RESVERATROL AUGMENTS PACLITAXEL TREATMENT IN MDA-MB-231 AND PACLITAXEL-RESISTANT MDA-MB-231 BREAST CANCER CELLS

Alyssa A. Sprouse

Submitted to the faculty of the University Graduate School
in partial fulfillment of the requirements
for the degree
Doctor of Philosophy
in the Department of Pharmacology & Toxicology,
Indiana University

August 2014

Accepted by the Graduate Faculty, of Indiana University, in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

Brittney-Shea Herbert, Ph.D., Chair

Doctoral Committee

David A. Flockhart, M.D., Ph.D.

Richard Nass, Ph.D.

Karen E. Pollok, Ph.D.

May 23, 2014

Ahmad R. Safa, Ph.D.

DEDICATION

For the family I was given and the family I have chosen.

For Zoey.

ACKNOWLEDGEMENTS

I would like to thank the Indiana University Simon Cancer Center Flow Cytometry Core facility for their services, Norma Lopez for her assistance with the senescence staining, Dr. Harlan Shannon for his guidance in designing the drug combination experiments, and the members of the Herbert laboratory for helpful discussions on this project. In addition, I would like to thank the IBMG program and the staff of the Department of Pharmacology & Toxicology for keeping me organized and for being helpful and supportive every step of the way. Thank you to the Department of Pharmacology & Toxicology faculty and students for their encouragement, discussion and constructive criticism that have facilitated my growth as a scientist and a professional. A special thank you to my mentor and thesis committee members for their helpful discussion, suggestions and guidance. This work was supported by the Indiana University Simon Cancer Center and the Indiana Genomics Initiative (INGEN) supported in part by the Lilly Endowment, Inc. I am also grateful for the philanthropic support in memory of Carol Herbert made to the Herbert laboratory through IUSCC. Finally, I would like to thank my friends and family for their unwavering love and support.

Alyssa A. Sprouse

RESVERATROL AUGMENTS PACLITAXEL TREATMENT IN MDA-MB-231 AND PACLITAXEL-RESISTANT MDA-MB-231 BREAST CANCER CELLS

Resveratrol has been shown to inhibit cell growth and induce apoptosis, as well as augment chemotherapeutics and irradiation in multiple cancer types. However, it is unknown if resveratrol is beneficial for treating drug-resistant cancer cells. To study the effects of resveratrol in triple negative breast cancer cells that are resistant to the common cancer drug, paclitaxel, a novel paclitaxel-resistant cell line was generated from the MDA-MB-231 breast cancer cell line. The resulting cell line, MDA-MB-231/PacR, exhibited a 12-fold increased resistance to paclitaxel but remained sensitive to resveratrol treatment. Resveratrol treatment reduced cell proliferation and colony formation and increased senescence and apoptosis in both the parental MDA-MB-231 and MDA-MB-231/PacR cell lines. Importantly, resveratrol treatment augments the effects of paclitaxel in both cell lines. The expression of the drug efflux transporter gene, *MDR1*, and the main metabolizing enzyme of paclitaxel gene, *CYP2C8*, was increased in the resistant cells. Moreover, pharmacological inhibition of the protein products of these genes, P-glycoprotein and CYP2C8, decreased paclitaxel resistance in the resistant but not in the parental cells, which suggests that the increase of these proteins are important contributors to the resistance of these cells. In conclusion, these studies imply that resveratrol, both alone and in combination with paclitaxel, may be useful in the treatment of paclitaxel-sensitive and paclitaxel-resistant triple negative breast cancers.

Brittney-Shea Herbert Ph.D., Chair

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ABC	ATP-binding cassette
Akt	protein kinase B
AMPK	adenosine monophosphate-activated protein kinase
ANOVA	analysis of variance
AP-1	activator protein 1
ATP	adenosine triphosphate
BCA	bicinchoninic acid
Bcl-2	B-cell lymphoma 2
Bcl-xL	B-cell lymphoma-extra large
BCRP	breast cancer resistance protein
BRCA	breast cancer susceptibility protein
BSA	bovine serum albumin
CAM	complementary and alternative medicine
cAMP	cyclic adenosine monophosphate
cDNA	complementary deoxyribonucleic acid
CBP	CREB-binding protein
COX-2	cyclooxygenase-2
C_T	threshold cycle
CYP	cytochrome p450
DAPI	4’,6’-diamino-2-phenylindole
DBC1	deleted in breast cancer 1
DCIS	ductal carcinoma in situ
DMEM	Dulbecco’s Modified Eagle Medium
DMSO	dimethyl sulfoxide

DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
ECL	enhanced luminol-based chemiluminescent
EDTA	Ethylenediaminetetraacetic acid
EGTA	ethylene glycol tetraacetic acid
EGFR	epidermal growth factor receptor
ER	estrogen receptor
FAS	apoptosis antigen 1
FITC	fluorescein isothiocyanate
HBSS	Hank's balanced salt solution
HER2	human epidermal growth factor receptor 2
HRP	horseradish peroxidase
IC_{50}	half maximal inhibitory concentration
IP	immunoprecipitation
MAP	microtubule-associated protein
MAPK	mitogen-activated protein kinase
MBC	metastatic breast cancer
MDR1	multidrug resistance protein 1
mRNA	messenger ribonucleic acid
MRP1	multidrug resistance-associated protein 1
NFκB	nuclear factor kappa-light-chain-enhancer of activated B cells
NP-40	nonyl phenoxypolyethoxylethanol
OHP	hydroxypaclitaxel
p21	cyclin-dependent kinase inhibitor 1
p53	cellular tumor antigen p53
P-AMPK	phosphorylated-adenosine monophosphate-activated protein kinase

PBS	phosphate buffered saline
PBST	phosphate buffered saline with tween
PCR	polymerase chain reaction
P-gp	P-glycoprotein
PI	propidium iodide
PKA	protein kinase A
PR	progesterone receptor
PVDF	Polyvinylidene fluoride
qPCR	real-time, quantitative polymerase chain reaction
RES	resveratrol
RIPA	radioimmunoprecipitation assay buffer
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
RT-PCR	reverse transcriptase polymerase chain reaction
SDS	sodium dodecyl sulfate
siRNA	small interfering ribonucleic acid
SIRT1	sirtuin 1
SLC	solute carrier
SLCO	solute carrier organic anion transporter
TBE	Tris/Borate/EDTA
TBST	Tris buffered saline with tween
TNBC	triple negative breast cancer
Tris	tris(hydroxymethyl)aminomethane
VEGF	vascular endothelial growth factor
XIAP	inhibitor of apoptosis protein 3

CHAPTER 1: INTRODUCTION and LITURATURE REVIEW

I. Breast Cancer

Cancer is a diverse group of diseases that are characterized in general by uncontrolled cell growth [1]. Although all cancers involve deregulation of genes that control cell growth, there are a wide range of causes and genes involved that are often specific to the tissue of origin. Though some of these genetic variations are hereditary, 90-95% are sporadic [2]. The tissue from which the abnormal cells originate identifies cancers though it may spread to many parts of the body, denoting metastatic disease.

Cancer originating from the breast, known as breast cancer, is the second most common cancer and is the fifth cause of cancer deaths in the world [3, 4]. In the United States, breast cancer is the second leading cause of cancer death among women [2]. One in eight women in the United States will develop breast cancer in their lifetime, and of these, approximately fifteen percent will succumb to the disease [5]. It was estimated that in 2013 alone more than two hundred thousand women and two thousand men would be diagnosed with breast cancer, and more than forty thousand people would die from the disease [5].

Breast cancers are almost exclusively carcinomas, which derive from epithelial cells; breast sarcomas, which derive from mesenchymal cells, are possible, though they are rare [6]. Carcinoma breast cancers can be divided into two main types: ductal carcinoma in situ (DCIS) and metastatic breast cancer (MBC). DCIS originates from the breast duct linings and is a non-invasive cancer, which may or may not ever progress to an invasive cancer. It has been suggested that only approximately one-third of DCIS cases will progress making the identification of the most likely subtypes to progress crucially important [7]. MBCs are cancers that have broken through the ductal or

glandular tissue from which they originated and have grown into the surrounding tissue. The stage of the disease is important for prognosis and treatment options and is determined by three measures: tumor size and distance of spreading within the breast, the spread to nearby lymph nodes, and the presence of distant metastases. Staging is ranked from 0 to IV with stage 0 being in situ and stage IV being the most advanced invasive cancer. Finally, breast cancers are defined by sub-type. Breast cancer sub-type is loosely defined by the expression of estrogen receptor (ER), progesterone receptor (PR), and whether or not the tumor overexpresses human epidermal growth factor receptor 2 (HER2). The luminal A subtype makes up approximately 40% of breast cancers and generally consists of ER+ and/or PR+ and HER2- tumors that are slow-growing and less aggressive [8]. The luminal B sub-type represents 10-20% of breast cancers most of which are ER+ and/or PR+ with either overexpression of HER2 or a high proliferation rate [8, 9]. The HER2-enriched sub-type is characterized by ER- and PR- cancers that overexpress HER2 and are highly invasive and aggressive [8]. Finally, 10% to 20% of breast cancers are basal-like, which have the worse prognosis of all sub-types [9]. The majority of basal-like breast cancers are triple negative breast cancers (TNBC), which are ER-, PR-, and do not overexpress HER2 [10].

There are many known risk factors for breast cancer. The most important risk factors for women to develop breast cancer relate to life-long hormone exposure and include: menopausal status, the age of menopause and menarche, and time of pregnancies. One of the most important risk factor for breast cancers, as for many other cancers, is age; risk increases with increasing age. Personal or family history of breast cancer is another major factor increasing risk of developing breast cancer. There are inherited genetic mutations, such as in the *BRAC1* and *BRCA2* genes, which increase the risk for breast cancer. Although these mutations are only present in 1% of the population, it is estimated that 5-10% of breast cancers result from these mutations and

so are considered inherited rather than sporadic [11]. Due to knowledge of these risk factors, women at high risk for breast cancer can be treated with tamoxifen, an estrogen receptor antagonist, or prophylactic surgery to prevent the development of the disease. The recent decreases in mortality for breast cancer have been attributed to both early detection, promoted by awareness and regular screening, and improvements in treatment [12].

Treatment options are dependent on the stage and subtype of breast cancer [13]. Surgical removal of the tumor is almost always the first treatment for breast cancer; the only exception to this is high stage, inoperable tumors that are first treated with chemotherapy, known as neaoadjuvant treatment, in an attempt to shrink the tumor and to make surgery a treatment option. There are two different options of breast cancer surgery: breast-conserving, removal of the tumor only, and total mastectomy, removal of all breast tissue. Choice between these surgeries is determined by the stage of disease and risk factors present. The treatment that follows surgery is dependent on the surgery performed, the stage and the cancer type. DCIS is treated with tamoxifen after a total mastectomy and with tamoxifen and radiation therapy after breast-conserving surgery. Stage I, II, and some stage III cancers are initially treated similarly to DCIS, but adjuvant, or post-surgery, chemotherapy and targeted treatments are added to the possible tamoxifen and radiation treatment. The combination of treatments is determined by the status of the axillary nodes, sub-type of the cancer, menopausal status of the patient, and RNA expression profiling information. More aggressive treatment is necessary in axillary node- positive and pre-menopausal patients. For the remaining stage III and all stage IV cancers, many tumors are inoperable. Treatment often includes hormone therapy, targeted therapies and chemotherapy simultaneously. Radiation therapy and surgery are also important parts of treatment though the timing of these treatments is less clear than in lower staged cancers.

For patients with TNBC, surgery along with radiation and traditional chemotherapy are the standard of care as discussed above. However, although great strides have been made over the last decade with targeted therapies, therapies that directly target ER and HER2, these therapies are ineffective in TNBCs, which lack ER and do not overexpress HER2. Due to the lack of targeted therapies, these patients have a poor prognosis [5].

A. Triple Negative Breast Cancer (TNBC)

TNBCs are associated with lower survival rates than other breast cancers [14]. Partially this is due to the recently decreased mortality rates of other breast cancers resulting from improvements of treatments that are not available to patients with TNBCs. Interestingly, pathologically complete response rates are higher in triple negative compared to other breast cancer sub-types [15]. However, TNBCs are highly aggressive and more likely than other sub-types to recur which result in lower survival rates [16]. A common measure of the aggressiveness of cancer is the differentiation status. Cancers that are not well differentiated, or look very different from the tissue of origin cells, tend to grow much faster and therefore spread faster [17]. TNBCs are much more likely to be poorly differentiated or undifferentiated compared to other breast cancers [18].

TNBCs are also associated with younger age and more advanced stage at diagnosis. In addition, TNBCs are more common and have worse prognosis among African Americans and Hispanics compared to other ethnic groups [18]. Some of this health disparity is undoubtedly due to differences in access to medical care, treatment or socioeconomic status [19-21]. However, it has been shown that access to medical care cannot completely remove this disparity [22-24], and there is a biological disadvantage for survival in African American women [10, 25, 26].

There are several active areas of research working toward improved treatments for TNBCs. The use of platinum agents in combination with standard therapy is currently being studied in cancers that have a mutation in *BRCA1*, a gene important in double-strand DNA break repair, as these cancers cannot repair the damage to DNA caused by platinum agents [27, 28]. This may be a promising treatment regimen in TNBCs as cancers with *BRCA1* mutations are often TNBCs. PARP inhibitors have also shown some success, but will possibly only work in *BRCA1* and *BRCA2* mutant cancers as they rely on synthetic lethality, which is the targeting of multiple pathways that would compensate if only one were targeted [29, 30]. An anti-epidermal growth factor receptor (EGFR) antibody has also been attempted, though the response rate was only 20% as the EGFR pathway was deactivated by the treatment in only 25% of the cases suggesting compensating mechanisms [31]. In addition, there is promise in the use of anti-angiogenic therapies for TNBCs [32, 33]. While all of these approaches are currently in clinical trials, none of these have yet made it to the clinic.

Due to health disparities, poor prognosis and lack of treatments, more research needs to be done to improve the treatment of TNBCs. In addition to these problems, resistance to chemotherapy is a concern for all cancers. However, in TNBCs, which rely entirely on chemotherapy treatment, drug resistance is of even larger concern. Though the pathologically complete response rate is high in TNBCs at 22%, the prognosis for cancers that do not respond to chemotherapy is very poor [34]. This suggests a high level of intrinsic resistance. Interestingly, approximately one third of metastatic breast cancer patients with intrinsic taxane resistance are patients with TNBCs [35]. Furthermore, the high rate of recurrence among TNBCs suggests an increased opportunity for acquired multi-drug resistance.

B. Cancer Drug Resistance

Cancer drug resistance limits the effectiveness of chemotherapeutics. It is estimated that 90% of treatment failure in metastatic cancers is due to drug resistance [36]. There are two different types of drug resistance: intrinsic and acquired [37]. Intrinsic drug resistance denotes that treatment is ineffective because resistance-mediating factors were already present in a tumor prior to treatment. Acquired resistance develops during treatment by adaptive responses or mutations in a previously sensitive tumor; resistance can also be acquired by the selection of a resistant population of cells, which can cause recurrence.

There are many known mechanisms of cancer drug resistance (Figure 1). Many mechanisms affect the balance of drug entry and exit to prevent drug accumulation, as accumulation of chemotherapeutics in cells is crucial for drugs to bind to their molecular targets. Cancer cells can alter drug accumulation by inhibiting drug uptake or, more commonly, increasing drug efflux. However, there are other ways of achieving a decrease of drug that is free to bind to molecular targets such as compartmentalizing the drug away from the target or altering drug metabolism so the active form of the drug is not available. Aside from drug availability, alteration of drug targets, DNA damage repair mechanisms and cell crisis response mechanisms can all lead to cancer cell survival. Increases in drug targets may allow cancer cells to compensate in the presence of a drug. In addition, the mutation of a drug target that inhibits drug binding can render a drug ineffective. Cancer cells are well known to hijack endogenous mechanisms to promote survival. A cancer cell that is capable of repairing DNA damage efficiently may be resistant to drugs that directly cause DNA damage. In addition, cancers that have hijacked cell cycle arrest or apoptosis pathways may be capable of escaping the cell death that chemotherapeutics should cause.

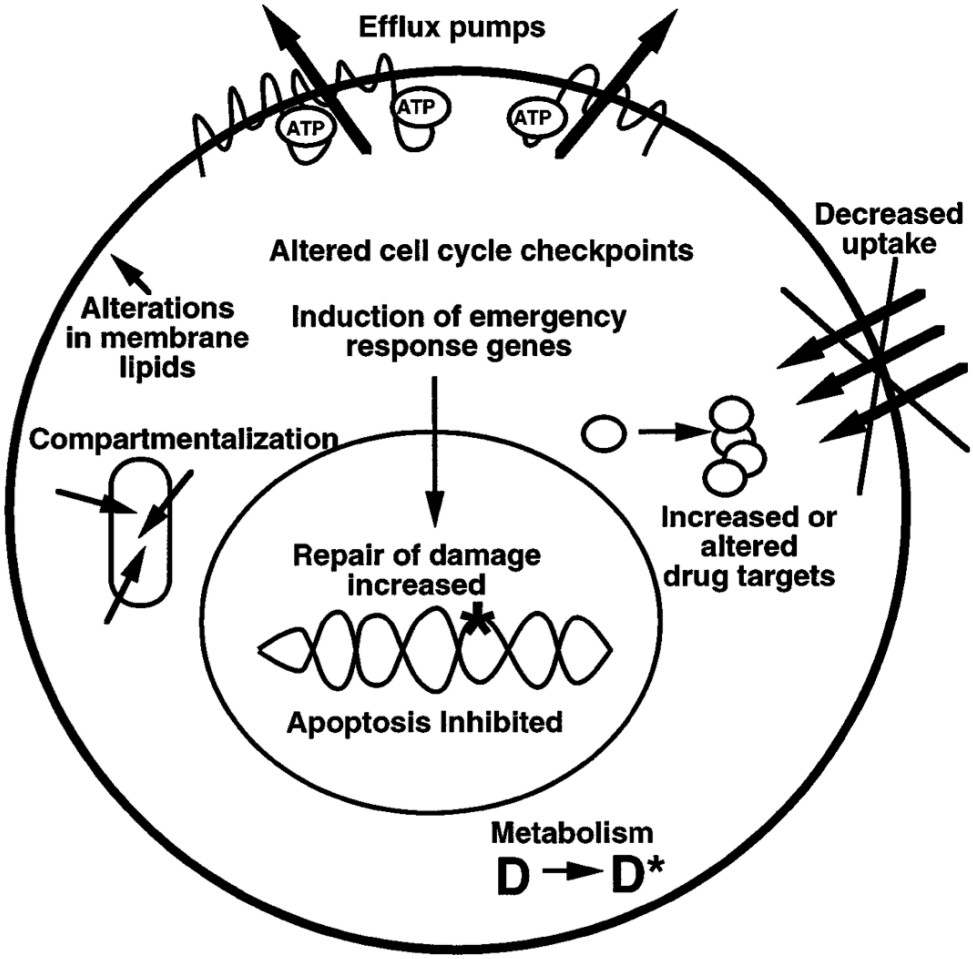


Figure 1. Mechanisms of Cancer Drug Resistance. Possible mechanisms by which cancer cells can evade drug-induced cell death. Resistance in cells may be a single mechanism or a combination of mechanisms (D=Drug; D*=Drug metabolite). Originally published in [38].

Due to toxicity to normal cells, doses of chemotherapeutics may be limited, which rules out any possibility of simply giving more drug to overcome some of these resistance mechanisms. However, there are several methods to overcome resistance to a drug [39]. Treating with a different drug to which the cells are sensitive is a simple

approach. However, many drug resistance mechanisms can cause multidrug resistance, conferring resistance to multiple drugs, limiting this approach. To avoid this problem, patients are standardly treated with a “cocktail” of chemotherapy drugs providing multiple drugs at once with different modes of entry and cellular targets and so a multiple hit approach, maximizing cancer cell killing. Another possibility to overcoming drug resistance is combining chemotherapy with another drug that will make the cells more sensitive to the original drug or otherwise enhances the effect of the chemotherapeutic. These combination therapies are molecularly targeted to improve response to chemotherapeutics and have shown varying levels of success [37].

It is important to study resistance of specific drugs in specific cancers as both factors impact the development and, therefore, the reversion of drug resistance. One of the first line treatments for breast cancer is the mitotic inhibitor from the taxane drug class, paclitaxel. Paclitaxel is a very successful drug; however, some breast cancers are intrinsically resistant and others can acquire resistance to the drug. Consequently, there is a need for methods to overcome paclitaxel resistance particularly in TNBCs, which rely heavily on chemotherapy treatment.

II. Paclitaxel

Taxanes are a class of microtubule-stabilizing agents or mitotic inhibitors [40]. Microtubules are protein polymer filaments, which are important in cellular functions such as cell shape, movement, signaling, division and mitosis [41]. These filaments are hollow cylinders made up of α and β tubulin heterodimers and display dynamic instability and treadmilling behavior [42]. Dynamic instability is the process of constant lengthening and shortening of the microtubules by the association and dissociation of α/β tubulin; this process can be held in stead state by balancing the lengthening and shortening of the

microtubules or can be overpowered by one or the other causing an imbalance in the process [41, 43, 44]. Treadmilling describes the lengthening of one end of the tubule concurrent with the shortening at the other end [45]. These processes occur in all cells and are regulated by microtubule-associated proteins (MAPs) such as tau, which binds and stabilizes microtubules [46]. The dynamic polymerization and depolymerization of microtubules is essential for cell division and chromosome segregation during mitosis [43]. Taxanes bind to β-tubulin on a site that is only accessible when tubulin is assembled [42, 47, 48]. When taxanes are bound to assembled tubulin, they suppress microtubule dynamics by stabilizing the microtubule and preventing disassembly [41]. This stabilization prevents the cells from undergoing mitosis and induces mitotic catastrophe and apoptosis [41, 49].

The first drug in the taxane family to be discovered, paclitaxel, was first isolated in 1969 from the bark of the pacific yew tree (*Taxus brevifolia*) [50]. After decades of improving synthesis methods, Taxus species cell cultures are now used with elicitor compounds to produce paclitaxel [51]. However, the difficulty of producing paclitaxel continues to create a shortage of the drug [52]. Intravenously administered paclitaxel presents nonlinear pharmacokinetics, is metabolized in the liver and is eliminated through the biliary system [53, 54]. Due to the high hydrophobicity of paclitaxel, it must be administered in a formulation of alcohol and Cremophor ® EL (polyoxyethylated castor oil) to assist delivery; this formulation can cause severe hypersensitivity reactions; though, the frequency of these reactions has been reduced with shorter infusion times and premedication with corticosteroids and anti-histamines [52]. In addition, paclitaxel has severe side effects such as neutropenia and neuropathy that significantly limit dosage [55]. However, paclitaxel can be administered with acceptable toxicity and is still considered a successful treatment for non-small-cell lung, ovarian and breast cancers in some patients [56-58].

Paclitaxel is a first-line treatment for breast cancer and has a response rate between 25 and 69% [59]. Paclitaxel can be given as a single agent but is often given in combination with an anthracycline, such as doxorubicin, as it improves disease-free survival and overall survival [60, 61]. In addition, paclitaxel is often given with an anthracycline and an alkylating agent, such as cyclophosphamide [62]. Paclitaxel given as a 1-hour infusion weekly, rather than as a 3-hour infusion every 3 weeks, improves both disease-free survival and overall survival [63]. For HER2 positive breast cancers, neoadjuvant paclitaxel and doxorubicin followed by cyclophosphamide, methotrexate, and fluorouracil given in combination with adjuvant and neoadjuvant trastuzumab, a HER2 inhibitor, improves clinical and pathological response by 22% [64]. Paclitaxel is currently in a clinical trial, CALGB-40603 (NCT00861705), in TNBCs to evaluate neoadjuvant chemotherapy combination of carboplatin with the standard paclitaxel and doxorubicin plus cyclophosphamide treatment. Although paclitaxel is an efficacious drug, resistance is a problem as with all cancer drugs tested to date.

A. Paclitaxel Resistance in Breast Cancers

Both intrinsic and acquired taxane resistance are common in cancers [45]. There are many mechanisms of paclitaxel resistance that have been previously shown to potentially be relevant in breast cancers. Importantly, though all of these mechanisms have been seen *in vitro*, none have yet been convincingly shown to be a clinically relevant mechanism of paclitaxel resistance in breast or any other cancer with the exception of the up-regulation of multidrug resistance proteins [65, 66]. Due to this clinical relevance, the up-regulation of multidrug resistance proteins is of particular importance and will be discussed at length. In addition, the next most likely mechanism to be relevant to resistance is changes in drug metabolism, which will also be discussed

at length. Resistance through drug target alteration has been shown with mutations in the paclitaxel target protein, β-tubulin [67-69]. In addition, changes in the expression profile of the seven different β-tubulin isotypes can affect paclitaxel action as isotypes βIII and βIV require more bound paclitaxel to stabilize the microtubule [70]. Variation of the expression of MAPs, which regulate microtubule dynamics, can also confer resistance to paclitaxel. Examples are high levels of microtubule stabilizing proteins, such as tau and stathmin, or low levels of destabilizing proteins, such as MAP4 [71-73]. Deregulation of the cell cycle, specifically in the spindle assembly checkpoint, and associated proteins can also lead to resistance [74-79]. Furthermore, changes in levels of apoptosis-related proteins such as p53, Bcl-2, Bcl-xL, Akt, survivin, XIAP, and NFκB to inhibit apoptosis can cause resistance [80-85]. The upregulation of HER2 can also confer paclitaxel resistance, which seems to show some clinical significance in the beneficial effect of combining trastuzumab with paclitaxel [86]. It is important to note that, for the present work, the upregulation of HER2 and the changes of p53 will not be mechanisms of concern, as the cell line used in this study does not overexpress HER2 and harbors mutant p53 protein. Though HER2 overexpression would not be an important clinical factor for TNBCs, it is possible that changes in p53 could be important clinically for TNBCs that harbor wild-type p53 protein. Importantly, most of these mechanisms have yet to be shown to have clinical relevance with the exception of multidrug resistance proteins and the alteration of drug metabolism, which are the most likely candidates for a resistance mechanism

B. Multidrug Resistance Proteins

ATP binding cassette (ABC) proteins are transporters that utilize ATP to transport a specific substrate or group of substrates across the cell membrane. Substrates for

these proteins can be metal ions, sugars, amino acids, peptides, proteins, hydrophobic compounds and metabolites [87]. These transporters are believed to operate by alternating access from one side of the membrane to the other by a conformational change [88]. ATP hydrolysis provides the energy to switch the protein from facing inside the cell when a substrate is bound to outward facing to then release the substrate. Drug interactions are a possibility for transporters with multiple substrates as substrates can act as competitive inhibitors [38]. ABC transporters that transport cancer drugs have been shown to be important in resistance by preventing cancer drug accumulation in cells. There are 48 known human ABC genes that are separated into seven distinct subfamilies based on organization of domains and amino acid homology which leads to differences in substrate specificity [87]. For example, the MDR proteins transport a variety of hydrophobic compounds whereas the MRP proteins transport organic anions, such as glutathione conjugates of compounds [87]. P-glycoprotein (P-gp, MDR1, ABCB1), BCRP (ABCG2), and MRP2 (ABCC2) have all been shown to be widely expressed in tumors and linked to drug resistance [89, 90]. The ability to inhibit these proteins has the potential to greatly improve chemotherapy treatment and so have been studied for many years [91].

P-gp was the first ABC efflux pump to be identified and has been studied extensively. P-gp is located on chromosome 9q31.1 and is ubiquitously expressed, though it is most prominently expressed in the liver and blood-brain barrier. P-gp is a highly promiscuous transporter, which binds electrically neutral and positively charged hydrophobic drugs [38]; hundreds of substrates have been identified, including several chemotherapeutics [89, 90]. It has been shown that treatment with several anti-cancer drugs in colon cancer cells can induce pregnane X receptor, which induces P-gp expression and decreases drug accumulation [92]. In addition, inhibition of P-gp has

been shown to restore drug sensitivity to resistant cells in culture that overexpress the protein [93].

Notably, paclitaxel is a substrate of P-gp, and P-gp has been implicated in paclitaxel resistance in breast cancer [94]. The analysis of 60 cells lines revealed that the lower the P-gp gene expression, the higher the sensitivity to paclitaxel [95]. In cell lines derived from breast carcinoma specimens, a similar correlation between high P-gp expression and high paclitaxel resistance was also shown [96]. P-gp is expressed in normal breast tissue, though usually in lower levels than in in cancer tissue [97]. Approximately 40% of untreated breast cancers express P-gp compared to 52% of cancers that have been treated with a P-gp substrate [97]. Interestingly, the level of P-gp positive cancers is higher in cancers within one month post-treatment at 56% compared to only 36% of cancers more than one month post-treatment suggesting that the effect of chemotherapy is transient [97]. Perhaps most striking is a comparison of tumors pre- and post-neoadjuvant treatment; only 43% of tumors were positive for P-gp prior to treatment compared to 64% positive afterward, which is a 37% induction of P-gp expression [97]. High expression of P-gp has been associated with poor response of locally advanced breast cancer to neoadjuvant chemotherapy, though the therapy in these studies did not include paclitaxel [98, 99]. Of critical importance, tumors with high P-gp expression demonstrate faster drug clearance and lower drug accumulation in the tumor [100-105].

Thus far, no P-gp inhibitors have made it to clinical use in combination with chemotherapeutics, though many clinical trials have been conducted with various inhibitors and chemotherapy regimens. When pooled, four studies of the P-gp inhibitor verapamil in advanced breast cancer patients refectory to anthracycline-containing treatment regimens, showed 15% re-sensitization [97]. However, later studies proved verapamil to have a dose limiting and life-threating cardiotoxicity [106]. The P-gp

inhibitor, biricodar, showed a partial response of 11% in combination with paclitaxel in locally advanced breast cancer patients refractory to paclitaxel [107]. Two other inhibitors, zosuquidar and tariquidar, have shown disappointing clinical results in breast cancer suggesting there may be redundancy in efflux pumps that must be addressed [37, 108, 109]. Recently the importance of the dynamics between P-gp and the membrane has become apparent, which may lead a new generation of inhibitors [91]. In summary, although there have been only minor clinical successes in reversing drug resistance with P-gp inhibitors, P-gp remains an important potential target to re-sensitize breast cancers that are refractory to or likely to be resistant to paclitaxel treatment as P-gp has been shown to be an important factor in tumors and targeting of P-gp can reverse some clinical drug resistance.

C. Drug Metabolism

Drug metabolism is separated into three phases although not all drugs have to go through all phases. Phase I metabolism reactions are often catalyzed by cytochrome P450 (CYP) enzymes and include oxidation, reduction, hydrolysis, cyclization, or decyclization reactions. Some of the resulting polar metabolites can be excreted at this point. Otherwise, the metabolite will have to undergo a phase II metabolism reaction, which include methylation, sulfation, acetylation, glucuronidation, glutathione conjugation, or glycine conjugation, catalyzed by a transferase to produce highly polar conjugate molecules that are usually less active and must be actively transported due to the addition of large anionic groups [110]. In phase III metabolism, these conjugates can be further metabolized and excreted from cells by ABC transporters with the anionic groups acting as an affinity tag for the transporters [111, 112]. Outside of the cell the conjugates and metabolites are either further metabolized or excreted [113].

CYP enzymes are a family of mono-oxygenases, which catalyze organic substance oxidation. Most commonly, CYPs insert one oxygen atom into the aliphatic position of an organic substrate and reduce the other oxygen atom to water [114]:

$$RH + O_2 + NADPH + H^+ \rightarrow ROH + H_2O + NADP^+$$

Generally membrane-associated proteins , human CYPs are located primarily in the endoplasmic reticulum but can also be located in the inner membrane of the mitochondria or the plasma membrane [115]. These enzymes are present in most tissues, though the expression is specific to each CYP and its function [116]. CYPs can play important roles in the formation and metabolism of lipids, steroids or xenobiotic substances such as toxic chemicals and drugs and may metabolize one, few or many substrates [117]. Total CYP activity accounts for approximately 75% of total drug metabolism [118]. Although some drugs can be activated by this metabolism, most drugs are deactivated by CYPs.

Changes in drug metabolism mechanisms in cancer cells can play a role in drug resistance [36, 37]. For drugs that must undergo metabolism for activation, a decrease in metabolism will decrease the amount of active drug available inside cells rendering the cells more resistant to the drug. More commonly drugs are inactivated by metabolism, and with an increase of metabolism there is a decrease in the amount of active drug available to bind to intracellular targets. Therefore, a decrease or increase of metabolism enzyme expression or activity could affect how cancer cells react to drugs. Changes in these enzymes can lead to cancer drug resistance or higher sensitivity to drugs. Therefore, manipulating these mechanisms with combination therapies provides a strategy for overcoming drug resistance. Conversely, it is also possible that drug combinations could dangerously increase drug toxicities. Importantly, this makes the study of individual drug metabolism pathways in individual cancers important to overcoming cancer drug resistance as well as predicting harmful drug-drug interactions.

Paclitaxel has two main metabolites in humans: 6α-hydroxypaclitaxel (6α-OHP) and C3'-hydroxypaclitaxel (C3'-OHP) (Figure 2). 6α-OHP is formed by CYP2C8 and C3'-OHP is formed by CYP3A4 [119]. 6α-OHP has been shown to be 30-fold less active than paclitaxel [120], and C3'-OHP shown to have no activity [121]. 6α-OHP is the most important metabolite making up approximately 60% of excreted paclitaxel compared to only 10% of C3'-OHP [122]. There is one other metabolite, which has very low abundance and was originally reported to be di-hydroxypaclitaxel [123] but was shown in a later study to be C2-hydroxypaclitaxel formed by CYP3A4 [124]. It has also been seen that induction of CYP3A4 due to concomitant drug use can change the metabolite profile of paclitaxel in a patient [54]. Crucially, it has been shown that it is possible for drug interactions to affect CYPs and, therefore, the efficacy of paclitaxel [125]. Enhanced ability of a cell to metabolize paclitaxel would protect a cell from the toxic effects of paclitaxel, and due to the larger contribution of CYP2C8 in paclitaxel metabolism it is likely that this enzyme would be of greater importance. Consequently, overexpression of CYP2C8 in a cancer cell could explain a high tolerance for paclitaxel and so confer resistance.

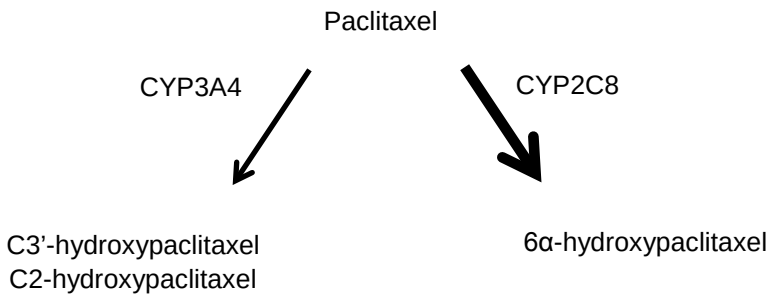


Figure 2. Metabolism of Paclitaxel in Humans. The major metabolism pathway through CYP2C8 produces 6α-hydroxypaclitaxel, a 30-fold less active metabolite. The metabolites produced by CYP3A4 are inactive and much less abundant. Adapted from [124, 126].

The *CYP2C8* gene is located on chromosome 10q24 along with the other CYP2C family members [127]. The *CYP2C8* gene has several polymorphisms, and though the high inter-individual variability in paclitaxel pharmacokinetics is well known [128], it has yet to be strongly linked to *CYP2C8* polymorphisms [129]. Interestingly, it has been suggested that the lack of consensus on a link between paclitaxel pharmacokinetics and *CYP2C8* polymorphisms may be due to the lack of studies that simultaneously consider polymorphisms of *ABCB1* and *CYP2C8*. CYP2C8 protein is highly expressed in the human liver making up approximately 7% of the total microsomal CYP content [130, 131]. CYP2C8 has many endogenous and exogenous substrates and carries out the phase I oxidative metabolism of at least 5% of drugs cleared by the liver [132]. CYP2C8 metabolizes drugs in several drug classes such as thiazolidinediones, meglitinides, NSAIDs, anti-malarials and taxanes; CYP2C8 is often a secondary mechanism of metabolism, but it can be the primary mechanism, as it is with paclitaxel, choloroquine, rosiglitazone and repaglinide [129]. In addition to the liver, CYP2C8 mRNA has been shown to be expressed in several other tissues including the kidney, intestine, adrenal gland, brain, mammary gland, ovary, heart and, notably, in breast cancer tumors [116, 133-136]. Importantly, overexpression of CYP2C8 has been shown to contribute to acquired paclitaxel resistance by increasing paclitaxel metabolism in colon cancer cells with no contribution of CYP3A4 [137]. Taken together, these data suggest alteration of paclitaxel metabolism may be an important mechanism of paclitaxel resistance in breast cancers.

III. Dietary Supplements

Complementary and alternative medicine (CAM) encompasses a wide range of health treatments that are not used in conventional medicine and are often supported by

little or no scientific evidence. In western countries, 40-50% of all cancer patients use some type of CAM [138, 139]. The most common user is female, highly educated and of high socioeconomic status. Therefore, the most prevalent CAM users are women with breast cancer [139, 140]. There are many reasons for people to choose CAM therapies; stress reduction, decreasing side effects, improving strength and the immune system, the desire of a ‘more holistic’ treatment and autonomy are all important factors reported [139, 141-145]. In addition, many hope to prevent cancer recurrence or to fight cancer directly. CAM therapies present many problems, which are confused by the fact that very few people disclose their CAM use to their oncologists [146, 147]. CAM therapies can cause side effects of their own accord, but they can also cause dangerous drug interactions. Perhaps the most dangerous problem for cancer patients is the postponing or omission of proper treatment. Finally, CAM therapies can have a large financial impact on both individuals and health care systems.

Several of the most common CAMs used by breast cancer patients fall into the dietary supplements category [148]. The category of dietary supplements encompasses a range of products from vitamins to botanicals. In the United States, dietary supplements are a multi-billion dollar per year industry [149]. The use of dietary supplements has been steadily rising in the United States. One study in 2002 showed, 14% of the general population and 16% of prescription drug users concurrently take some kind of dietary supplement [150]. In 2005, it was estimated that the prevalence of botanicals use in the United States is approximately 12.1-18.6% [151]. However, by 2011 it was reported that 50% of Americans reported using dietary supplements, and approximately 20% of adults were using botanicals [152]. Importantly, dietary supplements are not regulated like foods or drugs but instead fall under the Dietary Supplement Health and Education Act of 1994, which restricts the authority of the FDA over these products [153]. Therefore, many of these products have been insufficiently

tested for safety, efficacy and often quality control. Though viewed by the public as generally safe, there are many examples of botanicals causing harmful herb-drug interactions [154].

However, botanicals can also have beneficial health effects and provide the basis of many medications. It is estimated that from 1 in 3 to 1 in 2 of currently used drugs were derived from plants [155]. Indeed, over 60% of clinically used anti-cancer agents are derived from natural sources such as plants, marine organisms and microorganisms [156]. Vinblastine and vincristine were discovered in the 1950s, which spurred an extensive program initiated by the National Cancer Institute in 1960 producing most of these discoveries including taxanes and camptothecins. However, since these compounds have reached the clinic, no other plant-derived anti-cancer agents have made it to general use [156].

To conclude, botanicals and their derivatives have the potential to be both harmful and beneficial alone and in combination with prescription drugs. Considering that women with breast cancer are the most common CAM users, more research is needed to ensure the safety of patients taking botanicals. In addition, in light of the need for more treatments, especially for TNBCs, more research is needed to determine if any of these botanicals or their derivatives can improve treatments. Resveratrol is a highly studied, botanically derived chemical of particular interest, which has been shown to have a wide variety of health effects.

IV. Resveratrol

Resveratrol is made by a variety of plant species in response to fungal infection or exposure to ultraviolet light, and therefore classified as a phytoalexin [157]. Assaults cause a hormone response in plants, which induces gene expression of resveratrol

synthase to produce resveratrol [158]. Resveratrol functions as a plant pathogen toxin as well as and inducer of plant cell death when stress cannot be counteracted [159]. Due to widespread production in plants it is also present in the human diet though in very low amounts and is perhaps best known to be in grapes, peanuts and red wine [160]. Resveratrol is a polyphenol and a stilbenoid, a chemical with a stilbene backbone (Figure 3). Highly lipophilic and insoluble in water, resveratrol is highly soluble in ethanol and DMSO and so many studies are performed using one of these solutes [161]. It is stable in solution and human plasma with the exception of extended exposure to light or high pH environments [162].

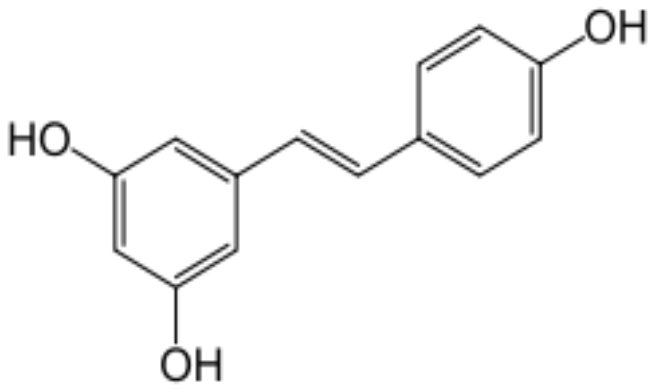


Figure 3. Chemical Structure of Resveratrol. Trans-3,4',5-trihydroxystilbene is a highly lipophilic polyphenol stilbenoid.

Resveratrol is being studied for prevention or delaying progression of aging, cardiovascular diseases, neurodegenerative diseases, as well as cancer prevention and treatment [163]. Generally, the protective effects are produced with low nanomolar to micromolar concentrations of resveratrol whereas anti-cancer effects are produced with high micromolar concentrations. This biphasic effect of resveratrol coincides with previous work showing that cancer cells treated with low concentrations of resveratrol increase proliferation compared to higher concentrations that cause cancer cell death

[164]. The anti-oxidant and anti-inflammatory effects of resveratrol seem to be important factors for the beneficial cardiovascular, neuroprotective, and anti-aging effects [163]. Resveratrol demonstrates protective effects at low μM concentrations in relation to multiple neurodegenerative disorders, which are a group of progressive disorders sharing inflammatory status and accumulation of reactive oxygen species causing neuron damage and death such as Alzheimer, Parkinson and Huntington Disease. The anti-inflammatory effects, reduction of oxidative stress, inhibition of apoptosis, and effects on neurological function are all important in neurodegenerative disease protection effects of resveratrol [163]. There are many targets that have been shown to be important in these actions many of which are also important in the cardioprotective, anti-ageing and anti-cancer effects. Of particular interest is the involvement of the activation of SIRT1 through the AMPK pathway, which is also important for the cardioprotective effects [163]. For resveratrol-induced chemoprevention, regulation of carcinogen metabolism as well as cell proliferation inhibition and apoptosis induction are important [165]. Resveratrol has been shown to prevent or delay the onset of cancer [166, 167]. In breast cancer, resveratrol has been shown to exhibit anti-initiation, anti-promotion and anti-progression activities in both hormone-sensitive and hormone-resistant breast cancers [168]. These effects of cancer prevention seem to be related to xenobiotic metabolism regulation as well as anti-inflammatory, anti-proliferative and pro-apoptotic effects [168]. Details of anti-cancer activity of resveratrol will be discussed at length below. Despite extensive pre-clinical work with resveratrol, human safety and efficacy studies are scarce [169]. However, many studies have been conducted attempting to elucidate the metabolism and complicated pharmacokinetics of resveratrol in humans.

A. Resveratrol Pharmacokinetics

Resveratrol is metabolized very rapidly in humans [170]. Free resveratrol and its metabolites are both highly bound to plasma proteins, which would suggests poor availability [171]. However, it has been shown that protein-bound resveratrol can still be transported into cells as free resveratrol through carrier-mediated transport [172]. The predominate metabolites consist of two sulfated and two glucuronidated forms, Sulfotransferases form the sulfated metabolites, and glucuronidated metabolites are formed by the UDP-glucoronosyltransferases 1A family [173]. In humans, the sulfated metabolite, resveratrol-3-*O*-sufate, is the most prominent [174]. Importantly, resveratrol metabolites have shown little or no anti-cancer activity with the exception of some resveratrol sulfates showing low cytotoxicity in breast cancer cells and one study showing activity in colon cancer cells [175-178]. However, physiologically relevant amounts of sulfate metabolites have been shown to be regenerated into resveratrol and cause cell proliferation inhibition of cultured colon cancer cells through autophagy and senescence [178]. Therefore, it is likely that any effects of resveratrol treatment are due to resveratrol activity and not the activity of metabolites.

Resveratrol is very well tolerated in humans [179]. After 8 days of daily oral resveratrol dosing in healthy volunteers, no toxicities presented with up to 1 gram per day, and only mild gastrointestinal toxicities were observed with 5 grams per day. The half-life of resveratrol has been seen to vary from 2.9 to 11.5 hours [180]. A seemingly poor oral bioavailability, which has been attributed to poor intestinal absorption or extensive intestinal metabolism, has been a major barrier to the potential clinical use of resveratrol [181-184]. However, resveratrol demonstrates high intestinal absorption, as approximately 70% enters enterocytes by passive diffusion in humans [185]. In rodents it has been shown that resveratrol is highly metabolized in the enterocytes, and MRP2 and

BCRP, but not P-gp, then excrete the resveratrol metabolites back into the intestinal lumen limiting intestinal absorption [186, 187]. In addition, resveratrol is highly excreted in urine, and has highly variable excretion in feces, which suggests the occurrence of the enterohepatic cycle [185]. Enteric recirculation is further supported by a secondary peak in resveratrol plasma concentration at 6 hours following the primary peak at 1 hour [185]. Interestingly, although resveratrol excretion shows high inter-individual variability, all the subjects had a portion, between 2 and 30%, of resveratrol that was not recovered in either the urine or feces; the fate of which was unknown. These phenomena create a confusing picture of resveratrol pharmacokinetics, which has spurred interest in resveratrol analogues and drug delivery systems to improve bioavailability.

The problem of bioavailability has been especially troubling for the anti-cancer effects of resveratrol that only occur at high micromolar concentrations. However, recently a series of studies have emerged suggesting that bioavailability may not be as major of a problem as once believed. Previously, plasma levels of resveratrol and its metabolites have been shown to be very low with gram doses resulting in only low micromolar amounts in plasma [170]. However, a new study has shown plasma concentrations of resveratrol metabolites in humans to be higher than previous estimations due to a lack of metabolite standards [178]. Critically, this study showed in a mouse model that the sulfated metabolite of resveratrol, which has the highest plasma concentrations of all the resveratrol forms, enters cells and can be converted back to free resveratrol. The uptake of the sulfate metabolite is likely dependent on anion transporter SLC22A9 and anion-transporting polypeptides SLCO1B1 and SLCO1B3 [178]. This mechanism of resveratrol regeneration resulted in a sustained exposure to resveratrol and suggests that resveratrol can be regenerated from conjugates as long as they are present, which can be at least 24 hours [174]. At 1 gram of resveratrol per day, 20 to 30 μM concentrations of resveratrol sulfates can be attained in human plasma,

which is within the range of inhibiting cell proliferation in cancer cells [179]. When resveratrol and resveratrol metabolite levels were measured in the colorectal tissue of colon cancer patients, resveratrol was shown to have accumulated to high micromolar, up to 640 μM, amounts [188]. Although there has been considerable interest in developing resveratrol analogs and delivery systems to promote delivery of efficacious concentrations to target tissues, these studies suggest these extra efforts may not be necessary.

B. Resveratrol Anti-cancer Pharmacodynamics

The anti-cancer effects of resveratrol have been extensively studied in many *in vitro* and *in vivo* human cancer models. Resveratrol has been shown to inhibit angiogenesis, invasion and metastasis, and cell proliferation and to induce cell cycle arrest and apoptosis in various cancers [189, 190]. Interestingly, many of the effects of resveratrol appear to be cell type specific. For example, the type of cell cycle arrest caused by resveratrol, if any, is dependent on cancer cell-type [161]. Additionally, due to phytoestrogen properties, resveratrol may stimulate growth in breast cancers that express ER [191]; however, this effect has been contested [192]. This contradiction may be explained by low resveratrol concentrations inducing proliferation whereas high concentrations suppress proliferation [193]. Finally, resveratrol has been shown to cause senescence, an essentially irreversible form of cell proliferation arrest [194], and autophagy, the effect and relevance of which is unclear [195], in various cancer cells [196]. Autophagy is a multistep process in which autophagolysosomes are formed so cells can destroy proteins or damaged organelles; this process is normally used by cells to promote survival during stress, however, it can also cause cells to undergo cell death [197]. Although resveratrol inhibits proliferation and induces cell death in many cancer

cells, the mechanisms through which resveratrol acts vary widely [161] (Figure 4). Some of the pathways resveratrol has been commonly shown to inhibit are the NF-κB, MAPK, and AP-1 pathways [161]. Resveratrol has also been shown to activate other pathways such as the p53 and FAS pathways [161]. In addition, resveratrol has extensively been shown to suppress protein kinases, growth factors, cell-cycle proteins as well as COX-2 and lipooxygenase [161]. Recently, evidence has shown that resveratrol has the ability to target cancer stem cells, which are capable of self-renewal and differentiation and are believed to the root of tumor heterogeneity [198], through inhibition of fatty acid synthase [199], inhibition of pluripotency maintain factors and epitherlial-mesenchymal transition [200, 201] and metabolic reprogramming [201]. With so many varied effects caused by resveratrol, it is important to study the effects of resveratrol in different cellular backgrounds.

A wide variety of resveratrol effects have been seen even within TNBC cell lines. In MDA-MB-468 cells, resveratrol inhibited TGF-α, PC-cell derived growth factor and insulin-like growth factor 1 receptor mRNA expression and increased TGF-β mRNA [202]. In the 4T1 triple negative cell line, resveratrol inhibited proliferation in culture, but in mice no inhibition of growth or metastasis was seen [203]. Most TNBC data has been obtained using MDA-MB-231 cells. Proliferation inhibition by resveratrol in MDA-MB-231 cells has been attributed to a decrease of reactive oxygen species [204]. Resveratrol has been shown to cause non-apoptotic cell death in MDA-MB-231 cells by decreasing expression and kinase activities of positive G1/S and G2/M cell cycle regulators and inhibiting ribonucleotide reductase activity with no effect on the low expression of p21, p27 or mutant p53 levels and causing no cell cycle arrest [205]. In another study, apoptosis was seen in MDA-MB-231 and attributed to an induction and increased nuclear localization of COX-2; this study also saw an increase of p53 phosphorylation though the significance of this is unclear considering the mutant status of p53 in these

cells [206]. However, resveratrol-induced proliferation inhibition and apoptosis in MDA-MB-231 cells has also been attributed to an increase of serine palmitoyltransferase and neutral sphingomyelinase leading to ceramide accumulation [207]. Resveratrol has also been shown to inhibit the promoter activity of NF-κB, a transcription factor often constitutively active in cancer cells, in a dose dependent manner in MDA-MB-231 cells [208]. Notably, resveratrol was shown to inhibit MDA-MB-231 tumor growth and cause cell death as well as decrease extracellular VEGF, a marker of angiogenesis, in an ectopic mouse model of TNBC [209]

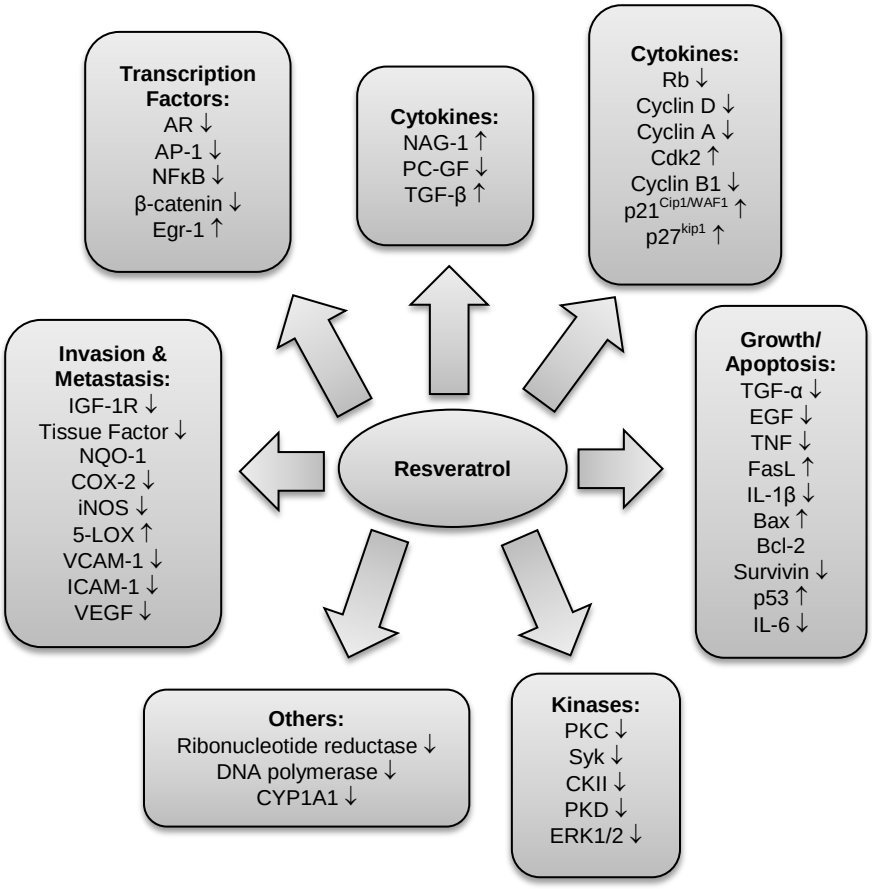


Figure 4. Molecular Mechanisms of Resveratrol Anti-cancer Activity. Adapted from [161]. The known molecular targets of resveratrol in various human cancers are shown grouped by type of target or physiological importance.

One mechanism of particular interest for resveratrol-induced apoptosis that has been generated through various works is through SIRT1 activation (Figure 5). Resveratrol is a well-known activator of the class III histone deacetylase protein, SIRT1. Recently, it was shown that resveratrol acts through the cAMP/PKA/AMPK pathway [210]. This pathway causes a phosphorylation event that dissociates SIRT1 from the negative protein regulator Deleted in Breast Cancer 1 (DBC1) [211]. Furthermore, SIRT1 activation by resveratrol has been shown to directly deacetylate the survivin promoter, decreasing survivin transcription [212]. Survivin is an inhibitor of apoptosis protein that binds and inhibits activated pro-apoptosis caspase 3 and caspase 7. Decreased survivin protein levels allow activated caspase 3 and caspase 7 to induce apoptosis as well as paclitaxel-induced apoptosis [81]. This SIRT1/survivin mechanism provides one of many possible mechanisms of action of resveratrol alone and in combination with other treatments.

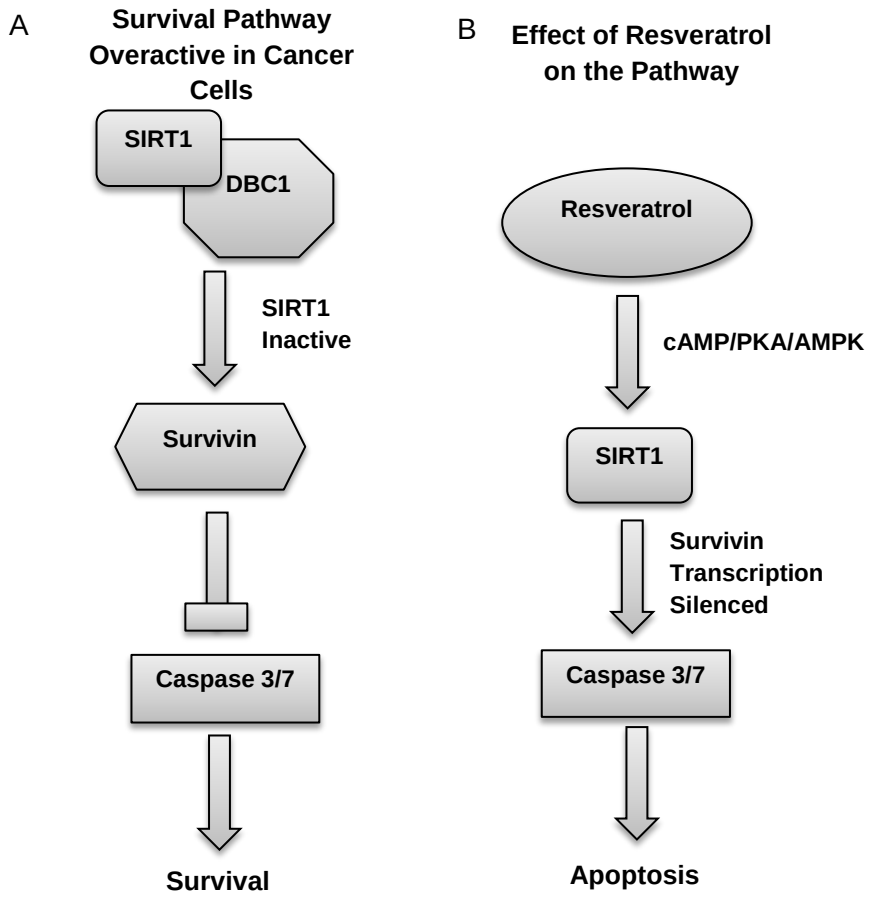


Figure 5. Hypothesized Mechanism of Resveratrol Induced Apoptosis. A) The survival pathway, which is hypothesized to be overactive in cancer cells, where DBC1 binds to SIRT1 and renders SIRT1 inactive allowing for the transcription of survivin. Survivin can then inhibit activated caspase 3 and 7 leading to cell survival. B) The hypothesized effect of resveratrol on the survival pathway where action on the cAMP/PKA/AMPK pathway causes SIRT1 and DBC1 dissociation. Free SIRT1 can then deacetylate the survivin promoter, which silences survivin expression allowing activated caspase 3 and 7 to cause apoptosis.

C. Resveratrol Combination Therapy

There is evidence to suggest that resveratrol has the potential to cause both harmful and beneficial effects when combined with other drugs. Resveratrol is well known to inhibit a variety of CYPs, which could cause both harmful and beneficial drug interactions when taken in high doses [213]. One example of a potential positive CYP interaction is resveratrol was shown to inhibit CYP17A1 and was suggested to be useful in prostate cancer chemoprevention [214]. An example of a CYP interaction that could cause beneficial or harmful effects is the ability of resveratrol to inactivate CYP3A4, which has many substrates [215]. In addition, positive combination effects of resveratrol and irradiation has been seen in multiple cancer cell lines [216]. Resveratrol has also been shown to sensitize multiple cancer types to various chemotherapeutics and other agents [217, 218].

Resveratrol has been shown to have a positive combination effect with paclitaxel in Non-Hodgkin Lymphoma, multiple myeloma, non-small cell lung cancer, lung cancer and human oral epidermoid carcinoma cells [217]. However, the combination effect was attributed to different mechanisms including decreased Bcl-xL expression [219], an increase in p21 [220], a decrease of survivin [221], and decreases in P-gp and Bcl-2 expression [222]. Conversely, it has also been shown that resveratrol can attenuate paclitaxel effects in neuroblastoma and bladder cancer cells in other studies [223-225]. These attenuation effects were also attributed to different mechanisms depending on the cell type such as decreased caspase-7 and caspase-3 expression, decreased PARP cleavage, Bcl-2 activity, decrease of reactive oxygen species, and effects on the cell cycle. Of particular interest, in one study resveratrol attenuated paclitaxel treatment in some breast cancer cells such as triple negatives, but not other breast cancer cells

[226]. These studies suggest resveratrol combination therapy may be cell type specific and clearly more research in the area is warranted.

V. Preliminary Work and Study Objectives

In a previous study from our laboratory, resveratrol was shown to have a biphasic effect on the growth of TNBC cells regardless of BRCA1 status [164]. An effect of promoting cell proliferation at low resveratrol concentrations and inhibiting growth at high resveratrol concentrations was observed by analysis of cell morphology, growth, survival and cell cycle. Interestingly, cells containing truncated, non-functional, mutant BRCA1 were more sensitive to resveratrol than cells with wild-type BRCA1. The difference in resveratrol effect suggests a role of BRCA1 in resveratrol action and further suggests resveratrol may be particularly useful in the small subset of TNBCs with mutant BRCA1. Notably, this data showed for the first time that low micromolar concentrations of resveratrol could sensitize TNBC cells to paclitaxel (Figure 6). This positive combination effect was not different between cells with mutant and wild-type BRCA1.

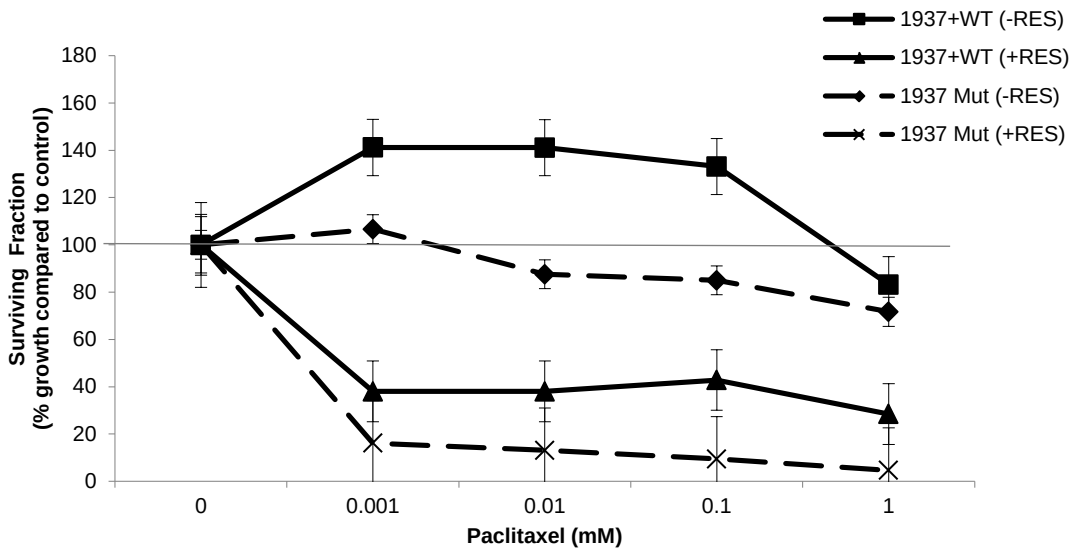


Figure 6. Resveratrol Sensitizes HCC1937 Cells with Mutant or Wild-type BRAC1 to Paclitaxel. From [164]. Surviving fraction of HCC1937 cells with mutant or wild-type BRAC1 after a 24-hour simultaneous treatment of varying concentrations of paclitaxel and 10 μM resveratrol (n=3). Error bars denote standard deviation. Alone 10 μM resveratrol treatment had no effect on cell growth after 24 hours.

This work spurred our laboratory to formulate a hypothesis that resveratrol could be used in TNBC cells both sensitive and resistant to paclitaxel to improve response to paclitaxel. To study paclitaxel resistance in TNBCs a cellular model was developed that mimics the selection of resistant cells in a tumor, which can lead to the recurrence of a resistant tumor. The objectives of this study were to determine the effects of resveratrol as a single agent and the effects of resveratrol in combination with paclitaxel treatment in paclitaxel-sensitive and -resistant cells. Here data is presented that shows that resveratrol induced cell proliferation inhibition, senescence, and apoptosis in paclitaxel-sensitive and -resistant TNBC cells. Importantly, the data show that resveratrol can be used in combination with paclitaxel to re-sensitize the resistant cells back to the parental

level and also to decrease the resistance of both resistant and parental cells below the original parental level. This effect of resveratrol on the paclitaxel sensitivity of these cells may be linked to the known ability of resveratrol to inhibit P-gp and CYP2C8 both of which were found in this study to be involved in the paclitaxel-resistance of our model. Overall, this work suggests that resveratrol can be used alone and in combination with paclitaxel in TNBCs regardless of the status of paclitaxel sensitivity.

CHAPTER 2: MATERIALS and METHODS

I. CELL CULTURE

A. Cell Culture and Reagents

MDA-MB-231 cells (ATCC; Manassas, VA) were cultured in DMEM media (Corning cellgro; Corning, NY) with 10% Hyclone Cosmic Calf Serum (Thermo Fisher Scientific; Waltham, MA) at 37°C, 5% CO_2 and 95% humidity. For regular cell sub-culture, cells were washed with 1X Hank's Balanced Salt Solution (HBSS) and removed from the plate with 0.05% trypsin treatment for approximately 30 seconds. Resveratrol and paclitaxel (Sigma-Aldrich; St. Louis, MO) were dissolved in DMSO and further diluted with 1X HBSS. All treatments were conducted in the dark to preserve the stability of both light-sensitive drugs.

B. Mycoplasma Testing

Cells were periodically tested with the PCR-based VenorGeM Mycoplasma Detection Kit (Sigma-Aldrich; St. Louis, MO) per manufacturer's instruction to ensure mycoplasma contamination was not present. 100 µL samples of media from cell culture were boiled at 95°C for 5 minutes, centrifuged and put on ice. 25 µL of PCR Mastermix (1 U *Taq* Polymerase, and 10% each of 10X reaction buffer, primer/nucleotide mix, and internal control) was mixed for each reaction. 23 µL of Mastermix was mixed with 2 µL of sample, positive control or DEPC-water as a negative control. Samples were run on an Mastercycler PCR machine (Eppendorf; Hamburg, Germany) with the following thermal cycle program: 1 cycle 94°C for 2 minutes; 39 cycles 94°C for 30 seconds, 55°C for 1

minute, 72°C for 30 seconds; cool down to 4°C. Samples were then mixed 5 µL to 1 µL of 6X loading buffer and run on an ethidium bromide agarose gel (1.5% agarose in 1X TBE, 0.02% ethidium bromide) for 20 minutes at 100V. The gel was then photographed using a GDS-8000 gel imaging system (UVP; Upland, CA).

II. RESISTANT CELL LINE GENERATION

To generate the paclitaxel-resistant line, an intermittent, stepwise method of treatment was used [227]. MDA-MB-231 cells were treated with the determined 24-hour IC_{30} of paclitaxel for 3-4 days. The drug was then removed for 3-4 days before treating again. This interval lasted for 2-4 weeks before the treatment was increased to the IC_{40}. This process was repeated until the cells were growing successfully under IC_{60} treatment of paclitaxel. After these cells were obtained, they were no longer grown in the presence of paclitaxel. Single-cell derived clones were obtained by limiting dilution in which cells were serially diluted across a 96-well plate and single clones were expanded. Expansion was conducted by trypsinization of cells and plating cells onto progressively larger plates.

III. CELL PROLIFERATION ASSAYS

A. Methylene Blue Cell Proliferation Assay

Cells were plated on 96 well plates at 2,000-10,000 cells per well, allowed to attach to the plate overnight, and treated with paclitaxel or resveratrol for 1-5 days. The media was aspirated, and cells were fixed in methanol for 15 minutes and then stained with 50 µL of 0.05% methylene blue stain (Ricca Chemical; Arlington, TX) for 10 minutes

[228]. The plates were washed with deionized water, dried and de-stained with 100 µL of 0.5 M hydrochloric acid solution. The absorbance was then measured using a plate reader at 610 nm. For IC_{50} determinations, cells were treated with a serial dilution of paclitaxel or resveratrol. GraphPad Prism version 4.00 for Windows (GraphPad Software; San Diego, CA) was used to calculate IC_{50} values.

B. Cell Count Proliferation Assay

To measure cell proliferation, total cell counts were completed. In each well of 12-well plates 15,000 cells were plated, allowed to attach to the plate overnight, and treated with resveratrol or DMSO vehicle control. Cells were collected by trypsinization and counted with a Z series Coulter Counter Cell and Particle Counter (Beckman Coulter, Inc.; Pasadena, CA) at 24, 48 and 72 hours post-treatment.

C. Cell Survival Assays

Two colony formation assays were performed. First, a plating efficiency assay was conducted as previously shown with modification [229]. Cells were plated at a density of $3x10^5$ on T25 flasks, allowed to attach overnight, and treated with resveratrol or DMSO vehicle control for 3 days. Cells were then collected by trypsinization and plated on 6-well plates at 30 cells per well. Cells were allowed to grow for 17 days undisturbed. Second, a clonogenic cell survival assay was conducted as previously described with modifications [230]. Cells were plated in 6-well plates at 30 cells per well, allowed to attach to the plate overnight, and treated with resveratrol or DMSO vehicle control for 3 days. Treatment was then removed, and the cells were allowed to grow for 17 days undisturbed. For both experiments, after the 17-day incubation, plates were

then washed with 1X HBSS and fixed with methanol for 15 minutes, stained with crystal violet for 1 hour and washed with deionized water. Colonies consisting of 50 cells or more were then counted and scored.

D. Checkerboard Drug Combination Assay

Combination assays were completed using a checkerboard method such that cells plated onto 96-well plates at a density of 4,000 cells per well were treated for 5 days with a serial dilution of paclitaxel across the plate horizontally and a dilution of resveratrol across the plate vertically. The plates were processed using the methylene blue cell proliferation method as described above. It is important for the data analysis that the serial dilution for both drugs is the same; for example, a one to two dilution was used for both drugs. This provides constant drug ratios across the diagonal of the plate. The amount of resveratrol used was the same for both cell lines (2.5-40 μM). The amount of paclitaxel used was different for both cell lines. An amount of paclitaxel was chosen to provide a full paclitaxel curve for each cell line. The parental MDA-MB-231 cells, therefore, were treated with less paclitaxel (0.244-31.25 nM) than the resistant MDA-MB-231/PacR cells (3.906-500 nM). It is important to note that differences in drug ratio were due to a change in paclitaxel concentration rather than resveratrol ratio. However, as it is the drug ratios that are compared, paclitaxel concentrations used for the two cell lines were chosen to provide four drug ratios in common between the two cell lines. This allowed for direct comparison of effects of these drug ratios between the two cell lines. The method can be used to determine if a drug combination is antagonistic, additive, or synergistic by graphing the data as an isobologram with the axes representing the concentration of one drug versus the other. For example, the IC_{50} value of resveratrol alone is plotted on the y-axis and IC_{50} value of paclitaxel alone on

the x-axis; these two points are connected to provide the line of additivity. Each drug ratio is represented on the graph as a single point based on the IC_{50} value for both drugs at this ratio. Any point that falls on the line of additivity denotes an additive effect, below the line denotes synergistic effect, and above the line denotes an antagonistic effect of the drug combination. Antagonism denotes a reduction of drug efficacy, an additive effect suggests an efficacy that is equal to the effects of the two drugs alone added together, and synergy denotes an improvement of effect of both drugs compared to each drug alone. The data were analyzed by determining the IC_{50} value for the constant drug ratio curves using the Chou Talalay method [231, 232].

E. Inhibitor Assay

For the P-gp and CYP2C8 inhibitor assays, the methylene blue method was used as described above. Cells were treated for 5 days with a serial dilution of paclitaxel either alone or in combination with 1 μM verapamil (Sigma-Aldrich; St. Louis, MO), 25 μM trimethoprim (Sigma-Aldrich; St. Louis, MO) or both. GraphPad Prism version 4.00 for Windows (GraphPad Software; San Diego, CA) was used to calculate IC_{50} values. IC_{50} values from different trials were averaged and compared using Microsoft Excel 2011 version 14.4.1.

IV. MOLECULAR TECHNIQUES

A. Cell Cycle Analysis

To analyze the cells' cycle phases following resveratrol treatment, cells were plated at a density of $1x10^6$ in T75 flasks, allowed to attach to the plate overnight, and

treated with resveratrol or DMSO vehicle control for 48 hours. All cells in the media and those attached to the plate were collected using trypsinization and centrifugation. Cells were resuspended in 1X HBSS to wash the pellet and cells were counted. After centrifugation and removal of the 1X HBSS cells were resuspended in 0.5 mL 1X PBS and kept on ice. Cells were fixed and permeablized by adding 4.5 mL of 70% ethanol dropwise while vortexing. Cells were then stored at -20°C. To stain for flow cytometry analysis, samples were centrifuged for 5 minutes and the supernatant was decanted. Cells were gently washed with 1X PBS and centrifuged, and the supernatant was decanted. Cells were resuspended in 100 uL binding buffer (0.1% TritonX-100 in PBS, 0.0002% DNase-free RNase A) and then stained with 5 µL propidium iodide (PI) staining solution (BD Biosciences; Franklin Lakes, NJ). An unstained control was prepared for each cell line with cells not treated with resveratrol; these controls are important for setting up flow cytometry conditions. Cells were gently vortexed and incubated at room temperature for 15 minutes in the dark. Cells were then diluted with 400 µL binding buffer. Samples were put through a 35 µm cell strainer and vortexed before mounting sample tube to flow cytometer. Fluorescence was measured using a LSRII 561 nM laser flow cytometer (BD Biosciences; San Jose, CA), and data was analyzed with FlowJo (TreeStar; Ashland, OR).

B. Senescence Staining

Cells were plated at 15,000 cells per well onto 24-well plates, allowed to attach to the plate overnight, treated with resveratrol or DMSO vehicle control for 3 days, and then stained using the Senescence Detection Kit (EMD Millipore; Billerica, MA) per manufacturer's instructions with modifications. Culture media was aspirated, and the plates were washed with 1X PBS. Cells were then fixed with 200 µL of Fixative Solution

at room temperature for 10 minutes. Cells were washed twice with 1X PBS and the stained at 37 °C overnight with 200 µL Staining Solution. The activity of β-galactosidase is a hallmark of senescent cells. The staining solution of this kit contains X-gal, which is cleaved by β-galactosidase to produce a blue dye. Therefore, blue cell staining denotes a positive result or senescent cell, and no staining denotes a negative result. Positive and negative cells were counted and scored from images provided using a phase-contract microscope at 20X. The experiment was conducted three separate times, and three representative fields for each treatment from each experiment were counted and scored.

C. Immunofluorescence

Cells were plated on 8-well glass chamber slides at 40,000 cells per well and were allowed to attach overnight. Media was removed and wells were washed with 1X HBSS. Cells were fixed to the plate with 100% methanol for 10 minutes and washed three times with 1X PBS for 5 minutes. The cells were then permeabilized with 0.2% Triton-X100 in 1X PBS for 10 minutes. Cells were washed again as before. The chamber was then removed and the cells were mounted to the slide with Vectashield Mounting Medium with DAPI (Vector Laboratories; Burlingame, CA). A coverslip was then sealed to the slide. Slides were then visualized with a fluorescence microscope.

D. Annexin V Apoptosis Flow Cytometry

For apoptosis analyses, 1,000,000 cells were plated in T75 flasks, allowed to attach to the plate overnight, and treated with resveratrol or DMSO vehicle control for 3 or 5 days. All cells in the media and attached to the plate were collected using

trypsinization and centrifugation. Cells were stained with FITC-conjugated Annexin V and PI using the FITC Annexin V Apoptosis Detection Kit I (BD Biosciences; Franklin Lakes, NJ) according to manufacturer's instructions. Collected cells were washed twice with 1X HBSS, and 1,000,000 cells were resuspended in 100 µL of 1X Binding Buffer. To stain the cells, 5 µL of FITC Annexin V and 5 µL of PI were added to the samples. An unstained, FITC Annexin V only, and PI only control were prepared for each cell line with cells not treated with resveratrol; these controls are important for setting up flow cytometry conditions. Samples were then gently vortexed and incubated for 15 minutes at room temperature in the dark. Samples were diluted with 400 µL of 1X Binding Buffer, put through a 35 µm cell strainer, and vortexed before mounting sample tube to flow cytometer. Fluorescence was measured using a LSRII 561 nM laser flow cytometer (BD Biosciences; San Jose, CA), and data was analyzed with FlowJo (TreeStar; Ashland, OR).

E. Immunoblotting

Cells were plated on T75 flasks, allowed to attach overnight, and treated with resveratrol or DMSO vehicle control for 2, 3 or 5 days. Cells were collected by trypsinization, washed with 1X HBSS, pelleted, and snap frozen in an -80°C freezer. Pellets were resuspended in RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 37 mM β glycerol phosphate, 47 mM NaF, 1% NP-40, 0.1% SDS, 0.5% Na deoxycholate, 10% glycerol) supplemented with protease inhibitor cocktail (Hoffmann-La Roche; Switzerland) and phosphatase inhibitor cocktail (Thermo Fisher Scientific; Waltham, MA) and were lysed by sonication for 10 seconds at 30 amperes. The protein content of the samples was estimated using a Pierce BCA Protein Assay Kit. A standard curve of protein concentration was created with lysis buffer, water and 2mg/mL BSA. Protein

samples were diluted 1:10 and 10 µL of standards and samples were added to a 96-well plate. 200 µL of a mix of 50 parts Reagent A to 1 part Reagent B was added to each well and mixed well. The plate was incubated at 37°C for 30 minutes. The plate was then cooled to room temperature and read on a plate reader at 562 nm. The standard curve was then used to estimate protein concentration of prepared samples. Ten or 25 µg of protein was mixed 1:1 with 2X Laemmli Buffer (4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.008% bromphenol blue and 0.125 M Tris HCl pH 6.8) and boiled at 95°C for 5 minutes. Samples were run on 6, 10 or 12% polyacrylamide gels using 1X Tris/glycine SDS (0.25 M Tris base, 0.96 M glycine, 0.5% SDS, pH 8.3) running buffer. PVDF membranes (Pall Corporation; Port Washington, NY) and 1X Transfer Buffer (0.25 M Tris base, 0.192 M glycine, 20% methanol, pH 8.3) were used for overnight (13V) or 2-hour (80V) transfer. Blotting was conducted using 5% milk in 0.1% Tween PBST or 5% BSA in 0.1% Tween TBST for phosphorylated proteins. All blots were incubated with primary antibody (1:1000-1:5000) at 4°C overnight and secondary antibody (1:5000-1:10,000) for 0.5-1 hour at room temperature with gentle agitation. Primary antibodies SIRT1 (Active Motif; Carlsbad, CA), DBC1, Survivin, Caspase 7, Caspase 3, AMPK, and P-AMPK (Cell Signaling; Danvers, MA) and secondary antibodies anti-mouse and anti-rabbit (Thermo Fisher Scientific; Waltham, MA) were used according to manufacturer's suggestion. Pierce ECL Western Blotting Reagent (Thermo Fisher Scientific; Waltham, MA) was used to visualize bands on X-ray film per manufacturer's instructions. An X-ray film developer was used to develop the film, and ImageJ was used for densitometry quantification.

F. siRNA Knockdown

Caspase 7 and Non-Targeting Dharmacon ON-TARGET*plus* SMARTpool siRNA constructs were used with Dharmacon lipofection transfection reagent (Thermo Fisher Scientific; Waltham, MA) according to manufacturer's instructions. Dharmacon ON-TARGET*plus* SMARTpool siRNAs, which contain a pool of 3-4 siRNAs, were reconstituted with 1X siRNA buffer (60 nM KCl, 6 mM HEPES-pH 7.5, and 0.2 mM $MgCl_2$) for a final stock concentration of 5 µM. The siRNA and siRNA buffer solution was mixed gently by pipetting without introduction of bubbles and incubated for 30 minutes at room temperature with gentle agitation. Aliquotes of siRNA were stored at -20°C. Cells were plated on 6-well plates at 200,000 cells per well and allowed to attach overnight. Cells were transfected with 25 nM siRNA and 0.1% transfection reagent. Transfection media was prepared by gently mixing siRNA and transfection reagent individually with serum-free media and incubating for 5 minutes at room temperature. The two mixtures were then gently mixed together and incubated for 20 minutes at room temperature. Complete media was added to the mixture and cells were treated with 1 mL of transfection media for 96 hours. The transfection media was then removed and the cells were then treated with resveratrol or DMSO vehicle control for 48 hours before the cells were collected and analyzed by immunoblotting as described above.

G. Co-immunoprecipitation

Cells were plated on 10 cm dishes and left to attach overnight. The cells were then treated for 2 hours with resveratrol or DMSO vehicle control and collected by scraping in 1 mL of 1X PBS. The cells were pelleted in a centrifuged at 10,000 rpm for 5 minutes and PBS was aspirated. The pellets were resuspended in co-

immunoprecipitation lysis buffer (25mM Tris, pH 8.0, 150mM NaCl, 0.5 mM EDTA, 0.5 mM EGTA, 1% NP-40) supplemented with protease inhibitor cocktail (Hoffmann-La Roche; Switzerland). The samples were lysed on ice for 30 minutes with vortexing every 10 minutes. The samples were then spun down as before and the supernatant was put into a new tube. The protein concentration was then determined as described in the "Immunoblotting" section. Along with untreated and treatment samples, a "lysate and bead only" control was prepared with untreated cells as a background control. 1500 µg of protein was added to a new sample tube for each sample. 2.5 µg of SIRT1 antibody was added to all but the "lysate and bead only" control. The total volume of each sample was brought up to 1 mL with lysis buffer, and the samples were incubated at 4°C with gentle agitation overnight. Protein A/G PLUS-Agarose Immunoprecipitation Reagent (Santa Cruz Biotechnology; Santa Cruz, CA) was used to collect the SIRT1-SIRT1 Antibody complex. A slurry of A/G beads in PBS was made to provide enough beads to coat the bottom of an Eppendorf tube by adding 37.5 µL of bead slurry. This 37.5 µL of bead slurry was then added to each sample and incubated for 2 hours at 4°C with gentle agitation. To collect beads, samples were spun down for 30 seconds at 8,000 rpm and supernatant was aspirated. Beads were rinsed 3 times with 1 mL cold 1X PBS on ice and gentle agitation for 10 minutes. After final rinse, 10 µL of cold PBS and 10 uL of 2X Laemmli Buffer was added to the beads. Samples were boiled on a heat block for 7 minutes at 95°C with vortexing three times during boiling. Samples were then spun down and the supernatant was loaded onto a polyacrylamide gel to be analyzed by Immunoblotting as was described above.

H. RNA Extraction

PCR based assays were used to explore possible mechanisms of resistance, which required RNA extracts from cell samples. Untreated cells were collected by trypsinization, washed, pelleted and snap frozen in an -80°C freezer. For all PCR based assays, RNase Away Decontamination Solution (Thermo Fisher Scientific; Waltham, MA) was used to keep the working area free of RNase contamination. RNA extraction was performed according to manufacturer's instructions using the RNeasy Mini Kit (Qiagen; Holland). Cell pellets were resuspended in 350 µL of Buffer RLT and the cell lysates were homogenized by passing the lysates through a blunt 20-gauge needle 5 times. Before addition to the column, 350 µL of 70% ethanol was then added to the lysates and mixed well. The lysates were then transferred to RNeasy Mini Kit spin columns placed in a 2 mL collection tube and centrifuged for 15 seconds at 10,000 rpm to transfer sample to the spin column membrane. Flow-through was discarded and 700 µL Buffer RW1 was added to the columns to wash the membrane. Samples were centrifuged again and flow-through was discarded. After adding 500 µL Buffer RPE to the columns to further wash the membrane, the columns were spun and flow-through discarded again. Another 500 µL Buffer RPE was added to the columns, and the sample was centrifuged for 2 minutes at 10,000 rpm. The spin columns were then placed in new 2 mL collection tubes and centrifuged at 13,200 rpm for 1 minute. Finally, the spin columns were placed in new 1.5 mL collection tubes, 40 µL of RNase-free water was added directly to the spin column membranes, and the samples were spun at 10,000 rpm for 1 minute to elute RNA from the membrane. Another 40 µL was added to the column and the spin repeated to ensure elution of all RNA. RNA concentration and purity of the eluate was determined using a NanoDrop spectrophotometer.

I. cDNA Synthesis

The RT^2 First Strand Kit (Qiagen SABiosciences; Holland) was used to synthesize cDNA from RNA samples for both the RT^2 Profiler PCR Array and the RT^2 qPCR Primer Assay according to manufacturer's instructions. Genomic DNA was eliminated from the RNA samples by mixing 0.5 µg of RNA with 2 uL of Buffer GE and RNase-free water to 10 µL total volume. This genomic DNA elimination mix was incubated at 42°C for 5 minutes and then placed immediately on ice for at least 1 minute. Reverse-transcription mix (4 µL 5X Buffer BC3, 1 µL Control P2, 2 µL RE3 Reverse Transcriptase Mix, and 3 µL RNase-free water) was prepared for each reaction and 10 µL was added to each tube containing 10 µL genomic DNA elimination mix and mixed gently. Samples were incubated at 42°C for exactly 15 minutes. The reaction was then immediately stopped by incubating at 95°C for 5 minutes. Finally, 91 µL RNase-free water was mixed gently into each sample, and the samples were placed on ice or stored at -20°C.

J. RT^2 Profiler PCR Array

The Human Cancer Drug Resistance RT^2 Profiler PCR Array (Qiagen SABiosciences; Holland) was used according to manufacturer's instructions. The array was performed a total of three times from the same cDNA sample for each cell line. The PCR components (50% 2X RT^2 SYBR Green Mastermix, 3.8% cDNA and 46.2% RNase-free water) were mixed for a 96-well array for each cell line at room temperature and 25 µL of the mixture was added to each well of the array using a 8-channel pipettor changing pipet tips following each pipetting step to avoid cross-contamination. The array plate was tightly sealed with optical thin-wall 8-cap strips. The plates were centrifuged

for 1 minute at 10,000 rpm at room temperature to remove bubbles and were placed on ice until ready to run real-time PCR. Real-time PCR was performed on an ABI 7500 machine with the following program: 1 cycle 95°C for 10 minutes; 40 cycles 95°C for 15 seconds, 60°C for 1 minute (perform fluorescence data collection); dissociation curve analysis. The 7500 Software version 2.0.6 was used to determine C_T values and the SABiosciences PCR Array Data Analysis Template Excel was used to analyze the data.

K. RT^2 qPCR Primer Assay

Primers for ABCB1, CYP2C8, and actin (Qiagen SABiosciences; Holland) were used in combination with the corresponding RT^2 Primer Assay (Qiagen SABiosciences; Holland) reagents and used according to manufacturer's instructions. Three biological replicates were run in triplicate. The PCR components (50% RT^2 SYBR Green Mastermix, 4% cDNA, 4% 10 μM RT^2 qPCR Primer, 42% RNase-free water) were mixed for a total volume of 25 μL per reaction and were added to 96-well PCR plates. The plates were sealed with adhesive film, centrifuged for 1 minute at 10,000 rpm at room temperature to remove bubbles, and placed on ice until ready to run real-time PCR. Real-time PCR was performed on an ABI 7500 machine with the same program used for the PCR Array. The 7500 Software version 2.0.6 was used to determine C_T values and the 2^(-$\Delta\Delta C_T$) method [233] was used to determine fold changes of gene expression between the parental, MDA-MB-231 cells, and the resistant MDA-MB-231/PacR and MDA-MB-231/PacR-Hi cells. P-values and standard deviations for PCR Array and real-time RT-PCR validation were calculated based on a Student's t-test of the replicate 2^(-ΔC_T) values for each gene in the control group and treatment groups as suggested by the manufacturer.

V. STATISTICS

Student's T-Test, two- and one-way ANOVA with Bonferroni correction were used to determine p-values where appropriate using GraphPad Prism4 software where $p<0.05$ was considered statistically significant.

CHAPTER 3: RESULTS

I. PACLITAXEL-RESISTANT CELLULAR MODEL GENERATION

To provide a cellular model of acquired paclitaxel resistance in MDA-MB-231 breast cancer cells, a paclitaxel resistant cell line was developed by an intermittent, stepwise treatment with paclitaxel (Figure 7). A continuous, stepwise method was also attempted but the resulting population did not develop as much resistance, based on IC_{50} analysis at a 24-hour paclitaxel treatment time, or grow as well as the population derived from the intermittent, stepwise method. Therefore, we continued experiments with the population derived by intermittent, stepwise paclitaxel treatment. The cells were no longer cultured in the presence of paclitaxel after the final population was acquired. The limiting dilution cloning method was used to provide a more homogeneous population and yielded 29 clones that could be successfully established as resistant cell lines. These cells were originally assessed with a 24-hour paclitaxel treatment. The cells did not lose paclitaxel-resistance even after up to 20 passages post-cloning.

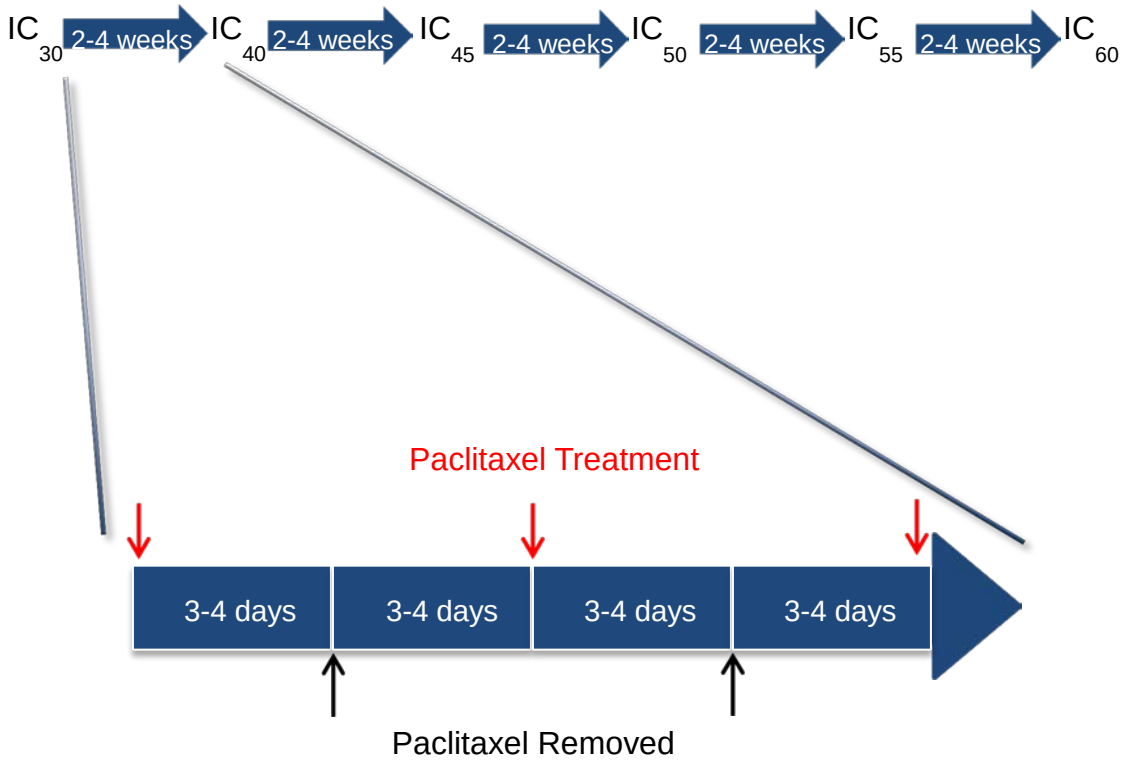


Figure 7. Schematic of Drug Resistant Cell Line Generation. Cells were treated with a stepwise increase of the 24-hour paclitaxel IC_{50} calculated from the methylene blue proliferation assay. Between each stepwise increase was a 2 to 4 week period of intermittent treatment. This intermittent treatment was conducted with repeatedly treating the cells with paclitaxel for 3 to 4 days, removing the drug, and allowing the cells to recover for 3 to 4 days. The resulting population was then cloned using the limiting dilution method.

II. RESVERATROL INDUCES CELL PROLIFERATION INHIBTION, SENESCENCE, AND APOPTOSIS

A. Resveratrol Inhibits Cell Proliferation

To determine the effects of resveratrol in these paclitaxel-sensitive and -resistant TNBC cells, the effects of resveratrol on cell proliferation of parental MDA-MB-231 and highly resistant MDA-MB-231/PacR cells were tested. To determine any correlation between paclitaxel resistance and resveratrol sensitivity, the 24-hour paclitaxel IC_{50} and 72-hour resveratrol IC_{50} were graphed against each other and a 'line of best fit' analysis was performed in Excel (Figure 8). The 'line of best fit' analysis resulted in a correlation coefficient of 0.046, suggesting no correlation. Due to the fact that a 24-hour time point of resveratrol does not provide a full IC_{50} curve, a longer time point of 5 days was used for further experiments. Of the 29 clones, the 12-fold increased resistance clone, MDA-MB-231/PacR, was selected for further study. The 5-day IC_{50} value of the parental MDA-MB-231cells was 5.1 ± 2.3 nM compared to 61.5 ± 9.5 nM for the MDA-MB-231/PacR cells. In addition, another clone was chosen for use in resistance mechanism experiments to provide comparison and validate the importance of the mechanism in more than one clone. The clone chosen, MDA-MB-231/PacR-Hi, was observed to have a 5-day IC_{50} value of 117.5 ± 42.7 nM. Though the population doubling of the MDA-MB-231/PacR cells was increased to 52 hours compared to the MDA-MB-231 cells at 35 hours, 10 and 100 μM resveratrol significantly inhibited the ability of both cell populations to proliferate (Figure 9). Treatment with 100 μM resveratrol in a colony formation and clonogenic cell survival assay completely inhibited colony formation in both cell lines, indicating that resveratrol inhibited the ability of a single cell to proliferate (Figure 10A-B). In both assays, 10 μM resveratrol treatment decreased colony formation, but the

decrease was only statistically significant for the surviving fraction in the MDA-MB-231/PacR cells. The difference in population doubling time between the two cell lines is illustrated by the larger colony size seen in the MDA-MB-231/PacR cells (Figure 10C).

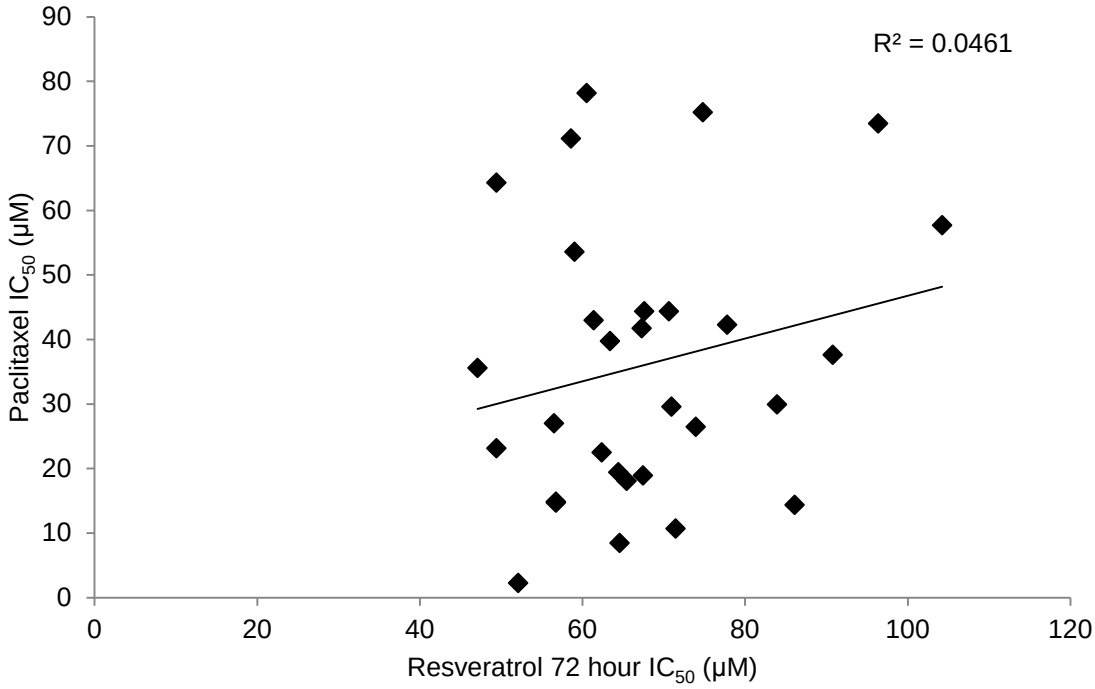


Figure 8. Paclitaxel Resistance is Not Correlated to Resveratrol Sensitivity. Each paclitaxel-resistant clone generated graphed by 24-hour paclitaxel IC_{50} versus 72-hour resveratrol IC_{50}. Line represents the line of best fit, and the R^2 is the correlation coefficient of the line of best fit.

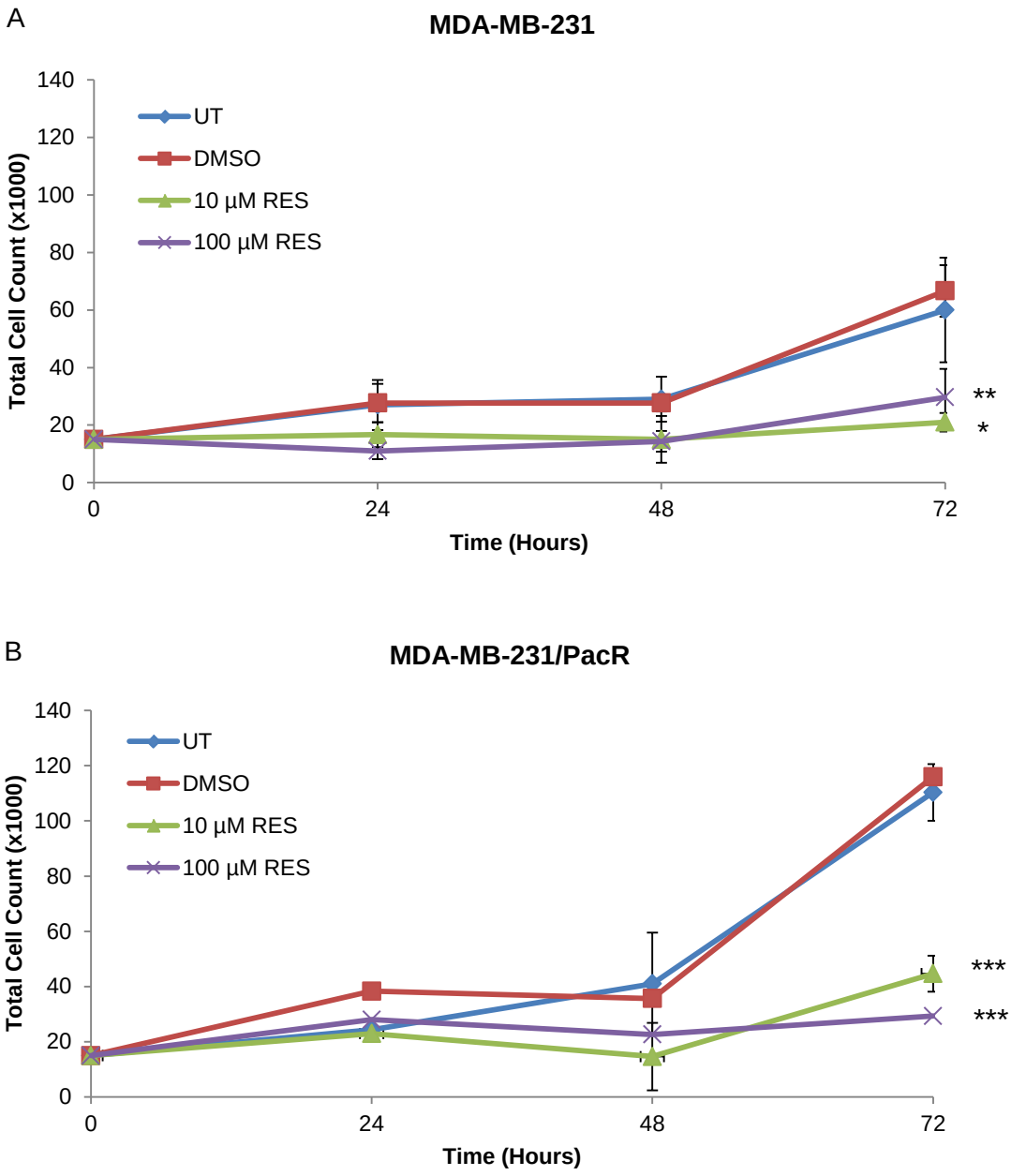


Figure 9. Resveratrol Inhibits Cell Proliferation. A) Total cell counts from plates seeded with $1.5x10^4$ cells 24-72 hours post resveratrol treatment of MDA-MB-231 and B) MDA-MB-231/PacR. cells (n=3) (UT=untreated; *p<0.05; **p<0.01; ***p<0.001; *p-values compare treatment to DMSO control).

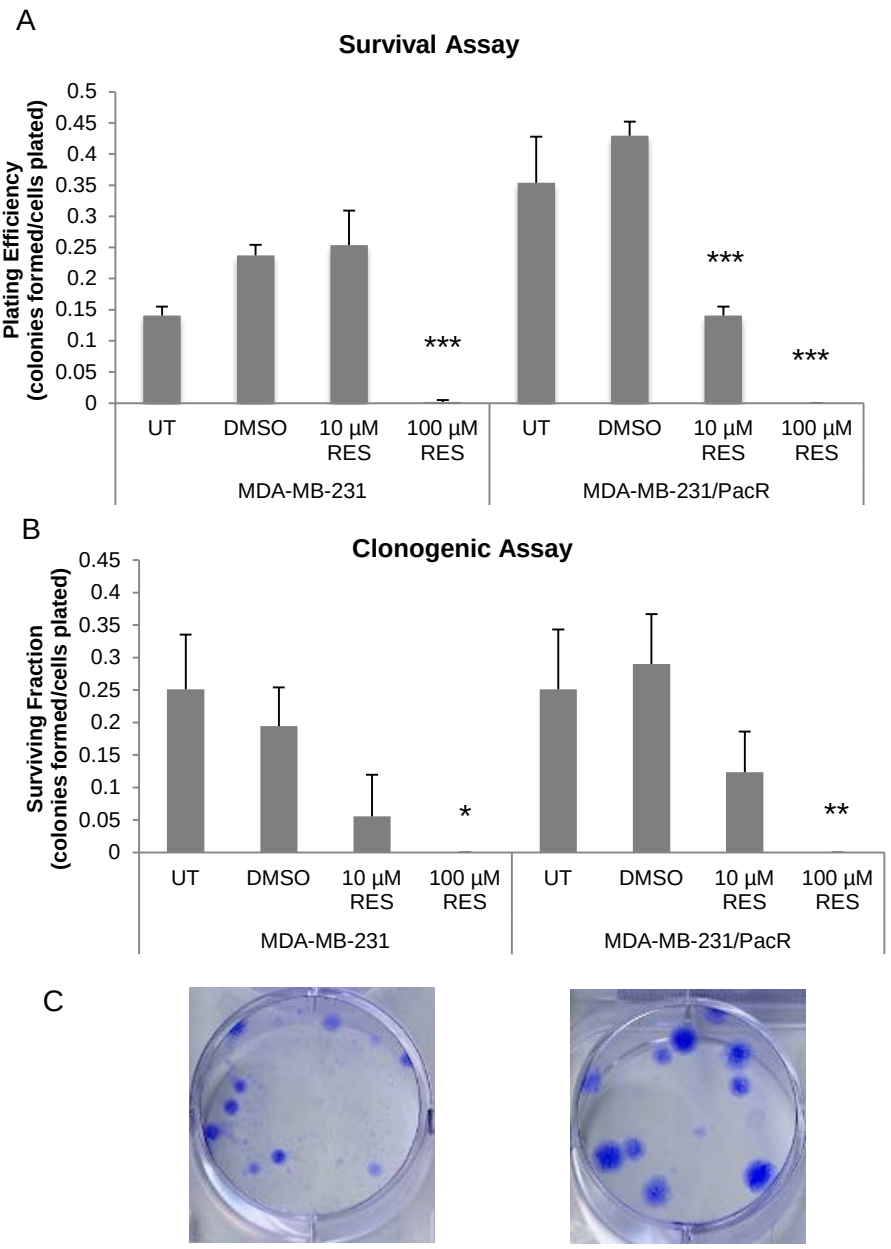


Figure 10. Resveratrol Inhibits Colony Formation. A) Plating efficiency and B) Surviving fraction of cells plated at low density after 72 hours of resveratrol treatment followed by a 17-day incubation without drug (n=3) and C) representative pictures of untreated colonies from clonogenic, colony formation assay. Error bars signify standard deviation (UT=untreated; *p<0.05; **p<0.01; ***p<0.001; *p-values compare resveratrol treatment to DMSO control).

B. Resveratrol Induces Sub-G1 Phase Accumulation

Due to the inhibition of cell proliferation observed in both cell lines by resveratrol, we next analyzed the effect of resveratrol on cell cycle profiles in parental MDA-MB-231 and MDA-MB-231/PacR cells. We chose a 48-hour time point based on the cell proliferation data which indicates a difference between vehicle treated and resveratrol treated is just becoming evident. In addition, a higher concentration, 300 μM, of resveratrol was added to evaluate the effects of resveratrol at concentrations previously used in anti-cancer studies [234, 235] and shown to accumulate in tissues [178]. At 48 hours, neither 100 nor 300 μM resveratrol treatment in either cell line caused any accumulation in G1, G2/M or S phase compared to vehicle control (Figure 11). There was a decrease of cells in G1 phase after 300 μM resveratrol treatment in both cell lines. This population decreased from 46% to 4% in MDA-MB-231 cells and 41% to 8% in MDA-MB-231/PacR cells. Importantly, there was accumulation in Sub-G1 phase in both cell lines, which suggests apoptosis. In the MDA-MB-231 cell line, the Sub-G1 phase increased from 3% in the untreated and DMSO treated to 18% and 49% in the 100 and 300 μM treated cells, respectively. In the MDA-MB-231/PacR cell line, the Sub-G1 phase increased from 8% in the untreated and DMSO treated to 23% and 34% in the 100 and 300 μM treated cells, respectively. In addition, in both cell lines 300 μM resveratrol caused accumulation of a peak with higher DNA content than G2/M phase, but not enough to denote cell aggregation or mitotic catastrophe. The population increased from 10% to 24% in the MDA-MB-231 and 9% to 26% in the MDA-MB-231/PacR cells. Due to the presence of micronuclei in both cell populations (Figure 12), as well as literature suggesting micronucleated calls can collect between G1 and G2 phase and after G2/M phase [236], we hypothesize this peak represents a population of micronucleated cells.

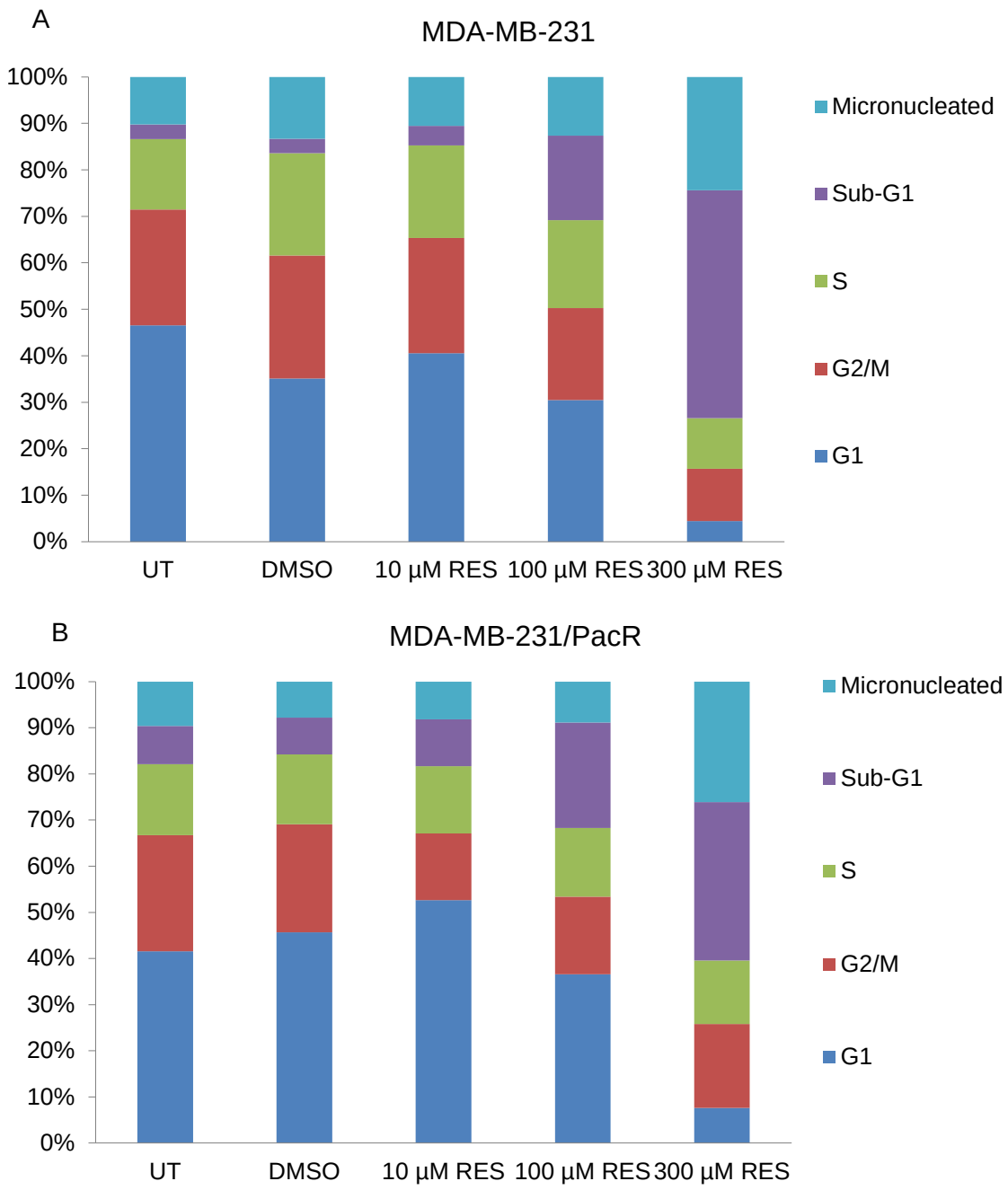
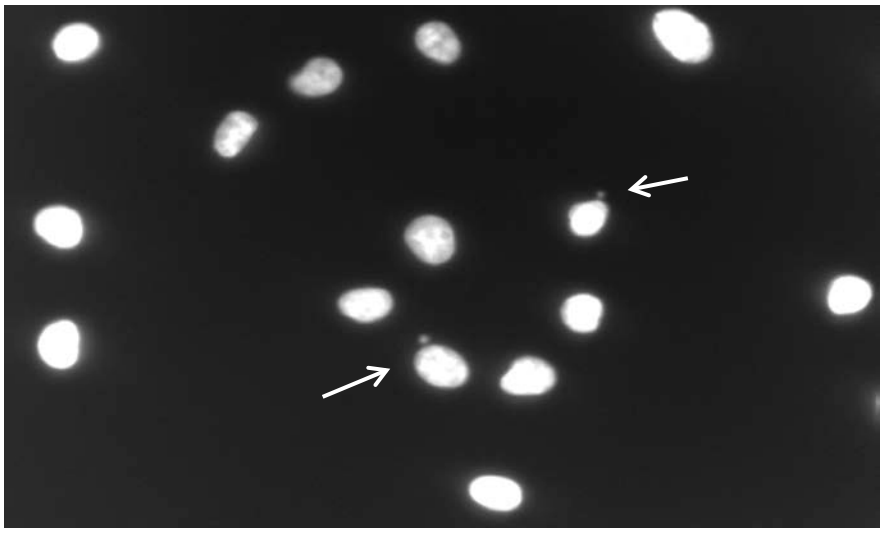


Figure 11. Resveratrol Induces Sub-G1 Accumulation. Cell cycle analysis measureing PI staining with flow cytometry showing percent of cells in each phase after 48 hours of resveratrol treatment in A) MDA-MB-231 and B) MDA-MB-231/PacR cells (n=4).

A

MDA-MB-231



B

MDA-MB-231/PacR

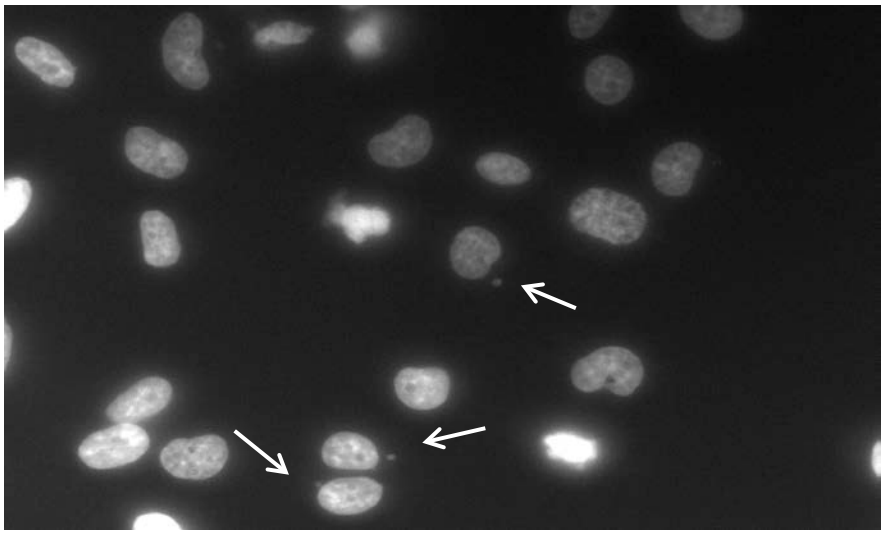


Figure 12. Micronuclei are Present in MDA-MB-231 & MDA-MB-231/PacR Cells. A) DAPI staining of the nuclei (40X magnification) of MDA-MB-231 cells and B) MDA-MB-231/PacR cells. White arrows indicate micronuclei.

C. Resveratrol Induces Senescence

Because we observed inhibition of cell proliferation without a prominent cell cycle arrest with 10 or 100 μM resveratrol treatment, we hypothesized resveratrol treatment was causing senescence in some of the cells. Due to the greatest cell proliferation difference occurring at 3 days, we used this treatment time to look for senescent cells. After 100 μM resveratrol treatment, 37% of MDA-MB-231 and 38% of MDA-MB-231/PacR cells were observed to be senescent by positive β-galactosidase staining (Figure 13). A treatment of 10 μM resveratrol caused a slight increase in senescence in both cell lines, but this increase was only statistically significant in the MDA-MB-231/PacR cells. In these pictures, the hallmark cellular morphology of senescent cells, an enlarged and flattened appearance [237], can also be seen in the blue stained, β-galactosidase positive cells.

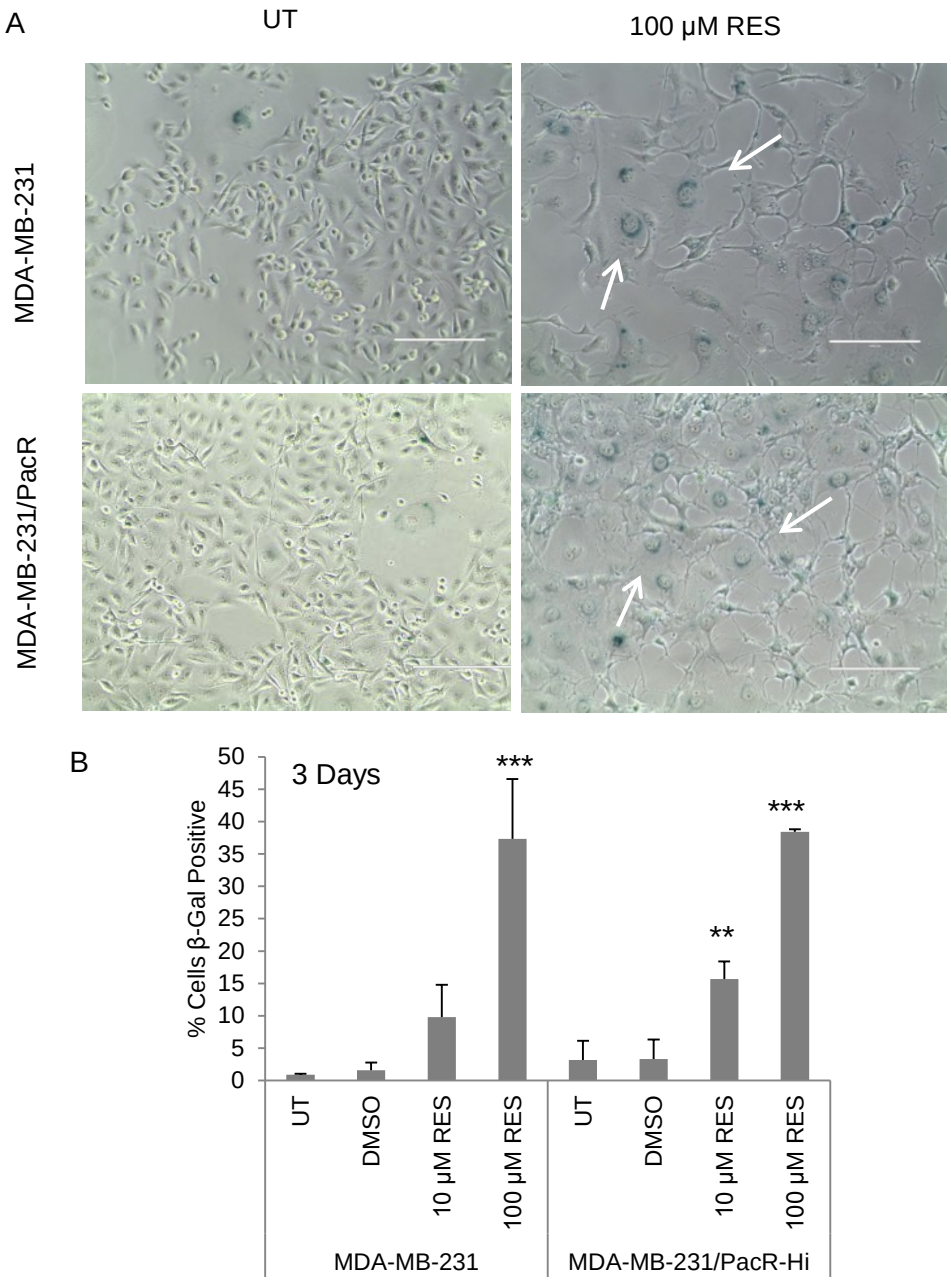


Figure 13. Resveratrol Induces Senescence. A) Representative fields of β-Galactosidase senescence staining after 48 hours of resveratrol treatment. White arrows point to blue stained, positive cells. Scale bar represents 200 μm. B) Quanitifcation of percent positive β-Galactosidase cells from three representative fields (n=3). Error bars signify standard deviation (*p<0.05; **p<0.01; ***p<0.001; *p-values compare treatment to DMSO control).

D. Resveratrol Induces Apoptosis

To ensure that the accumulated sub-G1 phase cells were apoptotic, Annexin V flow cytometry was conducted. This method differentiates between early apoptosis and late apoptosis/necrosis. After 3 days of resveratrol treatment, apoptosis was observed in both cell lines at 300 μM and in MDA-MB-231 cells at 100 μM resveratrol as well (Figure 14A). In MDA-MB-231 cells treated with 300 μM resveratrol, 59% late apoptosis/necrosis and 37% early apoptosis was observed for a total of 96% of cells undergoing apoptosis. In MDA-MB-231/PacR cells, 71% late apoptosis/necrosis and 18% early apoptosis was observed for a total of 89% of cells undergoing apoptosis. At this 3-day time point, 100 μM of resveratrol caused a statistically significant increase only in MDA-MB-231 cells when early and late apoptosis were added together ($p<0.001$). After 5 days of resveratrol treatment, both 100 and 300 μM treatment caused early and late apoptosis in both cell lines (Figure 14B). 300 μM resveratrol caused a total 96% of the cells to undergo apoptosis in both cell lines with 52% and 44% of MDA-MB-231 cells and 58% and 38% of MDA-MB-231/PacR cells being late and early apoptotic, respectively. When treated with 100μM resveratrol for 5 days, the percentage of total apoptotic cells was 64% of MDA-MB-231 cells ($p < 0.001$), made up of 46% late and 18% early apoptotic, and 53% of MDA-MB-231/PacR cells ($p < 0.01$), made up of 37% late and 16% early apoptotic cells.

To determine the importance of caspase 3 and caspase 7 in resveratrol-induced apoptosis, immunoblotting analysis was utilized. Procaspase, the inactive form, and cleaved caspase, the active form, were both measured. Caspase activation is a late apoptosis event. Due to the early and late apoptosis seen at 3 and 5 days we used these as well as a 48-hour time point to obtain a broad idea of caspase activity after resveratrol treatment. After 48 hours of 300 μM resveratrol treatment, procaspase 7 was

significantly decreased and cleaved caspase 7 was significantly increased for both cell lines (Figure 15). Slight activation of caspase 7 was seen with 100 μM of resveratrol treatment at this 48-hour time point though it was not statistically significant. After 3 and 5 days of resveratrol treatment, the activation of caspase 7 decreases in the 300 μM treatment, which is likely due to activated caspases being degraded after activation. In contrast, Caspase 7 activates in the 100 μM treatment group for both cell lines at 3 and 5 days (Figure 16). In addition, at 48 hours there was a decrease of procaspase 3 and slight but significant activation of caspase 3 with 300 μM treatment (Figure 17).

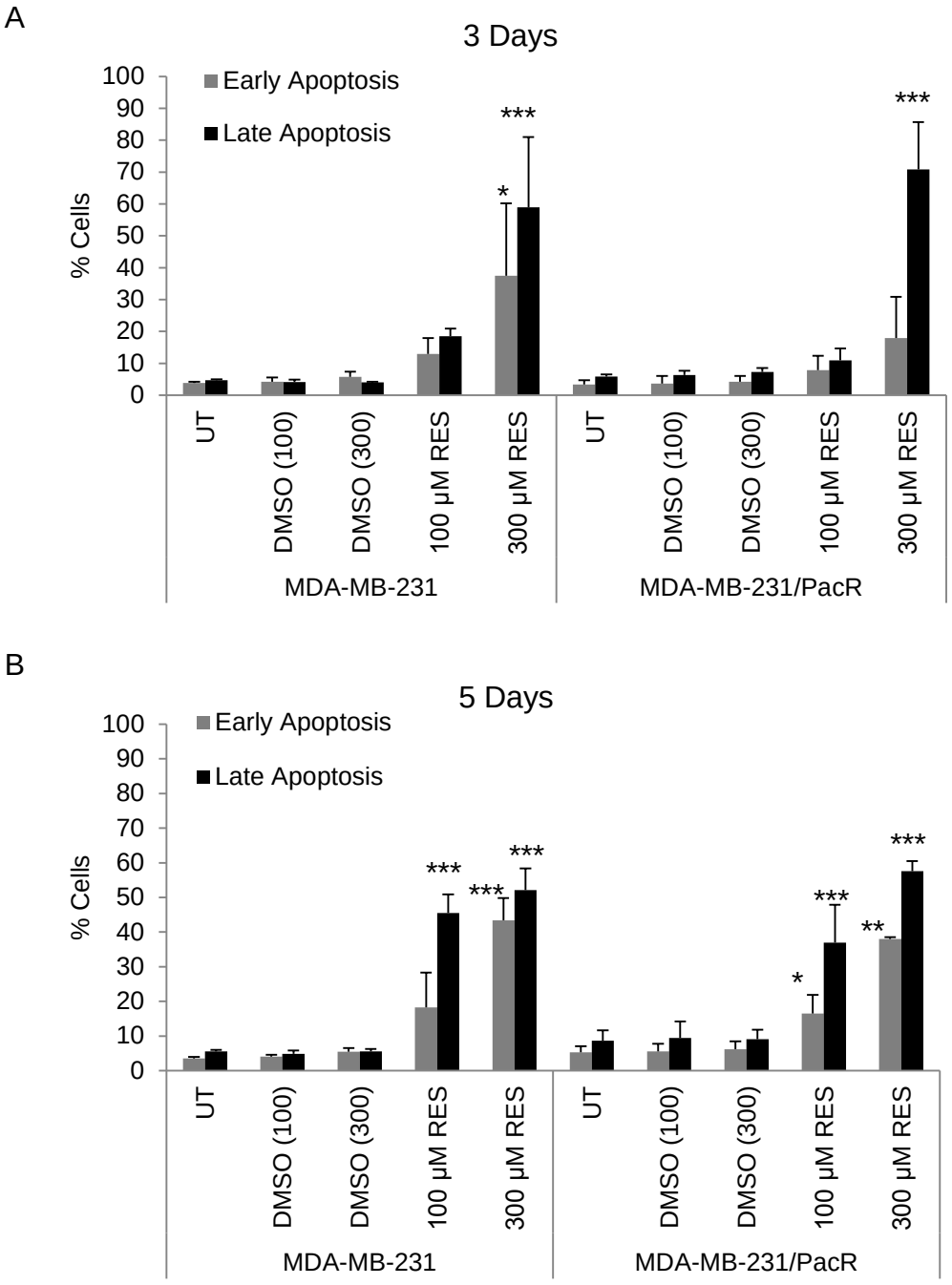


Figure 14. Resveratrol Induces Apoptosis. A) Percent of cells undergoing early (gray) and late (black) apoptosis measured by Annexin V/PI flow cytometry after 3 days and B) 5 days of resveratrol treatment (n=3). Error bars signify standard deviation (*p<0.05; **p<0.01; ***p<0.001; *p-values compare treatment to respective DMSO control).

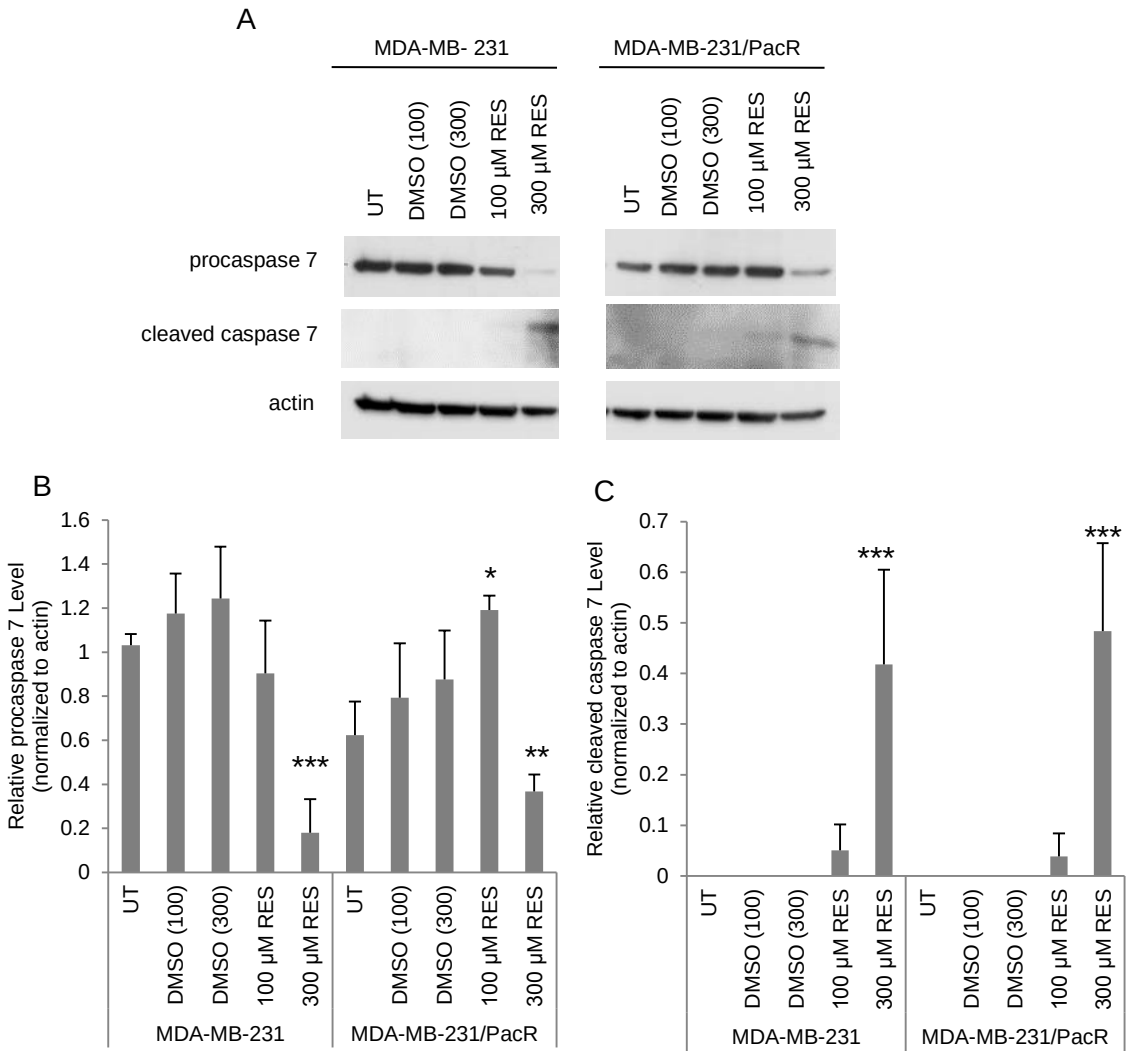


Figure 15. Caspase 7 Activation Occurs at 48 Hours with 300 µM Resveratrol Treatment. A) Representative immunoblot from three independent experiments of caspase 7 at 48 hours after resveratrol treatment. B) Densitometry quantification normalized to actin of procaspase 7 and C) cleaved caspase 7 (n=3). Error bars signify standard deviation (*p<0.05; **p<0.01; ***p<0.001; *p-values compare treatment to respective DMSO control).

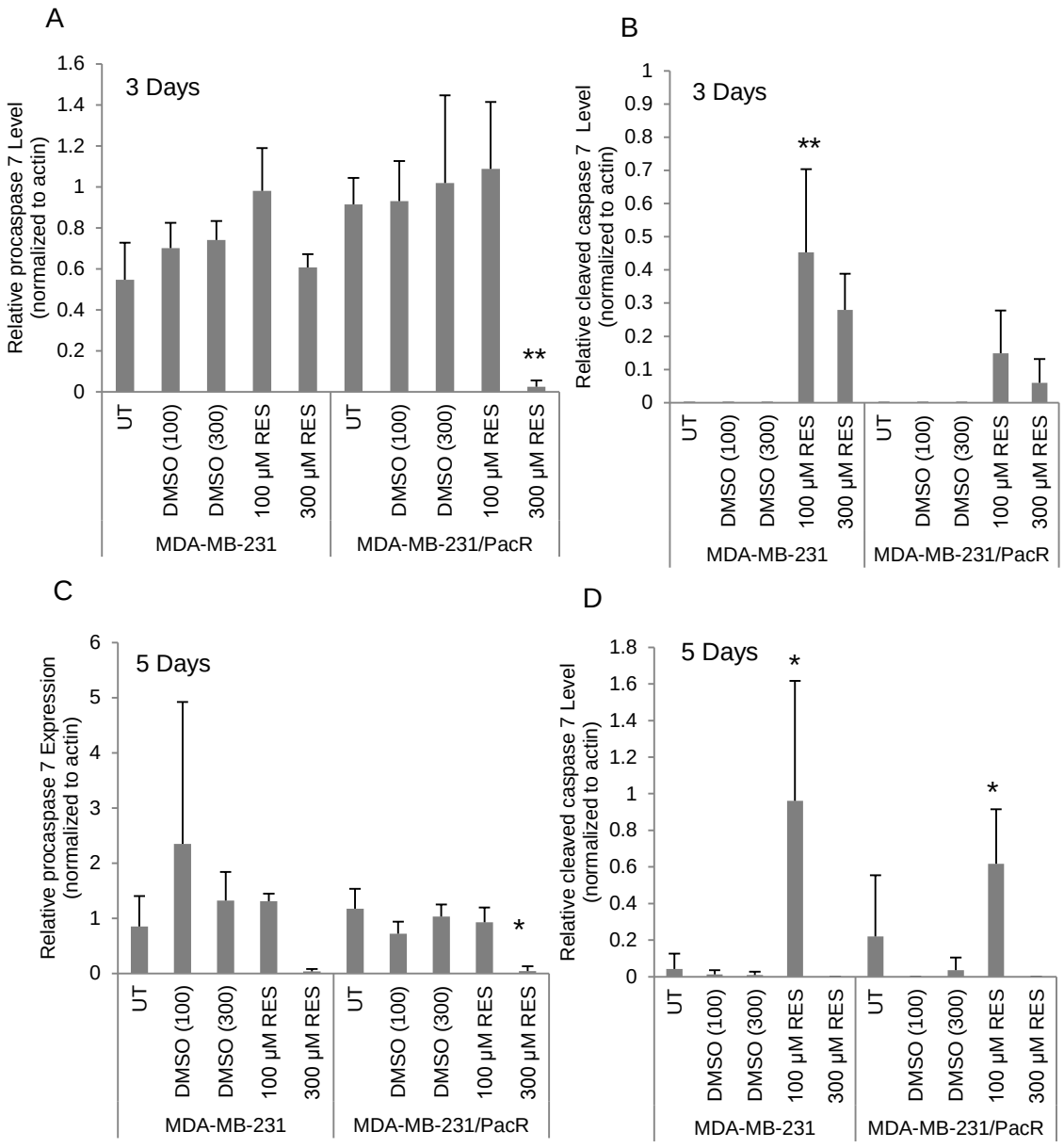


Figure 16. Caspase 7 Activation Occurs at 3 & 5 Days with 100 µM Resveratrol Treatment. Densitometry quantification normalized to actin of A) procaspase 7 and B) cleaved caspase 7 at 3 days and C) procaspase 7 and D) cleaved caspase 7 at 5 days (n=3). Error bars signify standard deviation (*p<0.05; **p<0.01; ***p<0.001; *p-values compare treatment to respective DMSO control).

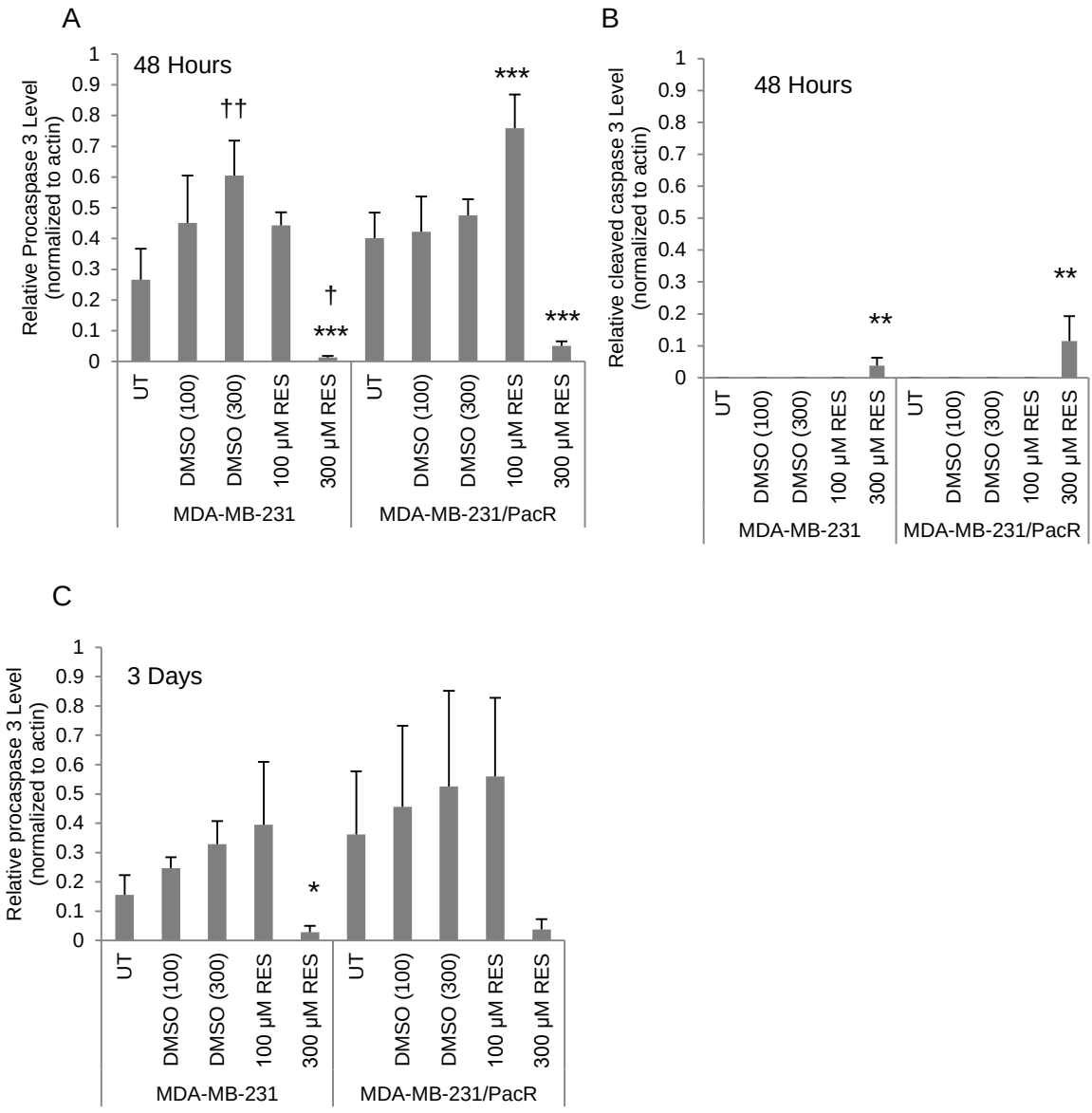


Figure 17. Caspase 3 Activation Occurs Before 48 Hours with 300 µM Resveratrol Treatment. Densitometry quantification normalized to actin of A) procaspase 3 and B) cleaved caspase 3 at 48 Hours and C) procaspase 3 at 3 days (n=3). Error bars signify standard deviation (*p<0.05; **p<0.01; ***p<0.001; *p-values compare treatment to respective DMSO control; †p-values compare untreated and DMSO control).

E. Resveratrol Decreases Survivin Protein Expression at High Concentration

To explore possible mechanisms of resveratrol action, survivin and SIRT1 pathways were examined as these have been shown to be important in other cancer cells [212]. Complete inhibition of survivin protein expression was achieved with 300 μM resveratrol at 48 hours and persisted for at least 5 days (Figure 18). However, while apoptosis occurred at 3 and 5 days of 100 μM treatment, there was no significant difference in survivin protein levels, suggesting survivin may not be critical in resveratrol-induced apoptosis in these cells at this lower concentration (Figure 18C-D). Furthermore, the decrease of survivin with 300 μM resveratrol may not be caused by caspase 7 cleavage activity as neither 40% caspase 7 knockdown in MDA-MB-231 cells nor 70% in MDA-MB-231/PacR cells resulted in any recovery of survivin levels (Figure 19). This result was further supported by the activation of caspase 7 in both cell lines treated with 100 μM resveratrol (Figure 16C-D) with no resulting decrease in survivin expression (Figure 19B). Resveratrol showed no effect on the expression of SIRT1 or the protein regulator of SIRT1, DBC1, in these cell lines (Figure 20). To determine activation of AMPK after 2 hours of resveratrol treatment, levels of phosphorylation on threonine 172 were measured by immunoblotting as this phosphorylation highly correlates with kinase activity [238]. Although 300 μM resveratrol did activate AMPK (Figure 21), which has been shown to cause dissociation of SIRT1 and DBC1 [210], co-immunoprecipitation studies showed little to no association of SIRT1 and DBC1 in these cells (Figure 22). In MDA-MB-231 cells there was little to no binding in any of the three separate co-immunoprecipitation experiments conducted. The binding of SIRT1 and DBC1 in the MDA-MB-231/PacR cells was more pronounced, but very erratic. Although binding was seen in the untreated DMSO (100) and 100 μM resveratrol group in the first trial, the second trial only week binding in untreated and DMSO (300). In the final trial,

association was seen between SIRT1 and DBC1 in only the DMSO (100), 100 μM and 300 μM resveratrol groups. To ensure that our co-immunoprecipitation protocol was not the cause of the erratic binding, a breast cancer cell line, the MDA-MB-468 cells, that has been shown to have SIRT1 and DBC1 binding was used (Figure 23). The three independent experiments with the MDA-MB-468 cells demonstrated more consistent association between SIRT1 and DBC1. These data provide a possible mechanism for both 100 and 300 μM resveratrol action that requires further investigation (Figure 24).

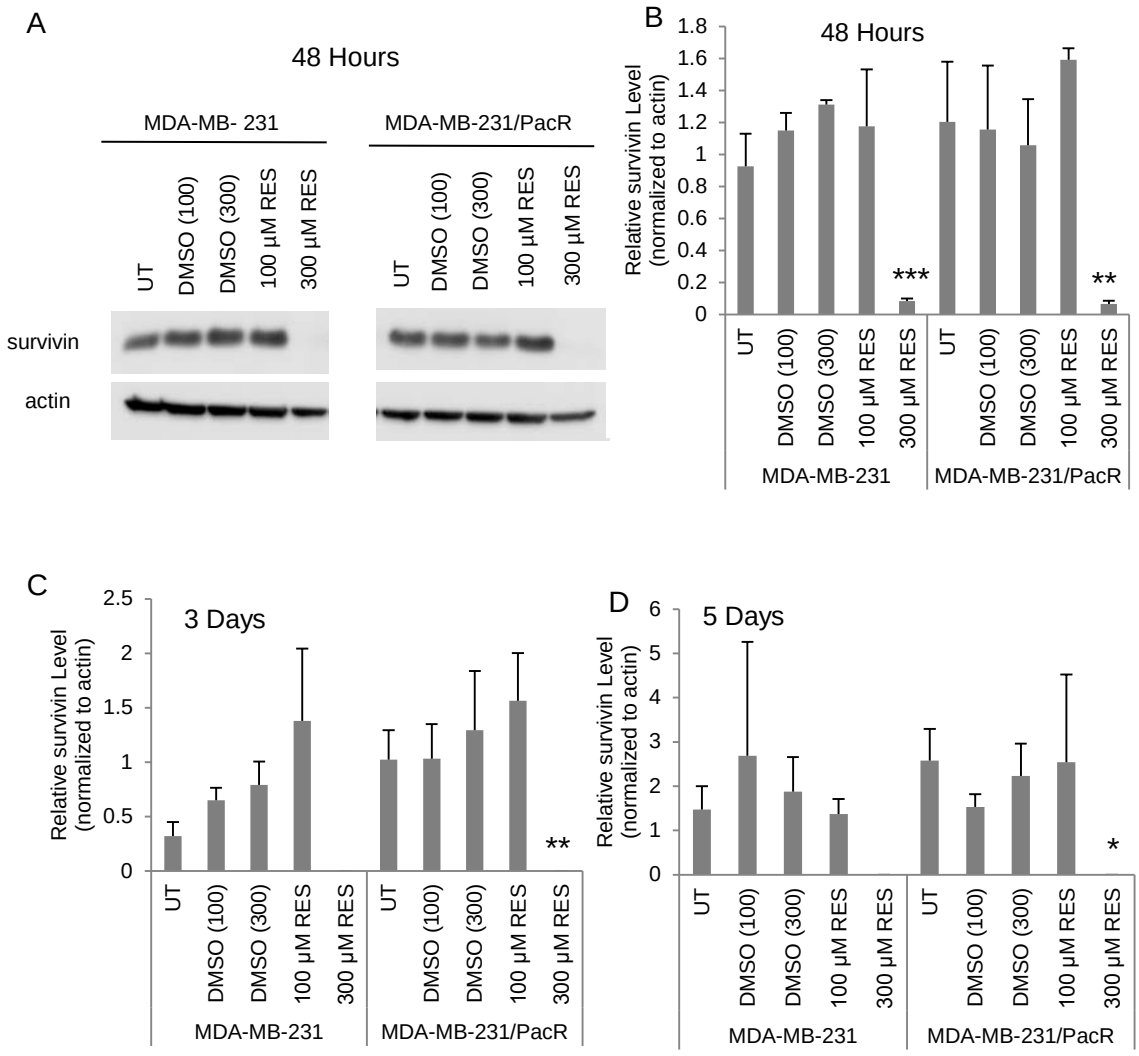


Figure 18. Resveratrol Inhibits Survivin Expression at High Concentration. A) Representative immunoblot of survivin at 48 hours after resveratrol treatment B) Densitometry quantification normalized to actin of survivin after 48 hours C) 3 days and D) 5 days (n=3). Error bars signify standard deviation (*p<0.05; **p<0.01; ***p<0.001; *p-values compare treatment to respective DMSO control).

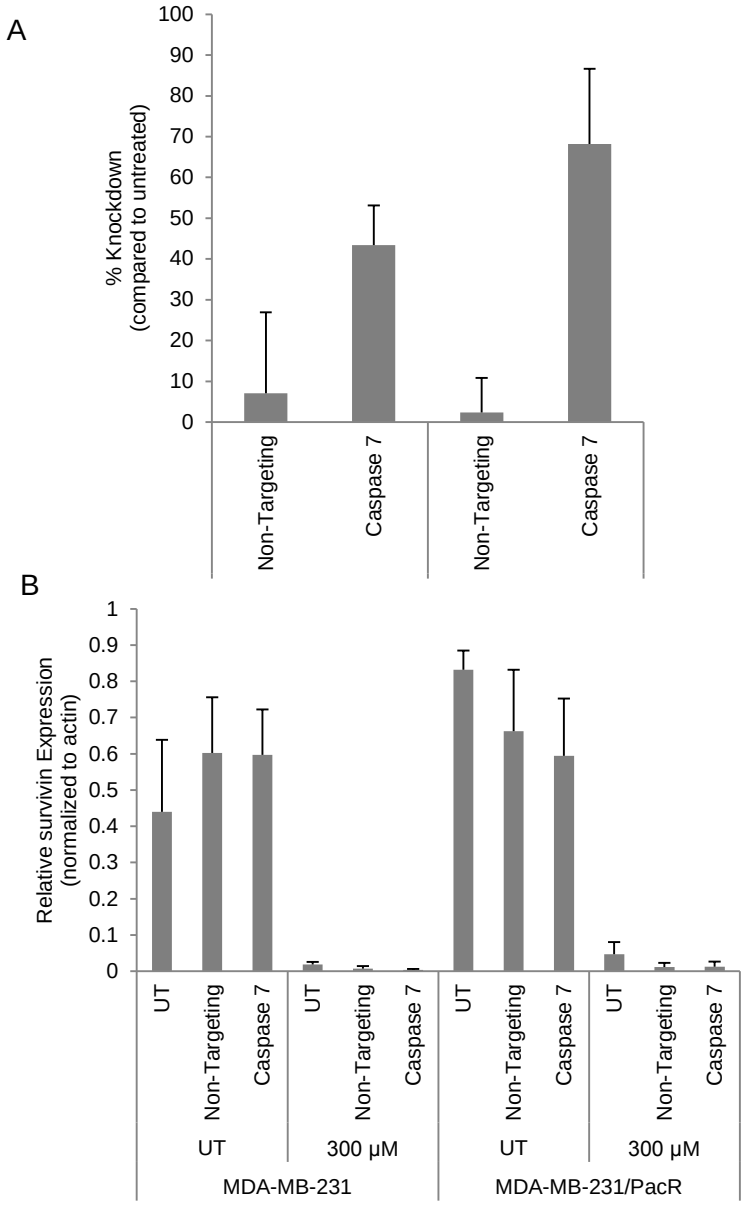


Figure 19. Caspase 7 Knockdown Does Not Affect Resveratrol-Induced Survivin Decrease. A) Percent knockdown achieved in three independent experiments using a pool of 4 siRNAs targeting Caspase 7 at 48 hours post a 96 hour transfection (n=3). B) Densitometry quantification normalized to actin of survivin expression following 96 hour siRNA transfection and subsequent 48-hour resveratrol treatment (n=3). Error bars signify standard deviation (*p<0.05; **p<0.01; ***p<0.001; *p-values compare treatment to respective DMSO control).

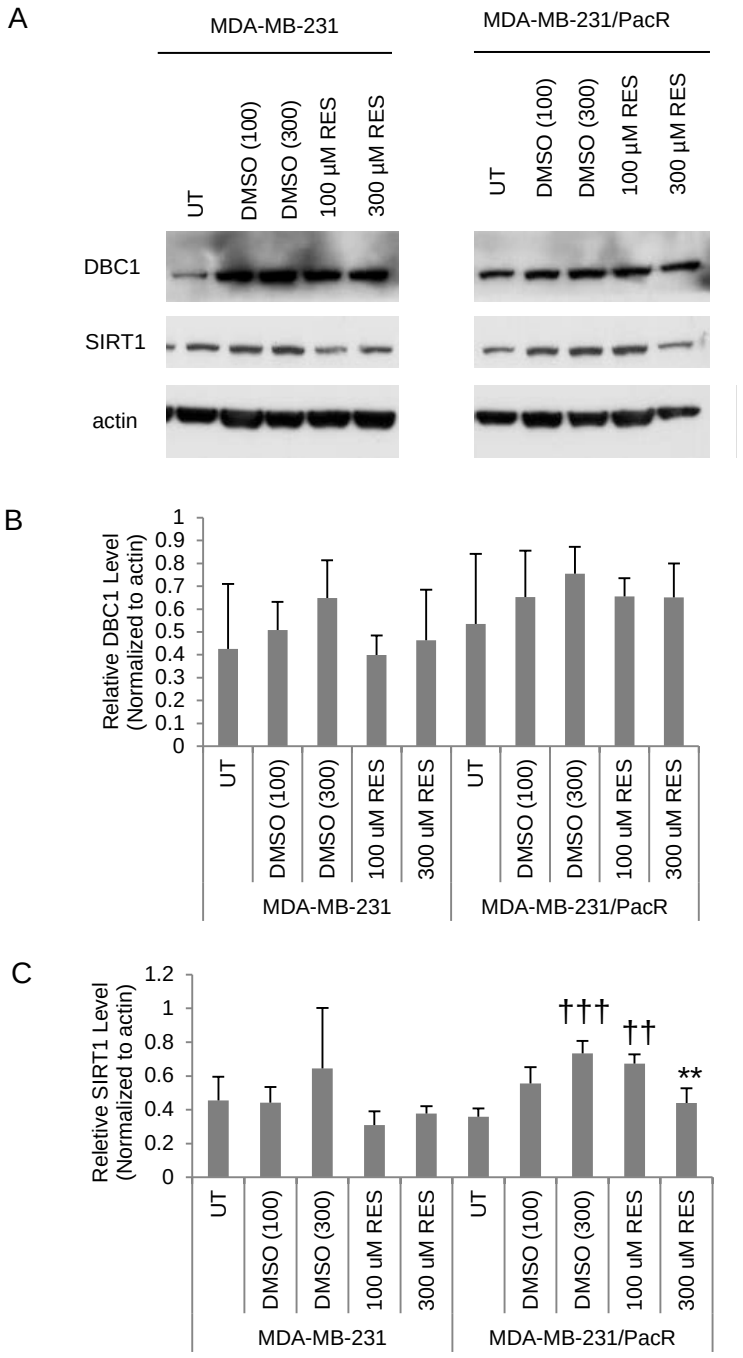


Figure 20. Resveratrol Does Not Affect Protein Expression of DBC1 & SIRT1. A) Representative immunoblots of SIRT1 and DBC1 after 2 hours of resveratrol treatment. B) Densitometry quantitation normalized to actin of DBC1 and C) of SIRT1. Error bars signify standard deviation (*p<0.05; **p<0.01; ***p<0.001; *p-values compare treatment to respective DMSO control; †p-values compare untreated and DMSO control).

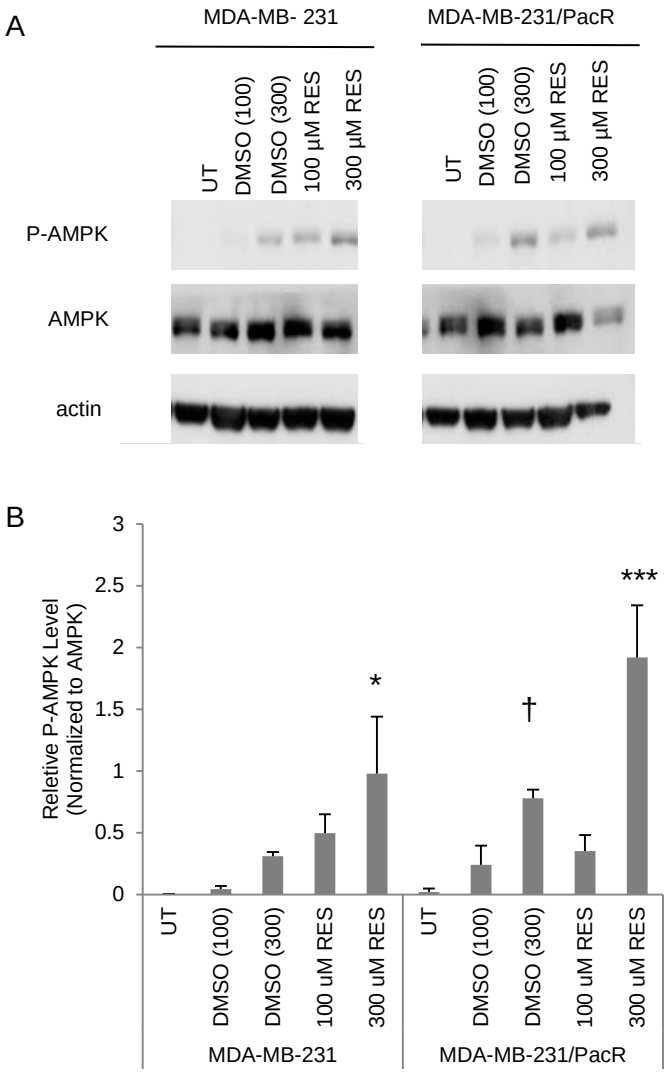


Figure 21. Resveratrol Activates AMPK. A) Representative immunoblots of P-AMPK and AMPK after 2 hours of resveratrol treatment. B) Densitometry quantitation of P-AMPK normalized to total AMPK (*p<0.05; **p<0.01; ***p<0.001; *p-values compare treatment to respective DMSO control; †p-values compare untreated and DMSO control).

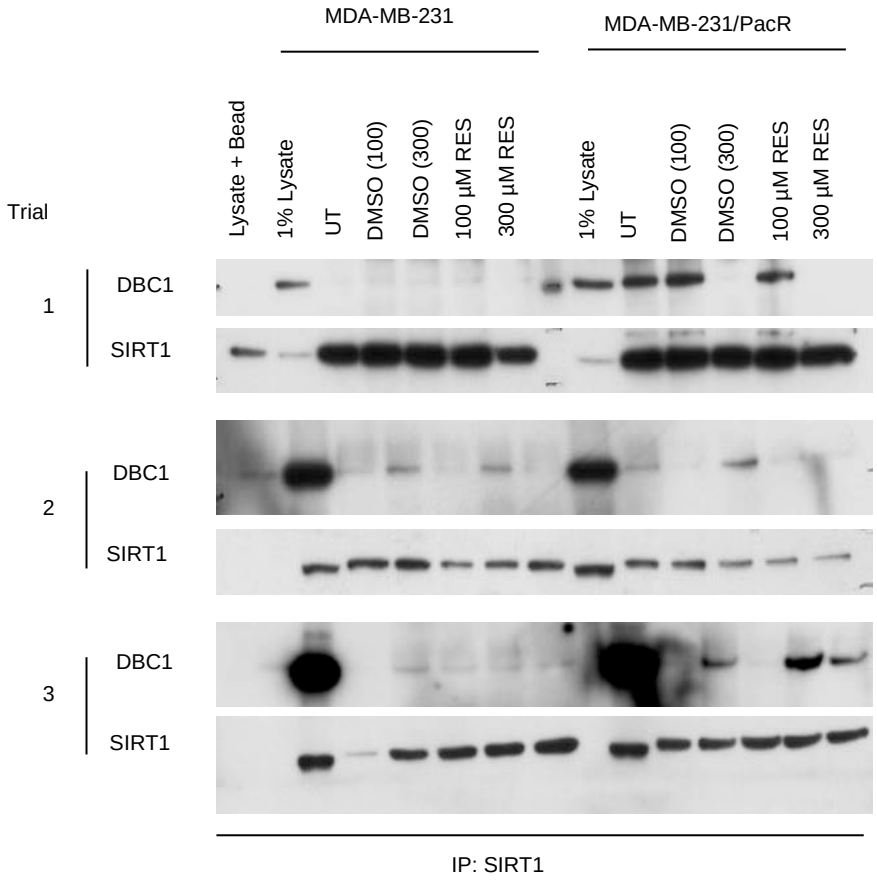


Figure 22. Resveratrol Does Not Affect Binding of DBC1 & SIRT1. Three independent trails of the immunoprecipitation of SIRT1 from cell lysates treated with resveratrol for 2 hours were conducted. Shown are the immunoblots from each trial probed for both SIRT1 to show immunoprecipitation and DBC1 to show any co- immunoprecipitation. Far left labels signify the trial number shown. A 1% lysate control and lysate and bead only control were included for each trial.

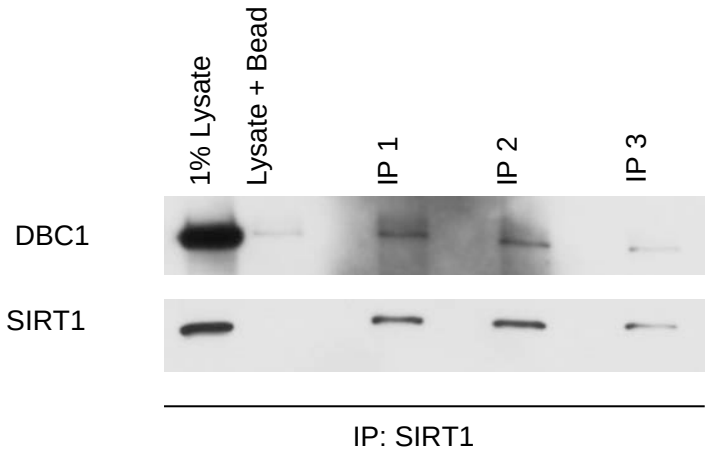


Figure 23. SIRT1 & DBC1 Co-immunoprecipitate in MDA-MB-468 cells. Three independent trails of the immunoprecipitation of SIRT1 from MDA-MB-468 cell lysates treated with resveratrol for 2 hours were conducted. Shown is the immunoblot showing each trail (IP 1-3 = immunoprecipitation trail 1-3) probed for both SIRT1 to show immunoprecipitation and DBC1 to show any co- immunoprecipitation. A 1% lysate control and lysate and bead only control were included.

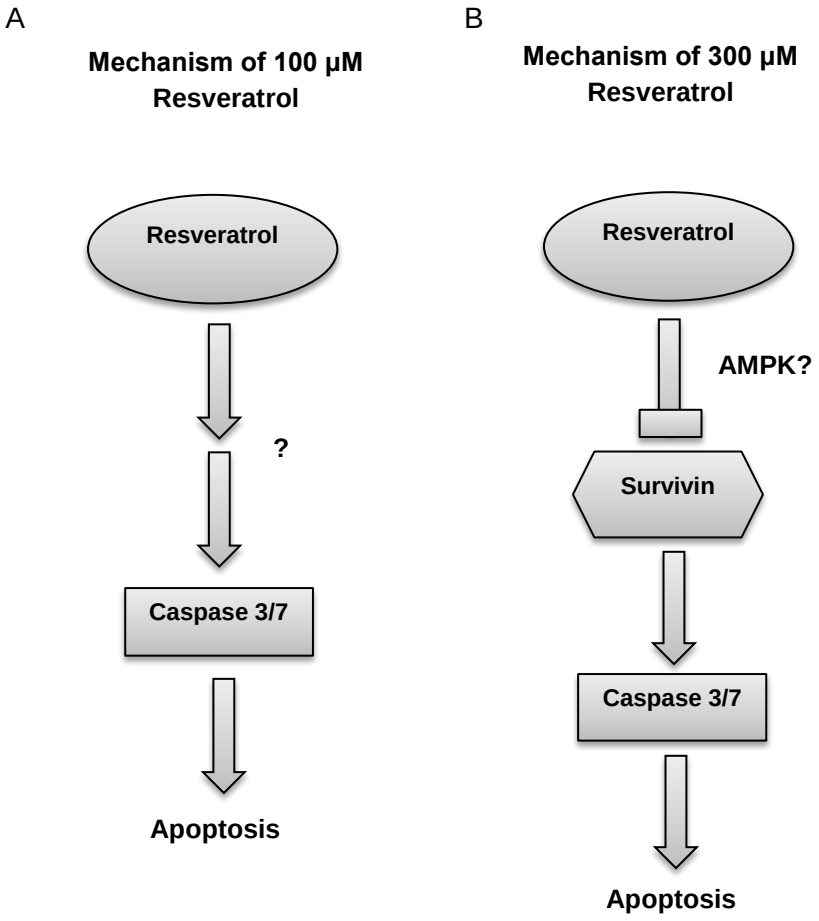


Figure 24. Mechanisms of Resveratrol Induced Apoptosis. A) Possible mechanism of 100 µM resveratrol treatment where resveratrol activates caspase 3 and 7 to lead to apoptosis with no contribution from survivin. B) Possible mechanism of 300 µM resveratrol treatment based on the data provided in this study showing the inhibition of survivin expression with AMPK activation possibly upstream of survivin. The inhibition of survivin allows activated caspase 3 and 7 to cause apoptosis. A "?" implies a gap in mechanism understanding.

III. RESVERATROL AUGMENTS PACLITAXEL TREATMENT

To determine whether resveratrol treatment could re-sensitize the resistant cells to paclitaxel, checkerboard drug combination assays were conducted as previously described [231, 232]. The addition of micromolar (2.5-40 μM) amounts of resveratrol simultaneously with paclitaxel resulted in a significant lowering of the IC_{50} value of paclitaxel in both MDA-MB-231and MDA-MB-231/PacR cells without any change in resveratrol IC_{50} (Figure 25). By comparing the effects the same ratios had on the different cell lines, it appeared that less resveratrol was required to achieve similar fold changes in IC_{50} in the resistant cells compared to the parental cells. A combination treatment of 1 part paclitaxel to 2,560 parts resveratrol produced the same IC_{50} value in both the MDA-MB-231 and MDA-MB-231/PacR cells; this IC_{50} value was significantly lower than the IC_{50} value of paclitaxel alone in MDA-MB-231 cells. In both cells lines, there were some ratios that caused a decrease of IC_{50} value of both drugs, though more resveratrol was required in the MDA-MB-231 cells compared to the MDA-MB-231/PacR cells to achieve this (Table 1). Most combinations did not give a synergistic effect, but rather appear additive or even antagonistic when represented in an isobologram due to the IC_{50} value of resveratrol not decreasing (Figure 26). Some combination ratios resulted in similar IC_{50} values for both cell lines. In contrast, other combination ratios significantly decreased the IC_{50} of the resistant cells but had no effect on the parental cells suggesting that resveratrol may act on two different mechanisms to have a combination effect, one that is specific to the resistant cells and one common to both parental and resistant. Due to these observations, we hypothesized that the mechanism of resistance of the MDA-MB-231/PacR cells was important for the effect of resveratrol in combination with paclitaxel in these cells. These data were contrary to a previous report that showed attenuation of paclitaxel by resveratrol with a 48-hour simultaneous

treatment [226]. Interestingly, we see similar attenuation at 24 hours in the MDA-MB-231 cells at both 10 and 100 μM resveratrol treatment (Figure 27). For the MDA-MB-231/PacR cells, 10 μM resveratrol treatment has no effect on paclitaxel whereas there is no attenuation is seen with 100 μM resveratrol treatment.

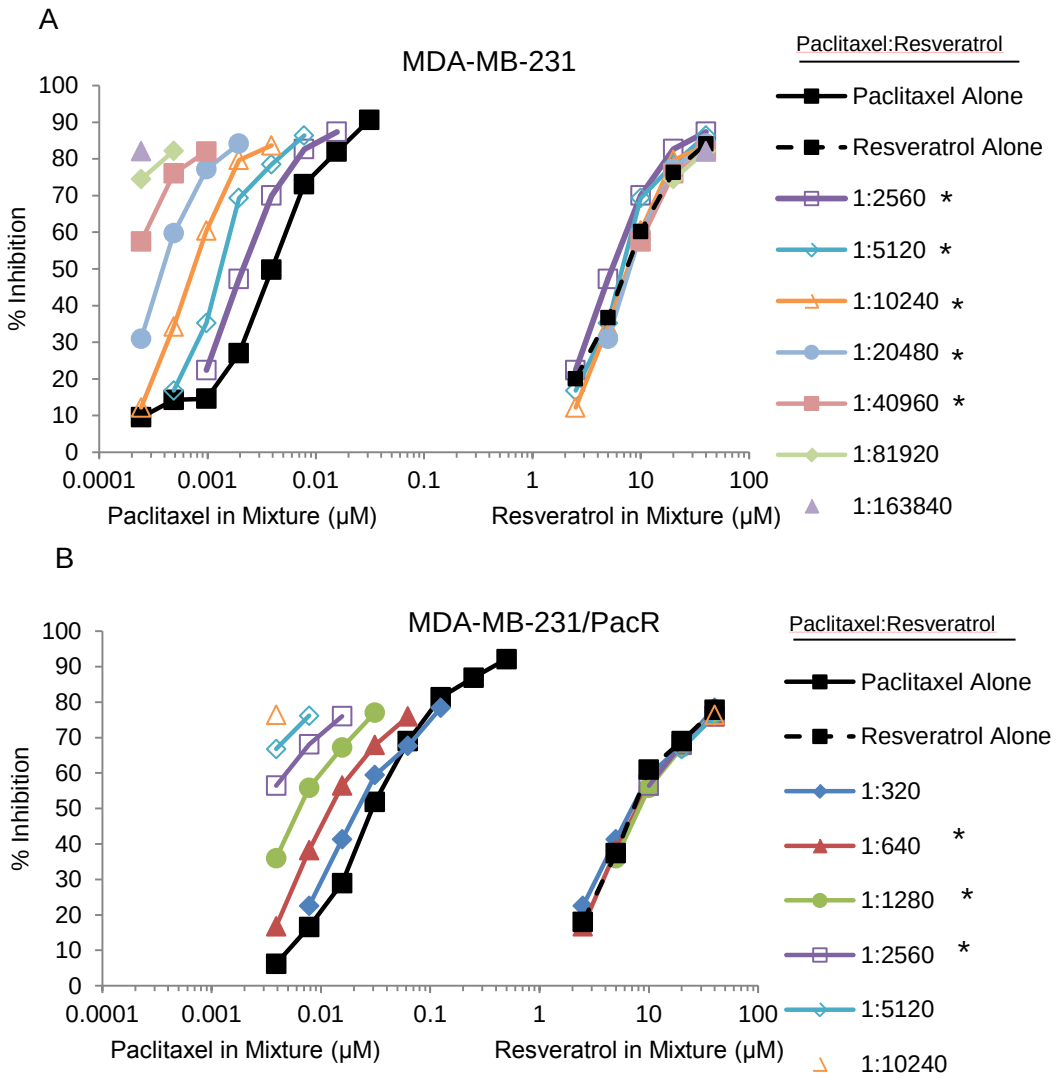


Figure 25. Resveratrol Augments Paclitaxel Treatment in MDA-MB-231 & MDA-MB-231/PacR Cells. Dose response curves of the constant drug ratios from the 5-day checkerboard combination assay in A) MDA-MB-231 and B) MDA-MB-231/PacR cells (n=3). Each drug ratio has two graphs one representing the % inhibition versus the amount of paclitaxel in the mixture as well as one representing the % inhibition versus the amount of resveratrol in the mixture. Standard deviations for individual point were

less than 50% (*p<0.05; **p<0.01; ***p<0.001; *p-values compare combination treatment IC_{50} values to the paclitaxel alone treatment IC_{50} value).

	Paclitaxel: Resveratrol	Paclitaxel IC_{50} (nM)	Fold Decrease compared to Paclitaxel Alone	Resveratrol IC_{50} (μM)	Fold Decrease compared to Resveratrol Alone
MDA-MB-231	Alone	4.40 ± 1.49	-	7.79 ± 0.57	-
	1:40960	0.16 ± 0.06***	28.19	6.39 ± 2.58	1.22
	1:20480	0.41 ± 0.09***	10.75	8.38 ± 1.83	0.93
	1:10240	0.80 ± 0.11***	5.53	8.15 ± 1.08	0.96
	1:5120	1.48 ± 0.24***	2.97	7.60 ± 1.22	1.02
	1:2560	2.42 ± 0.38*	1.82	6.20 ± 0.98	1.26
	1:1280	2.91 ± 0.42	1.51	3.73 ± 0.53*	2.09
	1:640	3.34 ± 0.42	1.32	2.14 ± 0.27**	3.64
	1:320	2.41 ± 0.21*	1.82	0.77 ± 0.07***	10.08
MDA-MB-231/PacR	Alone	37.64 ± 13.29	-	9.07 ± 1.57	-
	1:2560	2.52 ± 0.61***	14.93	6.45 ± 1.57	1.41
	1:1280	6.77 ± 1.23***	5.56	8.66 ± 1.58	1.05
	1:640	15.28 ± 2.11*	2.46	9.78 ± 1.35	0.93
	1:320	25.67 ± 4.21	1.47	8.21 ± 1.35	1.10
	1:160	35.96 ± 10.01	1.05	5.75 ± 1.60	1.58
	1:80	25.61 ± 9.37	1.47	2.05 ± 0.75***	4.43
	1:40	2.51 ± 2.57***	15.00	0.10 ± 0.10***	90.41
	1:20	0.16 ± 0.27***	240.95	0.00 ± 0.01***	2904.51

Table 1. Combination Treatment Decreases Paclitaxel IC_{50}. IC_{50} values for paclitaxel and resveratrol plus or minus the standard deviation and the fold decrease of IC_{50} compared to each drug alone of all combination treatments (*p<0.05; **p<0.01; ***p<0.001; *p-values compare IC_{50} values of combination treatment to individual drug alone).

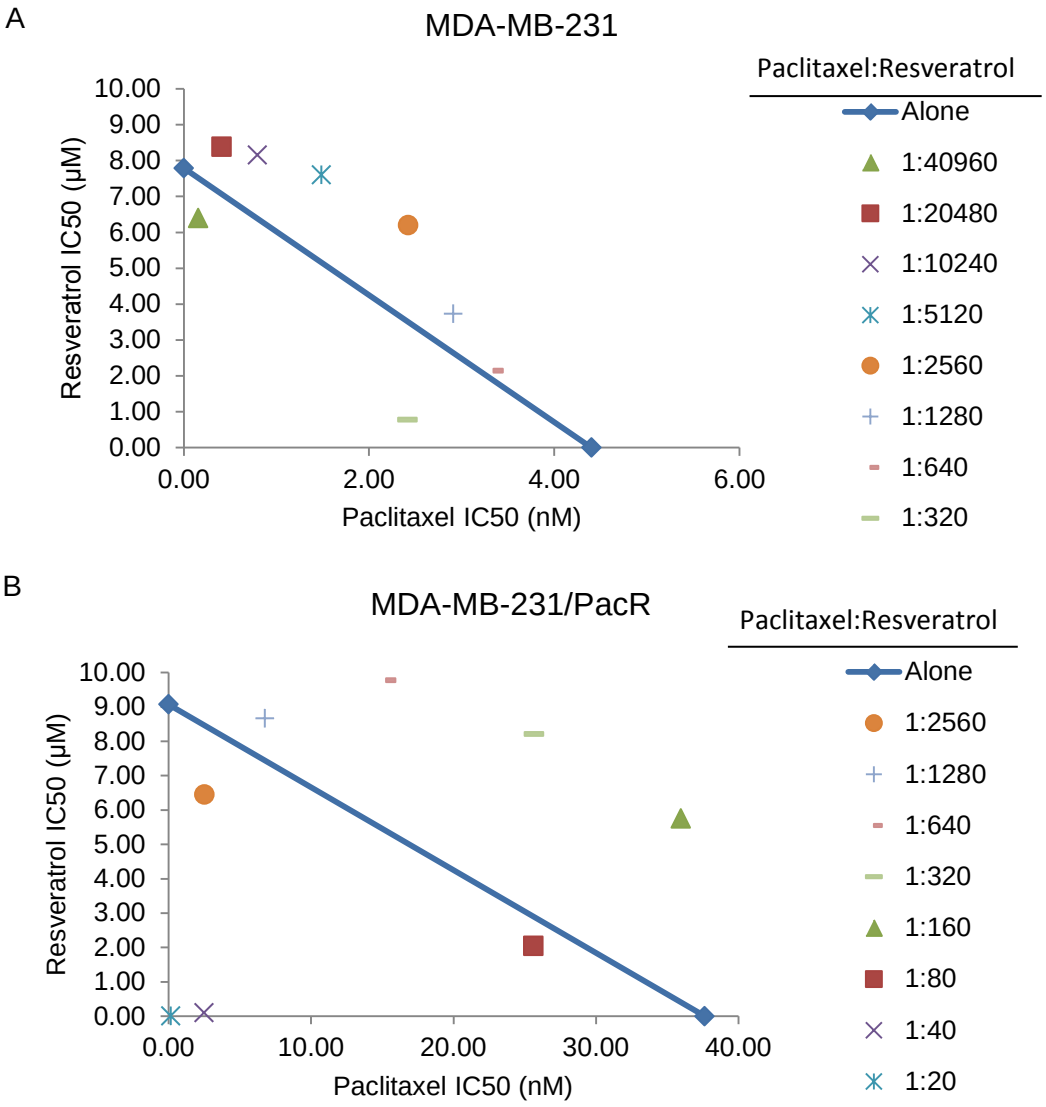


Figure 26. The Combination of Resveratrol and Paclitaxel is not Synergistic. Isobologram graphs showing the combination effect status of each drug ratio from the 5-day checkerboard combination assay in A) MDA-MB-231 and B) MDA-MB-231/PacR cells. The average IC_{50} values of resveratrol alone on the y-axis and of paclitaxel alone on the x-axis are connected to provide the line of additivity. Each drug ratio is a single point based on the average IC_{50} value for both drugs at this ratio. Any point that falls on the line of additivity denotes an additive effect, below the line denotes synergistic effect, and above the line denotes an antagonistic effect of the drug combination.

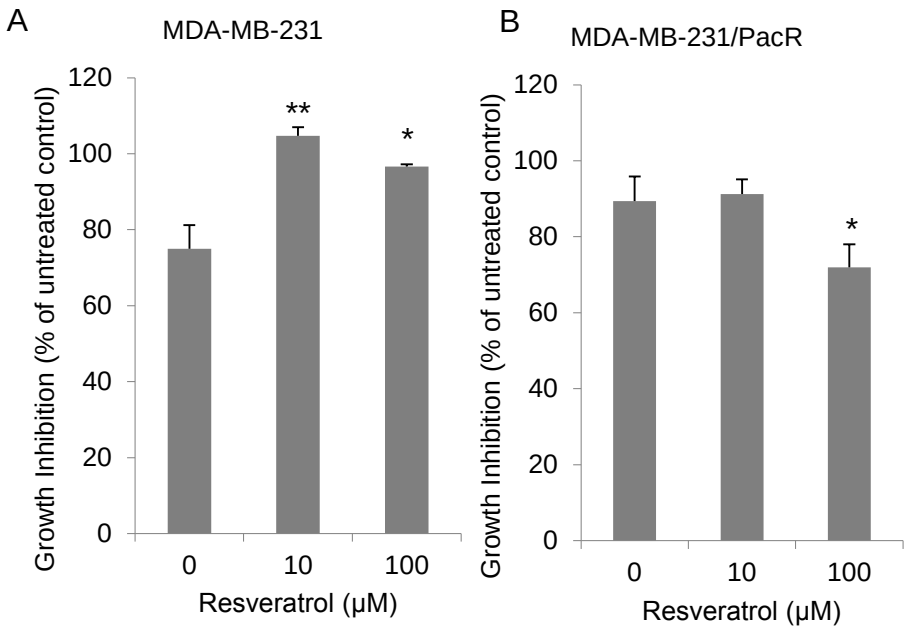


Figure 27. Resveratrol Can Attenuate Paclitaxel Treatment with Short-term, Simultaneous Treatment. Treatment with a 24-hour simultaneous treatment of 100 nM paclitaxel treatment with 0, 10, or 100 μM resveratrol. Resveratrol A) attenuates paclitaxel in MDA-MB-231 cells and B) has no effect on MDA-MB-231/PacR cells at 10 μM resveratrol with only a small decrease of growth inhibition at 100 μM resveratrol (n=3). Error bars represent standard deviation (*p<0.05; **p<0.01; ***p<0.001; *p-values compare resveratrol treatment to paclitaxel alone treatment).

IV. UPREGULATION OF P-GLYCOPROTEIN & CYP2C8 CONTRIBUTES TO PACLITAXEL RESISTANCE IN MDA-MB-231/PacR CELLS

To determine the mechanism of resistance in these cells, a commercial PCR array for human cancer drug resistance genes was used. Analysis of the PCR array identified two genes to be highly upregulated in the resistant MDA-MB-231/PacR cells compared to the parental MDA-MB-231 that are known to be factors in paclitaxel resistance, *ABCB1* and *CYP2C8* (Table 2). The full list of genes and the expression changes are shown in Appendix A. These two genes were focused on because of their known relevance to paclitaxel resistance in breast cancer. The over-expression *ABCB1* and *CYP2C8* was validated by real-time RT-PCR and the levels were compared between the parental MDA-MB-231 and the MDA-MB-231/PacR or MDA-MB-231/PacR-Hi cells. *ABCB1* was overexpressed in MDA-MB-231/PacR and MDA-MB-231/PacR-Hi cells compared to the parental line (Figure 28A). *CYP2C8* was also significantly upregulated in both cells lines compared to the parental cells (Figure 28B). Interestingly, there was also a significantly higher expression of *CYP2C8* in the MDA-MB-231/PacR cells compared to the MDA-MB-231/PacR-Hi cells.

Finally, the functional importance of the gene expression data was tested with inhibitors of the protein products of the genes. Verapamil was used to inhibit P-gp, the protein product of *ABCB1*; and trimethoprim was used to selectively inhibit CYP2C8 [239]. Inhibition of P-gp or CYP2C8 alone decreased the resistance of the MDA-MB-231/PacR cells to paclitaxel, as measured by proliferation assays (Figure 29). The paclitaxel IC_{50} for MDA-MB-231/PacR cells alone was 61 nM. After trimethoprim treatment, the IC_{50} decreased to 40 nM. After verapamil treatment the IC_{50} decreased to 8 nM. Simultaneous inhibition of both P-gp and CYP2C8 did not further lower the IC_{50} value of the MDA-MB-231/PacR cells at 9 nM, likely because verapamil alone lowered

the paclitaxel IC_{50} to nearly that of the parental MDA-MB-231 cells, which is 5 nM. Interestingly, only inhibition of P-gp significantly decreased the resistance to paclitaxel of the MDA-MB-231/PacR-Hi cells from the paclitaxel IC_{50} of 117 nM to 12 nM. There was no statistical difference between the paclitaxel IC_{50} of MDA-MB-231 and the IC_{50} values of the MDA-MB-231/PacR or MDA-MB-231/PacR-Hi cells after verapamil or the verapamil and trimethoprim treatment. Additionally, there were no differences between the paclitaxel IC_{50} values in any of the cells lines between the verapamil and the verapamil and trimethoprim treatments. Therefore, it seems that although P-gp, CYP2C8, and possibly other factors are relevant to the resistance, P-gp is likely the more important factor in both the MDA-MB-231/PacR and MDA-MB-231/PacR-Hi cells.

Symbol	Description	Function	Fold Change	T-TEST	Fold Up- or Down-Regulation
			MDA-MB-231/PacR / MDA-MB-231	p-value[a]	MDA-MB-231/PacR / MDA-MB-231
ABCB1	ATP-binding cassette, sub-family B (MDR/TAP), member 1	Xenobiotic metabolism	181.99	0.21	181.99
CYP2C8	Cytochrome P450, family 2, subfamily C, polypeptide 8	Xenobiotic metabolism	12.51	0.16	12.51
CYP1A1	Cytochrome P450, family 1, subfamily A, polypeptide 1	Xenobiotic metabolism	5.06	0.16	5.06
ATM	Ataxia telangiectasia mutated	S/T protein kinase	3.35	0.01	3.35
TNFRSF11A	Tumor necrosis factor receptor superfamily, member 11a	NFKB activator	3.11	0.00	3.11
APC	Adenomatous polyposis coli	Tumor repressor	3.11	0.05	3.11
CYP2E1	Cytochrome P450, family 2, subfamily E, polypeptide 1	Xenobiotic metabolism	0.03	0.01	-31.07
ERBB4	V-erb-a erythroblastic leukemia viral oncogene homolog 4 (avian)	Receptor tyrosine kinase	0.15	0.02	-6.71
AR	Androgen receptor	Androgen receptor	0.21	0.04	-4.80
ABCC3	ATP-binding cassette, sub-family C, member 3	Xenobiotic metabolism	0.23	0.14	-4.27
ABCG2	ATP-binding cassette, sub-family G, member 2	Xenobiotic metabolism	0.24	0.11	-4.11
FOS	FBJ murine osteosarcoma viral oncogene homolog	Transcription factor	0.32	0.06	-3.16

[a]P-values were calculated with a Student's T-Test of the replicate 2^(- ΔCt) values for each gene

Table 2. PCR Array Identified Two Genes, *ABCB1* & *CYP2C8*, Implicated in Breast Cancer Paclitaxel Resistance.

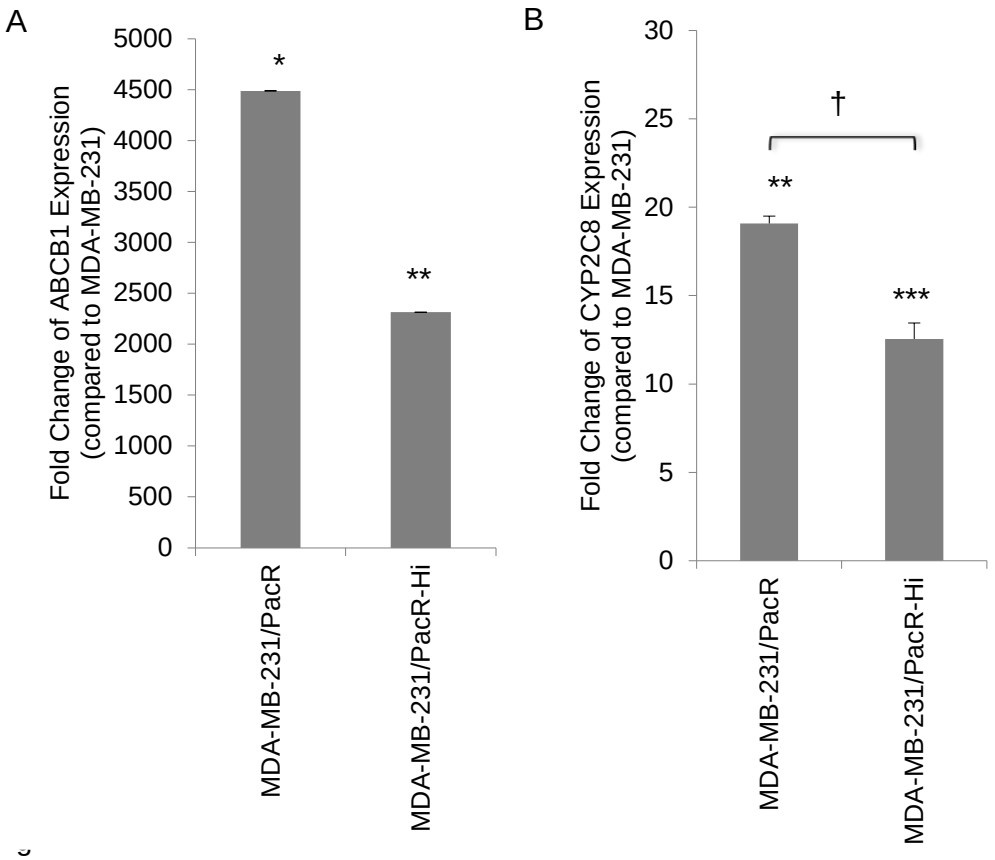


Figure 28. ABCB1 & CYP2C8 are overexpressed in MDA-MB-231/PacR & MDA-MB-231/PacR-Hi Cells. Fold change of gene expression from qPCR measuring mRNA levels in MDA-MB-231/PacR and MDA-MB-231/PacR-Hi cells compared to MDA-MB-231 cells A) of ABCB1 B) of CYP2C8 (n=3). Error bars signify standard deviation. P-values were calculated with a Student's T-Test of the replicate 2^(- ΔCt) values for each gene and compare resistant cell line to parental line (*p<0.05; **p<0.01; ***p<0.001; * p-value compares fold changes of each resistant line and the parental line; † p-value compares fold changes of MDA-MB-231/PacR to that of MDA-MB-231/PacR-Hi).

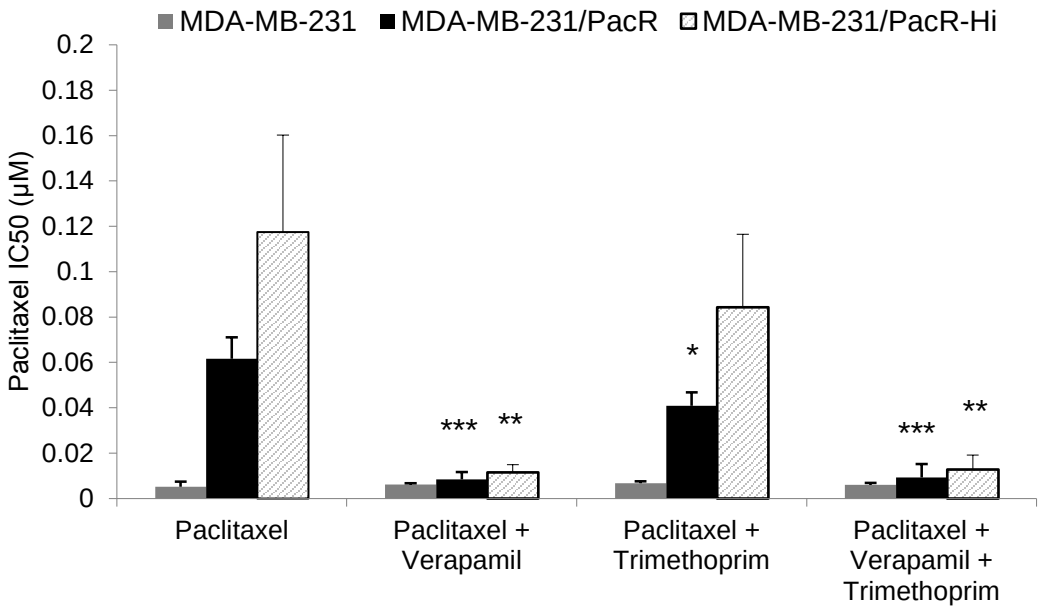


Figure 29. Inhibition of P-glycoprotein & CYP2C8 Re-sensitize MDA-MB-231/PacR Cells to Paclitaxel. The 5-day IC_{50} values for MDA-MB-231, MDA-MB-231/PacR and MDA-MB-231/PacR-Hi cells with paclitaxel alone or in simultaneous combination with the P-gp inhibitor, verapamil (1 µM), the CYP2C8 inhibitor, trimethoprim (25 µM), or both (n=3). Error bars signify standard deviation (*p<0.05; **p<0.01; ***p<0.001; *p-values compare inhibitor treatment to paclitaxel alone treatment). There was no statistical difference between the MDA-MB-231 and MDA-MB-231/PacR or MDA-MB-231/PacR-Hi cells after verapamil or the verapamil and trimethoprim treatment. Additionally, there were no differences between any of the cells lines between the verapamil and the verapamil and trimethoprim treatments

CHAPTER 4: DISCUSSION and FUTURE DIRECTIONS

I. RESISTANT CELLULAR MODEL GENERATION

A novel, paclitaxel-resistant TNBC cell line was successfully developed with a 12-fold resistance to paclitaxel compared to parental cells. The intermittent, stepwise treatment method (Figure 7) developed a more resistant and better growing population of resistant cells compared to a continuous treatment method. Furthermore, this intermittent treatment method mimics acquired paclitaxel resistance that can occur in patients after intermittent paclitaxel treatment of a primary tumor where any remaining cancer cells are paclitaxel resistant and can then recur as a paclitaxel-resistant tumor. Clinically relevant acquired drug resistance spans from 2- to 12-fold increased resistance compared to cells from tumors prior to treatment [240]. Therefore, this cellular model more closely mimics what is observed clinically when compared to other laboratory models, where resistance is so high it is not clinically meaningful [240]. Importantly, this clinically relevant resistance is maintained though the cells are no longer cultured in paclitaxel unlike other paclitaxel-resistant cell lines that must be continuously grown in the presence of the drug to maintain resistance [240]. Though generated cellular models are useful for studying cancer resistance, it is important that results from such a model system are further studied for their clinical relevance [37].

II. RESVERATROL INDUCES CELL PROLIFERATION INHIBITION, SENESCENCE, AND APOPTOSIS

The data presented here suggest that resveratrol induces cell proliferation inhibition, senescence, and apoptosis in TNBC cells regardless of paclitaxel sensitivity.

The low R^2 value, the correlation coefficient of the line of best fit, of the clone paclitaxel and resveratrol IC_{50} values analysis indicates that there is no correlation between paclitaxel resistance and resveratrol sensitivity (Figure 8). Furthermore, there was no statistically significant difference between the paclitaxel-resistant and -sensitive cells in any of the studies conducted with resveratrol alone.

In MDA-MB-231 and MDA-MB-231/PacR cells, resveratrol inhibited cell proliferation and induced senescence and apoptosis (Figures 9-22). In cell count proliferation (Figure 9) and colony formation assays (Figure 10), 100 μM resveratrol treatment completely inhibited the ability of the parental and resistant cells to proliferate. In the cell count assay, 10 μM resveratrol treatment significantly decreased proliferation in both cell lines (Figure 9) but only significantly decreased colony formation in the resistant cells in the survival assay (Figure 10A). The differential effect of colony formation at 10 μM resveratrol between the two methods suggests resveratrol may affect the ability of MDA-MB-231/PacR, but not MDA-MB-231, cells to attach to the plate after treatment. In addition, the proliferation data suggest that although 10 μM resveratrol was able to inhibit proliferation after 3 days (Figure 9), this effect was not irreversible as evidenced by the ability of colonies to form after removal of resveratrol (Figure 10). This is in contrast to the effect of 100 μM resveratrol treatment, where cell counts showed that cells plated were still attached to the plate though they had not proliferated.

Resveratrol alone did not induce cell cycle arrest (Figure 11), which has previously been shown in multiple cancer cell lines [161]. It is possible, however, that arrest was missed and occurred before the Sub-G1 accumulation occurred with 300 μM resveratrol at 48 hours, as no other time points were assessed. Additionally, it is possible that lower concentrations including, 10 and 100 μM, of resveratrol would have shown cell cycle arrest at time points longer than 48 hours. Interestingly, a peak was observed in both cell populations that may suggest micronucleation in these cells. As

discussed previously, micronucleated cells can accumulate between G1 and S phase as well as after G2/M phase due to their DNA content [236]. Though we did find the presence of micronucleated cells in the untreated cell lines (Figure 12), measuring the increase of micronucleation after 300 μM resveratrol treatment using immunofluorescence was not possible as very few cells remain attached to a slide. Micronucleated cells, a sign of genotoxic stress, are common in cancer cell populations; however, the fate of micronucleated cells is unclear. Micronuclei may re-incorporate into the nucleus, the cell may destroy the micronucleus and continue to proliferate though divisions are likely limited, or cell may undergo apoptosis [241]. Consequently, the significance of resveratrol treatment increasing miconucleation is uncertain.

In the context of the senescence and apoptosis data, the proliferation and cell cycle data is further explained. After 3 days of treatment, 37% of MDA-MB-231 and 38% of MDA-MB-231/PacR cells treated with 100 μM resveratrol were senescent (Figure 13). As senescent cells can arrest in G1, G2/M or S phase [194], this increase in senescence can explain why there is no cell proliferation and no cell cycle arrest concurrently. Additionally, at the same 3-day time point, 31% of MDA-MB-231 and 19% of MDA-MB-231/PacR cells treated with 100 μM resveratrol are undergoing apoptosis (Figure 14). Therefore, after 3 days of 100 μM resveratrol treatment, approximately 60% of both cell lines have lost their ability to proliferate as they are either senescent or apoptotic. It is also possible that by 3 days after treatment, there is also some cell cycle arrest or autophagy that explains why the remaining cells are not proliferating according to the cell proliferation data. In further support of additional mechanisms of action, 10 μM resveratrol treatment in both cell lines showed no cell cycle arrest and little senescence induction while cell proliferation was significantly inhibited, though this proliferation inhibition was partially reversible as suggested by colony formation. By 5 days after 100 μM resveratrol treatment, 64% and 53% of MDA-MB-231 and MDA-MB-231/PacR cells,

respectively, have undergone apoptosis, and so nearly all cells in both cell lines are not able to divide. After 48 hours of 300 μM resveratrol treatment, 49% and 34% of MDA-MB-231 and MDA-MB-231/PacR cells respectively, accumulated in Sub-G1 phase, which is consistent with late apoptosis (Figure 12). Correspondingly, the apoptosis data shows that by 3 days 300 μM resveratrol treatment, nearly all of the cells in both cell lines are in either early or late apoptosis (Figure 14).

Though the method of measuring apoptosis used in this study cannot distinguish between late apoptosis and necrosis, the presence of early apoptosis and the activation of caspase 3 and caspase 7 indicate that the cells are likely undergoing apoptosis. With 300 μM resveratrol treatment, a large activation of caspase 7 can be seen at 48 hours. For 100 μM resveratrol treatment, caspase 7 activation began at 48 hours (Figure 15) and increased through 3 and 5 days suggesting a slow acting caspase response (Figure 16). In contrast, the 48-hour caspase 3 data suggest this time point is likely the end of activation as none is seen at 3 or 5 days, and so this is a faster process than caspase 7 activation (Figure 17). In addition, these data imply caspase 3 is less important than caspase 7 in the 100 μM resveratrol as procaspase 3 never decreases and cleaved caspase 3 was never detected at this treatment concentration. It is important to note that the early and late apoptosis seen at 5 days may represent the heterogeneity of the cell population as this time point would suggest delayed onset apoptosis or other forms of cell death such as autophagy or necroptosis.

The mechanism of resveratrol-induced apoptosis in this model does not appear to be dependent on the decrease of survivin expression, though a dose-dependent decrease of survivin has been shown in multiple other human cancer cell lines [212, 242-244]. Survivin protein levels were only decreased by 300 μM resveratrol treatment (Figure 18). This decrease was complete by 48 hours and was sustained at least through 5 days. Treatment with 100 μM resveratrol did not decrease survivin even after

5 days. This is interesting because while apoptosis was observed at 3 and 5 days of 100 μM treatment, there was no significant difference in survivin protein levels, suggesting survivin may not be critical in resveratrol-induced apoptosis in these cells at this lower concentration (Figure 18C-D). It is possible there is a dose-dependent relationship with a small window of efficacy between 100 and 300 μM, but it is also possible the decrease is an off-target effect of resveratrol. Importantly, knockdown of caspase 7 did not result in any recovery of survivin protein levels (Figure 19), which implies that the decrease of survivin is not due to the cleavage activity of caspase 7 leading to the degradation of survivin protein, which is further supported by the fact that although caspase 7 activation is seen after 3 and 5 days of 100 μM resveratrol treatment, there is no decrease of survivin levels. Therefore, survivin may only play a role at high concentration resveratrol treatment or may be an off-target effect of resveratrol in these cells.

Resveratrol did not affect protein levels of SIRT1 or DBC1 (Figure 20) but did activate AMPK (Figure 17), as expected if resveratrol were activating SIRT1 by interrupting the binding of SIRT1 and DBC1. However, we observed little to no binding between SIRT1 and DBC1 in the MDA-MB-231 cells (Figure 22) in accordance with a previous study which determined the amount of binding between SIRT1 and DBC1 in various breast cancer cell lines [245]. However, some stronger binding between SIRT1 and DBC1 was seen in the MDA-MB-231/PacR cells though this binding was very erratic across independent experiments, making this data difficult to interpret. To ensure the results seen were not due to an ineffective protocol, the experiment was repeated with a cell line recognized by Sung et al. to have association between SIRT1 and DBC1. MDA-MB-468 cells showed much more consistent binding between SIRT1 and DBC1 suggesting that the lack of binding in the co-immunoprecipitation experiment in MDA-MB-231 was due to little SIRT1 and DBC1 association (Figure 23). However, this does not explain the erratic binding seen in and MDA-MB-231/PacR cells. Together these

data suggest the mechanism of resveratrol-induced apoptosis in these cells is not dependent on decreased survivin expression, changes in SIRT1 and DBC1 levels, nor the binding of SIRT1 and DBC1. However, it is possible that resveratrol acts through SIRT1 activation in these cells by allosteric activation [246] or altering SIRT1 localization [247]. These data provide a mechanism that is still not clearly understood (Figure 24).

Taken together, these studies on the efficacy and mechanism of resveratrol in this cellular model of paclitaxel-sensitive and -resistant TNBCs point out that resveratrol is capable of inhibiting cell proliferation at low micromolar amounts and causes apoptosis at higher concentrations. Although the mechanism of resveratrol-induced apoptosis is not completely understood, it is possible that survivin and SIRT1 play a role as suggested previously [212]. Notably, the effects of resveratrol were not significantly different between the paclitaxel-resistant and -sensitive cells, suggesting resveratrol may be useful in treating TNBCs regardless of paclitaxel sensitivity.

These findings highlight the need for further examination of the mechanism of resveratrol as a single agent in these cell lines. It is possible that the cells accumulated after G2/M phase as well as in S phase are micronucleated cells. Whether the population is indeed micronucleated cells could be determined with simultaneous flow cytometry and imaging. This method combined with cell sorting may also help determine what the fate of the cells may be and how it is important in the context of resveratrol treatment. In addition, a more complete time course of cell cycle analysis after resveratrol treatment would further elucidate the timeline of resveratrol effects. Determination of possible autophagy caused by resveratrol could explain the gap in cell proliferation, senescence and apoptosis data. Further examination to better understand what role, if any, survivin has in resveratrol treatment is also needed. Whether there is a dose-dependent response between 100 and 300 μM concentrations of resveratrol can be determined with immunoblotting, and whether survivin is required for resveratrol-

induced apoptosis can be assessed with stable survivin knockdowns. However, further survivin studies will not provide information on the mechanism of resveratrol-induced apoptosis at 100 μM treatment as survivin does not appear to important at this concentration. Therefore, further studies need to be conducted to determine the mechanism of resveratrol at this lower concentration as well. Considering previous work on resveratrol action, there are many possibilities for this mechanism that can be explored, though perhaps the most likely to be important in this model are activation of ceramide synthesis [207] and inhibition of NF-κB promoter activity [208] as these have been shown to be important factors in MDA-MB-231 cells. Ceramide is important mediator in cellular stress response, and the accumulation of ceramide is growth inhibitory and pro-apoptotic [248]. The inhibition of the oncogenic NF-κB, which is constitutively activated in MDA-MB-231 cells and many other cancers, can promote cancer cells to stop proliferating or undergo apoptosis [249]. Finally, there is currently no way to directly measure SIRT activity within a cell line, which makes determining resveratrol action on SIRT1 difficult. A common measure of SIRT1 activity in cells is to measure the amount of p53 that is acetylated using immunobloting with an antibody specific for aceteylated p53; lower acetylation of p53 suggests higher SIRT1 activity as p53 is a substrate for SIRT1. This method was attempted in the present study without success, possibly due to the mutated p53 in the cells. A fluorometric assay using a cell extract can measure SIRT1 activity directly; however, in the presence of resveratrol, the fluorescent probe has been shown to affect the results of these assays [250]. An important study would be the examination of the acetylation status of the survivin promoter after resveratrol treatment if survivin is shown to be important in resveratrol-induced apoptosis. A decrease of acetylation would be good evidence of SIRT1 activation by resveratrol as has been previously shown [212]. In addition to this, SIRT1 knockdown would determine the importance of SIRT1 in resveratrol-induced apoptosis.

Therefore, chromatin immunoprecipitation and stable SIRT1 knockdown would be the best options for further experiments into SIRT1 activation by resveratrol.

III. RESVERATROL AUGMENTS PACLITAXEL TREATMENT

Importantly, our data show that resveratrol can augment the effects of paclitaxel when used in simultaneous combination (Figure 25). This is contrary to a previous report showing resveratrol to attenuate paclitaxel treatment in MDA-MB-231 cells [226]. Interestingly, Fukui et al. used a 48-hour simultaneous treatment, and previous experiments from the present study showed a 24-hour time point of resveratrol treatment either had no effect or caused similar attenuation of paclitaxel treatment (Figure 27). The present study suggests at a 5-day time point resveratrol enhances paclitaxel treatment. The IC_{50} values of paclitaxel for both the parental and the resistant cells were lowered significantly with the addition of resveratrol. Notably, addition of resveratrol decreased the paclitaxel IC_{50} value of the resistant MDA-MB-231/PacR cells to the IC_{50} value of the parental line. Moreover, the IC_{50} value could be lowered below that of the parental line with more resveratrol. A trend emerged in these data suggesting the more resveratrol that there is in the combination solution, the lower the resulting paclitaxel IC_{50} value. This was true in both the parental and the resistant cells. It is most likely that higher amounts of resveratrol in the MDA-MB-231/PacR cells could lower the IC_{50} of paclitaxel to similar levels as the combination treatment achieved in the MDA-MB-231 cells. In addition, lower concentrations of resveratrol decreased the IC_{50} value of both drugs compared to the single drugs alone (Table 1). In fact, with concentrations of resveratrol and paclitaxel similar to those used by Fukui et al., this study shows that after 5 days the combination of resveratrol and paclitaxel is synergistic (Figure 26). It is important to note that although the isobologram analysis shows that these combinations are largely

antagonistic (Figure 26), this was due to the resveratrol IC_{50} remaining the same while the paclitaxel IC_{50} decreased. Consequently, this is not the most useful measure of combination success in these circumstances. Critically, the fact that synergy was not widely achieved is not of concern as a desirable decrease of paclitaxel IC_{50} was achieved and necessary amounts of resveratrol are attainable and well tolerated in humans as well as inexpensive. Both high concentrations of resveratrol decreasing the paclitaxel IC_{50} with no change in resveratrol IC_{50} and lower concentrations of resveratrol decreasing both paclitaxel and resveratrol IC_{50} could be useful clinical tools. Interestingly, some ratios that significantly lowered the resistance of the MDA-MB-231/PacR cells had no effect on the parental cells. Considered in the context of the trend of the data, this suggests resveratrol may be acting of two different mechanisms; one that is specific to the resistance of the cells and one that is common to both the parental and resistant cells. Together, these data suggest resveratrol could be used to treat TNBCs in combination with paclitaxel to sensitize paclitaxel-resistance cancers and decrease the dose of paclitaxel needed without changing the efficacy of treatment. This proposed regimen could potentially both improve outcomes for patients with paclitaxel-resistant cancers and decrease the general toxicity caused by paclitaxel in patients with both paclitaxel-resistant and -sensitive cancers.

Further combination studies are needed to ensure the trend is correct and the effects of higher amounts of resveratrol in MDA-MB-231/PacR cells as well as the effects of lower amounts of resveratrol in MDA-MB-231 cells. It will be important to determine if the combination of resveratrol and paclitaxel not only inhibits cell proliferation, as was estimated with the checkerboard combination assay, but also induces apoptosis. Whether the combination can only inhibit growth or can also induce apoptosis will have important implications in the clinical use of the combination. Better understanding of the combination effect will help determine the correct treatment

administration and regimen for *in vivo* studies, which will need to be conducted to demonstrate that the combination is effective in tumors. Determination of the combination mechanism will also be important in order to provide a pharmacodynamic target that can then be used in animal and human studies to ensure that the intended target is hit by the treatment.

Due to the classification of resveratrol as a dietary supplement, clinical trials are a possibility. However, there are important points to consider before human trials are attempted. The first consideration is that, although resveratrol alone is very well tolerated in humans, it is possible that the combination of resveratrol and paclitaxel could increase the toxicity of paclitaxel. Due to the possibility of increasing paclitaxel accumulation by decreasing P-gp expression, liver toxicity is of concern. Importantly, liver toxicity can be closely monitored in human subjects. Perhaps of greatest concern is the dose limiting neurotoxicity of paclitaxel and the effects of the combination treatment on peripheral neurons, which can be tested *in vitro* using either dorsal root ganglia from rats or those derived from induced pluripotent stem cells from humans. Furthermore, as a possible alternative to doing the extensive mouse studies before clinical trials, the cells used in this study could be used in 3-dimentional cultures to better estimate the *in vivo* relevance of the combination study. Cells are grown in matrigel or low-attachment dishes with serum free media containing growth factors to promote proliferation and will form mammospheres in the media [251]. The mammary stem cells in the culture created provide the multiple cell types and the structure of a tumor that would be present *in vivo* [252]. This system mimics tumor drug delivery and effects more closely than cells grown in 2-dimentional cultures [252].

However, the determination of efficacy and toxicity in mice may still be a critical step between cell line and human studies. For these studies, the formulation of resveratrol will have to be carefully considered prior to these *in vivo* studies. In addition,

pilot studies would need to be conducted to determine any changes in pharmacokinetics when the drugs are combined and the best administration method of resveratrol, oral gavage, which mimics oral dosing in humans, or intraperitoneal injection, which is common among resveratrol studies. It is likely that daily dosage of resveratrol would be used due the pharmacokinetic properties, and that paclitaxel in PBS would be administered as an intraperitoneal injection once a week as this regimen is commonly used in mice as it mimics the dosing in humans. Depending on whether the treatment proves to inhibit cell proliferation only or also causes apoptosis will dictate whether these studies focus on prevention of tumor growth or also tumor shrinking. These studies would likely be conducted with orthotopic xenograft mouse models implanting the cells used in this study into the mammary fat pad of the highly immunodeficient NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ (NSG) mice [253] and comparing parental and resistant cells, as well as mice treated with vehicle, paclitaxel, resveratrol or a combination of paclitaxel and resveratrol. As an alternative to this model, a more clinically relevant model may be patient-derived xenografts, where human breast tumor tissue samples are implanted into the mammary fat pad of mice [254].

IV. UPREGULATION OF P-GLYCOPROTEIN & CYP2C8 CONTRIBUTES TO PACLITAXEL RESISTANCE IN MDA-MB-231/PacR CELLS

These data show *ABCB1* and *CYP2C8* genes were highly overexpressed in the resistant cells compared to the parental cells (Table 2, Figure 28). These two genes were focused on because of their known relevance to paclitaxel resistance in breast cancer; however, other genes identified by the PCR array may also play a role in the resistance mechanism. Only the genes represented in Table 2 will be further discussed here; see Appendix A for all genes tested. It is unlikely that CYP1A1 as it has no

reported connection of paclitaxel resistance. However, it is possible that the decrease of CYP2E1 plays some role as it has been reported that the presence of CYP2E1 can augment paclitaxel [255]. Though the over expression of *ABCC3* and *ABCG2* has been shown to confer paclitaxel resistance in cancer cells [256, 257], the expression of these genes decreases in the resistant cells compared to the parental cells. The increased expression of NFκB activator, TNFRSF11A, should not be relevant as NFκB is constitutively active in these cells [258]. The remaining gene changes in Table 2, ATM, APC, ERBB4, AR, and FOS, have not been shown to be relevant to triple negative breast cancers. Therefore, the overexpression of the *ABCB1* and *CYP2C8* genes are the most likely factors to be importantin the resistance mechanism.

The product of *ABCB1*, P-gp, is an efflux pump that can remove paclitaxel from cells. CYP2C8 is the main metabolizing enzyme for paclitaxel that forms a 30-fold less active metabolite, 6α-OHP [123]. Interestingly, though overexpression of *CYP2C8* has previously shown to be involved in paclitaxel resistance, this was a transient induction that was reversed when the cells were no longer grown in the presence of paclitaxel [137], which is contrary to the more permanent gene amplification seen in this cell model of paclitaxel resistance. The increased expression of P-gp and CYP2C8 could allow these resistant cells to both pump paclitaxel out more quickly and to break it down to a less active metabolite more efficiently than the parental line. This effect would prevent the accumulation of paclitaxel in the cell, resulting in the need for much higher concentrations of paclitaxel to kill the cells. Importantly, inhibition of P-gp resulted in a decrease of paclitaxel resistance of MDA-MB-231/PacR and MDA-MB-231/PacR-Hi cells (Figure 26). Interestingly, inhibition of CYP2C8 was capable of decreasing the paclitaxel resistance of MDA-MB-231/PacR cells but not MDA-MB-231/PacR-Hi cells. Considering that the MDA-MB-231/PacR-Hi cells have higher resistance, lower *CYP2C8* gene expression, and less sensitivity to the CYP2C8 inhibitor compared to the MDA-MB-

231/PacR cells implies that CYP2C8 is a less important factor in the mechanism of paclitaxel resistance than P-gp. Additionally, the MDA-MB-231/PacR cells may have another factor contributing to resistance, as verapamil did not entirely remove paclitaxel resistance though higher verapamil may have removed all resistance. Collectively, these results suggest upregulation of P-gp is the most important factor in the paclitaxel resistance of these cells and pharmacological inhibition of P-gp can overcome the paclitaxel resistance acquired in these cells.

Resveratrol has been shown to decrease the formation of the less active 6α-OHP, the main paclitaxel metabolite formed by CYP2C8 [259]. And, critically, resveratrol has been shown to inhibit *ABCB1* gene expression in multiple cancer cell models and, thereby, increase cellular accumulation of P-gp substrate drugs [222, 260-262]. These mechanisms may explain the re-sensitizing ability of resveratrol to lower the paclitaxel IC_{50} in the resistant cells. Importantly, the inhibitors for P-gp and CYP2C8 do not affect the paclitaxel IC_{50} in the parental cells (Figure 29) suggesting that the function of P-gp and CYP2C8 are not important to paclitaxel sensitivity in the parental cells, which further supports the suggestion that there is a mechanism of the resveratrol-paclitaxel combination that is independent of the mechanism by which resveratrol re-sensitizes the resistant cells to paclitaxel.

Based on our findings, it will be important to validate that resveratrol is affecting the function of P-gp and CYP2C8 as well as paclitaxel accumulation in these cells using functional assays. Determination of increased P-gp protein levels is important and was attempted in this study. However, the immunoblotting was not successful and needs to be further optimized. To ensure the importance of *ABCB1* gene expression, qPCR of resveratrol treated MDA-MB-231/PacR cells should be conducted to determine whether resveratrol treatment decreases *ABCB1* gene expression in these cells alone and in combination with paclitaxel. Further, a fluorescent paclitaxel accumulation assay using

labeled paclitaxel, Oregon green 488 paclitaxel, can be conducted to determine accumulation in cells and efflux into media in the cells alone and with simultaneous resveratrol treatment. To determine the importance of CYP2C8, a fluormetric CYP2C8 assay can be conducted using dibenzylfluoresin, which is cleaved to a fluorescent metabolite by CYP2C8, CYP2C9, CYP2C19 and CYP3A4. This reagent should show the difference in CYP2C8 metabolism because, of the cytochromes that cleave this substrate, only CYP2C8 was shown to be different between the parental and resistance cells in the PCR array. These studies will be particularly important for predicting whether the combination of resveratrol and paclitaxel will increase toxicities *in vivo*. If resveratrol increases paclitaxel accumulation, it is possible that higher paclitaxel accumulation could occur in other tissues that highly express P-pg and CYP2C8, such as the liver, which could lead to an increase in paclitaxel toxicity. In addition, genetic manipulation of both cell lines will be necessary to ensure the importance of these genes in the resistance. Both knocking out expression in the resistant cells and overexpressing the genes in the parental cells would be necessary to show these genes are critical for resistance in this model.

Furthermore, any additional mechanisms that could account for the combination effect of resveratrol and paclitaxel seen in the parental cells need to be elucidated. The data suggest that resveratrol is acting on two different mechanisms, one to decrease the resistance of the resistant cells and another to further sensitize resistant and parental cells to paclitaxel. This data will also be important in determining whether this combination therapy will only be useful in TNBCs or in other cancers as well. Considering data from other cancer cell lines showing a positive resveratrol and paclitaxel combination, it is possible the decrease of Bcl-xL [219] or Bcl-2 [222] or the increase of p21 expression could be involved in promoting paclitaxel-induced apoptosis [220].

SUMMARY

A novel paclitaxel-resistant TNBC cell line was developed with a stable and clinically relevant resistance. The paclitaxel resistance had no effect on resveratrol efficacy. Resveratrol was capable of inhibiting cell proliferation and causing apoptosis in TNBC cells regardless of paclitaxel sensitivity. Importantly, resveratrol augmented paclitaxel treatment in both paclitaxel-sensitive and paclitaxel-resistant TNBC cells *in vitro*. The effect of resveratrol on the paclitaxel sensitivity of the resistant cells may be linked to the known ability of resveratrol to inhibit P-gp and CYP2C8 both of which were found to be involved in the paclitaxel-resistance of our model. Overall these data show for the first time that resveratrol can re-sensitize resistant TNBC cells to paclitaxel and can decrease the concentration of paclitaxel needed to produce the same effect as paclitaxel alone.

This study has some noteworthy limitations. Perhaps the most important limitation is the use of a laboratory generated cell line model of paclitaxel resistance, the properties of which may or may not correlate with the clinical presentation of paclitaxel resistance. In addition, although resveratrol was shown to induce cell proliferation inhibition, senescence, and apoptosis, the mechanism of resveratrol in these cells is still not completely understood. Furthermore, resveratrol clearly augments paclitaxel treatment in these TNBC cells, though the mechanism of the combination is unknown. Finally, this study does not address the global applicability of this treatment for cancers and so may prove to only be useful in a very small subset of patients

This study has raised many questions that need to be answered. The most critical of which are the mechanism of action of the combination and whether resveratrol augments paclitaxel treatment *in vivo*. It will be necessary to determine whether the resistance of the resistant cells is due to the inhibition of paclitaxel accumulation, and

whether resveratrol acts in these cells by increasing drug accumulation. In addition, the mechanism of resveratrol and paclitaxel combination that is common to both the parental and resistant cells will have important implications for the clinical application of this combination. A clinical trial is a possibility due to the classification of resveratrol as a dietary supplement. However, toxicity studies using 2- and 3-dimentional cell culture, studies identifying pharmacodynamic targets, and perhaps also animal studies will be important steps between cell line studies and human trials because there is a possibility that the combination could increase paclitaxel toxicity particularly in tissues that highly express P-gp, such as the liver and in peripheral neurons. The most critical studies will be the combination treatment in patients with TNBC and, depending on the mechanism of the combination, other cancers as well. This work and the questions it has raised have the important clinical implication that resveratrol may be a useful clinical tool as a single agent or in combination with paclitaxel for patients with TNBC to reverse paclitaxel resistance as well as decrease the dosages of highly toxic paclitaxel administered without affecting efficacy.

APPENDIX: Complete PCR Array Data

Symbol	Description	Fold Change	T-TEST	Fold Up- or Down-Regulation
		MDA-MB-231/PacR / MDA-MB-231	p-value	MDA-MB-231/PacR / MDA-MB-231
ABCB1	ATP-binding cassette, sub-family B (MDR/TAP), member 1	181.99	0.21	181.99
ABCC1	ATP-binding cassette, sub-family C (CFTR/MRP), member 1	0.60	0.94	-1.65
ABCC2	ATP-binding cassette, sub-family C (CFTR/MRP), member 2	0.70	0.81	-1.43
ABCC3	ATP-binding cassette, sub-family C (CFTR/MRP), member 3	0.23	0.14	-4.27
ABCC5	ATP-binding cassette, sub-family C (CFTR/MRP), member 5	1.07	0.44	1.07
ABCG2	ATP-binding cassette, sub-family G (WHITE), member 2	0.24	0.11	-4.11
AHR	Aryl hydrocarbon receptor	0.59	0.83	-1.70
AP1S1	Adaptor-related protein complex 1, sigma 1 subunit	0.86	0.65	-1.16
APC	Adenomatous polyposis coli	3.11	0.05	3.11
AR	Androgen receptor	0.21	0.04	-4.80
ARNT	Aryl hydrocarbon receptor nuclear translocator	1.83	0.32	1.83
ATM	Ataxia telangiectasia mutated	3.35	0.01	3.35
BAX	BCL2-associated X protein	0.99	0.46	-1.01
BCL2	B-cell CLL/lymphoma 2	1.17	0.34	1.17
BCL2L1	BCL2-like 1	0.64	0.75	-1.56
BLMH	Bleomycin hydrolase	0.79	0.79	-1.27
BRCA1	Breast cancer 1, early onset	1.15	0.47	1.15
BRCA2	Breast cancer 2, early onset	1.13	0.45	1.13
CCND1	Cyclin D1	0.78	0.70	-1.28
CCNE1	Cyclin E1	0.87	0.90	-1.15
CDK2	Cyclin-dependent kinase 2	2.60	0.19	2.60
CDK4	Cyclin-dependent kinase 4	1.74	0.40	1.74
CDKN1A	Cyclin-dependent kinase inhibitor 1A (p21, Cip1)	0.83	0.71	-1.21
CDKN1B	Cyclin-dependent kinase inhibitor 1B (p27, Kip1)	2.15	0.06	2.15
CDKN2A	Cyclin-dependent kinase inhibitor 2A (melanoma, p16, inhibits CDK4)	N/A	N/A	N/A
CDKN2D	Cyclin-dependent kinase inhibitor 2D (p19, inhibits CDK4)	0.93	0.60	-1.08
CLPTM1L	CLPTM1-like	0.74	0.61	-1.35
CYP1A1	Cytochrome P450, family 1, subfamily A, polypeptide 1	5.06	0.16	5.06

Symbol	Description	Fold Change	T-TEST	Fold Up- or Down-Regulation
		MDA-MB-231/PacR / MDA-MB-231	p-value	MDA-MB-231/PacR / MDA-MB-231
CYP1A2	Cytochrome P450, family 1, subfamily A, polypeptide 2	2.67	0.58	2.67
CYP2B6	Cytochrome P450, family 2, subfamily B, polypeptide 6	2.14	0.22	2.14
CYP2C19	Cytochrome P450, family 2, subfamily C, polypeptide 19	1.51	0.28	1.51
CYP2C8	Cytochrome P450, family 2, subfamily C, polypeptide 8	12.51	0.16	12.51
CYP2C9	Cytochrome P450, family 2, subfamily C, polypeptide 9	2.28	0.12	2.28
CYP2D6	Cytochrome P450, family 2, subfamily D, polypeptide 6	2.00	0.16	2.00
CYP2E1	Cytochrome P450, family 2, subfamily E, polypeptide 1	0.03	0.01	-31.07
CYP3A4	Cytochrome P450, family 3, subfamily A, polypeptide 4	2.83	N/A	2.83
CYP3A5	Cytochrome P450, family 3, subfamily A, polypeptide 5	0.59	0.77	-1.69
DHFR	Dihydrofolate reductase	1.07	0.75	1.07
EGFR	Epidermal growth factor receptor	0.59	0.90	-1.70
ELK1	ELK1, member of ETS oncogene family	0.59	0.66	-1.69
EPHX1	Epoxide hydrolase 1, microsomal (xenobiotic)	0.88	0.69	-1.14
ERBB2	V-erb-b2 erythroblastic leukemia viral oncogene homolog 2, neuro/glioblastoma derived oncogene homolog (avian)	0.90	0.64	-1.11
ERBB3	V-erb-b2 erythroblastic leukemia viral oncogene homolog 3 (avian)	0.53	0.70	-1.88
ERBB4	V-erb-a erythroblastic leukemia viral oncogene homolog 4 (avian)	0.15	0.02	-6.71
ERCC3	Excision repair cross-complementing rodent repair deficiency, complementation group 3 (xeroderma pigmentosum group B complementing)	1.28	0.24	1.28
ESR1	Estrogen receptor 1	1.21	0.77	1.21
ESR2	Estrogen receptor 2 (ER beta)	0.54	0.02	-1.85
FGF2	Fibroblast growth factor 2 (basic)	0.89	0.51	-1.12
FOS	FBJ murine osteosarcoma viral oncogene homolog	0.32	0.06	-3.16
GSK3A	Glycogen synthase kinase 3 alpha	1.35	0.52	1.35
GSTP1	Glutathione S-transferase pi 1	0.69	0.49	-1.46

Symbol	Description	Fold Change	T-TEST	Fold Up- or Down-Regulation
		MDA-MB-231/PacR / MDA-MB-231	p-value	MDA-MB-231/PacR / MDA-MB-231
HIF1A	Hypoxia inducible factor 1, alpha subunit (basic helix-loop-helix transcription factor)	0.70	0.32	-1.44
IGF1R	Insulin-like growth factor 1 receptor	1.12	0.55	1.12
IGF2R	Insulin-like growth factor 2 receptor	1.35	0.23	1.35
MET	Met proto-oncogene (hepatocyte growth factor receptor)	0.40	0.07	-2.48
MSH2	MutS homolog 2, colon cancer, nonpolyposis type 1 (E. coli)	1.69	0.12	1.69
MVP	Major vault protein	1.10	0.86	1.10
MYC	V-myc myelocytomatosis viral oncogene homolog (avian)	1.37	0.37	1.37
NAT2	N-acetyltransferase 2 (arylamine N-acetyltransferase)	1.86	0.06	1.86
NFKB1	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1	0.91	0.64	-1.10
NFKB2	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 2 (p49/p100)	2.44	0.02	2.44
NFKBIB	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, beta	1.74	0.16	1.74
NFKBIE	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, epsilon	1.50	0.18	1.50
PPARA	Peroxisome proliferator-activated receptor alpha	1.40	0.29	1.40
PPARD	Peroxisome proliferator-activated receptor delta	1.08	0.53	1.08
PPARG	Peroxisome proliferator-activated receptor gamma	1.39	0.00	1.39
RARA	Retinoic acid receptor, alpha	0.56	0.41	-1.78
RARB	Retinoic acid receptor, beta	2.61	0.00	2.61
RARG	Retinoic acid receptor, gamma	1.89	0.10	1.89
RB1	Retinoblastoma 1	1.60	0.05	1.60
RELB	V-rel reticuloendotheliosis viral oncogene homolog B	1.40	0.59	1.40
RXRA	Retinoid X receptor, alpha	1.07	0.96	1.07
RXRB	Retinoid X receptor, beta	1.67	0.01	1.67
SOD1	Superoxide dismutase 1, soluble	1.65	0.02	1.65
SULT1E1	Sulfotransferase family 1E, estrogen-preferring, member 1	1.77	0.57	1.77

Symbol	Description	Fold Change	T-TEST	Fold Up- or Down-Regulation
		MDA-MB-231/PacR / MDA-MB-231	p-value	MDA-MB-231/PacR / MDA-MB-231
TNFRSF11A	Tumor necrosis factor receptor superfamily, member 11a, NFKB activator	3.11	0.00	3.11
TOP1	Topoisomerase (DNA) I	1.42	0.03	1.42
TOP2A	Topoisomerase (DNA) II alpha 170kDa	1.29	0.17	1.29
TOP2B	Topoisomerase (DNA) II beta 180kDa	1.59	0.02	1.59
TP53	Tumor protein p53	1.87	0.04	1.87
TPMT	Thiopurine S-methyltransferase	0.91	0.63	-1.09
UGCG	UDP-glucose ceramide glucosyltransferase	1.38	0.05	1.38
XPA	Xeroderma pigmentosum, complementation group A	1.82	0.36	1.82
XPC	Xeroderma pigmentosum, complementation group C	1.83	0.34	1.83
ACTB	Actin, beta	0.83	0.24	-1.21
B2M	Beta-2-microglobulin	1.11	0.37	1.11
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	0.94	0.66	-1.07
HPRT1	Hypoxanthine phosphoribosyltransferase 1	0.82	0.07	-1.21
RPLP0	Ribosomal protein, large, P0	1.41	0.07	1.41
HGDC	Human Genomic DNA Contamination	-	-	-
RTC	Reverse Transcription Control	-	-	-
RTC	Reverse Transcription Control	-	-	-
RTC	Reverse Transcription Control	-	-	-
PPC	Positive PCR Control	-	-	-
PPC	Positive PCR Control	-	-	-
PPC	Positive PCR Control	-	-	-

REFERENCES

1. Hanahan, D. and R.A. Weinberg, *Hallmarks of cancer: the next generation.* Cell, 2011. **144**(5): p. 646-74.

2. Society, A.C., *Cancer Facts & Figures 2014.* 2014.

3. Bray, F., et al., *Global estimates of cancer prevalence for 27 sites in the adult population in 2008.* Int J Cancer, 2013. **132**(5): p. 1133-45.

4. Ferlay, J., et al., *Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008.* Int J Cancer, 2010. **127**(12): p. 2893-917.

5. Society, A.C. *Breast Cancer Facts & Figures 2013-2014*. 2013.

6. Al-Benna, S., et al., *Diagnosis and management of primary breast sarcoma.* Breast Cancer Res Treat, 2010. **122**(3): p. 619-26.

7. Allred, D.C., *Ductal carcinoma in situ: terminology, classification, and natural history.* J Natl Cancer Inst Monogr, 2010. **2010**(41): p. 134-8.

8. Perou, C.M. and A.L. Borresen-Dale, *Systems biology and genomics of breast cancer.* Cold Spring Harb Perspect Biol, 2011. **3**(2).

9. Voduc, K.D., et al., *Breast cancer subtypes and the risk of local and regional relapse.* J Clin Oncol, 2010. **28**(10): p. 1684-91.

10. Carey, L.A., et al., *Race, breast cancer subtypes, and survival in the Carolina Breast Cancer Study.* Jama, 2006. **295**(21): p. 2492-502.

11. Schwartz, G.F., et al., *Proceedings of the international consensus conference on breast cancer risk, genetics, & risk management, April, 2007.* Breast J, 2009. **15**(1): p. 4-16.

12. Berry, D.A., et al., *Effect of screening and adjuvant therapy on mortality from breast cancer.* N Engl J Med, 2005. **353**(17): p. 1784-92.

13. Institute, N.C. *National Cancer Institute: PDQ® Breast Cancer Treatment*. February 21, 2014 [cited 2014 March 19]; Available from: http://www.cancer.gov/cancertopics/pdq/treatment/breast/healthprofessional.

14. Carey, L.A., *Directed therapy of subtypes of triple-negative breast cancer.* Oncologist, 2011. **16 Suppl 1**: p. 71-8.

15. Rouzier, R., et al., *Breast cancer molecular subtypes respond differently to preoperative chemotherapy.* Clin Cancer Res, 2005. **11**(16): p. 5678-85.

16. Carey, L.A., et al., *The triple negative paradox: primary tumor chemosensitivity of breast cancer subtypes.* Clin Cancer Res, 2007. **13**(8): p. 2329-34.

17. Elston, C.W. and I.O. Ellis, *Pathological prognostic factors in breast cancer. I. The value of histological grade in breast cancer: experience from a large study with long-term follow-up.* Histopathology, 2002. **41**(3a): p. 154-61.

18. Bauer, K.R., et al., *Descriptive analysis of estrogen receptor (ER)-negative, progesterone receptor (PR)-negative, and HER2-negative invasive breast cancer, the so-called triple-negative phenotype: a population-based study from the California cancer Registry.* Cancer, 2007. **109**(9): p. 1721-8.

19. Ayanian, J.Z., et al., *The relation between health insurance coverage and clinical outcomes among women with breast cancer.* N Engl J Med, 1993. **329**(5): p. 326-31.

20. McWhorter, W.P. and W.J. Mayer, *Black/white differences in type of initial breast cancer treatment and implications for survival.* Am J Public Health, 1987. **77**(12): p. 1515-7.

21. Breen, N., et al., *The relationship of socio-economic status and access to minimum expected therapy among female breast cancer patients in the National Cancer Institute Black-White Cancer Survival Study.* Ethn Dis, 1999. **9**(1): p. 111-25.

22. DeSantis, C., A. Jemal, and E. Ward, *Disparities in breast cancer prognostic factors by race, insurance status, and education.* Cancer Causes Control, 2010. **21**(9): p. 1445-50.

23. Sprague, B.L., et al., *Socioeconomic status and survival after an invasive breast cancer diagnosis.* Cancer, 2011. **117**(7): p. 1542-51.

24. Wojcik, B.E., M.K. Spinks, and S.A. Optenberg, *Breast carcinoma survival analysis for African American and white women in an equal-access health care system.* Cancer, 1998. **82**(7): p. 1310-8.

25. Hershman, D.L., et al., *Treatment quality and outcomes of African American versus white breast cancer patients: retrospective analysis of Southwest Oncology studies S8814/S8897.* J Clin Oncol, 2009. **27**(13): p. 2157-62.

26. Dunn, B.K., et al., *Health disparities in breast cancer: biology meets socioeconomic status.* Breast Cancer Res Treat, 2010. **121**(2): p. 281-92.

27. Silver, D.P., et al., *Efficacy of neoadjuvant Cisplatin in triple-negative breast cancer.* J Clin Oncol, 2010. **28**(7): p. 1145-53.

28. Byrski, T., et al., *Pathologic complete response rates in young women with BRCA1-positive breast cancers after neoadjuvant chemotherapy.* J Clin Oncol, 2010. **28**(3): p. 375-9.

29. Comen, E.A. and M. Robson, *Inhibition of poly(ADP)-ribose polymerase as a therapeutic strategy for breast cancer.* Oncology (Williston Park), 2010. **24**(1): p. 55-62.

30. Tutt, A., et al., *Oral poly(ADP-ribose) polymerase inhibitor olaparib in patients with BRCA1 or BRCA2 mutations and advanced breast cancer: a proof-of-concept trial.* Lancet, 2010. **376**(9737): p. 235-44.

31. Carey, L.A., et al., *TBCRC 001: randomized phase II study of cetuximab in combination with carboplatin in stage IV triple-negative breast cancer.* J Clin Oncol, 2012. **30**(21): p. 2615-23.

32. Miller, K., et al., *Paclitaxel plus bevacizumab versus paclitaxel alone for metastatic breast cancer.* N Engl J Med, 2007. **357**(26): p. 2666-76.

33. Lang, I., et al., *Bevacizumab plus paclitaxel versus bevacizumab plus capecitabine as first-line treatment for HER2-negative metastatic breast cancer: interim efficacy results of the randomised, open-label, non-inferiority, phase 3 TURANDOT trial.* Lancet Oncol, 2013. **14**(2): p. 125-33.

34. Liedtke, C., et al., *Response to neoadjuvant therapy and long-term survival in patients with triple-negative breast cancer.* J Clin Oncol, 2008. **26**(8): p. 1275-81.

35. Wysocki, P.J., et al., *Primary resistance to docetaxel-based chemotherapy in metastatic breast cancer patients correlates with a high frequency of BRCA1 mutations.* Med Sci Monit, 2008. **14**(7): p. SC7-10.

36. Longley, D.B. and P.G. Johnston, *Molecular mechanisms of drug resistance.* J Pathol, 2005. **205**(2): p. 275-92.

37. Holohan, C., et al., *Cancer drug resistance: an evolving paradigm.* Nat Rev Cancer, 2013. **13**(10): p. 714-26.

38. Gottesman, M.M., *Mechanisms of cancer drug resistance.* Annu Rev Med, 2002. **53**: p. 615-27.

39. Fojo, T. and S. Bates, *Strategies for reversing drug resistance.* Oncogene, 2003. **22**(47): p. 7512-23.

40. Schiff, P.B., J. Fant, and S.B. Horwitz, *Promotion of microtubule assembly in vitro by taxol.* Nature, 1979. **277**(5698): p. 665-7.

41. Jordan, M.A. and L. Wilson, *Microtubules as a target for anticancer drugs.* Nat Rev Cancer, 2004. **4**(4): p. 253-65.

42. Downing, K.H. and E. Nogales, *Crystallographic structure of tubulin: implications for dynamics and drug binding.* Cell Struct Funct, 1999. **24**(5): p. 269-75.

43. Mollinedo, F. and C. Gajate, *Microtubules, microtubule-interfering agents and apoptosis.* Apoptosis, 2003. **8**(5): p. 413-50.

44. Mitchison, T. and M. Kirschner, *Dynamic instability of microtubule growth.* Nature, 1984. **312**(5991): p. 237-42.

45. Zhou, J. and P. Giannakakou, *Targeting microtubules for cancer chemotherapy.* Curr Med Chem Anticancer Agents, 2005. **5**(1): p. 65-71.

46. Honore, S., E. Pasquier, and D. Braguer, *Understanding microtubule dynamics for improved cancer therapy.* Cell Mol Life Sci, 2005. **62**(24): p. 3039-56.

47. Diaz, J.F., et al., *Changes in microtubule protofilament number induced by Taxol binding to an easily accessible site. Internal microtubule dynamics.* J Biol Chem, 1998. **273**(50): p. 33803-10.

48. Abal, M., J.M. Andreu, and I. Barasoain, *Taxanes: microtubule and centrosome targets, and cell cycle dependent mechanisms of action.* Curr Cancer Drug Targets, 2003. **3**(3): p. 193-203.

49. Roninson, I.B., E.V. Broude, and B.D. Chang, *If not apoptosis, then what? Treatment-induced senescence and mitotic catastrophe in tumor cells.* Drug Resist Updat, 2001. **4**(5): p. 303-13.

50. Kingston, D.G., *The shape of things to come: structural and synthetic studies of taxol and related compounds.* Phytochemistry, 2007. **68**(14): p. 1844-54.

51. Tabata, H., *Production of paclitaxel and the related taxanes by cell suspension cultures of Taxus species.* Curr Drug Targets, 2006. **7**(4): p. 453-61.

52. Binder, S., *Evolution of taxanes in the treatment of metastatic breast cancer.* Clin J Oncol Nurs, 2013. **17 Suppl**: p. 9-14.

53. Monsarrat, B., et al., *Hepatic metabolism and biliary excretion of Taxol in rats and humans.* J Natl Cancer Inst Monogr, 1993(15): p. 39-46.

54. Monsarrat, B., et al., *Modification of paclitaxel metabolism in a cancer patient by induction of cytochrome P450 3A4.* Drug Metab Dispos, 1998. **26**(3): p. 229-33.

55. Nabholtz, J.M., et al., *Multicenter, randomized comparative study of two doses of paclitaxel in patients with metastatic breast cancer.* J Clin Oncol, 1996. **14**(6): p. 1858-67.

56. Einzig, A.I., *Review of phase II trials of Taxol (paclitaxel) in patients with advanced ovarian cancer.* Ann Oncol, 1994. **5 Suppl 6**: p. S29-32.

57. Millward, M.J., et al., *Phase II trial of a 3-hour infusion of paclitaxel in previously untreated patients with advanced non-small-cell lung cancer.* J Clin Oncol, 1996. **14**(1): p. 142-8.

58. Fountzilas, G., et al., *A phase II study of paclitaxel in advanced breast cancer resistant to anthracyclines.* Eur J Cancer, 1996. **32a**(1): p. 47-51.

59. Paridaens, R., et al., *Paclitaxel versus doxorubicin as first-line single-agent chemotherapy for metastatic breast cancer: a European Organization for Research and Treatment of Cancer Randomized Study with cross-over.* J Clin Oncol, 2000. **18**(4): p. 724-33.

60. Gonzalez-Angulo, A.M. and G.N. Hortobagyi, *Optimal schedule of paclitaxel: weekly is better.* J Clin Oncol, 2008. **26**(10): p. 1585-7.

61. De Laurentiis, M., et al., *Taxane-based combinations as adjuvant chemotherapy of early breast cancer: a meta-analysis of randomized trials.* J Clin Oncol, 2008. **26**(1): p. 44-53.

62. Citron, M.L., et al., *Randomized trial of dose-dense versus conventionally scheduled and sequential versus concurrent combination chemotherapy as postoperative adjuvant treatment of node-positive primary breast cancer: first report of Intergroup Trial C9741/Cancer and Leukemia Group B Trial 9741.* J Clin Oncol, 2003. **21**(8): p. 1431-9.

63. Sparano, J.A., et al., *Weekly paclitaxel in the adjuvant treatment of breast cancer.* N Engl J Med, 2008. **358**(16): p. 1663-71.

64. Gianni, L., et al., *Efficacy and safety of neoadjuvant pertuzumab and trastuzumab in women with locally advanced, inflammatory, or early HER2-positive breast cancer (NeoSphere): a randomised multicentre, open-label, phase 2 trial.* Lancet Oncol, 2012. **13**(1): p. 25-32.

65. McGrogan, B.T., et al., *Taxanes, microtubules and chemoresistant breast cancer.* Biochim Biophys Acta, 2008. **1785**(2): p. 96-132.

66. Murray, S., et al., *Taxane resistance in breast cancer: mechanisms, predictive biomarkers and circumvention strategies.* Cancer Treat Rev, 2012. **38**(7): p. 890-903.

67. Wiesen, K.M., et al., *Wild-type class I beta-tubulin sensitizes Taxol-resistant breast adenocarcinoma cells harboring a beta-tubulin mutation.* Cancer Lett, 2007. **257**(2): p. 227-35.

68. Berrieman, H.K., M.J. Lind, and L. Cawkwell, *Do beta-tubulin mutations have a role in resistance to chemotherapy?* Lancet Oncol, 2004. **5**(3): p. 158-64.

69. Wang, Y., et al., *Mutations at leucine 215 of beta-tubulin affect paclitaxel sensitivity by two distinct mechanisms.* Biochemistry, 2006. **45**(1): p. 185-94.

70. Derry, W.B., et al., *Taxol differentially modulates the dynamics of microtubules assembled from unfractionated and purified beta-tubulin isotypes.* Biochemistry, 1997. **36**(12): p. 3554-62.

71. Rouzier, R., et al., *Microtubule-associated protein tau: a marker of paclitaxel sensitivity in breast cancer.* Proc Natl Acad Sci U S A, 2005. **102**(23): p. 8315-20.

72. Orr, G.A., et al., *Mechanisms of Taxol resistance related to microtubules.* Oncogene, 2003. **22**(47): p. 7280-95.

73. Alli, E., et al., *Effect of stathmin on the sensitivity to antimicrotubule drugs in human breast cancer.* Cancer Res, 2002. **62**(23): p. 6864-9.

74. Anand, S., S. Penrhyn-Lowe, and A.R. Venkitaraman, *AURORA-A amplification overrides the mitotic spindle assembly checkpoint, inducing resistance to Taxol.* Cancer Cell, 2003. **3**(1): p. 51-62.

75. Chabalier, C., et al., *BRCA1 downregulation leads to premature inactivation of spindle checkpoint and confers paclitaxel resistance.* Cell Cycle, 2006. **5**(9): p. 1001-7.

76. Sudo, T., et al., *Dependence of paclitaxel sensitivity on a functional spindle assembly checkpoint.* Cancer Res, 2004. **64**(7): p. 2502-8.

77. Swanton, C., et al., *Regulators of mitotic arrest and ceramide metabolism are determinants of sensitivity to paclitaxel and other chemotherapeutic drugs.* Cancer Cell, 2007. **11**(6): p. 498-512.

78. Takahashi, T., et al., *Cyclin A-associated kinase activity is needed for paclitaxel sensitivity.* Mol Cancer Ther, 2005. **4**(7): p. 1039-46.

79. Wang, T.H., H.S. Wang, and Y.K. Soong, *Paclitaxel-induced cell death: where the cell cycle and apoptosis come together.* Cancer, 2000. **88**(11): p. 2619-28.

80. Dong, Q.G., et al., *The function of multiple IkappaB : NF-kappaB complexes in the resistance of cancer cells to Taxol-induced apoptosis.* Oncogene, 2002. **21**(42): p. 6510-9.

81. Fujie, Y., et al., *Oxaliplatin, a potent inhibitor of survivin, enhances paclitaxel-induced apoptosis and mitotic catastrophe in colon cancer cells.* Jpn J Clin Oncol, 2005. **35**(8): p. 453-63.

82. Knuefermann, C., et al., *HER2/PI-3K/Akt activation leads to a multidrug resistance in human breast adenocarcinoma cells.* Oncogene, 2003. **22**(21): p. 3205-12.

83. Schmidt, M., et al., *p53 expression and resistance against paclitaxel in patients with metastatic breast cancer.* J Cancer Res Clin Oncol, 2003. **129**(5): p. 295-302.

84. Strobel, T., et al., *BAX enhances paclitaxel-induced apoptosis through a p53-independent pathway.* Proc Natl Acad Sci U S A, 1996. **93**(24): p. 14094-9.

85. Wang, Z., et al., *Differential effect of anti-apoptotic genes Bcl-xL and c-FLIP on sensitivity of MCF-7 breast cancer cells to paclitaxel and docetaxel.* Anticancer Res, 2005. **25**(3c): p. 2367-79.

86. Yu, D., et al., *Overexpression of ErbB2 blocks Taxol-induced apoptosis by upregulation of p21Cip1, which inhibits p34Cdc2 kinase.* Mol Cell, 1998. **2**(5): p. 581-91.

87. Dean, M., Y. Hamon, and G. Chimini, *The human ATP-binding cassette (ABC) transporter superfamily.* J Lipid Res, 2001. **42**(7): p. 1007-17.

88. Sharom, F.J., *The P-glycoprotein efflux pump: how does it transport drugs?* J Membr Biol, 1997. **160**(3): p. 161-75.

89. Eckford, P.D. and F.J. Sharom, *ABC efflux pump-based resistance to chemotherapy drugs.* Chem Rev, 2009. **109**(7): p. 2989-3011.

90. Sharom, F.J., *ABC multidrug transporters: structure, function and role in chemoresistance.* Pharmacogenomics, 2008. **9**(1): p. 105-27.

91. Sharom, F.J., *Complex Interplay between the P-Glycoprotein Multidrug Efflux Pump and the Membrane: Its Role in Modulating Protein Function.* Front Oncol, 2014. **4**: p. 41.

92. Harmsen, S., et al., *PXR-mediated induction of P-glycoprotein by anticancer drugs in a human colon adenocarcinoma-derived cell line.* Cancer Chemother Pharmacol, 2010. **66**(4): p. 765-71.

93. Tsuruo, T., et al., *Overcoming of vincristine resistance in P388 leukemia in vivo and in vitro through enhanced cytotoxicity of vincristine and vinblastine by verapamil.* Cancer Res, 1981. **41**(5): p. 1967-72.

94. Schinkel, A.H., et al., *Normal viability and altered pharmacokinetics in mice lacking mdr1-type (drug-transporting) P-glycoproteins.* Proc Natl Acad Sci U S A, 1997. **94**(8): p. 4028-33.

95. Alvarez, M., et al., *Generation of a drug resistance profile by quantitation of mdr-1/P-glycoprotein in the cell lines of the National Cancer Institute Anticancer Drug Screen.* J Clin Invest, 1995. **95**(5): p. 2205-14.

96. Mechetner, E., et al., *Levels of multidrug resistance (MDR1) P-glycoprotein expression by human breast cancer correlate with in vitro resistance to taxol and doxorubicin.* Clin Cancer Res, 1998. **4**(2): p. 389-98.

97. Leonessa, F. and R. Clarke, *ATP binding cassette transporters and drug resistance in breast cancer.* Endocr Relat Cancer, 2003. **10**(1): p. 43-73.

98. Vishnukumar, S., et al., *P-glycoprotein expression as a predictor of response to neoadjuvant chemotherapy in breast cancer.* Indian J Cancer, 2013. **50**(3): p. 195-9.

99. Chintamani, et al., *Role of p-glycoprotein expression in predicting response to neoadjuvant chemotherapy in breast cancer--a prospective clinical study.* World J Surg Oncol, 2005. **3**: p. 61.

100. Mubashar, M., et al., *99mTc-sestamibi imaging in the assessment of toremifene as a modulator of multidrug resistance in patients with breast cancer.* J Nucl Med, 2002. **43**(4): p. 519-25.

101. Kostakoglu, L., et al., *Association of tumor washout rates and accumulation of technetium-99m-MIBI with expression of P-glycoprotein in lung cancer.* J Nucl Med, 1998. **39**(2): p. 228-34.

102. Del Vecchio, S., A. Ciarmiello, and M. Salvatore, *Clinical imaging of multidrug resistance in cancer.* Q J Nucl Med, 1999. **43**(2): p. 125-31.

103. Yoon, J.H., et al., *Double-phase Tc-99m sestamibi scintimammography to assess angiogenesis and P-glycoprotein expression in patients with untreated breast cancer.* Clin Nucl Med, 1999. **24**(5): p. 314-8.

104. Sun, S.S., et al., *Expression of mediated P-glycoprotein multidrug resistance related to Tc-99m MIBI scintimammography results.* Cancer Lett, 2000. **153**(1-2): p. 95-100.

105. Cayre, A., et al., *Single static view 99mTc-sestamibi scintimammography predicts response to neoadjuvant chemotherapy and is related to MDR expression.* Int J Oncol, 2002. **20**(5): p. 1049-55.

106. Ozols, R.F., et al., *Verapamil and adriamycin in the treatment of drug-resistant ovarian cancer patients.* J Clin Oncol, 1987. **5**(4): p. 641-7.

107. Toppmeyer, D., et al., *Safety and efficacy of the multidrug resistance inhibitor Incel (biricodar; VX-710) in combination with paclitaxel for advanced breast cancer refractory to paclitaxel.* Clin Cancer Res, 2002. **8**(3): p. 670-8.

108. Ruff, P., et al., *A randomized, placebo-controlled, double-blind phase 2 study of docetaxel compared to docetaxel plus zosuquidar (LY335979) in women with metastatic or locally recurrent breast cancer who have received one prior chemotherapy regimen.* Cancer Chemother Pharmacol, 2009. **64**(4): p. 763-8.

109. Pusztai, L., et al., *Phase II study of tariquidar, a selective P-glycoprotein inhibitor, in patients with chemotherapy-resistant, advanced breast carcinoma.* Cancer, 2005. **104**(4): p. 682-91.

110. Jakoby, W.B. and D.M. Ziegler, *The enzymes of detoxication.* J Biol Chem, 1990. **265**(34): p. 20715-8.

111. Konig, J., et al., *Conjugate export pumps of the multidrug resistance protein (MRP) family: localization, substrate specificity, and MRP2-mediated drug resistance.* Biochim Biophys Acta, 1999. **1461**(2): p. 377-94.

112. Homolya, L., A. Varadi, and B. Sarkadi, *Multidrug resistance-associated proteins: Export pumps for conjugates with glutathione, glucuronate or sulfate.* Biofactors, 2003. **17**(1-4): p. 103-14.

113. Commandeur, J.N., G.J. Stijntjes, and N.P. Vermeulen, *Enzymes and transport systems involved in the formation and disposition of glutathione S-conjugates. Role in bioactivation and detoxication mechanisms of xenobiotics.* Pharmacol Rev, 1995. **47**(2): p. 271-330.

114. Meunier, B., S.P. de Visser, and S. Shaik, *Mechanism of oxidation reactions catalyzed by cytochrome p450 enzymes.* Chem Rev, 2004. **104**(9): p. 3947-80.

115. Neve, E.P. and M. Ingelman-Sundberg, *Cytochrome P450 proteins: retention and distribution from the endoplasmic reticulum.* Curr Opin Drug Discov Devel, 2010. **13**(1): p. 78-85.

116. Nishimura, M., et al., *Tissue distribution of mRNA expression of human cytochrome P450 isoforms assessed by high-sensitivity real-time reverse transcription PCR.* Yakugaku Zasshi, 2003. **123**(5): p. 369-75.

117. Nebert, D.W. and D.W. Russell, *Clinical importance of the cytochromes P450.* Lancet, 2002. **360**(9340): p. 1155-62.

118. Guengerich, F.P., *Cytochrome p450 and chemical toxicology.* Chem Res Toxicol, 2008. **21**(1): p. 70-83.

119. Rahman, A., et al., *Selective biotransformation of taxol to 6 alpha-hydroxytaxol by human cytochrome P450 2C8.* Cancer Res, 1994. **54**(21): p. 5543-6.

120. Harris, J.W., et al., *Isolation, structural determination, and biological activity of 6 alpha-hydroxytaxol, the principal human metabolite of taxol.* J Med Chem, 1994. **37**(5): p. 706-9.

121. Sparreboom, A., et al., *Isolation, purification, and biological activity of mono- and dihydroxylated paclitaxel metabolites from human feces.* Cancer Chemother Pharmacol, 1995. **36**(4): p. 299-304.

122. Cresteil, T., et al., *Taxol metabolism by human liver microsomes: identification of cytochrome P450 isozymes involved in its biotransformation.* Cancer Res, 1994. **54**(2): p. 386-92.

123. Harris, J.W., et al., *Metabolism of taxol by human hepatic microsomes and liver slices: participation of cytochrome P450 3A4 and an unknown P450 enzyme.* Cancer Res, 1994. **54**(15): p. 4026-35.

124. Vaclavikova, R., et al., *Different in vitro metabolism of paclitaxel and docetaxel in humans, rats, pigs, and minipigs.* Drug Metab Dispos, 2004. **32**(6): p. 666-74.

125. Monsarrat, B., et al., *Biotransformation of taxoids by human cytochromes P450: structure-activity relationship.* Bull Cancer, 1997. **84**(2): p. 125-33.

126. Kang, M.H., et al., *The P-glycoprotein antagonist PSC 833 increases the plasma concentrations of 6alpha-hydroxypaclitaxel, a major metabolite of paclitaxel.* Clin Cancer Res, 2001. **7**(6): p. 1610-7.

127. Gray, I.C., et al., *A 2.4-megabase physical map spanning the CYP2C gene cluster on chromosome 10q24.* Genomics, 1995. **28**(2): p. 328-32.

128. Somlo, G., et al., *High-dose paclitaxel in combination with doxorubicin, cyclophosphamide and peripheral blood progenitor cell rescue in patients with high-risk primary and responding metastatic breast carcinoma: toxicity profile, relationship to paclitaxel pharmacokinetics and short-term outcome.* Br J Cancer, 2001. **84**(12): p. 1591-8.

129. Daily, E.B. and C.L. Aquilante, *Cytochrome P450 2C8 pharmacogenetics: a review of clinical studies.* Pharmacogenomics, 2009. **10**(9): p. 1489-510.

130. Rendic, S. and F.J. Di Carlo, *Human cytochrome P450 enzymes: a status report summarizing their reactions, substrates, inducers, and inhibitors.* Drug Metab Rev, 1997. **29**(1-2): p. 413-580.

131. Shimada, T., et al., *Interindividual variations in human liver cytochrome P-450 enzymes involved in the oxidation of drugs, carcinogens and toxic chemicals: studies with liver microsomes of 30 Japanese and 30 Caucasians.* J Pharmacol Exp Ther, 1994. **270**(1): p. 414-23.

132. Totah, R.A. and A.E. Rettie, *Cytochrome P450 2C8: substrates, inhibitors, pharmacogenetics, and clinical relevance.* Clin Pharmacol Ther, 2005. **77**(5): p. 341-52.

133. Klose, T.S., J.A. Blaisdell, and J.A. Goldstein, *Gene structure of CYP2C8 and extrahepatic distribution of the human CYP2Cs.* J Biochem Mol Toxicol, 1999. **13**(6): p. 289-95.

134. Thum, T. and J. Borlak, *Gene expression in distinct regions of the heart.* Lancet, 2000. **355**(9208): p. 979-83.

135. Delozier, T.C., et al., *Detection of human CYP2C8, CYP2C9, and CYP2J2 in cardiovascular tissues.* Drug Metab Dispos, 2007. **35**(4): p. 682-8.

136. Knupfer, H., et al., *CYP2C and IL-6 expression in breast cancer.* Breast, 2004. **13**(1): p. 28-34.

137. Garcia-Martin, E., et al., *Acquired resistance to the anticancer drug paclitaxel is associated with induction of cytochrome P450 2C8.* Pharmacogenomics, 2006. **7**(4): p. 575-85.

138. Horneber, M., et al., *How many cancer patients use complementary and alternative medicine: a systematic review and metaanalysis.* Integr Cancer Ther, 2012. **11**(3): p. 187-203.

139. Molassiotis, A., et al., *Use of complementary and alternative medicine in cancer patients: a European survey.* Ann Oncol, 2005. **16**(4): p. 655-63.

140. Eschiti, V.S., *Lesson from comparison of CAM use by women with female-specific cancers to others: it's time to focus on interaction risks with CAM therapies.* Integr Cancer Ther, 2007. **6**(4): p. 313-44.

141. Eng, J., et al., *A population-based survey of complementary and alternative medicine use in men recently diagnosed with prostate cancer.* Integr Cancer Ther, 2003. **2**(3): p. 212-6.

142. Davidson, R., et al., *Psychological characteristics of cancer patients who use complementary therapies.* Psychooncology, 2005. **14**(3): p. 187-95.

143. Hann, D., et al., *Long-term breast cancer survivors' use of complementary therapies: perceived impact on recovery and prevention of recurrence.* Integr Cancer Ther, 2005. **4**(1): p. 14-20.

144. Verhoef, M.J., et al., *Complementary therapies for cancer patients: assessing information use and needs.* Chronic Dis Can, 2009. **29**(2): p. 80-8.

145. Hanf, V., *Complementary Methods in Breast Cancer Therapy.* Breast Care (Basel), 2009. **4**(1): p. 5-6.

146. Robinson, A. and M.R. McGrail, *Disclosure of CAM use to medical practitioners: a review of qualitative and quantitative studies.* Complement Ther Med, 2004. **12**(2-3): p. 90-8.

147. Yates, J.S., et al., *Prevalence of complementary and alternative medicine use in cancer patients during treatment.* Support Care Cancer, 2005. **13**(10): p. 806-11.

148. Hubner, J. and V. Hanf, *Commonly Used Methods of Complementary Medicine in the Treatment of Breast Cancer.* Breast Care (Basel), 2013. **8**(5): p. 341-347.

149. Garcia-Cazarin, M.L., et al., *Dietary Supplement Research Portfolio at the NIH, 2009-2011.* J Nutr, 2014.

150. Kaufman, D.W., et al., *Recent patterns of medication use in the ambulatory adult population of the United States: the Slone survey.* Jama, 2002. **287**(3): p. 337-44.

151. Tindle, H.A., et al., *Trends in use of complementary and alternative medicine by US adults: 1997-2002.* Altern Ther Health Med, 2005. **11**(1): p. 42-9.

152. Bailey, R.L., et al., *Dietary supplement use in the United States, 2003-2006.* J Nutr, 2011. **141**(2): p. 261-6.

153. 103-417, U.P.L., *Dietary Supplement Health and Education Act of 1994*, r. Congress, Editor. 1994.

154. Hermann, R. and O. von Richter, *Clinical evidence of herbal drugs as perpetrators of pharmacokinetic drug interactions.* Planta Med, 2012. **78**(13): p. 1458-77.

155. Barrett, B., D. Kiefer, and D. Rabago, *Assessing the risks and benefits of herbal medicine: an overview of scientific evidence.* Altern Ther Health Med, 1999. **5**(4): p. 40-9.

156. Cragg, G.M. and D.J. Newman, *Plants as a source of anti-cancer agents.* J Ethnopharmacol, 2005. **100**(1-2): p. 72-9.

157. Langcake, P. and R.J. Pryce, *A new class of phytoalexins from grapevines.* Experientia, 1977. **33**(2): p. 151-2.

158. Schroder, G., J.W. Brown, and J. Schroder, *Molecular analysis of resveratrol synthase. cDNA, genomic clones and relationship with chalcone synthase.* Eur J Biochem, 1988. **172**(1): p. 161-9.

159. Dixon, R.A., *Natural products and plant disease resistance.* Nature, 2001. **411**(6839): p. 843-7.

160. Signorelli, P. and R. Ghidoni, *Resveratrol as an anticancer nutrient: molecular basis, open questions and promises.* J Nutr Biochem, 2005. **16**(8): p. 449-66.

161. Aggarwal, B.B., et al., *Role of resveratrol in prevention and therapy of cancer: preclinical and clinical studies.* Anticancer Res, 2004. **24**(5A): p. 2783-840.

162. Iranshahy, M., et al., *Method Validation for the One-Month Stability Study of trans-Resveratrol in Human Plasma.* Jundishapur J Nat Pharm Prod, 2013. **8**(2): p. 65-9.

163. Tome-Carneiro, J., et al., *Resveratrol and clinical trials: the crossroad from in vitro studies to human evidence.* Curr Pharm Des, 2013. **19**(34): p. 6064-93.

164. Mangum, C., J.A. Cameron, and B.S. Herbert, *Effects of Resveratrol on Triple Negative (ER-, PR-, HER2-) Breast Cancer Cells.* FASEB J., 2008. **22**: p. 898.45.

165. Kundu, J.K. and Y.J. Surh, *Cancer chemopreventive and therapeutic potential of resveratrol: mechanistic perspectives.* Cancer Lett, 2008. **269**(2): p. 243-61.

166. Baur, J.A. and D.A. Sinclair, *Therapeutic potential of resveratrol: the in vivo evidence.* Nat Rev Drug Discov, 2006. **5**(6): p. 493-506.

167. Jang, M., et al., *Cancer chemopreventive activity of resveratrol, a natural product derived from grapes.* Science, 1997. **275**(5297): p. 218-20.

168. Le Corre, L., et al., *Resveratrol and breast cancer chemoprevention: molecular mechanisms.* Mol Nutr Food Res, 2005. **49**(5): p. 462-71.

169. Vang, O., et al., *What is new for an old molecule? Systematic review and recommendations on the use of resveratrol.* PLoS One, 2011. **6**(6): p. e19881.

170. Cottart, C.H., et al., *Resveratrol bioavailability and toxicity in humans.* Mol Nutr Food Res, 2010. **54**(1): p. 7-16.

171. Burkon, A. and V. Somoza, *Quantification of free and protein-bound trans-resveratrol metabolites and identification of trans-resveratrol-C/O-conjugated diglucuronides - two novel resveratrol metabolites in human plasma.* Mol Nutr Food Res, 2008. **52**(5): p. 549-57.

172. Jannin, B., et al., *Transport of resveratrol, a cancer chemopreventive agent, to cellular targets: plasmatic protein binding and cell uptake.* Biochem Pharmacol, 2004. **68**(6): p. 1113-8.

173. Aumont, V., et al., *Regioselective and stereospecific glucuronidation of trans- and cis-resveratrol in human.* Arch Biochem Biophys, 2001. **393**(2): p. 281-9.

174. Brown, V.A., et al., *Repeat dose study of the cancer chemopreventive agent resveratrol in healthy volunteers: safety, pharmacokinetics, and effect on the insulin-like growth factor axis.* Cancer Res, 2010. **70**(22): p. 9003-11.

175. Aires, V., et al., *Resveratrol metabolites inhibit human metastatic colon cancer cells progression and synergize with chemotherapeutic drugs to induce cell death.* Mol Nutr Food Res, 2013. **57**(7): p. 1170-81.

176. Kenealey, J.D., et al., *Resveratrol metabolites do not elicit early pro-apoptotic mechanisms in neuroblastoma cells.* J Agric Food Chem, 2011. **59**(9): p. 4979-86.

177. Miksits, M., et al., *Antitumor activity of resveratrol and its sulfated metabolites against human breast cancer cells.* Planta Med, 2009. **75**(11): p. 1227-30.

178. Patel, K.R., et al., *Sulfate metabolites provide an intracellular pool for resveratrol generation and induce autophagy with senescence.* Sci Transl Med, 2013. **5**(205): p. 205ra133.

179. Patel, K.R., et al., *Clinical trials of resveratrol.* Ann N Y Acad Sci, 2011. **1215**: p. 161-9.

180. Boocock, D.J., et al., *Phase I dose escalation pharmacokinetic study in healthy volunteers of resveratrol, a potential cancer chemopreventive agent.* Cancer Epidemiol Biomarkers Prev, 2007. **16**(6): p. 1246-52.

181. Andlauer, W., et al., *Assessment of resveratrol bioavailability in the perfused small intestine of the rat.* Drugs Exp Clin Res, 2000. **26**(2): p. 47-55.

182. Brill, S.S., et al., *Glucuronidation of trans-resveratrol by human liver and intestinal microsomes and UGT isoforms.* J Pharm Pharmacol, 2006. **58**(4): p. 469-79.

183. Kuhnle, G., et al., *Resveratrol is absorbed in the small intestine as resveratrol glucuronide.* Biochem Biophys Res Commun, 2000. **272**(1): p. 212-7.

184. Sabolovic, N., et al., *Resveratrol is efficiently glucuronidated by UDP-glucuronosyltransferases in the human gastrointestinal tract and in Caco-2 cells.* Biopharm Drug Dispos, 2006. **27**(4): p. 181-9.

185. Walle, T., et al., *High absorption but very low bioavailability of oral resveratrol in humans.* Drug Metab Dispos, 2004. **32**(12): p. 1377-82.

186. Juan, M.E., E. Gonzalez-Pons, and J.M. Planas, *Multidrug resistance proteins restrain the intestinal absorption of trans-resveratrol in rats.* J Nutr, 2010. **140**(3): p. 489-95.

187. Alfaras, I., et al., *Involvement of breast cancer resistance protein (BCRP1/ABCG2) in the bioavailability and tissue distribution of trans-resveratrol in knockout mice.* J Agric Food Chem, 2010. **58**(7): p. 4523-8.

188. Patel, K.R., et al., *Clinical pharmacology of resveratrol and its metabolites in colorectal cancer patients.* Cancer Res, 2010. **70**(19): p. 7392-9.

189. Cal, C., et al., *Resveratrol and cancer: chemoprevention, apoptosis, and chemo-immunosensitizing activities.* Curr Med Chem Anticancer Agents, 2003. **3**(2): p. 77-93.

190. Aluyen, J.K., et al., *Resveratrol: potential as anticancer agent.* J Diet Suppl, 2012. **9**(1): p. 45-56.

191. Gehm, B.D., et al., *Resveratrol, a polyphenolic compound found in grapes and wine, is an agonist for the estrogen receptor.* Proc Natl Acad Sci U S A, 1997. **94**(25): p. 14138-43.

192. Mgbonyebi, O.P., J. Russo, and I.H. Russo, *Antiproliferative effect of synthetic resveratrol on human breast epithelial cells.* Int J Oncol, 1998. **12**(4): p. 865-9.

193. Nakagawa, H., et al., *Resveratrol inhibits human breast cancer cell growth and may mitigate the effect of linoleic acid, a potent breast cancer cell stimulator.* J Cancer Res Clin Oncol, 2001. **127**(4): p. 258-64.

194. Blagosklonny, M.V., *Cell cycle arrest is not yet senescence, which is not just cell cycle arrest: terminology for TOR-driven aging.* Aging (Albany NY), 2012. **4**(3): p. 159-65.

195. Gewirtz, D.A., *The four faces of autophagy: implications for cancer therapy.* Cancer Res, 2014. **74**(3): p. 647-51.

196. Delmas, D., E. Solary, and N. Latruffe, *Resveratrol, a phytochemical inducer of multiple cell death pathways: apoptosis, autophagy and mitotic catastrophe.* Curr Med Chem, 2011. **18**(8): p. 1100-21.

197. Zhang, X., et al., *Plant natural compounds: targeting pathways of autophagy as anti-cancer therapeutic agents.* Cell Prolif, 2012. **45**(5): p. 466-76.

198. Lobo, N.A., et al., *The biology of cancer stem cells.* Annu Rev Cell Dev Biol, 2007. **23**: p. 675-99.

199. Pandey, P.R., et al., *Resveratrol suppresses growth of cancer stem-like cells by inhibiting fatty acid synthase.* Breast Cancer Res Treat, 2011. **130**(2): p. 387-98.

200. Shankar, S., et al., *Resveratrol inhibits pancreatic cancer stem cell characteristics in human and KrasG12D transgenic mice by inhibiting pluripotency maintaining factors and epithelial-mesenchymal transition.* PLoS One, 2011. **6**(1): p. e16530.

201. Shen, Y.A., et al., *Resveratrol Impedes the Stemness, Epithelial-Mesenchymal Transition, and Metabolic Reprogramming of Cancer Stem Cells in Nasopharyngeal Carcinoma through p53 Activation.* Evid Based Complement Alternat Med, 2013. **2013**: p. 590393.

202. Serrero, G. and R. Lu, *Effect of resveratrol on the expression of autocrine growth modulators in human breast cancer cells.* Antioxid Redox Signal, 2001. **3**(6): p. 969-79.

203. Bove, K., D.W. Lincoln, and M.F. Tsan, *Effect of resveratrol on growth of 4T1 breast cancer cells in vitro and in vivo.* Biochem Biophys Res Commun, 2002. **291**(4): p. 1001-5.

204. Damianaki, A., et al., *Potent inhibitory action of red wine polyphenols on human breast cancer cells.* J Cell Biochem, 2000. **78**(3): p. 429-41.

205. Pozo-Guisado, E., et al., *The antiproliferative activity of resveratrol results in apoptosis in MCF-7 but not in MDA-MB-231 human breast cancer cells: cell-specific alteration of the cell cycle.* Biochem Pharmacol, 2002. **64**(9): p. 1375-86.

206. Tang, H.Y., et al., *Resveratrol-induced cyclooxygenase-2 facilitates p53-dependent apoptosis in human breast cancer cells.* Mol Cancer Ther, 2006. **5**(8): p. 2034-42.

207. Scarlatti, F., et al., *Resveratrol induces growth inhibition and apoptosis in metastatic breast cancer cells via de novo ceramide signaling.* Faseb j, 2003. **17**(15): p. 2339-41.

208. Mezzanotte, L., et al., *A New Multicolor Bioluminescence Imaging Platform to Investigate NF-kappaB Activity and Apoptosis in Human Breast Cancer Cells.* PLoS One, 2014. **9**(1): p. e85550.

209. Garvin, S., K. Ollinger, and C. Dabrosin, *Resveratrol induces apoptosis and inhibits angiogenesis in human breast cancer xenografts in vivo.* Cancer Lett, 2006. **231**(1): p. 113-22.

210. Nin, V., et al., *Role of deleted in breast cancer 1 (DBC1) protein in SIRT1 deacetylase activation induced by protein kinase A and AMP-activated protein kinase.* J Biol Chem, 2012. **287**(28): p. 23489-501.

211. Kim, J.E., J. Chen, and Z. Lou, *DBC1 is a negative regulator of SIRT1.* Nature, 2008. **451**(7178): p. 583-6.

212. Wang, R.H., et al., *Interplay among BRCA1, SIRT1, and Survivin during BRCA1-associated tumorigenesis.* Mol Cell, 2008. **32**(1): p. 11-20.

213. Detampel, P., et al., *Drug interaction potential of resveratrol.* Drug Metab Rev, 2012. **44**(3): p. 253-65.

214. Oskarsson, A., et al., *Inhibition of CYP17A1 activity by resveratrol, piceatannol, and synthetic resveratrol analogs.* Prostate, 2014.

215. Chan, W.K. and A.B. Delucchi, *Resveratrol, a red wine constituent, is a mechanism-based inactivator of cytochrome P450 3A4.* Life Sci, 2000. **67**(25): p. 3103-12.

216. Kma, L., *Synergistic Effect of Resveratrol and Radiotherapy in Control of Cancers.* Asian Pac J Cancer Prev, 2013. **14**(11): p. 6197-6208.

217. Gupta, S.C., et al., *Chemosensitization of tumors by resveratrol.* Ann N Y Acad Sci, 2011. **1215**: p. 150-60.

218. Singh, C.K., J. George, and N. Ahmad, *Resveratrol-based combinatorial strategies for cancer management.* Ann N Y Acad Sci, 2013. **1290**(1): p. 113-21.

219. Jazirehi, A.R. and B. Bonavida, *Resveratrol modifies the expression of apoptotic regulatory proteins and sensitizes non-Hodgkin's lymphoma and multiple myeloma cell lines to paclitaxel-induced apoptosis.* Mol Cancer Ther, 2004. **3**(1): p. 71-84.

220. Kubota, T., et al., *Combined effects of resveratrol and paclitaxel on lung cancer cells.* Anticancer Res, 2003. **23**(5A): p. 4039-46.

221. Zhao, W., et al., *Resveratrol down-regulates survivin and induces apoptosis in human multidrug-resistant SPC-A-1/CDDP cells.* Oncol Rep, 2010. **23**(1): p. 279-86.

222. Quan, F., et al., *Reversal effect of resveratrol on multidrug resistance in KBv200 cell line.* Biomed Pharmacother, 2008. **62**(9): p. 622-9.

223. Mao, Q.Q., et al., *Resveratrol confers resistance against taxol via induction of cell cycle arrest in human cancer cell lines.* Mol Nutr Food Res, 2010. **54**(11): p. 1574-84.

224. Nicolini, G., et al., *Anti-apoptotic effect of trans-resveratrol on paclitaxel-induced apoptosis in the human neuroblastoma SH-SY5Y cell line.* Neurosci Lett, 2001. **302**(1): p. 41-4.

225. Rigolio, R., et al., *Resveratrol interference with the cell cycle protects human neuroblastoma SH-SY5Y cell from paclitaxel-induced apoptosis.* Neurochem Int, 2005. **46**(3): p. 205-11.

226. Fukui, M., N. Yamabe, and B.T. Zhu, *Resveratrol attenuates the anticancer efficacy of paclitaxel in human breast cancer cells in vitro and in vivo.* Eur J Cancer, 2010. **46**(10): p. 1882-91.

227. Godwin, A.K., et al., *High resistance to cisplatin in human ovarian cancer cell lines is associated with marked increase of glutathione synthesis.* Proc Natl Acad Sci U S A, 1992. **89**(7): p. 3070-4.

228. Oliver, M.H., et al., *A rapid and convenient assay for counting cells cultured in microwell plates: application for assessment of growth factors.* J Cell Sci, 1989. **92 (Pt 3)**: p. 513-8.

229. Goldblatt, E.M., et al., *The telomerase template antagonist GRN163L alters MDA-MB-231 breast cancer cell morphology, inhibits growth, and augments the effects of paclitaxel.* Mol Cancer Ther, 2009. **8**(7): p. 2027-35.

230. Munshi, A., M. Hobbs, and R.E. Meyn, *Clonogenic cell survival assay.* Methods Mol Med, 2005. **110**: p. 21-8.

231. Chou, T.C. and P. Talalay, *Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors.* Adv Enzyme Regul, 1984. **22**: p. 27-55.

232. Chou, T.C., *Drug combination studies and their synergy quantification using the Chou-Talalay method.* Cancer Res, 2010. **70**(2): p. 440-6.

233. Schmittgen, T.D. and K.J. Livak, *Analyzing real-time PCR data by the comparative C(T) method.* Nat Protoc, 2008. **3**(6): p. 1101-8.

234. Joe, A.K., et al., *Resveratrol induces growth inhibition, S-phase arrest, apoptosis, and changes in biomarker expression in several human cancer cell lines.* Clin Cancer Res, 2002. **8**(3): p. 893-903.

235. Juan, M.E., et al., *Resveratrol induces apoptosis through ROS-dependent mitochondria pathway in HT-29 human colorectal carcinoma cells.* J Agric Food Chem, 2008. **56**(12): p. 4813-8.

236. Frackowiak, S., et al., *Preservation of chromosome integrity during micronucleation induced by colchicine in PtK1 cells.* Chromosoma, 1986. **94**(6): p. 468-474.

237. Campisi, J., *Aging, cellular senescence, and cancer.* Annu Rev Physiol, 2013. **75**: p. 685-705.

238. Hawley, S.A., et al., *Characterization of the AMP-activated protein kinase kinase from rat liver and identification of threonine 172 as the major site at which it phosphorylates AMP-activated protein kinase.* J Biol Chem, 1996. **271**(44): p. 27879-87.

239. Wen, X., et al., *Trimethoprim and sulfamethoxazole are selective inhibitors of CYP2C8 and CYP2C9, respectively.* Drug Metab Dispos, 2002. **30**(6): p. 631-5.

240. McDermott, M., et al., *In vitro Development of Chemotherapy and Targeted Therapy Drug-Resistant Cancer Cell Lines: A Practical Guide with Case Studies.* Front Oncol, 2014. **4**: p. 40.

241. Bhatia, A. and Y. Kumar, *Cancer cell micronucleus: an update on clinical and diagnostic applications.* Apmis, 2013. **121**(7): p. 569-81.

242. Fulda, S. and K.M. Debatin, *Sensitization for anticancer drug-induced apoptosis by the chemopreventive agent resveratrol.* Oncogene, 2004. **23**(40): p. 6702-11.

243. Hayashibara, T., et al., *Resveratrol induces downregulation in survivin expression and apoptosis in HTLV-1-infected cell lines: a prospective agent for adult T cell leukemia chemotherapy.* Nutr Cancer, 2002. **44**(2): p. 193-201.

244. Bhardwaj, A., et al., *Resveratrol inhibits proliferation, induces apoptosis, and overcomes chemoresistance through down-regulation of STAT3 and nuclear factor-kappaB-regulated antiapoptotic and cell survival gene products in human multiple myeloma cells.* Blood, 2007. **109**(6): p. 2293-302.

245. Sung, J.Y., et al., *Balance between SIRT1 and DBC1 expression is lost in breast cancer.* Cancer Sci, 2010. **101**(7): p. 1738-44.

246. Hubbard, B.P., et al., *Evidence for a common mechanism of SIRT1 regulation by allosteric activators.* Science, 2013. **339**(6124): p. 1216-9.

247. Jin, Q., et al., *Cytoplasm-localized SIRT1 enhances apoptosis.* J Cell Physiol, 2007. **213**(1): p. 88-97.

248. Hannun, Y.A., C. Luberto, and K.M. Argraves, *Enzymes of sphingolipid metabolism: from modular to integrative signaling.* Biochemistry, 2001. **40**(16): p. 4893-903.

249. Escarcega, R.O., et al., *The transcription factor nuclear factor-kappa B and cancer.* Clin Oncol (R Coll Radiol), 2007. **19**(2): p. 154-61.

250. Borra, M.T., B.C. Smith, and J.M. Denu, *Mechanism of human SIRT1 activation by resveratrol.* J Biol Chem, 2005. **280**(17): p. 17187-95.

251. Manuel Iglesias, J., et al., *Mammosphere formation in breast carcinoma cell lines depends upon expression of E-cadherin.* PLoS One, 2013. **8**(10): p. e77281.

252. Pampaloni, F., E.G. Reynaud, and E.H. Stelzer, *The third dimension bridges the gap between cell culture and live tissue.* Nat Rev Mol Cell Biol, 2007. **8**(10): p. 839-45.

253. Shultz, L.D., et al., *Human lymphoid and myeloid cell development in NOD/LtSz-scid IL2R gamma null mice engrafted with mobilized human hemopoietic stem cells.* J Immunol, 2005. **174**(10): p. 6477-89.

254. Siolas, D. and G.J. Hannon, *Patient-derived tumor xenografts: transforming clinical samples into mouse models.* Cancer Res, 2013. **73**(17): p. 5315-9.

255. Gut, I., et al., *Cytotoxicity of cyclophosphamide, paclitaxel, and docetaxel for tumor cell lines in vitro: effects of concentration, time and cytochrome P450-catalyzed metabolism.* Arch Toxicol, 2000. **74**(8): p. 437-46.

256. Ji, N., et al., *Developing multidrug-resistant cells and exploring correlation between BCRP/ABCG2 over-expression and DNA methyltransferase.* Acta Biochim Biophys Sin (Shanghai), 2010. **42**(12): p. 854-62.

257. O'Brien, C., et al., *Functional genomics identifies ABCC3 as a mediator of taxane resistance in HER2-amplified breast cancer.* Cancer Res, 2008. **68**(13): p. 5380-9.

258. Nakshatri, H., et al., *Constitutive activation of NF-kappaB during progression of breast cancer to hormone-independent growth.* Mol Cell Biol, 1997. **17**(7): p. 3629-39.

259. Vaclavikova, R., et al., *Paclitaxel metabolism in rat and human liver microsomes is inhibited by phenolic antioxidants.* Naunyn Schmiedebergs Arch Pharmacol, 2003. **368**(3): p. 200-9.

260. Nabekura, T., S. Kamiyama, and S. Kitagawa, *Effects of dietary chemopreventive phytochemicals on P-glycoprotein function.* Biochem Biophys Res Commun, 2005. **327**(3): p. 866-70.

261. Al-Abd, A.M., et al., *Resveratrol enhances the cytotoxic profile of docetaxel and doxorubicin in solid tumour cell lines in vitro.* Cell Prolif, 2011.

262. Kim, T.H., et al., *Resveratrol enhances chemosensitivity of doxorubicin in multidrug-resistant human breast cancer cells via increased cellular influx of doxorubicin.* Biochim Biophys Acta, 2014. **1840**(1): p. 615-25.

CURRICULUM VITAE

Alyssa A. Sprouse

EDUCATION

2009 – 2014	Doctor of Philosophy, Pharmacology, Indiana University, Indianapolis, IN (GPA 3.78)
2004 – 2008	Bachelor of Science, Biology, University of Dayton, Dayton, OH (GPA 3.41)

LICENSURE AND CERTIFICATION

2010	Collaborative Institutional Training Initiative (CITI) Responsible Conduct of Research Module
2010	CITI Animal Care and Use Training

PROFESSIONAL ORGANIZATIONS

2014 –	Trainee Member, American Society for Clinical Pharmacology and Therapeutics
2014 –	Trainee Member, International Society of Pharmacometrics
2013 –	Member, American Association for the Advancement of Science
2011 –	Member, Association for Women in Science
2011 –	Associate Member, American Association for Cancer Research

HONORS AND AWARDS

2013	Mary Frances Picciano Dietary Supplement Research Practicum Travel Award *(see Grants and Fellowships)*
2012	Indiana University Purdue University Research Day First Place Award for Professional and Graduate Student Poster Competition
2009 – 2011	Indiana University School of Medicine Fellowship *(see Grants and Fellowships)*
2009	University of Dayton's Biology Department Learn, Lead and Serve Award
2007	University of Dayton Learn, Lead and Serve Grant *(see Grants and Fellowships)*
2005, 2007	University of Dayton Dean's List
2005 – 2009	University of Dayton Presidential Scholarship
2005 – 2009	Ohio Student Choice Grant

LABORATORY EXPERIENCE

2010 – 2014	Indiana University School of Medicine, Indianapolis, IN, Department of Pharmacology and Toxicology Supervisor: Dr. Brittney-Shea Herbert, Ph.D. *Researcher* Characterizing the mechanism of paclitaxel resistance, the mechanism of action of resveratrol, and the use of resveratrol and paclitaxel in combination in derived paclitaxel-resistant triple negative breast cancer cells.
2010	Indiana University School of Medicine, Indianapolis, IN Department of Pharmacology and Toxicology Supervisor: Dr. Jian-Ting Zhang, Ph.D. *Rotation Student* Examined proteome changes in derived gemcitabine-resistant pancreatic cancer cells compared to the parental cells.
2010	Indiana University School of Medicine, Indianapolis, IN Department of Pharmacology and Toxicology Supervisor: Dr. Karen Pollok, Ph.D. *Rotation Student* Examined the anti-proliferatrive effects of Nutlin-3a in breast and brain cancer cells.
2009	Indiana University School of Medicine, Indianapolis, IN Department of Pharmacology and Toxicology Supervisor: Dr. Brittney-Shea Herbert, Ph.D. *Rotation Student* Extracted *Echinacea angustifolia* to examine anti-proliferation effects in breast cancer cells.
2008 – 2009	University of Dayton, Dayton, OH Department of Biology Supervisor: Dr. Yiling Hong *Researcher* Examined the redifferenetiation effects of chemically defined media on melanoma cells and the growth inhibition effects of *Echinacea angustifolia* extract on breast cancer cells.
2007 – 2009	University of Dayton, Dayton, OH Department of Biology Supervisor: Dr. Yiling Hong *Laboratory Assistant* General laboratory maintenance and purchasing.

UNIVERSITY SERVICE

2011	Pharmacology and Toxicology Techniques Student Journal Club Initiator and Coordinator. Activities included starting a techniques journal club, giving presentations, and coordinating fellow student presenters.
2010 – 2014	Pharmacology and Toxicology New Student Orientation Poster Presentations.
2010 – 2014	Pharmacology and Toxicology Journal Club Series Presenter. Activities include leading journal club discussions.
2010 – 2014	Student Ambassador to prospective students visiting Indiana University School of Medicine. Activities include Building Guide, Poster Presentations, accompaniment to dinners and lunches.

OTHER PROFESSIONAL ACTIVITIES:

Invited Talks and Seminars:

2014	Division of Clinical Pharmacology Special Seminar, *Resveratrol Augments Paclitaxel Treatment in MDA-MB-231 and Paclitaxel-resistant MDA-MB-231 Breast Cancer Cells.* Indianapolis, IN (Seminar)
2013	Department of Pharmacology and Toxicology Student Seminar Series, *Resveratrol Activity in Paclitaxel-sensitive and -resistant Triple Negative Breast Cancer Cells*. Indianapolis, IN (Seminar)
2013	Department of Pharmacology and Toxicology Student Seminar Series, *Molecular Mechanisms of Paclitaxel Resistance and Resveratrol Sensitivity in MDA-MB-231 Breast Cancer Cells*. Indianapolis, IN (Seminar)
2013	American Association for Cancer Research Annual Meeting, *Molecular Mechanisms of Paclitaxel Resistance and Resveratrol Sensitivity in MDA-MB-231 Breast Cancer Cells*. Abstract #876, Washington, D.C. (Poster)
2012	IUSM Cancer Biology Research Club, *Molecular Mechanisms of Paclitaxel Resistance and Resveratrol Sensitivity in MDA-MB-231 Breast Cancer Cells*. Indianapolis, IN (Seminar)
2012	IUSCC Cancer Research Day, *Effects of Resveratrol on Paclitaxel-sensitive and -resistant Triple Negative Breast Cancer Cells,* Indianapolis, IN (Poster)
2012	Department of Pharmacology and Toxicology Student Seminar Series, *Effects of Resveratrol on Paclitaxel-sensitive and -resistant Triple Negative Breast Cancer Cells*. Indianapolis, IN (Seminar)

2012	IUPUI Research Day, *Effects of Resveratrol on Paclitaxel-sensitive and -resistant Triple Negative Breast Cancer Cells*. Indianapolis, IN (Poster)
2012	AACR Annual Meeting, *Effects of Resveratrol on Paclitaxel-sensitive and -resistant Triple Negative Breast Cancer Cells.* Abstract #5676, Chicago, IL (Poster)
2011	IUSM Cancer Biology Research Club, *Effects of Resveratrol on Paclitaxel-sensitive and -resistant Triple Negative Breast Cancer Cells.* Indianapolis, IN (Seminar)
2011	Department of Pharmacology and Toxicology Student Seminar Series, *Resveratrol as a Cancer Therapeutic*. Indianapolis, IN (Seminar)
2009	University of Dayton Stander Symposium, *Reprogramming Melanoma and Breast Cancer Cells with Chemically Defined Media and Echinacea Extract.* Dayton, OH (Poster)

Attended Talks and Seminars:

2013	Mary Frances Picciano Dietary Supplement Research Practicum, Bethesda, MD
2013	AACR 2013 Annual Meeting, Washington D.C.
2012	IUSCC Cancer Research Day, Indianapolis, IN
2012	IUPUI Research Day, Indianapolis, IN
2012	AACR 2012 Annual Meeting, Chicago, IL
2011	Purdue University Center for Cancer Research Drug Delivery and Cancer: Challenges and New Directions for Cancer Therapy, West Lafayette, IN
2011	Purdue University Breast Cancer Discovery Group Retreat, West Lafayette, IN
2011	Amelia Project Giving Wings to Research, Indianapolis, IN
2011	Purdue University International Symposium on Breast Cancer Prevention: Nutrition, Communication, and Public policy, West Lafayette, IN
2009 – 2014	Department of Pharmacology and Toxicology Seminar Series

GRANTS AND FELLOWSHIPS

Fellowships:

2009 – 2011 Indiana University School of Medicine Fellowship

Grants:

2013 Mary Frances Picciano Dietary Supplement Research Practicum Travel Award

2007 University of Dayton Learn, Lead and Serve Award

PEER REVIEWED PUBLICATIONS

Koziel JE, Fox MJ, Steding CE, **Sprouse AA**, Herbert BS (2011). Medical genetics and epigenetics of telomerase. *J Cell Mol Med*, 15:457-67; PMID: 21323862.

Sprouse AA, Steding CE, Herbert BS (2011) Pharmaceutical regulation of telomerase and its clinical potential. *J Cell Mol Med.* 16(1):1-7; PMID: 21973217.

Mangum CN*, **Sprouse AA***, Herbert BS (submitted 2013) Effects of Resveratrol on Triple Negative, Mutant BRCA1 Breast Cancer Cell Growth and Paclitaxel Augmentation. *BMC Cancer*. (*co-first authors)

Sprouse AA, Herbert BS (submitted 2014). Resveratrol Augments Paclitaxel Treatment in MDA-MB-231 and Paclitaxel-resistant MDA-MB-231 Breast Cancer Cells. *Mol Pharm.*

ABSTRACTS

2013 American Association for Cancer Research Annual Meeting, *Molecular mechanisms of paclitaxel resistance and resveratrol sensitivity in MDA-MB-231 breast cancer cells*. Abstract #876, Washington D.C.

2012 IUSCC Cancer Research Day, *Effects of Resveratrol on Paclitaxel-sensitive and -resistant Triple Negative Breast Cancer Cells,* Indianapolis, IN

2012 IUPUI Research Day, *Effects of Resveratrol on Paclitaxel-sensitive and -resistant Triple Negative Breast Cancer Cells.,* Indianapolis, IN

2012 American Association for Cancer Research Annual Meeting, *Effects of Resveratrol on Paclitaxel-sensitive and -resistant Triple Negative Breast Cancer Cells.* Abstract #5676, Chicago, IL