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The HER3 Receptor: Role as an Intervention Target in Ovarian Cancer

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Dedicated to my parents

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1. Introduction

Intercellular communication is a key concept of multicellular organisms that has evolved to manage the need of regulating complex processes like embryonic development, tissue differentiation, wound repair, and response to infections. The ability of the different cells of a metazoan organism to communicate with their specific environment, and therefore to coordinate their individual behaviour, is mainly mediated by growth factors, cytokines, and hormones, which bind to their corresponding receptors and activate a variety of signalling pathways. This finally results in a well coordinated cell specific response for the welfare of the whole organism regardless of an individual cell. Disturbance and unrestricted activation by aberrant signalling due to mutation, overexpression, and/or sequestration of important pathway components or the presence of active autocrine loops may, in contrast, result in the development of severe diseases like cancer, diabetes, immune deficiencies or cardiovascular diseases.

A large part of this cell-to-cell communication is conveyed by growth factors. Growth factors are small proteins/peptides that are released by cells and deliver their specific biologic messages by binding to their corresponding receptors on surrounding cells or even at distant sites like a classical hormone. These growth factor receptors, with the exception of the insulin receptor family, are monomeric transmembrane proteins. Binding of a specific ligand results in dimerisation, autophosphorylation and thereby activation of the receptor and of a plethora of different downstream signalling pathways.

1.1 The epidermal growth factor receptor (EGFR) family

The 90 protein tyrosine kinases encoded by the human genome can be divided into 58 receptor as well as 32 cytosolic tyrosine kinases. Both groups are further subdivided into different families based on sequence similarities as introduced by Hanks and Hunter (Hanks and Hunter 1995).

The mammalian EGF receptor family of receptor tyrosine kinases consists of four closely related receptors namely the EGFR (HER1) (Ullrich et al. 1984), HER2

(ErbB2/Neu) (Coussens et al. 1985; Schechter et al. 1985), HER3 (ErbB3) (Kraus et al. 1989; Plowman et al. 1990) and HER4 (ErbB4) (Plowman et al. 1993). These four receptors exhibit approximately 40% - 45% sequence identity and arose from a series of gene duplications early in vertebrate evolution (Stein and Staros 2000). They share a common structure with a ligand binding extracellular domain, a membrane spanning helix, and a tyrosine kinase domain flanked by a carboxyterminal tail which contains several tyrosine phosphorylation sites (Yarden and Sliwkowski 2001; Citri and Yarden 2006). The complexity of the EGF receptor ligand network is imposed by a multiplicity of ligands, such as the epidermal growth factor (EGF), the transforming growth factor α (TGFα), epiregulin, amphiregulin (AR), betacellulin, the heparin-binding EGF (HB-EGF) as well as epigen, which all bind to the EGFR while the four members of the neuregulin (NRG) family bind to HER3 and HER4.

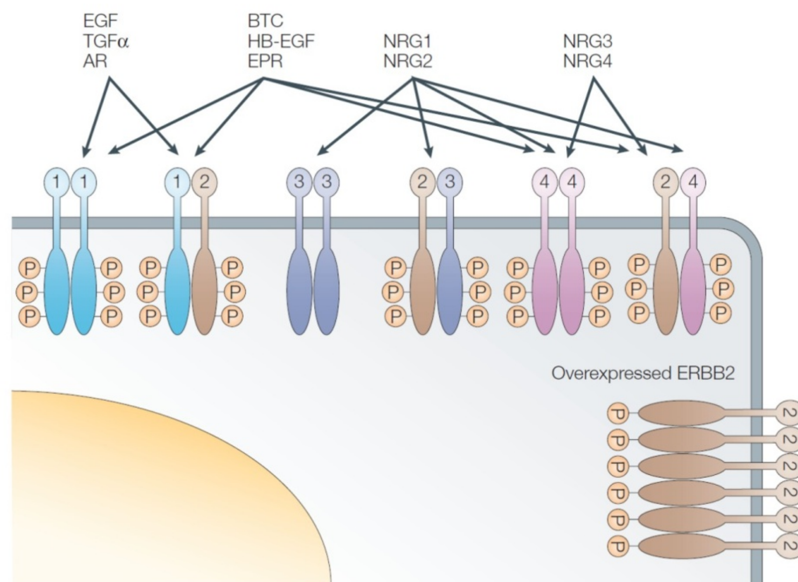


Fig. 1: The EGF receptor family and its ligands.
Binding of the epidermal growth factor family of ligands to corresponding receptors induces receptor dimerisation and phosphorylation of specific tyrosine residues within the cytoplasmic tail of the receptor. These phosphorylated tyrosine residues serve as docking sites for binding of interaction partners thereby activating downstream signalling pathways. (Figure was adopted from (Hynes and Lane 2005)).

1.1.1 Epidermal growth factor receptor (EGFR, ErbB1)

The epidermal growth factor receptor was the first member of the ErbB family of receptors to be identified. In 1978, it could be shown by Carpenter and colleagues that the addition of epidermal growth factor (EGF) to A-431 membrane preparations stimulated the phosphorylation of membrane components having molecular weights of 170,000 and 150,000 (Carpenter et al. 1978). Only two years later, it has been discovered that the receptor (tyrosine) kinase activated via EGF has the potential to phosphorylate tyrosine residues of intrinsic membrane proteins as well as exogenous added histones (Ushiro and Cohen 1980). Finally, the amino acid sequence of the human EGFR deduced from cDNA and the close similarity to the v-erbB oncogene, a truncated version of the EGFR, was elucidated in 1984 (Downward et al. 1984; Downward et al. 1984; Ullrich et al. 1984). Today it is well known that ligand induced dimerization of the monomeric receptor increases the activity of the intracellular tyrosine kinase domain, which subsequently results in trans-phosphorylation of several tyrosine residues in the homo- or heterodimerization partner (Schlessinger 2000). Moreover, adaptor proteins like growth-factor-receptor-bound 2 (GRB2) and Src-homology-2-containing (Shc) are recruited to the phosphorylated receptor resulting in activation of a variety of downstream signalling pathways (Citri and Yarden 2006). Unlike the ErbB3/HER3 receptor, which has six putative phosphatidylinosiol-3-kinase (PI3K) binding sites, no direct interaction of PI3K and EGFR has been reported and the PI3K-AKT pathway is activated via the small GTPase Ras (Schulze et al. 2005; Citri and Yarden 2006).

1.1.2 HER2 (ErbB2, neu)

The HER2 receptor is the second member of the EGF receptor family and has been discovered in 1985 as the homolog to the *neu* oncogene, which was identified one year before (Schechter et al. 1984; Coussens et al. 1985). So far, there is no known ligand that binds to the HER2 receptor but due to its open confirmation the receptor is continually available as dimerisation partner for other family members like the EGFR or HER3 (Cho et al. 2003; Garrett et al. 2003). In 1987, two years after the discovery of

the HER2 receptor, it could be shown that the receptor is overexpressed in approximately 30% of breast tumors with a two-fold or greater amplification of the *HER2* gene (Slamon et al. 1987). Moreover, this amplification correlated to patient overall survival as well as time to relapse. In addition to breast cancer, overexpression and/or amplification of the HER2 receptor has been reported in ovarian cancer, gastric carcinoma, and in tumors of the esophagus (Slamon et al. 1989; Berchuck et al. 1990; Jaehne et al. 1992; Pils et al. 2007; Stoecklein et al. 2008). Interestingly, it could be demonstrated in esophageal cancer that *HER2* gain confers high risk for early death only when present in disseminated tumor cells, but seems to have no relevance in the primary tumor (Stoecklein et al. 2008). In 1998, trastuzumab (Herceptin), a monoclonal antibody directed against the extracellular domain of the HER2/ErbB2 receptor, was approved for the treatment of metastatic breast cancer and received expanded FDA approval for adjuvant treatment of early breast cancer in combination with chemotherapy in 2006.

1.1.3 HER3 (ErbB3)

The HER3 (ErbB3) receptor is a 180 kDa glycoprotein (Kraus et al. 1989; Plowman et al. 1990) with an extracellular domain that consists, as in other members of the EGFR family, of four subdomains (I – IV). However, compared to the EGF receptor, where domain III is the main contributor to ligand binding, it is domain I in the HER3 receptor (Singer et al. 2001; Kani et al. 2005). Furthermore, HER3 does not homodimerise and its ligands bind to the receptor with a higher affinity compared to the binding of epidermal growth factor (EGF) to EGFR (Berger et al. 2004; Kani et al. 2005). Ligand-induced downregulation of the HER3 receptor is mediated by the RING finger E3 ubiquitin ligase Nrdp1 (neuregulin receptor degradation protein-1). Nrdp1 was identified in a yeast two-hybrid screen with the entire intracellular domain of HER3 as bait, and cellular Nrdp1 levels have been assumed to be critical in regulating steady-state levels of the receptor (Diamonti et al. 2002). Nrdp1 itself is stabilised by the ubiquitin-specific protease USP8, which becomes phosphorylated and therefore stabilised by AKT in a ligand and PI3K dependent manner (Wu et al. 2004; Cao et al. 2007). Furthermore, loss of Nrdp1 enhances the growth of breast tumor cell lines and

Nrdp1 expression inversely correlates with HER3 levels in human breast cancer (Yen et al. 2006). Despite structural similarities of HER3 and other EGF receptor family members, substitutions in the amino acids sequence of the kinase domain seems to be responsible for the impaired kinase activity of this protein (Hanks and Quinn 1991; Guy et al. 1994; Pinkas-Kramarski et al. 1996; Sierke et al. 1997; Stein and Staros 2000). Even though using a chimeric EGFR/HER3 receptor two groups reported an induction of HER3 autophosphorylation upon EGF stimulation (Kraus et al. 1993; Prigent and Gullick 1994) Moreover, recent findings by van der Horst *et al.* show that HER3 seems to have a high substrate specificity and is able to phosphorylate PYK2 (van der Horst et al. 2005). However, HER3 represents an important heterodimerisation partner for other EGF receptor family members. In this regard, dimerisation, preferentially with HER2, can be initiated upon ligand binding which results in activation of both receptors as well as downstream signalling components. An important role of this heterodimer-mediated signalling in normal development has been shown in genetically modified mice, where expression of ablated HER2 or HER3 genes resulted in defects in the development of the sympathetic nervous system (Britsch et al. 1998). This HER2/HER3 heterodimer reflects the most potent mitogenic signalling complex among the ErbB network despite the fact that both partners are incapable of signalling on its own (Wallasch et al. 1995; Pinkas-Kramarski et al. 1996). In this respect, HER3 and HER2 overexpression is associated in breast cancer and studies show that HER3 cooperates with HER2 to effectively transform NIH 3T3 cells (Alimandi et al. 1995; Witton et al. 2003).

The cytoplasmic part of the HER3 receptor possesses six potential PI3K binding sites and is therefore the preferred dimerisation partner when signalling occurs via the PI3K-AKT pathway (Schulze et al. 2005). Compensatory HER3 signalling as well as activation of the PI3K-AKT pathway in the presence of tyrosine kinase inhibitors targeting other EGF-receptor family members results in cellular survival (Sergina et al. 2007). Moreover, expression of the HER3 receptor correlates with tumor progression and reduced patient survival in malignant melanoma and metastases (Reschke et al. 2008). Furthermore, HER3 is associated with decreased survival in cancer of the ovary (Tanner et al. 2006). Interestingly, HER3 and Integrin α6β4 seem to be functionally associated, as reported by Folgiero and colleagues, whereas only HER3 and P-AKT positivity, as well as tamoxifen sensitivity, influences patient outcome in ERβ1-negative breast carcinomas (Folgiero et al. 2008).

1.1.4 HER4 (ErbB4)

The HER4 receptor represents the fourth member of the EGFR family of receptor tyrosine kinases (Plowman et al. 1993). This RTK can be activated by a multitude of ligands including betacellulin, epiregulin, HB-EGF, NRG1 and NRG2 (Riese et al. 1996; Elenius et al. 1997; Komurasaki et al. 1997; Crovello et al. 1998; Falls 2003). Unlike HER2 and HER3 but similar to EGFR, HER4 represents a fully functional receptor with soluble ligands and an active kinase domain compared to the HER2 or the HER3 receptor, respectively. Moreover, the HER4 receptor has the potential to homo and/or heterodimerise with other EGFR family members. Ligand binding and dimerisation then induces downstream signalling pathways and/or proteolytic cleaveage of HER4 in a γ-secretase-dependent manner and the receptor translocates to the nucleus (Ni et al. 2001; Williams et al. 2004). The role of the ErbB4 receptor in cancer development and progression is only poorly understood and sometimes contradictory. On the one hand, there are reports that link overexpression of HER4 with a positive outcome in breast cancer, but on the other hand, several studies connect the overexpression of ErbB4 with enhanced cell growth and tumor formation in vitro and in vivo (Witton et al. 2003; Junttila et al. 2005; Maatta et al. 2006; Lynch et al. 2007).

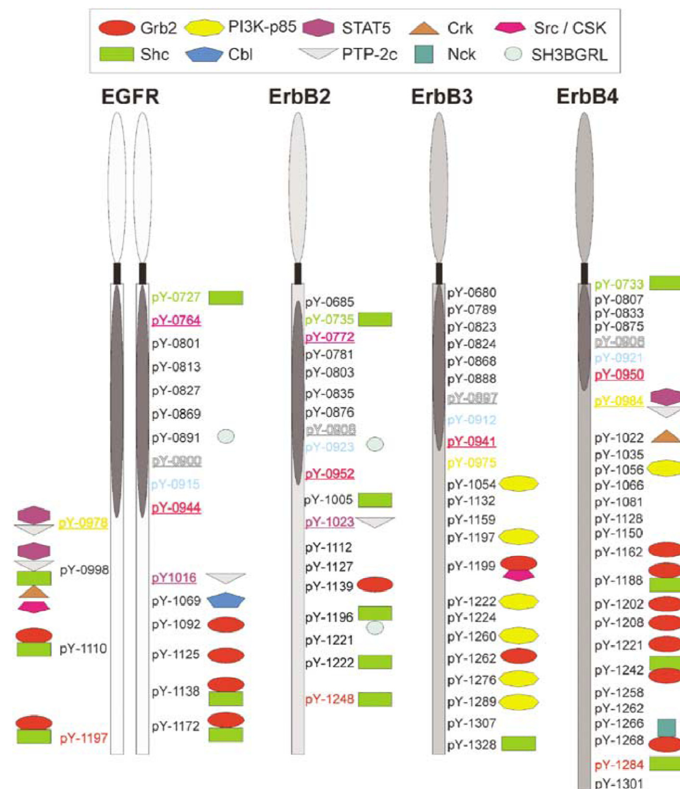


Fig. 2: Potential cytoplasmic interaction partners of EGF-receptor family members. Interaction partners of the EGF-receptor family identified by pull-down experiments using synthetic pepides with phosphorylated tyrosine residues and subsequent analysis of interaction partners by mass spectrometry. (Figure was taken from (Schulze et al. 2005)).

1.2 Neuregulin isoforms

Up to now, four neuregulin genes (NRG1-4) have been described generating at least 26 different NRG ligand isoforms by alternative splicing (Holmes et al. 1992; Wen et al. 1992; Marchionni et al. 1993; Carraway et al. 1997; Chang et al. 1997; Fischbach and Rosen 1997; Zhang et al. 1997; Harari et al. 1999). These ligand isoforms are implicated in a variety of developmental, physiological and pathological processes of the nervous system and the heart (Esper et al. 2006). Interestingly, loss of NRG1, HER2, or HER4 function induced either by gene deletion or mutation results in early embryonic lethality in mice. This is caused by cardiac development defects whereas HER3 deficiency (mice dying at day E13.5) results in a distinct cardiac phenotype (Gassmann et al. 1995; Lee et al. 1995; Meyer and Birchmeier 1995; Erickson et al. 1997). NRG1 ligands are able to bind to the HER3 as well as to the HER4 receptor

while different NRG2 isoforms seem to either activate HER3 or HER4 (Carraway et al. 1994; Carraway et al. 1997; Hobbs et al. 2002). This contrasts to NRG3 and NRG4, which seem to interact and activate the HER4 receptor only. Neuregulins are able to regulate proliferation, migration, angiogenesis, induction of apoptosis and cellular survival *in vitro* depending on the cell type and the respective neuregulin isoform (Lewis et al. 1996; Aguilar et al. 1999; Le et al. 2001; Venkateswarlu et al. 2002). Heregulin, for example, was shown to drive progression and neovascularisation of breast tumors *in vivo* (Atlas et al. 2003). Ligands of the NRG family can either act in an autocrine, a juxtacrine, or a paracrine manner. Most of the known isoforms are synthesized as transmembrane precursor molecules that can be cleaved by different metalloproteases which results in the release of the NRG ectodomain.

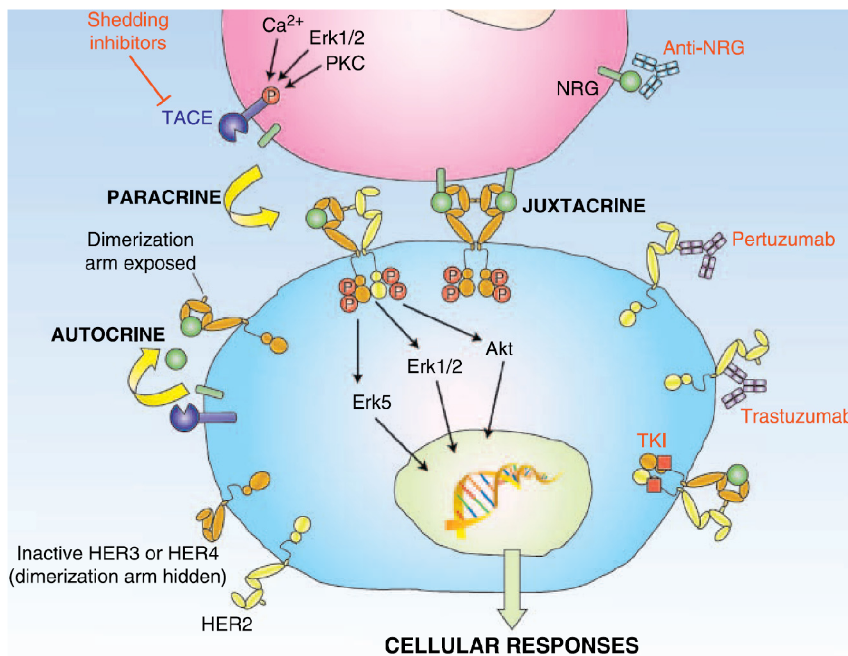


Fig. 3: Juxtacrine, paracrine and autocrine mode of action of neuregulin dependent receptor activation. Ligands of the neuregulin family can activate their corresponding receptors via different modes of action. In the membrane-bound situation the interaction is mediated by direct cell-cell contacts and the appropriate receptor is activated. In contrast to this, shedding of transmembrane neuregulins by cell surface metalloproteases like ADAM17/TACE (a disintegrin and metalloprotease 17) results in ectodomain release and autocrine or paracrine ligand binding. (Figure was modified from (Montero et al. 2008)).

1.3. AKT/PKB kinase and the PI3K-AKT pathway

The AKT serine-threonine kinase, which is also called protein kinase B (PKB) was identified in 1991 as a kinase related to PKA and PKC as well as by a different group as the retroviral oncogene (v-AKT) of the AKT8 retrovirus (acute transforming retrovirus) (Bellacosa et al. 1991; Coffer and Woodgett 1991; Jones et al. 1991). The AKT family consists of three members, namely AKT1, AKT2 and AKT3, regulating a wide variety of cellular processes like survival, proliferation, angiogenesis, migration, metabolism, and glucose homeostasis. They represent important and critical signalling nodes with a multitude of downstream targets regulated either directly or indirectly by these kinases. A crucial feature of AKT kinases, more precisely its anti-apoptotic role which is achieved by inhibiting the function of several pro-apoptotic proteins, is of pivotal interest in cancer research. In conjuction with its anti-apoptotic role, constitutively activated AKT1 does not induce tumors when it is expressed alone, whereas the activation of AKT1 greatly accelerates HER2-mediated mammary tumorigenesis in genetically modified mice (Hutchinson et al. 2001; Schwertfeger et al. 2001; Hutchinson et al. 2004). Therefore, the fundamental PI3K-AKT pathway is often deregulated in human cancer. In many ovarian cancers, for example, the PI3K pathway is activated by amplification of signalling components like *AKT2* or of the PI3K catalytic subunit, alpha isoform *(PI3KCA)* as well as by inactivating mutations of the phosphatase and tensin homolog *(PTEN)* gene (Bellacosa et al. 1995; Nakayama et al. 2006; Nakayama et al. 2007; Abubaker et al. 2009). Moreover, activation of this pathway is often associated with resistance to cytotoxic drugs.

PI3Ks can be divided into three different classes with the class I enzymes being the best studied. Class IA PI3K heterodimers consists of a regulatory subunit (p85) as well as a catalytic subunit (p110). Upon receptor activation e.g. by ligand binding, the p85 regulatory subunit either directly binds to the activated receptor or interacts with an adaptor protein, which subsequently results in the conversion of phosphatidylinositol-(4,5)-bisphosphate (PIP2) to phophatidylinositol-(3,4,5)-triphosphate (PIP3) by the catalytic subunit p110. The AKT kinase then binds to the freshly synthesized PIP3s via its Pleckstrin Homology (PH) domain and translocates to the cell membrane were it becomes phosphorylated and activated by the 3-phosphoinositide dependent protein kinase-1 (PDK1) and “PDK2”. The identity of the “PDK2” kinase responsible for the phosphorylation of the Ser473 residue of AKT was controversial for a long time and

many potential candidates like ILK and DNA-PK have been postulated (Persad et al. 2001; Feng et al. 2004). Recently, it has become evident that the mammalian target of rapamycin (mTOR) in complex with the rapamycin-insensitive companion of mTOR (Rictor) seems to be the long sought "PDK2" (Sarbassov et al. 2005) and responsible for the full activation of AKT.

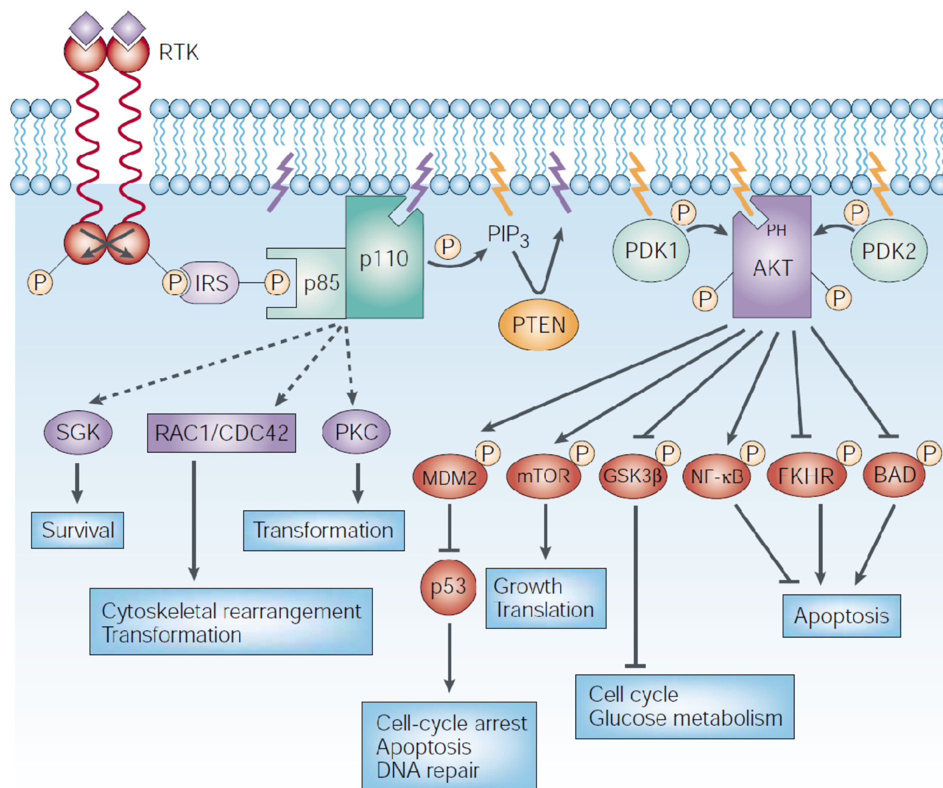


Fig. 4: The PI3K-AKT signalling pathway
Activation of PI3K by receptor tyrosine kinases (RTKs) or by G-protein coupled receptors (GPCRs) results in conversion of phosphatidylinositol-(4,5)-bisphosphate (PIP2) to phosphatidylinositol-(3,4,5)-triphosphate (PIP3). AKT binds to PIP3 via its Pleckstrin homology (PH) domain and thereby translocates to the cell membrane where it becomes phosphorylated at Thr308 and Ser473 by PDK1 and the mTORC2 (PDK2) complex, respectively. Subsequently, activated AKT phosphorylates a multitude of downstream targets regulating cellular fate (Figure was taken from (Vivanco and Sawyers 2002)).

1.4 Apoptosis (programmed cell death)

In a multicellular organism life and death has to be held in a balanced equilibrium. Disturbance of this tightly controlled fragile balance between cells generated by mitosis and cells undergoing apoptosis can finally result in the development of cancer. In contrast to necrosis, which is induced by several factors like infection, infarction, or other forms of cellular injury, apoptosis is the controlled process of programmed cell death which does not harm the surrounding tissue and only affects the apoptotic cell. The remaining cellular debris of a apoptotic cell will then be removed by phagocytes and the old cell is replaced by a new one (Savill and Fadok 2000). This programmed cell death plays a fundamental role in multicellular organisms; it is an integral part in tissue development, cellular homeostasis, lymphocyte development, and protection of cancer (Meier et al. 2000; Werlen et al. 2003). The first morphological description of apoptosis can be traced back to the drawings of Walther Flemming in the year 1885, showing cell shrinkage, nuclear fragmentation, and apoptotic body formation (Cotter 2009). Today, the widely accepted characteristics of apoptotic cells visible from the outside are cell rounding, retraction of contacts with neighbouring cells, blebbing of the plasma membrane, as well as important events happening inside the cell, such as the condensation and fragmentation of the nucleus, hydrolysis of nuclear DNA into small fragments, and the release of cytochrom c from the intermembrane space of the mitochondrium (Kerr et al. 1972; Williams et al. 1974; Wyllie et al. 1980). All different apoptotic pathways finally converge at one point, namely the activation of caspases. The first mammalian caspase of the large family of cysteinyl aspartate proteases to be identified was caspase-1 or ICE, discovered by its relation to CED-3 in C. elegans (Thornberry et al. 1992; Yuan et al. 1993). The caspase family can be subdivided into "initiator caspases" (e.g. caspase-8, -9 and -10) capable of activating the "effector caspases" (e.g. caspase-3, -6 and -7) which are responsible for cleavage of downstream targets (Riedl and Salvesen 2007). This apoptotic caspase cascade can either be induced by a number of external stimuli (extrinsic pathway) via activation of cell surface "death receptors" or by the so-called intrinsic or mitochondrial pathway (Parone et al. 2003; Wajant 2003). Activation of this intrinsic or BCL-2-regulated pathway predominantly leads to the activation of caspase-9 while the extrinsic or "death receptor" pathway recruits and activates caspase-8 at the cell surface. Caspases are synthesized as inactive zymogens, which consist of an N-terminal pro-domain, a large subunit, and a small

subunit. The pro-domain of caspase-2 and -9 are characterised by the caspase recruitment domain (CARD), whereas caspase-8 and -10 contain two tandem repeats of the death effector domain (DED). These domains play an essential role in the process of caspase activation because they serve as a platform for the homotypic interaction with other DED or CARD domains in activating interactors. Activation of the inactive zymogens by proteolytic cleavage then results in a hetero-tetrameric complex composed of two large and two small subunits (Kurokawa and Kornbluth 2009).

Another important family of proteins involved in the mitochondrial apoptotic pathway with either pro- or anti-apoptotic members is the BCL-2 (B-cell lymphoma-2) family. Soon after the discovery of the first family member (BCL-2), the interesting nature of the BCL-2 protein could be enlightened in more detail (Bakhshi et al. 1985; Tsujimoto et al. 1985; Cleary et al. 1986). Vaux and colleagues could show that the BCL-2 protein indeed has the potential to cooperate with c-myc to promote proliferation of B-cell precursor cells, but when it was overexpressed in an interleukin-3 (IL-3) dependent lymphoid and myeloid cell line cultivated in the absence of IL-3, BCL-2 promotes survival rather than proliferation (Vaux et al. 1988). Today, several BCL-2 family members which inhibit apoptosis like BCL-2 itself are known (BCL-XL, BCL-W, MCL1, BCL-B and BCL-2A1), whereas another class of the BCL-2 family promotes apoptosis (BAX, BAK). These pro-apoptotic effects are initialised by the permeabilisation of the outer mitochondrial membrane, which results in the subsequent release of cytochrome c and DIABLO. A third class of the BCL-2 family, namely the BH-3-only proteins (BAD, BLK, BID, HRK, BIM, BMF, NOXA and PUMA), promotes apoptosis by binding and regulating the anti-apoptotic BCL-2 protein. A multitude of death signals like DNA-damage, oncogene activation, microtubule disruption, and many more induce apoptosis via the BCL-2-regulated pathway. This results in permeabilisation of the outer mitochondrial membrane, release of multiple pro-apoptotic factors, and subsequent activation of the caspase cascade.

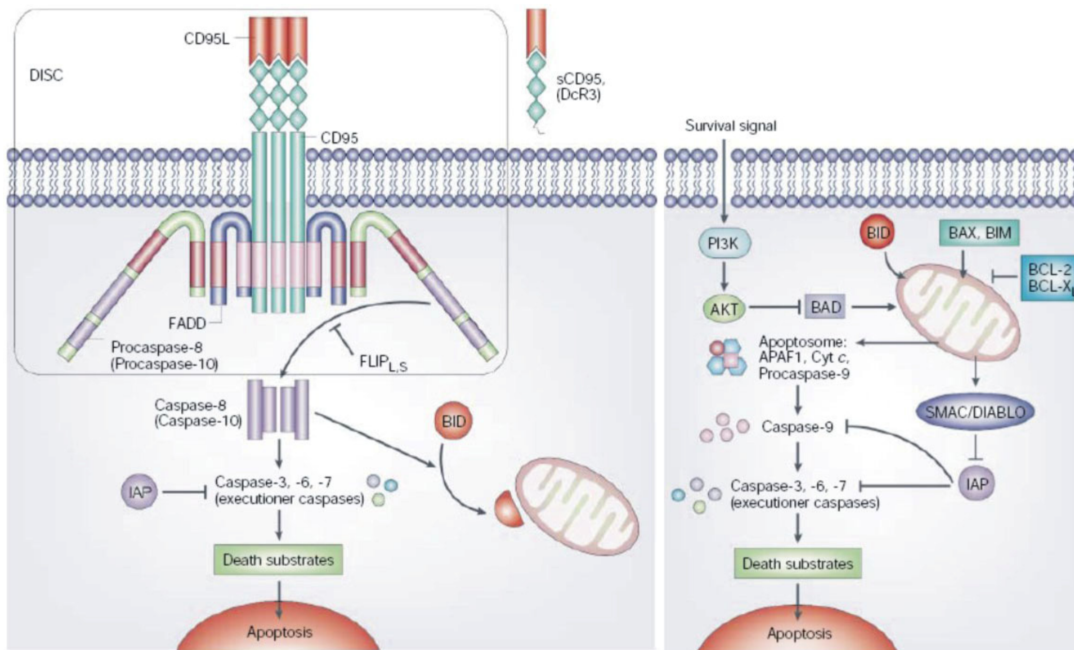


Fig. 5: The Extrinsic and intrinsic apoptotic pathway
Simplified illustration of the death receptor and the mitochondrial apoptotic pathway. Activation of the death receptor by ligand binding results in the formation of the death-induced signalling complex (DISC). Caspase-8 then is recruited to the DISC via the Fas-associated death domain protein (FADD) and autocatalytically activated. In contrast to this, the intrinsic or mitochondrial pathway is activated by the release of cytochrome c into the cytosol, which can be a result of chemotherapeutic or irradiation induced stress. Cytochrome c then binds to the apoptotic protease activating factor 1 (APAF1) to form the apoptosome which subsequently results in activation of caspase-9. Activation of the PI3K-AKT can inhibit the intrinsic apoptotic pathway by inactivating the pro-apoptotic BCL2-family member BAD. (Figure was modified from (Igney and Krammer 2002).

1.5 Chemotherapeutic drugs

Working for the State Department of Defence with the mission to investigate the potential therapeutic value of several chemical warfare agents in 1942, Louis Goodman and Alfred Gilman treated a patient with advanced non-Hodgkins lymphoma with nitrogen mustard and observed a remarkable although temporary improvement (Gilman 1963; Chabner and Roberts 2005). This observation as well as further studies established the important principle of chemotherapeutic drug treatment taking advantage of a higher vulnerability of tumors compared to normal tissue. Soon after, more and better alkylating agents like cyclophosphamide, ifosfamide, melphalan, and

chlorambucil became standard regimen components for the treatment of lymphomas, leukaemias, and other types of cancer (Chabner and Roberts 2005). Like alkylating agents, platinum based compounds form covalent bonds with electron-rich atoms like deoxyribonucleic acid (DNA) and proved efficiency in treating many different solid tumors and lymphomas. Cisplatin, the first platinum based compound that went into clinical trials in the early 70s was discovered by Rosenberg and colleagues while studying the effect of electric current on bacterial growth (Rosenberg et al. 1969; Higby et al. 1973; Lippman et al. 1973; Higby et al. 1974). While carboplatin, a platinum compound related to cisplatin, differs from cisplatin in its spectrum of toxicity, the activity of both platinum-based drugs is similar. Both chemotherapeutics interact with the DNA and form intrastrand crosslinks as well as adducts resulting in conformational changes of DNA. This interferes with replication and finally results in induction of apoptosis. Other chemotherapeutic drugs like anthracyclins, camptothecins, and etoposide target DNA topoisomerases (I and II) and represent some of the most active agents in the treatment of cancer. Doxorubicin, for example, the successor of daunorubicin, which was originally isolated from Streptomyces peucetius a soil-based microbe, acts mainly by intercalating into DNA, by affecting topoisomerase II, an enzyme responsible for the induction of DNA double strand breaks, and by the formation of hydroxyl radicals. While giving only an overview of the plethora and potential of chemotherapeutic drugs in the treatment of cancer, we should not underestimate the history of a second approach of chemotherapeutic drug therapy, namely the development of folate antagonists, as well as pyrimidine and purine antimetabolites. Antifolates such as methotrexate act by interfering with one or more biosynthetic steps involving folate coenzymes; and they were first used in 1944 to treat children with acute lymphoblastic leukaemia. In contrast, pyrimidine and purine antimetabolites like 5-fluorouracil, 5-azacytidine, and gemcitabine have evolved from the assumption that nucleic acids are involved in growth control. Gemcitabine, for example, which is used for the treatment of several solid tumors like pancreatic, lung, breast, and ovary, undergoes intracellular conversion to gemcitabine triphosphate and competes with deoxyctadine triphosphate for incorporation into DNA. The last “class” of chemotherapeutic drugs mentioned here, like vinca alkaloids and taxanes, targets microtubules which are components of the mitotic spindle and of the cellular cytoskeleton.

1.5.1 Chemotherapeutic resistance

Chemotherapeutic drug resistance is a major problem in the management of a variety of different cancers. For this reason it is of decisive importance to elucidate the cellular mechanisms underlying the characteristics of acquired or intrinsic chemotherapeutic resistance/insensitivity to improve treatment strategies and hence the survival of cancer patients.

Two different types of mechanisms that give rise to the tumor-cell specific drug resistance or insensitivity can be distinguished. Both mechanisms listed below only represent the resistance capabilities of tumor cells and not of the tumor as an entity. In this regard, the importance of tumor vascularisation and hepatic drug clearance as well as the protective function of the tumor microenvironment are not introduced here.

One mechanism that results in reduced drug sensitivity depends on an impaired accumulation of the cytotoxic drug inside the tumor cell. This can either be achieved by decreased cellular drug uptake, as it has been reported for folate antagonists and platinum-based compounds, by an increased efflux of the chemotherapeutic drugs via upregulation of ATP-binding cassette (ABC) transporters or by activation of the detoxifying system (Ishida et al. 2002; Lin et al. 2002; Szakacs et al. 2006). Platinum-based drugs, for example, require the assistance of transporters to enter the cell, and the mechanisms mentioned above decrease the concentration of the chemotherapeutic compound inside the cell, which may result in cellular survival.
The huge superfamily of ABC transporters represents the largest family of transmembrane proteins consisting of 48 human ABC genes, which can further be subdivided into seven distinct subfamilies (ABCA-ABCG) (Dean et al. 2001). The first member of the ABC family to be identified was the P-glycoprotein (P-gp), the product of the *MDR1* gene (Juliano and Ling 1976; Riordan and Ling 1979; Chen et al. 1986). Hydrophobic substrates like doxorubicin and vinblastine are transported by the ABCB1 (P-gp) transporter and several studies report an increased expression of this surface antigen in multidrug resistant human cancer cell lines as well as a low ABCB1 expression in drug-sensitive cells (Kartner et al. 1983; Kartner et al. 1985). Like the ABCB1, the ABCC1 (MRP1) transporter confers resistance to several chemotherapeutic drugs and was first identified in the doxorubicin resistant small-cell lung cancer cell line

NCI-H69 (Cole et al. 1992). Another member of this huge family of transporters is the ABCG2 or BCRP1 protein. ABCG2 has been discovered in mitoxantrone resistant cell lines that did not overexpress ABCB1 or ABCC1 and promoted resistance to anthracycline chemotherapeutic drugs (Allikmets et al. 1998; Doyle et al. 1998).

The second mechanism of cellular drug resistance introduced here relies on cellular changes like increased repair of DNA damage, alterations in cell cycle and/or reduction of apoptosis by the activation of anti-apoptotic pathways (McCubrey et al. 2008). In this regard, the activation of the PI3K-AKT pathway is an important acquirement of cancer cells to escape cell death upon exposure to toxic stimuli. In esophageal squamous cell carcinoma, for example, phosphorylation of AKT was significantly higher among patients who received chemotherapy compared to the control group and this increase was associated with poor prognosis. Moreover, 9 out of 37 patients even showed a direct increase in P-AKT expression levels after chemotherapy which was analysed by immunostaining for phospho-AKT (Yoshioka et al. 2008). Furthermore, IHC staining revealed increased levels of P-AKT significantly more frequent in a radiation-resistant compared to a radiation-sensitive group in cervical cancer (Kim et al. 2006).

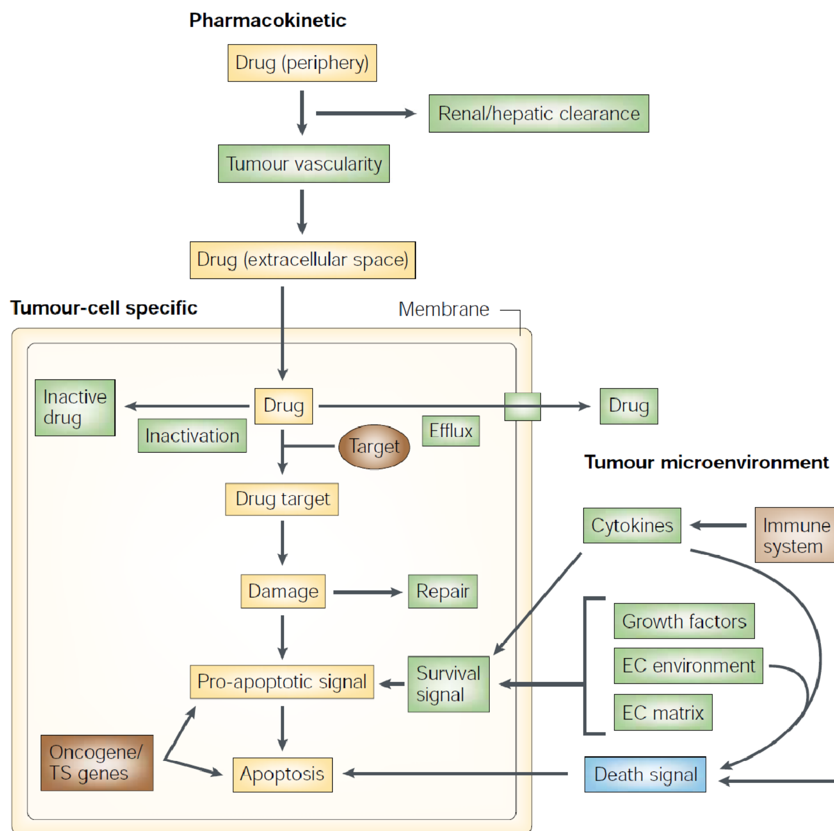


Fig. 5: Mechanism of drug resistance in cancer cells

Several mechanisms can be responsible for chemotherapeutic drug resistance or decreased cellular sensitivity. Besides the important role of the tumor microenvironment and the distribution and/or drug clearance (pharmacokinetic), which will not be a part of this thesis, the tumor-cell specific "response" after drug uptake is also of great importance. For example, cells can become less sensitive to drug treatment by decreasing drug uptake, by upregulation of ABC transporters, and by increasing drug efflux, by activation of the detoxifying system and/or by increased DNA repair or activation of pathways that promote cellular survival (Figure was taken from (Agarwal and Kaye 2003)).

1.6 Targeted therapies

The discovery of cellular oncogenes and first insights into the plethora of different signal-transduction pathways controlling cellular behaviour offers the possibility of developing rationally designed drugs. These are able to selectively target critical functional nodes in the oncogenic network, which play an essential role in tumor maintenance and/or progression. For this reason, the identification of the nodal points is

a crucial step for the generation of cancer drugs which are supposed to effectively kill tumor cells but sparing normal cells.

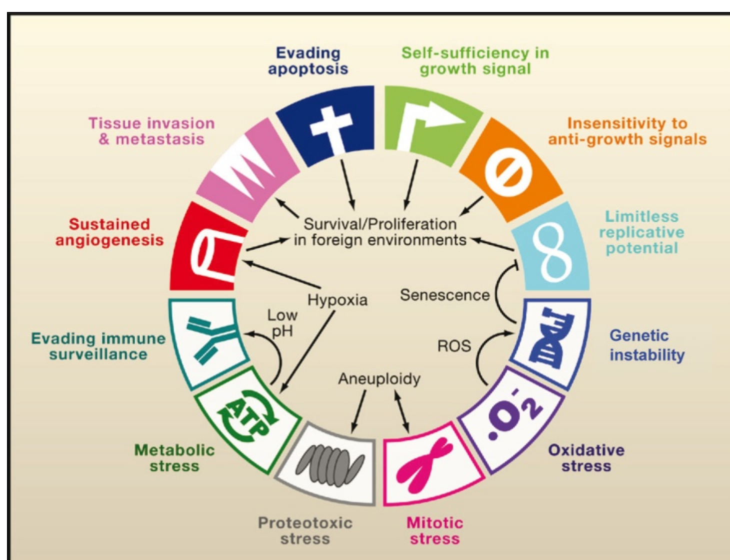


Fig. 6: The Hallmarks of Cancer
This figure includes the hallmarks of cancer originally proposed by Hanahan and Weinberg plus genetic instability, the evasion of immune surveillance postulated by Kroemer and Pouyssegur as well as a set of additional hallmarks such as metabolic stress, proteotoxic stress, mitotic stress, and oxidative stress proposed by Luo *et al.* (Hanahan and Weinberg 2000; Kroemer and Pouyssegur 2008; Luo et al. 2009). These hallmarks represent points of vantage to specifically target cancer cells while sparing non-tumorigenic cells (Figure was modified from (Luo et al. 2009)).

1.6.1 Small-molecule therapeutics

Imatinib (Gleevec) is the first FDA approved small-molecule therapeutic, a tyrosine kinase inhibitor (TKI) used in the treatment of chronic myelogenous leukaemia (CML) and advanced gastrointestinal stromal tumors (GIST) (Deininger et al. 2005). The efficacy of imatinib in treating chronic phase CML by inhibiting the kinase activity of the chimeric Bcr-Abl oncoprotein is an outstanding example of the potential of small-molecule therapeutics, but also demonstrates the strict oncogene addiction of some tumors. Besides imatinib, several TKIs targeting members of the EGFR family and other tyrosine kinases have been developed and approved by the FDA. Lapatinib (Tykerb), for example, which was designed as a dual kinase inhibitor that blocks the

kinase activity of the HER2 and the EGF receptor, has been approved in combination with capecitabine for the treatment of HER2 positive, advanced or metastatic breast cancer after treatment with an anthracycline, a taxane and trastuzumab. Erlotinib (Tarceva), a selective EGF receptor tyrosine kinase inhibitor, is clinically applied for the treatment of lung and pancreatic cancer patients, whereas gefitinib (Iressa), another TKI developed against the EGFR, is indicated as monotherapy of locally advanced or metastatic non-small cell lung cancer (NSCLC) after platinum-based and docetaxel chemotherapies having failed. Moreover, in 2009, gefitinib received marketing authorisation in Europe for the treatment of locally-advanced or metastatic NSCLC with activating mutations of the EGFR across all lines of therapy. In contrast to these single or dual kinase inhibitors, sunitinib (Sutent) and sorafenib (Nexavar) represent multi-kinase inhibitors targeting several kinases which results in an anti-angiogenic and anti-tumor activity.

1.6.2 Therapeutic monoclonal antibodies

Several monoclonal antibodies that target cell surface receptors have been developed and subjected to clinical trials. In contrast to TKIs, these antibodies can block ligand binding and/or receptor homo- or heterodimerisation, can influence receptor internalisation and degradation and/or induce antibody-dependent cellular cytotoxisity (Nahta and Esteva 2006). The use of monoclonal antibodies (mABs) for cancer therapy has first been made possible through the development of the hybridoma technique by Milstein and Köhler in 1975, which allowed the unlimited production of higly specific mABs *in vitro* (Kohler and Milstein 1975). The humanized monoclonal antibody trastuzumab (Herceptin) was the first oncogene-targeted therapy. In 1998, it was approved by the US Food and Drug Administration (FDA) for metastatic disease. Furthermore, trastuzumab obtained approval for adjuvant treatment of early breast cancer in combination with chemotherapy in 2006. This therapeutic antibody is indicated for the treatment of the approximately 20 – 30 % of breast tumors overexpressing the HER2 receptor. The development of trastuzumab was based on an important discovery by Denis Slamon, Axel Ullrich, and colleagues, who discovered that the *HER2* gene is amplified in about 30% of primary breast carcinoma tumors

(Slamon et al. 1987). Moreover, it was shown that *HER2* overexpression results in cellular transformation and tumorigenesis of NIH 3T3 cells (Hudziak et al. 1987). Shortly after, the murine antibody 4D5 directed against the extracellular domain of HER2 was developed and was shown to inhibit the growth of breast cancer cell lines overexpressing this receptor (Hudziak et al. 1989). Since then, a plethora of therapeutic mABs for the treatment of different cancers have been developed and approved by the FDA. Cetuximab (Erbitux), for example, is a chimeric monoclonal therapeutic antibody directed against the EGFR. It is indicated for the treatment of advanced squamous cell carcinoma of the head and neck either in combination with radiation therapy or as single agent after failure of platinum-based chemotherapy. In addition, cetuximab is indicated as single agent or in combination with the topoisomerase I inhibitor irinotecan (Camptosar) in EGFR-expressing metastatic colorectal cancer.

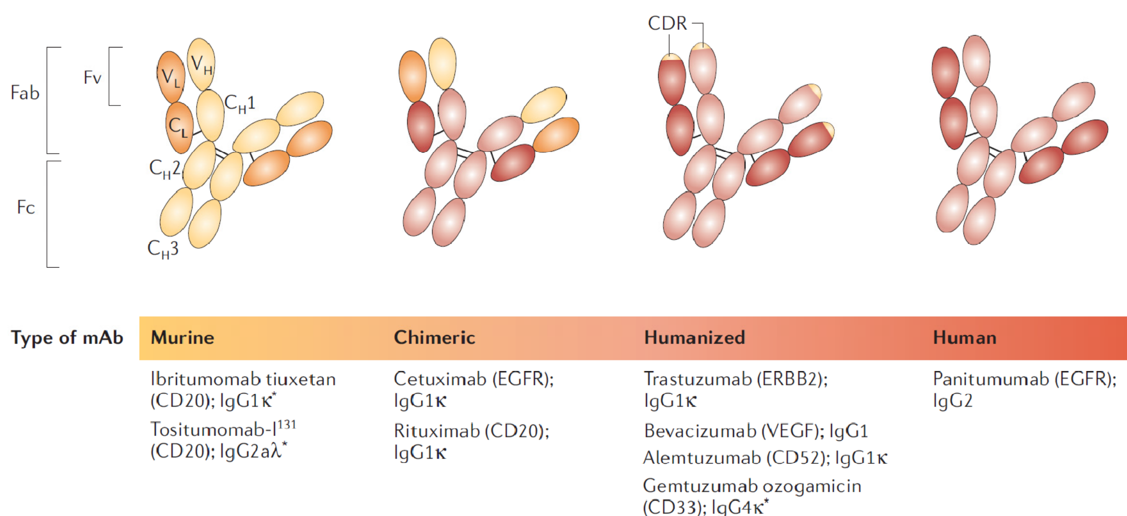


Fig. 7: Classification of therapeutic monoclonal antibodies.
Cetuximab and Rituximab are chimeric monoclonal antibodies that contain variable regions derived from murine source whereas the constant regions are human. In humanized mABs like trastuzumab only the complementarity-determining regions (CDRs), the part of the antibody that determines specificity, is of murine origin. In contrast, human monoclonal antibodies are completely derived from a human source. Most approved therapeutic monoclonal antibodies belong to the IgG1 subclass. The choice of IgG subclass is a key aspect as the different subclasses differ in half-life and immune-effector functions like complement-dependent cytotoxicity (CDC) and antibody-dependent cellular cytotoxicity (ADCC) (Figure was taken from (Imai and Takaoka 2006)).

1.7 Ovarian cancer

With 21.650 estimated new cases in the U.S. in 2008 and 9.660 cases in Germany in 2004, ovarian cancer ranks on position eight in the U.S. behind cancer of the breast, lung, colon, uterine corpus, non-hodgkin lymphoma, thyroid, and melanoma of the skin, as opposed to position five in Germany. It accounts for as many as 15.520 estimated deaths in the U.S. in 2008 and 5.479 deaths in Germany in 2004 (Jemal et al. 2008).

Approximately 80% to 90% of all ovarian cancers represent epithelial ovarian carcinomas while ovarian germ cell tumors account for only 5% and stromal tumors (tumors of the connective tissue) for the remaining 5% to 7%. The single cell layer that covers the ovary or lines cysts beneath the ovarian surface is supposed to be the origin of epithelial ovarian cancers (Feeley and Wells 2001). Most likely, the development of epithelial ovarian tumors can be explained by the existence of ovulatory cycles. In the course of these recurrent cycles, the epithelial surface ruptures and has to be repaired via proliferation or migration of surrounding epithelial cells. Therefore, each proliferative cycle potentially provides the possibility for the accumulation of the multiple genetic alterations which are necessary for the development of epithelial ovarian cancer.

Epithelial tumors of the ovary can further be subdivided into serous carcinomas, mucinous tumors, endometrioid carcinomas, clear cell carcinomas, Brenner tumors, and mixed epithelial tumors. The serous type accounts for approximately 75% of epithelial ovarian tumors, followed by the mucinous, the endometrioid, clear cell carcinomas, mixed epithelial tumors, Brenner, and tumors of undifferentiated histology.

While the majority of patients with ovarian cancer initially respond to first line treatment with a platinum-based compound and a taxane, most women develop a recurrent disease. These tumors are classified as either “sensitive” or “resistant” tumors depending on the time of the recurrence after the initial therapy. Disappointingly, there is no curative treatment for most of these patients so far and aims for a second-line treatment are “only” improvement of progression-free and overall survival as well as quality of life (Pfisterer et al. 2003). Moreover, approximately 20% to 30% of ovarian cancer patients never show any sign of clinical remission despite receiving first-line chemotherapy and are therefore classified as “refractory” tumors (Cannistra 2004).

Ovarian cancer reflects a heterogeneous disease and no predominant pathway is deregulated in most cancer patients. Nevertheless, activation of the PI3K-AKT pathway

either by amplification, mutation, deletion or epigenetic silencing of signalling components has been shown to drive oncogenesis and to promote resistance to cytotoxic chemotherapy in many cancer of the ovary (Shayesteh et al. 1999; Altomare et al. 2004; Levine et al. 2005; Huang and Hung 2009). A wide variation regarding overexpression of the EGFR can be observed in different studies published (Meden et al. 1995; Baekelandt et al. 1999). Nevertheless, one publication reports an association between patient outcome and expression of the EGFR (Psyrri et al. 2005). In the case of HER2, only 11% out of 837 recurrent ovarian or primary peritoneal cancers were classified as 2+ or 3+ positive by IHC analysis (Bookman et al. 2003). In contrast, a strong expression of the HER3 receptor could be observed in 53% out of 116 patients with a predominant cytoplasmic and only weak membrane staining pattern whereas another study reported a strong membrane expression of HER3 in 16 out of 98 tumors (Rajkumar et al. 1996; Tanner et al. 2006).

1.8 Single Nucleotide Polymorphisms (SNPs)

A Single Nucleotide Polymorphism represents a small genetic variation occurring in the DNA sequence of different persons with a frequency of more than one percent in respect to the entire human population. Most human SNPs are found outside of the coding sequence whereas SNPs within the coding sequence of a specific protein may result in the functional alterations of the protein and might therefore predispose its carriers to disease. Furthermore, these SNPs might eventually alter treatment response when carriers of the polymorphism are compared with wild-type carriers. In this regard, a SNP within the gene coding for the fibroblast growth factor receptor 4 (FGFR4) and the resulting conversion of the corresponding codon from glycine to arginine (G388R) has been shown to be associated with reduced disease free survival but not with tumor initiation in breast cancer patients (Bange et al. 2002; Thussbas et al. 2006). Moreover, high expression of FGFR4 in connection with the R388 allele was associated with poor clinical outcome in head and neck squamous cell carcinoma (Streit et al. 2004). Furthermore, the FGFR4 R388 allele was associated with tumor progression as well as tumor initiation in prostate cancer (Wang et al. 2004). Recently, a publication by Seitzer and colleagues highlighted the impact of the corresponding R385 allele in a WAP-

TGFα mouse mammary carcinoma model on mammary tumor progression whereas no effect could be observed in a MMTV-PymT mouse model emphasizing the importance of the oncogenic background (Seitzer et al. 2010). Another human SNP was identified in the coding region of the transmembrane domain of the HER2 receptor by Papewalis and colleagues in 1991 (Papewalis et al. 1991). Since then, several papers with different or even contradictory results have been published about the HER2 codon 655 polymorphism and its association with breast cancer risk.

2. Aims of the study

The anti-apoptotic PI3K-AKT signalling pathway has emerged as an important player promoting chemotherapeutic drug resistance. Moreover, HER3 mediated activation of the PI3K-AKT pathway has recently been shown to promote cellular survival in the presence of tyrosine kinase inhibitors such as gefitinib (Engelman et al. 2007; Sergina et al. 2007). Reasoned in the common occurrence of chemoresistance, a reportedly high expression of HER3, and the frequent activation of the PI3K-AKT pathway, ovarian cancer represents an excellent model to study cellular escape mechanisms upon chemotherapeutic treatment.

In the first part of this study, we aimed to determine the involvement of the HER3-PI3K-AKT pathway in doxorubicin resistance. We intended to analyse if the abrogation of this important signalling pathway increases doxorubicin induced apoptosis, and might reverse chemotherapeutic drug resistance. The relevance of this signalling mechanism is to be investigated in different cell lines, which have been established from previously untreated or pre-treated ovarian cancer patients.

In the second part of this thesis, we planned to analyse the potential role of a recently discovered genetic alteration of the HER3 receptor. In this regard, alterations in down-stream signaling events as well as in proliferation and apoptosis are to be analysed.

3. Material and Methods

3.1 Materials

3.1.1 Laboratory Chemicals

Acrylamide	Serva, Heidelberg
Agarose	BRL, Eggenstein
Aprotinin	Sigma, Taufkirchen
APS (Ammonium peroxodisulfate)	Bio-Rad, München
Bisacrylamid	Roth, Karlsruhe
BSA (Bovine serum albumin)	Sigma, Taufkirchen
Coomassie G250	Serva, Heidelberg
Crystal violet	Sigma, Taufkirchen
Deoxynucleotides (dG/A/T/CTP)	Roche, Mannheim
DTT (Dithiothreitol)	Sigma, Taufkirchen
Ethidium bromide	Sigma, Taufkirchen
Formaldehyde	PolySciences, Eppenstein
HEPES (N-(2-Hydroxyethyl)piperazine-N-(2-ethanesulfonic acid))	Serva, Heidelberg
Iodoacetamide	Sigma, Taufkirchen
L-Glutamine (GibCo)	Invitrogen, Eggenstein
Lipofectamine RNAiMAX	Invitrogen, Eggenstein
Lysozyme	Sigma, Taufkirchen
Oligofectamine	Invitrogen, Eggenstein
Penicillin/Streptomycin	Gibco, Eggenstein
PMSF (Phenylmethanesulfonyl fluoride)	Sigma, Taufkirchen
Ponceau S	Sigma, Taufkirchen
Propidium Iodide	Sigma, Taufkirchen
SDS (Sodium dodecyl sulfate)	Roth, Karlsruhe
Sodium azide	Serva, Heidelberg
Sodium fluoride	Sigma, Taufkirchen
Sodium orthovanadate	Sigma, Taufkirchen
TEMED (N,N,N',N'-Tetramethylethylenediamine)	Serva, Heidelberg
Thiazolyl Blue Tetrazolium Bromide (MTT)	Sigma, Taufkirchen
Triton X-100	Serva, Heidelberg
Tween 20	Sigma, Taufkirchen
Xylol	Merck, Darmstadt

All other chemicals were purchased in analytical grade from Merck (Darmstadt).

3.1.2 Enzymes

AffinitScript Reverse Transcriptase	Stratagene, USA
AMV Reverse Transcriptase	Roche, Mannheim
REDTaq ReadyMix	Sigma, Taufkirchen
Trypsin	Invitrogen, Eggenstein

3.1.3 “Kits“ and other materials

Caspase-Glo 3/7 Assay	Promega, USA
Cell culture materials	Greiner, Solingen Nunclon, Dänemark Falcon, UK Corning, USA
Cellulose nitrate 0.45 µm	Schleicher & Schüll, Dassel
Enhanced Chemi Luminscent (ECL) Kit	PerkinElmer/NEN, Köln
Hyperfilm MP	Amersham Pharmacia, Freiburg
Micro BCA Protein Assay Kit	Pierce, Sankt Augustin
Parafilm	Dynatech, Denkendorf
Protein A-Sepharose	Amersham Pharmacia, Freiburg
Protein G-Sepharose	Amersham Pharmacia, Freiburg
QIAquick Gel Extraction Kit (50)	Qiagen, Hilden
QIAquick PCR Purification Kit (50)	Qiagen, Hilden
QIAGEN RNeasy Mini Kit	Qiagen, Hilden
Sterile filter 0.22 µm, cellulose acetate	Nalge Company, USA
Sterile filter 0.45 µm, cellulose acetate	Nalge Company, USA
Whatman 3MM	Whatman, Rotenburg/Fulda

3.1.4. Chemotherapeutic drugs

Dacarbazine	Sigma, Taufkirchen
Doxorubicin	Sigma, Taufkirchen
Cisplatin	Max-Planck Apotheke, Martinsried

Cyclophosphamide	Sigma, Taufkirchen
Etoposide	Calbiochem, UK
Fluorouracil	Sigma, Taufkirchen

3.1.5 Small molecule inhibitors

Batimastat (BB94)	British Biotech, Oxford, UK
Erlotinib	Vichem Chemie, Hungary
Lapatinib	Vichem Chemie, Hungary
Wortmannin	Sigma, Taufkichen
LY294002	Cell Signaling, MA
UO126	Promega, USA

3.1.6 Growth factors and ligands

Heregulin-α	R&D Systems, Wiesbaden
Heregulin-β1	R&D Systems, Wiesbaden
Heregulin-β2	Biomol, Hamburg

3.2 Cell culture media

Cell culture media and additives were obtained from Invitrogen (Eggenstein). Media were supplemented to the requirements of each cell line.

- Dulbecco's Modified Eagle Media (DMEM) with 4.5 mg/ml Glucose, 10% FBS, 2 mM L-Glutamine, 1mM sodiumpyruvate

- Minimum essential media (MEM), 10% FBS, 2 mM L-Glutamine, 0,1 mM non essential amino acids (NEAA)

- RPMI 1640, 10% FBS, 2mM L-Glutamine

- RPMI 1640, 15% FBS, 2mM L-Glutamine

- McCoy's 5a, 10% FBS, 2mM L-Glutamine

- Freeze media: 90% heat-inactivated FBS, 10% DMSO

3.3 Stock solutions and commonly used buffers

Acrylamide solution (30/0,8%)	30% (w/v) Acrylamid 0.8% (w/v) Bisacrylamid
Ammoniumbicarbonate buffer (ABC)	50 mM NH_4HCO_3
DNA loading buffer (6x)	0.05% Bromphenol blue 0.05% Xylencyanol 30% Glycerol 100 mM EDTA pH 8.0
HNTG	20 mM HEPES, pH 7.5 150 mM NaCl 0.1% TritonX-100 10% Glycerol 10 mM $Na_4P_2O_7$
Laemmli buffer (3x)	100 mM Tris/HCl pH 6.8 3% SDS 45% Glycerol 0.01% Bromphenol blue 7.5% ß-Mercaptoethanol
NET	50 mM Tris/HCl pH 7.4 5 mM EDTA 0.05% Triton X-100 150 mM NaCl
PBS	137 mM NaCl 27 mM KCl 80 mM Na_2HPO_4 1.5 mM KH_2PO_4 pH 7.4
Propidium-Iodide (PI) buffer	0.1% Na-Citrate 0.1% Triton 20 µM Propidium Iodide

Stage-Tip sample buffer	1% TFA 5% CAN
Stage-Tip buffer A	0.5% acetic acid 0.1% TFA
Stage-Tip buffer B	0.5% acetic acid 80% ACN
SD-Transblot	50 mM Tris/HCl pH 7.5 40 mM Glycine 20% Methanol 0.004% SDS
“Strip” buffer	62.5 mM Tris/HCl pH 6.8 2% SDS 100 mM β-Mercaptoethanol
Stop-Solution (MTT)	9.5% SDS 5% 2-Butanol 0.012 M HCL
TAE	40 mM Tris/Acetate pH 8.0, 1 mM EDTA
TE10/0.1	10 mM Tris/HCl pH 8.0 0.1 mM EDTA pH 8.0
Tris-Glycine-SDS	25 mM Tris/HCl pH 7.5 200 mM Glycine 0.1% SDS
Triton X-100 lysis buffer	50 mM HEPES, pH 7.5 150 mM NaCl 1 mM EDTA 10% Glycerin 1% Triton X-100 10 mM $Na_4P_2O_7$ 2 mM Na_3VO_4 10 mM NaF 1 mM PMSF 100 µg/l Aprotinin

3.4 Eukaryotic cell lines

Cell line	Description origin	Reference
OVCAR3	human ovary adenocarcinoma (ascites)	(Hamilton et al. 1983) (Louie et al. 1986)
OVCAR4	human ovary adenocarcinoma	(Louie et al. 1986) (Schilder et al. 1990)
OVCAR5	human ovary adenocarcinoma	(Louie et al. 1986) (Schilder et al. 1990)
OVCAR8	human ovary adenocarcinoma	(Schilder et al. 1990)
SKOV3	human ovary adenocarcinoma (ascites)	(Fogh et al. 1977)
SKOV6	human ovary adenocarcinoma	(Shih et al. 1994)
SKOV8	human ovary adenocarcinoma (ascites)	(Provencher et al. 1993)
2774	ovarian carcinoma of endometrioid type (ascites)	(Freedman et al. 1978)
A2780	human ovary adenocarcinoma	(Louie et al. 1986) (Schilder et al. 1990)
AsPC-1	human pancreatic adenocarcinoma	ATCC
PaTu8902	human pancreatic grade II adenocarcinoma	(Elsasser et al. 1993)
PaTu8988t	human pancreatic adenocarcinoma metastatic to liver	(Elsasser et al. 1992)
PaTu8988s	human pancreatic adenocarcinoma metastatic to liver	(Elsasser et al. 1992)
Capan-1	human pancreas adenocarcinoma metastatic to liver	ATCC
Capan-2	human pancreas adenocarcinoma	ATCC

Colo357	human pancreatic adenocarcinoma metastatic to lymph nodes	(Morgan et al. 1980)
CFPAC	human pancreatic adenocarcinoma metastatic to liver	(McIntosh et al. 1988)
PancTU	human pancreatic adenocarcinoma	(Kalthoff et al. 1993)
SW850	potential cervix carcinoma	(Moore et al. 2001)

3.5 Antibodies

3.5.1 Primary antibodies

Antibody	**Immunogen origin**	**Reference**
p-Akt/PKB	Rabbit, polyclonal; phospho-Akt (Ser-473); recognizes p-Akt of human, rabbit and rat origin	NEB, Frankurt/M.
EGFR	Sheep, polyclonal; part of cytoplasmic domain of the human EGF	UBI, Lake Placid
p-EGFR	(Y-1173) Rabbit, monoclonal; recognizes endogenous EGFR phosphorylated at Y1173	Cell Signaling, MA
HER2	Rabbit, polyclonal; peptide between AA 1243-1255 of human HER-2	Millipore, Temecula
p-HER2	(Y1248) Rabbit, monoclonal; synthetic phospho-peptide between AA 1243-155	Millipore, Temecula
HER3 (2F12)	Mouse, monoclonal; peptide between AA 1295-1323 of human HER3	Millipore, Temecula
HER3 (1B4C3)	Mouse, monoclonal; directed against the extracellular domain of human HER3	E. van der Horst
HER3 (C-17)	Rabbit, polyclonal; directed against a c-terminal peptide of human HER3	Santa Cruz, CA

HER3 (H3.105.5)	Extracellular domain of recombinant human HER3	Millipore, Temecula
P-HER3 (Y1289)	Rabbit, monoclonal; recognizes phospho-tyrosine 1289 of human HER3	Cell Signaling, Temecula
HER4	Rabbit, monoclonal, recognizes residues near the c-terminus of human HER4	Cell Signaling, MA
HER4 (sc-283)	Rabbit, polyclonal, raised against a peptide corresponding to AA 1291-1308 of human HER4	Santa Cruz, CA
PI3K p85	Rabbit polyclonal; recognizes the SH2-domain of human p85	UBI, Lake Placid
Tubulin	Mouse, monoclonal; ascites	Sigma, Taufkirchen
P-Tyr (4G10)	Mouse, monoclonal; recognizes phospho-tyrosine residues	UBI, Lake Placid
VSV (P5D4) (Isotype control)	Mouse, monoclonal; recognises an epitope of eleven amino acids derived from the vesicular stomatitis virus glycoprotein VSV-G	Roche, Mannheim

3.5.2 Secondary antibodies

For immunoblot analysis corresponding secondary antibodies conjugated with horseradish peroxidase (HRP) were used.

Antibody	**Dilution**	**Origin**
Goat anti-mouse-HRP	1:10 000	Sigma, Taufkirchen
Goat anti-rabbit-HRP	1:50 000	Bio-Rad, München
Goat anti-sheep-HRP	1:25 000	Jackson ImmunoResearch Labs, USA
Goat anti-mouse Fab fragment	1:25 000	Amersham Biosciences, UK

Goat anti-rabbit Fab fragment	1:25 000	Amersham Biosciences, UK

3.5.3 Therapeutic monoclonal antibodies

Trastuzumab (Herceptin)	Max-Planck Apotheke, Martinsried
Cetuximab (Erbitux)	Max-Planck Apotheke, Martinsried

3.6. Oligonucleotides

3.6.1 siRNAs

HER2 sense 5’ GGGAAACCUGGAACUCACCtt 3’
HER2 antisense 5’ GGUGAGUUCCAGGUUUCCCtg 3’

HER3 sense_1 5’ GGCUAUGUCCUCGUGGCCAtt 3’
HER3 antisense_1 5’ UGGCCACGAGGACAUAGCCtg 3’

HER3 sense_2 5’ GGCAGUGUGUCCUGGGACUtt 3’
HER3 antisense_2 5’ AGUCCCAGGACACACUGCCtg 3’

HER3 sense_3 5´ GGUCUACGAUGGGAAGUUUtt 3’
HER3 antisense_3 5´ AAACUUCCCAUCGUAGACCtg 3’

HER3 sense_4 5’ GAAUGAAUUCUCUACUCUAtt 3’
HER3 antisense_4 5’ UAGAGUAGAGAAUUCAUUCat 3’

HER4 sense 5´ GGAAGAGCAUCAAAAAGAAtt
HER4 antisense 5´ UUCUUUUUGAUGCUCUUCCtt

GL-2 sense 5’ CGUACGCGGAAUACUUCGAtt 3’
GL-2 antisense 5’ UCGAAGUAUUCCGCGUACGtt 3’

ADAM17 sense_1 5’ AGUUUGCUUGGCACACCUUtt 3’
ADAM17 antisense_1 5’ AAGGUGUGCCAAGCAAACUtt 3’

ADAM17 sense_2 5' AGUAAGGCCCAGGAGUGUUtt 3'
ADAM17 antisense_2 5' AACACUCCUGGGCCUUACUtt 3'

ADAM17 sense_3 5' AGCCCUGUACAGUAGGAUUtt 3'
ADAM17 antisense_3 5' AAUCCUACUGUACAGGGCUtt 3'

3.6.2 RT-PCR-Primers

NRG1 Fwd 5' GGCAAGAAGAAGGAGCGAGG 3'

NRG1α Rvse 5' GAATCCAGGTTGGCACTTGC 3'
NRG1β1 Rvse 5' CCGCCTCCATAAATTCAATCC 3'
NRG1β2 Rvse 5' TCCTCCGCCTTGTAGAAGC 3'

EGFR Fwd 5' GTGGGGCCGACAGCTATGAGATGG 3'
EGFR Rvse 5' TGCTTGGTCCTGCCGCGTATGAT 3'

HER2 Fwd 5' CACATGACCCCAGCCCTCTACAGC 3'
HER2 Rvse 5' CACGGCACCCCCAAAGGCAAAAAC 3'

HER3 Fwd 5' CTCCGCCCTCAGCCTACCAGTT 3'
HER3 Rvse 5' TGCTCCGGCTTCTACACATTGACA 5'

HER4 Fwd 5' CAGTGTGAGAAGATGGAAGAT 3'
HER4 Rvse 5' CTTTTTGATGATCTTCCTTCTAAC 3'

MDR1 Fwd 5' GCAAAGCTGGAGAGATCCTCACCA 3'
MDR1 Rvse 5' CAACATTTTCATTTCAACAACTCCTGC 3'

MRP1 Fwd 5' AATGCGCCAAGACTAGGAAG 3'
MRP1 Rvse 5' ACCGGAGGATGTTGAACAAG 3'

BCRP1 Fwd 5' CCAGTTCCATGGCACTGGCCATA 3'
BCRP1 Rvse 5' CAGGGCCACATGATTCTTCCACA 3'

GAPDH Fwd 5' ACCACAGTCCATGCCATCAC 3'
GAPDH Rvse 5' TCCACCACCCTGTTGCTGTA 5'

Tubulin Fwd 5' AAGTGACAAGACCATTGGGGGAGG 3'
Tubulin Rvse 5' GGGCATAGTTATTGGCAGCATC 3'

3.7 Methods of mammalian cell culture

3.7.1 General cell culture techniques

Cell lines were grown in a humidified 93% air, 7% CO2 incubator at 37°C and routinely assayed for mycoplasma contamination. Before seeding, cells were counted with a Coulter Counter (Coulter Electronics) and corresponding cell number was calculated.

3.7.2 Cell culture in SILAC media

For the mass spectrometry (MS)-based study, OVCAR3 cells were grown for at least six cell doublings in media containing either 45 mg/l unlabeled L-arginine and 76 mg/l unlabeled L-lysine (Arg^0, Lys^0) or equimolar amounts of L-[U-$^{13}C_6$,$^{14}N_4$]arginine and L-[$^2H_4$]lysine (Arg^6, Lys^4), or L-[U-$^{13}C_6$,$^{15}N_4$] and L-[U-$^{13}C_6$,$^{15}N_2$]lysine (Arg^{10}, Lys^8) (Cambridge Isotope Laboratories or Sigma-Isotec) as well as dialyzed FBS (Gibco). The media was supplemented with penicillin and streptomycin (Invitrogen).

3.7.3 RNA interference

Cells were seeded 24 h prior to siRNA transfection in 6 cm, 6 well or 12 well plates at densities of 2 x 10^5 cells/plate, 7 x 10^4 cell/well or 2 x 10^4 cells/well, respectively. Transfection of 21-nucleotide siRNA duplexes (Ambion) was carried out using OligofectAMINE (Invitrogen) or Lipofectamine RNAiMAX (Invitrogen) and OPTI-MEM media (GIBCO) without FBS. After four hours media was changed to normal media containing FBS, and after additional 24 h cells were used for further experiments.
FACS analysis (sub-G1 content): Cells were then incubated with indicated amount of doxorubicin for 72 h.
Western blot analysis: Cells were then treated with indicated concentrations of doxorubicin for 24 h and 48 h before lysis.
Caspase 3/7-Glo assay: Cells were subsequently trypsinised and seeded in 96 well plates as described in 3.8.11.

3.7.4 Treatment of cells for western blot analysis

Cells were seeded in 6 cm plates at a density of 2 x 10^5 cells/plate. After 24 h media was changed to fresh media and cells were subsequently treated with indicated concentrations of doxorubicin or other chemotherapeutic drugs, TKI inhibitors or corresponding amount of DMSO. Cells which have been stimulated with NRG1 ligand isoforms were put on 0% FBS for 24 h and were then stimulated for the indicated time with 50 ng/ml of the corresponding ligand before cellular lysis.

3.8 Methods of Biochemistry and Cell Biology

3.8.1 Lysis of cells with Triton X-100 lysis buffer

Cells were washed with PBS and subsequently lysed for 10 minutes on ice with lysis buffer containing 50 mM HEPES pH 7.5, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 10% glycerol, 10 mM sodium pyrophosphate, 2mM sodium orthovanadate, 10mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride, and 10 µg/ml aprotinin. Lysates were then pre-cleared by centrifugation at 13000 rpm for 10 minutes at 4°C and finally stored at -20°C or directly subjected to western blot analysis.

3.8.2 Determination of total protein concentration in cell lysates

The overall protein concentration was determined using the Micro-BCA Protein Assay Kit (Pierce, Sankt Augustin) according to the supplied standard protocol.

3.8.3 Immunoprecipitation of proteins

Cell lysates which have been adjusted to an equal protein concentration were pre-cleared with 20 µl of protein A- or G-Sepharose for one hour. In the mean time, respective antibodies were pre-coupled to 40 µl Sepharose beads in lysis buffer for one hour and washed twice with lysis buffer. After combining, pre-cleared lysates and pre-coupled antibody-beads were incubated at 4°C for four hours and the precipitates were then washed three times with 700 µl lysis buffer, suspended in 3x Laemmli buffer, boiled for 10 minutes, and directly subjected to western blot analysis.

3.8.4 Cell lysis with NP-40 and anti-HER3 immunoprecipitation

SILAC-encoded OVCAR3 cells were washed once with ice-cold PBS and were subsequently lysed in buffer containing 50 mM Tris pH 7.5, 150 mM NaCl, 1% NP40, 0.1% sodium deoxycholate, 1 mM EDTA, 1 mM sodium orthovanadate, 1 mM PMSF, 0.1 µg/ml aprotinin and 10 mM NaF. Lysates were pre-cleared by centrifugation at 13000 rpm for 10 min. For each anti-HER3 immunoprecipitation reaction 10 µg anti-HER3 antibody was coupled to 40 µl protein A-Sepharose beads by pre-incubating for one hour in NP-40 lysis buffer. After washing 2x with lysis buffer, the anti-HER3 containing beads were added to the pre-cleared (1 h; 40 µl protein A-Sepharose beads) cell lysates (9.18 mg total protein amount) and incubated for four hours. Precipitates were subsequently washed 4x with lysis buffer and precipitated proteins were eluted by incubating 10 minutes with 80 µl 0.5% LDS buffer (Invitrogen) containing 50 mM DTT at 70°C. Elution fractions were pooled and concentrated by a factor of three in a vacuum concentrator (Eppendorf).

3.8.5 SDS-polyacrylamide-gelelectrophoresis (SDS-PAGE)

SDS-PAGE was conducted as described previously (Sambrook et al. 1990). The following proteins were used as molecular weight standards:

Protein	MW (kD)	Protein	MW (kD)
Myosin	205	Ovalbumin	42.7
ß-Galactosidase	116.25	Carboanhydrase	29
Phosphorylase b	97.4	Trypsin-Inhibitor	21.5
BSA	66.2	Lysozym	14.4

3.8.6 Western blotting

For western blot analysis total proteins were transferred to nitrocellulose membranes (Schleicher & Schuell) for three hours at 0.8 mA/cm^2 using a "Semidry”-Blot device in the presence of Transblot-SD buffer (Gershoni and Palade 1982). Following transfer, proteins were stained with Ponceau S (2 g/l in 2% TCA) in order to visualize marker protein bands. The membrane was then de-stained in water, blocked in NET-gelatin for several hours and incubated at 4°C overnight with the corresponding primary antibody diluted in NET-gelatin. The next day, membranes were washed three times with NET-gelatin and incubated with horseradish peroxidase-conjugated anti-rabbit secondary antibody diluted in NET-gelatin for one hour at room temperature. After additional

washing (three times), detection was performed by using enhanced chemiluminescence (ECL; Western Lightning, Perkin Elmer) on X-ray films.

3.8.7 In-gel protein digestion for MS-based study

Immunoprecipitates were separated by SDS-PAGE, using NuPAGE Novex Bis-Tris gels (Invitrogen) according to the manufacturer's instructions. The colloidal Blue Staining Kit (Invitrogen) was used to stain the gel with Coomassie blue. The protein-containing lanes were cut into 8 slices. In-gel digestion was performed as described (Shevchenko et al. 2006). Gel slices were cut into small pieces and washed with 50 mM ammonium bicarbonate (ABC) / 50% ethanol until cubes were fully destained. Gel pieces were dehydrated with ethanol and rehydrated with 50 mM ABC containing 10 mM DTT. Thiol groups on proteins were reduced for one hour at 56°C. The reduced thiol groups were then alkylated by adding 55 mM IAA in 50 mM ABC for one hour at 25°C in the dark. Gel pieces were again washed twice with a 50 mM ABC / 50% ethanol solution, dehydrated with 100% ethanol and dried in a concentrator 5301 (Eppendorf). Each gel fraction was re-hydrated in 50 mM ABC solution containing trypsin (1:100 wt/wt) and samples were digested at 37°C over night. Supernatants were collected and residual peptides were extracted by incubating twice with 30% acetonitrile (MeCN) in 3% TFA followed by two incubations with 100% MeCN. All extracts of a respective fraction were combined and the samples were concentrated in a concentrator 5301 (Eppendorf) to remove all MeCN. Then, samples were desalted and enriched using in-house made C_{18} STAGE Tip columns (Shevchenko et al. 2006; Rappsilber et al. 2007). Eluted peptides were concentrator 5301 (Eppendorf) to a final volume of 3-4 µl. For MS-analysis samples where then mixed 1:1 with 1% TFA and 5% MeCN.

3.8.8 RNA isolation and RT-PCR analysis

Total RNA was isolated using the RNeasy Mini-Kit (Qiagen) and reverse transcribed using AMV Reverse Transcriptase (Roche). 0.5-5 µg of RNA and 1 µl of random primer in a volume of 10 µl were incubated for 2 minutes at 68°C, followed by 10 minutes incubation at room temperature. After addition of 1 µl RNase inhibitor, 4 µl of 5x AMV RT buffer, 4 µl dNTPs (2.5 mM each) and 1 µl AMV RT the volume was adjusted to 20 µl. The reaction mix was incubated at 42°C for one hour and cDNA was purified using the Qiagen PCR purification kit (Qiagen) afterwards. REDTaq ReadyMix (Sigma-Aldrich) was used for PCR amplification reactions according to the manufacturer's recommendations. PCR products were subjected to electrophoresis on 1-2% agarose gels and DNA was visualized by ethidium bromide staining.

3.8.9 Cell proliferation assay

Cultured cells were seeded into 96-well plates at a density of 1000 cells/well in 4% FBS. The next day cells were treated with 10 µg/ml blocking antibody for one hour before addition of ligands at a concentration of 50 ng/ml. Cellular metabolism was assessed after 72 h of cultivation using the 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. Briefly, 20 µl of MTT solution (5 mg/ml in PBS) was added to the cells. After four hours at 37°C the formazan crystals formed were solubilized by addition of 50 µl stop solution (10% SDS, 5% butanol and 0.01 M HCl). Absorbance for each well was read at 570 nm using a microplate reader.

3.8.10 Flow cytometry (PI-assay)

Cells were seeded at a density of 2×10^4 or 3×10^4 cells/well into 12-well plates. The next day, media was changed and cells were treated with inhibitors, doxorubicin or corresponding amount of DMSO. After 72 h of cultivation in the presence of inhibitor and FBS, the supernatant (media) was collected and combined with trypsinised cells, centrifuged, and incubated with 0.01% Triton, 0.1% sodium citrate and 0.02 mM propidium iodide (Sigma) in the dark at 4°C. After two hours, cells were analysed by flow cytometry (FACS-Calibur, BD Bioscience) using the CellQuest Pro Software. The sub-G1 population was counted as the apoptotic population and represented as fraction of the total cells counted.

3.8.11 Flow cytometry (doxorubicin accumulation)

Cells were seeded at a density of 7×10^4 cells in 6 cm plates. The next day, media was changed and cells were treated with inhibitors and doxorubicin at indicated concentrations or with corresponding amount of DMSO control. After 24 h, cells were processed as described in 3.8.10 with the difference that buffer (0.01% Triton, 0.1% sodium citrate) without propidium iodide was used. Cells were subsequently analysed by flow cytometry (FACS-Calibur, BD Bioscience) using the CellQuest Pro Software. Based on the fluorescence of doxorubicin (λ_{ex} 470 nm; λ_{em} 585 nm in ethanol) its accumulation can be measured by using the FL2 FACS channel.

3.8.12 Caspase 3/7-Glo assay

Cells were seeded at a density of 2000 - 3000 cells/ well into 96 well plates in a volume of 50 µl. The next day cells were treated with inhibitors, doxorubicin or DMSO control. After additional 24 h, 50 µl of Caspase 3/7-Glo assay buffer was added and incubated

for one hour in the dark at room temperature. 50 µl was then transferred to a 96 white microwell plate and subsequently measured in a microplate luminometer (LB96V, EG&G Berthold). The Caspase 3/7-Glo assay represents a luminescent assay that measures activities of executioner caspases-3 and -7. The luminescence signal is proportional to the amount to the caspase activity present.

3.8.13 Statistical analysis

The t-test was used to compare data between two groups. $P < 0.05$ can be considered as statistically significant.

3.8.14 MS analysis on the LTQ-Orbitrap

MS analyses were done as described previously (Olsen et al. 2006; Daub et al. 2008). Briefly, peptide separations were done on 15 cm analytical columns (75 µm inner diameter) in-house packed with 3 µm C_{18} beads (Reprosil-AQ Pur, Dr. Maisch) using a nanoflow HPLC system (Agilent Technologies 1100), which was coupled online to a LTQ-Orbitrap mass spectrometer (Thermo Fisher Scientific) via a nanoelectrospray ion source (Proxeon Biosystems). The LTQ-Orbitrap was operated in the data-dependent mode to automatically switch between full scan MS in the orbitrap analyzer (with resolution R=60,000 at m/z 400) and the fragmentation of the five most intense peptide ions by either MS/MS or multi-stage activation in the LTQ part of the instrument, the latter being triggered on neutral loss species at 97.97, 48.99, or 32.66 m/z below the precursor ion for 30 ms (Schroeder et al. 2004). For all measurements with the orbitrap detector, a lock-mass strategy was used for internal calibration as described (Olsen et al. 2005).

3.8.15 Peptide identification and quantification

MaxQuant (http://www.maxquant.org/) was utilized for peptide identification and quantification. All MS raw files from the respective experiment were collectively processed with the MaxQuant software suite. MaxQuant performs peak list generation, SILAC-based quantification, estimation of false discovery rates, peptide to protein group assembly, and data filtration and presentation as described (Cox and Mann 2008). Data was searched against a concatenated forward and reversed version of the human International Protein Index (IPI) database version 3.37 containing 69141 protein entries and 175 frequently detected contaminants (such as porcine trypsin, human keratins and

Lys-C) using the Mascot search engine (Matrix Science; version 2.2.04). Cysteine carbamidomethylation was set as a fixed modification and methionine oxidation, protein *N*-acetylation, loss of ammonia from N-terminal glutamine as well as phosphorylation of serine, threonine and tyrosine residues were allowed as variable modifications. Spectra resulting from isotopically labeled peptides, as revealed by presearch MaxQuant analysis of SILAC partners, were searched with the fixed modifications Arg^6 and Lys^4 or Arg^{10} and Lys^8, respectively, whereas spectra for which a SILAC state could not be assigned before database searching were searched with Arg^6, Arg^{10}, Lys^4 and Lys^8 as variable modifications. The accepted mass tolerance was set to 5 p.p.m for precursor ions and to 0.5 Da for fragment ions. The minimum required peptide length was 6 amino acids and up to three missed cleavage sites and three isotopically labeled amino acids were permitted. The accepted FDR was 1% for both protein and peptide identifications, and the cut-off for the posterior error probability (PEP) of peptides was set to 10%.

4. Results

4.1 HER3/ErbB3 in ovarian cancer cells and its role in doxorubicin (chemo) sensitivity

4.1.1. Doxorubicin induces phosphorylation of AKT in four out of nine ovarian cancer cell lines

Although most of ovarian cancer patients initially respond well to the standard chemotherapeutic regimens (platinum-based compound and a taxane), the majority of them sooner or later develop a recurrent disease. Platinum “sensitive” tumors classified by recurrence diagnosed at least 6 months after initial treatment are normally treated with the same drugs again. In contrast, platinum “resistant” tumors characterised by recurrence within 6 months after initial therapy are treated with either Doxil (liposomal doxorubicin) Topotecan or Gemzar (gemcitabine) as second line therapy. Finally, ovarian tumors that never respond to first line therapy are classified as platinum “refractory”. In these cases, chances of a response to other chemotherapeutic drugs are generally low.

However, activation and/or overexpression of the PI3K-AKT pathway can be frequently observed in ovarian cancers and has been shown to be involved in chemoresistance (Bellacosa et al. 1995; Shayesteh et al. 1999; Woenckhaus et al. 2007; Fraser et al. 2008). Based on these informations, nine ovarian cancer cell lines were treated with different concentrations of doxorubicin and the phosphorylation of AKT was investigated by western blot using a phospho-serine specific anti AKT antibody. Notably, four out of nine tested ovarian cancer cell lines showed a clear increase in AKT phosphorylation upon addition of doxorubicin (OVCAR3, OVCAR4, SKOV6 and SKOV8) while there was no increase detectable in the remaining five cell lines (OVCAR5, OVCAR8, SKOV3, 2774 and 2780).

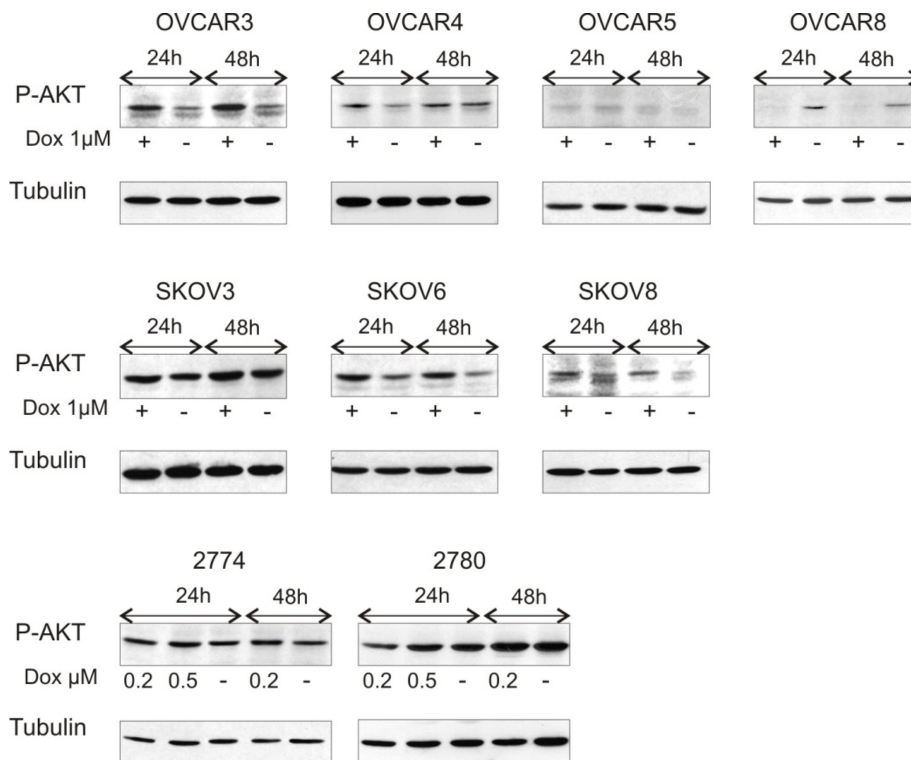


Fig. 8: Doxorubicin induces an increase in AKT phosphorylation.
Phosphorylation of AKT kinase (P-Ser 473) was analysed 24 as well as 48 hours after addition of doxorubicin at indicated concentrations. Lower concentrations of doxorubicin were used for the 2774 and 2780 ovarian cancer cell line based to their high doxorubicin sensitivity and the lack of enough viable cells at higher concentrations after 24 and 48 hours.

4.1.2. Inhibition of PI3K activity blocks the doxorubicin induced increase in AKT phosphorylation and induces apoptosis

Based on the observed doxorubicin induced increase in AKT phosphorylation, we further analysed a potential involvement of the phosphoinositide 3-kinase (PI3K) or a member of the PI3K-like family of kinases (PIKK) frequently reported as potential upstream activators of AKT (Burgering and Coffer 1995; Franke et al. 1995; Feng et al. 2004; Viniegra et al. 2005). Therefore, the PI3K inhibitor LY294002 shown to inhibit all major PI3K subclasses and DNA dependent protein kinase (DNA-PK) was utilised for the treatment of OVCAR3 and OVCAR4 cells in combination with doxorubicin (Vlahos et al. 1994; Rosenzweig et al. 1997). As anticipated, the PI3K inhibitor LY294002 completely abrogated the doxorubicin mediated increase in AKT serine 473

phosphorylation in both cell lines at a concentration of 20 µM, without any effect being observable at lower concentrations.

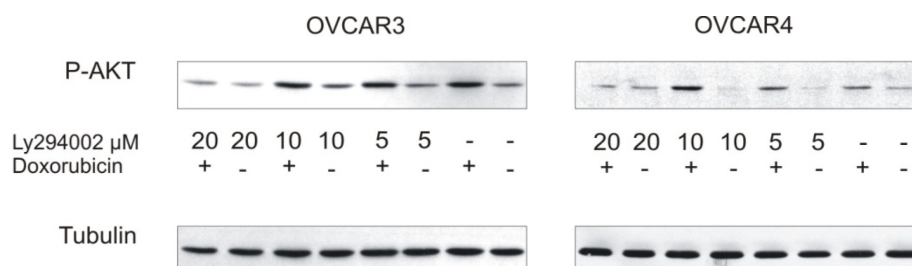


Fig. 9: The PI3K inhibitor LY294002 completely abolishes the doxorubicin induced AKT phosphorylation in the OVCAR3 and OVCAR4 cell line at a concentration of 20 µM. Cells were treated with or without doxorubicin (1 µM) for 24 hours in combination with indicated concentrations of the PI3K inhibitor LY294002 and compared to control cells treated plus/minus doxorubicin and DMSO. Phosphorylation of AKT was analysed via immunoblot by using a phospho-specific AKT antibody. Tubulin is shown as loading control.

Activation of the PI3K-AKT pathway which represents a key signalling cascade involved in cell survival as well as other important cellular mechanisms has been correlated with a decreased sensitivity to chemotherapeutic drugs (Dudek et al. 1997; Vivanco and Sawyers 2002). In this respect, we analysed the induction of apoptosis by performing a propidium-iodide assay (PI assay). The sub-G1 content was measured by FACS analysis representing the apoptotic fraction of total cells counted. Moreover, activation of the caspase cascade was monitored by the Caspase 3/7-Glo assay (Promega). Cells were incubated with the PI3K inhibitor LY294002 and doxorubicin alone or LY294002 was added in combination with doxorubicin. As anticipated, a clear increase in apoptosis was observed in both assays when cells were treated with LY294002 in combination with doxorubicin. In contrast, single treatments with either LY294002 or doxorubicin did not induce activation of caspases, whereas a minor increase in sub-G1 content was observed upon doxorubicin only.

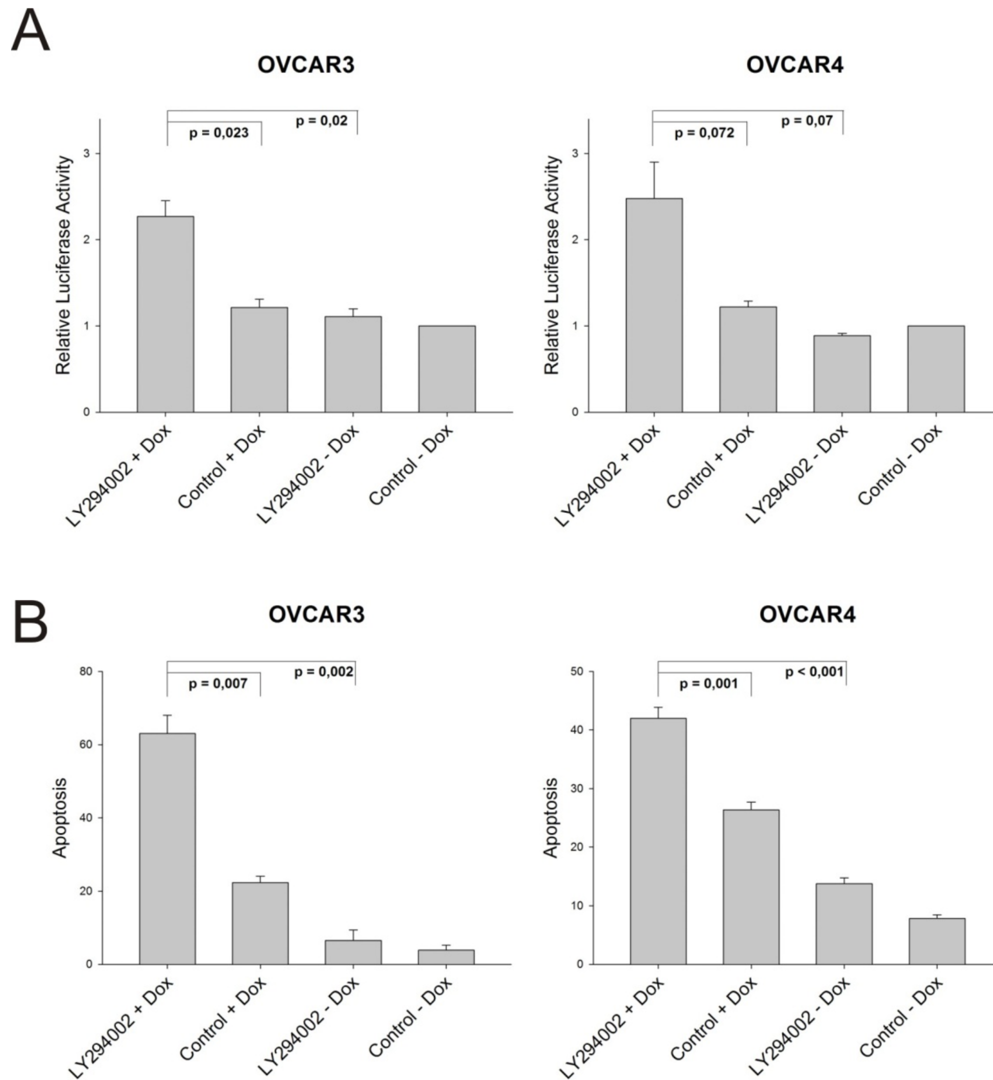


Fig. 10: Combination treatment of OVCAR3 and OVCAR4 cells with the PI3K inhibitor LY294002 and doxorubicin (Dox) significantly increases apoptosis. (A) Cells were treated with 20 µM of LY294002 in combination with doxorubicin (OVCAR3 = 1 µM and OVCAR4 = 2 µM) and the induction of apoptosis (analysed after 24 h) was compared to single treatments and DMSO control. Relative luciferase activity reflects the activation of executioner caspases-3 and -7 (Caspases 3/7-Glo assay) relative to the caspase activity of DMSO control cells. Mean values and SEM (standard error of the mean) of three independent experiments are shown. **(B)** Induction of apoptosis measured by the propidium-iodide (PI) assay. Sub-G1 content (%) was analysed 72 hours after treatment with 20 µM of LY294002 in combination with doxorubicin (OVCAR3 = 1 µM and OVCAR4 = 2 µM) and was compared to single treatments and DMSO control. Mean values (n = 3) and SEM are indicated.

4.1.3. Expression analysis of EGFR family members in ovarian cancer cell lines

Based on the promising results with the PI3K inhibitor LY294002, we aimed to identify the potential activator of the PI3K-AKT pathway in our ovarian cancer cell line model system. Previous reports have highlighted the important role of the HER3 receptor and its six potential PI3K binding sites in promoting TKI resistance by the activation of the HER3-PI3K-AKT signalling cascade (Schulze et al. 2005; Engelman et al. 2007; Sergina et al. 2007). Moreover, a positive expression of the HER3 receptor was inversely correlated with disease-free survival in ovarian cancer (Tanner et al. 2006). Therefore, we analysed the expression of the HER3 receptor in the nine ovarian cancer cell lines. Additionally, the expression of other EGFR family members representing potential HER3 heterodimerisation partners was analysed by immunoblot and RT-PCR. Interestingly, a moderate (OVCAR3/4) to high expression (SKOV6/8) of HER3 was measurable in all cell lines where we previously detected a doxorubicin mediated increase in AKT phosphorylation (Figure 1).

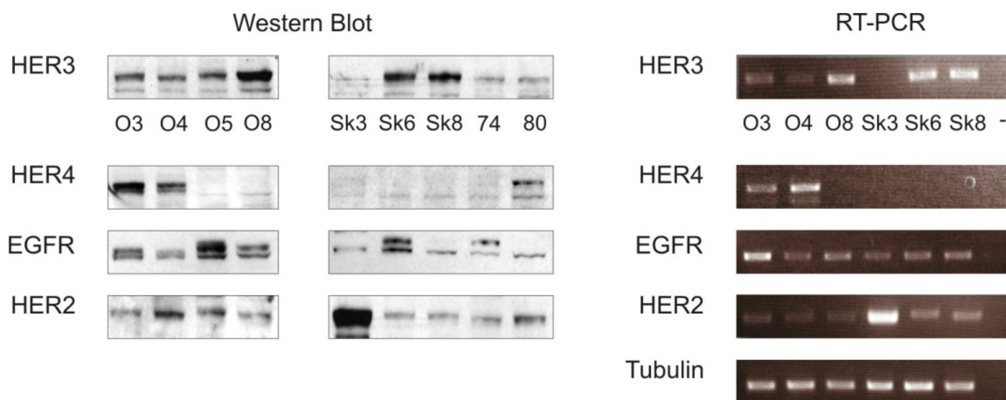


Fig. 11: Expression of EGF receptor family members in ovarian cancer cell lines. The ovarian cancer cell lines OVCAR3 (O3), OVCAR4 (O4), OVCAR5 (O5), OVCAR8 (O8), SKOV3 (Sk3), SKOV6 (Sk3), SKOV8 (Sk8), 2774 (74) and 2780 (80) were analysed for their expression of EGFR, HER2, HER3 and HER4 by western blot analyses and by RT-PCR as described in materials and methods.

4.1.4 Doxorubicin induces phosphorylation of HER3 and activated HER3 interacts with the PI3K regulatory subunit p85

Due to a detectable expression of the HER3 protein in all P-AKT positive (doxorubicin induced) ovarian cancer cell lines (Figure 1), we next analysed a potential increase in HER3 phosphorylation upon exposure to doxorubicin. Therefore, cells were again treated with doxorubicin for 24 and 48 hours, and the phosphorylation of HER3 was measured by using a phospho-specific HER3 antibody detecting the phospho-tyrosine (P-Tyr) 1289, which represents a potential PI3K binding site (Schulze et al. 2005). Interestingly, with the exception of SKOV8, all previously tested cell lines with an doxorubicin mediated increase in the AKT phospho-signal also exhibited a considerable increased HER3 phosphorylation when compared to the untreated control. In contrast, all P-AKT negative cell lines (upon addition of doxorubicin) did not demonstrate an increase in HER3 phosphorylation. Furthermore, no doxorubicin induced tyrosine phosphorylation of neither the EGFR nor the HER2 receptor was detected in the OVCAR3, OVCAR4, and SKOV6 cell line (data not shown). The increase in HER3 phosphorylation detected in three cell lines as well as immunoblots of OVCAR8, a "none responding" cell line, are shown in Figure 5.

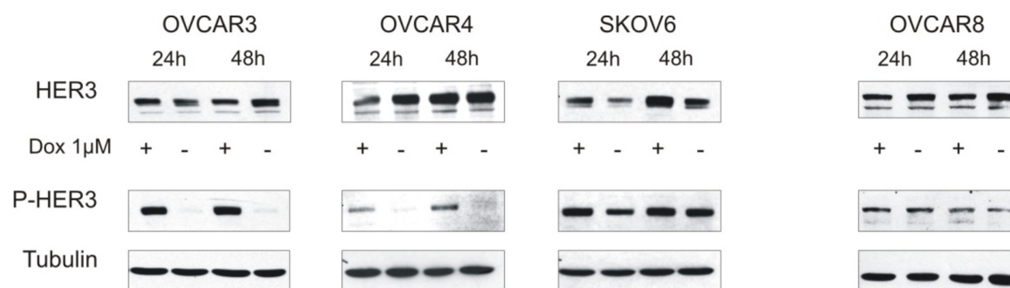


Fig. 12: Doxorubicin induces phosphorylation of HER3 in three out of nine tested ovarian cell lines. Cells were treated with or without doxorubicin (1 μM) for the indicated time and the phosphorylation of HER3 was detected by a phospho-specific antibody (P-Tyr 1289). Total amounts of HER3 were determined by the HER3 antibody (2F12, Millipore) while Tubulin is shown as loading control. The OVCAR8 cell line is shown as negative control representing non responding cell lines in this context.

To further validate the potential involvement of the HER3 receptor as a potential activator of AKT via the PI3K-AKT pathway, we tested, whether the PI3K regulatory subunit p85 can be immunoprecipitated with HER3. Therefore, cells were incubated with or without 1 µM of doxorubicin for 24 hours and HER3 was precipitated by using the HER3 specific antibody (2F12, Millipore). As expected, a strong increase in HER3 phosphorylation analysed by the P-Tyr antibody 4G10 was detected in doxorubicin treated cells when compared to untreated control cells. Moreover, the amount of co-precipitated p85 (PI3K subunit) was considerably higher in both tested cell lines. Furthermore, we validated these results with the homemade, HER3 specific antibody 1B4C3 that binds to the extracellular part of the HER3 receptor whereas the commercially available HER3 antibody 2F12 recognises the C-terminal part.

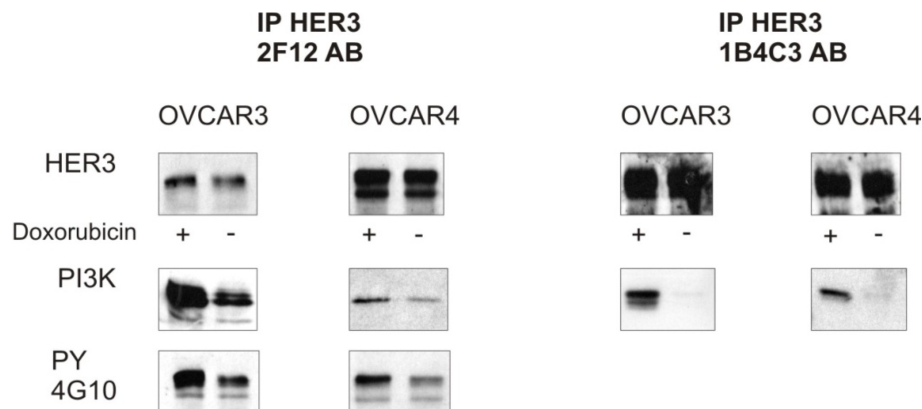


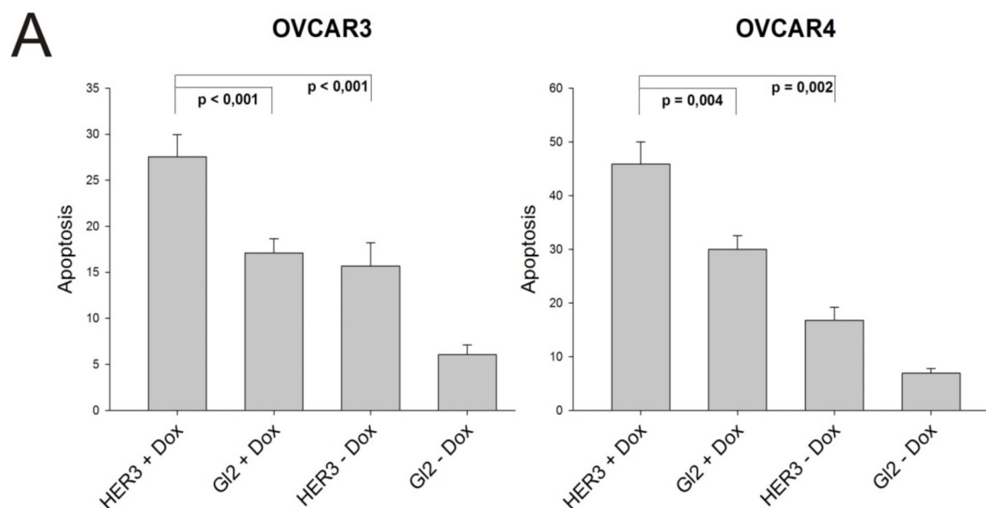
Fig. 13: The PI3K subunit p85 can be effectively precipitated with HER3 when cells were previously treated with doxorubicin. Cells were treated with or without doxorubicin (1 µM) for 24 hours. The HER3 receptor was immunoprecipitated by the HER3 specific antibody (Millipore, 2F12) which recognises the intracellular part of the receptor or by the homemade antibody 1B4C3 detecting the N-terminal part of HER3. Immunoblots for total HER3, phospho-tyrosine (P-Tyr, 4G10) as well as for p85 (regulatory subunit of PI3K) are shown.

4.1.4. Downregulation of HER3 increases doxorubicin mediated apoptosis

To validate the importance of HER3 in the context of chemo-sensitivity in the ovarian cancer cell lines, *HER3* was transiently downregulated by RNAi. Therefore, both cell lines were either treated with *HER3* specific siRNA or with control siRNA against luciferase (Gl2). Knock down experiments were performed separately with two

different *HER3* specific siRNAs in combination with the Oligofectamine transfection reagent (Invitrogen). Additionally, the obtained results were validated by using a mixture of three different *HER3* siRNAs combined with the RNAiMax transfection reagent (Invitrogen). Apoptosis (sub-G1 content) was measured 72 hours after addition of doxorubicin to siRNA treated cells (Figure 7 A and C). This corresponds to 96 hours after initiation of the *HER3* knock down. As anticipated, the downregulation of *HER3* in combination with doxorubicin was superior compared to single treatments. In contrast to this, no beneficial effect on apoptosis could be observed in the OVCAR8 cell line used as negative control in this experiment (negative control = no increase in P-HER3 and P-AKT upon addition of doxorubicin). Moreover, an early induction of apoptosis was analysed by the Caspase 3/7-Glo assay. Therefore, both cell lines were incubated with a *HER3* specific siRNA or control siRNA and were analysed 24 hours after addition of doxorubicin (Figure 7 B). As expected, this revealed a strong increase in caspase activation when doxorubicin was added to cells with downregulated levels of HER3.

As visualised in Figure 7 D, total amounts of HER3 could be effectively dimished in all three cell lines. Furthermore, the knock down completely abrogated the doxorubicin mediated increase in HER3 phosphorylation in the OVCAR3 and OVCAR4 cell line.



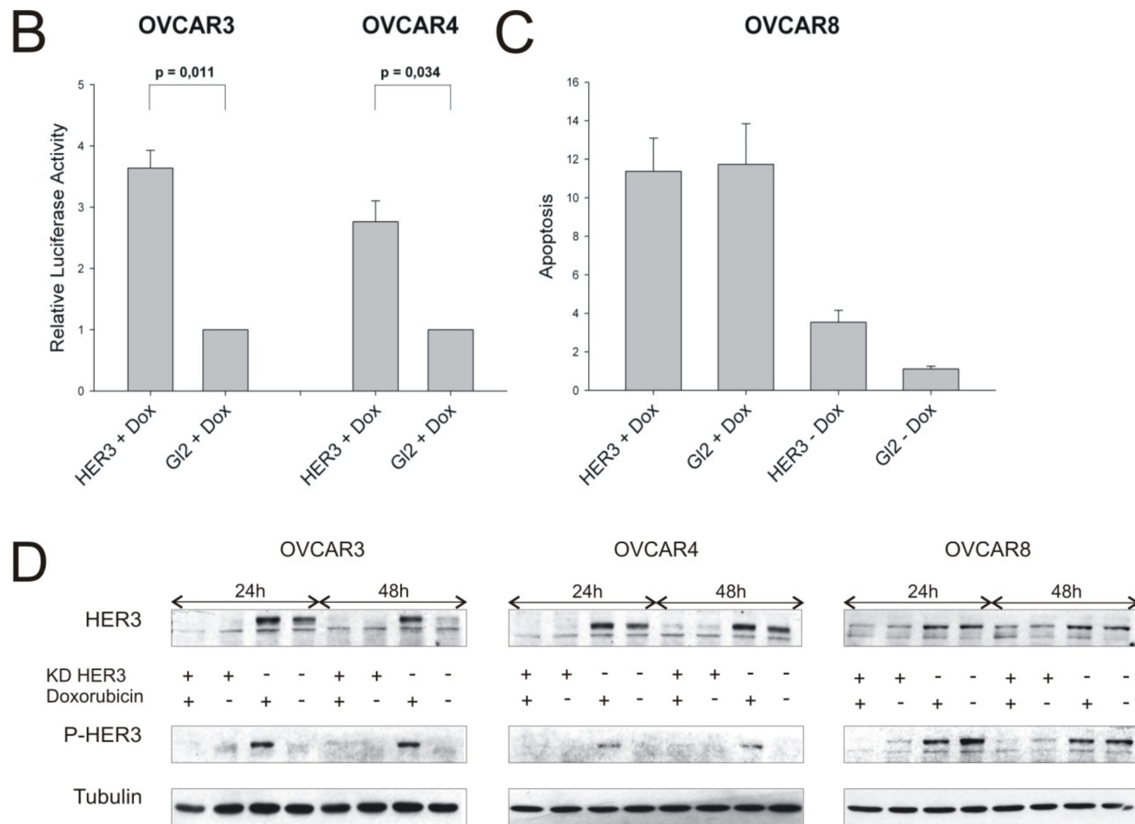
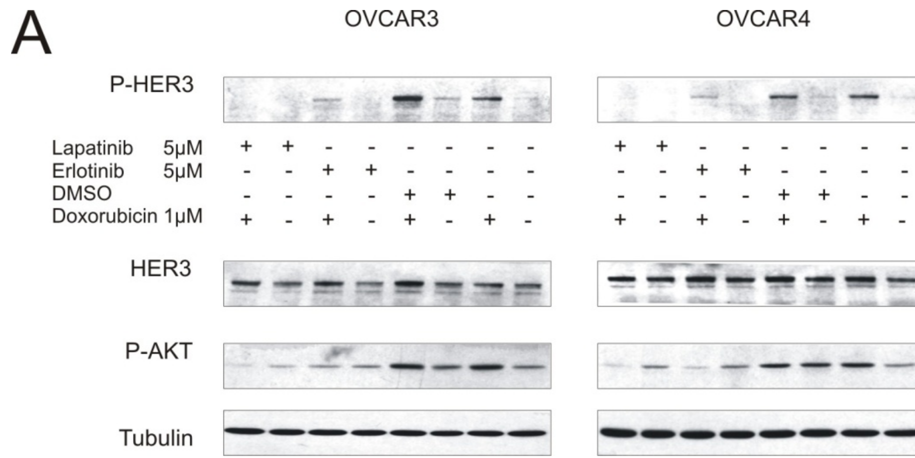


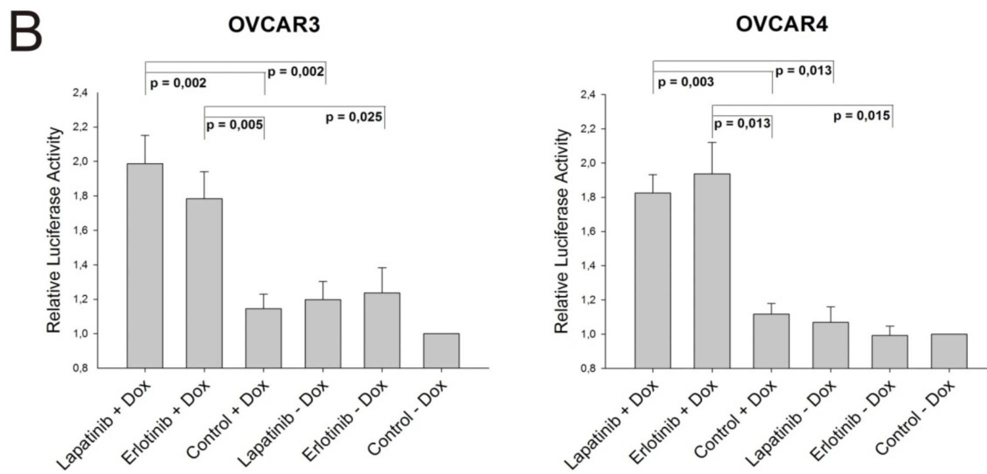
Fig. 14: Downregulation of *HER3* significantly increases apoptosis of OVCAR3 and OVCAR4 cells. (A) Apoptosis (sub-G1 content in %) was analysed 72 hours after addition of doxorubicin. Mean values ± SEM for the *HER3* knock down (HER3) in combination with doxorubicin (Dox), as well as siRNA control (Gl2) plus/minus doxorubicin are illustrated. All experiments were performed at least five times. **(B)** Mean values ± SEM (n = 3) of caspase activity (Caspase 3/7-Glo assay) for the combination *HER3* knock down plus doxorubicin treatment relative to siRNA control (Gl2) and doxorubicin. **(C)** Sub-G1 content was measured as described for the OVCAR3 and OVCAR4 cell line. OVCAR8 served as "negative" control in this experiment **(D)** Downregulation of *HER3* compared to *Gl2* control after 24 and 48 hours of doxorubicin treatment. Immunoblot analysis of total HER3 and phospho-HER3 (P-Tyr 1289) are demonstrated. Tubulin served as loading control.

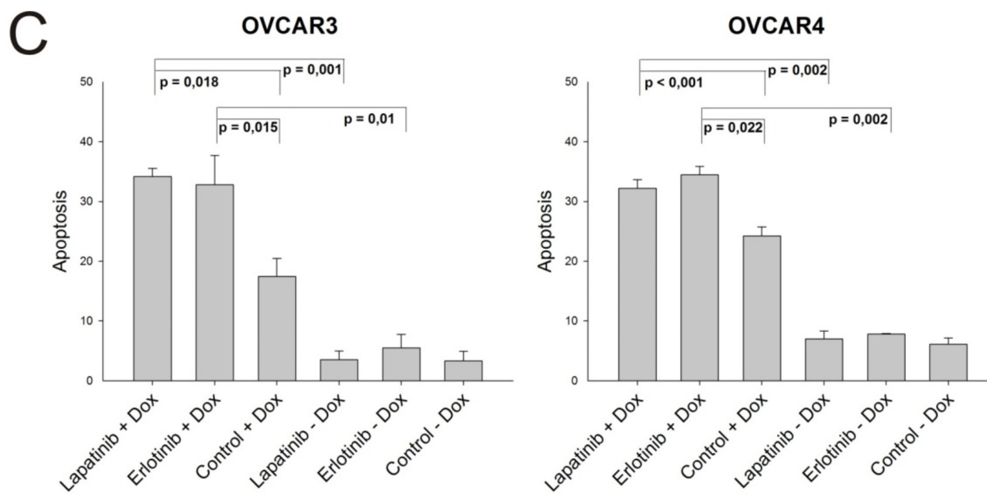
4.1.5. Lapatinib or Erlotinib effectively blocks the doxorubicin mediated increase in HER3 and AKT phosphorylation and enhances apoptosis

It is widely accepted that the HER3 receptor has an impaired kinase activity or high substrate specificity. Nevertheless, it represents an important heterodimerisation partner for other members of the EGFR family like EGFR itself or HER2. We therefore aimed to identify the potential dimerisation partner of HER3 in our ovarian chemo-

insensitivity cell line model system. Thus, we targeted two well known HER3 dimerisation partner namely the EGFR and the HER2 receptor. In this regard, two specific tyrosine kinase inhibitors, lapatinib (Tykerb) and erlotinib (Tarceva) were employed in the experiments. Lapatinib is a dual kinase inhibitor which is approved by the FDA for the treatment of breast cancer. It is capable of inhibiting the kinase activity of the EGFR and HER2. In contrast, erlotinib is dislodged as a selective inhibitor of the EGFR. Nevertheless, it has already been shown that besides their main targets, both inhibitors also have the potential to inhibit other members of the EGFR family. Erlotinib, for example, also has an inhibitory effect on the HER2 receptor mediated by direct interaction with the receptor, whereas lapatinib binds to HER4 as well (Wood et al. 2004; Schaefer et al. 2007; Qiu et al. 2008). By using these inhibitors, we first tested whether the combinatorial treatment of either lapatinib or erlotinib with doxorubicin abrogates the detectable increase in HER3 phosphorylation and activation of AKT. In addition, we analysed the efficacy of this combinatorial treatment on the level of apoptosis induction in OVCAR3 and OVCAR4 cells to further evaluate a potential clinical application of this combinatorial approach. Therefore, both cell lines were treated with lapatinib or erlotinib in combination with doxorubicin, and the activation of executioner caspases was measured after 24 hours, whereas DNA fragmentation was analysed after 72 hours. Interestingly, both inhibitors effectively blocked the doxorubicin induced increase in HER3 and AKT phosphorylation in both cell lines with lapatinib being superior to erlotinib. Moreover, treatment with lapatinib as well as erlotinib resulted in about two fold increased caspase activation when compared to untreated cells. In contrast, single administration of these inhibitors or doxorubicin revealed only minor effects. Furthermore, an increase in apoptosis was observed in the PI assay performed 72 hours after addition of drugs. As opposed to this, neither the addition of lapatinib nor erlotinib in combination with doxorubicin resulted in a significant increase in apoptosis in the “control” cell line OVCAR8 when compared to doxorubicin administered as single drug. These results are remarkable, because both cell lines exhibit only a moderate expression of the EGFR and HER2 receptor (see Figure 4). Moreover, it has been frequently reported that targeting the EGFR or HER2 by lapatinib or erlotinib reduces the number of cells progressing through S phase by inducing a G1 arrest. Based on this, one would expect that treatment with lapatinib or erlotinib interferes with doxorubicin induced cytotoxisity.


A
OVCAR3
OVCAR4
P-HER3
Lapatinib 5µM
Erlotinib 5µM
DMSO
Doxorubicin 1µM
HER3
P-AKT
Tubulin


B
OVCAR3
OVCAR4
Relative Luciferase Activity
p = 0,002
p = 0,002
p = 0,005
p = 0,025
p = 0,003
p = 0,013
p = 0,013
p = 0,015
Lapatinib + Dox
Erlotinib + Dox
Control + Dox
Lapatinib - Dox
Erlotinib - Dox
Control - Dox


C
OVCAR3
OVCAR4
Apoptosis
p = 0,018
p = 0,001
p = 0,015
p = 0,01
p < 0,001
p = 0,002
p = 0,022
p = 0,002
Lapatinib + Dox
Erlotinib + Dox
Control + Dox
Lapatinib - Dox
Erlotinib - Dox
Control - Dox

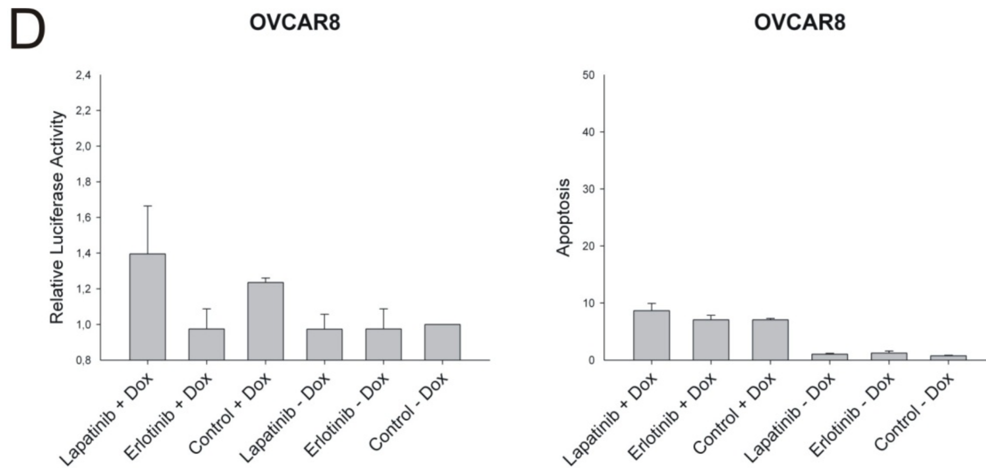


Fig. 15: Addition of lapatinib or erlotinib blocks the doxorubicin induced increase in HER3 and AKT phosphorylation and enhances apoptosis. (A) OVCAR3 and OVCAR4 cells were treated with lapatinib (5 µM), erlotinib (5 µM) or DMSO in combination with doxorubicin (1 µM). Cells were lysed 24 hours after addition of drugs and 50 µg of total protein was analysed by SDS-PAGE. Representative immunoblots for P-HER3, P-AKT, total HER3, and Tubulin are shown. **(B)** Mean values ± SEM (n = 5 for OVCAR3 and n = 4 for OVCAR4) of caspase activity (Caspase 3/7-Glo assay) relative to untreated control. Therefore, cells were incubated for 24 hours with lapatinib (10 µM), erlotinib (10 µM) or DMSO in combination with 1 µM (OVCAR3) and 2 µM (OVCAR4) of doxorubicin. **(C)** Mean values ± SEM (n = 3) of sub-G1 content (%) of cells treated with lapatinib or erlotinib (5 µM) in combination with doxorubicin (1 µM = OVCAR3, 2 µM = OVCAR4) for 72 hours. **(D)** Caspase activity and sub-G1 content was analysed as described for the OVCAR3 and OVCAR4 cell line. OVCAR8 served as “negative” control in this experiment.

4.1.6 Doxorubicin up-regulates the expression of different NRG1 isoforms

Our next step was to examine whether the doxorubicin mediated increase in HER3 phosphorylation is elicited by an autocrine or paracrine activation loop. Therefore, we investigated a potential upregulation of *NRG1* gene expression upon treatment with doxorubicin. Both cell lines were incubated with 1 µM of doxorubicin for 14 and 24 hours and total RNA was isolated and reversely transcribed as described in material and methods. Isoform-specific primers were used for PCR mediated amplification of *NRG1*. As anticipated, the expression of *NRG1 β1* and *β2* was induced in both cell lines upon treatment with doxorubicin. In contrast, upregulated expression of the *NRG1 α* isoform was detected only in OVCAR3 cells.

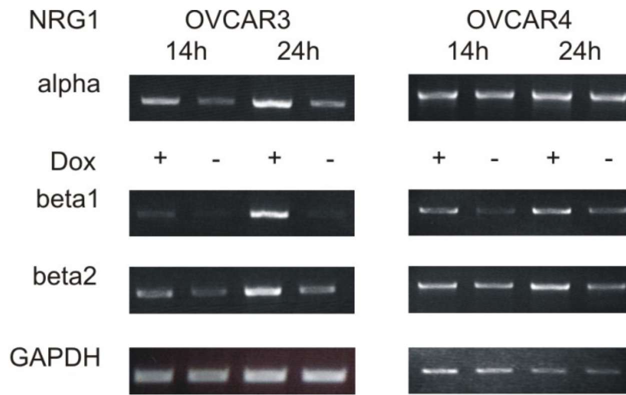


Fig. 16: Expression of NRG1 isoforms is upregulated upon doxorubicin treatment
RNA was extracted from OVCAR3 and OVCAR4 cells after doxorubicin (1 µM) treatment for indicated time and reverse transcribed (see materials and methods). Expression of ligands was analysed by semiquantitative RT-PCR by using NRG1 α, NRG1 β1 and NRG1 β2 specific primers. GAPDH serves as loading control.

4.1.7 Batimastat (BB94) completely abrogates the doxorubicin induced HER3 phosphorylation and significantly increases apoptosis

The metalloprotease inhibitor batimastat (BB94) is a broadband inhibitor of the ADAM (a disintegrin and metalloprotease) family of metalloproteases. This inhibitor was once the lead compound of British Biotech but it failed in clinical trials on showing no improvement of survival or quality of life in cancer patients. Nevertheless, batimastat is widely used for *in vitro* experiments with the goal to investigate the potential involvement of a metalloprotease in ligand shedding. Therefore, batimastat was used in combination with doxorubicin to potentially block shedding of HER3 ligands, whose expression is upregulated upon doxorubicin treatment (see Figure 9). In this regard, cells were treated with doxorubicin in combination with batimastat for 24 hours and phosphorylation of HER3 was analysed by western blot. Interestingly, the doxorubicin mediated increase of the HER3 phospho-signal was completely abrogated when cells were simultaneously incubated with doxorubicin and batimastat compared to doxorubicin only. This is the reason why we further tested if there is also a detectable increase in apoptosis upon this combinatorial treatment as one might conclude from our previous results. As anticipated, a strong induction of caspase activation was measured

in both cell lines, whereas only a marginal apoptotic effect (caspase activation) was detectable upon single treatment with batimastat or doxorubicin.

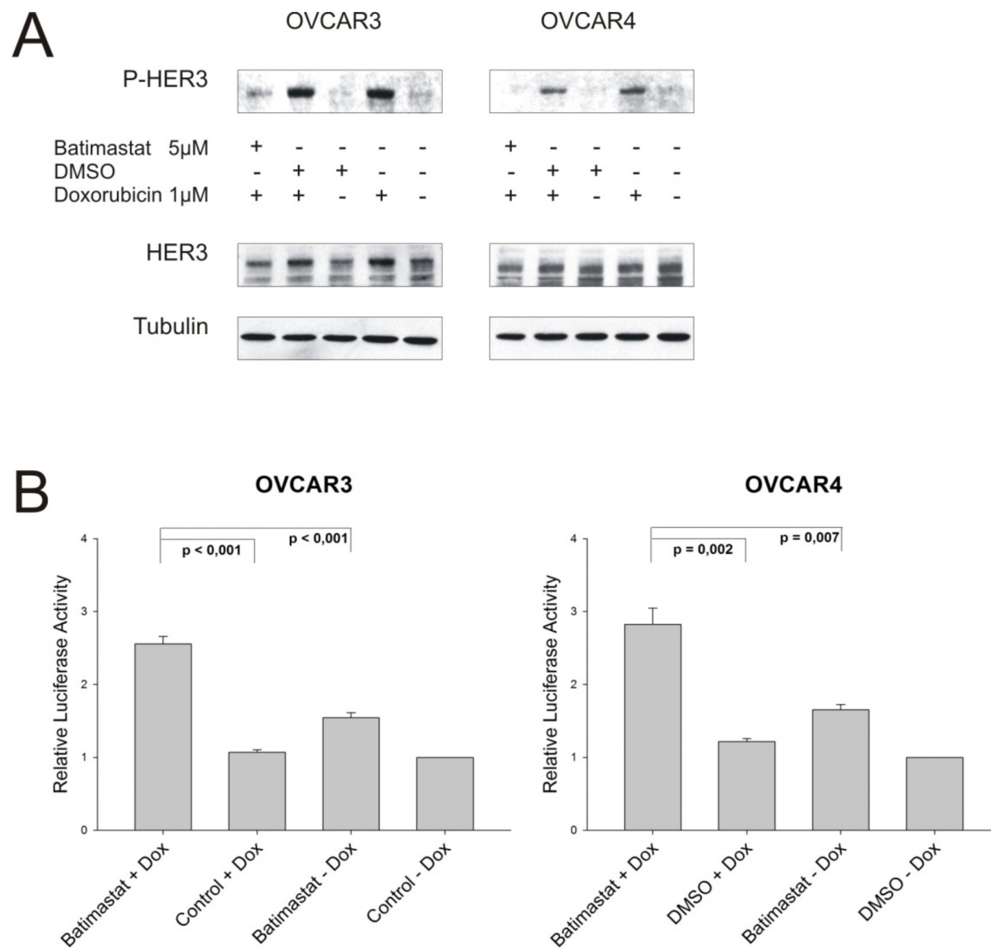


Fig. 17: Batimastat completely blocks the doxorubicin induced phosphorylation of HER3 and increases apoptosis. (A) Representative immunoblots of cells treated with batimastat (5 µM) in combination with doxorubicin (1 µM) for 24 hours compared to controls (DMSO plus doxorubicin or single treatments). Phosphorylation of HER3 (P-Tyr 1289), total HER3 protein amount as well as Tubulin serving as loading control was analysed. **(B)** Mean values ± SEM (n = 6 for OVCAR3 and n = 5 for OVCAR4) of caspase activity (Caspase 3/7-Glo assay) relative to DMSO control. Cells were seeded in 96 well plates and treated for 24 hours with or without batimastat (5 µM) and/or doxorubicin (OVCAR3 = 1 µM, OVCAR4 = 2 µM) and were subsequently analysed.

4.1.8 Reduction of cellular ADAM17 by RNAi diminishes the doxorubicin induced phosphorylation of HER3

The abrogation of HER3 phosphorylation upon treatment with batimastat plus doxorubicin together with the encouraging observations of apoptosis induction prompted us to further elucidate the potential involvement of a metalloprotease in the context of HER3 ligand shedding and receptor activation. Several groups have already shown that ADAM17 deficient cells are defective in shedding of different EGFR ligands like TGFα, HB-EGF and amphiregulin (Peschon et al. 1998; Merlos-Suarez et al. 2001; Sunnarborg et al. 2002). Recently, the major role of ADAM17 in shedding of the three EGFR ligands mentioned above and epiregulin as a new substrate has been strengthened by Sahin and colleagues (Sahin et al. 2004). In addition, no contribution of other ADAMs besides ADAM17 could be detected in TGFα, amphiregulin and HB-EGF shedding. Moreover, in NSCLC, activation of the HER3 receptor correlated with the expression of ADAM17 but not with ADAM10 and only the downregulation of ADAM17 but not ADAM9, ADAM10 or ADAM15 had an effect on HER3 and AKT activity in A549 cells (Zhou et al. 2006). Therefore, we investigated whether ADAM17 is also involved in shedding of HER3 ligands in our system. This was analysed by RNAi mediated reduction of cellular ADAM17 levels. As anticipated, the doxorubicin induced activation of the HER3 receptor was completely blocked in both cell lines upon knock down of ADAM17 with a comparable decline in signal intensity as observed for the downregulation of HER3.

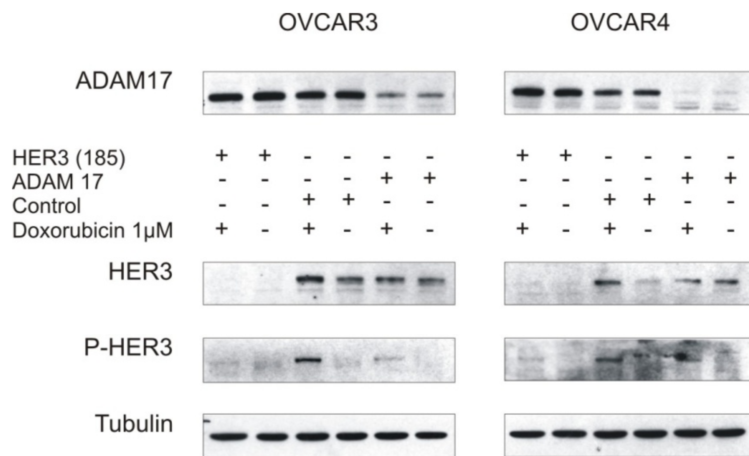


Fig. 18: Knock down of *ADAM17* effectively abolishes doxorubicin induced HER3 activation. Downregulation of *ADAM17* and *HER3* was performed as described. Cells were subsequently treated with or without doxorubicin (1 μM) for 24 hours before cellular lysis. Representative imunoblots for ADAM17, P-HER3, total HER3 and Tubulin are shown.

4.1.9 Exogenous addition of recombinant HER3 ligands partially reverses the apoptotic effect of batimastat plus doxorubicin

Based on these interesting observations, we tried to reverse the activation of the apoptotic cascade induced by the addition of batimastat in combination with doxorubicin. If our considerations were right, the exogenous induced re-activation of the HER3-PI3K-AKT cascade upon stimulation with HER3 ligands should result in a decrease in apoptosis in cells previously treated with doxorubicin and batimastat. Therefore, cells were incubated with doxorubicin in combination with batimastat and different NRG1 isoforms were added after 14 and 19 hours. Activation of the caspase cascade was then monitored after 24 hours. Interestingly, with the exception of NRG1 α in OVCAR4 cells, the addition of exogenous HER3 ligands partially reversed doxorubicin induced apoptosis either when administered as single ligand or as combination. However, there was no complete reduction of apoptosis observable by the exogenous addition of ligands, which might be due to the artificial character of this experiment.

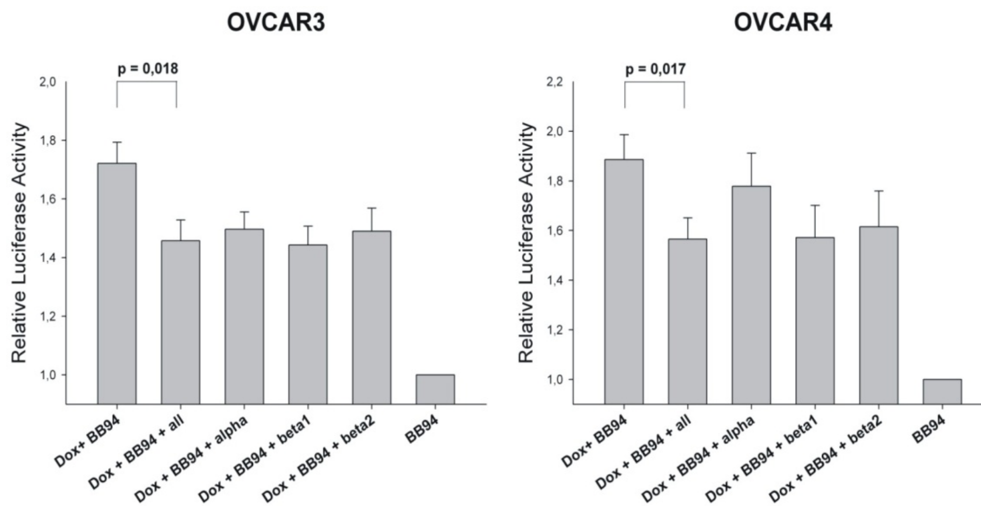


Fig. 19: Exogenous addition of HER3 ligands partially reverses the increase in caspase activation induced by doxorubicin in combination with batimastat (BB94). Mean values ± SEM (n = 6 for OVCAR3 and n = 5 for OVCAR4) of caspase activation (Caspase 3/7-Glo assay) relative to batimastat (BB94) single treatment. Cells were incubated with doxorubicin and batimastat as indicated and different NRG1 isoforms (α, β1, β2) were added after 14 and 19 hours at a concentration of 50 ng/ml. The activation of caspases was analysed 24 hours after addition of drugs.

4.1.10 HER3 blocking antibody treatment completely abrogates the doxorubicin induced increase in HER3 and AKT phosphorylation

Encouraged by the previous results, we further aimed to nail down the doxorubicin induced increase of the HER3-PI3K-AKT pathway to NRG1 mediated HER3 activation. Therapeutic monoclonal antibodies targeting the HER3 receptor are in clinical trials and might soon become available as a novel treatment option. In this regard, we utilised the highly specific commercially available HER3 blocking antibody 105.5 (Millipore) previously used by several groups and shown to inhibit ligand mediated HER3 phosphorylation (Chen et al. 1996). Initially, we tested whether this monoclonal HER3 antibody is capable of blocking the NRG1 mediated increase in HER3 and AKT phosphorylation in OVCAR3 and OVCAR4 cells, and whether it abrogates the receptor activation induced by NRG1 isoforms (NRG1 α, NRG1 β1 and NRG1 β2). As expected, a remarkable reduction in HER3 and AKT phosphorylation was detected (see Figure 13). In more detail, a strong reduction of phospho-signals was

observed upon antibody treatment and subsequent stimulation with NRG1 β1 or NRG1 β2 ligands. Based on these promising results, we slightly modified the experimental set up. Instead of stimulating cells with exogenous ligands cells were incubated with doxorubicin for 24 hours and the HER3 blocking antibody was added for an additional time of two hours before lysis. As anticipated, the HER3 blocking antibody completely inhibited the doxorubicin induced increase of HER3 and AKT phospho-signals in both cell lines, whereas no effect was detected with the isotype control antibody.

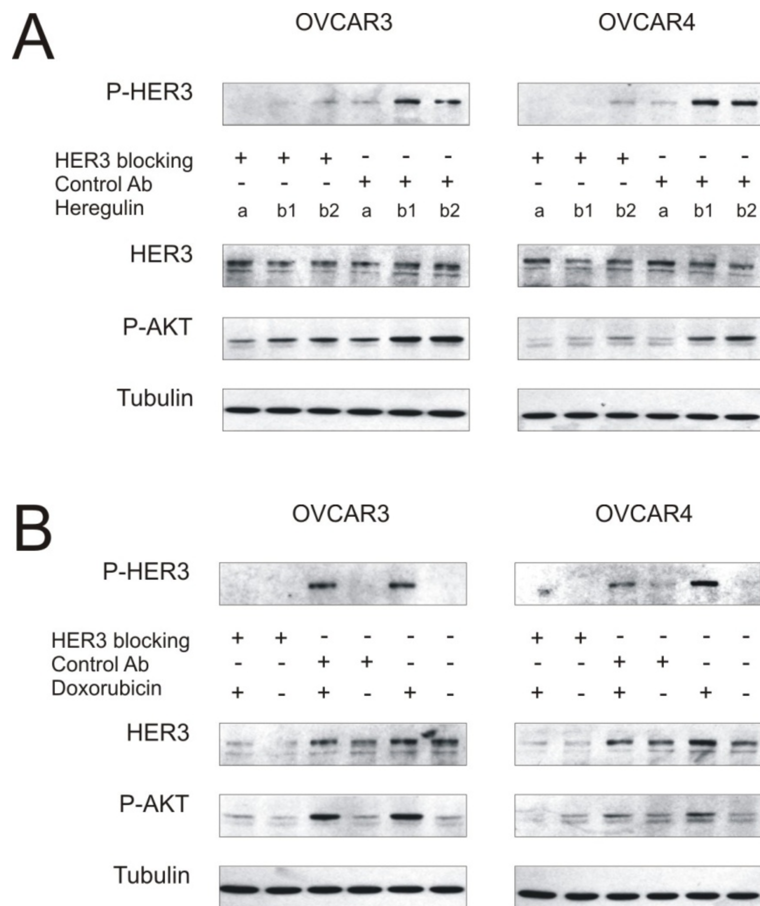


Fig. 20: HER3 blocking antibody treatment effectively abolishes NRG1 as well as doxorubicin mediated phosphorylation of HER3 and AKT. (A) Cells were treated with the HER3 blocking antibody 105.5 (10 µg/ml) or a IgG1 isotype control antibody for two hours. Cells were then stimulated with different HER3 ligand isoforms (50 ng/ml) for 40 min. **(B)** OVCAR3 and OVCAR4 were incubated with doxorubicin (1 µM) for 24 hours and treated with the HER3 blocking antibody (10 µg/ml) for two hours before cellular lysis. Representative immunoblots of P-HER3, total HER3 P-AKT and Tubulin are visualised.

4.1.11 Media from doxorubicin incubated OVCAR3 cells increases HER3 phosphorylation in MCF7 breast cancer cells

A tumor is a mass of heterogenous cells carrying DNA abnormalities that initiated the malignant behaviour as well as many additional genetic lesions that emerged during tumorigenesis due to a constant selective pressure. In this respect, we wanted to know whether the doxorubicin induced expression of HER3 ligands and their assumed secretion, even if only a few tumor cells would show this resonse, has the potential to activate the HER3-PI3K-AKT survival pathway in surrounding cells. For this experiment, we used the well established breast cancer cell line MCF7 with reportedly high levels of membranous HER3, as read out. Moreover, the HER3 receptor of MCF7 cells can be effectively phosphorylated by different NRG1 isoforms (own observations). Therefore, media from OVCAR3 cells cultivated in the presence of doxorubicin for 24 hours was removed and utilised for the stimulation of MCF7 breast cancer cells for 20 and 40 min. As expected, this media potentially activated the HER3 receptor in MCF7 cells whereas media from untreated OVCAR3 cells did not induce receptor activation. Thus, this data further reveals that HER3 ligands, expressed and cleaved by metalloproteases upon doxorubicin treatment, are soluble and capable to induce activation of the HER3-PI3K-AKT pathway in an autocrine and/or paracrine manner.

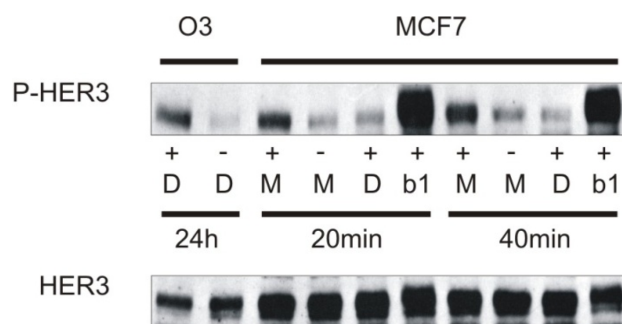


Fig. 21: Media from doxorubicin treated OVCAR3 cells potently induces HER3 phosphorylation in MCF7 breast cancer cell line. The breast cancer cell line MCF7 was incubated for the indicated time with media from previously treated OVCAR3 cells (with or without 1 µM of doxorubicin (D) for 24 hours) and phosphorylation of HER3 (P-Tyr 1289) was analysed by western blot. Controls as shown are doxorubicin (D) for 20 and 40 min as well as control stimulation of MCF7 cells with NRG β1 (50 ng/ml) for the indicated time.

4.1.12 Mass spectrometry-based search for potential HER3 dimerisation partners and interacting proteins upon doxorubicin treatment

With the exception of the HER2 receptor, which exists in an “open” and therefore active conformation, members of the EGF receptor family normally exist as monomers in a “closed” inactive conformation (Cho et al. 2003; Garrett et al. 2003). Therefore, the HER2 receptor is constitutively ready for dimerisation. In contrast, this conformation has to be induced by ligand binding to the extracellular domain of the receptor in other EGFR family members. It has been frequently reported that the HER3 receptor lacks an active kinase function due to sequence substitutions. In addition, it is not capable to form homodimers upon activation but nevertheless seems to be the preferred dimerisation partner of other EGFR family members (Pinkas-Kramarski et al. 1996; Berger et al. 2004). Although the HER2/HER3 receptor heterodimer has been reported to be the most potent signalling dimer of the EGFR family, it is not clear how the HER2 receptor can be phosphorylated and activated within a single HER2/HER3 complex. To elucidate the mechanism of HER3 receptor activation in our cell culture model system, we employed a mass spectrometry-based approach. We pulled down the receptor by immunoprecipitation with the commercially available HER3 specific antibody (2F12) to identify potential HER3 interactors upon doxorubicin treatment (see Figure 15). These experiments were performed in collaboration with Felix Oppermann, PhD student in the group of Henrik Daub. Serial depletion experiments were conducted to distinguish between potential HER3 interactors and proteins that unspecifically bind to Protein-A sepharose beads (Trinkle-Mulcahy et al. 2008). Briefly, the eluate of the first IP reaction conducted with the lysate of untreated (no doxorubicin) 0/0 SILAC (stable isotope labelling with amino acids in cell culture) labelled cells (see materials and methods) was combined with the eluate of the second IP (6/4 labelled) and the eluate of the first IP (10/8 labelled) of doxorubicin treated cells. To strengthen the obtained data, a label switch experiment was performed. A total of twenty P15 plates of 0/0 SILAC labelled cells and ten P15 plates for 6/4 and 10/8 were used to obtain a total protein amount of 9.18 mg deployed for each HER3 immunoprecipitation. For a more detailed description of the experimental settings see material and methods.

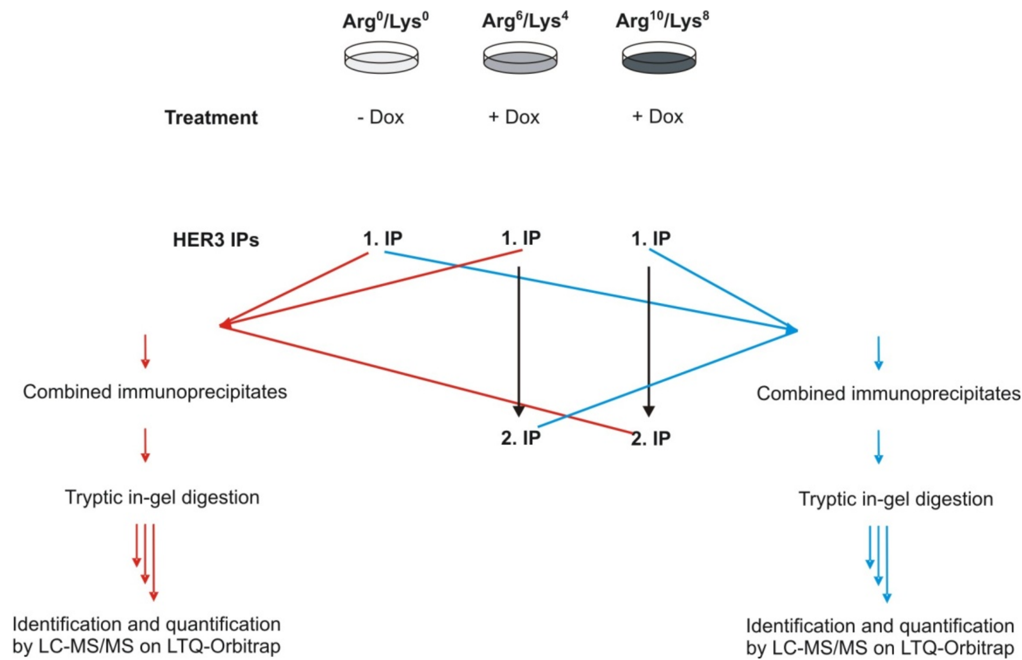


Fig. 22: Work-flow illustration of the mass spectrometry-based search for HER3 dimerisation and interacting partners upon treatment with doxorubicin. OVCAR3 cells were cultivated in SILAC-encoded media and thereupon treated with or without doxorubicin (1 µM) for 24 hours. HER3 IP´s were conducted with lysates of SILAC labelled cells with the HER3 specific commercially available antibody (2F12). Lysates of doxorubicin treated cells were subsequently used for a second IP reaction, performed with the supernatant of the first IP. Immunoprecipitates were then combined, as indicated, in-gel digested and identified by mass spectrometry.

To verify that the doxorubicin mediated induction of HER3 phosphorylation worked as expected in SILAC-encoded OVCAR3 cells, 50 µg total lysates were loaded on a 10 % gel and analysed by western blot for the expected increase in HER3 and AKT phosphorylation (Figure 16 A). We further tested whether most HER3 receptors were already pulled down by the first IP reaction and analysed if residual HER3 was left for the second IP (Figure 16 B). Therefore, 50 µg of total protein before and after each IP step was subjected to immunoblot analysis. As anticipated, the HER3 receptor was effectively depleted from the lysates of SILAC labelled OVCAR3 cells after the fist IP reaction leaving only a small fraction of residual HER3 in the lysate, which could not be futher depleted by the second IP.

Fig. 23: Doxorubicin effectively induces HER3 and AKT phosphorylation in SILAC-encoded OVCAR3 cells and HER3 could be successfully depleted. (A) Immunoblot analysis of the doxorubicin (1 µM; 24 hours) mediated increase of HER3 and AKT phosphorylation in SILAC-encoded OVCAR3 cells. 50 µg of total lysates utilised for the HER3 depletion experiment were loaded on a 10 % gel and analysed by immunoblot. **(B)** Control blots to analyse the effcicacy of the HER3 IP reactions. Total lysates (50 µg) before and after each IP were subjected to SDS-PAGE and analysed by western blot. EGFR is shown as loading control and was not affected by the IP performed with the anti-HER3 specific antibody (2F12, Millipore).

To identify significant differences in relative protein abundance, the relative ratios of the protein quantifications from the two biological replicate experiments were analysed for their normal distribution to account for the combined biological and technical variation in the quantitative MS analyses. Protein abundance was considered as significantly different ($p < 0.05$) in case ratios differed from the mean by 2 σ as determined from the 'ratio of ratios' distributions of the biological replicate analyses (data not shown). Thereof, values of more than 1.37 or less than 0.75 indicate increased or reduced cellular protein detection upon treatment with doxorubicin. In addition, values of less than 0.69 indicate proteins that are co-immunoprecipitated in the anti-HER3 IP and therefore represent potential HER3-interacting proteins.

We did not identify the potential heterodimerisation partner of the HER3 receptor responsible for the doxorubicin mediated increase in HER3 phosphorylation. However, we detected the HER4 receptor as specific HER3 interaction partner in the untreated state, and this HER3/HER4 interaction seems to decline when cells are treated with doxorubicin for 24 hours. In contrast, the p85 regulatory as well as the p110 catalytic PI3K subunit were identified as binding partners of HER3 with a more than fivefold increase upon doxorubicin addition. Notably, the HER3 receptor itself accumulated more than 1.5 fold in the doxorubicin treated sample.

Table 1: Putative HER3 interaction partners in doxorubicin treated OVCAR3 cells
Potential HER3 interaction partners in doxorubicin treated OVCAR3 cells as identified by mass spectrometry. Proteins below threshold of 0.7 for 2nd IP/ 1st IP for both experiments are indicated. Out of this list, only few proteins are present above the threshold of 1.4 for doxorubicin (Dox)/ DMSO control (Control).

Accession No.	Protein Names	Experiment I Normalized Ratio (2nd IP/1st IP)	Experiment II Normalized Ratio (2nd IP/1st IP)	Experiment I Normalized Ratio Dox /Control	Experiment II Normalized Ratio Dox /Control
IPI00790342	60S ribosomal protein L6	0.57	0.53	0.78	0.85
IPI00220628	A-kinase anchor protein 9	0.54	0.44	0.64	0.91
IPI00004233	Antigen KI-67	0.38	0.43	1.61	2.30
IPI00852708	BAT2D1 protein	0.49	0.51	1.00	1.21
IPI00217466	Histone H1.3;Histone H1c	0.55	0.62	0.66	0.63
IPI00031386	PI3-kinase p110 subunit alpha	0.44	0.46	5.67	4.40
IPI00807573	PI3-kinase p85 subunit alpha	0.50	0.59	5.76	5.02
IPI00026497	Poly [ADP-ribose] polymerase 2	0.54	0.57	1.31	1.19
IPI00043990	Probable ATP-dependent RNA helicase DDX31	0.70	0.57	0.38	0.43
IPI00005826	Probable E3 ubiquitin-protein ligase HERC2	0.26	0.59	1.10	1.25
IPI00298285	Receptor tyrosine-protein kinase erbB-3 precursor	0.41	0.37	1.42	1.49
IPI00016371	Receptor tyrosine-protein kinase erbB-4 precursor	0.65	0.57	0.27	0.28
IPI00028412	Sjoegren syndrome/scleroderma autoantigen 1	0.39	0.59	1.81	1.39
IPI00166957	Zinc finger and BTB domain-containing protein 2	0.29	0.34	0.71	0.66

To validate the obtained results of the putative HER3/HER4 receptor interaction, and to investigate why this interaction seems to be lost upon doxorubicin treatment, further IP experiments with the HER3 specific as well as with a HER4 specific antibody (sc-283, Santa Cruz) were performed (Figure 17 A). We thereby validated the HER4 receptor as HER3 dimerisation partner with a HER3/HER4 interaction only in the "unstimulated" (no doxorubicin) state. Interestingly, the apparently decline in HER4 interaction observed by mass spectrometry seems to be due to a decrease of total HER4 protein amounts upon doxorubicin treatment. Nevertheless, the residual HER3/HER4 interaction could be responsible for the doxorubicin mediated increase in HER3 phosphorylation. In this regard, we tested whether the doxorubicin induced phosphorylation of the HER3 receptor can be blocked by RNAi mediated downregulation of HER4, but no effect on HER3 phosphorylation could be observed (Figure 17 B).

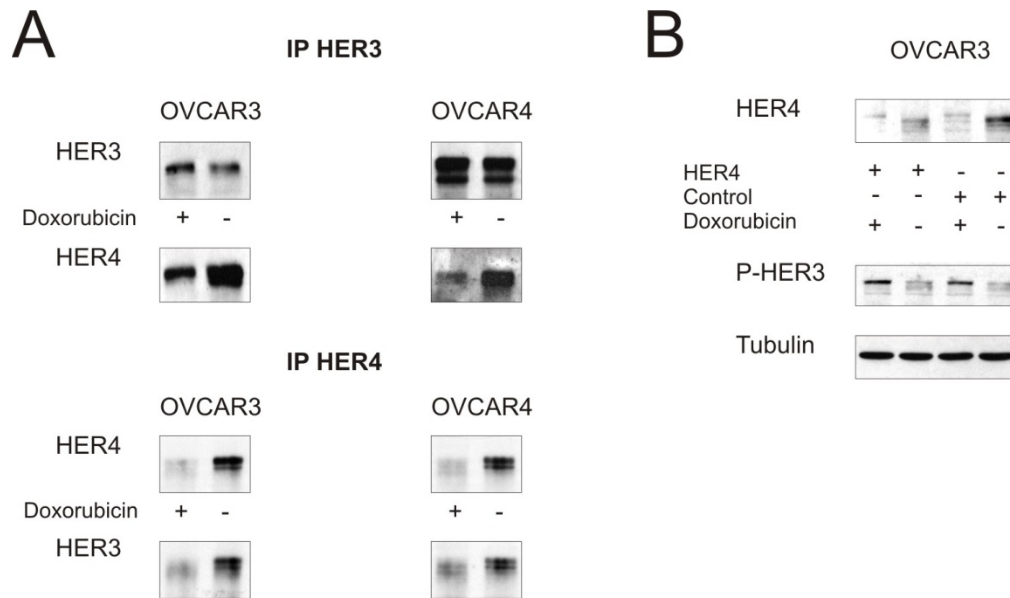


Fig. 24: HER4 effectively co-imunoprecipitates with HER3 in the basal state but its downregulation does not affect HER3 phosphorylation. (A) HER3 was immunoprecipitated by the HER3 specific antibody (Millipore, 2F12) and the HER4 receptor could be effectively co-precipitated in the basal (untreated) state. This interaction was lost upon doxorubicin treatment (24 hours, 1 µM) in both cell lines. Vice versa, HER3 was co-immunoprecipitated with the HER4 receptor (Santa Cruz, sc-283) only from lysates of untreated cells. **(B)** Downregulation of *HER4* by siRNA does not influence the doxorubicin induced increase in HER3 phospho-signal. Knock down was performed with *HER4* specific siRNA or control (Luciferase, Gl2), cells were treated with or without doxorubicin 1 µM and lysed after 24 hours. Representative immunoblots of HER4, P-HER3 (P-Tyr 1289) and Tubulin as loading control are shown.

4.1.13 Trastuzumab effectively inhibits the doxorubicin induced phosphorylation of HER3 and AKT while cetuximab does not

Paradoxically, it was not possible to detect the potential HER3 interaction/dimerisation partner in the doxorubicin induced state responsible for the activation of HER3 and its downstream signalling components via mass spectrometry. Moreover, no other EGFR family member except HER3 showed an increase in phosphorylation upon addition of doxorubicin as analysed by the use of phospho-specific EGFR and HER2 antibodies (data not shown). However, co-treatment with either lapatinib or erlotinib in combination with doxorubicin (Figure 8 A) completely abrogated the induced HER3 phosphorylation.

While both of these inhibitors were developed against the EGFR only or the EGFR and HER2, it has recently been reported that erlotinib inhibits the HER2 receptor by direct interaction whereas lapatinib also binds to HER4 (Wood et al. 2004; Schaefer et al. 2007; Qiu et al. 2008). Based on this TKI unspecifity, we tried to identify the potential HER3 dimerisation partner by using specific therapeutic monoclonal antibodies like trastuzumab (Herceptin) or cetuximab (Erbitux). Trastuzumab, on the one hand, is a humanized monoclonal antibody directed against the HER2 receptor, and is approved by the FDA as adjuvant treatment for breast cancer in combination with doxorubicin, cyclophosphamide, or paclitaxel. Furthermore, trastuzumab is indicated as single agent in node-positive or node-negative HER2 overexpressing breast tumors following anthracycline based therapy. On the other hand, cetuximab, a monoclonal antibody that binds to the EGF receptor, is indicated for the initial treatment of advanced squamous cell carcinoma of the head and neck in combination with radiation therapy as well as for treatment of recurrent or metastatic squamous cell carcinoma. Moreover, cetuximab is used as single agent for the treatment of EGFR-expressing metastatic colorectal cancer in patients that already received or are intolerant to chemotherapy.

However, we treated both ovarian cancer cell lines with doxorubicin for 24 hours and incubated them with either trastuzumab or cetuximab two hours before cells were lysed. Moreover, potential differences in the HER3 phospho-signal upon antibody treatments were compared to the effects observed with the 105.5 (HER3 blocking Ab), which has proven very effective in inhibiting the doxorubicin induced increase of HER3 phosphorylation (Figure 13). Notably, on incubating cells with trastuzumab, the doxorubicin mediated increase of the HER3 phosphorylation was completely abolished. The detectable decline of the HER3 phosphorylation was comparable to the decrease we observed by using the 105.5 blocking antibody directed against HER3 itself. In contrast, there was no effect on the HER3 phospho-signal intensity to be observed with the EGFR blocking antibody cetuximab or with the isotype control antibody, as expected.

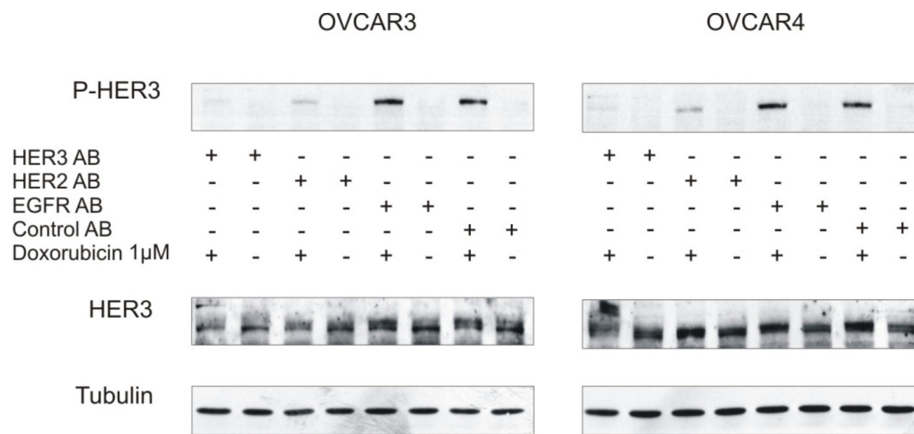


Fig. 25: Trastuzumab effectively abolishes doxorubicin mediated HER3 phosphorylation whereas cetuximab has no effect. OVCAR3 and OVCAR4 cells were incubated with or without doxorubicin 1 μM for 24 hours and were subsequently treated with either the HER3 blocking (HER3 AB), trastuzumab (HER2 AB), cetuximab (EGFR AB) or isotype control (Control AB) antibody at concentrations of 10 μg/ml for an additional time of two hours before cell lysis. Representative immunoblots of p-HER3 (P-Tyr 1289), HER3 and Tubulin are shown.

4.1.14 Downregulation of HER2 unveils HER2 as being responsible for the doxorubicin induced activation of HER3

To further validate the interesting results we obtained with the HER2 blocking antibody trastuzumab, we transiently downregulated the HER2 receptor in both cell lines and then treated them with doxorubicin. Briefly, both cell lines were incubated with specific siRNAs against the HER2, HER4, or the HER3 receptor. In this experiment, HER4 and HER3 served as negative and positive controls, respectively. Therefore, 24 hours after the knock down was carried out cells were incubated with doxorubicin and the phosphorylation of HER3 as well as AKT was analysed by western blot. As anticipated, downregulation of HER2 abrogated the doxorubicin mediated increase of the HER3 and AKT phospho-signal. Moreover, the important role of the HER2 receptor in our cell culture model system was further enlightened by the strong increase in apoptosis detected upon downregulation of HER2 and subsequent treatment with doxorubicin. In contrast, knock down of the HER4 receptor plus the addition of doxorubicin did neither result in a beneficial effect in regard to apoptosis nor did it affect the phosphorylation of HER3 or AKT.

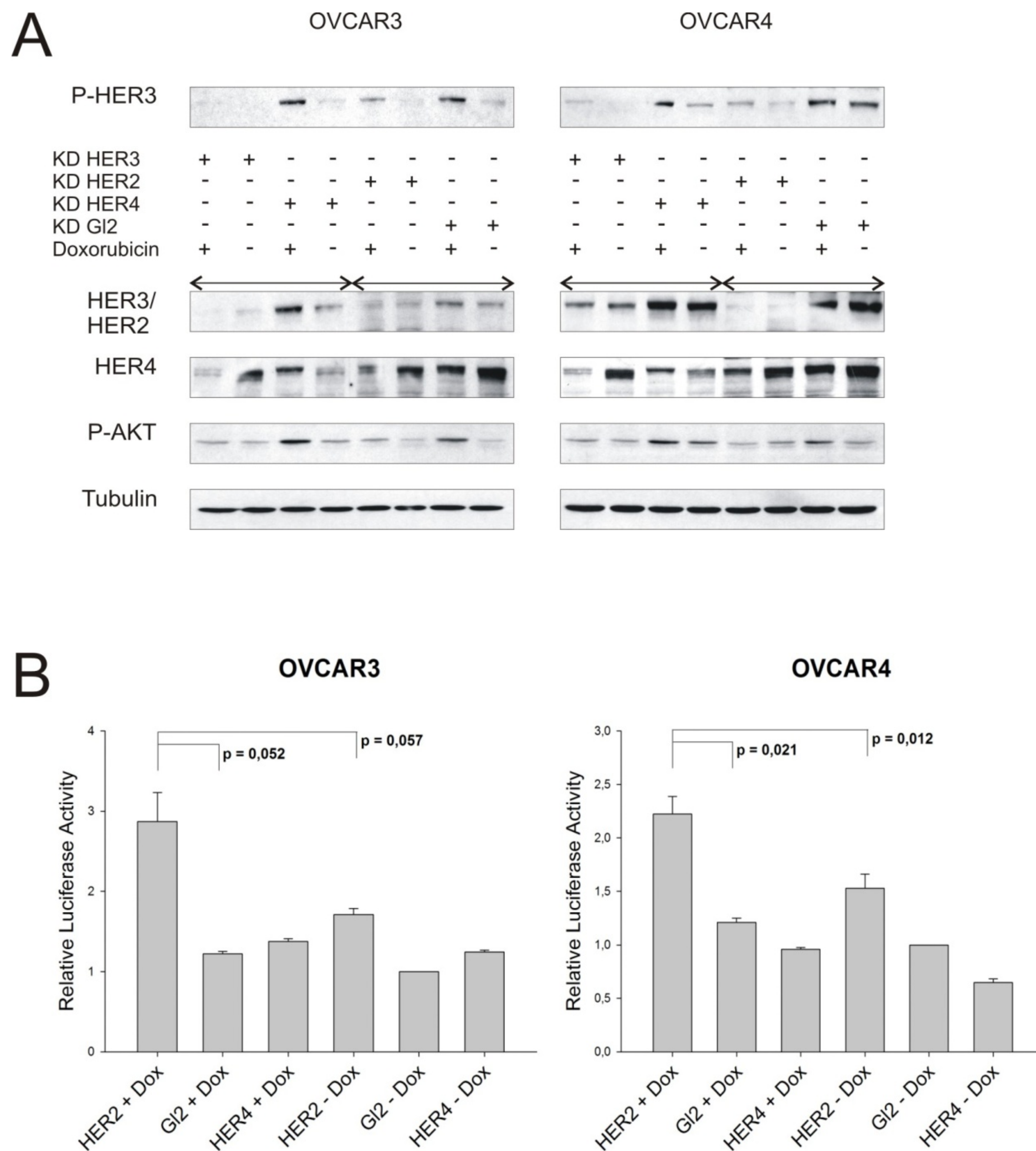


Fig. 26: Downregulation of HER2 effectively abolishes doxorubicin induced phosphorylation of HER3 and AKT and significantly increases apoptosis.

(A) Representative immunoblots of the siRNA mediated downregulation of the *HER2*, *HER3* and the *HER4* receptor compared to siRNA control (Gl2). Cells were stimulated ± doxorubicin 1 µM and were lysed after 24 hours (48 hours after the knock down was initiated). 50 µg of total protein was subjected to SDS-PAGE, blotted and probed with corresponding antibodies.

(B) Mean values ± SEM (n = 3) of caspase activation (Caspase 3/7-Glo assay) upon downregulation of *HER2* or *HER4* in combination with doxorubicin relative to siRNA control. Cells were transfected with specific siRNAs for *HER2* or *HER4* and incubated with or without doxorubicin (1 µM OVCAR3 and 2 µM OVCAR4) for additional 24 hours before analysis.

4.1.15 Cisplatin treatment increases HER3 phosphorylation while other chemotherapeutic drugs do not

As mentioned before, doxorubicin is used as second line treatment for recurrent ovarian cancer. In contrast, the administration of a platinum-based compound in combination with a taxane represents the standard first line treatment. Back in the 1980s, when most of the well characterised ovarian cancer cell lines were established, the standard chemotherapeutic drug treatment was different (Agarwal and Kaye 2003).
In Table 1, we grouped the tested cell lines depending on the chemotherapeutic pre-treatment of cancer patients from where these cells have been established. However, it was not possible to obtain information about the origin of the SKOV6 cell line.

Table 2: Analysed ovarian cancer cell lines are grouped depending on chemotherapeutic pre-treatment of patients from where they have been established. The increase in phosphorylation of HER3 and AKT is indicated and the nine different ovarian cancer cell lines are grouped based on chemotherapeutic pre-treatment of cancer patients from where cell lines have been established (Dox = doxorubicin; Cis = cisplatin; Cyclo = cyclophosphamide; Carbo = carboplatin).

Ovarian cancer cell lines used

pretreated ovarian cancer patients		pAKT	pHER3
OVCAR3	Dox/Cis/Cyclo	+	+
OVCAR4	Dox/Cis/Cyclo	+	+
OVCAR8	Carbo h.d.	-	-
SKOV3	Thiotepa	-	-
SKOV8	Dox/Cis/Cyclo	+	-
unknown origin			
SKOV6		+	+
untreated ovarian cancer patients			
OVCAR5		-	-
2774		-	-
2780		-	-

Interestingly, the OVCAR3 and OVCAR4 cell line were established from cancer patients who have been pre-treated with a combination of doxorubicin, cyclophosphamide, and cisplatin (Freedman et al. 1978; Hamilton et al. 1983; Louie et

al. 1986; Schilder et al. 1990; Provencher et al. 1993). Based on these available information, we analysed whether the observed activation of the HER2-HER3-PI3K-AKT pathway might represent a generalised mechanism for chemotherapeutic drug insensitivity, or whether the activation of this anti-apoptotic pathway is a cellular response restricted only to doxorubicin. Therefore, both cell lines were treated with cisplatin, etoposide, fluorouracil, cyclophosphamide, dacarbazine, as well as doxorubicin as positive control. Interestingly, out of this six chemotherapeutic drugs tested, only the addition of doxorubicin and cisplatin resulted in a clear increase in HER3 phosphorylation. As anticipated, no increase in P-HER3 was detected in the OVCAR8 cell line upon addition of cisplatin.

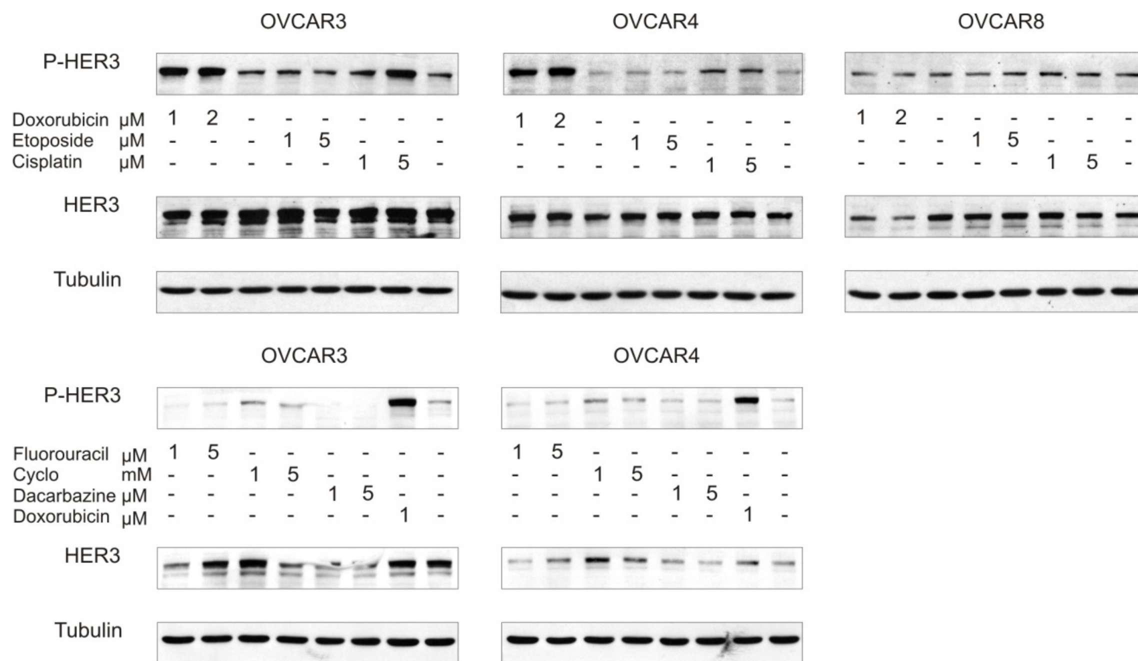


Fig. 27: Cisplatin increases phosphorylation of HER3 whereas etoposide, fluorouracil, cyclophosphamide and dacarbazine do not. Cells were treated with indicated concentrations of different chemotherapeutic drugs for 24 hours. Phosphorylation of HER3 was analysed by the P-HER3 specific antibody (P-Tyr 1289). Tubulin served as loading control.

4.1.16 Inhibition of the HER2-HER3-PI3K-AKT pathway increases the accumulation of doxorubicin

While performing several *in vitro* experiments, we observed that pellets of lysed cells previously treated with doxorubicin and inhibitors against components of the HER2-HER3-PI3K-AKT pathway, displayed a more pronounced red staining (based on the red colour of doxorubicin) when compared to pellets of cells which were treated with doxorubicin only. Based on this observation as well as on the information provided by recent publications which reported a potential crosstalk between the PI3K-AKT pathway and the expression of different ABC family members, further experiments were performed (Kuo et al. 2002; Lee et al. 2004). Due to the fluorescence activity of doxorubicin, it is possible to directly measure and compare the amount of accumulated doxorubicin upon several different treatment combinations. Therefore, cells were either treated with lapatinib, erlotinib, the PI3K inhibitor LY294002, the MEK inhibitor UO126, or DMSO in combination with doxorubicin for 24 hours, and nuclear accumulation of doxorubicin was analysed by FACS. It is well known that doxorubicin induces a strong G2 phase arrest, and therefore this G2 peak was used to compare differences in fluorescence intensity which reflects variable amounts of intercalated doxorubicin (Siu et al. 1999). Notably, treatment with lapatinib, erlotinib, or LY294002 resulted in a clear shift of the G2 peak (Figure 21), whereas the MEK inhibitor UO126 had no effect. To validate that the observed peaks indeed reflect the G2 phase of doxorubicin arrested cells, the same treatment combinations were used for a slightly modified experiment where propidium-iodide (PI) was added to the hypotonic FACS buffer (data not shown). As expected, the observed peaks in the PI handled samples reflected the signals of Figure 21 without the shift of G2 peaks. Therefore, it seems as if we can modify the cellular accumulation of doxorubicin by affecting HER2-HER3-PI3K-AKT signalling.

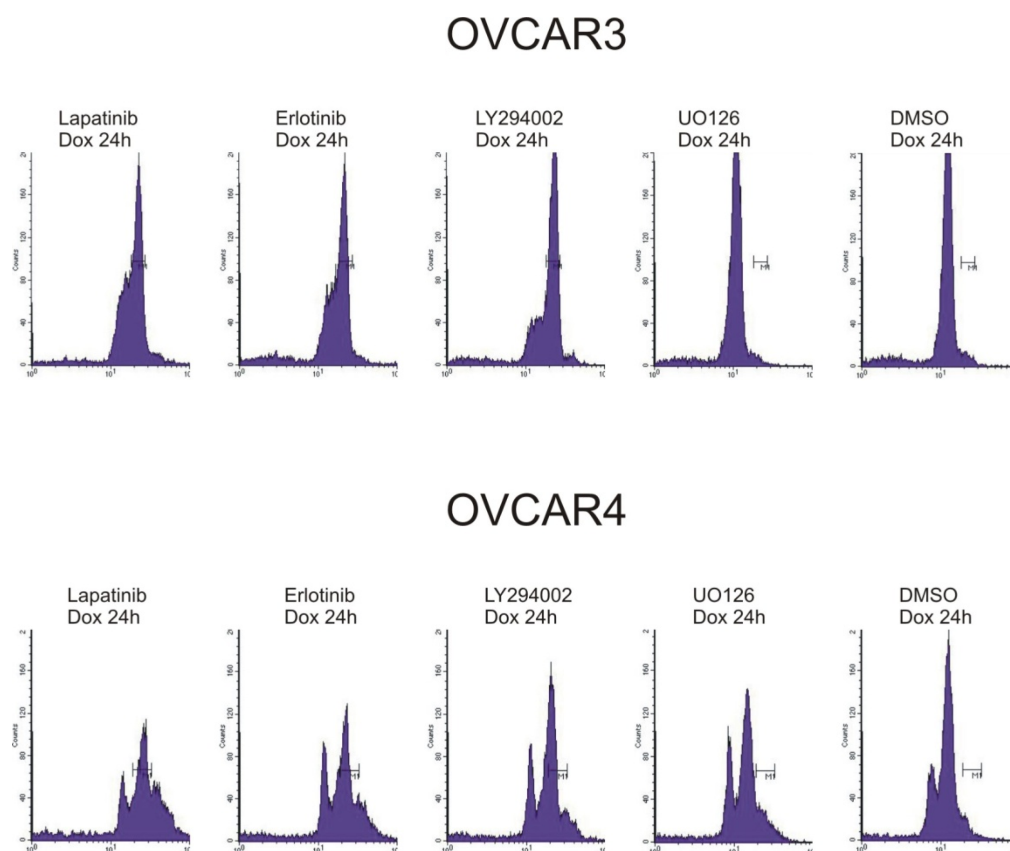


Fig. 28: Treatment of lapatinib, erlotinib, LY294002 but not UO126 increases cellular accumulation of doxorubicin. Representative histograms of cells treated with either lapatinib (10 µM), erlotinib (10 µM), LY294002 (20 µM), UO126 (10 µM) or DMSO in combination with 1 µM doxorubicin for 24 hours. The amount of nuclear doxorubicin was analysed by FACS analysis as it has been described in materials and methods. Indicated bar (M1) matches to the G2 peak of lapatinib plus doxorubicin treated cells.

4.1.17 Expression of MDR1, MRP1 and BCRP1 does not correlate with cellular sensitivity to the combinatorial drug treatment

Based on the interesting results depicted in Figure 21 and on recent publications reporting a direct interaction of lapatinib and erlotinib with members of the ABC family of transporters, we investigated the expression of *MDR1, MRP1,* and *BCRP1* in OVCAR3, OVCAR4, and OVCAR8 cells (Collins et al. 2009). Therefore, we analysed the basal expression levels of these important ATP-binding cassette transporters, which have frequently been linked to multidrug-resistance, by RT-PCR. Furthermore, a potential increase in expression upon drug (doxorubicin) treatment for 20 hours was

tested. Interestingly, the basal expression of *MDR1* was highest in the OVCAR8 cell line, which was used as “negative” control in the previous experiments, whereas the OVCAR3 cell line exhibits the lowest expression of *MDR1*. No clear difference could be observed for *MRP1* and the expression of *BCRP1* was slightly lower in the OVCAR8 cell line compared to OVCAR3 and OVCAR4. In the doxorubicin treated state, expression of *MDR1* was upregulated in the OVCAR3 and OVCAR8 cell line. *MRP1* strongly increased in OVCAR3 and was slightly affected in OVCAR8 whereas *BCRP1* expression was downregulated in all three cell lines upon doxorubicin treatment. To summarise this, expression patterns of *MDR1*, *MRP1,* and *BCRP1* did not show a correlation with the observed sensitivity to the combinatorial treatments (Figure 8), and the observed effects do not seem to be related to a direct inhibitor-transporter interaction.

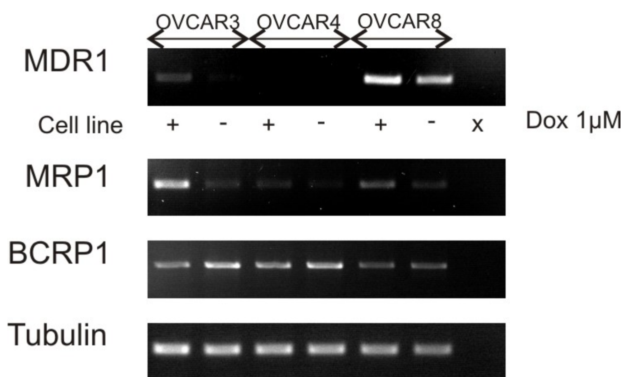


Fig. 29: Expression levels of three ABC family members in the basal and in the doxorubicin treated state. Basal expression levels of the ABC family members *MDR1*, *MRP1,* and *BCRP1* were analysed by RT-PCR in the OVCAR3, OVCAR4, and OVCAR8 cell line. Doxorubicin mediated changes in expression of this three ABC family members were investigated after 20 hours of doxorubicin treatment.

4.2 The S1119C HER3 polymorphism in pancreatic cancer cells

A previously unknown *HER3* single nucleotide polymorphism (SNP) resulting in a serine to cysteine conversion at position 1119 was detected in as many as 36 different cancer cell lines by a cDNA-based sequence analysis of the entire tyrosine kinase transcriptome performed within 254 established tumor cell lines (Ruhe et al. 2007). In 4 out of 17 (24%) pancreatic, 6 out of 53 (11%) melanoma, 4 out of 6 (50%) prostate as well as several other cancer cell lines (for further information visit **http://tykiva.bii.a-star.edu.sg/SOGdb/cgi-bin/sogweb.pl**), the above mentioned polymorphism was found in a heterozygous or homozygous manner. This S1119C conversion is located in the cytoplasmic part of the receptor outside of the predicted kinase domain, and no functional involvement of this residue has been reported so far. Nevertheless, we analysed whether this sequence alteration does somehow affect the signalling properties of the HER3 receptor. Furthermore, the effectiveness of a direct anti-HER3 or an indirect (chemotherapeutic) treatment strategy was evaluated in cell lines carrying either a serine or a cysteine at position 1119.

4.2.2 HER3 blocking antibody effectiveness does not correlate with expression of the S1119 or C1119 HER3 allele

In proliferation experiments which have been initially performed with pancreatic, prostate as well as melanoma cell lines (data not shown), pancreatic cancer cells have proven to be most sensitive towards the treatment with the HER3 specific blocking antibody 105.5. Based on these findings, we compared five pancreatic cancer cell lines including one homozygous S1119 carrier, three heterozygous C1119 carriers and the CFPAC cell line, homozygous for the C1119 allele (Figure 23). Therefore, cells were seeded in media with a reduced amount of FCS and the HER3 blocking antibody (105.5, Millipore), or the respective isotype control was added the next day at a concentration of 10 µg/ml. One hour later, cells were stimulated with 50 ng/ml of either NRG1 β1 or β2 and the efficacy of the HER3 blocking antibody in regard to the inhibition of cell proliferation was determined after 72 hours.

With the exception of PaTu 8902 (C1119 heterozygous), ligand induced proliferation of cells was effectively blocked by the HER3 blocking antibody. However, no direct correlation between treatment response and *HER3* codon 1119 sequence alteration was detected.

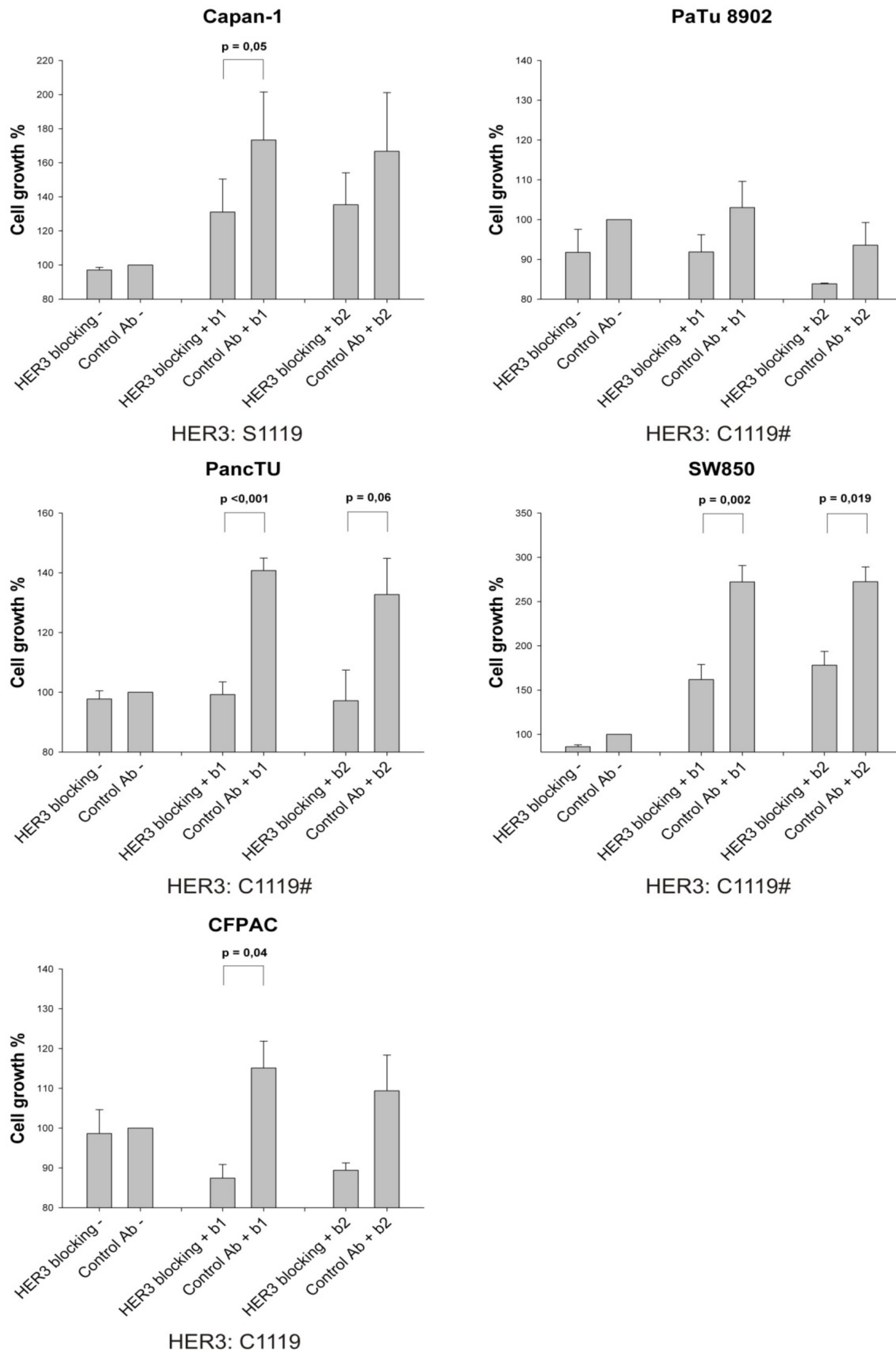


Fig. 30: Sensitivity to inhibition of proliferation by the HER3 blocking antibody 105.5 does not seem to relate to the HER3 S1119C polymorphism. Mean values and standard errors (n = 3) of pancreatic cancer cell lines seeded in 96 well plate with reduced amount of FBS (4%) stimulated with HER3 ligands and incubated with the HER3 blocking antibody 105.5. Cells were treated with the HER3 blocking antibody (105.5, Millipore) at a concentration of 10 µg/ml or with the corresponding isotype control antibody. One hour later, cell lines were either stimulated with 50 ng/ml of Heregulin β1 (b1) or β2 (b2) and the effects on cellular growth were analysed 72 hours later. # = heterozygous

4.2.3 Stimulation with HER3 ligands induces phosphorylation of HER3 and AKT in tested cell lines

Ligand induced activation of the HER3 receptor by NRG1 isoforms could result in differences of HER3 as well as AKT activity between those cell lines carrying the HER3 S1119 or the C1119 in a heterozygous or homozygous manner. Moreover, durability of the ligand induced HER3-PI3K-AKT pathway activation could be influenced by a potential impaired receptor internalisation based on the HER3 sequence alteration. In an attempt to analyse this possibility, we have chosen a relatively simple approach to gain insight into HER3-PI3K-AKT signalling differences between the five pancreatic cancer cell lines mentioned. Therefore, cells were starved for 24 hours and were subsequently stimulated with 50 ng/ml of either NRG1 α or β1 for 40 min before lysis. Moreover, remaining HER3 and AKT phospho-signal intensities initially induced by stimulation with HER3 ligand isoforms for 40 min were analysed after a additional starvation period of one hour.

Interestingly, no basal phosphorylation of HER3 and AKT was detectable after cells have been starved for 24 hours prior to the experiment. As visualised in Figure 24, the subsequent stimulation with recombinant NRG1 β1 resulted in a strong increase of HER3 and AKT phospho-signals in all five cell lines analysed. In contrast, the exogenous stimulation with the NRG1 α isoform increased phosphorylation of HER3 and AKT only marginally. However, the subsequent starvation of cells for an additional time of one hour resulted in significant reduction of P-HER3 and P-AKT signal intensity levels in all cell lines with no apparent differences between S1119 or C1119 carriers.

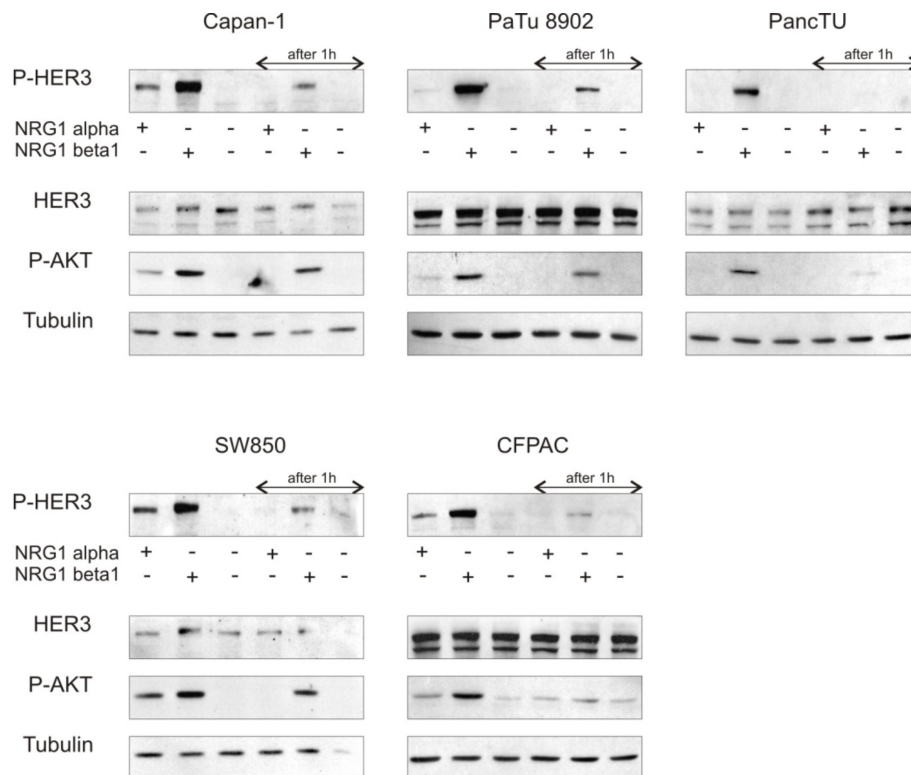


Fig. 31: Phosphorylation of HER3 and AKT can be induced by HER3 ligand stimulation. Cells have been starved with 0% FBS for 24 hours and stimulated with HER3 ligands at a concentration of 50 ng/ml for 40 min or have been stimulated for 40 min and subsequently starved for an additional time of one hour before lysis. Phosphorylation of HER3 and AKT was analysed by western blot using phospho-specific HER3 and AKT antibodies. Tubulin served as loading control.

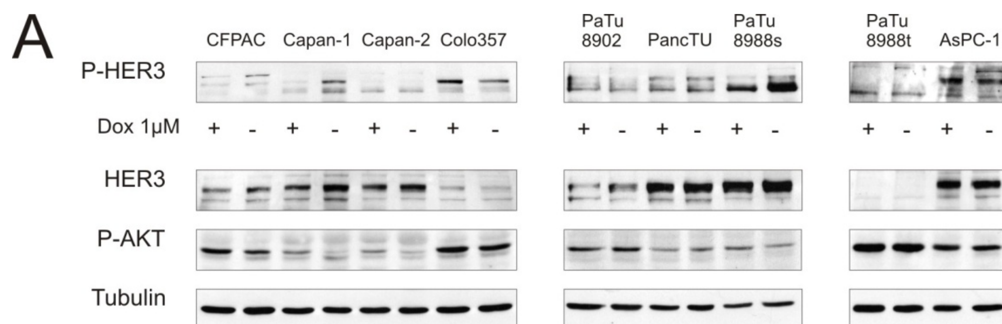
4.2.3 Doxorubicin mediated changes in HER3 and AKT phosphorylation do not correlate with the expression of the S1119 or C1119 allele

Based on the results we obtained with our ovarian cancer cell line model (see Chapter 4.1), we analysed, whether a doxorubicin mediated increase in HER3 and/or AKT phosphorylation is also detectable in the pancreatic cancer cell lines mentioned and whether the *HER3* S1119C polymorphism may affect this cellular response. Moreover, the sensitivity of these pancreatic cancer cell lines in regard to the cytotoxic effect of doxorubicin was analysed by measuring the induction of apoptosis (Caspase 3/7-Glo assay). It is important to mention that the SW850 cancer cell line was excluded from our panel of cell lines, since the pancreatic origin of these cells is not completely

clarified (Moore et al. 2001). Moreover, we added several pancreatic cell lines carrying the S1119 wt allele as well as the PaTu 8988s cell line, for which *HER3* sequence information of codon 1119 is not available.

Pancreatic cancer cells were incubated with 1 µM doxorubicin (as indicated) for 24 hours and the phosphorylation of HER3 and AKT was analysed by immunoblot (Figure 25 A). Interestingly, a slight increase in AKT phospho-signal was observed in CFPAC, Capan-1, Capan-2 as well as Colo357. However, we did not detect a doxorubicin mediated increase in HER3 phosphorylation (P-Tyr 1289) in any of the tested pancreatic cancer cell lines. To further analyse whether another PI3K binding site of HER3 might be affected by this doxorubicin treatment, we immunoprecipitated the HER3 receptor and analysed its total tyrosine phosphorylation (4G10, P-Tyr antibody). In this experiment, only those cells with an increased AKT phospho-signal upon doxorubicin addition were tested. However, we could not detect an increase in total HER3 phosphorylation.

Furthermore, we analysed the apoptotic sensitivity of cells carrying either the S1119 or the C1119 allele. Therefore, cells were incubated with 1 µM of doxorubicin for 40 hours and the induction of apoptosis was measured (Figure 25 C). However, there was no obvious correlation between cellular sensitivity and the *HER3* sequence alteration detectable.



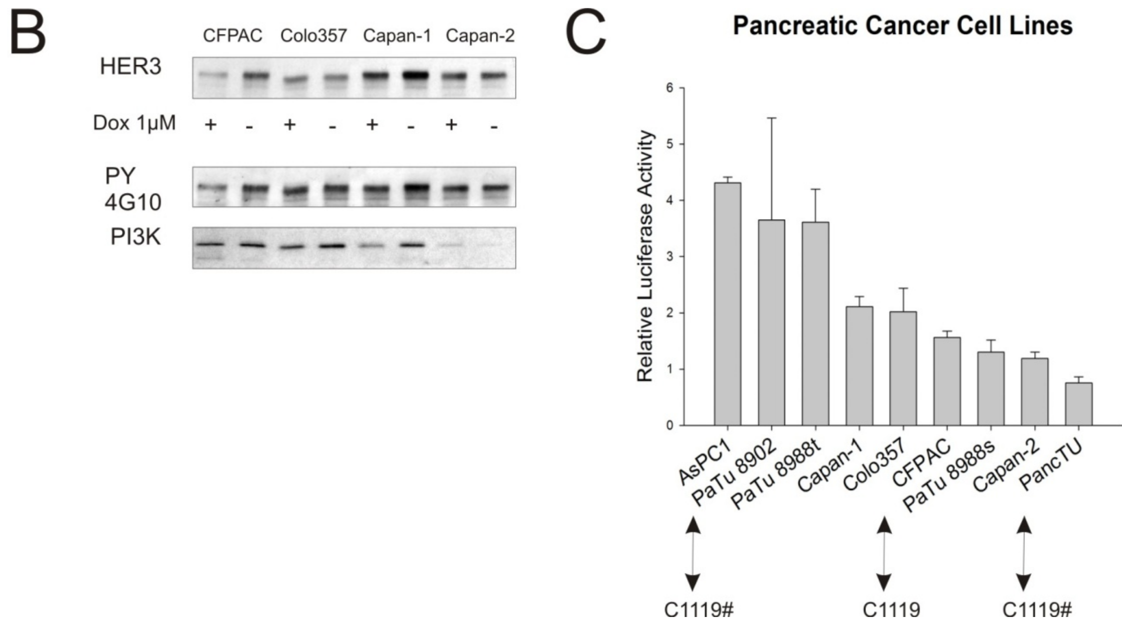


Fig. 32: Phosphorylation of HER3 is not increased upon addition of doxorubicin. No difference in doxorubicin induced apoptosis could be detected between S1119 and C1119 carrying pancreatic cancer cell lines. (A) Cells were treated with or without doxorubicin for 24 hours at a concentration of 1 µM and the phosphorylation of HER3 (P-Tyr 1289) and AKT (P-Ser 473) was analysed by western blot. **(B)** HER3 was immunoprecipitated by the 2F12 antibody from lysates of cells treated with 1 µM doxorubicin for 24 hours. Total HER3 phosphorylation was detected by the P-Tyr specific antibody 4G10. Interaction of HER3 with the p85 (PI3K subunit) after doxorubicin treatment was also analysed. **(C)** Induction of apoptosis (Caspase 3/7-Glo assay) was measured after 40 hours of doxorubicin (1 µM) treatment. Mean values and standard errors (n = 3) are shown relative to untreated corresponding cells.

5. Discussion

5.1 The HER3-PI3K-AKT pathway represents a potential target in ovarian cancer chemoresistance

In 2009, an estimated number of approximately 1.5 million new cancer cases have occurred with about half a million deaths caused by this devastating disease (Jemal et al. 2009). Despite the development of new anti-cancer therapeutics like TKI and therapeutic monoclonal antibodies, classical chemotherapy still is the principle care for the treatment of most cancers. However, chemotherapeutic drug resistance often occurs and the management of multi-drug resistant recurrent or refractory tumors represents a difficult and insuperable hurdle for treating clinicians. In this regard, ovarian tumors represent a paramount example. Although first-line response rates are about 80% most patients relapse and the 5-year survival rate of patients with advanced ovarian cancer is only 30% due to the development of drug resistance (Agarwal and Kaye 2003). Therefore, comprehensive insight into the mechanisms underlying drug resistance in ovarian cancer as well as other cancer types is of great importance for the development of novel and more efficient treatment strategies.

5.1.1 AKT is activated by the HER3-PI3K-AKT pathway in doxorubicin treated ovarian cancer cells

The PI3K-AKT pathway has emerged as an important anti-apoptotic pathway which is frequently activated in a variety of human cancers (Vivanco and Sawyers 2002). Activation of this signalling cascade has been involved in promoting resistance against several chemotherapeutic drugs (Li et al. 2005; Winograd-Katz and Levitzki 2006; Liu et al. 2007; Yu et al. 2008). Moreover, the fundamental role of the HER3 receptor as upstream activator of the PI3K-AKT pathway was recently elucidated to mediate insensitivity to TKI treatment (Engelman et al. 2007; Sergina et al. 2007). Upon mitogen stimulation, the full activation of AKT is mediated via phosphorylation of Thr308 and Ser473 by PDK1 and mTORC2, respectively (Sarbassov et al. 2005). Furthermore, DNA-PK has the potential to directly phosphorylate AKT at Ser473 upon

DNA damage (Bozulic et al. 2008; Toulany et al. 2008). In contrast to growth factor mediated AKT activation, DNA-PK dependent (direct) phosphorylation of AKT seems to represent an early cellular response to γ-irradiation or doxorubicin exposure.

Most studies on chemotherapeutic drug resistance are based on chemo-insensitive cancer cell lines generated by a continuous cellular exposure with the respective chemotherapeutic drug. Many of these reports focus on differences in the cellular expression levels of important anti-apoptotic proteins between these insensitive cell lines and their respective sensitive counterparts (Liu et al. 2007; Hui et al. 2008; Servidei et al. 2008). In our experiments, we examined nine available ovarian cancer cell lines originated from either untreated or pre-treated patients resembling the natural occurrence of chemotherapeutic resistance (Freedman et al. 1978; Hamilton et al. 1983; Louie et al. 1986; Schilder et al. 1990; Provencher et al. 1993). Interestingly, in the OVCAR3 and OVCAR4 cell line, established from patients previously treated with a combination of cisplatin, adriamycin (doxorubicin) and cyclophosphamide, a clear increase in HER3 and AKT was observed upon exposure to doxorubicin or cisplatin. In contrast, it was not possible to detect an increase in phosphorylation in cell lines which have been established from previously untreated patients (OVCAR5, 2774 and 2780). However, contrary to our expectations, there was no activation of HER3 or AKT to be observed in the OVCAR8 and SKOV3 cell line established from patients treated with high dose of carboplatin or thiotepa, respectively. As mentioned before, AKT can be either activated by ligand mediated growth factor activation, subsequent recruitment of PI3K and phosphorylation of AKT by PDK1 and mTORC2 or by direct phosphorylation of Ser473 induced by DNA-PK. However, in the OVCAR3 and OVCAR4 cell line the doxorubicin induced long-term increase in AKT phosphorylation was dependent on activation of the HER3-PI3K-AKT pathway and could be effectively blocked by using HER3 specific siRNA. Moreover, cellular apoptosis significantly increased upon combination of the PI3K inhibitor LY294002 with doxorubicin as well as by downregulation of the HER3 receptor by different siRNAs and subsequent doxorubicin treatment.

5.1.2 HER3 activation is dependent on ADAM17 and can be effectively blocked by batimastat

Due to the detected long-term doxorubicin mediated increase in HER3-PI3K-AKT pathway activity, we assumed and confirmed that the increase in HER3 phosphorylation correlates with an upregulated expression of HER3 ligands. Moreover, by stimulating MCF7 breast cancer cells with media from OVCAR3 cells, previously incubated with doxorubicin, we could further show that these HER3 ligands seem to be freely soluble and therefore have the potential to activate the HER3 receptor in an autocrine or paracrine way. All ligands of the EGFR family are generated as membrane-anchored precursor proteins that can be proteolytically cleaved by metalloproteases and are thereby released from the cell (Blobel 2005). Batimastat represents a broadband inhibitor of the ADAM family of metalloproteases and it has frequently been reported to block shedding of EGFR ligands (Dong et al. 1999; Borrell-Pages et al. 2003). We therefore enlightened the involvement of a metalloprotease by successfully abrogating the doxorubicin mediated phosphorylation of HER3 with batimastat. Furthermore, the combinatorial treatment of batimastat and doxorubicin significantly increased apoptosis when compared to single treatments. The importance of ADAM17 mediated shedding of several ligands of the EGFR family like TGFα, HB-EGF, amphiregulin and epiregulin has been elucidated by several groups (Peschon et al. 1998; Merlos-Suarez et al. 2001; Sunnarborg et al. 2002; Sahin et al. 2004). Moreover, Zhou and colleagues recently demonstrated that activation of the HER3 receptor correlates with the expression of ADAM17 in NSCLC and downregulation of ADAM17 reduced HER3 as well as AKT activity in A549 cells (Zhou et al. 2006). In concordance to this, downregulation of ADAM17 completely abrogated the doxorubicin induced increase in HER3 phosphorylation in our experiments. These results therefore fully support our assumption that the doxorubicin mediated activation of the anti-apoptotic HER3-PI3K-AKT pathway is induced by an upregulated expression of HER3 ligands and the subsequent involvement of a metalloprotease (ADAM17) in the postulated autocrine ligand activation loop.

5.1.3 Activation of the HER3-PI3K-AKT pathway is dependent on the activity of the HER2 receptor

Due to the lack of certain residues, which are essential for the catalytic kinase activity of other EGFR family members, it is widely assumed that the HER3 receptor has no intrinsic kinase activity (Hanks and Quinn 1991; Guy et al. 1994; Pinkas-Kramarski et al. 1996; Sierke et al. 1997). Nevertheless, HER3 serves as an important dimerisation partner for other EGFR family members, especially HER2 (Alimandi et al. 1995; Wallasch et al. 1995; Pinkas-Kramarski et al. 1996; Holbro et al. 2003). Therefore, we aimed to identify the dimerisation partner of HER3 responsible for the doxorubicin mediated activation of the HER3-PI3K-AKT axis in our system. Two already approved TKIs targeting the HER2 and EGFR (lapatinib, dual kinase inhibitor) or only the EGFR (erlotinib) were used in combination with doxorubicin (Pollack et al. 1999; Xia et al. 2002). Interestingly, treatment with either lapatinib or erlotinib resulted in the complete abrogation of the doxorubicin mediated increase in HER3 phosphorylation. Moreover the combinatorial treatment of either lapatinib or erlotinib in combination with doxorubicin significantly increased apoptosis when compared to single treatments. This was in striking contrast to our "negative" control cell line (OVCAR8) where no beneficial effects on apoptosis were detected. Considering the complete loss of HER3 phosphorylation and the significant increase in apoptosis observed with both inhibitors one might assume that the EGFR is responsible for the doxorubicin induced increase in HER3 phosphorylation. However, lapatinib as well as erlotinib have the potential to inhibit other members of the EGFR family besides their main targets. Lapatinib, for example, binds to the inactive form of the HER4 receptor, and erlotinib also inhibits the HER2 receptor (Schaefer et al. 2007; Guix et al. 2008; Qiu et al. 2008). Therefore, we decided to further analyse potential dimerisation/interaction partners upon doxorubicin treatment by utilising state of the art mass-spectrometry. One general hurdle when performing immunoprecipitations linked to subsequent mass spectrometry analysis with the goal to identify potential interaction partners is the huge number of detected proteins that unspecifically bind to the sepharose beads and do not represent specific interaction partners (Trinkle-Mulcahy et al. 2008). For that reason, we circumvented this problem by performing serial depletion experiments as visualised in the results part (Figure 15). This analysis confirmed the PI3K subunits p85 and p110 as specific HER3 interaction partners in the doxorubicin induced state. However, the small number of detected

interaction partners can be explained by the fact that HER3 is characterised by a large number of PI3K binding sites and serves as the main heterodimerisation partner when signalling occurs via the PI3K-AKT pathway (Schulze et al. 2005). Nevertheless, the HER4 receptor was identified by mass spectrometry as interaction/heterodimerisation partner of HER3 in the untreated state. This observation could further be validated. However, siRNA mediated downregulation of HER4 had no effect on the doxorubicin induced activation of HER3. Interestingly, a recent publication provides a possible explanation for the occurrence and importance of the HER3/HER4 interaction in the untreated state (Jura et al. 2009). Based on a structural analysis of the HER3 kinase domain, Jura and colleagues postulated that the formation of active heterodimers with other members of the EGFR family is restricted by the formation of heterooligomers between the HER3 and the HER4 receptor. Regarding HER4, a pleithora of studies have been performed and published with the goal to understand the significance of this receptor in cancer development and progression. Nevertheless, published results are diverse or even contradictory. On the one hand, several publications highlighted a pro-proliferative function of the ligand activated HER4 receptor in different cancer cell lines or when overexpressed in 3T3 cells (Weiss et al. 1997; Kainulainen et al. 2000; Starr et al. 2006; Eto et al. 2010). Moreover, HER4 seems to play an important role in MMP7 mediated mammary gland tumorigenesis (Lynch et al. 2007). On the other hand, expression of HER4 in breast cancers was associated with a favourable therapeutic outcome by a pleithora of publications (Suo et al. 2002; Witton et al. 2003; Tovey et al. 2004). Furthermore, the existence of at least four HER4 isoforms which are differentially susceptible to sequential proteolysis by TACE and γ-secretase even complicates the picture. Therefore, inconsistencies between publications might be due to the detection of these different isoforms (Koutras et al. 2009). However, a tremendous reduction of total HER4 protein (180 kDa size) was observed in our experiments upon cellular exposure to doxorubicin. As this decrease of HER4 protein was detected in both cell lines, the model postulated by Jura *et al.* represents an interesting explanation. Further experiments could enlighten the functional role of this interaction in more detail.

However, the HER2 dependency of the doxorubicin induced activation of the HER3-PI3K-AKT pathway was further elucidated by the use of specific blocking antibodies. Precisely, positive results, which have been obtained by using trastuzumab (HER2 Ab) to block the doxorubicin induced phosphorylation of HER3 as well as the ineffectivity

of cetuximab (EGFR Ab), absolutely supported our data of the TKI treatment experiments in this context. Based on this data as well as the results we obtained by using lapatinib and erlotinib, the HER2 dependence in activating the anti-apoptotic HER3-PI3K-AKT signalling cascade could be clearly demonstrated.

By screening monoclonal antibodies directed against the extracellular domain of the HER2 receptor, the murine antibody 4D5 was identified as a potent growth inhibitor of breast cancer cell lines overexpressing HER2 (Hudziak et al. 1989). Subsequent experimental strategies resulted in the development of a humanised version of the murine 4D5 antibody and finally its approval by the FDA in 1998 for the treatment of metastatic breast cancer (Carter et al. 1992). Different mechanisms of action seem to be responsible for the efficacy of trastuzumab in patients with high levels of HER2 (Nahta and Esteva 2006). Internalisation of the HER2-trastuzumab complex might represent one mechanism how antibody treatment affects signalling of HER2-overexpressing cells (Hudziak et al. 1989; Kumar et al. 1991). Nevertheless, it is still a matter of debate if trastuzumab indeed mediates downregulation of surface HER2 receptor (Austin et al. 2004). Interestingly, a recent publication by Juntilla and colleagues reported that trastuzumab effectively reduced levels of phosphorylated HER3 in a HER2 overexpresssing breast cancer xenograft model (Junttila et al. 2009). Moreover, trastuzumab was more effective than pertuzumab (Omnitarg) in abrogating constitutive ligand-independent HER2/HER3 interactions in HER2 overexpressing cells. Furthermore, trastuzumab affected ligand-induced HER2/HER3 complex formation in MCF7 cells with a moderate HER2 expression (Agus et al. 2002). Therefore, the observed abrogation of HER3 phosphorylation upon doxorubicin treatment and the subsequent addition of trastuzumab might be due to a decrease in HER2/HER3 interaction although we could not directly detect this heterodimer in our mass-spectrometry experiments. A possible explanation for this could be the instability of the HER2/HER3 heterodimer as reported by Junttila *et al.* for the ligand-independent complex. In this regard, chemical crosslinking experiments of HER2/HER3 by 3,3-dithiobis[sulfosuccinimidylpropionate] (DTSSP) could overcome this problem (Junttila et al. 2009). Nevertheless, according to our results, trastuzumab might represent an interesting option in the treatment of recurrent ovarian cancers when combined with doxorubicin. Based on this, we initiated xenograft studies with the goal to analyse a potential clinical application of trastuzumab as well as lapatinib in combination with doxorubicin.

Finally, we validated the importance of HER2 in activating the HER3-PI3K-AKT pathway upon cellular exposure to doxorubicin as well as the anti-apoptotic function of this activated pathway by performing siRNA mediated *HER2* knock down experiments. In this regard, downregulation of *HER2* abrogated the doxorubicin mediated increase in HER3 and AKT phosphorylation and increased apoptosis, whereas knock down of *HER4* did not.

5.1.4 Doxorubicin and cisplatin induces phosphorylation of HER3 while other chemotherapeutic drugs do not

Chemotherapeutic treatment failure in cancer patients can be explained by either intrinsic or acquired mechanisms of drug resistance. In contrast to the intrinsic insensitivity, which is already present prior to initial exposure to chemotherapeutic drugs, acquired resistance is based on genetic or epigenetic alterations during drug treatment. The ovarian cancer cell lines that were used in our experiments can be grouped according to the chemotherapeutic pre-treatment of patients at the time of cell line establishment. Interestingly, both cell lines (OVCAR3 and OVCAR4) responding with a strong activation of the HER3-PI3K-AKT pathway have been derived from pre-treated ovarian cancer patients. Moreover, these cells responded with an increase in HER3 phosphorylation to treatment with either doxorubicin or cisplatin, drugs previously used for the treatment of the corresponding patients, whereas there was no increase observed in cell lines established from untreated patients. Therefore, the detected HER3 activation might represent a mechanism of acquired chemotherapeutic drug insensitivity. This assumption would further explain why we could not detect an activation of the HER3-PI3K-AKT pathway in several breast cancer cell lines analysed (data not shown), which were established before chemotherapeutic drug treatment of patients. We have initiated an immunohistochemical (IHC) analysis of HER3 phosphorylation levels in ovarian tumors derived from patients which were either treated only with a platinum-based (first-line treatment) drug or a platinum-based compound and doxorubicin as second-line treatment compared to tumors of chemotherapeutically untreated patients. These experiments are important to provide additional information on the *in vivo* importance of this anti-apoptotic pathway in regard to chemoresistance in ovarian cancer.

5.1.5 Inhibition of the HER2-HER3-PI3K-AKT increases the cellular concentration of doxorubicin

The importance of the PI3K-AKT pathway and the plethora of its downstream targets, which are involved in the regulation of cell survival or death, have frequently been reported (Luo et al. 2003; Engelman 2009). However, few publications highlighted a potential crosstalk between this pathway and the regulation of ABC family member expression. For example, Kuo and colleagues demonstrated that upregulation of *MDR1* expression was detectable upon treatment of HepG2 cells with the active metabolite of hepatocarcinogen 2-acetylaminofluorecnce (2-AAF) (Kuo et al. 2002). Moreover, this increased expression was dependent on NF-κB activity downstream of PI3K and AKT. Furthermore, a study with prostate cancer cell lines revealed a direct correlation between PI3K activity and expression of *MRP1* but not of *MDR1*. These reports are in excellent accordance with our observations of an increased amount of intercalated doxorubicin when the HER3-PI3K-AKT pathway was inhibited in the OVCAR3 and OVCAR4 cell line. However, this effect was not visible when cells were treated with the MEK inhibitor UO126, which served as a negative control. In this regard, further experiments with the goal to examine expression changes of ABC family members by an activated as well as by the inhibited HER3-PI3K-AKT pathway would be of great interest in our ovarian cancer model.

In contrast to this, two recent publications reported a direct interaction of lapatinib and erlotinib with ABCB1 (P-gp) and ABCG2 (BCRP1) by measuring accumulation of chemotherapeutic agents after TKI treatment (Shi et al. 2007; Dai et al. 2008). However, due to the observed increase in apoptosis in OVCAR3 and OVCAR4 cells upon downregulation of HER3 or HER2 and combined doxorubicin treatment, this TKI-ABC transporter interaction does not seem to be the main cause for the observed apoptotic effects after addition of lapatinib or erlotinib plus doxorubicin in our system. Moreover, expression levels of *MDR1, MRP1* and *BCRP1* in untreated (without doxorubicin) as well as in treated (plus doxorubicin) OVCAR3, OVCAR4 and OVCAR8 cells do not seem to correlate with the cellular sensitivity to either lapatinib or erlotinib. In more detail, it has been frequently reported that doxorubicin is a P-gp substrate (Ueda et al. 1987; Gottesman et al. 2002). The OVCAR8 cell line used as "negative control" in a variety of apoptosis assays revealed the highest expression of *MDR1* compared to OVCAR3 and OVCAR4. Moreover, expression of *MRP1* another

ABC family member able to export doxorubicin, was higher in OVCAR8 compared to OVCAR4 (Cole et al. 1992). Finally, after incubating cells with doxorubicin, the expression of BCRP1 was approximately the same in all three cell lines tested.

5.2 The HER3 S1119C polymorphism in pancreatic cancer cells

Single nucleotide missense polymorphisms in components of different signalling pathway like the p85α regulatory subunit, the HER2, the FGFR4, the EPHB2 and many more have been frequently reported and associated with cancer initiation and/or progression (Almind et al. 2002; Bange et al. 2002; Streit et al. 2004; Ruhe et al. 2007; Li et al. 2008; Zogopoulos et al. 2008). Although the HER3 S1119C conversion detected in 36 human cancer cell lines is located outside of the predicted kinase domain, this sequence variation could still influence signalling properties of the receptor and was therefore investigated in more detail. We therefore compared different pancreatic cancer cell lines carrying either the S1119 or the C1119 allele in a heterozygous or homozygous fashion. It has been shown by several publications that, to some extent, the effectiveness of therapeutic monoclonal antibodies like cetuximab is attributable to the antibody-induced downregulation and subsequent degradation of the target receptor (Sunada et al. 1986). However, in contrast to the EGFR, the HER3 receptor is not rapidly internalised, which represents the first step of the endocytic degradation pathway (Baulida et al. 1996; Baulida and Carpenter 1997). More recently, it has been demonstrated that downregulation of HER3 is mediated by the AKT-USP8-Nrdp1 stability cascade in a ligand dependent manner (Wu et al. 2004; Cao et al. 2007). In this regard, we analysed HER3 and AKT phosphorylation intensities and the decline of the signal after subsequent starvation of cells in pancreatic cancer cell lines carrying either the S1119 or the C1119 allele. However, it was not possible to detect a difference by immunoblot analysis when cells were initially stimulated by NRG1 β1 or after a following starvation period of one hour performed to analyse the decline of signals. Moreover, there was no correlation with the S1119C polymorphism apparent although the addition of NRG1 α slightly increased phosphorylation of HER3 and AKT only in three out of the five tested cell lines. Nevertheless, we further investigated the influence of the S1119C HER3 polymorphism on doxorubicin sensitivity because the HER3-

PI3K-AKT pathway reflects an important anti-apoptotic pathway and activation of this signaling cascade was observed in ovarian cancer cell lines upon treatment with doxorubicin (see Chapter 4.1 of this thesis). However, we did not detect an increased activation of the HER3-PI3K-AKT pathway in any of the tested cell lines. Additionally, sensitivity of pancreatic cancer cell lines to cytotoxic stress was not dependent on the expression of either the S1119 or the C1119 HER3 allele.

6. Summary

In the first part of this thesis, we demonstrated that doxorubicin treatment results in an activation of the HER3-PI3K-AKT pathway in cancer cell lines which have been established from chemotherapeutically pre-treated ovarian cancer patients. We further unveiled that the doxorubicin mediated induction of this anti-apoptotic signalling cascade is based on an upregulated expression of HER3 ligands and their subsequent shedding by ADAM17 since addition of batimastat, downregulation of ADAM17 and an anti-HER3 blocking antibody treatment completely inhibited the activation of this pathway. Importantly, the doxorubicin mediated increase in HER3-PI3K-AKT activity seems to be dependent on the HER2 receptor, although we could not detect a direct interaction between HER3 and HER2. This is concluded according to our observations that treatment with lapatinib or erlotinib, the addition of trastuzumab but not cetuximab, and the downregulation of HER2 but not HER4 completely abrogated the doxorubicin mediated increase of HER3 signalling. Notably, cellular apoptosis was markedly increased by blocking the activity of this anti-apoptotic pathway at several levels. This could be observed upon incubation of cells with doxorubicin in combination with either batimastat, downregulation of HER3 or HER2 but not HER4, upon lapatinib or erlotinib treatment, and upon addition of the PI3K inhibitor LY294002. Based on these results and the likewise observed cisplatin mediated increase in HER3 phosphorylation, we postulate that activation of the HER3-PI3K-AKT pathway represents a major mechanism of acquired chemoresistance in ovarian cancer cells.

In the second part of this study, we investigated the functional significance of the HER3 S1119C polymorphism, which was discovered by the "Singapore Oncogenome Project". Therefore, we analysed several pancreatic cancer cell lines expressing either the S1119 or the C1119 allele in a heterozygous or homozygous fashion. The impact of the mentioned polymorphism on cellular proliferation upon treatment with an anti-HER3 specific blocking antibody, ligand induced signal transduction as well as cellular sensitivity to cytotoxic drug treatment was analysed. However, we could not detect a direct correlation between the cellular responses and the expression of either the S1119 or the C1119 allele.

7. Zusammenfassung

Im ersten Teil dieser Doktorarbeit konnten wir zeigen, dass eine Behandlung ovarialer Krebszelllinien mit Doxorubicin, welche aus Tumoren von Patientinnen mit einer vorhergehenden chemotherapeutischen Behandlung etabliert worden sind, die Aktivierung des HER3-PI3K-AKT Signalwegs hervorruft. Zudem konnten wir darlegen, dass die Doxorubicin abhängige Induktion dieser anti-apoptotischen Signalkaskade auf eine hochregulierten Expression von HER3 Liganden und der anschließenden ADAM17 vermittelten Spaltung zurückzuführen ist. Dies konnte anhand der Zugabe von Batimastat, der Herunterregelung von ADAM17 sowie der Behandlung mit einem gegen den HER3 Rezeptor gerichteten blockierenden Antikörper gezeigt werden, wodurch die Aktivierung dieses Signalwegs komplett unterbunden wurde. Interessanterweise scheint die Doxorubicin basierte Zunahme der HER3-PI3K-AKT Aktivität abhängig vom HER2 Rezeptor zu sein, obwohl wir keine direkte Interaktion zwischen dem HER3 und dem HER2 Rezeptor nachweisen konnten. Diese Schlussfolgerung basiert auf unseren experimentellen Ergebnissen, die zeigen, dass nach der Behandlung sowohl mit Lapatinib oder Erlotinib, der Zugabe von Trastuzumab, aber nicht Cetuximab, als auch durch die Herunterregulierung von HER2, aber nicht von HER4, die Doxorubicin induzierte HER3 Signalweiterleitung unterbunden ist. Beachtenswert in diesem Zusammenhang ist, dass wir eine Zunahme der Apoptose induzieren konnten, wenn die Aktivierung dieser Signalkaskade an verschiedenen Signalpunkten unterbunden wurde. Dies konnte sowohl für die Kombination Doxorubicin mit entweder Batimastat, der Herunterregelung von HER3 oder HER2, aber nicht HER4, nach Zugabe von Lapatinib oder Erlotinib, als auch in Kombination mit dem PI3K Inhibitor LY294002 gezeigt werden. Basierend auf diesen Ergebnissen und einer ebenfalls beobachteten Zunahme der HER3 Phosphorylierung nach Zugabe von Cisplatin postulieren wir, dass es sich bei der beschriebenen Aktivitätszunahme der HER3-PI3K-AKT Signalkaskade um einen Mechanismus der erworbenen chemotherapeutischen Resistenz in ovarialen Krebszellen handelt.

Im zweiten Teil dieser Studie untersuchten wir die funktionelle Auswirkung des HER3 S1119C Polymorphismus, welcher im Zuge des “Singapore Oncogenome Project“ entdeckt wurde. Hierzu analysierten wir Krebszelllinien des Pankreas, welche entweder

das S1119 oder das C1119 Allel exprimieren. Des Weiteren testeten wir die Auswirkung dieses HER3 Polymorphismus auf die Zellproliferation nach Behandlung mit einem spezifisch gegen den HER3 Rezeptor gerichteten Antikörpers, auf die von Liganden induzierten Signalweiterleitung und auf die zelluläre Sensitivität gegenüber zytotoxischen Medikamenten. Jedoch konnten wir keine Korrelation zwischen den Zellantworten und der Expression entweder des S1119 oder des C1119 Allels sowohl heterozygot als auch homozygot feststellen.

8. References

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9. Appendix

Abbreviations

ABC	ATP-binding cassette
ADAM	A disintegrin and metalloprotease
ATCC	American type culture collection
BB94	Batimastat
BCRP1	Breast cancer resistance protein 1
BSA	Bovine serum albumin
°C	Degree Celsius
cDNA	Copy DNA
Carbo	Carboplatin
Cis	Cisplatin
Cyclo	Cyclophosphamide
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethylsulfoxide
DNA	Desoxyribonucleic acid
Dox	Doxorubicin
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
FACS	Fluorescence activated cell sorting
FBS	Fetal bovine serum
FDA	US Food and Drug Administration
Gl2	Gl2-Luciferase
h	Hour
HB-EGF	Heparin-binding EGF-like growth factor
HER2, 3, 4	Human EGF Receptor 2, 3, 4
IgG1	Immunoglobulin G1
IP	Immunoprecipitation
kDa	Kilodalton
LC-MS/MS	Liquid chromatography based mass spectrometry
µ	Micro
m	Milli
M	Molar
MDR1	Multidrug resistance 1
MRP1	Multidrug resistance-associated protein 1
MS	Mass spectrometry
NSCLC	Non-small cell lung cancer
Ab	Antibody
min	Minute
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
P-gp	P-glycoprotein
PI	Propidium-iodide

PI3K	Phosphoinositide 3-kinase
PMSF	Phenylmethylsulfonylfluoride
P-Ser	Phospho-serine
P-Tyr	Phospho-tyrosine
RNA	Ribonucleic acid
RNAi	RNA interference
rpm	Rotations per minute
RT	Room temperature
RTK	Receptor tyrosine kinase
Ser	Serine
SDS	Sodium dodecylsulfate
SEM	Standard error of the mean
SILAC	stable isotope labeling with amino acids in cell culture
siRNA	Short interfering RNA
SNP	Single nucleotide polymorphism
TEMED	N, N, N‘, N‘-Tetramethyletylendiamine
TGFα	Transforming growth factor alpha
Tyr	Tryosine
WT	Wildtype

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