THE EFFECTS OF SOLUTE CARRIER ORGANIC ANION TRANSPORTERS ON JADOMYCIN PHARMACOLOGY IN BREAST CANCER

by

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TABLE OF CONTENTS

LIST OF TABLES v

LIST OF FIGURES vi

ABSTRACT viii

LIST OF ABBREVIATIONS USED ix

ACKNOWLEDGEMENTS xiii

CHAPTER 1. INTRODUCTION 1

1.1 Development of Cancer 1

1.2 Tumor Suppressor and Proto-Oncogenes 2

1.3 Tumor Suppressor Gene Modification 2

1.3.1 Oncogene Development 3

1.4 The Hallmarks of Cancer 6

1.5 Breast Cancer 8

1.6 Risk Factors 8

1.7 Classification of Breast Cancer 10

1.8 Breast Cancer Treatments 13

1.8.1 Surgery 13

1.8.2 Radiation therapy 13

1.8.3 Chemotherapy 13

1.8.4 Hormone Therapy 14

1.8.5 Targeted Therapy 15

1.9 Multi-Drug Resistant Breast Cancer 16

1.10 Multi-drug Resistance Mechanisms 16

1.10.1 Increased Drug Efflux Transporter Expression 16
1.10.2 Decreased Drug Uptake Transporter Expression 16
1.10.3 Increased Drug Metabolism and Detoxification 17
1.10.4 Activation of DNA Repair Mechanisms 17
1.10.5 Apoptosis Inhibition 18
1.10.6 Alteration of Drug Target 18
1.11 Jadomycins 18
1.12 Cytotoxicity of Jadomycins 20
1.13 Mechanisms of Jadomcyins Anticancer Activity 23
1.14 Solute Carrier Transporters and Drug Uptake 25
1.15 Objectives and Project Overview 28
CHAPTER 2. MATERIALS AND METHODS 30
2.1 Chemicals 30
2.2 The Production of Jadomycins 30
2.3 Cell Lines 30
2.4 Western Blots 31
2.5 Flow Cytometry 32
2.6 MTT Assay 33
2.7 RNA Collection, Reverse Transcriptase Reaction, and Quantitative Polymerase Chain Reaction 33
2.8 Gene Knockdown using Lentivirus 35
2.9 Statistical Analysis 36
CHAPTER 3. RESULTS 37

3.1 SLCO Gene Expression Profiles of Breast Cancer Cells and Breast Epithelial Cells..... 37

3.1.1 The Impact of Drug Resistant Phenotype on SLCO Expression within each Cell Type. 37

3.1.2 The Impact of Drug-Resistant Phenotype on SLCO Expression Across Cell Type.....38

3.1.3 Impact of Breast Cancer Cell Type on Relative Expression of SLCOs within Cell Type. 40

3.1.4 The Impact of Breast Cancer Cell Type on SLCO Expression. 41

3.2 Protein Expression of SLCO4A1 and SLCO3A1 in Breast Cancer Cells. 43

3.2.1 Detection of *SLCO3A1* and *SLCO4A1* by Western Blot and FACS Analysis 43

3.3 Lentiviral Knockdown of *SLCO3A1* and *SLCO4A1* Expression in MCF7 Cells. 43

3.3.1 Confirmation of Lentiviral Transduction. 44

3.3.2 Gene Expression of *SLCO4A1* and *3A1* in Knockdown Cell Lines and Control Cell Lines. 45

3.3.3 Effect of *SLCO4A1* and *SLCO3A1* Knockdown on Jadomycin Cytotoxicity. 45

CHAPTER 4. DISCUSSION 49

4.1 Understanding SLCO Expression Profiles..... 49

4.2 Influence of Transporter Expression in Jadomycin Cytotoxicity..... 51

4.3 Future Experiments and Limitations..... 53

CHAPTER 5. CONCLUSION..... 55

REFERENCES 56

LIST OF TABLES

Table 1: Risk factors in developing breast cancer with age..... 8

Table 2: Breast cancer molecular subtypes review..... 10

Table 3: IC_{50} values of cytotoxic drugs against control and ABC transporter overexpressing cells as evaluated by cell viability tests in MCF7 cells. This data represents an excerpt of previously published data. 22

Table 4: IC_{50} values of cytotoxic drugs against control and ABC transporter overexpressing cells as evaluated by cell viability tests in 231 cells. This data represents an excerpt of previously published data..... 22

Table 5: SLCO transporter expression, function, and substrate specificity..... 26

Table 6: PCR primers designed to evaluate gene expression of SLCO family and housekeeping genes. 34

LIST OF FIGURES

Figure 1: Five common mechanisms in which EGFR-related mutagens lead to malignancies 5

Figure 2: Breast cancer stages 0, I, II, III, IV characteristics and common treatment methods 12

Figure 3: Incorporation of amino acids into jadomycins through a non-enzymatic biosynthetic pathway in S. venezuelae 19

Figure 4: Chemical structures of a library of jadomycins, differing by the R group 20

Figure 5: Overview of the proposed mechanisms of action of jadomycins 25

Figure 6: Hypothesized impact of knockdown on jadomycin transport 29

Figure 7: The impact of drug resistant phenotype on SLCO expression within each cell type. mRNA expression of SLCO transporters are evaluated in drug resistant MCF7 cells and MCF7-CON cells 38

Figure 8: The impact of drug resistant phenotype on SLCO expression across cell type. The mRNA expression of SLCOs were measured in MCF7-CON cells and three MCF7 MDR cells 39

Figure 9: Impact of breast cancer cell type on relative expression of SLCOs within cell type. mRNA expression of SLCO genes were measured in different breast cancer cell types 41

Figure 10: The impact of breast cancer cell type on SLCO expression across each cell type. The mRNA expression of SLCOs were measured in a panel of breast cancer cells and breast epithelial cells 42

Figure 11: Western blot scanned at 800 nm to detect GAPDH control protein 43

Figure 12: Confirmation of lentiviral transduction of MCF7 cells 44

Figure 13: The effect of lentiviral shRNA transduction on the expression of SLCO3A1 and SLCO4A1 45

Figure 14: The effect of SLCO3A1 and 4A1 lentiviral shRNA treatment on the dose-response toxicity of jadomycin S in MCF7 cells 46

Figure 15: The effect of SLCO3A1 and 4A1 lentiviral shRNA treatment on the IC_{50} values of jadomycin S in MCF7 cells 46

Figure 16: The effect of SLCO3A1 and 4A1 lentiviral shRNA treatment on the dose-response toxicity of doxorubicin in MCF7 cells 47

Figure 17: The effect of SLCO3A1 and 4A1 lentiviral shRNA treatment on the IC_{50} values of doxorubicin in MCF7 cells 47

Figure 18: Cell viability assay of jadomycin S (10 μM) at different timepoints in different knockdown cell lines and control cell lines. 48

Figure 19: Cell viability assay of doxorubicin (10 μM) at different timepoints in different knockdown cell lines and control cell lines. 48

ABSTRACT

Breast cancer is the most commonly diagnosed cancer in women, and often becomes multidrug-resistant (MDR). Jadomycins are experimental chemotherapeutics that maintain cytotoxicity in MDR breast cancer cells and investigating the jadomycin uptake mechanism is critical. We hypothesized that solute carrier transporters (SLCO) mediate jadomycin uptake, facilitating jadomycin cytotoxicity in MDR breast cancer cells. The objectives were to determine the expression patterns of 11 SLCOs in seven breast cancer cell lines (using quantitative polymerase chain reaction), and then determine the impact of SLCO knockdown on jadomycin cytotoxicity (using lentivirus transduction and MTT assay).

The expression of the SLCOs varied with breast cancer cell type and MDR status. Knockdown cells of the highest expressed SLCOs, *SLCO4A1* and *SLCO3A1* in MCF7-CON cells did not alter jadomycin S or doxorubicin cytotoxicity. The lack of effect of knocking down individual SLCOs suggests that several SLCO transporters may govern jadomycin uptake allowing for their broad-spectrum activity in MDR breast cancer.

LIST OF ABBREVIATIONS USED

ABC	ATP binding cassette
ABL1	Abelson murine leukemia viral oncogene homolog 1
AhR	Aryl hydrocarbon
AI	Aromatase inhibitor
ANOVA	Analysis of variance
APC	Adenomatous polyposis coli
ATP	Adenosine triphosphate
BRCA1/2	Breast cancer susceptibility gene 1 and 2
BSA	Bovine serum albumin
BT474	Breast tumor 474
c-CBL	Casitas B-lineage lymphoma
CCNE1	Cyclin E1
CDK	Cyclin-dependent kinase
cDNA	Complimentary DNA
CK	Cytokeratin
CML	Chronic myelogenous leukemia
CTLS	Cytotoxic T lymphocytes
CYP	Cytochrome P450
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DOX	Doxorubicin
DS DNA	Double stranded DNA
E2F	E2 transcription factor
EGFR	Epidermal growth factor receptor
EMT	Epithelial-to-mesenchymal transition
ErbB2	Receptor tyrosine-protein kinase
ETP	Etoposide
FACS	Fluorescence-activated cell sorting
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase

GDP	Guanosine diphosphate
GFP	Green fluorescent protein
GI_{50}	Growth inhibition 50
Grb2	Growth factor receptor-bound protein 2
GTP	Guanosine triphosphate
H460	Human non-small-cell lung cancer
HDAC	Histone deacetylases
HepG2	Human hepatocellular carcinoma
HER2	Epidermal growth factor receptor 2
HRMS	High resonance mass spectrometry
IM-9	Human lymphoblast cell line derived from multiple myeloma
IM-9/BCL-2	Human lymphoblast cell line derived from multiple myeloma with Bcl-2 overexpression
IR	Infrared spectroscopy
KRAS	Kirsten Rat sarcoma
LB	Lennox broth
LFS	Li-Fraumeni syndrome
LRMS	Low-resolution mass spectrometry
MADR2	Mad-related gene 2
MCF 10A	Michigan Cancer Foundation 10A
MCF7	Michigan Cancer Foundation-7
MDA MB 231	M.D. Anderson metastasis breast cancer 231
MDA-MB-435	M.D. Anderson metastasis breast cancer 435
MDR	Multidrug-resistant
MGMT	O(6)-methylguanine methyltransferase
MITX	Mitoxantrone
mRNA	Messenger ribonucleic acid
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NAC	N-acetylcysteine
NCI	National Cancer Institute

NK	Natural killer
NMR	Nuclear magnetic resonance
NSEP1	Nuclease sensitive element binding protein 1
OATP	Organic anion transporter polypeptide
OD_{600}	Optical density at a wavelength of 600 nm
PCNA	Proliferating cell nuclear antigen
PEI	Polyethylenimine
PI	Propidium iodide
PKC	Protein kinase c
PPIA	Peptidylprolyl isomerase A
PR	Progesterone receptors
PTEN	Phosphate and tensin homolog
PXR	Pregnane X receptor
qPCR	Quantitative polymerase chain reaction
RB	Retinoblastoma-susceptibility gene
ROS	Reactive oxygen species
SEM	Standard error of the mean
SERM	Selective estrogen receptor modulators
SHRNA	Small hairpin RNA
SKBR3	Sloan Kettering breast cancer
SLCO	Solute carrier organic anion
SOD1/2	Superoxide dismutase 1/2
STS	Steroid sulfatases
TBST	Tris-buffered saline
TGF-A/B	Transforming growth factor alpha/beta
TGI	Total growth inhibition
TLC	Thin layer chromatography
TOP2A/B	Topoisomerase 2 alpha/beta
TP53	Tumor protein 53
TPA	Tetradecanoyl phorbol acetate
TXL	Taxol

UV-VIS	Ultra violet visible spectroscopy
VEGF	Vascular endothelial growth factor
v-MYB	Avian myeloblastosis viral oncogene homolog
WT1	Wilms' tumor 1

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CHAPTER 1. INTRODUCTION

1.1 Development of Cancer

Cancer is a group of diseases characterized by excessive cellular proliferation and invasion of heathy tissue by abnormal cells. The development of cancer is linked to factors such as obesity, age, and exposure to carcinogens; yet all cancers can ultimately be attributed to genetic instabilities. These genetic changes in the cells lead to abnormal protein function and expression, providing cancer cells the ability to grow excessively. All cancer cells begin as normal healthy cells before obtaining the cancer phenotype. Indeed, it is the genes that allow the cell to repair damaged tissues, maintain proper cellular growth, and ensure healthy tissue development that are culprits for cancer formation. Changes in the expression levels of these genes and protein function lead to uncontrolled growth.

The transformation of healthy tissue to a malignancy is a multi-step process termed carcinogenesis. The first step is initiation which is a genetic alteration that leads to the mutation of a single cell, resulting from a spontaneous error or carcinogen exposure. The initiation stage occurs when the DNA damage is not corrected by cellular repair mechanisms, deeming the initiation stage irreversible. For example, exposure to polycyclic hydrocarbons found in cigarette smoke can induce structural genomic changes such as N^2 guanine and adenine adducts.[1] Following the initiation step is promotion, which occurs when preneoplastic cells replicate, increasing the population of cells that are capable of malignant conversion.[2,3] Initiated cells are harmless unless proliferation occurs, and may remain quiescent until exposed to a promoter.[4] Promoters alter normal growth factors within the cell and can change gene expression.[4] These molecules bind to nuclear receptors, acting as DNA ligands that bind to regulatory regions of genes, altering their expression.[1] For example, in skin cells, tetradecanoyl phorbol acetate (TPA) is a tumor promoter that targets protein kinase c (PKC), which controls genes for cell cycle progression. Changes in PKC can lead to excessive cellular proliferation, in turn playing a role in tumorigenesis.[5,6] The third and final stage is progression, which requires irreversible genetic mutation resulting in phenotypic changes characteristic of cancer cells such as acquiring the capability to metastasize, excessively grow, and alter

biochemical signaling.[2–4] Progression is further characterized by the evolution of genetic changes, which leads to stages of cancer into higher degrees of malignancy.[1]

1.2 Tumor Suppressor and Proto-Oncogenes

Cancer is driven by the accumulation of genetic alterations of tumor suppressor genes and proto-oncogenes. Tumor suppressor genes are those that reduce cell proliferation, repair DNA, and trigger apoptosis in abnormal cells. Cancer can arise when tumor suppressor genes become inactivated, and cellular mechanisms that suppress growth are turned off.[7] Proto-oncogenes are the opposite, such that when malignancies arise upon activation of proto-oncogenes, yielding what is known as oncogenes. Proto-oncogenes assist in cellular division and growth and are tightly regulated in normal cells, but when oncogenes develop, there is disorderly cellular growth and enhanced cell survival. [7,8]

1.3 Tumor Suppressor Gene Modification

Approximately 50 tumor suppressor genes have been identified in various types of cancer.[9] Loss-of-function tumor suppressor gene mutations are recessive, meaning that both copies of the gene must be altered in for a phenotypic change to occur. This concept is known as the two-hit hypothesis and is responsible for most inherited cancers.[8,10] The two-hit hypothesis was first proposed in 1971 by geneticist Alfred Knudson when he explored the genetic mechanisms underlying retinoblastoma, a juvenile retinal cancer.[11] Retinoblastoma is caused by a mutation in the retinoblastoma-susceptibility gene (RB), which inhibits cellular proliferation.[12] In normal cells, RB suppresses a family of transcription factors known as E2F, which assists with DNA synthesis. [10] Knudson discovered that individuals who inherited one allele of the mutated gene would only require one mutation in the healthy gene to cause loss of function, which would occur much more rapidly than if two normal genes were inherited and both alleles needed to encounter spontaneous gene damage.[10]

This concept was supported when Knudson studied unilateral and bilateral retinoblastoma. There was a delayed onset for those with unilateral retinoblastoma, which suggests nonhereditary association, as both alleles acquiring spontaneous mutations is a slower process. In comparison, bilateral retinoblastomas occur at an early age suggesting

a hereditary association consistent with single gene mutations. Individuals with the tumor suppressor gene (RB1) mutation often developed multiple tumors and sooner.[11]

Another commonly known tumor suppressor gene is the TP53 gene, which codes for the p53 protein. The p53 protein acts in the nucleus of the cell to ensure that damaged DNA either gets repaired or forces the cell into apoptosis.[13] When DNA is damaged, the p53 protein is rapidly produced, and this will increase transcription of a cyclin-dependent kinase (CDK) inhibitor called p21.[10] This inhibitor prevents cell cycle progression by blocking p21-cyclin complexes as well as binding to proliferating cell nuclear antigen (PCNA). This allows time for damaged DNA to be repaired, and if it cannot apoptosis is triggered. If p53 is inactive, cells with DNA damage will not only proliferate, but increase the risk of genome instability and frequency of mutations.[10] P53 mutations are common in cancers, with 38%-50% of ovarian, esophageal, colorectal, head and neck, larynx, and lung cancers having the abnormality.[13] TP53 mutations in germ cells are the cause of Li-Fraumeni Syndrome (LFS), which pre-disposes carriers to developing carcinomas, breast cancers, brain tumors, and adrenal corticol carcinomas.[13]

Other important tumor suppressor genes that have been identified are: phosphate and tensin homolog (PTEN), Wilms' tumor 1 (WT1), adenomatous polyposis coli (APC), Mad-related gene 2 (MADR2), breast cancer susceptibility gene 1 and 2 (BRCA1, BRCA2).[10]

1.3.1 Oncogene Development

Proto-oncogenes encode for proteins that assist with cell division, inhibit cell differentiation, and prevent cell death.[14] Oncogenes are dominant genes, meaning they only require one allele to be mutated for cancer to form, and they often are not associated with inherited cancers.[8] Genetic mutations that activate oncogenes can occur through point mutations, insertions, or deletions that lead to an overactive gene product or exist in the promoter region that can result in a variety of hazardous phenotypic changes.[8]

Many of the 40 known proto-oncogenes are transmembrane proteins that play a large role in signaling mechanisms of extracellular ligands.[14] These ligands can be growth factors that cause conformational changes to the membrane receptors, triggering an intracellular series of events that lead to cell proliferation, growth, or angiogenesis.[14] For

example, epidermal growth factor receptor (EGFR) is a tyrosine kinase receptor that is commonly over expressed in various carcinomas. EGFR can bind to several different types of ligands and dimerizes upon binding. This leads to the phosphorylation of tyrosine residues, which can trigger intracellular signaling pathways downstream of the receptor. Numerous EGFR pathways lead to cell proliferation, motility, and survival.[15] There are five different ways in which EGFR effects can be elevated, leading to a cancerous phenotype, as illustrated in Figure 1. First, there can be an increased production of ligands. Transforming growth factor alpha (TGF-α) and EGF are commonly co-expressed with EGFR in various types of cancer, causing uncontrolled EGFR activation and cancer development. Second, there can be an increased EGFR expression. Elevated levels of EGFR correlate with decreased survival of individuals with head and neck, ovarian, esophageal, and bladder cancer. Third, mutations can occur in the EGFR protein that makes it a more effective receptor. These can occur in the extracellular domain, intracellular domain, or the specific tyrosine kinase intracellular domain. Fourth, there can be defective downregulation of EGFR. Downregulation occurs when the protein is internalized and degraded by the lysosome. EGFR mutants evade this mechanism by avoiding c-CBL binding, an enzyme essential for downregulation. Fifth, EGFR cross-talk can occur, which is specific communication between EGFR and other receptors that influence signaling frequency. For example, the EGFR-ErbB2 heterodimer seems to be the strongest and most potent inducer of growth signaling when compared to other dimers.[15]

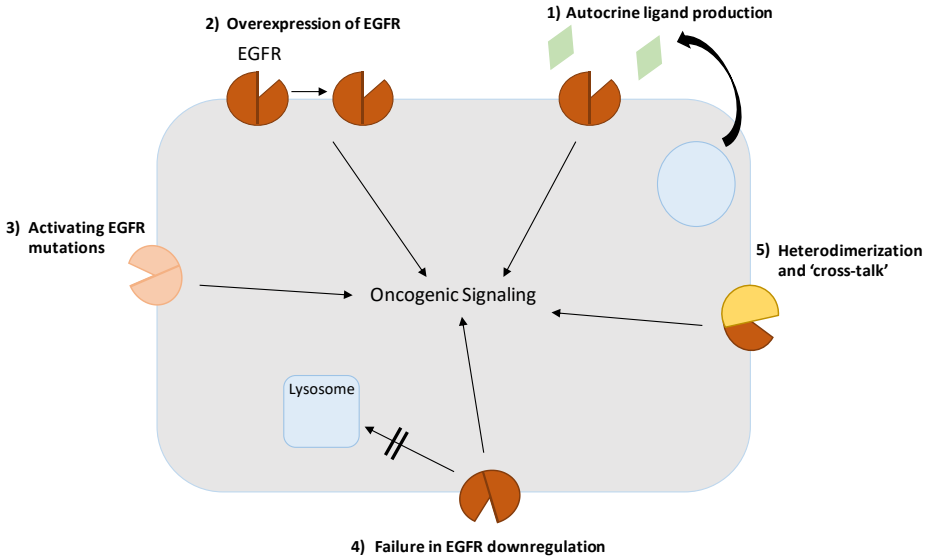

Figure 1: Five common mechanisms in which EGFR-related mutagens lead to malignancies. 1) An increased production of ligands can lead to increased activation. 2) An increase in EGFR expression can cause uncontrolled EGFR activation. 3) Certain mutations occurring in the EGFR protein can it a more effective receptor. 4) Defective downregulation of EGFR due to failure of internalization and degradation. 5) EGFR cross-talk can occur, which is specific communication between EGFR and other receptors that influence signaling frequency.[15]

Some proto-oncogenes produce intracellular proteins that are triggered by growth receptors, such as KRAS, which is signaled by the EGFR.[14] KRAS is a GTPase transducer protein that remains inactive until switched on by GTP binding, which is triggered by interacting with the Grb2 protein as a result of extracellular signaling via EGFR-ligand binding.[16] The GTP-activated KRAS is then able to transmit a signal to the nucleus, resulting in growth, migration, and cellular differentiation.[15,16] This proto-oncogene becomes an oncogene when the KRAS protein mutates in such a way that it does not hydrolyze GTP to GDP, therefore remaining in the active form.[16]

Another causation of proto-oncogene to transform into oncogenes is chromosomal translocation. For example, the Philadelphia chromosome, which results from chromosome 9 (carrying the ABL1 gene) and 22 (carrying the BCR gene) fusing together. The ABL1 gene provides the protein with high protein kinase activity, triggering numerous other cell cycle regulating proteins.[14] The Philadelphia chromosome is associated with numerous types of leukemia such as chronic myelogenous leukemia (CML).[13,17]

1.4 The Hallmarks of Cancer

In 2000 Hanahan and Weinberg published six hallmarks of cancer and added two more hallmarks as well as two enabling characteristics in 2011.[19,20] These characteristics can be seen as the driving force of malignancies.

Sustaining proliferative signaling is the first hallmark to review. Cancer cells can achieve self-sufficient growth signaling by three different mechanisms: releasing growth factors to stimulate own growth (autocrine) or for neighboring cells (paracrine), increasing cell surface receptors, or influencing downstream signaling pathways to be permanently activated.[8]

Evading growth suppressors is a hallmark of cancer that allows malignancies to become insensitive to signals that either push the cell into quiescence or signals that prevent the cell from ever returning to the cell cycle.[8] An example of this type of signaling is when transforming growth factor beta (TGF-β) binds to its receptor and triggers a signaling cascade of intercellular proteins (RB, CDK, and CDK inhibitors) that controls cell cycle.[8]

Resisting cell death allows cancer cells to grow uncontrollably and resist not only the organisms' apoptosis signaling but different types of anti-cancer treatments. In healthy cells when DNA is damaged the proliferation level of the cells is reduced in order for DNA to be repaired, but if it is not repaired, the cell undergoes apoptosis. Apoptosis can be triggered extrinsically and intrinsically, and cancer cells can either ignore external signals to cell death or increase intrinsic anti-apoptotic signaling in relation to pro-apoptotic signaling.[8]

Sustained angiogenesis allows for the growth of new blood vessels. Cancer cells require nutrients and oxygen and require higher levels of some molecules (e.g. glucose) due to increased growth rates and different metabolic actions, which can be delivered by new blood vessels.[21] Cancer cells force pro-angiogenesis signals to overcome anti-angiogenesis signals. For example, pro-angiogenesis signals such as vascular endothelial growth factor (VEGF) or anti-angiogenesis signals such as thrombospondin-1.[8]

Cellular immortalization exists in cancer cells to a degree due to increased levels of the telomerase enzyme. In normal cells, there is a finite number of cellular replications due to the shortening of telomeres.[8] Tumors with this higher telomerase enzyme expression have the ability to re-build shortening telomeres to ensure complete transcription of genes.[8]

Invasion and metastasis involve primary tumor cells overtaking and overwhelming the local surrounding healthy tissue and spreading to other areas in the organism. A concept that is essential for metastasis of carcinomas is epithelial-to-mesenchymal transition (EMT), a process in which epithelial cells gain migratory properties by detaching from the basement membrane. Patterns have been observed between primary tumor and metastasis colonies that allow certain tumor cells to selectively invade different tissues.[8]

Reprogramed energy metabolism reflects on the idea that cancer cells metabolize glucose in a much different way than normal cells. Nonmalignant cells utilize oxidative phosphorylation to generate the maximum amount of ATP per glucose molecule. Cancer cells, however, acquire ATP through aerobic glycolysis, a much less efficient way to process glucose which results in the production of lactate, even in the presence of oxygen.[8,22] There are several hypothesis for this, what is known as the Warburg effect, however, reasons for this route of glucose metabolism are still unknown.[23]

Evading immune destruction is the second emerging hallmark of cancer. The immune system has mechanisms to fight against malignant and pre-malignant cells. An immune surveillance mechanism is always searching for developing cancer cells and the most prominent evidence for this is the increased levels of certain cancers in immunocompromised patients.[24] Thus, malignant cells that are present and develop into tumors have somehow evaded immune destruction. Cancer gains the ability to avoid immune surveillance by a process referred to as “immunoediting”. Immunoediting occurs when the immune system can target and eliminate most of the cancerous cells with the exception of some mutated cells. These mutations may include gaining the ability to inactivate cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells by producing certain immunosuppressive factors or recruiting immunosuppressive inflammatory cells.[19,25] These mutated cells can then proliferate and form tumors.[19]

Two enabling characteristics have been determined in the most recent version of hallmarks of cancer: genome instability and inflammation. *Genome instability* refers to the idea that the genome of cancer cells become increasingly unstable during proliferation and continues to gain mutations that can lead to more advantageous phenotypes.[8,19] *Inflammation* utilizes immune system recruitment to reinforce tumor growth.[8,19]

1.5 Breast Cancer

On average, 72 Canadian women are diagnosed with breast cancer each day.[26] Breast cancer is the second leading cause of cancer-related death among women.[27] Breast cancer most commonly begins in the ducts or the lobes of the breast tissue, the most common type of breast cancer being ductal carcinomas. Lobular carcinoma are found in the lobes of the breast and is more frequently bilateral in comparison to other type of breast cancers.[28]

1.6 Risk Factors

There are several defined risk factors for developing breast cancer. This includes age, family link to breast cancer, endogenous and hormonal differences, obesity, and alcohol consumption. As age increases so does the risk of developing breast cancer. Fifty-one percent of breast cancer cases occur in women between the ages of 50 to 69.[29] Table 1 displays the probability of developing breast cancer in different age groups.[26] The rate of risk of developing hormone-dependent breast cancer decreases after menopause due to a drop hormone production.[27]

Table 1: Risk factors in developing breast cancer with age.[115]

Age	**10-year probability**	**or 1 in:**
20	0.1%	1,567
30	0.5%	220
40	1.5%	68
50	2.3%	43
60	3.4%	29
70	3.9%	25
Lifetime risk	12.4%	8

Having a higher number of first-degree family members with breast cancer increases the risk of development, as one may carry a genetic predisposition to certain oncogenes or tumor suppressor gene mutations.[30] Women with mutations in the BRCA1 and

BRCA2 gene have a significantly higher risk of developing breast cancer.[30] The BRCA1/2 genes are tumor suppressor genes that produce proteins that repair double-strand DNA breaks, and are the best known genes linked to breast cancer. Lifetime breast cancer risk for women with BRCA1 and BRCA2 mutations are 65% to 81%, and 45% to 85%, respectively.[30] Often individuals with a family history will be advised to seek genetic counseling to estimate potential risk.[27]

There are numerous endogenous and hormonal factors that alter breast cancer risk. For example, an early menarche puts women at a higher risk for developing breast cancer and later onset of menopause also increases risk. A two-year menarche delay reduces risk by 10% and a five-year menopause delay grants a 17%.[27,30] The increased number of cycles and exposure to hormones increases risk.[31] Other signs of hormone changes that may decrease risk are giving birth at a young age, giving birth at all, breast feeding, and decreased levels of circulating testosterone.[30]

There appears to be a relationship between hormone replacement therapy used in women for treatment of menopause symptoms as well as oral contraceptives and breast cancer incidence. These breast cancers are often hormone receptor positive. Patients that use hormone therapies in comparison to those who do not have a slightly higher risk of developing breast cancer when exposed over a five year period.[30,32]

Obesity plays a large role in the increased risk of postmenopausal breast cancer and is also associated with poor prognosis of early0staged breast cancer.[33] There are several biological pathways that link excess adipose tissue found in obese or overweight individuals to breast cancer formation.[34] For example, peripheral adipose tissue can convert androgens to estrogens, increasing estrogen levels which assists proliferation of hormone-dependent breast cancer.[34] Obesity is also linked to increase levels of insulin in the blood, which is linked to mitogenic, anti-apoptotic, and pro-angiogenic signaling, pushing cancer cells into active replication and tumor formation.[34]

When 10 grams of alcohol is consumed daily, there is a 7-10% increase risk for breast cancer in adult women.[35] There is an alcohol-induced increase of estradiol and estrone in the blood after consuming approximately 2.5 drinks daily.[35] Radiation is a mutagen and a carcinogen that can lead to genetic damages.[30] Radiation at a young age can play a large

role in cancer development, but after the age of 30, radiation exposure has a minimal effect.[27]

1.7 Classification of Breast Cancer

Classifying cancer is necessary to ensure that the patient gets the most appropriate and effective treatment for their disease type. There are numerous qualities that the patient and cancer cells can possess that needs to be evaluated properly to categorize the malignancy. This includes things like patient age, tumor size, hormone receptor profile, immunohistochemistry, lymph node size.[36] It is essential not only to look at individual qualities, but analyze all available information together, as it is common that patients with similar malignant phenotypic qualities can respond drastically different to the same treatments.[36]

One of the most well-known biomarkers for breast cancer are cell membrane hormone receptors. Commonly estrogen receptors (ER), progesterone receptors (PR), and human epidermal growth factor receptor 2 (HER2) and are detected using gene expression profiling, as cancer cells with positive hormone receptor profiles often respond positively to hormone therapy.[36–38] The presence or absence of these receptors allow for subtyping into one of the following groups: luminal A, luminal B, HER2, or basal-like. These subtypes are summarized in Table 2.[36] Ki67 index is a commonly used tool to categorize subtypes. Ki67 is strongly associated with tumor cell growth and proliferation, and the fraction of Ki67 positive tumor cells is referred to as the Ki-labelling index.[39] A high Ki67 index is associated with high proliferation rates and is correlated to abnormal p53 expression, large tumors, development at a young age, and negative ER/PR expression.[38,40,41]

Table 2: Breast cancer molecular subtypes review.[36]

Molecular subtype	Biomarker profile	Qualities
Luminal A	ER+ and/or PR+, HER2-, low Ki67 index	Approximately 70% of invasive breast cancers, good prognosis, luminal B has higher histological grade and proliferation rates than luminal A and may overexpress HER2, sensitive to endocrine
Luminal B	ER+ and/or PR+, HER2+; ER+ and/or PR+, HER2-, high Ki67 index	

		therapies, variable chemotherapy response
HER2+	ER-, PR-, HER2+	Approximately 15% of invasive breast cancers, higher grade and lymph node positive, more aggressive than luminal, poor prognosis, responds to trastuzumab and anthracycline-based chemotherapy.
Basal-like	ER-, PR-, HER2-, and CK5/6, CK14, CK17, laminin EGFR	Approximately 15% of invasive breast cancer, referred to as 'triple negative breast cancer, related to BRCA1 mutation, high levels found in premenopausal African American women, does not respond to endocrine treatment or trastuzumab, sensitivity to platinum-based chemotherapy.

Luminal A is the most common breast cancer subtype, consisting of 50 – 60% of all breast cancers. These cancers tend to have a good prognosis with high survival rates, low recurrence rates, and treatment is often hormone-based therapy. Luminal A cancer cells are ER+, occasionally express PR, and have low expression of proliferative genes as evaluated by Ki67 expression.[38]

Luminal B is more aggressive then luminal A, with a higher Ki67 index, higher recurrence rate, lower survival rates, and a worse prognosis. [38,41] The main differences between luminal A and luminal B subtypes is the expression of proliferation genes, where luminal B highly expresses genes such as cyclin E1 (CCNE1), nuclease sensitive element binding protein 1 (NSEP1), or avian myeloblastosis viral oncogene homolog (v-MYB).[38]

The HER2 receptor is a proto-oncogene and is in the family of four-membrane tyrosine kinases.[38] Having a HER2+ cancer is indicative of a more aggressive form of cancer as these cancers have a high Ki67 index, high histological, and nuclear grades. Some HER2+ cancers are also ER+ but often are lowly expressed. HER2+ cancers have an increased resistance to endocrine therapy and a greater likelihood of lymph node involvement.[42]

The basal-like carcinomas are considered to have the worst prognosis among these four listed subcategories.[43] Premenopausal African American women are more likely to develop this form of breast cancer.[44] Basal-like carcinomas do not express any of the hormone receptors mentioned. Therefore, they are ER-, PR-, HER2- and referred to as triple negative. They do have high levels of myoepithelial markers, CK5/6, CK14, CK17, and laminin, leading to the name basal-like breast cancer.[38] Higher than normal levels of these markers suggest excessive cellular growth and stress.[25] Basal-like breast cancers also are linked to the BRCA1 gene mutation which further links this subtype to TP53, EGFR, and P cadherin abnormalities.[38]

It is essential to understand which category a malignancy falls into, in order to apply the most effective treatment. For example, BRCA1 gene mutated cells lack double-strand DNA break repair mechanisms, which may increase a basal-like breast cancer's sensitivity to drugs that target double strand DNA.[38] HER2+ breast cancer also has a higher sensitivity to certain cytotoxic treatments such as doxorubicin, possibly due to the higher co-expression of topoisomerase-2, the respective target.[38]

The different stages of breast cancer evaluate how far the disease has progressed and is evaluated by tumor size, lymph node involvement, and presence of metastasis.[45] There are five stages of breast cancer: stage 0, I, II, III, IV.[46] These stages and the most common treatments are described in Figure 2.[45,46]

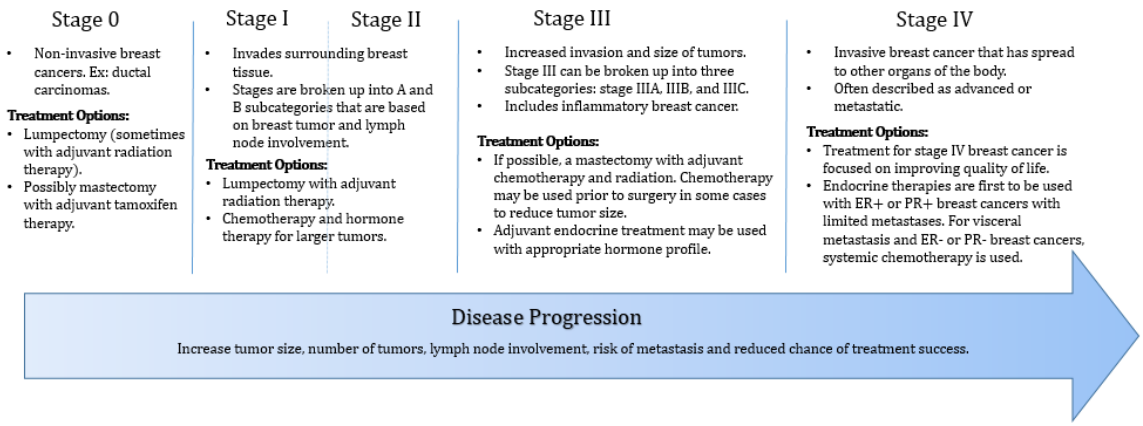

Figure 2: Breast cancer stages 0, I, II, III, IV characteristics and common treatment methods.[45,46]

1.8 Breast Cancer Treatments

The treatment options for breast cancer depends on patient characteristics including the subtypes and stages, certain genomic markers, patients age, health, menopausal status, and presence of mutagens.[47] Not only does different stages of cancer lead to different treatment plans, but also different goals for treatment are set. For example, the intention of treating early stages of cancer is eliminating the cancer and preventing metastasis, but for late stage metastatic cancer the intention of treatment is to improve the quality of life.[45] Generally there are five categories of breast cancer treatment: surgery, radiation, chemotherapy, hormonal therapy, and targeted therapy.

1.8.1 Surgery

Surgery is removal of the tumor from the breast as well as investigating axillary lymph nodes for cancer. A lumpectomy is when the tumor as well as a small amount of surrounding healthy tissue is removed, leaving most of the breast intact. Lumpectomies are often used in combination with radiation when cancer is invasive, and is sometimes referred to as breast-conserving surgery.[47] Mastectomy is the removal of the entire breast.[47] Lumpectomy with adjuvant radiation has equivalent survival rates to a mastectomy, so when the surgery can be achieved with accuracy, it is recommended.[48]

1.8.2 Radiation therapy

Radiation is the use of high energy x-rays to kill cancer cells.[47] External beam radiation therapy is when radiation is applied from outside the body. Intra-operative radiation involves the use of a probe in operation and applying radioactive sources inside the tumor is referred to as brachytherapy.[47] Radiation may be given after surgery or before surgery to shrink the tumor. Recurrence is reduced when radiation is used in combination with surgery and/or chemotherapy.[47]

1.8.3 Chemotherapy

Chemotherapy is the use of anti-cancer drugs that cause cell death, often by damaging DNA and preventing replication.[47] Platinum-based drugs such as cisplatin and

carboplatin are used in many different cancers including breast cancer. These compounds often induce genomic instability by forming a DNA-platinum adduct.[47]

Taxanes have been considered fundamental for the treatment of breast cancer. Two of the most common taxanes, paclitaxel and docetaxel, bind to tubulin, preventing stabilization of microtubules inducing cell cycle arrest.[49] They do, however, have slightly different effects in that docetaxel has more potent anti-cancer activity and higher levels of apoptosis. Taxanes are subject to multi-drug resistance due to efflux via ABC transporters, drastically changing efficacy of the treatment.[49,50]

Doxorubicin and epirubicin fall into the anthracycline category of breast cancer chemotherapies. Doxorubicin intercalates DNA, inhibits topoisomerase-II DNA repair as well as generates free radicals resulting in DNA and protein damage in cancer cells.[51] A major downfall of doxorubicin as a chemotherapy treatment is cardiotoxicity. There are numerous mechanisms in which doxorubicin is linked to cardiotoxicity, one of which is oxidative stress. Doxorubicin contains a quinone, which causes redox reactions increasing levels of ROS and lipid peroxidation in cardiomyocytes. Doxorubicin treatment can also alter gene expression of contractile proteins such as α-actin, myosin, and troponin-I. Decreased expression is associated with myofibrillar loss and reduced contractile function. DNA damage and apoptosis by inhibition of topoisomerase IIβ further contribute to cardiomyopathy.[52,53] The formation of a doxorubicin metabolite interferes with iron and calcium regulations of the proton pump of the mitochondria, which causes death of cardiomyocytes.[51] Another limiting problem of doxorubicin is resistance development. Increased ABC transporter expression causes doxorubicin to be pumped out of the cell, and reduce the treatment efficacy.[51]

1.8.4 Hormone Therapy

Hormone therapy is the first line of defense for most ER+ breast cancers. Tumors that are hormone receptor positive use estrogen and progesterone to stimulate proliferation. There are numerous different hormone therapy approaches that decreases the uptake of estrogen and progesterone into the cell.[54]

Selective estrogen receptor modulators (SERMs) serve as anti-estrogens and competitively bind to ERs. The binding of SERMs to the ligand binding domain causes a

conformational change that prevents binding of co-factors and blocks trans-activation of the receptors.[54] The most common and successful SERM is tamoxifen, a non-steroidal mixed antagonist-agonist of the ER that reduces the risk of recurrence and is used for the treatment and prevention of breast cancer.[45,47,54]

Aromatase inhibitors (AIs) work by reducing estrogen produced throughout the body. When women reach menopause, androgens are converted to estrogens by the aromatase enzyme in order to compensate for the dip in hormone production from the ovaries. Use of aromatase inhibitors block this conversion, starving the cells of hormone uptake.[47,54] This group of drugs, therefore, are commonly used in post-menopausal women and are generally tolerated better than SERMs. Third generation AIs include anastrozole, letozole, and exemstane.[54]

1.8.5 Targeted Therapy

Targeting the inhibition or knockdown of tumor dependent pathways can be effective breast cancer treatment.[55] Targeting specific molecules or proteins involved in promoting malignant phenotypes has led to the discovery of numerous beneficial treatments.

Approximately 15% of cancers over express the HER-2 protein.[48] Trastuzumab is a monoclonal antibody that binds to the extracellular domain of the HER-2 receptor, blocking intracellular signaling.[48,55] It is prescribed for the use against tumors that are non-metastatic as well as HER-2 positive and often is used in combination with chemotherapies.[47] There are concerns with cardiotoxicity using trastuzumab, but it appears to be treatable and reversible, however it should not be used in combination with other drugs that induce cardiotoxicity such as doxorubicin.[55]

Targeting different receptors in the receptor tyrosine kinase family is popular. Cetuximab is an EGFR inhibitor that competitively binds with the extracellular domain of the receptor and prevents activation, preventing signal transduction. Cetuximab in combination with cisplatin has shown evidence to reduce risk of disease progression and increase progression-free survival.[55] Gefitinib reversibly inhibits to EGFR tyrosine kinase phosphorylation, which reduces downstream signaling. Alone, gefitinib has slight benefits in advanced tumors, but responses are positive for the use of gefitinib in

combination with certain chemotherapeutic agents like doxorubicin, tamoxifen and hormone therapies.[55]

1.9 Multi-Drug Resistant Breast Cancer

Chemotherapy drugs that can effectively kill cancer cells, unfortunately also destroy healthy tissue. Therefore, it is essential to remain within the therapeutic index of the drug. This can be challenging in cases of multi-drug resistance (MDR), inherent or acquired, as cells can become desensitized to different structurally and mechanistically unrelated drugs. There are many different mechanisms that can induce MDR, and six will be discussed: increased expression of drug efflux transporters, decreased expression of drug uptake transporters, increased drug metabolism, activation of DNA repair, inhibition of apoptosis, and alteration of drug target.[56]

1.10 Multi-drug Resistance Mechanisms

1.10.1 Increased Drug Efflux Transporter Expression

Increased drug efflux from the cell can be attributed to increased expression of ABC transporters, a large family of transporters that hydrolyze ATP to pump substrates out of the cell.[57] A high expression of these transporters is correlated with a general poor prognosis.[58] The first transporter in this category to be identified was P-glycoprotein (also known as ABCB1)), which transports both taxanes and anthracyclines and is encoded by the MDR1 gene.[58]

1.10.2 Decreased Drug Uptake Transporter Expression

Transporters in the solute carrier (SLC) superfamily have been shown to uptake numerous chemotherapy drugs such as gemcitabine, nucleoside analogs, paclitaxel, and some platinum drugs.[56] Thus by reducing the drug uptake mechanisms cancer cells can reduce intracellular drug concentrations resulting in decreased drug efficacy. For example, the effect of a common chemotherapeutic, methotrexate, is reduced with decreased expression of reduced-folate carrier (SLC19A1). Expressing human SLC19A1 in transporter-deficient Chinese hamster ovary cells led to restoration of methotrexate

transport and efficacy.[59] Also, children with acute lymphoblastic leukemia with an SLC19A1 mutation had higher methotrexate plasma levels than other genotypes.[60] Cells that develop this resistance may be the result of mutations to the transporter gene that alters binding of the substrate drug or expression levels of the transporter.[61]

1.10.3 Increased Drug Metabolism and Detoxification

Phase I microsomal cytochrome P450 (CYP) enzymes metabolize exogenous substrates including chemotherapeutics. CYPs are most highly expressed in the liver but are also present in extrahepatic tissues malignant tissues. Since the metabolic action of CYPs most commonly leads to drug detoxification, genetic or environmental factors that increase the rate or efficiency of metabolizing enzymes can increase the deactivation of chemotherapies leading to drug resistance. However, in the case of pro-drugs this could lead to increased concentrations of the active metabolite.[56] Drugs can be inactivated through phase II metabolism for example, through conjugation to glutathione. Thus increased production of glutathione appears to be an existing mechanism of action for drug resistance in numerous types of cancer.[62]

1.10.4 Activation of DNA Repair Mechanisms

Causing DNA breaks, whether it is through forming direct DNA adducts or inhibiting DNA repair mechanisms, is a common target for chemotherapeutic drugs. Resistance, however, can arise when DNA is repaired regardless or the cell becomes tolerant to the damages through bypassing the lesions. Enhanced DNA repair mechanisms have been considered as major mechanisms of drug resistance for cisplatin, melphalin, and other various alkylating agents.[62] For example, in malignant glioma there is resistance to alkylating agents due to DNA repair by O(6)-methylguanine methyltransferase (MGMT).[56] The presence of genomic mutations that alter DNA repair mechanisms provide cancer cells the capability to overcome DNA breaks. Specialized mutagenic DNA polymerases play a role in DNA repair and bypassing lesions through what is referred to as translesion synthesis.[62]

1.10.5 Apoptosis Inhibition

Evading apoptosis is a hallmark of cancer, which remains a barrier for many chemotherapies. The apoptotic cascade is triggered by the release of cytochrome c from the mitochondria or activation of tumor necrosis factor in response to ligand binding. This leads to caspases that initiate the morphological and biochemical changes of apoptosis.[56] The bcl-2 genes are involved in the homeostasis of pro- and anti-apoptotic factors, so mutations in this area of the genome can prevent treatment success.[63] Over expression of anti-apoptotic signals may overtake pro-apoptotic signals triggered by treatment. P53 plays a large role in cell cycle control and apoptosis, and mutations correlate to drug resistance in malignancy cells.[62]

1.10.6 Alteration of Drug Target

Many chemotherapeutic drugs work by binding to essential enzymes, blocking or promoting activity. Changes in the quantity or shape of a target can influence the efficacy of a drug and can result in resistance. Mutations that influence sensitivity to drugs have been seen in enzymes such as dihydrofolate reductase, thymidylate synthase, and topoisomerase I and II.[64] For example, resistance to the BCR-ABL kinase inhibitor imatinib mesylate arises from mutations in the BCR-ABL kinase.[64]

The discovery of drugs that are not subject to multi-drug resistance continues to intrigue researchers. The five-year survival rate for women with metastatic cancer is 26%, so further treatment methods, especially those for late-stage breast cancers, continue to be in high demand.[65]

1.11 Jadomycins

Numerous natural products are produced by soil bacteria, and many of these have been developed into clinically used therapeutics. The soil bacterium, Streptomyces venezuelae ISP5230, under stress conditions using either heat, phage, or ethanol shock is responsible for the biosynthesis of the jadomycins. [66–68] Jadomycins are characterized as members of the angucycline family, a group of natural products that have a polyaromatic backbone biosynthesized by a type II polyketide synthase. The gene cluster responsible for the biosynthesis of the jadomycins has been identified in S. venezuelae ISP5230 and the

function of many of the gene products has been determined through homology modelling or gene deletion. Of note, and which is unique to the jadomycin biosynthetic gene cluster, is that the biosynthesis contains a non-enzymatic step that incorporates amino acids present in the minimal media used for secondary metabolite production, directly into the jadomycin skeleton. This provides a route for numerous different jadomycin derivatives to be developed (Figure 3).[69] The non-enzymatic step in jadomycin biosynthesis often results in an oxazolone ring this is formed by a reaction between a biosynthetic aldehyde precursor and the amino acid present in media. This results in the side chain of the amino acid becoming a side chain on the oxazolone ring. Based on the properties of the amino acid used, different cyclization processes will occur, resulting in a different ring structure. For example, use of L-serine results in a jadomycin lacking an oxazolone ring, and instead produces a differentially structured five membered ring with a carboxylic acid side chain. This is a result of the cyclization by the primary alcohol present in L-serine, rather than the carboxylic acid.[66,70]

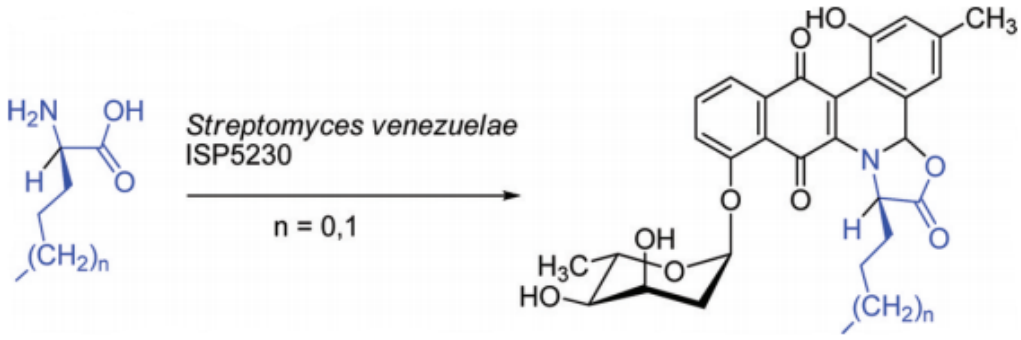

Figure 3: Incorporation of amino acids into jadomycins through a non-enzymatic biosynthetic pathway in *S. venezuela*e.[82]

Synthesis of jadomycins begins with the formation of the polyketide core. Genes jadA, jadB, and jadC code for enzymes that contribute to the polyketide synthetase core. This enzyme initiates building the polyketide structure using acetate and nine malonate molecules.[71,72] The polyketide structure is modified with jadD, jadE, and jadI enzymes which assist ring catalyzation.[72] The oxidative opening of the 5,6-bond of the angucyclinone intermediate is completed with oxygenases encoded by jadF, jadG, and jadH.[73–75] This reaction leads to amino acid incorporation, that is likely a non-enzymatic

step.[76,77] Finally, the L-digitoxose residue appears to be attached by a glycosyltransferase which is encoded by the jadS enzyme.[78] Structures of jadomycins are shown in Figure 4.

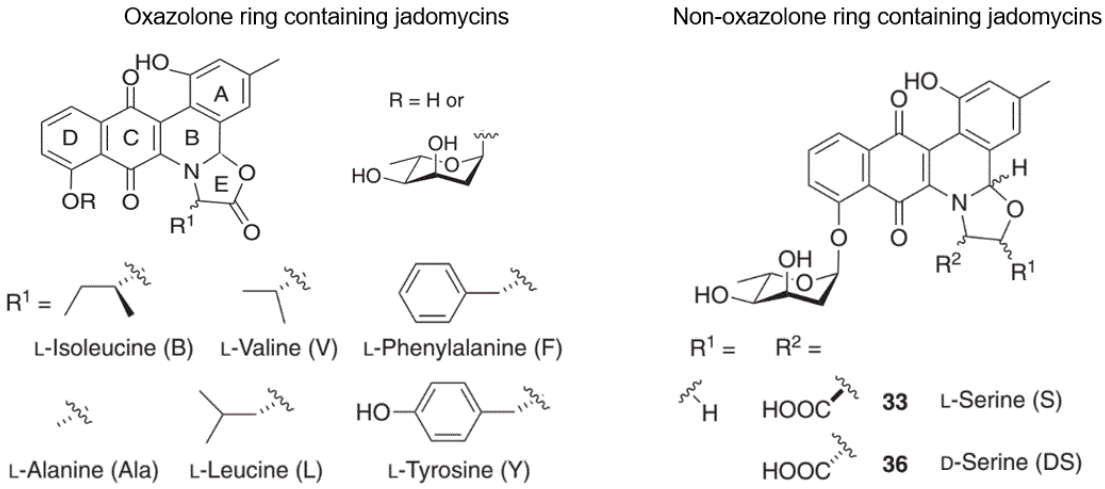

Figure 4: Chemical structures of a library of jadomycins, differing by the R group.[70]

Jadomycins have been shown to have antimicrobial and anticancer activity. Structure-activity relationships have identified that anticancer effects of jadomycins depends on the specific side chain as well as the cell line tested.[79]

1.12 Cytotoxicity of Jadomycins

In 2008, Jakeman *et al* evaluated the microbial activities of jadomycin B and derivatives against *S. aureus, S. epidermidis, Pseudomonas aeruginosa, Enterococcus faecalis,* and *Bacillus subtilis*. It was found that jadomycin G and N were least effective amongst the jadomycins tested. There are unique structural differences in these two compounds compared to the others, in that jadomycin G is lacking an oxazolone-binded substituent and jadomycin N is lacking the oxazolone ring completely. When evaluating the MRSA strain, jadomycin B, L, and F were most active. Jadomycin B and L both have aliphatic side chains where F has a phenyl ring substituent.[76]

Further evaluations were conducted with jadomycins in different cancer cell lines. In T-47D and MDA-MB-435 breast cancer cells jadomycin structure-activity relationships were different than in antimicrobial studies. It was found that jadomycin S was most active out of all 19 studied. Overall jadomycins with small polar or alkyl side chains were

most active, aromatic side chains were least active, and the stereochemistry of the α-carbons did not significantly change jadomycin activity.[76,80] Furthermore, it was identified that jadomycins were more effective in rapidly proliferating cancer cells. This could indicate that jadomycins have a level of selection against healthy cells, as cancer cells have higher growth rates than healthy cells.

Zheng *et al* investigated 6 different jadomycins against four different cancer cell lines: HepG2 (human hepatocellular carcinoma), IM-9 (human lymphoblast cell line derived from multiple myeloma), IM-9/Bcl-2 (human lymphoblast cell line derived from multiple myeloma with Bcl-2 overexpression), and H460 (human non-small-cell lung cancer). Jadomycin S was most potent against all excluding H460, where jadomycin F was the most potent in this cell line. This study reported that all jadomycins tested are cytotoxic against cancer cells.[81]

Two different jadomycins with norvaline and norleucine incorporated into the oxazolone ring were tested by the NCI against 60 different cancer cell lines. These cell lines included leukemia, lunch carcinoma, colon, central nervous system, melanoma, ovarian, renal, prostate, and breast cancer cells. Both derivatives inhibited the growth of the majority of cell lines tested and the median GI_{50} (concentration where growth inhibition is half the maximum value) and TGI (the concentration of drug that causes total growth inhibition) were in the low micromolar range.[82] Nine jadomycin triazole derivatives were also tested in the same 60 cancer cell lines by NCI. The low cytotoxicity of non-triazole containing derivatives suggested the importance of triazole moiety for activity. All but one of the compounds inhibited the growth of most of the cell lines. The median GI_{50} and TGI were in the low micromolar range.[79]

Jadomycins maintain their cytotoxicity in cells that are MDR and over express ABC transporters. Issa *et al* showed that jadomycins do not lose their cytotoxicity in taxol (MCF7-TXL), etoposide (MCF7-ETP), and mitoxantrone (MCF7-MITX) resistant MCF7 cells, which overexpress ABCB1, ABCC1, and ABCG2 respectively, and in triple negative MDA-MB-231 that overexpress ABCB1 transporters. The jadomycin potency in MDR cancer cells is only slightly lower than potency relative to the MCF7-CON cells. In comparison the reduction in the potency of known ABC transporter substrates docetaxel, etoposide, and mitoxantrone when comparing the MDR and control breast cancer cells.

IC_{50} values displaying this concept is shown in Table 3.[66–68] These results suggest that jadomycins may have potential as clinical agents against MDR cancer cells. It is essential that jadomycin activity be explored further due to the limited treatments available for MDR breast cancer.

Table 3: IC_{50} values of cytotoxic drugs against control and ABC transporter overexpressing cells as evaluated by cell viability tests in MCF7 cells. This data represents an excerpt of previously published data.[66]

	Drug Sensitive		**Drug Resistant**	
Control Drugs	**MCF7-CON**	**MCF7-TXL**	**MCF7-ETP**	**MCF7-MITX**
Docetaxel	4.0 ± 1.4	83.5 ± 23.1(20.4)*	-	-
Etoposide	41.8 ± 12.2	-	156 ± 17.2(3.7)*	-
Mitoxantrone	1.6 ± 0.5	-	-	117.4 ± 16.6(69.9)*
Jadomycins				
Jadomycin B	4.4 ± 0.8	5.8 ± 0.6(1.3)	156 ± 17.2(1.8)	9.9 ± 2.6(2.2)
Jadomycin S	1.9 ± 0.8	3.6 ± 1.0(1.8)	3.6 ± 0.7(1.8)	5.2 ± 0.8(2.6)
Jadomycin F	0.9 ± 0.3	3.1 ± 0.3(3.2)*	7.2 ± 0.5(3.3)*	7.6 ± 0.3(3.7)*

* data points are significantly different than the corresponding control cells.

Table 4: IC_{50} values of cytotoxic drugs against control and ABC transporter overexpressing cells as evaluated by cell viability tests in 231 cells. This data represents an excerpt of previously published data.[68]

Control Drugs	**231-CON**	**231-TXL**
Docetaxel	0.6 ± 0.2	4.6 ± 1.1*
Mitoxantrone	0.3 ± 0.1	2.1 ± 0.6*
Jadomycins		
Jadomycin B	2.8 ± 0.5	2.5 ± 0.4
Jadomycin S	2.6 ± 0.7	2.8 ± 0.2
Jadomycin F	3.0 ± 1.0	3.2 ± 0.3

* data points are significantly different than
the corresponding control cells.

1.13 Mechanisms of Jadomcyins Anticancer Activity

In 2015, Hall *et al* evaluated the effects of jadomycin B, S, SPh, and F on reactive oxygen species (ROS) production, which can induce DNA breaks, reducing cellular proliferation.[67] Intracellular ROS levels were increased when MCF7 cells were treated with jadomycin. The antioxidant N-acetyl cysteine (NAC) blocked jadomycin-generated ROS, which lead to increased cell viability and decreased potency.[67] ROS production appears to be driven by the presence of copper. Jadomycins provide the electrons to reduce of Cu(II) to Cu(I), which leads to production of superoxide, hydroxyl radicals, and H_2O_2.[83] A copper chelating agent D-Pen decreased jadomycin cytotoxicity and copper donor $CuSO_4$ increased cytotoxicity, supporting copper-dependent ROS production.[67] Inhibition of cytosolic SOD1, an enzyme that converts superoxide to H_2O_2, led to increased jadomycin cytotoxicity whereas blocking mitochondrial SOD2 had no impact on jadomycin cytotoxicity, suggesting that jadomycin induces superoxide production in the cytosol as opposed to the mitochondria.[67] Furthermore, when jadomycin-induced ROS was inhibited, treatment retained 100% efficacy yet with lower potency. This suggested the existence of ROS-independent mechanisms. [67,68]

ROS-independent jadomycin mechanisms were supported when investigating jadomycins' effect on double-stranded (DS) DNA damage and apoptosis while altering ROS activity. Jadomycins caused DS DNA damage as it induced γH2AX (phosphorylated histone H2AX), a marker for DS DNA damage. Jadomycins also induced apoptosis that was measured by propidium iodide (PI) and Annexin V staining. These effects were not blocked or enhanced by ROS neutralization (n-acetyl cystine (NAC)) or induction (auranofin), suggesting that jadomycin-induced DS DNA damage and apoptosis act independent of ROS.[68,84,85]

Aurora B kinase is a protein that assists in cellular replication by facilitating in the attachment of the mitotic spindle to the centromere, inhibiting this protein has shown potential as chemotherapeutic drugs.[86,87] In 2008 Fu *et al* suggested that jadomycin B had properties to be an effective aurora B kinase inhibitor.[86] It was discovered that jadomycin

B inhibited the growth of IPl1-321, an aurora B kinase inhibitor sensitive yeast. In *in vitro* enzyme assays jadomycin B dose-dependently inhibited aurora B kinase and prevented the phosphorylation of histone H3, a downstream target of aurora B kinase.[67,86] It was also found that levels of histone H3 phosphorylation inhibition was maintained when ROS activity was inhibited with NAC, suggesting that inhibition of aurora B kinase is ROS independent.[88] It is also known, however, that DNA damage leads to aurora B kinase inhibition.[89] This suggests that aurora B kinase may be a secondary indirect effect caused by the direct DNA damage initiated by jadomycins. Consistent with this we have demonstrated in unpublished works that jadomycins B,S, and F are very low potency inhibitors of aurora B kinase in *in vitro* kinase assays.

In 2015 Martinez-Farina *et al* used WaterLOGSY NMR spectroscopy to show that jadomycin DS binds to topoisomerase IIβ.[90] Topoisomerase II control the topology of DNA strands, as it winds and unwinds strands during replication, allowing for enzymes such as polymerase to have access to nucleic bases.[91] This provided evidence that jadomycins could potentially act as topoisomerase inhibitors as an additional mechanism of action. Jadomycins have been shown to reduce the expression of TOP2A and TOP2B genes that encode for topoisomerases IIα and IIβ and lower topoisomerase IIβ protein levels. Jadomycin B and F specifically poisoned topoisomerase IIβ as opposed to jadomycin S, which had no effect on type II topoisomerase.[68] While the mechanisms of jadomycin cytotoxicity have not been resolved completed, there is ample evidence to support intracellular targets (Figure 5).

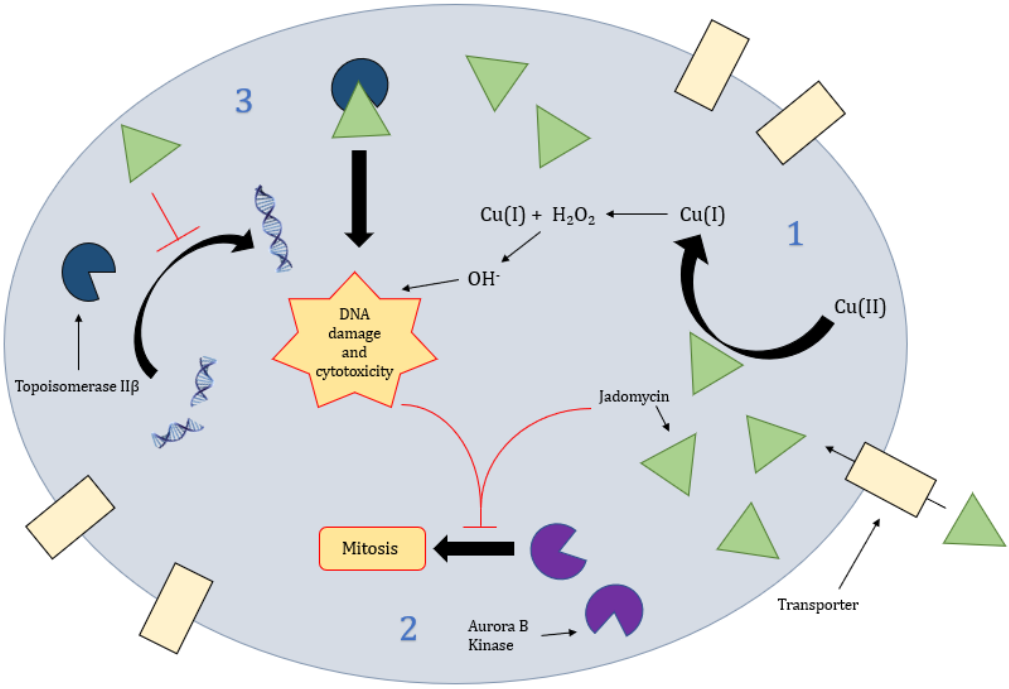

Figure 5: Overview of the proposed mechanisms of action of jadomycins. 1) Production of ROS. 2) Inhibition of aurora B kinase. 3) Inhibition of topoisomerase IIβ.[67,68,88]

1.14 Solute Carrier Transporters and Drug Uptake

The solute carrier organic anion superfamily (SLCO) is a group of plasma membrane transporters that assists with the uptake of substrates into the cell and are within the solute carrier (SLC) transporters family, The influx of essential substances such as sugars, peptides, amino acids via SLC transporters suggest that they could be a potential target to facilitate drug uptake.[92]

The solute carrier organic anion family has eleven members that transport large and fairly hydrophobic organic anions.[93] Table 5 displays tissue distribution and substrate specificity of the different SLCO transporters. Substrates of SLCO transporters are often amphipathic, organic, have a molecular weight greater than 300 Da, and can be anions or carry a neutral charge.[94] This suggests that jadomycins could possibly be substrates for these transporters.

Table 5: SLCO transporter expression, function, and substrate specificity.[93,116,117]

SLCO Transporter	Tissue expression	Substrates
SLCO1A2	Expressed in the brain, liver, lung, kidney, and testes.	Bilirubin, bromosulphophthalein, estradiol-17β-glucuronide, estrone-3-sulphate, imatinihb, lopinavir.
SLCO1B1	Liver.	Atorvastatin, bilirubin, bromosulphophthalein, fluorescein, dehydroepiandrosterone-3-sulfate, estradiol-17β-glucuronide, estrone-3-sulphate.
SLCO1B3	Liver (Central vein).	Benzylpenicillin, bilirubin, dehydroepiandrosterone-3-sulfate, estradiol-17β-glucuronide, estrone-3-sulphate, docetaxel, paclitaxel, methotrexate.
SLCO1C1	Brain and testes.	Dehydroepiandrosterone-3-sulfate, estradiol-17β-glucuronide, estrone-3-sulphate, thyroxine (T4), triiodothyronine (T3).
SLCO2A1	Brain, colon, heart, kidney, liver, lung, ovary, pancreas, placenta, prostate, skeletal muscle, spleen and small intestine.	Latanoprost acid, PGH_2, PGE_1, PGE_2, $PGF_{2\alpha}$, thromboxane B_2.
SLCO2B1	Intestines, placenta, keratinocytes, breast, heart, skeletal muscle, brain, and highest in the liver.	Atorvastatn. benzylpenicillin, dehydroepiandrosterone-3-sulfate, estrone-3-sulphate.

SLCO3A1	Lung, spleen, leukocytes, thyroid, with highest expression in the testes, brain, and heart.	Benzylpenicillin, estrone-3-sulphate, PGE_1, PGE_2, $PGF_{2\alpha}$, thyroxine (T4),
SLCO4A1	Lung, liver, skeletal muscle, kidney, pancreas, with highest expression in the heart and placenta.	Benzylpenicillin, estradiol-17β-glucuronide, estrone-3-sulphate, thyroxine (T4), triiodothyronine (T3), reveser triiodothyronine (rT3).
SLCO4C1	Kidney	cAMP, digoxin, estrone-3-sulphate, thyroxine (T4), triiodothyronine (T3).
SLCO5A1	Fetal brain, prostate, skeletal muscle, and thymus.	Straplatin, known substrates are limited.
SLCO6A1	Testes, with low expression in the spleen, brain, fetal brain, and placenta.	Dehydroepiandrosterone-3-sulfate, thyroxine (T4), triiodothyronine (T3), taurocholate.

SLCO transporters are beginning to be explored as therapeutic targets for cancer therapy. SLCO transporter expression is altered due to modified nutrient requirements, and this overexpression can be utilized to uptake chemotherapeutics. *SLCO1B1* has high expression in lung and colon human carcinomas and is also known to take up chemotherapeutics flavopiridol, methotrexate, and atrasentan.[92] *SLCO1B3* also is upregulated in prostate, gastric, colorectal, pancreatic, and breast cancers and is responsible for the uptake of anticancer drugs like taxel derivatives, imatinib, SN-38, and methotrexate.[92]

SLCO transporters may play a large role in the progression of hormone receptor positive breast carcinomas. Steroid sulfatases (STS) catalyzes the hydrolysis of steroid sulfates to a biologically active form, for example, activating estrone-3-sulfate to estrogen. It was shown that increased intracellular estrone-3-sulfate levels assists with

cancer cell proliferation and survival. Inhibiting *SLCO4A1* and *SLCO3A1* reduced estrone-3-sulfate levels, suppressing cellular proliferation.[95,96] *SLCO3A1* and *SLCO4A1* are both highly expressed in ER+ MCF7 and T-47D breast cancer cells, as they assist the uptake of hormone precursors such as estrone-3-sulfate.[95–97] As seen in Table 5, numerous SLCO transporters mediate the uptake of this estrogen precursor, linking expression of these transporter to cell survival.[92] *SLCO2A1*, *SLCO5A1*, and *SLCO4C1* have higher mRNA levels in MCF7 cells when compared to non-transformed breast epithelial MCF-10A cells.[97] It is plausible that drugs that are substrates of these transporters will have a level of selectivity for cancer cells over healthy cells or could reduce hormonal activation of cell growth and proliferation by competitively inhibit the uptake of estrogen precursors.

The function, expression, and substrate specificity of SLCO transporters suggest a possible uptake mechanism for jadomycins into the cell and require exploration.

1.15 Objectives and Project Overview

It was hypothesized that jadomycins are taken up into the cell through SLCO transporters and uptake by SLCOs will influence jadomycin cytotoxicity. This study involved two objectives. The first objective was to compare the mRNA and protein expression of 11 SLCO transporters (*SLCO1A2, 1B1, 1B3, 1C1, 2A1, 2B1, 3A1, 4A1, 4C1, 5A1,* and *6A1)* in a panel of breast cancer cells. For objective one there were two sub aims. The first was to determine if the gene expression of SLCO transporters were influenced by the drug-resistance status of MCF7 cells. To assess this question SLCO transporter expression was compared in drug-sensitive MCF7-CON versus drug-resistant MCF7-TXL, MCF7-MITX, MCF7-ETP that overexpress ABCB1, ABCG2, and ABCC1, respectively. The second aim was to determine if the gene expression of SLCO transporter were influenced by the hormone receptor status of breast cancer cells. For this sub-aim SLCO transporter expression was compared in triple negative MBA-MD-231; ER+, PR+, HER2+ BT474; ER-, PR-, HER2+ SKBR3; and breast epithelial MCF-10A cells. The second objective was to determine how the loss of function of SLCO transporters influences the accumulation and cytotoxicity of jadomycins. The most highly expressed transporters were selected for gene knockdown using commercially available

lentivirus shRNA vectors for evaluation of objective 2. Following knockdown of transporters, the cells were treated with jadomycins to evaluate how the reduction in transporter expression influences jadomycin cytotoxicity. Theoretically, when a knockdown occurs, jadomycin cytotoxicity (measured using cell viability assays) should be reduced, providing evidence that jadomycin uptake is facilitated by the corresponding transporter. This process can be seen in Figure 6.

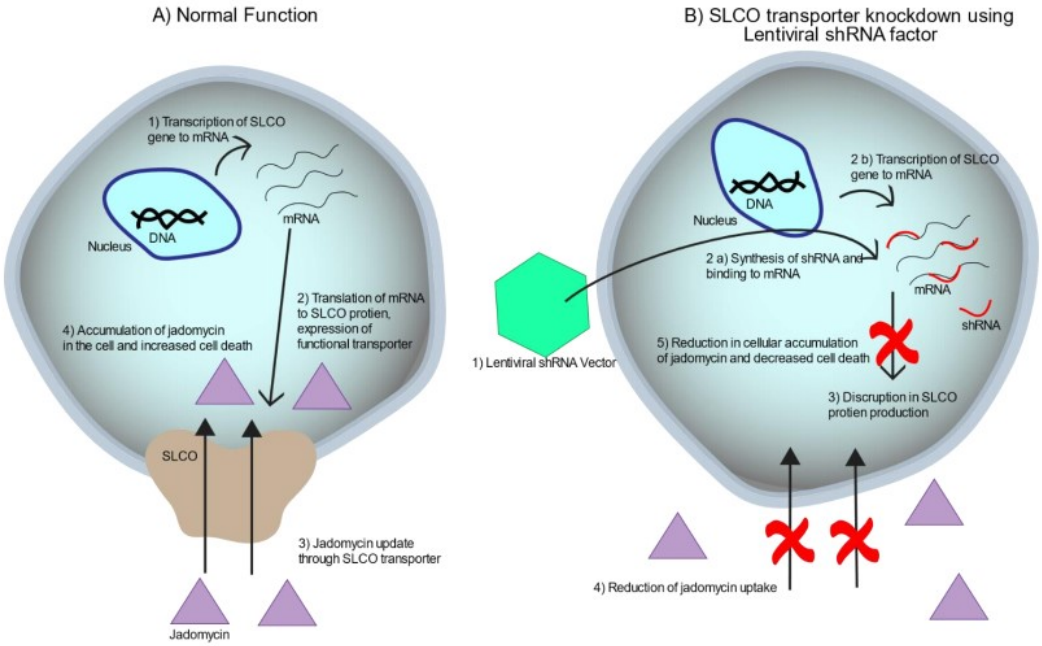

Figure 6: Hypothesized impact of knockdown on jadomycin transport. **A)** depicts a normal functioning cell treated with jadomycin and undisturbed expression of a SLCO transporter. Cell **B)** is the result of SLCO knockdown using lentiviral shRNA vectors.

It is essential to understand the mechanisms of jadomycin uptake in order to determine which types of breast cancer cells may be best targeted by jadomycins. Ultimately, this information will help guide future preclinical and potentially clinical testing of jadomycins.

CHAPTER 2. MATERIALS AND METHODS

2.1 Chemicals

Paclitaxel, mitoxantrone dihydrochloride, LB broth (Lennox), ampicillin, glycerol, BSA (bovine serum albumin), sodium azide, MTT, dimethyl sulfoxide (DMSO), methanol, doxorubicin hydrochloride, polyethylenimine, and polybrene were all purchased from Sigma Aldrich (Oakville, ON, Canada).

2.2 The Production of Jadomycins

Jadomycin analogues isoleucine (B), serine (S), and phenylalanine (F) were synthesized and purified by the David Jakeman laboratory (College of Pharmacy, Dalhousie University, Halifax, N.S.) using an established method.[66,76,79,82] *Streptomyces venezuelae* ISP5230 was grown with 60 mM of the amino acids isoleucine, serine, or phenylalanine until the OD_{600} read 0.6. The mixture was then shocked with 100% ethanol (3% v/v) and is shaken until A_{526} reaches between 0.5 and 1.0. The mixture was filtered and passed through a reverse-phase capture C_{18} column. The crude natural product was eluted from the column with methanol and was purified using column chromatography. Jadomycins B, S and F were characterized using thin layer chromatography (TLC), ultra violet visible spectroscopy (UV-Vis), infrared spectroscopy (IR), nuclear magnetic resonance spectroscopy (NMR), as well as low resolution mass spectrometry (LRMS) and high resonance mass spectrometry (HRMS).[77,79,80,83]

2.3 Cell Lines

MCF7 breast cancer cell lines were provided by Drs. Robert Robey and Susan Bates (National Cancer Institute Bethesda, MD). The BT474, SKBR3, and MDA-MB-231 cell lines were provided by Dale Corkery, Chansey Veinotte, and Drs. Graham Dellaire and Jason Berman (Dalhousie University and the IWK Health Centre, Halifax, Nova Scotia, Canada). The MCF10A cells were provided by Dr. Brent Johnston (Dalhousie University, Halifax, Nova Scotia, Canada). HEK-293T cells were provided by Pak Phi Poon (EGAD Core Facility, Life Sciences Research Institute, Dalhousie University, Halifax, Nova Scotia, Canada). Drug resistant MCF7 cell lines overexpressing ABCB1 (MCF7-TXL), ABCG2 (MCF7-MITX), ABCC1 (MCF7-ETP) were generated through selection media

containing taxol, mitoxantrone, and etoposide, and were also obtained from Dr. Robert Robey and Susan Bates.[66,98]

The MCF7-CON, MCF7-TXL, MCF7-MITX, MCF7-ETP, MDA-MB-231, BT474, and SKBR3 cell lines were cultured in phenol red-free Dublecco's modified Eagle's medium (DMEM) (Thermo Scientific, Ottawa, Ontario, Canada), supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin (Invitrogen, Burlington, ON, Canada) as wells 1% sodium pyruvate (Sigma, St. Louis, MO, USA). HEK-293T cells were cultured in the same medium mixture excluding the 1% sodium pyruvate. MCF7-TXL, MCF7-MITX, MCF7-ETP sublines were additionally supplemented with 400 nM paclitaxel, 100 nM mitoxantrone, 4 μM etoposide, respectively. MCF10A cells were cultured in Dublecco's modified Eagle's medium/Ham's Nutrient Mixture F12 (Sigma Alrich, Oakville, ON, Canada) supplemented with 5% (v/v) horse serum, 1 ng/mL cholera toxin, 10 μg/mL human insulin, 10 ng/mL epidermal growth factor, 0.5 μg/mL hydrocortisone, and 1% (v/v) penicillin-streptomycin, all purchased from Sigma Aldrich (Oakville, ON, Canada). Cells were stored in an incubator at 5% CO_2, humidified, at 37ºC. Medium for all cells were changed every 3-4 days. TXL, MITX, and ETP treatments for respective MDR cell lines were stopped one week prior to any experiments.

2.4 Western Blots

MCF7-CON, MCF7- TXL, and MCF7-MITX cells were grown in 6 well plates until they reached exponential growth. Crude membrane protein collection was initiated by washing the plate twice with 10 mL of cold PBS. Cold PBS was added to the plate and a cell scraper was used to detach the cells. The cell solution was then added into a 50 mL tube. The plate was rinsed with cold PBS again and added to the tube. The cells were pelleted by centrifuging at 1000 rpm for 10 minutes (4ºC). Supernatant was removed, and the pellet was resuspended in 1.25 mL of 50 mM Mannitol solution (50 mM Mannitol; 1 mM tris-base, pH 7.4 with HEPES). Protease inhibitors (Sigma Aldrich, Oakville, ON, Canada) were added at 1:500. Using a 1 mL syringe fitted with a 27-gauge needle, the cells were sheered by passing them through 10 times. Sheered cells were put into a microcentrifuge tube and centrifuged at 1000 rpm for 5 minutes (4ºC). The supernatant

was retained and put into a new centrifuge tube. The supernatant was centrifuged at 14000 RMP for 30 minutes (4ºC). The supernatant was removed, and the pellet was resuspended in a total volume of 100 µL in 50 mM Mannitol solution. The protein concentration was quantified using a Lowry assay.

Crude membrane protein (1.125 µg) was loaded into each well. Proteins were separated by 10% or 7.5% SDS-PAGE. Proteins were transferred to nitrocellulose membranes. The membranes were incubated overnight in a 1:1000 dilution of goat polyclonal anti-GAPDH from Abcam (Toronto, Ontario, Canada) with 1:1000 dilution of rabbit polyclonal anti-SLCO3A1 antibody from Abcam (Toronto, Ontario, Canada) or a 1:500 dilution of rabbit polyclonal anti-SLCO4A1 antibody from Abcam (Toronto, Ontario, Canada) at (4ºC). Membranes were washed 4 times with TBST, and incubated again for 1 hour with rocking at room temperature with IRDye 680RD conjugated donkey α-rabbit and IRDye 800CW conjugated donkey α-goat purchased from Mandel Scientific (Guelph, Ontario, Canada). Membranes were scanned at 700 and 800 nm infrared wavelengths using a Licor Odyssey (Mandel Scientific).

2.5 Flow Cytometry

MCF7-CON cells were seeded at 100,000 cells per well into a 6 well plate and incubated overnight. The cells were harvested using 1 mL TryPLE Express in 4 mL FACS capable tubes. Cells were washed with 1-2 mL PBS. The membrane was permeabilized using 0.05% triton for 10 minutes three times, separating incubation times by rinsing with PBS. 50 µL of 1 µg/mL rabbit polyclonal anti-SLCO3A1 antibody, rabbit polyclonal anti-SLCO4A1, or rabbit polycolonal IgG isotype control from Abcam (Toronto, Ontario, Canada) was added to FACS tubes and mixed gently. Tubes were incubated on ice for at least 1 hour. Cells were washed three times with 2 mL of FACS buffer to remove excess antibodies. FACS buffer consisted of 500 mL PBS, 5 g BSA, and 1 g sodium azide. In the dark, 50 µL of 1:4000 goat α-rabbit Alexa Fluor 488 (Sigma Aldrich, Toronto, Ontario, Canada) was added to the cells and incubated at room temperature for at least 30 minutes. Cells were washed three times with 2 mL of FACS buffer to remove extra antibodies. 300 µL of 1% paraformaldehyde made in PBS was added to the cells and incubated for at least 30 minutes before reading. Flow cytometry

was read on a BS FACS Calibur in the Dalhousie University's flow cytometry facility. Live cells were gated and threshold on FSC was set to 52.

2.6 MTT Assay

MTT assays were used to evaluate the concentration-dependence of jadomycin S (0.1 – 10.0 μM) and doxorubicin (1.0 – 10.0 μM) cytotoxicity. MTT assays were also used to evaluate the time dependence of jadomycin S and doxorubicin cytotoxicity treatments each at 10 μM at 8 different timepoints (24, 12, 8, 6, 4, 2, 1, 0.5 hours). All MTT assays were tested in MCF7-CON, *SLCO3A1* knockdown MCF7 cells (MCF7-CON 3A1 KD), *SLCO4A1* knockdown MCF7 cells (MCF7-CON 4A1 KD), and non-silencing control MCF7 (MCF7-CON NS) cells. Cells were seeded at 5000 cell/well of a 96 well plate and incubated at 5% CO_2 and 37ºC overnight. After appropriate treatments, MTT solution was added to wells for a final concentration of 0.83 mg/ml and were incubated for 1 hour. Wells were aspirated completely and 100 μL of dimethyl sulfoxide (DMSO) was used to solubilize formazan product. The absorbance of formazan was measured at 550 nm using a Biotek Synergy HT plate reader (Bioteck, Winooski, Vermont, USA). Cell viability was calculated by dividing the average absorbance of the test wells by the average absorbance of vehicle treated wells and multiplying by 100%. IC_{50} values are characterized by the drug concentration required to reduce the cell viability by 50%.

Equation 1 $$Y = 100/(1+10^{(LogIC_{50}-X)*HillSlope)})$$

Values are calculated from the $\log_{10}$ concentration versus normalized response curve using equation 1 in GraphPad Prism software. The measured absorbance at 550 nm is Y and X is the drug concentration.

2.7 RNA Collection, Reverse Transcriptase Reaction, and Quantitative Polymerase Chain Reaction

MCF7-CON, MCF7-TXL, MCF7-MITX, MCF7-ETP, MDA-MB-231, SKBR3, and BT474 cell lines were all seeded at 300,000 cells in 3 wells of a 6 well plate and incubated at 5% CO_2 and 37ºC overnight. RNA was collected directly from untreated plates using Aurum total RNA mini kit (Bio-rad, Mississauga, Ontario, Canada)

according to manufacturer's instructions. Isolated RNA was reverse transcribed to complementary DNA (cDNA) using Super Script II Reverse Transcriptase (Life Technologies). Polymerase chain reaction (PCR) was used to amplify cDNA using 11 different PCR primer pairs for *SLCO1A2, 1B1, 1B3, 1C1, 2A1, 2B1, 3A1, 4A1, 4C1, 5A1,* and *6A1* (Sigma Alrich). Primers were designed using Primer BLAST and are listed in Table 6. Primers were validated through melt-curve analysis as well as amplicon product size agarose gels. Complimentary DNA was amplified via quantitative PCR using 125 nM primers in a volume of 20 µL using a SYBER Green PCR Kit using a Step-One Plus real-time PCR thermocycler (Applied Biosystems). PCR cycling parameters consist of dissociation, amplification, and melting curve. Dissociation is run at 95 ºC for 10 minutes for 1 cycle. Amplification was run at 95ºC for 20 seconds, 60 ºC for 18 seconds, and 72 ºC for 30 seconds for 4 cycles. The melting curve was run at 95 ºC for 1 minute, 65 ºC for 30 seconds, and 95 ºC for 30 seconds for 1 cycle. A total of 35 cycles were run. Gene expression was normalized using three housekeeping genes: *glyceraldehyde 3-phosphate dehydrogenase (GAPDH), Beta-actin (β-actin), Peptidylprolyl Isomerase A (PPIA)* via the $\Delta\Delta C_t$ method.

Table 6: PCR primers designed to evaluate gene expression of SLCO family and housekeeping genes.

Gene	**Forward Primers (5' – 3')**	**Reverse Primers (5' – 3')**
SLCO4A1	GACCTGCCTCTCTCCATCTG	CTCAGGCTGAACTGGGACTC
SLCO3A1	AAATCCTTCGCCTTCATCCT	CCACTCATGGTCTTCCAGGT
SLCO5A1	AGGGTTCTTGCTGGGAGTAC	ATTCTCCCTGGGTGCTGAAA
SLCO1B3	AGTCATTGGCTTTGCACTGG	ACACAAGGAAACCAAGCCAC
SLCO2B1	ACAGAAGGAGCAAGTGACCCA	CCCATTGTGGCTTTGGTTTCC
SLCO1B1	GCACCTCACATGTCATGCTG	GACAAGCCCAAGTAGACCCT
SLCO2A1	TCGGGTCTCATTTCCAGCTT	TGGCCAAGGTGTACTGGTAG
SLCO4C1	TTCTGTGCCCACACACAAGT	CCAATCCACCAAGCTCCCAA
SLCO1C1	ACTCCCATTCAGCCTTTGGG	CAGAAAGGCACAGCTGCAAG
SLCO1A2	TGGATGTGTGGTTATGGGCT	GGAGTTTCACCCATTCCACG
SLCO6A1	TTCATCTGGCCTGGTAGCAA	TTCCTGCTATTCCCTGCACA
GAPDH	GAGTCAACGGATTTGGTCGT	TTGATTTTGGAGGGATCTCG
B-actin	GGACTTCGAGCAAGAGATGG	AGCACTGTGTTGGCGTACAG
PPIA	ACCGCCGAGGAAAACCGTGT	CTGTCTTTGGGACCTTGTCTGCA

2.8 Gene Knockdown using Lentivirus

SLCO3A1, *SLCO4A1*, as well as packaging genes psPAX2 and pMD2G GIPZ shRNA clones were purchased from Gene Analysis & Discovery (Dalhousie University) and the GIPZ non-silencing shRNA control was provided by Nichole McMullen of Dr. Roy Duncan laboratory (Dalhousie University, Halifax, Nova Scotia, Canada). All clones came in the form of plasmids packaged in *E.coli*. Single colonies were selected and grown in media consisting of 1 liter of 2x LB (low-salt) with ampicillin (100 µg/ml). Glycerol at 8% was added for long-term storage at -80ºC. The plasmid-expressing *E. coli* cultures were grown in a volume of media that was 20% the volume of the container, shaking, at 30ºC for 14-16 hours. QIAfilter Plasmid Midi Kit (Qiagen) was used to isolate and purify DNA plasmid from the bacteria, using the manufacturer protocols. DNA purity was validated and quantified by measuring absorbance at 260 nm and 280 nm. Purity was measured by the ratio of absorbance at 260 nm to 280 nm, where the ideal ratio exists between 1.8 and 2.0. DNA specificity was validated through restriction enzyme digestion using Thermo Scientific™ FastDigest™ restriction enzymes KpnI and SacII and run on agarose gels.

Viral particles containing appropriate shRNA were then produced using HEK-293T cells. 150,000 cells were seeded into a 6 well plate and were incubated at 5% CO_2 and 37ºC until cells were approximately 80% confluent. Two different tubes were prepared separately; tube one contained 500 µL DMEM medium (Thermo Scientific, Ottawa, Ontario, Canada) as well as 2 µg psPAX2, 1 µg pMD, and 3.3 µg of SLCO3A1, 4A1, or the non-silencing (NS) shRNA plasmid. Tube two contained 500 uL DMEM medium and 18 µL of 25 kDa polyethylenimine (PEI) (1 mg/ml). The tubes were incubated at room temperature separately for 5 minutes, then they were mixed and incubated at room temperature for 15 minutes. Media was removed from cells and the tube mixture was added directly to the cells. Cells were incubated at 5% CO_2 and 37ºC for 6 hours, and then the mixture was removed and replaced with 1 mL fresh DMEM. The media was collected after 24 hours and filtered through a 0.45 µM filter and stored at 4ºC. Fresh media was added once more and left an additional 24 hours before harvesting. Harvested media collected at 24 and 48 hours both contain viral particles containing shRNA. The plasmid vectors contains the green fluorescence protein (GFP) gene to allow for positive

culture identification. GFP fluorescence microscopy was used to identify completion of transduction.

MCF7-CON cells were seeded into a 6 well plate at 150,000 cells/well and incubated at at 5% CO_2 and 37ºC overnight. Polybrene was added to 1 mL of the viral particle media mixture for a final concentration of 8 µg/mL. Media in the 6 well plate was removed and 1 mL of the viral particle and polybrene mixture was added to the cells. The cells were then incubated at at 5% CO_2 and 37ºC until green fluorescent protein (GFP) was expressed (approximately 5 days). Once majority of the cells expressed GFP, the media was changed to normal MCF7-CON media supplemented with 1.5 µg/mL puromycin, which acts as a selection agent. Puromycin selection was carried out for at least 2 weeks, until all non-transfected cells were eliminated. Resulting knockdown cell lines were propagated and cryostored at -80ºC.

2.9 Statistical Analysis

Gene expression experiments and cell viability experiments were conducted in triplicate or quadruplicate. Data was expressed as mean ± SEM. One-way ANOVA was used to compare experiments with one independent variable. A Bonferroni test was used for *post-hoc* analysis of the significant ANOVA. A difference in mean values between groups was significant when $P \leq 0.05$. A two-way ANOVA was used to compare experiments with two independent variables. When comparing SLCO transporters within each cell types, *SLCO4A1* was used as a reference gene where expression = 1. When comparing SLCO transporters between drug resistant and drug sensitive breast cancer cell types, MCF7-CON cells were used as a reference where SLCO expression = 1. When comparing SLCO transporters across breast cancer cell types with different hormone receptor profiles, MCF-10A cells were used as a reference where SLCO expression = 1.

CHAPTER 3. RESULTS

3.1 SLCO Gene Expression Profiles of Breast Cancer Cells and Breast Epithelial Cells.

It was first necessary to evaluate gene expression of all SLCO transporters in the panel of breast cancer cells prior to knockdown to determine which transporters have high enough expression to have functional importance.

3.1.1 The Impact of Drug Resistant Phenotype on SLCO Expression within each Cell Type.

Analysis of MCF7-CON cells indicated that the highest expressed transporters are *SLCO4C1* and *SLCO1C1* (Figure 7). There was no significant difference between any transporters and reference gene *SLCO4A1*. In MCF7-TXL cells *SLCO4A1* had the highest relative expression and was significantly higher than *SLCO5A1*, *1B3, 2B1, 1B1, 2A1, 1A2*, and *1C1*. *SLCO4C1* was second highest expressed, being significantly different than *SLCO5A1*, *1B3, 2B1, 1B1, 2A1,* and *1A2.* Neither *SLCO4A1* or *SLCO4C1* was significantly higher in expression than *SLCO3A1*, which was also highly expressed. In MCF7-MITX cells *SLCO4A1* was the highest expressed transporter, followed by *SLCO3A1.* Both *SLCO4A1* and *SLCO3A1* were significantly different than all other transporters. In MCF7-ETP cells, *SLCO4A1* also had the highest expression and was significantly higher than all other transporters. *SLCO3A1* had the next highest expression and was significantly higher than all other transporters except *SLCO4C1*, which followed in the next highest expressed transporters. In summary, all MDR breast cancer cells have *SLCO4A1, 3A1,* and *4C1* as their highest expressed transporters, where as in MCF7-CON cells, only the *SLCO1C1* transporter is significantly higher than any other transporter.

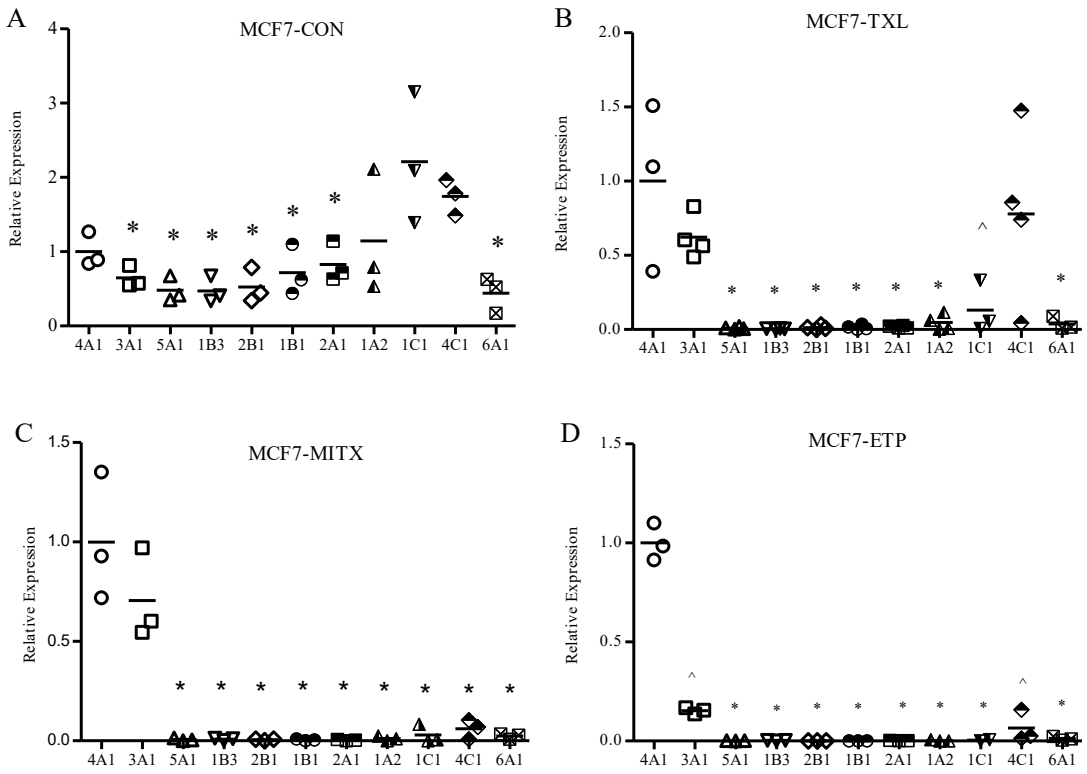

Figure 7: The impact of drug-resistant phenotype on SLCO expression within each cell type. mRNA expression of SLCO transporters is evaluated in drug-resistant MCF7 cells and MCF7-CON cells. Values are reported as fold change and each bar represents the mean and standard error of the mean of 3 or 4 independent replicates. **A)** Expression measured in MCF7-CON cells. Transporters 3A1, 5A1, 1B3, 2B1, 1B1, 2A1, and 6A1 were all significantly different than 1C1 (*). **B)** Expression measured in MCF7-TXL cells. Transporters that were significantly different than both 4A1 and 4C1 are denoted as (*), where the transporter (1C1) that was significantly different than 4A1 alone is denoted by (^). **C)** Expression measured in MCF7-MITX cells. Transporters were significantly different than both 4A1 and 3A1 are denoted by (*). **D)** Expression measured in MCF7-ETP cells. All transporters are significantly different than 4A1 (^). Transporters that were significantly different than both 4A1 and 3A1 are denoted by (*). Statistical analysis was conducted using a one-way ANOVA followed by Bonferroni post test. A value of $P \leq 0.05$ was considered significant.

3.1.2 The Impact of Drug-Resistant Phenotype on SLCO Expression Across Cell Type.

Expression of SLCO transporters were also analyzed individuality, comparing four cell types: MCF7-CON, MCF7-ETP, MCF7-MITX, and MCF7-TXL (Figure 8). When comparing the *SLCO4A1* transporter, there was no significant difference in expression between MCF7-CON cells and any MDR breast cancer cells tested. *SLCO3A1* transporter

expression was highest in MCF7-TXL cells, followed by MCF7-MITX. Both cell lines had a significantly higher expression of *SLCO3A1* than in MCF7-ETP. *SLCO4C1* transporter had highest expression in MCF7-TXL cells, a significantly higher level than the three other cell types. There is no change in relative expression between any MCF7 cells in several transporters: *SLCO1B1, 2B1, 1B3, 5A1, 1A2, 1C1, 2A1*, and *6A1*.

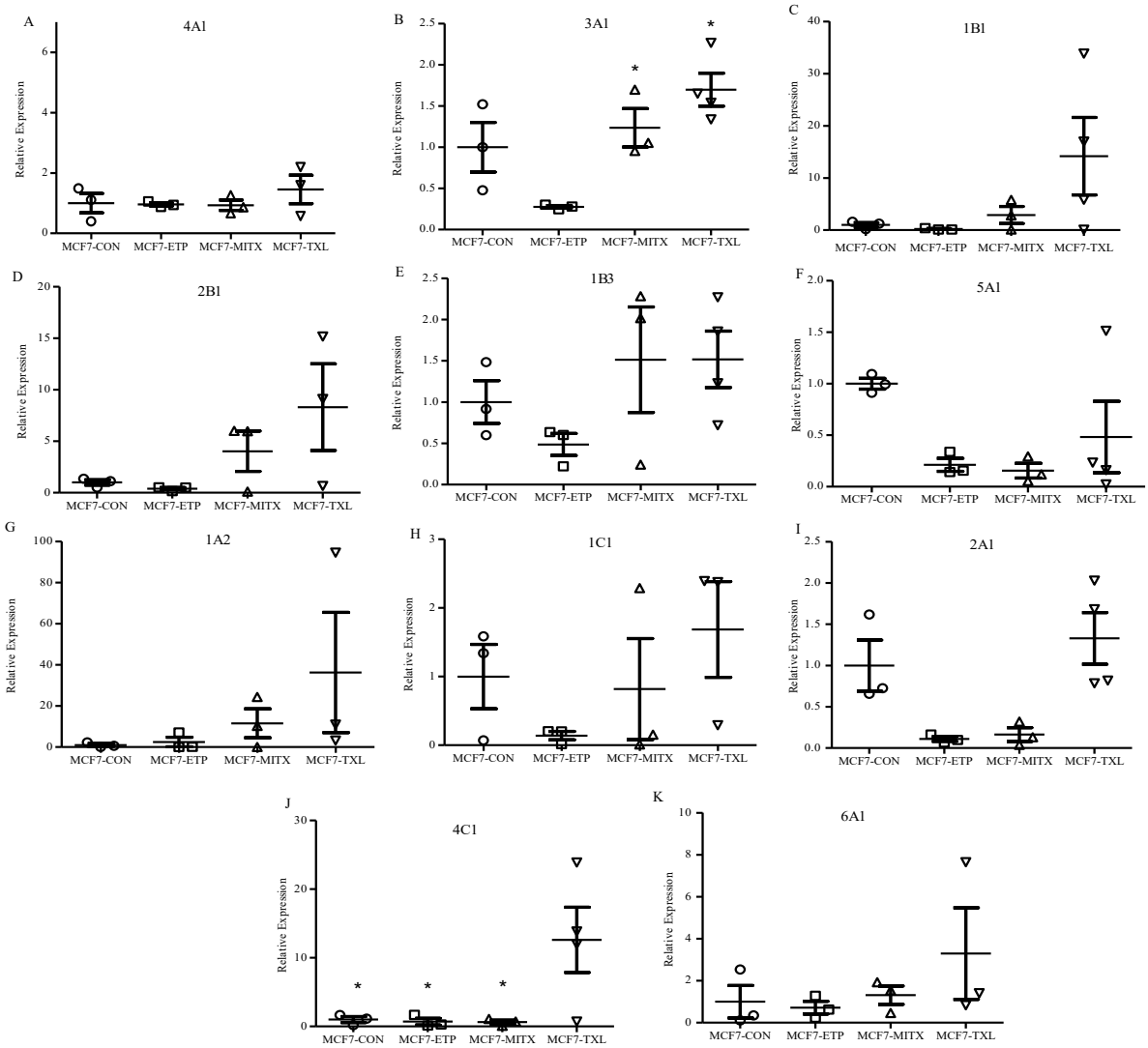

Figure 8: The impact of drug resistant phenotype on SLCO expression across cell type. The mRNA expression of SLCO **A)** *4A1* **B)** *3A1* **C)** *1B1* **D)** *2B1* **E)** *1B3* **F)** *5A1* **G)** *1A2* **H)** *1C1* **I)** *2A1* **J)** *4C1* **K)** *6A1* were measured in MCF7-CON cells and three MCF7 MDR cells. Values were reported as fold change values and each bar represents the mean and standard error of the mean of 3-5 independent experimental replicates. In panel B, the * denotes cells where *SLCO3A1* expression was significantly higher than in the MCF7-

ETP cells. In panel J, the * denotes cells where *SLCO4C1* was significantly lower than in the MCF7-TXL cells. Statistical analysis was conducted using a one-way ANOVA followed by Bonferroni post test. A value of $P \leq 0.05$ was considered significant.

3.1.3 Impact of Breast Cancer Cell Type on Relative Expression of SLCOs within Cell Type.

In the non-cancerous breast epithelial MCF-10A cells, the reference gene *SLCO4A1* was the highest expressed transporter as every other transporter is significantly lower, except *SLCO3A1* (Figure 9). *SLCO3A1* was significantly higher than all transporters with the exception of *SLCO4A1* and *2A1*. In SKBR3 (ER-, PR-, HER2+) cells *SLCO4A1* had a significantly higher expression than all other transporters. In 231-CON (ER-, PR-, HER2-) cells, the highest expressed transporter was *SLCO1B3*. *SLCO3A1* was the next highest expressed transporter, which was significantly higher than four transporters (*2B1, 1B1, 1A2, 6A1*). *SLCO2B1* transporters were tested repeatedly and always yielded undefined results, suggesting that expression was too low to be detected. In BT474 (ER+, PR+, HER2+) cells the highest transporter expressed is *SLCO1B3*, like 231-CON cells, however, there is no significant differences between any transporter expression in BT474.

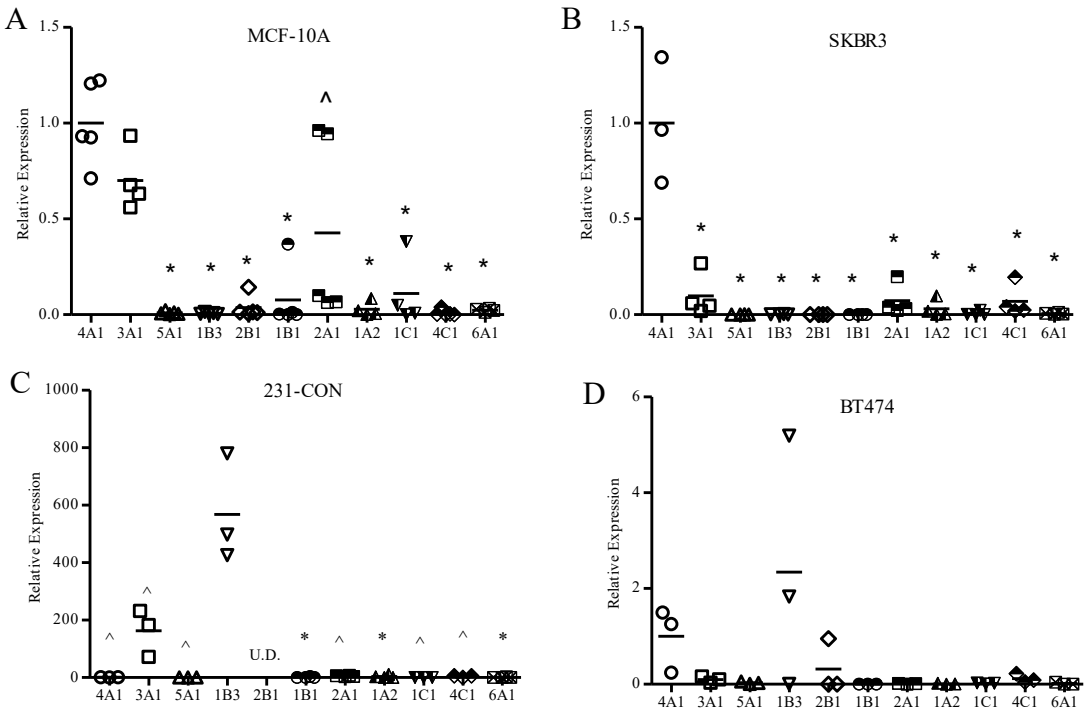

Figure 9: Impact of breast cancer cell type on relative expression of SLCOs within cell type. mRNA expression of SLCO genes were measured in different breast cancer cell types. Results were reported as fold change values and each bar represents the mean and standard error of the mean of 3 or 4 independent replicates. **A)** SLCO mRNA expression measured in MCF-10A cells All transporters that were significantly different than both 4A1 and 3A1 are denoted by (*), and transporters that were significantly different than just 4A1 are denoted by (^). **B)** SLCO mRNA expression measured in SKBR3. All transporters were significantly different than 4A1 and is denoted by (*). **C)** SLCO mRNA expression measured in 231-CON cells. All transporters that were significantly different than both 1B3 and 3A1 are denoted by (*), and transporters that are significantly different than just 1B3 are denoted by (^). 2B1 is undetermined in 231-CON cells. **D)** SLCO mRNA expression measured in BT474 cells and reported as fold change values. There were no significant differences. Statistical analysis was conducted using a one-way ANOVA followed by Bonferroni post test. A value of $P \leq 0.05$ was considered significant.

3.1.4 The Impact of Breast Cancer Cell Type on SLCO Expression.

When evaluating expression of *SLCO3A1*, both SKBR3 and BT474 had significantly lower expression than the reference cell line, MCF-10A (Figure 10B). The expression of the *SLCO2B1* transporter was significantly higher in the MCF-10A cells compared to the 231-CON, SKBR3, and BT474 (Figure 10D). *SLCO1B3* expression was significantly higher in the BT474 cells compared to the MCF-10A and SKBR3 cells (Figure 10E).

SLCO5A1 expression was significantly higher in BT474 cells compared to the 231-CON and SKBR3 cells (Figure 10F). Transporters *SLCO4A1*, *1B1*, *1A2*, *1C1*, *2A1*, *4C1*, and *6A1* expression was not different between cell types.

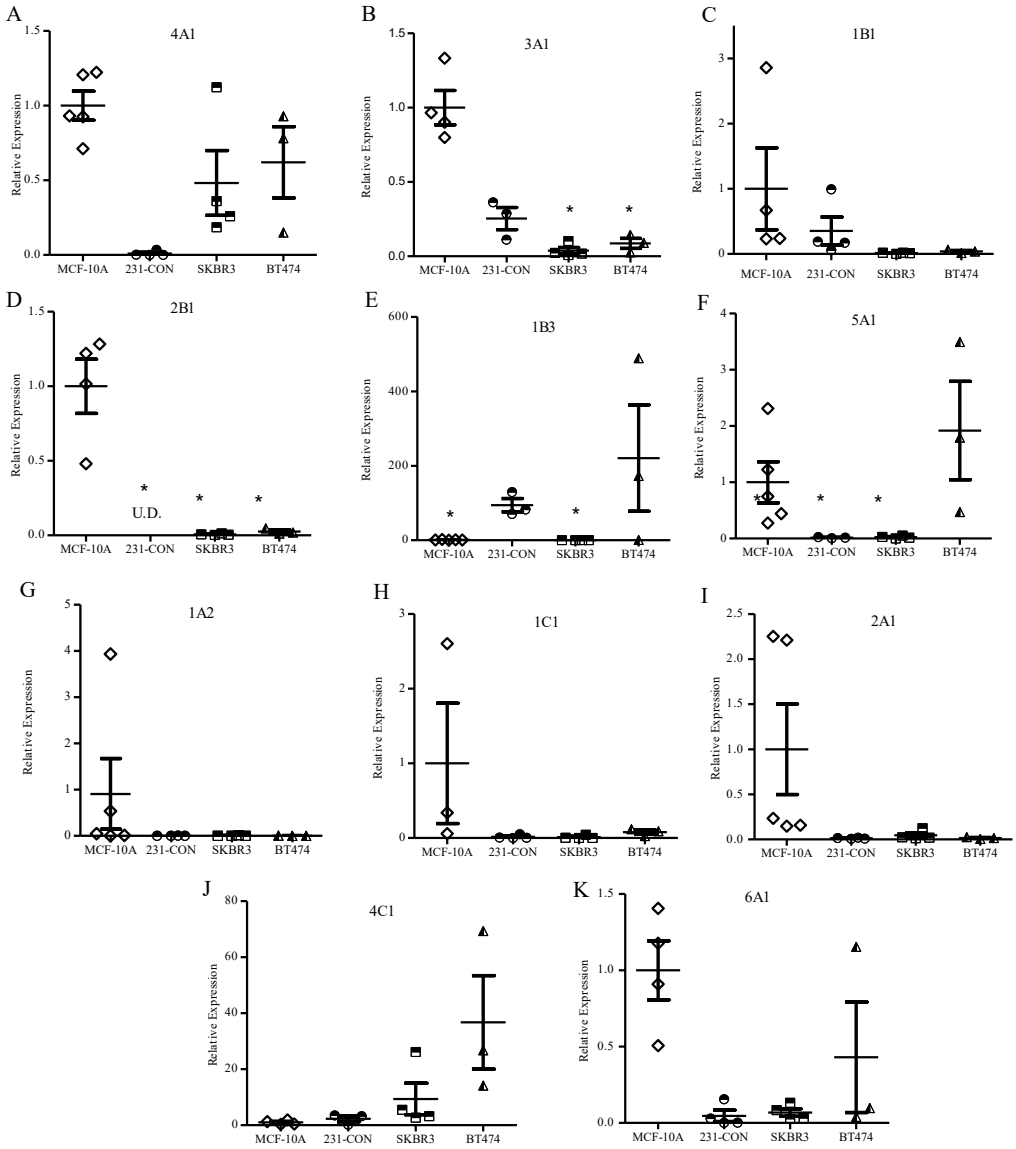

Figure 10: The impact of breast cancer cell type on SLCO expression across each cell type. The mRNA expression of SLCO **A)** *4A1* **B)** *3A1* **C)** *1B1* **D)** *2B1* **E)** *1B3* **F)** *5A1* **G)** *1A2* **H)** *1C1* **I)** *2A1* **J)** *4C1* **K)** *6A1* were measured in a panel of breast cancer cells and breast epithelial cells. Values were reported as fold change and each bar represents the mean and standard error of the mean of 3-5 independent experimental replicates. In panel B the * denotes the cells where *SLCO3A1* expression was significantly different than MCF7-10A. In panel D the * denotes the cells where *SLCO2B1* expression was significantly different than MCF7-10A. Cell type 231-CON is undefined (U.D.). In panel E, the * denotes the cells where *SLCO1B3* expression was significantly different from

BT474. In panel F, the * denotes the cells where *SLCO5A1* expression was significantly different from BT474. Statistical analysis was conducted using a one-way ANOVA followed by a Bonferroni post test. A value of $P \leq 0.05$ was considered significant.

3.2 Protein Expression of SLCO4A1 and SLCO3A1 in Breast Cancer Cells.

3.2.1 Detection of *SLCO3A1* and *SLCO4A1* by Western Blot and FACS Analysis

Generally, *SLCO4A1* and *SLCO3A1* were the highest expressed transporters across cell lines therefore, these transporters were selected for the assessment of protein detection. *SLCO3A1* western blots in cell membrane fractions from MCF7-CON, -MITX, and -TXL cells did not show positive detection of the *SLCO3A1* transporters. Observed bands should be present at 85 kDa, with two additional bands at 35 and 37 kDa.[99] At 800 nm bands were seen at 50, 37 and approximately 32 as shown in Figure 11. This corresponds to desired bands for GAPDH detection.[100] While western blots for *SLCO4A1* were also attempted, a technical notification was received from ABCAM that the *SLCO4A1* used could not detect the SLCO4A1 in western blot.

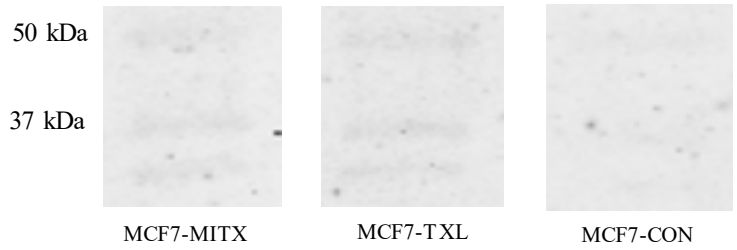

Figure 11: Western blot scanned at 800 nm to detect GAPDH control protein. Bands seen at 50, 37, and 32 indicate the presence of the GAPDH protein.

Preliminary results of one trial show the presence of *SLCO3A1* in MCF7-CON cells. Percent of gated cells for IgG isotype control was 18.41 compared to the *SLCO3A1* antibody which was 37.12. These results are promising for improved detection sensitivity of *SLCO3A1* via flow cytometry versus western blotting.

3.3 Lentiviral Knockdown of *SLCO3A1* and *SLCO4A1* Expression in MCF7 Cells.

Generally, *SLCO4A1* and *SLCO3A1* were the highest expressed transporters across cell lines, therefore, these transporters were selected for initial knockdown experiments.

MCF7-CON cells were used for initial knockdown experiments as the lentivirus protocol needed to be optimized.

3.3.1 Confirmation of Lentiviral Transduction.

To confirm the successful transduction of MCF7 cells, fluorescent microscopy was used to screen for the GFP reporter construct that is contained on the lentiviral plasmid. GFP was expressed in MCF7 CON cells that were transduced with lentiviral vectors containing *SLCO3A1* shRNA (MCF7 3A1 KD), *SLCO4A1* shRNA (MCF7 4A1 KD), and the non-silencing control shRNA (MCF7 NS CON) cells. GFP was not seen in MCF7-CON cells. Images are shown in Figure 12.

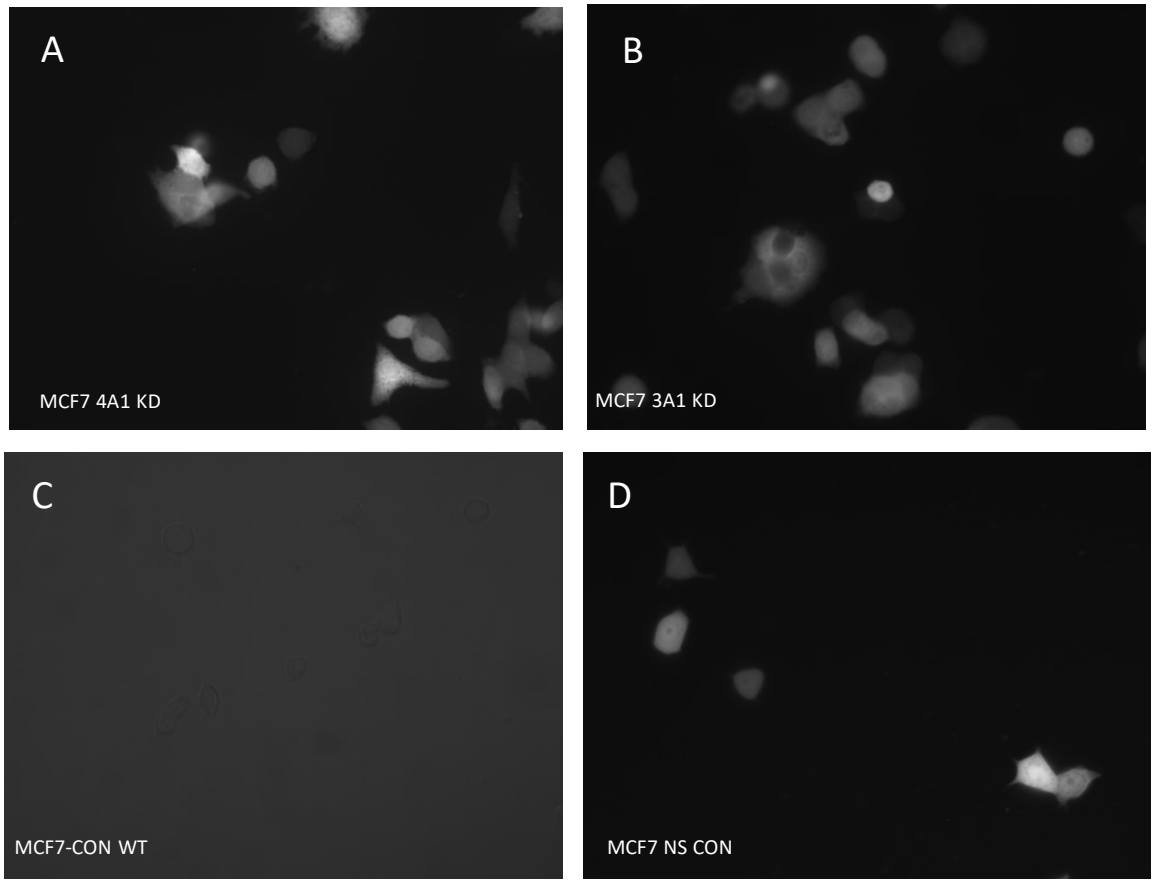

Figure 12: Confirmation of lentiviral transduction of MCF7 cells. Fluorescent imaging of GFP expressing cells SLCO4A1 KD, SLCO3A1 KD, and MCF7 NS CON. No GFP expressing cells are seen in MCF7-CON cells.

3.3.2 Gene Expression of *SLCO4A1* and *3A1* in Knockdown Cell Lines and Control Cell Lines.

Expression of *SLCO3A1* was evaluated in MCF7 3A1 KD cells and compared to MCF7 NS CON cells and MCF7 CON cells. Transduction with the *SLCO3A1* shRNA caused a significant downregulation of *SLCO3A1* compared to MCF7-CON and the MCF7 NS-CON (Figure 13A). In comparison the expression of *SLCO4A1* in MCF7 4A1 KD cells was not significantly downregulated between MCF7-CON and the MCF7 NS-CON (Figure 13B).

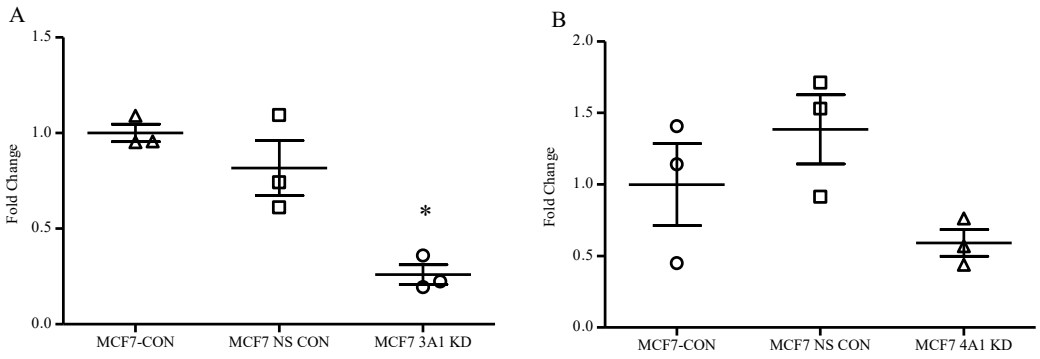

Figure 13: The effect of lentiviral shRNA transduction on the expression of *SLCO3A1* and *SLCO4A1*. Figure **A)** shows the expression of *SLCO3A1* in control cells and 3A1 knockdown cells. MCF7 3A1 KD was significantly different than both MCF7-CON and MCF7 NS CON, denoted by (*). Figure **B)** shows the expression of *SLCO4A1* in control cells and 4A1 knockdown cells.

3.3.3 Effect of *SLCO4A1* and *SLCO3A1* Knockdown on Jadomycin Cytotoxicity.

MFC7-CON, MCF7-NS CON, MCF7 3A1 KD, and MCF7 4A1 KD cells were treated with jadomycin S concentrations (0.1, 1, 2, 4, 8 μM) and doxorubicin (0.1, 0.5, 1, 5, 10 μM) for 72 hours. The cell viability was measured using the MTT assays and results are reported as percent cell viability (Figure 14 and 16) as well as IC_{50} values (Figure 15 and 17). The IC_{50} of jadomycin S 2.18 μM ± 0.07 and doxorubicin 0.836 μM ± 0.45 in MCF7-CON, which corresponds to reported values of 1.97 μM ± 0.85 and 1.11 μM ± 0.14, respectively.[66,101] There were no significant effects of the NS control, SLCO3A1, or

4A1 lentiviral shRNA treatments on cell viability or IC_{50} values compared to the MCF7-CON cells.

MTT cell viability assay results at earlier timepoints 0.5, 1, 2, 4, 6, 8, 12 and 24 hours also failed to show a significant effect of *SLCO3A1* or *SLCO4A1* lentiviral shRNA treatments on cell viability compared to the MCF7-CON cells (Figures 18 and 19).

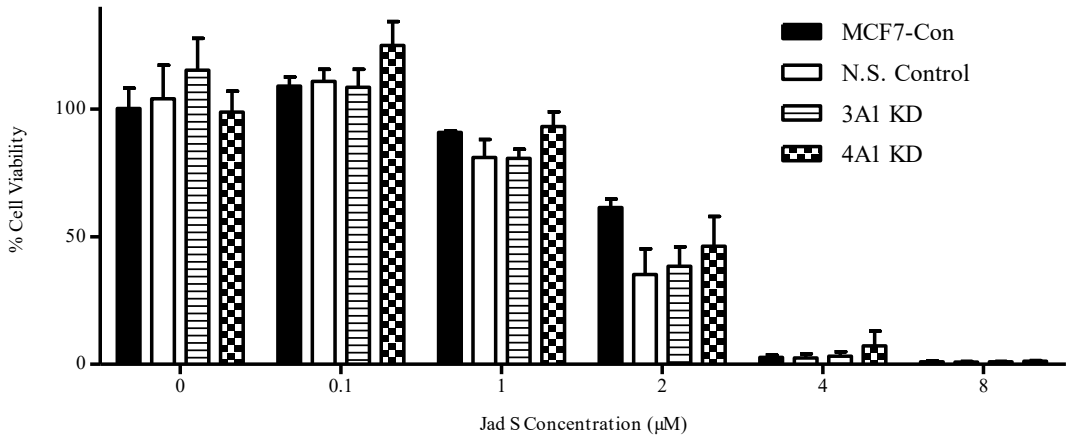

Figure 14: The effect of *SLCO3A1* and *4A1* lentiviral shRNA treatment on the dose-response toxicity of jadomycin S in MCF7 cells. Each bar represents the mean and standard error of the mean of 4 independent replicates. Statistical analysis was conducted using a one-way ANOVA followed by a Bonferroni post test. A value of $P \leq 0.05$ was considered significant.

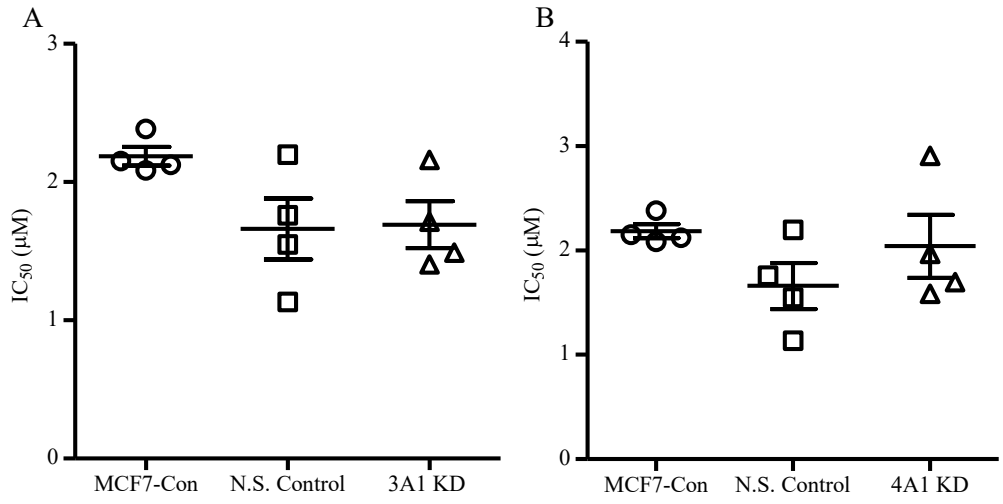

Figure 15: The effect of *SLCO3A1* and *4A1* lentiviral shRNA treatment on the IC_{50} values of jadomycin S in MCF7 cells. Each bar represents the mean and standard error of the

mean of 4 independent replicates. Statistical analysis was conducted using a one-way ANOVA followed by a Bonferroni post test. A value of $P \leq 0.05$ was considered significant.

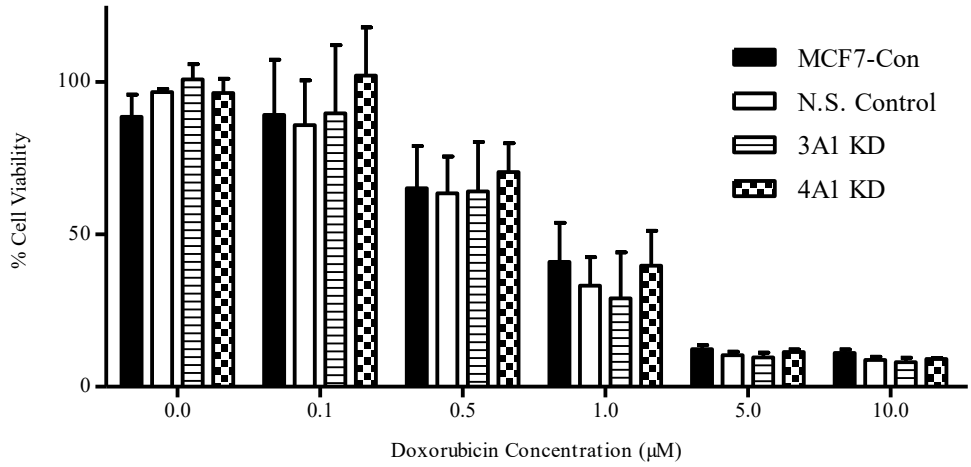

Figure 16: The effect of *SLCO3A1* and *4A1* lentiviral shRNA treatment on the dose-response toxicity of doxorubicin in MCF7 cells. Each bar represents the mean and standard error of the mean of 3 independent replicates. Statistical analysis was conducted using a one-way ANOVA followed by a Bonferroni post test. A value of $P \leq 0.05$ was considered significant.

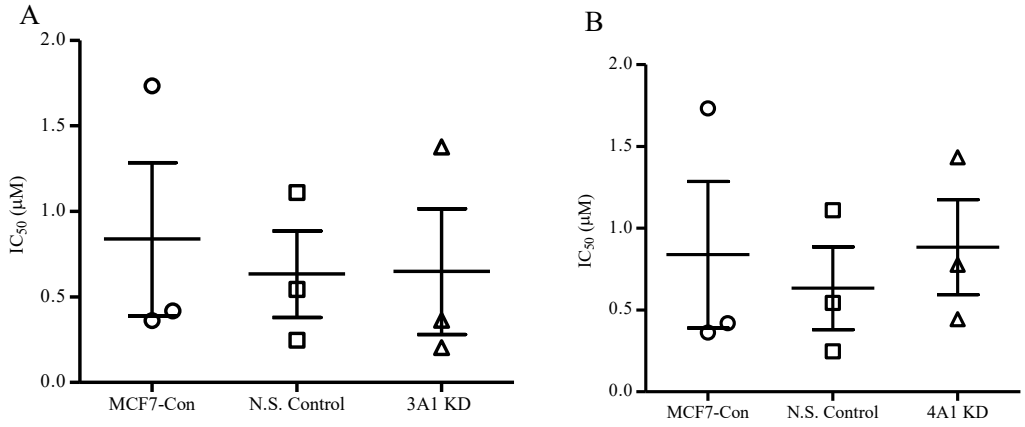

Figure 17: The effect of *SLCO3A1* and *4A1* lentiviral shRNA treatment on the IC_{50} values of doxorubicin in MCF7 cells. Each bar represents the mean and standard error of the mean of 4 independent replicates. Statistical analysis was conducted using a one-way ANOVA followed by a Bonferroni post test. A value of $P \leq 0.05$ was considered significant.

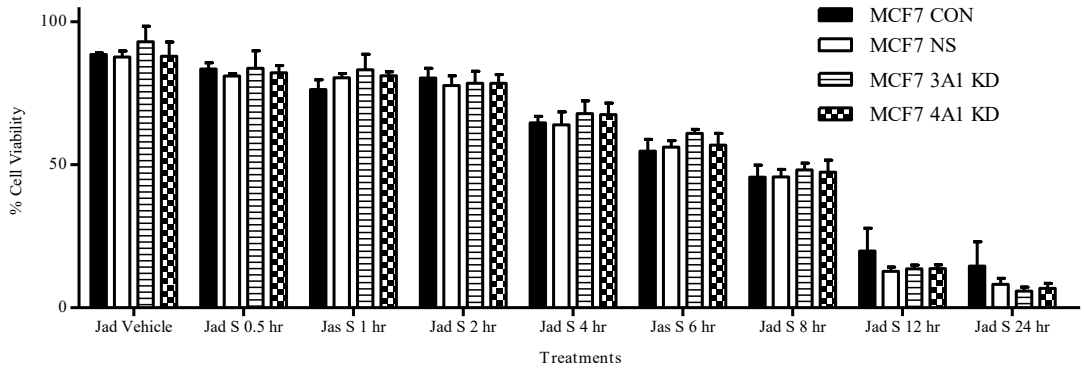

Figure 18: Cell viability assay of jadomycin S (10 μM) at different timepoints in different knockdown cell lines and control cell lines.

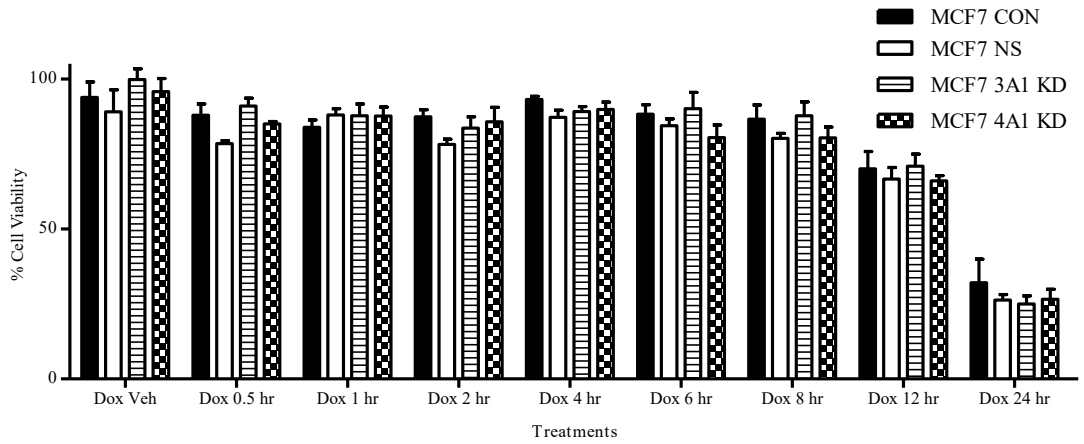

Figure 19: Cell viability assay of doxorubicin (10 μM) at different timepoints in different knockdown cell lines and control cell lines.

CHAPTER 4. DISCUSSION

4.1 Understanding SLCO Expression Profiles

Drug discovery and design is a multi-step process, including steps such as structure activity relationship development, mechanism elucidation, and formulation. The more that can be known about how a drug works, the more effective and tailored treatment can become. For example, personalized cancer treatments are increasing in popularity, especially using tumor genotyping tools and assays.[102] If it is found that drug targets are highly expressed in the tumor, it is more likely that the treatment will be effective. If a transporter is determined to be responsible for the uptake of jadomycins, this is one more protein that can be clinically evaluated for expression in the tumor to determine if a jadomycin might be an appropriate treatment.

The pattern of gene expression of SLCO transporters changed between the MCF7-CON cells and all MDR MCF7 cells, suggesting that SLCO transporter expression is dependent on expression of ABC transporters. The highest transporter expressed in MCF7-CON cells was *SLCO1C1*; whereas MDR MCF7 cells consistently express *SLCO4A1* highest amongst the transporters, followed by *3A1* and *4C1*. There was no change in *SLCO4A1, 3A1, 1B1, 2B1, 1A2, 1C1, 4C1,* and *6A1* in MDR MCF7 expression cells when compared to MCF7-CON cells.

Membrane transporters are responsible for intracellular solute concentrations, and the two major superfamilies are SLC and ABC.[103] It is possible that this shift in SLCO transporter expression in MDR MCF7 cells versus the MCF7 cells is linked to an increase in the function of the ABC efflux transporters. ABC efflux transporters do not only export drugs, but also amino acids, sugars, nucleosides, vitamins, peptides, lipids, and more.[57] An increase in these ABC transporters may cause a loss of essential biomolecules. This decrease in necessary biomolecules may have been compensated by a corresponding uptake via SLCO transporters, triggering an increased expression as seen in the data. Numerous substrates are shared between SLCO transporters and ABC transporters, certain prostaglandins and steroids for example, further supporting this theory.[104–107] *SLCO3A1* and *SLCO4C1* both have significantly higher expression in MCF7-TXL cells than in MCF7-CON cells. This suggests a relationship between

SLCO3A1 and *SLCO4C1* to the ABCB1 transporter that is overexpressed in MCF7-TXL cells. In 2007 an interaction was already established between *SLCO4C1* and ABCB1, as they both transported the type 2 diabetes drug, sitagliptin in and out of renal proximal tubules, respectively.[108] Relationships such as this suggest that there are more potentially shared substrates, creating a link between the two transporters.

It was anticipated that there would be a significant upregulation of SLCO transporters in cancer cells due to the high demand for nutrients and a known link of SLC transporters to cancer.[109] However, MCF-10A cells appeared to be expressing equal, and in several cases, higher SLCO transporter expression than most cancer cell lines. In 2016, Bhutia *et al* reported that SLC transporters can not only act as tumor promoters, but also tumor suppressors. There are four transporters belonging to the SLC gene family that have been identified as tumor suppressors. These transporters move molecules linked to inhibition of histone deacetylases (HDAC) such as butyrate, propionate, and pyruvate.[110] Pyruvate has been identified as a substrate for *SLCO2A1*, and the results above show that MCF-10A has the highest expression of *SLCO2A1* over all other cell lines, as shown in Figure 10D. It is possible that other transporters of high expression in both cancerous and non-cancerous cells can uptake tumor promoters or suppressors depending on the needs of the cells.

Some SLCO transporters expression is regulated by hormones and ligands. The phenotype differences in the cell types studied would cause them to have different intracellular hormone and ligand concentrations. These can act as regulatory factors, playing a direct impact on SLCO transporter expression. This will support the findings that the cancer cells studied have variable SLCO transporter expressions across cell type. For example, *SLCO1A2* is regulated by nuclear receptors. *SLCO1A2* is upregulated in human breast cancer cells by rifampin, which binds to the PXR nuclear receptor. *SLCO1A2* expression also is significantly influenced by the steroid and xenobiotic receptor (SXR) in breast carcinoma cell lines. *SLCO1A2* is also upregulated in response to increase levels of bile acid levels, which would target the small intestine and stomach. *SLCO1A2* was also shown to be upregulated by androgen absence in prostate cancer cells.[111] The *SLCO4C1* promoter was found to be activated by an AhR receptor ligand, 3-methylcholanthrene. In hepatocytes, SLCO1B transporters are frequently upregulated by

exposure to hepatocyte growth factors. [93] This variety of regulatory factors suggest that is it plausible that there are hormones and growth factors that are taken up by other transporters in breast cancer cells, or varying levels of regulatory factors among the breast cancer panel, leading to the changes in SLCO expression seen among breast cancer cell types.

4.2 Influence of Transporter Expression in Jadomycin Cytotoxicity

The gene expression data shows that *SLCO3A1* was effectively knocked down when compared to MCF7-CON, and was significantly different than MCF7 NS CON. However, data shows that *SLCO4A1* gene expression was not significantly different than either control cell line. Perhaps the specific shRNA was not effective and additional shRNAs for *SLCO4A1* need to be tried. The baseline expression may not have been high enough to observe an effect in the MCF7 cells, and other cancer cells with higher *SLCO4A1* expression will require testing. Insignificant changes in *SLCO3A1* and *4A1* expression between MCF7 NS CON and MCF7 CON cells, indicating that the virus transduction is causing specific SLCO gene expression.

SLCO3A1 expression had no significant effect on cytotoxicity of jadomycin S in the 72-hour studies. This suggests that *SLCO3A1* does not uptake jadomycin S. Alternatively, the long incubation time may have allowed for drug uptake to reach a steady state level in the MCF7 3A1 KD cells, resulting in similar toxicity levels in knockdown cells and control cells. To investigate this further we conducted cytotoxicity assays over short time periods. However, no differences were observed, ruling out a time-dependent effect of *SLCO3A1* knockdown on jadomycin S uptake and toxicity. The final possibility is that even if there was reduced jadomycin S uptake, the function of other SLCOs or uptake transporters could compensate. This is likely given that overlapping substrate specificity of the different SLCOs.

Although there was not a significant reduction on gene expression of *SLCO4A1* in MCF7 4A1 KD cells, we decided to examine cytotoxicity in the 4A1 KD cells in case the functional assay was more sensitive at detecting differences in the knockdown. We observed no change in jadomycin S cytotoxicity. However, given the lack of *SLCO4A1* knockdown, we cannot conclude if jadomycin S is transported by *SLCO4A1* or not.

Although results show that *SLCO4A1* and *3A1* knockdown cells do not significantly influence jadomycin toxicity, it is possible that jadomycin uptake is non-selective and may be compensated by other transporters in the family or the SLC family. *SLCO* transporters are responsible for the influx of numerous different anticancer drugs. For example, *SLCO1B3* plays a role in methotrexate, docetaxel, glutathione, and paclitaxel uptake.[93,110] *SLCO1B1* also plays a role in methotrexate uptake as well as atrasentan.[112] Transporters *SLCO1A2*, *1B1*, and *1B3* have a long list of reported substrates, much longer than *SLCO4A1* and *SLCO3A1*. This could potentially be due to these transporters having low specificity or being simpler to study and identify. Therefore, they may be suitable to investigate next.

Non-selective uptake of jadomycin S is further supported by the fact that jadomycin S IC_{50} values are similar between various cell lines. We reported the IC_{50} values of jadomycin S in MCF7-CON, BT474, SKBR3, and MDA-MB-231 are 3.38 μM ± 0.09, 3.09 μM ± 0.54, 3.08 μM ± 0.73, 2.79 μM ± 0.48, respectively.[67] The similar IC_{50} values between cell lines that have various transporter expression further suggests that jadomycin uptake can be performed by different transporters.

All cell viability assays were conducted using jadomycin S, that has serine as an R group. It is essential in the future to conduct the same experiments with jadomycin B and F, as their R side chains are hydrophobic aliphatic and hydrophobic aromatic, respectively. It is possible that a significant result will be seen using these jadomycin treatments, as SLCO transporters take up organic compounds, and having an organic sidechain theoretically should assist with uptake.

The doxorubicin IC_{50} values of the MCF7 NS, MCF7 3A1 KD, and MCF7 4A1 KD cells are anticipated to all be approximately equal between cell lines. Results did show that there was no significant difference between any cell lines, however, the pattern seen in doxorubicin IC_{50} values were mirrored in jadomycin S results described above. This data suggests that jadomycin S is unaffected by *SLCO4A1* and *3A1*, unless doxorubicin also is transported via *SLCO4A1* and *3A1* transporters or other transporters compensated for the knockdown. In 2009, Okabe *et al* profiled SLCO and SLC22 genes in the NCI-60 cancer cell lines to identify the uptake of doxorubicin, leading to the discovery that SLC22A4 resulted in an increased cellular uptake of doxorubicin.[113] SLC22A16 was also

identified as a mediator of doxorubicin uptake.[114] Since other transporters in the same family were found to transport doxorubicin, it is possible that *SLCO4A1* and *SLCO3A1* may still play a role in doxorubicin transport, making it an unsuitable control.

All cell viability assays were conducted after 15 passages, and at this time the SLCO transporter mRNA levels in knockdown cells may have changed. This is likely due to the puromycin selectivity treatments being halted. Once puromycin is stopped the cells may gradually lose the knockdown phenotype. The cells should be administered with puromycin throughout culture to ensure that the knockdown phenotype is maintained.

4.3 Future Experiments and Limitations

The experiments in this thesis were thoughtfully and efficiently designed. However, there were several limitations to the experiments conducted. The MCF7-CON cells that were used for knocked down did not have the highest *SLCO3A1* and *4A1* transporter expression among cell lines. There were other cell lines such as MCF7-MITX and MCF7-TXL that have a higher expression of *SLCO3A1.* Using a different cell line may result in larger functional changes after the knockdown, leading to larger differences in cytotoxicity between cell lines. Only two SLCO transporters, *SLCO3A1* and *4A1*, were assessed in this project. Possible uptake by other transporters would allow for jadomycins to exude their cytotoxic effect. Elucidating which transporters are responsible for jadomycin uptake may require inhibiting or knocking down multiple transporters at a time.

It is possible that knockdown of the transporters is lost with time if puromycin treatments are not continued throughout the culture. Cytotoxicity assays should be conducted in cells that are consistently treated with puromycin throughout the culture. Additionally, we are lacking a cytotoxic positive control that specifically binds to *SLCO3A1* and *4A1.* It may be necessary to develop a cell line that knocks down numerous transporters at a time to overcome this obstacle.

In future experiments, the transporter protein expression in knockdown and control cells will be characterized using FACS or western blot. This will ensure that the transporter levels are being altered, as qPCR can only validate that mRNA levels are changing. Expression of mRNA do not necessarily always correspond with protein levels.

Apoptosis, ROS, and accumulation experiments will be conducted in the knockdown cell lines to detect the influence and presence of jadomycin intracellularly. Jadomycins have an impact on both apoptosis and ROS levels; these can further evaluate the impact that transporter expression has on jadomycin activity. Accumulation assays will directly show the impact of transporter expression on the uptake. Due to chemical differences, other jadomycins should be investigated. Substrates for transporters is dependent on chemical structure, therefore, transporter uptake will vary.

CHAPTER 5. CONCLUSION

Jadomycins have already shown much potential as anticancer agents *in vitro*, especially due to the fact that these drugs are effective against MDR breast cancer cells. Although the mechanism of action of jadomycins are becoming more understood, the route of uptake is still unknown. It is critical to investigate this in order to better utilize jadomycins. SLCO transporters are a family of transmembrane proteins that can uptake amphiphilic organic compounds into cells. It was hypothesized that jadomycins were taken up into the cell through these SLCO transporters. This study included evaluating the gene expression of SLCO transporters in a panel of breast cancer cell subtypes followed by knocking down *SLCO4A1* and *SLCO3A1* and evaluating how the physiological change led to changes in cytotoxicity. Knockdown of *SLCO3A1* was successful, however *SLCO4A1* expression between knockdown cells and control cells is not significantly different. Therefore, we cannot conclude if jadomycin S is or is not an *SLCO4A1* substrate.

Results show that breast cancer cells express SLCOs, and the pattern of expression is dependent on drug resistance and hormone receptor profile. There is no significant difference between IC_{50} values of knockdown and control cell lines in jadomycin or doxorubicin, therefore jadomycin and doxorubicin cytotoxicity is not influenced by loss of *SLCO3A1* function. Jadomycin S retains its potency despite the knockdown of highly expressed drug uptake *SLCO3A1* transporter. This is potentially a beneficial property that may contribute to jadomycin effectiveness in a variety of breast cancer cells despite their different drug uptake transporter profiles.

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