MOLECULAR MECHANISMS OF TAXANE RESISTANCE

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By

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ABSTRACT

Treatment with the taxanes (Paclitaxel or Docetaxel) is often the therapy of choice for women with breast cancer. In most cases, the taxanes can arrest cell proliferation at the G2/M phase of mitosis and cause cell death. However, some tumors develop resistance during the course of treatment, which is a major concern for patients, physicians and scientists. Several mechanisms have been proposed to explain how resistance to the taxanes occurs, but this phenomenon remains incompletely understood. We hypothesize that acquired taxane resistance in breast cancer cells may be associated with cellular transporters and when challenged with cytotoxic concentrations of taxane drugs may die through multiple cell death mechanisms.

Using the MDA-MB-231 breast cancer cell line, we developed cells that are resistant to either Docetaxel (DocR) or Paclitaxel (PacR). We then characterized the cell lines and found that DocR cells were cross resistant to Paclitaxel but PacR cells were only partially cross resistant to Docetaxel. It was confirmed by DNA fingerprinting that the genetic lineages of sensitive and resistant cell lines were similar. In addition, the resistant cell lines were found to proliferate more rapidly compared to sensitive cells, simultaneously having a greater number of cells in the S phase of the cell cycle.

While β -tubulin isotype expression changes and mutations have been implicated in conferring resistance to the taxanes, this was not the case in our cell lines. Of the transporters, Pgp, a member of the ABC superfamily of transporters was found to be upregulated in both resistant cell lines compared to sensitive cells, however siRNA experiments to inhibit expression of Pgp showed only a minor reduction of cell number in resistance cells, indicating the possibility that other ABC or SLC superfamily transporters may play a role in the resistance phenotypes.

Finally, levels of cell death and the response to IC_{50} concentrations of the taxane drugs were measured in sensitive and resistant cells. Clear differences in the use of alternative cell death pathways mechanisms emerged. Sensitive cells have higher levels of apoptosis compared to resistant cells determined by both SubG1 and Annexin V assays. There was no significant difference in caspase activation between sensitive and resistant cells by both western blots of protein expression and poly-caspase activation assays. Paclitaxel resistant cells had the highest levels of mitochondrial membrane permeability compared to CTL and DocR cells as was determined by JC-1 assays. Although both sensitive and resistant cells change their morphology consistent with what is described as induction of mitotic catastrophe at IC_{50} concentrations of either taxane drug, PacR cells followed by DocR were largest in cell size. Using propidium iodide dye, both resistant cell lines showed higher levels of necrosis compared to sensitive cells with the PacR cells having the highest levels. Finally, autophagy levels as determined by protein expression of cleaved LC3 and p62 were highest in PacR cells and when autophagy was inhibited with

Beclin-1 siRNA, PacR cells showed a reduction in cell number in both Paclitaxel and Docetaxel compared to CTL and DocR cells.

In conclusion, it appears that several cell death pathways are used by sensitive and taxane resistant MDA-MB-231 breast cancer cells and that clinical use of inhibitors or inducers of different cell death pathways in conjunction with standard taxane chemotherapy may improve the success of taxane therapy in women with breast cancer in the clinic.

DEDICATION

I would like to dedicate this thesis to my family, friends and colleagues whose support made this thesis possible.

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CHAPTER 1: INTRODUCTION

1.1 Breast Cancer Statistics and Risk Factors

Breast cancer is the most common cancer in women worldwide (1). There are over one million new cases of breast cancer each year and over 400,000 deaths globally (1). Breast cancer incidence rates are almost 3 times higher, and mortality rates almost 2 times higher, in developed countries compared to developing countries (1). The highest incidence rates are found in Northern and Western Europe, North America, Australia, New Zealand, Uruguay and Argentina. Incidence is lowest in Africa, Asia and most of central and South America (2).

In the United States, breast cancer is the most commonly diagnosed cancer in women (3). There were an estimated 192,370 new breast cancer cases in 2009 accounting for 27% of all newly diagnosed cancers in women with the highest incidence rates in California, New York and Texas (4). Breast cancer is the second leading cause of death from cancer in women with approximately 40,170 deaths due to breast cancer in 2009 or 15% of all cancer related deaths in women (3, 4). Although incidence and mortality due to breast cancer remains high, there has been a decline in incidence. There was a 2.2% decrease in annual percentage of breast cancer incidence from 1999-2005 and a 1.8% decrease in mortality from 1998-2005 in women (4, 5). Reduction in death rates is thought to be due to advances in awareness, prevention, early detection, early diagnosis and treatment (6, 7). While there is no evidence that clinical examination, self examination or breast ultrasounds are effective tools for early detection, randomized controlled trials of screening using mammography have shown a significant reduction of mortality by 20-35% for women between the ages of 50 and 69 years (8). For

younger women with increased breast density and those at high risk for developing breast cancer, MRIs may be the more sensitive and specific tool used for screening (7, 9).

Breast cancer incidence and death rates vary by racial and ethnic groups. From 2001-2005 breast cancer incidence was highest in White women (130.6 per 100,000 population age adjusted to the 2000 US standard population), followed by African American (117.5), Hispanic (90.1) and Asian American and Pacific Islander (89.6), with the lowest rates found in American Indian and Alaskan native women (75.0) (4). Higher breast cancer incidence rates in White women are thought to be due to older age at first birth and use of hormone replacement therapy (HRT) (10). Mortality rates from breast cancer, however, do not follow the same trend and were highest in African Americans (33.5) followed by White (24.4), American Indian and Alaskan native (17.1), Hispanic (15.8) and Asian and Pacific Islander women (12.6) (4). Higher breast cancer mortality rates in African American women may be due to differences in timely diagnosis through mammography and unequal access to timely, high-quality treatment (10).

Most breast cancer cases are thought to be due to sporadic somatic mutations. However, 5-10% of breast cancer cases are hereditary, the majority which involve mutations in the BRCA1 and BRCA2 genes (11). A number of other factors have also been associated with increased breast cancer risk and include early menarche and late menopause, hormone exposure, nulliparity, low parity and late age first pregnancy, high postmenopausal weight and high adult weight gain (obesity), alcohol consumption, a history of benign proliferative breast disease and exposure to high levels of ionizing radiation. However the greatest risk factor remains age (12-15). Female breast cancer incidence increases rapidly until age 50 and then more slowly after menopause probably because of lower levels of circulating estrogens (1, 2, 16). Interestingly, over 50

epidemiological studies have been conducted on physical activity and breast cancer risk. These studies show that physical activity lowers breast cancer risk for both pre and postmenopausal women, women of all racial and ethnic backgrounds, and for women living throughout the world (12). Although Vitamin D has been implicated in some studies with reduced breast cancer risk, thus far, associations of Vitamin D, calcium, and breast cancer risk remain inconclusive (17).

1.2 Prevention and Treatment of Breast Cancer

Breast cancer treatment generally involves surgery followed by adjuvant (post surgery) therapy to lower the risk that the breast cancer will return after the primary treatment. Surgery can involve lumpectomy or mastectomy (18). While neoadjuvant or preoperative therapy was initially used for inoperable breast cancer, increasingly, neoadjuvant therapy is also being used for operable breast cancer to improve surgical options and acquire early information on response and biology of the tumor (19, 20). Adjuvant therapy can be local or systemic and can be radiation, hormonal therapy, cytotoxic chemotherapy or targeted therapy used singularly or in combination (19, 21). Radiotherapy such as chest wall irradiation after mastectomy, breast irradiation after lumpectomy, and irradiation of lymph nodes plays an important role in adjuvant breast cancer treatment (22). Randomized studies have shown that adjuvant radiotherapy can reduce local-regional recurrence of breast cancer by up to 70% and improves long term survival in lymph node positive patients (23).

Clinicians generally categorize breast cancer into estrogen receptor positive (ER+), estrogen receptor negative (ER-) and HER-2 (human epidermal growth factor receptor 2) overexpressing categories (24). Around 70% of primary breast cancer tumor cells overexpress ER compared to

7% of normal breast epithelial cells (25). The triple negative subtype (ER/PR and HER2-negative) has been shown to have the worst overall survival and disease free survival compared to ER/PR-positive, HER2-negative breast tumors (1, 24). If a patient's tumor is hormone receptor positive, hormonal adjuvant therapy is administered as either an antiestrogen or an aromatase inhibitor. Antiestrogens include selective estrogen receptor modulators (SERMs) such as Tamoxifen and selective estrogen receptor downregulators (SERDs) like Fluevestrant (26). Aromatase inhibitors that prevent the biosynthesis of estrogen include Anastrozole, Letrozole and Exemestane; these drugs have for the most part replaced Tamoxifen as the preferred treatment for hormone receptor positive breast cancer in postmenopausal women (27). The endocrine treatment of ER-positive breast cancers with Tamoxifen, Fluevestrant and aromatase inhibitors were some of the first target based therapies in breast cancer treatment (28). Tamoxifen has not only been used to treat breast cancer but also been shown to reduce ER-positive breast cancer risk by up to 49% in women that are at high risk for breast cancer but do not have the disease yet. Tamoxifen does not affect the incidence of ER-negative breast tumors (29).

Systemic adjuvant chemotherapy can significantly increase disease free and overall survival in breast cancer patients (30). Determining the type of systemic chemotherapy depends on a patient's age and overall health, the size of the primary tumor, lymph node status and the stage of the cancer, microscopic appearance of tumor cells, hormone receptor and HER-2 status (19, 31). Patients that have low or undetectable expression of steroid hormone receptors (<10% of cells are positive for steroid receptor expression and lack of progesterone receptors) are strong candidates for cytotoxic chemotherapy. Some of the most common chemotherapy drugs used are

the anthracyclines that are anti-tumor antibiotics like doxorubicin, and epirubicin, mitotic inhibitor drugs such as the taxanes Paclitaxel and Docetaxel, alkylating agents that damage DNA like cyclophosphamide, and antimetabolites that interfere with DNA and RNA growth such as 5-fluorouracil and methotrexate (7, 32). Chemotherapy drug treatment for patients with advanced metastatic breast cancer can be administered as single drugs or as polychemotherapy (in combination with other drugs). Currently, the anthracycline doxorubicin and or the taxanes are preferred as first line treatments in metastatic breast cancer patients (33). However, whether combination chemotherapy or sequential single agent chemotherapy should be used remains controversial (31).

Using DNA microarray technology, the molecular classification of breast cancers based on gene expression variations has categorized breast cancers into five distinct subtypes, i.e., luminal A, luminal B, normal-like, HER-2 like and basal-like. Normal-like breast cancers resemble normal breast tissue, HER-2-like overexpress HER-2, luminal A and B are ER-positive and basal-like are triple negative (estrogen, progesterone and HER-2 receptor negative) (9). The distribution of these breast cancer subtypes varies across different populations. ER-positive, luminal A breast cancers were found to be predominant in Asian, White and postmenopausal African American women, whereas ER-negative, basal like breast cancers were found to be more prevalent in Indigenous African and premenopausal African American women (9).

After the identification of HER-2 overexpression in some breast cancers Trastuzumab, a monoclonal antibody to HER-2, was incorporated in combination with standard adjuvant chemotherapy, which has led to a 50% reduction of recurrence in patients with that subtype of

breast cancer (9). Other targeted therapies that have been shown to be successful are the dual EGFR and HER-2 tyrosine kinase inhibitor (Lapatinib) and the anti-angiogenic VEGF (vascular endothelial growth factor) monoclonal antibody (Bevacizumab) (28, 34).

The use of gene expression profiling to identify predictive and prognostic genomic markers for breast cancer is likely to significantly increase in the future and could likely lead to more specific, less toxic and personalized treatments (35, 36). A molecule being considered as a prognostic biomarker in breast cancer is p53, a tumor suppressor gene involved in apoptosis and regulation of normal cell growth, due to gene sequencing and a mutation that was found in p53 and which showed p53 to be an independent negative prognostic marker in breast cancer (37). Already, Oncotype Dx, a 21 gene reverse-transcription –PCR assay and MammaPrint, a 70 gene expression profile signature based on research conducted at the Netherlands Cancer Institute, have the potential to identify lymph node negative patients to distinguish patients who do not require additional chemotherapy, thereby sparing some patients from the toxicities of some therapies (9, 30, 37). Several other studies are currently underway to determine whether or not gene expression profiling is an effective prognostic tool. For example, two large, prospective, randomized trials TAILORx (Trial Assigned Individualized Options for Treatment (Rx)) and MINDACT (Microarray In Node negative Disease may Avoid Chemotherapy Trial) are currently in progress (30, 38). Future drug development will probably also include the use of biomarkers that identify individual and ethnic variabilities of drug pharmacogenetics, pharmacokinetics and pharmacodynamics (9).

1.3 Taxane Chemotherapy Drugs

Taxane Structure (39)

Figure 1.3 Taxane Chemotherapy Drugs. Structure of Paclitaxel, Docetaxel and the molecular precursor 10-Deacetylbaccatin III from which both drugs can be synthesized (39).

Throughout history, therapies derived from nature have been used to treat illnesses. Millions of potential candidate compounds can be derived from plants, animals, marine organisms and microorganisms (32). Of the 87 approved anticancer drugs, 62% are of natural origin or modeled on natural product precursors (40). In the 1960s, the National Cancer Institute began a large scale screening program to find natural products with anticancer properties. This program resulted in the discovery of Paclitaxel, a compound derived from the bark of the Pacific Yew tree (*Taxus brevifolia*) (32, 41). Because the bark of the tree was a limited resource, in 1986 the semi-synthetic analog Docetaxel was developed from a molecular precursor, 10-deacetyl baccatin III derived from the needles of the European Yew tree (*Taxus baccata*) (32, 42). Today, both drugs are synthesized and supply is no longer an issue (32). The taxanes, Paclitaxel (Taxol®) and Docetaxel (Taxotere®), are important and widely used cancer chemotherapy drugs in the treatment of both early and invasive, metastatic human breast cancer, in addition to ovarian, lung, prostate, head and neck, bladder and esophagus cancers (7, 31, 39, 43-46). The response rate to the taxanes when they are used a first line treatment for breast cancer is between 25-69% (47). A meta-analysis of clinical trials supports the use of the taxanes in adjuvant chemotherapy in women with operable early breast cancer. Both overall survival and disease free survival showed an improvement compared to women that received adjuvant treatment without the taxanes. No recommendations were made regarding dosage and scheduling of taxane treatment in this analysis (48). Similarly, randomized trials comparing women with metastatic breast cancer that had received regimens that included the taxanes with those that did not, showed an improvement of overall survival, time to progression and overall response (49).

1.4 Mechanisms of Action of Taxane Drugs

Microtubules are hollow dynamic structures (25nm in diameter) involved in a number of cellular functions and play a particularly crucial role during cell division (50). Microtubules comprise heterodimers of α and β -tubulin (50 kDa in size) within protofilaments that polymerize and depolymerize (grow and shrink by adding or removing tubulin subunits) in a dynamic fashion according to the necessary cellular functions that they perform (50, 51). While primarily thought of as components of mitotic spindle and centrosomes during mitosis, microtubules have other functions in cells. These functions include maintenance of cell shape, cellular motility and adhesion, intracellular transport; microtubules may also modulate the interaction of growth factors with cell surface receptors (41). Microtubules radiate out from microtubule organizing centers in centrosomes in the cytoplasm of interphase cells, during which the turnover of tubulin is the slowest (50). At the onset of the cell cycle, turnover of spindle microtubules can be 4-100 times faster than at interphase, which requires structural and regulatory coordination of MAPs (microtubule associated proteins) and microtubule interacting proteins (50).

When a taxane drug binds to microtubules these structures stabilize and increase tubulin assembly without MAPs and GTP, which are normally required for the assembly process (52, 53). This increased polymerization and stabilization leads to arrest of the cell cycle at the G2/M phase, which inhibits mitosis and eventually leads to apoptosis (54). Mitotic block at metaphase/anaphase also occurs and causes the formation of an incomplete metaphase plate of chromosomes and an abnormal organization of spindle microtubules (55). During the G2/M transition the taxanes, and in particular Docetaxel causes the phosphorylation or dephosphorylation of several cyclin dependant cyclins (*e.g.* cdc2) and kinases (*e.g.* Cyclin B1)

(44). In addition, inhibition of cyclin dependent kinases by increased expression of p21 has been associated with Paclitaxel treatment (44).

The main antitumor effects of both taxanes are considered to be due to the drugs binding to the microtubule β -tubulin subunit, but some studies have shown that the taxanes can inhibit cell growth at concentrations that are lower than those required for microtubule disruption. These observations imply that alternative mechanisms may also be present (44). Some of these microtubule independent events are thought to include inactivation of the antiapoptotic proteins Bcl-2 and Bcl-xL via their phosphorylation and the activation of proapoptotic Bax (44, 56).

Both taxanes may also induce apoptosis by p53-dependent and independent pathways (44). The role of p53 as a predictive marker of clinical response particularly to the taxane drugs is not clear and some studies have shown response to the taxane drugs in the presence of a p53 mutation which may indicate that the taxane mechanism of action is independent of p53 (37, 57, 58). However, p53 mutant cancer cell lines are more resistant to taxane induced apoptosis and according to cell type, different cell death signaling pathways can be activated through initiation from different cell compartments including the nucleus, cytoplasmic elements and the plasma membrane (44).

The main structural differences between the taxane drugs are the substitutions at the C10 position of the taxane ring and at position C13 on the ester side chain. The C13 position is what allows the drugs to bind to microtubules and leads to their antitumor effects (59). Functional differences include the fact that Docetaxel affects both mitotic spindle and centrosome

organization and acts on three phases of the cell cycle (S/G2/M) compared to Paclitaxel, which suppresses microtubule dynamics by allowing microtubule attachment but changes the tension across the kinetochore during mitosis which affects only the G2/M phases of the cell cycle (47, 60, 61). Pre-clinical studies have also suggested that Docetaxel is 100-fold more potent than Paclitaxel at achieving Bcl-2 phosphorylation and apoptotic cell death (62). This may be because Docetaxel has an almost two-fold greater affinity for the β -tubulin subunit (1.9) compared to Paclitaxel (1.0), and is twice as potent causing maximum microtubule polymerization at 0.2 μ M concentration compared to 0.4 μ M for Paclitaxel (61). Docetaxel also inhibits depolymerization of microtubules at about twice the efficacy of Paclitaxel and does not change the number of protofilaments in microtubules. In contrast, Paclitaxel induces polymerization of microtubules with 12 instead of the normal 13 protofilaments (42). In addition, there is greater uptake of Docetaxel into tumor cells leading to longer retention times which may account for the fact that there is incomplete cross-resistance between the two drugs (61). Incomplete cross-resistance between the two taxane drugs was found in clinical trials of patients that had breast, ovarian or non-small cell lung cancer specifically regarding Paclitaxel drug resistant patients that were subsequently treated with Docetaxel (61). Another difference between the two drugs is that Docetaxel is predominantly metabolized by cytochrome P450 subclasses CYP3A4/5, whereas Paclitaxel is metabolized by CYP2C8/CYP3A4 to inactivate hydroxylated metabolites (63). The hydroxylated metabolites of the taxanes are either inactive or less potent than the parental drugs (61). In addition, the toxicity profiles of the two drugs differ, Paclitaxel treatment has been associated with the development of peripheral neuropathies and myalgias/arthralgias whereas Docetaxel treatment can result in cumulative fluid retention that can be dose limiting (39).

1.5 Resistance to Taxane Drugs

Chemotherapy treatment is beneficial to many patients. Nonetheless, up to 50% of patients fail to benefit due to either intrinsic or acquired multidrug resistance (64). Dose escalated taxane resistance can result in multifactorial resistance mechanisms including overexpression of transporters such as P-glycoprotein (Pgp/MDR1/ABCB1), mutations in β -tubulin genes, differential β -tubulin isotype expression, changes in expression of microtubule associated proteins (MAPs), or changes in proteins in the apoptotic pathway such as increased expression of Bcl-2 and Bcl-xL (44, 65, 66).

Both α and β -tubulin are encoded by different genes and have cell and tissue specific expression patterns (50). There are 6 main isotypes of β -tubulin with tissue specific distribution (Table 1.1).

Table 1.1 β -tubulin isotypes Adapted From (50)

Tubulin Isotype	Chromosome Localization	Tissue Distribution
β I	6q21.33	Constitutive expression; predominant isotype in cells
β II	6p25.2	Major isotype of neurons; low levels in lung, kidney, spleen, stomach and thymus; increased levels in prostate adenocarcinoma
β III	16q24.3	(same as β II)
β IVa	19p13.3	Brain specific
β IVb	9q34.3	Constitutive expression with highest levels in testis; increased levels in liver cancer
β VI	20q13.32	Haematopoiesis-specific cell types: megakaryocytes and platelets

Paclitaxel's binding site is in the interior facing lumen of microtubules and the binding is thought to involve a hydrogen bond with serine 275 in all β -tubulin isotypes except class III and VI (50). P53 and pro and antiapoptotic Bcl-2 family proteins are attached to, or carried by, microtubules. However how mutations in β -tubulin DNA, differential expression of β -tubulin isotypes or posttranslational modifications of β -tubulin affect the ability of the taxane drugs to bind and cause the transport or release proteins involved in apoptosis is unclear. Changes in β -tubulin and MAPs are associated with resistance in some systems (50).

Drug resistance can occur by specific resistance to a single drug or resistance to a wide variety of drugs, known as multiple drug resistance (MDR). MDR can be conferred by several mechanisms, including from the activation of detoxifying systems such as DNA repair and activation of cytochrome P450 3A (67). Another mechanism of MDR is through the overexpression of transmembrane glycoproteins such as the ATP-dependent 170- kDa cell surface transport protein P-glycoprotein (Pgp), encoded by the ABCB1 gene, which is part of the ABC (ATP Binding Cassette) superfamily of 49 transport genes (7 ABCA-ABCG subfamilies) (61, 68-70). The induction of both cytochrome P450 3A and Pgp in coordination has been reported (67).

ABC transporters use the hydrolysis of ATP to bring nutrients into cells or pump toxins, drugs and lipids out of cells (69). ABC transporters consist of a minimum of four domains, two transmembrane domains within the plasma membrane lipid bilayer and two ABC cytoplasmic nucleotide binding domains (69). The ABC domains have highly conserved sequences compared to the variable transmembrane domains that probably reflect the diversity of the

compounds that these transporters can transport (69). The ABCA subfamily members are predominantly found in the central nervous and hematopoietic systems and are involved in lipid homeostasis and transport (71). The ABCB subfamily is involved in drug transport, intracellular peptide transport, and major histocompatibility (MHC) class I antigen presentation. The ABCC subfamily has the largest number of drug transporters but also include a family member that is a chloride ion channel (71). The ABCD subfamily encodes peroxisome half transporters. ABCE and ABCF family members are thought to be involved in mRNA translation and some members of the ABCG subfamily are involved in drug transport as well (71).

Because of the high degree of homology in the sequences of the ABC transporter superfamily members, the National Cancer Institute conducted a study to establish a database and profile 48 different ABC transporters in over 60 different cell lines representing leukemias, melanomas, ovarian, breast, prostate, lung, renal, colon and central nervous system cancers to determine the role of the transporters in chemoresistance and or chemosensitivity (72). It was found that certain transporters were expressed across many cell lines, whereas others were tissue specific and the distribution of the transporters appears to be independent of sequence homology (72). It was also found that more than half of the ABC transporters could play a role in drug resistance and several transporters of unknown function may influence the response of cancer cells to treatment (72).

Given the similarities between the ABC superfamily members, a combination of inhibitors may be required to effectively block their functions. Some studies are now screening the expression of all ABC transporter members in tumor cell lines and clinical samples (71). Whereas

resistance due to the transporters can occur from reduced uptake of drugs or increased efflux, for hydrophobic drugs such as Docetaxel and Paclitaxel, entry into cells is predominantly by diffusion but can be enhanced with transporters. Efflux via transporters is what most likely causes some form of taxane chemoresistance (70, 73). Transporters and channels also can indirectly modulate sensitivity and resistance to chemotherapy drugs by providing nutrients to cells, and by modifying electrochemical gradients across membranes, apoptosis, and the efficiency of drug diffusion across membranes (70).

Of the ABC transporter members, the most studied ABC transporter is Pgp, which not only maintains cholesterol distribution across membranes but also exports lipophilic chemotherapeutic agents (69). Substrates of Pgp are thought to enter the Pgp transporter after partitioning into the plasma membrane rather than directly from the extracellular or intracellular aqueous phase. The drug binding domain is thought to be able to accommodate two substrates simultaneously (73). It has also been suggested that Pgp can cause an increase in intracellular pH, leading to the depolarization of the plasma membrane electrical potential by acting as a proton pump or a chloride channel thereby reducing the intracellular accumulation of weak bases or reducing pH dependent binding of molecules to intracellular targets (74).

A meta-analysis of 31 breast cancer trials found that Pgp was expressed in 41% of tumors and that treatment with chemotherapy drugs increased the expression of Pgp, which resulted in a 3-fold reduction in response to chemotherapy, the clinical relevance of these findings remains controversial (75). Regarding the role that Pgp in response to taxane chemotherapy, both Docetaxel and Paclitaxel are substrates for the Pgp transporter, and activation of this pump is

thought to be a major mechanism of taxane resistance (43). *In vitro* studies suggest that Docetaxel may be a weaker substrate for Pgp than Paclitaxel, which suggests that Pgp may be less likely as a primary resistance mechanism and that other mechanisms of resistance would be important to explore (76). Other ABC genes implicated with resistance to the taxanes are ABCB4 (MDR2/3) and ABCB11 (BSEP/SPGP) for which Paclitaxel is a substrate and ABCC2 (MRP2), ABCC10 (MRP7) for which both taxanes are substrates (67, 70, 77).

Overexpression of MDR efflux proteins results in decreased accumulation of anticancer drugs within the cancer cell. However, the precise role of ABC transporters in clinical breast cancer remains controversial (78, 79). Drugs such as Verapamil (a calcium channel blocker) can inhibit the Pgp resistance mechanism in cancer cells (80). Verapamil has also been shown to inhibit ABCB4 and ABCB11 transport in addition to ABCC1 (MRP1) (70, 81-83). Since Verapamil is a substrate for Pgp, by binding to Pgp, Verapamil competitively inhibits the binding of other substrates (73, 84). In NCI/ADR-RES cells that highly express Pgp, 100 μ M Verapamil was shown to increase 100 nM [14 C] Paclitaxel intracellular concentration by 8-fold and decrease its efflux (73). In Paclitaxel resistant and sensitive hepatocarcinoma cells (HepG2), uptake of [3 H] Paclitaxel in resistant cells was found to be half that of sensitive cells (85). With the introduction of Verapamil, in the resistant cells, Pgp mRNA was found to be increased and OATP1B3 (influx transporter) mRNA decreased. Verapamil caused an increase in intracellular [3 H] Paclitaxel particularly in the resistant cells presumably due to the decreased efflux capacity of Pgp (85). Verapamil has also been shown to increase the binding of ATP to the Pgp transporter that saturates Pgp with ATP, stimulates ATPase activity, and may limit the availability of ATP for

other substrates or cause ATP hydrolysis and depletion as one of its other mechanisms of inhibition of Pgp (73).

Unfortunately, Verapamil has cardiovascular effects and must be used at high doses in order to achieve *in vivo* plasma concentrations that reverse MDR; doses of only 0.4-1.2 μ M are considered safe and effective for its desired cardiovascular effects but may not fully reverse MDR (74). The high doses required to effectively reverse MDR (2-4 μ M) can be life threatening (74). Verapamil has two enantiomers (R-Verapamil and S-Verapamil) that differ in pharmacokinetic and pharmacodynamic properties (86). R-Verapamil is the predominant form of Verapamil used in clinic trials because it has lower cardiovascular effects (79).

Because of the undesirable side effects of first generation ABC transporters inhibitors such as Verapamil, second and third generation Pgp/ABCB1 inhibitors were generated. The second generation inhibitor Valspodar and third generation inhibitors such as Zosuquidar have proved to be disappointing in ovarian and breast cancer clinic trials (71). If Pgp is what mediates resistance to the taxanes, Verapamil or other Pgp inhibitors should be able to restore sensitivity, however this has been difficult to implement in the clinic due to toxicity, low efficacy of inhibitors and pharmacokinetic interactions and results have been inconclusive (75, 77, 79). In addition, standardization of clinical trial methodology, the use of immunohistochemistry to detect *in situ* Pgp protein expression, cell line and tissue positive and negative controls and clear evidence of drug-resistance reversal in clinical trials will help to clarify the role of Pgp and Verapamil (79).

Another superfamily of transporters is the solute-carrier (SLC) transporter that contains at least 362 genes (55 families) (Table 1.2). SLC genes include passive transporters, symporters and antiporters as well as mitochondrial, and vesicular transporters. Unlike the ABC superfamily of transporters, SLC transporters do not require ATP (70, 87).

Table 1.2 SLC Transporter Gene Families Adapted From (87)

Protein Family	Description	Number of Members
SLC4, 8, 9, 12, 20, 24, 26, 34	Inorganic cation and anion transport	8
SLC1, 7, 15, 17, 32, 36, 38, 43	Amino acid and or oligopeptides transport	8
SLC2, 5, 37, 45	Glucose and other sugar transport	4
SLC10, 13, 16, 47	Bile salts and organic anions transport	4
SLC11, 30, 31, 39, 40, 41	Metal ions transport	6
SLC6, 14, 18, 22, 42, 44	Urea, ammonium, neurotransmitters, biogenic amines and choline transport	6
SLC19, 23, 33, 46	Vitamins and cofactors transport	4
SLC28, 28, 35	Nucleosides and nucleotides transport	3
SLC25, UCP1, 2, 3	Mitochondrial transport	4
SLC27, SLCO2	Fatty acids, prostaglandins and steroid sulphates transporters	2
SLCO1, 2, 3, 4, 5, 6	Organic anion transporting polypeptides (OATPs), prostaglandins, steroid sulphates, thyroid hormone transport	6

There is about 20-25% amino acid sequence homology mostly in the consensus domain shared between transporters within the same family (87). Over half of the SLC genes remain to be characterized and understanding their role in pharmacology and cancer chemotherapy is important (87). Both Paclitaxel and Docetaxel are substrates of and enter cells via the OATP1B3 transporter that is encoded by the SLCO1B3 gene. Paclitaxel is also a substrate of OAT2, encoded by the SLC22A7 gene. The role of SLC transporters in taxane resistance remains to be elucidated (88-90).

1.6 Cell Death and the Taxane Drugs

Changes in the onset of apoptosis and other types of cell death can contribute to the development of cancer (91, 92). Many chemotherapy treatments, through their antitumor effects, cause apoptosis. While antiapoptotic mechanisms have been implicated in conferring drug resistance, not all cells respond to anticancer therapy with an apoptotic mechanism. Thus, alternative cell death pathways are being considered (93). Pharmacological agents that target specific cell death pathways have been developed, including Bcl-2 antagonists to restore the apoptotic pathway in resistant tumors (94-96). Early phase clinical trials of molecules that are death receptor ligands are also underway in order to induce caspase-8 dependent apoptosis in cancer cells (97). The main issue with these agents is that they have led to studies that focus on only one or two genes or proteins that may not be important in the clinic and that do not account for activation of different cell death mechanisms at the same time and that overlap and share signaling pathways. These different cell death mechanisms include apoptosis, necrosis, mitotic catastrophe and autophagy that can be caspase independent mechanisms of programmed cell death (98, 99).

More recently, clinical trials have been initiated to explore the modulation of alternative cell death pathways. For example, a double blind, placebo controlled, randomized phase III clinical trial of carmustine and radiation therapy with or without daily chloroquine (an autophagy inhibitor) showed that median survival in patients treated with chloroquine was 24 months compared to 11 months in patients treated with placebo (97). Pharmaceutical enhancement of necrotic cell death to increase immunity has not been a focus of drug development. However PARP inhibitors that inhibit necrosis and simultaneously enhance apoptosis have been developed as chemosensitizers and are in early phase clinical trials (97). Another approach has been to activate mitotic catastrophe by using KSP inhibitors (kinesin spindle protein) or use Chk1 inhibitors (checkpoint kinase) to disrupt the G2/M mitotic checkpoint and sensitize p53 deficient cells to DNA damaging chemotherapy drugs (100).

Apoptosis, a type I programmed cell death, is defined by morphological changes due to organized chromatin condensation, fragmentation of the cell nucleus, formation of apoptotic bodies, cell shrinkage and ruffling of the cell membrane (101, 102). This process requires energy from ATP to occur (103). Apoptosis is mediated by two major pathways, the *extrinsic death receptor pathway* and the *intrinsic mitochondrial pathway* (104, 105). The *extrinsic death receptor pathway* is due to signals from death receptors such as the Tumor Necrosis Factor receptor superfamily (TNF), TNF-related apoptosis inducer ligand (TRAIL) receptors and FasLigand (FasL). These signals ultimately activate caspases, a family of cysteine-dependent aspartate-specific proteases, that act as death effector molecules, which then initiate DNA cleavage and catabolism of the cytoskeleton (91, 93, 104, 106). Stimulation of the death receptors specifically leads to activation of the initiator caspase-8, which propagates the

apoptosis signal by cleaving downstream caspases such as caspase 3 (93). Cancer cells and resistant tumors can evade the extrinsic apoptotic pathway by increasing antiapoptotic molecules and/or decreasing proapoptotic molecules, as well as downregulating or removing death receptor expression at the plasma membrane (93).

The *intrinsic mitochondrial pathway* incorporates both pro-apoptotic and anti-apoptotic members of the Bcl-2 family of proteins that affect changes in mitochondrial permeability, the release of cytochrome *c*, and the simulation of caspase-9 that then cleaves and activates caspases-3, -6, and -7 (104, 107). The Bcl-2 family has 20 members that share a Bcl-2 homology (BH) domain and includes five antiapoptotic proteins (*e.g.*, Bcl-2 and Bcl-xL) and two groups of proapoptotic proteins the three member Bax family and the eight member BH3-only families (108). The prosurvival or antiapoptotic members Bcl-2 and Bcl-xL are thought to inhibit apoptosis due to some but not all cytotoxic signals. These members are associated with the outer mitochondrial membrane, the endoplasmic reticulum (EnR), and the nuclear envelope (108). Mitochondrial permeability occurs due to apoptotic stimuli that lead to activation and translocation of the proapoptotic Bcl-2 family member Bax to the mitochondria where it changes conformation, oligomerizes, and then inserts into the outer mitochondrial membrane causing the formation of openings that allow cytochrome *c* and other proteins in the intermembrane space to be released into the cytoplasm (105, 109). The antiapoptotic Bcl-2 family members, including Bcl-2, prevent oligomerization, bind to the Bcl-2-homology-3 domains on Bax and other proapoptotic members, and thereby prevent mitochondrial membrane permeabilization and apoptosis (105). Calcium is also an important regulator of cytochrome *c* release and can cause phosphorylation of other Bcl-2 proapoptotic proteins (105). Some studies have shown that

cytochrome *c* release does not always lead to apoptosis if the caspases are defective.

Cytochrome *c* can also be released through caspase independent mitotic catastrophe and mitotic catastrophe can be inhibited by Bcl-2 (105). Overexpression and mutations of antiapoptotic molecules such as Bcl-2 and Bcl-xL, or downregulation and mutation of proapoptotic Bax, have been implicated with increased tumorigenesis and chemoresistance (93, 109, 110).

Cytotoxic drugs have also been shown to modulate NF- κ B, which depending on the stimulus and cell types, can activate either proapoptotic death receptors or antiapoptotic genes such as Bcl-xL (109). Paclitaxel can induce permeabilization of the mitochondrial membrane by affecting Bcl-2 through hyperphosphorylation and subsequent opening of the permeability transition pore on mitochondria (93). Permeabilization of the outer mitochondrial membrane due to cytotoxic drugs including Paclitaxel can also be due to the compromise of redox or energy balance and perturbations of intermediate metabolism such as increasing reactive oxygen species or decreasing their detoxification, depleting ADP, ATP and NADPH and inhibiting the respiratory chain. Each of these activities can all lead to increased mitochondrial membrane permeabilization (93). The extrinsic receptor pathways and the intrinsic mitochondrial pathway are linked at several different levels including the activation of caspase-8 via the death receptors that leads to the activation of proapoptotic Bid, a Bcl-2 family protein with a BH3-only domain, which then translocates to the mitochondria to activate the release of cytochrome *c* (93).

Necrosis, in contrast to apoptosis, is an apparently passive process that does not require ATP and occurs in response to changes outside of the cell (103). Necrosis is marked by energy depletion, cellular edema, vacuolization of the cytoplasm, breakdown of the plasma membrane and

membrane lipids, loss of function of ion pumps/channels, and an inflammatory response due to the release of cell contents into the surroundings (100, 103, 111). Cells that die by necrosis also have changes in nuclear morphology but not the same chromatin condensation and fragmentation of DNA as occurs in apoptosis (103). While apoptosis involves the Bcl-2 family of proteins and the caspases, necrosis is induced by depletion or reduction of cellular energy content, intracellular calcium overload, generation of reactive oxygen species (ROS), and activation of nonapoptotic proteases and calcium dependent proteases (100). Recent studies have suggested that the activation of apoptosis may inhibit necrosis because caspases cleave and inactivate proteins required for programmed necrosis (type 3 cell death) such as PARP (poly(ADP-ribose) polymerase) (103). Necrosis can also occur in cells that have bioenergetic stress, which are the same conditions that can stimulate autophagy as a survival mechanism to boost ATP levels, suggesting that these two mechanisms can occur in parallel (103). Regardless of the initial stimulus of cell death, mitochondrial membrane permeability and intracellular energy levels determine whether or not cells die by apoptosis or necrosis and can be modulated by pre-depleting cells of ATP which then switches cell fate from apoptosis to necrosis even when apoptosis stimulators are used (112).

Mitotic catastrophe occurs during mitosis as a result of DNA damage or abnormal spindle formation. Defective cell cycle checkpoints lead to missegregation of chromosomes and/or cell fusion during mitosis and result in tetraploidy, incomplete cytokinesis, and giant nonviable cells. Multiple micronuclei are produced resulting from the formation of nuclear envelopes around clusters or fragments of chromosomes (91, 100, 113, 114). Mitochondrial membrane permeability and caspase activation have been reported in conjunction with mitotic catastrophe

but others suggest the process is caspase independent (113). In prostate cancer cells, drug induced mitotic catastrophe is thought to occur independently of p53 and involves the release of cytochrome *c* which takes place between caspase-2 and -3 activation (115). This potential molecular pathway is thought to be dependent on DNA damage and cell cycle arrest at the G2/M phase however it is not known if Docetaxel acts through this mechanism and if Docetaxel induced mitotic catastrophe is an end point or followed by apoptosis (115). In addition, a reduction in the G2/M cell fraction but an increase in S phase, SubG1 cells and tetraploid population with DNA content of 4N to 8N appeared in PC3 cells in response to Docetaxel treatment probably due to incomplete cytokinesis but not in DU145 cells which showed DNA content lower than 4N probably due to complete cell division (115).

While apoptosis is considered to be the primary mechanism of cell death due to the taxanes, both taxane drugs can induce cell line and dose specific mixtures of apoptosis and mitotic catastrophe cell death (116). In MCF-7 and MCF-10 human breast cells, Paclitaxel has a concentration dependent effect; at low concentrations, apoptosis and necrosis were found, in contrast to mitotic arrest and necrosis at high concentrations (116). In MDA-MB-231 human breast cancer cells, Paclitaxel can induce multinucleated nonapoptotic cell death (116). In MDA-MB-231, MCF-10A and MCF-7 cells, mitotic catastrophe was found to be the predominant mechanism of cell death due to Docetaxel with no significant levels of apoptosis (116).

Autophagy is a catabolic cellular degradation pathway that sequesters and degrades cytoplasmic organelles, proteins and other contents through the initiation of double membrane phagosome formation. The phagosomes form autophagosomes which fuse with lysosomes to produce

autophagolysosomes where digestion of cellular content with lysosomal hydrolases takes place (96). This bulk process is also known as macroautophagy (117). Another form of autophagy is called chaperone mediated autophagy (CMA) and occurs when a pentapeptide motif in a target protein (KFERQ) is recognized by Hsc70, which facilitates the delivery of the protein to LAMP2A, both a receptor and a pore for substrate uptake into lysosomes (117, 118). The third form of autophagy is the least understood. Microautophagy occurs when portions of lysosomal membranes directly engulf nearby cytoplasm (117).

The macroautophagy process (hereby referred to as autophagy) is regulated by several Atg proteins that can be divided into four groups. Group 1 are involved in the initiation, nucleation, and expansion steps of autophagosome formation and involves the Atg1 complex (119). The second group is the Beclin-1/class III PI3K complex, which is involved in the nucleation phase of autophagosome formation (119). The third group involves Atg5 and Atg12, which are ubiquitinated by E1 like enzymes Atg7 and Atg10 to allow for autophagic vacuoles to form; LC3 (ortholog of yeast Atg8) is also involved (119, 120). The last group is controlled by Atg1 and Atg9 and is involved in the recycling of Atg proteins in and out of autophagosomes membranes during the formation of the autophagosomes. Rab GTPases are involved in the maturation of autophagosomes (119).

Intermediary structures of the autophagy pathway, such as LC3-II/LC3-I (microtubule associated protein 1 light chain 3 beta) an autophagosome marker and p62 (SQSTM1/sequestome-1) which links LC3 and ubiquitinated structures destined for autophagy, can be used to monitor autophagic activity by using antibodies to LC3 (96, 97, 121). The amount of LC3II localized to the

autophagosome membrane, the product of posttranslational modification of LC3-I, correlates with the number of autophagosomes (97, 121). The direction of damaged or unfolded proteins to the autophagy pathway involves p62, which binds to polyubiquitinated proteins by oligomerization and binds to LC3 on the autophagosome to target these aggregates to the autophagosome (122, 123). This sequestering process is thought to limit the impact of toxic proteins in cells. The inability to eliminate p62 through the autophagy process is, therefore, considered to be toxic to cells because of failure of the protein quality control mechanism that involves p62 (122).

Autophagy can be a bulk nonspecific mechanism degradation process, or specifically target mitochondria, catalase, peroxisomes, endoplasmic reticulum and protein aggregates. Nonetheless, the exact mechanism that specific proteins and organelles are targeted remains to be fully determined (96). Liver-specific autophagy defects in mice that cause damaged mitochondria and high levels of oxidative stress to accumulate, the activation of a DNA damage response in cells, has been attributed to persistence of p62 (122). Failure of autophagy-defective cells to eliminate p62 also results in changes in NF- κ B regulation and gene expression; p62 is a known activator of NF- κ B, that leads to increased tumorigenesis and liver damage (122, 124). Similarly, brain targeted autophagy deficiency can lead to neurodegeneration, which may indicate that autophagy's role in eliminating damaged organelles and proteins is an important quality control mechanism during stress and aging (122).

Autophagy is also considered to be an alternative energy source for survival in periods of metabolic stress or a type 2 programmed cell death when excessive "self-cannibalization" occurs

(91, 96, 103, 108, 125, 126). Metabolic and therapeutic stresses that are known to induce autophagy include interruption of growth factor signaling pathways, inhibition of proteasomal degradation, activation of mitogen-activated protein kinase signaling, accumulation of intracellular calcium and endoplasmic reticulum stress (97). Autophagy is also induced and localized in advanced tumors that have central hypoxic tumor regions where it supports cell survival (122, 127). Direct biochemical associations between stressors and known autophagy genes have yet to be fully characterized (97). However, both Bcl-2 and Bcl-xL can associate with Beclin-1 (ortholog of yeast Atg6), and inhibit autophagy through Beclin-1's BH3 domain (119, 126, 128). Beclin-1 is a 60 kDa protein that has been identified in the trans-Golgi network, the endoplasmic reticulum, at the mitochondria, and the perinuclear membrane (119). Endoplasmic reticulum targeted Bcl-2, but not mitochondrial Bcl-2 can inhibit autophagy (128).

The association of Bcl-xL with Beclin-1 can cause cells to be susceptible to both autophagy and apoptosis. In addition, Beclin-1 and class III PI3K have been found to be substrates for caspases -3, -7, and -8 indicating further cross talk between autophagy and apoptosis (129). When apoptosis is blocked due to the absence of Bax or Bak, the autophagic survival pathway is prolonged compared to the response to cytotoxic drugs which leads to autophagic cell death (126). Autophagy can also lead to either a caspase independent cell death or be caspase activating (97). Defects in the function of proapoptotic Bax and Bak in bone marrow derived immortalized cells and when apoptosis was inhibited using a caspase-8 inhibitor in mouse fibroblasts, autophagic cell death was caused (127). However cell death due to caspase-8 inhibition is prevented by the inhibitor of autophagy, 3-methyladenine (3-MA) (130).

Genetic inhibition of autophagy with Beclin-1 or Atg5 RNA interference, or through the constitutive activation of the class I PI3K-AKT-mTOR signaling pathway, can prevent the survival response even when apoptosis is blocked due to cell death by necrosis (96, 127). 3-methyladenine, a general inhibitor of phosphatidyl-inositol 3-kinases, has been shown to inhibit pre-autophagosome formation and reduced cell viability in Trastuzumab-refractory HER2 amplified breast cancer cells. However, this inhibitor has several off target effects and can interfere with many other cellular processes besides autophagy (103, 121). It is however still unclear exactly how the duality between apoptosis and autophagy is controlled, whether autophagy plays a tumor suppressor or protective role, or whether autophagy should be promoted or inhibited in clinical therapy. Thus, before clinical therapies are developed, molecules that specifically positively or negatively regulate autophagy must be identified in order to better understand how autophagy affects cancer cell death (126, 127). In addition, determining which of the various cell death or survival pathways is used specifically by taxane resistant and sensitive cells is important and will be more closely examined in this thesis.

1.7 Hypothesis and Aims

We hypothesize that acquired taxane resistance in breast cancer cells may be associated with cellular transporters and when challenged with cytotoxic concentrations of taxane drugs may die through multiple mechanisms.

The hypothesis was tested in two aims:

Aim 1: Develop, characterize and determine the role of transmembrane transporters and β -tubulin in the taxane resistant phenotype.

Aim 2: Determine cell death mechanisms implicated with taxane sensitivity and resistance.

CHAPTER 2: Development and Characterization of Taxane Resistant Breast Cancer Cells

2.1 Introduction

Tumors that do not respond to chemotherapeutic agents from the very beginning of treatment are considered to be *de novo* or intrinsically resistant. Tumors that initially respond but fail to continue to respond during the course of treatment are considered examples of acquired resistance (131). Cell lines resistant to taxanes can be generated by a single high dose exposure to a drug or in a dose escalating manner (65). We have used the latter method to select our resistant MDA-MB-231 cells, starting with a low concentration (0.01 nM) for each taxane. Our models of acquired resistance to the taxane drugs were developed in the presence of 1 μ M Verapamil, to help eliminate the development of Pgp-mediated resistance phenotype. In this chapter we explore the possibility of cross-resistance of both taxane resistant cell lines to the drug that they were not exposed to in the development of their resistant phenotype. We also, determine whether or not sensitive and resistant cells still retain genetic similarity by DNA fingerprinting and determine the role of both β -tubulin isotype expression changes and β -tubulin DNA sequence mutations in the sensitive and resistance phenotypes.

2.2 Materials and Methods

2.2.1 Cell Culture and Reagents

MDA-MB-231 cells are derived from human invasive ductal carcinoma of the breast and are a model of estrogen receptor-negative (ER-) and progesterone receptor-negative (PR-) breast cancer that is p53 deficient (59, 132). MDA-MB-231 cells were originally obtained from the Lombardi Comprehensive Cancer Center's Tissue Culture Shared Resource and the cells were propagated routinely in Modified IMEM (Invitrogen Corporation, Carlsbad, CA) supplemented with 5% Fetal Bovine Serum (Gemini Bio Products, West Sacramento, CA) and 1 μ M Verapamil hydrochloride (Sigma-Aldrich, St. Louis, MO). Establishment and characterization of the two taxane resistant sublines were developed by serial passaging. All of the cell cultures were maintained in a humidified 95% air/5% CO₂, 37°C incubator and were shown to be free of *Mycoplasma* spp. contamination.

Selection Procedure: Cell lines resistant to taxanes were generated by a dose escalating method to select our resistant MDA-MB-231 cells, starting with a low concentration (0.01 nM) for each taxane and 1 μ M Verapamil, a Pgp inhibitor (65). The concentration of either Paclitaxel (Sigma-Aldrich, St. Louis, MO) or Docetaxel (Sanofi-Aventis, Bridgewater, NY) was increased step-wise after three successive passages of cells at each dose (133). At each dose level several aliquots of cells were frozen and stored in liquid nitrogen. Following this step-wise selection, we obtained two stably resistant MDA-MB-231-derived breast cancer cell lines (Paclitaxel at 7 nM and Docetaxel and 2 nM).

2.2.2 Cell proliferation assays

For cross-resistance proliferation assays, sensitive (CTL), Docetaxel Resistant (DocR) and Paclitaxel Resistant (PacR) MDA-MB-231 cells were seeded at a density of 1×10^4 cells/well into 24-well plates in drug free media with 1 μ M Verapamil for 24 hours before treatment with either no drug or (1-60 nM) of Docetaxel or Paclitaxel for 72 hours. For all cells, Verapamil was maintained in the media throughout the experiment. Cells were then trypsinized with 0.5% Trypsin (Invitrogen, Grand Island, NY), resuspended in Phosphate Buffered Saline pH 7.4 (Invitrogen, Carlsbad, CA), and counted in a Beckman Coulter counter (Beckman Coulter Corp., Fullerton, CA) to assess cell number. At least three independent experiments were done in quadruplicate, and data are normalized to sensitive cells without drug treatment. The proliferation data were repeated in triplicates. Data are presented as the mean $\pm$ SE for a representative experiment.

For the 9 day proliferation assay, CTL, DocR and PacR MDA-MB-231 cells were plated into 6-well plastic tissue culture plates at a density of 1×10^5 cells/well in drug free media with 1 μ M Verapamil for 24 hours before treatment with either 7 nM Paclitaxel for Paclitaxel resistant cells, 2 nM Docetaxel for Docetaxel resistant cells or no drug for the sensitive cells. For all cells, Verapamil was maintained in the media throughout the experiment. Cells were then trypsinized with 0.5% Trypsin (Invitrogen, Grand Island, NY), resuspended in Phosphate Buffered Saline pH 7.4 (Invitrogen, Carlsbad, CA), and counted in a Beckman Coulter counter (Beckman Coulter Corp., Fullerton, CA) to assess cellular number at 24 hours, 3, 6 and 9 days post-treatment. At least three independent experiments were done in quadruplicate, and data are normalized to vehicle-treated cells. Data are presented as the mean $\pm$ SE for a representative experiment.

2.2.3 DNA Fingerprinting

DNA fingerprinting was performed by the Lombardi Comprehensive Cancer Center Analysis Shared Resource according to the following protocol (134) using PowerPlex 1.2 System (Promega, Madison, WI). PCR amplification was performed according to the manufacturer's protocol and allele size was determined by electrophoresis of the PCR products in 6% denatured polyacrylamide gels and compared to ROX 500 size standards (Applied Biosystems, Foster City, CA) using an automated sequencer ABI 377 (Applied Biosystems, Foster City, CA). Different size alleles were analyzed using GENESCAN v.3.1 and GENOTYPER v.2.1 software (Applied Biosystems, Foster City, CA). The experiments were repeated twice for confirmation.

2.2.4 β -tubulin isotype expression by RT-PCR

RNA isolated from three independent passages of sensitive, Docetaxel and Paclitaxel resistant cell lines as described above and in (135) and purified using the RNeasy Mini Kit (Qiagen, Valencia, CA). 6 μ g of total RNA was reverse transcribed and specific oligonucleotide primers and concentrations of magnesium chloride were used as in (136). The β -tubulin isotypes were analyzed by PCR according to the protocol and primer sequences provided in (136) using β_2 -microglobulin as an internal loading control. The PCR was carried out using the (Promega, Madison, WI) GoTaq Flexi kit. After an initial denaturation at 94°C for 10 min, PCR was carried out for the relevant number of cycles as in (136) for class I, II, III, IVa, IVb and VI β -tubulin isotypes using the following conditions: 94°C for 30s, the appropriate annealing temperature for 30s, 72°C for 90s, and a 10 min extension step at 72°C. PCR products were electrophoresed using a 2% agarose gel (Invitrogen, Carlsbad, CA). Gel Images were captured

using the Alpha EaseFC (FluorChem 8900) software (Alpha Innotech Corporation, San Leandro, CA).

2.2.5 β -tubulin sequence mutations

Genomic DNA was extracted using the QIAamp DNA Mini Kit protocol and reagents (Qiagen, Valencia, CA); the protocol and primer sequences were as described in (136) and obtained from (Integrated DNA Technologies, Coralville, IA) and the protocol as described. DNA sequencing was performed by the Lombardi Comprehensive Cancer Center Macromolecular Analysis Shared Resource. Sequence analysis was performed using data from the National Center for Biotechnology Information GenBank. Sequences of each exon of each cell line were compared to existing NCBI's Nucleotide BLAST β -tubulin sequence (ref NM 178014.2, Homo Sapiens Tubulin, beta TUBB). In addition NCBI's Nucleotide BLAST, the β -tubulin sequence of each cell line was compared to the others (CTL vs. DocR vs. PacR). Mutation Surveyor software (Softgenetics, State College, PA) was used for DNA variant analysis of the β -tubulin sequence. Single Nucleotide polymorphisms were found using the Ensembl Genome Browser for the Homo Sapiens TUBB gene (ENSG00000196230).

2.2.6 Cell Cycle Assay

Cells were seeded at a density of 1×10^5 cells/well into 6-well plates in drug free media with 1 μ M Verapamil for 24 hours before treatment with IC₅₀ levels of either Paclitaxel or Docetaxel drug or no drug treatment for 48 hours. IC₅₀ levels were determined from proliferation assay data using SigmaPlot 8.0 (Systat, San Jose, CA). CTL IC₅₀ for Paclitaxel was found to be 2.0 nM, CTL IC₅₀ for Docetaxel drug was 0.6 nM. PacR IC₅₀ for Paclitaxel drug was 19.4 nM and for

Docetaxel drug was 8.1 nM. DocR IC₅₀ for Paclitaxel drug was 172.3 nM and for Docetaxel drug was 38.4 nM. Cells were then trypsinized with 0.5% Trypsin (Invitrogen, Grand Island, NY), resuspended in Phosphate Buffered Saline pH 7.4 (Invitrogen, Carlsbad, CA) and then analyzed for alterations in cell cycle via fluorescence activated cell sorting, which was done by the Lombardi Comprehensive Cancer Center Flow Cytometry Shared Resource using ModFit software (Verity Software House, Topsham, ME). Data are presented as the mean $\pm$ SE for four independent experiments.

2.2.7 Statistical Analysis

All statistical tests were performed using SigmaStat software (Systat, San Jose, CA). Student's *t* test was used to compare two groups in which the data are normally distributed; a Wilcoxon *t* test was used to compare groups in which data are not normally distributed, and the Mann-Whitney Rank Sum test for data that does not have equal variance. For multiple comparisons, ANOVA was used with a *post hoc t* test for multiple comparisons.

2.3 Results

2.3.1 Cross-Resistance

Following the step-wise selection generation of acquired taxane resistant breast cancer models, we obtained two stably resistant MDA-MB-231-derived breast cancer cell lines (Paclitaxel at 7 nM and Docetaxel at 2 nM). Given the structural similarity of the two taxanes, there is a potential for cross-resistance to develop. Therefore, we performed cell proliferation assays to compare a) sensitivity of the parental cell line to each drug, b) sensitivity of each resistant cell line to the drug it was selected against, and c) sensitivity of each resistant cell line to the other taxane. Sensitive (CTL) and 7 nM Paclitaxel resistant cells (PacR) remain sensitive to Docetaxel, while the 2 nM Docetaxel MDA-MB-231 cells (DocR) are resistant to Docetaxel. CTL cells are also sensitive to Paclitaxel while the dose-response curve for PacR cells to this curve is significantly shifted. In contrast, DocR cells are completely cross-resistant to Paclitaxel. These data suggest that there are different factors that contribute to Docetaxel resistance as opposed to Paclitaxel resistance (Fig. 2.1).

Figure 2.1 Cross-Resistance

A

B

Figure 2.1 Cross-Resistance. Full cross-resistance to Paclitaxel of Docetaxel resistant cells (DocR) (A) and partial cross-resistance of Paclitaxel resistant cells (PacR) to Docetaxel (B). In Paclitaxel drug, ANOVA of CTL cells, $p = 0.003$, DocR $p \leq 0.001$, PacR $p \leq 0.001$; at 1 nM Paclitaxel drug, PacR cells have significantly higher cell numbers compared to CTL and DocR cells (Student's t -test, $p \leq 0.001$). DocR cells also have higher cell numbers compared to CTL (Mann-Whitney Rank Sum test, $p = 0.029$). In Docetaxel drug, ANOVA of sensitive (CTL) cells $p = 0.001$, DocR $p \leq 0.001$, PacR $p \leq 0.001$; at 1 nM DocR and PacR cells have significantly higher cell numbers compared to CTL (Student's t -test, $p \leq 0.001$). Data are presented as the mean $\pm$ SE for a representative experiment.

2.3.2 DNA Fingerprinting

To confirm that the genetic lineage of all three cell lines was similar, we performed DNA fingerprinting. Using markers at 9 different loci (CSF1PO, TPOX, TH01, vWA, D16S539, D7S820, D13S317, D5S818 and the Y-specific Amelogenin) we confirmed that the CTL, PacR, and DocR cells are identical in origin (Fig. 2.2) in addition to determination of allele sizes by PCR (134).

Figure 2.2 DNA Fingerprinting

Figure 2.2 DNA Fingerprinting. DNA Fingerprinting analysis of sensitive CTL, Paclitaxel resistant PacR and Docetaxel resistant DocR MDA-MB-231 cells showing the results at markers D16S539, D7S820, D13S317, D5S818 (A) and CSF1PO, TPOX, TH01, vWA and Y-specific Amelogenin (B) with allele sizes exhibited under each allele. No significant differences are found between sensitive and taxane resistant breast cancer cells.

2.3.3 β -tubulin isotype expression

One possible mechanism for both Paclitaxel and Docetaxel resistance is altered expression of six different β -tubulin isotypes in breast cancer cells. Overexpression of class II β -tubulin in CHO and NIH 3T3 cells was not shown to confer Paclitaxel resistance (50). In addition, overexpression of class I or IVb in CHO cells did not change Paclitaxel sensitivity and siRNA experiments knocking down class II and IVb β -tubulin isotypes in NSCLC cell lines didn't affect Paclitaxel sensitivity either (50). In MCF-7 and MDA-MB-231 breast cancer cells however, class III β -tubulin is a biomarker of resistance to Docetaxel (50, 137).

In contrast, in the clinic, patients with advanced breast cancer, class II β -tubulin expression correlated with lower levels of Docetaxel response (50). High levels of class III and IV β -tubulin have been reported to destabilize microtubules and be associated with Paclitaxel resistance in breast cancer (138, 139). High expression of class III β -tubulin has also been shown to be a predictive biomarker of clinical Paclitaxel resistance in breast cancer. Tumors with high levels of both class I, II and class III β -tubulin also show a lower response rate to Docetaxel in breast cancers (50, 136, 140, 141).

The main difference between class I and class III β -tubulin is an amino acid substitution that leads to a change in the three dimensional conformation preventing stable Paclitaxel binding to class III β -tubulin (137). Class III β -tubulin also causes microtubules to be more dynamic, and therefore resistant to the stabilizing effects of the taxane drugs (137). Recently, studies have

shown that overexpression of class III β -tubulin can be induced by cellular stressors such as hypoxia and glucose deprivation in ovarian cancer cells. Interestingly, the promoter region of class III β -tubulin has p53 and NF- κ B binding sites but whether these transcription factors can regulate β III-tubulin expression is unclear (50). In the CTL, PacR and DocR breast cancer cells, no differential statistically significant β -tubulin isotype expression was found by RT-PCR densitometry, however, the isotype IVa appears to be lower in both CTL and PacR cells compared with DocR cells (Fig. 2.3).

Figure 2.3 RT-PCR of β -tubulin Isotypes

Figure 2.3 RT-PCR of β -tubulin Isotypes. RT-PCR of β -tubulin isotypes I-VI in Sensitive (Ctrl.), Docetaxel resistant (DocR) and Paclitaxel Resistant (PacR) cells. β_2 -microglobulin was used as a positive control. No statistically significant differences in isotype expression are found

between taxane sensitive and resistant breast cancer cells. Data are presented as the mean $\pm$ SE for experiments.

2.3.4 β -tubulin sequence mutations

The β I-tubulin gene is located at chromosome 6p23.1 and consists of 4 exons that encode a protein of 445 amino acids (142, 143). Both Paclitaxel and Docetaxel share a common binding site on β -tubulin. The binding sites of Paclitaxel to β -tubulin were found to be on the N-terminal 1-31 amino acids that are encoded by exons 1 and 2 and amino acids 217-231 (144-146). Other drugs that bind the taxane binding site include epothilones, discodermolide and eleutherobin (47). A possible mechanism for both Paclitaxel and Docetaxel resistance is mutation of the class I β -tubulin gene. However, commonly found mutations in the β 1-tubulin gene in breast cancers indicated in the literature were not found when sequencing of the coding region of the β 1-tubulin gene was performed by PCR in our sensitive and resistant cell lines. These include point mutations from T to A resulting in Asparagine 26 to Glutamine substitution in the Paclitaxel binding region of β 1-tubulin in epidermoid breast cancer cells resistant to Paclitaxel (KB-15-PTX/099), single mutation of β 1-tubulin from A to G resulting in a change from Glutamate 198 to Glycine in MDA-MB-231 breast cancer cells resistant to Paclitaxel that do not express Pgp and a somatic missense mutation in codon 306 of exon 4 of β 1-tubulin, changing CGC to TGC resulting in a change from Arginine to Cysteine in breast cancer tissues (51, 59, 79).

Commonly found mutations that may contribute to taxane resistance or sensitivity in breast cancer were not found in our cell lines but possible novel mutations cannot be excluded (Fig. 2.4). Point mutations can cause defective and sometimes harmful protein products (147). There are several different types of mutations that can occur, such as missense mutations that involve a single nucleotide change that causes a change in a codon that results in a different amino acid.

These mutations may or may not play a role in the structure and function of a protein (148).

Another type of mutation is a nonsense mutation that involves a nucleotide change that results in a stop codon which produces a truncated protein (148). A frameshift mutation involves the insertion or deletion of a nucleotide. Such a mutation disrupts the reading frame and can also result in the production of a stop codon that leads to a truncated protein (148).

Mutation Surveyor software can detect DNA variants from sequence data by comparing a sample to a known GenBank reference sequence in addition to quantifying alleles. The Mutation Surveyor software also determines if the sequence variations found are common single nucleotide polymorphisms. Examples of mutations found β 1-tubulin of CTL, DocR and PacR cells using Mutation Surveyor software were mutations not in the taxane binding region of β 1-tubulin, such as missense mutations in exon 4a of CTL, PacR and DocR cells that resulted in a change of amino acids 181 (Valine to Isoleucine) and 183 (Proline to Serine), a heterozygous frameshift mutation at amino acid 424, and a nonsense mutation in exon 4c at amino acid 405 of sensitive and resistant cells. The sequence of each exon across the cell lines was also compared and in both resistant cells at amino acid 368 in exon 4d, there was a sequence and codon change. However, this change did not correspond to a change in the amino acid, which remained Isoleucine so it is not relevant to the resistance phenotype. Thus far, there is no data to indicate that β 1-tubulin mutations are clinically relevant and they are considered to be rare events (50, 65).

Figure 2.4 β -tubulin Sequence Mutations

Figure 2.4 β -tubulin Sequence Mutations. Mutation Surveyor software analysis showing novel mutations of class I β -tubulin. Possible missense mutations was found in exon 4a of PacR cells (A), a possible heterozygous frameshift mutation in CTL cells (B), and a possible nonsense mutation in exon 4c of CTL cells (C). Lane one in each frame represents the codons, the second lane represents the protein that the codon sequence will code for, the 3rd lane is the reference sequence, the 4th lane is the sample sequence and the 5th lane is the mutation that is found followed by the 6th lane which describes the type of mutation.

2.3.5 Cell Cycle

Cell cycle progression was measured in the sensitive and resistant cell lines by performing cell cycle assays at IC₅₀ levels of either Paclitaxel or Docetaxel drug compared to no drug treatment. For the G1 phase, without drug treatment, DocR cells were found to have higher numbers of cells compared to CTL cells without drug treatment ($p=0.029$). PacR cells without drug treatment showed the opposite, a lower number of cells in G1 compared to CTL cells ($p=0.029$) and DocR cells ($p\leq 0.001$) without drug treatment. In addition, PacR cells had lower numbers of cells in the G1 phase compared to CTL cells in IC₅₀ Docetaxel drug as well ($p=0.029$). In contrast, for the S phase of the cell cycle, both taxane resistant cell lines showed a significantly higher percentage of cells in S phase without drug treatment ($p=0.029$). For the G2/M phase, without drug treatment, both resistant cell lines had lower levels of cells in this phase compared to CTL cells ($p=0.029$) with DocR cells having lower G2/M phase cells compared to PacR cells as well ($p\leq 0.001$). PacR cells had lower levels of G2/M cells compared to CTL cells at IC₅₀ levels of Paclitaxel drug as well ($p=0.029$) (Fig. 2.5). It may be that the increased levels of S phase in both taxane resistant cells but not CTL is indicative of enhanced proliferation.

Figure 2.5 Cell Cycle Analysis with and without IC₅₀ Paclitaxel and Docetaxel at 48 hours.

Figure 2.5 Cell Cycle Analysis with and without IC₅₀ Paclitaxel and Docetaxel at 48 hours.

Cell cycle analysis of CTL, DocR and PacR cells in the G1 phase (A), ANOVA p=0.011, DocR vs. CTL without drug treatment (Mann-Whitney Rank Sum test p=0.029), PacR cells vs. CTL cells (Mann-Whitney Rank Sum test p=0.029) and DocR cells (Student's *t* test p≤0.001) without drug treatment. PacR cells vs. CTL in IC₅₀ Docetaxel drug (Mann-Whitney Rank Sum test p=0.029). For the S phase of the cell cycle (B), ANOVA p=0.014, both DocR and PacR vs. CTL

cells (Mann-Whitney Rank Sum test $p=0.029$). For the G2/M phase of the cell cycle (C), ANOVA $p=0.006$, without drug treatment, DocR and PacR vs. CTL cells (Mann-Whitney Rank Sum test $p=0.029$) and DocR vs. PacR cells (Student's t test $p\leq 0.001$). PacR cells vs. CTL cells at IC_{50} levels of Paclitaxel drug (Mann-Whitney Rank Sum test $p=0.029$). Data are presented as the mean $\pm$ SE for four independent experiments.

2.3.6 Proliferation

In addition to cell cycle analysis, a basal proliferation assay was conducted in order to determine the proliferation rates over a 9 day period of each cell line. Initial cell numbers were similar up to day 3 but from day 6 to day 9 significant differences appeared. Docetaxel resistant cells were found to have significantly higher cell numbers, 4.9-fold higher ($p=0.005$) compared to sensitive cells at day 9. Paclitaxel resistant cells also had higher cell numbers, by 2.0-fold, ($p=0.05$) compared to CTL MDA-MB-231 cells (Fig. 2.6).

Figure 2.6 Cell Proliferation – 9 days

Figure 2.6 Cell Proliferation – 9 days. Cell proliferation assay over a 9 day period shows that DocR cells proliferate the fastest and have the highest cell number, ANOVA $p=0.003$ followed by PacR cells, ANOVA $p=0.003$ compared to CTL ANOVA $p=0.003$. DocR cells compared to CTL have significantly higher cell numbers at day 9 (Student's t test $p=0.005$) as do PacR cells compared to CTL, (Student's t test $p=0.05$). Data are presented as the mean $\pm$ SE for a representative experiment.

2.4 Discussion

While Paclitaxel and Docetaxel drugs are similar structurally, Docetaxel appears to be the more potent of the two drugs. In addition, cells that are resistant to Docetaxel appear to be cross-resistant to Paclitaxel whereas the same does not hold for Paclitaxel resistant cells treated with Docetaxel. Although the sensitive and resistant cell lines respond differently to either taxane drug, they still retain a similar genetic lineage background as was confirmed by DNA fingerprinting. When compared over a period of 9 days at baseline (sensitive cells grown without drug, DocR cells grown in 2 nM of Docetaxel drug and PacR cells grown in 7 nM Paclitaxel drug) it appears that both resistant variants proliferate more rapidly than sensitive cells. In addition, both resistant variants have higher cell numbers in the S phase of the cell cycle compared to CTL cells. As predicted, the variants also have but numbers of cells in the G1 and G2/M phases, implying a higher rate of proliferation.

Regarding expression of β -tubulin, it is still unclear how the differential expression of β -tubulin isotypes and microtubule proteins are regulated. Determining which isotypes can be used as biomarkers for clinical response to chemotherapy drugs will be an important step. However, in our breast cancer models of taxane sensitivity and acquired resistance, differential expression of β -tubulin isotypes did not appear to be involved in resistance to either drug. In addition to β -tubulin, microtubule associated proteins (MAPs) have been implicated in taxane resistance. MAPs such as MAPT and MAP4 can bind and stabilize microtubules against depolymerization and increased expression of MAPs is associated with resistance (50). While MAPT is predominantly expressed in neuronal tissue, MAPT expression has been found to be associated

with breast cancers that are less responsive to Paclitaxel. The steric hindrance that MAPT causes can limit Paclitaxel's access to the inner luminal surface of microtubules (50). Induction of p53 can inhibit MAP4, which leads to reduced sensitivity to Paclitaxel (50). Using gene expression microarray technology described in chapter 3, MAP4 was found to be upregulated in DocR and PacR vs. CTL cells by 1.32 and 1.56-fold respectively ($p=0.032$, $p=0.019$) however MAPT expression was not differentially expressed therefore whether or not MAPs are involved in our models of taxane resistance remains to be determined.

The role of γ -actin still needs to be determined in the resistant and sensitive phenotypes. Pharmacological studies and gene expression microarray studies of several cancer cell lines have shown that disruption of the microtubule cytoskeleton can affect the actin cytoskeleton and regulators of the actin cytoskeleton such as γ -actin and the LIM kinase 2 gene are being considered as predictive biomarkers for drug resistance to certain cytotoxic drugs. Nonetheless, their role in resistance to the taxanes is still unclear and they were not found to be differentially expressed either taxane resistant cell lines by microarray (50, 149).

Regarding, β -tubulin mutations, although known mutations to class I β -tubulin in breast cancer did not appear in the DNA sequence of our sensitive and resistant breast cancer models, the proposed mutations have yet to be shown to have clear clinical relevance(65) . Possible novel mutations could have been identified in our models but were not in the taxane binding region and so may not be functionally relevant however it is not known if these changes could affect conformation and indirectly affect taxane binding.

CHAPTER 3: Role of Transporters in the Taxane Resistance Phenotype

3.1 Introduction

Membrane transporters and ion channels make up 4% of the human genome, with 406 genes that are ion channels and 883 genes that are transporters. 350 of the transporters are classified as intracellular transporters (70). Of these transporters, both the ABC and SLC superfamilies have been implicated in conferring resistance to the taxanes. Resistance due to the transporters can occur from reduced uptake of drugs or increased efflux. For hydrophobic drugs such as Docetaxel and Paclitaxel, their efflux via transporters is what most likely causes chemoresistance (70, 73).

The ABC transporters use the hydrolysis of ATP to bring nutrients into cells or toxins, drugs and lipids out of cells (69). Of the ABC transporter members, Pgp has been strongly implicated in taxane resistance and substrates for Pgp are usually hydrophobic drugs with a polyaromatic backbone and a neutral or positive charge (69, 70). Other ABC genes implicated with resistance to the taxanes are ABCB4 (MDR2/3) and ABCB11 (bile salt transporter BSEP/sister of Pgp SPGP which can also be inhibited by Verapamil like Pgp) for which Paclitaxel is a substrate and ABCC2 (MRP2), ABCC10 (MRP7) for which both taxanes are substrates (67, 70, 77). Drugs such as Verapamil can inhibit the Pgp resistance mechanism in cancer cells and has been used to help eliminate the development of this mechanism of resistance in the taxane resistant MDA-MB-231 models.

The other group of transporters is the solute-carrier (SLC) transporter superfamily. These transporters are passive transporters that do not require ATP, unlike the ABC superfamily, and commonly transport and mediate chemosensitivity for hydrophilic drugs (70, 87). Both Paclitaxel and Docetaxel have been shown to be substrates of, and enter cells via the OATP1B3 transporter encoded by the SLCO1B3 gene. Paclitaxel has also been shown to be a substrate of OAT2, encoded by the SLC22A7 gene. The precise role of both the ABC and SLC transporters in taxane resistance in the clinic remains to be determined (88-90).

3.2 Materials and Methods

3.2.1 Cell Culture and Reagents

See Materials and Methods section 2.2.1

3.2.2 Gene Expression Microarrays

RNA was isolated from three independent passages of parental sensitive, and Docetaxel and Paclitaxel resistant cell lines as described (135) and purified using the RNeasy Mini Kit (Qiagen, Valencia, CA). Total RNA was prepared and hybridized to Affymetrix Human U133A GeneChips (Affymetrix, Santa Clara, CA) by the Lombardi Comprehensive Cancer Center Analysis Shared Resource to screen over 14,500 genes (22,283 probe sets). Data analysis was performed to obtain the most informative genes. To reduce the dimensional data set from which to identify candidates for gene network building, we excluded genes with intensity of ≤ 1.20 between the resistant and sensitive cells. We then used a data driven approach that applies both false discovery rate (FDR) and miss-rate estimations to describe the remaining dataset and to identify a final data set for analysis using p-values of $p \leq 0.05$ (150, 151). Using the vertebrate TRANSFAC database, 53 differentially expressed ABC and SLC family transporter genes were analyzed for common transcription factor binding sites in their 5000 basepair upstream DNA promoter regions (152).

3.2.3 PCR

RNA was isolated from four independent passages of sensitive, Docetaxel and Paclitaxel resistant cell lines as described above and in (135) and purified using the RNeasy Mini Kit

(Qiagen, Valencia, CA). 0.5 µg RNA was reverse transcribed to generate first strand cDNA using RT² First Strand Kit (SuperArray Bioscience Corporation, Bethesda, MD) and labeled using RT² SYBR Green/ROX qPCR Master Mix (SuperArray Bioscience Corporation, Bethesda, MD) before being added to the 384 well RT² Profiler Drug Transporters PCR Array (Human) (SuperArray Bioscience Corporation, Bethesda, MD) and run using ABI Instruments 7900HT. The Human Drug Transporters array contains a total of 84 transporters, 29 ABC family members, 46 SLC family members, and 9 other transporters. The full list of genes can be found on the SA Bioscience website (http://www.sabiosciences.com/rt_pcr_product/HTML/PAHS-070A.html). Analysis was performed using RT² Profiler PCR Array Data Analysis Template from the SA Bioscience website.

3.2.4 Cell Lysis and Immunoblotting

For the determination of Pgp protein expression, cells were grown in T75 flasks and cultured in normal growth media with 1 µM Verapamil for the sensitive cells, 7 nM Paclitaxel for Paclitaxel resistant cells and 2 nM Docetaxel for Docetaxel resistant cells. Cells were then lysed in modified radioimmunoprecipitation assay buffer [150 mmol/L NaCl, 50 mmol/L Tris (pH 7.5), 1% Igepal CA-630, and 0.5% deoxycholate] supplemented with Complete Mini protease inhibitor cocktail tablets (Roche, Mannheim, Germany) and 1 mmol/L sodium orthovanadate phosphatase inhibitor (Sigma-Aldrich, St. Louis, MO). Lysates were clarified by centrifugation and total protein was quantitated using the bicinchoninic acid assay purchased from Pierce (Rockford, IL). Whole cell lysate (20µg) was resolved by PAGE using NuPAGE 12% precast gels (Invitrogen, Carlsbad, CA). Proteins were then transferred to nitrocellulose membranes, which were probed with the Pgp antibodies overnight at 4°C: (1:500; Sigma-Aldrich, St. Louis,

MO). Membranes were then incubated with horseradish peroxidase–conjugated secondary antibodies for 1 hour at room temperature before enhanced chemiluminescence (GE Healthcare, Buckinghamshire, UK)) and exposure to film (X-OMAT Blue XB-1; Kodak, Rochester, NY). To confirm equal loading of the gels, membranes were reprobed with antibodies for β -actin (1:5000; Sigma-Aldrich, St. Louis, MO).

3.2.5 P-Glycoprotein siRNA

Pgp siRNA and Non Targeting siRNA were obtained from Qiagen (Valencia, CA). Western Blots were performed to confirm knockdown using the above method. Cells were then seeded at a density of 1×10^4 cells/well into 24-well plates in drug free media with 1 μ M Verapamil for 24 hours before treatment with Pgp or Non Targeting siRNA for 24 hours followed by either Paclitaxel or Docetaxel or no drug for 48 hours. At least three independent experiments were done in quadruplicate, and data were normalized to Non Targeting siRNA treated cells. The siRNA proliferation data were repeated in triplicates. Data are presented as the mean $\pm$ SE for a representative experiment.

3.2.6 Statistical Analysis

See Materials and Methods section 2.2.7

3.3 Results

3.3.1 Molecular profiling

Molecular profiling using gene expression microarray technology is a useful tool for the identification of diagnostic, prognostic, or predictive genes as well as the identification of genes for the building of signaling networks (153-155). Gene expression microarrays are the most common high throughput approach and the Affymetrix oligonucleotide GeneChip platform is currently the leading microarray platform (156, 157). Because of the large number of genes, to reduce the dimensionality of the data set, principal component analysis and hierarchical clustering were performed to group together samples and genes that are similar (158, 159). It was found that the gene expression pattern of CTL cells was distinguishably different and separate from both PacR and DocR cells and there was an overlap in gene expression between the DocR and PacR cell lines (Fig. 3.1). Of the 22,283 probe sets, in DocR cells compared to CTL, 2,169 probe sets were found to be differentially expressed at a fold change of >1.45 and $p \leq 0.05$. PacR cells compared to CTL cells had 2,263 probe set differential expression at a fold change of >1.45 and $p \leq 0.05$ while a DocR and PacR cell comparison only showed 527 probe sets that were differentially expressed at a fold change of >1.45 and $p \leq 0.05$ (Fig. 3.2).

Figure 3.1 Principal Component Analysis and Hierarchical Clustering

Figure 3.1 Principal Component Analysis and Hierarchical Clustering. Principal Component Analysis (A) and Hierarchical Clustering (B) of gene expression data from 3 passages of CTL, PacR and DocR MDA-MB-231 cells.

Figure 3.2 Microarray Probe Set Expression Differences of Taxane Sensitive and Resistant Cell Lines

Figure 3.2 Microarray Probe Set Expression Differences of Taxane Sensitive and Resistant Cell Lines. Probe set expression differences between DocR and CTL cells (A), PacR and CTL

cells (B) and DocR and PacR cells (C) at >1.45 , < 1.45 and ≥ 1.20 and < 1.20 fold changes and $p \leq$ or > 0.05 .

3.3.2 Molecular Profiling of Transporters

In the microarray data, both the ABC and SLC transporter superfamily members were found to be differentially expressed. Of the ABC superfamily, 7 ABC transporter members were differentially expressed between DocR and CTL cells at a fold change of >1.45 and $p \leq 0.05$. Of the ABC transporters, those that have been characterized as being transporters of the taxanes were Pgp (ABCB1), which was upregulated by > 40 -fold ($p=0.00003$ value), ABCB4 which was upregulated by 3.81-fold ($p=0.0044$), and ABCC10 was downregulated by 1.48-fold ($p=0.022$). PacR cells had 11 ABC transporters differentially expressed compared to CTL cells; fold change >1.45 , $p \leq 0.05$. Pgp was also found to be upregulated by 2.79-fold ($p=0.0015$ value) in PacR cells compared to CTL cells while ABCC10 was downregulated by 1.57-fold ($p=0.014$). Both taxane resistant cell lines did not differentially express ABCB11 and ABCC2. Both DocR and PacR cells had 30 differentially expressed SLC transporters at a fold change of ≥ 1.45 and $p \leq 0.05$ but only 6 SLC transporters overlapped between the two resistant cell lines. The OATP1B3 and OAT2 transporters (SLCO1B3 and SLC22A7 genes), which are known to transport the taxanes, were not differentially expressed.

3.3.3 PCR of Transporters

Using a transporter PCR array, several of the transporters that were differentially expressed by microarrays were validated including the upregulation of Pgp mRNA in both resistant cells lines. In DocR cells compared to CTL cells, Pgp was found to be upregulated by >100 -fold ($p<0.001$). In addition, ABCB4 was confirmed to be upregulated by 16.64-fold but without a significant p-value ($p=0.095$). ABCB11, ABCC10 and ABCC2 were downregulated but their values did not

reach significance. In DocR cells, of the SLC transporters, SLCO1B3 was found to be upregulated by 5.12-fold ($p=0.05$), while SLC22A7 was not significantly changed. In the PacR cells compared to CTL, Pgp was upregulated by >100-fold ($p<0.001$) and ABCB4 ABCB11, ABCC2 and ABCC10 were all not significantly changed. In PacR cells, of the SLC transporters, SLC22A7 was found to be downregulated by 4.36 ($p=0.011$) while SLCO1B3 was upregulated but without a significant p-value by 3.00-fold ($p=0.161$) (Fig. 3.3)

Figure 3.3 PCR Validation of Transporter Gene Expression at Baseline

Figure 3.3 PCR Validation of Transporter Gene Expression at Baseline. Validation of ABC and SLC transporter expression by SuperArray PCR. DocR vs. CTL cells (A) and PacR vs. CTL cells (B) at baseline.

3.3.4 P-glycoprotein Expression, Inhibition, and Taxane Resistance

Increased protein expression of Pgp in both resistant cell lines compared to CTL cells was also validated using western blots (Fig. 3.4). To determine if Pgp was functionally relevant in the resistance phenotypes, Pgp protein expression was knocked down using siRNA and proliferation assays were performed (Fig. 3.4). These experiments indicated a slight increase in cell number in Pgp siRNA treatment of CTL cells 1-60 nM of Paclitaxel ($p=0.038$, $p=0.029$, $p\leq 0.001$, $p\leq 0.001$, $p=0.003$ respectively). Conversely, DocR cells experienced a reduction in cell number following treatment with Pgp siRNA with 10 nM ($p=0.019$), 40 nM ($p\leq 0.001$) and 60 nM ($p=0.002$) Paclitaxel. PacR cells also had a reduction in cell number in Pgp siRNA at 10 and 20 nM of Paclitaxel ($p\leq 0.001$) but a slightly higher cell number at 60 nM ($p=0.004$) (Fig. 3.5). In 1-60 nM of Docetaxel, CTL cells had slightly higher cell numbers in Pgp siRNA ($p\leq 0.001$, $p=0.006$, $p=0.001$, $p=0.005$, $p\leq 0.001$ respectively). DocR cells in Pgp siRNA had a reduction in cell number at 10, 20 and 40 nM of Docetaxel ($p=0.001$, $p=0.011$ and $p=0.029$ respectively). However, PacR cells in Pgp siRNA had slightly higher cell numbers at 10-60 nM of Docetaxel ($p=0.029$) (Fig. 3.6).

When these proliferation assays were performed without Verapamil, CTL cells also had higher cells numbers in Pgp siRNA treatment at 1-60 nM Docetaxel ($p=0.008$, $p=0.005$, $p=0.001$, $p=0.0017$, and $p\leq 0.001$ respectively). Both taxane resistant cells showed a reduction in cell number in both Paclitaxel and Docetaxel. PacR cells had lower cell numbers following treatment with Pgp siRNA with 20 nM ($p\leq 0.001$) and 40nM ($p=0.001$) Paclitaxel (Fig. 3.7) and 10-60 nM of Docetaxel ($p\leq 0.001$, $p\leq 0.001$, $p\leq 0.001$ and $p=0.005$ respectively) (Fig.3.8). DocR

cells had significantly lower numbers of cells in 10-60 nM of Paclitaxel ($p=0.006$, $p=0.001$, $p=0.008$, and $p=0.001$ respectively) and Docetaxel ($p\leq 0.001$, $p\leq 0.001$, $p\leq 0.001$, and $p=0.024$ respectively) (Fig. 3.6). These data suggest that although Pgp expression is significantly increased in the resistant cells, particularly in DocR cells, Pgp inhibition varies in its ability to reduce cell numbers in DocR and PacR cells.

Figure 3.4 P-glycoprotein Protein Expression and siRNA Knockdown

Figure 3.4 P-glycoprotein Protein Expression and siRNA Knockdown. Western blot showing protein expression in CTL, PacR and DocR cells at baseline. Densitometry reading shows DocR have significantly higher Pgp to β-Actin ratio (Student's *t* test $p \leq 0.001$) (A). Data are presented as the mean $\pm$ SE for three independent experiments.

Pgp siRNA showing knockdown of Pgp protein expression compared to Non-Targeting siRNA (NT) (B).

Figure 3.5 P-glycoprotein siRNA Proliferation Assays in Paclitaxel with Verapamil

Figure 3.5 P-glycoprotein siRNA Proliferation Assays in Paclitaxel with Verapamil. Pgp siRNA proliferation assays in Paclitaxel drug of CTL (A), DocR (B) and PacR (C). CTL, DocR and PacR cells in Pgp and Non Targeting (NT) siRNA by ANOVA, $p \leq 0.001$. CTL cells in Pgp siRNA treatment with 1 nM (Student's t test $p = 0.038$), 10 nM (Mann-Whitney Rank Sum test $p = 0.029$), 20 nM and 40 nM (Student's t test $p \leq 0.001$), and 60 nM (Student's t test $p = 0.003$) Paclitaxel. DocR cells following treatment with Pgp siRNA with 10 nM (Student's t test $p = 0.019$), 40 nM (Student's t test $p \leq 0.001$), and 60 nM (Student's t test $p = 0.002$) Paclitaxel.

PacR cells in Pgp siRNA treatment with 10 nM and 20 nM (Student's *t* test $p \leq 0.001$), and 60 nM (Student's *t* test $p = 0.004$) Paclitaxel. Data are presented as the mean $\pm$ SE for a representative experiment.

Figure 3.6 P-glycoprotein siRNA Proliferation Assays in Docetaxel with Verapamil

Figure 3.6 P-glycoprotein siRNA Proliferation Assays in Docetaxel with Verapamil. Pgp siRNA proliferation assays in Docetaxel drug of CTL (A), DocR (B) and PacR cells (C). CTL cells by ANOVA in Pgp and NT siRNA $p=0.002$. CTL cells in Pgp siRNA treatment with 1 nM (Student's t test $p \leq 0.001$), 10 nM (Student's t test $p=0.006$), 20 nM (Student's t test $p=0.001$), 40 nM (Student's t test $p=0.005$), and 60 nM (Student's t test $p \leq 0.001$) Docetaxel. DocR cells by ANOVA in Pgp siRNA $p \leq 0.001$ and Non Targeting (NT) siRNA $p=0.003$. DocR cells following Pgp siRNA treatment with 10 nM (Student's t test $p=0.001$), 20 nM (Student's t test $p=0.011$), and 40 nM (Mann-Whitney Rank Sum test $p=0.029$) Docetaxel. PacR cells by ANOVA in Pgp

and NT siRNA $p \leq 0.001$. PacR cells in Pgp siRNA treatment with 10-60 nM (Mann-Whitney Rank Sum test $p = 0.029$) Docetaxel. Data are presented as the mean $\pm$ SE for a representative experiment.

Figure 3.7 P-glycoprotein siRNA Proliferation Assays in Paclitaxel without Verapamil

Figure 3.7 P-glycoprotein siRNA Proliferation Assays in Paclitaxel without Verapamil. Pgp siRNA proliferation assays without Verapamil in Paclitaxel drug of CTL (A), DocR (B) and PacR cells (C). CTL cells by ANOVA in Pgp siRNA treatment $p=0.008$ and Non Targeting (NT) siRNA $p\leq 0.001$. CTL cells in Pgp siRNA treatment with 1-60 nM (Student's t test $p=0.035$, $p=0.016$, $p=0.003$, $p=0.021$, $p=0.004$) Paclitaxel. DocR cells by ANOVA in Pgp siRNA treatment $p=0.007$ and NT siRNA $p=0.034$. DocR cells in Pgp siRNA treatment with 10 nM (Student's t test $p=0.006$), 20 nM (Student's t test $p=0.001$), 40 nM (Student's t test $p=0.008$),

and 60 nM (Student's *t* test $p=0.001$) Paclitaxel. PacR cell by ANOVA in Pgp and NT siRNA $p\leq 0.001$. PacR cells in Pgp siRNA treatment with 20 nM (Student's *t* test $p\leq 0.001$), and 40 nM (Student's *t* test $p=0.001$) Paclitaxel. Data are presented as the mean $\pm$ SE for a representative experiment.

Figure 3.8 P-glycoprotein siRNA Proliferation Assays in Docetaxel without Verapamil

Figure 3.8 P-glycoprotein siRNA Proliferation Assays in Docetaxel without Verapamil. Pgp

siRNA proliferation assays without Verapamil in Docetaxel drug of CTL (A), DocR (B) and PacR (C) cells. CTL cells by ANOVA in Pgp siRNA treatment $p \leq 0.001$ and Non Targeting NT siRNA $p = 0.001$. CTL cells in Pgp siRNA treatment with 1-60 nM (Student's t test $p = 0.008$, $p = 0.005$, $p = 0.001$, $p = 0.0017$, $p \leq 0.001$) Docetaxel. DocR cells in Pgp and NT siRNA by ANOVA $p \leq 0.001$. DocR cells following treatment with Pgp siRNA with 10 nM (Student's t test

$p \leq 0.001$), 20 nM (Student's t test $p \leq 0.001$), 40 nM (Student's t test $p \leq 0.001$), and 60 nM (Student's t test $p = 0.024$) Docetaxel. PacR cells in Pgp and NT siRNA by ANOVA $p \leq 0.001$. PacR cells in Pgp siRNA treatment with 10 nM (Student's t test $p \leq 0.001$), 20 nM (Student's t test $p \leq 0.001$), 40 nM (Student's t test $p \leq 0.001$), and 60 nM (Student's t test $p = 0.005$) Docetaxel. Data are presented as the mean $\pm$ SE for a representative experiment.

3.3.5 Other Transporters and transcription factors

While the Pgp (ABCB1) resistance mechanism appears to have a modest role in the resistance phenotype, over 70 other transmembrane transporters of both the ABC and SLC families were also differentially expressed by microarray, of which several were subsequently validated by PCR. Because of the high number of differentially expressed transporters, a high throughput approach was taken to see if common transcription factors that bind the promoter regions of most of the differentially expressed transporters could be commonly regulating these transporter families. Enrichment motif analysis was undertaken using the TRANSFAC's database of transcription factors and 18 different transcription factors were found to be enriched. These transcription factors commonly bind the promoter regions of the majority of the transporters identified by microarray and PCR. Of the 18 transcription factors, through motif enrichment two were found to have the greatest number of binding sites in the majority of the transporter promoter regions, namely PAX4 and TBX5 (Fig. 3.9). However, by microarray data, western blots and PCR, these transcription factors were not detected.

A

Panel A displays a scatter plot of VSTBX5_Q5 (green circles) across a grid of gene symbols (y-axis) and genomic coordinates (x-axis). The y-axis lists genes from ABCD3 to ABCD4, with many genes having multiple isoforms indicated by subscripts. The x-axis ranges from -5000 to 0. The plot shows a dense distribution of green circles, indicating the presence of VSTBX5_Q5 across many genes and genomic regions.

B

Panel B displays a scatter plot of VSPAX4_Q3 (black stars) across a grid of gene symbols (y-axis) and genomic coordinates (x-axis). The y-axis lists genes from ABCD3 to ABCD4, with many genes having multiple isoforms indicated by subscripts. The x-axis ranges from -5000 to 0. The plot shows a sparse distribution of black stars, indicating the presence of VSPAX4_Q3 across fewer genes and genomic regions compared to Panel A.

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3.4 Discussion

To determine the role of the ABC and SLC superfamilies of transporters, a high throughput approach using gene expression microarrays to measure differential gene expression patterns between CTL, DocR and PacR cells was completed. It was found that the general gene expression profile of CTL cells was substantially different from both PacR and DocR cells. The differential gene expression pattern of DocR and PacR cell lines at fold changes of >1.45 and $p \leq 0.05$, were small compared to DocR vs. CTL and PacR vs. CTL cells. Because our cells were grown in Verapamil, a known Pgp inhibitor, we had attempted to reduce the likelihood that the mechanism of resistance to the taxanes would be through Pgp. Nonetheless, Pgp was overexpressed in both taxane resistant models by gene expression microarrays, PCR, and at the protein level by western blot, particularly in DocR cells. Knocking down Pgp expression with siRNA had a different effect in sensitive and resistant cells with Verapamil. Sensitive cells that did not overexpress Pgp had slightly higher cell numbers with Pgp siRNA whereas DocR cells, which had the highest Pgp expression levels, had a reduction in cell number in both Paclitaxel and Docetaxel. PacR cells however, had a reduction in cell number only in Paclitaxel but not Docetaxel. However, when the Pgp siRNA experiments were performed without Verapamil, the reduction in cell number was present in both resistant cell lines and increased DocR cells in both Paclitaxel and Docetaxel drugs. This indicates that Verapamil may not be able to fully reverse MDR in resistant cells.

While the Pgp (ABCB1) mechanism appears to play a role in the resistance phenotype, over 70 other transmembrane transporters of both the ABC and SLC families were also differentially expressed by microarray (fold change >1.45 , $p \leq 0.05$). However, their differential expression

was not found to be significant by PCR analysis (67, 70, 77, 88-90). Over half of the ABC and SLC transporters still need to be characterized and their role in taxane resistance remains to be elucidated (87). Given the similarities between the ABC superfamily members and the large number of potential SLC transporters, a combination of inhibitors and siRNA library may be required to effectively block their functions and determine what role they play in the taxane resistance phenotypes (71).

Chapter 4. Cell Death Mechanisms and Taxane Resistance

4.1 Introduction

Paclitaxel and Docetaxel exert their cytotoxic effects primarily by disrupting spindle microtubule dynamics during cell division leading to cell cycle arrest and apoptosis (47). The taxanes have been shown to increase both overall survival and disease free survival in early and metastatic breast cancer patients. Nonetheless, even with response to treatment, time to progression is short, and life is often prolonged for only a few months before relapse occurs (47). One of the main problems in the clinic is resistance to taxane treatment and a number of resistance mechanisms have been proposed. These mechanisms include the overexpression of efflux ABC transporters, the downregulation of influx SLC transporters, changes in β -tubulin isotype expression, and mutations in the β -tubulin sequence. Changes in cell death mechanisms have also been implicated in conferring resistance to the taxanes; in this chapter we explore the role of cell death and taxane resistance (91, 92). While changes in apoptosis have been implicated in resistance to the taxanes and other chemotherapy drugs, the role of alternative cell death mechanisms such as necrosis, mitotic catastrophe, and autophagy and resistance to the taxanes will also be explored.

The taxanes induce cell death by apoptosis through multiple signaling pathways that remain to be fully characterized. The two main pathways that lead to cell death by apoptosis are the *intrinsic mitochondrial pathway* and the *extrinsic death receptor pathway*. The intrinsic pathway is activated by many stimuli including DNA damage and cell cycle deregulation. The signaling leads to increased mitochondrial permeability, which results in the release of cytochrome *c* and

activation of caspases -9, -3 and -7. The pathway is regulated by the Bcl-2 family members. The extrinsic pathway involves the activation of plasma membrane receptors that lead to the activation of caspase -8, which can also activate the intrinsic pathway through activation of the proapoptotic Bcl-2 family member Bid that subsequently activates other proapoptotic members Bax or Bak (47). Anti apoptotic Bcl-2 and Bcl-xL can block the release of proapoptotic Bax, Bid, Bim, and Bad into the cytoplasm by heterodimerizing with them, which prevents mitochondrial permeability and the release of cytochrome *c*. The relative concentrations of either pro or antiapoptotic Bcl-2 family members can determine whether cells survive or die by apoptosis due to taxane treatment (47). The role of phosphorylation of the Bcl-2 family members has also been implicated in either activation or inhibition of apoptosis. Although many studies have suggested that phosphorylation of Bcl-2 leads to enhanced cell death by apoptosis, others have shown that phosphorylation can increase anti-apoptotic activity (47). Bcl-2 has specifically been identified as a Paclitaxel binding protein via the screening of a library of phage displayed peptides (160). The C-13 side of chain of Paclitaxel has been shown to bind to the loop region of Bcl-2. Clinically, the phosphorylation of Bcl-2 has been implicated with increased sensitivity to both Paclitaxel and Docetaxel compared to tumors with reduced Bcl-2 phosphorylation (161, 162). In addition, changes in the intrinsic mitochondrial pathway have been specifically linked to acquired Paclitaxel resistance. In MCF-7 breast cancer cells that have resistance to Paclitaxel induced apoptosis, overexpression of antiapoptotic Bcl-2 and Bcl-xL was found. In addition, MDA-MB-468 breast cancer cells that also have resistance to Paclitaxel induced apoptosis, a reduction in expression of proapoptotic Bcl-xL and Bim was found. In both of these cases, the use of an ABT-737, a small molecule Bcl-2 antagonist, restored sensitivity to

Paclitaxel indicating that the intrinsic mitochondrial pathway that is controlled by Bcl-2 family members may be important in inducing Paclitaxel resistance (163).

NF- κ B, a transcription factor involved in cell proliferation and inhibition of apoptosis, can translocate to the nucleus and activate the transcription of antiapoptotic genes such as Bcl-2 and Bcl-xL (164). Akt signaling regulates NF- κ B and its prosurvival function, and NF- κ B has been shown to be upregulated following Paclitaxel treatment resulting in reduced apoptosis. However, some studies suggest that NF- κ B has a propapoptotic role in Paclitaxel induced apoptosis (164-166). The PI3K/AKT pathway not only regulates NF- κ B but also a number of other genes involved in cell survival, cell growth and cell cycle progression such as mTOR, proapoptotic Bcl-2 family members, and caspases. This pathway is often dysregulated in cancer, which suggests a role in response to chemotherapy treatment (167-169). In ovarian cancer, increased activation of Akt was found to confer resistance to Paclitaxel and the use of a PI3K/AKT inhibitor LY294002 has been shown to increase sensitivity to Paclitaxel in lung and esophageal cancer cell lines (170, 171).

Interestingly, Bcl-2, Bcl-xL, NF- κ B, and PI3K are not only involved in apoptosis but also autophagy. Autophagy is a catabolic cellular degradation pathway that sequesters and degrades cytoplasmic organelles, proteins and other contents through the formation of multi membrane autophagic vacuoles (autophagosomes) that fuse with lysosomes to produce autophagolysosomes where digestion of cellular content takes place (172). Autophagy is also considered to be a mechanism for the generation of alternative energy production for survival in periods of metabolic stress due to decreased extracellular nutrients or intracellular metabolites from the loss

of growth factor signaling. Autophagy is also considered to be a type 2 programmed cell death pathway when excessive self digestion occurs (172). HeLa and HCT116 cancer cells cultured in the absence of nutrients induce autophagy but when autophagy is blocked with either 3-MA or chloroquine apoptosis results due to failure to adapt to a lack of nutrients (120, 172, 173).

Apoptosis can occur when autophagy is inhibited but this is not always the case. Malignant glioma cells treated with DNA damaging agents like etoposide and temozolomide have a large amount of autophagy that, when inhibited, results in non-apoptotic cell death with micronucleation (172, 174). In addition, cancer cells in which both apoptosis and autophagy are suppressed have been shown to die by necrosis (172, 175). Others have shown that induction of autophagy can lead to necrotic cell death (172). In addition, reactive oxygen species (ROS) can promote mitochondrial membrane permeability and stimulate the proteolytic activity of Atg4 that then activates autophagy (172, 176). The type of initiating stressor is probably what determines which cell death process will predominate. Metabolic stress and the removal of damaged organelles and proteins would probably initiate the survival autophagy response which would create a high ATP state (172). Pre-treatment of cells with rapamycin (an inducer of autophagy) has been shown to cause a decrease in mitochondrial mass and the reduction of susceptibility of cells to mitochondrial membrane permeability dependent apoptotic stimuli (172).

The Beclin-1/class III PI3K complex is involved in the nucleation phase of autophagosome formation (119). P62 is involved in the targeting of proteins to autophagosomes, however, failure of cells to eliminate p62 also results in changes in NF- κ B regulation and gene expression, as p62 is a known activator of NF- κ B (122, 124). Both Bcl-2 and Bcl-xL can associate with Beclin-1 and inhibit autophagy through Beclin-1's BH3 domain (119, 126, 128). Genetic inhibition of

autophagy with Beclin-1 or Atg5 RNA interference, or through the constitutive activation of the class I PI3K-AKT-mTOR signaling pathway, can prevent the survival response; cell death can then occur by necrosis (96, 127). Another inhibitor of autophagy, 3-MA, has been shown to inhibit pre-autophagosome formation and reduce cell viability in Trastuzumab-refractory HER2 amplified breast cancer cells (103, 121).

While apoptosis is considered to be the primary mechanism of cell death due to the taxanes, both taxane drugs can induce cell line and dose specific mixtures of apoptosis and mitotic catastrophe cell death (116). Mitotic catastrophe is considered to be a death that occurs during mitosis as a result of DNA damage or abnormal spindle formation and involves defective cell cycle checkpoints that leads to missegregation of chromosomes and/or cell fusion during mitosis and may result in tetraploidy, incomplete cytokinesis and giant nonviable cells with multiple micronuclei resulting from the formation of nuclear envelopes around clusters or fragments of chromosomes (91, 100, 113, 114). Mitochondrial membrane permeability and caspase activation have been reported in conjunction with mitotic catastrophe but others suggest the process is caspase independent (113)

Unlike apoptosis, necrosis is a passive process that does not require ATP and occurs in response to changes outside of the cell (103). Necrosis is marked by energy depletion, cellular edema, vacuolization of the cytoplasm, breakdown of the plasma membrane and membrane lipids, loss of function of ion pumps/channels and an inflammatory response due to the release of cell contents into the surroundings (100, 103, 111). Studies have suggested that the activation of apoptosis may inhibit necrosis because caspases cleave and inactivate proteins required for

programmed necrosis (103). Necrosis can also occur in cells that have bioenergetic stress, the same conditions that can stimulate autophagy as a survival mechanism to boost ATP levels, suggesting that these two mechanisms can occur in parallel (103). Therefore it appears that several cell death mechanisms may be involved in taxane sensitive and resistant cells and these will be determined in this chapter.

4.2 Materials and Methods

4.2.1 Cell Culture and Reagents

See Materials and Methods section 2.2.1

4.2.2 Gene Expression Microarrays

See Materials and Methods section 3.2.2 for microarrays. Apoptosis gene pathways were visualized using KEGG pathway enrichment analysis of genes that were significantly differentially expressed ($p < 0.05$). KEGG analysis was performed by the Virginia Polytechnic Institute and State University (VA Tech) Computational Bioinformatics and Bioimaging laboratory. Gene expression data (fold changes) were integrated into KEGG pathways for apoptosis with red color on a node representing upregulation, and green representing downregulation. Red dashed lines connect genes belonging to the same KEGG orthology group and black solid lines connect genes having a relation as defined in KEGG pathways. Bigger size nodes represent genes with significant fold changes ($p < 0.05$).

4.2.3 PCR

RNA was isolated from sensitive, Docetaxel and Paclitaxel resistant cell lines as described above and in (135) and purified using the RNeasy Mini Kit (Qiagen, Valencia, CA). 1.0 μ g RNA was reverse transcribed to generate first strand cDNA using RT² First Strand Kit (SuperArray Bioscience Corporation, Bethesda, MD) and labeled using RT² SYBR Green/ROX qPCR Master Mix (SuperArray Bioscience Corporation, Bethesda, MD) before being added to the 96 well RT² Profiler Apoptosis PCR Array (Human) (SuperArray Bioscience Corporation, Bethesda, MD) and run using ABI Instruments 7900HT. The Apoptosis array contains 84 genes including genes

that are TNF receptors and their ligands, the Bcl-2, caspase, IAP, Traf, CARD, death domain, death effector domain and CIDE families. The full list of genes can be found on the SA Bioscience website: (http://www.sabiosciences.com/rt_pcr_product/HTML/PAHS-012A.html). Analysis was performed using RT² Profiler PCR Array Data Analysis Template from the SA Bioscience website.

4.2.4 Cell Lysis and Immunoblotting

For the determination of baseline Bcl-2, Phospho-Bcl-2, Bcl-xL, Survivin, Caspase- 2, -3, -4, -7, -8, -9, Parp, Beclin-1, LC3, XBP-1, protein expression, cells were grown in T75 flasks and cultured in normal growth media with 1 μ M Verapamil for the sensitive cells. Resistant cells, in addition to Verapamil were also grown in 7 nM Paclitaxel for Paclitaxel resistant cells and 2 nM Docetaxel for Docetaxel resistant cells (baseline concentrations). For the determination of protein expression of Bcl-2, Bcl-xL, Bax, Caspase-3, -7, -8, -9, Beclin-1, LC3, p62/SQSTM1, NF- κ B, XBP-1 with and without IC₅₀ concentrations of either taxane, CTL, PacR and DocR cells were grown in T75 flasks and cultured in normal growth media with 1 μ M Verapamil, or IC₅₀ concentrations of Paclitaxel or Docetaxel drug for 48 hours. Cells were then lysed in modified radioimmunoprecipitation assay buffer [150 mmol/L NaCl, 50 mmol/L Tris (pH 7.5), 1% Igepal CA-630, and 0.5% deoxycholate] supplemented with Complete Mini protease inhibitor cocktail tablets (Roche, Mannheim, Germany) and 1 mmol/L sodium orthovanadate phosphatase inhibitor (Sigma-Aldrich, St. Louis, MO). Lysates were clarified by centrifugation and total protein was quantitated using the bicinchoninic acid assay purchased from Pierce (Rockford, IL). Whole cell lysate (25 μ g) was resolved by PAGE using NuPAGE 12% precast gels (Invitrogen, Carlsbad, CA). Proteins were then transferred to nitrocellulose membranes, which were probed with the

antibodies overnight at 4°C. Antibodies for Bcl-2, Phospho Bcl-2, Bcl-xL, Bax, Survivin, Caspase -2,-3, -7, -8, -9, Parp, Beclin-1 were obtained from Cell Signaling Technology (1:500; Danvers, MA). Caspase -4, XBP-1, and p62/SQSTM1 were obtained from Abcam (1:500; 1:2000, and 1:1000 respectively; Cambridge, MA). LC3 was obtained from Sigma-Aldrich (1:1000; St. Louis, MO), and NF-κB (p65) from Millipore (1:1000; Billerica, MA). Membranes were then incubated with horseradish peroxidase–conjugated secondary antibodies for 1 hour at room temperature before enhanced chemiluminescence (GE Healthcare, Buckinghamshire, UK) and exposure to film (X-OMAT Blue XB-1; Kodak, Rochester, NY). To confirm equal loading of the gels, membranes were reprobed with antibodies for β-actin (1:5000; Sigma-Aldrich, St. Louis, MO).

4.2.5 Apoptosis and Necrosis Assays

For apoptosis and necrosis, cells were seeded at a density of 1×10^5 cells/well into 6-well plates in drug free media with 1 μM Verapamil for 24 hours before treatment with IC₅₀ levels of either Paclitaxel or Docetaxel or no drug treatment for 48 hours. Cells were subsequently harvested and stained as described in the TACS Annexin V Kit (Trevigen, Gaithersburg, MD). Apoptosis was measured by fluorescence activated cell sorting of green stained Annexin V-FITC cells.

Necrosis was measured by fluorescence activated cell sorting of red stained propidium iodide (PI) cells. Additionally, apoptosis was measured by FACS sorting of cells in the SubG1 fraction.

Annexin, PI and SubG1 cell sorting and analyses were conducted by the Lombardi

Comprehensive Cancer Center Flow Cytometry Shared Resource. Necrosis was also measured by alternatively staining cells with trypan blue dye (Invitrogen, Carlsbad, CA) and counted using

Invitrogen's Countess[®] Automated Cell Counter. Data are presented as the mean $\pm$ SE for four independent experiments.

4.2.6 Mitochondrial Membrane Permeability and Activity Assays

For mitochondrial membrane permeability, cells were seeded at a density of 1×10^5 cells/well into 6-well plates in drug free media with 1 μ M Verapamil for 24 hours before treatment with IC₅₀ levels of either Paclitaxel or Docetaxel drug or no drug treatment for 12 hours. Cells were subsequently stained with JC-1 dye solution and harvested as described in the MitoProbe JC-1 Assay Kit (Invitrogen, Carlsbad, CA). Green fluorescence was measured by a Wallac Victor2 1420 Multilable Counter (Perkin-Elmer, Boston, MA). Data are presented as the mean $\pm$ SE for three independent experiments.

For mitochondrial activity, cells were seeded at a density of 1×10^5 cells/well into 6-well plates in drug free media with 1 μ M Verapamil for 24 hours before treatment with IC₅₀ levels of either Paclitaxel or Docetaxel or no drug treatment for 48 hours. Cells were subsequently stained with MitoTracker[®] Red 580 dye solution and harvested as described in the MitoTracker Red FM kit (Invitrogen, Carlsbad, CA). Data are presented as the mean $\pm$ SE for four independent experiments.

4.2.7 Caspase Activation Assays

To determine caspase activity, cells were seeded at a density of 1×10^5 cells/well into 6-well plates in drug free media with 1 μ M Verapamil for 24 hours before treatment with IC₅₀ levels of either Paclitaxel or Docetaxel or no drug treatment for 48 hours. Cells were subsequently stained

with FLICA reagent and harvested as described in the Vybrant[®] FAM Poly Caspases Assay kit (Invitrogen, Carlsbad, CA). Data are presented as the mean $\pm$ SE for three independent experiments.

4.2.8 3-Methyladenine Autophagy Inhibition

Cells were seeded at a density of 1×10^4 cells/well into 24-well plates in drug free media with 1 μ M Verapamil for 24 hours before treatment with 5 mM 3-methyladenine (Sigma-Aldrich, St. Louis, MO) dissolved in Phosphate Buffered Saline or PBS alone for 24 hours followed by either Paclitaxel or Docetaxel or no drug for 7 days. Cells were then trypsinized with 0.5% Trypsin (Invitrogen, Grand Island, NY), resuspended in Phosphate Buffered Saline pH 7.4 (Invitrogen, Carlsbad, CA), and counted with a Beckman Coulter counter (Beckman Coulter Corp., Fullerton, CA). At least three independent experiments were done in quadruplicate, and data were normalized to PBS treated cells. Proliferation data were repeated in triplicates. Data are presented as the mean $\pm$ SE for a representative experiment.

4.2.9 Beclin-1 siRNA

Beclin-1 siRNA (BECN1) was obtained from Thermo Scientific (Lafayette, CO) and Non Targeting siRNA was obtained from Qiagen (Valencia, CA). Western Blots were performed to confirm knockdown using the above method. Cells were then seeded at a density of 1×10^4 cells/well into 24-well plates in drug free media with 1 μ M Verapamil for 24 hours before treatment with Beclin-1 or Non Targeting siRNA for 24 hours followed by either Paclitaxel or Docetaxel or no drug for 48 hours. Cells were then trypsinized with 0.5% Trypsin (Invitrogen, Grand Island, NY), resuspended in Phosphate Buffered Saline pH 7.4 (Invitrogen, Carlsbad,

CA), and counted with a Beckman Coulter counter (Beckman Coulter Corp., Fullerton, CA). At least three independent experiments were done in quadruplicate, and data were normalized to Non Targeting siRNA treated cells. The siRNA proliferation data were repeated in triplicates. Data are presented as the mean $\pm$ SE for a representative experiment.

4.2.10 Light Microscopy and Mitotic Catastrophe

Cells were seeded onto 18 x 18 microscope cover glass in 6-well plates in drug free media with 1 μ M Verapamil for 24 hours before treatment with IC₅₀ levels of either Paclitaxel or Docetaxel or no drug treatment for 48 hours. Cells were then washed three times with 100% alcohol (5 minutes each) (Warner Graham Company, Cockeysville, MD), followed by 2 times with 95% alcohol (3 minutes each) (Warner Graham Company, Cockeysville, MD) then stained with Harris Hematoxylin (1 minute) (Fisher Scientific, Pittsburg, PA), rinsed with distilled water and then dipped 3 times in 1% Li₂CO₃ (Sigma-Aldrich, St. Louis, MO) followed by distilled water. Slides were then dehydrated two times 95% alcohol and two times with 100% alcohol (1 minute each) followed by Xylene (1 minute) (Sigma-Aldrich, St. Louis, MO) and mounted on microscope slides using Permount (Fisher Scientific, Pittsburg, PA) and visualized at a magnification of 100x using Olympus BX61 microscope (Olympus, Center Valley, PA) and Olympus DP70 camera (Olympus, Center Valley, PA).

4.2.11 Cell Size

To determine cell size, cells were seeded at a density of 1×10^5 cells/well into 6-well plates in drug free media with 1 μ M Verapamil for 24 hours before treatment with IC₅₀ levels of either Paclitaxel or Docetaxel or no drug treatment for 48 hours. Cells were then trypsinized with 0.5%

Trypsin (Invitrogen, Grand Island , NY), resuspended in Phosphate Buffered Saline pH 7.4 (Invitrogen, Carlsbad, CA), and counted by cell size with a Beckman Coulter counter (Beckman Coulter Corp., Fullerton, CA). At least three independent experiments were done and data was normalized to vehicle treated CTL cells. Data are presented as the mean $\pm$ SE for three independent experiments.

4.2.12 Breast Cancer Clinical Samples

We used prospectively collected, clinically annotated breast tumor biopsies obtained through a translational research protocol led by Dr. Minetta Liu, MD (IRB 2000-310: “A Pilot Study to Establish a Standardized Protocol for Gene Microarray Analysis in Patients Receiving Neoadjuvant Chemotherapy for Breast Cancer: Identifying Factors Predictive of a Response to Paclitaxel”). Serial core needle biopsies of the breast primary were obtained under ultrasound guidance at four specified time points from chemotherapy-naïve breast cancer patients receiving up to four cycles of single agent Paclitaxel (175 mg/m² Q3weeks) in the preoperative setting. The defined time points were at baseline, at 48 hours after initial drug exposure, upon completion of the first treatment cycle, and upon completion of the last sequential cycle of Paclitaxel. Two to four Tru-Cut® biopsies were obtained at each time point using a 14 gauge, disposable, core-cutting needle under sterile conditions with local anesthesia. Each core was immediately placed in 500 μ l of RNALater™ solution (Qiagen, Valencia, CA) at room temperature and labeled with standard patient identifiers. All samples were ultimately transferred to the Tissue and Histopathology Shared Resource at Georgetown University for storage at -20°C and subsequent processing. Each core was washed for 5 minutes with 500 μ l of DEPC treated PBS (ribonuclease-free; 4°C) and then embedded separately in an OTC block. A longitudinal

section of all core biopsy specimens was stained with Hematoxylin and Eosin, reviewed by the study pathologist, and compared with the routine histopathologic analysis of formalin-fixed specimens to confirm the presence and relative proportion of malignant tissue in the biopsy specimen. All specimens cleared for gene microarray analysis were stored in the OTC block at -80°C. RNA extraction and microarrays were performed as in Materials and Methods section 3.2.2.

4.2.13 Statistical Analysis

See Materials and Methods section 2.2.7

4.3 Results

4.3.1 Molecular Profiling of Cell Death Genes

Molecular profiling using gene expression microarray technology was used to determine the differential expression of genes involved in cell death that had fold changes of >1.45 and $p \leq 0.05$ at baseline (CTL cells grown without drug, DocR in 2 nM Docetaxel and PacR cells in 7 nM Paclitaxel). In the microarray data, several Bcl-2 family members were found to be differentially expressed in the taxane resistant cells compared to sensitive cells. However, the pattern of differential expression of apoptosis genes that was visualized using KEGG representation was not completely identical the two resistant cell lines (Fig. 4.1). Proapoptotic Bcl-2 family members Bax and Bid were downregulated in DocR and PacR cells by 1.50 and 1.48-fold ($p=0.011$, $p=0.005$ respectively) and 2.08 and 1.49-fold respectively ($p=0.005$, $p=0.007$ respectively) compared to CTL cells. PacR also had reduced expression of proapoptotic Bad and Bak by 1.53-fold ($p=0.0001$, $p=0.005$ respectively) and antiapoptotic Bcl-xL was downregulated by 1.87-fold ($p=0.04$). In addition, in both DocR and PacR cells compared to CTL cells, caspase-4 was upregulated by 1.67 and 1.66-fold respectively ($p=0.04$, $p=0.05$) and Parp1 was upregulated by 1.72 and 1.47-fold respectively ($p=0.0006$, $p=0.0014$). Autophagy Atg genes were not significantly differentially expressed by microarray however Akt1 was downregulated in DocR compared to CTL cells by 1.46-fold ($p=0.005$) and in both DocR and PacR vs. CTL cells, and class III PI3K was upregulated by 2.79 and 2.99-fold respectively ($p=0.018$, $p=0.031$).

Figure 4.1 KEGG Representation of Apoptosis Genes

Figure 4.1 KEGG Representation of Apoptosis Genes. KEGG representation of differentially

expressed genes involved in Apoptosis in DocR vs. CTL cells (A) and PacR vs. CTL cells (B) with fold changes ≥ 1.20 and $p < 0.05$ at baseline. The greater the fold differences, the larger the node and darker the color.

4.3.2 PCR of Apoptosis Genes

Several genes involved in apoptosis that were differentially expressed by microarrays were validated using an apoptosis specific PCR array including proapoptotic Bax and Bid that were downregulated in DocR compared to CTL cells by 1.03 and 1.80-fold respectively. PacR cells compared to CTL downregulated proapoptotic Bax and Bid by 2.42 and 1.49-fold respectively and Bad and Bak were downregulated by 4.73 and 3.29-fold respectively. Antiapoptotic Bcl-xL was downregulated by 1.49-fold in PacR cells vs. CTL. In addition, caspase-4 was upregulated in DocR and PacR cells by 2.50 and 1.17-fold respectively. Although caspase-2 was not differentially expressed by microarray, in the PCR data caspase-2 was the second most downregulated gene in DocR compared to CTL cells by >100-fold. Akt1 was the most downregulated gene in DocR vs. CTL cells by >100-fold compared to only 1.39-fold downregulation in PacR compared to CTL cells (Fig. 4.2). However, to determine whether or not these fold changes represent significant p-values, further PCR array replicates must be completed.

Figure 4.2 PCR Validation of Apoptosis Gene Expression at Baseline

Figure 4.2 PCR Validation of Apoptosis Gene Expression at Baseline. Validation of Apoptosis Gene Expression using SuperArray PCR of DocR vs. CTL cells (A) and PacR vs. CTL cells (B) at baseline.

4.3.3 Western Blots of Cell Death Genes

The expression of several proteins that are involved with different cell death pathways was determined by western blots at baseline (Fig. 4.3). There were no significant differences in protein expression of antiapoptotic Bcl-2 and Bcl-xL between the CTL, PacR, and DocR cells; phosphorylated Bcl-2 expression was not detected in any of the cell lines. High protein expression of Survivin in ovarian cancer clinical samples has been associated with clinical resistance to Paclitaxel, and the stable transfection of Survivin into human ovarian carcinoma cells caused a 4-6 fold increase in resistance to both Paclitaxel and Docetaxel drug (177). Thus we measured protein expression of Survivin by western blots in the taxane sensitive and resistant cell lines. However no significant differences in the CTL, PacR and DocR cells were detected. Neither were any differences in protein expression of caspase-2,-3,-4, -7 or Parp detected. However, slightly higher levels of cleaved caspase-8 (41 kDa) and -9 (37 kDa) were found in both PacR and DocR cells. Of the genes involved in autophagy, no differences in protein expression were found between CTL, PacR, and DocR cells of Beclin-1 and LC3I and II levels. Because ER stress has been shown to regulate autophagy and XBP-1 regulates the transcription of several genes involved in ER quality control, redox homeostasis and the oxidative stress response, and ablation of XBP-1 has been shown to increase autophagy, we determined whether or not XBP-1 protein expression was different in the CTL, PacR and DocR cells (178). However no differences in protein expression of unspliced and spliced XBP-1 were found.

Figure 4.3 Western Blots of Cell Death Protein Expression at Baseline

Figure 4.3 Western Blots of Cell Death Protein Expression at Baseline. Western blots of protein expression of Bcl-2, Survivin, Bcl-xL, caspases-3, -7, -2, -4, -8, -9, Parp, Beclin-1, LC3, and XBP-1. Data are presented as the mean $\pm$ SE for three independent experiments.

4.3.4 Mitochondrial Permeability and Activity assays

To determine mitochondrial membrane permeability (MMP) levels in the sensitive and resistant cell lines, CTL, DocR and PacR cells were stained with JC-1 dye at IC₅₀ levels of either Paclitaxel or Docetaxel drug compared to no drug treatment at 12 hours. JC-1 is a cationic dye that accumulates in mitochondria based on mitochondrial potential which is indicated by a fluorescent emission shift from green (~525nm) to red (~590nm). Mitochondrial depolarization is indicated by a decrease in red/green fluorescence intensity ratio due to increased formation of red fluorescent J-aggregates. The ratio of green to red fluorescence is dependent only on mitochondrial potential and not on mitochondrial size, shape and density. Without drug treatment, PacR cells were found to have the highest MMP compared to CTL and DocR cells ($p<0.05$). DocR cells also had higher levels of MMP compared to CTL cells ($p<0.05$). At IC₅₀ of Paclitaxel, PacR cells also had higher levels of MMP compared to CTL and DocR cells ($p=0.032$, $p=0.043$ respectively). In addition, PacR cells also appeared to have higher levels of MMP at IC₅₀ concentrations of Docetaxel compared to DocR cells ($p\leq 0.001$) (Fig. 4.4).

Mitochondrial activity was determined using MitoTracker Red 580 dye, which passively diffuses across the plasma membrane and accumulates in active mitochondria. Although no statistically significant differences were found between the sensitive and both resistant models without drug treatment and at IC₅₀ concentrations of either taxane, it appears that DocR cells may have slightly lower mitochondrial activity levels compared to both CTL and PacR cells (Fig. 4.5).

Figure 4.4 Mitochondrial Membrane Permeability Assays with and without IC₅₀ Paclitaxel and Docetaxel at 12 hours

Figure 4.4 Mitochondrial Membrane Permeability Assays with and without IC₅₀ Paclitaxel and Docetaxel at 12 hours. Mitochondrial membrane permeability determined by JC-1 staining of CTL, DocR, and PacR cells at 12 hours. Without drug treatment (A), ANOVA $p=0.004$, DocR vs. CTL (Student's t test $p<0.05$), PacR cells vs. CTL cells (Student's t test $p<0.05$) and PacR vs.

DocR cells (Student's *t* test $p < 0.05$). At IC_{50} levels of Paclitaxel drug (B), ANOVA $p = 0.033$, PacR vs. CTL cells (Student's *t* test $p = 0.032$) and PacR vs. DocR cells (Student's *t* test $p = 0.043$). At IC_{50} levels of Docetaxel drug (C), PacR vs. DocR cells (Student's *t* test $p \leq 0.001$). Data are presented as the mean $\pm$ SE for three independent experiments.

Figure 4.5 Mitochondrial Activity Assays with and without IC₅₀ Paclitaxel and Docetaxel at 48 hours

Figure 4.5 Mitochondrial Activity Assays with and without IC₅₀ Paclitaxel and Docetaxel at 48 hours. Mitochondrial activity determined by MitoTracker Red staining of CTL, DocR, and PacR cells at 48 hours. Without drug treatment (A), at IC₅₀ levels of Paclitaxel drug (B) and at

IC₅₀ levels of Docetaxel drug (C). No statistically significant differences between the cell lines with and without IC₅₀ taxane drug treatment. Data are presented as the mean $\pm$ SE for four independent experiments.

4.3.5 Annexin V Apoptosis Assays

Early in the apoptosis process, the inner and outer plasma membranes flip and expose phosphatidylserine (PS) to the outer cell surface (179). This allows Annexin-V-FITC dye to bind to PS and detection of early levels of apoptosis in cells (179). CTL, DocR, and PacR cells were stained with Annexin V-FITC without drug treatment and at IC₅₀ levels of either Paclitaxel or Docetaxel drug at 48 hours. Cells were then sorted with FACS to determine relative early apoptosis level differences between the sensitive and resistant cells. Without drug treatment and at IC₅₀ levels of either Paclitaxel or Docetaxel, CTL cells had the highest levels of apoptosis compared to DocR and PacR cells without drug treatment ($p=0.029$). At IC₅₀ concentrations of Docetaxel, PacR cells had slight higher levels of early apoptosis compared to DocR cells ($p=0.049$) (Fig. 4.6).

Figure 4.6 Annexin V Early Apoptosis Assay with and without IC₅₀ Paclitaxel and Docetaxel at 48 hours

Figure 4.6 Annexin V Early Apoptosis Assay with and without IC₅₀ Paclitaxel and Docetaxel at 48 hours. Early apoptosis levels determined by Annexin V-FITC staining of CTL, DocR, and PacR cells at 48 hours. Without drug treatment (A), ANOVA $p=0.013$, CTL vs. DocR and PacR cells (Mann-Whitney Rank Sum test $p=0.029$). At IC₅₀ levels of Paclitaxel drug (B), ANOVA $p=0.002$, CTL vs. DocR and PacR cells (Mann Whitney Rank Sum test $p=0.029$). At IC₅₀ levels of Docetaxel drug (C), ANOVA $p=0.001$, CTL vs. DocR and PacR cells (Mann

Whitney Rank Sum test $p=0.029$) and PacR vs. DocR cells (Student's t test $p=0.049$). Data are presented as the mean $\pm$ SE for four independent experiments.

4.3.6 SubG1 Apoptosis Assays and Western Blots

Another method used to determine apoptosis levels was the determination of DNA content below the G1 region of normal diploid cells or SubG1 fraction of CTL, DocR, and PacR cells (179).

Without drug treatment and at IC₅₀ concentrations of Paclitaxel drug at 48 hours, CTL cells had the highest levels of apoptosis compared to DocR and PacR cells without drug treatment ($p=0.029$). At IC₅₀ concentrations of Docetaxel drug, there were no significant differences in apoptosis levels by SubG1 fraction of cells between the sensitive and resistant cells (Fig. 4.7).

Western blots of antiapoptotic Bcl-2 showed a decrease in expression of Bcl-2 in PacR cells, compared to CTL cells but no significant differences in of expression in DocR cells compared to CTL. However, antiapoptotic Bcl-xL protein expression was not significantly different between sensitive and resistant cells. Proapoptotic protein expression of Bax was also not significantly different between sensitive and resistant cells and although NF- κ B expression appeared to be lower in the resistant cells compared to CTL cells, the difference was not statistically significant (Fig. 4.8).

Figure 4.7 SubG1 Apoptosis Assay with and without IC₅₀ Paclitaxel and Docetaxel at 48 hours

Figure 4.7 SubG1 Apoptosis Assay with and without IC₅₀ Paclitaxel and Docetaxel at 48 hours. Apoptosis by SubG1 fraction of cells of CTL, DocR, and PacR cells at 48 hours.

Without drug treatment (A), ANOVA $p=0.011$, CTL vs. DocR and PacR cells (Mann-Whitney

Rank Sum test $p=0.029$). At IC₅₀ levels of Paclitaxel drug (B), ANOVA $p=0.011$, CTL vs.

DocR and PacR cells (Mann-Whitney Rank Sum test $p=0.029$). At IC₅₀ levels of Docetaxel drug

(C), no statistically significant differences between sensitive and resistant cells. Data are presented as the mean $\pm$ SE for four independent experiments.

Figure 4.8 Western Blots of Protein Expression of Bcl-2, Bcl-xL, NF-κB and Bax with and without IC₅₀ Paclitaxel and Docetaxel at 48 hours

antiapoptotic Bcl-2 and Bcl-xL (A) and NF- κ B and proapoptotic Bax at 48 hours with (+) and without (-) IC₅₀ concentrations of Docetaxel (+Doc) or Paclitaxel (+Pac) (B). For Bcl-2 densitometry, ANOVA $p=0.037$, PacR without drug vs. CTL no Drug (Student's t test $p=0.013$). No statistically significant protein expression differences by densitometry of Bcl-xL, NF- κ B and Bax. Data are presented as the mean $\pm$ SE for three independent experiments.

4.3.7 Caspase Activation Assays and Western Blots

One of the events that occur during the early stages of apoptosis is activation of the caspases. A poly caspases assay was used to detect active caspases by using a FLICA fluorescent reagent that associates with a fluoromethyl ketone (FMK) moiety, which reacts covalently with a cysteine in a caspase specific amino acid sequence. The recognition sequence is Valine-Alanine-Aspartic acid and a carboxyfluorescein group is attached as a reporter. The FLICA reagent interacts with the enzymatic reactive center of activated caspases via the recognition sequence and then attaches covalently to the FMK moiety. The amount of green fluorescent signal (488 nm) determines the amount of activated caspases-1, -3, -4, -5, -6, -7, -8, and -9.

Without drug treatment and at IC_{50} concentrations of Docetaxel at 48 hours, there were no significant differences in caspase activation levels between sensitive and resistant cells. At IC_{50} concentrations of Paclitaxel, DocR cells had slightly higher levels of caspase activation compared to PacR cells ($p=0.041$) (Fig. 4.9). Western blots of caspases -3, -7, -8, and -9 did not show significant differences in whole caspase protein levels or the cleaved products except in caspase-7, where slightly higher cleaved levels of caspase-7 (30 kDa) were found in the PacR and DocR cells compared to CTL cells (Fig. 4.10).

Figure 4.9 Poly-Caspase Activation Assay with and without IC₅₀ Paclitaxel and Docetaxel at 48 hours

Figure 4.9 Poly-Caspase Activation Assay with and without IC₅₀ Paclitaxel and Docetaxel

at 48 hours. Poly-caspase activation assay at 48 hours of CTL, DocR, and PacR cells without drug treatment (A), and with IC₅₀ levels of Docetaxel drug (C), do not show significant differences in caspase activation. With IC₅₀ of Docetaxel drug (B), ANOVA $p=0.004$, DocR vs. PacR cells (Student's t test $p=0.041$). Data are presented as the mean $\pm$ SE for three independent experiments.

Figure 4.10 Western Blots of Protein Expression of Caspases with and without IC₅₀ concentrations of Paclitaxel and Docetaxel at 48 hours

Figure 4.10 Western Blots of Protein Expression of Caspases with and without IC₅₀ concentrations of Paclitaxel and Docetaxel at 48 hours. Western blots of protein expression of caspases-3, -7, -8, and -9 at 48 hours. Data are presented as the mean ± SE for three independent experiments.

4.3.8 Mitotic Catastrophe by Microscopy and Cell Size

To determine whether cells were dying by mitotic catastrophe, cells were stained with Hematoxylin dye and visualized under a light microscope at magnification of 100x. Without drug treatment, the nuclei of CTL, DocR, and PacR cells appear normal in morphology. However, with the addition of IC₅₀ concentrations of Docetaxel and Paclitaxel at 48 hours, in sensitive and resistant cells, significant nuclear morphological changes occur and giant cells with multiple micronuclei appear (Fig. 4.11).

Comparing median cell size differences between CTL, DocR, and PacR cells also showed differences. Without drug PacR cells were the largest vs. CTL ($p \leq 0.001$) (PacR average median cell size = 17.45 μm), and larger than DocR cells (DocR average median cell size = 15.38 μm) ($p=0.007$), (CTL average median cell size = 13.93 μm). DocR cells were also larger compared to CTL cells without drug treatment ($p=0.042$). At IC₅₀ concentrations of Paclitaxel drug, PacR cells were also the largest in size (PacR average median cell size = 17.06 μm) compared to CTL cells (CTL average median cell size = 14.35 μm) ($p=0.002$) and appeared also to be larger than DocR cells but these differences were not statistically significant (DocR average median cell size = 15.64 μm). At IC₅₀ concentrations of Docetaxel drug, PacR cells were again the largest (PacR average median cell size = 17.15 μm) compared to CTL (CTL average median cell size = 14.26 μm) ($p \leq 0.001$) and DocR cells (DocR average median cell size = 15.52 μm) ($p=0.024$) (Fig. 4.12).

Figure 4.11 Mitotic Catastrophe Morphology by Light Microscopy with and without IC₅₀ Paclitaxel and Docetaxel at 48 hours

Figure 4.11 Mitotic Catastrophe Morphology by Light Microscopy with and without IC₅₀ Paclitaxel and Docetaxel at 48 hours. Mitotic Catastrophe morphology with light microscopy

of CTL, DocR, and PacR cells without drug treatment at 48 hours (A), CTL cells with IC_{50} concentrations of Docetaxel or Paclitaxel (B), DocR cells with IC_{50} concentrations of Docetaxel (C) or Paclitaxel and PacR cells IC_{50} concentrations of Docetaxel or Paclitaxel (D).

Figure 4.12 Cell Size with and without IC₅₀ Paclitaxel and Docetaxel at 48 hours

Figure 4.12 Cell Size with and without IC₅₀ Paclitaxel and Docetaxel at 48 hours.

Comparison of average median cell sizes of CTL, DocR, and PacR cells at 48 hours. PacR cells are the largest with and without taxane drug treatment. Without drug treatment (A), ANOVA $p \leq 0.001$, PacR vs. CTL cells (Student's t test $p \leq 0.001$), PacR vs. DocR cells (Student's t test $p = 0.007$) and DocR vs. CTL cells (Student's t test $p = 0.042$). At IC₅₀ concentrations of Paclitaxel (B), ANOVA $p = 0.007$, PacR vs. CTL cells (Student's t test $p = 0.002$). At IC₅₀ concentrations of

Docetaxel (C), ANOVA $p=0.001$, PacR vs. CTL cells (Student's t test $p\leq 0.001$) and PacR vs. DocR cells (Student's t test $p=0.024$). Data are presented as the mean $\pm$ SE for three independent experiments.

4.3.9 Necrosis by Propidium Iodide and Trypan Blue Dyes

During necrosis, the plasma membrane loses integrity, which allows cells to be stained by propidium iodide that was used to determine levels of necrosis in taxane sensitive and resistant cell lines at 48 hours. Without drug treatment, PacR cells had the highest necrosis levels compared to CTL cells ($p=0.029$) and DocR cells had the second highest levels of necrosis when compared to CTL cells ($p=0.029$). At IC_{50} of Paclitaxel, PacR cells had the highest levels of necrosis compared to CTL and DocR cells ($p=0.029$, $p=0.012$ respectively). At IC_{50} concentrations of Docetaxel, PacR cells also had the highest levels of necrosis compared to CTL cells ($p=0.029$), followed by DocR compared to CTL cells ($p=0.029$) (Fig. 4.13).

Another method used to determine loss of membrane integrity and necrosis is cellular exclusion or uptake of trypan blue dye. CTL, DocR, and PacR cells were stained with trypan blue after treatment with IC_{50} concentrations of either Paclitaxel, Docetaxel drug or vehicle for 48 hours, the total number cells stained blue were then counted and the percent of cells dying by necrosis was determined (Fig. 4.14). Using this method, no statistically significant differences were found between the sensitive and resistant cell lines without drug treatment and with IC_{50} concentrations of Paclitaxel or Docetaxel. This could be due to the fact that trypan blue staining not only stains necrotic cells but also cells that are in the late stages of apoptosis (180).

Figure 4.13 Necrosis by Propidium Iodide Dye with and without IC₅₀ Paclitaxel and Docetaxel at 48 hours

Figure 4.13 Necrosis by Propidium Iodide Dye with and without IC₅₀ Paclitaxel and

Docetaxel at 48 hours. Necrosis by propidium iodide dye of CTL, DocR, and PacR cells at 48 hours. PacR cells have the highest necrosis levels with and without taxane drug treatment.

Without drug, ANOVA $p=0.007$, both PacR and DocR vs. CTL cells (Mann-Whitney Rank Sum

test $p=0.029$). At IC_{50} concentrations of Paclitaxel, ANOVA $p \leq 0.001$, PacR and DocR cells vs. CTL (Mann-Whitney Rank Sum test $p=0.029$) and PacR vs. DocR cells (Student's t test $p=0.012$). At IC_{50} concentrations of Docetaxel ANOVA $p=0.001$, PacR and DocR vs. CTL cells (Mann-Whitney Rank Sum test $p=0.029$). Data are presented as the mean $\pm$ SE for four independent experiments. Data are presented as the mean $\pm$ SE for four independent experiments.

Figure 4.14 Necrosis by Trypan Blue dye with and without IC₅₀ Paclitaxel and Docetaxel at 48 hours

Figure 4.14 Necrosis by Trypan Blue Dye with and without IC₅₀ Paclitaxel and Docetaxel at 48 hours. Necrosis by trypan blue dye of CTL, DocR, and PacR cells at 48 hours. No statistically significant differences between the cell lines with and without IC₅₀ concentrations of Paclitaxel or Docetaxel. Data are presented as the mean $\pm$ SE for four independent experiments.

4.3.10 Autophagy Expression, Inhibition, and Taxane Resistance

To determine levels of autophagy in the sensitive and resistant cells, protein expression of LC3, p62, Beclin-1, and XBP-1 was measured by western blots with and without IC₅₀ concentrations of either Paclitaxel or Docetaxel at 48 hours. Increased protein expression of the cleaved LC3 (LC3II) and decreased p62 expression was found in PacR compared to CTL cells, indicating higher levels of autophagy may be occurring. No protein expression differences were found of either Beclin-1 or XBP-1 across the cell lines (Fig. 4.15).

In order to determine if the role of autophagy was a survival or cell death mechanism, particularly in PacR cells that had increased protein expression of autophagy markers of cleaved LC3 and p62, autophagy was inhibited using 3-MA (Fig. 4.16 and 4.17) or Beclin-1 siRNA (BECN1) (Fig. 4.18 and 4.19). With 5 mM 3-MA for 7 days, proliferation assays in Paclitaxel drug of CTL, DocR, and PacR cells showed that CTL cells in 3-MA had slightly higher cell numbers at 1 nM ($p=0.029$), 10 nM ($p=0.004$), 20 nM ($p=0.029$), 40 nM ($p=0.003$) and at 60 nM ($p=0.019$) compared to cells in PBS vehicle. No statistically significant differences of DocR and PacR cell number was seen when comparing 3-MA or vehicle treatment. In Docetaxel, CTL cells in 3-MA again showed slightly higher cell numbers at 1 nM ($p\leq 0.001$), 10 nM ($p=0.019$), 20 nM ($p=0.001$) and 40 nM ($p=0.006$) compared to cells in vehicle. DocR cells in 3-MA however showed lower number of cells at 10 nM ($p=0.031$). PacR cells in 3-MA had lower cell numbers at 1 nM ($p=0.021$), but lower cell numbers with vehicle at 20 nM ($p\leq 0.001$) and 40 nM ($p=0.004$).

When proliferation assays were performed after knockdown of Beclin-1 with siRNA and compared to a non targeting siRNA, CTL cells in BECN1 siRNA had higher cell numbers at 10 nM ($p=0.024$), at 20 nM ($p=0.006$), at 40 nM ($p=0.029$), and at 60 nM ($p\leq 0.001$). DocR in BECN1 siRNA also had higher cell numbers at 20 nM ($p=0.001$). In contrast, PacR in BECN1 siRNA had lower cell numbers at 10 nM ($p=0.004$), at 20 nM, at 40 nM ($p\leq 0.001$), and at 60 nM ($p=0.029$). At 60 nM, PacR cells with BECN1 siRNA had a 2-fold reduction in cell number. Proliferation assays in Docetaxel showed the same pattern in CTL cells in BECN1 siRNA, which had higher cell numbers at 1 nM ($p=0.001$), at 10 nM ($p=0.014$), and at 60 nM ($p=0.013$). DocR cells in BECN1 siRNA at 20 nM had higher cell numbers ($p=0.044$) but lower cell numbers at 60 nM ($p=0.043$). PacR cells in Docetaxel drug and BECN1 siRNA showed the same pattern as in Paclitaxel drug, namely a reduction in cell number at 10 nM ($p=0.029$), at 20 nM ($p=0.011$), at 40 nM ($p=0.026$), and at 60 nM ($p=0.008$). At 60 nM of Docetaxel, PacR cells with BECN1 siRNA had a 1.4-fold reduction in cell number.

Figure 4.15 Western Blots of Protein Expression of LC3, p62, Beclin-1, and XBP-1 with and without IC₅₀ Concentrations of Paclitaxel and Docetaxel at 48 hours

Figure 4.15 Western Blots of Protein Expression of LC3, p62, Beclin-1, and XBP-1 with and without IC₅₀ Concentrations of Paclitaxel and Docetaxel at 48 hours. Cleaved LC3 (LC3II) by densitometry ANOVA p=0.020, cleaved LC3 in PacR with Paclitaxel drug vs. CTL

cells without drug (Student's t test $p=0.045$). Data are presented as the mean $\pm$ SE for three independent experiments.

Figure 4.16 3-MA Proliferation Assays in Paclitaxel - 7 days

Figure 4.16 3-MA Proliferation Assays in Paclitaxel - 7 days. Proliferation assays of inhibition of autophagy with 5 mM 3-methyladenine for 7 days compared to PBS vehicle in Paclitaxel of CTL (A), DocR (B), and PacR (C) cells. CTL cells by ANOVA in 3-MA p=0.004 and PBS vehicle p=0.005. CTL cells in 3-MA at 1 nM (Mann-Whitney Rank Sum test p=0.029),

at 10 nM (Student's *t* test $p=0.004$), at 20 nM (Mann-Whitney Rank Sum test $p=0.029$), at 40 nM (Student's *t* test $p=0.003$), and at 60 nM (Student's *t* test $p=0.019$). No statistically significant differences of DocR and PacR cells in 3-MA or vehicle treatment. Data are presented as the mean $\pm$ SE for a representative experiment.

Figure 4.17 3-MA Proliferation Assays in Docetaxel -7 days

Figure 4.17 3-MA Proliferation Assays in Docetaxel - 7 days. Proliferation assays with 5 mM 3-methyladenine for 7 days compared to PBS vehicle of CTL (A), DocR (B), and PacR (C) cells in Docetaxel. CTL cells in 3-MA by ANOVA $p=0.006$, PBS vehicle ANOVA $p=0.003$. CTL cells in 3-MA at 1 nM (Student's t test $p\leq 0.001$), at 10 nM (Student's t test $p=0.019$), at 20 nM (Student's t test $p=0.001$), and at 40 nM (Student's t test $p=0.006$). DocR cells in by

ANOVA in 3-MA $p \leq 0.001$, and PBS (Student's *t* test $p = 0.018$). DocR in 3-MA at 10 nM (Student's *t* test $p = 0.031$). PacR cells by ANOVA in 3-MA and PBS $p \leq 0.001$. PacR in 3-MA at 1 nM (Student's *t* test $p = 0.021$), in PBS at 20 nM (Student's *t* test $p \leq 0.001$), and at 40 nM (Student's *t* test $p = 0.004$). Data are presented as the mean $\pm$ SE for a representative experiment.

Figure 4.18 Beclin-1 siRNA Knockdown and Proliferation Assays in Paclitaxel

Figure 4.18 Beclin-1 siRNA Knockdown and Proliferation Assays in Paclitaxel. Western blot of Beclin-1 siRNA showing knockdown of Beclin-1 protein expression compared to Non Targeting (NT) siRNA (A). Proliferation assays with Beclin-1 siRNA compared to NT siRNA of CTL (B), DocR (C), and PacR (D) cells in Paclitaxel. CTL cells in BECN1 and NT siRNA by ANOVA $p \leq 0.001$. CTL cells in BECN1 siRNA at 10 nM (Student's t test $p = 0.024$), at 20 nM

(Student's *t* test $p=0.006$), at 40 nM (Student's *t* test $p=0.029$), and at 60 nM (Student's *t* test $p\leq 0.001$). No statistically significant differences of DocR cells by ANOVA. DocR in BECN1 siRNA at 20 nM (Student's *t* test $p=0.001$). PacR cells by ANOVA in BECN1 siRNA $p\leq 0.001$ and in NT siRNA $p=0.003$. PacR in BECN1 siRNA at 10 nM (Student's *t* test $p=0.004$), at 20 nM and 40 nM (Student's *t* test $p\leq 0.001$), and at 60 nM (Student's *t* test $p=0.029$). At 60 nM, PacR cells with BECN1 siRNA show a 2-fold reduction in cell number. Data are presented as the mean $\pm$ SE for a representative experiment.

Figure 4.19 Beclin-1 siRNA Proliferation Assays in Docetaxel

Figure 4.19 Beclin-1 siRNA Proliferation Assays in Docetaxel. Proliferation assays with Beclin-1 siRNA compared to NT siRNA of CTL (A), DocR (B), and PacR (C) cells in Docetaxel. CTL cells in BECN1 siRNA by ANOVA $p \leq 0.001$, and NT siRNA by ANOVA $p = 0.001$. CTL cells in BECN1 siRNA at 1 nM (Student's t test $p = 0.001$), at 10 nM (Student's t test $p = 0.014$), and at 60 nM (Student's t test $p = 0.013$). DocR cells in BECN1 siRNA by ANOVA $p = 0.003$. DocR in BECN1 siRNA at 20 nM (Student's t test $p = 0.044$), and at 60 nM

(Student's *t* test $p=0.043$). PacR cells by ANOVA in BECN1 siRNA $p\leq 0.001$. PacR in BECN1 siRNA at 10 nM (Student's *t* test $p=0.029$), at 20 nM (Student's *t* test $p=0.011$), at 40 nM (Student's *t* test $p=0.026$) and at 60 nM (Student's *t* test $p=0.008$). At 60 nM of Docetaxel drug, PacR cells with BECN1 siRNA show a 1.4-fold reduction in cell number. Data are presented as the mean $\pm$ SE for a representative experiment.

4.3.11 Breast Cancer Clinical Samples

Forty breast cancer patients participated in a clinical study to determine responsiveness to Paclitaxel drug. Serial core needle biopsies from their breast cancer tumors were obtained from chemotherapy-naïve breast cancer patients receiving single drug Paclitaxel in the preoperative setting at four different time points. The defined time points were at baseline before treatment with Paclitaxel, at 48 hours after initial drug exposure, upon completion of the first treatment cycle, and upon completion of the last sequential cycle of Paclitaxel. Upon completion of the last cycle of chemotherapy, patients were classified as responders by the National Cancer Institute's RECIST (Response Evaluation Criteria in Solid Tumors) criteria. Approximately 70% of the patients achieved a clinical response with Paclitaxel. An average of three core biopsy specimens were obtained from each subject at each required time point, and approximately 50% of these biopsies contain >70% tumor DNA, with at least one adequate tissue sample for approximately 69% of the time points at which biopsies were obtained.

Microarray data from core needle biopsies of 12 patients that were categorized to be responders to Paclitaxel treatment and 2 non responders at baseline, before Paclitaxel treatment, have been analyzed. The microarray data comparing gene expression differences between responders and non responders before Paclitaxel treatment indicated that over 2323 probe sets were differentially expressed (fold change >1.45; $p \leq 0.05$), which is similar to the 2189 probe set differences between the *in vitro* CTL and PacR MDA-MB-231 cells (Fig. 4.20). KEGG pathway enrichment analysis was performed on the differentially expressed apoptosis genes from clinical samples. The expression data (fold changes) was integrated into the KEGG pathway for

apoptosis (Fig. 4.21) and of particular interest was the significant downregulation of Bcl-2 in responders by an average of 7.07 fold ($p=0.024$) that was not seen in *in vitro* CTL cells. When comparing genes that were differentially expressed between the *in vitro* CTL and PacR cells, with those that were differentially expressed in the responder and nonresponder clinical samples, 74 genes were found to be commonly differentially expressed. The expression pattern of genes related to apoptosis (Bcl-2 family) and autophagy (Atg family) in the *in vitro* models were not found to be an accurate predictor of response in the clinical setting. This could be due to the positive ER+/PR+ status of the clinical samples (most were hormone receptor positive), whereas the *in vitro* models are ER-/PR- hormone receptor negative cells, and the very small number of cases in this interim analysis. In addition, the cell line model of acquired resistance to Paclitaxel was developed after lengthy exposure to Paclitaxel drug whereas the baseline clinical samples were of patients that had never been exposed to Paclitaxel.

The clinical project is still ongoing and microarrays from the remaining 28 patients at baseline and all 40 patients' microarrays from the other 3 specific time points remain to be completed and await permission from the Lombardi Comprehensive Cancer Center clinicians to complete the analysis of the remaining 148 microarrays. This study may ultimately help to determine what gene expression changes occur in the development of acquired resistance to Paclitaxel treatment.

Figure 4.20 Microarray Probe Set Expression Differences Clinical Samples and *in vitro* Cell Lines

Figure 4.20 Microarray Probe Set Expression Differences Clinical Samples and *in vitro* Cell Lines. Probe set expression differences between *in vitro* CTL and PacR cells and clinical responders and non responder breast cancer tumors with patients before Paclitaxel treatment at >1.45 fold changes and $p \leq 0.05$.

responder clinical breast cancer tumor samples before treatment with Paclitaxel with fold changes ≥ 1.20 and $p < 0.05$ at baseline. Antiapoptotic Bcl-2 expression is reduced in responders to Paclitaxel treatment.

4.3 Discussion

The taxanes have been associated with a number of different types of cell death. Determining which types of cell death are or are not occurring in the models that were developed of acquired taxane resistance in MDA-MB-231 breast cancer cells has important clinical implications. To determine the expression pattern of cell death genes, a high throughput approach using gene expression microarrays to determine differential gene expression patterns between CTL, DocR, and PacR cells was completed and it was found that the gene expression pattern of apoptosis genes by microarray and PCR of PacR and DocR cells at fold changes of >1.45 and $p \leq 0.05$ compared to CTL cells was not identical.

Although a pattern has emerged regarding the differences in cell death patterns in PacR cells compared to CTL and DocR cells, it appears that several different cell death mechanisms can occur in taxane sensitive and resistant cells. CTL cells seem to favor apoptosis whereas mitochondrial permeability, necrosis (which does not depend on ATP), autophagy as a survival response (which can generate ATP) and cell size appear to be highest in PacR cells. Although DocR cells also appear to have higher necrosis levels, autophagy does not appear to play the same role as in PacR cells.

In addition, the role of ATP, Ca^{2+} and the PI3K-AKT-mTOR pathway may need to be elucidated particularly in DocR cells which may explain why they have a different cell death pattern from PacR cells. Further studies that elucidate the possible interactions between autophagy and apoptosis, necrosis and mitotic catastrophe and their respective genes are also needed to best

determine what inhibitors or inducers of cell death pathways can be used to enhance response to taxane chemotherapy treatment in the clinic. Cell size may also be affected by the PI3K-AKT-mTOR pathway and MCF-7 breast cancer cells transfected with constitutively active Akt1 or Akt3 showed an increase in size that was due to mTOR dependent increase in protein synthesis and mTOR independent inhibition of protein degradation (181). Although, both DocR and PacR cells had reduced expression of Akt1 by microarray, Akt3 expression was slightly higher in PacR cells compared to CTL cells, by 1.26-fold ($p=0.0166$), and in DocR cells, Akt3 was upregulated by 2.24-fold ($p=0.0356$).

Recently, it has been shown that necrosis can affect mitochondria, but differently from apoptosis. During intrinsic mitochondrial apoptosis, proapoptotic Bcl-2 family members Bax and Bak can cause permeabilization of the outer mitochondrial membrane, the release of cytochrome *c*, and caspase activation. Mitochondrial events caused by necrosis however involve the opening of a pore in the inner mitochondrial membrane (mitochondrial permeability transition pore, MPTP), which is regulated by cyclophilin D, a peptidyl-prolyl *cis-trans* isomerase D (PPID), that is found in the mitochondrial matrix (182). The formation of a MPTP causes a loss of electrical potential across the inner mitochondrial membrane that is needed to drive ATP production, mitochondrial swelling and can lead to the rupture of the outer mitochondrial membrane (OMM) (182). This may explain why both mitochondrial permeability and necrosis but not apoptosis are highest in PacR cells.

It has also been shown that Bnip3L, a BH3-only like protein, which is found in the OMM and the endoplasmic reticulum (EnR) can stimulate apoptosis or necrosis (182). Depending on where

Bnip3L is localized, a different outcome can occur. When it is localized at the mitochondria, Bnip3L stimulates Bax/Bak outer mitochondrial membrane permeabilization, cytochrome *c* release, caspase activation and apoptosis. However, when Bnip3L is localized at the endoplasmic reticulum, cyclophilin D dependent MPTP occurs leading to cytochrome *c* release that is Bax/Bak independent, necrotic features and OMM rupture (183). It is thought that endoplasmic reticulum signaling may involve EnR Ca^{2+} release and mitochondrial uptake, particularly in mitochondria that are tethered to EnR (182). In the microarray data, while PPID was found to be upregulated in both DocR and PacR cells compared to CTL cells by 2.43-fold and 2.48-fold ($p=0.008$, $p=0.001$ respectively), Bnip3L was only upregulated in PacR cells by 2.62-fold ($p=0.009$) thus the role of these genes in taxane resistance may need to be further elucidated.

Comparing the *in vitro* MDA-MB-231 breast cancer models of acquired Paclitaxel resistance and sensitivity that were developed in the laboratory to the clinical phenotype by gene expression microarrays is currently ongoing. Genes involved in apoptosis and autophagy in the *in vitro* models do not appear to be good predictors of response or non response to Paclitaxel in the initial 12 responder and 2 non responder needle core biopsies of breast cancer tumors microarrays before Paclitaxel treatment. A better comparison between the *in vitro* models and the clinical samples would be to look exclusively at the microarray data of clinical samples that are hormone receptor negative (ER-/PR-) and at the last time point, after the last cycle of Paclitaxel treatment when the data becomes available. Thus far, of interest in the clinical sample microarray data set is a decrease in antiapoptotic Bcl-2 gene expression seen in responders to Paclitaxel treatment.

CHAPTER 5: DISCUSSION

5.1 Taxane Resistance Mechanisms

Systemic adjuvant chemotherapy can significantly increase disease free and overall survival in breast cancer patients (30). Clinical trials have demonstrated that the use of the taxanes in adjuvant breast cancer therapy for patients with early or metastatic breast cancer can improve clinical outcome (47-49). However, one of the main problems encountered with use of the taxanes is resistance.

There are many proposed mechanisms of dose escalated or acquired resistance to the taxanes including overexpression of transporters such as Pgp, mutations in β -tubulin genes, differential β -tubulin isotype expression, changes in expression of microtubule associated proteins (MAPs), or changes in proteins in the apoptotic pathway such as increased expression of Bcl-2 and Bcl-xL.

A number of these proposed mechanisms were investigated. While differential expression of β -tubulin isotypes and mutations that have been described to contribute to taxane resistance in breast cancer cells and tissues were not found by PCR in either sensitive or taxane resistant cells, the role of microtubule associated proteins is not clear. Slightly increased gene expression of MAP4 was found in the taxane resistant phenotypes and its role remains to be elucidated in future studies. Although mechanisms of resistance to either Paclitaxel or Docetaxel that involve β -tubulin do not appear to contribute significantly to the resistant phenotypes, resistance to the

taxanes appears to involve a number of other mechanisms that may explain the difficulty in overcoming resistance in the clinic.

In addition, the taxane resistant models are not the same, as is seen in the differences in gene expression profiles and in the lack of complete cross-resistance of PacR cells to Docetaxel. Many transporters are differentially expressed between taxane resistant and sensitive breast cancer cells and the expression pattern is different between PacR and DocR cells. The role of transmembrane transporters appears to be an important mechanism in taxane resistance in the *in vitro* resistant models, given the number of both ABC and SLC superfamily genes that were differentially expressed between sensitive and taxane resistant cells. In particular, the non ATP dependent SLC family of transporters had a larger number of differentially expressed genes.

The pattern of differential expression of transporters was not identical between DocR and PacR cells. However, Pgp was found to be overexpressed in both resistant cell lines by gene expression microarray, qPCR, and at the protein level by western blot. Pgp siRNA only partially reduced cell numbers in select conditions, indicating that Pgp may not act alone in conferring resistance. One way to better determine what role the other transporters may play, even if the taxanes are not known substrates for them, would be through the use of a siRNA library.

Furthermore, the use of Verapamil in the breast cancer cells may also be contributing to the differential expression of transporters. The level of overexpression of Pgp and differential expression of ABC and SLC transporters may be a mechanism by which the resistant cells try to overcome Verapamil's effects. Considering that Verapamil can cause ATP depletion of cells,

this could explain the predominance of differential expression of SLC transporters, that do not require ATP, in both resistant cell lines.

Changes in the onset of apoptosis and other types of cell death can also contribute to the development of cancer and resistance (91, 92). The antitumor effects of many chemotherapy treatments result in apoptosis. Nonetheless, not all cells respond to anticancer therapy with an apoptotic mechanism, which is why alternative cell death pathways should be considered (93). Pharmacological agents that target specific cell death pathways have been developed. However, these agents have led to studies that focus on only one or two genes or proteins that may not be important in the clinic, and that do not account for concurrent activation of different cell death mechanisms that overlap and share common signaling pathways. These different cell death mechanisms include apoptosis, necrosis, mitotic catastrophe, and autophagy (98, 99).

The different methods of cell death were investigated and it was found that sensitive cells have higher levels of apoptosis with and without IC_{50} concentrations of either Paclitaxel or Docetaxel. In contrast, PacR cells had the highest mitochondrial permeability and the lowest expression of antiapoptotic Bcl-2 and NF- κ B. Antiapoptotic Bcl-xL, and proapoptotic Bax, with and without IC_{50} concentrations of either taxane, were not significantly changed. Neither were there significant differences in caspase activation or protein expression of whole and cleaved caspases -3,-7,-8,-9 when measured by western blots in sensitive and resistant cell lines.

Mitotic catastrophe by light microscopy and the morphological appearance of giant multi and micronucleated cells appears to be present in both sensitive and resistant cell lines in IC_{50}

concentrations of either taxane. However, with and without IC₅₀ concentrations of either taxane, the PacR cells had the largest cell size, followed by DocR cells, when compared to CTL cells. Necrosis, which does not require ATP, was found to be highest in PacR cells followed by DocR cells with and without IC₅₀ concentrations of either taxane. The predominance of Necrosis as a cell death mechanism in the resistant cell lines could correlate with the increase in Pgp and other ABC transporter expression that require ATP in both resistant cell lines. The concurrent use of Verapamil could lead to ATP depletion causing the cells to die by an energy independent necrotic mechanism.

Autophagy, which can be either a survival (energy generating) or a cell death mechanism, was found to have a different role in sensitive and taxane resistant cells. PacR cells, which had reduced Bcl-2 expression, also had higher expression of LC3II and lower levels of p62 by protein expression with and without IC₅₀ concentrations of either taxane, indicating higher levels of autophagy in PacR cells compared to CTL or DocR cells. Beclin-1 and both total and spliced XBP-1 were not differentially expressed. Inhibition of autophagy with 3-MA did not reverse the resistance phenotypes but Beclin-1 siRNA caused a decrease in cell number in PacR cells by 2-fold in Paclitaxel and 1.4-fold in Docetaxel, indicating that autophagy could be a survival mechanism. There was also a slight increase in cell number of CTL cells and DocR cells with Beclin-1 siRNA, indicating that autophagy may be a cell death mechanism in these cells.

The concurrent presence of multiple cell death mechanisms or survival pathways indicates that there may be extensive cross talk among these various mechanisms, as has already been suggested between antiapoptotic Bcl-2 localized at the endoplasmic reticulum, antiapoptotic Bcl-

xL and Beclin-1, p62 and NF- κ B. In addition, endoplasmic reticulum localized Bnip3L and differences in calcium (Ca^{2+}) levels could explain the differences in necrosis levels between the two taxane resistant models but this remains to be determined. Verapamil could also be affecting the differential predominance of cell death types in sensitive and resistant cells by modulating intracellular Ca^{2+} and ATP levels. In the resistant cells that significantly overexpress Pgp and other ABC transporters, ATP depletion and changes in Ca^{2+} levels could be occurring due to the Verapamil present. Intracellular Ca^{2+} and ATP levels could explain the differences in mitochondrial membrane permeability, apoptosis, necrosis and autophagy between sensitive and resistant cells. In addition, the differential expression of transporters could also be contributing to changes in plasma membrane electrical chemical gradients which could affect other intracellular downstream targets. Further studies to elucidate cross talk between transporters, proteins at the endoplasmic reticulum and the mitochondria and the various cell death mechanisms will need to be conducted and could also contribute to the difficulty in overcoming resistance in the clinic.

5.3 Major Discoveries

- *P-glycoprotein is overexpressed in both taxane resistant phenotypes but its knockdown only partially reduces cell number in taxane resistant cells. Many other transporters of both the ABC and SLC superfamilies are associated with resistance and some of these may contribute to the resistance phenotype.*
- *Cell death patterns are different between sensitive and resistant cells, with apoptosis being the preferred method of cell death in taxane sensitive cells. Mitochondrial membrane permeability, necrosis, autophagy, and cell size are highest in PacR cells. Mitotic catastrophe occurs in sensitive and resistant cells at IC₅₀ concentrations of either of the taxanes.*
- *Autophagy can be either a survival or a cell death mechanism. Inhibition of autophagy with Beclin-1 siRNA can either enhance cell numbers as in sensitive and DocR cells or reduce cell numbers as in PacR cells.*

5.4 Future Directions

5.4.1 siRNA Library to Transporters

Although Pgp activity was inhibited with Verapamil in taxane sensitive and resistant MDA-MB-231 breast cancer cells, Pgp was overexpressed in both taxane resistant phenotypes by gene expression microarrays, qPCR, and protein expression by western blot. Pgp knockdown with siRNA caused only partial reduction in cell number, implicating some of the other 70 or more transporters of the ABC and SLC superfamilies in the resistance phenotypes. To best determine if the differential expression of the other transporters are involved, an siRNA library would probably be the best method to knockdown each of the transporters and determine whether or not they contribute to either or both of the taxane resistant phenotypes.

5.4.2 Molecular Mechanisms of Docetaxel Resistance

DocR cells and PacR cells may not use the same mechanism of resistance. While DocR cells are cross resistant to Paclitaxel, PacR cells are not fully cross resistant to Docetaxel. In addition, there were differences in expression patterns of both ABC and SLC transporters and determining if they play a different role in DocR resistance compared to PacR resistance would be clarified through transporter siRNA library experiments.

Differential expression of β -tubulin isotypes and known mutations in breast cancer cells and tissues in the sequence of β -tubulin do not appear to contribute to either of the taxane resistant phenotypes, however it is not clear if microtubule associated proteins could be playing a role in

resistance to DocR such as MAP4 which was found to be differentially expressed in both DocR and PacR cells compared to CTL cells by microarray.

The PI3K/AKT/mTOR pathway may play a different role in DocR cells compared to PacR cells and perhaps knocking down the expression of class III PI3K with siRNA, or overexpressing the downregulated Akt1, could elucidate differences in DocR and PacR resistance mechanisms.

Differences in the PI3K-AKT-mTOR pathway between the two resistant phenotypes could also affect cell size and cell growth. While DocR cells had the highest levels of proliferation, DocR cells had both the second highest cell size, the lowest expression of Akt1, and the highest expression of Akt3 by microarray.

Differential gene expression of necrosis inducing PPID was found in DocR and PacR cells; Bnip3L upregulation was only found in PacR cells. Endoplasmic reticulum localized Bnip3L and Ca^{2+} levels may explain the differences in necrosis levels between the two taxane resistant models and remains to be determined.

Finally, the role of metabolic stress or energy levels could be contributing to the differences in Docetaxel resistance phenotype compared to the Paclitaxel resistance phenotype. Many SLC transporters that do not require ATP were differentially expressed in DocR cells, and mitochondria membrane permeability was lower in DocR cells. Necrosis, which also does not require ATP, was also lower in DocR cells compared to PacR cells, and autophagy, which can generate ATP, appeared to be a survival mechanism in PacR but not DocR cells.

Interestingly, the differential expression of stem cell markers in DocR cells could also be explored because cancer stem cells have self renewal capacity, drive tumorigenicity, recurrence and metastasis (184-186). Differential expression of putative cancer stem cell surface markers CD24 and CD44 was found in both DocR and PacR cells compared to CTL cells by microarrays. Upregulation of CD24 by 3.23-fold ($p=0.001$) and 2.00-fold ($p=0.026$) in DocR and PacR cells respectively, and downregulation of CD44 by 1.49-fold ($p=0.006$) and 1.40-fold ($p=0.011$) in DocR and PacR cells compared to CTL cells respectively, was found. Suz12, a marker of cancer stem cell transition from pluripotent to differentiated state, was only found to be differentially expressed in PacR cells (upregulated by 1.50-fold, $p=0.016$) but not DocR cells (187).

5.4.3 Clinical Samples and Paclitaxel Resistance

The determination of gene expression differences between patients that respond or do not respond to Paclitaxel treatment is currently in progress. Thus far microarrays of 12 responders and 2 non responders before Paclitaxel treatment were analyzed and the remaining patient samples remain to be arrayed. Determination of estrogen and progesterone receptor status of the patient breast cancer tumor samples must be obtained. Future gene expression profile comparisons will be conducted between microarray data from ER-/PR-negative sensitive breast cancer cells *in vitro* (CTL) and responders to Paclitaxel treatment and acquired Paclitaxel resistant cells (PacR) with clinical non responders to Paclitaxel treatment after the last treatment cycle of Paclitaxel. Previous gene expression profile comparisons were of patients before Paclitaxel treatment and without prior knowledge of hormone receptor status which yielded very few common genes that were differentially expressed in both the clinical samples and *in vitro* cell lines.

5.5 Conclusion

Resistance to either Paclitaxel or Docetaxel appears to be multifactorial, which could explain why it is a difficult problem to overcome in the clinic. The taxane resistant models are not the same, as is seen in the differences in gene expression profiles as well as the lack of complete cross-resistance of PacR cells to Docetaxel. Many transporters are differentially expressed between taxane resistant and sensitive breast cancer cells and the expression pattern is different between PacR and DocR cells. The role of transporters other than Pgp in the ABC superfamily and the SLC superfamily of transporters still remains to be elucidated.

Cell death mechanisms also differ between sensitive and resistant cells, with apoptosis being the predominant form of cell death in sensitive but not taxane resistant cells. Several other types of cell death were found to be present. Mitochondrial membrane permeability, necrosis, autophagy, and cell size were highest in Paclitaxel resistant cells followed by Docetaxel resistant cells compared to sensitive cells. Mitotic catastrophe however occurs in both sensitive and resistant cells. Autophagy appears to have a different role in the two taxane resistant models. Autophagy appears to be a survival mechanism in PacR but not DocR cells. The use of inducers or inhibitors of alternative cell death pathways are currently being tested in clinical trials.

Autophagy inhibitors in cases of Paclitaxel resistance, or inducers of apoptosis in both taxane resistant models, could be complements to taxane treatment. However, further studies to determine the exact interactions between apoptosis, necrosis, mitotic catastrophe, and autophagy and their respective genes is needed to best determine what inhibitors or inducers of cell death pathways can be used to enhance response to taxane chemotherapy treatment in the clinic.

Appendix A: Cell Cycle Genes, CDC25C, p53 and Taxane Resistance

A.1 Introduction

CDC25C has been described as a “mitotic trigger” and is a dual specificity protein phosphatase that catalyzes the removal of inhibitory phosphates on Cdc2, which then activates cdc2/cyclin B (188). The cdc2/cyclin B complex in turn activates CDC25C in a autocatalytic feedback loop by phosphorylating the Ser214 residue on CDC25C, which then blocks inhibitory phosphorylation of Cdc25C on its Ser216 residue and leads to initiation of mitosis (188, 189). Activity of CDC25C is induced during G2/M transition and is also thought to be a substrate of cyclin A/Cdk2 complexes (189).

Recent studies of G2/M cell cycle genes in taxane resistance have begun to explore the possible role of CDK2. However, small inhibitory RNA (siRNA) directed against CDK2 in several human cancer cell lines results in little cell cycle delay (190). Other studies have shown that transfection of dominant-negative (DN)-CDK2 can evoke resistance to Paclitaxel but re-expression of wild-type CDK2 does not restore sensitivity to the Paclitaxel (191). Thus, the effect of signaling by CDK2, at least as it relates to taxane resistance, likely involves additional functionally relevant events. The role of CDC25C was not addressed in these studies; therefore its role and its expression in resistance and sensitivity was novel and was to be determined in addition to the role of other cell cycle genes (CDK2, cdc2, Cyclin A, Cyclin B1) and p21 and p53.

A.2 Materials and Methods

A.2.1 Cell Culture and Reagents

See Materials and Methods Section 2.2.1

A.2.2 Gene Expression Microarrays

See Materials and Methods section 3.2.2. Gene pathway interconnectivity was visualized using Pathway architect software from Agilent Technologies (Santa Clara, CA).

A.2.3 PCR

RNA was isolated from three independent passages of sensitive, Docetaxel and Paclitaxel resistant cell lines as described above and in (135) and purified using the RNeasy Mini Kit (Qiagen, Valencia, CA). 1.0 µg RNA was reverse transcribed to generate first strand cDNA using (Promega, Madison, WI) products. For each cDNA sample, a qPCR reaction and a standard curve was obtained using TaqMan Universal PCR Master mix and TaqMan primers (Applied Biosystems, Carlsbad, CA): CDC25C, Hs00156411_m1 and RPLP0 (housekeeping gene), Hs99999902_m1. Each reaction (10 µl) was run in triplicate on the ABI Prism 7900 HT Sequence Detection System using the manufacturer's absolute quantification protocol. Expression data for each reaction was determined relative to RPLPO expression.

A.2.4 Cell Lysis and Immunoblotting

For the determination of baseline CDK2, CDC25C, Cyclin A, Cyclin B1, Cdc2, p21, p53 protein expression, cells were grown at baseline conditions in T75 flasks and cultured in normal growth

media with 1 μ M Verapamil for the sensitive cells, 7 nM Paclitaxel for Paclitaxel resistant cells and 2 nM Docetaxel for Docetaxel resistant cells. Cells were then lysed in modified radioimmunoprecipitation assay buffer [150 mmol/L NaCl, 50 mmol/L Tris (pH 7.5), 1% Igepal CA-630, and 0.5% deoxycholate] supplemented with Complete Mini protease inhibitor cocktail tablets (Roche, Mannheim, Germany) and 1 mmol/L sodium orthovanadate phosphatase inhibitor (Sigma-Aldrich, St. Louis, MO). Lysates were clarified by centrifugation and total protein was quantitated using the bicinchoninic acid assay purchased from Pierce (Rockford, IL). Whole cell lysate (25 μ g) was resolved by PAGE using NuPAGE 12% precast gels (Invitrogen, Carlsbad, CA). Proteins were then transferred to nitrocellulose membranes, which were probed with the antibodies overnight at 4°C. Antibodies for p53, CDC25C, Cyclin A, Cyclin B1, Cdc2, p21, were obtained from Cell Signaling Technology (1:1000; 1:1000, 1:2000, 1:2000, 1:1000, 1:1000 respectively, Danvers, MA). CDK2 was obtained from BD Biosciences (1:1000; San Jose, CA). Membranes were then incubated with horseradish peroxidase–conjugated secondary antibodies for 1 hour at room temperature before enhanced chemiluminescence (GE Healthcare, Buckinghamshire, UK) and exposure to film (X-OMAT Blue XB-1; Kodak, Rochester, NY). To confirm equal loading of the gels, membranes were reprobed with antibodies for β -actin (1:5000; Sigma-Aldrich, St. Louis, MO).

A.2.5 P53 siRNA

P53 siRNA was obtained from Thermo Scientific (Lafayette, CO) and Non Targeting siRNA was obtained from Qiagen (Valencia, CA). Western Blots were performed to confirm knockdown using the above method. Cells were then seeded at a density of 1×10^4 cells/well into 24-well plates in drug free media with 1 μ M Verapamil for 24 hours before treatment with p53 or Non

Targeting siRNA for 24 hours followed by either Paclitaxel or Docetaxel or no drug for 48 hours. At least three independent experiments were done in quadruplicate, and data were normalized to Non Targeting siRNA treated cells. The siRNA proliferation data were repeated in triplicates. Data are presented as the mean $\pm$ SE for a representative experiment.

A.2.6 Statistical Analysis

See Materials and Methods section 2.2.7

A.3 Results

A.3.1 Molecular Profiling of Cell Cycle Genes

Initial exploration of the microarray data from the comparison of genes expressed in PacR and DocR cells and those that are sensitive to either taxane showed significant differential upregulation of CDC25C by 2-fold ($p=0.001$) in PacR vs. DocR cells. In addition, expression of both CDK2 and Cyclin B is significantly increased in Paclitaxel-resistant vs. Docetaxel-resistant cells (1.56-fold; $p=0.031$ and 1.50-fold; $p=0.007$, respectively). P53 also appeared to be differentially expressed in DocR compared to CTL cells as shown by microarray gene data (downregulated 1.40-fold; $p=0.034$). A quick virtual analysis of CDC25C interactions using the Pathway Architect software program confirmed that CDC25C has direct interactions with Cyclin A and Cyclin B, which are involved in progression of the cell cycle at G2/M, and suggested the presence of a small CDK2 signaling component that might be important in the differential sensitivity of the DocR and PacR cells to Docetaxel (Fig. A1). When the cell lines were rearrayed, CDC25C no longer was found to be differentially expressed and therefore CDC25C and the cyclins were no longer pursued as resistance mechanisms.

Figure A1 Pathway Architect Representation of Molecular Interactions of CDC25C and Other Cell Cycle Genes

Figure A1 Molecular interactions of CDC25C with other cell cycle genes by Pathway Architect.

A.3.2 Western Blots and PCR of Cell Cycle Proteins

Western blots for CDC25C, CDK2, Cyclin B1, Cyclin A, CDC2, p21 also showed little difference between our resistant and parental cell lines (Fig. A2). Differential expression of p21, another important gene involved in the cell cycle revealed inconsistent expression depending on the passage that protein was extracted from. PCR of CDC25C showed slightly elevated CDC25C in DocR cells and not PacR cells which was contrary to our microarray data (Fig. A3).

Figure A2 Western Blots of Protein Expression of Cell Cycle Proteins at Baseline

Figure A2 Western Blots of Protein Expression of Cell Cycle Proteins at Baseline. Protein expression by western blots of cell cycle proteins CDK2, CDC25C, Cyclin A, Cdc2, Cyclin B1 and p21 at baseline.

Figure A3 PCR Validation of CDC25C at Baseline

Figure A3 PCR Validation of CDC25C at Baseline. PCR of CDC25C in CTL, DocR and PacR cells as a ratio to RPLP0 at baseline. Data are presented as the mean $\pm$ SE for three independent experiments.

A.3.3 P53 siRNA

P53 siRNA experiments showed that p53 siRNA was not able to reverse the resistance phenotypes in either the DocR or the PacR cells which is consistent with the fact that MDA-MB-231 breast cancer cells having a point mutation in p53 rendering it nonfunctional (Fig. A4 and A5). However, PacR cells in p53 siRNA and 1 and 60 nM of Paclitaxel had slightly higher cell numbers compared to Non Targeting siRNA ($p=0.033$ and $p=0.029$ respectively). In P53 siRNA and Docetaxel, CTL cells at 10 nM and at 60 nM had slightly higher cell numbers ($p=0.016$ and $p=0.011$ respectively). PacR cells also had slightly higher cell numbers with p53 siRNA at 40 nM and at 60 nM ($p=0.007$ and $p=0.029$ respectively) of Docetaxel.

Figure A4 P53 siRNA Knockdown and Proliferation Assays in Paclitaxel.

Figure A4 P53 siRNA Knockdown and Proliferation Assays in Paclitaxel. Western blots of p53 protein siRNA knockdown (A). P53 siRNA proliferation assays of CTL, DocR and PacR

cells with p53 and Non Targeting (NT) siRNA in Paclitaxel (B). CTL cells with p53 and NT siRNA by ANOVA, $p \leq 0.001$. DocR cells in p53 siRNA by ANOVA $p \leq 0.001$ and in NT siRNA $p = 0.003$. PacR cells in p53 and NT siRNA by ANOVA $p \leq 0.001$. PacR cells in p53 siRNA at 1 nM (Student's *t* test $p = 0.033$), and at 60 nM (Mann-Whitney Rank Sum test $p = 0.029$).

Figure A5 P53 siRNA Proliferation Assays in Docetaxel

Figure A5 P53 siRNA Proliferation Assays in Docetaxel. P53 siRNA proliferation assays of CTL, DocR and PacR cells with p53 and NT siRNA in Docetaxel. CTL cells by ANOVA with p53 siRNA $p=0.001$ and in NT siRNA $p\leq 0.001$. CTL cells at 10 nM (Student's t test $p=0.016$), and at 60 nM (Student's t test $p=0.011$). DocR cells by ANOVA in p53 siRNA $p=0.002$, NT siRNA $p\leq 0.001$ and for PacR cells by ANOVA in both p53 and NT siRNA, $p\leq 0.001$. PacR cells at 40 nM (Student's t test $p=0.007$), and at 60 nM (Mann-Whitney Rank Sum test $p=0.029$).

Data are presented as the mean $\pm$ SE for a representative experiment.

A.4 Discussion

CDC25C has been described as a “mitotic trigger”, is a substrate of cyclin A/Cdk2 complexes, and can interact with other cell cycle genes such as Cdc2 that activates cdc2/cyclin B (188, 189). In addition, other genes are thought to be important in the cell cycle such as CDK2 and p21 (190). Initial microarray data from the comparison of genes expressed in CTL, PacR and DocR cells showed a differential upregulation of CDC25C by in PacR vs. DocR cells. In addition, expression of both CDK2 and Cyclin B is also significantly increased in PacR vs. DocR cells. P53 also appeared to be downregulated in DocR compared to CTL cells as shown by microarray gene data. Western blots for CDC25C, CDK2, Cyclin B1, Cyclin A, CDC2, p21 showed little difference between our resistant and parental cell lines and expression of p21, revealed inconsistent expression depending on the passage that protein was extracted from. PCR of CDC25C showed slightly elevated CDC25C in DocR cells and not PacR cells which was contrary to the initial microarray data. However, when the cell lines were rearranged, CDC25C no longer was found to be differentially expressed and therefore CDC25C and the cyclins were no longer pursued as resistance mechanisms. In addition, p53 siRNA experiments did not show a reduction in cell number and either CTL, PacR or DocR cells, which may be due to the fact that MDA-MB-231 breast cancer cells have a point mutation in p53 that renders it nonfunctional.

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