1937 (CRL-2336)

HCC-1937 are adherent cells of the duct in the mammary gland too. EGP2 and cytokeratin 19 are expressed in this cell line. Neither the estrogen receptor, nor the progesterone receptor, nor the p53 or the Her2/neu is produced in HCC-1937. Furthermore there is a mutation in the breast cancer 1 gene (BRCA1) (http://www.lgcstandards-atcc.org). The gene cluster of this cell line corresponds to the basal A type (Neve et al. 2006). Again, pictures of the different proteins and receptors of HCC-1937 were taken. The photos showed that this cell lines is negative for ER, PR, Her2/neu, p53 and CK5/6 and is positive for EGFR and Ki-67 (see Figure 2).



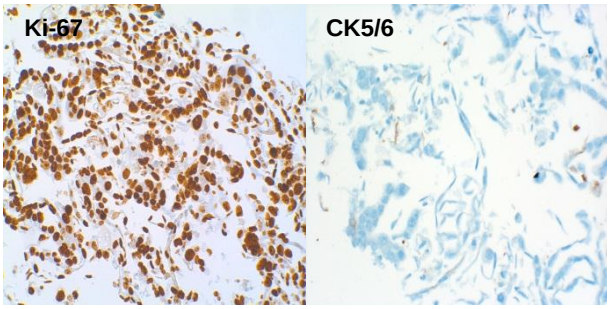
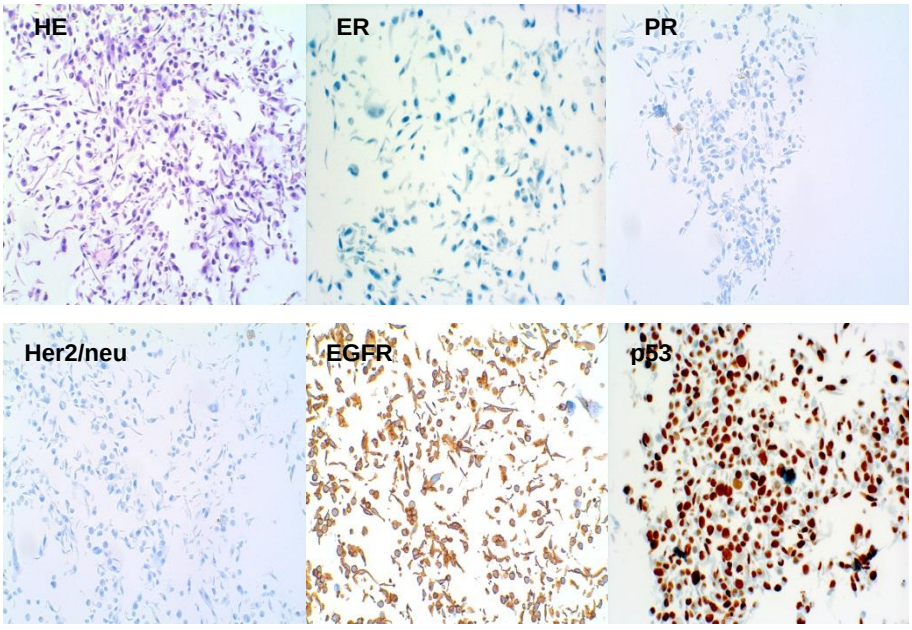


Figure 2: Photos of receptors and proteins of HCC-1937. Paraffin blocks were made with HCC-1937 followed by staining for HE, ER, PR, Her2/neu, EGFR, p53, Ki-67 and CK5/6. Legend: HE – Hematoxylin and Eosin; ER – estrogen receptor; PR – progesterone receptor, Her2/neu – human epidermal growth factor; EGFR – epidermal growth factor receptor; CK5/6 – cytokeratin 5/6. The pictures are kindly provided by Dr. Zsuzsanna Bagó-Horváth.

1.3.1.3 MDA-MB-231 (HTB-26)

These are adherent cells of the mammary gland with an adenocarcinoma. MDA-MB-231 cells are expressing the EGFR and the transforming growth factor receptor alpha (TGF alpha) (http://www.lgcstandards-atcc.org). Furthermore this cell line is negative for the receptors of estrogen, progesterone and Her2/neu and is positive for the protein p53. MDA-MB-231 belongs to the gene cluster basal B (Neve et al. 2006). Pictures were taken from this cell line too. The photos demonstrated that MDA-MB-231 are negative for ER, PR, Her2/neu and CK5/6 and are positive for EGFR, p53 and Ki-67 (see Figure 3).



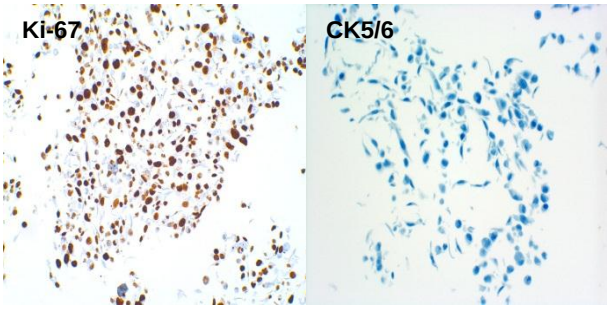


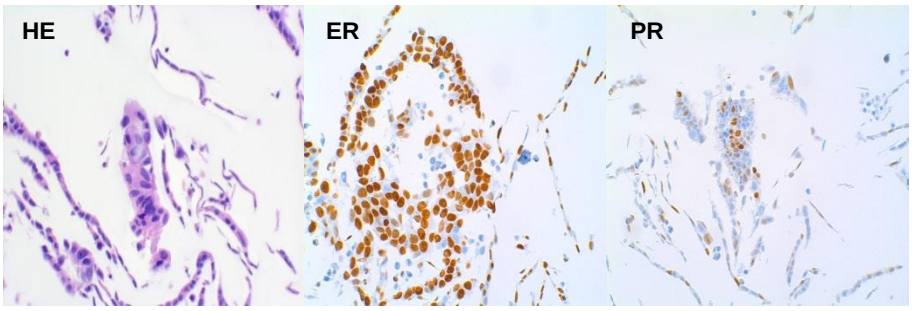
Figure 3: Photos of receptors and proteins of MDA-MB-231. Paraffin blocks were made with MDA-MB-231 followed by staining for HE, ER, PR, Her2/neu, EGFR, p53, Ki-67 and CK5/6. Legend: HE – Hematoxylin and Eosin; ER – estrogen receptor; PR – progesterone receptor, Her2/neu – human epidermal growth factor; EGFR – epidermal growth factor receptor; CK5/6 – cytokeratin 5/6. The pictures are kindly provided by Dr. Zsuzsanna Bagó-Horváth.

1.3.2 Reference breast cancer cells

Three different breast cancer cells with epithelial, luminal or basal origin were used for the experiments (listed below).

1.3.2.1 MCF-7 (HTB-22)

MCF-7 are adherent cells of the mammary gland with an adenocarcinoma. This cell line is positive for the receptors of estrogen (http://www.lgcstandards-atcc.org) and progesterone and slightly positive for Her2/neu. The gene cluster of MCF-7 is luminal (Neve et al. 2006). Pictures of this cell line were taken too, to verify the data. Our data of the photos of MCF-7 illustrated that this cell line is negative for EGFR, CK5/6, p53 and Her2/neu and is positive for Ki-67, ER and PR (see Figure 4).



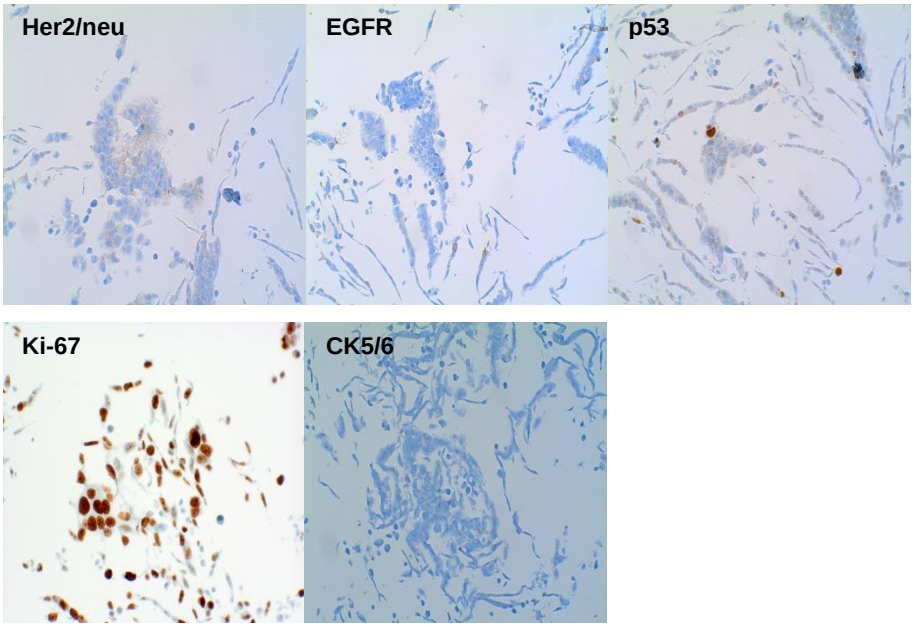
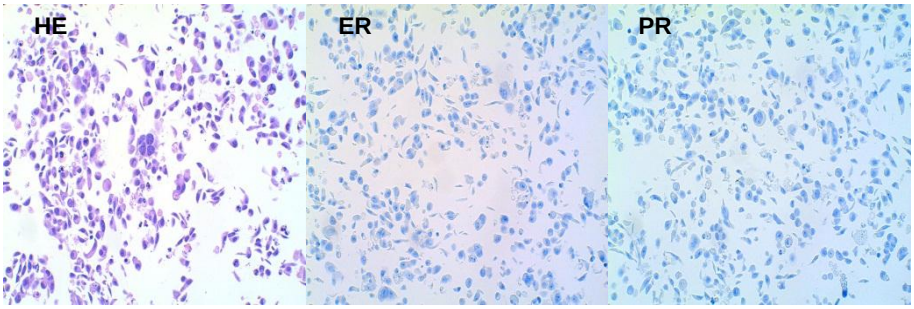


Figure 4: Photos of receptors and proteins of MCF-7. Paraffin blocks were made with MCF-7 followed by staining for HE, ER, PR, Her2/neu, EGFR, p53, Ki-67 and CK5/6. Legend: HE – Hematoxylin and Eosin; ER – estrogen receptor; PR – progesterone receptor, Her2/neu – human epidermal growth factor; EGFR – epidermal growth factor receptor; CK5/6 – cytokeratin 5/6. The pictures are kindly provided by Dr. Zsuzsanna Bagó-Horváth.

1.3.2.2 SKBR-3 (HTB-30)

SKBR-3 are adherent cells of the mammary gland with an adenocarcinoma (http://www.lgcstandards-atcc.org). They are negative for the hormone receptors of estrogen and progesterone and are positive for Her2/neu. These cells belong to the epithelia gene cluster (Neve et al. 2006). Pictures of different receptors and proteins of this cell line were taken too. The photos demonstrated that SKBR-3 cells are positive for Her2/neu, p53, Ki-67 and EGFR and are negative for ER, PR and CK5/6 (see Figure 5).



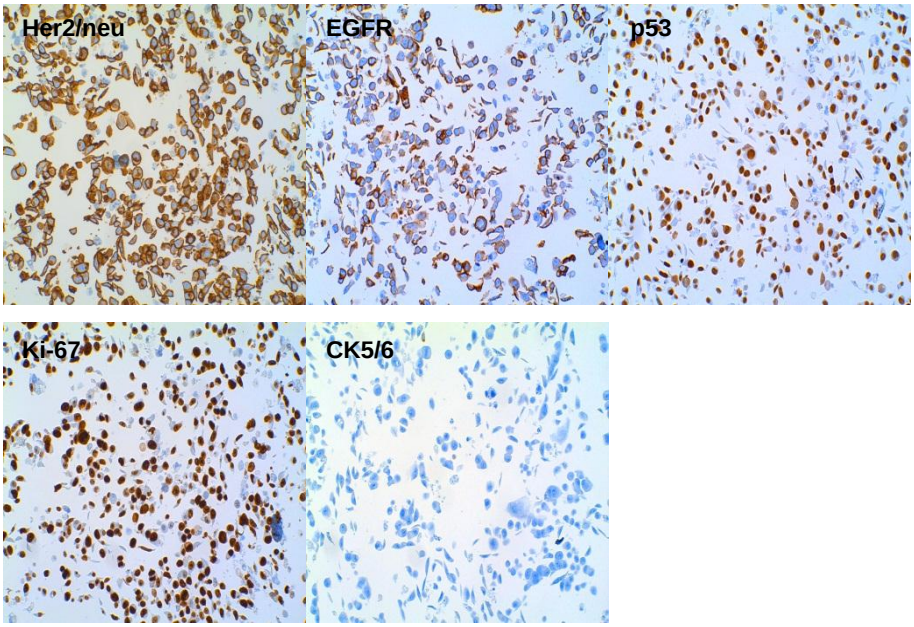
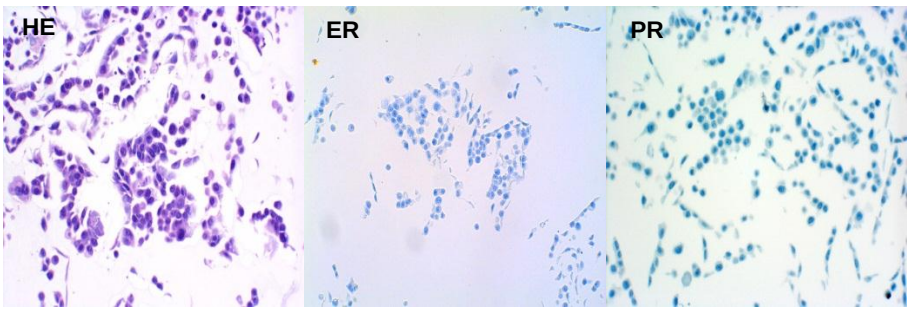


Figure 5: Photos of receptors and proteins of SKBR-3. Paraffin blocks were made with SKBR-3 followed by staining for HE, ER, PR, Her2/neu, EGFR, p53, Ki-67 and CK5/6. Legend: HE – Hematoxylin and Eosin; ER – estrogen receptor; PR – progesterone receptor, Her2/neu – human epidermal growth factor; EGFR – epidermal growth factor receptor; CK5/6 – cytokeratin 5/6. The pictures are kindly provided by Dr. Zsuzsanna Bagó-Horváth.

1.3.2.3 MDA-MB-468 (HTB-132)

The MDA-MB-468 are also adherent cells of the mammary gland with an adenocarcinoma. This cell line is positive for the EGFR and the TGF alpha (http://www.lgcstandards-atcc.org). The gene cluster of MDA-MB-468 is basal A and the cell line is negative for the hormone receptors of estrogen and progesterone. MDA-MB-468 is slightly positive for Her2/neu, which is the difference compared to the real basal-like breast cancer cells. Therefore this cell line has properties of both cancer types (Neve et al. 2006). Again, photos were taken. The photos showed that these cells are positive for p53, Ki-67, CK5/6 and EGFR and are negative for ER, PR andHer2/neu (see Figure 6).



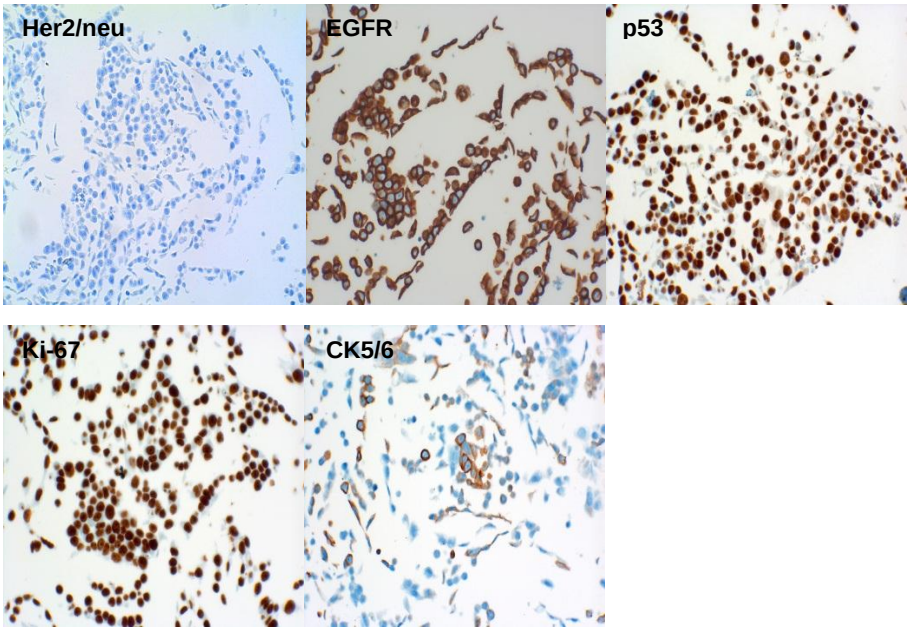


Figure 6: Photos of receptors and proteins of MDA-MB-468. Paraffin blocks were made with MDA-MB-468 followed by staining for HE, ER, PR, Her2/neu, EGFR, p53, Ki-67 and CK5/6. Legend: HE – Hematoxylin and Eosin; ER – estrogen receptor; PR – progesterone receptor, Her2/neu – human epidermal growth factor; EGFR – epidermal growth factor receptor; CK5/6 – cytokeratin 5/6. The pictures are kindly provided by Dr. Zsuzsanna Bagó-Horváth.

1.3.3 Overview of the used cancer cells

The most important molecular characteristics of the three basal-like breast cancer cells and the three reference breast cancer cells are summarized in Table 1 below.

Table 1: Characteristics of the used breast cancer cell lines.

Cell lines	Cluster	ER	PR	Her2/neu	EGFR	p53	Ki-67	CK5/6
HCC-38	**basal B**	**-**	**-**	**-**	**+**	**+**	**+**	**+**
HCC-1937	**basal A**	**-**	**-**	**-**	**+**	**-**	**+**	**-**
MDA-MB-231	**basal B**	**-**	**-**	**-**	**+**	**+**	**+**	**-**
MCF-7	**luminal**	**+**	**+**	**(-)**	**-**	**-**	**+**	**-**
SKBR-3	**epithelial**	**-**	**-**	**+**	**+**	**+**	**+**	**-**
MDA-MB-468	**basal A**	**-**	**-**	**(-)**	**+**	**+**	**+**	**+**

1.4 Y-box binding protein 1 (YB-1)

The Y-box binding protein 1 is a member of the cold shock proteins family (Lasham et al. 2003). High amounts of this transcription and translation factor are found in 73% of basal-like breast cancer cells. Stratford and co-workers could show that this protein can be activated by either the p90 ribosomal S6 kinase (RSK), or by activated Akt or by protein kinase C α (PKCα). This transcription factor is active after the phosphorylation of serine 102 (Stratford et al. 2008). YB-1 is located either in the nucleus or the cytoplasm. Normally, this protein is found in the cytoplasm. In particular during cellular stress, for example after the treatment with cytostatica, YB-1 is translocated into the nucleus where it can work as transcription factor (Lage et al. 2008). After activation of this protein, YB-1 can interact with EGFR, ER-α, Her-2 and c-MYC (Stratford et al. 2008; Lage et al. 2008). Stratford and co-workers could further show that YB-1 is mainly activated by RSK (which is activated via the mitogen activated protein kinase (MAPK) pathway). RSK1 is the more important kinase compared to RSK2 in relation to the YB-1 phosphorylation at serine 102 (Stratford et al. 2008). Phosphorylated YB-1 binds to the EGFR promoter in the nucleus and leads to a high expression of this receptor. Therefore the MAPK pathway is very active (Stratford et al. 2007). Experiments have shown that YB-1 negatively regulates the tumor suppressor p53. The more YB-1 is available, the less p53 can be active and vice versa (Lasham et al. 2003).

1.5 Signaling pathways

1.5.1 Mitogen activated protein kinase (MAPK) pathway

The MAP kinases are serine/threonine kinases, which are activated by an extracellular stimulus. The stimulus is transduced via a signaling cascade from the surface into the nucleus. Three different major groups of theses kinases are classified in mammalian cells: 1) ERKs (extracellular signal-regulated kinases), 2) JNKs (c-Jun amino-terminal kinases) and 3) p38 MAPKs. All groups have a three-tiered kinase cascade in common. This means that a MAPKKK activates a MAPKK, which further activates a MAPK (Johnson et al. 2002). For my diploma thesis, I focused on the ERK pathway. This pathway consists of Ras (G-protein), Raf (MAPKKK), MEK1/2 (MAPKK) and ERK1/2 (MAPK). After binding of the stimulus to

the receptor on the cell surface, the bound GDP of Ras is replaced by GTP. As a result this protein is activated. By binding this protein to Raf, Raf is converted into the active form too. Hence Raf is able to phosphorylate MEK1 and MEK2 at two different serine residues, leading to the activation of this kinase. The same two serines in MEK1 can also be phosphorylated by for example Tpl2, but to a much lower extent. ERK1/2, the last kinases in this cascade, can then be activated by either MEK1 or MEK2 by phosphorylation of threonine and tyrosine residues of the MAPK (Kolch 2000). The MAPK can furthermore activate the p90 ribosomal S6 kinase (RSK), which then is able to activate the Y-box binding protein 1 via phosphorylation at the serine 102 (Stratford et al. 2008). The ERK pathway is needed for cell proliferation, cell survival, tumorigenesis, differentiation and development of the cells. Cancer cells often have mutations in proteins within this pathway, leading to a consistent activation of the ERK pathway and further to more proliferation of the cells. This leads to the assumption that anticancer drugs should downregulate this pathway (Johnson et al. 2002).

1.5.2 Nuclear factor 'kappa-light-chain-enhancer' of activated B-cells(NF-κB) pathway

This pathway can be distinguished between the canonical (NF-κB essential modulators (NEMO) dependent) and the non-canonical (NEMO independent) NF-κB signaling pathway. Recent studies led to the assumption that there occur more interactions between these two different pathways than it was always thought (Shih et al. 2011). Inflammation, immunity, apoptosis and cell proliferation are the processes which are triggered by the NF-κB pathway (Viatour et al. 2005). The transcription factor NF-κB can exist as homodimer or heterodimer of the Rel homology domain (RHD). These dimers can be built by five monomers (RelA, RelB, cRel, p50 and p52) (Shih et al. 2011). For my diploma thesis, the dimer RelA (p65):p50, which is part of the canonical way, was the most important one. At first the stimulus (e.g. TNF-α) binds to the corresponding receptor on the cell surface. This leads to the binding of some adaptors and the IκB kinase (IKK) complex to the receptor. The inhibitory molecule IκB-α is also bound to the IKK complex. After activation of this complex, the inhibitory molecule is at first phosphorylated, then

ubiquitinilated and at last degraded via the proteasome. After the depletion of this molecule, the p50:p65 complex can be phosphorylated and transduced into the nucleus. The NF-κB can bind the DNA in the unphosphorylated or in the phosphorylated state. In addition the whole NF-κB-IκB complex can be found either in the cytoplasm (mainly) or in the nucleus. The complex of adaptors, IKKs and IκB-α furthermore can activate Tpl2 (MAPKKK) to phosphorylate the p105 subunit, which then is ubiquitinilated and degraded. As a result free Tpl2 can further phosphorylate NF-κB (which then is transported into the nucleus) on the one hand, or activate the MAPKK, MEK1, on the other hand. This kinase in turn is able to activate ERK and then RSK, which again can phosphorylate NF-κB for example (Viatour et al. 2005). The NF-κB signaling pathway seems to play an important role in various cancer types. Often there is a mutation somewhere within this pathway, which leads to a permanent active transcription factor. This further leads to survival, invasion and metastasis of these cancer types. For example, basal-like breast cancer cells are cancer types, which usually show a high activity of this transcription factor (Yamaguchi et al. 2009).

1.5.3 Phosphatidylinositol-3-kinase (PI3K) – Akt – mammalian target of Rapamycin (mTOR) pathway

This signaling pathway is very important for the cells because it is necessary for many essential functions like cell proliferation, cell survival, cell differentiation transcription and translation (McAuliffe et al. 2010). After the binding of the stimulus to the receptor on the cell surface, the lipid kinase PI3K is activated and generates phosphatidylinositol-3,4,5-triphosphate ($PI(3,4,5)P_3$) (PIP3) (Osaki et al. 2004). This leads to the activation of phosphoinositide-dependent kinase (PDK) 1, which causes the phosphorylation of Akt (also called Protein Kinase B (PKB)). The phosphorylated form of this serine/threonine kinase can activate mTOR, which is able either to activate Akt again via a feedback loop or phosphorylate the p70 S6 kinase (McAuliffe et al. 2010). Another possibility of active Akt is the phosphorylation of the IKK complex. This means that the serine/threonine kinase is able to activate the NF-κB pathway, leading to survival of the cells (Ozes et al. 1999). Further interactions of the PI3K-Akt-mTOR pathway with other pathways, for example the MAPK pathway,

seem to occur (Osaki et al. 2004). This pathway seems to play an important role in human cancer. Often the tumor suppressor gene phosphatase and tensin homolog (PTEN) is mutated or lost, leading to a permanently activated PI3K-Akt-mTOR pathway. This causes the proliferation and survival of cancer cells, for example of basal-like breast cancer cells (McAuliffe et al. 2010).

1.5.4 Interactions of the signaling pathways

After stimulation of one of the described pathways, this pathway is usually not the only activated one. There are some interactions between these signaling pathways which are known for sure and some which are assumed (Kolch 2000; Stratford et al. 2008; Viatour et al. 2005; McAuliffe et al. 2010; Ozes et al. 1999; Osaki et al. 2004). Some of these interactions concerning the MAPK pathway, the PI3K-Akt-mTOR pathway and the NF-κB pathway are shown in Figure 7 below.

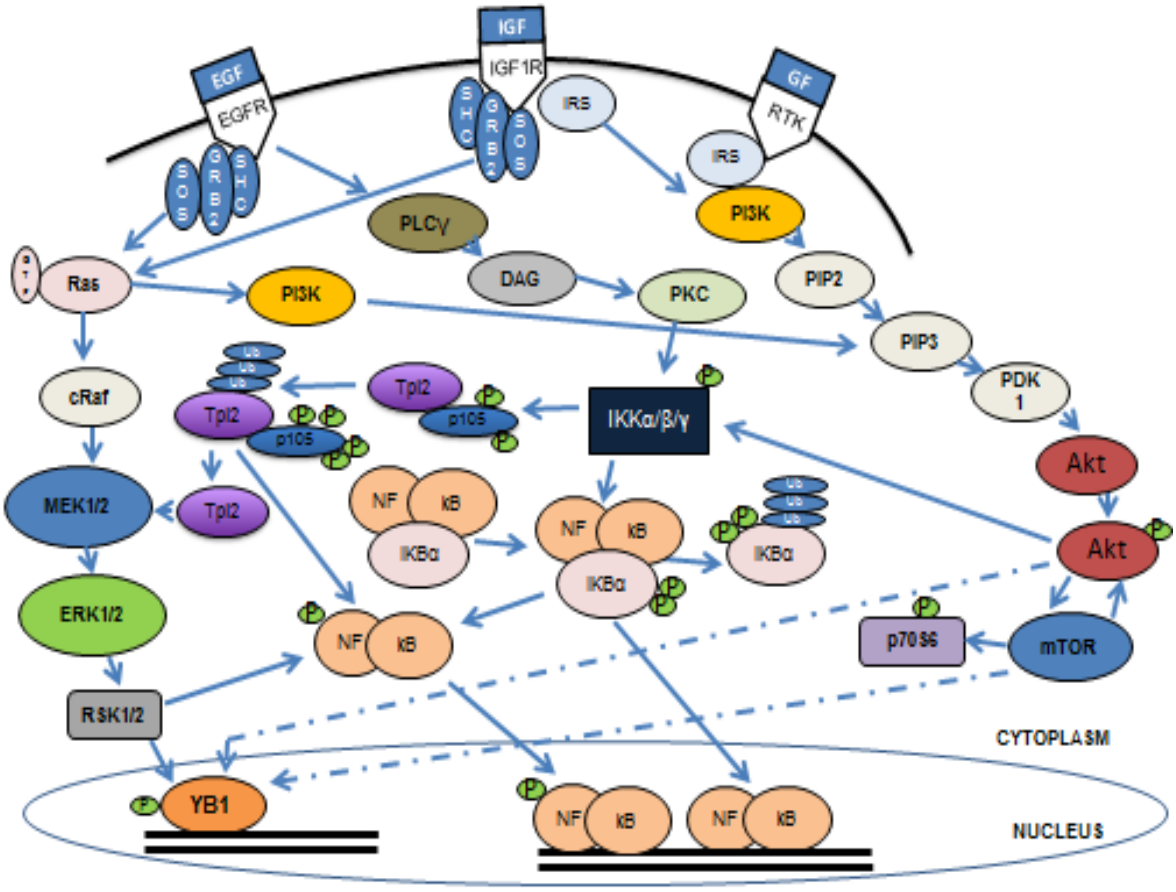


Figure 7: Interactions of the MAPK pathway, the PI3K-Akt-mTOR pathway, the canonical NF-κB pathway and the Tpl2 dependent NF-κB pathway. Legend: The blue arrows stand for known interactions, the blue dotted arrows stand for interactions which are not known for sure.

1.6 Inhibitors

1.6.1 Cetuximab (Erbitux)

This substance is a mouse/human chimeric monoclonal antibody (IgG1) against the extracellular domain of the EGFR, which blocks the activation of the receptor tyrosine kinase. This inhibitor is often used for treatment of colon and breast cancer types with an overexpression of the EGF receptor. Xu and co-workers could further show that cetuximab is not effective if there is an overexpression of the EGFR on the one hand and an overexpression of, for example, Her2 on the other hand (Xu et al. 2005).

1.6.2 PD98059

PD98059 is an inhibitor of the mitogen activated protein kinase kinases, MEK1 and MEK2, and leads furthermore to no activation of the MAP kinases, ERK1 and ERK2. Monno and co-workers could show in their experiments that the level of phosphorylated ERK is reduced in MCF-7 cells after the treatment with this substance (Monno et al. 2000).

1.6.3 U0126, U0124

U0126 is an inhibitor of MEK1 and MEK2 without affecting ERK1, ERK2 or ATP. This substance is able to suppress the amount of active MAPK in MCF-7 cells, but not the total amount of the kinase (Kurokawa et al. 2000). U0124 is a negative control for U0126.

1.6.4 ERK Inhibitor II (FR180204)

This substance is an inhibitor of ERK1 and ERK2. Ohori and co-workers were able to show that this ERK Inhibitor can be used to treat mouse collagen-induced arthritis (CIA). A reduction of immune response can be achieved by inhibiting ERK with this substance (Ohori et al. 2007).

1.6.5 ERK Inhibitor III

The phosphorylation of ERK1 and ERK2 is not affected by this inhibitor. This substance only avoids the phosphorylation of the ERK substrates RSK1 and Elk-1 via the EGF-stimulated pathway (http://www.emdchemicals.com).

1.6.6 SL0101

SL0101 is an inhibitor of the p90 ribosomal S6 kinase. In addition to this function, it also affects the proliferation of some cells. This substance does not have an effect on the survival of MCF-10A, a normal breast cancer cell line. The treatment of MCF-7 with SL0101 leads to a suppression of their growth (Troussard et al. 2006). Furthermore this inhibitor leads to a lower amount of the phosphorylated protein YB-1^{S102}, therefore a lower amount of YB-1 binding the promoter and thus leading to less production of the EGFR (Stratford et al. 2008).

1.6.7 Akt Inhibitor IV

This substance avoids the phosphorylation and therefore the activation of Akt. The binding of Akt Inhibitor IV instead of ATP occurs at a kinase upstream of Akt. No impact on the protein PI3 kinase of this substance could be detected (http://www.emdchemicals.com).

1.6.8 Rapamycin

Rapamycin is an inhibitor of the serine-threonine kinase mammalian target of rapamycin (mTOR). It leads to a suppression of the protein translation and to a disturbance of a proper cell cycle activity. Furthermore the phosphorylation of the p70 S6 kinase and the 4E binding protein 1 cannot take place. Noh and co-workers could show that after the treatment with rapamycin, 12 out of 15 different breast cancer cell lines were not able to proliferate normally (Noh et al. 2004).

1.6.9 Wortmannin

This fungal metabolite is a selective, permeable and irreversible inhibitor of the catalytic activity of the PI3 kinase (Weng et al. 1999; Okada et al. 1994). The growth

of MCF-7 cells is suppressed in a similar way either by treatment of the cells with wortmannin or an overexpression of PTEN (Weng et al. 1999).

1.6.10 PQ401

PQ401 is a diaryl urea compound which binds at the kinase domain, the β-subunit, of the IGF1R. This substance leads to a blocking of the autophosphorylation of this receptor in MCF-7 cells and furthermore to no growth of these breast cancer cells. In addition, the treatment of MCF-7 breast cancer cells with PQ401 leads to more activation of the caspase. It seems that this substance induces the Akt antiapoptotic pathway after inhibiting the IGF1R (Gable et al. 2006).

1.6.11 Violacein

The purple-colored pigment violacein is obtained from the *Chromobacterium violaceum*, which is found in the Amazon in Brasil. This inhibitor plays a very important role in the treatment of cancer because of its antiviral, antibacterial, antitumoral, antileishmanial and antiulcerogenic effects. There are four known modes of actions of violacein, like the inhibition of the phosphorylation of IκB-α, less production of the two NF-κB subunits p50 and p65, blocking the NF-κB transport into the nucleus and at last a downregulation of the amount of phosphorylated Akt. These efficacies lead to the inhibition of cell growth and to an induction of apoptosis in colon cancer cells. Violacein does not only alter the activity of the NF-κB pathway, furthermore a suppression of the PI3-kinase–Akt–mTOR pathway can be observed. It seems that this pigment does not have an impact on the MAPK pathway (Kodach et al. 2006).

1.6.12 Disulfiram

This substance belongs to the dithiocarbamate family. This inhibitor can bind copper and the aldehyde dehydrogenase is suppressed by this substance (Chen et al. 2006). Important characteristics of this antioxidant for the treatment of cancer are the altering of the redox potential and the blocking of reactive oxygen intermediates. The first feature of this substance leads to a downregulation of the amount of NF-κB which can bind the DNA in the nucleus. The other mentioned characteristic causes

the depletion of IκB-α (Wu et al. 2005). Furthermore disulfiram is used as substance for alcohol withdrawal (Chen et al. 2006).

1.6.13 BAY 11-7082

BAY 11-7082 is an inhibitor of the NF-κB pathway. This substance avoids the phosphorylation of IκB-α at the one hand and blocks the TNF-α-induced NF-κB activation on the other hand. It leads to apoptosis in colon cancer cells and therefore BAY 11-7082 plays an important role in treatment of this cancer type (Scaife et al. 2002).

1.6.14 MG-132

This substance blocks the activity of the 26S proteasome (Morelli et al. 2003). MG-132 treated MDA-MB-231 breast cancer cells have a reduced NF-κB binding activity. Furthermore this substance leads to less production of VEGF mRNA in MDA-MB-231 (Shibata et al. 2002).

1.6.15 Tpl2 Kinase Inhibitor

The Tpl2 kinase exists in two isoforms and functions as an oncogene (Gantke et al. 2011). It is a cell-permeable substance and inhibits the serine/threonine kinase Tpl2. The blocking of this kinase causes the suppression of the TNF-α production and the downregulation of signaling (Gavrin et al. 2005).

1.7 Objectives of my work

Among the different subtypes of breast cancer, basal-like breast cancer shows particular characteristics. This cancer type is triple negative for the receptors estrogen, progesterone and Her2/neu. Thus, this carcinoma cannot be treated with anti-estrogens or Her2 neutralizing antibodies and is hardly susceptible to cytotoxic therapies (Cleator et al. 2007). Nevertheless, my laboratory could show previously that some cytotoxic compounds such as platinum derivates, taxanes and anthracyclines have effects on this subtype. In the last years research in tumor biology led to the assumption that signaling pathways and transcription factors may offer another option of treatment of this aggressive breast cancer (Johnson et al. 2002; Yamaguchi et al. 2009; McAuliffe et al. 2010).

The long-term objective of my work was to identify alternative therapies based on signaling pathways for this mamma carcinoma. This goal should be reached by identifying the key signaling pathways and transcription factors which are needed for the survival of these cancer cells. The used inhibitors are listed in Figure 8 below. For the experiments three basal-like breast cancer cells (HCC-38, HCC-1937, MDA-MB-231) and three reference mamma carinomas (MCF-7, SKBR-3, MDA-MB-468) were used.

Following methods were done:

- Dose-response curves (MTT-assays) and adjustment via non-linear regression with inhibitors of different signaling pathways alone or in combination as well as in combination with cytotoxins (calculation of the IC_{50}: inhibiting concentration of a substance for 50% of the cells).
- Immunoblotting with cell lysates of inhibitor treated cell lines for quantitative analysis of the signaling pathways and the transcription factors.

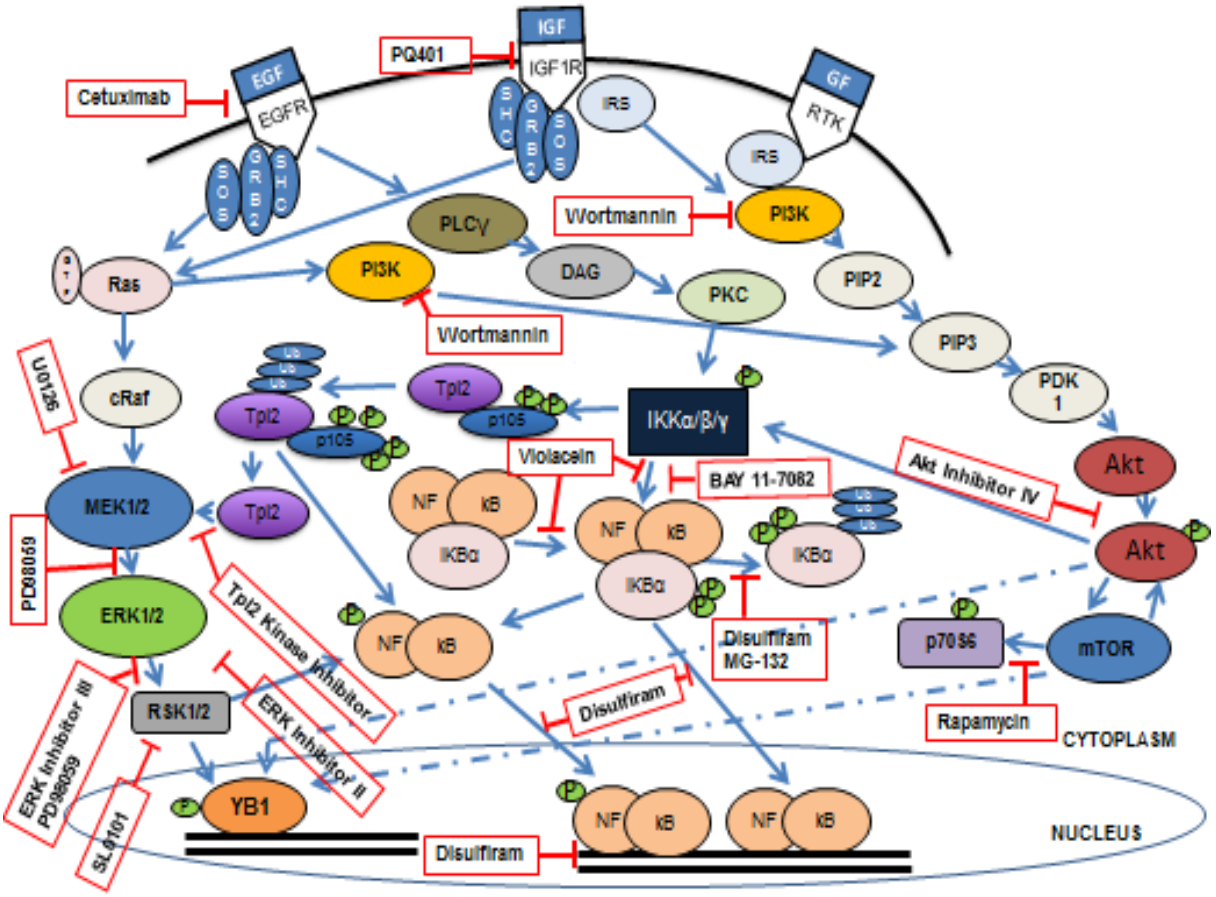


Figure 8: Interactions of the MAPK pathway, the PI3K-Akt-mTOR pathway, the canonical NF-κB pathway and the Tpl2 dependent NF-κB pathway. Legend: The blue arrows stand for known interactions, the blue dotted arrows stand for interactions which are not known for sure. The red boxes are the used inhibitors.

2 Materials and Methods

2.1 Materials

2.1.1 Equipment

Table 2: List of equipment used in this work.

Device	Device name	Supplier
heating block	Hybaid Omni Gene	genXpress
scale	BP2100S	Sartorius
pH – meter	PHM 83 Autocal	Radiometer
pH – electrode		Metrohm
centrifuge 1	EBA 12	Hettich
rotor 1	1412	Hettich
centrifuge 2	Rotanta 46 RSC	Hettich
rotor 2	5624	Hettich
vortexer	L24	Labinco
spectral photometer	U-2900	Hitachi
quartz cuvettes		Hellma
X – ray films	Amersham Hyperfilm® ECL	GE Healthcare
X – ray film cassette		Kodak
developer	E.O.S.	Agfa
blotting apparatus	TransBlot SD Cell	Bio Rad
PVDF – membrane		Thermo Scientific
Whatman paper		Whatman
SDS – Page apparatus	MiniProtean	Bio Rad
1.5 ml tubes		Sarstedt

Device	Device name	Supplier
tips		Greiner bio – one
sterile plugged tips		ART
pipettor	Pipetus®	Hirschmann Laborgeräte
pipettes		Costar
cell scraper		Sarstedt
magnetic stirrer	M20/1	Framo Gerätetechnik
incubator	Cytoperm2	Heraeus
laminar flow	Maxisafe 2020	Thermo Scientific
microscope 1	Nikon TMS	Nikon
microscope 2	BH-2	Olympus
water bath		Memmert
counting chamber	Bürker – Türk	Marienfeld
syringes		Gilson
cryo tubes		Nalgene
Asys Reader	Asys Expert Plus	Asys
96 – well plates		Corning
6 – well plates		Corning
15 ml tubes		Corning
50 ml tubes		Corning
30 ml tubes	Sterilin tubes	Bertoni
sonicator	Microson XL 2000	Misonix

2.1.2 Cell lines

Table 3: List of used cell lines.

Cell line	**ATCC®number**
HCC-38	CRL-2314
HCC-1937	CRL-2336
MDA-MB-231	HTB-26
MCF-7	HTB-22
SKBR-3	HTB-30
MDA-MB-468	HTB-132

2.1.3 Materials for cell culture methods

2.1.3.1 Reagents

Table 4: List of used reagents in cell culture methods.

Product	**Supplier**
RPMI 1640-Glutamax + L-glutamine	Invitrogen
Gentamycin	Invitrogen
Fetal calf serum (FCS)	PAA
Accutase	PAA
Trypsin	PAA
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich
Ethanol (100%)	Merck
Dye Solution	Promega
Solubilization Solution/ Stop Mix	Promega

2.1.3.2 Growth medium

For all experiments the following growth medium was used.

Table 5: Composition of used growth medium.

Amount / Concentration	**Product**
500 ml	RPMI 1640-Glutamax
4 mM	L-glutamine
5 mg	Gentamycin
10 % (heat inactivated)	FCS
➔ stored at 4°C	

2.1.3.3 Inhibitors and cytotoxins

Table 6: List of used inhibitors and cytotoxins.

Product	**Supplier**	**Solvent**
Cetuximab	cytotostatica pharmacy, AKH	DMSO
PD98059	Merck	DMSO
U0126	Merck	DMSO
U0124	Merck	DMSO
ERK Inhibitor II	Merck	DMSO
ERK Inhibitor III	Merck	DMSO
SL0101	Merck	Ethanol
PQ401	Tocris	DMSO
InSolution Akt Inhibitor IV®	Merck	DMSO
Wortmannin	Merck	DMSO
Rapamycin	Merck	DMSO
Violacein	Sigma-Aldrich	DMSO
Tetraethylthiuram disulfide (Disulfiram)	Sigma-Aldrich	DMSO

Product	Supplier	Solvent
MG-132	Enzo Life Science	DMSO
Tpl2-Kinase Inhibitor	Merck	DMSO
BAY 11-7082	Sigma-Aldrich	DMSO
Epirubicin	cytotostatica pharmacy, AKH	
Docetaxel	cytotostatica pharmacy, AKH	
Cisplatin	cytotostatica pharmacy, AKH	

2.1.4 Materials for molecular biological methods

2.1.4.1 Reagents

Table 7: List of used reagents in molecular biological methods.

Product	Supplier
Acrylamide/bis (97/3), 30 %	Merck
Ammonium peroxodisulfate (APS)	Fluka
Bromphenol blue	Merck
Complete Mini EDTA-free protease Inhibitor Cocktail Tablets	Roche
1,4 Dithiothreitol (DTT)	Merck
Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich
Glacial acetic acid	Merck
Glycerol	Merck
Glycine	Merck
Potassium chloride	Merck
Methanol	Merck
Sodium acetate	Merck
Sodium chloride	Merck

Product	Supplier
Poinceau S	Sigma-Aldrich
PhosSTOP Phosphatase Inhibitor Cocktail Tablets	Roche
Phosphate bufferd saline (PBS)	Invitrogen
Sodiumdodecylsulfat (SDS)	Pharmacia
SuperSignal® West Dura Extended Duration Substrate	Thermo Scientific
SuperBlock® Blocking Buffer	Thermo Scientific
Temed	Bio Rad
Tris base	Merck
Tween-20	Merck
NuPAGE® LDS Sample Buffer (4x)	Invitrogen

2.1.4.2 Antibodies and Markers

Table 8: List of used markers, primary and secondary antibodies.

Product	Supplier	Size (kDa)
MagicMark™ XP Western Protein Standard	Invitrogen	
Precision Plus Protein™ Dual Color Standards	Bio-Rad	
GAPDH (FL-335): sc-25778	Santa Cruz	37
Rabbit monoclonal [EP2708Y] to YB1 (ab76149)	Abcam	49
Phospho-YB1 (Ser102) (C34A2) Rabbit mAb	Cell Signaling	49
RSK-2 (E-1): sc-9986	Santa Cruz	80
IKK-α	Cell Signaling	85
IKK-β	Cell Signaling	87
phospho-IKK-α/β	Cell Signaling	85/87
NF-κBp65	Cell Signaling	65

Product	Supplier	Size (kDa)
phospho-NF-κBp65	Cell Signaling	65
IκB-α	Cell Signaling	39
phospho- IκB-α	Cell Signaling	41
MEK1/2	Cell Signaling	45
Goat anti rabbit IgG HRPO (H+L)	Pierce	
Goat anti mouse IgG HRPO (H+L)	Pierce	

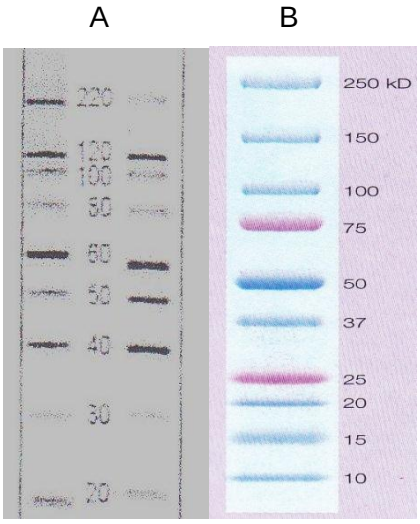


Figure 9: Used protein markers. (A) MagicMark™ XP Western Protein Standard, (B) Precision Plus Protein™ Dual Color Standards.

2.1.4.3 Buffers

All buffers were prepared after the Standard Operating Procedure of the laboratory.

0.5 M Tris-HCl (pH 6.8) buffer

Table 9: Chemical composition of 0.5 M Tris-HCl (pH 6.8) buffer.

Concentration (Amount)	Product/Supplier	Storage
0.5 M (6.05 g/ 100 ml ddH_2O)	Tris base (Merck)	RT
➔ pH adjusted to 6.8 with HCl and sterile filtered		

1 M Tris-HCl (pH 9.1) buffer

Table 10: Chemical composition of 1 M Tris-HCl (pH 9.1) buffer.

Concentration (Amount)	Product/Supplier	Storage
1 M (12.1 g/ 100 ml ddH_2O)	Tris base (Merck)	RT
➔ pH adjusted to 9.1 with HCl and sterile filtered		

20% SDS buffer

Table 11: Chemical composition of 20% SDS buffer.

Concentration (Amount)	Product/Supplier	Storage
20% (20 g/ 100 ml ddH_2O)	SDS (Pharmacia)	RT
➔ sterile filtered		

40% APS buffer

Table 12: Chemical composition of 40% APS buffer.

Concentration (Amount)	Product/Supplier	Storage
40% (400 mg/ 1 ml ddH_2O)	APS (Fluka)	-20°C

5x Tris-Glycine (pH 8.3) buffer

Table 13: Chemical composition of 5x Tris-Glycine (pH 8.3) buffer.

Concentration (Amount)	Product/Supplier	Storage
125 mM (15.1 g/1 l ddH_2O)	Tris base (Merck)	RT
1250 mM (94 g/ 1 l ddH_2O)	Glycine (Merck)	
0.5% (50 ml/ 1 l ddH_2O)	10% SDS (Pharmacia)	
➔ pH adjusted to 8.3 with HCl and sterile filtered		

Transfer buffer

Table 14: Chemical composition of Transfer buffer.

Concentration (Amount)	Product/Supplier	Storage
39 mM (2.9 g/1 l ddH_2O)	Glycine (Merck)	4°C
48 mM (5.8 g/ 1 l ddH_2O)	Tris base (Merck)	
0.037% (3.7 ml/ 1 l ddH_2O)	10% SDS (Pharmacia)	
20% (200 ml/ 1 l ddH_2O)	Methanol (Merck)	
➔ sterile filtered		

2x SDS-lysis buffer (non-denaturing)

Table 15: Chemical composition of 2x SDS-lysis buffer (non-denaturing).

Concentration (Amount)	Product/Supplier	Storage
50 mM (1 ml/10 ml ddH_2O)	0.5 M Tris-HCl pH 6.8	-20°C
6% (3 ml/ 10 ml ddH_2O)	20% SDS (Pharmacia)	
20% (2 ml/ 10 ml ddH_2O)	100% Glycerol (Merck)	
1.85 mM (37 µl/ 10 ml ddH_2O)	0.5 M EDTA (Sigma-Aldrich)	
1 Tablet	PhosSTOP Phosphatase Inhibitor Cocktail (Roche)	
1 Tablet	Complete Mini EDTA-free Protease Inhibitor Cocktail (Roche)	

10x Tris buffered saline (TBS) (pH 7.4) buffer

Table 16: Chemical composition of 10x Tris buffered saline (TBS) (pH 7.4) buffer.

Concentration (Amount)	Product/Supplier	Storage
136.9 mM (80 g/1 l ddH_2O)	Sodium chloride (Merck)	RT
5.4 mM (4 g/ 1 l ddH_2O)	Potassium chloride (Merck)	
49.5 mM (60 g/ 1 l ddH_2O)	Tris base (Merck)	
➔ pH adjusted to 7.4 with HCl and sterile filtered		

0.5% Glacial acetic acid solution

Table 17: Chemical composition of 0.5% Glacial acetic acid solution.

Concentration (Amount)	Product/Supplier	Storage
5% (5 ml/100 ml ddH_2O)	Glacial acetic acid (Merck)	RT

0.1% Poinceau S solution

Table 18: Chemical composition of 0.1% Poinceau S solution.

Concentration (Amount)	Product/Supplier	Storage
0.1% (0.1 g/100 ml ddH_2O)	5% Glacial acetic acid	RT
➔ sterile filtered, reusable		

2x probe buffer (denaturing)

Table 19: Chemical composition of 2x probe buffer (denaturing).

Concentration (Amount)	Product/Supplier	Storage
72 mM (100 mg/10 ml ddH_2O)	DTT (Merck)	-20°C
5 ml	100% Glycerol (Merck)	
1 pipette tip	Bromphenol blue (Merck)	

2.2 Methods

2.2.1 Cell culture methods

2.2.1.1 Cell Proliferation Assay

The CellTiter 96® Non-Radioactive Cell Proliferation Assay (MTT-Assay [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]) was performed to determine the dose-response relationship in the used cellular systems.

- **harvesting and counting cells**

At first the growth medium was discarded and Accutase was added for 8 minutes at 37°C, 5% CO_2:95% air to detach the cells. After harvesting the cells they were centrifuged at 287 g for 5 minutes at room temperature. Then the cells were counted using Bürker-Türk counting chambers to reach the amount of 10^4 cells in 100 µl per well in a 96-well microtiterplate. All experiments were performed in triplicates.

- **serial dilution of inhibitors/ cytotoxin**

A serial dilution was made with the used inhibitors and cytotoxins. 25 µl of the substances were mixed with the cells in the 96-well plate (no inhibitors/ cytotoxins in well A, increasing inhibitor/ cytotoxin concentration from well B to H). Growth medium was added into the wells to reach the end volume of 150 µl. The microtiterplates were put into the incubator (37°C, 5% CO_2:95% air) for 72 hours.
Following inhibitor/cytotoxin concentrations were used for the experiments (see Table 20).

Table 20: Concentration range and incubation time of used substances.

Substance	Concentration range	Incubation time
Rapamycin	0.1 nM - 100 µM	72 h
Cetuximab	0.01 ng/ml – 10 µg/ml	72 h
Violacein	1 nM – 1 mM	72 h
Akt Inhibitor IV	0.01 nM – 10 µM	72 h
Wortmannin	0.1 nM – 0.1 mM	72 h

Substance	Concentration range	Incubation time
Disulfiram	4 nM – 4 mM	72 h
SL0101	0.1 nM – 0.1 mM	72 h
PQ401	0.1 nM – 0.1 mM	72 h
ERK Inhibitor II	0.1 nM – 0.1 mM	72 h
ERK Inhibitor III	0.1 nM – 0.1 mM	72 h
PD98059	0.1 nM – 0.1 mM	72 h
U0126	0.1 nM – 0.1 mM	72 h
U0124	0.1 nM – 0.1 mM	72 h
Tpl2 Kinase Inhibitor	0.1 pM – 0.1 µM	72 h
BAY 11-7082	0.1 nM – 0.1 mM	72 h
MG-132	0.1 nM – 0.1 mM	72 h
Akt Inhibitor IV + Rapamycin	0.01 µM – 0.1 µM (A) + 1 µM – 10 µM (R)	72 h
Akt Inhibitor IV + Violacein	0.01 µM – 0.1 µM (A) + 0.1 nM – 1 nM (V)	72 h
Akt Inhibitor IV + Wortmannin	0.01 µM – 0.1 µM (A) + 0.01 µM + 1 µM (W)	72 h
PD98059 + ERK Inhibitor II	0.1 µM + 10 µM (P) + 1 µM – 10 µM (E)	72 h
ERK Inhibitor II + SL0101	1 µM – 10 µM (E) + 1 µM – 10 µM (S)	72 h
Wortmannin + ERK Inhibitor II	0.01 µM - 1 µM (W) + 1 µM – 10 µM (E)	72 h

Substance	Concentration range	Incubation time
Akt Inhibitor IV + PD98059	0.01 µM – 0.1 µM (A) + 0.1 µM – 10 µM (P)	72 h
Akt Inhibitor IV + ERK Inhibitor II	0.01 µM – 0.1 µM (A) + 1 µM – 10 µM (E)	72 h
Akt Inhibitor IV + SL0101	0.01 µM – 0.1 µM (A) + 1 µM – 10 µM (S)	72 h
PD98059 + Wortmannin	0.1 µM – 10 µM (P) + 0.01 µM - 1 µM (W)	72 h
PD98059 + Violacein	0.1 µM – 10 µM (P) + 0.1 nM – 1 nM (V)	72 h
Epirubicin + Akt Inhibitor IV	0.1 nM – 0.1 µM (E) + 0.01 µM – 0.1 µM (A)	72 h
Epirubicin + Violacein	0.1 nM – 0.1 µM (E) + 0.1 nM – 1 nM (V)	72 h
Epirubicin + PD98059	0.1 nM – 0.1 µM (E) + 0.1 µM – 10 µM (P)	72 h
Epirubicin + Wortmannin	0.1 nM – 0.1 µM (E) + 0.01 µM - 1 µM (W)	72 h
Docetaxel + Akt Inhibitor IV	0.01 nM – 10 nM (D) + 0.01 µM – 0.1 µM (A)	72 h
Docetaxel + Violacein	0.01 nM – 10 nM (D) + 0.1 nM – 1 nM (V)	72 h
Docetaxel + Wortmannin	0.01 nM – 10 nM (D) +	72 h

Substance	Concentration range	Incubation time
	0.01 µM - 1 µM (W)	
Docetaxel + PD98059	0.01 nM – 10 nM (D) +	72 h
	0.1 µM – 10 µM (P)	
Cisplatin + Akt Inhibitor IV	0.01 µM – 10 µM (C) +	72 h
	0.01 µM – 0.1 µM (A)	
Cisplatin + Violacein	0.01 µM – 10 µM (C) +	72 h
	0.1 nM – 1 nM (V)	
Cisplatin + Wortmannin	0.01 µM – 10 µM (C) +	72 h
	0.01 µM - 1 µM (W)	
Cisplatin + PD98059	0.01 µM – 10 µM (C) +	72 h
	0.1 µM – 10 µM (P)	
Violacein + Tpl2 Kinase Inhibitor	0.1 nM – 1 nM (V) +	72 h
	0.1 nM – 1 nM (T)	
Violacein + BAY 11-7082	0.1 nM – 1 nM (V) +	72 h
	0.01 µM – 1µM (B)	
Violacein + MG-132	0.1 nM – 1 nM (V) +	72 h
	1 nM – 10 nM (M)	
BAY 11-7082 + Tpl2 Kinase Inhibitor	0.01 µM – 1µM (B) +	72 h
	0.1 nM – 1 nM (T)	
BAY 11-7082 + MG-132	0.01 µM – 1µM (B) +	72 h
	1 nM – 10 nM (M)	
MG-132 + Tpl2 Kinase Inhibitor	1 nM – 10 nM (M) +	72 h
	0.1 nM – 1 nM (T)	

- **addition of dye solution and solubilizer**

The viable cells were counted indirectly by performing the MTT-Assay, to assess the cytotoxicity of the used inhibitors/ cytotoxins. 15 µl of a dye solution, a chromogenic dye which contains a tetrazolium salt, were added into each well of the microtiterplate. The plate was incubated (37°C, 5% CO_2:95% air) for 4 hours. The living cells are uptaking the tetrazolium salt into the mitochondria and are converting it into a formazan product. After the incubation time 100 µl of the solubilizer were added into each well to dissolve the formazan.

- **detection and evaluation**

After 24 hours the extinction of the wells of the microtiterplates were measured using Anthos–Reader 2001. The wavelength of 570 nm and a reference wavelength of 690 nm were chosen to detect the amount of the produced, blue-coloured, formazan. The evaluation was done by drawing sigmoid nonlinear dose-response curves using GraphPadPrism 4.0 software. The IC_{50} value – the inhibitor/ cytotoxin concentration leading to 50% growth inhibition compared to untreated cells – was calculated by this program.

2.2.1.2 Inhibition of cells for cell lysates

The used cell lines were inhibited with different substances to prepare cell lysates for Western blotting.

- **harvesting and counting cells**

The cells were harvested and counted like written in 2.2.1.1 harvesting and counting cells. A cell count of 3x 10^4 cells in 1 ml per well of a 6-well plate was requested in this approach.

- **addition of inhibitors**

The concentrations of the inhibitors were chosen very low, that the majority of the cells were still viable. The inhibitors were diluted until the requested concentration was reached. 1.5 ml of the solution was added into each well of the plate. The 6-well plates were put into the incubator (37°C, 5% CO_2:95% air) for 72 hours.

2.2.2 Molecular biological methods

2.2.2.1 Western blotting

All steps were performed after the Standard Operating Procedure of the laboratory.

- **cell lysis**

All steps for cell lysis were done on ice. The growth medium was discarded and the cells were washed twice with cold 1x PBS. 20 µl/ 50 µl 2x SDS-lysis buffer were put onto the cells in the 6-well plate/ cell flask. The cells were detached by using cell scrapers and transferred into 1.5 ml tubes.

- **sonication**

For the complete lysis of the cells and the degradation of genomic DNA, the cells were sonicated. The sonicater was setted in pulsed mode and approximately 30 pulses were given to the cell lysates. This procedure was done until the lysates were homogeneous.

- **spectral photometry**

A 1:100 dilution of the probes with ddH_20 were made and 500 µl of the dilution were measured against the blank at 260 nm. The amount of protein was calculated with the following formula: Extinction 0.1 µg = 1.3 µg/µl protein.

- **electrophoresis**

The SDS-PAGE-gel was prepared like written in the tables below.

➔ *10% separating gel:*

Table 21: Chemical composition of 10% separating gel.

Reagents	For one gel	For two gels
Acrylamide/bis (97/3) 30%	1.5 ml	3 ml
ddH_2O	1.5 ml	3 ml
1 M Tris-HCl (pH 9.1)	1.9 ml	3.8 ml
10% SDS	50 µl	100 µl
40% APS	10 µl	20 µl
Temed	2.5 µl	5 µl

The gel apparatures were filled with the separating gel and a 1:2 dilution of isopropanol with ddH_2O was put on top of the gel. After one hour the separating gel was compact.

➔ *4% stacking gel:*

Table 22: Chemical composition of 4% stacking gel.

Reagents	**For one gel**	**For two gels**
Acrylamide/bis (97/3) 30%	0.325 ml	0.65 ml
ddH_2O	0.625 ml	1.25 ml
0.5 M Tris-HCl (pH 6.8)	1.55 ml	3.1 ml
10% SDS	25 µl	50 µl
40% APS	4 µl	8 µl
Temed	2.5 µl	5 µl

The isopropanol/ddH_20 mixture was removed and the stacking gel was put on top of the separation gel. The combs were put into the gel and after 30 minutes of polymerization the combs were removed.
10 µg of protein and 4x loading buffer were substituted with 1x Tris-glycine-buffer to reach the end volume of 20 µl. The probes were denatured at 100°C for 3 minutes, kept on ice and then centrifuged for some seconds. Then the whole volume of the probes and 2.5 µl of the markers were loaded onto the gel. The used buffer for electrophoresis was 1x Tris-glycine-buffer and the gel was running at 150 Volt for about one hour.

- **transfer**

For the transfer of the proteins from the gel onto the membrane a semi-dry blotting method was used. 3 transfer buffer conditioned Whatman papers were put onto the positive electrode. The methanol and transfer buffer conditioned membrane was then put onto the papers. Onto the membrane the conditioned gel was put afterwards. At the top 3 transfer buffer conditioned Whatman papers were put. At last the negative electrode was put onto them.

➔ *Assembly:*

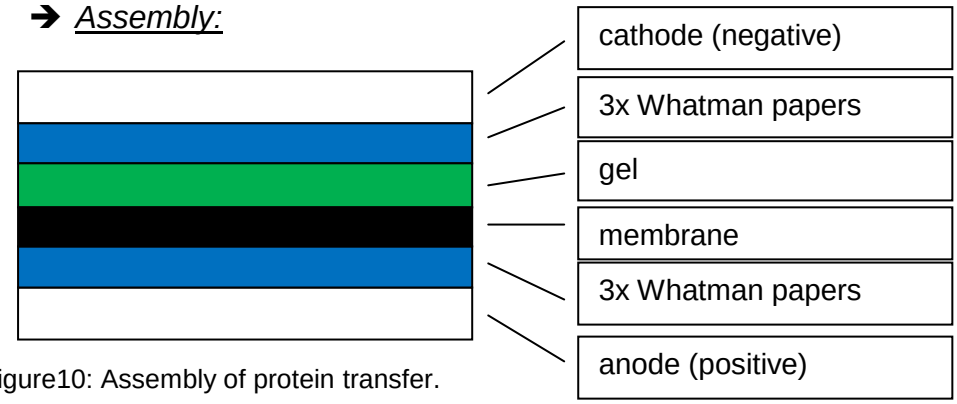


Figure10: Assembly of protein transfer.

For transfer following conditions were chosen: 0.8 mA/ cm^2 membrane, 1 hour.

- **Poinceau S staining**

As a control, if the transfer did work, the membrane was washed 3 times with ddH_20. Afterwards Poinceau S solution was put onto the membrane for 2-3 minutes. The membrane was washed again with ddH_20 for two times. Now the transferred protein bands were visible.

- **blocking**

At first all binding sites on the membrane were blocked with 15 ml SuperBlock Blocking Buffer + 0.05% Tween 20 at 4°C over night.

- **primary antibody**

The primary antibody was diluted in SuperBlock Blocking Buffer + 0.05% Tween 20. The antibodies were diluted like written in the instruction leaflet and are listed in Table 23 below. 15 ml of diluted primary antibody was put onto the membrane for one hour at room temperature. The membrane was gently shaking this hour.

Table 23: Used primary antibody dilutions.

Primary antibody	**Dilution**
GAPDH	1:5000
YB-1	1:10000
phospho-YB-1^{S102}	1:1000

Primary antibody	Dilution
RSK-2	1:100
IKK-α	1:1000
IKK-β	1:1000
phospho-IKK-α/β	1:1000
NF-κBp65	1:1000
phospho-NF-κBp65	1:1000
IκB-α	1:1000
phospho- IκB-α	1:1000
MEK1/2	1:1000

- **washing**

The used washing buffer was 1x TBS (pH 7.4) + 0.05% Tween 20. The membrane was washed 5 times for 10 minutes with this buffer at room temperature. The membrane was gently shaking the whole time.

- **secondary antibody**

The stock concentration of the secondary antibodies (0.8 mg/ml) were diluted with SuperBlock Blocking Buffer + 0.05% Tween 20 to reach the end concentration of 1:100000 to 1:250000 (see Table 24). 15 ml of diluted secondary antibody was put onto the membrane for one hour at room temperature. The membrane was gently shaking this hour.

Table 24: Used secondary antibody dilutions.

Secondary antibody	Dilution
Goat anti rabbit IgG HRPO (H+L)	1:200000
Goat anti mouse IgG HRPO (H+L)	1:100000

- **washing**

The used washing buffer was 1x TBS (pH 7.4) + 0.05% Tween 20. The membrane was washed 5 times for 10 minutes with this buffer at room temperature. The membrane was gently shaking the whole time.

- **development**

For development of the protein bands Luminol/Enhancer Solution and Stable Peroxidase Solution were mixed in ratio 1:1. 0.125 ml/cm^2 membrane were put onto the membrane and incubated at room temperature for 5 minutes. The membrane was gently shaking the whole time. The membrane was dried between two Whatman papers and put into a plastic foil. The foil was put into a film cassette and an X-ray film was put onto the membrane for seconds to minutes until the protein bands were visible. The X-ray film was put into the developer. For orientation of the size of the protein bands, the bands of the markers were titled with their sizes.

3 Results

3.1 Cytotoxicity Assays

3.1.1 Cytotoxicity assays of one inhibitor

These experiments were done to establish the IC_{50} value, the concentration inhibiting 50% of the exposed cells. For the experimental procedure see chapter 2.2.1.1.

Cetuximab

This antibody against the extracellular domain of the EGFR should inhibit the activation of the receptor tyrosine kinase (Xu et al. 2005). In our approach, cetuximab showed no activity in the investigated cell lines. The IC_{50} values were calculated with more than10 µg/ml for the BLBC and the reference cells (see Figure 11).

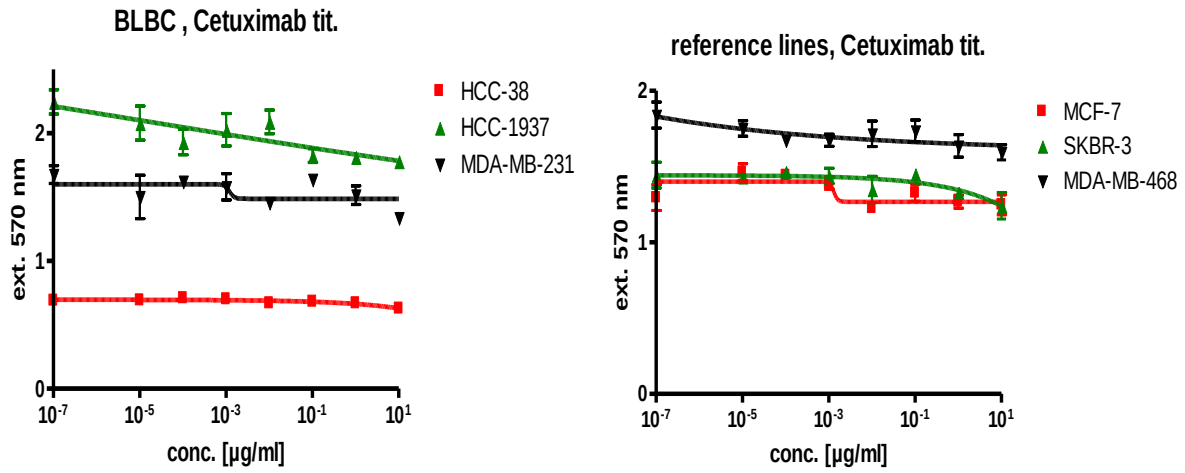
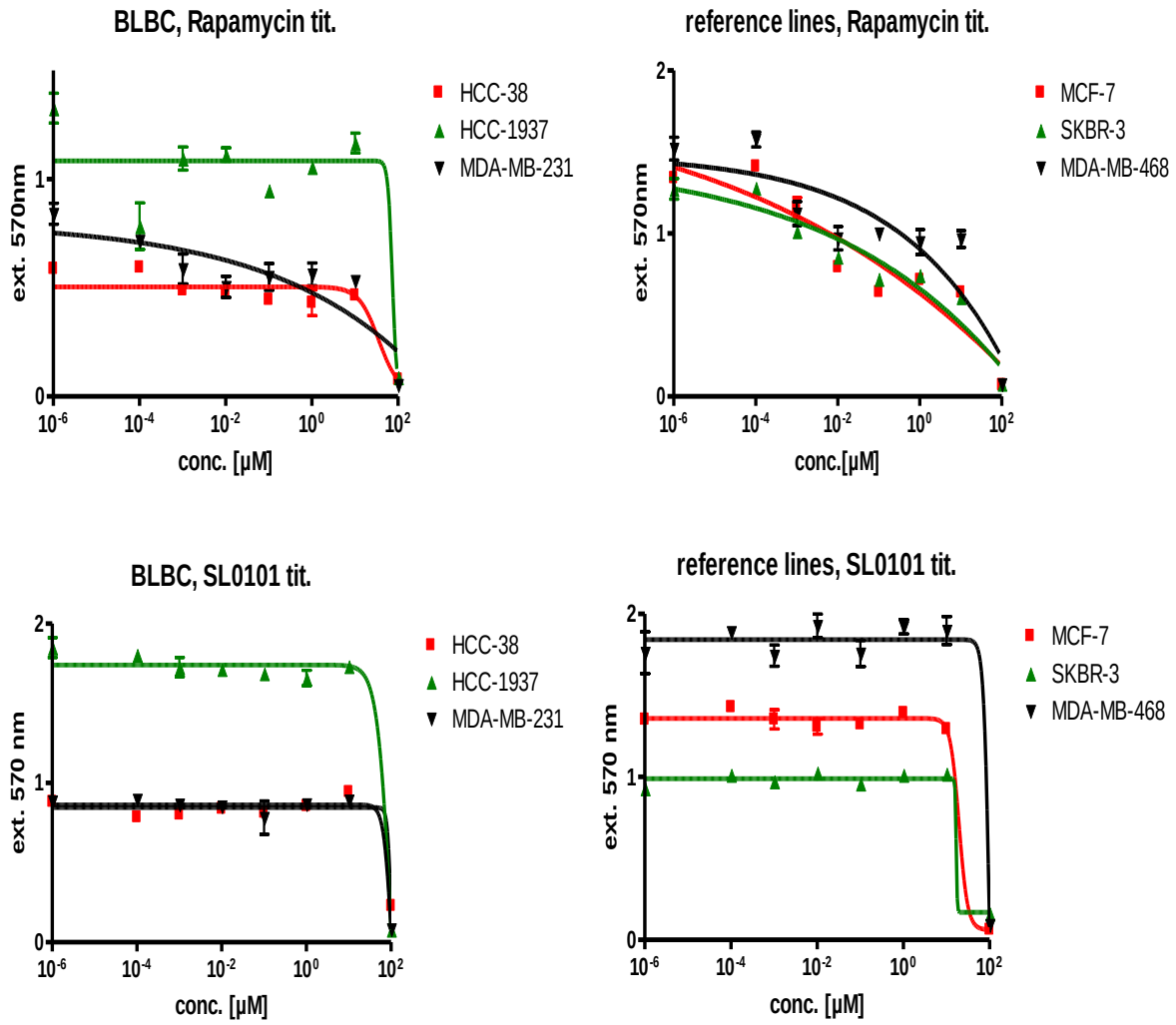


Figure 11: Cytotoxicity assay of Cetuximab. The BLBC and the reference cells were treated with 0.01 ng/ml – 10 µg/ml Cetuximab and incubated for 72 h at 37°C. Legend: BLBC: basal-like breast cancer cells, ext.570: extinction at a wavelength of 570 nm, conc. [µg/ml]: concentration of the substance in µg per one ml, tit.: titrated.

Rapamycin, SL0101, ERK Inhibitor II, ERK Inhibitor III, U0126

MEK1/2, ERK1/2, RSK and mTOR were blocked with the corresponding inhibitors, for further investigation of the MAPK pathway. Rapamycin had little activity in the BLBC up to concentrations of 10 µM, but inhibited cells significantly at 100 µM. The same effect could be observed in the ERK Inhibitor II treated reference lines and in all six cell lines treated with SL0101, ERK Inhibitor III and U0126 (except U0126 treated HCC-38). A minor activity was reached in rapamycin treated reference cell lines with continuous killing of the cells. No significant killing of the BLBC up to concentrations of 100 µM could be observed after treatment with ERK Inhibitor II (see Figure 12).



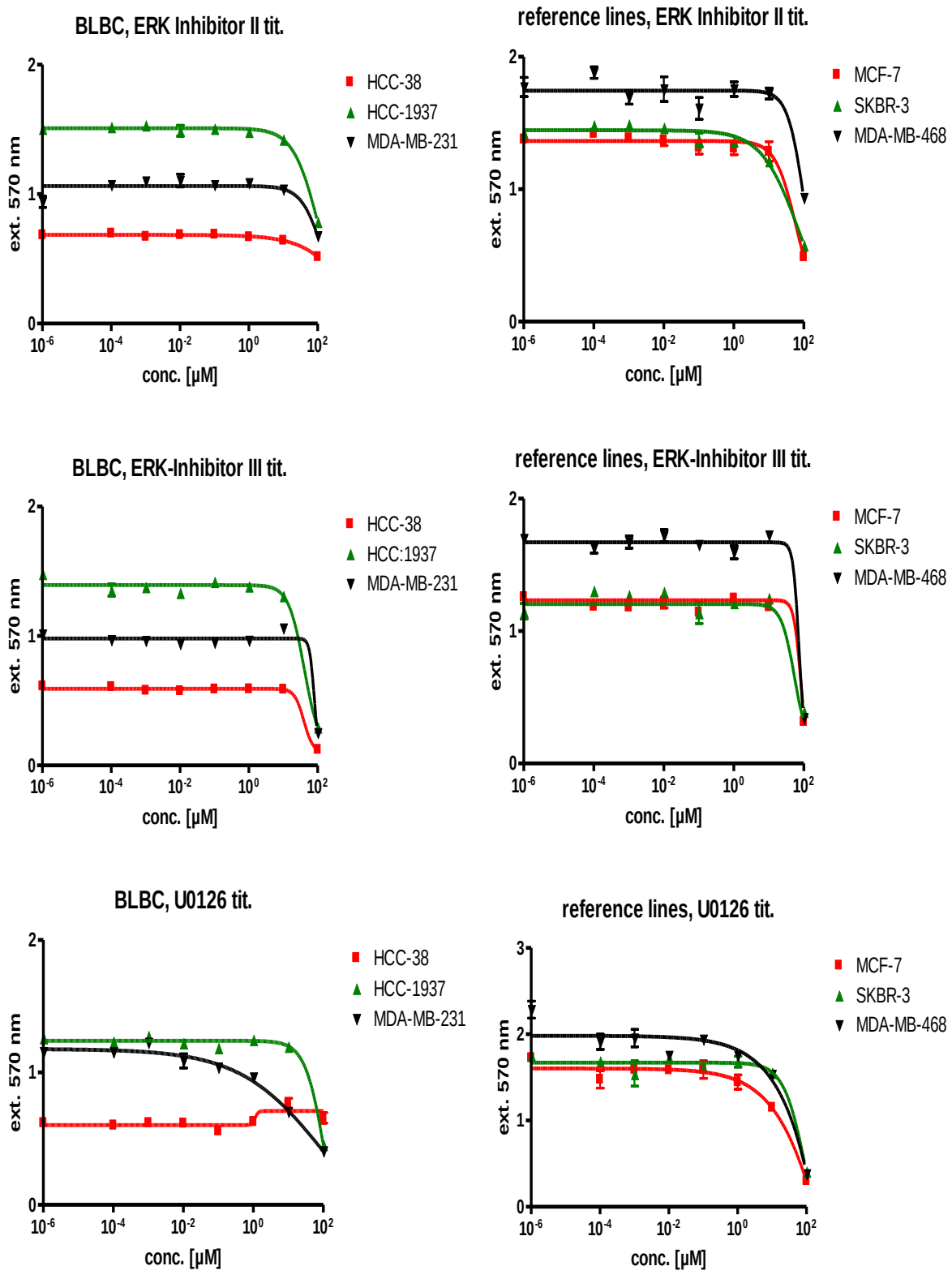


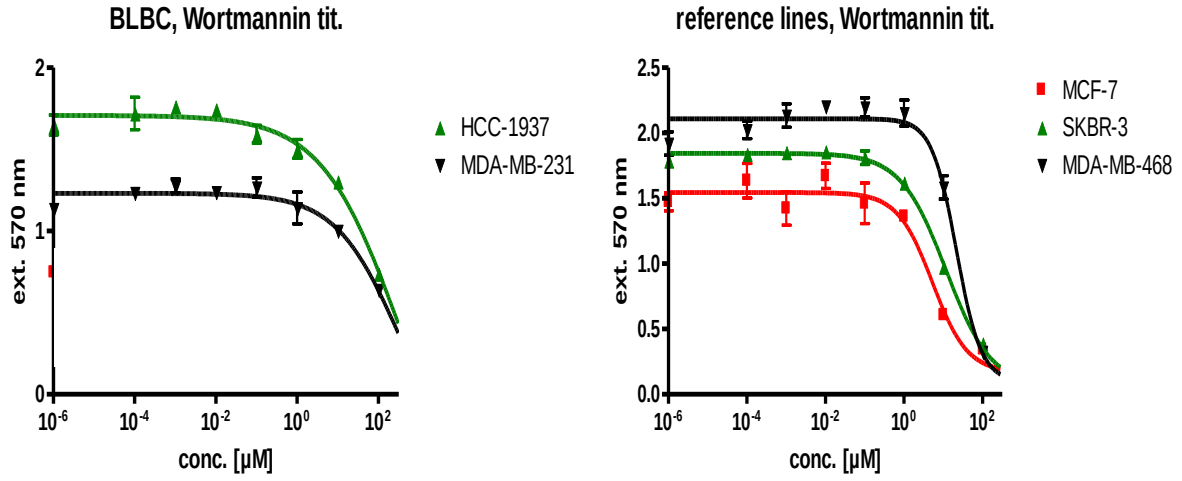
Figure 12: Cytotoxicity assay rapamycin, SL0101, ERK Inhibitor II, ERK Inhibitor III and U0126. The BLBC and the reference cells were treated with 0.1 nM–100 µM rapamycin, 0.1 nM–0.1 mM SL0101, 0.1 nM–0.1 mM ERK Inhibitor II, 0.1 nM–0.1 mM ERK Inhibitor III, 0.1 nM–0.1 mM U0126 and

incubated for 72 h at 37°C. Legend: BLBC: basal-like breast cancer cells, ext.570: extinction at a wavelength of 570 nm, conc. [µM]: concentration of the substance in µM, tit.: titrated.

None of the used inhibitors of the MAPK pathway was able to kill the breast cancer cells at low concentrations. There was no significant difference between the BLBC and the reference lines.

Wortmannin

This metabolite acts as inhibitor of the catalytic activity of PI3 kinase (Weng et al. 1999; Okada et al. 1994). In the used cellular systems, little activity of wortmannin could be observed in the BLBC up to concentrations of 10 µM. A more significant inhibition could be reached at a concentration of 100 µM. The reference cell lines could be inhibited significantly with a wortmannin concentration of 10 µM (see Figure 13).



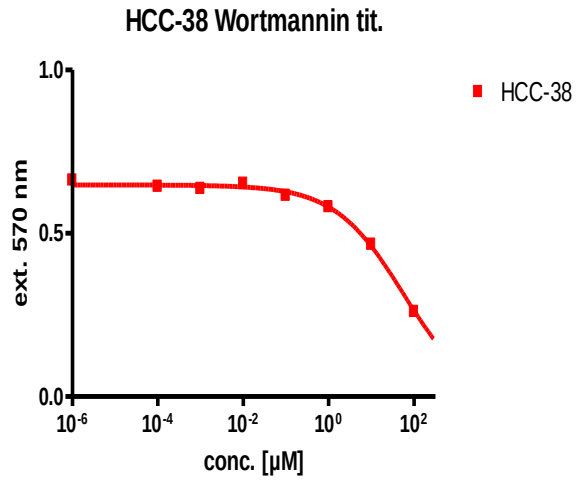


Figure 13: Cytotoxicity assay of wortmannin. The BLBC and the reference cells were treated with 0.1 nM–0.1 mM wortmannin and incubated for 72 h at 37°C. Legend: BLBC: basal-like breast cancer cells, ext.570: extinction at a wavelength of 570 nm, conc. [µM]: concentration of the substance in µM, conc., tit.: titrated.

Disulfiram

The two known modes of action of this substance are the NF-κB suppression and the IκB-α inhibition (Chen et al. 2006). Although the obtained data points were very fluctuating and the slopes of the investigated cell lines were very different, disulfiram seemed to show an inhibitory effect on all six breast cancer cell lines. The IC_{50} value was observed, using low or very low micromolar concentrations of this substance (see Table 25).

Table 25: Calculated IC_{50} values of the used cell lines inhibited with disulfiram.

Cell lines	**IC_{50}**
HCC-38	8.86 µM
HCC-1937	0.3 µM
MDA-MB-231	0.39 µM
MCF-7	0.71 µM
SKBR-3	7.58 µM
MDA-MB-468	0.29 µM

No significant difference could be observed between the basal-like breast cancer cells and the reference cells (see Figure 14).

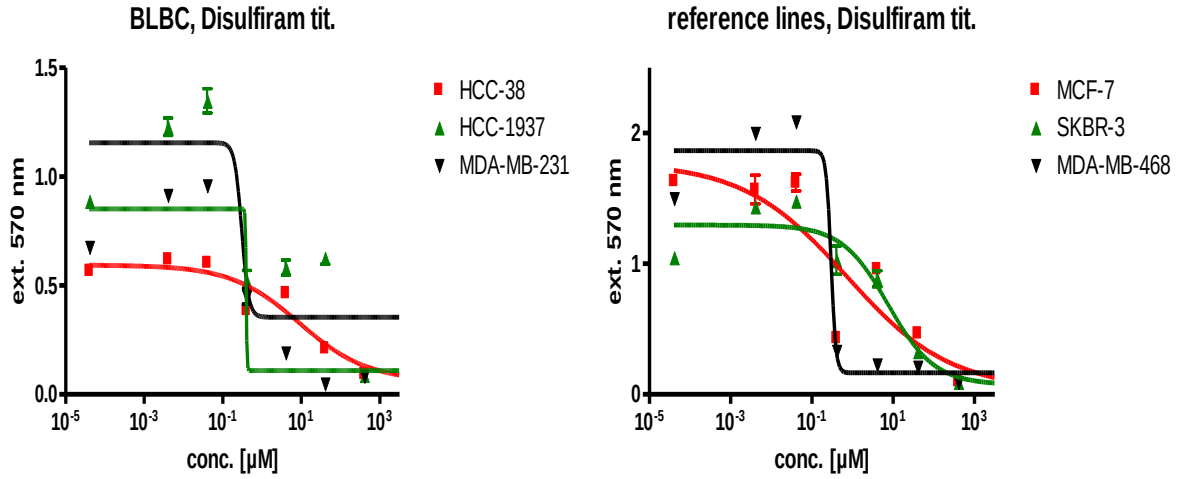


Figure 14: Cytotoxicity assay of disulfiram. The BLBC and the reference cells were treated with 4 nM–4 mM disulfiram and incubated for 72 h at 37°C. Legend: BLBC: basal-like breast cancer cells, ext.570: extinction at a wavelength of 570 nm, conc. [µM]: concentration of the substance in µM, tit.: titrated.

Akt Inhibitor IV

The substance Akt Inhibitor IV prevents the autophosphorylation of Akt (http://www.emdchemicals.com). In the used cellular systems 50% inhibition of the cells could be reached at very low micromolar concentrations by the use of Akt Inhibitor IV (see Table 26).

Table 26: Calculated IC_{50} values of the used cell lines inhibited with Akt Inhibitor IV.

Cell lines	**IC_{50}**
HCC-38	0.95 µM
HCC-1937	0.9 µM
MDA-MB-231	0.64 µM
MCF-7	0.77 µM
SKBR-3	0.46 µM
MDA-MB-468	0.25 µM

The activity of this substance was almost the same in all six breast cancer cell lines (see Figure 15).

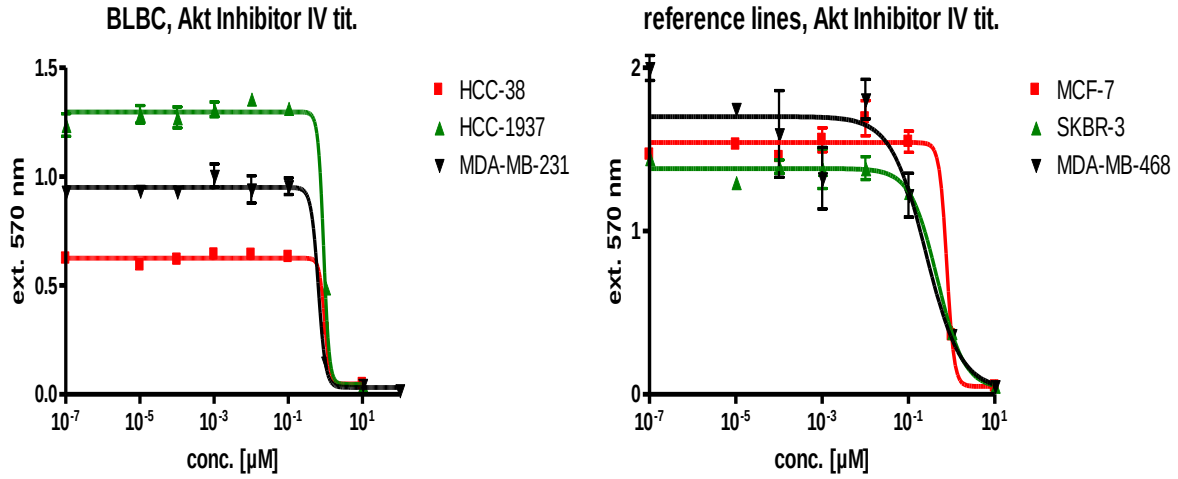


Figure 15: Cytotoxicity assay of Akt Inhibitor IV. The BLBC and the reference cells were treated with 0.01 nM – 0.01 mM Akt Inhibitor IV and incubated for 72 h at 37°C. Legend: BLBC: basal-like breast cancer cells, ext.570: extinction at a wavelength of 570 nm, conc. [µM]: concentration of the substance in µM, tit.: titrated.

In comparison to rapamycin, the other used inhibitor of the PI3K-Akt-mTOR pathway, Akt Inhibitor IV was able to inhibit the cell growth at lower concentrations.

Violacein

The purple-colored pigment violacein has four known modes of actions (inhibition of the phosphorylation of IκB-α, reduced production of the two NF-κB subunits p50 and p65, blocking the NF-κB transport into the nucleus) (Kodach et al. 2006). In addition, violacein inhibits the PI3-kinase–Akt–mTOR pathway. In our cellular systems, violacein has shown high activity in all cell lines. IC_{50} values at very low micromolar or even at nanomolar concentrations were observed (see Table 27).

Table 27: Calculated IC_{50} values of the used cell lines inhibited with violacein.

Cell lines	**IC_{50} (average out of 2 experiments)**
HCC-38	0.59 µM
HCC-1937	75 nM
MDA-MB-231	0.51 µM
MCF-7	38 nM
SKBR-3	0.125 µM
MDA-MB-468	0.11 µM

There was no significant difference between BLBC and the reference breast cancer cell line (one experiment out of two is shown) (see Figure 16).

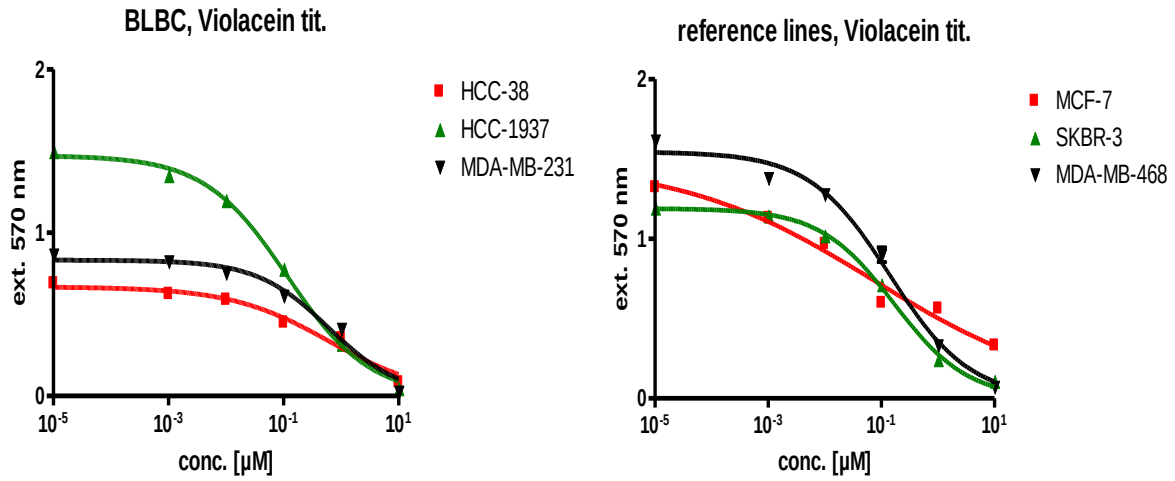


Figure 16: Cytotoxicity assay of violacein. The BLBC and the reference cells were treated with 1 nM–1 mM violacein and incubated for 72 h at 37°C. Legend: BLBC: basal-like breast cancer cells, ext.570: extinction at a wavelength of 570 nm, conc. [µM]: concentration of the substance in µM, tit.: titrated.

BAY 11-7082

This substance functions as an inhibitor of the phosphorylation of IκB-α and the suppression of NF- κB activation (Scaife et al. 2002). The IC_{50} value was observed at low micromolar concentrations in the breast cancer cells by the use of BAY 11-7082 (see Table 28).

Table 28: Calculated IC_{50} value of the used cell lines inhibited with BAY 11-7082.

Cell lines	**IC_{50} (average out of 2 experiments)**
HCC-38	1.495 µM
HCC-1937	3.405 µM
MDA-MB-231	0.17 µM
MCF-7	1.64 µM
SKBR-3	2.645 µM
MDA-MB-468	1.51 µM

Again, no significant difference between the BLBC and the reference breast cancer cell lines was observed (one experiment out of two is shown) (see Figure 17).

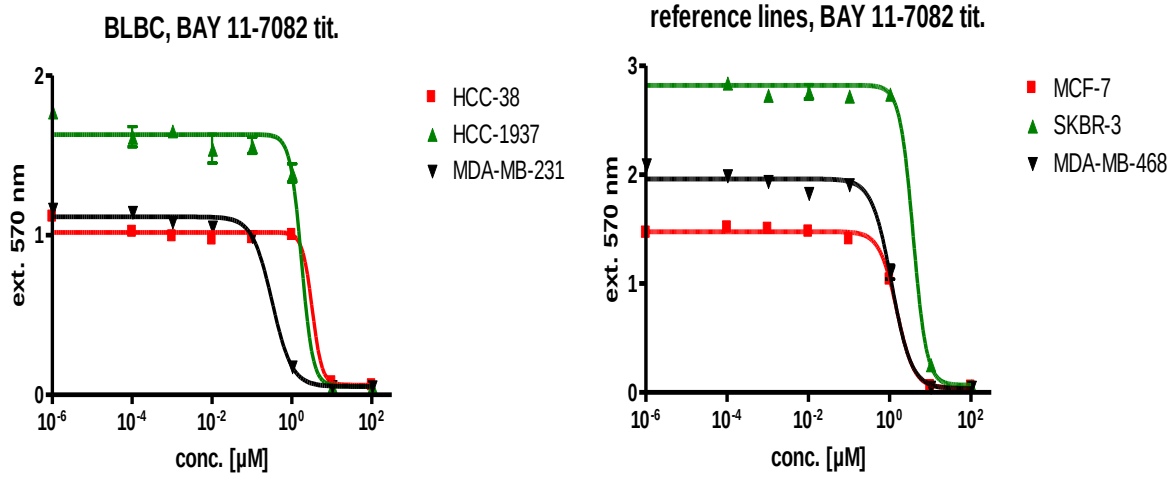


Figure 17: Cytotoxicity assay of BAY 11-7082. The BLBC and the reference cells were treated with 0.1 nM – 0.1 mM BAY 11-7082 and incubated for 72 h at 37°C. Legend: BLBC: basal-like breast cancer cells, ext.570: extinction at a wavelength of 570 nm, conc. [µM]: concentration of the substance in µM, tit.: titrated.

MG-132

This proteasome inhibitor was tested too, for further investigations of the NF-κB pathway. A growth inhibition of all used breast cancer cell lines was observed at very low micromolar concentrations by the use of MG-132 (see Table 29).

Table 29: Calculated IC_{50} value of the used cell lines inhibited with MG-132.

Cell lines	**IC_{50} (average out of 2 experiments)**
HCC-38	0.18 µM
HCC-1937	0.14 µM
MDA-MB-231	0.26 µM
MCF-7	0.485 µM
SKBR-3	0.145 µM
MDA-MB-468	0.13 µM

The six breast cancer cell lines were reacting very similar after the addition of this inhibitor (one experiment out of two is shown) (see Figure 18).

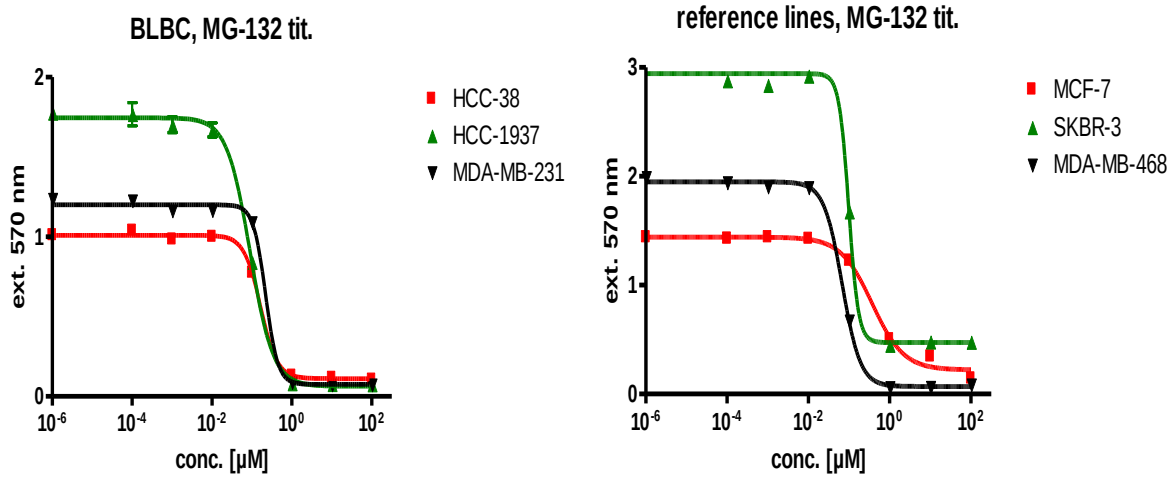


Figure 18: Cytotoxicity assay of MG-132. The BLBC and the reference cells were treated with 0.1 nM–0.1 mM MG-132 and incubated for 72 h at 37°C. Legend: BLBC: basal-like breast cancer cells, ext.570: extinction at a wavelength of 570 nm, conc. [µM]: concentration of the substance in µM, tit.: titrated.

The activity of MG-132 was similar to the other used NF-κB inhibitors BAY 11-7082 and violacein.

Tpl2 Kinase Inhibitor

This inhibitor suppresses the activity of the kinase Tpl2 (Gavrin et al. 2005). The IC_{50} value in the BLBC and the reference cells was observed at nanomolar concentrations of Tpl2 Kinase Inhibitor (see Table 30). In contrast to all the other substances (disulfiram, Akt Inhibitor IV, violacein, BAY 11-7082, MG-132; Figures 14-18), a complete eradication of the cells at higher concentrations was not obtained in any of the used cells. In this case a growth arrest could be obtained in all six cell lines.

Table 30: Calculated IC_{50} value of the used cell lines inhibited with Tpl2 Kinase Inhibitor.

Cell lines	**IC_{50} (average out of 2 experiments)**
HCC-38	55.5 nM
HCC-1937	25.5 nM
MDA-MB-231	45.5 nM
MCF-7	21.5 nM
SKBR-3	28.5 nM
MDA-MB-468	13.5 nM

Similar activity of Tpl2 Kinase Inhibitor was observed in the six breast cancer cell lines (one experiment out of two is shown) (see Figure 19).

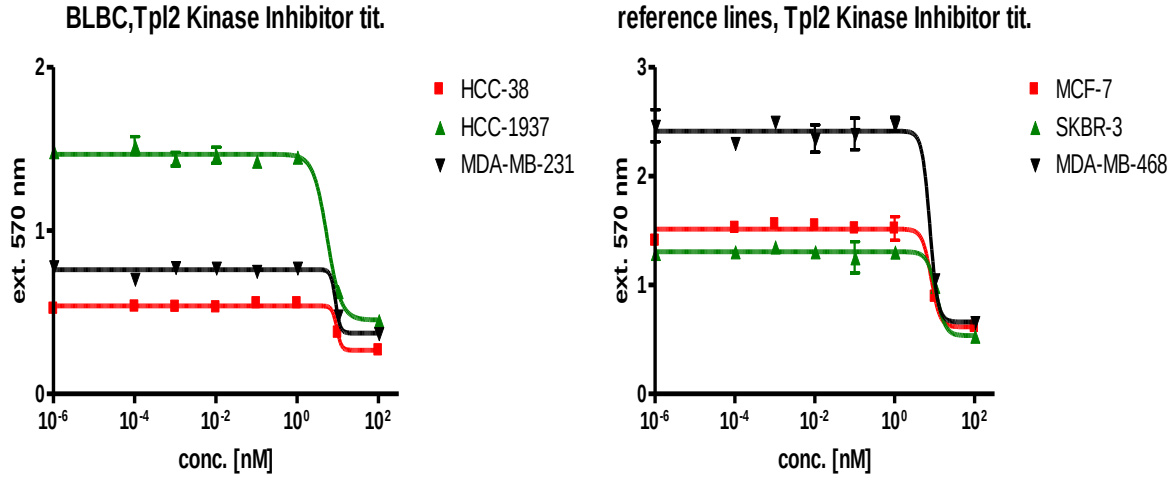


Figure 19: Cytotoxicity assay of Tpl2 Kinase Inhibitor. The BLBC and the reference cells were treated with 0.1 pM – 0.1 µM Tpl2 Kinase Inhibitor and incubated for 72 h at 37°C. Legend: BLBC: basal-like

breast cancer cells, ext.570: extinction at a wavelength of 570 nm, conc. [µM]: concentration of the substance in µM, tit.: titrated.

Tpl2 Kinase Inhibitor was the NF-κB pathway inhibitor with the most activity in the used cellular systems.

Cytotoxicity assays with the substances PD98059, U0124 and PQ401 were done too. No or no significant activity of this substance could be reached (data not shown).

3.1.2 Cytotoxicity assays of two inhibitors or one inhibitor and one cytotoxin combination

These experiments were done to find out if lower IC_{50} values could be reached when combinations of two inhibitors or one inhibitor and one cytotoxin were used. Two concentrations of each inhibitor/ cytotoxin were chosen for these approaches. The higher one represents the concentration at which the substance alone was barely working in the cell lines. The lower concentration was chosen a power of ten below the higher one, in case the inhibition starts earlier if combinations of two substances were used. For the experimental procedure see chapter 2.2.1.1. Instead of calculating the IC_{50} value with the GraphPadPrism 4.0 software, a bar diagram was made out of the data, which were obtained from the Anthos Reader apparatus.

Akt Inhibitor IV + Violacein

The combination of Akt Inhibitor IV (inhibitor of Akt phosphorylation) and violacein (multiple mode of action inhibitor of NF-κB pathway) led to an earlier inhibition of the reference cell lines (MCF-7, SKBR-3 and MDA-MB-468) at the higher concentrations, compared to the single use of these two substances. Unfortunately this effect could not be seen in the basal-like breast cancer cell lines (HCC-38, HCC-1937, MDA-MB-231) (see Figure 20).

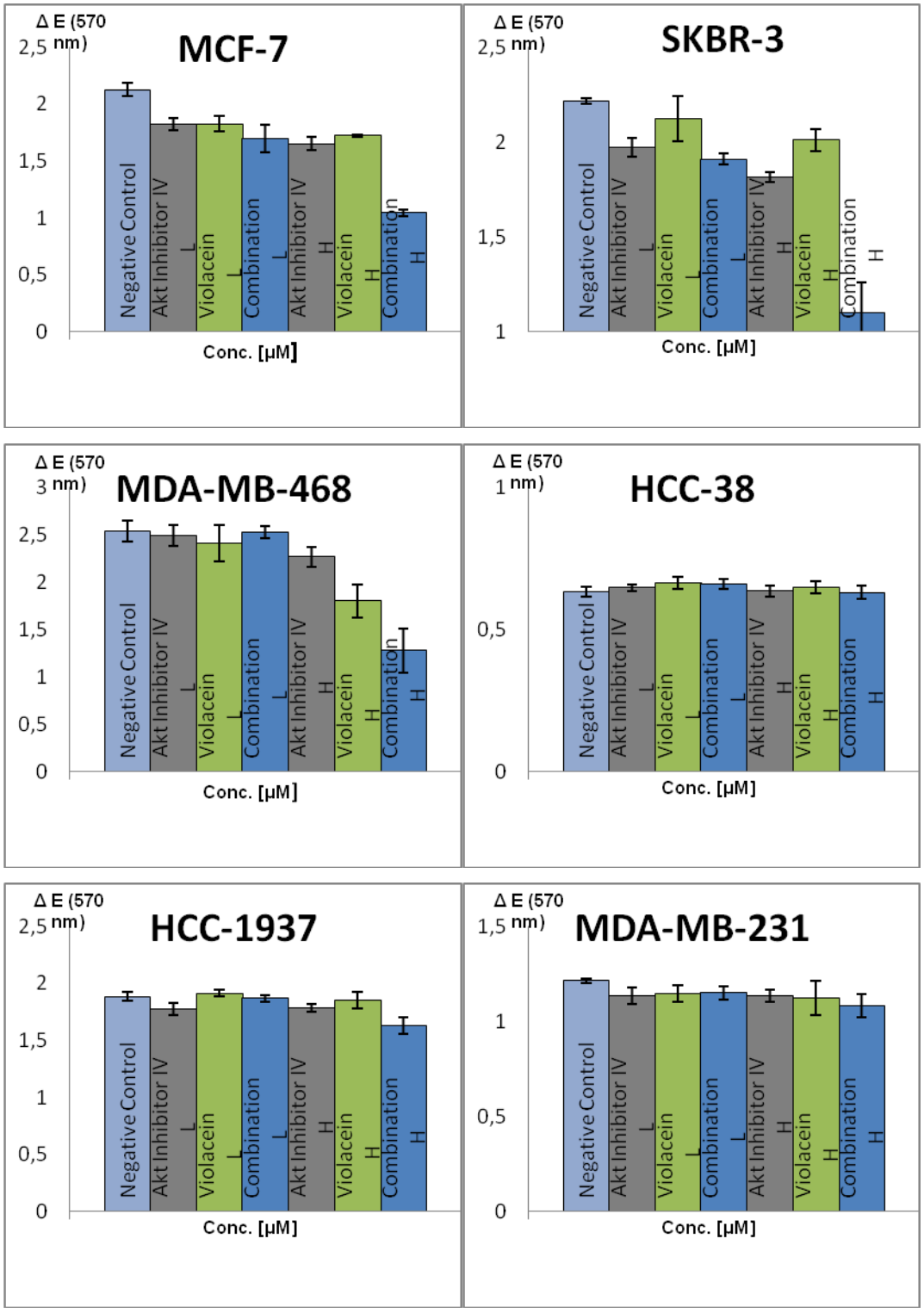


Figure 20: Cytotoxicity assay of Akt Inhibitor IV and violacein. The cell lines were treated with the two inhibitors and incubated for 72 h at 37°C. Legend: ΔE(570nm): extinction at a wavelength of 570 nm, conc. [µM]: concentration of the substances in µM, Akt Inhibitor IV L: 0.01 µM (all cell lines), Akt Inhibitor IV H: 0.1 µM (all cell lines), violacein L: 0.0001 µM (all cell lines), violacein H: 0.001 µM (all cell lines), Combination L: 0.01 µM Akt Inhibitor IV + 0.0001 µM violacein (all cell lines), Combination H: 0.1 µM Akt Inhibitor IV + 0.001 µM violacein (all cell lines).

For further investigations of the communication between the pathways, more combinations with different inhibitors of the three pathways (MAPK pathway, PI3K-Akt-mTOR pathway, NF-κB pathway) and cytotoxins were done. Unfortunately no higher activity in the used cellular systems was observed by these combinations. The following substances were combined in a concentration range of 0.0001 µM to 10 µM (data not shown).

- → Akt Inhibitor IV + rapamycin
- → Akt Inhibitor IV + wortmannin
- → ERK Inhibitor II + PD98059
- → ERK Inhibitor II + SL0101
- → ERK Inhibitor II + wortmannin
- → Akt Inhibitor IV + PD98059
- → Akt Inhibitor IV + ERK Inhibitor II
- → Akt Inhibitor IV + SL0101
- → PD98059 + wortmannin
- → PD98059 + violacein
- → Epirubicin + PD98059
- → Epirubicin + Akt Inhibitor IV
- → Epirubicin + wortmannin
- → Epirubicin + violacein
- → Docetaxel + PD98059
- → Docetaxel + Akt Inhibitor IV
- → Docetaxel + wortmannin
- → Docetaxel + violacein
- → Cisplatinum + PD98059
- → Cisplatinum + Akt Inhibitor IV
- → Cisplatinum + wortmannin
- → Cisplatinum + violacein
- → violacein + Tpl2 Kinase Inhibitor
- → violacein + BAY 11-7082
- → violacein + MG-132
- → BAY 11-7082 + Tpl2 Kinase Inhibitor
- → BAY 11-7082 + MG-132
- → MG-132 + Tpl2 Kinase Inhibitor

3.2 Quantitative Analysis of Proteins

The same secondary antibodies and one or both of the markers listed in table were used for all Western blotting experiments. GAPDH was chosen as loading control. For experimental procedure see chapter see 2.2.2.1.

3.2.1 Quantitative analysis of YB-1, phospho-YB-1^{S102} and RSK2 in untreated breast cancer cells

With the first experiments I wanted to find out if there is a difference in the amount of the transcription factor YB-1, the active form of this transcription factor, phospho-YB-1^{S102}, and the protein RSK2 in the untreated used breast cancer cells.

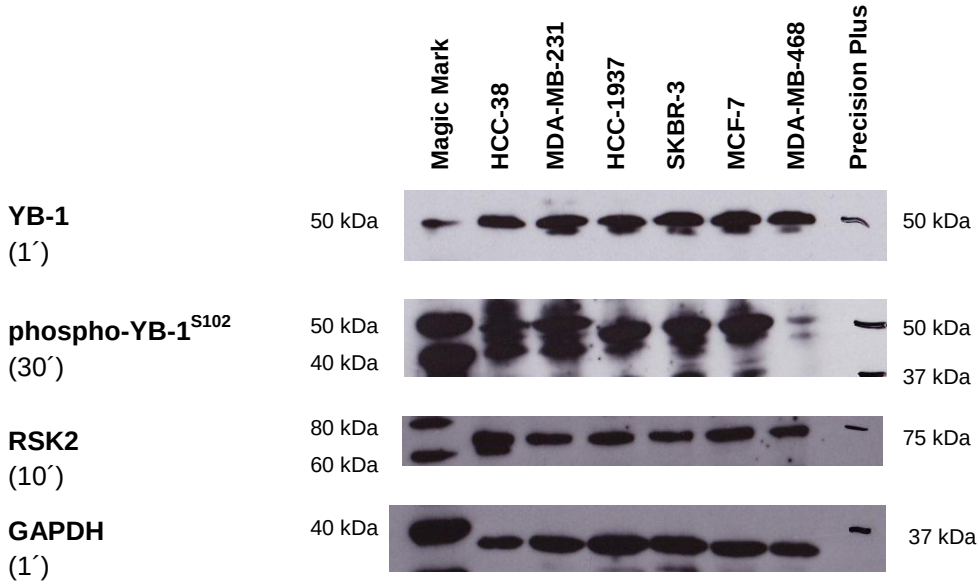


Figure 21: Detection of the proteins YB-1, phospho-YB-1^{S102}, RSK2 and GAPDH. 10 µg of the cell lines were used for a 10% SDS-Page gel and transferred onto a PVDF-membrane. The first antibody was diluted in SuperBlock Blocking Buffer 1:10000 for YB-1, 1:1000 for phospho-YB-1^{S102}, 1:100 for RSK2 and 1:5000 for GAPDH. GAPDH, YB-1 and phospho-YB-1^{S102} were incubated with Goat anti rabbit IgG (H+L) HRPO and RSK2 with Goat anti mouse IgG (H+L) HRPO. As exposure time 1 minute for YB-1 and GAPDH, 10 minutes for RSK2 and 30 minutes for phospho-YB-1^{S102} were chosen.

There was no difference in the level of YB-1 in the untreated breast cancer cells. A higher amount of the phosphorylated form of this transcription factor was observed in the cell lines HCC-1937, SKBR-3 and MCF-7 (lanes 4-6). Due to the fact that the antibody against phospho-YB-1^{S102} showed unspecific binding (lanes 2-3) and thus

was not very informative; the detection of this protein was not reproducible and was therefore not done in the following experiments. Furthermore it seemed that untreated HCC-38 expressed more RSK2 protein compared to the other untreated cell lines.

3.2.2 Quantitative analysis of YB-1 and RSK2 in inhibitor treated breast cancer cells

The following experiments were done to find out if the treatment of the cells with different inhibitors, from which it was supposed that they could have an effect on the proteins YB-1 and RSK2, alters the amount of their expression compared to the untreated cells. The concentrations of the inhibitors were chosen very low that only a small amount of the cells were killed.

3.2.2.1 Akt Inhibitor IV treated cells

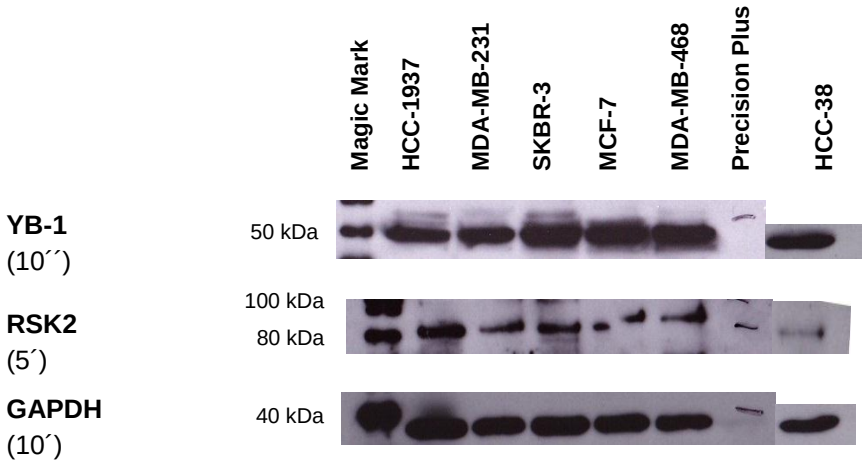


Figure 22: Detection of the proteins YB-1, RSK2 and GAPDH. 10 µg of the cell lines were used for a 10% SDS-Page gel and transferred onto a PVDF-membrane. The first antibody was diluted in SuperBlock Blocking Buffer 1:10000 for YB-1, 1:100 for RSK2 and 1:5000 for GAPDH. GAPDH and YB-1 were incubated with Goat anti rabbit IgG (H+L) HRPO and RSK2 with Goat anti mouse IgG (H+L) HRPO. As exposure time 10 seconds for YB-1, 10 minutes for GAPDH and 5 minutes for RSK2 were chosen (45´ for HCC-38 – incubated at a different blot).

The basal-like breast cancer cells (HCC-38, HCC-1937, MDA-MB-231) were expressing less YB-1 protein compared with the reference cells (MCF-7, SKBR-3, MDA-MB-468) and the untreated cells after the treatment with Akt Inhibitor IV (see

Figure 21). The signal of the RSK2 band seemed to be a little less intense in HCC-38 compared to the others. Maybe this was due to the fact that this cell line was done on a separate gel, which in general produced weak signals. More RSK2 protein was produced by HCC-1937 compared to all other treated cells. No difference in the amount of this protein in the untreated and the Akt Inhibitor IV treated cells was observed (see Figure 21).

3.2.2.2 Violacein treated cells

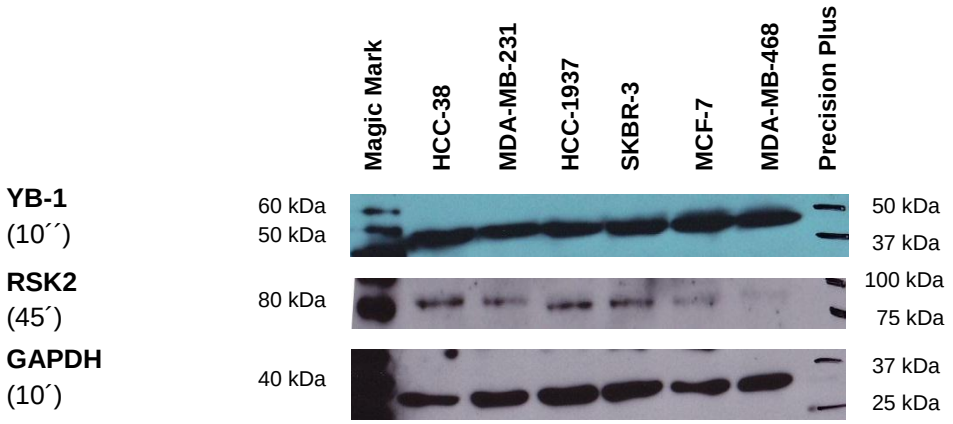


Figure 23: Detection of the proteins YB-1, RSK2 and GAPDH. 10 µg of the cell lines were used for a 10% SDS-Page gel and transferred onto a PVDF-membrane. The first antibody was diluted in SuperBlock Blocking Buffer 1:10000 for YB-1, 1:100 for RSK2 and 1:5000 for GAPDH. GAPDH and YB-1 were incubated with Goat anti rabbit IgG (H+L) HRPO and RSK2 with Goat anti mouse IgG (H+L) HRPO. As exposure time 10 seconds for YB-1, 10 minutes for GAPDH and 45 minutes for RSK2 were chosen.

It seemed that the treatment with violacein did not influence the expression of YB-1 compared with the untreated cells (see Figure 21). The bands of RSK2 were very weak in all cell lines, especially in MCF-7 and MDA-MB-468. In MDA-MB-468 there was almost no signal visible. Maybe the production of this protein was downregulated after the treatment with violacein in comparison to the untreated ones (see Figure 21).

3.2.2.3 Disulfiram treated cells

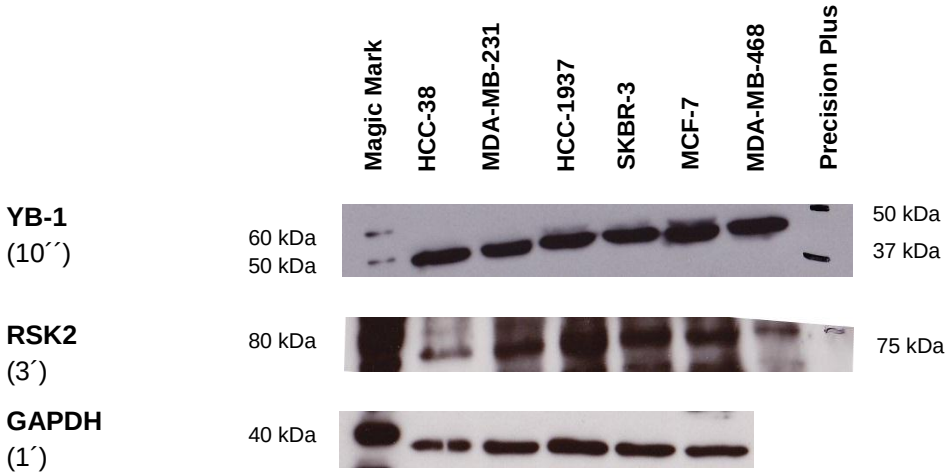


Figure 24: Detection of the proteins YB-1, RSK2 and GAPDH. 10 µg of the cell lines were used for a 10% SDS-Page gel and transferred onto a PVDF-membrane. The first antibody was diluted in SuperBlock Blocking Buffer 1:10000 for YB-1, 1:100 for RSK2 and 1:5000 for GAPDH. GAPDH and YB-1 were incubated with Goat anti rabbit IgG (H+L) HRPO and RSK2 with Goat anti mouse IgG (H+L) HRPO. As exposure time 10 seconds for YB-1, 1 minute for GAPDH and 3 minutes for RSK2 were chosen. No MDA-MB-468 was incubated with GAPDH.

Due to the lack of cell lysate from MDA-MB-468, no loading control with GAPDH could be done with this cell line. The treatment with disulfiram did not influence the amount of produced YB-1 compared with the untreated cells (see Figure 21). HCC-1937 was producing more RSK2 and HCC-38 as well as MDA-MB-468 were producing less RSK2 compared with the other cell lines and the untreated ones (see Figure 21).

3.2.3 Quantitative analysis of NF-κB pathway members in untreated breast cancer cells

With the first experiment I wanted to find out if there is a difference in the amount of NF-κB pathway proteins in the untreated basal-like breast cancer cells and the untreated reference breast cancer cells.

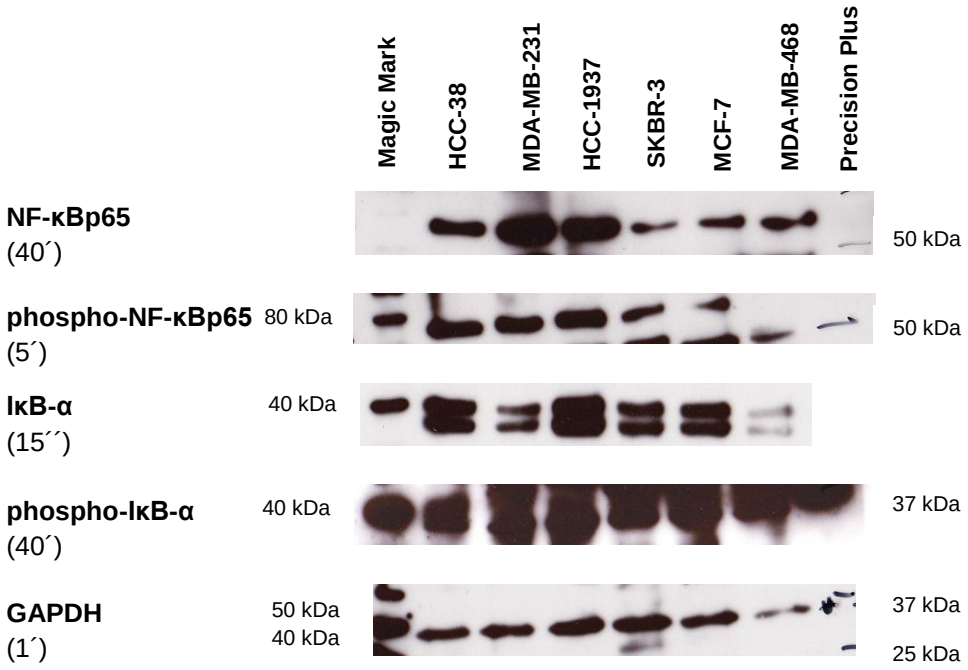


Figure 25: Detection of the proteins NF-κBp65, phospho-NF-κBp65, IκB-α, phospho-IκB-α and GAPDH. 10 µg of the cell lines were used for a 10% SDS-Page gel and transferred onto a PVDF-membrane. The first antibody was diluted in SuperBlock Blocking Buffer 1:1000 for NF-κBp65, phospho-NF-κBp65, IκB-α, phospho-IκB-α and 1:5000 for GAPDH. GAPDH, NF-κBp65, phospho-NF-κBp65 and phospho-IκB-α were incubated with Goat anti rabbit IgG (H+L) HRPO and IκB-α with Goat anti mouse IgG (H+L) HRPO. As exposure time 15 seconds for IκB-α, 1 minute for GAPDH, 5 minutes for phospho-NF-κBp65 and 40 minutes for NF-κBp65 and phospho-IκB-αwere chosen.

The basal-like breast cancer cells expressed much more phoshporylated as well as unphosphorylated NF-κBp65 than the reference cells. After 5 minutes there was no phospho-NF-κBp65 signal visible in the cell line MDA-MB-468, but a weak signal could be seen after 40 minutes (data not shown). Much more IκB-α was present in HCC-38 and HCC-1937 than in the other cells. Only a weak signal could be seen in MDA-MB-468. The amount of the phosphorylated form of IκB-α was almost the same in all cells (maybe a little bit more in HCC-1937). The band of MDA-MB-468

incubated with GAPDH was less intense because less than 10 µg lysate were loaded. The antibodies IKK-α, IKK-β and phospho-IKK-α/β were used as well but no informative results could be obtained (data not shown).

3.2.4 Quantitative analysis of NF-κB pathway members in inhibitor treated breast cancer cells

These experiments were done to find out if the inhibitors, which had a suppressive effect on the cells in the cytotoxicity assays (see Figures 15-19), alter the cells on protein level as well compared to the untreated state. Again the concentrations of the inhibitors were chosen very low that only a small amount of cells were killed by the substances.

3.2.4.1 Violacein treated cells

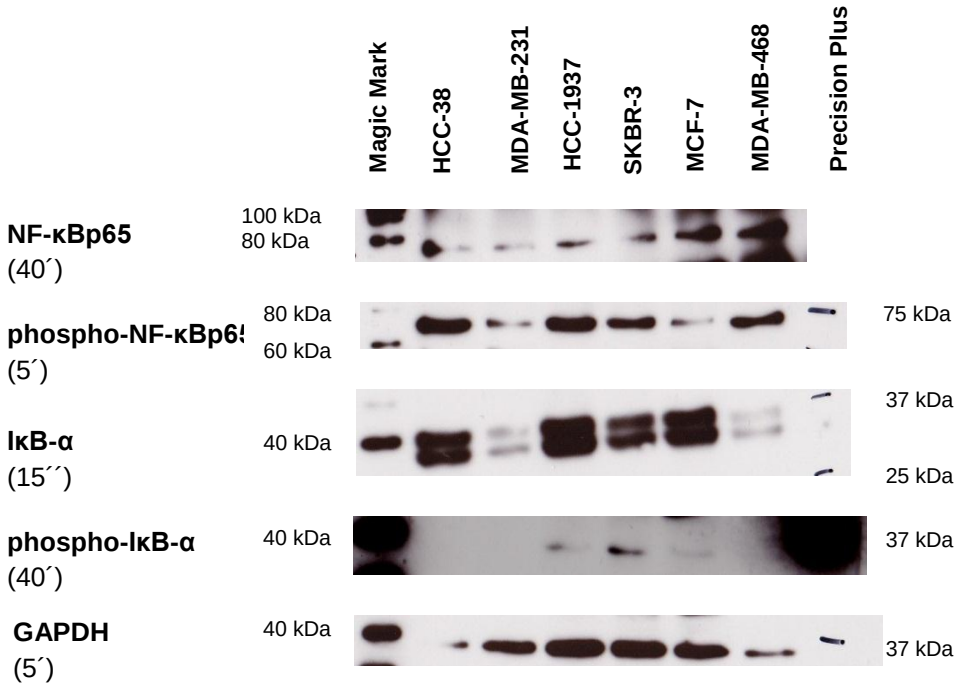


Figure 26: Detection of the proteins NF-κBp65, phospho-NF-κBp65, IκB-α, phospho-IκB-α and GAPDH. 10 µg of the cell lines (inhibited with 1 nM violacein) were used for a 10% SDS-Page gel and transferred onto a PVDF-membrane. The first antibody was diluted in SuperBlock Blocking Buffer 1:1000 for NF-κBp65, phospho-NF-κBp65, IκB-α, phospho-IκB-α and 1:5000 for GAPDH. GAPDH, NF-κBp65, phospho-NF-κBp65 and phospho-IκB-α were incubated with Goat anti rabbit IgG (H+L) HRPO and IκB-α with Goat anti mouse IgG (H+L) HRPO. As exposure time15 seconds for IκB-α, 5

minutes for phospho-NF-κBp65 and GAPDH and 40 minutes for NF-κBp65 and phospho-IκB-α were chosen.

The inhibition with violacein led to a much weaker signal of NF-κBp65 in the basal-like breast cancer cells. The signal in the reference cells stayed almost the same as in the untreated cells (see Figure 25). The phosphorylated NF-κBp65 was only downregulated in the cell line MDA-MB-231, maybe a little in MCF-7 as well. SKBR-3 and MDA-MB-231 were producing less IκB-α compared with the untreated state (see Figure 25). The amount of phospho-IκB-α protein was less in all cell lines in comparison with the untreated cells (see Figure 25). In this Blot HCC-38 and MDA-MB-468 had a weaker GAPDH signal than they normally had.

3.2.4.2 Tpl2 Kinase Inhibitor treated cells

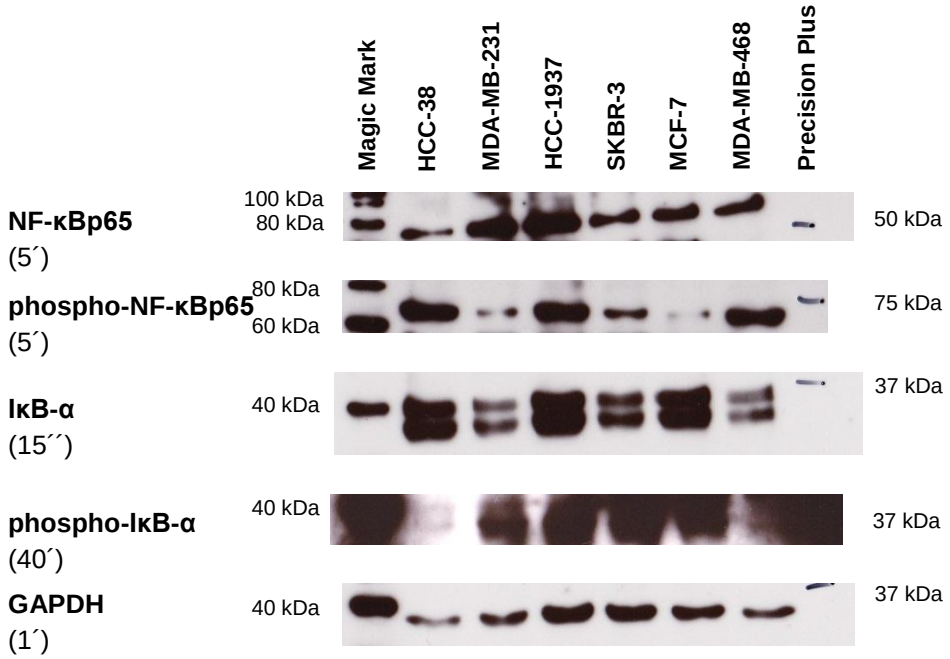


Figure 27: Detection of the proteins NF-κBp65, phospho-NF-κBp65, IκB-α, pospho-IκB-α and GAPDH. 10 µg of the cell lines (inhibited with 1 nM Tpl2 Kinase Inhibitor) were used for a 10% SDS-Page gel and transferred onto a PVDF-membrane. The first antibody was diluted in SuperBlock Blocking Buffer 1:1000 for NF-κBp65, phospho-NF-κBp65, IκB-α, phospho-IκB-α and 1:5000 for GAPDH. GAPDH, NF-κBp65, phospho-NF-κBp65 and phospho-IκB-α were incubated with Goat anti rabbit IgG (H+L) HRPO and IκB-α with Goat anti mouse IgG (H+L) HRPO. As exposure time 15 seconds for IκB-α, 1 minute for GAPDH, 5 minutes for phospho-NF-κBp65 and NF-κBp65 and 40 minutes for phospho-IκB-α were chosen.

Compared with the untreated control, the treatment with Tpl2 Kinase Inhibitor did not alter the amount of NF-κBp65 in the cell lines very much (see Figure 25). Maybe a little more protein was expressed in the reference cell lines and in MDA-MB-231. MCF-7 and MDA-MB-231 were producing less phospho-NF-κBp65, MDA-MB-468 seemed to be expressing a little more of this protein compared with the untreated cells (see Figure 25). HCC-38 and MDA-MB-468 were producing less phospho-IκB-α compared with the untreated state (see Figure 25). No difference in the IκB-α signal could be seen in the cell lines after the treatment with Tpl2 Kinase Inhibitor (see Figure 25).

3.2.4.3 MG-132 treated cells

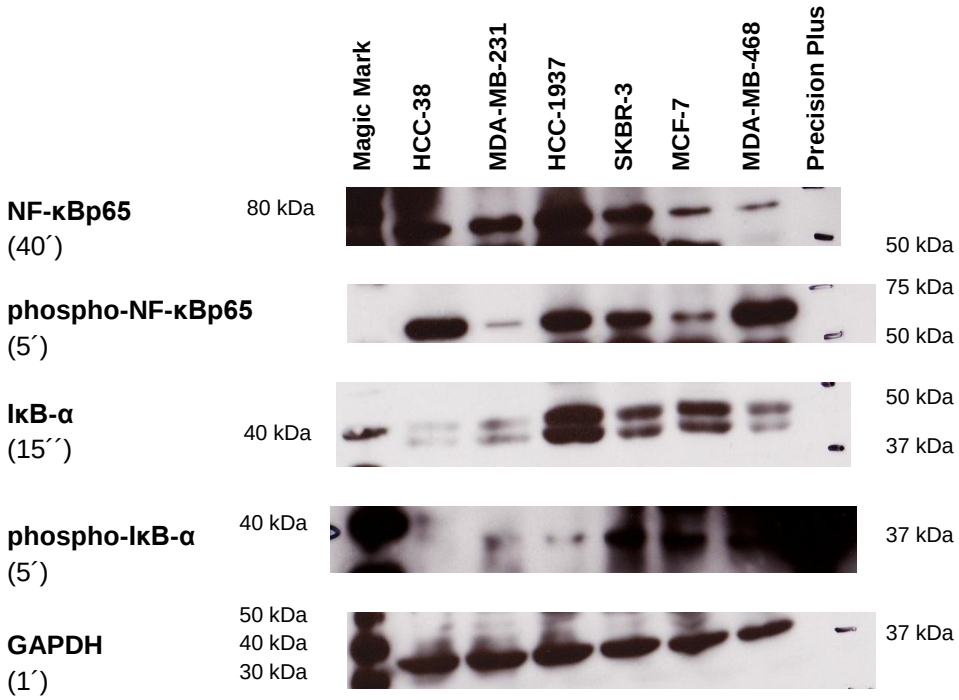


Figure 28: Detection of the proteins NF-κBp65, phospho-NF-κBp65, IκB-α, phospho-IκB-α and GAPDH. 10 µg of the cell lines (inhibited with 0.01 µM MG-132) were used for a 10% SDS-Page gel and transferred onto a PVDF-membrane. The first antibody was diluted in SuperBlock Blocking Buffer 1:1000 for NF-κBp65, phospho-NF-κBp65, IκB-α, phospho-IκB-α and 1:5000 for GAPDH. GAPDH, NF-κBp65, phospho-NF-κBp65 and phospho-IκB-α were incubated with Goat anti rabbit IgG (H+L) HRPO and IκB-α with Goat anti mouse IgG (H+L) HRPO. As exposure time 15 seconds for IκB-α, 1 minute for GAPDH, 5 minutes for phospho-NF-κBp65 and phospho-IκB-α and 40 minutes for NF-κBp65 were chosen.

It seemed that the inhibition with MG-132 led to a weaker NF-κBp65 expression, except in HCC-38 in comparison with the untreated cells (see Figure 25). The signal of the phosphorylated NF-κBp65 was weaker in all cell lines too, except in MDA-MB-468 with a higher expression of this protein. Compared with untreated controls, the IκB-α signal was downregulated in all cell lines, except in MDA-MB-468 where it was upregulated (see Figure 25). A downregulation of phospho-IκB-α in all breast cancer cell lines was observed after treatment with MG-132 when compared with untreated controls (see Figure 25).

3.2.4.4 BAY 11-7082 treated cells

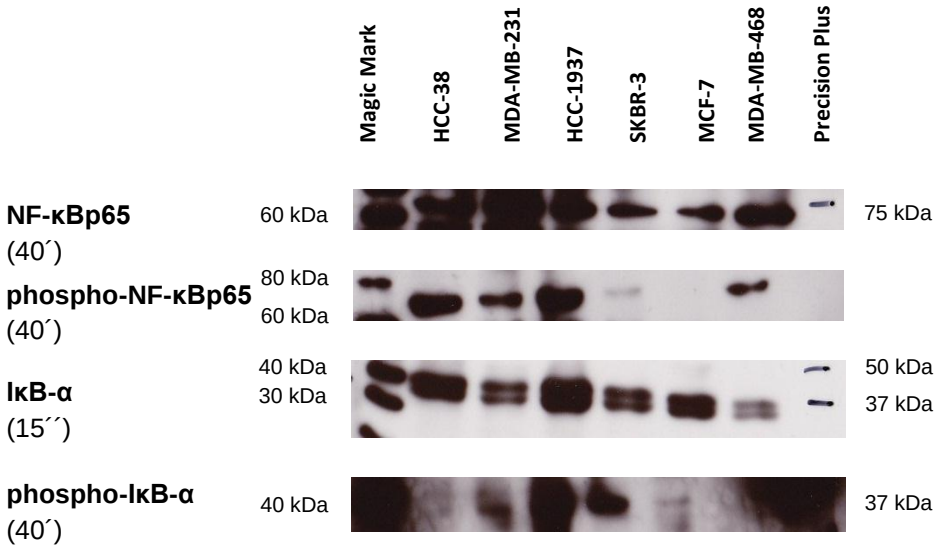


Figure 29: Detection of the proteins NF-κBp65, phospho-NF-κBp65, IκB-α and phospho-IκB-α. 10 µg of the cell lines (inhibited with 0.1 µM BAY 11-7082 (HCC-38, SKBR-3 1 µM)) were used for a 10% SDS-Page gel and transferred onto a PVDF-membrane. The first antibody was diluted in SuperBlock Blocking Buffer 1:1000 for NF-κBp65, phospho-NF-κBp65, IκB-α and phospho-IκB-α. NF-κBp65, phospho-NF-κBp65 and phospho-IκB-α were incubated with Goat anti rabbit IgG (H+L) HRPO and IκB-α with Goat anti mouse IgG (H+L) HRPO. As exposure time 15 seconds for IκB-α, and 40 minutes for NF-κBp65, phospho-NF-κBp65 and phospho-IκB-α were chosen.

In the basal-like breast cancer cell lines less NF-κBp65 was expressed after the treatment with BAY 11-7082. In the reference cell lines the amount increased, except in MCF-7. Phospho-NF-κBp65 was downregulated in MDA-MB-231, MCF-7 as well as SKBR-3 and upregulated in MDA-MB-468 compared with the untreated cells (see Figure 25). In HCC-38 and HCC-1937 the amount of the protein stayed the same.

The treatment of the cells with this inhibitor did not alter the protein level of unphosphorylated and phosphorylated IκB-α compared with the untreated control (see Figure 25). GAPDH could not be blotted due to technical problems.

3.2.4.5 Akt Inhibitor IV treated cells

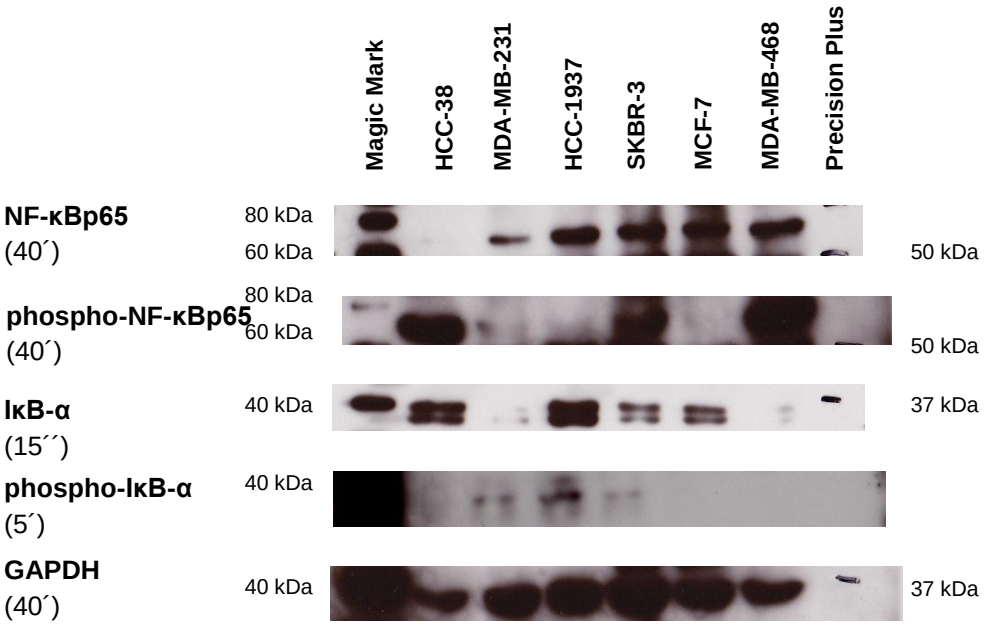


Figure 30: Detection of the proteins NF-κBp65, phospho-NF-κBp65, IκB-α, phospho-IκB-α and GAPDH. 10 µg of the cell lines (inhibited with 0.1 µM Akt Inhibitor IV (SKBR-3, MDA-MB-468 0.01 µM)) were used for a 10% SDS-Page gel and transferred onto a PVDF-membrane. The first antibody was diluted in SuperBlock Blocking Buffer 1:1000 for NF-κBp65, phospho-NF-κBp65, IκB-α, phospho-IκB-α and 1:5000 for GAPDH. GAPDH, NF-κBp65, phospho-NF-κBp65 and phospho-IκB-α were incubated with Goat anti rabbit IgG (H+L) HRPO and IκB-α with Goat anti mouse IgG (H+L) HRPO. As exposure time 15 seconds for IκB-α, 5 minutes for phospho-IκB-α and and 40 minutes for NF-κBp65, phospho-NF-κBp65 and GAPDH were chosen.

After the inhibition with Akt Inhibitor IV all basal-like breast cancer cell lines were producing less NF-κBp65 compared with the untreated cells (see Figure 25). SKBR-3 was slightly upregulating and MCF-7 was slightly downregulating the protein. In MDA-MB-468 the amount of NF-κBp65 stayed the same. While all cell lines express less phospho-NF-κBp65, in MDA-MB-468 more of this protein was present compared with the untreated control (see Figure 25). The expression of IκB-α was lower in all cells except in MDA-MB-468 after the treatment with this inhibitor. In comparison with

the untreated cells, the phosphorylated form of this protein was downregulated in all six breast cancer cell lines (see Figure 25).

3.2.5 Quantitative analysis of MEK1/2 in untreated and Tpl2 Kinase Inhibitor treated breast cancer cells

In the non-canonical NF-κB pathway the Tpl2 kinase leads to an activation of MEK1. The following experiment was performed to verify if a downregulation of MEK1/2 could be observed after the treatment with Tpl2 Kinase Inhibitor.

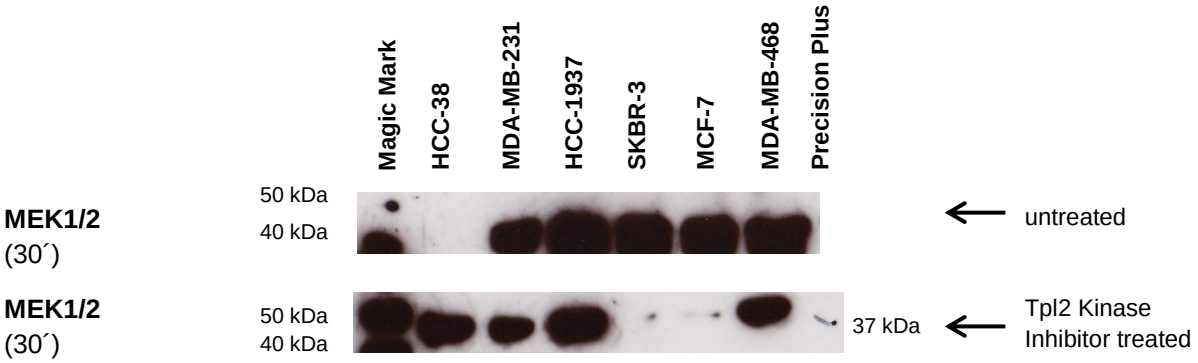


Figure 31: Detection of MEK1/2 in untreated and Tpl2 Kinase Inhibitor treated breast cancer cells. 10 µg of the cell lines (untreated and treated with 1 nM Tpl2 Kinase Inhibitor) were used for a 10% SDS-Page gel and transferred onto a PVDF-membrane. The first antibody was diluted 1:1000 in SuperBlock Blocking Buffer. The Blots were incubated with Goat anti rabbit IgG (H+L) HRPO. As exposure time 30 minutes were chosen.

The MEK1/2 expression in SKBR-3 and MCF-7 was downregulated after the treatment with Tpl2 Kinase Inhibitor. HCC-38 did not produce this protein in the naive state but seemed to gain this ability after treatment. The amount of MEK1/2 stayed the same in the other untreated and Tpl2 Kinase Inhibitor treated cell lines.

4 Discussion

Given that BLBC is hardly sensitive to treatments usually administered in other types of breast cancer, there is urgent need for alternative therapeutic strategies. The main objective of my diploma work was to investigate novel experimental therapies for basal-like breast cancer based on inhibitors of signaling pathways.

First of all, I investigated the MAPK pathway because it was known from literature, that EGFR is frequently overexpressed and that the transcription factor YB-1 is expressed in 73% of basal-like breast cancer cells (Stratford et al. 2008). Unexpectedly, none of the used inhibitors of the MAPK pathway, i.e. cetuximab, PD98059, U0126, ERK inhibitor II and III, and SL0101, was able to eradicate the investigated cancer cells (see Figures 11-12).

Being much more efficient, a low IC_{50} value using MTT-assays could only be obtained by inhibition of the NF-κB pathway. With this strategy, several compounds were effective, such as disulfiram, Akt Inhibitor IV, violacein, BAY 11-7082, MG-132 and Tpl2 Kinase Inhibitor (see Figures 14-19 and Tables 25-30). A marginally suppressive effect could be observed by inhibiting IGF1-R with PQ401 (data not shown).

As there is currently no inhibitor of YB-1 available, this putatively relevant transcription factor could not be assessed by MTT-assays. To analyze the effect of other active inhibitors on the expression of YB-1, I investigated untreated cells and cells treated with disulfiram, Akt Inhibitor IV and violacein by Western blotting. The data showed that there is no difference in the expression of YB-1 in the untreated basal-like breast cancer cells compared with the untreated reference cells. Likewise, the level of RSK2, the precursor protein of YB-1, was almost the same in all six breast cancer cell lines (see Figure 21). Unfortunately, no informative results could be obtained for the activated form of this transcription factor, phospho-YB-1^{S102}, due to technical problems in combination with unspecific binding of phospho-YB-1^{S102} (see Figure 21). After the treatment with Akt Inhibitor IV less YB-1 was produced in all cells and the amount of RSK2 was not altered (see Figure 22). This result was expected insofar as it was known from literature, that phosphorylated Akt directly activates YB-1 (Stratford et al. 2008). Unfortunately, a statement with respect to the

activated form of this transcription factor, which might be an important one, is limited to the observation that the binding of phospho-YB-1^{S102} is very unspecific. However, with the generated data it can be shown that the treatment with the NF-κB pathway inhibitors has no impact on the amount YB-1 and RSK2 in the BLBC as well as in the reference cells (see Figures 23-24).

As the next focus, cytotoxicity assays with combinations of two inhibitors or one inhibitor and one cytotoxic agent were performed to determine if an earlier inhibition of the cells could be reached with a combination of two substances. In fact, a lower IC_{50} value was only observed by combination of Akt Inhibitor IV and violacein when compared with the IC_{50} values of these two substances alone (see Figure 20). However, this effect was limited to the reference cell lines (MCF-7, SKBR-3, MDA-MB-468), but was not observed in the basal-like breast cancer cells (HCC-38, HCC-1937, MDA-MB-231) (see Figure 20). No increase in inhibition efficiency or a neutralizing effect could be obtained with all other tested 27 combinations. These combinations addressed single pathways by using two inhibitors within the pathway, the combination of two pathways by using two corresponding inhibitors or the combination of signaling inhibitors with cytotoxic agents (data not shown).

Among the investigated signaling pathways believed to be essential for BLBC in vitro, it seemed that the NF-κB pathway is the only pathway which is needed for the survival of these cancer cells. Therefore, Western Blots were done to investigate the NF-κB pathway in detail. First of all the untreated cells were blotted with the antibodies IKK-α, IKK-β, phospho-IKK-α/β, NF-κBp65, phospho-NF-κBp65, IκB-α and phospho-IκB-α, which are all members of the canonical NF-κB pathway. In case of IKK-α, IKK-β and phospho-IKK-α/β no informative results could be obtained and therefore no statement about these proteins can be done (see Figure 25). However, basal-like breast cancer cells were overexpressing NF-κBp65 and the phosphorylated form of this protein as well. The cell lines HCC-38 and HCC-1937 were producing more IκB-α, but all cell lines expressed the same amount of phospho-IκB-α (see Figure 25). These data show that the BLBC are more regulated by the canonical NF-κB pathway than the reference cells are. Further Immunoblots were done with the six cell lines treated with the inhibitors violacein, Tpl2 Kinase Inhibitor, MG-132, BAY 11-7082 and Akt Inhibitor IV. NF-κBp65 was downregulated in the violacein treated basal-like breast cancer cells. The phosphorylated form of this

protein was only suppressed in one BLBC (MDA-MB-231) and in one reference cell (MCF-7). The phospo-IκB-α was downregulated in all six cells whereas the unphosphorylated form was only inhibited in SKBR-3 and MDA-MB-231 (see Figure 26). It is known that the pigment isolated from *Chromobacterium violaceum* inhibits the expression of the p65 subunit partially and the p50 subunit completely, but it is not known on which level this inhibition takes place. Further it is known that this substance inhibits the phosphorylation of IκB-α (Kodach 2006). The downregulation of phospho-IκB-α, which was observed in my experiment, was anticipated. However, it was not expected that the unphosphorylated form of this protein was suppressed in two cell lines as well, because violacein should not have an effect on this NF-κB pathway family member. A reason for the downregulation of NF-κBp65 but no alteration of the phosphorylated form, could be the fact that phospho-NF-κB cannot only be generated by the unphosphorylated form, but also by the Tpl2 kinase dependent pathway. In this pathway Tpl2 activates RSK1 via MEK1 and ERK1/2 and this protein phosphorylates free NF-κB. This transcription factor can bind the DNA in the nucleus in either phosphorylated or unphosphorylated state. My data suggest that after the treatment with violacein, less unphosphorylated NF-κB is binding the DNA.

After treatment of the cells with Tpl2 Kinase Inhibitor the amount of NF-κBp65 was not altered. The phosphorylated transcription factor subunit was downregulated in one BLBC (MDA-MB-231) and in one reference line (MCF-7). An upregulation of this protein took place in the cell line MDA-MB-468 (see Figure 27). While no alteration in the NF-κBp65 levels was anticipated, it was unexpected that a downregulation of the phosphorylated form only took place in one BLBC and one reference cell instead of all six cell lines. It is known, that the Tpl2 Kinase Inhibitor suppresses the activation of MEK1 from Tpl2 (Gavrin et al. 2005). Therefore, phospho-NF-κB should normally not be generated via this pathway. This is why only the activated form of this transcription factor should be suppressed by this inhibitor. The amount of IκB-α was not altered by the treatment of the cells with Tpl2 Kinase Inhibitor. Phospho-IκB-α was downregulated in HCC-38 and MDA-MB-468 (see Figure 27). By current knowledge, one would not expect a modulation of phospho-IκB-α in these two cell lines as Tpl2 acts downstream of this protein. This hitherto unnoticed interaction might point at an interaction between the canonical and non-canonical NF-κB pathway which are not yet characterized at this level.

Except HCC-38, all cell lines treated with the proteasome inhibitor MG-132, were producing less NF-κBp65. Further all treated cells except MDA-MB-468 were expressing lower levels of the phosphorylated form of the protein. The phospho-IκB-α was downregulated after exposure to MG-132 in all cell lines and the unphosphorylated IκB-α was suppressed in five of the six breast cancer cells (see Figure 28). This proteasome inhibitor normally prevents the degradation of phosphorylated IκB-α and therefore the release of free NF-κB from the binding complex (Morelli et al. 2003). Concerning NF-κBp65, phosphorylated and unphosphorylated IκB-α, the data of this experiment were very surprising as one would expect either an increase of these proteins or at least no downregulation when compared with the cells without treatment with MG-132. Normally, more of the NF-κB-IκB-α and NF-κB-phospho-IκB-α complexes should be present, if the degradation of phosphorylated IκB-α is prevented by this inhibitor, which was not the case. In contrast, the data obtained for phospho-NF-κBp65 were in agreement with the theory, because if no unphosphorylated NF-κB is released from the complex, no phosphorylation of this protein can take place via this canonical pathway. Thus, the proteasome inhibitor MG-132 has very likely some other effects, which are not yet known soliciting the need for detailed investigations on the comprehensive mechanism of action of this inhibitor.

The substance BAY 11-7082 inhibits the phosphorylation of IκB-α (Scaife et al. 2006). As for violacein a downregulation of phospho-NF-κB and phospho-IκB-α and no alteration in the amount of NF-κBp65 and IκB-α was expected. In contrast to my expectations, NF-κBp65 was suppressed in all basal-like breast cancer cells and in one reference cell line (MCF-7), while the other two reference cells were producing more of this protein. Furthermore phospho-NF-κB was downregulated in MDA-MB-231, MCF-7 as well as SKBR-3 and upregulated in MDA-MB-468. The level of phospho-NF-κB stayed the same in HCC-38 and HCC-1937. No alteration in the amount of unphosphorylated and phosphorylated IκB-α could be detected in all cell lines (see Figure 29). Similar to observations reported above, an explanation for these unexpected results could be hitherto unknown interactions between pathways. For example, BAY 11-7082 may also have additional effects to the inhibition of the IκB-α phosphorylation.

The last inhibitor I used for treatment of the breast cancer cells was Akt Inhibitor IV. While NF-κBp65 was suppressed in the BLBC and MCF-7, SKBR-3 was producing more of this protein after the treatment with this substance. No alteration could be observed in the cell line MDA-MB-468. The production of phospho-NF-κBp65 was inhibited in all breast cancer cells, except in MDA- MB-468. The phosphorylated IκB-α was downregulated in all six cell lines and less IκB-α was produced in all cancer cells, except in MDA-MB-468 (see Figure 30). Concerning the downregulation of phospho-IκB-α and phospho-NF-κBp65, these results were in accordance with my expectations. Normally, Akt Inhibitor IV prevents the phosphorylation of Akt, which directly activates the IKK complex (http://www.emdchemicals.com).

MDA-MB-468 was not reacting as a basal-like cancer cell nor as a reference cell. This cell line is a basal-like breast cancer cell which is not triple negative. This seems to be the reason why the data of this cell line often did not match with the data of the real basal-like breast cancer cells or the reference breast cancer cells.

According to the results of the Tpl2 Kinase Inhibitor in the cytotoxicity assays and the Immunoblotting (see Figures 19 and 27), it had to be investigated if this substance really prevents the production of MEK1. This was done by Western blotting with Tpl2 Kinase Inhibitor treated cells in comparison to untreated cells. The antibodies used for these approaches detected unphosphorylated MEK1/2 and phospho-MEK1/2. Unfortunately the results for the phosphorylated form of this protein were not informative and therefore no statement in respect to the activated state of MEK1/2 can be done (data not shown). However the amount of MEK1/2 was only downregulated in two reference cell lines (MCF-7 and SKBR-3). No alteration in the expression level of this protein could be observed in the other four cell lines (see Figure 31). Maybe the signal of MEK1 was masked by the signal of MEK2 in this experiment. Another explanation for these data is that MEK1 is not the only target from Tpl2. For detailed information of the Tpl2 kinase dependent NF-κB pathway more experiments would be useful. One possibility would be the use of MEK1 and MEK2 specific antibodies to be sure which subunit of the two MEKs is detected and to address possible differences between these subunits.

Conclusion

After investigation of the MAPK pathway, the PI3K-Akt-mTOR pathway and the NF-κB pathway, as pathways known to be relevant for vital cellular functions, the NF-κB signaling pathway seems to be the most important one for the survival of the basal-like breast cancer cells. Except disulfiram, all inhibitors of this pathway led either to a complete eradication or to a growth stop of the cells in the cytotoxicity assays at a low micromolar concentration (IC_{50}: 0.01 µM – 3.4 µM). By Immunoblotting upon exposure with different NF-κB pathway inhibitors, it could further be shown that the canonical NF-κB pathway is very complex. Moreover, the obtained data suggest that interactions with other signaling pathways might take place, because some of the used substance did affect the canonical NF-κB pathway members in ways not in agreement with current knowledge. For detailed information about this pathway and the communication with other pathways more experiments are needed. No statement about the importance of the transcription factor YB-1 can be done by these experiments, because no informative results could be obtained for phospho-YB-1^{S102}. Maybe the activated form of this protein plays a key role in the survival of the basal-like breast cancer cells too, but a good working antibody would be needed for these approaches. It would be a very worthwhile endeavor to investigate if this transcription factor can not only be activated by the MAPK pathway and the PI3K-Akt-mTOR pathway, but also by the NF-κB signaling pathway. In conclusion, the results of these experiments lead to the assumption that alternative therapeutic strategies for basal-like breast cancer should be based on NF-κB pathway inhibition.

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6 Abbreviations

´	minutes
´´	seconds
°C	degrees Celsius
µg	microgram
µl	microliter
µM	micromolar
AMP	adenosine monophosphate
APS	ammonium peroxodisulfate
ATCC	American type culture collection
ATF-3	cyclic AMP dependent transcription factor 3
ATP	adenosine triphosphate
BAP1	BRCA-1 associated protein 1
BLBC	basal-like breast cancer
Bmi1	BMI1 polycomb ring finger oncogene
BRCA-1	breast cancer antigen 1
CD133	prominin 1
CDC2	cyclin-dependent kinase 1
c-Jun	jun proto-oncogene
CK	cytokeratin
c-kit	tyrosine kinase KIT
c-myc	v-myc myelocytomatosis viral oncogene homolog (avian)
cm^2	square centimeter
ddH_2O	double distilled water
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DTT	1,4 Dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EGP2	epithelial glycoprotein 2
Elk1	E twenty-six (ETS)-like transcription factor 1

ER	estrogen receptor
ERK	extracellular signal-regulated kinase
ext.	extinction
FCS	fetal calf serum
fos	FBJ murine osteosarcoma viral oncogene homolog
g	gram
GATA-3	GATA binding protein 3
GDP	guanosine diphosphate
GTP	guanosine-5'-triphosphate
h	hours
HE	hematoxylin
Her2 (ErbB2)	human epidermal growth factor receptor 2
IC_{50}	inhibitory concentration leading to 50% growth inhibition
IGF1R	insulin-like growth factor 1 receptor
IgG	immunglobulin G
IκB	inhibitory kappa B protein
IKK	IκB kinase complex
JNK	c-Jun amino-terminal kinase
kDa	kilodalton
l	liter
m	molar
mA	milliampere
MAD2	mitotic arrest deficient 2
MAD2L1	MAD2 mitotic arrest deficient-like 1 (yeast)
MAPK	mitogen activated protein kinase
MAPKK	mitogen activated protein kinase kinase
MAPKKK	mitogen activated protein kinase kinase kinase
ml	milliliter
mM	millimolar
mTOR	mammalian target of rapamycin
MTT	3-(4,5- Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NEMO	nuclear factor kappa B essential modulators
NF-κB	nuclear factor 'kappa-light-chain-enhancer' of activated B-cells

nM	nanomolar
nm	nanometer
PBS	phosphate buffered saline
PDK1	phosphoinositide-dependent kinase 1
PI3K	phosphatidylinositol-3-kinase
PIP3	phosphatidylinositol-3,4,5-triphosphate ($PI(3,4,5)P_3$)
PKB (Akt)	protein kinase B
PKC	protein kinase C
PR	progesterone receptor
PTEN	tumor suppressor gene phosphatase and tensin homolog
PVDF	polyvinylidene fluoride
RAD21	RAD21 homolog (S.pombe)
RHD	Rel homology domain
RSK	p90 ribosomal S6 kinase
RT	room temperature
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SLUG	snail homolog (Drosophila)
Sox2	sex determining region Y - box 2
TBS	tris buffered saline
TGF	transforming growth factor
tit.	titrated
TNF	tumor necrosis factor
TP53	tumor protein p53
Tpl2	tumor progression locus 2
VEGF	vascular endothelial growth factor
YB-1	Y-box binding protein 1
$YB\text{-}1^{S102}$	Y-box binding protein 1 phosphorylated at serine 102

7 Abstract

About 15% of all breast cancer cells are triple negative for the hormone receptors estrogen, progesterone and Her2/neu (human epidermal growth factor receptor 2). Almost all of these are basal-like breast cancer (BLBC) cells. As this subtype of mamma carcinoma cannot be treated with anti-estrogens or Her2 neutralizing antibodies, taxanes, antracyclines and platinum derivatives are currently tested in clinical trials. Inhibition of signaling pathways may offer another option in this aggressive subtype of breast cancer.

To investigate which pathways play a key role for the cells, dose response curves (MTT-assay) with different inhibitors, cytotoxins and combinations of both were established. HCC-38, HCC-1937 and MDA-MB-231 were used as representative BLBC cells with epithelial or luminal breast cancer cells as reference (MCF-7, SKBR-3 and MDA-MB-468). The following inhibitors were used: cetuximab (against EGFR), PD98059 and U0126 (against MEK1/2), ERK Inhibitor II (against ERK1/2), ERK Inhibitor III and SL0101 (against RSK), Akt Inhibitor IV (against Akt), wortmannin (against PI3K), rapamycin (against mTOR), PQ401 (against IGF1R), violacein, disulfiram, MG-132, Tpl2 Kinase Inhibitor and BAY11-7082 (all NF-κB inhibitors). The MTT-assays showed that the inhibition of NF-κB strongly inhibited the BLBC cells. Quantitative analysis of the canonical NF-κB pathway members (IKKα, IKKβ, phospho-IKKα/β, NF-κBp65, phospho-NF-κBp65, IκB-α and phospho-IκB-α) were done with lysates of the inhibited cells by Immunoblotting. The blotting of the untreated cells showed that the basal-like cells were overexpressing NF-κBp65 and phospho-NF-κBp65 compared with the reference cells. The expression of NF-κBp65 was downregulated in BLBC after treatment with violacein, MG-132, BAY11-7082 and Akt Inhibitor IV. Phospho-NF-κBp65 did not show consistent results in the BLBC cells. The IKK proteins were below the detection limit of the Western blot. IκB-α and phospho-IκB-α were downregulated in almost all cells after the treatment with violacein, MG-132 and Akt Inhibitor IV.

These data show that the NF-κB pathway is an important signaling pathway for the survival of the basal-like breast cancer cells. In response to the tested inhibitors, the regulation of this pathway seems to be very complex suggesting a communication between the canonical NF-κB pathway and other signaling pathways as MG-132,

Tpl2 Kinase Inhibitor and BAY11-7082 did not downregulate their target proteins as expected. All used NF-κB inhibitors, except disulfiram, were effective in the MTT-assays resulting either in complete eradication or growth stop of the cells (IC_{50}: 0.01 µM – 3.4 µM). In conclusion, these experiments show that alternative therapies for basal-like breast cancer based on the NF-κB pathway may be a promising approach.

8 Zusammenfassung

Ungefähr 15% aller Brustkrebszellen sind dreifach negativ für die Hormonrezeptoren Östrogen, Progesteron und Her2/neu (human epidermal growth factor receptor 2). Fast alle von diesen sind Mammakarzinome vom Basaltyp (BLBC). Da dieser Brustkrebssubtyp nicht mit Antiöstrogenen oder Her2 neutralisierenden Antikörpern behandelt werden kann, werden Taxane, Antrazykline und Platinderivate im Moment in klinischen Studien getestet. Eine andere Möglichkeit der Therapie dieses aggressiven Subtyps, könnte die Inhibierung verschiedener Signalwege darstellen.

Um herauszufinden welche Signalwege eine Schlüsselrolle für diese Zellen spielen, wurden Dosis-Wirkungskurven (MTT-Assay) mit verschiedenen Inhibitoren, Zytotoxinen und Kombinationen beider erstellt. HCC-38, HCC-1937 und MDA-MB-231 wurden als repräsentative Brustkrebszellen des Basaltyps herangezogen. Epitheliale oder luminale Mammakarzinome (MCF-7, SKBR-3 und MDA-MB-468) dienten als Referenzzellen. Folgende Inhibitoren wurden für die Experimente verwendet: cetuximab (gegen EGFR), PD98059 und U0126 (gegen MEK1/2), ERK Inhibitor II (gegen ERK1/2), ERK Inhibitor III und SL0101 (gegen RSK), Akt Inhibitor IV (gegen Akt), wortmannin (gegenPI3K), rapamycin (gegen mTOR), PQ401 (gegen IGF1R), violacein, disulfiram, MG-132, Tpl2 Kinase Inhibitor und BAY11-7082 (alle NF-κB Inhibitoren). Mittels der Dosis-Wirkungskurven konnte gezeigt werden, dass die Inhibierung von NF-κB das Überleben der BLBC verhindert. Eine quantitative Analyse der kanonischen NF-κB Signalwegmitglieder (IKKα, IKKβ, phospho-IKKα/β, NF-κBp65, phospho-NF-κBp65, IκB-α und phospho-IκB-α) wurde mit Lysaten der inhibierten Zellen mittels Immunoblotting erreicht. Es konnte gezeigt werden, dass die unbehandelten Basalzellen die Proteine NF-κBp65 und phospho-NF-κBp65 im Vergleich zu den Referenzzellen überexpremieren. NF-κBp65 wurde durch die Behandlung der Zellen mit violacein, MG-132, BAY 11-7082 und Akt Inhibitor IV runterreguliert. Phospho-NF-κBp65 reagierte nach der Behandlung mit den Substanzen in den Zelllinien nicht immer gleich. Über die IKK Proteine konnte keine Aussage getroffen werden, da sie unter dem Detektionslimit lagen. Die Expression von IκB-α und phospho-IκB- α wurde nach der Behandlung mit violacein, MG-132 und Akt Inhibitor IV in fast allen Zellen verringert.

Diese Ergebnisse zeigen, dass der NF-κB Signalweg einen wichtigen Weg für das Überleben der Basalzellen darstellt. Die Regulation dieses Signalweges scheint sehr kompliziert zu sein und es liegt die Vermutung nahe, dass es eine Kommunikation zwischen dem kanonischen NF-κB Signalweg und anderen Signalwegen gibt, da manche verwendeten Substanzen (MG-132, Tpl2 Kinase Inhibitor und BAY 11-7082) nicht immer in der Lage waren die betreffenden Proteine zu inhibieren. Alle verwendeten Inhibitoren, außer Disulfiram, haben bei den MTT- Experimenten entweder zu einem Wachstumsstopp oder zu einer Eradikation der Zellen geführt (IC_{50}: 0,01 µM – 3,4 µM). Zusammenfassend kann gesagt werden, dass es vielversprechend scheint, alternative Therapien für das Mammakarzinom vom Basaltyp auf dem NF-κB Signalweg basieren zu lassen.

9 Curriculum Vitae

GENERAL INFORMATIONS

Name	Simone Klingenbrunner
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EDUCATION

2005 - 2012	Study of Molecular Biology at the University of Vienna
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LABORATORY EXPERIENCE

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November 2010	Center for Brain Research Supervisor: Univ. Prof. Mag. Dr. Johannes Berger
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