THE ANTICANCER ACTIVITY AND MECHANISMS OF ACTION OF JADOMYCINS IN MULTIDRUG RESISTANT HUMAN BREAST CANCER CELLS

by

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Submitted in partial fulfilment of the requirements
for the degree of Doctor of Philosophy

at

Dalhousie University
Halifax, Nova Scotia
June 2017

DEDICATION

To Liz.

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ABSTRACT

Breast cancer is the most common cancer in women, and approximately one third of all breast cancers will ultimately metastasize. Metastatic breast cancer is classified as an incurable disease. The development of multidrug resistance (MDR) is largely responsible for the difficulty associated with its treatment.

Jadomycins are natural products biosynthesized by the bacteria *Streptomyces venezuelae* International Streptomyces Project (ISP) 5230, which have anticancer activity but poorly defined mechanisms of action. Preliminary research in our laboratory showed jadomycins retain their cytotoxic potency in MDR-MCF7 breast cancer cells that overexpress certain ATP-binding cassette (ABC) drug efflux transporters. My research goal was to more completely characterize jadomycin cytotoxicity profiles and pharmacological mechanisms to better understand their potential applications in the treatment of MDR breast cancer.

First I show multiple jadomycin analogues retain their cytotoxic potency in ABCB1, ABCC1, or ABCG2-overexpressing MCF7 and ABCB1-overexpressing MDA-MB-231 versus control MCF7 and MDA-MB-231 breast cancer cells. Inhibitors of ABCB1, ABCC1, and ABCG2 minimally affect jadomycin cytotoxicity, suggesting jadomycins are poor ABC transporter substrates.

I then show jadomycins have multiple cytotoxicity mechanisms that are influenced by breast cancer cell type. Jadomycins increase intracellular reactive oxygen species (ROS) in MCF7 and MDA-MB-231 cells. In MCF7 cells, inhibition of the antioxidant thioredoxin reductase with auranofin potentiates jadomycin cytotoxicity. Conversely, neutralization of ROS with the antioxidant N-acetylcysteine decreases jadomycin potency, but not efficacy, evidencing a ROS-independent cytotoxicity mechanism. In MDA-MB-231 cells, jadomycins cause DNA double strand breaks and apoptosis. These effects are not blocked by ROS neutralization or enhanced by antioxidant inhibition, further evidencing ROS-independent mechanisms of cytotoxicity. Additional assays show that ROS-independent mechanisms include aurora B kinase inhibition and type II topoisomerase inhibition, which can lead to DNA double strand breaks and apoptosis. These cytotoxicity mechanisms are preserved in the ABC-transporter overexpressing MCF7 and MDA-MB-231 cells, thus helping explain how jadomycins remain effective in these cells.

My research is the first to detail the polypharmacology of jadomycins' anticancer activity. Additionally, I describe the promising jadomycin activity against ABC-transporter overexpressing MDR breast cancer cells, supporting further research into how these natural products may be used as MDR metastatic breast cancer treatments.

LIST OF ABBREVIATIONS USED

231-CON	Control MDA-MB-231 cell line
231-TXL	Paclitaxel-resistant, *ABCB1*-overexpressing MDA-MB-231 cell line
3-AT	3-amino-1,2,4-triazole
4T1	Mouse mammary carcinoma cell line
A549	Human alveolar epithelial cancer cell line
ABC	ATP-binding cassette
B	Isoleucine
BRCA1/2	Breast cancer susceptibility genes 1/2
BT474	Human invasive ductal carcinoma cell line
Cas9	CRISPR-associated protein 9
CM-DCF	5-(and 6-)chloromethyl-2'7'-dichlorohydrofluorescein
CM-DCFH_2-DA	5-(and 6-)chloromethyl-2'7'-dichlorodihydrofluorescein diacetate
CRISPR	Clustered regularly interspaced short palindromic repeats
DDC	Sodium diethyldithiocarbamate
DNL	D-norleucine
DNV	D-norvaline
D-Pen	D-penicillamine
DS	D-serine
EC_{50}	Half maximal effective concentration
EA	Ellagic acid
ER	Estrogen receptor
F	Phenylalanine
FBS	Fetal bovine serum
G	Glycine
GAPDH	Glyceraldehyde phosphate dehydrogenase

GPx	Glutathione peroxidase
GSH	Glutathione
GS-SG	Glutathione disulfide
GST	Glutathione-*S*-transferase
γH2AX	Phosphorylated histone H2AX
H640	Human non-small-cell lung cancer cell line
HEK-293	Human embryonic kidney cells - 293
HeLa	Cervical cancer cell line
HepG2	Human hepatocellular carcinoma cell line
HER2	Human epidermal growth factor receptor 2
His3	Histone H3
IC_{50}	Half maximal inhibitory concentration
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
ISP	International Streptomyces project
kDNA	Kinetoplast DNA
Ko-143	Ko-143 hydrate
L	Leucine
LDH	Lactate dehydrogenase
MCF7	Michigan Cancer Foundation – 7 / Human invasive ductal carcinoma cell line
MCF7-CON	Control MCF7 cell line
MCF7-ETP	Etoposide-resistant, *ABCC1*-overexpressing MCF7 cell line
MCF7-MITX	Mitoxantrone-resistant, *ABCG2*-overexpressing MCF7 cell line
MCF7-TXL	Paclitaxel-resistant, *ABCB1*-overexpressing MCF7 cell line
MDA-MB-231	MD Anderson series metastatic breast 231 / Human invasive ductal carcinoma cell line

MDA-MB-435	MD Anderson series metastatic breast 435 / Human invasive ductal carcinoma cell line
MDR	Multidrug resistance
miRNA	MicroRNA
MK-571	MK-571 sodium salt hydrate
mTOR	Mammalian target of rapamycin
MTT	Thiazolyl blue methyltetrazolium bromide
NAC	N-acetyl cysteine
Nrf2	Nuclear factor (erythroid-derived 2)-like 2
PARP	Poly(ADP-ribose) polymerases
PD-1	Programmed death-1
PD-L1	Programmed death-ligand 1
P-His3(Ser10)	Histone H3 phosphorylated at serine 10
PI3K	Phosphoinositide 3-kinase
PPIA	*Peptidylprolyl isomerase A*
PR	Progesterone receptor
Prx	Peroxiredoxin
PTEN	Phosphatase and tensin homologue
qPCR	Quantitative real time polymerase chain reaction
ROS	Reactive oxygen species
S	Serine
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SERM	Selective estrogen-receptor modulator
SKBR3	Human invasive ductal carcinoma cell line
SLCO	Solute carrier organic anion
SOD1/2	Superoxide dismutase 1/2
SPhG	*s*-phenylglycine

T	Threonine
T47D	Human invasive ductal carcinoma cell line
TNF	Tumour necrosis factor
TP53	Tumour protein p53
Trx	Thioredoxin
TrxR	Thioredoxin reductase
TXL	Paclitaxel (Taxol)
VRP	Verapamil
W	Tryptophan
Z-VAD	N-Benzyloxycarbonyl-Val-Ala-Asp(O-Me) fluoromethyl ketone (Z-VAD[OMe]-FMK)

ACKNOWLEDGEMENTS

I would first like to thank my kind, understanding, and very patient supervisor and mentor, Dr. Kerry Goralski. You have always been there to support me, and I thank you sincerely for believing in me even when I did not believe in myself. I would not have been able to finish this adventure without your support.

Thank you to my lab mates, both past and present. Sebastian, Mark, Vicky, Jay, Heather, Matt, Nick, and Leah, you have all taught me many things and have had a lasting impact on my life, both professionally and personally. I am a better person and scientist for having known you all.

To Sandi and Luisa, simply put you are both amazing people. You two have helped me countless times throughout my time at Dalhousie. I hope you both know just how appreciated you are by everyone in Pharmacology and myself. Thank you.

To my departmental committee members, Drs. Kishore Pasumarthi and Jim Fawcett, thank you for always having my best interests at heart. You have both been extremely understanding during my greatest times of struggle, and have always kept in mind what was best for me as a student and human being.

Thank you to the Department of Pharmacology and everyone in it. Our department is filled with intelligent, hard-working, and friendly people, and I thank you all for being such a supportive group. To the College of Pharmacy, thank you for adopting me as one of your own, and for all of the help you have provided me throughout my years in Kerry's lab.

Last of all, I would like to give a huge thanks to all of my friends and family who have supported me throughout my studies. I would not have been able to complete this path I have chosen without your help along the way, for which I will be forever grateful.

CHAPTER 1.00.00: INTRODUCTION

1.01.00: PREFACE

Breast cancer remains the most commonly diagnosed cancer in Canadian females, with approximately 1 in 9 Canadian women expected to be diagnosed with some form of the disease in their lifetime [1]. Largely due to improved screening programs and increased awareness, breast cancer is now often caught in its early stages when it is most treatable [2]. However, late stage metastatic breast cancer remains an incurable disease, primarily due to the development of multidrug resistance (MDR) [3,4]. My research has focussed on a group of compounds called jadomycins, natural products biosynthesized by the soil bacteria *Streptomyces venezuelae* ISP5230, which are able to largely retain their cytotoxic potency in certain MDR breast cancers cells [5,6]. The first chapter of this dissertation will serve to introduce the prevalence and pathology of cancer in general followed by breast cancers, with a focus on late stage, metastatic, MDR breast cancers. I will then provide a description of jadomycin analogues and what was known pertaining to their anticancer activity prior to my research. The second, third, and fourth chapters will describe my original research detailing the cytotoxic potency of jadomycins in MDR breast cancers and the multiple mechanisms of action through which they act. The final chapter will discuss the implications of these results on future jadomycin research and their potential as anticancer treatments.

1.02.00: AN INTRODUCTION TO CANCER

Cancers are complex tissues composed of multiple and distinct types of cells that interact with each other. Even the healthy cells surrounding a tumour that were once thought to be simple bystanders to the cancer development (tumourigenesis) are now known to be actively recruited by the cancerous cells, forming the tumour-associated stroma (the supportive framework of the tumour that consists of important connective tissue, such as blood vessels). This forms a tumour micro-environment vital to the cancer cells' growth and proliferation that contains more than just the cancerous tissue itself [7].

The development of cancer involves dynamic changes to the genome [8]. Analyses of cancerous tumours at various stages suggest that they become increasingly aggressive through the accumulation of multiple genetic changes. These changes typically fall into two categories: gain-of-function mutations in proto-oncogenes, and loss-of-function mutations in tumour suppressor genes [9].

Proto-oncogenes are genes that, when working properly, help cells to grow, such as during fetus development or to create new tissue after an injury. Mutations can occur that cause these proto-oncogenes to be activated more than normal or permanently "switched on". When this happens these genes are re-labelled as oncogenes. The activation of oncogenes can result in cells proliferating out of control, which is one of the steps required in the development of a cancerous (malignant) tumour [2]. The genes that encode for human epidermal growth factor 2 (*ERBB2*), phosphatidylinositol 3-kinase (*PI3KCA*), C-Myc (*MYC*), and cyclin-D1 (*CCND1*) are examples of oncogenes that are frequently dysregulated in cancer, and in particular breast cancer [9].

When working properly tumour suppressor genes slow down or stop cell division, repair damaged DNA, and signal cells to undergo apoptosis (programmed cell death) when they are damaged beyond repair. In this way tumour suppressor genes prevent cells from proliferating out of control and becoming cancerous. Mutations to the DNA of tumour suppressor genes can decrease their activity or turn them off completely, allowing for once healthy cells to bypass these checkpoints and grow out of control [2]. Examples of tumour suppressor genes include tumour protein 53 (*TP53*), phosphatase and tensin homolog (*PTEN*), ataxia telangiectasia mutated serine/threonine kinase (*ATM*), RAD50 double strand break repair protein (*RAD50*), and partner and localizer of BRCA2 (*PALB2*) [9].

Epigenetic dysregulation can also contribute to the development and growth of cancer cells through altered DNA methylation or histone modifications. For example, genes that encode enzymes that are involved in the modification of DNA histones can be mutated, causing inactivation of tumour suppressor genes in renal cell carcinoma [9,10].

Some gene mutations that can increase one's chances of developing cancer are genetic, in that they are passed on from generation to generation and are not the result of environmental factors. For example, mutations can occur in the tumour suppressing breast cancer susceptibility genes 1 and 2 (*BRCA1* and *BRCA2*), inhibiting their normal activity and increasing one's risk of developing breast or ovarian cancer. However, the majority of mutations that lead to cancer are not genetic, in that they occur after birth. A number of environmental factors have been determined to damage DNA and increase the risk of developing cancer, including smoking, radiation, obesity, certain hormones, chronic inflammation, and pollution. The development of cancer is a stepwise process,

whereby multiple mutations occur to the DNA typically over the course of many years. When enough mutations develop that inhibit tumour suppressor genes and promote oncogenes, cancer can occur [2,11].

1.03.00: THE HALLMARKS OF CANCER

As described by Hanahan and Weinberg in two separate seminal review papers, there are six "hallmarks of cancer" that describe the unique properties of cancerous cells that differentiate them from healthy cells, followed by two enabling characteristics and two emerging hallmarks, summarized in **Table 1** and described in greater detail below [7,8].

Table 1: The hallmarks, enabling characteristics, and emerging hallmarks of cancer [7].

Hallmarks	**Enabling characteristics**
Sustaining proliferative signaling	Genome instability and mutation
Evading growth suppressors	Tumour-promoting inflammation
Resisting cell death	
Enabling replicative immortality	**Emerging hallmarks**
Inducing angiogenesis	Reprogramming energy metabolism
Activating invasion and metastasis	Evading immune destruction

The hallmarks of cancer are as follows. **(1) Sustaining proliferative signaling**. Healthy cells carefully control the signals that instruct them to grow and proliferate, resulting in them dividing only when needed and in an orderly fashion. Cancer cells deregulate these signals, causing the cancer cells to continuously and uncontrollably proliferate. For example, cancer cells can develop the ability to produce growth factor ligands themselves allowing them to stimulate their own growth, or to send these growth factors to the normal cells within their associated tumour-supporting stroma to improve the tumour

microenvironment [12,13]. Increased proliferative signalling can also be the result of elevated levels of growth receptor proteins displayed at the cancer cell surface, making them more responsive to growth factors [7]. **(2) Evading growth suppressors**. Cancer cells have the ability to avoid cellular signals used to stop excessive growth and proliferation. The mutation of tumour suppressor genes can result in growth suppressors not being properly produced, resulting in the cell not receiving the signal to stop proliferating. Healthy cells also typically stop growing through what is called "contact inhibition", in that increased cell-to-cell contact between cells in a tissue or culture signals the healthy cells to stop proliferating. Many cancer cells do not display this contact inhibition, resulting in further proliferation [7]. **(3) Resisting cell death**. Healthy cells undergo apoptosis when they are damaged beyond repair. Many cancers are known to become resistant to these apoptotic signals, allowing them to evade death. The most common mechanism is through the loss of function of the TP53 tumour suppressor protein, eliminating a damage sensor from the cell that will no longer respond to apoptotic signals. In this way cells that would normally undergo apoptosis are able to continue proliferating, despite being heavily damaged, propagating the damaged cell line [7,14]. **(4) Enabling replicative immortality**. Normal cells have a limited number of times they can divide. Near the end of their proliferative-lifecycle these cells enter senescence, a state of viability without proliferation, or crisis, where they die. Some cells are able to surpass these mechanisms, leading to what is termed immortalization. There is evidence that this immortalization is in large part the result of increased telomerase activity, which is overexpressed in approximately 90% of cancers. Telomerase is an enzyme that adds repeat telomere sequences to the ends of DNA, protecting it during

progressive cell division cycles. By extending telomeric DNA, this increased telomerase is able to counter the progressive telomere erosion that occurs in healthy cells, preventing the cancerous cells from entering senescence or crisis [7,15]. **(5) Inducing angiogenesis**. In adults, the development of new vasculature (angiogenesis) to carry blood is largely quiescent, other than during wound healing or female reproductive cycling. Conversely, during tumour development, an "angiogenic switch" is almost always activated, resulting in the formation of new vasculature to the tumour site to provide nutrients for the ever-expanding cells [16]. This chronic activation of angiogenesis is the result of altered expression of angiogenic regulators, such as increased expression of the angiogenesis inducer vascular endothelial growth factor-A or decreased expression of the angiogenesis inhibitor thrombospondin-1 [7,17,18]. **(6) Activating invasion and metastasis**. Late stage, metastatic cancers are known to have altered shapes and mechanisms that control their attachment to other cells or to the extracellular matrix. The best characterized example is the loss-of-function of E-cadherin in many metastatic cancers, a protein that aids in cell-cell or cell-extracellular matrix adhesion. Loss of such protein activity allows for cancer cells to detach from the primary tumour, travel through the nearby blood or lymphatic vessels, escape from the lumina of these vessels, and ultimately colonize elsewhere in the body forming micrometastases [7,19].

Following these hallmarks, two "enabling characteristics" of cancer cells were proposed. **(1) Genome instability and mutation**. The rate of mutation in normal cells is typically quite low, due to the ability of the genome maintenance systems to detect and resolve DNA defects. The gene alterations present in tumourigenic cells cause them to be more predisposed to further mutations than healthy cells, such as through decreased

expression of DNA repair proteins, making it easier for these cells to develop the above-described hallmark capabilities of cancers [7,20]. **(2) Tumour-promoting inflammation**. Tumour cells have long been associated with immune system activity and inflammation, processes which are potentiated by increased oxidative stress [21], and there is now significant evidence that these immune cells have tumour-promoting effects and stimulate the acquisition of hallmark capabilities in early cancer cells [7]. Chronic inflammation can contribute to the development of hallmark capabilities by supplying bioactive molecules to the tumour microenvironment, such as growth factors, survival factors, and proangiogenic factors through the presence of immune cells [7,22,23,24].

The final two "emerging hallmarks" are: **(1) Reprogramming energy metabolism**. The unfettered growth that is observed with cancer cells requires an immense amount of energy, and therefore requires adjustments to the cells' energy metabolism to fuel this increased proliferation. This increase in energy production appears to occur through increased glycolysis, which occurs even in tumour cells with access to oxygen, and has been labelled aerobic glycolysis or colloquially termed the "Warburg effect". This is unusual since glycolysis is 18-fold less efficient than oxidative phosphorylation in the production of ATP and typically only occurs in cells when oxygen is unavailable. However, it is believed that this glycolytic-dependency aids the sustained proliferation of cancer cells as intermediates formed in glycolysis are used in various biosynthetic pathways, such as those that generate amino acids, nucleosides, and lipids, thus facilitating the creation of the macromolecules and organelles required for cell division [7,25]. **(2) Evading immune destruction**. While chronic inflammation has been linked to tumourigenesis, the immune system also plays an important role in identifying and

removing early- and late-stage cancer cells. Cancer cells that successfully form full-fledged tumours therefore must have some mechanism that allows them to avoid being detected and destroyed by immune cells. One of the key methods through which cancer cells develop resistance to immune system activity is a process called “immune editing” or “immunoediting”, whereby the immune system detects tumour cells and kills most of them, but fails to detect a few mutated cells that are only weakly immunogenic [26,27]. These remaining cells are then able to continue proliferating and spread undeterred by the body’s immune system. This immunoediting can occur through a variety of methods, such as modifying T-cell function, altering antigen presentation to be unrecognizable by immune cells, and increasing the production of immune suppressive mediators [7,27].

The hallmarks of cancer describe how cancer cells can develop and how they differ from normal cells, and are a general explanation covering all varieties of tumours. However, each type of cancer is unique with many differences separating them from cancers of other organs or tissues, resulting in many different prognoses and treatment options. The focus of my doctoral work has been on breast cancer and its treatment, and therefore the remainder of my dissertation will also be focussed on this disease.

1.04.00: INTRODUCTION TO BREAST CANCER

Breast cancer occurs when normal breast cells undergo multiple genetic mutations resulting in the phenotypes described above. The tumour is considered malignant (cancerous) if the cells are able to invade surrounding tissue or metastasize to other parts of the body. Benign (non-cancerous) tumours are more common than malignant cancers,

are not at risk of spreading, and do not pose the same threat as malignant tumours. The majority of breast cancers develop in the milk ducts (ductal carcinomas) and most others occur in the glands that produce milk, called lobules (lobular carcinomas). There are a few other breast cancer subtypes that can develop that are not ductal or lobular, such as inflammatory breast cancer or angiosarcoma, though these make up a small minority of all breast cancers diagnosed [2].

Breast cancer is the most commonly diagnosed malignant disease in women, with 1 in 9 Canadian women expected to be diagnosed with breast cancer at some point in their lives [1]. Breast cancer is the second-leading cause of cancer-related death (second to lung cancer) in women, with the total chance that any given woman will die of breast cancer at approximately 3%. The incidence of breast cancer increased steadily between approximately the years 1980 and 2000, when it began to decrease followed by a sharp 7% decrease between 2002 and 2003 [2]; this decline is believed to be the result of a reduction in the use of hormone therapy in post-menopausal women to alleviate their menopause symptoms, due to the discovery that hormone therapy increases a woman's risk of developing breast cancer [28]. Since 2004 the relative breast cancer incidence rate has remained relatively stable, while death rates from breast cancer have been decreasing since 1989. This is believed to be due to a combination of increased awareness and improved detection methods allowing for breast cancer to be discovered and treated earlier, along with improvements in available breast cancer treatments [1].

1.05.00: BREAST CANCER RISK FACTORS AND CAUSES

There are a variety of risk factors that have been linked with increasing one's risk of developing breast cancer that can be loosely categorized as (1) non-modifiable factors and (2) modifiable factors.

Some of the non-modifiable factors that increase one's risk of developing breast cancer are as follows. (i) Gender; women are >100 times more likely to develop breast cancer than men [2]. (ii) Ageing; most invasive breast cancers occur in women >55 years old [29]. (iii) Certain inherited genes and family history; the most common cause of hereditary breast cancer being mutations in *BRCA1* and/or *BRCA2*, two tumour suppressor genes that can lose their activity when mutated. Mutant *BRCA1* or *BRCA2* can result in a 46% to 87% chance of a woman developing breast cancer in their lifetime [30]. (iv) Race and ethnicity; Caucasian women have a slightly higher chance of developing breast cancer than African, Asian, Hispanic, or Native women [2]. (v) Breast tissue density; increased breast tissue density mainly occurs due to higher numbers of lobules and milk ducts, the most common sites in which breast cancer develops; women with dense breast tissue are estimated to have a 1.2 to a 4-fold increased risk of developing breast cancer versus women with low density breast tissue [31]. (vi) Starting menstruation at an early age (<12 years old) or starting menopause at a late age (>55 years old); this is believed to be at least partly due to an increased lifetime exposure of estrogen and progesterone, which can promote the development, growth, and proliferation of breast cancer cells [2,29]. (vii) Previous exposure to radiation therapy; being treated for another cancer, such as Hodgkin's lymphoma, previously in an individual's life with radiation therapy increases the risk of developing a future breast

cancer due to damage from the radiation to healthy cells [29]. (viii) Having had a previous breast tumour (even if benign); this is associated with an increased risk that a second unrelated breast cancer will develop later in the patient's life [29].

Modifiable risk factors that can be changed include: (i) Drinking alcohol; alcohol has been associated with an increased risk of developing breast cancer. However, more research is needed to better understand this correlation and the effect of moderate versus heavy drinking still needs to be ascertained [32]. (ii) Weight; being overweight or obese after menopause is known to increase risk, while the inverse is true in premenopausal women where obesity is associated with a decreased risk of developing breast cancer [33]. The increased risk observed in obese or overweight versus thin postmenopausal women is believed to be the result of increased exposure to estrogen, which is primarily produced by the fat cells after menopause [33]. Conversely, in premenopausal women most estrogen is produced by the ovaries, and the estrogen estradiol is quite lipophilic, and is therefore heavily sequestered in fat tissue [34]. This reduces the total amount of bioactive estrogen available in the body, lowering the obese or overweight premenopausal woman's exposure and therefore her risk of developing breast cancer [33,34]. (iii) Exercise; increased exercise has been linked to a decreased risk of developing breast cancer, which may be due to improved function of the immune system or exercise-induced alterations in ovarian function [35,36]. (iv) Age at first pregnancy; having no children or waiting until >30 years old can increase the risk of developing breast cancer. The protective effect of early pregnancy is believed to be the result of reduced susceptibility of the fully differentiated mammary glands to carcinogens due to a decrease in their cell proliferation, and due to a changed hormonal environment induced

by altered levels of estrogen, progesterone, and growth factors, for example, that occurs during pregnancy [37]. (v) Use of oral contraceptives; use of the birth control pill increases the risk of developing breast cancer, likely due to increased lifetime exposure to estrogen and progesterone [38]. (vi) Hormone therapy after menopause; as previously mentioned, this increased lifetime exposure to estrogen and progesterone has been linked to an increased risk of developing breast cancer [39]. (vii) Breastfeeding; breastfeeding for 1.5 to 2 years lowers the risk of developing breast cancer, which is thought to be due to a reduction in the total number of lifetime menstrual cycles or by lowering lifetime exposure to estrogen and progesterone [40].

As with other cancers, breast cancers are the result of multiple mutations to the DNA. Some DNA mutations are inherited, but most mutations that lead to breast cancer are acquired over one's lifetime. As described previously, *BRCA1* and *BRCA2* tumour suppressor gene mutations are the most commonly observed inherited gene mutations that can increase a woman's risk of developing breast cancer [30]. Other known cancer-associated mutations that can be inherited include those of the tumour suppressor genes *PTEN* [41] and *TP53* [42], though mutations in these genes are less commonly inherited than *BRCA* mutations. DNA mutations that are acquired throughout an individual's lifetime can result from exposure to radiation or carcinogens, for example, though most environmental causes of mutations are still unknown [2]. Gene mutations can also occur spontaneously, such as through mismatch errors during DNA replication [43].

BRCA mutations are considered "high-penetrance" since they often lead to cancer. While many women with high-penetrance mutations develop cancer, it is important to stipulate that most cancers are not caused by high-penetrance mutations. Instead, most

cancers result from multiple low-penetrance mutations or gene variations that are factors in cancer development that can build up over time until eventually the cells become cancerous. Each of these mutations has a small risk of cancer-development individually, but become significant as more and more mutations occur over time. These mutations can affect parameters such as hormone levels, metabolism, and angiogenesis to promote cancer cell growth or prevent the inhibition of this growth [2].

1.06.00: CLASSIFICATION OF BREAST CANCER

The majority of breast cancers are adenocarcinomas, cancers of epithelial cells that line breast tissue and originate in glandular tissue, with ductal and lobular carcinomas accounting for approximately 75 and 15% of all cases of breast cancer, respectively [2,44]. The remaining 10% of cases are mostly made up of mucinous, tubular, comedo, inflammatory, medullary, and papillary carcinomas [45]. Sarcomas, cancers of the muscle, fat, or connective tissue, can also occur in the breast, though they are very rare making up only ~0.1% of all cases of breast cancer [46].

Ductal carcinoma *in situ* (also known as intraductal carcinoma) is a non-invasive cancer "type", in that the cancerous cells have not invaded anywhere other than the inside of the ducts; the rest of the breast tissue and body are not affected by the cancer and it has not metastasized. However, it is currently impossible to accurately predict which cases of ductal carcinoma *in situ* will eventually spread and become metastatic, as some cases do and some do not [2]. About 20% of all new breast cancer cases are ductal carcinoma *in situ* [47], for which surgery results in a nearly 100% cure rate [48]. Invasive (or

infiltrating) ductal carcinoma is the most common type of breast cancer. Invasive ductal carcinoma starts in milk ducts and can penetrate the ductal walls, spreading into the fatty tissue of the breast. At this point it is possible for this cancer to metastasize out of the breast through lymph fluid or blood, spreading throughout the body. About 80% of invasive cancers are invasive ductal carcinoma. Invasive (or infiltrating) lobular carcinoma starts in the milk-producing lobules. Like invasive ductal carcinoma, it can also spread into the fatty tissue of the breasts and eventually metastasize. Invasive lobular carcinoma makes up about 10% of all invasive breast cancers [2,44].

Once the breast cancer has been typed it is "graded" based on how closely the cancer cells resemble healthy breast cells and how rapidly the cells are dividing. A lower grade means the breast cancer is less likely to spread, and higher grade means more likely; the grade values range between 1 and 3. Grade 1 cancer cells are "well differentiated", look most similar to normal cells with marked tubule formation and normal nuclear morphology, and are typically slow-growing. Grade 2 breast cancer cells are "moderately differentiated", look less like normal cells with some tubule formation and moderate nuclear pleomorphisms, and grow a little faster. Grade 3 cancer cells are "poorly differentiated", appear the least like normal cells with no tubule formation and marked nuclear pleomorphisms, and are typically fast-growing [49,50].

Breast cancers can then be further categorized based on their expression of the hormone receptors estrogen receptor (ER) and progesterone receptor (PR) and that of the transmembrane tyrosine kinase human epidermal growth factor receptor 2 (HER2; also known as receptor tyrosine-protein kinase ERBB2). Estrogen and progesterone hormones can promote the development, growth, and proliferation of breast cancer cells. Therefore,

an important step in typing a cancer is to test if it expresses these hormone receptors. Breast cancers that express ER are labelled ER-positive (ER+) and those that express PR are labelled PR-positive (PR+), otherwise they are labelled ER-negative (ER-) or PR-negative (PR-). Breast cancers can be both ER+ and PR+, positive for just one receptor, or negative for both. Hormone-receptor positive cancers tend to grow slower than hormone-receptor negative breast cancers and are more common in post-menopausal women, while hormone-negative cancers tend to grow more aggressively and occur more often in pre-menopausal women [2,51]. HER2 is involved in the regulation of apoptotic, proliferative, and metabolic pathways in cells, and its overexpression has been associated with about 20% of breast cancers [52,53]. HER2 is an oncogene, and therefore its overexpression can promote the growth and development of cancers. Breast cancers that overexpress HER2 gene and protein are known as HER2-positive (HER2+), while breast cancers that express normal levels of HER2 are labelled HER2-negative (HER2-); HER2+ cancers tend to grow more aggressively than HER2- cancers [54].

Breast cancers that test negative for ER, PR, and HER2 are known as triple-negative. This subtype comprises approximately 15% of all breast cancers, and the tumours are typically of a higher grade, larger size, and grow more quickly than non-triple-negative breast cancers [55]. The reasons for this increased aggressiveness are not yet well understood [56]. Additionally, triple-negative breast cancers are significantly more likely to occur in women <40 years of age and disproportionately affect women of African or Hispanic ancestry [57]. Triple-negative breast cancers do not respond to hormone therapies or HER2-targeting drugs; therefore it can be difficult to treat these cancers and they typically result in a poorer prognosis than breast cancers that test positive for ER,

PR, or HER2. Cytotoxic chemotherapy is currently the only treatment available for patients with advanced triple-negative breast cancer [58].

Breast cancers can be further divided into one of five "classifications": Luminal A, luminal B, basal, claudin-low, and HER2, the details of which are summarized in **Table 2** [59]. Breast cancer cells can be referred to as luminal-like or basal-like based on their genetic resemblance to healthy luminal or basal breast epithelial cells [60]. Ki67 is a protein strictly associated with cell proliferation and its expression helps classify breast cancer cells [61]. Claudin-low labelled cells have low levels of certain claudins, such as claudins 3, 4, and 7 [62], which are proteins expressed in epithelia and endothelia that form paracellular barriers and pores that determine tight junction permeability; low levels of claudins have been associated with poorer outcomes in breast cancer patients [63].

Table 2: Molecular classifications of breast cancer [59].

Classification	Receptor expression	Other characteristics
Luminal A	ER+, PR+/-, HER2-	Ki67 low, endocrine responsive, typically chemotherapy responsive.
Luminal B	ER+, PR +/-, HER2+	Ki67 high, typically endocrine responsive, variable response to chemotherapy. HER2+ are responsive to HER2-targeting drugs.
Basal	ER-, PR-, HER2-	Ki67 high, endocrine nonresponsive, often chemotherapy responsive
Claudin-low	ER-, PR-, HER2-	Ki67, claudin-3, claudin-4, and claudin-7 low. Intermediate response to chemotherapy.
HER2	ER-, PR-, HER2+	Ki67 high, responsive to HER2-targeting drugs and chemotherapy.

Last of all a patient's cancer can be "staged" which determines the size of the primary tumour and how far it has spread throughout the body; it is one of the most important factors when determining a patient's treatment options. The main questions physicians

are trying to answer when staging a cancer are: (i) is the cancer invasive or non-invasive, (ii) how big is the primary tumour and has it grown to nearby areas, (iii) has the cancer spread to nearby lymph nodes, and if so, how many, and (iv) has the cancer metastasized to any other parts of the body [2]? The stage of a cancer is determined using a "tumour-node-metastasis staging system", where the primary tumour (T), lymph nodes (N), and distant metastasis (M) are measured and graded. A value of 0 is given after the T, N, or M to signify the absence of primary tumour, cancer cells in the lymph nodes, or distant metastases, respectively, while higher numbers connote more aggressive pathophysiologies. An abbreviated summary of the tumour-node-metastasis staging system for breast cancer is shown in **Table 3**. This information is analyzed by clinicians and, depending on the combination of T, N, and M grading, the patient is given a cancer "stage" diagnosis which ranges between 0 to IV, with 0 being least and IV being most severe [64]. Where "relative" signifies relative to healthy people without cancer, the 5-year relative survival rates for each stage of breast cancer are: near 100% for stages 0 and I, evidencing why it is important for cancer patients to be diagnosed early while the cancer is most easily treated, 93% for stage II, and 72% for stage III. Stage IV, which is reserved for advanced, metastatic cases of breast cancer, only has a 5-year relative survival rate of 22% [2].

Table 3: Abbreviated tumour-node-metastasis staging system for breast cancer [64].

Primary tumour (T)	
TX	Primary tumour cannot be accessed
T0	No evidence of primary tumour
T1	Tumour ≤ 2 cm in greatest dimension
T2	Tumour > 2 cm but not > 5 cm in greatest dimension
T3	Tumour > 5 cm in greatest dimension
T4	Tumour of any size with direct extension to chest wall or skin
Regional lymph nodes (N)	
NX	Regional lymph nodes cannot be accessed (e.g. previously removed)
N0	No regional lymph node metastasis
N1	Metastasis in movable ipsilateral axillary lymph node(s)
N2	Metastasis in ipsilateral axillary lymph nodes fixed or matted, or in clinically apparent ipsilateral internal mammary nodes in the absence of clinically evident axillary lymph node metastasis
N3	Metastasis in ipsilateral infraclavicular lymph node(s), or in clinically apparent ipsilateral internal mammary lymph node(s) and in the presence of clinically evident axillary lymph node metastasis; or metastasis in ipsilateral supraclavicular lymph node(s) with or without axillary or internal mammary lymph node involvement
Distant metastasis (M)	
MX	Distant metastasis cannot be accessed
M0	No distant metastasis
M1	Distant metastasis

1.07.00: BREAST CANCER TREATMENT OPTIONS

The treatments for breast cancer can be generally categorized as local or systemic. Surgery and radiation therapy are local, since they only treat the tumour and immediate area without affecting the rest of the body. Anticancer drugs that make their way into the bloodstream and thereby affect the whole body are systemic treatments, and these include hormone therapy, targeted therapy, and chemotherapy [2].

1.07.01: SURGERY AND RADIATION THERAPY

How a particular breast cancer is treated depends on a variety of factors, such as invasiveness, gene expression, and the general health and age of the patient in question. Typically treatment will include surgery, though if the cancer is too advanced surgery may not be considered and systemic therapies will instead be used immediately. There are two main types of breast cancer surgery: (i) breast-conserving surgery, also called a lumpectomy, and (ii) a mastectomy, where the entire breast and sometimes surrounding breast tissue are removed. When possible, breast-conserving surgery is typically the preferred method as it allows the patient to keep most of the breast, though they will most likely also require radiation therapy to help prevent the cancer from returning. However, in more aggressive or advanced cases of breast cancer the doctor may recommend removing the breast in its entirety. Typically during a lumpectomy or mastectomy one or more nearby lymph nodes will be checked for cancer to see if it has spread. If cancer cells are found in these lymph nodes, further lymph-node removal surgery is likely recommended [65].

Radiation therapy is typically completed along with other breast cancer treatments, such as surgery, and its use depends on a variety of factors such as what type of surgery was completed, the spread of the cancer, and the patient's age and overall health. Briefly, radiation therapy treats cancer cells using high energy radiation, such as x-rays, to damage and destroy the tumour cells. With current technology, the radiation can be targeted to only damage the tumour and surrounding tissue. There are two main types of radiation therapy used for breast cancer: (i) External beam radiation, which is the most common form of radiation therapy where the radiation is focussed from an instrument

outside of the body on the area affected by cancer. (ii) Internal radiation, also called brachytherapy, which is where a device containing radioactive material is placed inside the breast tissue for a short time in the area affected by the cancer [66].

Many women receive systemic treatments after surgery and/or radiation therapy, which include hormone therapy, chemotherapy, and targeted therapy.

1.07.02: HORMONE THERAPY

Hormone therapy is used in breast cancers that are positive for the hormone receptors ER and/or PR. The most potent naturally occurring estrogens in humans and in order of potency are 17β-estradiol, estrone, and estriol [67]. Hormone therapy is systemic therapy that reduces the effects of these estrogens in the patient; it is typically used post-surgery (adjuvant), though it can also be used before surgery (neoadjuvant), and is often continued for up to five years [2]. The utility of estrogen-inhibiting or ER-inhibiting drugs is undisputed in ER+ breast cancer, but they are often also used with ER-, PR+ breast cancers, as these treatments can still be effective in such cases [68] which may be due to the fact that estrogens can bind to PR as well as ER [69]. Progesterone antagonists (antiprogestins) are also being researched and have shown early promise in PR+ cancers, though more safety and efficacy studies are needed before these drugs become more readily utilized in the treatment of breast cancer in humans [70,71]. There are currently multiple approved drugs that effectively inhibit estrogens' activity in the human body.

Tamoxifen is a pro-drug commonly used in hormone-receptor-positive breast cancers. It is metabolized to its active metabolites such as 4-hydroxy-tamoxifen and 4-hydroxy-*N*-

desmethyl-tamoxifen (endoxifen) by cytochrome P450 3A4 and 2D6, respectively, which work by inhibiting the activity of estrogen in breast cancer cells [72]. Tamoxifen is part of a category of drugs called selective estrogen-receptor modulators (SERMs) due to its anti-estrogenic properties in breast tissue but pro-estrogen activity in other tissues such as the uterus and bones [73]. Current data suggests that prolonged tamoxifen therapy (up to and over five years) can be beneficial for both pre- and post-menopausal women with hormone receptor-positive breast cancers [73]. Toremifene is another SERM which has a similar safety and efficacy profile to Tamoxifen; however, it is typically reserved for metastatic breast cancer patients. A minority of patients whose hormone receptor-positive breast cancer has not responded to tamoxifen may improve with toremifene [74]. Fulvestrant is a competitive ER-inhibitor used to treat hormone receptor-positive metastatic breast cancer in postmenopausal women that has progressed on other endocrine therapies. Fulvestrant is unique in that it is not a SERM, instead inhibiting ER activity everywhere in the body [75].

Aromatase inhibitors are another category of estrogen-lowering drugs that are used in post-menopausal women. Aromatase is a cytochrome P450 enzyme that synthesizes estrogens from androgenic substrates, such as estrone from androstenedione and estradiol from testosterone [76,77]. Before menopause, most estrogen is produced in the ovaries with mean plasma estradiol levels of 110 pg/mL. However, when the ovaries are not producing this estrogen, either in post-menopausal women or due to certain treatments in pre-menopausal women, plasma estradiol levels fall to approximately 7 pg/mL. This residual estrogen production is produced solely from aromatase enzymes in nonglandular sources, such as liver, muscle, brain, normal breast and breast-cancer tissue, and

particularly from subcutaneous fat which contains high levels of aromatase. Aromatase inhibitors block the production of estrogens by aromatases, but have no effect on the estrogens produced by the ovaries, hence why they are most effective in the treatment of hormone-receptor-positive breast cancers in post-menopausal women. There are currently three approved aromatase inhibitors available called letrozole, anastrozole, and exemestane [2,77].

For pre-menopausal women, shutting down the ability of the ovaries to produce estrogen is another option that can improve anti-cancer treatments. This is termed "ovarian ablation", which can improve the efficacy of aromatase inhibitors when used in combination and is typically reserved for metastatic breast cancer, though it is sometimes used in earlier stages. Ovarian ablation can be done in one of three ways: (i) oophorectomy, where the ovaries are surgically removed, (ii) treatment with luteinizing hormone-releasing hormone analogs, which are drugs that inhibit the signal sent to the ovaries to produce estrogen, causing temporary menopause. This is done more often than oophorectomy as it is less invasive and its effects on estrogen production are not permanent [78], and (iii) some chemotherapy drugs can damage the ovaries, stopping the production of estrogen. This damage is often reversible after discontinuing treatment; however, it can be permanent. Chemotherapy typically is not used for the purpose of inhibiting estrogen production in the ovaries, though it is sometimes a beneficial side effect when treating hormone-receptor positive breast cancers [2].

1.07.03: TARGETED THERAPY

Targeted therapies are treatments that specifically target HER2, ER, or PR and their associated biochemical pathways in cancers positive for these receptors. Targeted therapies work differently from chemotherapy which attacks all rapidly proliferating cells in the body, and from hormone therapy which inhibits the effects of female sex hormones.

There are currently three approved HER2-targeting agents available, trastuzumab, pertuzumab, and lapatinib (trade names Herceptin, Perjeta, and Tykerb, respectively). [79]. Trastuzumab is a monoclonal antibody that binds to the extracellular domain of HER2 thus inhibiting HER2 downstream signaling, such as the activation of mitogen-activated protein kinase, serine/threonine kinase Akt, and cyclin-D1 which promote cell growth and proliferation. It also increases expression of the tumour suppressor p27 in HER2 overexpressing cancer cells. It has no effect in HER2-negative breast cancers [80]. It was the first HER2 targeted therapy approved by the United States Food and Drug Administration in 1998 [79]. Pertuzumab is also a monoclonal antibody which binds to the extracellular domain of HER2 which blocks the dimerization of HER2 with other HER family receptors, again inhibiting downstream signaling [81]. Lapatinib, which is not an antibody, inhibits the intracellular tyrosine kinase activity of HER2, blocking tyrosine kinase phosphorylation and thereby inhibiting HER2 downstream pathways [79].

For women with hormone-receptor positive cancers, hormone-receptor targeted therapies can be beneficial along with hormone therapy. Palbociclib and everolimus (trade names Ibrance and Afinitor, respectively) are two examples of hormone-receptor targeted therapies. Palbociclib inhibits cyclin-dependent kinases 4 and 6, which promote

progression from the gap 1 (G1) to synthesis (S) phase during the cell cycle, and on which hormone receptor positive breast cancer cells are dependent to proliferate. In combination with other therapies palbociclib can improve progression-free survival of hormone-receptor positive breast cancers [82]. Everolimus is an inhibitor of the mammalian target of rapamycin (mTOR), which is part of the phosphoinositide 3-kinase (PI3K)/Akt/mTOR pathway which regulates several cellular functions in cancers, including cell growth, proliferation, and survival. Increased activation of this pathway is one mechanism through which hormone-receptor positive cancers become resistant to hormone therapy [83]. As such everolimus has been found to be beneficial in the treatment of hormone receptor-positive, HER2-negative metastatic breast cancers that have progressed after hormone therapy [84].

1.07.04: CHEMOTHERAPY

Chemotherapy treats a woman's whole body for breast cancer, not just the breast tissue, and primarily works by killing rapidly proliferating cells. This systemic therapy therefore can have many side effects and be quite damaging to proliferative healthy tissue [2], and is less effective in indolent versus rapidly proliferating breast cancers [85]. Recently it has been discovered that chemotherapy can also affect the immune system, sensitizing the tumour to immune cells. This "immunogenic chemotherapy" is at least partially responsible for the anticancer activity observed with many chemotherapeutics [86].

Chemotherapy is given either intravenously or orally, and the drugs travel through the blood stream to reach cancer cells that may be present throughout the body.

Chemotherapy is typically given after surgery (adjuvant chemotherapy) with the goal to kill any cells that were left behind or perhaps were not seen with imaging tests, or before surgery (neoadjuvant therapy) to shrink the initial tumour to make surgery a more viable option while also killing cancer cells that have spread elsewhere in the body. Chemotherapy is also often used in advanced breast cancers that have spread past the breast and nearby lymph nodes, where surgery will no longer be a viable method to remove the bulk of cancerous tissue. Chemotherapy typically is most effective when multiple drugs are used at once, called combination therapy. The most common chemotherapeutics used in breast cancer are the anthracyclines (such as doxorubicin and epirubicin), taxanes (such as paclitaxel [Taxol] and docetaxel), 5-fluorouracil, cyclophosphamide, and carboplatin. For advanced, metastatic cases of breast cancer treatments may also include other chemotherapeutics such as mitoxantrone, capecitabine, gemcitabine, cisplatin, vinorelbine, ixabepilone, or eribulin [2].

There are many mechanisms through which the various chemotherapeutics induce cancer cell death. For example, anthracyclines such as doxorubicin damage DNA by inhibiting DNA synthesis, inducing reactive oxygen species (ROS), forming DNA adducts and cross-links between DNA base pairs, and inhibiting type II topoisomerases, ultimately leading to cancer cell apoptosis [87]. Alternatively, taxanes stabilize microtubules, cellular polymers that play a key role during mitosis. During mitosis the microtubules connect at the kinetochore before pulling apart sister chromatids, resulting in two sets of DNA to form two new cells. Taxanes stabilize these microtubule-kinetochore interactions preventing them from properly disassembling during cell division, triggering the cell to undergo apoptosis [88,89]. Mitoxantrone, which was

initially created as a less cardiotoxic alternative to doxorubicin [90], primarily causes cell death through its direct interactions with DNA; these include its inhibition of type II topoisomerases by creating a stable mitoxantrone-topoisomerase II-DNA complex [91] and its ability to induce DNA fragmentation, ultimately resulting in cell death [92].

By working through a variety of mechanisms, combination therapy with multiple chemotherapeutics can better treat a patient's cancer. Drug-resistant cancer cells typically innately exist in low levels in any given tumour due to random mutations. By treating with just one chemotherapeutic, this gives a competitive advantage to these resistant mutants, allowing them to grow and thrive in the absence of other, non-resistant cancer cells. Combination therapy eliminates any cancer cells that are resistant to any one of these drugs used in isolation, and because the likelihood of doubly or triply resistant cells is much lower than of singly resistant cells, this greatly increases the chance of the therapy successfully eliminating the cancer [93].

1.07.05: IMMUNOTHERAPY

Immunotherapy is the treatment of disease using substances that stimulate the immune response. This can be done using monoclonal antibodies, which target specific proteins that are largely expressed in cancer cells, signaling the immune system to eliminate the cells that express those proteins. For example, these treatments include "immune checkpoint inhibitors". Normal cells contain proteins called checkpoints, which signal immune T cells to not attack, preventing the immune system from targeting healthy tissue by mistake. These checkpoints can also be expressed in cancer cells, confusing the

immune system and preventing it from attacking the mutated tissue. Immune checkpoint inhibitors are monoclonal antibodies that block checkpoint proteins, preventing them from signaling the T cells to not attack, thereby allowing the immune system to detect and kill the cancerous cells [94]. The protein, programmed death-1 (PD-1), is an example of an immune checkpoint that is expressed by immune T cells and pro-B cells. It works by bonding with programmed death ligand-1 (PD-L1), which can be overexpressed in cancer cells, thus preventing the immune system from attacking these cancer cells [95]. Monoclonal antibodies that inhibit PD-1 or PD-L1, such as atezolizumab, pembrolizumab, and nivolumab, increase the immune system's response to these PD-L1 overexpressing cancer cells, allowing the patient's own immune system to target and kill the neoplastic tissue [94].

Immunotherapy works best in cancerous tissue that is innately heavily infiltrated by immune cells. Approximately 70% of breast cancers contain lymphocytic infiltration in the stroma, suggesting that immunotherapy could be beneficial in a significant number of breast cancer patients. To date, phase I trials have demonstrated promising results using anti-PD-1 and anti-PD-L1 antibodies with remarkably durable responses in heavily pretreated, metastatic, and triple-negative breast cancers (which typically have high levels of immune infiltration) with somewhat lower responses in ER-positive cancers (which typically have lower levels of immune infiltration). Due to these promising early results, as of early 2016 there were approximately fifty clinical trials either underway or about to begin to evaluate the ability of immune system-altering drugs to treat breast cancer patients [96].

1.07.06: SHORTCOMINGS OF CURRENT TREATMENT OPTIONS

Breast cancer treatment has greatly improved over the past few decades. Surgery has proven quite effective in treating early stage breast cancer, sometimes with no further treatment required. Radiation therapy has become more precise in its ability to only target the cancerous and directly surrounding tissue. Systemic therapies, including hormone, targeted, and chemotherapies have also greatly increased the long-term survivability of cancer [2]. For example, six months of adjuvant anthracycline-based polychemotherapy with fluorouracil, doxorubicin, and cyclophosphamide or fluorouracil, epirubicin, and cyclophosphamide significantly reduced the annual breast cancer death rate by about 38% in women <50 years of age and by about 20% for those aged 50-69 versus women who were not given these chemotherapeutic combination therapies. Similarly, use of adjuvant tamoxifen for 5-years reduces the annual breast cancer death rate by 31% in women with ER+ disease versus those who did not take adjuvant tamoxifen [51]. However, while there have been significant improvements in overall survival using the above-mentioned treatments, there are still thousands of patients who die every year because of their breast cancer, with approximately 5,000 deaths in Canada attributed to breast cancer in 2016 [1]. As previously mentioned, only ~22% of patients with metastatic, stage IV breast cancer survive at least five years after initial diagnosis, and as chemotherapy is the primary method of treating late stage breast cancer [2], this indicates that novel chemotherapeutic drugs are needed with improved anticancer efficacy to improve the prognosis of patients diagnosed with late-stage breast cancer.

Side effects are another major concern of current anticancer therapies which oftentimes are severe and debilitating and can cause irreparable damage. The side-effects of surgery

include pain or tenderness, the development of scar tissue, and change in the shape of the breast with breast conserving surgery or absence of the breast entirely with mastectomy. Radiation to the breast tissue can cause swelling, rashes and fatigue, nerve damage causing shoulder, arm, and hand numbness, and inhibit a woman's ability to breastfeed later in life [2]. Chemotherapeutics are especially damaging to various healthy organs in the patient, depending on the particular drug in question [97]. Cumulative neurotoxicity and hematopoietic toxicity are serious limiting side effects associated with taxanes [3]. Cardiotoxicity induced by anthracyclines, particularly doxorubicin, is a common and serious side effect. Cardiomyopathy caused by anthracyclines can appear during treatment or many years after, and typically appears clinically as congestive heart failure or arrhythmias, though severe damage can still occur even if the patient is asymptomatic. Sudden death from arrhythmia can occur years after completion of doxorubicin treatment [98]. Patients who are younger at the time of treatment carry a higher risk of developing cardiomyopathies than older patients, and females have a higher risk than males [99]. The use of the platinum-based chemotherapeutic cisplatin is limited due to severe nephrotoxicity and ototoxicity. Patients treated with cisplatin have a high probability of developing permanent hearing damage, and while renal function can improve somewhat with time after cessation of treatment, it rarely heals completely [97,100]. Infertility in men and pre-menopausal women is also a serious concern. Cytotoxic chemotherapy and radiotherapy may both damage gonadal tissue permanently, rendering the patient infertile. While radiotherapy of the breast tissue typically does not damage the gonads, if the cancer has spread and radiotherapy is directed to the abdomen and pelvis, the risk of complications increases [101]. Patients requiring chemotherapy with an alkylating agent

(which bind to DNA to prevent DNA replication), such as cyclophosphamide, have a high risk of menstrual irregularities, ovarian toxicity, and premature menopause as a consequence of their treatment [101]. The testes are also extremely sensitive to chemotherapy and radiation, with testicular dysfunction and infertility being among the most common side-effects in men treated with alkylating agents [97]. In addition to these serious, oftentimes permanent, and sometimes deadly side-effects of chemotherapy, less severe but still extremely uncomfortable and potentially debilitating side effects are common. These include hair loss, mouth sores, loss of appetite and weight loss, nausea and vomiting, diarrhea, fatigue, and increased chance of infections and easy bruising or bleeding due to chemotherapy-induced damage to the blood-forming cells of the bone marrow [2].

Second cancers are also a serious risk associated with cancer therapies, which can develop in patients years after the successful treatment of a first cancer. For example, secondary myelodysplasia, leukemia, as well as bone and bladder cancers have been associated with the use of certain chemotherapeutics, such as alkylating agents and topoisomerase II inhibitors [102,103,104,105]. These treatments work by damaging the DNA of cancer cells, thus causing cell death. However, irreversible damage can also be caused in healthy tissue by these drugs, causing DNA mutations that can increase the patient's chance of developing a second cancer later in life [106].

New breast cancer treatments are required with improved efficacy that will improve the long-term survivability of patients, especially in those with late-stage metastatic disease. Additionally, current chemotherapeutics can result in dangerous and permanent

debilitating toxicities, warranting continued research efforts into the development of novel drug treatments that induce fewer and less severe side effects.

1.08.00: METASTATIC BREAST CANCER

When breast cancer cells spread outside of the breast tissue to a new part of the body it is called metastatic breast cancer. Metastatic breast cancer can also be referred to as stage IV breast cancer. The most commons sites of breast cancer metastases are in bone, brain, liver, and lung tissue. For cancer cells to metastasize, they need to follow a series of steps that allows them to escape the primary breast tumour, spread throughout the body, and grow in another organ or tissue. These steps involve: (1) Spreading into, or invading, nearby normal tissue. (2) Spreading through the walls of nearby blood or lymph vessels. (3) Traveling through the lymphatic system or bloodstream, allowing the cancer cells to travel to almost anywhere in the body. (4) Stopping in small blood or lymph vessels at some other location in the body, traversing through the blood or lymph vessel wall, and moving into the surrounding tissue. (5) Surviving and proliferating in the new tissue to form a secondary metastatic tumour site. (6) Inducing angiogenesis around the tumour, providing a blood supply to the tumourigenic cells and allowing them to further grow and proliferate [107]. It is difficult for cancer cells to meet all of these criteria and successfully form micrometastases. In experimental settings, only ~0.01% of metastatic clonal cancer cells injected into circulation were able to form metastatic foci. However, as 90% of all human cancer-related deaths are due to metastases, this miniscule success rate is still high enough to make metastasis-formation a serious impediment in the successful treatment of cancers [108].

The majority of lymph from the breasts is drained through the axillary nodes (under the arm), supra- and infraclavicular nodes (around the collar bone), and internal mammary nodes (inside the chest near the breast bone). For this reason, these are the nodes that oncologists typically first check to see if a breast cancer has spread. If cancer cells have spread to a patient's lymph nodes, there is a greater probability that the cancer cells have also metastasized to other parts of the body [2].

Metastatic breast cancer remains an incurable disease, despite advances in available treatments, with a median survival time of 2 to 3 years post-diagnosis. One third of women with early stage breast cancer will eventually have their disease metastasize [3], and over 570,000 people are estimated to die from metastatic breast cancer every year worldwide [109]. For women with hormone- receptor positive metastases, systemic hormone therapies can be used to help reduce the growth and spread of further metastases, such as tamoxifen or aromatase inhibitors depending on the menopausal-status of the woman. Similarly, those with HER2+ cancers can receive systemic HER2 targeting drugs, as previously discussed. Typically chemotherapeutics are used in combination in such cases, and in women with triple-negative metastases chemotherapy is the sole option available [3].

Breast cancer is one of the most chemotherapy-sensitive types of solid tumours, and treatment with anthracyclines (such as doxorubicin) and taxanes (such as paclitaxel and docetaxel) remain the typical first-line chemotherapeutics in metastatic breast cancer. Due to the cardiotoxicity associated with doxorubicin, taxanes have emerged as the preferable treatment choice. Once the efficacy of these treatments begins to fail, later-line treatments include drugs such as capecitabine, eribulin, and ixabepilone. Gemcitabine,

platinum agents, and irinotecan can also be used. Improving overall survival and quality of life are key aims when treating metastatic breast cancer patients. Typically with chemotherapy for metastatic breast cancer, single agent sequential-treatment is preferable to combination therapy. While combination therapy often can achieve better response rate, this is at the cost of more severe side effects and has been found to have little impact on overall survival [3,110].

Drug resistance is the primary impediment to successfully treating patients with metastatic breast cancer [110]. The use of taxanes and doxorubicin can be limited due to the development of, or innate, MDR in metastatic breast cancer cells, such as through the overexpression of ATP-binding cassette (ABC)-transporters like ABCB1 [3]. MDR is a very significant problem because the cancer can no longer be effectively treated with these drugs [111]. Current practice is to use treatments post-anthracycline- or taxane-use that are from another drug class, with the hope of avoiding cross-resistance. However, the need remains for new chemotherapy treatments for women with metastatic breast cancer that are still effective in these drug-resistant cancer cells. Ideally, these new agents should belong to a novel class of drug, have a different mechanism of action, and improve overall survivability while simultaneously having less severe side effects when given as a monotherapy [110].

1.09.00: MULTIDRUG RESISTANCE IN CANCER

Failure of a patient's cancer to respond to a particular therapy can result for two general reasons: (i) host factors, such as poor absorption or rapid metabolism of the drug, that

impair the delivery of the anticancer drugs to the tumour cells, and (ii) genetic or epigenetic alterations within the cancer cells that affect their sensitivity to anticancer drugs [112,113].

Resistance to chemotherapy can occur prior to any drug treatment due to random mutations in the cancer cells that imbue a drug-resistant phenotype (called primary or innate resistance). For example, weakly ER-positive breast cancer cells that only express slightly higher levels of ER versus ER-negative cells are often treated using hormone therapy. These cells do not typically respond well to such treatments, and this low-expression of ER is therefore considered a form of innate drug-resistance [114]. Alternatively, drug-resistance can develop over time following exposure to chemotherapeutic agents that do not successfully kill all cancer cells present, giving a competitive edge to any remaining cells that happened to have an innate resistance, which grow to form drug-resistant tumours (called acquired resistance) [115]. After cancer cells become resistant to one drug, they often also show cross-resistance to other structurally and mechanistically unrelated drugs, a phenomenon known as MDR, due to the non-specific nature of the methods through which drug-resistance occurs [113].

These MDR mechanisms can occur through: (i) genetic mutations that alter the translation of certain MDR-associated proteins, (ii) alterations to the expression levels of small, noncoding RNAs called microRNA (miRNA) which bind to mRNA and prevent translation, thus silencing certain genes and promoting MDR, or (iii) altered epigenetic regulation, such as through DNA methylation, affecting gene transcription [116].

There are multiple mechanisms through which cancer cells can become resistant to different classes of drug simultaneously, which can occur in isolation or in combination, rendering many chemotherapeutics ineffective.

1.09.01: INCREASED DRUG EFFLUX

Resistance to natural-product lipophilic anticancer drugs is typically due to overexpression of ATP-dependent efflux pump proteins found in the cell membranes of cancer cells that have broad drug specificity. This type of drug resistance is so common that it is sometimes referred to as “classical multidrug resistance”. These pumps belong to a family of ABC transporters that have shared sequences and homologies. At least 48 human ABC genes have been identified and are divided into seven distinct subfamilies, labelled ABCA through to ABCG, followed by numbers to differentiate the various proteins [117]. Resistance to both taxanes and anthracyclines, the two classes of chemotherapeutics typically used for breast cancer, arises predominantly from decreased intracellular concentrations as the result of increased efflux through ABC-transporters [118].

The major mechanism of multidrug resistance is the overexpression of ABCB1, encoded by the gene *ABCB1* and also commonly referred to as permeability-glycoprotein or multidrug resistance protein 1; it is the best studied of the various ABC-transporters [112,119]. ABCB1 is a broad-spectrum multidrug efflux pump that consists of twelve transmembrane regions and two ATP-binding sites [120]. Binding of a substrate to the transmembrane domain of ABCB1 stimulates its ATPase activity, which causes a

conformational change that releases the substrate to the extracellular space [121]. Hydrolysis at the second ATP-binding site then resets the transporter so that it can again bind substrate, completing one catalytic cycle [122]. ABCB1 efficiently removes many chemotherapeutics and other pharmaceuticals through the lipid bilayer of cells, which can render treatments ineffective [113]. In breast cancer, the expression of ABCB1 is increased after certain therapies, including taxanes or anthracyclines, and this increase has been associated with a greater likelihood of further treatments failing [113,123].

Overexpression of other ABC-transporters can cause cancer cell MDR as well. One such transporter is ABCC1, also called multidrug-resistance-associated protein 1 [124]. ABCC1 has a similar structure to ABCB1, recognizes neutral and anionic lipophilic natural products and drugs, and is widely expressed in many human tissues and cancers [125]. A third ABC-transporter that has been associated with drug resistance, in particular resistance to the drug mitoxantrone, is ABCG2. ABCG2 is also known as mitoxantrone-resistance protein, breast cancer resistance protein, or ABC transporter in placenta. This transporter is believed to be a homodimer of two half-transporters, each containing an ATP-binding domain and six transmembrane units [113,126].

While multiple ABC-transporters, including those described above, have been associated with MDR in cancer cells, they are also expressed in healthy cells and typically serve to protect tissue from potential toxins. Consistent with their wide distribution throughout the body, it has become clear that they are largely involved in the transport of many endogenous substrates along with their ability to efflux cytotoxic drugs [113]. For example, ABC-transporters are involved in the regulation of central nervous system permeability. The blood-brain barrier protects the central nervous system from

toxins, and is formed by the microvascular endothelial cells of capillaries. ABCB1 is found at high concentrations along the luminal surface of these capillary cells and helps efflux cytotoxins from the cells back into the capillary, thus protecting the central nervous system [127]. Other ABC transporters such as ABCC1 are involved in the removal of metabolic waste from the central nervous system into the blood [128]. ABC transporters have also been found to be normally expressed in testicular tissue to protect sperm from toxins, and in the placenta to protect the developing fetus [129].

ABC-transporters are also widely expressed in the liver, gastrointestinal tract, and kidneys to help with the excretion of toxins from the body, thus protecting the whole organism [113]. For example, ABCB1 is found in the apical membranes of hepatocytes, where it transports toxins into the bile to be excreted [130]. ABCB1 in the gastrointestinal tract, situated in the apical membranes of mucosal cells, extrudes toxins that may have been ingested by the organism forming a first line of defence; though it also extrudes drugs that are ABCB1-substrates that have been ingested by a patient, thus decreasing their bioavailability [131]. ABCB1 is also known to efflux intravenous drugs through the gastrointestinal tract to eliminate them from the body [132].

1.09.02: REDUCED DRUG UPTAKE

Resistance to anticancer drugs can also be mediated by reduced uptake into the cells. Hydrophilic drugs that do not regularly pass the cell membrane through passive diffusion can instead “piggyback” on uptake transporters that bring nutrients into the cell, or on agents that enter the cell *via* endocytosis. Cells can become resistant to these drugs

through mutations that eliminate or modify these uptake transporters [112]. For example, resistance to toxic folate compounds such as methotrexate commonly occurs through mutations to the uptake folate binding protein or the reduced folate transporter, reducing their influx activity [133]. Similarly, cancer cell mutants with defective endocytosis are resistant to cancer drugs, such as immunotoxins, that must be internalized to induce cancer cell death and that enter the cells *via* endocytosis [112,134].

1.09.03: ACTIVATION OF DETOXIFICATION SYSTEMS

MDR can occur through the increased activation of detoxifying systems within the cells, such as through increased drug metabolism and deactivation with cytochrome P450 enzymes or glutathione-*S*-transferases (GSTs) [135,136]. These systems can modify, degrade, or complex the drug with other molecules during the metabolic process, ultimately inactivating the drug. Therefore increased activity of these detoxification systems can lead to MDR. Similarly, if a patient is treated with an anticancer pro-drug which requires being metabolized to become active, the activity of these detoxification systems can decrease, reducing the rate at which the non-toxic pro-drug is metabolized to its active form [135].

1.09.04: ACTIVATION OF DNA REPAIR SYSTEMS

MDR can develop due to increased activity of DNA repair systems. This type of MDR can occur after continued exposure to any drug that damages DNA, preventing the intended DNA damage from occurring and thereby preventing the cancer cell from dying

[113]. For example, resistance to DNA damaging alkylating agents can occur due to increased expression of the DNA repair protein *O*(6)-methylguanine methyltransferase, and is considered a significant barrier to the successful treatment of cancers that overexpress this protein [137]. Increased activity of these DNA repair systems has been observed alongside increased expression of efflux ABC-transporters, illustrating how multiple mechanisms can occur at the same time to imbue MDR in cancer cells [138].

1.09.05: BLOCKED APOPTOSIS

MDR can occur as the result of defective or absent apoptotic pathways. This can be due to genetic mutations, overexpression of certain miRNAs, or altered DNA methylation that affect signaling pathways that are involved in the initiation and/or execution of apoptosis in a cell [113,139,140]. For example, the PI3K/Akt pathway is an important signaling pathway that regulates many cell responses including cell proliferation and survival, and aberrations in this pathway occur in ~70% of breast cancers [141], which have been linked to tamoxifen and trastuzumab resistance in breast cancer cells along with *ABCB1*-upregulation [116]. When this pathway is hyperactivated, it can lead to increased cell proliferation and inhibition of apoptosis [141].

1.09.06: ALTERATION OF DRUG TARGETS

An anticancer drug's efficacy is influenced by its molecular target, and alterations to this target through mutations or modifications of its expression levels, can in turn alter the efficacy of the drug. These drug-target alterations can therefore lead to MDR [135].

For example, many anticancer drugs inhibit topoisomerase II, an enzyme that prevents DNA super- and under-coiling, thereby preventing DNA damage. Drugs that stabilize the DNA-topoisomerase II transient interaction prevent topoisomerase II from working properly, inducing DNA breaks which signal the cell to undergo apoptosis. Cancer cells can become resistant to this method of cell death by mutating the gene that encodes the topoisomerase II protein, thus forming a mutated version of topoisomerase II that is no longer targeted by these drugs [142]. **Figure 1** summarizes these mechanisms of drug resistance.

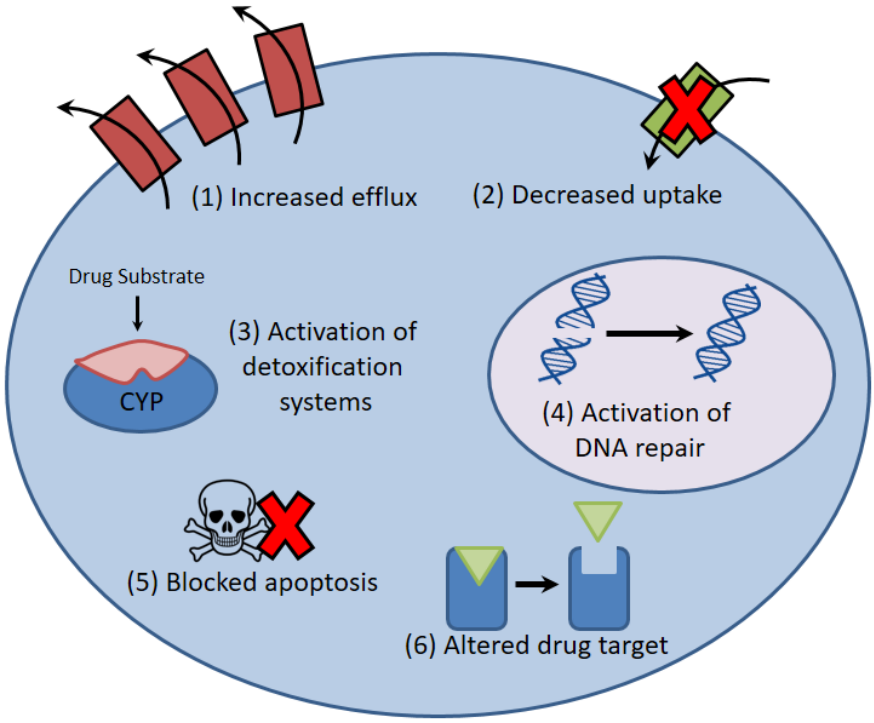

Figure 1: Mechanisms of multidrug resistance. These include: (1) increased drug efflux through ABC-transporters, (2) decreased influx through uptake transporters, (3) activation of detoxification systems such as drug metabolism *via* cytochrome P450 enzymes (labelled CYP above), (4) activation of DNA repair mechanisms, (5) inhibition of cellular apoptotic pathways, and (6) alteration of the drug target.

1.09.07: MULTIFACTORIAL MULTIDRUG RESISTANCE

An important principle in the development of MDR is that cancer cells are genetically heterogeneous. This means that while mutations in these cancer cells share a common phenotype that favours rapid and uncontrolled proliferation, many of these mutations are different between cells. Additionally, the rapid proliferation of these cells increases the chance for the development of further, unique mutations as the cancer progresses. Therefore, tumour cells that are exposed to chemotherapy will be naturally selected for those that have the ability to survive and grow in the presence of the drug regimen, and due to natural genetic heterogeneity these MDR cancer cells will likely display multiple mechanisms of resistance, making further treatment yet more challenging. This phenomenon is called “multifactorial multidrug resistance” [113].

1.09.08: TREATMENT OF MULTIDRUG RESISTANT CANCERS

The various mechanisms of MDR described above depict a major hurdle in the treatment of advanced breast cancer. One method that has been proposed to treat patients with MDR cancers is by using inhibitors of the ABC-transporters, in particular ABCB1, as this is the most commonly observed mechanism of MDR in cancer cells. The reasoning behind this is that by inhibiting these transporters the chemotherapeutic drugs will not be effluxed as readily, thus improving their potency and efficacy in treating the MDR cells. The general strategy to inhibit ABCB1 has been to develop compounds that either compete with the anticancer compounds for active transport or non-competitively inhibit ABCB1 [113].

The first generation of ABCB1 inhibitors included a variety of compounds such as verapamil, quinine, and cyclosporin A. However, the speculation that these inhibitors could improve cytotoxic potency of chemotherapeutics was replaced with the reality that they were in fact only weak inhibitors of ABCB1, and caused significant toxicities at high doses in patients such as hypotension, heart block, fluid retention, tinnitus, and vertigo, among others, depending on the specific inhibitor used [143,144].

Based on these results second-generation ABCB1-inhibitors were developed, such as the drug valspodar. While valspodar effectively inhibited ABCB1 and avoided the primary toxicity associated with the first-generation ABCB1-inhibitors, it did demonstrate significant toxicity stemming from drug-drug interactions with the toxic chemotherapeutics used; valspodar significantly inhibited the metabolism and clearance of the chemotherapeutics with which it was co-treated, elevating their plasma concentrations to unacceptable and difficult to predict levels [145].

A third generation of ABCB1-inhibitors was developed which included the drugs elacridar, tariquidar, and zosuquidar, which reportedly avoided the pharmacokinetic issues observed with the second-generation inhibitors. Unfortunately, the ability of these inhibitors to reverse MDR caused by ABCB1-overexpression was minimal. For example, a phase II trial testing tariquidar in combination with anthracycline- or taxane-containing regimens in patients with ABCB1-overexpressing MDR breast cancer resulted in a partial response in only 1 patient among the 17 who received the combination treatment (6% response rate). It was hypothesized that the use of these inhibitors was minimally effective due to the development of alternative mechanisms of MDR in the patients' tumours [146].

Fourth generation ABC-transporter inhibitors are currently being developed from natural products that have exhibited potential as chemosensitizers in the hopes of developing novel inhibitors that are less toxic and more effective than the previous three generations [147]. For example, the plant-derived compound curcumin has been shown to significantly reduce the growth of ABCB1-overexpressing colorectal cells in mice in combination with the chemotherapeutic capecitabine, compared with capecitabine or curcumin on their own [148]. In another study, curcumin reportedly reversed MDR in cells and animal models by inhibiting the expression and function of ABC-transporters and inhibiting ATPase activity [149]. While still in the early stages, this data suggests a possible role for curcumin as an inhibitor of ABC-transport and a potentiator of the cytotoxic activity of chemotherapeutics in MDR cancers [147]. Other natural products such as resveratrol [150], tannic acid [151], quercetin [152], and tea catechins [153] are also being studied as potential 4th generation ABC-transporter inhibitors. Currently these inhibitors are all in early, preclinical stages of research. However, based on promising cellular and *in vivo* trials completed thus far, reversing MDR in cancer cells by inhibiting ABC-transporters remains a possibility worth exploring that will hopefully prove successful with this newest generation of inhibitors [147].

An alternative method of treating MDR cancers that overexpress ABC-transporters is through simple avoidance. Instead of using ABC-transporter inhibitors in combination with chemotherapeutics that are known substrates of these efflux transporters, novel drug therapies that are poor ABC-transporter substrates could be used without the need for ABC-transporter inhibitors. Such compounds would not be recognized by ABC drug efflux transporters and would therefore not be eliminated from the MDR cells, thus

helping them to retain their anticancer activity [154]; however, susceptibility to other mechanisms of resistance could still arise. Such novel therapeutics are currently being developed. For example, a derivative of the compound indirubin called PH II-7 has been found to be effective in 18 different cancer cell lines and 5 drug resistant cell lines. Su, *et al* determined that PH II-7 was able to retain its cytotoxic properties in these MDR cells due to it not being effluxed by ABCB1 [155]. Similarly, the taxane analog DJ-927 overcomes ABCB1-induced MDR in various tumour cell lines both *in vitro* and *in vivo*. This compound was found to be a poorer substrate of ABCB1 than docetaxel and paclitaxel, suggesting a mechanism for how DJ-927 retains its cytotoxic activity in ABC-transporter overexpressing cancer cell lines [156].

By avoiding transporter-mediated efflux, cytotoxic drugs that are poor ABC-transporter substrates can retain their cytotoxic potency in ABC-transporter overexpressing MDR cancer cells (**Figure 2**). Based on the limited success of ABC-transporter inhibitors used in combination with chemotherapeutics, further research into novel cytotoxic compounds that are unaffected by the overexpression of these drug efflux transporters is needed.

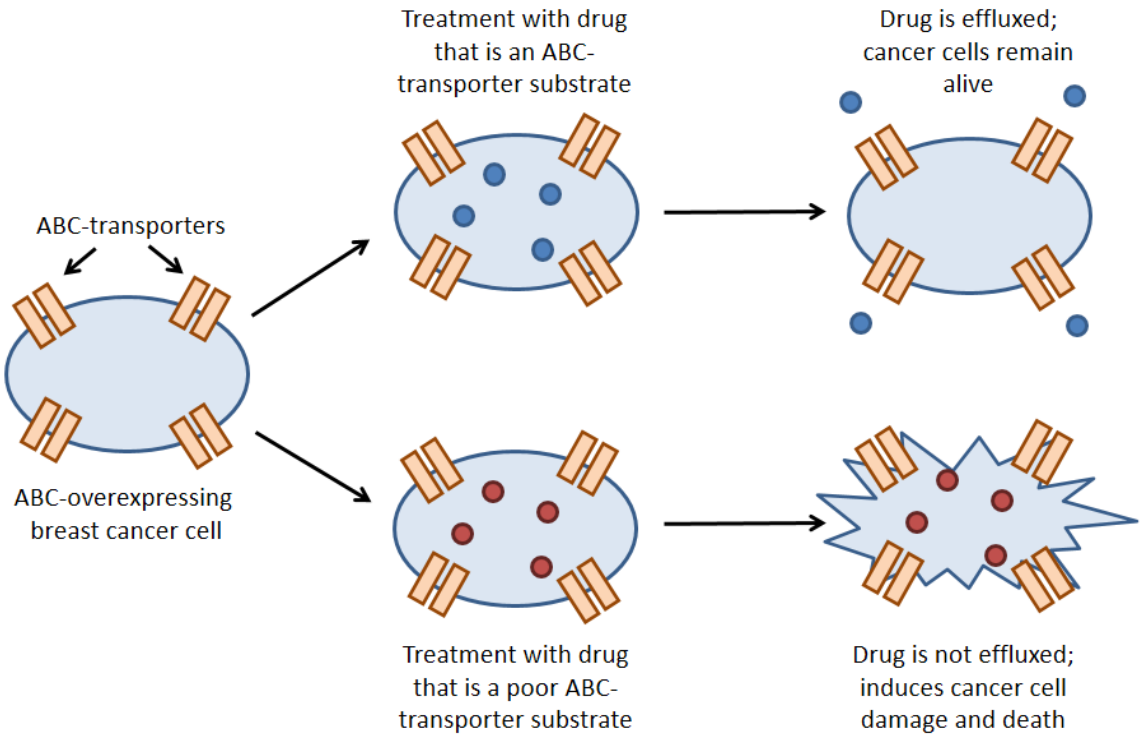

Figure 2: ABC-transporter substrate versus poor-substrate drugs in the treatment of ABC-transporter overexpressing MDR cancers. Drugs that are substrates of ABC-transporters can be effluxed from the cell, rendering the treatment ineffective and preventing the drug from killing the cancerous tissue. Drugs that are poor substrates of ABC-transporters are not effluxed, remain within the cells, and are thereby able to retain their cytotoxic potency in the ABC-transporter overexpressing MDR cancer cells.

1.10.00: THE USE OF NATURAL PRODUCTS FOR DRUG DEVELOPMENT

Natural products (compounds obtained from a variety of natural sources such as plants, fungi, and bacteria) have been a rich source of compounds for drug discovery for decades [157]. Historically, almost all medicinal preparations were derived from plants or animals. More recently, natural products or compounds derived from natural products make up a large portion of compounds that enter clinical trials to become approved drugs,

in particular novel anticancer and antimicrobial agents [158,159]. An analysis of all new medicines approved by the United States Food and Drug Administration between 1981 and 2010 showed that 34% of all small molecule drugs were natural products or derivatives of natural products [160], including the tubulin-binding anticancer drugs such as paclitaxel and vinblastine [161,162].

Bacteria especially have been important sources of novel natural products, with the Actinomycete genus *Streptomyces* being responsible for 70-80% of all newly developed antibiotics in the 1950s and 1960s and are currently responsible for approximately 34% of all isolated bioactive microbial metabolites [163]. Of particular relevance to my work is the thin-walled, colourless, hyaline, and monopodially-branched soil-bacteria *Streptomyces venezuelae* ISP5230 [164]. This organism can be used to biosynthesize (produce complex compounds within living organisms or cells) a large category of bioactive compounds called jadomycins [165].

1.11.00: BIOSYNTHESIS AND STRUCTURE OF JADOMYCINS

The first biosynthesized jadomycin analogue, simply labelled jadomycin at the time and later referred to as jadomycin A (**Figure 3a**), was created by a group of researchers from Saint Mary's University, Mount Saint Vincent University, and the Halifax location of the National Research Council in 1991 [166]. The proceeding glycosylated analogue, which contained a sugar (2,6-dideoxy-L-digitoxose) added to ring D of the jadomycin backbone (**Figure 3b**), was published in 1993 and labelled jadomycin B [167,168]. Since then, over 25 different jadomycin analogues have been biosynthesized and isolated [169,170].

Through the use of heat or ethanol shock, *S. venezuelae* ISP5230 can be manipulated to produce jadomycins through fermentation [167,171]. Jadomycins are pigmented, angucycline-derived antibiotics that contain a pentacyclic 8*H*-benz[*b*]oxazolo[3,2-*f*-]-phentathridine backbone, which contain five aromatic rings (labelled A through E in **Figure 3**) including a dihydropyridine (B) and an oxazolone ring (E) [166,167,171]. The nitrogen heteroatom of the oxazolone ring E derives from the incorporation of an amino acid provided in the growth medium, which incorporates itself into the jadomycin backbone during biosynthesis [172,173], and the 2,6-dideoxy-L-digitoxose sugar is appended onto ring D by an enzyme called JadS [174].

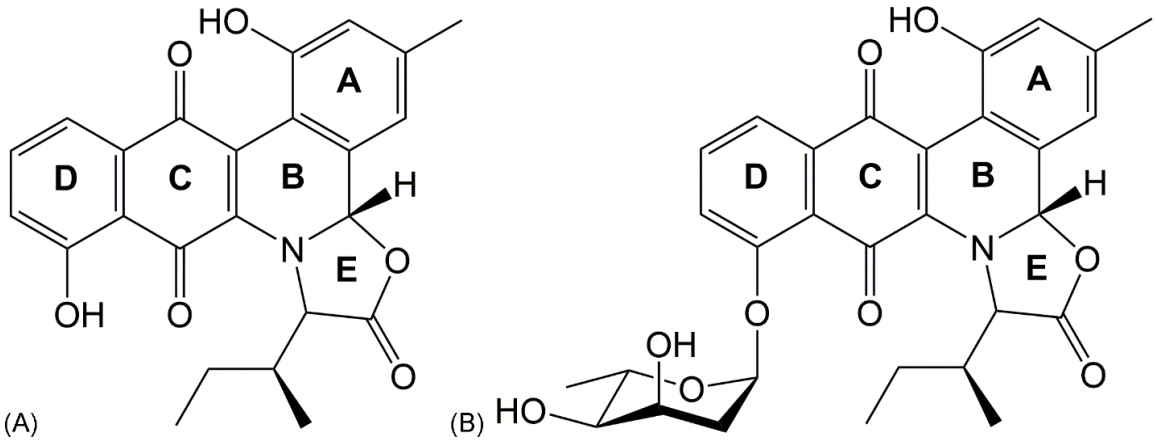

Figure 3: Structures of (A) jadomycin A and (B) jadomycin B, re-drawn from structures reported by Shan, *et al* [168]. The nitrogen heteroatom of the oxazolone ring E derives from the incorporation of an amino acid provided to the bacteria in the growth medium which is biosynthesized into the angucycline backbone. A sugar, 2,6-dideoxy-L-digitoxose, is incorporated into ring D in jadomycin B.

When jadomycin B was first biosynthesized using heat-shocked *S. venezuelae* ISP5230, the bacteria were grown in medium in which the only nitrogen source was the amino acid isoleucine [167]. It was noted that an isoleucine side group made up part of the oxazolone ring of jadomycin B, suggesting that the bacteria had metabolized the amino acid isoleucine and incorporated it into the jadomycin backbone. Doull, *et al* also documented that different pigmented product was biosynthesized by the *S. venezuelae* ISP5230 when different amino acids were used, which they presumed to be jadomycin B analogues in which the bacteria had incorporated a different amino acid [175].

The observation that *S. venezuelae* ISP5230 could biosynthesize novel jadomycin analogues by using different amino acids as a nitrogen source stimulated research by Jakeman, *et al* to create novel compounds and determine if they exhibited different chemical properties. Using a variety of natural and non-natural amino acids, it was determined that *S. venezuelae* ISP5230 could incorporate these amino acids in a non-enzymatic reaction to biosynthesize a variety of novel and unique jadomycin analogues [165,169,172,173,176].

1.12.00: THE BIOLOGICAL ACTIVITY OF JADOMYCINS

In initial pharmacological investigations, jadomycins were found to display antibacterial properties in several strains of *S. aureus*, *S. epidermis*, *Pseudomonas aeruginosa*, *Enterococcus faecalis*, and *Bacillus subtilis*. Interestingly, different analogues of jadomycins displayed different potencies in the various bacterial strains; for example jadomycins B, L, and F were found to be the most active of the 11 jadomycins

tested against methicillin-resistant *Staphylococcus aureus*. This evidences how different amino acids incorporated into the oxazolone ring of jadomycins can alter their activity, providing impetus to explore the bioactivity of various jadomycin analogues to determine if a certain amino-acid side group best exemplifies their biological activity [176].

The anticancer activity of jadomycins was first explored in 2005, when six jadomycin analogues were tested for activity against four different cancer cell lines: HepG2 (human hepatocellular carcinoma), IM-9 and IM-9/Bcl-2 (two human lymphoblast cell lines derived from multiple myeloma), and H640 (human non-small-cell lung cancer). Each jadomycin exhibited anticancer activity, but similar to the antimicrobial results observed by Jakeman, *et al*, there was differential activity between the jadomycins; specifically, jadomycin S was most potent in the HepG2, IM-9, and IM-9/Bcl-2 cell lines, while jadomycin F was most potent in the H460 cell line [177].

Borrisow, *et al*, tested the cytotoxicity of 19 jadomycin analogues in two breast cancer cell lines, T-47D (ER+, PR+, HER2-) and MDA-MB-435 (ER-, PR-, HER2+) [59,178,179], in a study published in 2007. The EC_{50} values for the treatments ranged between 1 and 30 μM, with the more potent analogues containing small polar side chains, such as jadomycin S, and the least potent analogues containing bulkier aromatic rings in the side chains, such as jadomycin W. This evidenced how the amino acid attached to the oxazolone ring in a jadomycin analogue can affect its anticancer activity, and also suggested which amino acids may best potentiate this activity. Additionally, the jadomycin analogues were consistently more toxic in the MDA-MB-435 versus the T-47D cells. Since the MDA-MB-435 cells are more highly proliferative than the T-47D cells, this evidenced a mechanism related to cell-cycle transit. Such an attribute suggests

that jadomycins could successfully target rapidly proliferating cancer cells over slower proliferating healthy cells; an important trait of any anticancer drug [169]. Nine jadomycin analogues have also been tested in MCF7 (ER+, PR+, HER2-) [179] breast cancer and HCT116 colorectal cancer cell lines, with IC_{50} values ranging from 0.97 to 66.8 μM. Supporting the results reported by Borrisow, *et al*, jadomycins with alkyl or small polar side groups showed the highest potencies against the cancer cells. In this same study, most jadomycins were found to be equally toxic to non-cancerous human mammary epithelial cells. An exception was jadomycin ornithine, which showed an approximately 2-fold reduction in potency in the non-cancerous versus cancerous breast cells. This suggests that the replacement of the hydroxyl functional group in the attached side chain, such as in jadomycins S or T, with an amino group could improve jadomycin selectivity [180]. Additionally, since Borrissow, *et al*, determined that jadomycins exhibit increased potency in more rapidly proliferating cells [169], jadomycins could still be selective for cancer cells *in vivo* since breast cancer cells typically proliferate approximately 11- to 15-fold faster than healthy breast cells in human breast cancer patients [181], warranting further research.

Two jadomycin analogues that contained D-norvaline (DNV) and D-norleucine (DNL) have been tested in a 60-cell line cancer screen by the National Cancer Institute. Jadomycins DNV and DNL were tested in six leukemia, nine non-small-cell lung carcinoma, six colon, six central nervous system, nine melanoma, seven ovarian, eight renal, two prostate, and six breast cancer cell lines at five doses spanning a 5 $\log_{10}$ concentration range. The percent growth and mean optical density were determined by the National Cancer Institute. Both analogues were found to be efficacious for almost all

of the cell lines tested, with the exception of the leukemia cells which displayed an as-of-yet unexplained innate resistance to the jadomycin treatments [182]. Similarly, seven novel jadomycin analogues containing a triazole moiety between the variable amino acid side group and oxazolone ring were biosynthesized and tested by the NCI in 60-cell line cancer screens. All of the compounds exhibited similar potencies, inhibiting the growth of the majority of the cell lines tested in the low micromolar range [183].

1.12.01: JADOMYCINS IN THE TREATMENT OF MULTIDRUG RESISTANT BREAST CANCER

Our laboratory previously examined the cytotoxic potency of multiple jadomycin analogues in drug-sensitive control MCF7 breast cancer cells compared with ABCB1-overexpressing paclitaxel-resistant (MCF7-TXL), ABCC1 overexpressing etoposide-resistant (MCF7-ETP), and ABCG2-overexpressing mitoxantrone-resistant (MCF7-MITX) MCF7 cell lines.

Using MTT cell viability assays, it was confirmed that the MCF7-TXL, MCF7-ETP, and MCF7-MITX cells were resistant to the cytotoxic effects of the ABCB1 substrate docetaxel, the ABCC1 substrate etoposide, the ABCB1 and ABCC1 substrate doxorubicin, and the ABCG2 substrate mitoxantrone. In comparison, the jadomycin analogues DNV, B, L, SPhG, F, S, T, and W all effectively induced breast cancer cell death in the three MDR MCF7 cell lines with only a small decrease in potency relative to the MCF7-CON cells; this decreased potency was significantly less than that observed

with the control drugs. This provided the first of our evidence that jadomycins largely retain their cytotoxic potency in ABC-transporter overexpressing MDR MCF7 cells [6].

Additionally, we determined that jadomycins are not inhibitors of ABCB1, ABCC1, or ABCG2 using human embryonic kidney (HEK)-293 cells stably transfected with these ABC-transporters. This is an important property to determine as it suggests jadomycins may be minimally susceptible to drug-drug interactions *in vivo* if they were to be used alongside other chemotherapeutics that are substrates for these transporters, and could therefore be viable candidates for chemotherapeutic combination therapies [6].

1.13.00: EARLY UNDERSTANDING OF JADOMYCINS' MECHANISMS OF ACTION

Before my work into jadomycins' mechanisms of action, only a rudimentary understanding of how they killed cancer cells was known. There are, however, a few preclinical studies that explored some possible mechanisms through which jadomycins could induce breast cancer cell death, which proved invaluable in the development of my own research project.

1.13.01: INHIBITION OF AURORA B KINASE

One potential jadomycin mechanism of action is through the inhibition of an enzyme called aurora B kinase [184]. There are three aurora kinases in humans, labelled aurora kinases A, B, and C [185]. Aurora B kinase is an important enzyme that is necessary for

correct microtubule-kinetochore attachment, chromosome alignment and segregation, and cytokinesis during cellular mitosis [186]. Additionally, the gene expression of both aurora kinases A and B are commonly overexpressed in a variety of primary cancer tumours [187]. Since aberrant aurora kinases lead to errors in chromosome alignment and segregation and because they are only expressed during mitosis, aurora kinase inhibition has little to no effect on quiescent cells. Therefore the inhibition of aurora B kinase is an attractive method for anticancer therapies [188].

Using computer-based virtual screening, Fu, *et al* identified jadomycin B as a possible aurora B kinase inhibitor, suggesting that jadomycin B could fit into the ATP-binding pocket of the enzyme and bind strongly to the residues surrounding this cleft, thus inhibiting its activity. In addition, this group then showed that jadomycin B directly inhibits the phosphorylating activity of aurora B kinase *in vitro*, and that it inhibits the phosphorylation of histone H3 on serine 10 (P-His3[Ser10]), a downstream target of aurora B kinase, in A549 lung carcinoma cells, HeLa cervical carcinoma cells, and HepG2 liver carcinoma cells. Together, this suggested that jadomycin B was an inhibitor of aurora B kinase [184], though further research was required to determine if aurora B kinase inhibition was a shared mechanism among various jadomycin analogues, and to determine if this mechanism contributes to jadomycin cytotoxicity in breast cancer cells.

1.13.02: DNA CLEAVAGE AND REACTIVE OXYGEN SPECIES INDUCTION

DNA damaging agents have a long history of use in chemotherapy. The earliest developed chemotherapeutics were nitrogen mustards and folate antagonists in the 1940s

[189,190], and both are effective cancer killing agents due to their DNA-damaging properties. DNA integrity is vital for maintaining cellular proliferation and function. DNA damage is detected by cell-cycle checkpoint proteins, which when activated halt the cell cycle to prevent the transmission of damaged DNA to new cells during mitosis. When DNA lesions occur during the S phase of the cell cycle it blocks replication fork progression, often leading to replication-associated DNA double strand breaks, which are among the most toxic forms of DNA damage. If this DNA damage is not repaired it typically results in cell death. Cancer cells often have more relaxed DNA damage-sensing and -repair mechanisms than healthy cells, and are able to ignore cell-cycle checkpoints that would tell a normal cell to halt proliferation. This is one way through which cancer cells are able to proliferate rapidly. However, this feature of cancer cells makes them susceptible to DNA damaging agents, since replicating damaged DNA increases the likelihood that cell death will ultimately be signaled. The concept of damaging DNA as a cancer treatment is widely exploited by various chemotherapeutics, including cisplatin, doxorubicin, 5-fluorouracil, etoposide, and gemcitabine [191]. One way that chemotherapeutics damage DNA is through the induction of ROS, which can react with DNA and cause cell lethal double strand breaks [192].

ROS such as hydrogen peroxide, hydroxyl radicals, or superoxide are constantly and naturally generated within biological systems [193]. In healthy cells, ROS levels are controlled by balancing the generation of ROS with their elimination *via* scavenging systems [194]. ROS perform many important roles in the body. They are involved in cell cycle progression [195], mediation of tumour suppressor genes such as *p53* [196,197], and protective mechanisms including apoptosis, phagocytosis, and detoxification

reactions [198]. Paradoxically, when intracellular ROS levels are heightened, damage can be done to proteins, lipids, and DNA; this is termed oxidative stress and can lead to the progression of cancer and other diseases [199,200,201]. Even higher, excessive levels of ROS can lead to irreparable damage and ultimately trigger cell death, and is a method used in anticancer chemotherapy [202]. The theory behind elevating intracellular levels of ROS through the use of anticancer drugs is based on the fact that cancer cells typically have innate levels of ROS higher than those observed in healthy cells, and are therefore already heavily dependent on their cellular antioxidant systems. These drugs increase ROS activity in the cancer cells past their tolerability threshold, inducing cell death. This is known as the "threshold concept for cancer therapy" [203,204]. Since healthy cells innately have lower levels of ROS they have a higher capacity to cope with the increased oxidative stress induced by certain anticancer drugs in comparison to tumour cells [204]. Using this method of chemotherapy, selectivity towards cancer cells versus healthy cells can be achieved [203].

Using *in vitro* acellular assays and purified, supercoiled bacterial plasmid DNA it was determined that jadomycin B cleaves DNA in the presence of Cu(II) ions. Ultraviolet-visible spectroscopy indicated that this cleavage does not involve direct jadomycin B-DNA binding, and instead it appears that jadomycin B forms a weak binding interaction with Cu(II) in the presence of DNA. It was noted that the Cu(II)-mediated cleavage was enhanced in the presence of ultraviolet light, which indicates that a jadomycin B radical cation and Cu(I) may be intermediates in DNA cleavage. Therefore Monro, *et al* suggested that jadomycin B may serve as a source of electrons for Cu(II) reduction, producing Cu(I) which in turn reacts with H_2O_2 to form extremely reactive hydroxyl

radicals, which then cause DNA cleavage. In addition, scavengers of the reactive hydroxyl radicals and superoxide inhibited jadomycin B's plasmid DNA cleaving properties, further suggesting that jadomycin B cleaves bacterial plasmid DNA through a Cu(II)-mediated induction of ROS *in vitro* [205].

In a follow-up study, it was found that two additional jadomycin analogues, L and SPhG, damaged DNA as well as jadomycin B. Interestingly, the changes in amino acid side groups had marked effects on the jadomycins' DNA damaging properties, where jadomycin L induced DNA double strand breaks at concentrations >20 μM in the absence of Cu(II), jadomycin B required Cu(II) ions to induce DNA damage, and jadomycin SPhG induced single-strand DNA breaks only after exposure to ultraviolet light. Additionally, when the amino acid side group was replaced with a simple hydrogen atom (labelled jadomycin G), all DNA damaging properties were lost. This further exemplifies how the activity of jadomycins can be affected by altering the attached amino acid *via* precursor-directed biosynthesis [206].

While this data shows that jadomycins can induce damage to bacterial plasmid DNA in acellular *in vitro* experiments, further trials were required to determine whether jadomycins induce ROS or damage DNA within cancer cells, and if the jadomycins cause cancer cell death as a result.

11.13.03: INDUCTION OF APOPTOSIS

The term apoptosis was first used in 1972 to describe a morphologically distinct form of programmed cell death [207] that allows an organism to remove unneeded or damaged

cells through a controlled and orderly process [208]. A wide range of anticancer agents induce apoptosis in cancerous cells, such as etoposide, mitoxantrone, and doxorubicin [209]. Apoptotic cells exhibit several biochemical modifications, such as protein cleavage, protein cross-linking, DNA breakdown, and phagocytic recognition [210], and morphological changes such as nuclear fragmentation, blebbing (bulging) at the cell surface, and disorganized cytoplasmic organelles, all with an intact cell membrane even late into the apoptotic process [211].

The majority of proteolytic cleavages that occur during apoptosis result from the action of a unique family of cysteine-dependent proteases called caspases [212]. Of the twelve known human caspases, six are confirmed to be involved in apoptosis mechanisms: caspases 3, 6, 7, 8, 9, and 10. These can be categorized into two separate classes: (1) Effector (or "downstream") caspases, which are responsible for the majority of proteolytic cleavages that disassemble the cell. The effector caspases are caspases 3, 6, and 7. (2) Initiator (or "upstream") caspases, which initiate the proteolytic cascade of the effector caspases. The initiator caspases are caspases 8, 9, and 10 [212,213,214].

Current apoptotic research suggests there are two main apoptotic pathways through which chemotherapeutics act: the extrinsic (or death receptor) pathway, and the intrinsic (or mitochondrial) pathway; however, there is evidence that these two pathways are not necessarily mutually exclusive and that reactions in one pathway can influence the other [208,215]. Briefly, the extrinsic pathways involve transmembrane receptor-mediated interactions. These so-called "death receptors" are members of the tumour necrosis factor (TNF) receptor gene superfamily [216]. When a ligand binds to its appropriate receptor,

such as TNFα binding to TNF receptor 1, a sequence of intracellular biochemical reactions is triggered that ultimately activates the initiator caspase 8 [208].

The intrinsic pathways involve a diverse array of non-receptor-mediated stimuli, such as radiation, ROS, or viral infections, which produce intracellular signals that act directly on intracellular targets, and are mitochondrial-initiated events. The apoptotic-inducing stimuli cause changes in the inner mitochondrial membrane which results in the release of pro-apoptotic proteins from the intermembrane space into the cytosol [217]. For example, cytochrome *c* is usually found in the intermembrane space of mitochondria, but can be released into the cytosol during intrinsic apoptosis where it forms a complex called an apoptosome with the protein Apaf-1, which then activates procaspase-9 thus continuing the apoptotic process [218,219]. The majority of anticancer drugs that induce apoptosis do so through this cytochrome c/Apaf-1/caspase-9 pathway [213].

A third apoptotic pathway is also possible that involves T-cell mediated cytotoxicity and perforin-granzyme-dependent killing of the cell, (granzymes are serine proteases that induce apoptosis in cells and are transported into a cell from the immune cells by being packaged with the protein perforin, which facilitates the delivery of the granzyme into the target cell). All three pathways ultimately converge on the same execution pathway, namely cleavage of the effector caspases, such as caspase 3 [208,220].

The major alternative to apoptosis is necrosis (though other forms of cell death are also possible, such as through autophagy), which is considered a toxic process where the cell is a passive victim and follows an energy-independent form of cell death. Necrosis is typically a more uncontrolled and passive process while apoptosis is controlled and energy-dependent. Necrosis typically occurs due to two main mechanisms: interference

with the energy supply of the cell or direct damage to the cell membrane. A variety of morphological changes occur during necrosis that ultimately cause a loss in cell membrane integrity, resulting in the release of the cytoplasmic contents into the surrounding tissue which often causes an inflammatory response [208]. Clinically, this is the most relevant difference between apoptosis and necrosis, as apoptotic cells do not release their cellular constituents into the surrounding tissue and are quickly phagocytosed by macrophages or adjacent normal cells, and therefore elicit essentially no inflammatory response [221,222].

Using propidium iodide and fluorescence-activated cell sorting (FACS) to detect DNA fragmentation that is characteristic of apoptosing cells [223], Fu, *et al* reported that jadomycin B induced apoptosis in A549 lung carcinoma cells at a concentration of 5 μg/mL, and that an increasing number of cells underwent apoptosis as the time of the jadomycin B treatment was increased (using 12, 24, and 48 hour time points). Fu, *et al* then confirmed their initial findings using chromatin condensation assays, in which apoptotic cells show brightly stained nuclei due to chromosome condensation which can be detected with the fluorescent dye Hoechst 33342, again showing that a 5 μg/mL dose of jadomycin B induces apoptosis in A549 cells. This is the first published work to suggest that a jadomycin causes apoptosis in cancer cells [184], which warranted further research into jadomycins' apoptosis-inducing properties.

1.13.04: BONDING WITH TOPOISOMERASE IIβ

Due to DNA's double helical structure, replication produces catenated DNA progenies that must be unlinked by topoisomerases in order to undergo cytokinesis. Topoisomerases prevent DNA supercoiling by regulating over- and under-winding during cell processes such as replication and transcription which occur during mitosis, with topoisomerase I repairing single strand breaks and type II topoisomerases repairing double strand breaks [224]. Topoisomerase inhibitors typically lead to DNA damage by preventing proper breaking and re-ligating, triggering pro-apoptotic caspases. Topoisomerase inhibitors are among the most efficient inducers of apoptosis, preferentially targeting rapidly proliferating cells, and are therefore effective anticancer therapies [224,225].

In a recently published study, Martinez-Farina, *et al* determined that the jadomycin analogue DS binds to topoisomerase IIβ [226], suggesting that topoisomerase-inhibition is a potential mechanism that could explain how jadomycins are able to induce DNA damage, warranting further studies.

1.14.00: OBJECTIVE AND HYPOTHESIS

Metastatic breast cancer remains an incurable disease primarily due to the development of MDR. The development of novel therapeutics that induce cancer cell death while avoiding ABC-transporter efflux is one method to treat such MDR tumour cells. We previously determined using MTT assays that multiple jadomycins largely retain their ability to reduce the viability of *ABCB1*-, *ABCC1*-, or *ABCG2*-overexpressing MDR MCF7 breast cancer cells, suggesting that they could be effective drugs in the treatment

of MDR metastatic breast cancer, warranting further research into their anticancer activity [6]. Additionally, only a simplistic understanding of the mechanisms through which jadomycins induce breast cancer cell death was known prior to my research. Understanding a drug's mechanisms of action helps to predict in which tumour types and patient subpopulations it is most likely to exhibit a clinical response. Such information is also useful in suggesting possible combination therapies with other chemotherapeutics, and in predicting potential mechanisms of disease resistance to the drug therapy [227]. Therefore, this warranted further study to better understand the mechanisms of action through which jadomycins induce breast cancer cell death.

I **hypothesized** that the ability to evade ABC transporter efflux combined with a polypharmacological mechanism of action both contribute to the retention of jadomycins' cytotoxic potency in MDR breast cancer cells. The **overall objective** of my research was to investigate the cytotoxicity of jadomycin analogues in drug-sensitive versus drug-resistant breast cancer cells, and to further elucidate the mechanisms through which jadomycins exert their anticancer activity. I investigate these objectives in three sequential studies, which are described in the following three chapters of this dissertation.

(1) Jadomycins were previously shown to reduce the viability of breast cancer cells, with *in vitro* evidence that jadomycin B inhibits aurora B kinase. The objectives of the first study were therefore to validate if jadomycins retain their cytotoxic potency in MDR MCF7 breast cancer cells that overexpress *ABCB1*, *ABCC1*, or *ABCG2* using a lactate dehydrogenase (LDH) assay for cell death, determine if jadomycin B inhibits aurora B kinase in MDR breast cancer cells, and explore novel cancer gene targets affected by jadomycin treatments.

(2) Data from study 1 suggested that ROS may be involved in jadomycins' anticancer activity. Therefore, the objectives of the second study were to determine if jadomycins induce ROS in MDR and control MCF7 breast cancer cells, the role these ROS play in jadomycins' cytotoxic activity, and which intracellular antioxidant pathways are involved in the metabolism of jadomycin-generated ROS using pharmacological inhibitors to better understand this mechanism of action.

(3) Data from study 2 suggested that jadomycin cytotoxicity is potentiated by the induction of ROS in MCF7 cells. We believed it was important to determine if this mechanism was retained in an additional and more aggressive triple-negative breast cancer cell line, MDA-MB-231, that also overexpressed *ABCB1*, and whether this ROS-induction was causing intracellular DNA damage and cell death *via* apoptosis. The objectives of the third study were therefore to determine if jadomycin cytotoxic potency is retained in control and MDR MDA-MB-231 breast cancer cells, to determine if jadomycins induce DNA damage and apoptosis in these cells, and to determine if these events are the result of jadomycin-induced ROS activity or a secondary mechanism, namely the inhibition of type II topoisomerases.

Jadomycins have shown promising biological activity and many analogues with different properties have been created *via* precursor-directed biosynthesis. Through my research I have confirmed that jadomycins retain their cytotoxic potency in MDR, ABC-transporter overexpressing breast cancer cells, and determined multiple intracellular mechanisms responsible for jadomycins' anticancer activity.

CHAPTER 2.00.00: EXPLORATION OF JADOMYCINS' CYTOTOXIC POTENCY AND MECHANISMS OF ACTION IN ABC-TRANSPORTER OVEREXPRESSING MCF7 BREAST CANCER CELLS

The work described in this chapter was conducted by myself unless otherwise indicated; all Figures and Table 2 were published as part of the paper "Jadomycins are cytotoxic to *ABCB1*-, *ABCC1*-, and *ABCG2*-overexpressing MCF7 breast cancer cells", on which I am the second author.

Original publication information:

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Anti-Cancer Drugs, 2014, Issue 3, Volume 25, Pages: 255-269

2.01.00: ABSTRACT

Multidrug resistance remains a major obstacle in the effective treatment of metastatic breast cancer. One mechanism by which multidrug resistance is conferred is through decreased intracellular drug accumulation due to the upregulation of ABC transporters. Jadomycins, polyketide-derived natural products produced by *Streptomyces venezuelae* ISP5230, inhibit the growth of human breast ductal carcinoma cell lines T47D and MDA-MB-435 and largely retain their ability to reduce cell viability, as measured with MTT assays, in ABC-transporter overexpressing MDR MCF7 versus MCF7-CON breast cancer cells. The 1st goal of the present study was to validate the effect of ABC-drug efflux transporter activity on jadomycin cytotoxicity using LDH cell death measuring assays in control versus *ABCB1*-, *ABCC1*-, or *ABCG2*-overexpressing MDR MCF7 cells. Seven jadomycin analogues (DNV, B, L, SPhG, F, S and T) effectively killed MCF7 control and *ABCB1*-, *ABCC1*-, or *ABCG2*-overexpressing MCF7 breast cancer cells as measured with LDH assays. The inhibition of ABCB1, ABCC1, or ABCG2 with verapamil, MK-571 sodium salt hydrate (MK-571), or ko-143 hydrate (ko-143), respectively, did not augment the cytotoxicity of jadomycins DNV, B, L, SPhG, F, S, or T in drug-resistant MCF7 cells, suggesting these jadomycins are not effluxed by these ABC-transporters. The 2nd goal was to explore the mechanisms of action through which jadomycins induce breast cancer cell death. This included an evaluation of the putative jadomycin mechanism of aurora B kinase inhibition and a PCR cancer gene array to identify potential new targets. Jadomycin B inhibited aurora B kinase activity in MCF7-CON and MDR MCF7 cells, and jadomycin S altered the expression of 21 cancer gene targets in MCF7-CON cells as measured by the PCR cancer gene array, 4 of which were

verified using quantitative PCR, *TrxR1*, *IGF1*, *BCL2*, and *HDAC11*. We conclude that jadomycins B, L, SPhG, F, S, and T are effective agents in the eradication of MCF7 breast cancer cells grown in culture, that their cytotoxicities are minimally affected by ABCB1, ABCC1, and ABCG2 efflux transporter function, and that their cytotoxic mechanisms of action include aurora B kinase inhibition and possibly additional targets as well, warranting further mechanism of action research.

2.02.00: INTRODUCTION

Breast cancer is the most commonly diagnosed form of malignancy in women [228], and it is estimated that 20-30% of breast cancers will eventually metastasize [4,229]. MDR affects virtually all drug therapies available for metastatic breast cancer [4,101]. The most frequently encountered mechanism of MDR is the decreased intracellular accumulation of cytotoxic drugs due to the upregulation of ABC drug efflux transporters [112,230,231].

One strategy to overcome MDR is to identify novel anticancer agents that are poor substrates of ABC transporters [154]. Work conducted previously in our laboratory suggested that jadomycins, polyketide derived natural products secreted by the soil bacteria *Streptomyces venezuelae* ISP5230 [165], may be useful MDR anticancer agents in this regard. Using MTT cell viability assays it was shown that seven jadomycin analogues, DNV, B, L, SPhG, F, S, and T, were all similarly cytotoxic in drug-sensitive MCF7-CON and drug-resistant *ABCB1*-, *ABCC2*-, or *ABCG2*-overexpressing MCF7 cells (labelled MCF7-TXL, -ETP, and -MITX, respectively). Furthermore, the toxicity of four jadomycins (B, DNV, L and S) in the ABC-transporter overexpressing MCF7 cells was not enhanced by chemical inhibitors of these transporters [6]. Based on these data, we hypothesized that jadomycin cytotoxicity is independent of ABC efflux transporter activity. In addition, given the evidence at the time supporting that jadomycins act through intracellular mechanisms (aurora B kinase inhibition, DNA damage, and induction of apoptosis [169,184,206]) it was crucial to verify whether ABC drug efflux

transporters differentially impact the pharmacological activity of jadomycins in order to properly determine their potential as anticancer drugs in MDR breast tumour cells.

Continuing this line of research I had three objectives: 1) to validate if jadomycins retain their cytotoxic properties in drug-resistant MCF7 cells using cell death measuring LDH assays and compare the results to those from the previously completed cell viability measuring MTT assays; 2) to evaluate the effect of inhibiting ABCB1, ABCC1, or ABCG2 transporters on the cytotoxicity of the remaining potent jadomycins F, T, and SPhG to thoroughly assess structural activity relationships; (3) to generate preliminary data on jadomycins' mechanisms of action in breast cancer cells. For the third objective I chose to evaluate the effect of jadomycins on aurora B kinase, which was a potential target based on our review of the literature at the time, and to complete PCR cancer gene arrays and quantitative PCR to identify potential new targets to guide my subsequent mechanism of action experimental focus.

2.03.00: MATERIALS AND METHODS

2.03.01: CHEMICALS

Paclitaxel, docetaxel, mitoxantrone dihydrochloride, MTT, dimethylsulfoxide, methanol, doxorubicin hydrochloride, VRP, MK-571, ko143, sodium lactate, phenazine methosulfate, β-nicotinamide adenine dinucleotide, and iodonitrotetrazolium chloride were purchased from Sigma Aldrich (Oakville, ON, Canada).

2.03.02: THE PRODUCTION OF JADOMYCINS

The jadomycin analogues D-norvaline (DNV), isoleucine (B), leucine (L), S-phenylglycine (SPhG), phenylalanine (F), tryptophan (W), serine (S), and threonine (T) were synthesized and purified using an established methodology [182,183,232]. These jadomycins are divided into three main categories: jadomycins derived from the assimilation of amino acids with hydrophobic aliphatic side chains (DNV, B, and L), hydrophobic aromatic side chains (SPhG, F, and W) and hydrophilic side chains (S and T) (**Figure 4**). Briefly, *S. venezuelae* ISP5230 colonies were grown in minimal culture medium that included the amino acid of interest as the sole nitrogen source. The *S. venezuelae* ISP5230 bacteria were shocked with a 3% final concentration of ethanol to induce secondary metabolism. The cultures were monitored spectrophotometrically at 600 and 526 nm for accurate measurement of cellular growth and natural product production, respectively. The crude products were collected using a reverse-phase C_{18} column. The crude extracts containing jadomycins were then purified *via* column chromatography. The identity of each purified jadomycin was confirmed using

ultraviolet-visible, infrared, and nuclear magnetic resonance spectroscopy and low resolution and high resolution mass spectrometry.

2.03.03: CELL LINES

The MCF7 cells and their derivative sub-lines were kindly provided by Drs. Robert Robey and Susan Bates (National Cancer Institute, National Institutes of Health, Bethesda, MD). Serial passages of MCF7 cells in medium containing increasing concentrations of paclitaxel (MCF7-TXL), etoposide (MCF7-ETP) and mitoxantrone (MCF7-MITX), were previously used to generate resistant sub-lines that were, respectively, characterized by the upregulation of *ABCB1*, *ABCC1*, and *ABCG2*, respectively [231,233,234]. The MCF7-CON and the MCF7-TXL, -ETP, and -MITX resistant sub-lines were cultured in phenol red free Dulbecco's modified Eagles medium (Thermo Scientific, Ottawa, ON, Canada) supplemented with 10% fetal bovine serum (FBS), 100 IU/mL penicillin, 250 μg/mL streptomycin (Invitrogen, Burlington, ON, Canada) and 1 mM sodium pyruvate (Sigma, St. Louis, MO, USA). Additionally, the media for MCF7-TXL, -ETP, and -MITX cells were continuously supplemented with 400 nM paclitaxel, 4 μM etoposide, and 100 nM mitoxantrone, respectively. The growth medium was changed every 2 or 3 days and the cells were maintained in a humidified atmosphere supplemented with 5% CO_2 at 37°C (standard conditions). All MCF7 cells were grown in drug-free culture medium for 1 week prior to experiments.

2.03.04: LACTATE DEHYDROGENASE ASSAYS

Cytotoxicity against MCF7-CON, -TXL, -ETP, and -MITX cells was tested using a LDH-release assay [235,236]. Briefly, the cells were seeded in 96-well plates (20,000 cells/well), incubated for 24 hours under standard conditions, treated with increasing concentrations of jadomycins (0.3-180 μM) or control drugs doxorubicin or mitoxantrone and then incubated for a further 48 hours. The supernatant was removed from each well and placed in a new plate. These cells were lysed with 0.1 % Triton X-100 and their LDH activity used as an index of cell death (LDH_D). The viable cells that remained adhered to the original plate were also treated with 0.1% Triton X-100 and their LDH activity measured (LDH_V). LDH activity was measured by treating 100 μL of cell lysate, supernatant, or commercial LDH standard solution (Cayman Chemical, MI, USA) with 100 μL of in-house LDH-reaction solution (25 mM sodium lactate, 147 μM phenazine methosulfate, 644 μM β-nicotinamide adenine dinucleotide and 326 μM iodonitrotetrazolium chloride in 200 mM Tris buffer, pH = 8). The plate was shaken (650 rpm, 5-10 minutes) and absorbance (490 nm) measured using a Biotek Synergy HT plate reader [235]. The LDH activity of each well was calculated using formula 1:

$$\text{LDH activity} = (A_{490} - \text{y-intercept})/\text{slope} \qquad (1)$$

Where A_{490} is the sample absorbance and the *y-intercept* and *slope* are values determined from the LDH standard curve. For each drug-treated sample the percentage of total cell death could then be calculated using equation 2:

$$\text{Total \% cell death} = 100*(LDH_D)/(LDH_D + LDH_V) \quad (2)$$

The concentration of drug that resulted in 50% cell death (EC_{50}) was calculated from the log_{10} concentration versus response curves using the following equation 3 for each jadomycin:

$$y = \text{bottom of curve} + (\text{top of curve} - \text{bottom of curve})/(1 + 10^{(\text{LogEC50} - x)*\text{Hill slope}}) \quad (3)$$

Where *y* is the percent-cell death and *x* is the drug concentration. The fold-resistance values for each particular drug were obtained by dividing their EC_{50} value in the drug-resistant MCF7 cells by their mean EC_{50} value found in the MCF7-CON cells [237].

2.03.05: MTT VIABILITY ASSAYS

To help determine if jadomycin cytotoxicity was affected by ABCB1, ABCC1, or ABCG2 efflux function, MTT cell viability assays were used after exposing drug-sensitive and drug-resistant MCF7 cells to various concentrations of jadomycins with or without the inhibitors of the ABC transporters, 7.5 μM VRP (ABCB1), 25 μM MK-571 (ABCC1) and 0.5 μM Ko-143 (ABCG2) [238,239,240]. The fold-reversal of MDR for a given drug treatment was calculated by dividing the IC_{50} for the drug-treated cells by the mean IC_{50} in the drug plus ABC transporter inhibitor-treated cells [241].

Briefly, MCF7-CON, MCF7-TXL, MCF7-ETP, and MCF7-MITX cells were seeded in 96-well plates at 5,000 cells/well in 100 μL of medium. The cells were allowed to adhere for 24 hours under standard conditions then treated with jadomycins (0.1 – 100 μM) or control cytotoxic drugs for 72 hours. After the incubation, 20 μL of MTT solution was added to the wells for 2 hours. The medium and MTT mixture was aspirated and the formazan-containing cells were dissolved in 100 μL of dimethylsulfoxide. Optical

density of formazan was measured at 550 nm on a Biotek Synergy HT plate reader (Biotek, Winooski, VT, USA). The percentage of cell viability was calculated as the absorbance of each test well divided by the average absorbance of the vehicle control wells multiplied by 100. The concentration that resulted in a 50% reduction in viability (IC_{50}) was calculated from the $\log_{10}$ concentration versus normalized response curves using equation 4:

$$y = 100/(1 + 10^{(\text{LogIC50-}x)*\text{Hill slope}}) \qquad (4)$$

Where y is the measured absorbance at 550 nm and x is the drug concentration.

2.03.06: WESTERN BLOT ANALYSIS

MCF7-CON, -TXL, and -MITX cells were grown in 6 well plates until they reached exponential growth. They were then treated in triplicate for 24 hours with vehicle, 5, or 10 μM jadomycin B (MCF7-CON) or vehicle, 10, or 20 μM jadomycin B (MCF7-TXL and -MITX). Cells were washed with cold PBS and lysed in 150 μL of radioimmunoprecipitation assay lysis buffer that contained phenylmethylsulfonyl fluoride, protease inhibitor cocktail, and sodium orthovanadate (Santa Cruz, cat. #SC-24948). The triplicate samples for each treatment were pooled and the protein content of the whole cell extracts were quantified using the Lowry method [242]. Twenty μg of each pooled sample was separated with 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were transferred to nitrocellulose membranes. The membranes were incubated overnight in a 1:1,000 dilution of monoclonal mouse anti-human P-His3(Ser10) antibody (Cell Signaling Technology, Danvers, MA) and

polyclonal goat anti-human β-actin antibody (Abcam Inc. Cambridge, MA) at 4 °C. Following washing, the membranes were incubated in 1:10,000 dilutions of IRDye 680RD conjugated donkey anti-mouse and IRDye 800CW conjugated donkey anti-goat secondary antibodies for 1 hour at room temperature. For the visualization of P-His3(Ser10) and β-actin the membranes were scanned at 700 and 800 nm infrared wavelengths, respectively using a Licor odyssey (Mandel Scientific, Guelph, ON). The pixel intensity of each P-His3(Ser10) was normalized to the intensity of the respective β-actin and these ratios were then expressed as a percentage of the vehicle-treated MCF7-CON cells.

2.03.07: RNA COLLECTION, REVERSE TRANSCRIPTION, AND PCR CANCER GENE ARRAY OR QUANTITATIVE REAL-TIME PCR

Total RNA was isolated from lysates of MCF7-CON cells treated with jadomycin S (10 μM) or jadomycin vehicle for 24 hours under standard conditions using the Aurum total RNA Mini Kit according to the manufacturer's instructions. Isolated RNA (0.5 μg) was reverse-transcribed to complementary DNA using Super Script II Reverse Transcriptase (Life Technologies). The complementary DNA was amplified *via* quantitative PCR using (a) the gene-specific primers provided in the Human Cancer Drug Targets PCR Array (Qiagen, Toronto, Canada) or (b) 125 nM gene-specific primers in a total volume of 20 μL using a SYBR Green PCR Kit using a Step One Plus real-time PCR thermocycler (Applied Biosystems). Gene expression was normalized (a) for the PCR cancer gene array using the average of the four housekeeping genes *glyceraldehyde phosphate dehydrogenase* (*GAPDH*), *β-actin*, *hypoxanthine phosphoribosyltransferase 1* (*HPRT1*),

and *ribosomal protein L13a* (*RPL13A*) as provided by the kit, or (b) for the qPCR trials using *GAPDH via* the $\Delta\Delta C_t$ method [243].

2.03.08: STATISTICAL ANALYSIS

For each cell culture experiment, the individual treatments were performed in triplicate, quadruplicate, or quintuplicate. Each cell culture experiment was repeated at least three times. All data are expressed as mean ± SEM. An unpaired t-test was used for statistical comparison of experiments involving two groups. A one-way ANOVA was used for multiple comparisons in experiments with one independent variable. A two-way ANOVA was used for multiple comparison procedures in experiments with two independent variables. A Bonferroni test was used for *post-hoc* analysis of the significant ANOVA. A difference in mean values between groups was considered to be significant when $P \leq 0.05$.

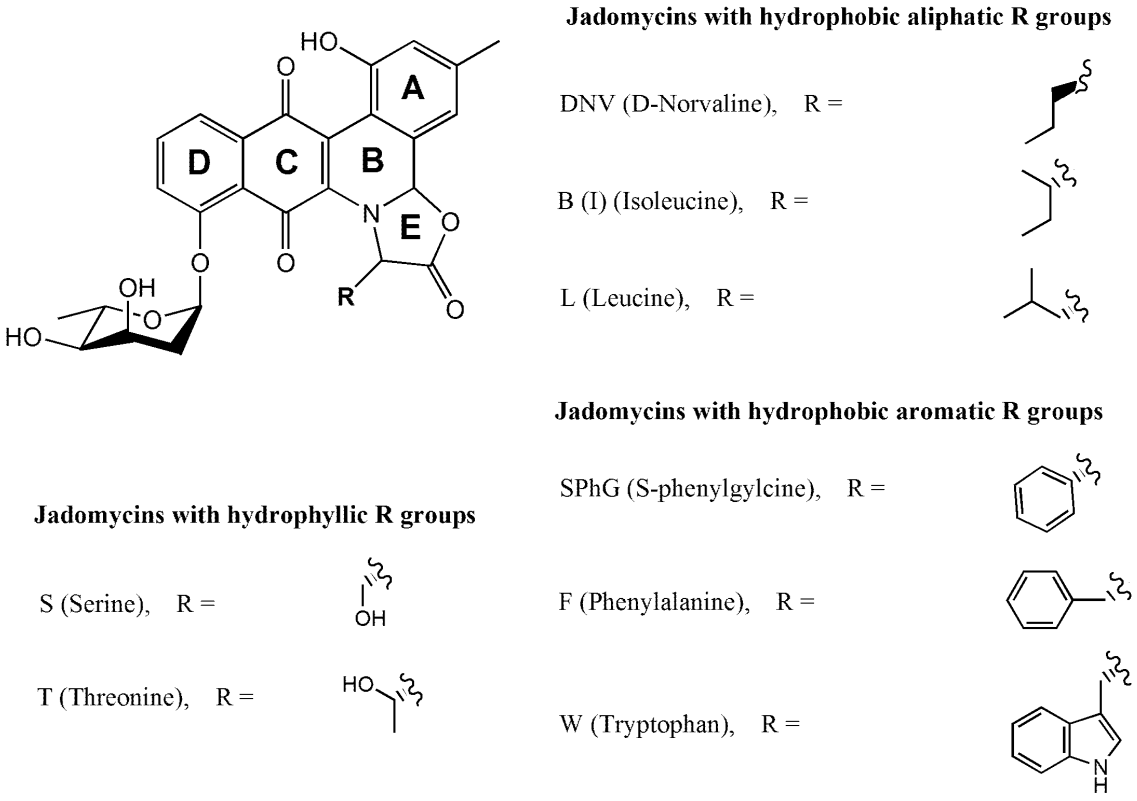


Figure 4: Structures of jadomycin analogues used for this study. The nitrogen heteroatom of the oxazolone ring E derives from the incorporation of an amino acid provided to the bacteria in the growth medium which is biosynthesized into the angucycline backbone. The specific amino acid used can alter the structure of the jadomycin as indicated by the various R-groups to produce various analogues. A sugar, 2,6-dideoxy-L-digitoxose, is incorporated into ring D.

2.04.00: RESULTS

2.04.01: JADOMYCINS ARE TOXIC TO DRUG-SENSITIVE AND DRUG-RESISTANT MCF7 CELLS AS MEASURED BY LDH ASSAYS

As a measure of drug cytotoxicity in MCF7 cells we employed LDH assays, which measure the LDH released into the medium from dead or dying cells. When compared to MCF7-CON cells, the control cytotoxic drugs, doxorubicin and mitoxantrone [145,244], demonstrated significantly lower potency (higher EC_{50} values) towards the corresponding MCF7-TXL, -ETP, and -MITX drug-resistant cells. Similar to what was seen previously with the MTT cell viability assays [6], LDH assays showed that jadomycins DNV, B, L, SPhG, F, S, and T effectively killed the MCF7-TXL, -ETP, and, -MITX resistant cell lines with only small increases (1.5-3.5) in fold-resistances relative to the MCF7-CON cells. Also similar to the MTT assays, jadomycin W was less potent than the other jadomycins. For the LDH assays, the declines in potency were significant for jadomycins DNV, S, and T in all three resistant cell lines, for jadomycin B in the MCF7-ETP and MCF7-MITX cells, for jadomycin L in the MCF7-ETP cells, and for jadomycin F in the MCF7-TXL cells (**Table 4**). Similar to the MTT assays, the LDH assays demonstrated significantly higher fold-resistances to doxorubicin in MCF7-TXL and -ETP cells (88.2-fold and 12.1 fold, respectively) or mitoxantrone in MCF7-MITX cells (>100 μM EC_{50} value) in comparison to all eight tested jadomycins (**Figures 5a-c**).

2.04.02: THE INHIBITION OF ABCB1, ABCC1, OR ABCG2 DOES NOT AUGMENT JADOMYCIN EFFECTS ON MCF7 CELL VIABILITY

To further explore if the anticancer effects of jadomycins are dependent or independent of ABCB1, ABCC1, and ABCG2 function, we compared the ability of ABCB1 (VRP), ABCC1 (MK-571), and ABCG2 (Ko-143) inhibitors to sensitize the MCF7 cell lines to jadomycin and control drug (doxorubicin and mitoxantrone) treatments [240,245,246]. As expected, VRP, MK-571, and Ko-143 significantly reduced the IC_{50} of doxorubicin in MCF7-TXL and -ETP cells and that of mitoxantrone in MCF7-MITX cells, from 92, 27, and 120 μM to 19.1, 6.8 and 3.9 μM, respectively (**Figures 6a-c**). In comparison, in the MCF7-TXL, -ETP, and -MITX cells, the respective ABCB1, ABCC1, and ABCG2 inhibitors tended to reduce the IC_{50} values of jadomycins by only 25 to 50 % (**Figures 6d-x**). These reductions in IC_{50} values in the resistant MCF7 cells were only significant in the cases of jadomycins SPhG and F in response to the addition of the ABCC1 inhibitor MK-571 (**Figures 6n** and **q**). Unexpectedly, MK-571 and Ko-143 significantly reduced the baseline IC_{50} values of doxorubicin and mitoxantrone in the MCF7-CON cells (**Figures 6b** and **c**). Similarly, the MCF7-CON cells were sensitized to jadomycin B by VRP (**Figure 6g**), to jadomycins DNV, B, L, SPhG and S by MK-571 (**Figures 6e, h, k, n,** and **t**) and to jadomycins B and L by Ko-143 (**Figures 6i** and **l**).

Given that the ABC transporter inhibitors sensitized MCF7-CON cells to control drugs and jadomycins in some cases, we employed a secondary analysis in which we compared the "fold-reversal" of IC_{50} values in response to the inhibition of ABC transporters in MCF7-CON to those in MCF7-TXL, -ETP, and -MITX resistant cells. The fold-reversal is a measure of the magnitude to which an ABC efflux transporter inhibitor can sensitize

cells to the effects of a given cytotoxic drug, thus reversing the MDR phenotype. The greater the fold-reversal value for a given drug in a given cell line, the more that drug is affected by the overexpression of the ABC-transporter in question, and the stronger the evidence suggesting that drug is a substrate of that transporter. The magnitude of fold-reversal exhibited by resistant cells in response to doxorubicin in MCF7-TXL and MCF7-ETP cells and mitoxantrone in MCF7-MITX cells was significantly higher than that observed in the MCF7-CON cells. In contrast, the fold-reversal of jadomycins was non-specific, in that it was unchanged in the resistant cells versus the control cells, with the exception of jadomycin SPhG which, to the contrary, displayed significantly higher fold-reversal in the MCF7-CON cells than in the MCF7-ETP resistant cells (**Figure 7**).

2.04.03: JADOMYCIN B INHIBITS AURORA B KINASE

Based on the similar jadomycin cytotoxicity profiles in the control and drug-resistant MCF7 cells, we hypothesized that the effects of jadomycins on their intracellular targets would be preserved in the MDR cell lines. We addressed this by examining the effect of jadomycin B on the inhibition of aurora B kinase-dependent P-His3(Ser10) in MCF7-CON, -TXL, and -MITX cells; jadomycin B was chosen as a representative analogue to best replicate the experiments completed by Fu, *et al* [184]. As measured by the normalized P-His3(Ser10)/β-actin ratio, P-His3(Ser10) was dose-dependently and significantly reduced versus the vehicle control (labelled 0 μM) by jadomycin B in MCF7-MITX cells (20 μM), MCF7-TXL cells (10 and 20 μM), and MCF7-CON cells (5 and 10 μM) (**Figure 8**).

2.04.04: JADOMYCINS B AND S INFLUENCE CANCER GENE TARGETS

To expand on our knowledge of jadomycins' mechanisms of action, we used a Human Cancer Drug Targets PCR Gene Array in MCF7-CON cells treated with jadomycin S (10 μM, 24 h) versus those treated with vehicle. Due to the similar bioactivity observed between jadomycin analogues, one analogue was chosen as a representative jadomycin. Jadomycin S was chosen due to its greater water solubility and biosynthetic yields versus the other jadomycins. By measuring the relative gene expression of 84 cancer treatment targets, we identified twenty-one potential "hits" (defined as an at least 3-fold change in the gene's regulation) in cells treated with jadomycin S versus vehicle control. This included 2 genes with increased expression and 19 genes with decreased expression, all of which are summarized in **Table 5**.

Based on these preliminary array results, we used qPCR to determine if jadomycins B and S (10 μM, 24 h), chosen as representative jadomycin analogues, significantly altered the expression of these gene hits and therefore warranted further study. The genes *TrxR1*, *BCL2*, and *HDAC11* were significantly altered by both jadomycins B and S versus vehicle, and *IGF1* was by jadomycin B (**Table 6)**. The primers used for these qPCR trials are listed in **Table 7**.

Table 4: EC_{50} values (μM) measuring the cytotoxic effects of control drugs and jadomycins in drug-sensitive and drug-resistant MCF7 cells as measured by LDH assays. Data represent the mean EC_{50}s ± SEM of at least three independent experiments performed in quadruplicate. The fold-resistance values were calculated by dividing the EC_{50} of the drug in the MCF7-TXL, -ETP, or -MITX resistant cell lines by the EC_{50} of the drug in the MCF7-CON cells. * EC_{50} value in MCF7-TXL, -ETP, or -MITX cells was significantly different from the corresponding EC_{50} value in the MCF7-CON cells, as determined by a one-way ANOVA, followed by a Bonferroni's multiple comparison test ($P < 0.05$).

EC_{50} ± SEM μM (fold-resistance)

		Resistant Cells		
Control Drugs	**MCF7-CON**	**MCF7-TXL**	**MCF7-ETP**	**MCF7-MITX**
Doxorubicin	1.1 ± 0.2	97.9 ± 9.1 (88.2)*	13.3 ± 4.9 (12.1)*	
Mitoxantrone	1.1 ± 0.3			>100

Jadomycins	**MCF7-CON**	**MCF7-TXL**	**MCF7-ETP**	**MCF7-MITX**
Jadomycin DNV	4.7 ± 0.2	13.3 ± 1.5 (2.8)*	11.3 ± 0.9 (2.3)*	12.3 ± 0.01 (2.5)*
Jadomycin B	4.3 ± 0.7	11.8 ± 1.1 (2.7)	13.9 ± 2.3 (3.2)*	13.7 ± 1.3 (3.1)*
Jadomycin L	6.9 ± 2.0	16.4 ± 3.3 (2.3)	17.8 ± 1.6 (2.5)*	12.6 ± 0.5 (1.8)
Jadomycin SPhG	5.7 ± 1.1	12.3 ± 3.2 (2.1)	13.3 ± 2.0 (2.3)	11.4 ± 1.2 (2.0)
Jadomycin F	4.4 ± 0.1	15.6 ± 1.7 (3.5)*	10.9 ± 2.9 (2.4)	11.4 ± 0.8 (2.5)
Jadomycin W	42.3 ± 3.0	88.9 ± 21.0 (2.1)	67.0 ± 6.9 (1.5)	94.2 ± 24.3 (2.2)
Jadomycin S	4.4 ± 0.3	12.0 ± 2.2 (2.7)*	10.7 ± 1.2 (2.4)*	11.3 ± 0.7 (2.5)*
Jadomycin T	4.8 ± 0.5	12.3 ± 0.5 (2.5)*	11.4 ± 2.0 (2.3)*	14.8 ± 1.0 (3.0)*

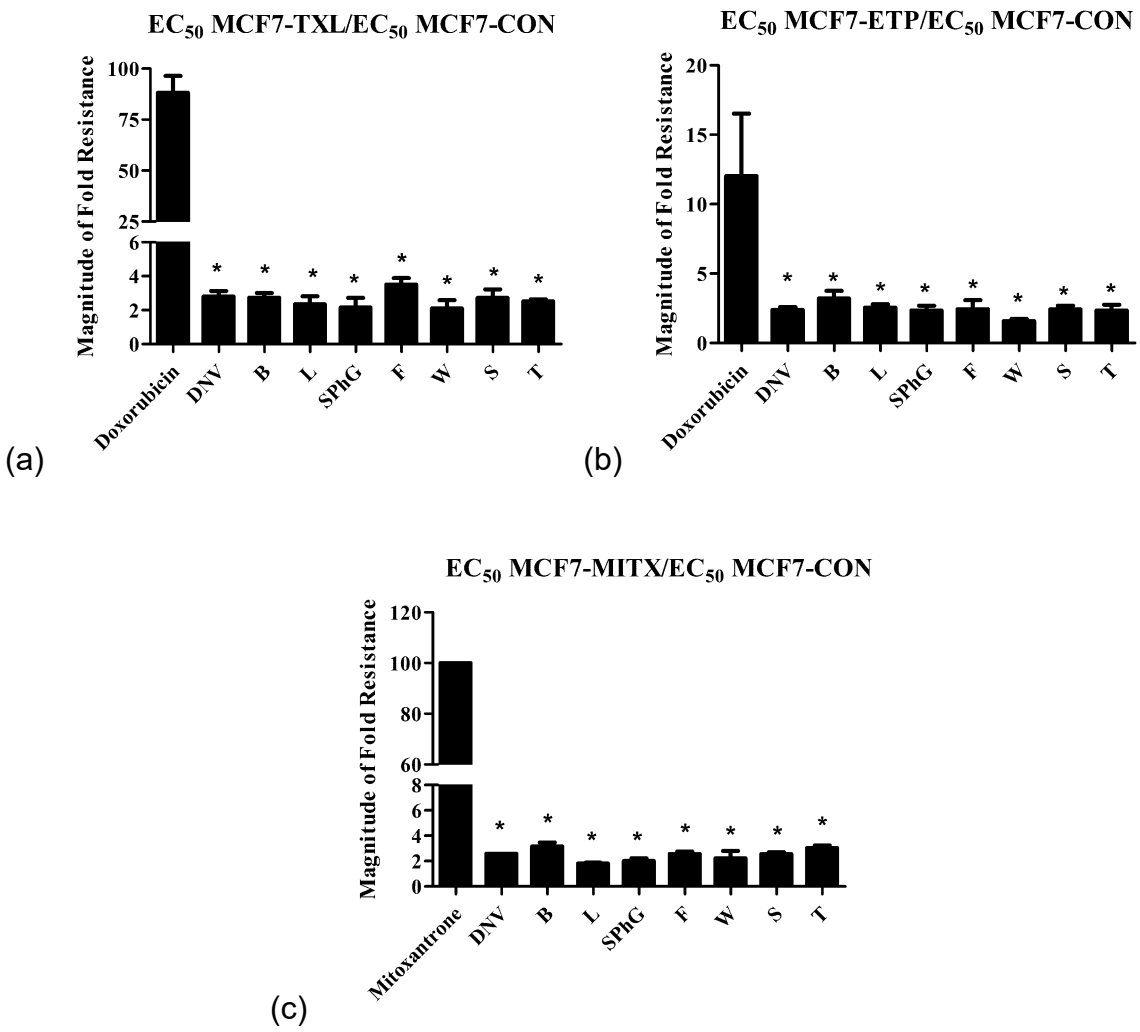
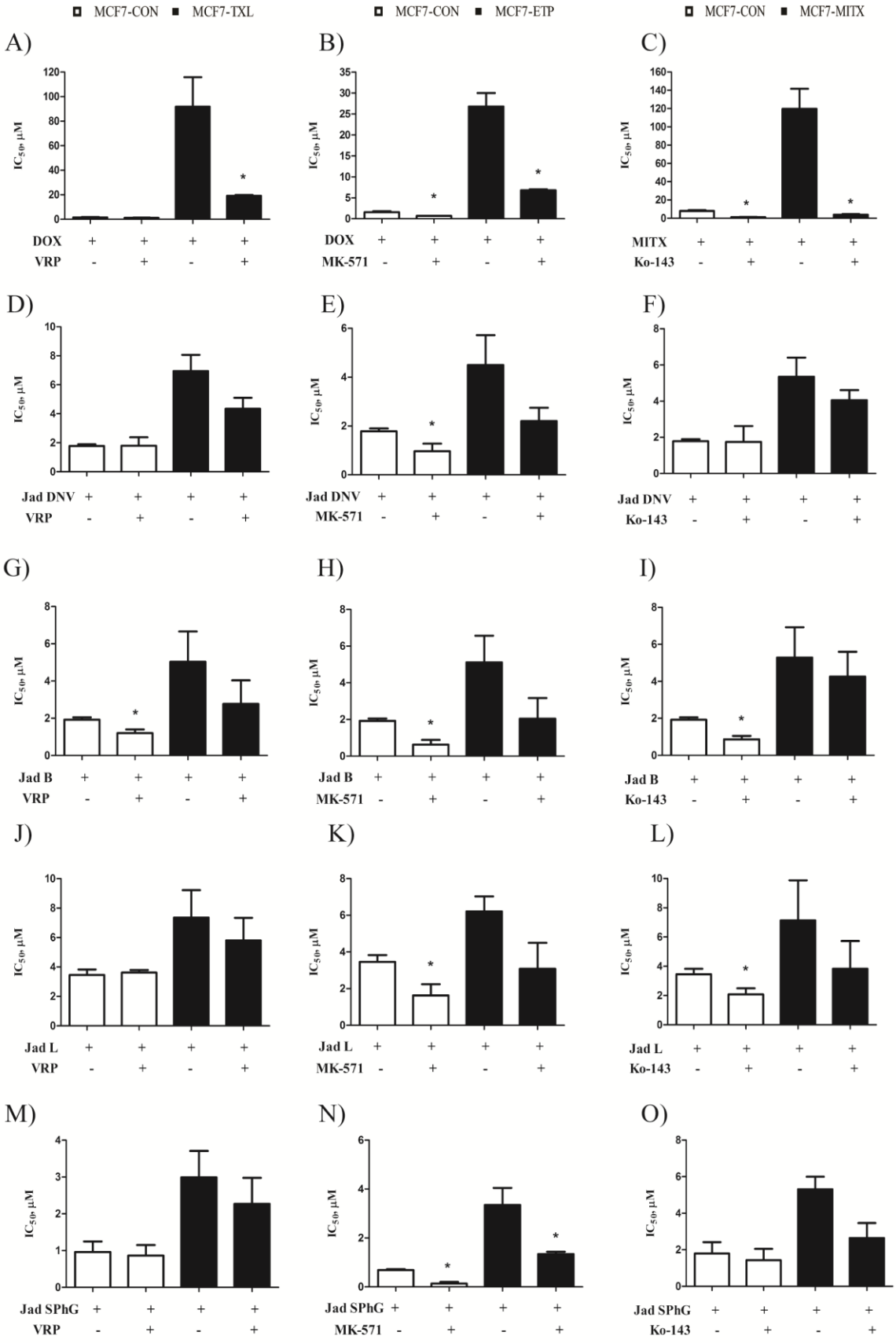


Figure 5: Fold-resistances of jadomycins and control drugs in MCF7-TXL, MCF7-ETP, and MCF7-MITX cells. EC_{50} values were obtained using LDH cytotoxicity assays for each drug, and the fold-resistance was calculated by dividing the EC_{50} values of a particular drug treatment in each of the resistant cell lines by the mean EC_{50} value calculated in control cells for that specific drug. The fold resistance values of control cytotoxic drugs were then compared to those of the jadomycins in MCF7-TXL, -ETP, and -MITX cells. * The fold-resistance of the jadomycin was significantly lower compared to the fold-resistance of the corresponding control cytotoxic agent as determined by a one-way ANOVA, followed by Bonferroni's multiple comparison test ($P < 0.05$).


MCF7-CON
MCF7-TXL
MCF7-ETP
MCF7-MITX
A)
B)
C)
D)
E)
F)
G)
H)
I)
J)
K)
L)
M)
N)
O)
IC50, µM
DOX
VRP
MK-571
MITX
Ko-143
Jad DNV
Jad B
Jad L
Jad SPhG

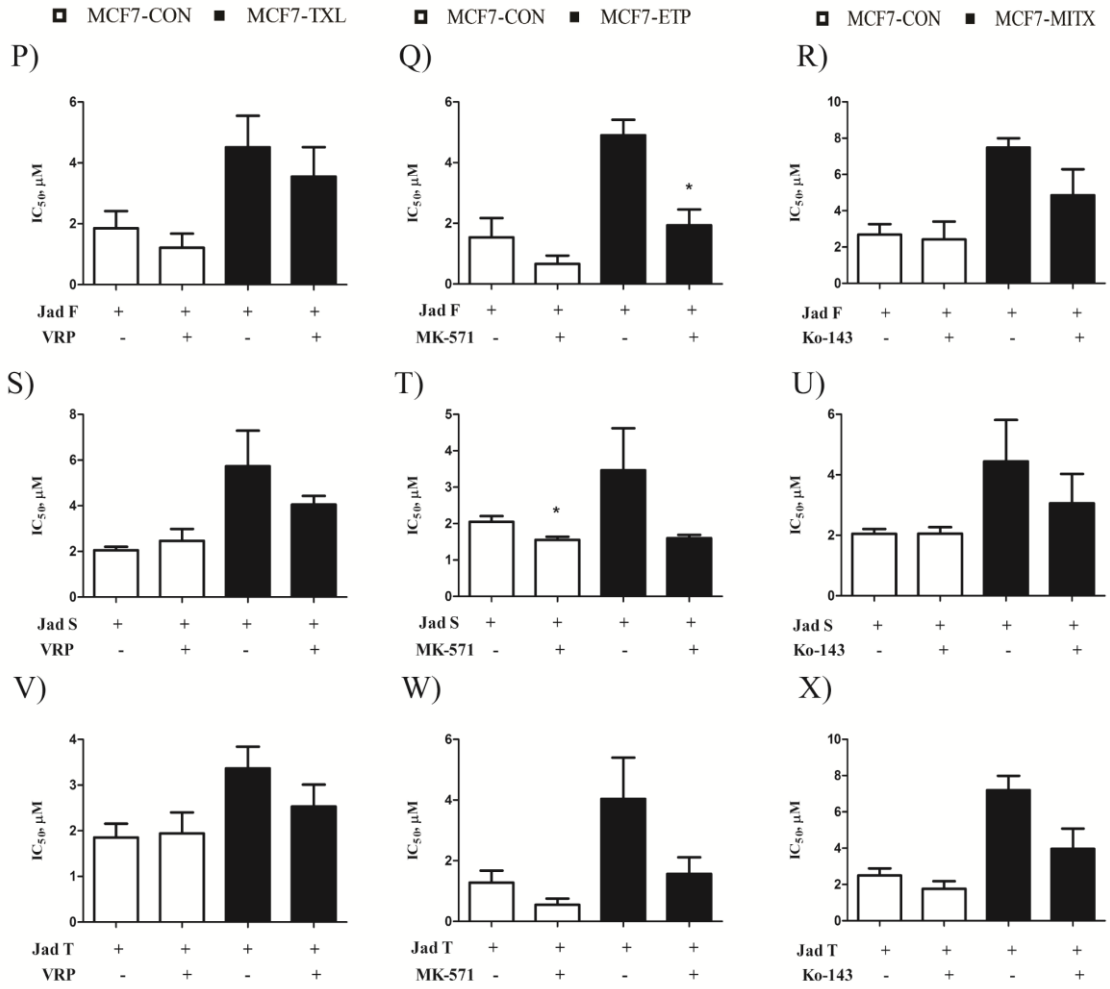


Figure 6: The effect of inhibiting ABCB1, ABCC1, or ABCG2 on jadomycin cytotoxicity in MCF7-CON and MCF7-TXL, -ETP, and -MITX cells. Cells were treated with the control drugs doxorubicin (DOX; a, b) or mitoxantrone (MITX; c) or jadomycins DNV (d-f), B (g-i), L (j-l), SPhG (m-o), F (p-r), S (s-u), or T (v-x) with and without inhibitors of ABCB1 (VRP), ABCC1 (MK-571), and ABCG2 (Ko-143). Cell viability was measured using MTT assays. Data represent the mean IC_{50} values ± SEM of at least three independent experiments performed in triplicate. * The IC_{50} value of the given drug treatment was significantly different in response to the inhibition of ABCB1, ABCC1, or ABCG2 as compared to the respective same-cell line control without inhibition, as determined by an unpaired t-test ($P < 0.05$). Note that the assays for jadomycins DNV, B, L, and S were previously completed by Mark Issa [5,6], while I completed the assays for jadomycins F, SPhG, and T. For a full structural comparison I have included the data from all jadomycin trials in this chapter.

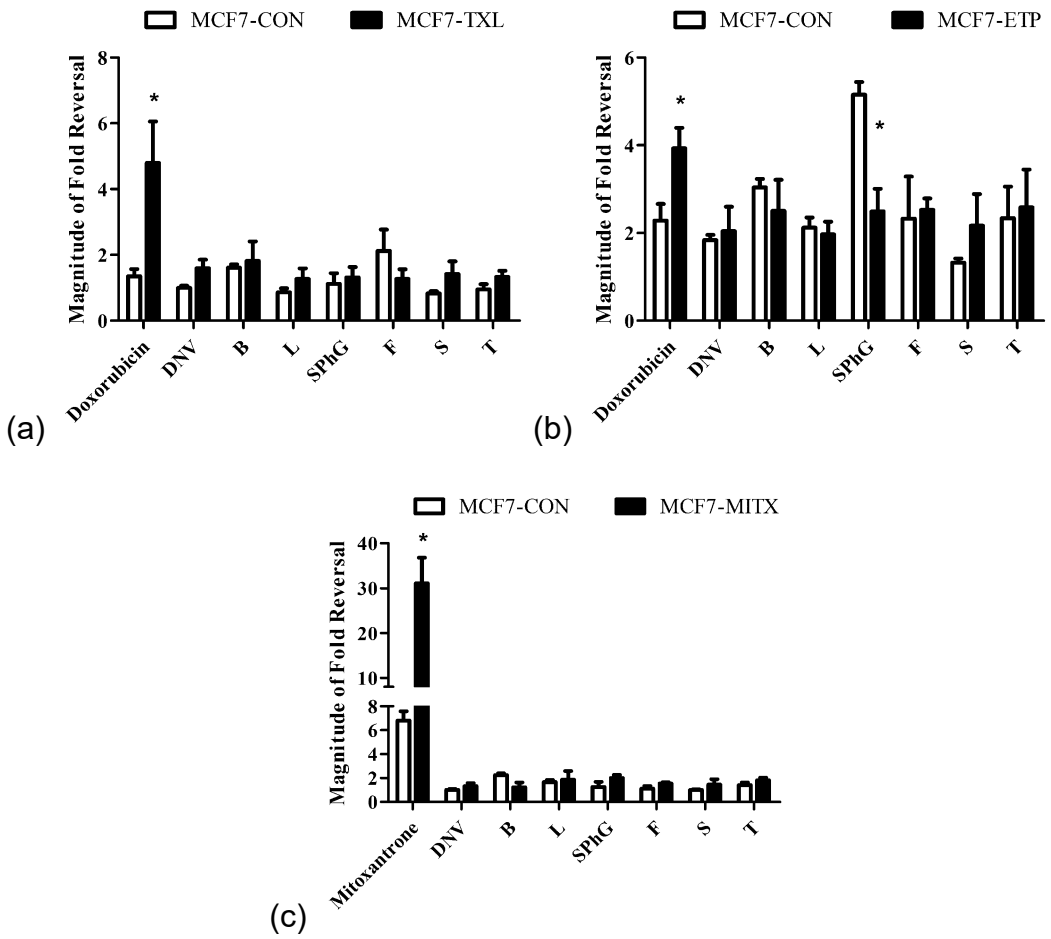


Figure 7: Analysis of the fold-reversal in IC_{50} values of control cytotoxic drugs and jadomycins in response to inhibition of ABCB1, ABCC1, or ABCG2. IC_{50} values were obtained using MTT viability assays in the presence or absence of VRP, MK-571, or Ko-143, which are inhibitors of ABCB1 (a), ABCC1, (b) or ABCG2 (c) transporters, respectively. The fold-reversal in a given cell line was calculated by dividing the IC_{50} value of each drug obtained in the absence of each ABC transporter inhibitor by the mean IC_{50} value of the drug in the presence of each inhibitor. For each drug the fold-reversal in resistant cells was compared to that in control cells. * For the corresponding drug, the fold-reversal was significantly different between the MCF7-CON and MCF7-TXL, -ETP, or -MITX cells, as determined by an unpaired t-test ($P < 0.05$). Note that the assays for jadomycins DNV, B, L, and S were previously completed by Mark Issa [5,6], while I completed the assays for jadomycins F, SPhG, and T. For a full structural comparison I have included the data from all jadomycin trials in this chapter.

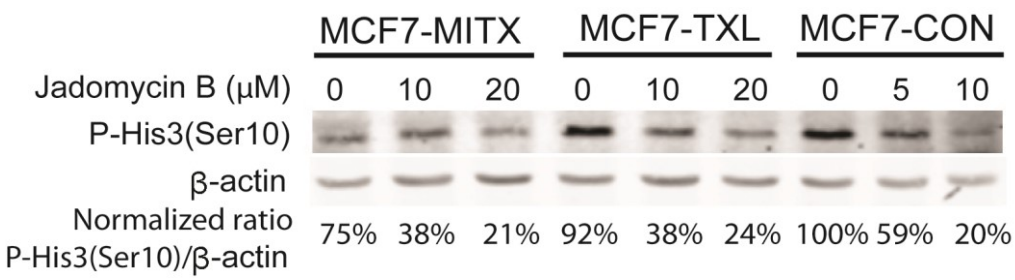


Figure 8: Jadomycin B inhibits aurora B kinase. MCF7-CON, -MITX, and -TXL cells in the exponential growth phase were treated with 0-20 μM jadomycin B for 24 hours, at which time whole cell protein lysates were prepared for western blot analysis of the Aurora B kinase target P-His3(Ser10) and loading control β-actin. The P-His3(Ser10) band intensity was normalized to that of the corresponding β-actin band intensity and these ratios were then expressed as a percentage of each vehicle-treated MCF7 cell line. Each band represents the immunodetectable P-His3(Ser10) and β-actin in the pooled protein from three sample replicates and is representative of three independent experiments. * For the corresponding MCF7 cell line, the normalized ratio of P-His3(Ser10)/β-actin for that particular jadomycin B treatment was significantly different versus the vehicle control (labelled 0 μM), as determined by a one-way ANOVA, followed by Bonferroni's multiple comparison test ($P < 0.05$).

Table 5: Genes up- or down-regulated by jadomycin S (10 μM, 24 h) versus vehicle control in MCF7-CON cells as measured by the Human Cancer Drug Targets PCR Array. Fold-regulation represents the gene fold-changes in a biologically meaningful way. Fold-change values greater than one indicate a positive- or up-regulation, and the fold-regulation is equal to the fold-change. Fold-change values less than one indicate a negative- or down-regulation, and the fold regulation is the negative inverse of the fold-change. A fold-regulation value of at least ±3 was chosen as the minimum value to suggest a potential "hit", thus warranting further studies into jadomycins' effects on that gene.

Functional Gene Grouping	**Genes Up-Regulated (Fold Up-Regulation)**	**Genes Down-Regulated (Fold Down-Regulated)**
Apoptosis		*BCL2* (-5.0)
Growth Factors and Receptors	*IGF1* (50.6)	*ERBB2* (-4.1), *ERBB3* (-3.4), *ERBB4* (-18.4), *KIT* (-28.2)
Drug Metabolism	*TrxR1* (11.7)	
Hormone Receptors		*ESR1* (-5.2), *PGR* (-13.6)
Receptor Tyrosine Kinase Signaling		*AKT1* (-7.4)
Cell Cycle		*CDC25A* (-6.5), *CDK2* (-3.4), *MDM4* (-4.1)
Type II Topoisomerases		*TOP2A* (-4.0), *TOP2B* (-4.2)
Transcription Factors		*IRF5* (-4.0)
Protein Kinases		*PRKCA* (-4.4)
RAS Signaling		*KRAS* (-5.3)
Histone Deacetylases		*HDAC11* (-4.5), *HDAC6* (-3.2)
Poly ADP-Ribose Polymerases		*PARP1* (-3.6)

Table 6: Verification of gene regulation changes caused by jadomycin B or S treatment (10 μM, 24 h) in MCF7-CON cells versus vehicle controls. ***** For the corresponding gene, the fold up- or down-regulation was significantly different between the jadomycin B or S and vehicle treated MCF7-CON cells as determined by a one-way ANOVA, followed by Bonferroni's multiple comparison test ($P < 0.05$, $n = 3$).

	Fold up- or down-regulation versus vehicle ± SEM	
Gene	**Jadomycin B**	**Jadomycin S**
TrxR1	16.9 ± 3.1 *	11.0 ± 0.7 *
IGF1	2.9 ± 0.4 *	2.5 ± 0.4
BCL2	-2.5 ± 0.2 *	-2.5 ± 0.2 *
ERBB2	-2.8 ± 0.2	-2.5 ± 0.8
ERBB3	-1.8 ± 0.1	-1.7 ± 0.4
ERBB4	-3.4 ± 0.2	-5.9 ± 2.3
KIT	-4.6 ± 0.3	-5.2 ± 2.0
ESR1	-2.4 ± 0.1	-3.0 ± 0.8
PGR	-4.0 ± 0.3	-5.0 ± 1.3
AKT1	-1.2 ± 0.1	-1.3 ± 0.2
CDC25A	-2.4 ± 0.2	-2.7 ± 1.2
CDK2	-1.5 ± 0.1	-1.5 ± 0.2
MDM4	3.9 ± 1.1	2.6 ± 0.8
TOP2A	-1.2 ± 0.1	-1.4 ± 0.2
TOP2B	-2.0 ± 0.1	-2.0 ± 0.5
IRF5	1.0 ± 0.1	1.0 ±0.1
PRKCA	1.1 ± 0.1	-1.1 ± 0.1
KRAS	-3.0 ±0.6	-3.1 ± 0.9
HDAC11	-2.0 ± 0.1 *	-2.1 ± 0.3 *
HDAC6	-1.2 ± 0.1	-1.3 ± 0.1
PARP1	-1.2 ± 0.1	-1.3 ± 0.2

Table 7: PCR primers used to verify the gene expression of the targets identified by the PCR cancer gene array in MCF7-CON cells treated with either jadomycin S or B (10 μM) or vehicle control for 24 hours.

Gene	**PCR forward primers (5'-3')**	**PCR reverse primers (5'-3')**
TrxR1	CCACTGGTGAAAGACCACGTT	AGGAGAAAAGATCATCACTGC
IGF1	TGCCAATGTGGTGCTATTGT	GAAAGGTGGTGGTGGCTAGA
BCL2	ATGTGTGTGGAGAGCGTCAA	GCCGTACAGTTCCACAAAGG
ERBB2	AAGGCGGACGCCTGATGGGT	ATAAGCCAAATTCTGTGCTG
ERBB3	CTGGGACTCTGAATGGCCTG	CCTGTCACTTCTCGAATCCA
ERBB4	TGTGAGAAGATGGAAGATGGC	GTTGTGGTAAAGTGGAATGGC
KIT	TGACTTACGACAGGCTCGTG	CCACTGGCAGTACAGAAGCA
ESR1	TGGAGATCTTCGACATGCTG	AGAGACTTCAGGGTGCTGGA
PGR	GTCAGTGGGCAGATGCTGTA	TGCCACATGGTAAGGCATAA
AKT1	CACACCACCTGACCAAGATG	CTCAAATGCACCCGAGAAAT
CDC25A	GAGATCGCCTGGGTAATGAA	TGCGGAACTTCTTCAGGTCT
CDK2	TATCTGTTCCAGCTGCTC	CTCGGTACCACAGGGTCACC
MDM4	AATGTCGCTTTAGATGAAGA	CTGTGCGAGAGCGAGAGTCTG
TOP2A	TGGCTGAAGTTTTGCCTTCT	GGCCTTCTAGTTCCACACCA
TOP2B	GAGTGGCTTGTGGGAATGTT	TGTGCTTCTTTCCAGGCTTT
IRF5	CTCCAATGGCCCTGCTCCCA	GAACTATTGAGAGGGCCACG
PRKCA	CGAGGAAGGAAACATGGAACTCAG	TTCCTGTCGGCAAGCATCAC
KRAS	AGAGTTAAGGACTCTGAAGA	TGTCGGATCTCCCTCACCAA
HDAC11	CCCAGACAGGAGGAACCATA	CTCCACACGCTCAAACAGAA
HDAC6	CGAGCTGATCCAAACTCCTC	ATCAGCCATGTCCTGACCTC
PARP1	GCTCCCAGGAGTCAAGAGTG	CAGATCAGGTCGTTCTGAGC
GAPDH	GAGTCAACGGATTTGGTCGT	TTGATTTTGGAGGGATCTCG
β-actin	GGACTTCGAGCAAGAGATGG	AGCACTGTGTTGGCGTACAG
PPIA	ACCGCCGAGGAAAACCGTGT	CTGTCTTTGGGACCTTGTCTGCA

2.05.00: DISCUSSION

Chemotherapy remains an important treatment method for metastatic breast cancer. Due to the development of the commonly observed ABC transporter-mediated MDR phenotype, currently available chemotherapeutics often prove ineffective. One approach in countering the problem of MDR is the development of cytotoxic therapies that are not affected by ABC-transporters. For example, ixabepilone is an epothilone analogue that acts similarly to paclitaxel but is a poor substrate for ABC transporters, thus its anticancer effects are minimally susceptible to ABC transporter overexpression [4,247]. Ixabepilone is currently approved for the treatment of metastatic breast cancer patients after failure of an anthracycline and a taxane pre-treatment [248]. GRN1005 is a paclitaxel-peptide derivative that is not transported by ABCB1, and therefore exhibits improved brain penetration [249]. GRN1005 has been tested in a phase 2 clinical trial involving breast cancer patients with brain metastasis, though the results have not yet been released [250].

Through the use of LDH assays, our study demonstrates that jadomycins show substantially smaller potency reductions in comparison to the anthracycline doxorubicin in *ABCB1* and *ABCC1* overexpressing MCF7-TXL and -ETP cells, respectively and in comparison to mitoxantrone in *ABCG2* overexpressing MCF7-MITX cells versus MCF7-CON cells. Furthermore, inhibitors of ABCB1, ABCC1, and ABCG2 selectively sensitized the resistant cell lines to doxorubicin and mitoxantrone but not to jadomycins as measured with the calculated fold-resistance values. These two key findings support that ABCB1, ABCC1, and ABCG2 transporters minimally affect the cytotoxicity of jadomycins in MCF7 cells. As a result, we believe that jadomycins are excellent

candidates for further study for the treatment of MDR forms of metastatic breast cancer in animal models.

Some jadomycins showed statistically higher EC_{50} values as measured by LDH assays in one or more of the ABC-overexpressing cell lines compared to the MCF7-CON cells. In all cases, these were of relatively small magnitude (2.3 - 3.5-fold increases) which were much lower than those of the control drugs (12.1 to 88.2-fold increases). A small loss in jadomycin potency in the resistant cell lines corroborates with the behaviour of other natural product anticancer drugs or their derivatives that circumvent the MDR phenotype mediated by ABC transporters [247,251,252]. For example, the epothilone derivative ixabepilone demonstrated a 2.2-fold reduction in potency in *ABCB1*-overexpressing colon carcinoma cells in comparison to drug-sensitive colon carcinoma cells, versus a 28-fold reduction in potency of paclitaxel [247]. Similarly, the microtubule-destabilizing agent pseudolaric acid B showed a 2.2-fold reduction in potency in *ABCB1*-overexpressing versus control MDA435/LL6 breast carcinoma cells, while doxorubicin showed a 17-fold reduction [251]. Therefore, molecules that are poor substrates for ABC transporters are still expected to be slightly less potent in *ABC*-transporter overexpressing cell lines versus drug-sensitive cell lines, possibly due to the development of other mechanisms of MDR (for example, reduced drug uptake or increased drug metabolism) that are unrelated to the efflux transporter proteins. While initial studies of ixabepilone showed a small reduction in potency in *ABCB1*-overexpressing cancer cells, this result did not preclude further investigations of this drug's potential in MDR cancers. This led to its eventual approval for clinical use in metastatic breast cancer resistant to taxanes and anthracyclines. The similarly minimal

potency reductions for jadomycin cytotoxicity in the *ABCB1*, *ABCC1*, and *ABCG2*-overexpressing MCF7-TXL, -ETP, and -MITX cells relative to the MCF7-CON cells are encouraging and support further testing of jadomycin effectiveness against *ABC*-transporter overexpressing metastatic breast cancer.

The inclusion of multiple jadomycin analogues in this study was important as it allowed for us to determine if structural differences influenced jadomycin cytotoxicity in MDR MCF7 cells. The lower efficacy and potency of jadomycin W suggested that the incorporation of an amino acid with a large aromatic R group into the jadomycin backbone may reduce its interactions with intracellular targets or possibly restrict its access to the intracellular environment. As a result of its much lower potency we did not pursue the analysis of jadomycin W. When comparing the data within each of the MCF7-CON, -TXL, -ETP, and -MITX cell lines, we observed that jadomycins derived from the assimilation of amino acids with hydrophilic (S and T), hydrophobic aliphatic (DNV, B, and L), or smaller hydrophobic aromatic R groups (SPhG and F) exhibited similar potencies. Previously, jadomycins B, S, and T were shown to be similarly potent in cancer cells [169,184]. Our LDH assays replicated these results in MCF7-CON cells and also showed that jadomycin potency is largely maintained in the three drug resistant MCF7 cell lines. The observation that jadomycin W was less potent than the other jadomycins used is consistent with previous reports that the amino acid side chain incorporated into the oxazolone ring can have a significant influence on jadomycin activity [169,183,184,206]. However, the similar toxicity profiles of jadomycins B, DNV, F, L, S, SPhG, and T indicates that the jadomycin structure may also be altered with little to no effect on cytotoxic potency or efficacy in cancer cells, both in drug-sensitive

MCF7-CON and MDR MCF7-TXL, -ETP, and -MITX breast cancer cells. This is of great value for drug development purposes since physicochemical properties of potential drugs, such as solubility and permeability, frequently present barriers to them becoming successful therapies. For instance, estramustine, an anticancer drug used for the treatment of prostate carcinoma, did not initially exhibit the desired aqueous solubility. The addition of a phosphate ester functionality to the estramustine molecule significantly improved its aqueous solubility, making it a more attractive drug-candidate [253]. Given that jadomycins are amenable to precursor-directed biosynthesis, they therefore have the potential to have their chemical structure changed to improve their pharmacokinetic profile while minimally affecting or even improving their anticancer activity.

Despite initial promising results, phase III clinical trials have demonstrated that the use of an ABC transporter inhibitor along with chemotherapy is currently not a feasible approach, mainly due to adverse drug-drug interactions [145,254,255,256]. Given that therapy for metastatic breast cancer frequently consists of combination therapy that include cytotoxic agents [257,258], it was deemed important to determine if jadomycins inhibit the ABCB1, ABCC1, or ABCG2-mediated transport of substrate molecules.

The observation that jadomycin potency was not enhanced in cells co-treated with the ABCB1, ABCC1, and ABCG2 inhibitors VRP, MK-571, and Ko-143 as measured by the calculated fold-resistances suggests that jadomycins are poor substrates of these transporters. This provides evidence that jadomycins are able to largely maintain their cytotoxic potency in ABC-transporter overexpressing breast cancer cells due to their ability to avoid efflux through them. Additionally, the experiments we completed previously in HEK cells stably transfected with ABCB1, ABCC1, and ABCG2 evidence

that jadomycins are not inhibitors of these transporters either [5,6]. These data suggest that jadomycins may not be susceptible to adverse drug-drug interactions involving ABC transporters should they ever be developed for therapeutic use. An exception was jadomycin DNV, which slightly enhanced the accumulation of the ABCC1 and ABCG2 substrates at 50 μM, indicating that it could be an inhibitor of these efflux transporters at higher concentrations.

The inhibition of Aurora B kinase has been identified as a possible mechanism causing jadomycin B toxicity [184], and therefore we began our jadomycin mechanism of action work by determining if jadomycin B inhibited aurora B kinase in control and drug-resistant MCF7 breast cancer cells. Our western blot data showing similar inhibition of the aurora B kinase target P-His3(Ser10) in MCF7-CON, -TXL, and -MITX cells by jadomycin B suggests that the largely-preserved cytotoxic potency of jadomycins in these MDR cells is in part due to these compounds maintaining their inhibitory potency of aurora B kinase. It will be interesting to determine if other jadomycin analogues also inhibit aurora B kinase in MDR breast cancer cells in future studies, as this may allow for a better understanding of which jadomycins best retain their anticancer activity in *ABC*-transporter overexpressing MDR cell lines.

A PCR Cancer Gene Array was then used to identify possible genes affected by jadomycin treatment in MCF7 cells, thus providing us with new directions in which to take our mechanistic experiments. Twenty-one genes were identified as possible targets worth exploring after treatment with jadomycin S. To confirm or disprove whether these genes were truly affected by jadomycins, all were further tested using repeated qPCR trials to determine which genes were significantly affected by both jadomycins B and S.

Through these trials the expression of 3 genes was found to be altered by both jadomycins, *TrxR1*, *BCL2*, and *HDAC11*, with *IGF1* being altered by jadomycin B. Of these four genes, *TrxR1* was most affected by the jadomycin treatments. *TrxR1* encodes thioredoxin reductase 1, a protein involved in the Trx/Prx antioxidant pathway and an important component of a cell's ability to neutralize potentially damaging ROS [259,260,261]. The increased expression of *TrxR1* caused by jadomycin treatment suggests a possible role played by ROS in jadomycin cytotoxicity, a hypothesis supported by previously published data proposing that jadomycins cause DNA damage by inducing ROS [205]. *BCL-2* encodes B-cell lymphoma 2 protein which is part of the Bcl-2-family of proteins and is involved in the regulation of all major types of cell death including apoptosis, necrosis, and autophagy. Inhibition of BCL-2 is regarded as an important cancer drug target [262]. *HDAC11* encodes histone deacetylase (HDAC) 11, a protein that deacetylates both nuclear histone and non-histone proteins including transcription factors; therefore it plays an important role in the regulation of many cell processes [263]. Inhibition of HDACs represents a promising approach in chemotherapy [264]. *IGF1* encodes insulin-like growth factor (IGF)-1, a ligand that binds to IGF-1 receptor. There is evidence that IGF-1 signaling may contribute to stages of cancer progression, including tumour growth, invasiveness of the cancer cells, and resistance to cancer treatments, and IGF-1 inhibitors are being studied as possible cancer treatments [265]. Since we only have qPCR data using a small sample size to suggest that these cancer gene targets are affected by jadomycin treatment, we cannot confidently conclude their importance in jadomycins' mechanisms of action. However, these changes do warrant further experimentation to be completed to fully elucidate their significance.

In summary, we have identified 6 jadomycins (B, F, L, S, SPhG, and T) that are potent cytotoxic agents of MCF7 breast cancer cells and do not inhibit ABC-transporters. The cytotoxic potency of these jadomycins was minimally reduced in *ABCB1*-, *ABCC1*-, and *ABCG2*-overexpressing MCF7-TXL, -ETP, and -MITX versus drug-sensitive MCF7-CON breast cancer cells. The mechanisms through which jadomycin B induces breast cancer cell death includes the inhibition of aurora B kinase, may involve ROS activity, and may include the inhibition of the cancer gene targets *BCL-2* and *HDAC11*. Based on their favourable pharmacological parameters, further investigations using other cancer cell models as well as animal models are justified to determine the therapeutic potential of jadomycins in treating MDR cancers.

CHAPTER 3.00.00: JADOMYCIN BREAST CANCER CYTOTOXICITY IS MEDIATED BY A COPPER-DEPENDENT, REACTIVE OXYGEN SPECIES-INDUCING MECHANISM

The work described in this chapter was conducted by myself and the data published in the paper "Jadomycin breast cancer cytotoxicity is mediated by a copper-dependent, reactive oxygen species-inducing mechanism", on which I am the first author. Minor edits have been made from the original published manuscript to tailor this chapter for my dissertation.

Original publication information:

Steven R. Hall, Heather L. Blundon, Matthew A. Ladda, Andrew W. Robertson, Camilo F. Martinez-Farina, David L. Jakeman, Kerry B. Goralski

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Pharmacology Research & Perspectives, 2015, Issue 2, Volume 3, e00110: Pages 1-16

3.01.00: ABSTRACT

Currently the mechanisms of jadomycin cytotoxicity are poorly understood; however, ROS-induced DNA cleavage is suggested based on bacterial plasmid DNA cleavage studies and our previously completed PCR cancer gene arrays. This study's objective was to determine if and how ROS contribute to jadomycin cytotoxicity in drug-sensitive MCF7-CON and paclitaxel-resistant MCF7-TXL breast cancer cells. As determined using an intracellular, fluorescent, ROS-detecting probe, jadomycins B, S, SPhG, and F dose-dependently increased intracellular ROS activity 2.5-5.9 fold. Co-treatment with the antioxidant N-acetyl cysteine (NAC) lowered ROS concentrations to below baseline levels and decreased the corresponding cytotoxic potency of the four jadomycins 1.9-3.3 fold, confirming a ROS-mediated mechanism. Addition of $CuSO_4$ enhanced, whereas addition of the Cu(II)-chelator D-penicillamine (D-Pen) reduced, the ROS generation and cytotoxicity of each jadomycin. Specific inhibitors of the antioxidant enzymes superoxide dismutase 1 (SOD1), GST, and thioredoxin reductase (TrxR), but not catalase, enhanced jadomycin-mediated ROS generation and anticancer activity. In conclusion, the results indicate that jadomycin cytotoxicity involves the generation of cytosolic superoxide *via* a Cu(II)-jadomycin reaction, a mechanism common to all jadomycins tested and observed in MCF7-CON and drug-resistant MCF7-TXL cells. The SOD1, glutathione (GSH), and peroxiredoxin/thioredoxin (Prx/Trx) cellular antioxidant enzyme pathways scavenged intracellular ROS generated by jadomycin treatment. Blocking these antioxidant pathways could serve as a strategy to enhance jadomycin cytotoxic potency in drug sensitive and multidrug resistant breast cancers.

3.02.00: INTRODUCTION

Our previous work has shown that jadomycins with hydrophobic aliphatic functional groups, isoleucine (B) and leucine (L), hydrophobic aromatic functional groups, phenylalanine (F) and *s*-phenylglycine (SPhG), and hydrophilic functional groups, serine (S) and threonine (T), are effective cytotoxic agents against hormone-receptor positive MCF7 breast cancer cells *in vitro* [6,176,206]. Furthermore, jadomycin potency was minimally affected by overexpression of the ABC drug efflux transporters *ABCB1*, *ABCC1*, or *ABCG2* [6]. Jadomycins therefore warrant additional pharmacological characterization in breast cancer cell models, including those with a multidrug resistant phenotype.

Only a basic understanding of the mechanisms behind jadomycin anticancer activity is currently known [6,169,184,206]. One possible mechanism of jadomycin cytotoxicity is indirect DNA cleavage, resulting from the generation of the ROS superoxide, singlet oxygen, hydroxyl radical, and H_2O_2, though this has only been tested in extracellular models using bacterial plasmids [205]. The observed increase in *TrxR1* gene expression further suggests a role played by ROS in breast cancer cells upon treatment with jadomycins. Additionally, through virtual screening, jadomycin B was proposed to act as an aurora B kinase inhibitor [184]. The ability of jadomycin B to block the activity of purified aurora B kinase and the phosphorylation of an aurora B kinase target protein in lung and breast cancer cells further supports this proposed function [6,184].

Many currently available anticancer drugs are cytotoxic to cancer cells through the generation of ROS [266]. Cancer cells have innate levels of ROS higher than those typically observed in healthy cells, and are therefore already heavily dependent on their

cellular antioxidant systems. Certain anticancer drugs increase ROS activity in the cancer cells past their tolerability threshold, inducing cell death. This is known as the "threshold concept for cancer therapy" [203,204]. Since healthy cells have lower innate levels of ROS, they have a higher capacity to cope with the increased oxidative stress induced by ROS-inducing anticancer drugs in comparison to tumour cells [204], and therefore selectivity towards cancer cells versus healthy cells can be achieved [203]. Thus, the hypothesis that jadomycin breast cancer toxicity could be achieved through ROS-activity modification is a viable option that needs to be tested experimentally.

The objectives of this study were to (1) determine if hormonal and HER2 receptor profiles affect jadomycin anticancer activity in breast cancer cells, (2) determine if jadomycins alter the activity of ROS within breast cancer cells and if jadomycin cytotoxicity is dependent on this ROS activity, (3) determine if jadomycins' ROS-inducing properties are retained in *ABCB1* overexpressing MDR breast cancer cells, and (4) to determine the specific ROS induced by jadomycin treatment and the antioxidant pathways involved in their elimination using pharmacological modulators of ROS homeostasis.

3.03.00: MATERIALS AND METHODS

3.03.01: CHEMICAL AND BIOLOGICAL MATERIALS

MTT, NAC, Triton X-100, H_2O_2 30% (w/w) in water, paclitaxel, dimethylsulfoxide, methanol, sodium lactate, phenazine methosulfate, β-nicotinamide adenine dinucleotide, iodonitrotetrazolium chloride, copper (II) sulfate ($CuSO_4$), D-Pen, MitoTEMPO, sodium diethyldithiocarbamate (DDC), ellagic acid (EA), and PCR primers were purchased from Sigma Aldrich (Oakville, Ontario, Canada). Auranofin, 3-amino-1,2,4-triazole (3-AT), and radioimmunoprecipitation assay lysis buffer containing phenylmethyl-sulfonyl fluoride, protease inhibitor cocktail, and sodium orthovanadate were purchased from Santa Cruz Biotechnology Inc. (Dallas, Texas, USA). Dulbecco's modified Eagle's medium was purchased from Fisher Scientific (Mississauga, Ontario, Canada). FBS, penicillin and streptomycin, sodium pyruvate, and 5-(and 6-)chloromethyl-2'7'-dichlorodihydrofluorescein diacetate (CM-$DCFH_2$-DA) were purchased from Life Technologies (Burlington, Ontario, Canada). Aurum total RNA Mini Kit and SYBR Green were purchased from Bio-Rad (Mississauga, Ontario, Canada). Monoclonal mouse anti-human P-His3(Ser10) antibody was purchased from Cell Signaling Technology (Danvers, Massachusetts, USA). Polyclonal rabbit anti-human histone H3 (His3) antibody was purchased from Abcam Inc. (Toronto, Ontario, Canada). IRDye 680RD conjugated donkey anti-mouse and IRDye 800CW conjugated goat anti-rabbit were purchased from Mandel Scientific (Guelph, Ontario, Can).

3.03.02: PRODUCTION OF JADOMYCINS

Jadomycins B, S, SPhG, and F were isolated and characterized as previously described [6,182,183,232].

3.03.03: CELL LINES

The MCF7-CON and -TXL breast cancer cells were kindly provided by Drs. Robert Robey and Susan Bates (National Cancer Institute, Bethesda, MD, USA). The BT474, SKBR3, and MDA-MB-231 cell lines were kindly provided by Dale Corkery, Chansey Veinotte, and Drs. Graham Dellaire and Jason Berman (Dalhousie University, Halifax, NS, Canada). Serial passages of MCF7 cells in medium containing increasing concentrations of TXL were previously completed to generate the resistant sub-line MCF7-TXL, which was characterized by its increased expression of *ABCB1* [6,233]. All breast cancer cells were cultured in phenol red-free Dulbecco's modified Eagle's medium supplemented with 10% FBS, 100 IU/mL penicillin, 250 μg/mL streptomycin, and 1 mM sodium pyruvate (10%-FBS standard assay medium). The medium for MCF7-TXL cells was additionally supplemented with 400 nM paclitaxel. The cells were maintained in a humidified, 95% air/5% CO_2 atmosphere at 37 °C (standard conditions), the growth medium was changed weekly and cells were split every 7 to 14 days. The MCF7-TXL cells were grown in drug-free culture medium for 1 week before experiments were begun.

3.03.04: MTT VIABILITY ASSAYS

MTT assays were used to evaluate the anticancer activity of jadomycins B, S, and F (0.256 - 30 μM) in MCF7-CON, BT474, SKBR3, and MDA-MB-231 breast cancer cells and completed according to our previously described methods [6].

3.03.05: ROS MEASURING ASSAYS

To quantify the presence of intracellular ROS in MCF7 cells, a fluorescent assay utilizing the ROS-reactive CM-DCFH$_2$-DA was used. CM-DCFH$_2$-DA passively diffuses through cell membranes where it is de-esterified to 5-(and 6-)chloromethyl-2'7'-dichlorodihydrofluorescein (CM-DCF), confining it within the cell. In the presence of ROS, CM-DCF is oxidized yielding a fluorescent compound that can be quantified as a general oxidative stress indicator [267]. MCF7 cells were seeded in black-sided, clear-bottomed 96-well plates at 40,000 cells/well in 100 μL of 10%-FBS standard assay medium. Cells were left to adhere for 24 hours under standard conditions, at which time they were ~90% confluent. After 24 hours, the medium was removed and 50 μL of 7.5 μM CM-DCFH$_2$-DA in Dulbecco's modified Eagle's medium supplemented with 1% FBS, 1% sodium pyruvate, and 1% penicillin/streptomycin (1%-FBS standard assay medium) was added to each well, excluding blanks, for 1 hour under standard conditions. The CM-DCFH$_2$-DA-containing medium was removed and cells were treated with 100 μL of jadomycin B, S, SPhG, or F (2.5-30 μM), H_2O_2 (125-2,000 μM), or vehicle in 1%-FBS standard assay medium for 24 hours in quintuplicate. After 24 hours fluorescence was read at excitation 485/20 nm and emission 528/20 nm using a Biotek Synergy HT

plate reader. Background fluorescence from blanks was subtracted and the fold-change in fluorescence was calculated *via* formula 5:

$$\text{Fold-increase} = \frac{F_{avg,treatment}}{F_{avg,vehicle}} \quad (5)$$

Where $F_{avg,treatment}$ is the average fluorescence for each sample, and $F_{avg,vehicle}$ is the average fluorescence of the vehicle control.

3.03.06: EFFECT OF NAC ON JADOMYCIN IC_{50} VALUES

MCF7-CON cells were co-treated with jadomycins and the antioxidant NAC to examine the role of intracellular ROS as a mechanism of jadomycin-mediated cytotoxicity [268]. MCF7-CON cells were seeded in 96-well plates at 20,000 cells/well in 100 µL of 1%-FBS standard assay medium and grown for 24 hours under standard conditions. The medium was removed and 80 µL of 1%-FBS standard assay medium containing NAC at a final concentration of 0.3-15 mM or H_2O vehicle was added to each well and left at standard conditions for 1 hour, followed by the addition of 20 µL of 1%-FBS standard assay medium containing jadomycin B, S, SPhG, or F at final concentrations of 1.25-35.0 µM or the 1:7 methanol:H_2O vehicle control (jadomycin vehicle) in quintuplicate for 72 hours under standard conditions. Medium was aspirated from each well, 50 µL of phosphate buffered saline added and aspirated to remove residual dead cells, and 100 µL of 0.1% (v/v) Triton X-100 in water added to kill the remaining viable cells followed by plate shaking at 500 RPM for 5 minutes. Following cell lysis with 0.1 % Triton X-100, LDH activity was measured by treating each well with 100 µL of in-house LDH reaction solution (25 mM sodium lactate, 147 µM

phenazine methosulfate, 644 µM β-nicotinamide adenine dinucleotide, and 326 µM iodonitrotetrazolium chloride in 200 mM Tris buffer (pH=8), the plate shaken at 500 RPM for 1 minute, and absorbance (490 nm) quantified using a Biotek Synergy HT plate reader as a measure of cell viability post-drug treatment [235,269,270]. The %-cell viability for each jadomycin or H_2O_2 treatment concentration was calculated *via* formula 6:

$$\%\text{-cell viability} = \frac{A_{490,avg,treatment}}{A_{490,veh,avg}} \times 100\% \quad (6)$$

Where $A_{490,avg,treatment}$ is the average absorbance for each treatment, and $A_{490,veh,avg}$ is the average absorbance of the vehicle control.

3.03.07: WESTERN BLOT ANALYSIS

Western blot analyses were performed as previously published [6] with the following modifications. MCF7-CON cells seeded in clear, 6-well plates were treated with 800 µL of 10%-FBS standard assay medium containing NAC (final concentration of 15 mM) or H_2O vehicle under standard conditions for 1 hour. This was followed by the addition of 200 µL of 10%-FBS standard assay medium containing 5-fold concentrated jadomycin B, S, SPhG, or F (final concentration of 10 µM), H_2O_2 (final concentration of 500 µM), or jadomycin vehicle in triplicate for 24 hours under standard conditions. A 20 µg aliquot of protein from each pooled sample was separated using a 12.5% SDS-PAGE and transferred to nitrocellulose membranes. Membranes were incubated overnight in a combination 1:500 dilution of a monoclonal mouse anti-human P-His3(Ser10) antibody

and 1:2,000 dilution of a polyclonal rabbit anti-human His3 antibody at 4°C. Membranes were then incubated in a combination 1:15,000 dilution of IRDye 680RD conjugated donkey anti-mouse and 1:15,000 dilution of IRDye 800CW conjugated goat anti-rabbit secondary antibodies. Membranes were scanned at 700 and 800 nm infrared wavelengths using a Licor Odyssey (Mandel Scientific). The integrated intensity and area of P-His3(Ser10) were measured and normalized to those of His3 for each protein sample. These ratios were then compared with those of the vehicle controls and calculated as a %-change in protein expression.

3.03.08: EFFECTS OF PRO- AND ANTI-OXIDANT CO-TREATMENTS ON JADOMYCIN-DEPENDENT ROS ACTIVITY AND CYTOTOXICITY

To determine how jadomycin-induced ROS activity is linked to jadomycin cytotoxicity in MCF7-CON or -TXL cells we developed an assay where the change in ROS activity and LDH activity were sequentially measured in the same cells following jadomycin treatment in the presence or absence of pharmacological modulators of ROS generation or deactivation. MCF7-CON and -TXL cells were plated and pre-treated with CM-$DCFH_2$-DA as described earlier for the ROS assays. This was followed by a 1 hour pre-incubation with the following compounds based on published effective final concentrations with some modifications for ideal dosing: 0.3-15 mM NAC [271], 10 μM $CuSO_4$, 1 mM D-Pen [272], 0.1-10 nM MitoTEMPO [273], 1 mM DDC [274], 10 mM 3-AT [275], 20 μM EA [276], or 1 μM auranofin [277], followed by a 24 hour treatment in quadruplicate or quintuplicate with jadomycin B (7.5-25 μM), S (12-35 μM), SPhG (7.5-

20 μM), or F (7.5-20 μM) or jadomycin vehicle. Sequentially, ROS activity and LDH activity assays were performed for each treatment as described in the earlier methods.

3.03.09: RNA ISOLATION, REVERSE TRANSCRIPTION, AND QUANTITATIVE REAL-TIME PCR

Total RNA was isolated from lysates of MCF7-CON cells treated with jadomycin B (10 μM) or jadomycin vehicle for 24 hours under standard conditions using the Aurum total RNA Mini Kit according to the manufacturer's instructions. Isolated RNA (0.5 μg) was reverse-transcribed to complementary DNA using Super Script II Reverse Transcriptase (Life Technologies). The complementary DNA was amplified *via* quantitative PCR using 125 nM gene-specific primers in a total volume of 20 μL using a SYBR Green PCR Kit and a Step One Plus real-time PCR thermocycler (Applied Biosystems) in duplicate for each primer set. Gene expression was normalized using the average of the three housekeeping genes *GAPDH*, *β-actin*, and *peptidylprolyl isomerase A (PPIA) via* the $\Delta\Delta C_t$ method [243].

3.03.10: STATISTICAL ANALYSIS

All data are presented as mean ± SEM. An unpaired *t* test was performed for dual comparisons in experiments with one independent variable. A one-way or two-way ANOVA was performed for multiple comparisons in experiments with one or two-independent variables, respectively. A Bonferroni's multiple comparison test was used for *post-hoc* analysis of the significant ANOVA. In the case of non-parametric data

involving one-independent variable, a Kruskal-Wallis one-way ANOVA followed by Dunn's multiple comparison test was used. A difference in mean values between groups was considered significant if $P \leq 0.05$.

3.04.00: RESULTS

3.04.01: JADOMYCINS ARE EQUALLY CYTOTOXIC AGAINST MCF7-CON, BT474, SKBR3, AND MDA-MB-231 BREAST CANCER CELLS

MTT cell viability measuring assays quantified the %-cell viability of MCF7-CON, BT474, SKBR3, and MDA-MB-231 breast cancer cells after being treated with various concentrations of jadomycins B, S, or F. The IC_{50} of each jadomycin in each cell line was calculated and used as a measure of drug potency (**Table 8**). The IC_{50} values of jadomycins B, S, and F were not significantly different in each of the four breast cancer cell lines tested. Given the similar jadomycin cytotoxicity profiles, we chose a single cell line (MCF7) to investigate their mechanisms of action in this chapter.

3.04.02: JADOMYCINS INCREASE ROS ACTIVITY IN MCF7-CON CELLS

Using the general intracellular ROS-detecting probe CM-$DCFH_2$-DA, jadomycin treatments in MCF7-CON breast cancer cells showed a dose-dependent increase in ROS activity (**Figure 9**). Jadomycin B and F concentrations of 20 or 30 μM and jadomycin S and SPhG concentrations of 30 μM significantly increased intracellular ROS activity in MCF7-CON in comparison to the jadomycin vehicle control. To verify the increase in fluorescence was solely due to increased intracellular ROS activity the autofluorescence of the four jadomycins and their ability to react directly with CM-$DCFH_2$-DA were tested, which showed no autofluorescence or reactivity (**Figure 10**).

3.04.03: NAC CO-TREATMENT DECREASES THE CYTOTOXIC POTENCY OF JADOMYCINS IN MCF7 CELLS

The antioxidant NAC is a cysteine precursor which is taken up into cells and converted to cysteine, the rate-limiting molecule in the synthesis of GSH, an integral component in a cell's antioxidant defense system [278]. In the absence of NAC, jadomycins B, S, F, and SPhG had equal mean IC_{50} values (4.0 – 5.3 μM) in MCF7-CON cells. In the presence of increasing concentrations of NAC, the concentration-cytotoxicity response curves, as measured by LDH assays, were right-shifted to varying degrees for jadomycins B, S, SPhG, and F (**Figures 11a**, **b**, **c**, and **d**, respectively). The treatment of MCF7-CON cells with 3-15 mM NAC alone resulted in a small reduction (17-25%) in cell viability (**Figure 11e**). The shift in jadomycin potency was quantified by a progressive yet saturable increase in jadomycin IC_{50} values with increasing NAC concentration (**Figure 11f**). Overall jadomycin SPhG was least affected by NAC as demonstrated by the IC_{50} values that were lower than those of jadomycins B, S, or F when co-treated with ≥ 3 mM NAC. While NAC reduced the potency of each jadomycin, greater than 95% loss in cell viability could still be attained using higher doses of jadomycin in the presence of NAC.

3.04.04: NAC DOSE-DEPENDENTLY DECREASES JADOMYCIN-MEDIATED ROS ACTIVITY WHILE SIMULTANEOUSLY INCREASING THE VIABILITY OF MCF7-CON AND MCF7-TXL CELLS

The results of our initial experiments suggested that ROS generation is a mechanism of jadomycin toxicity. The next experiments were designed to confirm the mechanistic link

between ROS generation and jadomycin toxicity by performing ROS assays and LDH cytotoxicity assays sequentially in the same cells. In MCF7-CON cells jadomycins B, S, SPhG, and F produced between a 1.4 and 2.6-fold increase in intracellular ROS which was associated with a 61 to 78 % loss in viable cells compared to vehicle treated cells in the absence of NAC. When co-treated with increasing concentrations of NAC, similar dose-dependent reductions in intracellular ROS activity and increases in %-cell viability were observed for each of the four jadomycins in MCF7-CON cells (**Figures 12a**, **b**). Comparable results were observed in MCF7-TXL cells, albeit using marginally higher concentrations of each jadomycin (**Figures 12c**, **d**).

3.04.05: INHIBITION OF AURORA B KINASE BY JADOMYCINS IS RETAINED WHEN ROS ARE INHIBITED

To assess if the second putative mechanism of jadomycin cytotoxicity (aurora B kinase inhibition) is dependent or independent of ROS generation we examined jadomycin inhibition of aurora B kinase activity with and without ROS neutralization using 15 mM NAC. Aurora B kinase activity was quantified by measuring the phosphorylation of the aurora B kinase downstream target P-His3(Ser10) [6,184] by western blotting (**Figures 13a**, **b**). Jadomycins B, S, SPhG, and F all inhibited phosphorylation of His3(Ser10) compared to the jadomycin vehicle control. A similar level of inhibition was maintained when ROS activity was inhibited with NAC. NAC co-treatment did not significantly alter the level of inhibition induced by any of the jadomycin treatments. The H_2O_2 positive control had no effect on P-His3(Ser10) in the presence or absence of NAC. The NAC

treatment did not alter P-His3(Ser10) compared to the NAC vehicle in the absence of jadomycin treatments.

3.04.06: JADOMYCIN-INDUCED ROS ACTIVITY AND CORRESPONDING CYTOTOXICITY ARE COPPER DEPENDENT

MCF7-CON cells co-treated with $CuSO_4$ and jadomycin S, SPhG, or F demonstrated 2.2 to 3.1-fold greater intracellular ROS activity compared to cells treated with those jadomycins alone, with a similar trend observed for jadomycin B (**Figure 14a**). The increased ROS activity was correlated with a further 34.5 to 51.6 % decrease in cell viability for all four jadomycin treatments relative to the vehicle-treated control cells. While 10 μM $CuSO_4$ increased ROS activity by 2.1-fold in the absence of jadomycins, this was not associated with any change in cell viability indicating the cytotoxicity was jadomycin dependent (**Figure 14b**). The ROS activity in cells treated with jadomycin B, S, or F and the copper chelator D-Pen decreased 1.6 to 2.3-fold compared to the respective controls. These reductions in intracellular ROS were associated with 41.1 to 57.9 % increases in %-cell viability for jadomycin B, SPhG, or F treated cells. D-pen treatment in the absence of jadomycins had no effect on ROS activity or %-cell viability (**Figures 14c**,**d**).

3.04.07: PRO- AND ANTI-OXIDANT CO-TREATMENTS ALTER JADOMYCIN-INDUCED ROS ACTIVITY AND CYTOTOXICITY

The TrxR inhibitor auranofin significantly increased ROS activity 2.5 to 3.8-fold and decreased MCF7-CON cell viability 43.7 to 76.8 % when co-treated with each of the four jadomycins in comparison to jadomycin alone. The SOD1 inhibitor DDC increased ROS activity in all but the jadomycin B co-treated cells 1.7 to 2.0-fold, though it consistently decreased cell viability for each jadomycin 41.2 to 68.2 %. The GST inhibitor EA did not alter ROS activity, but decreased cell viability in cells co-treated with jadomycins S or SPhG by 40.5 or 28.6 %, respectively. The catalase inhibitor 3-AT had no effect on ROS activity or cell viability when co-treated with any of the jadomycin analogues used. None of these inhibitors affected ROS activity or %-cell viability in the absence of jadomycin (**Figure 15**). The antioxidant, superoxide dismutase 2 (SOD2) mimetic MitoTEMPO at multiple concentrations did not affect ROS activity or cell viability when co-treated with any of the jadomycins (**Figure 16**).

The three co-treatments, which resulted in the largest ROS and cell viability changes, $CuSO_4$, auranofin, and DDC, all produced similar results in MCF7-TXL cells. $CuSO_4$ consistently increased ROS activity for each jadomycin treatment as well as for the no jadomycin control 1.9 to 2.8-fold, auranofin increased ROS activity 2.3 and 2.0-fold when co-treated with jadomycins S and SPhG, respectively, while DDC had no effect on ROS. $CuSO_4$ decreased cell viability for each jadomycin co-treatment, except SPhG, by 32.6 to 40.0 %. Auranofin and DDC decreased cell viability for each jadomycin treatment by 51.5 to 66.8 % and 37.9 to 55.3 %, respectively (**Figure 17**).

3.04.08: JADOMYCIN B TREATMENT INDUCES THE EXPRESSION OF *TRXR1*

To expand on our previous results indicating an increase in *TrxR1* expression with jadomycin B or S treatment (**Table 6**), qPCR was completed to determine if jadomycin B, chosen as a representative jadomycin treatment, altered the expression of various antioxidant encoding genes. A 24 hour treatment of jadomycin B versus jadomycin vehicle in MCF7-CON cells at standard conditions resulted in no change in *SOD1*, *SOD2*, *Trx*, *catalase*, or *nuclear factor (erythroid-derived 2)-like 2* (*Nrf2*) mRNA levels when compared with the housekeeping gene *GAPDH*. However, supporting our previous work, a significant 10.2-fold increase in *TrxR1* expression was observed (**Figure 18**). The PCR primer sequences used can be found in **Table 9**.

Table 8: IC_{50} values (μM) as determined by MTT assays after treatment with jadomycins in multiple breast cancer cell lines. Data show the mean IC_{50} ± SEM of at least three independent experiments performed in quadruplicate. The IC_{50} values for each jadomycin were equal in each of the four breast cancer cell lines tested as determined by one-way ANOVAs, followed by Bonferroni's multiple comparison tests ($P \leq 0.05$).

Cell line	**IC_{50} value (μM)**		
	Jadomycin B	**Jadomycin S**	**Jadomycin F**
MCF7-CON	2.58 ± 0.39	3.38 ± 0.09	3.59 ± 0.52
BT474	4.16 ± 0.54	3.09 ± 0.54	5.05 ± 1.62
SKBR3	3.82 ± 0.84	3.08 ± 0.73	4.70 ± 0.90
MDA-MB-231	1.76 ± 0.33	2.79 ± 0.48	3.25 ± 0.29

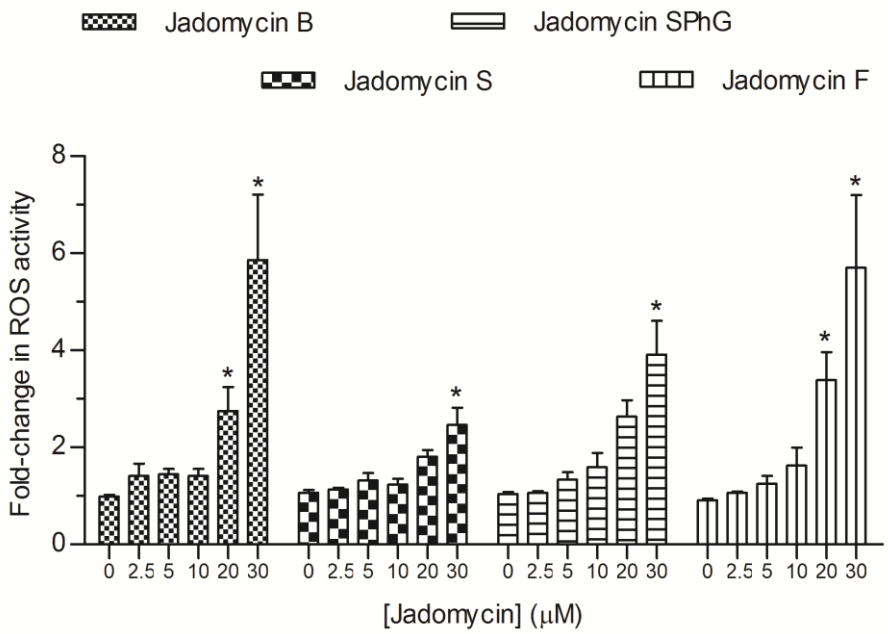
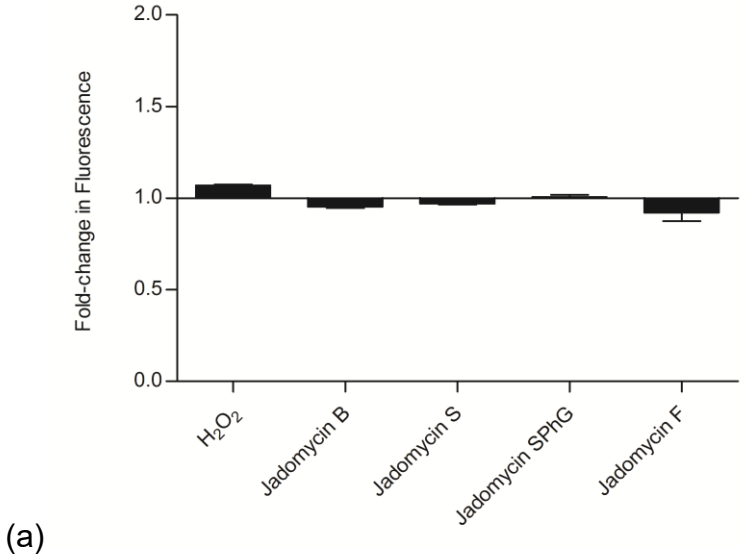
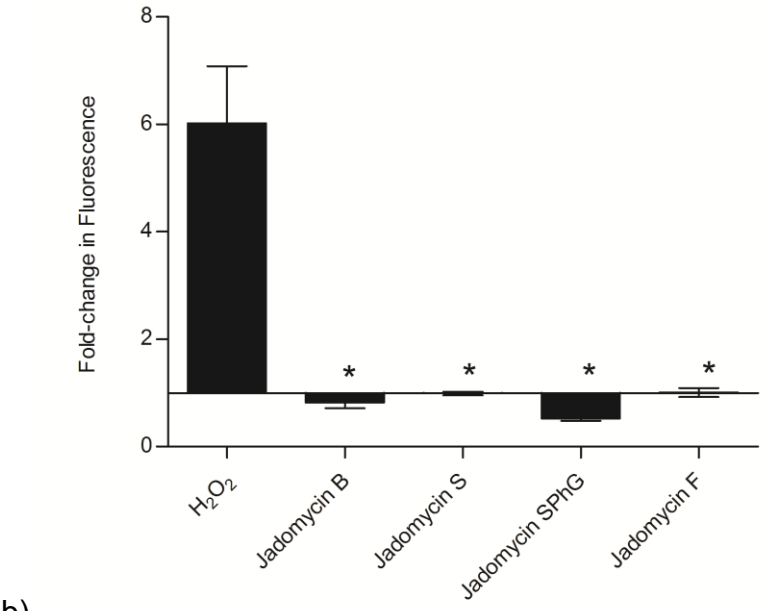


Figure 9: Jadomycins increase intracellular ROS activity in MCF7-CON cells. ROS activity was quantified by measuring the fluorescence of CM-DCF in MCF7-CON cells after 24 hours of being treated with jadomycins B, S, SPhG, or F (2.5-30 μM) or jadomycin vehicle. ROS activity was expressed as a fold-change relative to jadomycin vehicle, which was assigned a value of 1. Each bar represents the mean ± SEM of at least three independent experiments. * $P \leq 0.05$, the fold-change in ROS activity was significantly different when compared with the vehicle control (0 μM bar) as determined by a Kruskal-Wallis non-parametric one-way ANOVA, followed by the non-parametric Dunn's multiple comparison test.



(a)



(b)

Figure 10: Jadomycins B, S, SPhG, and F do not autofluoresce at the wavelengths used to detect CM-DCF fluorescence and they do not react with CM-DCFH$_2$-DA. The fluorescence (excitation 485/20 nm and emission 528/20 nm) of jadomycins (30 µM) and the H_2O_2 ROS control (2 mM) alone (**a**) and with CM-DCFH$_2$-DA (7.5 µM) (**b**) was measured in MCF7 cell medium. Each bar represents the mean ± SEM of three independent experiments. * $P \leq 0.05$, the fold-change in fluorescence was significantly different when compared with the H_2O_2 control as determined by a one-way ANOVA, followed by Bonferroni's multiple comparison test.

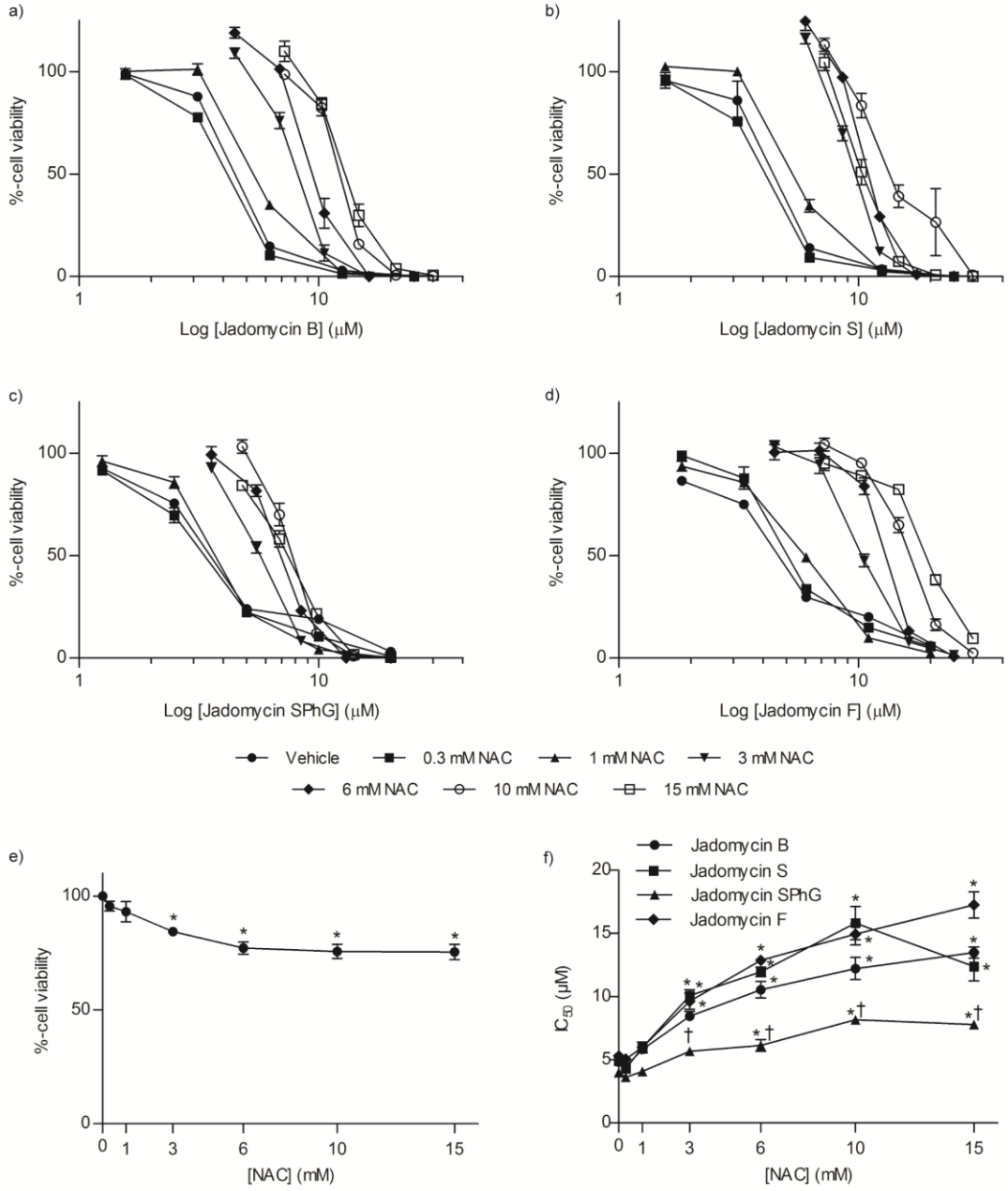


Figure 11: NAC co-treatment decreases the cytotoxic potency of jadomycins in MCF7 cells. MCF7-CON cells were treated with 1.25-35.0 μM jadomycin B **(a)**, S **(b)**, SPhG **(c)**, and F **(d)** in the presence and absence of NAC (0.3-15 mM) or NAC alone **(e)** for 72 hours. For each treatment the cell viability was measured using LDH assays and is expressed as %-cell viability relative to the vehicle treated control in the absence of NAC. From the concentration-response curves, the jadomycin IC_{50} values in the absence and presence of each NAC concentration were determined **(f)**. Each symbol represents

the mean ± SEM of at least three independent experiments. * $P \leq 0.05$, the IC_{50} value for the specified jadomycin was significantly different compared to the H_2O vehicle control (0 mM NAC bar). † $P \leq 0.05$, the IC_{50} value of jadomycin SPhG was significantly different compared to jadomycins B, S, and F at the indicated NAC concentration. A one-way **(e)** or two-way ANOVA **(f)**, followed by the Bonferroni's multiple comparison test, were used for the analyses.

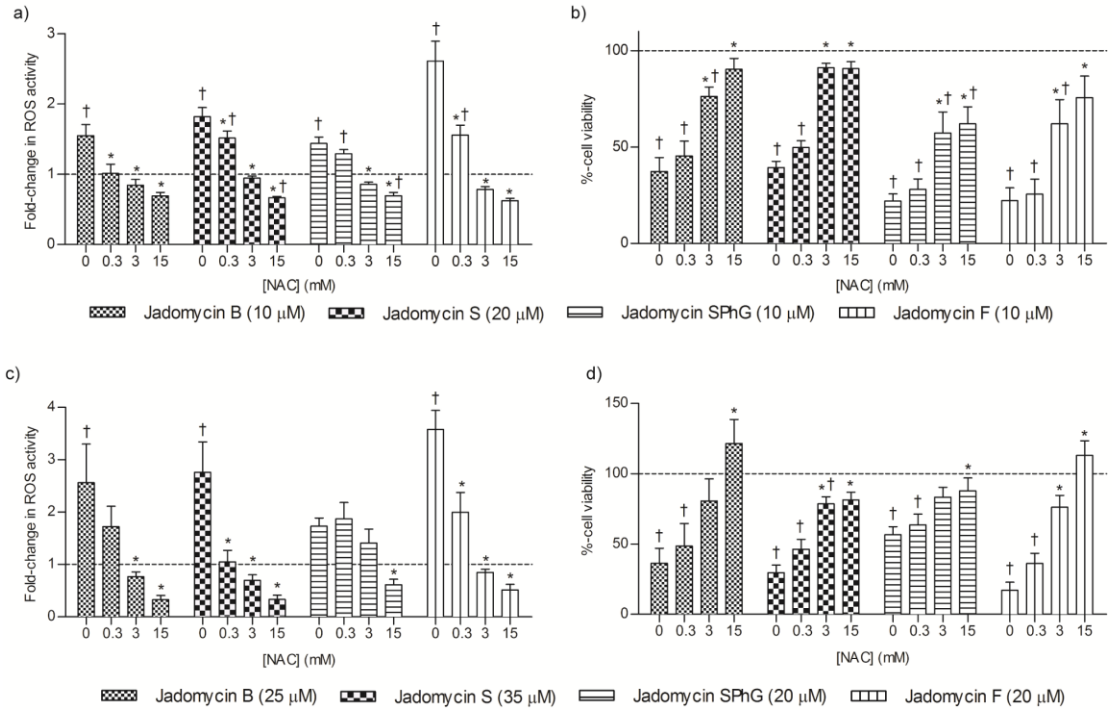


Figure 12: NAC dose-dependently decreases jadomycin-mediated ROS activity while simultaneously increasing the viability of MCF7-CON (**a** and **b**) and MCF7-TXL (**c** and **d**) cells. MCF7-CON or MCF7-TXL cells were treated for 24 hours with jadomycins B, S, SPhG, or F (10-35 μM) or the jadomycin vehicle control followed by co-treatment with NAC (0.3-15 mM) or H_2O vehicle control (0 mM NAC bar). ROS activity and cell viability were measured as described in the legends for Figures 1 and 2 and are respectively expressed as a fold-change in ROS activity **(a, c)** and %-cell viability **(b, d)** relative to the jadomycin vehicle/H_2O (no jadomycin or NAC) control, which was assigned a value of 1 for ROS activity or 100 % for the cell viability assays and represented by the horizontal dotted lines. Each bar represents the mean ± SEM of at least three independent experiments. * $P \leq 0.05$, the fold-change in ROS activity or %-cell viability was significantly different when compared with the H_2O vehicle control (0 mM NAC bar), and † $P \leq 0.05$, compared to jadomycin vehicle control (dotted line) determined by one-way ANOVAs, followed by Bonferroni's multiple comparison tests.

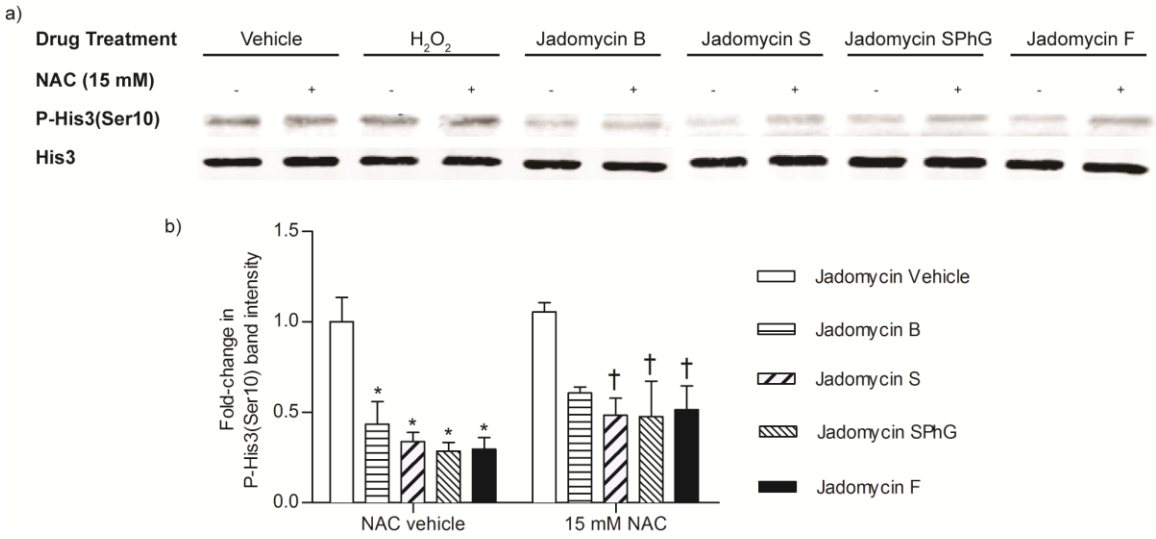


Figure 13: Jadomycins S, SPhG, and F inhibit phosphorylation of the aurora B kinase target His3(Ser10), independent of ROS activity. MCF7-CON cells were treated for 24 hours with jadomycins B, S, SPhG, or F (10 μM), H_2O_2 (500 μM) or jadomycin vehicle control and co-treated with NAC (15 mM) or H_2O vehicle control, after which total protein was collected. Levels of P-His3(Ser10) and His3 control were determined *via* western blotting **(a)**. Band intensities were measured and the fold-changes in P-His3(Ser10)/His3 ratios calculated relative to either jadomycin vehicle and H_2O vehicle controls or jadomycin vehicle and NAC (15 mM) alone **(b)**. * $P \leq 0.05$, the fold-change in protein band intensity was significantly different compared to the jadomycin vehicle and H_2O vehicle control (left side, clear bar). † $P \leq 0.05$, the fold-change in protein band intensity was significantly different compared to the jadomycin vehicle and NAC (15 mM) control (right side, clear bar). NAC co-treatment did not significantly alter the fold-change in band intensity for any of the jadomycin treatments. A two-way ANOVA, followed by the Bonferroni's multiple comparison test, were used for the analyses.

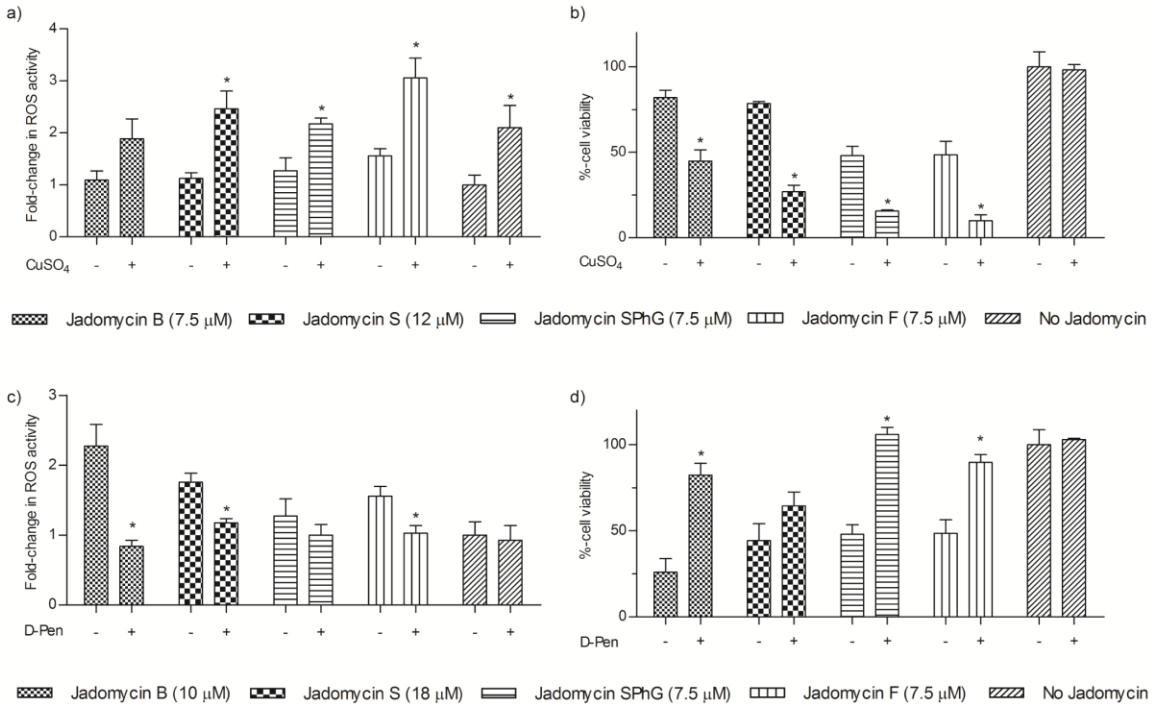


Figure 14: Jadomycin-induced ROS activity and corresponding cytotoxicity are copper dependent. MCF7-CON cells were treated for 24 hours with jadomycins B, S, SPhG, or F (7.5-18 μM) or the jadomycin vehicle control (labelled No Jadomycin) with (+) $CuSO_4$ (10 μM) or D-Pen (1 mM), or an H_2O vehicle (-). ROS activity is expressed as fold-change **(a, c)** and cell viability as a percentage **(b, d)** relative to the No Jadomycin control in the absence of $CuSO_4$ or D-Pen. Each bar represents the mean ± SEM of at least three independent experiments. * $P \leq 0.05$, for the indicated jadomycin, the fold-change in ROS activity or %-cell viability was significantly different in the presence versus absence of $CuSO_4$ or D-Pen as determined by an unpaired *t* test.

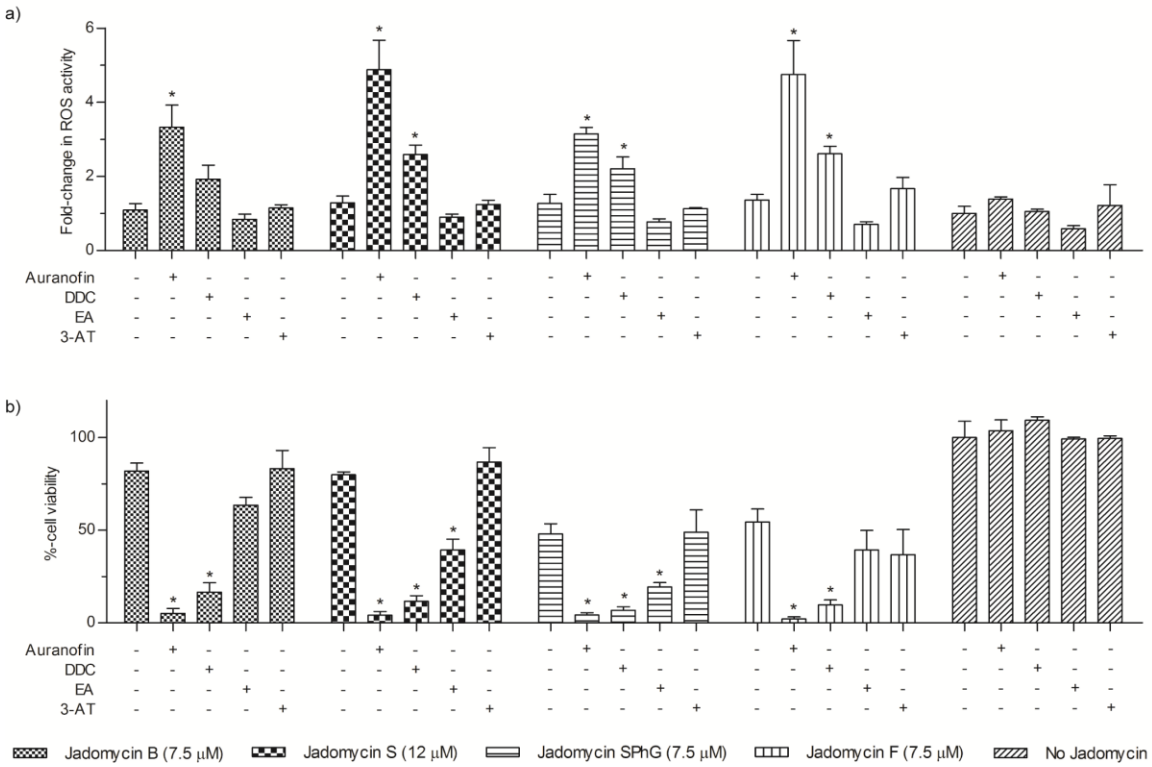


Figure 15: Antioxidant inhibitor co-treatments alter jadomycin-induced ROS activity and cytotoxicity in MCF7-CON cells. MCF7-CON cells were treated for 24 hours with jadomycins B, S, SPhG, or F (7.5-12 μM) or the jadomycin vehicle control (labelled No Jadomycin) with (+) auranofin (1 μM), DDC (1 mM), EA (20 μM), or 3-AT (10 mM) or a DMSO vehicle control (-). ROS activity is expressed as fold-change (**a**) and cell viability as a percentage **(b)** relative to the No Jadomycin control in the absence of auranofin, DDC, EA or 3-AT. Each bar represents the mean ± SEM of at least three independent experiments. * $P \leq 0.05$, for the indicated jadomycin, the fold-change in ROS activity or %-cell viability was significantly different in the presence versus the absence of auranofin, DDC or EA as determined by a one-way ANOVA, followed by Bonferroni's multiple comparison test.

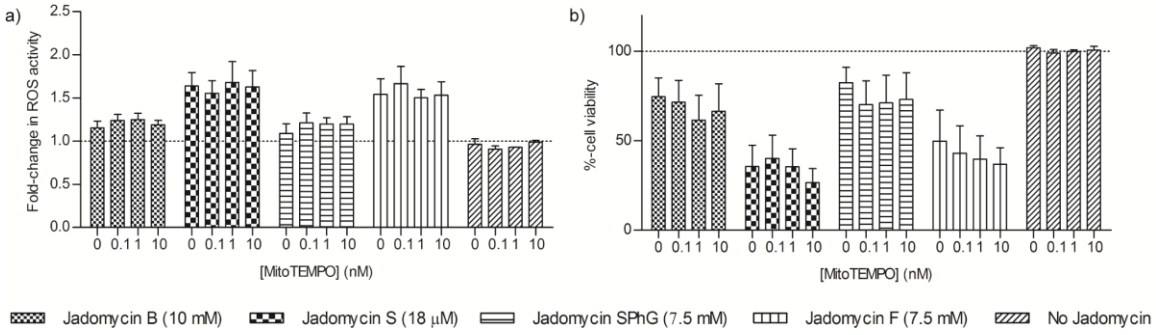


Figure 16: MitoTEMPO co-treatments do not alter jadomycin-induced ROS activity and cytotoxicity in MCF7-CON cells. After MCF7-CON cells were treated for 24 hours with jadomycin B, S, SPhG, or F (7.5-18 μM) or the jadomycin vehicle control (labelled No Jadomycin) and co-treated with MitoTEMPO (0.1-10 nM) or a H_2O vehicle control (bar labelled 0 nM), ROS activity was quantified by measuring the fluorescence of CM-DCF and cell viability was measured using LDH assays. Data are expressed as a fold-change in ROS activity (**a**) and %-cell viability (**b**), respectively. Each bar represents the mean ± SEM of three independent experiments. The fold-change in ROS activity and %-cell death were not significantly different when compared with the H_2O vehicle control as determined by a one-way ANOVA, followed by Bonferroni's multiple comparison test.

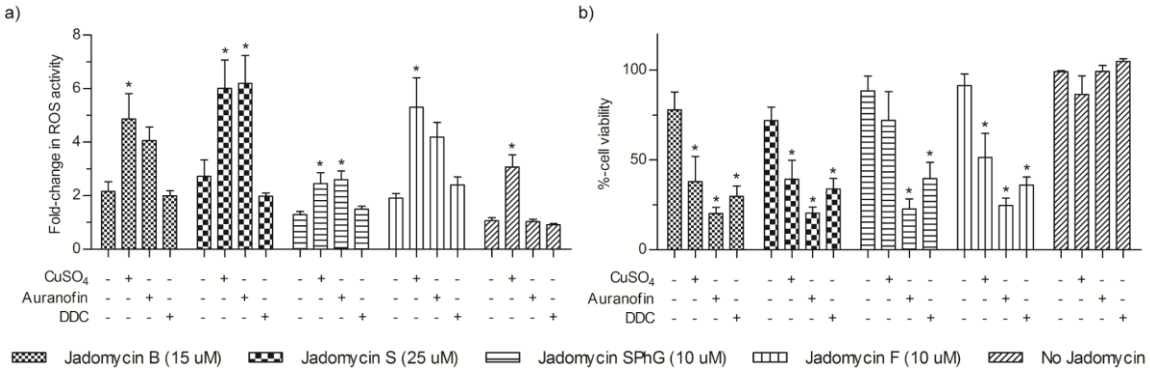


Figure 17: Pro-oxidant co-treatments alter jadomycin-induced ROS activity and cytotoxicity in MCF7-TXL cells. MCF7-TXL cells were treated for 24 hours with jadomycins B, S, SPhG, or F (10-25 μM) or the jadomycin vehicle control (labelled No Jadomycin) with (+) $CuSO_4$ (10 μM), auranofin (1 μM), or DDC (1 mM) or a DMSO vehicle control (-). ROS activity is expressed as fold-change (**a**) and cell viability as a percentage **(b)** relative to the no jadomycin control in the absence of $CuSO_4$, auranofin or DDC. Each bar represents the mean ± SEM of at least three independent experiments. * $P \leq 0.05$, for the indicated jadomycin, the fold-change in ROS activity or %-cell viability was significantly different in the presence versus absence of $CuSO_4$, auranofin or DDC as determined by a one-way ANOVA, followed by Bonferroni's multiple comparison test.

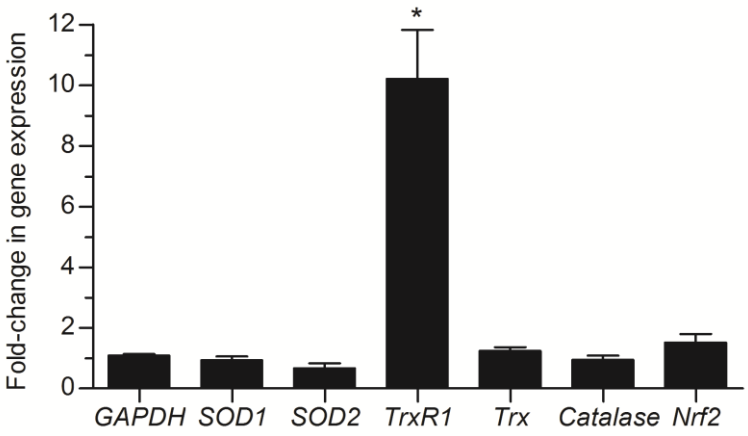


Figure 18: Jadomycin B treatment induces the expression of *TrxR1* in MCF7-CON cells. MCF7-CON cells were treated with either jadomycin B (10 μM) or jadomycin vehicle and the expression of the antioxidant encoding genes *SOD1*, *SOD2*, *TrxR1*, *Trx*, *Catalase*, and *Nrf2* and the housekeeping genes *GAPDH, β-actin*, and *PPIA* was measured *via* qPCR. The fold-change in gene expression, which represents the change in expression of a given gene in jadomycin B versus jadomycin vehicle treated cells, was calculated *via* the $\Delta\Delta C_t$ method and normalized to the average of the three housekeeping genes. Each bar represents the mean ± SEM of three independent experiments. * $P \leq 0.05$, the fold-change in gene expression was significantly different when compared with that of the *GAPDH* housekeeping control as determined by a one-way ANOVA, followed by Bonferroni's multiple comparison test.

Table 9: PCR primers used to determine the expression of antioxidant and housekeeping genes in MCF7-CON cells treated with either jadomycin B (10 μM) or vehicle control for 24 hours.

Gene	PCR forward primers (5'-3')	PCR reverse primers (5'-3')
SOD1	GGAGACTTGGGCAATGTGAC	CACAAGCCAAACGACTTCCA
SOD2	AAACCTCAGCCCTAACGGTG	CCACACATCAATCCCCAGCA
TrxR1	CCACTGGTGAAAGACCACGTT	AGGAGAAAAGATCATCACTGC
Trx	GGTGAAGCAGATCGAGAGCA	CCACGTGGCTGAGAAGTCAA
Catalase	ACTTCTGGAGCCTACGTCCT	AAGTCTCGCCGCATCTTCAA
Nrf2	ACACGGTCCACAGCTCATC	TGTCAATCAAATCCATGTCCTG
GAPDH	GAGTCAACGGATTTGGTCGT	TTGATTTTGGAGGGATCTCG
β-actin	GGACTTCGAGCAAGAGATGG	AGCACTGTGTTGGCGTACAG
PPIA	ACCGCCGAGGAAAACCGTGT	CTGTCTTTGGGACCTTGTCTGCA

3.05.00: DISCUSSION

Many anticancer drugs increase ROS activity within cancer cells, including vinblastine [279], paclitaxel [268], and doxorubicin [280]. While ROS are often considered oncogenic and certain ROS-inducing drugs are known to induce toxic side effects due to oxidative damage, ROS production remains a vital mechanism shared by many non-surgical methods of cancer treatment due to their effectiveness in inducing cancer cell death [281,282]. Monro, *et al*. determined that jadomycin B, in the presence of Cu(II), caused single strand cleavage of supercoiled bacterial plasmid DNA. This effect was blocked using the antioxidants catalase, superoxide dismutase, or hydroxyl radical and singlet oxygen scavengers, suggesting ROS were responsible for the DNA cleavage [205]. Using the rapidly proliferating MCF7 cell line, to which jadomycins are similarly toxic in comparison to the three other breast cancer cell lines tested, we demonstrated that four jadomycin analogues, B, S, F, and SPhG, chosen to represent the three structural classes of jadomycins (hydrophobic aliphatic, hydrophobic aromatic, and hydrophilic), increased intracellular ROS activity, supporting the findings of Monro, *et al*. Consistent with a ROS-mediated mechanism of action we then determined that the antioxidant NAC dose-dependently blocked jadomycin-generated ROS activity, which correlated with increased cell viability and reduced jadomycin potency. Despite this, higher concentrations of jadomycins were still able to attain close to 100% efficacy in the presence of NAC. One explanation for this efficacy preservation is that the ROS generated by the higher concentrations of jadomycins exceeded the GSH-generating capacity of the maximum tolerated dose of NAC. A second possibility is that a ROS-independent mechanism of cytotoxicity is triggered by higher concentrations of

jadomycin. This second putative mechanism could involve the inhibition of aurora B kinase, a protein often overexpressed in cancers and linked to tumour formation and progression [184,283] and for which jadomycin B has been previously shown to be an inhibitor [6,184]. Supporting this hypothesis, we showed that jadomycins similarly reduced the phosphorylation of the downstream aurora B kinase target His3(Ser10) both in the presence of elevated ROS and after ROS neutralization to baseline levels using NAC (15 mM). This observation, coupled with the fact that the ROS positive control H_2O_2 did not inhibit His3(Ser10) phosphorylation, supports that aurora B kinase inhibition by jadomycins could proceed independently of ROS generation. However, the exact role of the aurora B kinase pathway in jadomycin-mediated cytotoxicity remains to be determined.

Next, a pharmacological approach was used to determine which ROS are involved in jadomycin cytotoxicity and the potential mechanisms involved in ROS detoxification following jadomycin treatment. Monro, *et al*. proposed that jadomycin B serves as a source of electrons for the reduction of Cu(II) to Cu(I), which reacts further to form multiple ROS species including superoxide, hydroxyl radicals, and H_2O_2, leading to bacterial plasmid DNA cleavage [205]. The observation that the copper-chelating agent D-Pen [272] reduced whereas added $CuSO_4$ increased ROS production and cytotoxicity of jadomycins B, S, SPhG, and F indicated that jadomycin cytotoxicity in MCF7-CON and drug-resistant MCF7-TXL cells is Cu(II)-dependent. This Cu(II)-dependency varied slightly between jadomycins, suggesting the cytotoxic mechanisms of each analogue are not identical. Differences in jadomycin Cu(II)-dependency have also been previously observed, with jadomycin B being Cu(II)-dependent and jadomycin L being Cu(II)-

independent [206]. Endogenous copper in MCF7 cells was sufficient to mediate jadomycin cytotoxicity, while increasing the medium copper concentration enhanced jadomycin cytotoxicity, and *vice versa*. Since serum and tumour copper levels are elevated in various cancers [272] this Cu(II)-dependency of jadomycins indicates a possible exploitable route of cancer cell selectivity over healthy cells. Interestingly, $CuSO_4$ treatment alone produced a similar increase in intracellular ROS compared to $CuSO_4$ plus jadomycin co-treatments, with no effect on cell viability. Cu(II) is widely known to be involved in the production of ROS, particularly superoxide, hydroxyl radicals, and H_2O_2 [284]. This could indicate that the type of ROS being produced are different and more damaging in the presence of Cu(II) and jadomycin versus Cu(II) alone, such as higher levels of superoxide and less H_2O_2.

Our experiments showed how the cytosolic SOD1 inhibitor DDC [274] enhanced jadomycin-mediated ROS production and cytotoxicity whereas the mitochondrial antioxidant MitoTEMPO [273], a SOD2 mimic, had no effect. This supports increased cytosolic, but not mitochondrial, concentrations of superoxide as a primary mechanism of jadomycin-mediated killing of MCF7-CON and MCF7-TXL breast cancer cells. Furthermore, the augmenting effect of DDC on jadomycin-induced ROS generation and cell death indicates that SOD1 conversion of superoxide to H_2O_2 is an important cellular mechanism for jadomycin detoxification. The ROS-inducing anthracycline, doxorubicin, is known to have a high affinity for mitochondria. Since cardiac tissue is rich in mitochondria, doxorubicin is quite cardiotoxic [281]. By increasing only cytosolic superoxide it will be interesting to determine if jadomycins do not induce similar cardiotoxic side effects.

The reduction of H_2O_2 to H_2O can be completed through multiple antioxidant pathways. One is the Prx/Trx pathway, in which Prx reduces H_2O_2 to water, Trx reduces Prx, TrxR reduces Trx, and NADPH reduces TrxR, thus reactivating the pathway [259,260,261]. The TrxR inhibitor auranofin [277] increased ROS activity and decreased cell viability when co-treated with each jadomycin tested in MCF7-CON and -TXL cells, indicating that conversion of H_2O_2 to H_2O by the Prx/Trx pathway is vital for the detoxification of jadomycin-induced ROS. The importance of this pathway is further exemplified by the increased *TrxR1* expression in MCF7-CON cells treated with jadomycin B, a gene expression change that suggests an attempt by the breast cancer cells to increase Prx/Trx pathway activity to survive the treatment.

EA is an inhibitor of the cytosolic GSTs, which are multifunctional detoxifying enzymes involved in a second pathway of H_2O_2 reduction [276]. GSTs catalyze the conjugation of GSH to toxins, such as H_2O_2, after which glutathione peroxidases (GPx) reduce the GSH-H_2O_2 complex into two water molecules and glutathione disulfide (GS-SG) [276,285,286]. Despite reports of low expression of GST and GPx1 enzymes in MCF7 cells [287,288] the observed abilities of EA to enhance and NAC to inhibit jadomycin cytotoxicity supports that the GST/GPx system of H_2O_2 reduction is active *in vitro* in the detoxification of some jadomycins. Differences in the mechanisms of cytotoxicity between jadomycins are also further evidenced here as EA did not consistently alter the potency for each jadomycin tested. The third tested inhibitor, 3-AT, which inhibits H_2O_2 reducing catalase [275] showed no activity in MCF7-CON cells when co-treated with any of the four tested jadomycins. This is inconsistent with Monro, *et al*. who found the addition of catalase inhibited bacterial plasmid DNA cleavage

induced by jadomycin B [205]. We therefore hypothesize that the Prx/Trx and GST/GPx antioxidant systems are more heavily involved in the reduction of jadomycin-induced H_2O_2 within MCF7 cells than catalase. However, the results of Monro, *et al*. suggest that if the Prx/Trx and GST/GPx systems were inhibited, catalase could become involved.

A limitation of our study is that we only assessed the mechanism of jadomycin cytotoxicity in the MCF7 breast cancer cell line. We feel that this approach was justified given that none of the jadomycin treatments yielded a significant difference in cytotoxicity between the MCF7 (ER+, PR+, and HER2-), BT474 (ER-, PR+, HER2+), SKBR3 (ER-, PR-, HER2+), and MDA-MB-231 (ER-, PR-, HER2-) cell lines [179]. Furthermore, since cell viability was reduced independently of ER, PR, or HER2 status, it is likely the mechanism(s) through which jadomycins reduce cell viability is unrelated to those targets. However, to help address this limitation we have expanded our work into the triple-negative MDA-MB-231 breast cancer cells, which is the main cell line used in the proceeding Chapter 4.

In conclusion, jadomycins demonstrate potential as anticancer agents due to their ability to retain cytotoxicity in multidrug resistant MCF7-TXL breast cancer cells that overexpress drug efflux ABC-transporters versus control MCF7-CON cells [6]. Based on our results we have proposed a working model (**Figure 19**) describing how jadomycins induce breast cancer cell death *in vitro* by increasing cytosolic superoxide and H_2O_2 in a Cu(II)-dependent reaction, and that these ROS are reduced in the cytosol by SOD1 and the Prx/Trx and GST/GPx antioxidant pathways. Inhibition of these pathways presents viable co-treatment options that should be further tested for their ability to improve jadomycin potency and cancer cell selectivity in preclinical models.

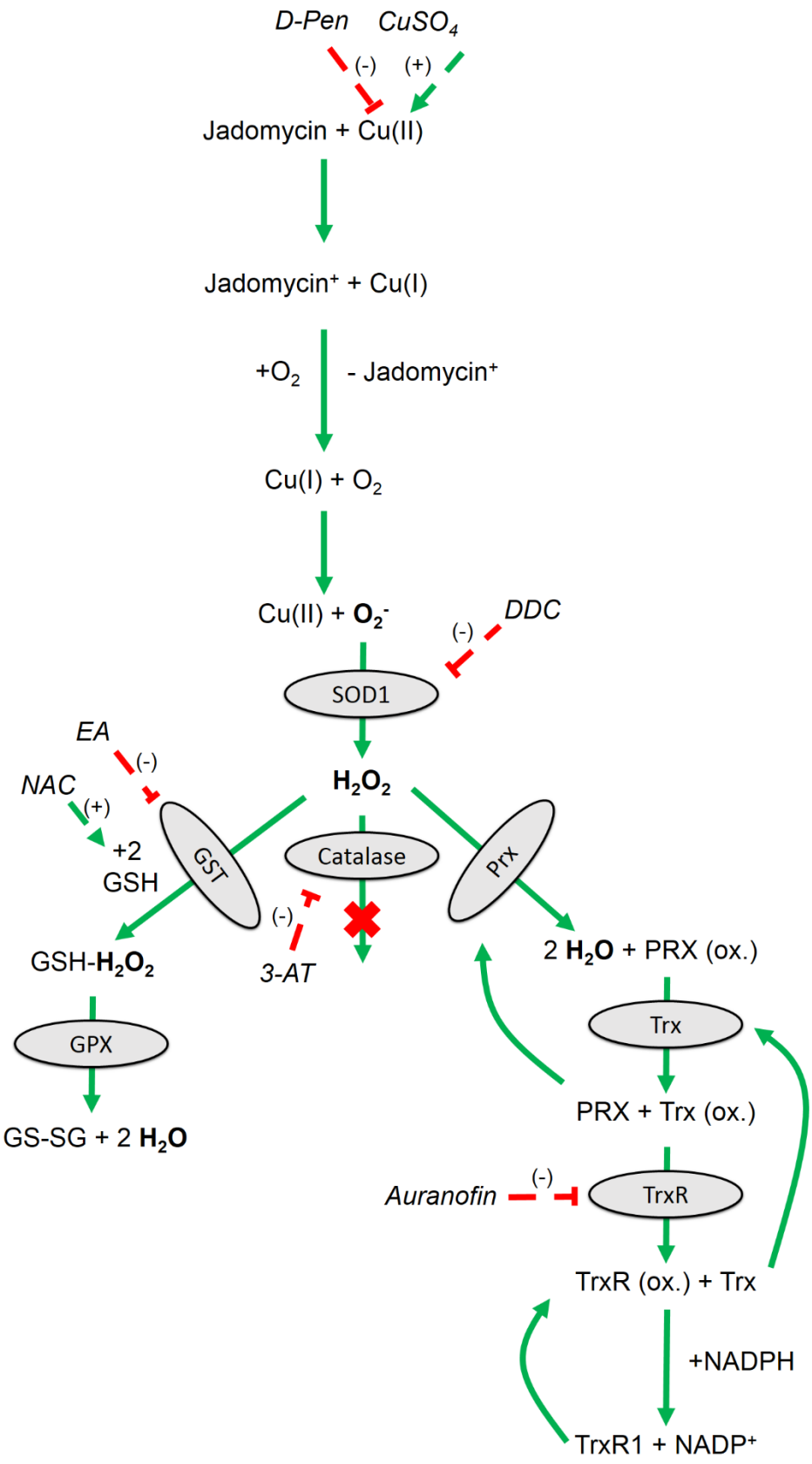

D-Pen
CuSO4
(-)
(+)
Jadomycin + Cu(II)
Jadomycin+ + Cu(I)
+O2
- Jadomycin+
Cu(I) + O2
Cu(II) + O2-
DDC
(-)
SOD1
EA
(-)
NAC
(+)
H2O2
+2
GSH
GST
Catalase
Prx
(-)
3-AT
GSH-H2O2
2 H2O + PRX (ox.)
GPX
Trx
GS-SG + 2 H2O
PRX + Trx (ox.)
Auranofin
(-)
TrxR
TrxR (ox.) + Trx
+NADPH
TrxR1 + NADP+

Figure 19: Proposed pathway of cytosolic jadomycin-induced ROS and their metabolism within MCF7 breast cancer cells. Jadomycin-mediated ROS generation and cytotoxicity is enhanced by $CuSO_4$ and blocked by the Cu(II)-chelating agent D-Pen, indicating intracellular ROS are being induced through a reaction between jadomycins and intracellular Cu(II). The ability of DDC to enhance jadomycin cytotoxicity implicates cytosolic superoxide as a primary mediator of jadomycin cytotoxicity. Furthermore, it indicates SOD1 conversion of superoxide to H_2O_2 is an important step in the neutralization of jadomycin-induced ROS. The abilities of the GST inhibitor EA and TrxR inhibitor auranofin to enhance and the GSH precursor NAC to inhibit jadomycin-mediated ROS generation and cytotoxicity indicates that H_2O_2 is also a mediator of jadomycin cytotoxicity, and that the subsequent conversion of H_2O_2 to H_2O by the GST/GPx and Trx/Prx antioxidant pathways are important for jadomycin detoxification. Lack of an effect by the catalase inhibitor 3-AT suggests this antioxidant pathway is not vital in the cellular metabolism of jadomycin-induced ROS in these cells. Italicized compounds represent inhibitors (-) or promoters (+) used with arrows depicting where they exert their effects within the ROS metabolism pathway. Species in bold represent ROS. Oxidised species are labelled with (**ox.**).

CHAPTER 4.00.00: JADOMYCINS INHIBIT TYPE II TOPOISOMERASES AND PROMOTE DNA DAMAGE AND APOPTOSIS IN MULTIDRUG RESISTANT TRIPLE NEGATIVE BREAST CANCER CELLS

The work described in this chapter was conducted by myself and the data has been submitted to the *Journal of Pharmacology and Experimental Therapeutics.* I am currently completing revisions as requested by the reviewers, after which we will resubmit the manuscript.

Submitted manuscript information:

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Submitted to the Journal of Pharmacology and Experimental Therapeutics on March 2, 2017. Reviewer comments and invite to resubmit received March 21, 2017.

4.01.00: ABSTRACT

Jadomycins are natural products that kill drug-sensitive and MDR breast cancer cells. To date the cytotoxic activity of jadomycins has never been tested in MDR breast cancer cells that are also triple-negative, a category of breast cancer cells that are particularly aggressive and difficult to treat. Additionally, there is only a rudimentary understanding of how jadomycins cause cancer cell death, which includes the induction of intracellular ROS. We first created a paclitaxel-resistant, triple-negative breast cancer cell line (231-TXL) from drug-sensitive MDA-MB-231 cells (231-CON). Using MTT cell viability measuring assays, jadomycins B, S, and F were found to be equipotent in drug-sensitive 231-CON and MDR 231-TXL cells, and using ROS-detecting assays these jadomycins were determined to increase ROS activity in both cell lines by up to 7.3-fold. Jadomycins caused DNA double strand breaks in 231-CON and 231-TXL cells as measured by γH2AX western blotting. Co-incubation with the antioxidant NAC or pro-oxidant auranofin did not affect jadomycin-mediated DNA damage. Jadomycins induced apoptosis in 231-CON and 231-TXL cells as measured by annexin V affinity assays, a process which was maintained when ROS were inhibited. This indicated that jadomycins are capable of inducing MDA-MB-231 apoptotic cell death independently of ROS activity. Using qPCR, western blotting, and direct topoisomerase inhibition assays, it was determined that jadomycins are type II topoisomerase inhibitors. We therefore propose novel mechanisms through which jadomycins induce breast cancer cell death independently of ROS-activity, namely through the inhibition of type II topoisomerases, induction of DNA damage, and apoptosis.

4.02.00: INTRODUCTION

Certain types of breast cancer are innately more difficult to treat than others. Breast tumour cells that lack or have little expression of estrogen receptor (ER) and progesterone receptor (PR) and do not overexpress human epidermal growth factor receptor 2 (HER2) are known as triple-negative breast cancers. Triple-negative breast tumours are typically of a larger size and higher grade than non-triple-negative breast tumours, with a higher rate of metastasis development and a lower overall survival rate. About 15% of all breast cancers are triple-negative, and they disproportionally affect women under the age of forty. Treatment options for triple-negative breast cancer are limited as hormone-receptor or HER2-targeted therapies are ineffective, and for advanced cases the only treatments available are cytotoxic chemotherapies [55,57,58]. With up to 30% of all cases of breast cancer ultimately metastasizing, and the high prevalence of MDR and triple-negative breast cancers [4,58], new and more effective treatments are needed.

We have shown that many jadomycin analogues are effective cytotoxic agents against ER-positive, PR-positive, HER2-negative MCF7 breast cancer cells, and that they largely retain their potency in MDR MCF7 breast cancer cells that overexpress the ABC drug efflux transporter genes *ABCB1*, *ABCC1*, or *ABCG2* [6]. We have also determined that jadomycins are equally cytotoxic in triple-negative MDA-MB-231 versus non-triple-negative MCF7, BT474, and SKBR3 breast cancer cells [289]. Jadomycins are therefore attractive compounds for the treatment of drug resistant and triple-negative breast cancers.

Currently we only have a basic understanding of how jadomycins exert their anticancer activity. We initially determined that these compounds induce intracellular ROS activity through a Cu(II)-dependent mechanism in drug-sensitive and MDR *ABCB1*-overexpressing MCF7 breast cancer cells, and that jadomycin potency can be altered when co-treated with anti- and pro-oxidants, suggesting that jadomycin anticancer activity is at least partially dependent on ROS. Interestingly, it was also found that when ROS were inhibited jadomycins still retained 100% cytotoxic efficacy in the breast cancer cells (albeit with lower potency), evidencing that jadomycins are also acting through ROS-independent mechanisms [289]. One such alternate mechanism is the inhibition of aurora B kinase, an important mitotic protein, which can lead to cancer cell death [6,184,289]. Jadomycins may also interact with topoisomerase IIβ, an enzyme that reduces DNA tension during replication, to which jadomycin DS was recently discovered to bond [226]. The polypharmacological nature of jadomycins' anticancer activity could help explain how these compounds evade drug resistance.

Our previous jadomycin mechanisms of action work was performed in MCF7 breast cancer cells [6,289]. To advance our understanding of how breast cancer cell type may influence jadomycins' mechanisms of action, the more aggressive triple-negative cell line, MDA-MB-231, was chosen for this study. Additionally, while we have shown that jadomycins kill MDR and drug-sensitive breast cancer cells [6], a better understanding of their intracellular cytotoxic targets and the method of cell death is still needed. Building on our past experiments that determined jadomycins are ROS-inducers and the fact that oxidative stress can cause DNA damage and apoptosis [290], we hypothesized that jadomycins damage DNA, ultimately leading to breast cancer cell apoptosis.

4.03.00: MATERIALS AND METHODS

4.03.01: CHEMICAL AND BIOLOGICAL MATERIALS

Thiazolyl blue methyltetrazolium bromide (MTT), NAC, methanol, propidium iodide, mitoxantrone, paclitaxel, doxorubicin, agarose, benzamide, $CaCl_2$, NaCl, Tris-HCl, HEPES, KCl, $MgCl_2$, adenosine triphosphate (ATP), bovine serum albumin, and phosphate buffered saline were purchased from Sigma Aldrich (Oakville, Ontario, Canada). Auranofin was purchased from Santa Cruz Biotechnology Inc. (Dallas, Texas, USA). Dulbecco's modified Eagle's medium, FBS, penicillin and streptomycin, sodium pyruvate, 5-(and 6-)chloromethyl-2'7'-dichlorodihydrofluorescein diacetate (CM-$DCFH_2$-DA), Super Script II Reverse Transcriptase, dithiothreitol, and TrypLE Express were purchased from Thermo Fisher Scientific (Burlington, Ontario, Canada). Annexin-V-FLUOS was purchased from Roche Diagnostics (Indianapolis, Indiana, USA). The cell fractionation kit, Z-VAD(OMe)-FMK, mouse monoclonal to γH2AX (phospho S139) antibody, mouse monoclonal to topoisomerase IIα antibody, rabbit polyclonal to topoisomerase IIβ antibody, and rabbit polyclonal to Histone H3 antibody were purchased from Abcam Inc. (Toronto, Ontario, Canada). Blocking buffer, IRDye 680RD-conjugated donkey anti-mouse antibody, and IRDye 800CW conjugated goat anti-rabbit antibody were purchased from Mandel Scientific (Guelph, Ontario, Canada). SsoAdvanced Universal SYBR Green Supermix was purchased from Bio-Rad (Mississauga, Ontario, Canada). Kinetoplast DNA, purified topoisomerase IIα, and 5x stop buffer were purchased from TopoGEN, Inc. (Buena Vista, Colorado, USA). Purified topoisomerase IIβ was kindly provided by Dr. Neil Osheroff (Vanderbilt University, Nashville, Tennessee, USA).

4.03.02: PRODUCTION OF JADOMYCINS

Jadomycins B, S, and F were isolated and characterized as previously described [6,182,183,232].

4.03.03: CELL LINES

The MDA-MB-231 (231-CON) breast cancer cells were kindly provided by Drs. David Hoskin and Anna Greenshields (Dalhousie University, Halifax, NS, Canada). Polyclonal MDA-MB-231 paclitaxel-resistant (231-TXL) cells were created in-house using slowly increasing concentrations of paclitaxel (Sigma Aldrich) over seven months until the cells could survive a final concentration of 470 nM, the same paclitaxel concentration used in our previously described MCF7-TXL cells [6]. All MDA-MB-231 cells were cultured in phenol red-free Dulbecco's modified Eagle's medium supplemented with 10% FBS, 100 IU/mL penicillin, 250 μg/mL streptomycin, and 1 mM sodium pyruvate (standard assay medium; Thermo Fisher Scientific), with the 231-TXL cells maintained with 470 nM paclitaxel. The cells were split and growth medium changed every 3-4 days up to a maximum of 35 passages. Cells were maintained in a humidified, 95% air/5% CO_2 atmosphere at 37 °C (standard conditions).

4.03.04: RNA ISOLATION, REVERSE TRANSCRIPTION, AND QUANTITATIVE REAL-TIME PCR

Total RNA was isolated from lysates of 231-CON and 231-TXL cells (a) with no drug treatment, or (b) treated with jadomycin B, S, or F (20 μM), mitoxantrone (1 μM), or 1:7 methanol:H_2O vehicle control (jadomycin vehicle) for 24 hours under standard conditions using the Aurum total RNA Mini Kit according to the manufacturer's instructions. Isolated RNA (0.5 μg) was reverse-transcribed to complementary DNA using Super Script II Reverse Transcriptase (Thermo Fisher Scientific). The complementary DNA was amplified *via* quantitative polymerase chain reaction (qPCR) using 125 nM gene-specific primers (Table 1) in a total volume of 20 μL using a SYBR Green PCR Supermix (Bio-Rad), and a Step One Plus real-time PCR thermocycler (Applied Biosystems, Foster City, California, USA) in duplicate for each primer set. Gene expression was normalized using the average of the three housekeeping genes *GAPDH*, *beta-actin*, and *PPIA via* the $\Delta\Delta C_t$ method [243].

4.03.05: MTT VIABILITY ASSAYS

MTT assays were used to evaluate the anticancer activity of jadomycins B, S, and F (0.1 – 20 μM) and the ABCB1 substrates mitoxantrone (0.1 nM – 50 μM) and doxorubicin (0.5 nM – 100 μM) in 231-CON and 231-TXL breast cancer cells and completed according to our previously described methods [6].

4.03.06: ROS MEASURING ASSAYS

To quantify the presence of intracellular ROS in 231-CON and 231-TXL cells, a fluorescent assay utilizing the ROS-reactive CM-$DCFH_2$-DA (Thermo Fisher Scientific) was used as previously described [289] with the following alterations: On day one, 20,000 cells were seeded in each well of a black-sided, clear bottomed 96-well plate. On day two, after the CM-$DCFH_2$-DA-containing medium was removed from each well, the cells were either (a) treated with 100 µL of jadomycin B, S, or F (2.5-40 µM) or vehicle in 1%-FBS standard assay medium for 24 hours in triplicate, or (b) pre-treated with 80 µL of medium control, NAC, or auranofin (final concentrations of 2.5 mM and 1 µM, respectively) for 1 hour and then treated with 20 µL of jadomycin B, S, or F (final concentrations of 5-20µM) in 1%-FBS standard assay medium for 24 hours in triplicate.

4.03.07: WESTERN BLOT ANALYSIS

231-CON or 231-TXL breast cancer cells were seeded in 6-well plates at 400,000 cells/well and left to adhere overnight in standard assay medium at standard conditions. They were then either (a) treated in triplicate for 24 h with medium control, jadomycin vehicle, jadomycin B, S, or F (15 µM), or mitoxantrone (1 µM), or (b) pre-treated in triplicate for 1 h with NAC, auranofin, or benzamide (2.5 mM, 1 µM, and 5 mM, respectively) then treated with jadomycin S (15 µM) or jadomycin vehicle for 24 h. The triplicate samples for each treatment were pooled, and the cytosolic, mitochondrial, and nucleic protein was then fractionated and collected using a Cell Fractionation Kit (Abcam Inc.; ab109719) as per the manufacturer's instructions. Protein content in each fraction

was measured using the Lowry method [242]. Nucleic protein was separated with a 15% or 6% SDS-PAGE (for γH2AX and type II topoisomerase II western blots, respectively) and transferred to a nitrocellulose membrane. Membranes were incubated overnight in either (a) a 1:1,000 dilution of mouse monoclonal to γH2AX (phospho S139) antibody (Abcam Inc.; ab26350) and rabbit polyclonal to Histone H3 antibody (ab1791), or (b) a 1:500 dilution of mouse monoclonal to topoisomerase IIα antibody (ab180393) and a 1:20,000 dilution of rabbit polyclonal to Histone H3 antibody, at 4 °C. Following washing, membranes were incubated in 1:10,000 dilutions of IRDye 680RD conjugated donkey anti-mouse and IRDye 800CW conjugated goat anti-rabbit secondary antibodies (Mandel Scientific; 926-68072 and 926-32211, respectively) for 1 h at room temperature. For visualization of protein bands membranes were scanned at 700 and 800 nm infrared wavelengths, using a LI-COR Odyssey scanner (Mandel Scientific). Pixel intensity of each tested protein band was normalized to the intensity of the respective Histone H3 bands using ImageJ, and these ratios expressed as a fold-change versus the medium-control treated MDA-MB-231 cells.

4.03.08: FLOW CYTOMETRIC ANALYSIS OF APOPTOSIS

Flow cytometric analysis of 231-CON and 231-TXL cells stained with annexin-V-FLUOS and propidium iodide was used to determine if jadomycins induced apoptosis. On day one, cells were seeded at 50,000 cells/well into 12-well flat-bottomed plates and left to adhere overnight. On day two, cells were treated with jadomycin B, S, or F (1.25 – 30 μM) or the positive control mitoxantrone (0.1 – 1 μM), with or without a 1-h pre-treatment with auranofin, benzamide, Z-VAD, or NAC (1 μM, 5 mM, 100 μM, and 2.5

mM, respectively) or vehicle control, in 500 µL of standard assay medium for 24 – 48 h, depending on which time point best exemplified the effects (or lack thereof) of the co-treatment. Nonadherent and adherent cells were combined in 5 mL round bottom tubes (Corning; Corning, New York, USA), which were harvested using TrypLE Express (Thermo Fisher Scientific). Cells were washed with phosphate buffered saline and labeled with annexin-V-FLUOS (Roche Diagnostics) diluted as per the manufacturer's instructions and propidium iodide (1 µg/mL; Sigma Aldrich) in detection buffer (10 mM HEPES, 140 mM NaCl, and 5 mM $CaCl_2$) for 15 minutes at room temperature. Each sample was then diluted with 300 µL of cold detection buffer and analyzed by flow cytometry using a FACSCalibur flow cytometer (BD Biosciences; Mississauga, Ontario, Canada). Percentage of healthy, early apoptotic, or late apoptotic/necrotic cells was analyzed using FCS Express 5 (De Novo Software, Glendale, California, USA).

4.03.09: TYPE II TOPOISOMERASE INHIBITION GEL ASSAY

The inhibition of topoisomerase IIα or IIβ activity was measured using the ATP-dependent decatenation reaction of kinetoplast DNA (kDNA) catenanes to open and closed circular decatenated kDNA [291]. Methods were based on those of Hasinoff, *et al* [292]. Individual reactions took place in 10 µL of 50 mM Tris HCl (pH = 8), 120 mM KCl, 10 mM $MgCl_2$, 0.5 mM ATP, 0.5 mM dithiothreitol, 30 µg/mL bovine serum albumin, 250-500 ng kDNA, 0.5 units of purified topoisomerase IIα enzyme or 20 ng/mL of purified topoisomerase IIβ, and jadomycins B, S, and F (10 – 640 µM), positive control doxorubicin (0.31 – 10 µM) or jadomycin vehicle. Reactions were incubated for 30 minutes at 37 °C and stopped using 5x stop buffer containing 5% sarkosyl, 0.125%

bromophenol blue, and 25% glycerol. The reaction products were separated by agarose gel (1% w/v) electrophoresis using TAE buffer. Both the agarose gel and the running TAE buffer contained 0.5 μg/mL of ethidium bromide. Gels were run at 135 V for 15 minutes, then destained in water for 10 minutes. Gels were photographed using an Olympus C-4000 Zoom camera under UV transillumination. Decatenated kDNA (TopoGEN) was run as a control, along with kDNA untreated with topoisomerase enzyme. The presence and brightness of the open circular and closed circular kDNA bands was used as a measure of topoisomerase IIα or IIβ activity, with intensity of these bands measured using ImageJ. The intensity of these bands for each given treatment was compared to that of the jadomycin vehicle (labelled 0 μM) for which there was no topoisomerase II inhibition, and relative topoisomerase IIα or IIβ inhibition was calculated.

4.03.10: STATISTICAL ANALYSIS

All data are presented as the mean value of at least three separate replicate trials with each trial's values displayed in scatter plots. An unpaired *t* test was performed for dual comparisons in experiments with one independent variable. A one-way or two-way analysis of variance (ANOVA) was performed for multiple comparisons in experiments with one or two-independent variables, respectively. A Bonferroni's multiple comparison test was used for *post-hoc* analysis of the significant ANOVA. A difference between mean values between groups was considered significant if $P \leq 0.05$.

4.04.00: RESULTS

4.04.01: 231-TXL CELLS OVEREXPRESS *ABCB1* AND JADOMYCINS ARE EQUIPOTENT IN 231-TXL VERSUS 231-CON CELLS

A 95,000-fold increase in the mRNA level of *ABCB1* was observed in the 231-TXL versus 231-CON cells, while no difference was seen in the expression of *ABCC1* or *ABCG2* (**Figure 20a**). Using MTT assays IC_{50} values of jadomycins B, S, and F were determined to be equal in both the drug-sensitive 231-CON and MDR 231-TXL breast cancer cells, while the IC_{50} values of the ABCB1 substrates mitoxantrone and doxorubicin were significantly higher in the 231-TXL versus 231-CON cells (**Figure 20b**).

4.04.02: JADOMYCINS INDUCE ROS ACTIVITY IN 231-CON AND 231-TXL CELLS WHICH CAN BE ALTERED USING ANTI- OR PRO-OXIDANT CO-TREATMENTS

Jadomycins B (40 μM), S (30 and 40 μM), and F (40 μM) significantly increased ROS in 231-CON cells in comparison to the jadomycin vehicle (**Figure 21a**). The antioxidant and glutathione precursor NAC and the pro-oxidant and thioredoxin reductase inhibitor auranofin were used to inhibit or enhance ROS levels in the cells following jadomycin treatments [289]. NAC (2.5 mM) and auranofin (1 μM) significantly decreased and increased, respectively, ROS activity in the 231-CON cells when co-treated with jadomycins S or F (40 μM), though not when co-treated with jadomycin B (40 μM) (**Figure 21b**). Since all jadomycins induced ROS, jadomycin S was chosen as a

representative jadomycin for this and all following replicative experiments involving 231-TXL cells. Jadomycin S was chosen due to greater water solubility and biosynthetic yields versus jadomycins B and F. Jadomycin S (20 and 40 μM) significantly increased ROS activity in the 231-TXL cells, and while NAC significantly decreased jadomycin S (40 μM) induced ROS activity, auranofin had no effect (**Figure 21c**).

4.04.03: JADOMYCINS INDUCE DNA DOUBLE STRAND BREAKS IN 231-CON AND 231-TXL CELLS

When double strand breaks occur within DNA it is always followed by the phosphorylation of histone H2AX; the amount of phosphorylated histone H2AX (γH2AX) in cells treated with cytotoxic agents can therefore be used as a measure of DNA double strand breaks [293]. In 231-CON cells, jadomycins B, S, and F (15 μM) and the control mitoxantrone (1 μM) significantly increased γH2AX protein levels versus the vehicle control, as measured using western blotting (**Figure 22a**). Jadomycin S (15 μM) significantly increased γH2AX protein expression in 231-TXL cells whereas mitoxantrone did not (**Figure 22b**). The induction of γH2AX protein expression in 231-CON cells by jadomycin S (15 μM) was not altered by co-treatment with the antioxidant NAC (2.5 mM) or pro-oxidant auranofin (1 μM), while co-treatment with benzamide (100 μM), an inhibitor of DNA repair poly(ADP-ribose) polymerases (PARPs) [294], significantly increased γH2AX protein expression. None of the co-treatments affected γH2AX levels on their own (**Figure 22c**).

4.04.04: JADOMYCINS INDUCE APOPTOSIS IN 231-CON AND 231-TXL CELLS

Apoptosis induced by cytotoxic drugs can be measured using annexin V affinity assays which differentiate healthy, early apoptotic, and dead (also labelled late apoptotic/necrotic) cells using fluorescently labelled annexin V and propidium iodide followed by FACS analysis [295,296]. Two examples of annexin V affinity assays can be seen in **Figure 23a**, depicting 231-CON cells treated with either the vehicle control (left hand side) or jadomycin S (20 μM; right hand side) for 36 hours. Healthy cells are in the lower-left quadrant (no fluorescence), early apoptotic in the lower-right (annexin V fluorescence), and late apoptotic/necrotic cells in the upper-right (annexin V and propidium iodide fluorescence).

Thirty-six hour treatments with jadomycins B and F (20 μM), jadomycin S (10 and 20 μM), and the control mitoxantrone (1 μM) induced significantly more early apoptosis versus the vehicle control (labelled 0 μM) in the 231-CON cells (**Figure 23b**). As well, these 36 h jadomycin B, S (10 and 20 μM), and F (5 and 20 μM) and mitoxantrone (0.1 μM) treatments significantly increased the number of late apoptotic/necrotic cells versus the vehicle control treatments (**Figure 23c**). In the 231-TXL cells, jadomycin S (20 μM) and mitoxantrone induced significantly greater early apoptosis versus the vehicle control (**Figure 23d**), while only jadomycin S induced significantly more late apoptosis/necrosis (**Figure 23e**).

4.04.05: JADOMYCIN CYTOTOXICITY IS ENHANCED BY AURANOFIN AND BENZAMIDE AND REDUCED BY Z-VAD

Jadomycins B (30 μM), S (20 μM), and F (30 μM) and mitoxantrone (1 μM) induced equal amounts of early apoptosis and late apoptosis/necrosis with or without the antioxidant NAC (2.5 mM) co-treatment after 36 hours (**Figure 24a**). The pro-oxidant auranofin (1 μM) had no effect on the amount of early apoptosis induced by jadomycins B, S, or F (5 μM), however it did significantly increase the number of late apoptotic/necrotic cells. Auranofin did not affect the cytotoxicity of mitoxantrone (0.1 μM) (**Figure 24b**). The PARP inhibitor benzamide (5 mM), while having no effect on late apoptosis/necrosis when co-treated with any of the jadomycin (5 μM) or mitoxantrone (0.1 μM) treatments, did significantly increase the amount of early apoptosis induced by jadomycin S after a 48 h treatment (**Figure 24c**). The cell permeable, irreversible pan-caspase inhibitor Z-VAD (100 μM) [297] had no effect on late apoptosis/necrosis, though it did significantly reduce the number of early apoptotic cells when co-treated with jadomycins B (30 μM), S (20 μM), and F (30 μM) or mitoxantrone (1 μM) for 36 h (**Figure 24d**). After 36 h co-treatments in the 231-TXL cells, Z-VAD significantly decreased jadomycin S-induced early apoptosis, while NAC, auranofin, and benzamide had no effect. Auranofin and benzamide both significantly increased the amount of late apoptosis/necrosis measured in the 231-TXL cells when co-treated with jadomycin S, while NAC and Z-VAD had no noticeable effect. No significant differences in early apoptosis or late apoptosis/necrosis were observed with any of the co-treatments when used with mitoxantrone (**Figure 24e**). None of these co-treatments had any effect on cell death on their own at the concentrations indicated.

4.04.06: JADOMYCINS ARE INHIBITORS OF TOPOISOMERASE IIα AND IIβ

The lack of effect of NAC and auranofin on jadomycin-induced DNA damage and early apoptosis suggested a ROS-independent mechanism. Martinez-Farina *et al* recently determined that the jadomycin analogue DS bonds to human topoisomerase IIβ protein [226], and our previously completed qPCR trials showed a minor 1.2 to 2-fold decrease in MCF7 cell regulation of topoisomerase genes when treated with 10 μM jadomycin S, albeit not significantly (**Table 6**). Therefore we chose to probe the possible involvement of topoisomerase inhibition by jadomycins as a ROS-independent mechanism of DNA damage and apoptosis. Jadomycins B, S, and F (20 μM, 36 h treatments) significantly reduced the expression of *TOP2A* and *TOP2B*, the genes that encode for topoisomerase IIα and IIβ, respectively, in 231-CON cells versus the vehicle control. A smaller but statistically significant decrease in *TOP1*, the gene that encodes topoisomerase I, was observed for jadomycin S with no significant changes for jadomycins B or F. The mitoxantrone control had no effect on *TOP1* expression, though it did cause a small *TOP2A* increase and *TOP2B* decrease versus the vehicle (**Figure 25a**). Jadomycin S (20 μM, 36 h) caused similar significant decreases in *TOP1*, *TOP2A*, and *TOP2B* expression in the 231-TXL cells while mitoxantrone had no effect (**Figure 25b**). The PCR primers used are listed in **Table 10.**

Jadomycins B, S, and F (15 μM, 24 h) and mitoxantrone (1 μM, 24 h) significantly lowered the levels of topoisomerase IIα protein versus the vehicle control (**Figure 25c**). Jadomycin S (15 μM, 24 h) but not mitoxantrone (1 μM, 24 h) decreased topoisomerase IIα in the 231-TXL cells (**Figure 25d**).

Using a protocol adapted from Topogen (Colorado, USA) and Hasinoff, *et al* [292], the ability of jadomycins and the known topoisomerase poison doxorubicin [298] to directly inhibit topoisomerases IIα and IIβ was measured. Jadomycins B, S, and F (10 – 640 μM) and doxorubicin (0.3125 – 10 μM) dose-dependently and directly inhibited both topoisomerases (**Figure 26a-d**). The topoisomerase IIα IC_{50} values of jadomycins S, F, and doxorubicin were significantly lower than that of jadomycin B, and the topoisomerase IIβ IC_{50} value for DOX was lower than that of jadomycin B. No drug was differentially potent in the inhibition of topoisomerase IIα versus IIβ (**Table 11**).

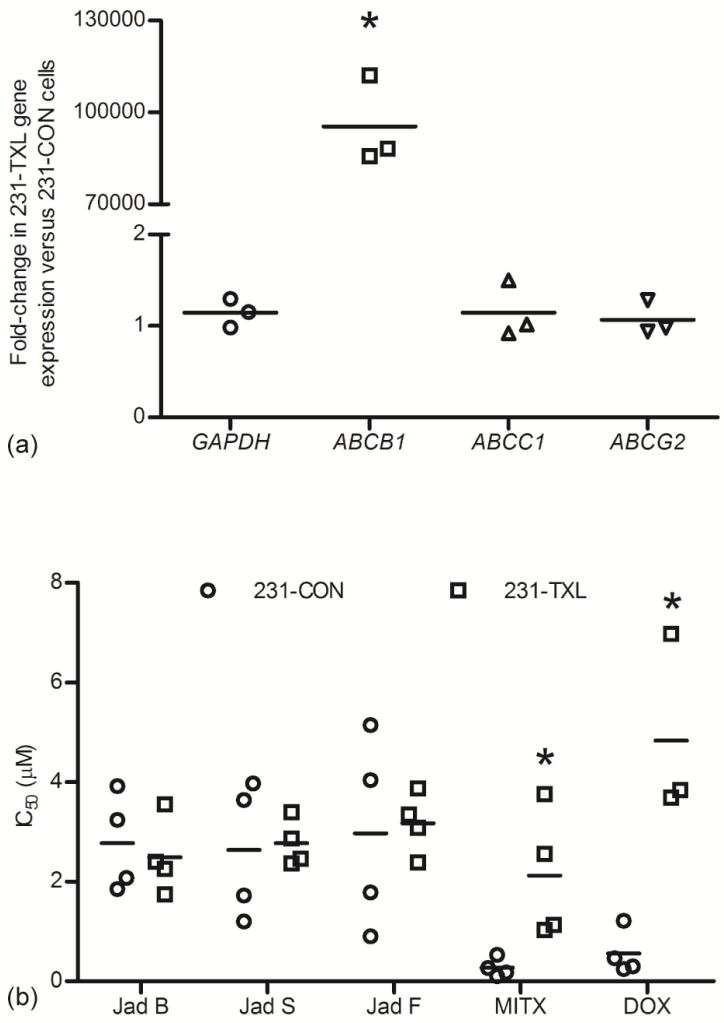


Figure 20: (a) Growth of MDA-MB-231 cells in paclitaxel selection medium for seven months generated the MDR breast cancer cell line 231-TXL that specifically overexpressed *ABCB1* versus drug sensitive 231-CON cells, as measured using qPCR. **(b)** The IC_{50} values of Jadomycins (Jads) B, S, and F (72 h treatments) in MTT assays were equal in 231-TXL cells versus 231-CON. The IC_{50} values of the control drugs mitoxantrone (MITX) and doxorubicin (DOX) were significantly higher in the 231-TXL cells versus 231-CON cells. Each bar represents the mean of at least three independent experiments. * $P \leq 0.05$, (a) the indicated gene's expression was significantly different from that of the *GAPDH* housekeeping control as determined by a one-way ANOVA followed by Bonferroni's multiple comparison test, or (b) the average IC_{50} value of the indicated drug treatment in 231-TXL cells was significantly different from that measured in the 231-CON cells as determined by an unpaired *t* test.

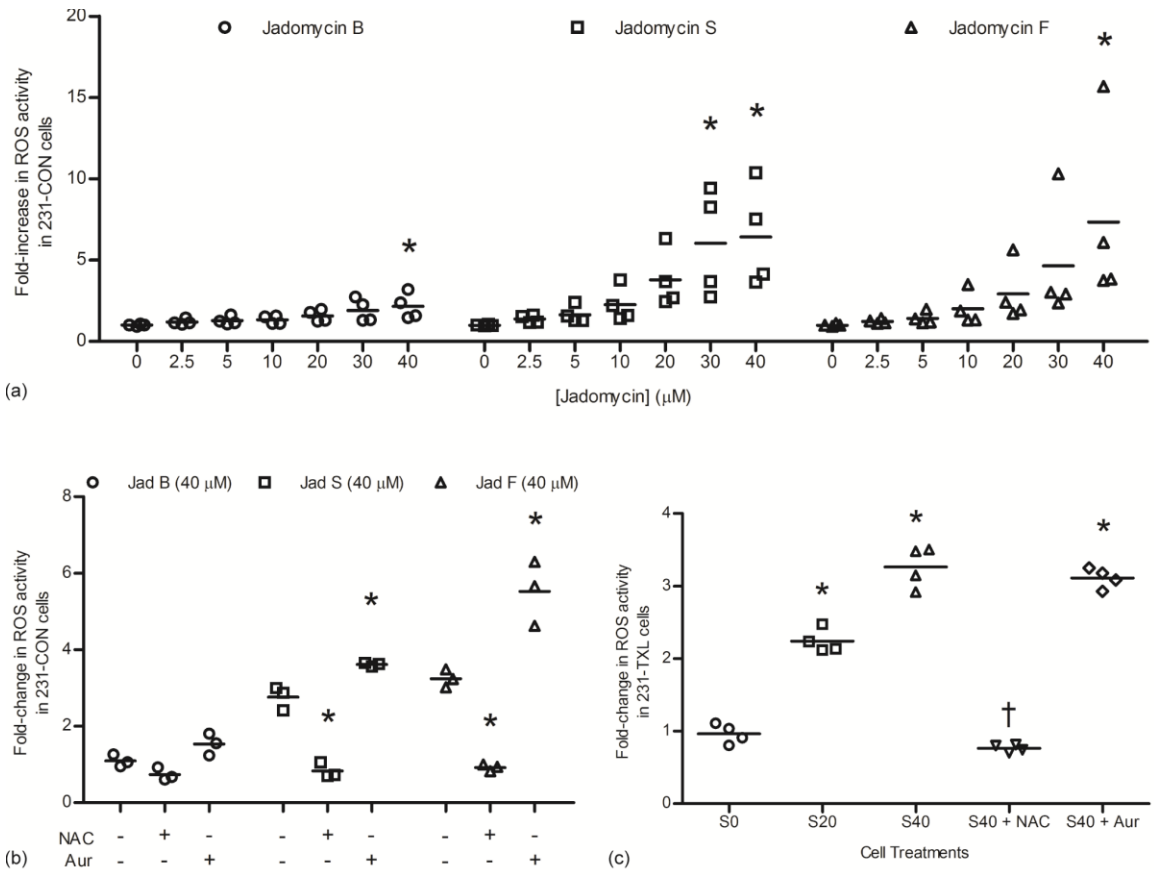


Figure 21: (a) Jadomycins (Jads) B, S, and F (2.5 - 40 μM) dose-dependently increased ROS activity in 231-CON cells versus vehicle control (0 μM). **(b)** The anti-oxidant N-acetyl cysteine (NAC, 2.5 mM) decreased and the pro-oxidant auranofin (Aur; 1 μM) increased intracellular ROS activity in Jad S and F treated 231-CON cells. **(c)** Jadomycin S (20 - 40 μM; S20 and S40) dose-dependently increased ROS activity in 231-TXL cells versus vehicle control (S0). NAC (2.5 mM) significantly decreased ROS activity induced by S40 in 231-TXL cells, while Aur (1 μM) had no effect. ROS activity was expressed as a fold-change relative to the medium-treated control cells. Each bar represents the mean of at least three independent experiments. * $P \leq 0.05$, (a and c) the fold-change in ROS activity was significantly different compared with the vehicle control, or (b) when compared with the no co-treatment control for that specific jadomycin. † $P \leq 0.05$, the fold-change in ROS activity is significantly different compared with S40. Determined by one-way ANOVAs, followed by Bonferroni's multiple comparison tests.

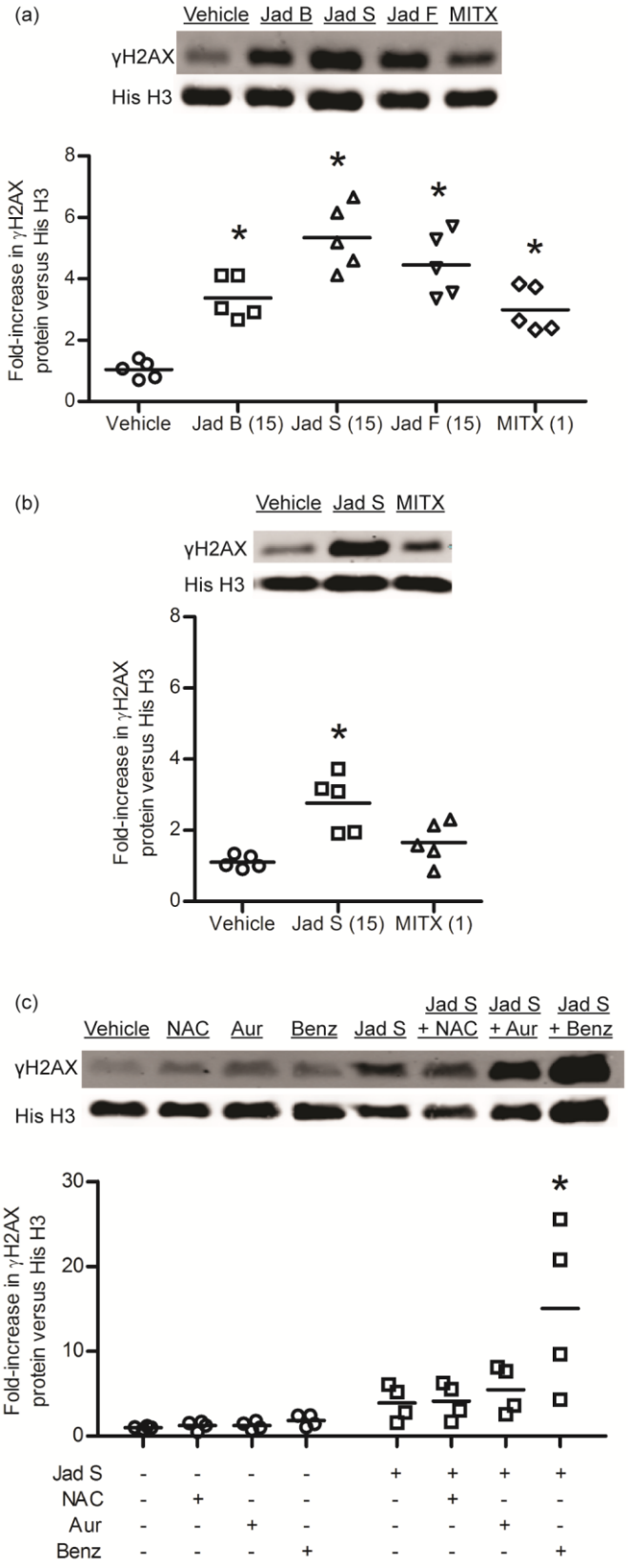

(a)
Vehicle
Jad B
Jad S
Jad F
MITX
γH2AX
His H3
Fold-increase in γH2AX protein versus His H3
Vehicle
Jad B (15)
Jad S (15)
Jad F (15)
MITX (1)
(b)
Vehicle
Jad S
MITX
γH2AX
His H3
Fold-increase in γH2AX protein versus His H3
Vehicle
Jad S (15)
MITX (1)
(c)
Vehicle
NAC
Aur
Benz
Jad S
Jad S + NAC
Jad S + Aur
Jad S + Benz
γH2AX
His H3
Fold-increase in γH2AX protein versus His H3
Jad S
NAC
Aur
Benz

Figure 22: (a) Jadomycins (Jads) B, S, and F (15 μM, 24 h) and mitoxantrone (MITX) increased the phosphorylation of histone H2AX (γH2AX; a marker of double strand DNA breaks) versus vehicle control in 231-CON cells. **(b)** In 231-TXL cells Jad S (15 μM, 24 h) but not MITX (1 μM) increased γH2AX protein expression versus vehicle control. **(c)** The PARP-inhibitor benzamide (Benz; 5mM), but not N-acetyl cysteine (NAC; 2.5 mM) or auranofin (Aur; 1 μM), further increased γH2AX in Jad S-treated (15 μM, 24 h) 231-CON cells. When administered as single treatments NAC, Aur, and Benz did not affect γH2AX levels. γH2AX protein expression was depicted as a fold-change relative to the medium-treated control cells. Each bar represents the mean of at least four independent experiments. * $P \leq 0.05$, (a and b) the fold-change in γH2AX protein expression was significantly different when compared with the vehicle or (c) when compared with the no co-treatment controls as determined by one-way ANOVAs, followed by Bonferroni's multiple comparison tests.

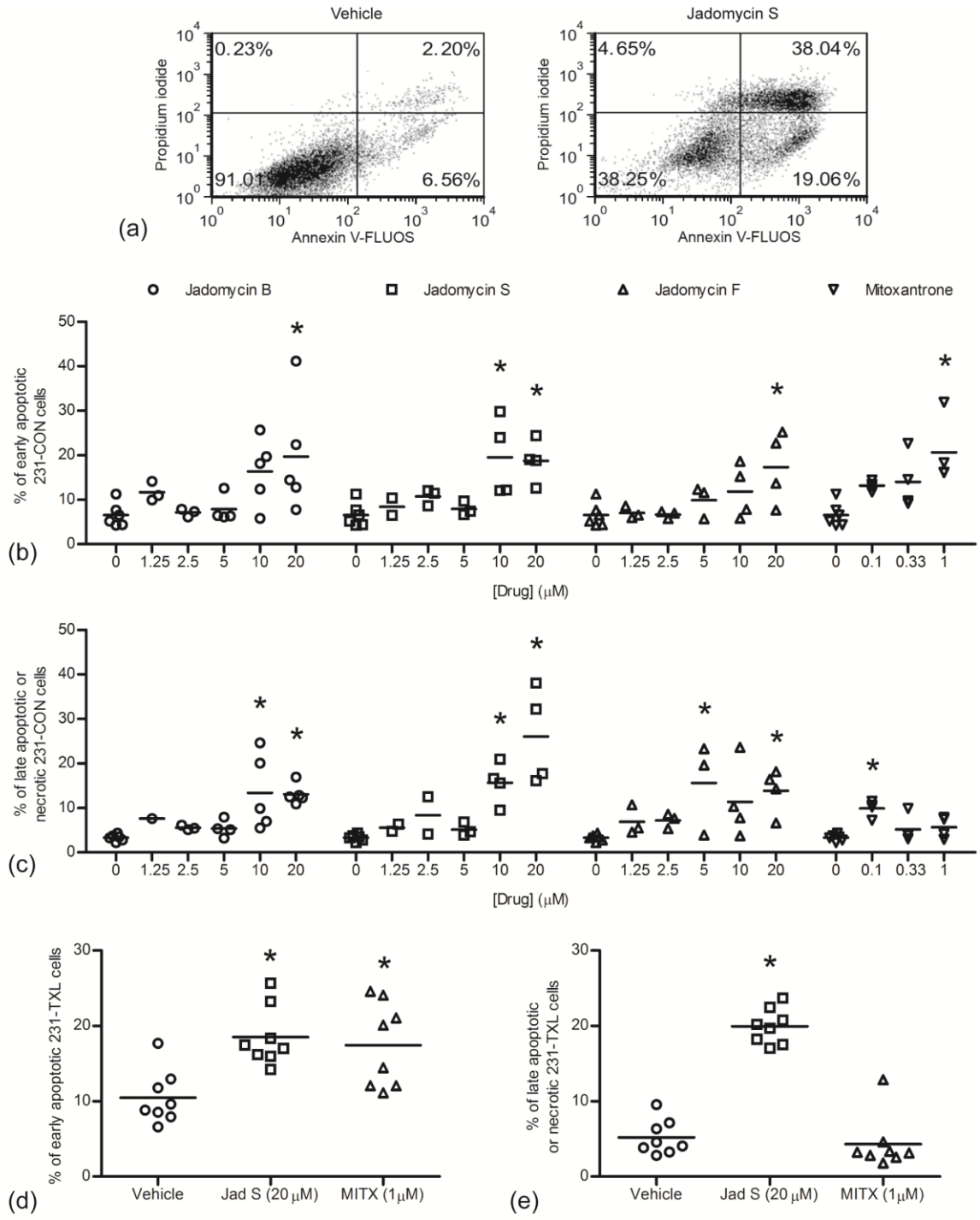


Figure 23: (a) Right-hand side (RHS) representative FACS figure shows how jadomycin S (20 μM; 36 h) induced more 231-CON cell death than jadomycin vehicle on the left-hand side (LHS). Lower LHS quadrants show the percentage of healthy cells, lower RHS quadrants show early apoptotic cells, and upper RHS quadrant shows late apoptotic/necrotic cells. Jadomycins B, S, or F (1.25-20 μM) or mitoxantrone (0.1-1 μM)

treatments for 36 h induced significantly greater **(b)** early apoptosis and **(c)** late apoptosis/necrosis versus vehicle (labelled 0 μM) in drug-sensitive 231-CON cells. **(d)** Jadomycin (Jad) S (20 μM) and mitoxantrone (MITX; 1 μM) significantly increased early apoptosis in multidrug-resistant 231-TXL cells versus the vehicle control after 36 h treatments, and **(e)** Jad S also increased late apoptosis/necrosis. Each bar represents the mean of at least three independent experiments. * $P \leq 0.05$, the %-early apoptosis or %-late apoptosis/necrosis was significantly different compared with the vehicle treatment controls as determined by one-way ANOVAs, followed by Bonferroni's multiple comparison tests.

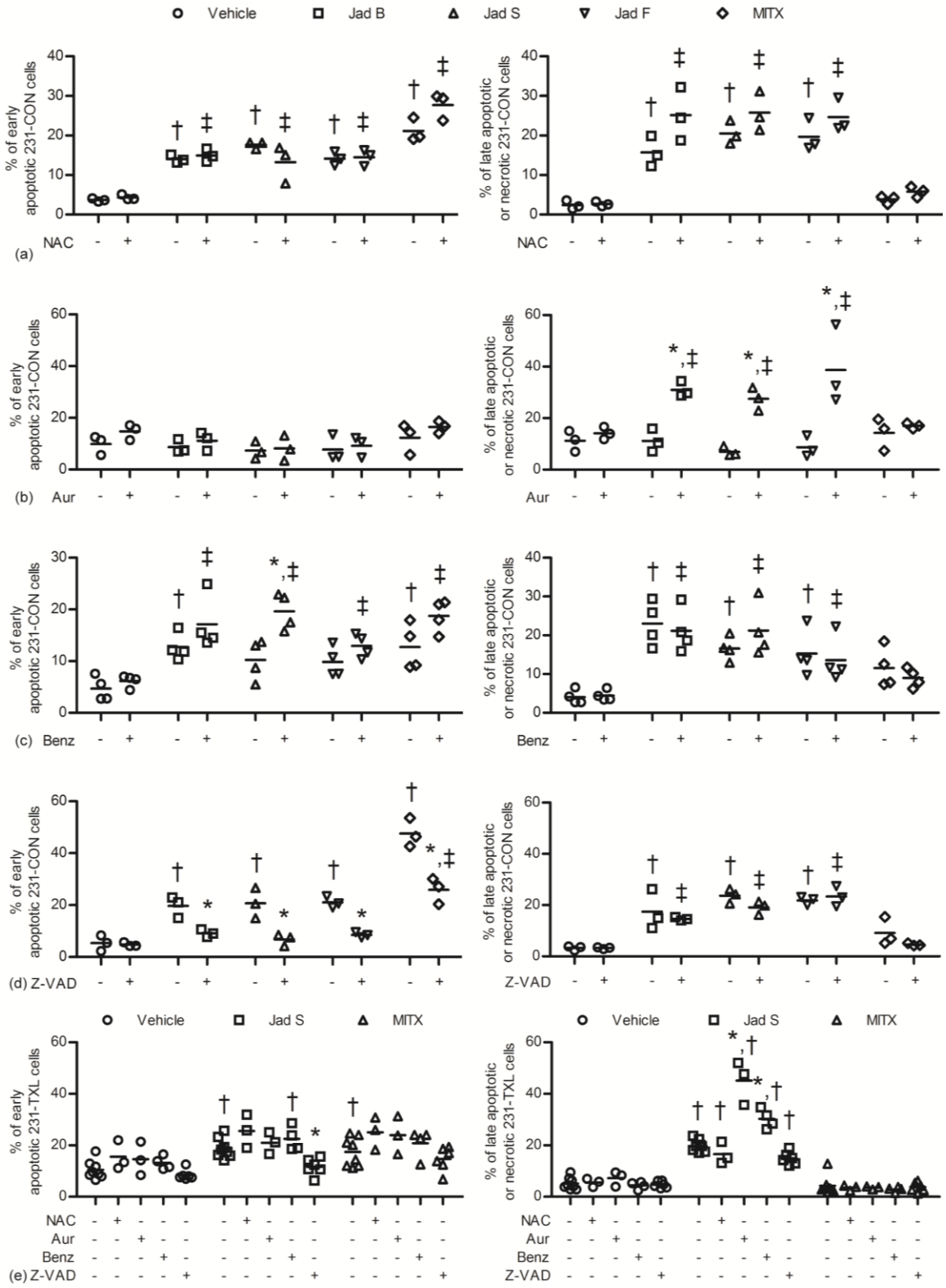


Figure 24: (a) NAC (2.5 mM) did not affect jadomycin (Jad) B, S, or F or mitoxantrone (MITX; 30, 20, 30, and 1 μM, respectively) induced early apoptosis or late

apoptosis/necrosis after 36 h in 231-CON cells. **(b)** Auranofin (Aur; 1 μM) did not affect early apoptosis with Jads B, S, or F (5 μM) or MITX (0.1 μM) after 24 h in 231-CON cells. It did increase late apoptosis/necrosis when co-treated with each Jad though not with MITX. **(c)** Benzamide (Benz; 5 mM) increased early apoptosis induced by Jad S (5 μM) after 48 h in 231-CON cells, with no effect on late apoptosis/necrosis. It had no significant effect with Jads B and F (5 μM) or MITX (0.1 μM). **(d)** Z-VAD (100 μM) significantly reduced early apoptosis induced by Jads B, S, and F and MITX (30, 20, 30, and 1 μM, respectively) after 36 h in 231-CON cells, while having no effect on late apoptosis/necrosis. **(e)** Z-VAD (100 μM) significantly decreased and NAC (2.5 mM), Aur (1 μM), and Benz (5 mM) did not affect early apoptosis when co-treated with Jad S (20 μM) in 231-TXL cells for 36 h. Aur and Benz increased Jad S induced late apoptosis/necrosis while NAC and Z-VAD had no effect. None of the co-treatments affected early apoptosis or late apoptosis/necrosis levels induced by MITX. No co-treatments had any effect on their own. Each bar represents the mean of at least three independent experiments. * $P \leq 0.05$, the %-early apoptosis or %-late apoptosis/necrosis of the drug treatment plus co-treatment was significantly different versus the drug treatment on its own as determined by (a-d) unpaired *t* tests or (e) one-way ANOVAs followed by Bonferroni's multiple comparison tests. † $P \leq 0.05$, the %-early apoptosis or %-late apoptosis/necrosis of the drug treatment with no co-treatment is significantly higher than that of the no treatment control, and ‡ $P \leq 0.05$, the %-early apoptosis or %-late apoptosis/necrosis of the drug treatment plus co-treatment is significantly higher than that of the co-treatment alone, as determined by two-way ANOVAs, followed by Bonferroni's multiple comparison tests.

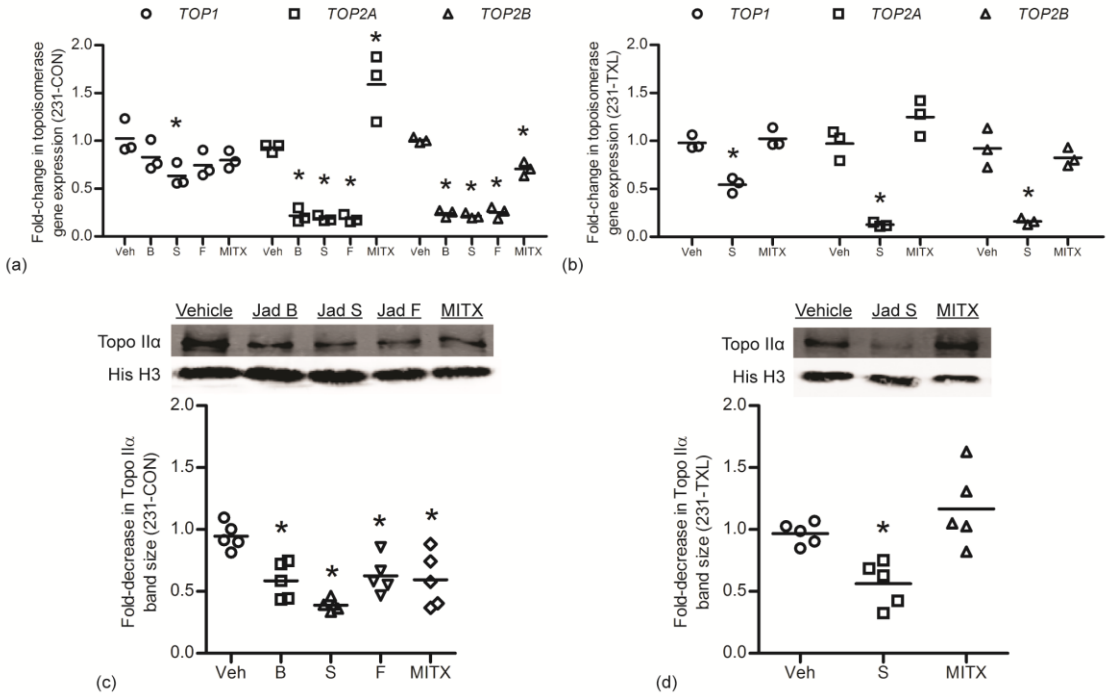


Figure 25: (a) Jadomycins (Jads) B, S, and F (20 μM) all significantly reduced the expression of *TOP2A* and *TOP2B* genes in 231-CON cells after 36 h. A small *TOP1* decrease was also observed with Jad S. The mitoxantrone (MITX) control (1 μM) did not alter *TOP1,* though it did increase *TOP2A* and decrease *TOP2B* expression. **(b)** Jad S (20 μM) significantly decreased *TOP1*, *TOP2A*, and *TOP2B* expression in 231-TXL cells after 36 h. MITX (1 μM) had no effect. **(c)** Jads B, S, and F (15 μM) and MITX (1 μM) significantly lowered topoisomerase (Topo) IIα protein detected after 24 h in 231-CON cells relative to the histone H3 (His H3) loading control. **(d)** Jad S (15 μM, 24 h) significantly lowered Topo IIα protein detected while MITX did not after 24 h in 231-TXL cells. Each bar represents the mean of at least three independent experiments. * $P \leq 0.05$, the value is significantly different from the vehicle control (Veh) as determined by a 1-way ANOVA, followed by Bonferroni's multiple comparison test.

Table 10: PCR primers used to determine the expression of ABC transporter and topoisomerase encoding genes in 231-CON and 231-TXL cells.

Gene	PCR forward primers (5'-3')	PCR reverse primers (5'-3')
ABCB1	AGGCCAACATACATGCCTTC	CCTTCTCTGGCTTTGTCCAG
ABCC1	AGGTGGACCTGTTTCGTGAC	TCCACCAGAAGGTGATCCTC
ABCG2	TTATCCGTGGTGTGTCTGGA	TTCCTGAGGCCAATAAGGTG
TOP1	AGTCCGGCATGATAACAAGG	GCCGAGCAGTCTCGTATTTC
TOP2A	TGGCTGAAGTTTTGCCTTCT	GGCCTTCTAGTTCCACACCA
TOP2B	GAGTGGCTTGTGGGAATGTT	TGTGCTTCTTTCCAGGCTTT
GAPDH	GAGTCAACGGATTTGGTCGT	TTGATTTTGGAGGGATCTCG
B-actin	GGACTTCGAGCAAGAGATGG	AGCACTGTGTTGGCGTACAG
PPIA	ACCGCCGAGGAAAACCGTGT	CTGTCTTTGGGACCTTGTCTGCA

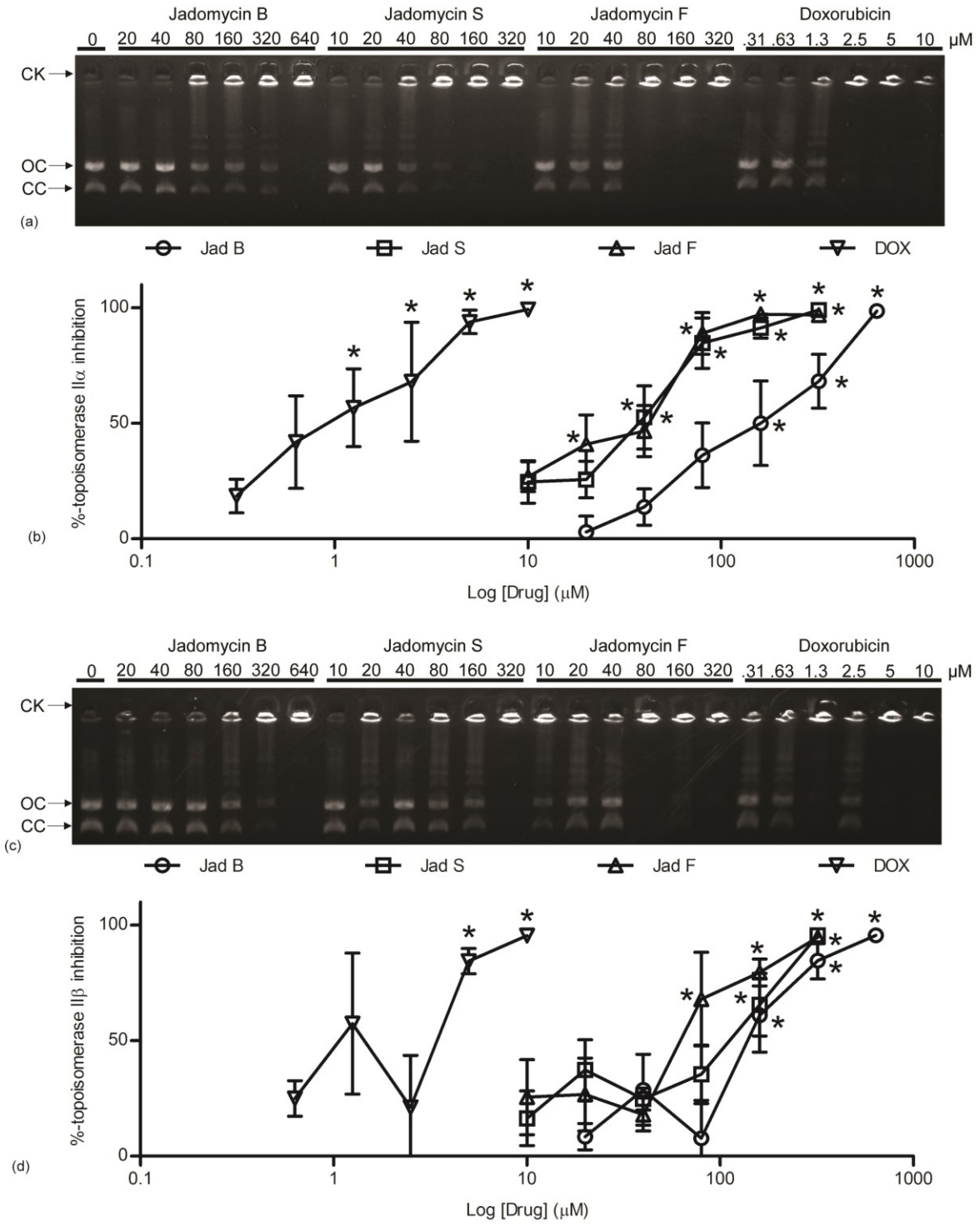


Figure 26: The conversion of catenated kDNA circles (CK) to open circular (OC) and closed circular (CC) decatenated kDNA by purified **(a)** topoisomerase IIα or **(c)** IIβ enzyme was dose-dependently inhibited by jadomycins (Jads) B, S, and F and doxorubicin (DOX). The size of the OC and CC bands for each treatment were calculated

for each Jad and DOX dose from which the % inhibition curves for topoisomerase IIα (**b**) or IIβ (**d**) were generated. Each point represents the mean of at least three independent experiments. * $P \leq 0.05$, the value is significantly different from the vehicle control (Veh) as determined by a 1-way ANOVA, followed by Bonferroni's multiple comparison test.

Table 11: IC_{50} values of jadomycins (Jad) B, S, and F and doxorubicin (DOX) for the inhibition of topoisomerases (Topo) IIα and IIβ, as measured with kDNA decatenation assays. Each value represents the mean of at least four independent experiments. * $P <$ 0.05, the IC_{50} value is significantly different from that of Jad B for the given topoisomerase and no drug treatment was significantly more potent at inhibiting one topoisomerase versus the other, as determined by a 2-way ANOVA followed by Bonferroni's multiple comparison test.

	IC_{50} ± SEM (μM)			
	Jad B	*Jad S*	*Jad F*	*DOX*
Topo IIα	180.0 ± 47.0	43.3 ± 12.7 *	31.8 ± 9.8 *	2.2 ± 0.6 *
Topo IIβ	146.9 ± 33.4	69.0 ± 11.0	59.3 ± 12.3	2.8 ± 0.9 *

4.05.00: DISCUSSION

By exposing control triple-negative 231-CON breast cancer cells to gradually increasing concentrations of paclitaxel, we successfully created a MDR cell line (231-TXL) that was resistant to the ABCB1 substrates mitoxantrone and doxorubicin [299,300], but not to jadomycins. This corroborates our earlier results describing how jadomycin potency is largely unaffected by ABC-transporter overexpression in MCF7 cells [6], providing further evidence of jadomycins' potential in ABC-transporter overexpressing MDR cancers.

We verified that jadomycins maintained their ROS-inducing properties in 231-CON and 231-TXL triple-negative breast cancer cells, as previously observed in MCF7 cells [289], evidencing that jadomycin-dependent ROS induction is independent of hormone receptor or HER2 expression profiles. While the antioxidant effects of NAC [278] were maintained in jadomycin-treated resistant 231-TXL cells, the pro-oxidant effects of auranofin [277] were not, suggesting these cells developed resistance to auranofin's ROS-inducing properties.

Since ROS can induce DNA double strand breaks [192], we hypothesized that jadomycins would cause double strand breaks in MDA-MB-231 breast cancer cells. The significant increases in γH2AX protein observed when 231-CON and 231-TXL cells were treated with jadomycins support this hypothesis, and the retention of this effect in 231-TXL cells treated with jadomycin S versus the loss observed with mitoxantrone further supports that jadomycins retain their anticancer properties in ABC-transporter overexpressing MDR cells. Interestingly, when 231-CON cells were treated with NAC or auranofin along with jadomycin S there was no additional change in γH2AX, while co-

treatment with the DNA repair PARP-inhibitor benzamide [294] resulted in significantly higher levels. This data confirms that jadomycins cause DNA double strand breaks, while also suggesting this damage occurs independently of ROS.

Increased ROS activity and double strand breaks within cells are common triggers of apoptosis [290,301]. Additionally, using chromatin condensation assays Fu *et al* provided evidence that jadomycin B induces apoptosis in lung carcinoma A549 cells [184]. Therefore we suspected that jadomycins would also induce apoptosis in breast cancer cells. The annexin V affinity assays supported this idea by showing significantly more early apoptotic 231-CON and -TXL cells when treated with jadomycins B, S, or F versus vehicle. Our data also expanded on the earlier results of Fu, *et al* [184] by showing that the ability to induce apoptosis is a common property of multiple jadomycins. Furthermore, jadomycins induced a significant increase in annexin V and propidium iodide dual-stained cells, signifying cells killed through either apoptosis *or* necrosis (labelled late apoptosis/necrosis) [296]. Therefore, while our data indicated jadomycins induced apoptosis, we cannot conclude whether cell death occurred solely through apoptosis or through a combination of apoptosis and necrosis.

To determine the importance of jadomycin-induced ROS in eliciting apoptosis, annexin V affinity assays were run with 231-CON and 231-TXL cells co-treated with NAC or auranofin along with jadomycins. The antioxidant NAC had no effect on jadomycin-induced early apoptosis or late apoptosis/necrosis, suggesting jadomycins induced apoptosis and cell death independently of ROS. Conversely, when 231-CON and 231-TXL cells were co-treated with auranofin, a significant increase in late apoptosis/necrosis was observed. Since auranofin did not increase ROS in 231-TXL cells, this suggests

auranofin augmented jadomycin-mediated cell death independently of ROS. It is plausible that auranofin instead increased jadomycin potency through a second mechanism, possibly through its inhibition of the ubiquitin-proteasome system which is involved with many cell processes including cell cycle regulation and DNA repair [302], though more experiments will have to be completed to confirm or disprove this possibility. Interestingly, NAC decreased and auranofin increased jadomycin potency in MCF7 breast cancer cells as previously measured with LDH cell death assays [289]. This suggests ROS may still play a role in jadomycin cytotoxic potency; however, as MCF7 cells appear to be more sensitive to ROS-inducing drugs than MDA-MB-231 cells [303], their effects may depend on the cancer cell line used.

The significantly greater induction of early apoptosis by jadomycin S in 231-CON cells and late apoptosis/necrosis in 231-TXL cells when co-treated with benzamide, which inhibited PARP proteins that are important mediators of DNA repair [294], provides evidence that jadomycins damage DNA. Additionally, since a significant difference was only seen with jadomycin S in 231-CON cells treated with and without benzamide and not B or F, this suggests that despite many similarities, structural differences can functionally alter jadomycin activity.

The pan-inhibitor of the apoptotic family of caspases, Z-VAD [297], significantly lessened jadomycin-induced early apoptosis, suggesting that jadomycins induce caspase-dependent apoptosis. In contrast, jadomycins still induced late apoptosis/necrosis after the Z-VAD co-treatments, evidencing that jadomycins may also induce cell death *via* caspase-independent mechanisms, such as necrosis or caspase-independent apoptosis [304]. Alternatively, Z-VAD can induce programmed necrosis, also known as

necroptosis, when used at high enough concentrations in certain cell lines [305,306,307]. However, the concentration of Z-VAD we used had no effect on 231-CON or 231-TXL cell viability on its own, and MDA-MB-231 cells have previously been found to be unaffected by this Z-VAD-induced necroptosis due to their not expressing the important necroptotic protein, receptor interacting protein kinase 3 (RIPK3) [308]. Therefore, we suspect Z-VAD-induced necroptosis was not a factor in our jadomycin plus Z-VAD experiments, and that the retained increases in late apoptosis/necrosis observed in these assays instead indicate that the jadomycins induce caspase-independent cell death as well as caspase-dependent apoptosis. Note that the induction of cancer cell death through multiple cell death mechanisms is not unusual, and is in fact typical of many chemotherapeutics [309,310].

The fact that similar results were seen in 231-TXL versus 231-CON cells with and without the co-treatments suggests that the mechanisms behind jadomycin cytotoxicity are largely preserved in the MDR cell line.

If ROS are not involved in the DNA double strand breaks and apoptosis induced by jadomycins, then what is the mechanism? Jadomycins are known to inhibit aurora B kinase [6,184,289], however this mechanism is not likely to induce DNA damage since the opposite is true: DNA damage inhibits aurora B kinase [311]. Alternatively, jadomycins could inhibit topoisomerases. Topoisomerases prevent DNA supercoiling by regulating over- and under-winding during cellular processes such as replication and transcription [224], and their inhibition can cause DNA damage and apoptosis [225].

The large decreases in *TOP2A* and *TOP2B* gene expression caused by jadomycins B, S, and F, with only a small *TOP1* decrease observed with jadomycin S, suggest that

jadomycins preferentially inhibit type II topoisomerase gene expression. The known topoisomerase II inhibitor mitoxantrone [224] slightly increased and decreased *TOP2A* and *TOP2B* respectively while having no effect on *TOP1*, evidencing that jadomycins may be more efficacious topoisomerase II inhibitors at the gene expression level. The decreased topoisomerase IIα enzyme levels observed in 231-CON and 231-TXL cells treated with jadomycins, as measured by western blotting, suggests the inhibition of topoisomerase II gene expression was followed by a decrease in topoisomerase protein synthesis. Alternatively, the depleted topoisomerase IIα detected and the previously observed DNA damage caused by jadomycin treatment are consistent with topoisomerase II poisons [312,313,314], suggesting jadomycins may have acted as such. Additional DNA cleavage trials are currently underway to confirm or disprove this possibility. The decreased levels of topoisomerase IIα observed in 231-TXL cells treated with jadomycin S suggest that this mechanism is retained in the *ABCB1*-overexpressing cells, versus mitoxantrone which loses its inhibitory properties.

The topoisomerase II inhibition gel assays showed that jadomycins B, S, and F and the topoisomerase II poison doxorubicin [298] dose-dependently and significantly inhibit the enzymes directly, with each treatment reaching 100% inhibitory efficacy. The higher IC_{50} value of jadomycin B versus those of jadomycins S and F for topoisomerase IIα suggests the structural differences in jadomycin analogues results in measurable variances of their inhibitory potency. Additionally, these direct topoisomerase II-inhibition IC_{50} values were higher than the concentrations required to inhibit topoisomerase II gene and IIα protein levels in cellular assays, and higher than the IC_{50} values measured through MTT cell viability assays. This suggests that the reduction of topoisomerase II gene and protein

expression would be most likely to occur in breast cancer cells exposed to jadomycin treatments, rather than direct enzyme inhibition. However, depending on the level of jadomycin accumulation in cells, direct inhibition of topoisomerase II enzymes is still possible.

In conclusion, jadomycins demonstrate potential as novel treatments for drug resistant breast cancer due to their ability to maintain their cytotoxic potency in MDR, triple-negative 231-TXL cells and as previously described in MDR MCF7 cells [6]. We now propose a novel anticancer mechanism describing how jadomycins inhibit type II topoisomerases, cause DNA double strand breaks, and induce apoptosis (**Figure 27**). Based on these studies jadomycins warrant further experimentation to discover more potent anticancer analogues, to better understand their polypharmacology, and to determine their effectiveness in the treatment of MDR breast cancer *in vivo*.

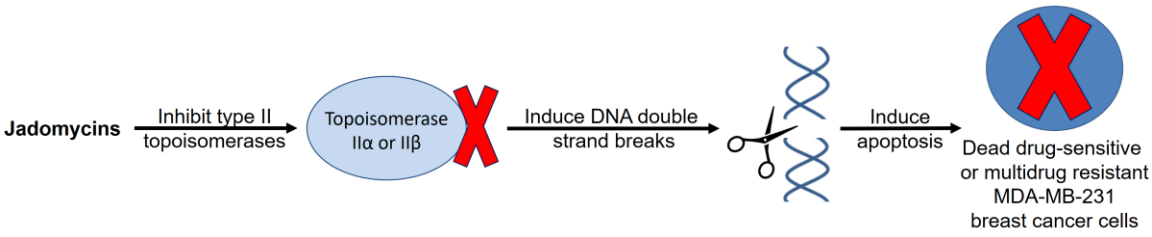


Figure 27: Putative novel pathway through which jadomycins are cytotoxic to drug-sensitive and drug-resistant breast cancer cells.

CHAPTER 5.00.00: DISCUSSION

Jadomycins represent a novel category of natural products that display promising anticancer activity. The research presented herein provides the first comprehensive studies of jadomycins' anticancer mechanisms of action and advances our understanding of their effectiveness in MDR breast cancer cells. The aim of Chapter 5 is to provide a general and overarching discussion of my project while providing perspectives on its limitations and possible future research directions.

5.01.00: JADOMYCINS ARE EFFECTIVE CYTOTOXIC AGENTS IN MDR BREAST CANCER CELLS

The first key finding of my research is that jadomycins largely retain their cytotoxic potency in ABC-transporter overexpressing MCF7 and MDA-MB-231 versus control breast cancer cells, despite the loss of potency observed with control drugs such as mitoxantrone and doxorubicin. The development of MDR in tumour cells is commonly due to overexpression of drug-effluxing ABC-transporters, rendering many treatments ineffective [117]. ABCB1 in particular is important to consider in studies of MDR, as it is the largest driver of drug-resistance in cancer cells [112]. Therefore the ability of jadomycins to retain their cytotoxic potency in ABC-transporter overexpressing breast cancer cells, such as in the *ABCB1*-overexpressing MCF7-TXL and 231-TXL cells on which I focussed in Chapters 3 and 4, exemplifies their potential value as MDR cancer treatments.

Our data supports that this retention in potency is the result of jadomycins being poor substrates of ABCB1, ABCC1, and ABCG2; however, to be certain we must first show if jadomycins are taken into the breast cancer cells when treated, or if instead the jadomycins interact with extracellular targets that lead to the intracellular effects we have observed. We suspect the cellular influx of jadomycins is mediated by solute carrier proteins, and experiments are currently underway to confirm or disprove this hypothesis.

The second key finding of my research is that jadomycins kill drug-sensitive and drug-resistant breast cancer cells through multiple mechanisms. Much of drug discovery over the past few decades has been largely focussed on the development of drugs intended to act against one specific target with high potency and selectivity. It was thought that by acting on one target a drug would have a direct therapeutic effect on its target while limiting its side effect profile due to an avoidance of off-target effects. Likewise, a multitarget or promiscuous drug was thought to be too unpredictable and potentially dangerous. It is now better recognized that these two beliefs are too simplistic to explain the mechanisms of action of drugs, and designing single drug molecules able to act simultaneously with multiple targets is gaining traction in drug discovery [315].

The ability of a drug to act on multiple targets to elicit a therapeutic response is called "polypharmacology". The theory behind polypharmacology is similar to that of combination therapy as seen in chemotherapy, where multiple cancer pathways are targeted to improve clinical outcomes. Targeting multiple pathways may potentiate drug efficacy, either additively or synergistically, and decrease the insurgence of drug resistant mutants. In addition, the inherent redundancy of biological networks (i.e. multiple oncogenes, tumour suppressor genes, or proteins that can repair DNA or induce

apoptosis) can impede the desired effects of a therapeutic that is specific for one target. Particularly complex diseases, such as cancer, that involve alterations in many proteins and pathways are therefore unlikely to be successfully treated by pharmacological interventions based on one target, while the modulation of an optimal array of targets typically proves more effective [315,316]. For example, the modulation of a single oncogenic pathway is unlikely to achieve durable disease remission, while targeting multiple pathways at once can significantly improve outcomes [317].

Jadomycins induce ROS in breast cancer cells which can potentiate their cytotoxic activity, depending on the sensitivity of the particular cell line being used to oxidative stress. Jadomycins also inhibit multiple cancer targets that are vital to cancer cell growth and proliferation, namely aurora B kinase and type II topoisomerases. Jadomycins also cause DNA damage in breast cancer cells, and ultimately induce apoptosis. If jadomycins were more specific to any one of these mechanisms, i.e. they only increased ROS activity, inhibited aurora B kinase, or inhibited topoisomerase IIα or IIβ, it is likely they would not have the same anticancer activity in drug-sensitive and drug-resistant breast cancer cells that we have observed. The known mechanisms that can influence jadomycin anticancer activity are summarized in **Figure 28**.

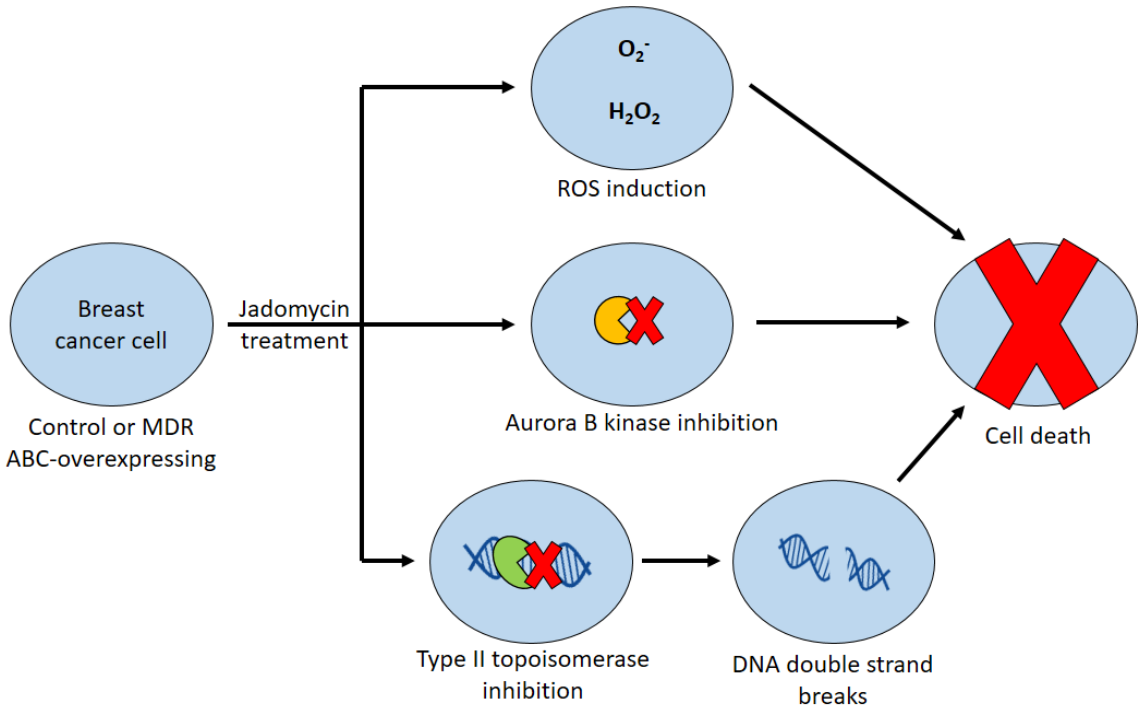


Figure 28: Summary of the known mechanisms of jadomycins' anti-breast cancer activity.

5.02.00: INFLUENCE OF MDR MDA-MB-231 VERSUS MCF7 CELL TYPE ON JADOMYCIN CYTOTOXIC POTENCY AND MECHANISMS OF ACTION

The cell lines in which I completed the majority of my experiments were MCF7 or MDA-MB-231 breast cancer cells. MCF7 cells were first established in 1973 at the Michigan Cancer Foundation, and are currently the most commonly used breast cancer cell line worldwide [318], largely because of their sensitivity to estrogen targeting drugs through high expression of ER [319]. Our group's initial work was largely focussed in MCF7 cells because of its widely accepted utility as a breast cancer cell model and because we were able to obtain three different ABC-transporter overexpressing MDR MCF7 cell lines from scientists at the National Institutes of Health in Maryland, USA.

However, because the molecular properties of different breast cancer cell lines can vary greatly, they often respond differently to drug treatments [59]. While jadomycin cytotoxicity was determined to be equipotent in breast cancer cell lines with various hormone receptor and HER2 statuses [289], these data provide no information regarding consistency of mechanism across cell lines; therefore, it was important to conduct mechanism of action studies in additional cell lines to provide a broader understanding of how jadomycins work. Triple-negative cells do not respond to targeted or hormone therapy, making them particularly challenging to treat; however, they can respond to chemotherapy [2]. Therefore, understanding how jadomycins act in MDR triple-negative breast cancer cells is of particular relevance. For these studies, I chose the MDA-MB-231 cell line, which is part of the MD Anderson series of breast cancer cell lines and is a commonly used model of triple-negative breast cancer [59,320].

While jadomycins undoubtedly retained their potency to a greater extent than control drugs in MDR MCF7 cells, a small 2- to 4-fold decrease in potency was observed (Chapter 2). In contrast, jadomycins B, S, and F were all equipotent in 231-TXL versus 231-CON cells (Chapter 4); a particularly promising discovery since chemotherapy is the primary treatment option for triple-negative breast cancers, which can be rendered ineffective due to the development of MDR. However, these results do beg the question as to why jadomycins appear to better retain their cytotoxic potency in MDR MDA-MB-231 versus MDR MCF7 cells.

Perhaps jadomycins better target MDR 231-TXL versus MDR MCF7 cells because MDA-MB-231 breast cancer cells proliferate faster, are more aggressive, and display a more invasive phenotype than MCF7 cells [59], and since chemotherapy targets rapidly

proliferating cells [2], jadomycins may better retain their cytotoxicity in such cells. It is also possible the MDR MCF7 cells we obtained from the National Institutes of Health display additional forms of MDR along with ABC-transporter overexpression that were not re-created in my in-house 231-TXL cell line, allowing the MCF7-TXL cells to exhibit a more poly-resistant phenotype. For example, preliminary qPCR trials previously completed by Mark Issa showed that the genes for some members of the solute carrier organic anion (SLCO) uptake transporter superfamily are altered in the MDR MCF7 cells, such as SLCO-2B1, -3A1, -4C1, and -5A1 (**Appendix I, Supplemental Figure 1**). It is not yet known whether jadomycins enter cells through any of these uptake transporters. Current experimental work in the laboratory is addressing this. However, if jadomycins are transported into breast cancer cells by SLCOs, it may help explain why jadomycin potency decreases slightly in the MDR MCF7 cells. Additionally, since I determined jadomycins induce DNA damage in breast cancer cells, it would also be interesting to determine if the MDR MCF7 cells have increased DNA repair mechanisms versus the MDR MDA-MB-231 cells, thus increasing the MCF7 cells' jadomycin-resistance.

Ultimately, with the currently available data I cannot conclude with certainty why jadomycin potency is unchanged in 231-TXL versus 231-CON cells while being slightly decreased in MCF7-TXL, -ETP, and -MITX versus MCF7-CON cells. Regardless, since jadomycins are able to largely retain their potency in multiple ABC-transporter overexpressing MDR breast cancer cell lines, in particular the MDR triple-negative cells, this provides evidence that jadomycins may prove to be viable treatment options for drug-resistant cancers.

5.03.00: JADOMYCIN CYTOTOXIC POTENCY IS DEPENDENT ON ROS IN MCF7 BUT NOT MDA-MB-231 CELLS

Evidence that jadomycins may induce ROS activity was first published by Monro, *et al* in 2011, using *in vitro* DNA cleavage assays [205]. As described in Chapter 3, jadomycins B, S, SPhG, and F all induced ROS-activity in MCF7-CON and -TXL breast cancer cells, and I determined that jadomycin cytotoxic potency was dependent on this ROS activity by co-treating the cells with anti- and pro-oxidants, such as NAC and auranofin, and observing the effects on cell death [289]. As described in Chapter 4, when similar experiments were repeated in MDA-MB-231 breast cancer cells, NAC was found to have no effect on jadomycin-induced cell death despite jadomycins inducing ROS in the 231-CON and -TXL cell lines and NAC significantly reducing this ROS activity. These data confirm that jadomycins induce ROS in multiple breast cancer cell lines; however, they also suggest that the impact of this ROS-induction on jadomycin cytotoxicity can differ depending on the cell line's sensitivity to alterations in intracellular oxidative stress. For example, MCF7 cells appear to be more sensitive to ROS-induction than MDA-MB-231 cells [303], possibly due to MCF7 cells displaying low levels of antioxidant enzymes such as GST and GPx1 [287,288]; therefore, this could explain why co-treatment with the antioxidant NAC decreases jadomycin potency in MCF7 but not MDA-MB-231 breast cancer cells.

A second key finding of these studies is that the TrxR inhibitor auranofin potentiated jadomycin cytotoxicity in drug-sensitive and drug-resistant MCF7 and MDA-MB-231 cells. Interestingly, this potentiating effect was associated with an increase in intracellular ROS in MCF7-CON, MCF7-TXL, and 231-CON cells but not in 231-TXL cells. This

suggested that auranofin was augmenting jadomycin cytotoxicity through a ROS-independent mechanism in the 231-TXL cells. One possible explanation is through the inhibition of the ubiquitin-proteasome system. This system is heavily involved in regulating cell cycle regulation and DNA repair, is upregulated in a number of cancers, and is inhibited by auranofin [302]. Additional experiments will have to be completed to confirm or disprove this possibility.

This data evidences that jadomycins induce ROS in multiple breast cancer cell lines. However, the effects of these induced ROS on jadomycin cytotoxicity can change depending on the properties of these cells. In some cancer cells this increased ROS may potentiate jadomycin cytotoxicity, as was observed in the MCF7 cells, while in other cells it may simply be a side effect of the jadomycin treatment that has no significant effect on the compound's cytotoxicity, as was observed in the MDA-MB-231 cells.

This suggests two possible avenues of combination therapy with jadomycins worth exploring. Firstly, since ROS can be quite damaging to healthy cells [198], if a patient's tumour cells are determined to not be sensitive to ROS activity, jadomycins could be used in combination with an antioxidant therapy, such as NAC, to reduce the total ROS produced, thus better protecting the healthy tissue while not affecting the jadomycin's cytotoxic potency. This idea has already been explored in children with acute lymphoblastic leukemia, which determined that patients treated with NAC and vitamin E as antioxidant adjuvant therapy reduced the severity of chemo- and radiotherapy-related side-effects versus patients who did not receive the antioxidant therapy [321].

Alternatively, if a patient's tumour is determined to be ROS-sensitive, a combination therapy including jadomycin and a pro-oxidant agent, such as auranofin, could increase

the jadomycin's potency against the tumour cells thereby improving treatment outcomes. The use of auranofin, as well as other TrxR1 inhibitors, has also already been proposed for combination therapies in the treatment of cancers [302,322], and a phase I/II study testing auranofin as a treatment for chronic lymphocytic leukemia has been completed, though the results have yet to be reported [323]. It should also be noted that auranofin may in fact be a useful co-treatment regardless of cell-sensitivity to ROS, based on our 231-TXL co-treatment results. Our group plans to test jadomycin and auranofin combination treatments in the 4T1 mouse model of breast carcinoma to better determine the utility of such a therapy *in vivo*.

5.04.00: JADOMYCINS MAY BE TYPE II TOPOISOMERASE POISONS

In Chapter 4, I described how jadomycins inhibit the expression of *TOP2A* and *TOP2B*, decrease topoisomerase IIα protein levels, and directly inhibit topoisomerase IIα and IIβ using kDNA decatenation assays.

These results are indicative of two possible situations. The first is that jadomycins are catalytic inhibitors (inhibitors that reduce the effectiveness of a catalyst) of type II topoisomerases, and the decreased protein expression of topoisomerase IIα as observed in my western blots is simply the result of the decreased gene expression. The second possibility is that jadomycins are acting as a more toxic type of topoisomerase inhibitor, called topoisomerase II poisons. The transient covalent complex that occurs between type II topoisomerases and DNA can be stabilized by topoisomerase II poisons, such as doxorubicin [313]. As a result, topoisomerase II signals are depleted in western blots due

to retention of the poison-topoisomerase-DNA complexes in the gel slots [314], which could explain the decreased topoisomerase IIα protein levels I observed in cells treated with jadomycins. Additionally, topoisomerase poisons cause DNA lesions such as double strand breaks, which do not occur with less toxic topoisomerase catalytic inhibitors [312], another mechanism that was observed in cells treated with jadomycins. This suggests jadomycins may be acting as topoisomerase II poisons. Jadomycins also inhibited topoisomerases IIα and IIβ directly, as determined with kDNA decatenation enzyme activity assays; however, this result is possible with both topoisomerase catalytic inhibitors and poisons.

To determine if jadomycins are topoisomerase II poisons, DNA cleavage assays are currently being conducted. Briefly, key characteristics of the covalent topoisomerase enzyme-DNA complex include the topoisomerase being covalently bound to the DNA and the presence of a break in the DNA substrate strand; this complex is also freely reversible. By using circular plasmid DNA as the substrate strand, the topoisomerase poisoning potential of a given compound can be tested by treating the plasmid DNA with purified topoisomerase enzyme and the compound in question. If linearized DNA is detected using an agarose gel, this shows that DNA breaks were created by the topoisomerase and not properly re-ligated due to the compound stabilizing the enzyme-broken DNA complex, thereby signifying the compound is a poison. If the compound is instead a catalytic inhibitor, any DNA breaks re-ligate back to their original form and no linearized DNA is observed [324].

These experiments will allow me to confirm whether jadomycins are acting as topoisomerase II poisons and thereby directly causing the observed DNA damage or if

they are acting as catalytic inhibitors, in which case the DNA damage is likely the result of an additional yet to be discovered mechanism; therefore, the results of these experiments will prove vital in directing future jadomycin research.

5.05.00: PROJECT LIMITATIONS AND RECOMMENDATIONS FOR FUTURE STUDIES

As described throughout my dissertation, my experiments were carefully designed, included proper controls and replicates, and allowed me to formulate a more detailed description of jadomycins' anti-MDR breast cancer activity than what was understood prior to my work. However, like any research project there were limitations. Below I have listed a few of these limitations along with suggestions about what can be done next, or explanations of what is already being done in our lab, to answer these remaining questions.

Limitation 1: Jadomycin uptake into breast cancer cells

As briefly discussed to in subchapter 5.01.00, while we have shown how jadomycins are minimally affected by ABC-efflux transporter overexpression and therefore are able to retain their anticancer activity in ABC-overexpressing MDR breast cancer cells, we have not yet confirmed whether jadomycins are entering the cells in the first place. Based on their inhibition of intracellular type II topoisomerases and aurora B kinase and induction of intracellular ROS and DNA damage, I hypothesize that jadomycins are being taken up into the cells and are thus able to induce breast cancer cell death.

However, it is not impossible that jadomycins are instead acting on extracellular targets that trigger intracellular pathways which in turn cause the results we have observed. Therefore, experiments to determine if jadomycins are entering the cancer cells and if so, through what transporters, have been initiated in our laboratory. These will include a jadomycin mass spectrometry method we have recently developed to quantify intracellular jadomycin levels, and the treatment of breast cancer cells with solute-carrier transporter inhibiting lentiviral shRNA vectors to decrease the expression of these drug-influx transporters and observe the effects on jadomycin cytotoxicity to determine which, if any, transporters actively uptake the jadomycins.

Limitation 2: Lack of *in vivo* data

As can be seen throughout my dissertation, the entirety of my published work has been *in vitro* and cellular research. These data have been illustrative of how the novel natural product jadomycins affect MDR breast cancer cells, have allowed us to attain a much clearer understanding of how jadomycins exert their anticancer effects, and have been vital in determining how to best test these compounds in an *in vivo* system; however no *in vivo* data was included. I did attempt to test jadomycin activity using the zebrafish embryo preclinical model, which showed that the toxic concentrations of jadomycins B, S, and F in the embryos were approximately 10-fold higher than their IC_{50} values in cell culture, which were encouraging results from a safety standpoint. However, I was ultimately unable to get any breast cancer cell line to successfully proliferate in the embryos and therefore was unable to test the jadomycins' anticancer activity *via* this method. Instead, to answer the question of jadomycin anticancer activity and toxicity *in*

vivo, along with determining their pharmacokinetic profile, our group is currently using Balb/C mice allotransplanted with 4T1 breast cancer cells as an animal breast cancer model, from which we will ascertain a better understanding of jadomycins' anticancer activity.

Limitation 3: The use of small molecule inhibitors

While using small molecule inhibitors to alter various biochemical pathways (e.g. auranofin, NAC, or benzamide) is a pharmacologically sound method of testing the effects of these pathways on the activity of the drug being tested, they are not without flaws. By using small molecule inhibitors, off-target effects can occur that alter or confound results. For example, auranofin is an inhibitor of TrxR1 which is a vital part of the Prx/Trx antioxidant pathway, which causes the compound's pro-oxidant activity [277]. It is for this reason that I initially used auranofin as described in Chapter 3, to observe the effects of TrxR inhibition on intracellular ROS activity and on jadomycin cytotoxic potency. However, as discussed in Chapter 4 and subchapter 5.03.00, auranofin is also an inhibitor of the ubiquitin-proteasome system, which is heavily involved in DNA repair [302]; therefore, while the data supports that auranofin-enhanced jadomycin cytotoxic potency is due to its pro-oxidant activity, it suggests that auranofin's ubiquitin-proteasome inhibition may also be important to its jadomycin cytotoxicity-enhancing properties. To get a better understanding of which cellular pathways have the greatest influence on jadomycins' anticancer activity, future experiments should include specific gene disruption techniques, such as using small interfering RNA to silence specific genes or the newly developed Clustered Regularly Interspaced Short Palindromic Repeats

(CRISPR) and CRISPR-associated protein 9 (Cas9) system, which uses the programmable DNA nuclease Cas9 to inhibit the activity of certain genes; either technique would silence a particular gene with greater specificity than what can be achieved with small molecule inhibitors [325]. These methods would give us a greater degree of confidence about exactly which pathways jadomycins are affecting and which pathways can be exploited to improve their activity.

Limitation 4: Lack of cancer cell selectivity data

The key to developing a successful chemotherapeutic is that it must selectively kill cancerous tissue over healthy tissue [326]. A limitation in my doctoral work is that I did not fully explore whether jadomycins display such selectivity. One set of MTT assays was completed in 231-CON cells versus healthy human mammary epithelial cells (HMECs), which found jadomycins to be equipotent in both the cancerous and healthy tissue (**Appendix I, Supplemental Figure 2**). However, the control drugs doxorubicin and mitoxantrone, which are both used clinically and known to exhibit cancer cell selectivity, were also equipotent in the two cell lines. This suggests that these assays are not adequate on their own to make any conclusions regarding jadomycins' putative cancer cell selectivity.

Since chemotherapeutics target rapidly proliferating cells [2] and jadomycins inhibit the important mitotic proteins aurora B kinase and type II topoisomerases, it is suspected that jadomycins and the control drugs were not selective towards the cancerous 231-CON cells versus the healthy HMECs in these experiments because both cell lines were

proliferating at similarly rapid rates. Future experiments comparing rapidly versus slowly proliferating cancerous and healthy cells will have to be completed to confirm or disprove this hypothesis. If determined to be true, this would suggest a method through which jadomycins may be selective for cancerous breast tissue *in vivo* and in the clinic, since breast cancer cells typically proliferate significantly faster than healthy breast cells [181].

Limitation 5: Use of MTT assays

MTT tetrazolium reduction assays were used throughout my doctoral research as a measure of cell viability to test the potency of jadomycins and other drugs. Viable cells with active metabolism convert MTT into a measureable purple formazan. For cells that are in the log phase of growth, the amount of formazan product is generally proportional to the number of metabolically active viable cells, in which case MTT assays can accurately measure cell viability. However, culture conditions that alter the metabolism of cells can affect the rate of MTT reduction into formazan, even if viability is unaffected. For example, when cells approach confluence and their growth becomes contact inhibited, metabolism can slow. This reduces the amount of MTT converted to formazan, despite the cells still being viable [327]. It is for this reason that I initially completed the LDH assays described in Chapter 2, which verified the accuracy of the MTT assays previously completed by Mark Issa [6]. In addition, the MTT assays were always done when the cells were in the log growth phase. For these reasons, I am confident in the accuracy of the MTT assay results described in my dissertation. However, additional cell viability measuring assays that have shown improved accuracy

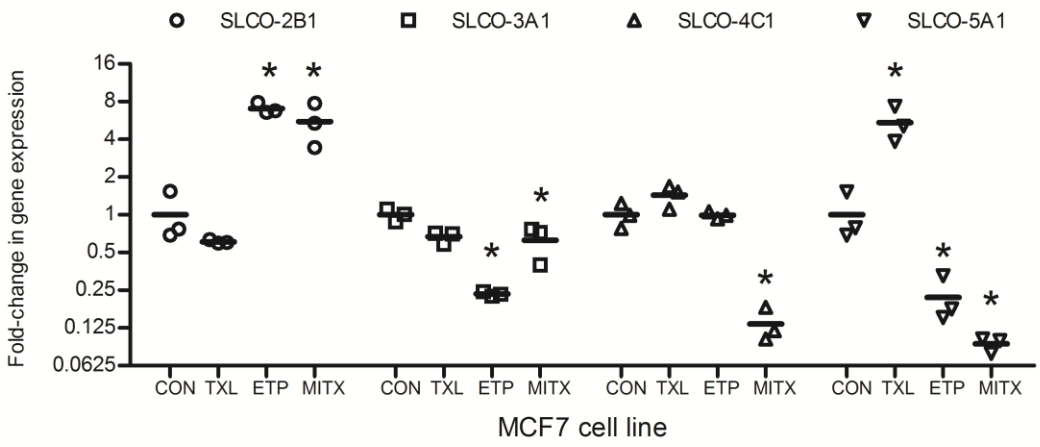
and reduced interference from glycolysis inhibitors versus MTT assays include the neutral red uptake, resazurin reduction, and sulforhodamine B assays, which could be explored as alternative methods of measuring cell viability in the future [328].

5.06.00: FINAL SUMMARY

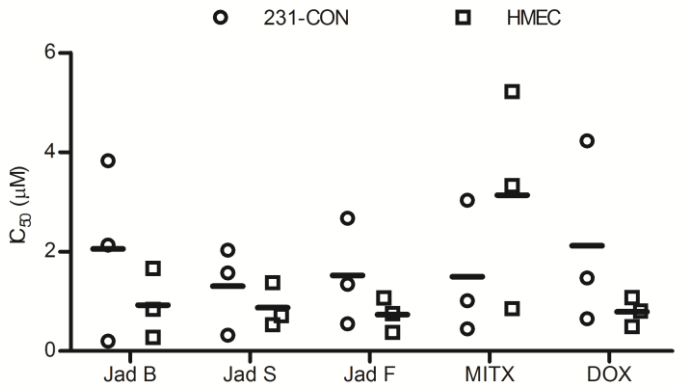
Jadomycins are natural products with promising anticancer activity. My research describes how jadomycins are largely equipotent in ABC-transporter overexpressing MDR- versus drug-sensitive control breast cancer cells, and how jadomycin potency is unaffected by the presence or absence of hormone receptors or HER2. Based on the high prevalence of MDR and of triple-negative breast cancers, both of which are difficult to treat effectively with currently available therapeutics, jadomycins offer a potential novel option worth investigating further.

I have also described in detail multiple mechanisms through which jadomycins exert their anticancer effects in both drug-sensitive and MDR breast cancer cells, information which can be used to determine in which cancer subtypes jadomycins may be most effective and to suggest potential combination therapies. In conclusion, my work will help guide future jadomycin and cancer science, and I am confident that jadomycin research will continue to exhibit promising results.

APPENDIX I: SUPPLEMENTAL SUPPORTING DATA



Supplemental Figure 1: Fold-increases and -decreases of SLCO transporters in MDR MCF7-TXL, -ETP, and -MITX versus MCF7-CON cells. * $P \leq 0.05$, the $\log_2$ of the fold-change in gene expression in the MDR cell line was significantly different compared with the MCF7-CON cells as determined by one-way ANOVAs, followed by Bonferroni's multiple comparison tests.



Supplemental Figure 2: Jadomycins (Jads) and control drugs, mitoxantrone (MITX) and doxorubicin (DOX), do not selectively reduce the viability of 231-CON breast cancer cells versus healthy HMECs. Cells were treated for 72 hours with various concentrations of each drug and then cell viability was measured with MTT assays, from which IC_{50} values were calculated as a measure of drug potency. No treatment was significantly different in 231-CON versus HMECs as determined by unpaired *t*-tests.

APPENDIX II: COPYRIGHT APPROVAL

1) Copyright approval to reuse 6 figures/tables from "Jadomycins are cytotoxic to *ABCB1*-, *ABCC1*-, and *ABCG2*-overexpressing MCF7 breast cancer cells", published in *Anti-Cancer Drugs*, 2014, in Chapter 2 of this dissertation.

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Author: Mark Issa, Steven Hall, Stephanie Dupuis, et al

Publication: Anti-Cancer Drugs

Publisher: Wolters Kluwer Health, Inc.

Date: Jan 1, 2014

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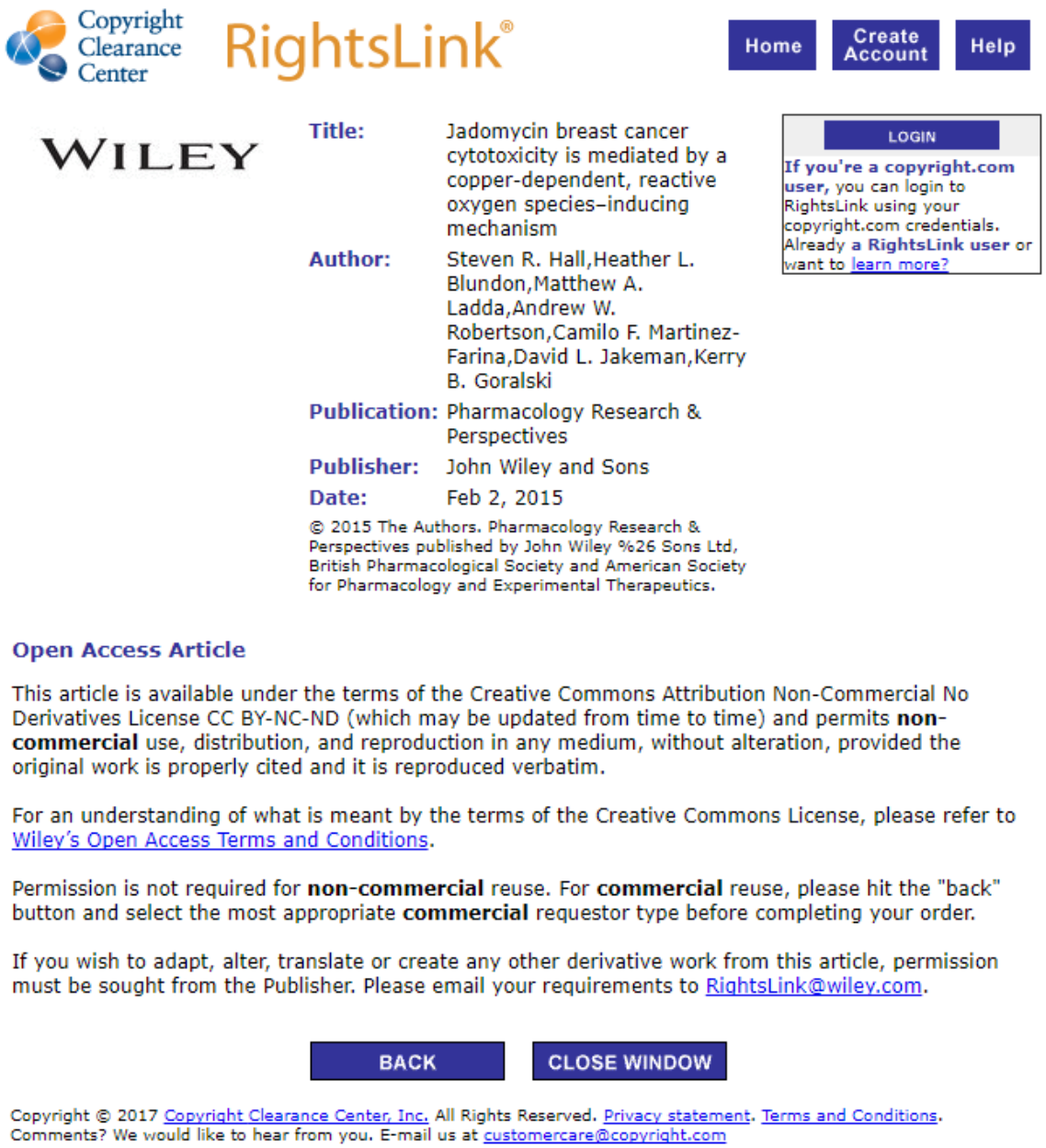

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Title: Jadomycin breast cancer cytotoxicity is mediated by a copper-dependent, reactive oxygen species–inducing mechanism

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Publication: Pharmacology Research & Perspectives

Publisher: John Wiley and Sons

Date: Feb 2, 2015

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