Raloxifene for the Treatment of Triple Negative Breast Cancer

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I would like to dedicate this manuscript to my stepdad Bernard without whom none of this would have been possible. I will always be indebted to you.

When you know a thing, to hold that you know it,
and when you do not know a thing, to allow that you do not know it –
this is knowledge
Confucius 551 – 479 v. Chr
**Abstract**

Triple negative breast cancer (TNBC) represents a subgroup of mammary cancers associated with particularly poor prognosis as they are refractory to currently available targeted therapy used to treat other breast cancers. Opposed In contrast to the normal 60 mg/day dose of raloxifene used to reduce the risk of estrogen receptor-α (ER)-positive breast cancer, low dose raloxifene was previously shown to be effective at reducing tumor growth in xenografts of TNBC that lack the ER expression. This study aimed to verify the TNBC growth-inhibition induced by low dose raloxifene and to investigate molecular changes within the tumor to gain insights into the mechanism of raloxifene action. For this female CD1 athymic nude mice (5-6 weeks old) were implanted with MDA-MB-468 cells (8 × 10⁶) and once a palpable tumor of approximately 200 mm³ had formed, the mice were randomly assigned into three treatment groups: 0.25% DMSO (vehicle; n=9), 0.5 mg/kg raloxifene (n=11) and 0.85 mg/kg raloxifene (n=12). Mice were treated daily for 10 weeks via oral gavage and the tumor volume was measured weekly. Upon necropsy, tumors were weighed, sliced in half and each half was appropriately prepared for immunohistochemistry (IHC) and Western blotting. Treatment with 0.85 mg/kg raloxifene treatment, resulted in a significant reduction in tumor volume of 32% compared to control, whereas the 0.5 mg/kg raloxifene dose decreased tumor size by 23%. These results were further supported by a reduction of up to 40% in tumor weight. Immunohistochemical analysis of tumor tissue showed that expression of Ki67 was significantly increased by at least 50% in tumors from both raloxifene groups. The results from the Western blot analysis showed that NF-κB expression was reduced by 37% in tumors treated with 0.85 mg/kg raloxifene. Furthermore, p38 activation was reduced by 80% in tumors treated with raloxifene compared to control. Expression of the ER was confirmed to be absent in the tumors tested. Surprisingly expression of EGFR was increased by 85% in tumors from the 0.5 mg/kg raloxifene group and by 115% in tumors from the 0.85 mg/kg raloxifene group. These results indicated that raloxifene reduced cell proliferation, mediated through a mechanism involving the inhibition of proliferative mediators NF-κB and p38 rather than acting as an ER antagonist. The possibility remains however that other proteomic or genomic alterations induced by raloxifene are responsible for the reduction in tumor growth. Overall, this study showed that raloxifene effectively reduced tumor growth in a xenograft model of TNBC at a dose 15-fold lower than the currently used dose in the clinic for ER-positive breast cancer.
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List of Abbreviations

4-OHT  4-hydroxytamoxifén
AKT  V-akt murine thymoma viral oncogene homolog 1
ANOVA  Analysis of variance
API  Activator protein
APS  Ammonium persulfate
Arg/R  Arginine
ATP  Adenosine triphosphate
BAD  Bcl- XL/Bcl-2-associated death
BCA  Bicinchoninic acid
BSA  Bovine serum albumin
CDK  Cyclin dependent kinase
CDKI  Cyclin dependent kinase inhibitor
cDNA  Complimentary DNA
Cys/C  Cysteine
Cmax  Peak plasma concentration
CORE  Continuing Outcomes Relevant to Evista
CYP  Cytochrome P450
DEPC  Diethylpyrocarbonate
DFS  Disease free survival
DMEM  Dulbecco's modified eagle media f-12 modification DMSO Dimethyl sulfoxide
DNA  Deoxyribonucleic acid
DTT  Dithiothreitol
EGF  Epidermal growth factor
EGFR  Epidermal growth factor receptor
EMT  Epithelial-to-mesenchymal transition
ER  Estrogen receptor
ERK1/2  Extracellular-signal regulated kinase 1/2
FDA  US Food and drug administration
GDP  Guanosine diphosphate
Gly/G  Glycine
Grb2  Growth factor receptor-bound protein 2
GTP  Guanosine triphosphate
HSP  Heat shock protein
IkB  Inhibitory NF-κB
IKK  IκB kinase
IHC  Immunohistochemistry
JNK  c-Jun N-terminal kinase
LBD  Ligand binding domain
MAPK  Mitogen activated protein kinase
Lys/K  Lysine
MAPKAPK-2  Mitogen-activated protein-kinase-activated protein kinase 2 MAPKK MAPK kinase
MEM  Minimum essential medium-α modification
MORE  Multiple outcomes of raloxifene evaluation
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>mTOR</td>
<td>Mammalian target of rapamycin</td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4,5-dimethylthiazil-2-yl)-2,5-diphenyltetrazolium Na2C4H4O6 Sodium tartrate</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium chloride</td>
</tr>
<tr>
<td>NaOH</td>
<td>Sodium hydroxide</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>OR</td>
<td>Odds ratio</td>
</tr>
<tr>
<td>PDK1</td>
<td>Phosphoinositide-dependent kinase 1</td>
</tr>
<tr>
<td>PH</td>
<td>Pleckstrin-homology</td>
</tr>
<tr>
<td>Phe/F</td>
<td>Phenylalanine</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositide-3-kinase PIKK PI3K-related kinase</td>
</tr>
<tr>
<td>PIP2</td>
<td>Phosphatidylinositol-4,5-diphosphate</td>
</tr>
<tr>
<td>PIP3</td>
<td>Phosphatidylinositol-3,4,5-triphosphate</td>
</tr>
<tr>
<td>PLD</td>
<td>Phospholipase D</td>
</tr>
<tr>
<td>PR</td>
<td>Progesterone receptor</td>
</tr>
<tr>
<td>PTB</td>
<td>Phosphotyrosine binding</td>
</tr>
<tr>
<td>PTEN</td>
<td>Phosphatase and tensin homolog on chromosome 10</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Realtime polymerase chain reaction</td>
</tr>
<tr>
<td>RAC</td>
<td>Related to protein kinase A and C</td>
</tr>
<tr>
<td>RHD</td>
<td>Rel homology domain</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RR</td>
<td>Relative risk</td>
</tr>
<tr>
<td>TKR</td>
<td>Tyrosine kinase receptor</td>
</tr>
<tr>
<td>S6K</td>
<td>S6 kinase</td>
</tr>
<tr>
<td>SAPK</td>
<td>Stress activated protein kinase</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>Sec</td>
<td>Second</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>Ser/S</td>
<td>Serine</td>
</tr>
<tr>
<td>SERM</td>
<td>Selective estrogen receptor modulator</td>
</tr>
<tr>
<td>SH2</td>
<td>Src homology-2</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering RNA</td>
</tr>
<tr>
<td>Sos</td>
<td>Son of sevenless</td>
</tr>
<tr>
<td>STAR</td>
<td>Study of tamoxifen and raloxifene</td>
</tr>
<tr>
<td>TCA</td>
<td>Trichloracetic acid</td>
</tr>
<tr>
<td>TEMED</td>
<td>N,N,N',N'-tetramethyl-ethane-1,2-diamine</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
</tr>
<tr>
<td>Thr/T</td>
<td>Threonine</td>
</tr>
<tr>
<td>Tmax</td>
<td>Time to reach the Cmax</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor-α</td>
</tr>
<tr>
<td>Tyr/Y</td>
<td>Tyrosine</td>
</tr>
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</table>
Chapter 1: Introduction

1.1 Breast Cancer

1.1.1 Epidemiology
GLOBOCAN estimated that there were 12.7 million worldwide cancer cases and 7.6 million cancer deaths in 2008. The continuous increases in cancer incidences are primarily because of the aging and growth of the world population (Ferlay et al., 2010). These factors, along with increases in cancer-causing behaviors, especially smoking in economically developing countries, have also contributed to the rise in the global burden of cancer. Breast cancer is the most frequently diagnosed cancer (23%, 1.38 million) and is the leading cause of cancer death among females (14%, 458,400) (Jemal et al., 2011). Around half of all breast cancer cases and ~60% of deaths are estimated to occur in economically developing countries (Ferlay et al., 2010). Following Western Europe, Australia and New Zealand together had the second highest incidence rate of breast cancer in the world (85.5 per 100,000) (Jemal et al., 2011). International variations in incidence rates are largely due to differences in reproductive and hormonal factors, and the availability of early detection services (Jemal et al., 2010). Reproductive factors that have been shown to increase breast cancer incidence include, a long menstrual history, nulliparity, recent use of postmenopausal hormone therapy or oral contraceptives, and late age at first birth (Hulka et al., 2001).

1.1.2 Classifications
Breast cancer is a very heterogeneous disease, both in its biological and clinical behavior (Hanahan, 2000). This is made apparent by the many different breast cancer classifications, all of which influence the way patients are treated. Breast cancer can be classified according to its histopathology (ductal or lobular origin (Elston et al., 1991)), its grade (degree of cancer cell differentiation (Genestie et al., 1998)), its stage (0 – 4; tumor size, lymph node status and metastasis (Singletary et al., 2002)), and its receptor status (Sotiriou et al., 2009). The latter will be introduced in detail, as it is the most relevant to the research discussed in this document. Differing hormonal receptor profiles introduce major complexities, and are in
many cases responsible for therapeutic failure in many patients (Constantinidou et al., 2010; Foulkes et al., 2010; Reis-Filho et al., 2008).

One of the coherent differences found between patients is whether the estrogen receptor-α (ER) is expressed in the breast tumor or not. The mitogenic, cholesterol derived hormone 17β-estradiol can bind to the ER and exerts its action through a complex ER signaling cascade (Matthews et al., 2003). Binding of a ligand (such as 17β-estradiol) induces conformational changes in the receptor, which leads to dimerization, migration into the nucleus, protein–DNA interactions, recruitment of co-regulator proteins as well as other transcription factors. Finally, a pre-initiation complex is formed which is necessary for transcription of protein-coding genes (Levin, 2005). These genes generally contribute to the regulation of cell proliferation and prevention of apoptosis (programmed cell death) (Cunha et al., 2004). The US Food and Drug Administration (FDA) has approved endocrine therapy with selective estrogen receptor modulators (SERMs) for the treatment of tumors expressing the ER, but not for patients with ER-negative tumors (Sengupta et al., 2008).

A tumor can also be positive for the HER-2 receptor, which is a member of the epidermal growth factor receptor (EGFR) family. This cell-surface receptor family consists of four closely related tyrosine kinase receptors namely the EGFR (or HER-1 or erbB-1), HER-2 (or erbB-2), HER-3 (or erbB-3) and HER-4 (or erbB-4). Expression of the HER-2 receptor allows treatment with the 2009 in New Zealand approved drug trastuzumab, which targets the HER-2. This monoclonal antibody blocks the cell-proliferative effects associated with HER-2 (Fenton, 2010). Furthermore, HER-2 is also the preferred dimerization partner of ligand bound EGFR (or HER-1) to convey signal transduction, leading to cell growth and differentiation (Olayioye, 2001). In 12 to 17% of all breast cancer patients however, the tumor tissue is negative for ER, the HER-2 receptor as well as the progesterone receptor (PR). This is referred to as triple negative breast cancer (TNBC) (Foulkes et al., 2010).

### 1.1.3 Triple negative and basal-like breast cancer

Expression of the ER and HER-2 provides important targets for the treatment of these breast cancers with tamoxifen and trastuzumab, respectively (Hudis, 2007; Shang et al., 2002). The subgroup of TNBC accounts for 15% of all invasive breast cancers and is more likely to be of higher histological grade and has a worse prognosis than any other breast cancer subtype (Constantinidou et al., 2010; Foulkes et al., 2010). Due to the lack of drug-targetable receptors no effective treatment regimens are currently available for TNBC so that surgery,
radiotherapy and (neo) adjuvant chemotherapy (antracycline plus taxane) are still the mainstay of treatment (Carey et al., 2007; Foulkes et al., 2010; Reis-Filho et al., 2008). TNBCs are distinct from basal-like breast cancers, even though ~80% of TNBCs are also basal-like breast cancers. These ER and HER-2 negative, basal-like tumors are characterized by gene expression profiles usually only found in the basal or myoepithelial cells of the human breast (Brenton et al., 2005). Distinctive genes were first identified by microarray analysis and include high-molecular-weight ‘basal’ cytokeratins (CK; CK5 ⁄ 6, CK14 and CK17), vimentin, p-cadherin, αB crystallin, fascin and caveolins 1 and 2 (Reis-Filho et al., 2008).

A commonly mutated gene in breast cancer patients is BRCA1, which encodes a protein called breast cancer type 1 susceptibility protein. This protein is responsible for repairing double-strand breaks in DNA (Hall et al., 1990). Consequently, loss of a functional BRCA1 gene, via a mutation, epigenetic silencing, or enhanced transcription of a negative regulator of BRCA1, promotes chromosomal instability and therefore increases the incidence of carcinogenesis (Fasano et al., 2009). It has been suggested that BRCA-mutations may impact upon the receptor profile of breast cancers as more than 75% of tumors arising in women carrying a mutation in this gene have a triple-negative phenotype, basal-like phenotype, or both (Fasano et al., 2009). BRCA1 mutation carriers may also be more resistant to common chemotherapy. A study using MCF-7 cells transfected with BRCA1-small interfering RNA (siRNA) showed that the suppression of BRCA1 significantly increased paclitaxel resistance by 9-fold (Chabalier et al., 2006).

The EGFR (HER-1) is overexpressed in up to 66% of basal-like and triple-negative tumors (Reis-Filho et al., 2008). EGFR-amplification is thought to be one of the reasons why patients with TNBC have a much worse disease outcome/survival compared to patients with an ER-positive phenotype, who lack EGFR amplification (Biswas et al., 2000; Corkery et al., 2009; Foulkes et al., 2010; Liu et al., 2011; Nicholson et al., 1990). Mutations of the EGFR-activating gene are very rare, however, in up to 25% of these cases, the EGFR gene is amplified (Reis-Filho et al., 2005; Reis-Filho et al., 2006). Overexpression of EGFR may prove to be a valid target for the treatment of TNBC, but results have been mixed so far. Many phase II studies of EGFR tyrosine-kinase inhibitors in metastatic breast cancers have had a maximal 5% response rate (Ueno et al., 2011). On the other hand, one randomized phase II study of 173 metastatic TNBC patients showed an overall response rate of 20% when either a combination of cetuximab, an anti-EGFR antibody, plus up to six 3-week
cycles of cisplatin, or cisplatin alone was given (Baselga et al., 2010). These data suggest that EGFR may be an important target for TNBC.

Risk factors for triple negative or basal-like breast cancers may differ from risk factors associated with other breast cancer types (Foulkes et al., 2010). In a study of 6370 female TNBC patients who were compared with 44,704 women with other breast cancers, it was found that TNBC patients were significantly more likely to be under age 40 (odds ratio, 1.53) and non-Hispanic black (odds ratio, 1.77) or Hispanic (odds ratio 1.23). Late-stage non-Hispanic black women with TNBC had the poorest survival rate, with a 5-year relative survival rate of only 14% (Bauer et al., 2007). Following initial therapy, peak risk of recurrence is between the first and third years and the majority of deaths occur in the first 5 years (Dent et al., 2007).

In a study of 1,424 patients (2,022 controls) it was shown that the risk for high grade basal-like (unlike low grade ER-positive) breast cancer rises with a higher body-mass index during pre-menopausal years, increasing parity, lower lifetime duration of breast feeding and also with an elevated ratio of waist-to-hip circumference (Millikan et al., 2008). These data together with the large study by Bauer et al. (2007) point towards a complex interplay of genetic and societal factors that together put African-American and Hispanic women at increased risk for both basal-like and TNBC (Foulkes et al., 2010).

1.1.4 MDA-MB-468 cells vs. MDA-MB-231 cells

Many different cancer cell lines exist and are available for research. The US National Cancer Institute (NCI)-60 Cell Line, is a list of 59 cancer cell lines derived from brain, blood and bone marrow, breast, colon, kidney, lung, ovary, prostate and skin cancers (NCI, 2012). The tumorigenic MDA-MB-468 cell line was isolated from a pleural effusion of a 51-year-old black female patient with metastatic adenocarcinoma of the breast (Cailleau et al., 1978). These cells exhibit an epithelial morphology and express the transforming growth factor alpha (TGF-α) and the EGFR. The EGFR is overexpressed and present at 1x10^6 per cell (Bates et al., 1990). However, ER, HER-2 and the PR are not expressed. The tissue donor was heterozygous for the metabolic pentose phosphate pathway enzyme glucose-6-phosphate dehydrogenase (G6PD) alleles. The cell line was identified to exhibit the class G6PD-A phenotype (Cailleau et al., 1978). Unlike patients with a G6PD deficiency, patients with G6PD-A show no hemolytic phenotype (Boultonwood et al., 2002). They rather exhibit differences in qualitative electrophoretic mobility relative to the wild-type molecule, due to a
change from asparagine to aspartic acid (Boultwood et al., 2002; Boyer et al., 1962). MDA-MB-468 cells have been described to grow in single or loosely attached groups of round cells that usually can be shaken off the flask-walls (Cailleau et al., 1978).

MDA-MB-231 cells were isolated from a pleural effusion from a metastatic adenocarcinoma of the breast from a 51-year-old Caucasian woman. This tumorigenic cell line exhibits epithelial morphology and also expresses TGF-α and the EGFR (Cailleau et al., 1978). However it does not express ER, HER-2 nor PR. MDA-MB-231 cells express the Wnt7b oncogene (Huguet et al., 1994). Unlike MDA-MB-468 cells, MDA-MB-231 cells showed a G6PD B phenotype, which like G6PD A show no hemolytic effects, have almost normal enzyme activity, a qualitative rapid electrophoretic mobility activity relative to the wild-type enzyme and a substitution mutation changing serine to phenylalanine (Boultwood et al., 2002; Boyer et al., 1962). MDA-MB-231 cells have been described as randomly growing spindle-shaped cells, which can become confluent and are distinct from fibroblasts (Cailleau et al., 1978). Importantly, the p53 gene as in most TNBC cells is mutated in both cell lines (Nigro et al., 1989). Alongside p53 mutations, Table 1-1 summarizes other important genetic mutations found in MDA-MB-468 and MDA-MB-231 cells.

| Table 1-1 Genetic mutations in two TNBC cell lines |  |
|---|---|---|---|
| **Gene** | **Mutation** | **Gene** | **Mutation** |
| **p53** | Arg273 → His | **p53** | Arg280 → Lys |
| **PTEN** | G253 → T (loss of expression) | **BRAF** | Gly464 → Val |
| **RB1** | codon 2787 deletion | **CDKN2A** | codon 471 deletion |
| **SMAD4** | codon 1659 deletion | **Kras** | Gly13 → Asp |
| **Vimentin** | Negative | **Vimentin** | Positive |

Summary of some genetic mutations identified in MDA-MB-468 cells and in MDA-MB-231 cells. Adapted from (Wellcome-Trust-Sanger-Institute, 2012)

Neither cell line expresses BRCA1 or BRCA2, nor do they carry any mutations in these genes (Elstrodt et al., 2006). In contrast, the ER-positive MCF-7 cells overexpress BRCA1
10-fold, and BRCA2 7-fold when compared with the BRCA1/2 mRNA levels of human mammary epithelial cells (Rauh-Adelmann et al., 2000).

Vimentin, a type III intermediate filament, plays a significant role in anchoring and supporting the position of organelles within the cytosol. Its expression is used as a marker of cells undergoing an epithelial-to-mesenchymal transition (EMT) during normal development and metastatic progression (Katsumoto et al., 1990). Lack of ER and presence of vimentin have been associated with adverse pathologic features such as an invasive ductal/lobular morphology, high tumor grade and poor prognosis in breast cancer (Chen et al., 2008; Heatley et al., 1993; Thompson et al., 1992). Furthermore, the relationships between the ER, vimentin and invasiveness in human breast cancer cell lines have been assessed. Using matrigel outgrowth assays, Boyden chamber chemoinvasion assays and assessment of invasiveness in nude mice, it was found that cells negative for ER, but positive for vimentin (MDA-MB-231 cells) were significantly more invasive in all tests compared to cells negative for both ER and vimentin (MDA-MB-468 cells) or cell lines positive for ER, but negative for vimentin such as MCF-7 cells (Thompson et al., 1992). These results were supported by a study assessing the malignant potential of the same cell lines (Zhang et al., 1991). It was shown that MDA-MB-231 cells in vitro as well as in vivo were consistently more aggressive with a higher malignant potential compared to MDA-MB-468 cells (Zhang et al., 1991)

1.2 Cell Signaling in TNBC

Molecular signaling networks play critical roles in the processes of tumor pathogenesis and progression. Such signaling molecules are now being recognized as therapeutic targets (Bianco et al., 2006). This section aims to discuss important signaling molecules, and their pathways that are relevant for this study and in general to TNBC.

1.2.1 Epidermal growth factor receptor

As mentioned above, 66% of patients with basal-like and triple negative breast cancers overexpress EGFR (HER-1 isoform) making it a valid target for therapeutic intervention (Reis-Filho et al., 2008). Downstream of EGFR are important cell-signaling pathways or molecules such as the phosphatidylinositol 3-kinases (PI3K)/AKT pathway, extracellular-signal-regulated kinases (ERK1/2) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (Figure 1-1C). EGFR (or HER-1 or erbB-1) is with three other
distinct, but structurally related family members (HER-2, HER-3 and HER-4), a tyrosine kinase receptor (TKR) involved in cell proliferation, differentiation and survival (Figure 1-1C) (Bianco et al., 2006; Prenzel et al., 2001; Voldborg et al., 1997). EGFR is a 170-kDa transmembrane protein, with four N-terminal extracellular domains, two of which form the ligand binding domains. The hydrophobic transmembrane anchors the receptor in the cellular membrane and connects the extracellular domain with the highly conserved tyrosine kinase, C-terminal, intracellular domain (Prenzel et al., 2001; Voldborg et al., 1997). EGFR can be bound by a number of different ligands, such as the epidermal growth factor (EGF), TGF-α, heparin-binding EGF, amphiregulin, betacellulin, epiregulin and neuregulin G2b (Normanno et al., 2003). Ligand binding induces receptor dimerization and autophosphorylation of tyrosine residues. The tyrosine residues of the C-terminal domain most commonly phosphorylated and required for activation are Tyr-992, Tyr-1045, Tyr-1068, Try-1086, Tyr-1148 and Try-1173 (Sibilia et al., 2007). They also allow the interaction with signal transducer proteins that have a Src homology-2 (SH-2) or phosphotyrosine binding (PTB) domains, such as PI3K (Mani et al., 2005). Following activation, E3 RING finger ubiquitin-ligase-CBL binds specific phosphotyrosine residues, and the ubiquitinated EGFR is internalized in clathrin-coated endosomes. These then fuse with lysosomes where proteases drive EGFR degradation. In the absence of ubiquitination by CBL, the receptor is recycled back to the cell membrane where it can undergo reactivation upon ligand binding (Mani et al., 2005).

1.2.2 PI3K/AKT pathway

The PI3K/AKT pathway has been linked to diverse cellular functions involving cell growth, proliferation, differentiation, motility, survival and intracellular trafficking (Figure 1-1C) (LoPiccolo et al., 2008). PI3K is a heterodimer consisting of a catalytic p110-subunit and an adaptor p85-subunit. The adaptor subunit associates directly with the phosphotyrosine residues of the activated EGFR via its SH-2 domain. This activates the catalytic domain of PI3K, converting phosphatidylinositol-4,5-diphosphate (PIP2), a plasma membrane lipid, to phosphatidylinositol-3,4,5-triphosphate (PIP3) (Vivanco et al., 2002). PIP3 binds to the pleckstrin homology (PH) domain of AKT, leading to its translocation to the cell membrane where it becomes activated. The tumor suppressor phosphatase and tensin homolog deleted on chromosome ten (PTEN; refer to Table 1-1), antagonizes PI3K by dephosphorylating PIP3, thereby preventing activation of AKT (LoPiccolo et al., 2008).
The serine/threonine kinase AKT is fully activated when Ser-473 in the hydrophobic motif is phosphorylated. AKT subsequently phosphorylates proteins with the commonly found RXRXX(S/T) consensus sequence (Obenauer et al., 2003). AKT reduces apoptosis by phosphorylating and thereby inactivating pro-apoptotic proteins such as BAD and caspase 9 (Bianco et al., 2006). Cell cycle progression can also be stimulated by AKT through inhibition of glycogen synthase kinase-3β (GSK-3β). This stabilizes the expression of cyclin D1, which is essential for cell cycle G1 to S-phase transition (Alao, 2007; LoPiccolo et al., 2008). Furthermore, AKT affects cell cycle progression through its inhibitory phosphorylation of the cyclin-dependent kinase inhibitor p27<sup>KIP1</sup>, opposing p27-mediated G1 arrest (Liang et al., 2002). Due to the wide array of biological effects mediated by AKT, it is commonly discussed as a drug target for many different cancer types (Courtney et al., 2010; Garcia-Echeverria et al., 2008).

**Figure 1-1 Summary cell signaling**

To illustrate the complex interactions between different pathways, the (A) Wnt/β-catenin, (B) p38 MAPK and (C) EGFR, PI3K/AKT-, ERK1/2-, and NF-κB-signaling pathways are shown together. Red crosses depict inhibitory interactions. Highlighted in yellow are proteins assessed in this study. Abbreviations: Activating transcription factor 2 (ATF-2), Mitogen-activated protein kinase kinase (MEK), MAP kinase-activated protein kinase 2 (MAPKAPK2), Murine Double Min 2 (MDM2), Phospholipase C (PLC), RAF proto-oncogene serine/threonine-protein kinase (Raf-1), Rat sarcoma (Ras). Adapted from: (Chen et al., 2009; Courtney et al., 2010; Hanahan, 2000; Karin, 2006; Olson et al., 2004; Romashkova et al., 1999; Ryan et al., 2000).
1.2.3 NF-κB

Of great importance to cancer research is NF-κB, a protein complex that promotes cell survival by controlling the transcription of many genes, whose products inhibit processes involved in apoptosis in both normal and cancerous cells (Figure 1-1C). The protein family of NF-κB contains five members: NF-κB1 (p105 and p50), NF-κB2 (p100 and p52), c-Rel, RelB, and RelA (p65), all of which share a Rel homology domain (RHD) (Ghosh et al., 2002). This domain mediates DNA binding, dimerization, and the interaction with specific inhibitors called inhibitor of kappa B (IKK or IκB kinase). The trimer IKK consists of two highly homologous kinase subunits IKKa and IKKB, and a non-enzymatic regulatory component, IKKy/NEMO (Karin et al., 2000). These inhibitors retain NF-κB dimers (p50/p65) in the cytoplasm, but upon phosphorylation and subsequent degradation of IKK proteins, NF-κB is released, and the dimer is able to enter the nucleus. Using the RHD, NF-κB can regulate transcription of diverse genes encoding cytokines, growth factors, cell adhesion molecules, and pro- and anti-apoptotic proteins (Luo et al., 2005).

Two distinct NF-κB pathways exist: the classical (canonical) pathway triggered in response to microbial and viral infections or exposure to proinflammatory cytokines, and the alternative pathway triggered by members of the TNF cytokine family (Luo et al., 2005). The classical pathway is responsible for the inhibition of apoptosis, whereas the alternative pathway is critical for the proliferation, survival and maturation of premature B cells and the development of secondary lymphoid organs, as well as osteoclastogenesis (Dejardin, 2006; Luo et al., 2005). Activation via the classical pathway leads to phosphorylation-induced degradation of the IKK trimer, which targets NF-κB dimers consisting of p65 and p50. This is mainly mediated by the IKKB subunit (of the IKK trimer) (Li et al., 1999b). This pathway can induce the expression of several anti-apoptotic/pro-survival B-cell lymphoma-2 (Bcl-2) family members such as Bcl-extra large (Bcl-XL) and Bcl-2 related protein A1/Bfl-1. Both of these prevent apoptosis by inhibiting membrane permeability transition and depolarization of the mitochondria, and cytochrome c release. Furthermore, NF-κB may target proteins from the TNF-α signaling cascade such as TNF receptor-associated factor 2 and 6 (TRAF2/6), and cellular inhibitor of apoptosis 1 and 2 (c-IAP1/2). This suppresses the pro-apoptotic effects of caspase-8, thereby increasing cell survival (Wang et al., 1998). AKT has also been shown to phosphorylate IKK, which indirectly increased the activity of NF-κB, stimulating increases in the transcription of pro-survival genes (Ozes et al., 1999; Romashkova et al., 1999).
Furthermore, NF-κB promotes cell cycle progression, by regulating the expression of genes involved in the cell cycle machinery such as cyclins D1, D2, D3 and cyclin E, and c-myc. Of particular importance for mammary gland development and breast carcinogenesis is NF-κB-induced cyclin D1 expression as shown in female mice lacking cyclin D1 (Yu et al., 2001). This cell cycle regulator is important as it forms active complexes together with its binding partners cyclin dependent kinase 4 and 6 (CDK4 and CDK6), promoting the transition from G1 to S phase by phosphorylating and inactivating the retinoblastoma protein (RB) (Alao, 2007).

1.2.4  p27

Cyclin dependent kinases (CDKs) are cell cycle regulating proteins that allow a cell to replicate. Normal progression through the cell cycle is monitored by checkpoints to ensure processes at each phase of the cell cycle have been accurately completed before progression into the next phase. By modulating the activity of CDKs, these checkpoints induce cell cycle arrest, to allow for proper repair of the defects. Mitogenic signals are first sensed by expression of the D-type cyclins (D1, D2, and D3), which bind and activate CDK4 and CDK6 during G1 phase (cells prepare to initiate DNA synthesis). The formed complex allows expression of E-type cyclins (E1 and E2) that bind and activate CDK2. CDK2-cyclin E completely inactivates pocket protein RB by phosphorylation. In the hypo-phosphorylated state, RB acts as a tumor suppressor by inhibiting cell cycle progression. The CDK2-cyclin E complex is essential for the G1 to S-phase transition (Malumbres et al., 2009). The activity of CDKs is further regulated by two families of inhibitors: INK4 proteins and the Cip and Kip family (p21, p27 and p57). p27^Kip1 is an important regulator for the progression from G1 to S-phase (Figure 1-1C). As cells exit the quiescence, p27^Kip1 bound to CDK2-cyclin E must be degraded, and newly synthesized p27^Kip1 together with p21^{waf1/cip1} facilitates the assembly, nuclear import and activation of D-type-CDKs such as cyclin D1-CDK4/6. Phosphorylation of p27^Kip1 regulates these effects. Upon cell cycle progression, the levels of p27^Kip1 drop rapidly (Alkarain et al., 2004).

Several pathways and proteins have been identified that can control the activity of p27^Kip1. EGFR has been shown to activate p27^Kip1 proteolysis in ER-negative SKBR-3 cells as well as in MCF-7 cells (Donovan et al., 2001; Lenferink et al., 2001). Furthermore, PI3K-dependent activation of AKT, phosphorylated p27^Kip1 on Thr-198, which leads to impaired nuclear import, and thus to the accumulation of p27^Kip1 in the cytoplasm (Liang et al., 2002).
1.2.5 ERK

The extracellular signal-regulated kinase (ERK) pathway is a major contributor to the control of cell proliferation, survival and differentiation (Figure 1-1C) (Kohno et al., 2006; Murphy et al., 2006; Seger et al., 1995). Binding of growth factors to transmembrane TKRs such as EGFR initiates the signaling by RAS (GTP binding protein) via growth factor receptor-bound protein 2 (Grb2) and son of sevenless (SOS), a guanine nucleotide exchange factor (Kohno et al., 2006; Seger et al., 1995). This is followed by sequential stimulation of several cytoplasmic protein kinases collectively referred to as the mitogen-activated protein kinase- (MAPK) signaling cascade (Seger et al., 1995). This leads to ERK 1 and ERK 2 (ERK1/2) activation by phosphorylation of conserved tyrosine and threonine residues. Phosphorylated-ERK (p-ERK) further phosphorylates other targets such as the transcription factor activator protein (AP)-1, caspase 9, BAD, BCl-2, c-myc, c-fos and many more (Kohno et al., 2006; Murphy et al., 2006). Due to the wide array of cell growth mediated signaling by ERK, it is a therapeutic target under development for several different cancers (Kohno et al., 2006; Shimizu et al., 2012).

1.2.6 p38

p38 also belongs to the MAPK family, however, it is structurally and functionally distinct from ERK1/2 and c-Jun N-terminal kinase (JNK) (Figure 1-1B). There are four different p38 MAPK members which share an approximate 60% amino acid sequence identity; p38α, p38β, p38γ, and p38δ (Risco et al., 2012). These MAPKs contrast in their expression patterns, substrate specificity, and sensitivities to chemical inhibitors (Meng et al., 2011).

Activation of p38 has been shown in response to many different extracellular stimuli such as UV-light, heat, osmotic shock, inflammatory cytokines (TNF-alpha & IL-1), and growth factors (CSF-1) (Zarubin et al., 2005). Furthermore, activation of p38 is not only dependent on stimulus, but also on cell type. One study has shown that insulin stimulates p38 in 3T3-L1 astrocytes, but in chick forebrain neuron cells, insulin causes downregulation of p38 (Zarubin et al., 2005). On the other hand, insulin has been shown to stimulate the activation of p38 in mouse embryonic stem cells (Han et al., 2005).

In the non-phosphorylated state, p38 is relatively inactive, but it becomes rapidly activated by phosphorylation of two Thr-Gly-Tyr motifs (Olson et al., 2004). As with all MAP kinases, p38 is also activated by dual kinases (MKK). The two main upstream kinases known to activate p38 are MKK3 and MKK6, both of which are also required for TNF-
induced p38 activation (Zarubin et al., 2005). Overexpression of MKK kinases (such as TAK1, ASK1/MAPKKK5, DLK/MUK/ZPK and MEKK4) leads to activation of both p38 and JNK pathways. An important downstream target of p38 is MAPAPK-2, which has been observed to activate heat shock protein 27 (Hsp27), lymphocyte-specific protein 1 (LSP1), cAMP response element-binding protein (CREB), transcription factor ATF1, SRF, and tyrosine hydroxylase (Zarubin et al., 2005). This eventually leads to a very complex signaling network, which is intricately entangled in cellular processes like cytokine production, cell differentiation, cell senescence, apoptosis, cell-cycle arrest and tumor suppression. The arrays of cellular responses have been shown to be heavily dependent on cell type and stimuli (Lenassi et al., 2006). These factors make the role of p38 in cancer development as well as treatment, very complex (Lenassi et al., 2006; Wagner et al., 2009).

High expression of p38 has been correlated with invasive and poor prognostic breast cancers. In a study of 11 intraductal, Grade II or III breast tumors, the levels of p38 enzyme activity were found to be approximately 40% higher compared to their adjacent control tissue. In a study of 96 lymph node-positive breast carcinoma patients, the levels of phosphorylated-p38 (P-p38), HER-2 and Ki67 were measured using immunohistochemistry (IHC) prior to initiation of adjuvant chemotherapy with fluorouracil, doxorubicin and cyclophosphamide. The results showed that p38 activation occurred in 20% of the 96 carcinomas. These carcinomas also had a high Ki67 index, which correlated with a significantly shorter progression free survival (Esteva et al., 2004). In a study of 42 effusions and 51 corresponding solid breast tumors (23 primary, 28 metastases), the expression of activated p38, ERK and JNK was investigated. It was shown that nuclear expression of P-p38 and P-JNK was significantly higher in effusions compared with primary tumors and lymph node metastasis (Davidson et al., 2006). Furthermore, quantitative analysis showed that a higher p38 activation ratio significantly correlated with shorter overall survival (60% lower). The authors concluded that p38 might be a potential prognostic marker for patients with breast cancer effusions (Davidson et al., 2006).

Recently, Chen et al. (2009) investigated the role of p38 in regulating breast cancer cell proliferation. For this, p38 signaling was blocked in several breast cancer cell lines (MDA-MB-468, MDA-MB-231 cells and MCF-7 cells) using dominant-negative constructs, siRNAs, and small molecule inhibitors of p38. It was shown that blockade of p38 in MDA-MB-468 cells (and other ER-negative, p53 mutant cell lines) reduced the cell number by approximately 90% over 9 days compared to control MDA-MB-468 cells. This observation was however not seen in ER-positive, p53 wild-type cells. The authors proposed that while
p38 may function as a regulator of survival in the context of p53 wild-type, it acts as a crucial regulator of cell proliferation when cells express mutant p53. These results showed that development of p38 specific inhibitors may be effective in breast cancer patients exhibiting a p53-mutant and triple-negative molecular profile (Chen et al., 2009).

Furthermore, studies have shown that a complex interaction between NF-κB and p38 exists (Bhat et al., 2002; Craig et al., 2000; Saha et al., 2007; Sakai et al., 2002; Vanden Berghe et al., 1998). The association between the two has been shown to be important for the induction of the interleukin-6 gene (Craig et al., 2000), whose protein product is commonly raised in metastatic cancer patients (Barton, 2005). Blocking p38 with specific inhibitors reduces expression of NF-κB target genes, as well as NF-κB-driven transcriptional activity (Saha et al., 2007). In the mouse fibrosarcoma cell line L929, the p38 inhibitor SB203580 (10 µM) was able to repress TNF-stimulated expression of the interleukin-6 gene, as well as of a NF-κB-dependent reporter gene construct (Vanden Berghe et al., 1998).

The association of NF-κB with coactivators following nuclear import depends on posttranslational modifications such as acetylation. Subsequently the formation of a transcription factor complex occurs, which can bind to DNA and initiate transcription (Chen et al., 2004). It has also been shown that p38 acetylates the NF-κB-coactivator p300 at lysine 310. The p38 inhibitor SB203580 (10 µM) prevented acetylation of p300 and transcriptional activity of NF-κB (Saha et al., 2007).

1.2.7 Beta-catenin

The Wnt/β-catenin pathway has been shown to play key roles in normal embryonic development as well as in tumorigenesis. This pathway also regulates cell proliferation, migration, and differentiation (Figure 1-1A) (King et al., 2012). Secreted Wnt glycoproteins bind to the low-density lipoprotein receptor-related protein 5/6 (LRP5/6) and to Frizzled, a seven-pass transmembrane receptor protein, to activate the Wnt/β-catenin signaling pathway (Dann et al., 2001; King et al., 2012). One of the key downstream elements of this pathway is β-catenin (King et al., 2012). Phosphorylation of cytosolic β-catenin leads to its degradation, thereby preventing its path to the nucleus (Orford et al., 1997). Wnt signaling is assumed to inhibit the phosphorylation of β-catenin at Ser-45, Ser-33, Ser-37, and Thr-41 thus inducing its cytosolic accumulation and allowing β-catenin to escape the degradation of the multiprotein complex composed of the tumor suppressor proteins adenomatous polyposis
coli (APC), Axin, and GSK-3β in order to enter the nucleus (Behrens et al., 1998; Orford et al., 1997; Salic et al., 2000). There it binds to transcription factor-4 (TCF-4), which then associates with the T-cell factor/lymphocyte enhancer factor (TCF/LEF) family of transcription factors to activate Wnt/β-catenin-responsive genes including c-myc, cyclin D1, and Axin2 (Behrens et al., 1996; Lustig et al., 2002; Seidensticker et al., 2000).

Wnt/β-catenin signaling has important implications in different stages of mammary gland development as well as in mammary tumorigenesis. Furthermore, this pathway has specifically been identified to play crucial roles in TNBC development and progression (King et al., 2012). One recent study using in situ breast cancer tissue microarrays containing luminal A, luminal B, HER-2-positive and ER-negative and basal-like breast cancers, analyzed β-catenin subcellular localization. In the 32 basal-like breast cancers analyzed, the expression of nuclear and cytosolic β-catenin was significantly different to all other tumor subtypes. High cytosolic β-catenin staining was also significantly associated with worse overall survival, with an adjusted hazard ratio of 2.91 (95% CI: 1.48–5.73; p = 0.002) (Khramtsov et al., 2010). Another study performed IHC on a tissue microarray containing 245 invasive breast carcinomas from uniformly treated patients. The results showed that β-catenin activation was preferentially and significantly only identified in TNBCs. This was also associated with poor clinical outcomes (Geyer et al., 2011). Collectively, this suggests that β-catenin may be an attractive pharmacological target for this aggressive breast cancer subtype (Khramtsov et al., 2010).

### 1.3 Apoptosis

To maintain homeostasis in a multicellular organism, an intricate balance between cell proliferation and cell death is essential. Several different types of cell death have been described: apoptosis, cell death associated with autophagy and necrosis. Regardless of cell death type, the final stage of a dying or dead cell is the engulfment by phagocytes. Clearance of dead cells is of absolute importance to maintain proper homeostasis of an organism (Cotter, 2009).

During apoptosis, well-defined morphological changes occur that allow its distinction from other cell death types. These include plasma membrane-blebbing, chromatin condensation, nuclear fragmentation, and formation of apoptotic bodies (budding). Cell death can also be characterized by biochemical criteria such as protein cleavage, protein cross-linking, DNA breakdown, differing kinetics of phosphatidylserine exposure on the outer leaflet of the
plasma membrane, changes in mitochondrial membrane permeability and the release of intermembrane space mitochondrial proteins. Furthermore, caspase-dependent activation and nuclear translocation of a caspase-activated DNase resulting in internucleosomal DNA cleavage also characterize apoptosis from other cell death types (Elmore, 2007; Krysko et al., 2008).

1.4 Angiogenesis

One requirement for a tumor to grow and progress is the sufficient and consistent supply of nutrients and oxygen, as well as the removal of metabolic wastes and carbon dioxide (Hanahan, 2000; Hanahan et al., 2011). This constant burden of a tumor to strive for survival and growth is in part achieved by angiogenesis (Figure 1-2). This process is responsible for the formation of a tumor-associated neovasculature. Angiogenesis requires existing blood vessels from which to sprout, whereas vasculogenesis is the production of tube-like assemblies from new endothelial cells (Hanahan, 2000). Following embryogenesis, these vascular activities become largely quiescent (except during wound healing and menstruation). During tumorigenesis however, a so called “angiogenic switch” is almost always turned on, leading to continuous angiogenesis. This instructs endothelial cells to shift from the normal, quiescent state to undergo active cell proliferation. This helps to sustain further neoplastic growth (Hanahan, 2000; Hanahan et al., 2011).

Angiogenesis is initiated by the recruitment of progenitor endothelial cells from the bone marrow, which then proliferate, migrate and invade the extracellular matrix (Greenberg et al., 2010). The newly formed blood vessels require maturation and stabilization. Inducers and inhibitors tightly regulate this entire process (Greenberg et al., 2010; Hanahan, 2011). A well-known inducer of angiogenesis with important roles in cancer development and treatment is the vascular endothelial growth factor-A (VEGF-A). This factor can bind to its TKRs (VEGFR-1-3) to mediate endothelial cell proliferation and regulation of vascular permeability, altering the extracellular matrix to further promote angiogenesis. Its expression can be upregulated by oncogenic signaling and hypoxia (Hanahan et al., 2011). Many growth factors and cytokines are the result of VEGF production such as EGFR, platelet derived growth factor, TNF-α, TGF, insulin-like growth factor, interleukin 1-α, and interleukin-6. Tumors are unable to grow any further than a few millimeters without new vasculature (Greenberg et al., 2010).
Figure 1-2 Angiogenesis and VEGF signaling

(A), the VEGFR has multiple downstream pathways that collectively, ultimately control angiogenesis. (B), simple schematic of angiogenesis, emphasizing the importance of sufficient blood and nutrient supply/waste disposal, for tumor growth. Glycoprotein CD105 is expressed in actively dividing endothelial cells and therefore aids as a marker of angiogenesis (also assessed in this study). Abbreviations used in (A): endothelial nitric oxide synthase (eNOS), nitric oxide (NO), Cell division control protein 42 (Cdc42), heat shock protein 27 (Hsp27), phospholipaseCγ (PLCγ), inositol 1,4,5-trisphosphate (IP3), Prostacyclin (PGI2). Proteins highlighted in yellow have been assessed in this study. Figure adapted from (Rini et al., 2005; Valdes et al., 2008).

The gold standard of assessing the neovascularization of tumors both preclinically and clinically, is by counting the microvessel density (MVD) within tumor sections (Nico et al., 2008). MVD correlates with VEGF expression in cancers, and in comparison with benign breast tissue the levels of VEGF and VEGFR are significantly higher in ductal carcinoma in situ and even higher in invasive disease (Guidi et al., 1997; Toi et al., 2001). In a study of 845 primary breast tumors of patients who developed a recurrence during the follow-up, the levels of VEGF were measured with ELISA. All of the patients received either tamoxifen (n = 618) or cyclophosphamide, methotrexate, 5-fluorouracil or 5-fluorouracil, adriamycin, cyclophosphamide chemotherapy (n = 227) as first-line systemic therapy after diagnosis of advanced disease. The results showed that increased expression of VEGFR was associated
with a significantly poorer response to hormonal or chemotherapy (Foekens et al., 2001). Another study investigated the differences between intratumoral levels of VEGF and survival of patients with TNBC and non-TNBC. It was shown that TNBC had significantly higher VEGF levels and a significantly shorter recurrence-free, and overall survival period with a shorter time from diagnosis to relapse and from relapse to death (7.5 months versus 17.5 months). Furthermore the levels of VEGF correlated with poor outcomes regardless of tumor size, nodal status, histologic grade, type of relapse (distant vs. local) or age of the patient (Linderholm et al., 2009).

Endoglin (CD105) is a 180 kDa homodimeric integral membrane glycoprotein which is vitally and primarily expressed in vascular endothelial cells of capillaries, arterioles and venules (Gougos et al., 1992). Its usefulness in cancer research comes from the fact that CD105 is expressed at low levels in resting, non-active endothelial cells; however, during angiogenesis its expression is upregulated. This also coincides with increases in cell cycle activation (G1 phase) and cell proliferation (S-phase and G2-M phase) (Burrows et al., 1995; Perez-Gomez et al., 2010). Therefore CD105 antibodies can be used as a marker to analyze MVD (Fonsatti et al., 2001). In a study of 905 breast carcinomas, the prognostic significance of angiogenesis as evaluated by CD105 expression was compared to CD31 expression (another angiogenic marker). The results showed that CD105 provided a significantly stronger prognostic indicator compared to CD31 vessel labeling (Dales et al., 2004).

1.5 Raloxifene

1.5.1 General drug information

Raloxifene hydrochloride (Evista) is a second generation selective estrogen receptor modulator (SERM) that is given orally at a recommended dose of 60 mg/day (Cauley et al., 2001).

Absorption: Peak plasma concentrations (T\text{max}) are reached after 30 min following administration, however, raloxifene has a considerably lower bioavailability (60%) compared to the first generation SERM tamoxifen (~100%) (Hochner-Celnikier, 1999; Snyder et al., 2000). Raloxifene undergoes extensive first-pass metabolism, forming glucuronide conjugates that are highly bound to plasma proteins albumin and \(\alpha_1\)-acid glycoprotein. Like estrogen, raloxifene, undergoes enterohepatic circulation (Hochner-Celnikier, 1999; Snyder et al., 2000), which would involve biliary excretion of glucuronides
into the intestine, followed by enzymatic hydrolysis by β-glucuronidase and reabsorption of the aglycone (Kemp et al., 2002). This further decreases the absolute bioavailability of raloxifene to just 2% (Snyder et al., 2000; Wiebe et al., 1988). A normal dose of 60 mg results in a mean peak C\text{max} of 0.5 ng/mL, whereas multiple doses of 60 mg are expected to produce a mean C\text{max} of 1.36 ng/mL. However, the area under the plasma concentration-versus time curve (AUC) does not change significantly when multiple doses are given (Snyder et al., 2000).

**Distribution:** The SERM raloxifene is widely distributed in tissues, and its volume of distribution is 2348 L/kg (not dose dependent) following a single oral dose of 30-150 mg. It is distributed into the liver, serum, lungs and kidneys (Snyder et al., 2000).

**Metabolism:** Raloxifene is metabolically activated and converted into the active form in the liver, lungs, spleen, bone, uterus and kidneys (Snyder et al., 2000). In the circulation, apart from traces of raloxifene only raloxifene conjugates 4′-β-glucuronide, 6′-β-glucuronide, and 6′,4-β-diglucuronide can be found. Due to the absence of other metabolites, this indicates that the parent compound is not metabolized by the cytochrome P450 enzyme system (Hochner-Celnikier, 1999; Knadler et al., 1995). Only small amounts of less than 1% of free raloxifene can be detected in the circulation following a single dose. The half-life (t\text{1/2}) of raloxifene at steady state has been shown to range from 15.8 to 86.6 h with an average of 32.2 h(Snyder et al., 2000).

**Elimination:** Raloxifene is primarily secreted in the feces (Knadler et al., 1995). Its glucuronide metabolites are eliminated in the biliary tract, and further broken down by bacteria to the parent drug (Snyder et al., 2000). Under 6% of glucuronide metabolites are excreted in the urine and less than 0.2% of raloxifene is eliminated in the urine unchanged (Snyder et al., 2000).
Figure 1-3 Structure of raloxifene

The 2D (left) and 3D (right) structure of raloxifene is shown. IUPAC name: [6-hydroxy-2-(4-hydroxyphenyl)-1-benzothiophen-3-yl]-[4-(2-piperidin-1-ylethoxy)phenyl]-methanone (C_{28}H_{27}NO_4S; 473.58 g/mol). This benzothiophene has a half-life at steady state ranging from 16.8 to 86.6 h. Chemical structures and chemical properties were obtained from PubChem (National Center for Biotechnology Information), CID 5035 (accessed February 2012).

In terms of important drug-drug interactions, cholestyramine (an anion exchange resin used to reduce total and LDL-cholesterol levels) interferes with the pharmacokinetics of raloxifene in a way that the absorption and enterohepatic circulation of raloxifene is reduced by 60%. For this reason, concomitant administration of cholestyramine and raloxifene should be avoided (Snyder et al., 2000). Interestingly and importantly tamoxifen has been shown to dangerously interact with warfarin (anticoagulant for deep vein thrombosis to maintain prothrombin time at ~20-25 sec) however, in contrast raloxifene displays no such interaction. In a study by Tenni et al. (1989) the effects of warfarin and tamoxifen in a female patient were assessed. The patient received three daily doses of warfarin (10, 10 and 5 mg) and seven weeks later received 40 mg of tamoxifen. The prothrombin time the following day increased from 19 sec to 50 sec causing her to develop a subdural hematoma. The potential mechanism may be the competition of both compounds for plasma protein binding (Tenni et al., 1989). On the other hand raloxifene (120 mg/day) did not cause such pronounced increases in prothrombin time. Only a 10% increase in the prothrombin time compared to warfarin given alone was observed (Miller et al., 2001). These studies show that raloxifene has significant advantages over the first generation SERM tamoxifen in terms of adverse side effects induced by drug-drug interactions. The FDA first approved raloxifene in 1997 for the prevention of osteoporosis. In 2007 raloxifene received FDA approval for the
reduction in the risk of invasive breast cancer in postmenopausal women with osteoporosis and in postmenopausal women at high risk for invasive breast cancer (NCI; ‘FDA Approval for Raloxifene Hydrochloride’). The first large clinical study to investigate the risk reduction of invasive breast cancer was the Multiple Outcomes of Raloxifene Evaluation (MORE) trial (Cummings et al., 1999). This randomized, placebo-controlled, double-blinded, multinational treatment study in 5,133 postmenopausal women, showed that after a median of four years of treatment, raloxifene reduced the incidence of invasive breast cancer by 71% compared with placebo (HR: 0.29; 95 percent CI: 0.15, 0.56) in women at high risk (Cummings et al., 1999). Of the 2,557 women treated with raloxifene, 11 developed invasive breast cancers compared to 38 women of 2,576 women treated with placebo. More specifically, raloxifene reduced the incidence most significantly in women who developed ER-positive breast cancers, with a relative risk of 0.10 (95% CI: 0.04-0.24). However, raloxifene did not change the risk of ER-negative breast cancers, with a relative risk of 0.88 (Cummings et al., 1999; Dickler et al., 2001).

The Continuing Outcomes Relevant to Evista (CORE) trial was a follow-up study conducted in a subset of 4,011 postmenopausal women who were originally enrolled in the MORE trial (Martino et al., 2004). Following a median of three additional raloxifene treatment years, the incidence of invasive and ER-positive breast cancer was reduced by 66% (HR: 0.34; 95% CI: 0.18 to 0.66) and 59% (HR: 0.41; 95% CI: 0.24 to 0.71) respectively. However, the incidence of invasive ER-negative breast cancer in women who received raloxifene was not statistically significantly different from that in women who received placebo (HR: 1.13, 95% CI: 0.29 to 4.35; p = 0.86) (Martino et al., 2004).

Although the study design of the double-blinded Study of Tamoxifen and Raloxifene (STAR) trial is questionable, including the chosen publication process by press release (Centres, 2006), the results nevertheless suggested that raloxifene (60 mg/day) was equally as effective as tamoxifen (20 mg/day) at reducing the risk of invasive (ER-positive) breast cancer in high risk patients (included 19,747 post-menopausal women of all races). The incidence for invasive ER-positive breast cancer with raloxifene treatment was 4.4 per 1000 women, compared to 4.3 in women treated with tamoxifen. The advantage of raloxifene over tamoxifen is due to its significant reduction in endometrial cancer risk (RR: 0.16; 95% CI: 0.09–0.29). The STAR trial confirmed previous results by the MORE and CORE study that raloxifene does not have have a statistically significantly effect the incidence of ER-negative breast cancer (Vogel, 2009).
In the international, multicenter, randomized, double-blinded, placebo-controlled Raloxifene Use for The Heart (RUTH) trial, 10,101 postmenopausal women with an increased risk of coronary heart disease (CHD), received either 60 mg/day of raloxifene (n=5044) or placebo (n=5057). The women were followed up for a median of 5.6 years. The results showed that raloxifene reduced the risk of vertebral fractures by 35%, but not of non-vertebral fractures (Barrett-Connor et al., 2006). In contrast, the results on cardiovascular disease were less certain. Treatment with raloxifene had no significant effect on the primary end point, coronary events; however, it significantly increased the risk of venous thromboembolism (VTE) by 44%. Unexpectedly the incidence of fatal stroke was 49% higher (20 more deaths) in the raloxifene group compared to the placebo group. The increase in fatal stroke was considered to be an “outlier” and has not been shown in any other raloxifene study and was therefore not explainable. On the other hand raloxifene decreased death-rates, from other non-cancer or non-cardiovascular diseases, even more significantly (37 fewer deaths in the raloxifene group). Furthermore, this study also showed that the risk of developing invasive ER-positive breast cancer was reduced by 55% in women that were treated with raloxifene. Conversely there were also no statistically significantly results regarding the effects of raloxifene on the incidence of ER-negative breast cancer (Barrett-Connor et al., 2006). Regardless of the increase in stroke, the results from all the above-mentioned clinical trials suggest that raloxifene, is overall, as safe as, and at least equally as effective as tamoxifen, at reducing the risk of invasive ER-positive breast cancer. Again it should be highlighted that the clinical trials using 60 mg/day of raloxifene were ineffective at reducing the incidence of developing ER-negative breast cancer.

1.5.2 Classical raloxifene/SERM mechanism

Two estrogen receptors exist (ER-α and ER-β) which are both members of the nuclear receptor superfamily, a family of ligand-regulated transcription factors (Nilsson et al., 2001). Both receptors share a high degree of sequence conservation within their DNA binding domains (DBD), where in particular the P-box, a motif situated within the DBD, is identical in amino acid sequence between the two receptors. This P-box is required for receptor–DNA recognition and specificity (Brzozowski et al., 1997; Nilsson et al., 2001). Another conserved region in both receptors is the ligand binding domain (LBD) where both receptors have been shown to display similar affinities for the endogenous 17β-estradiol (Kuiper et al., 1997). However, different natural compounds and subtype specific ligands have been reported to have differing affinities for the two receptors (Kuiper et al., 1998; Sun et al.,
In the absence of a ligand, the ER is associated with a large heat shock protein complex in either the nucleus or the cytoplasm (McDonnell et al., 2010). Binding of a ligand induces conformational changes in the receptor which leads to dimerization, protein–DNA interactions, recruitment of co-regulator proteins as well as other transcription factors, and finally the formation of a so called preinitiation complex which is necessary for transcription of protein-coding genes (Brzozowski et al., 1997; McDonnell et al., 2010; Nilsson et al., 2001). This way 17β-estradiol can regulate the transcription of certain genes positively or negatively, thereby affecting protein synthesis, cellular growth and metabolism (Nilsson et al., 2001; Rachez et al., 2001).

The ER-α is predominantly expressed in the uterus, liver, kidney, and heart, whereas the ER-β is expressed primarily in the ovary, prostate, lung, gastrointestinal tract, bladder, and hematopoietic and central nervous systems (Nilsson et al., 2001). Both receptors are co-expressed in several other tissues such as the mammary gland, epididymis, thyroid, adrenal, bone, and specific regions of the brain (Nilsson et al., 2001). Both receptors contain two transactivation functions (AFs), which are situated at either N- or the C-terminal domain (NTD or CTD) respectively (Ascenzi et al., 2006; Nilsson et al., 2001). AF-1 serves ligand independent activation functions whereas AF-2 has ligand dependent activation functions and is located within the LBD (at the CTD). These AF regions not only contribute to estrogen-mediated signaling but also mediate cell- and promoter-specificity. Interestingly it has been shown that specifically the AF-1 region is very active in ER-α on a variety of estrogen responsive promoters but activity is minimal in ER-β. It should also be mentioned that both receptors differ in their response to the synthetic anti-estrogens raloxifene and tamoxifen, which are both partial agonists at the ER-α, however they act as pure antagonists at the ER-β (Barkhem et al., 1998).

X-ray crystallographic data (3.1Å) published by Brzozowski et al. (1997) of the LBD of ER-α and ER-β indicated that the folding of the AF-2 region in both receptors is very similar in overall architecture. Upon binding of agonist 17β-estradiol the position of helix 12 changes its conformation so that it is positioned over the ligand-binding pocket and forms an interaction surface, which allows the recruitment of coactivators (Ascenzi et al., 2006; Brzozowski et al., 1997). Helix 3, 4 and 5 are responsible for forming a hydrophobic groove, which is important for the interaction with helix 12 when raloxifene is bound to the LBD (Brzozowski et al., 1997). This displacement of helix 12 disrupts the coactivator interaction surface, preventing their recruitment. This therefore impacts upon the expression of ER target genes (Brzozowski et al., 1997; Eiler et al., 2001).
Raloxifene has been shown to have the same binding affinity of 0.4 nM towards the ER-α as 17β-estradiol does. On the other hand, as raloxifene acts as pure antagonist towards ER-β, the binding affinity of raloxifene is significantly higher (20.2 nM) compared with the binding affinity of 0.11 nM for 17β-estradiol (Ascenzi et al., 2006; Escande et al., 2006).

1.5.3 Physiological effects of SERMs

The unique pharmacology exhibited by SERMs like raloxifene, allows them to act in the bone, liver and the cardiovascular system as agonist, and in tissue such as the breast and the brain as antagonist (Ascenzi et al., 2006). Unlike other SERMs, raloxifene acts as a mixed antagonist in the uterus (Williams-Brown et al., 2011). This tissue specific pharmacology arises in part from the varying expression levels of the ER-α and ER-β, which can homo- or heterodimerize. Knock out/down studies of ER-α or ER-β in mice have shown that these two receptors exert similar, but also unique physiological functions (Matthews et al., 2003; McDonnell et al., 2010; Riggs et al., 2003). ER-β is considered to oppose the physiological actions of 17β-estradiol activated ER-α (Matthews et al., 2003). It has been shown that the heterodimerization between ER-α and ER-β, reduces the transcription of ER-α genes (Lindberg et al., 2003). Therefore, considering that SERMs can bind both receptor subtypes, the net estrogenic response, regardless of being a positive or negative one, is expected to differ from tissue to tissue (Riggs et al., 2003). Furthermore, interactions of the ER with DNA are also influenced by coregulators, which are tissue specifically expressed and regulated (Riggs et al., 2003). These coregulators (over 300 identified interacting proteins) can either promote or repress transcriptional activities of the ER (McDonnell et al., 2010). Therefore, depending on the ligand induced conformational changes of the ER and the recruitment of specific coregulators, the effect a SERM may produce in a given tissue, becomes highly complex (Riggs et al., 2003).

1.5.4 Raloxifene in TNBC: Background studies

Independent of SERMs classical activity involving the ER, raloxifene has been shown to reduce cell viability not only in ER-negative breast cancer cells, but also in cancer cells of prostate, uterine leiomyoma, bladder and endometrial origin (Khosrokhavar et al., 2009; Stuart et al., 2010; Stuart et al., 2008; Todorova et al., 2010; Werner et al., 2005). Werner et al. (2005) aimed to elucidate the effects of raloxifene and other SERMs in ER-positive and
ER-negative breast cancer cells. The results showed that after six days of treatment with 1 µM raloxifene in MDA-MB-231, MCF-7 and T47D cells, an apoptosis to cell proliferation (A/P) ratio index of over 1 was observed. This suggested that regardless of ER expression, apoptosis was significantly induced by raloxifene (Werner et al., 2005). In MDA-MB-231 cells treated with 5-25 µM raloxifene, a dose-dependent, 60% reduction in cell survival was observed after 48 h (Todorova et al., 2010). Further evidence that raloxifene induces apoptosis in MDA-MB-231 cells was provided by Stuart et al. (2008). Concentrations of 5 µM raloxifene significantly decreased the cell number by ~30% after 7 days of treatment (Stuart et al., 2008). This coincided with increases in the proportion of cells in the G1-phase as well as increased numbers of apoptotic cells. Protein expression analysis showed that the expression of phosphorylated-AKT was reduced (Stuart et al., 2008). A more thorough mechanistic analysis showed that 4 µM raloxifene in MDA-MB-231 cells induced significant reductions in the expression of 40S ribosomal protein S6 kinase (p-S6K) and activated/phosphorylated-AKT after 12 h. Furthermore, the expression of p65 (a major subunit of the NF-κB dimer) was also significantly decreased (Stuart et al., 2010). Taurin et al. (2011) also provided evidence for effectiveness of raloxifene in ER-negative cell lines. The cell viability of MDA-MB-468, MDA-MB-231, Hs578t and SKBr-3 cells were all significantly reduced by raloxifene treatment (Taurin et al., 2011). This was accompanied by increases in the number of cells found in the G1-phase, as well as increases in the number of apoptotic cells. Proteins involved in cell proliferation, protein synthesis and apoptosis were also affected by raloxifene treatment (Taurin et al., 2011).

In a study of prostate cancer cells, it was shown that treatment with 1 µM raloxifene reduced the cell number of Du145 cells (ER-β positive only) by approximately 95% after four days (Kim et al., 2002b). Moreover, raloxifene significantly increased the number of apoptotic cells and caused caspase 9 activation (Kim et al., 2002b). In TSU-PR1 (ER-β positive only) bladder cancer cells, raloxifene treatment (1 µM) significantly reduced cell viability by ~75% compared to control after for four days (Kim et al., 2002a). This coincided with the induction of a ~15-kDa cleavage product of BAD as early as 24 h after raloxifene treatment (Kim et al., 2002a).

In human uterine leiomyoma cells raloxifene has been shown to exhibit a U-shaped dose response (Liu et al., 2007). Treatment with 1 nM raloxifene for 72 h significantly decreased cell viability, whereas 100 nM raloxifene increased the viability of leiomyoma cells (Liu et al., 2007). This was confirmed by measuring the percentage of proliferating cell nuclear
antigen-positive cells. Furthermore, only 1 nM raloxifene significantly decreased expression of the antiapoptotic Bcl-2 protein (Liu et al., 2007).

The effects of raloxifene in combination with other agents have also been investigated. In a chemopreventative study in rats, raloxifene (20 mg/kg diet) was combined with 9-cis-retinoic acid (60 mg/kg diet) (Anzano et al., 1996). Combination treatment reduced tumor incidence 30% more than raloxifene treatment alone (Anzano et al., 1996). Interestingly, in a xenograft model of TNBC (MDA-MB-231 cells), the efficacy of 0.85 mg/kg raloxifene to reduce tumor growth was significantly reduced when combined with 25 mg/kg epigallocatechin gallate (EGCG) (Scandlyn, 2011). These results indicated a negative interaction between the two drugs that was not detected in vitro previously (Stuart et al., 2010; Stuart et al., 2008). Importantly for this study, was the 79% reduction in tumor volume after 8 weeks of raloxifene treatment in this xenograft model (Scandlyn, 2011). Further evidence for the tumor suppressive ability of raloxifene was provided by a 10-week tumor-regression study (Taurin et al., 2011). Female athymic nude mice engrafted with MDA-MB-468 cells that received 0.85 mg/kg raloxifene had a reduced tumor size of 30% compared to the original size of the tumor at day 0. Compared to control, the tumor size was 70% smaller in animals treated with raloxifene after 10 weeks (Taurin et al., 2011).

Taking the appropriate normalization of body surface area into account, the human equivalent dose (HED) of raloxifene used by Scandlyn (2011) and Taurin et al. (2011) is approximately 15-fold lower than the currently clinically used dose of 60 mg/day. This was calculated using the following formula published by Reagan-Shaw et al., (2008): HED [mg/kg] = Animal dose [mg/kg] x \( \frac{[\text{Animal } K_m]}{[\text{Human } K_m]} \), where the \( K_m \) factor is calculated from the body surface area (BSA; m\(^2\)) and the weight (kg). Animal \( K_m = 3 \) and Human \( K_m = 37 \).

Interestingly, despite the very low doses used by Scandlyn (2011) and Taurin et al. (2011), raloxifene was significantly effective at suppressing tumor growth in two different xenograft models of TNBC.

Many different mechanisms by which raloxifene reduces cell viability have been identified in vitro. The most notable in ER-negative cells involve, AKT, NF-κB, BAD, Bcl-2, EGFR, G1-cell cycle arrest, cyclin D1 and S6K (Anzano et al., 1996; Davis et al., 2006; Khosrokhavar et al., 2009; Kim et al., 2002a; Kim et al., 2002b; Liu et al., 2007; Stuart et al., 2010; Olivier et al., 2006; Scandlyn et al. 2011; Stuart et al., 2008; Taurin et al., 2011; Todorova et al., 2010; Werner et al., 2005). These studies have also shown that raloxifene induces cytocidal effects independently of ER expression, but also that raloxifene acts via
multiple distinctive, cell specific and dose dependent mechanisms. Due to the complex action of raloxifene in ER-negative cells, and the fact that the majority of mechanistic studies have been performed in vitro, the evidence available that has shown effectiveness of low dose raloxifene to suppress tumor growth in TNBC xenografts is limited. Furthermore, the exact mechanisms by which raloxifene reduces tumor growth in vivo is currently unknown.

1.6 Project Rational and Aims

The work presented here is a continuation and verification of studies conducted by Taurin et al. (2011). Specifically, Taurin et al. (2011) demonstrated that raloxifene given orally at very low doses of 0.5 mg/kg and 0.85 mg/kg was effective at reducing tumor growth in xenograft models of TNBC after 10 weeks of daily treatment by up to 70% compared to control. The dose was established from earlier combination studies of tamoxifen (and raloxifene) with EGCG (Scandlyn et al., 2011). In these in vivo models of TNBC, the combination of EGCG (25 mg/kg) was effective with 75 µg/kg tamoxifen. As the human dose of tamoxifen is 3-fold higher than the 60 mg human dose of raloxifene, the first dose response study with raloxifene also started 3-fold higher at 0.225, 0.5 and 0.85 mg/kg. Focus however remained on the two higher doses as they were more effective at reducing the tumor growth than 0.225 mg/kg raloxifene (Taurin et al., 2011).

(1º) Drug efficacy

The primary goal of this study was to verify the effectiveness of low dose raloxifene in xenografts of TNBC.

Tumor growth study

- Investigate the effectiveness of daily oral 0.5 mg/kg or 0.85 mg/kg raloxifene in 5 – 6 week old, female, CD1 athymic nude mice inoculated with MDA-MB-468 cells, at reducing tumor growth compared to control over a 10 week treatment period
(2°) Mechanism of action

The secondary goal was to obtain insights into the \textit{in vivo} mechanism behind raloxifene-induced tumor-growth inhibition. To achieve this goal, the following two experimental procedures were used:

\textbf{Western blotting}

- Investigate how oral low dose raloxifene altered the expression and phosphorylation of important proteins involved in mitogenic (anti-apoptotic) and pro-apoptotic signaling in excised tumors (EGFR, AKT, NF-κB, p27, β-catenin, ERK1/2, p-ERK)
- Investigate the expression of stress activated proteins (p38, P-p38)

\textbf{Immunohistochemistry}

- Investigate how oral raloxifene altered the expression of Ki67 as a marker of cell proliferation in tumor sections
- Investigate the effects of raloxifene on angiogenesis (CD105)
- Investigate and confirm the absence of the ER in tumors
- Gain insights into whether raloxifene affects apoptosis (TUNEL assay)

(3°) Drug safety

The tertiary goal was to assess the general health and safety of low dose raloxifene in nude mice used in this study. To achieve this goal, the following procedures were performed:

\textbf{ALT activity assay}

- Investigate whether low dose raloxifene was hepatotoxic in any of the mice

\textbf{General animal health}

- Investigate animal weight changes and differences between control and treatment groups
- Investigate toxic effects of major organs (liver, kidneys, spleen and uterus)
Chapter 2: Materials and Methods

2.1 Materials:

β–actin and NF-κB antibodies were purchased from AbCam (N.S.W., Australia). MDA-MB-468 breast cancer cells were purchased from American Type Culture Collection (Manassas, VA, USA). AKT, P-AKT, EGFR, and rat anti-mouse CD105 primary antibodies, matrigel and diaminobenzidine (DAB) kit were purchased from BD Biosciences (San Diego, CA, USA). Ethylene-diaminetetra-acetic acid (EDTA), DPX mounting medium, glycerol, propan-2-ol, sodium bicarbonate and xylene were purchased from BDH Laboratory Supplies (Poole, England). Molecular weight (MW) markers, horseradish peroxidase (HRP) conjugated goat anti-mouse, goat anti-rabbit and streptavidin-biotin goat anti-rat IgG secondary antibodies and sodium dodecylsulfate (SDS) were purchased from BioRad Laboratories (CA, U.S.A.). Complete Mini EDTA-free Protease Inhibitor Cocktail Tablet was purchased from Calbiochem (Darmstadt, Germany). Optimal Cutting Temperature (OCT) compound was purchased from CellPath (Newton, UK). β-catenin, p27, p38, P-p38, F4/80 primary antibodies were purchased from Cell Signalling (USA). N-hexane was purchased from Fluka (Germany). Bovine serum albumin (BSA) and trypsin were purchased from Gibco (Auckland, NZ). Foetal bovine serum (FBS) was purchased from Invitrogen (Auckland, NZ). Acetone and sodium chloride were purchased from Merck (Darmstadt, Germany). Pam’s non-fat milk powder was purchased from New World supermarket (Pam’s Products, Auckland, NZ). Ethanol (96%) was purchased from Schlarlau Chemie (S.A., Spain). Acrylamide, β-mercaptoethanol, bisacrylamide, copper (II) sulfate pentahydrate (CuSO4.5H2O, 99%), deoxycholate, Dulbecco’s Modified Eagle’s media (DMEM), glycine for electrophoresis, hydrochloric acid (HCl, 37%), Mayer’s Haematoxylin, MEM media, methanol (99.8%), nonidet-P40, Nutrient Mixture F-12 Ham media, eosin, poly-L-lysine, Ponceau red stain, sodium bicarbonate, soyabean trypsin inhibitor, TEMED, Trizma base, triton X-100 and trypsin were purchased from Sigma Aldrich (St Louis, MO, USA). Plasma alanine aminotransferase (ALT) reagent was purchased from ThermoFisher (USA). SuperSignal West Pico Chemiluminescent Substrate was purchased from Thermo Scientific (Rockford, IL, USA).
2.2 Cell Maintenance

MDA-MB-468 breast cancer cells were maintained in DMEM/HamF12 phenol red free media (pH 7.4), supplemented with 10% FBS, 100 U/mL penicillin, 100 µg/mL streptomycin, 25 ng/mL amphotericin B and 0.2% sodium bicarbonate. Cells were cultured in 75 cm² flasks and incubated in 5% CO₂/95% humidified air at 37°C. Solutions were heated to and maintained at 37°C. All procedures were conducted in sterile conditions. Once the cells reached 90% confluence, they were passaged and washed with 5 mL phosphate buffered saline solution (PBS). This was followed by incubation for 2 min in trypsin solution (2.69 mM EDTA, 1g/L trypsin, 0.14 M NaCl, 76.78 mM tris HCl; pH 8.0) at 37°C, to allow cells to detach from the bottom of the flask. The same volume of complete growth media was added, and the cell suspension was transferred into a 50 mL conical tube. Cells were then centrifuged at 1200 rpm for 3 min at 4°C. The supernatant was discarded and the cell pellet resuspended in fresh supplemented growth media.

2.3 In Vivo Studies

2.3.1 Animal housing and care

Female athymic nude mice (5-6 weeks old) were purchased from the Hercus Taieri Resource Unit (Dunedin, NZ). All procedures were approved by the University of Otago (AEC# 91/07). Mice were housed in pathogen-free conditions with sterile woodchip bedding and access to sterile food (Relsame rodent diet, Dunedin, NZ) and water ad libitum. The temperature was maintained between 21-24°C, and the housing scheduled for 12 h light/dark cycles.

2.3.2 MDA-MB-468 Xenograft

The mouse xenograft was generated using a cell suspension of 8 x 10⁶ (passage number 10) MDA-MB-468 cells/200 µL matrigel in DMEM growth media. After three days acclimatization, mice were inoculated with 100 µL of the cell suspension into the lower right flank. Tumors were then left to grow for 14 days until they reached a size of approximately 200 mm³. Mice were then randomly assigned into three groups: 0.25% DMSO-vehicle (n = 9), 0.5 mg/kg raloxifene (n = 11) and 0.85 mg/kg raloxifene (n = 12). Mice were dosed daily by oral gavage for 10 weeks. Mice were weighed daily and monitored for weight gain and
general animal health. The tumor volume (height x length x width) was measured weekly by
the same two examiners using electronic calipers. The tumor volume was expressed as the
mean ± SEM in mm$^3$.

2.3.3 Tissue and blood collection

After the 10 weeks of treatment, all mice were euthanized by CO$_2$ inhalation. Blood was
drawn immediately from the inferior vena cava using a 20-gauge needle and a heparinized
syringe, and then immediately placed on ice. Upon necropsy, tumors were weighed and
immediately cut in half with a razor blade. One half was used for immunohistochemistry and
accordingly prepared by freezing it in Optimal Cutting Temperature (OCT) compound
wrapped in tin foil. These were then placed into a beaker of N-hexane for 30 sec and then
into liquid nitrogen. Labeled tin foil squares were then stored at -20ºC until required. The
other half was required for Western blotting and was, just like other dissected major organs
(liver, kidney, spleen and uterus) placed in labeled Eppendorf© tubes, and directly frozen in
liquid nitrogen. Tubes were stored at -80ºC until required. Organ weights were expressed as
a percentage of total body weight.

2.3.4 Assessment of alanine aminotransferase levels:

The collected blood samples were centrifuged (5000 rpm, 4ºC) for 5 min (Eppendorf 5810R
centrifuge) and plasma was transferred into a second set of Eppendorf© tubes. Plasma ALT,
an indicator of hepatotoxicity, was measured using a commercially available kit
(ThermoFisher). 100 µL of plasma was transferred into a plastic cuvette, to which 1 mL of
ALT reagent reconstituted and warmed to 37ºC was added. The absorbance was measured at
340 nm on a spectrophotometer (BioRad Benchmark Plus Microplate Spectrophotometer)
each min, for three min. ALT activity, expressed in international units per liter (IU/L) was
determined using the following equation: $\Delta$ absorbance x (TV X 1000) / (6.3 x SV x P),
where $\Delta$ absorbance = (Abs0min – Abs3min), TV = total volume in the cuvette (1.1 mL), SV
= volume of sample added (0.1 mL) and P = path length (1 cm).
2.4 Western Blotting

2.4.1 Preparation of protein extracts

Tumor samples were ground into a thin powder at just under -80°C, to prevent the tissue from warming in order to limit protein degradation by proteases (Espina et al., 2006). The tumor-powder was homogenized in 0.5 mL of buffer (20 mM Tris-HCl pH 8, 137 mM NaCl, 10% glycerol, 1% TritonX-100, 2 mM EDTA, 0.5% Na deoxycholate and one complete Mini EDTA-free Protease Inhibitor Cocktail Tablet) at 4°C. Samples were then sonicated for 3 x 7 sec and incubated on ice for 30 min followed by further centrifugation at 16,000 g for 10 min, at 4°C. Protein concentration was determined and adjusted equally across all samples using the bicinchoninic acid (BCA) assay (Smith et al., 1985).

2.4.2 BCA protein assay

Two dilutions of each sample were prepared (1:25 and 1:50) using 1% TritonX-100 with 20 µL of each diluted sample added to a 96-well plate in triplicate. The 4,4’-dicarboxy-2,2’-biquinoline (BCA) solution was prepared by combining bicinchoninic acid solution with 4% CuSO₄·5H₂O, (w/v) in a 50:1 ratio. To each sample, 200 µL of BCA solution was added. A standard curve was made using concentrations of 0-500 µg/mL BSA in a 96-well plate which was then incubated for 30 min at 37°C. Absorbance was determined using a Biorad Benchmark Plus microplate reader with an absorbance of 562 nm. The data was obtained using the Microplate Manager Software (version 5.2.1). The standard curve was used to determine the protein concentrations of the samples. Samples were adjusted to 10 µg/µL of protein using lysis buffer. For electrophoresis, 4X sample buffer (20 mM Tris-HCl, 137 mM NaCl, 10% glycerol, 1% nonidet P-40, 2 mM EDTA) was added to the protein extracts. Samples were placed in a heat block (95-100°C) for 5 min, cooled on ice for 5 min, and centrifuged for a approximately 20 sec (up to 5000 g). Samples were stored at -20°C until required for gel electrophoresis.

2.4.3 Gel Electrophoresis

Sodium dodecylsulfate-polyacrylamide gel (SDS-PAGE) electrophoresis was used to separate the proteins from the tumor samples according to their molecular weight (MW) (Laemmli, 1970). Depending on the MW of the protein under investigation, the resolving gel
consisted of either a 10% (5 mL acrylamide/bisacrylamide solution, 3.75 mL lower tris buffer, 500 µL of 50% glycerol, 75 µL APS, 5.75 mL ddH2O and 10 µL TEMED) or 17% acrylamide gel. A 4% acrylamide stacking gel (0.85 mL acrylamide/bisacrylamide solution, 1.25 mL upper tris buffer, 50 µL APS, 2.9 mL dH2O, 2.5 µL TEMED) was used regardless of the acrylamide percentage of the resolving gel. Each well of the polyacrylamide gel was loaded with 10, 15 or 20 µg of tumor protein extracts. A Precision Plus Protein™ MW marker (30 – 250 kDa) was loaded into the first well to identify protein MW. Gels were run at 100 V in running buffer (25 mM tris-base, pH 8.3, 0.192 M glycine, 0.1% (w/v) SDS) using a Bio-Rad Mini-Protean III apparatus for 20 min. Once the dye had migrated through the end of the stacking gel the voltage was increased to 150V and run until the dye front had reached the end of the resolving gel.

2.4.4 Transfer and staining

Once protein samples were separated by SDS-PAGE the proteins were transferred to a PVDF membrane soaked in transfer buffer (25 mM tris-base, pH 8.3, 0.192 M glycine and 10% methanol). Proteins were transferred for 90 min at 100 V, using a Bio-Rad wet transfer system. Following protein transfer, the PVDF membranes were washed in ddH2O and stained with Ponceau red stain for 30 sec while sitting on a shaker. Membranes were then rinsed repeatedly in ddH2O to remove the Ponceau red stain, followed by incubation in blocking buffer (TBS (0.025 mM tris-base, 0.1 M NaCl, pH 7.4) and 2 % BSA) for 1 h at room temperature (RT). Membranes were then washed with TBS and incubated with the appropriate primary antibody overnight at 4°C on a shaker. Primary antibodies were diluted (in TBS, 2 % BSA) to the following concentrations: EGFR (1:2000), AKT (1:2000), P-AKT (1:1500), NF-κB (1:2000), p38 (1:2000), P-p38 (1:2000), p27 (1:2000), β-catenin (1:2000), ERK1/2 (1:2000), p-ERK (1:2000) and β-actin (1:5000).

Following incubation, each membrane was washed with TBST (0.05% tween 20, 0.025 mM tris-base, 0.1 M NaCl, pH 7.4) 5 times for ~5 min each wash. Membranes were then incubated in 5% non-fat milk powder dissolved in TBS with HRP conjugated goat anti-mouse or goat anti-rabbit secondary antibody at RT for 1 h. Membranes were then washed again in TBST 5 times for ~5 min each wash. This step removed the secondary antibody and reduced non-specific binding. Protein bands were visualized by incubating membranes for 4 min in SuperSignal West Pico Chemiluminescent Substrate (1.8 mL).
2.4.5 **Densitometry**

The digital chemiluminescence images were taken using a Versadoc (BioRad) imaging system and quantified using Quantity One software 4.6.5 Basic (BioRad). The GS710 BioRad scanner was set to scan x-ray blue film at 63.5 × 63.5 resolution. Using the ‘Volume Rect Tool’, boxes were drawn around each single band of the corresponding expected MW of the protein under assessment and also of the background. The ‘Volume Analysis Report’ was set to give the density of the band (rather than volume) as suggested by Gassman *et al.* (2009). The density values were exported to Microsoft® Excel (2007) and the background density was subtracted from the protein-band density and the following value was divided by the corresponding band of the β-actin/control density. In cases where duplicate or triplicate Western blots were performed, changes were graphed as a percentage of control. Proteins that were assessed both in their native and phosphorylated-state were graphed as:

\[
\frac{\text{Phosphorylated protein}}{\text{native protein}} \div (\beta-\text{actin})
\]

2.5 **Immunohistochemistry**

To prepare tumor samples for immunohistochemistry (IHC), tumors were sliced into 10 μm thick sagittal sections at approximately -19 to -21°C (depending on RT) using a Leica CM1850 cryostat. Three tumor slices (one positive control) were placed on a single poly-l-lysine-coated microscope slide. Slides were dried under a fan for approximately 30 min at RT and then stored at -20°C until required. Boxes containing tumor slides were wrapped in tin foil to reduce damage of sections from condensation occurring during opening and closing of the boxes. Immunohistochemistry was used to determine the expression of Ki67, CD105, and ER. Apart from differing anti-body dilutions the method of staining was the same as previously developed (Allen, 2010).

2.5.1 **Primary antibody binding (IHC-Part I)**

Slides containing the tumor sections were thawed for 30 min at RT and then washed twice for 5 min each time in 1X PBS. Tumors were fixed by repetitively applying 4% acetone for 10 min at RT and once slides were dry, the sections were marked using a DAKO pen (hydrophobic barrier) and left for 10 min to dry.

As a positive control for the ER antibody, MCF-7 cells were fixed to glass slides, dried for 30 min and then incubated in 4% paraformaldehyde for 10 min at RT and allowed to dry.
Glass slides were washed three times in 1X PBS for 2 min and incubated in 0.1 M glycine for 15 min at RT. To block endogenous peroxidase activity, the slides were incubated for 20 min in 0.3% hydrogen peroxide in methanol at RT, followed by three washing steps in 1X PBS, each 2 min long.

Antigen retrieval was only used for the Ki67 antibody (and also tried for F4/80), and therefore this part was omitted for the other antibodies. To retrieve the antigen, slides were placed in 90-95°C citrate buffer for 10 min. The slides were then left in the citrate buffer to cool down for 20-25 min and washed twice for 5 min each in 1X PBS.

The remaining steps were the same for all antibodies. In a humidified chamber, tumor sections were incubated for 1 h in blocking solution (1.5% goat serum, 0.2% BSA, 0.001% avidin D, 1X PBS) and then rinsed with 1X PBS for 5 min. Sections were then incubated overnight in a humidified chamber at 4°C with the appropriate antibody: CD105 (1:100, rat anti-mouse; 22 h), Ki67 (1:200, rat anti-mouse; 16 h), and ER, (1:150, rat anti-mouse; 20 h).

2.5.2 **Secondary antibody binding & staining (IHC-Part II)**

Once the overnight incubation time had finished, the primary antibody was removed and tumor sections were washed twice for 2 min and twice for 5 min using 1X PBS at RT. Following this, the secondary goat anti-rat antibody was applied at RT for 30 min in the following dilutions: 1:200 for CD105; 1:500 for Ki67; and 1:500 for ER.

Slices were then washed twice for 5 min each time with 1X PBS. Streptavidin-biotin solution was applied to the tumor-specimens for 30 min at RT and sections were again washed with 1X PBS three times for 5 min. Sections were stained with DAB for either 20 sec (Ki67), 6 min (ER-α) or 20 min (CD105) and then washed twice with ddH₂O for 5 min. Tumor sections were washed once more with ddH₂O three times for 2 min and then counterstained with Mayer’s hematoxylin for 15 sec and then rinsed thoroughly with ddH₂O. This was followed by applying 0.1% sodium bicarbonate solution twice for 10 sec and finally rinsed with ddH₂O for 30 sec. To dehydrate the tumor sections, slides were covered in 70% ethanol for 5 min, then in 96% ethanol for 5 min and again for 10 min. To further dehydrate and remove the hydrophobic marking of the DAKO pen, slides were soaked in xylene twice for 5 min and left to dry completely (at least 1 h) until the coverslips were mounted using DPX-medium. Slides were left to dry for 36 – 48 h.
2.5.3 ApopTag® Peroxidase In Situ Apoptosis Detection Kit

Apoptotic cells were detected by terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) for which the manufacturers protocol (Millipore) was used. Briefly, the tumor sections were fixed in 1% paraformaldehyde, washed in two changes of 1X PBS, post-fixed in ethanol:acetic acid and washed again in PBS. Sections were then quenched in 3% hydrogen peroxide and rinsed with PBS. The ‘EQUILIBRATION BUFFER’ was directly applied to the specimen and incubated for 5 min, followed by incubation for 1 h at 37°C with the ‘WORKING STRENGTH TdT ENZYME’. At RT, the ‘WORKING STOP/WASH BUFFER’ was applied to sections, which were then washed 3 times in 1X PBS. Tumor sections were covered in ‘ANTI-DIGOXIGENIN PEROXIDASE CONJUGATE’ and incubated for 30 min and again washed in four changes of 1X PBS. Specimens were stained for 8 min using DAB. The remaining steps were exactly the same as with all the other antibodies (refer to Part 2 above). Due to the limited availability of this kit, only one tumor from each group was analyzed for apoptosis.

2.6 Analysis of IHC Results

Tumor sections were photographed using a Zeiss Axioplan MC80 BX microscope, Zeiss AxioCam HRC digital camera and Zeiss Axiovision 3.1 software (Carl Zeiss Vision GmBH, Germany).

2.6.1 Ki67

Pictures (2 – 10, depending on tumor size) of the entire tumor were taken at 50 times magnification and merged together using Microsoft PowerPoint 2007. These were exported as JPEG images in a randomized way to fully blind the assessor. The 57 tumor images (most tumors in duplicates) were then opened in ImageJ, where the area of the entire tumor was measured using the 'freehand selection' tool. This was followed by pressing 'analyze' and 'measure' to get an arbitrary number (pixels) that was subtracted from the total area of cells stained brown/positive for Ki67. Results were graphed as a percentage of total tumor size.
2.6.2 **CD105**

Pictures (6-30, depending on tumor size) of the entire tumor-sections were taken at 100 × magnification and merged together using Microsoft PowerPoint 2007. A grid over the image was made to simplify counting and an independent person was asked to completely randomize the order of the 59 tumors sections (most in duplicates). Pictures were horizontally printed and while looking at the image (blinded) in PowerPoint at 400% zoom, positive brown-staining, in particular those tumors with a circular lumen, were counted and numbers were written in the individual windows of the grid. This allowed for a better understanding of what exactly was counted and what was left out. An independent, fully blinded assessor with a set of 15 randomly picked tumors repeated this process to confirm the results.

2.6.3 **Estrogen receptor-α**

For this experiment only one tumor slide from each treatment group was chosen (based purely on the quality of the section). Fixed MCF-7 cells acted as a positive control. Pictures were taken at 100 and 639 × magnification, at three randomly selected parts of the tumor sections (in duplicate).

2.6.4 **ApopTag® apoptotic cells**

Pictures (2 – 8) of the 6 analyzed tumor sections were taken at 50 × magnification, and using the Zeiss Axiovision 3.1 software, a 50 or 100 μm scale was drawn on the image. The multiple images taken were then merged back together in Microsoft PowerPoint 2007. A grid was drawn over the tumor with squares in sizes of either 50 x 50 μm or 100 x 100 μm. Counting the squares allowed me to calculate to work out the approximate area in mm² of the tumor section. The TdT enzyme positive staining of each entire tumor was counted of as described for CD105, and the results were graphed as a ratio of TdT positive cells to the total cell/tumor area. The formula used was: \( \frac{\text{Tumor area (mm}^2\text{)}}{\text{No. of TdT positive cells}} \)

Due to poor quality of the duplicate tumor section from the control group, the duplicate section was excluded from further analysis. Therefore, no error bar (SEM) is shown in Figure 3-16 of the vehicle group.
2.7  Statistical Analysis

The tumor growth/volume measured weekly for 10 weeks, was analyzed using a repeated measures two-way ANOVA coupled with the Bonferroni post hoc test in which $p < 0.05$ denoted a statistically significant difference. Tumor, animal and individual organ weights, plasma ALT levels as well as the differences in protein expression as determined by Western blotting, and all IHC results were analyzed using a one-way ANOVA coupled with Bonferroni’s post hoc test in which $p < 0.05$ denoted a statistically significant difference.
Chapter 3: Results

3.1 Drug Efficacy

3.1.1 Tumor growth

To determine the efficacy of low dose raloxifene towards tumors with a triple-negative phenotype, mice bearing (200 mm$^3$) MDA-MB-468 cell xenografts were treated for 10 weeks with 0.5 or 0.85 mg/kg of raloxifene, or 0.25% DMSO. Tumor volumes were measured weekly to allow the establishment of a tumor growth curve (Figure 3-1). Initially there were 13 control mice and 14 in each of the two raloxifene treatment groups. Throughout the study several mice had to be sacrificed due to eye infections. The final animal count at the end of the 10 week study was: vehicle n = 9; 0.5 mg/kg raloxifene n = 11; 0.85 mg/kg raloxifene n = 12.

The tumor growth curve showed that after three weeks of treatment the first noticeable differences in tumor-growth characteristics appeared. Tumors from the vehicle group increased in volume more rapidly compared to tumors from both raloxifene treatment groups. At the end of the 10-week study, tumors from the 0.85 mg/kg raloxifene group (256.3 ± 47 mm$^3$) were 32% smaller compared to the vehicle group (378.2 ± 27 mm$^3$). Tumors from mice treated with 0.5 mg/kg of raloxifene had approximately 23% smaller tumor volumes compared to the vehicle group. In comparison to the initial average tumor volume across all mice (208.3 ± 4 mm$^3$), tumor size in the control group increased by ~45%, in the 0.5 mg/kg raloxifene group by ~37% and in the 0.85 mg/kg raloxifene group by ~18%. Statistical analysis showed that both raloxifene doses had a significant effect over time when compared to vehicle ($p < 0.03$). It is interesting to note that the average tumor size from the 0.85 mg/kg raloxifene group plateaued after 7 weeks (261.9 ± 38 mm$^3$) of treatment.
Figure 3-1 Tumor growth following treatment with raloxifene

Female athymic nude mice were inoculated with MDA-MB-468 cells and treated orally for 10 weeks either with 0.25% DMSO-vehicle-control (n=9), 0.5 mg/kg raloxifene (Ral; n=11) or 0.85 mg/kg raloxifene (n=12). Results are represented as mean ± SEM. Data was analyzed using a repeated measures two-way ANOVA coupled with the Bonferroni post hoc test. * 0.5 mg/kg and 0.85 mg/kg of raloxifene groups were significantly different to vehicle, p<0.03.

3.1.2 Tumor weight

Upon euthanasia, the tumor as well as major organs were dissected and weighed. The results showed 0.85 mg/kg raloxifene significantly (p<0.05) decreased the tumor weight by ~40% and 0.5 mg/kg raloxifene by ~19% compared to vehicle treated mice. It should be pointed out, that during the process of measuring the tumors, several errors occurred with the scale, which was not tared (zero) properly. Therefore there were two measurements in the 0.85 mg/kg raloxifene group, which were not included in the analysis (Figure 3-2).
Dissected tumors from a xenograft model of TNBC after 10 weeks of vehicle-DMSO (n=9), 0.5 mg/kg (n=11) or 0.85 mg/kg (n=10) raloxifene (Ral) treatment. Results are represented as mean ± SEM. Data was analyzed using a one-way ANOVA coupled with the Bonferroni post hoc test. *0.85 mg/kg raloxifene was significantly different to vehicle control $p < 0.05$.

### 3.2 General Observations of Animal Health

#### 3.2.1 Body weight

To determine the overall animal health, the animals were weighed daily throughout the 10-week study (Figure 3-3). There were no indications of compromised animal health and no significant differences between any of the animal weight measurements within, or between the three treatment groups.

#### 3.2.2 Organ weight and ALT activity

To determine major organ functionality, liver, kidney, spleen and uterus were dissected and weighed. Plasma ALT activity was also measured in blood samples taken upon euthanasia. All these physiological parameters were normal and not significantly different between the three treatment groups (Table 3-1).
Weekly animal body weight measurements in grams (g), of vehicle-DMSO (n=9), 0.5 mg/kg (n=11) or 0.85 mg/kg (n=12) raloxifene (Ral) treatment in a xenograft model of TNBC. Data was analyzed using a one-way ANOVA coupled with the Bonferroni post hoc test. Results are represented as mean ± SEM.

**Table 3-1 Organ weight and ALT activity**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Liver</th>
<th>Kidney</th>
<th>Spleen</th>
<th>Uterus</th>
<th>ALT activity (IU/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>5.86 ± 0.2</td>
<td>1.46 ± 0.1</td>
<td>0.57 ± 0.1</td>
<td>0.31 ± 0.1</td>
<td>63.4 ± 8.2</td>
</tr>
<tr>
<td>0.5 mg/kg Ral</td>
<td>5.53 ± 0.2</td>
<td>1.41 ± 0.1</td>
<td>0.69 ± 0.1</td>
<td>0.4 ± 0.1</td>
<td>64.5 ± 5.4</td>
</tr>
<tr>
<td>0.85 mg/kg Ral</td>
<td>5.82 ± 0.1</td>
<td>1.46 ± 0.1</td>
<td>0.73 ± 0.1</td>
<td>0.3 ± 0.1</td>
<td>71.3 ± 7.7</td>
</tr>
</tbody>
</table>

Organ weight as a percentage of body weight (mean ± SEM) and ALT activity (IU/L) from tumor bearing mice treated for 10 weeks either with vehicle-DMSO (n=9), 0.5 mg/kg (n=11) or 0.85 mg/kg (n=12) raloxifene (Ral). Data was analyzed using a one-way ANOVA coupled with the Bonferroni post hoc test. Results are represented as means ± SEM. No statistically significant differences were observed between any of the treatment groups and the vehicle group.
3.3 Mechanistic Analyses

3.3.1 Ki67 expression

To determine the effects of raloxifene on actively proliferating cells the antibody Ki67 was used. Figure 3-4 shows that the mean area of Ki67 positive cells in the tumors was approximately 47% lower in the 0.5 mg/kg raloxifene treated group, compared with the vehicle group. The higher, 0.85 mg/kg raloxifene dose, reduced the number of Ki67 positive cells by 53% compared to vehicle. Raloxifene at both doses was shown to have a statistically significant \((p < 0.05)\) effect on Ki67 expression. It should be pointed out however, that not all tumors could be quantified for Ki67 expression, mainly due to poor quality tumor sections, particularly from the large tumors. Furthermore, one tumor from the 0.85 mg/kg raloxifene group had a Ki67 expression value of 19.8 ± 18.6%. Due to the large discrepancies between the duplicates, this tumor was not included in the analysis.

![Ki67 expression graph](image)

Figure 3-4 Immunohistochemical analysis of Ki67

Ki67 expression in 10 μm thick DAB stained tumor sections from a MDA-MB-468 cell xenograft model of triple negative breast cancer. Pictures of each tumor were taken at 50× magnification and analyzed blinded using ImageJ. Data is expressed as Ki67 positive, proliferating cells (stained brown) as a percentage of total tumor cells after treatment with vehicle (DMSO; \(n = 5\)), 0.5 mg/kg (\(n = 6\)) or 0.85 mg/kg (\(n = 7\)) of raloxifene (Ral). Results are represented as means ± SEM. Data was analyzed using a one-way ANOVA coupled with the Bonferroni post hoc test. *Both raloxifene treated groups were significantly different to vehicle control \(p < 0.05\).
3.3.2 NF-κB expression

To investigate the mechanism by which raloxifene reduced cell proliferation, Western blotting was used to determine changes in NF-κB expression, as this transcription complex has important roles in cell proliferation. The results showed that NF-κB expression was decreased by approximately 37% in mice treated with 0.85 mg/kg of raloxifene (63.5±10.3%), compared to the control group (100 ± 10.8%). This decrease however, was not statistically significant \( (p = 0.0739) \). In the 0.5 mg/kg raloxifene group (99.5±15%), no changes in NF-κB expression were observed (Figure 3-5).

![Western blot analysis of NF-κB](image)

**Figure 3-5 Western blot analysis of NF-κB**

Proteins were extracted from tumors obtained from a xenograft model of TNBC treated daily for 10 weeks with 0.25% DMSO, 0.5 or 0.85mg/kg of raloxifene (Ral). Western blots were quantified by densitometry (graph). Triplicate results are shown as a percentage of control. All proteins were normalized to β-actin of each blot (only one shown for simplicity). The gap in the third vehicle blot denotes a sample for which there was not enough sample to run a 3rd time. Data was analyzed using a one-way ANOVA coupled with the Bonferroni post hoc test. Results are represented as mean ± SEM.

3.3.3 p38 expression

To further investigate the effects of raloxifene on cell proliferation, the protein expression of p38 was assessed. Interestingly, p38 has also been shown to interact with NF-κB, which may affect cell proliferation by reducing NF-κB-driven transcriptional activity and/or attenuation
of NF-κB target gene expression (Saha et al., 2007; Vanden Berghe et al., 1998). The results showed that p38 expression compared to control was increased by around 30 and 50% in mice treated with 0.5 mg/kg and 0.85 mg/kg of raloxifene, respectively (Figure 3-6A). These differences were not statistically significant. On the other hand, activated phosphorylated-p38 was decreased significantly (p<0.01) by both raloxifene doses, compared to vehicle treated mice. The 0.5 mg/kg raloxifene dose caused a decrease of ~20% compared to vehicle treated mice and the 0.85 mg/kg raloxifene dose caused a significant (p < 0.01) reduction of over 90% (Figure 3-6B). In figure 3-6C, the ratio of activated and native p38 is shown. The activation/phosphorylation of the available native p38 was significantly (p < 0.05) decreased by 80% in mice treated with 0.85 mg/kg of raloxifene compared to control. The lower, 0.5 mg/kg raloxifene dose on the other hand only reduced activation of p38 by 15% compared to vehicle-control.
Proteins were extracted from tumors obtained from a xenograft model of TNBC treated daily for 10 weeks with 0.25% DMSO, 0.5 or 0.85mg/kg of raloxifene (Ral). Western blots were quantified by densitometry (graph). Duplicate results are shown as a percentage of control. (A) p38 expression, (B) phosphorylated-p38 (C) ratio between the phosphorylated and the total protein. All protein bands were normalized to β-actin of each blot (only one shown for simplicity). Results are represented as means ± SEM. The gap in the third vehicle blot denotes a or depleted tumor-protein sample. Results significantly different compared to control using a one-way ANOVA with a Bonferoni post hoc test: * p < 0.05; ** p < 0.01.

Figure 3-6 Western blot analysis of P38 and p-P38
3.3.4  p27 expression

To determine whether reductions in cell proliferation were associated with changes in cell cycle proteins, the expression of p27\textsuperscript{Kip1} was assessed. This CDK inhibitor can prevent cyclin D1 activation, which can also be affected by NF-κB and p38 (Vlach \textit{et al.}, 1997). The results shown in figure 3-7 revealed that neither raloxifene dose had any significant effects on p27\textsuperscript{Kip1} expression.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{p27_expression.png}
\caption{Western blot analysis of p27}
Proteins were extracted from tumors obtained from a xenograft model of TNBC treated daily for 10 weeks with 0.25% DMSO, 0.5 or 0.85mg/kg of raloxifene. Western blots were quantified by densitometry (graph). All protein bands were normalized to β-actin of each blot (only one shown for simplicity). The top band of the two visible bands is p27. Data was analyzed using a one-way ANOVA coupled with the Bonferroni post hoc test. Results are represented as means ± SEM.
\end{figure}

3.3.5  EGFR expression

As EGFR is overexpressed in MDA-MB-468 cells, expression analysis of this protein provides a good starting point to assess a different pathway by which raloxifene may have affected cell proliferation. Contrary to what was expected, the results shown in figure 3-8 showed that EGFR expression was significantly increased by 85% (\(p < 0.05\)) in mice treated with 0.5 mg/kg raloxifene and by 115% (\(p < 0.01\)) in mice treated with 0.85 mg/kg raloxifene, compared to control.
Proteins were extracted from tumors obtained from a xenograft model of TNBC treated daily for 10 weeks with 0.25% DMSO, 0.5 or 0.85mg/kg of raloxifene (Ral). Western blots were quantified by densitometry (graph). Triplicate results are shown as a percentage of control. All protein bands were normalized to β-actin of each blot (only one shown for simplicity). Results are represented as means ± SEM. Results significantly different to control with one-way ANOVA with post hoc test: * p < 0.05; ** p < 0.01.

3.3.6  **AKT expression**

To investigate the consequences of the significant increases in EGFR expression, the important downstream target, AKT was assessed with Western blotting. The tumors of mice treated with 0.5 or 0.85 mg/kg of raloxifene, had AKT expression values of 0.42 ± 0.12 and 0.26 ± 0.07, respectively. In the control tumors, AKT expression was 9% and 44% lower compared to raloxifene treated mice (Figure 3-9A). Using a phosphorylation-specific antibody, activation of AKT was also assessed. In figure 3-9B it is shown that both raloxifene doses caused a reduction in p-AKT of 35% compared to control. The ratio between AKT and p-AKT showed that there was no difference between 0.5 mg/kg raloxifene and control, however, the 0.85 mg/kg caused a reduction of around 65% compared to control (Figure 3-9C). Due to large animal variations, none of the differences between AKT, p-AKT or their ratio to each other were significantly different between the treatment groups.
Figure 3-9 Western blot analysis of AKT and p-AKT

Proteins were extracted from tumors obtained from a xenograft model of TNBC treated daily for 10 weeks with 0.25% DMSO, 0.5 or 0.85mg/kg of raloxifene (Ral). Western blots were quantified by densitometry (graph). (A) AKT expression, (B) phosphorylated-AKT (C) ratio between the phosphorylated and the total protein. All protein bands were normalized to β-actin of each blot (only one shown for simplicity). Data was analyzed using a one-way ANOVA coupled with the Bonferroni post hoc test. Results are represented as means ± SEM.
3.3.7 ERK and β-catenin expression

To further try to explain the increase in EGFR expression, the expression of another important downstream pathway protein, ERK1/2, which is involved in cell proliferation, was assessed. Total ERK1/2 expression, as shown in figure 3-11A, was decreased by 5% and 20% in tumors treated with 0.5 mg/kg and 0.85 mg/kg raloxifene respectively. A specific antibody that detects ERK phosphorylation (Figure 11B) at threonine-980 showed that treatment with raloxifene decreased the expression of p-ERK by 14% and 45%, respectively. Compared to control, the expression-ratio of p-ERK over total ERK1/2 was decreased by 12% and 53% with 0.5 mg/kg and 0.85 mg/kg raloxifene, respectively. However, no changes were statistically different.

As cell proliferation is also controlled by β-catenin (e.g. regulation of cyclin D1), the expression of this Wnt signaling protein was also assessed. The results showed that compared to the vehicle group, β-catenin expression was increased by approximately 330 and 340% in animals treated with 0.5 mg/kg and 0.85 mg/kg of raloxifene respectively (Figure 3-10). These results were nevertheless not significantly different compared to control.

Figure 3-10 Western blot analysis of β-catenin

Proteins were extracted from tumors obtained from a xenograft model of TNBC treated daily for 10 weeks with 0.25% DMSO, 0.5 or 0.85mg/kg of raloxifene. Western blots were quantified by densitometry (graph). Duplicate results are shown as a percentage of control. All protein bands were normalized to β-actin of each blot (only one shown for simplicity). The gap in the second vehicle blot denotes a depleted tumor-protein sample. Data was analyzed using a one-way ANOVA coupled with the Bonferroni post hoc test. Results are represented as mean ± SEM.
Proteins were extracted from tumors obtained from a xenograft model of TNBC treated daily for 10 weeks with 0.25% DMSO, 0.5 or 0.85 mg/kg of raloxifene (Ral). Western blots were quantified by densitometry (graph). (A) ERK1/2 expression, (B) phosphorylated-ERK (C) ratio between the phosphorylated and the total protein. All protein bands were normalized to β-actin of each blot (only one shown for simplicity). Data was analyzed using a one-way ANOVA coupled with the Bonferroni post hoc test. Results are represented as means ± SEM.
3.3.8 CD105

To determine whether raloxifene treatment had any effects on angiogenesis, an important contributor of tumor growth, immunohistochemical analysis of CD105 expression was performed. Blinded counting of CD105 positive staining of the tumor pictures, taken at 100× magnification, showed that raloxifene increased CD105 expression, however, this was not statistically significant (Figure 3-12). The tumors of animals treated with 0.5 or 0.85 mg/kg of raloxifene, had 82.2 ± 16.4 and 77.5 ± 14.9 CD105-positive stains, respectively. In the control tumors, CD105 positive staining was up to 17% lower compared to raloxifene treated mice. An independent, fully blinded assessor confirmed these results. However, of the nine control tumors assessed for CD105 expression, only four tumor sections were of sufficient quality to be included in the analysis. Figure 3-13 shows one example image from each treatment group, highlighting the CD105 positive staining.

![Figure 3-12 Immunohistochemical analysis of CD105](image)

**Figure 3-12 Immunohistochemical analysis of CD105**

Immunohistochemistry of 10µm thick DAB stained tumor sections from a MDA-MB-468 cell xenograft model of triple negative breast cancer treated daily for 10 weeks with 0.25% DMSO (n=4), 0.5 mg/kg (n=6) or 0.85 mg/kg (n=9) of raloxifene (Ral). Photographs of the entire tumor section were taken at 100× magnification and brown, luminal stains were counted whilst blinded to the treatment group. Data was analyzed using a one-way ANOVA coupled with the Bonferroni post hoc test. Results are represented as mean ± SEM.
Immunohistochemistry of 10µm thick DAB stained tumor sections from a MDA-MB-468 cell xenograft model of triple negative breast cancer treated daily for 10 weeks with 0.25% DMSO, 0.5 mg/kg or 0.85 mg/kg of raloxifene. Photographs of the entire tumor sections were taken at 100× magnification (small images bottom, right corner of each larger image). (A) Shows a 380% increased size of an image of a tumor section taken from a control treated mouse. (B) Shows a 240% increased size of an image of a tumor section taken from a 0.5 mg/kg raloxifene treated mouse. (C) Shows a 240% increased size of an image of a tumor section taken from a 0.85 mg/kg raloxifene treated mouse. The white arrows point towards DAB stained (brown), luminal stains, which were counted as CD105 positive (blinded to treatment group).

To investigate whether there was relationship between tumor size and CD105 expression in this tumor model, a linear regression analysis was performed. The results in Figure 3-14 showed that there was no correlation between the tumor volume measured at week 10, and their respective positive staining for CD105 expression.
Figure 3-14 Linear regression of tumor volume vs. CD105 positive staining

Tumors from a xenograft model of TNBC, treated daily for 10 weeks with either 0.25% DMSO (n=4), 0.5 mg/kg (n=6) or 0.85 mg/kg (n=9) of raloxifene underwent IHC for CD105 expression (Figure 3-12). The number of luminal CD105 positive stains of individual tumor sections, versus their final tumor volume in week 10 is shown in this linear regression. Goodness of Fit: $r^2=0.0042$; not statistically significant as $p=0.7922$. Total number of values = 19.

3.3.9 Estrogen receptor-α

Of great importance for this study was the absence of ER expression. To verify this, MCF-7 cells and tumor sections from the TNBC xenograft were incubated with a primary ER antibody for immunohistochemical analysis. Staining indicated that in all treatment groups, no specific staining of the antigen occurred (Figure 3-15). MCF-7 cells on the other hand were stained completely brown. It should be pointed out that this analysis was only performed on one tumor from each treatment group and should ideally be performed in all tumor samples.
**Figure 3-15 Immunohistochemical analysis of ER expression**

ER-α expression (brown) in cultured MCF-7 cells and 10 µm thick DAB stained tumor tissue from a MDA-MB-468 cell xenograft model of triple negative breast cancer. ER-α expressing cells are visible in brown, and cells negative for the ER are blue. The small images in the bottom-right corner are pictures of the tumor/MCF-7 cells at 100× magnification, which were further magnified to 639×. (A) cultured, untreated MCF-7 cells, (B) vehicle treatment, (C) 0.5mg/kg raloxifene treatment. (D) 0.85 mg/kg raloxifene treatment.

### 3.3.10 Apoptosis

To gain insight into whether raloxifene induced apoptosis in this xenograft model of TNBC, an *in situ* apoptosis kit was used (TUNEL assay). Due to limited availability of the apoptosis kit, only one tumor from each group (n = 1/group) was assessed for apoptosis. This preliminary work was performed to investigate whether expression analysis of pro-apoptotic proteins may help solve the mechanism by which raloxifene reduces tumor growth. Tumor sections were chosen purely based on the quality of the tumor sections.

It was thought to be interesting to compare the degree of apoptosis, with that of cell proliferation and whether there would be an inverse relationship between the two. A graph of this is shown in Figure 3-16. The results showed that the 0.5 mg/kg and the 0.85 mg/kg raloxifene dose had ratios of TdT positive/apoptotic cells to total tumor area (mm²) of 638.1
± 12.6 and 835.8 ± 99.8, respectively. Compared to the vehicle treated mouse this accounted for a respective 36% and 52% increase in the number of apoptotic cells. The percentage of Ki67 positive cells found in these specific tumor samples showed that raloxifene treatment with 0.5 mg/kg and 0.85 mg/kg decreased Ki67 positive cells by 63% and 36% compared to the vehicle treated mouse. It should be emphasized that the units for both measurements are different and cannot be directly compared. These results provide only a rough idea, and no conclusions can be drawn from this, due to the small sample size.

Figure 3-16 Analysis of three tumors for their apoptotic and proliferating cell numbers

Analysis was performed using the ApopTag® Peroxidase In Situ Apoptosis Detection Kit (TUNEL method) of three 10 µm thick DAB stained tumor sections from a MDA-MB-468 cell xenograft model of triple negative breast cancer. Of the same tumors, the Ki67 expression is shown. Pictures of each tumor were taken at 50× magnifications and analyzed either using PowerPoint (apoptosis) or ImageJ (proliferation). In the left columns (dotted bars) are TdT positive/apoptotic cells as ratio of total tumor-cell area and in the right columns are Ki67 positive cells (bars filled with big squares) as a percentage of total tumor cells. Mice were treated with vehicle (DMSO; n=1), 0.5 mg/kg (n=1) or 0.85 mg/kg (n=1) of raloxifene (Ral). Shown are average values of a duplicate measurement from each tumor. Results are represented as mean ± SEM. No error bar is shown for the apoptosis-vehicle group as the duplicate was of poor quality.
Chapter 4: Discussion

4.1 Critical Evaluation of Experimental Techniques

This study made use of a xenograft model of TNBC using MDA-MB-468 cells. Daily oral treatment for 10 weeks with DMSO, or low-dose raloxifene commenced once a palpable tumor of approximately 200 mm$^3$ was reached. Weekly tumor-volume measurements allowed assessment of treatment effectiveness in preventing tumor growth in comparison to the control treatment. In this experiment, the cancer cells were subcutaneously injected into the rear, right flank of the animal. However, cells can also be transplanted orthotopically into the mammary gland, which provides a more favorable microenvironment. But there are also crucial differences in the mouse and human mammary stroma which should be considered (Hovey et al., 1999).

As early as 1969, the observation was made that athymic T-cell deficient mice might be a useful tool in studying human cancer in vivo, as these mice did not reject the large, foreign human cancer cells (Rygaard et al., 1969). Xenograft models have since been used extensively for cancer gene validation and the testing of novel compounds, mainly due to their ease of use and low cost (Sharpless et al., 2006). Despite these advantages, there are obvious shortcomings for the use of this model system. Problematic is the strongly compromised immune response of nude mice, which does not allow assessment of the drugs’ influence or interactions with the immune system. Most important however is the fact that xenograft models, model cancer as if it was a disease of homogenous rogue cells (Vargo-Gogola et al., 2007). However, breast cancer is not a single disease, but a collection of breast diseases that have diverse histopathologies, genetic and genomic variations, and clinical outcomes (Hanahan, 2000; Sharpless et al., 2006). It is the complex interplay of distinct and heterogeneous neoplastic and host components that maintain the tumor. Therefore, a xenograft model fails to recapitulate the complex and evolving tumor-host stroma interactions, as well as the effects of the microenvironment on drug action (Sharpless et al., 2006). Due to this complexity and heterogeneity, no single experimental model is expected to mimic all characteristics of this disease. Therefore it is the combination and integration of multiple different systems that together may come closest to representing the clinical observations (Vargo-Gogola et al., 2007).

It is also interesting to compare what are considered to be effective treatments in the pre-clinical and the clinical setting. Pre-clinically, a treatment is commonly considered effective
if it significantly delays the tumor growth compared to the control group. In contrast, the standardized Response Evaluation Criteria in Solid Tumors (RECIST criteria) for evaluating treatment efficacy in human clinical trials requires a tumor shrinkage of over 50% to be considered a response (Sharpless et al., 2006). Therefore results of xenograft studies may be more clinically representative when both, tumor growth and tumor regression studies are performed.

It is important to make note of the spread of a single weekly data point (large SEM error bar). The tumors were measured by the same two people each week, one of whom was very experienced, and the other was new to tumor volume measurements. The average was taken from both measurements and graphed in conjunction with the SEM. The use of electronic calipers is a very accurate (± 0.02 mm according to manufacturer) way of measuring, however, due to the large discrepancies observed in the data it seems unlikely that this was due to an inaccurate measuring tool, but was caused by animal handling methods. The specific way in which the electronic calipers were used might also contribute to the varying tumor measurements. It may therefore be desirable that a more detailed standardized method of animal handling and caliper-use is established and adhered to rigidly throughout the 10-week study. Furthermore, it may help to measure the tumor volume at least twice weekly (Crawford et al., 2008). This, although a very laborious task would have the advantage of revealing the consistency of tumor measurements, as the tumor volumes would not be expected to grow/shrink so quickly, especially in xenografts using MDA-MB-468 cells which have a generally low invasive capacity (compared to MDA-MB-231 cells) (Sheridan et al., 2006; Thompson et al., 1992). Additionally such a study may gain further reliability if the treatment groups were blinded to the assessors. Although, unblinding is likely to occur due to the noticeably larger tumors of the vehicle group, it may still reduce any subjective bias towards a particular treatment group.

Immunohistochemistry was used to determine the effects of raloxifene on cell proliferation, angiogenesis, and to gain insights into ER expression and apoptosis. IHC is a commonly used method in cancer diagnostics, for example for the determination of the receptor status of breast cancer samples. Advantages of this method are the short procedure time, its low cost and its linkage to clinical outcome and response to treatment (Schnitt, 2001). The use of IHC has also been identified to be superior to ligand-binding assays (Harvey et al., 1999). However, IHC is heavily dependent on formalin fixation time and the numerous antibodies available which vary in sensitivity and specificity (Gown, 2008). Additionally, there is neither a uniformly accepted threshold for positivity, nor a standard scoring system (%)
positive cells, % strongly positive cells, 0-3+, etc.) (Schnitt, 2001). Needless to say, the quality of the tumor section is critical, too.

A serious problem that was noticeable throughout all IHC studies herein, was that the sections taken from some of the larger tumors were often of very poor quality due to crinkles and/or ripples. This affected tumor-sections particularly from the vehicle group, reducing the number of included tumors, hence the statistical power. The crinkles/ripples occurred despite the relatively thick slicing of 10 µm. In comparison with other available literature on xenograft models, this is at least twice as thick (4-5 µm, (Ricci-Vitiani et al., 2010; Roomi et al., 2005; Saffran et al., 2001; Sawaoka et al., 1998; Shah et al., 2006; Sirotnak et al., 2000)), and it might be an improvement to consider using thinner tumor sections for future IHC experiments. This might also enhance antibody specificity, and reduce background staining. An interesting observation made was that some tumors differed from others, in that they did not form a continuous layer of cells throughout the tumor. These tumors only had a cellular layer at the tumor periphery with the rest being filled by a white appearing, fluid-like mixture, likely to be the BD Matrigel™ matrix. As there was no pattern confining these tumors to a particular treatment group, the analysis of the results did not require adjustments.

Western blotting was used to gain a better understanding of the molecular events of tumor growth inhibition in response to raloxifene treatment. The combination of IHC and Western blotting would more reliably determine the mechanism of raloxifene action as the results from IHC can be overlapped and backed up by Western blotting as both techniques assess the expression of a particular protein of interest. IHC may additionally provide information about the cellular- and tissue/tumor-localization, however this possible in this study due to the poorly cut tumor sections.

Sometimes the reproducibility of the Western blots performed in this study was questionable. It is likely that the discrepancies occurred during tumor-sample loading onto the gel. Minor differences between gel compositions or the age of the gels may also have contributed to these observations.

The qualitative process of Western blotting can only be semi-quantitative in combination with densitometry, which then allows for statistical analysis of the data. The method of densitometry is frequently not documented, and the various procedures that can be applied vary significantly in the end-result (i.e. a significant result can be insignificant and vice versa depending on the method used) (Gassmann et al., 2009). The method of densitometry used for the analysis of Western blots from this study aimed to follow the recommended guidelines from Gassmann et al. (2009), who published an extensive study on various
methods of densitometry (refer to ‘Methods’ for detail). Although it was suggested that entire blots should be presented, this was not done due to the many repeats performed and the difficulty of presenting all of them in an orderly way.

4.2 Raloxifene Efficacy in TNBC

Low dose raloxifene was shown to be an effective treatment to reduce the tumor growth of TNBC xenografts. Particularly the 0.85 mg/kg raloxifene dose, significantly reduced the tumor volume over the daily, 10 week treatment-period. Despite large animal variations in the tumor growth between treatment groups, mice treated with 0.85 mg/kg of raloxifene had 32% smaller tumors after 10 weeks of treatment compared to control. These results were in line with tumor weight measurements, which showed that tumors from the 0.85 mg/kg raloxifene group were approximately 40% lighter than tumors from the control group. It should be noted that two mice (out of 12) treated with 0.85 mg/kg raloxifene were not at all responsive to the therapy, as their tumors continuously increased over the 10 week. Interesting was the observation that 0.85 mg/kg raloxifene completely halted any tumor growth/change in the last 3 weeks of the study. This may suggest that peak effectiveness of raloxifene is reached after ~7 weeks of treatment.

Raloxifene has been shown to reduce cell viability in many different ER-negative cells, including breast, prostate, myeloma, uterine leiomyoma, bladder and endometrial cancer cells (Khosrokhavar et al., 2009; Stuart et al., 2010; Stuart et al., 2008; Todorova et al., 2010; Werner et al., 2005).

The results of this study are most convincingly supported by previous findings by Taurin et al. (2011), who also investigated the effects of low dose raloxifene on xenograft tumor growth. For this, five to six week old athymic female nude mice were inoculated with MDA-MB-468 cells. Once the tumors reached a volume of 400-500 mm$^3$, the mice were randomized (n = 5) and treated daily with 0.85 mg/kg of raloxifene for 10 weeks. The results showed that raloxifene reduced tumor size by 30% compared to the original size of the tumor at day 0 and by 70% after ten weeks compared to mice treated with vehicle (Taurin et al., 2011).

In vitro studies showed that raloxifene decreased the cell viability of MDA-MB-468 cells (EC$_{50}$ of 6.8 µM), MDA-MB-231 cells (EC$_{50}$ of 9.5 µM), Hs578t cells (EC$_{50}$ of 6.8 µM) and SKBr-3 cells (EC$_{50}$ of 10.6 µM) (Taurin et al., 2011). Using flow cytometry, cell cycle analysis demonstrated an increased proportion of cells in the G1 phase in all four cell lines.
Furthermore, after 48 h of raloxifene treatment, apoptosis was increased by 8-fold in MDA-MB-468, MDA-MB-231, and Hs578t cells and by 6-fold in SKBr-3 cells. Western blotting showed that raloxifene affected the expression and phosphorylation patterns of proteins involved in cell proliferation (NF-κB, β-catenin, EGFR, and AKT), protein synthesis (4EBP-1 and mTOR) and apoptosis (caspase 3) (Taurin et al., 2011).

Further evidence that raloxifene induces apoptosis in MDA-MB-231 cells was provided by Stuart et al. (2008). Concentrations of 5 µM raloxifene significantly decreased the cell number by ~30% after 7 days of treatment. Apoptosis (measured by flow cytometry) was significantly induced in 8% of raloxifene treated cells after 36 h. Cell cycle analysis showed that a significant 8% increase in the proportion of cells in G1-phase was observed after 24 h of raloxifene treatment. Western blotting was employed to assess whether raloxifene treatment had effects on the expression of important cell signaling proteins. Treatment for 18 h reduced the expression (~10%) of phosphorylated AKT, but this change in expression was not statistically significant (Stuart et al., 2008).

Werner et al. (2005) also aimed to elucidate the effects of raloxifene and other SERMs in ER-positive and ER-negative breast cancer cells (passage number unknown). The effects of the treatment combinations were assessed in terms of cell proliferation (measured by quantification of cyclin D1 mRNA) and ratio to apoptosis (measured by using the Nicoletti method - a flow cytometric method for measuring the percentage of apoptotic nuclei after propidium iodide staining). Six days of incubation with raloxifene (1 µM) in MDA-MB-231, MCF-7 and T47D cells showed an apoptosis to cell proliferation (A/P) ratio index of over 1. This suggested a significant induction (fold-change unknown) of apoptosis with low concentrations of raloxifene. Although the mechanism under which apoptosis induction occurred was not assessed, the results highlight that the apoptotic effects induced by raloxifene in MDA-MB-231 cells, are not dependent on ER expression (Werner et al., 2005).

In an early study by Thompson et al. (1988) the effects of raloxifene (and tamoxifen) were compared to those of 17β-estradiol in MCF-7 and MDA-MB-231 cells on cell proliferation, cell invasiveness, chemotactic responsiveness and collagenase production (type IV collagenase assay). Interestingly, tamoxifen and its active metabolite 4-hydroxytamoxifen (0.1 µM) markedly reduced not only the proliferation (by ~60%) of MCF-7 cells but also increased cellular invasiveness by ~30%. In stark contrast, raloxifene (0.1 µM) also reduced cell proliferation (by ~60%) but did not stimulate the invasiveness of the cells. Four-day treatment with the SERMs and 17β-estradiol in MDA-MB-231 cells showed that neither
treatment had any significant effects on cell proliferation nor cell invasiveness or chemotactic responsiveness (Thompson et al., 1988).

In a publication by Khosrokhavar et al. (2009), MDA-MB-231 cells were treated with raloxifene for 7 days at concentrations ranging from 1 µM to 1 pM to assess cell viability using a spectrophotometric Resazurine-based method. None of the six concentrations tested had any significant effects on cell viability. In contrast, T-47D cells (ER and Her-2 positive) were considerably more responsive to raloxifene treatment. A 40% reduction in cell viability was observed after a 7 day incubation with 0.1 nM raloxifene (Khosrokhavar et al., 2009). Arguably, concentrations of 1 µM raloxifene or less seem insufficient to have a significant effect on cell viability in ER-negative cells. A recent publication by Todorova et al. (2010) showed that 5-25 µM of raloxifene caused a dose-dependent reduction in cell survival in MDA-MB-231 cells by up to 60%. Interestingly this 60% reduction (results of three independent experiments) after seven days of incubation with 25 µM raloxifene was not statistically significant, making the reliability of the results questionable (Todorova et al., 2010). It should also be pointed out that the passage number of MDA-MB-231 cells greatly differed. In the study by Todorova et al. (2010), the cells used were at passage 26. Khosrokhavar et al. (2009) used the same cell line but with a passage number between 44 and 51. Studies have shown that with increases in passage number or over-subculturing, alterations in cell morphology, response to stimuli, growth rates, protein expression, transfection and signaling occurs (Chang-Liu et al., 1997; Esquenet et al., 1997; Sambuy et al., 2005; Yu et al., 1997). Long-term subculturing places selective pressure on cell line traits, favoring genotypic and phenotypic changes, allowing cells to for example, grow faster (Hughes et al., 2007). With this in mind, the aforementioned studies’ results may not be comparable, as it is possible that the over-subcultured MDA-MB-231 cells used by Khosrokhavar et al. (2009) may have led to changes in the intracellular expression of proteins critical for raloxifene action and its associated cell death induction.

In a study of prostate cancer cells, it was shown that ER-positive, PC3 and PC3M cells were more resistant to raloxifene treatment (1 µM to 1 pM) than ER-negative Du145 cells (ER-β positive only) (Kim et al., 2002b). The number of Du145 cells was decreased by approximately 95% after four days of raloxifene (1 µM) treatment. Using an apoptotic TUNEL assay it was shown that raloxifene significantly increased apoptotic cells and caused caspase 9 activation in Du145 cells. Conversely in PC3 cells, raloxifene caused caspase 8
activation, indicating that raloxifene-induced apoptosis is likely to involve multiple and independent pathways depending on cell type (Kim et al., 2002b).

In the bladder cancer cell line TSU-PR1 (ER-β positive only), raloxifene treatment at concentrations ranging from 1 µM to 1 pM, induced apoptosis, including changes in nuclear morphology, DNA fragmentation, and cytochrome c release, in a dose dependent manner (Kim et al., 2002a). Cell viability of TSU-PR1 cells was significantly reduced by \( \sim 75\% \) compared to control after treatment with 1 µM raloxifene for 4 days. Furthermore, raloxifene induced a \( \sim 15\)-kDa cleavage product of BAD as early as 24 h after raloxifene treatment, but no changes in Bax, Bcl-2, and Bcl-XL expression. This showed raloxifene treatment induced cytocidal effects in bladder cancer cells independent of ER expression, but rather through cleavage of BAD, which leads to cytochrome c release and subsequent induction of apoptosis (Kim et al., 2002a).

Raloxifene efficacy was also investigated in human uterine leiomyoma cells. In this interesting study by Liu et al. (2007), twelve uterine leiomyoma tissues were obtained from premenopausal women with regular menstrual cycles who underwent abdominal hysterectomy or myomectomy. The tissue samples were then prepared for \textit{in vitro} culturing. Treatment with 1 nM raloxifene for 48 and 72 h significantly decreased cell viability by \( \sim 8\% \) and 15\%, respectively. On the other hand, treatment with 10 and 100 nM raloxifene for 48 h increased the viability of leiomyoma cells by 10\%. The percentage of proliferating cell nuclear antigen (PCNA)-positive cells was decreased by \( \sim 10\% \) after 48 h of treatment with 1 nM raloxifene. Treatment for 48 h with 10 and 100 nM raloxifene increased the percentage of PCNA-positive cells by 10\% and 13\%, respectively compared to control. TUNEL assay further confirmed these findings. The percentage of apoptotic nuclei was increased by \( \sim 9\% \) in cells treated with 1 nM raloxifene, however, no changes in apoptotic cells were found at the other raloxifene concentrations. Compared with untreated control cultures, treatment with 1 nM raloxifene significantly decreased Bcl-2 protein expression in cultured leiomyoma cells, whereas treatment with raloxifene at concentrations of 10 and 100 nM did not affect the expression of this anti-apoptotic protein.

Another study opposing a simple dose response model exhibited by raloxifene was in 21 postmenopausal women with metastatic (stage IV), ER-positive breast carcinomas (Gradishar et al., 2000). Patients were treated with raloxifene 150 mg twice daily until tumor progression was observed. A partial response was observed in 19\% of the patients after a median of 20.7 months of treatment. Although, the high raloxifene doses were well tolerated
and safe in these highly selected patients, the authors concluded that there was no need to further investigate high dose raloxifene as monotherapy (Gradishar et al., 2000).

Raloxifene has also been investigated in combinatorial studies. Combination therapy of raloxifene (1 µM) with cisplatin (0.27 µg/mL) has been investigated in the 5637 bladder cancer cell line (ER-β positive only). The results showed that after four days of treatment with both agents a synergistic effect was observed, reducing the cell viability by 75% compared to control. A study conducted in 300 virgin female Sprague-Dawley rats was aimed to evaluate the chemopreventative activity of raloxifene (20 mg/kg diet) in combination with 9-cis-retinoic acid (9cRA, 60 mg/kg diet) (Anzano et al., 1996). The well accepted rat model of breast cancer induced by the carcinogen nitro-somethylurea (NMU, 50 mg/kg body weight) was used. After 140 days of treatment, the tumor incidence in vehicle treated mice was 96% (3.2 tumors and an average tumor burden of 12.6 g). In rats treated individually with raloxifene tumor incidence was reduced to 55% (1 tumor per rat and an average tumor burden of 2.3 g). 9cRA alone reduced the tumor incidence to 79%. The combination treatment of raloxifene and 9cRA reduced the tumor incidence to approximately 20% (0.5 tumors per rat and average tumor burden of 0.6 g) (Anzano et al., 1996).

Scandlyn et al. (2011) first conducted studies of raloxifene in MDA-MB-231 xenografts. Mice either received tamoxifen (75 µg/kg, p.o.), raloxifene (0.85 mg/kg, p.o.), EGCG (25 mg/kg, i.p.), or combinations of tamoxifen + EGCG or raloxifene + EGCG (Scandlyn, 2011). The tumor volume of mice treated with the combination of tamoxifen + EGCG was significantly reduced by 71% compared to control after 10 weeks of daily treatment, but on the other hand, individual treatment of either tamoxifen or EGCG failed to suppress tumor growth. In contrast, raloxifene was more effective at reducing the tumor volume individually, rather than when used in combination with EGCG. The tumor volume was reduced by 79% by raloxifene treatment alone and 54% by raloxifene + EGCG after 8 weeks. This suggested that EGCG interfered with raloxifene’s metabolism. However, the activities of hepatic uridine diphospho-glucuronosyltransferase (UGT) enzymes, which metabolize raloxifene and EGCG, were not altered by EGCG treatment (Scandlyn, 2011). This very interesting finding suggests that EGCG’s conjugation, which was given at a high dose, saturated the UGT enzyme, therefore not allowing conjugation of raloxifene. If active glucuronide metabolites of raloxifene are responsible for tumor growth suppression, then 98% of the dose would be active (only 2% remaining as the parent compound to act as a SERM (Snyder et al., 2000)), unless conjugation by UGT was prevented by a saturated UGT.
enzyme (e.g. high EGCG). The enzyme(s) responsible, as well as the active glucuronide metabolites remain to be identified. One should not exclude the possibility that metabolites produced in the mouse are more active/different than their human counterparts.

One study has compared the metabolism and disposition of raloxifene in liver and intestinal microsomes from female humans and female Sprague-Dawley rats (70-110 days old) (Jeong et al., 2005). The results showed that total intrinsic clearance of the major raloxifene metabolites 4'-β-glucuronide and 6'-β-glucuronide in human intestinal microsomes was 3- to 6-fold higher compared to rat intestinal microsomes. In contrast to intestinal microsomes, the rate of glucuronide clearance was the same as in liver microsomes. Regardless of the species, intrinsic clearance from small intestinal microsomes was 2- to 5-fold higher than that of hepatic microsomes. RT-PCR revealed that rat microsomes lacked expression of the UGT1A10 and UGT1A8 enzymes (Jeong et al., 2005; Komura et al., 2011). This may explain the much lower bioavailability of raloxifene in humans (2%) compared to rats (39%) (Jeong et al., 2005). Mizuma (2009) also highlighted that intestinal glucuronidation catalyzed by UGTs, may have a greater impact on oral bioavailability of raloxifene than hepatic glucuronidation does. In humans, particularly UGT1A8 and UGT1A10 are of major importance for raloxifene conjugation (Mizuma, 2009). Unlike the rat, mice do express intestinal UGT1A10, making the mouse the more representative animal of raloxifene metabolism in humans compared to the rat (Komura et al., 2011).

A big problem noted in this study was the unexpected slow tumor growth in the control group. This compromised mechanistic analyses of raloxifene action by IHC and Western blotting. In comparison with other studies using MDA-MB-468 xenograft, the tumors herein grew at a significantly lower rate. One study showed that untreated six-week-old female athymic NCR/c (nu/nu) nude mice inoculated with $1 \times 10^7$ MDA-MB-468 cells bore tumors of approximately 480 mm$^3$ after 35 days treated with 0.1% DMSO (Kahan et al., 2000). In another study using four- to six-week-old nu/nu athymic female mice, $1 \times 10^7$ MDA-MB-468 cells were subcutaneously injected in the right flank. Mice treated with DMSO-control bore tumors of ~800 mm$^3$ after 35 days, and of approximately 1900 mm$^3$ after 50 days (Caldas-Lopes et al., 2009). Another study using NMRI-nu (nu/nu) mice, between 8 to 10 weeks old, were grafted subcutaneously in the right flank with $5 \times 10^6$ MDA-MB-468 cells in 0.1 mL of PBS. Control animals (n = 9) bore tumors with a volume between 300 and 1000 mm$^3$ after 50 days. After ~80 days, the majorities of the tumors had grown to approximately 1500 mm$^3$ (Rousseau et al., 2011). Although $8 \times 10^6$ MDA-MB-468 cells were inoculated in this experiment, however, it seems unlikely that a difference of $2 \times 10^6$ cells explains the
coherent differences in tumor growth kinetics between this and other studies. The most plausible explanation is that the genetic variability between the outbred mice used in this study and the inbred mice used in the above-mentioned studies, is the major contributing factor for the notable discrepancies in the tumor growth kinetics. Such genetic differences could be identified using Microarray chips (see Future Directions). This may also provide some explanation for the observation that tumors of the vehicle-group shrunk considerably in the final two weeks of the study. Technical explanations for this lower tumor growth rate might be that the matrigel was not properly prepared, or that a significant proportion of the cells selected for inoculation were of a dormant subpopulation that were inactive in vivo. Tumors would therefore grow at considerably lower rate (Benton et al., 2011; Hedley et al., 2009). One group has reduced this stalling effect in tumor growth by injecting more matrigel, this however, is not applicable once treatment has started (Fridman et al., 1991).

4.3 Safety of Raloxifene

As expected, low dose raloxifene did not have any toxic effects on liver, kidney, spleen or uterus. Their weight remained the same in all three groups. Animal body weight did also not differ between the treatment groups. Plasma alanine aminotransferase (ALT) levels did not increase upon treatment with raloxifene. Raloxifene is cleared primarily by glucuronidation and sulfation of the phenolic groups and not by oxidative metabolism, which would form reactive hepatotoxic quinone or quinone methide metabolites (Liebler, 2008; Park et al., 2011).

4.4 Mechanistic Analyses

4.4.1 Ki67 expression

Analysis of Ki67 expression showed that both raloxifene doses equally reduced Ki67 expression. As the smaller sized tumors were easier to slice the sections produced from them were of significantly higher quality, allowing better examination of the protein staining. As the 0.5 mg/kg raloxifene group was a mix of relatively large and small tumors, there was a random inclusion of smaller sized tumors from this group, but as the large tumor sections could not be analyzed for Ki67 staining due to their poorer quality a bias was introduced. Thus Ki67 expression was probably lower than the actual expression in the 0.5 mg/kg
raloxifene treatment group. This may also explain the similarity observed between both raloxifene doses on the effects of Ki67 expression. Regardless of this, raloxifene decreased tumor cell proliferative activity by approximately 50%. It is well known that a key feature of tumor growth/progression is cell proliferation. The nuclear protein Ki67 is expressed in all phases of the cell cycle except the G0 phase, and its immunohistochemical analysis is frequently discussed as a potential prognostic marker of early breast cancer (Gerdes et al., 1984). In 17 out of 18 studies with over 200 patients, a statistically significant association between Ki67 expression and prognosis was identified. This provided strong evidence for a biological relationship between Ki67 expression and patient prognosis, however, the cutoffs to distinguish ‘Ki67 high’ staining from ‘Ki67 low’ staining varied from 1% to 28.6%, which significantly limits clinical use of Ki67 (Urruticoechea et al., 2005). A recent publication by the “Recommendations from the International Ki67 in Breast Cancer Working Group”, makes comprehensive recommendations on preanalytical and analytical assessment as well as interpretation and scoring of Ki67 expression. Their aim was to “harmonize methodology”, and ultimately to improve the validity of this marker in clinical practice (Dowsett et al., 2011). Suggestions were made to fix sections stained for Ki67 in neutral buffered formalin for 4-48 h to achieve best results (Dowsett et al., 2011). However, in this experiment sections stained for Ki67 were fixed in acetone for only 10 min. Furthermore, counting mitotic figures of stained sections, or measuring the incorporation of labeled nucleotides into DNA, may also be used to confirm the results. It should also be stated that the intensity of Ki67 expression can vary throughout the cell cycle, raising concern about misclassification of cycling cells as resting cells (Dowsett et al., 2011). Unfortunately the Ki67 expression analysis done in this study was performed prior to the publication by Dowsett et al. (2011) and could therefore not be executed to the standard described there.

Although tamoxifen has been shown to significantly decrease Ki67 expression regardless of ER expression (Clarke et al., 1993), studies with raloxifene have been less conclusive. Ki67 expression in tumors of 20 postmenopausal women with invasive ductal, ER-positive, Her-2-negative breast cancers who were treated with 60 mg/day raloxifene for 28 days, was analyzed (Lopes-Costa et al., 2010). The results showed that raloxifene treatment significantly decreased Ki67 expression by 46%, when comparing the staining prior to, and following the 28-day treatment period (Lopes-Costa et al., 2010). This study, alongside another recent study from the same research-group was performed in patients with ER-
positive breast cancer (da Silva et al., 2009). The results are therefore not comparable to the results from the TNBC xenograft herein.

In a phase II, double-blind, placebo-controlled, randomized clinical trial of postmenopausal women, Dowsett et al. (2001) investigated the effects of raloxifene on Ki67 expression. Of the 143 patients, approximately equal numbers either received placebo, a single-daily oral dose of 60 mg or a twice-daily oral dose of 300 mg of raloxifene for 14 consecutive days. Of the patient sample, 83.2% (119 patients) were ER-positive and 16.8% (24 patients) were ER-negative (Dowsett et al., 2001). Baseline Ki67 measurements were significantly higher (by 40%) in ER-negative patients compared to ER-positive patients. Comparison of the endpoint and baseline Ki67 measurements in ER-positive patients showed that there was a median decrease of 21% among patients treated with 60 mg/day. A median decrease of 14% in Ki67 expression was noted in patients treated with 600 mg/kg of raloxifene. Statistical significance was only observed in the lower 60 mg/day treatment group, which points towards an inverse dose response effect of raloxifene. On the other hand, Dowsett et al. (2001) concluded that there was no significant effect of raloxifene on Ki67 expression in ER-negative tumors; however, the data for this was not shown. It was mentioned that due to errors in sample size calculation, authors focused only on ER-positive tumors. The authors stated: “it was a priori known that raloxifene was most likely to have an effect in patients with ER-positive tumors” (Dowsett et al., 2001). Therefore, no definite conclusions of the effects of raloxifene on Ki67 expression in ER-negative breast cancers can be made from this study. No studies investigating the effects of raloxifene on Ki67 expression in MDA-MB-468 cells or such xenograft models have been repeated.

**4.4.2 NF-κB expression**

Of central importance to many key cellular biochemical processes is NF-κB (Maeda et al., 2008). This transcription factor regulates the expression of several hundred cellular genes, involved in immune and inflammatory responses, differentiation, oxidative stress responses, cell adhesion, apoptosis and cell proliferation (see [www.nf-kb.org](http://www.nf-kb.org); under Target Genes). NF-κB is a potential drug target for cancer therapy (regardless of ER expression) due to its involvement in the regulation of genes associated with cell proliferation such as cyclin D1 (Guttridge et al., 1999), c-myc (Duyao et al., 1990), and VEGF (Chilov et al., 1997), as well as genes involved in apoptosis such as Bax (Grimm et al., 2005), Bcl-2 (Catz et al., 2001) and Fas-Ligand (Matsui et al., 1998). The expression of NF-κB was therefore assessed in the tumor samples of this study.
In mice treated with 0.85 mg/kg raloxifene, a decrease in NF-κB expression of 37% compared to vehicle-control was observed. This decrease was not statistically significant mainly due to the considerable animal variation. Additionally there were some inconsistencies between the three replicates, which are likely to be attributable to inconsistent loading of the SDS-PAGE gel. Nevertheless, the findings of this study are supported by a mechanistic study of raloxifene in MDA-MB-231 cells by Stuart et al. (2010). A detailed Western blotting analysis was performed to identify the effects of raloxifene on cell signaling molecules including proteins involved in cell survival or apoptosis (Stuart et al., 2010). Cells were treated with 4 µM of raloxifene and protein expression was assessed at three different time points: 6, 12 and 18 h. The expression of p65 (a major subunit of the NF-κB dimer) was significantly decreased by ~22% after 6 h and ~30% after 12 h (Stuart et al., 2010).

A study conducted in multiple myeloma JJN-3 cells (ER-α and ER-β positive), showed that treatment with 5 µM of raloxifene for 72 h inhibited cell proliferation by 50% (Olivier et al., 2006). Electrophoretic mobility shift assays confirmed that raloxifene at the given concentration decreased NF-κB DNA-binding activity in JJN-3 cells after 30 min, and furthermore, it completely inhibited all interactions after 16 h. Tamoxifen at the same concentration showed a significantly less pronounced inhibition of NF-κB DNA-binding activity. Immunofluorescence staining was performed to determine the subcellular localization of NF-κB subunits and showed that raloxifene treatment rapidly induced p65 relocalization from the nucleus to the cytoplasm. However, analysis of nuclear extracts using immunoprecipitation showed that NF-κB’s p65 subunit associated strongly with the ER after 5 min of raloxifene treatment (Olivier et al., 2006). Using a chromatin immunoprecipitation assay, the binding of p65 to NF-κB-regulated promoters was evaluated in the presence of raloxifene on the mip-1α gene promoter. This gene promoter was chosen because it is a NF-κB-regulated gene whose expression was significantly decreased after raloxifene treatment in JJN-3 cells. It was shown that 5 µM raloxifene treatment for 2 h led to the dissociation of p65 and ER binding to the mip-1α gene promoter (Olivier et al., 2006). The authors of this study concluded that raloxifene may induce a conformational change in the ER, which results in decreased NF-κB DNA-binding activity and, consequently, in the release and translocation of p65 from the nucleus to the cytoplasm (Olivier et al., 2006). It is interesting to note the different effects of raloxifene on p65 in MDA-MB-231 and JJN-3 cells, and the importance of the ER. Comparison between the time-onset of p65 inhibition observed by Stuart et al. (2010) and Olivier et al. (2006) show considerable differences between the two.
cell lines. It may be that the ER is critical for a much quicker and more pronounced response on NF-κB’s p65 subunit. However, other cell line specific responses cannot be excluded by these findings.

An interesting study by Ryan et al. (2000) showed that induction of p53 caused activation of NF-κB, which correlated with the ability of p53 to induce apoptosis (Ryan et al., 2000). On the contrary, inhibition of NF-κB activity abolished p53-induced cell death, indicating that NF-κB is essential in p53-mediated cell death. It was concluded that therapeutic inhibition of NF-κB is effective and desirable only in p53-null or defective tumors. However, in tumors that retain wild type p53, p53 itself is an important mediator of chemosensitivity. Therefore targeting NF-κB in these wild type p53 tumors may be more counterproductive, as therapeutic inhibition of NF-κB is likely to result in the repression of p53-mediated tumor cell death (Ryan et al., 2000).

Moreover, it should be emphasized that complete inhibition of NF-κB has been discussed to be more favorable only for acute treatment settings, as NF-κB in inflammatory cells serves an important immune function, and its absence can therefore result in severe immunodeficiency (Karin, 2006). In this study, NF-κB was not completely abolished, which may be a favorable response in terms of adverse side effects. The results from this study may therefore provide a starting point for a further, more comprehensive analysis of possible NF-κB target genes/proteins. One could assess the binding of NF-κB to specific promoter regions of target genes involved in cell proliferation using EMSA, and/or dual fluorescence in situ hybridization (FISH). Furthermore, real-time quantitative-PCR (RT-PCR) and Western blotting could be used to evaluate changes in the expression of genes/proteins important for cell proliferation, such as cyclin D1 (Hui et al., 2003; Joyce et al., 1999).

### 4.4.3 p38 expression

Studies have shown that p38 can directly regulate the transcriptional activity of NF-κB. Therefore the expression of this mitogen protein kinase was also assessed (Goebeler et al., 2001; Sakai et al., 2002). In this study, 0.85 mg/kg of raloxifene significantly decreased the ratio of phosphorylated-p38 to total p38 by approximately 80%. Due to the cell context-specific and cell type-specific manner under which p38 can affect cell proliferation, differentiation, survival and migration, its role in cancer treatment and development is still somewhat controversial (Sosa et al., 2011; Wagner et al., 2009).
In cervical cancer HeLa cells, treatment with nocodazole (3 µM), taxol (1 µM), vincristine (1 µM), or vinblastine (1 µM) for various times (0–30 h), showed that p38 activation was required for chemotherapeutic drug-induced cell death (Deacon et al., 2003). In another study of cisplatin (66 µM) doxorubicin (1.7 µM) and taxol (100 nm), it was investigated whether the role of p38 is treatment and/or cancer cell-type specific (Losa et al., 2003). In this large study, the three chemotherapeutic agents were tested for up to 6 h in: Human Embryonic Kidney (HEK) 293 cells, epidermoid carcinoma A431 cells, human diploid fibroblast strain IMR-90, Cercopithecus aethiops (African green monkey) kidney fibroblast cell line Cos-7, human keratinocyte cell line HaCaT, head-and-neck squamous carcinoma cells HN19 and HN30 as well as Hela cells (Losa et al., 2003).

Cisplatin was shown to have the strongest cell line dependent effects on p38 activation, detectable as early as 1 h after treatment. Treatment with doxorubicin in the various cell lines showed that this chemotherapeutic agent produced cell-type specific responses regarding p38 phosphorylation. This activation was clearly found in Hela and HEK 293 cells, to a lesser extent in HaCaT, IMR90, A431 and HN19 cells, but not at all in HN30 or Cos cell lines (Losa et al., 2003). In contrast to this effect taxol did not cause p38 phosphorylation in any of the cell lines. Using specific inhibitors of p38 (10 µM of either SKF86002 or SB 203580) it was shown that cell viability was increased by p38 inhibition prior to cisplatin treatment. From this data, the authors concluded that low activation of p38 correlated with a treatment-resistant phenotype (Losa et al., 2003). It should be pointed out that treatment with cisplatin was at a much higher concentration (at least 38-fold) compared to both other drugs, which may be important for the differential effects observed.

These two studies by Deacon et al. (2003) and Losa et al. (2003) support the notion of cell-line and treatment specific responses of p38. Of greater relevance to TNBC, was the study by Chen et al. (2009), who noted that out of the of the four different mammalian p38 isoforms identified (p38α, p38β, p38γ, p38δ), p38α and p38δ were most abundantly expressed in 37 human breast tumors as well as in MCF-7, T47D, MDA-MB-361, MDA-MB-231, MDA-MB-453, MDA-MB-468, BT549, HCC1937 and SKBR-3 breast cancer cell lines (Chen et al., 2009). Furthermore, the expression of a dominant-negative p38 mutant (generated by mutating Thr-180 to Ala and Tyr-182 to Phe which are both required for phosphorylation/activation of p38) showed cell type specific responses. In TNBC cell-lines, MDA-MB-468 and MDA-MB-231 cells (p53MUT), the expression of the dominant-negative p38 significantly inhibited cell proliferation by ~90 and ~75%, respectively. On the other hand in ER-α positive MCF-7 cells (p53WT) the expression of the p38 mutant did not
suppress cell proliferation. Additionally, the dominant-negative p38 mutant in MDA-MB-468 cells also inhibited anchorage-dependent and –independent proliferation. Cell cycle distribution studies showed that the anti-proliferative effects caused by the p38 mutant MDA-MB-468 cells were due to inhibition of G1/S cell cycle progression (Chen et al., 2009). Protein and RNA measurements concluded that this was due to the blockage of cyclin D1 in the MDA-MB-468 cells expressing dominant-negative p38. Furthermore, application of siRNA (10 nM) to silence either p38α or p38δ showed that proliferation of MDA-MB-468 cells after 4 days of transfection with p38α-siRNA was significantly reduced by ~50%, but not by transfection with p38δ-siRNA (Chen et al., 2009). From these results it was concluded that p38α is the major mediator of proliferation signaling in MDA-MB-468 cells.

Further confirmation was provided by the use of two specific p38 inhibitors (SB203580 and AZ10164773) in the various breast cancer cell lines listed above (Chen et al., 2009). Among the cell-lines tested, TNBC cells with p53 mutations were the most sensitive to both p38 inhibitors. The IC₅₀ of SB203580 and AZ10164773 was 9 µM and < 0.1 µM, respectively in MDA-MB-468 cells. To achieve the same 50% inhibition of cell proliferation in MCF-7 cells, 30 µM of SB203580 and 20 µM of AZ10164773 were required. Importantly, cell proliferation of normal breast cells (HMECs) and immortalized breast cells (MCF-10A) was not inhibited by treatment with either p38 inhibitor (Chen et al., 2009).

To confirm the importance of p53 for p38-mediated cell proliferation, siRNA was used to effectively inhibit p53WT mRNA expression in MCF-7 cells. The results showed that blockage of p53 significantly sensitized MCF-7 cells to AZ10164773 (IC₅₀ of <0.1µM) (Chen et al., 2009). This led the authors to conclude that loss of p53 expression caused breast cancer cells to become dependent on p38 for their growth (Chen et al., 2009). The protein phosphatase Wip1, which is stimulated by p53, is able to dephosphorylate p38, thereby inactivating it. Wip1 was also knocked down using siRNA in MCF-7 (Chen et al., 2009). The IC₅₀ of AZ10164773 in Wip1-silenced MCF-7 cells was 1 µM, indicating that loss of Wip1 sensitizes p53WT cells to p38 inhibition. The authors finally concluded that targeting p38 might be clinically useful in patients with highly aggressive TNBCs that are p53MUT (Chen et al., 2009).

The most common overall conclusion regarding p38 and cancer treatment is that it acts tissue- and treatment-specifically (Bradham et al., 2006; Chen et al., 2009; Lenassi et al., 2006; Losa et al., 2003; Olson et al., 2004; Wagner et al., 2009). Interestingly, breast cancer patients with high levels of p38 have also been correlated with tamoxifen-resistance, and highly invasive and therefore poor prognostic breast cancer (Davidson et al., 2006; Esteva et
al., 2004; Gutierrez et al., 2005; Salh et al., 2002). The literature therefore does not exclude the possibility that raloxifene may act through p38 to reduce cell proliferation in this xenograft model of TNBC.

4.4.4 P27

It has been shown that 5 µM raloxifene significantly increased the proportion of MDA-MB-231 cells in G1-phase by 8% after 24 h of treatment (Stuart et al., 2010). Therefore, to further understand the effects of raloxifene on cell proliferation in this xenograft study, the expression of the cell cycle regulating protein p27\textsuperscript{Kip1} was assessed. This protein is associated with G1-specific cyclin-CDK complexes and inhibits their catalytic activity. Increased expression of p27\textsuperscript{Kip1} is associated with G1-cell cycle arrest (Vlach et al., 1997). Additionally, overexpression of p27\textsuperscript{Kip1} in highly proliferative human breast cancers was accompanied by overexpression of cyclin D1, which is essential for the transition from G1 to S-phase (Fredersdorf et al., 1997). Cyclin D1 can also be regulated by p38 and NF-κB (Guttridge et al., 1999; Hinz et al., 1999; Thoms et al., 2007). Western blot analysis of p27\textsuperscript{Kip1} showed that raloxifene however did not affect the expression of this protein, also indicating that both p38 and NF-κB were not interacting with p27\textsuperscript{Kip1} to decrease cell proliferation.

4.4.5 EGFR signaling

As EGFR is commonly overexpressed, and regarded as an important drug target for TNBCs, expression of this TKR was also assessed (Ueno et al., 2011). As its name suggests, the EGFR initiates important signal transduction cascades, which ultimately lead to cell proliferation. Western blot analysis showed that raloxifene treatment at both concentrations significantly increased EGFR expression by up to 115% compared to control. This interesting observation was contrary to what was expected, as high EGFR expression is usually associated with increases in tumor growth due to stimulation of cell proliferation (Dua et al., 2010; Yun et al., 2008).

One could argue that the observed increase in EGFR expression induced by raloxifene, may be a survival response of the cancer-cells to prevent/reduce tumor-shrinkage by enhancing cell proliferation through EGFR-signaling. Phosphorylation, required for EGFR activation may be inhibited by raloxifene treatment either in a direct or indirect manner. However, to verify this, the phosphorylation-status of EGFR should be assessed using an antibody that is
specific for one of the many phosphorylation sites found on the EGFR. Of greatest relevance are tyrosine 845, 1068, 1045, 1148 and 1173. Tryosine-845 is involved in stabilizing the activation loop, maintaining the active state enzyme and providing a binding surface for substrate proteins (Hackel et al., 1999). Phosphorylation at position 1068 allows the Grb2 adapter protein to bind the activated EGFR. This is a link to the activation of Ras and ERK1/2 (Rojas et al., 1996). The tyrosine residue pair 1148 and 1173 provides a docking site for the Shc scaffold protein and are also involved in MAP kinase signaling activation (Zwick et al., 1999).

To further understand the downstream consequences of increased EGFR expression, AKT was analyzed using Western blotting. This non-specific serine/threonine protein kinase is involved in important processes surrounding cell survival (by inhibiting apoptosis), protein synthesis and general tissue growth (Luo et al., 2003). Expression analysis showed that raloxifene did not significantly affect the expression of this protein. Although there was a tendency towards lower expression of AKT and p-AKT (and their ratios) in the 0.85 mg/kg raloxifene group, no conclusions could be drawn as animal variation was very high.

Similar observations were made when ERK1/2, another important mediator of cell proliferation downstream of EGFR, was analyzed. No statistically significant changes were observed in ERK expression. However, it should be stated that there was also a tendency towards a reduction in the ratio between ERK1/2 and p-ERK expression in the 0.85 mg/kg raloxifene group. Again, no conclusions about the effects of raloxifene on this pathway could be taken from this data. As with AKT, analysis of ERK1/2 expression was only analyzed by a single Western blot and should ideally be repeated.

4.4.6 β-catenin expression

The Wnt/β-catenin pathway has been shown to play a key role in normal embryonic development and in tumorigenesis. This pathway also regulates cell proliferation, migration, and differentiation (King et al., 2012). As a key downstream component of the Wnt/β-catenin signaling pathway, the expression of β-catenin was investigated. It was shown that expression of β-catenin expression was greatly increased (at least 330%) in both animal groups receiving raloxifene compared to control. These increases were not statistically different, due to high variations in β-catenin expression between mice. As with EGFR, increased expression of this protein is usually associated with increased cell proliferation, as β-catenin constitutively interacts with one or more target genes, leading to persistent
activation of cell growth signals (Cadigan et al., 1997; King et al., 2012; Polakis, 1999; Wodarz et al., 1998).

Several studies have identified overlap between β-catenin and EGFR signaling, which may explain the increases in expression observed in both proteins in this experiment. One study has shown that β-catenin can directly heterodimerize with the EGFR in a murine mammary tumor virus (MMTV)-Wnt-1 transgenic model of mammary carcinoma (Schroeder et al., 2002). Wnt overexpression has also been shown to activate signaling via the EGFR (Faivre et al., 2007). Another study showed that EGFR activation could transactivate β-catenin, which subsequently led to tumor cell invasion in human glioblastoma cells and A431 human epidermoid carcinoma cells (Ji et al., 2009). One could therefore argue that the increase in β-catenin expression observed in this experiment was a result of the increased expression of EGFR (or vice versa). This would indicate that raloxifene caused an upregulation of cell signaling events that promote cell proliferation. However, this was actually not the case as tumors in raloxifene treated mice were smaller and Ki67 expression was also significantly reduced in raloxifene treated animals.

One could propose that raloxifene may inhibit or cause a dysfunction in the ubiquitin–proteasome system, as this degradation complex controls the abundance and activity of both β-catenin and EGFR (Paul, 2008). This could be verified with selective proteasome inhibitors such as lactacystin or, in case of β-catenin, with a GSK-3β activity assay (Fenteany et al., 1998; Welsh et al., 1993). The serine/threonine kinase GSK-3β is one of the central players in the destruction complex, which acts as a negative regulator of β-catenin by phosphorylating it. Studies have reported a GSK-3β specific down-regulation mediated by other proteins such as Dsh, GSK-3β binding protein/frequently rearranged in advanced T-cell lymphomas-1 (GBP/Frat-1), PKC, Axin and insulin, and thereby stimulating the signaling function of β-catenin (Cook et al., 1996; Kishida et al., 1998; Li et al., 1999a; Welsh et al., 1993). It may be possible that GSK-3β was downregulated in the xenografts of this study, resulting in β-catenin accumulation.

As with the EGFR-analysis, the antibody used to assess β-catenin expression was not able to differentiate between phosphorylated and non-phosphorylated β-catenin. The possibility cannot be excluded that the majority of the detected β-catenin was in the phosphorylated state primed for degradation. Therefore, the expression of β-catenin should be assessed with specific antibodies targeting the serine or threonine phosphorylation sites on β-catenin. Additionally the subcellular localization of β-catenin should be determined as well, and to confirm that β-catenin binds to the promoter region of the TCF/LEF element to exert its
proliferative actions (Sena et al., 2006). This could be done using a chromatin immunoprecipitation (ChIP) assay or an electrophoretic mobility shift assay (EMSA) (Hart et al., 1999; Jho et al., 2002).

4.4.7 CD105 expression

It was unlikely that the drop in cell proliferation can solely be explained by investigating only certain molecular pathways, directly controlling cell proliferation. Therefore angiogenesis, a more indirect pathway that arguably may affect tumor growth, was assessed (Greenberg et al., 2010). Immunohistochemical analysis of CD105 showed that raloxifene did not have any significant effects on the expression of this angiogenic marker. CD105 positive staining was up to 17% higher in animals treated with raloxifene compared to control animals, however, it should be pointed out that only four tumors of the control group were of sufficient quality to be included in the analysis. Furthermore, linear regression analysis of CD105 positive staining versus tumor volume revealed that there was no correlation between the two variables ($r^2 = 0.0042$). Analysis of CD105 should be repeated on thinner and better quality tumor slices, as this was the major limitation for a consistent and representative investigation of angiogenesis.

Studies of raloxifene-mediated changes in angiogenesis are limited, and to date only a single relevant study regarding this topic has been published in a peer-reviewed journal. The aim of this study by da Silva et al. (2009) was to investigate the effects of raloxifene on CD34 (angiogenesis) and Ki67 expression, and whether there was a correlation between the two measurements. For this, 16 postmenopausal women with ER-positive, stage II breast carcinoma received 60 mg/day of raloxifene for 28 days prior to definitive surgery. Immunohistochemical analysis of CD34 showed that compared to the measurements taken prior to raloxifene treatment, the numbers of positively stained microvessels were reduced by almost 50% after the 28-day treatment period. The percentage of Ki67 positively stained nuclei was also decreased by 37% (da Silva et al., 2009). This suggested a correlation between MVD and cell proliferation. It is important to note that this study was performed in biopsies of ER-positive breast cancer patients, as estrogen deprivation itself causes marked reductions in angiogenesis (Morales et al., 1995). Tamoxifen, on the other hand has been shown to inhibit angiogenesis in ER-negative fibrosarcoma tumors in 26 ovariectomized female Fischer 344 rats (100 g). Prior to tumor implantation (72 h), animals received 25 mg of tamoxifen, and after 10 days without any further treatment, tumors were dissected and investigated for MVD using an antibody against tissue-transglutaminase (a specific marker
for tumor vasculature). It was found that compared to control, tamoxifen treated rats had a 43% lower MVD (Blackwell et al., 2000). Although the significance of this study may be hampered by the fact the animals were pretreated, it nevertheless suggests a possibility that raloxifene may also act via an ER-independent mechanism to reduce angiogenesis, by acting on VEGF or its receptors. VEGF is believed to be one of the most important mediators of angiogenesis and is critical for tumor growth. VEGF has a multitude of effects, notably its mitogenic effects on vascular endothelial cells and induction of vascular permeability. Such angiogenic effects of VEGF are mediated by VEGFR-1 and VEGFR-2 (Figure 1-2) (Hanahan et al., 2011). It may therefore be interesting to perform semi-quantitative protein analysis using Western blotting and/or mRNA analysis using RT-PCR of the VEGFR from the tumors of this xenograft study.

4.4.8 Estrogen receptor-α

Immunohistochemical analysis confirmed the absence of ER expression. Thus, the SERM raloxifene did not act as an ER antagonist, nor did treatment cause ‘ER-restoration’ in the MDA-MB-468 cell-xenograft model of TNBC after 10 weeks of daily oral treatment. Some studies have reported re-expression of the ER in MDA-MB-231 cells and MCF-7 cells, selected for loss of the ER (Oesterreich et al., 2001; Sharma et al., 2006). Specifically, Sharma et al. (2006) showed that it is possible to restore ER expression and sensitize MDA-MB-231 cells to tamoxifen treatment. However, to achieve this, cells had to be pretreated with demethylating agent 5-aza-2′-deoxycytidine or trichostatin A (histone deacetylase inhibitor). It seems therefore highly unlikely that raloxifene treatment on its own restored ER expression. Previous work by Taurin et al. (2011) also confirmed the absence of ER expression after 10 weeks of 0.85 mg/kg raloxifene in the same xenograft model. Future experiments should be performed with one of the three clinically validated antibodies (1D5, 6F11 and SP1 clones) for the detection of the ER, both by IHC and Western blotting (Gown, 2008; Ogle et al., 1997). In this study a monoclonal ER antibody was used with an immunogen corresponding to the amino acid residues at 247-261 of the DNA binding domain of the ER. According to the manufacturer, using Western blotting only a predicted band size at 68 kDa is detectable (Abcam, Datasheet ab16460).

Apart from the major 66 kDa ER, several different splice variants have been identified and cloned. One of these splice variants is ER-α36, which may in part and in conjunction with other splice variants, explain the heterogenic effects of estrogen signaling (Wang et al., 2005). Recently, 10 out of 12 human TNBC specimens were found to express ER-α36,
predominantly on the plasma membrane and the cytoplasm (Zhang et al., 2011). Additionally, EGFR was detected in six specimens, four of which also co-expressed ER-α36, indicating that a particular subset of TNBC patients, co-expresses both EGFR and ER-α36, but not the full-length ER (Zhang et al., 2011). Moreover, MDA-MB-231 and MDA-MB-436 cells both lacking the ER, were found to express high levels of ER-α36 and EGFR (Zhang et al., 2011). Using small hairpin RNAs to silence ER-α36, as well as inhibitors of EGFR (BiBx, AF1478 and Gefitinib), Src (PP2), and 17β-estradiol, it was shown that ER-α36 interacted only with EGFR in the absence of 17β-estradiol (Zhang et al., 2011). On the other hand in the presence of 17β-estradiol, ER-α36 interacted with Src. Interesting to note was that ER-α36 in ER-negative cells was capable of retaining the mitogenic responses of estrogen, which suggested that non-genomic estrogen signaling contributes to the development and progression of ER-negative breast cancer that express ER-α36 (Zhang et al., 2011).

The nude mice used in this study were not ovariectomized (circulating plasma 17β-estradiol levels of 74-128pg/mL (Seibert et al., 1983)), opening the possibility that ER-α36 would associate with Src. Src is proto-oncogenic, non-receptor tyrosine kinase and its expression is four- to 30-fold higher in breast cancers compared to normal epithelial breast cells (Verbeek et al., 1996). It also plays a role in several signaling pathways that are involved in cell proliferation and survival (Tryfonopoulos et al., 2011). In a study using MDA-MB-468 cells, treatment with 10 μM of the Src inhibitor PP1, caused effective inhibition of ERK activation (no densitometry) (Ren et al., 2004). Jallal et al. (2007) used female BALB/c nu/nu mice which were inoculated with MDA-MB-231 cells into the mammary fat pad to test the effects of Src inhibition on tumor growth. The results showed that after 9 weeks of treatment with oral SKI-606 (Src inhibitor) there was a 54% reduction in tumor size compared to control (Jallal et al., 2007). It should be highlighted that the dose of 150 mg/kg used was very high, making the results questionable.

It may therefore be plausible that raloxifene treatment in this MDA-MB-468 xenograft reduced cell proliferation by inhibiting Src, mediated by interactions with ER-α36. However, ER-α36 expression has only been shown in MDA-MB-231, MDA-MB-435 and MCF-7 breast cancer cell lines, ER-positive and ER-negative human breast cancer tissue, Hec1A endometrial carcinoma cells, gastric adenocarcinoma cell lines BGC-823 and SGC7901 cells, gastric cancer cell line MKN-45 and human colorectal cancer tissue (Deng et al., 2010; Jiang et al., 2008; Lee et al., 2008; Lin et al., 2009; Lin et al., 2010; Tong et al., 2010). Expression of ER-α36 therefore remains to be verified in MDA-MB-468 cells. Although
actually lacking any experimental data to back up this hypothesis, investigating Src and/or ER-α36 expression may provide a starting point for a mechanistic analysis of raloxifene in TNBC.

4.4.9 Apoptosis

To gain an insight into whether raloxifene may not only affect cell proliferation, but also apoptosis, the number of apoptotic cells as a ratio of the tumor area was assessed. This was performed only on three tumors slides chosen based on the quality of the sections. These preliminary results showed that raloxifene treatment increased the number of apoptotic cells in a dose dependent manner by up 52% compared to control. To get an idea of whether there was an inverse correlation between apoptosis and cell proliferation, the same tumors assessed for apoptosis were analyzed together with the results from their Ki67 expression analysis. This indicated that cell proliferation was at least 63% decreased by raloxifene treatment compared to control. These findings were supported by a study investigating 21 formalin-fixed, paraffin-embedded invasive breast cancers for Ki67 expression and its correlation to anti-apoptotic genes, notably Bcl2. The results showed that anti-apoptotic genes were downregulated only in tumors that had a 40% or greater amount of Ki67 immunostaining (Tan et al., 2005). Several in vitro studies have also shown raloxifene induces apoptosis in TNBC cells. In MDA-MB-231 cells, 5 μM raloxifene significantly induced apoptosis, as measured by flow cytometry in ~8% of cells after 36 h (Stuart et al., 2008). In MDA-MB-468, MDA-MB-231 and Hs578t cells, 7-10 μM raloxifene increased apoptosis by 8-fold after 48 h compared to control (Taurin et al., 2011). Regardless of the previous findings in in vitro studies, as only three tumors were assessed for apoptosis in this study, further verification is essential to be able to draw any conclusions about the effects of raloxifene on apoptosis in this xenograft model of TNBC. Furthermore it should be stated that proper blinding was not fully achievable for apoptosis analysis due to the small number of tumors under assessment.

4.5 Future Directions

The daily oral dose of 60 mg/day used in the major clinical trials (CORE, MORE, STAR and RUTH) were approximately 15 to 25–fold higher than the human equivalent dose (HED) used in this study (0.5, 0.85 mg/kg) (Reagan-Shaw et al., 2008). All those clinical trials concluded however that raloxifene, although effective at reducing invasive ER-positive
breast cancer, did not cause a reduction in ER-negative breast cancers compared to placebo. In regards to the results of this and previous studies in the Rosengren laboratory, this may suggest either (1), that raloxifene has a U-shaped dose-dependent effect in ER-negative tumors, appearing only to suppress ER-negative tumor growth at a very low dose; or (2), that the TNBC xenografts are not representative of the complex clinical setting (commonly suggested as a reason why so many drugs fail in the clinic (Garber, 2009; Sharpless et al., 2006)); or (3), that possible active metabolites in the mouse are different from humans. To verify these hypotheses, it would be crucial to repeat this 10-week study with doses that better represents the currently clinically used dose on top of the effective 0.85 mg/kg raloxifene dose used in this study. Using the same formula utilized to calculate the HEDs of 0.5 mg/kg and 0.85 mg/kg raloxifene, the 60 mg/day human dose would approximate to 12.5 mg/kg in the mouse (Reagan-Shaw et al., 2008). Differences in tumor growth inhibition between the 12.5 mg/kg and the 0.85 mg/kg raloxifene dose could be further analyzed using IHC and Western blotting. To determine metabolites of raloxifene in the mouse, blood should be collected at various time points following administration (0, 0.25, 0.5, 1, 2, 5, 8, 12 and 24 h) to allow analysis using liquid chromatography mass spectrometry. The mRNA levels of intestinal UGT1A10 and UGT1A8 should also be determined.

To further verify whether the results are representative of the clinic, triple-negative tumorgrafts may be used. Tumorgrafts are established by using human tumor fragments from patients that are directly implanted into immunodeficient mice (Decaudin, 2011). Downfalls of such models are that interspecies incompatibilities in receptor-ligand interactions between engrafted human tumor cells and the surrounding mouse tissue can contribute to the failure of tumorgraft establishment and its growth (Rangarajan et al., 2003). The overall “take rate” for breast cancer tumorgrafts is only 12-20%, however, tumorgrafts mirror parent human tumors much more closely than xenografts (Garber, 2009). It has been shown that tumorgrafts can predict treatment response in 90% of patients and drug resistance in 97% (Fiebig et al., 2004).

Drug resistance is a major problem in cancer treatment (Majumder et al., 2011). The underlying mechanism of SERM resistance is poorly understood (Cook et al., 2011). Only a few studies have focused on raloxifene-resistance, however they all used models of ER-positive raloxifene-resistant MCF-7 cells (Liu et al., 2003; O'Regan et al., 2006). To investigate whether tumors from the TNBC xenograft would remain responsive to raloxifene therapy, mice with established tumors (100 -200 mm³) could be treated for 5 – 8 weeks, or any time-period long enough to see an effect compared to control, and then treatment could
be halted for several weeks (3-6). If tumors were not under remission, one would expect them to increase significantly during this drug-washout period. Following the treatment-free period, raloxifene therapy could then be recommenced for a few weeks (4-6) to investigate drug response on tumor growth. This method has recently been used by Caldas-Lopes et al. (2009), to investigate the effects of 75 mg/kg of PU-H71 (Hsp90 inhibitor) on tumor growth in TNBC.

As this study has shown strong p38 inhibition in the tumors of mice treated with raloxifene, the importance of p38 for tumor growth inhibition by raloxifene should be verified by inoculating MDA-MB-468 dominant-negative p38 cells (as used by Chen et al., 2009) into the same xenograft model used in this study. Previous work by Scandlyn et al. (2011) has shown that raloxifene at the same doses was similarly effective at reducing the tumor volume in a xenograft model using MDA-MB-231 cells. It would therefore be interesting to assess whether raloxifene in the MDA-MB-231 cell xenografts also acted upon p38. This, according to the conclusions made by Chen et al. (2009), would be plausible as MDA-MB-231 cells are also p53 mutant (Wasielewski et al., 2006). Due to the heterogeneity of breast cancer, Vargo-Gogola et al. (2007) has suggested that only by combining multiple different systems (such as using different cell lines, tumor growth and tumor regression studies, genetically modified animal models etc.) an adequate representation of human breast cancer can be created.

To determine the effects of raloxifene on gene expression, an “Affymetrix GeneChip® Exon Array System for Mice” could be used to analyze gene expression and alternative splicing variations between raloxifene and vehicle treated tumors. This method in conjunction with the appropriate statistical analysis would allow further confirmation and identification of pathways and genes relevant to raloxifene mediated tumor suppression in these TNBC xenografts (Ma et al., 2010).

### 4.6 Conclusion

Experimental evidence was provided herein supporting low dose raloxifene in the treatment of TNBC. Mice dosed with 0.85 mg/kg raloxifene had significantly smaller tumors after 10 weeks of oral treatment compared to control, without any toxic side-effects noted. Immunohistochemical analysis revealed that the smaller tumor size was associated with significant reductions in cell proliferation, probably mediated through reductions in NF-κB and p38 expression. Although limited to only a few tumors, IHC confirmed the absence of
the ER. With these results, a potential mechanism of raloxifene action in triple-negative, p53 mutant tumors was established (Figure 4-1) supported mainly by studies from Ryan et al. (2000) and Chen et al. (2009). This however requires further confirmation and may only provide a starting point for additional mechanistic research on this matter.

**Figure 4-1 Hypothesized mechanism of raloxifene in TNBC**

In wild type p53 cells, extracellular stimuli can activate p38, which then phosphorylates and activates p53, leading to p53-dependent transcription and apoptosis (not shown). However, in p53 mutant cells (shown here), p53-dependent apoptosis cannot occur and no negative feedback regulation involving Wip1 (which in p53\(^{WT}\) cells dephosphorylates p38) exists. Signaling via p38 therefore continues to activate proliferative pathways and stimulates cell cycle progression and cell proliferation through mediators such as ATF-2 and MAPKAPK-2 (not shown). In such p53\(^{Mut}\) cells (MDA-MB-468 cells), inhibition of p38 would therefore suppress cancer cell growth (Chen et al., 2009). NF-κB has been shown to be essential for p53-mediated apoptosis, as inhibition of NF-κB activity abolished p53-induced cell death. Therefore, therapeutic inhibition of NF-κB would be more/only effective in p53\(^{Mut}\) cells (Ryan et al., 2000). Raloxifene may have either directly or indirectly via p38, reduced NF-κB expression, and thereby further enhancing effects of raloxifene-mediated p38 inhibition. This may ultimately lead to decreases in cell proliferation and possibly increases in apoptosis. Adapted from Chen et al. (2009). Red crosses depict pathway(Armstrong et al., 1994)s/processes that are inhibited or reduced.

Moreover, TUNEL assay of 3 tumors showed a possible link to apoptosis induction by raloxifene treatment. However this remains to be verified in all other tumors. There were also some not fully explainable observations, such as the significant increases in EGFR expression, as well as the high levels of β-catenin. These findings should be further pursued
with phosphorylation specific antibodies or microarray analysis (‘Future Directions’ section). Overall, the fact that raloxifene was effective at reducing tumor growth with a dose 15-fold lower than the clinically given dose, warrants further research with the potential to identify novel drug-targetable pathways for TNBC.
Bibliography


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