THE CYTOTOXIC EFFECTS OF NOVEL JADOMYCINS IN DRUG-SENSITIVE
AND DRUG-RESISTANT MCF7 BREAST CANCER CELLS

by

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Abstract

Multidrug resistance refers to the simultaneous resistance to structurally and mechanistically unrelated cytotoxic drugs. Chronic administration of cytotoxic drugs to patients with metastatic breast cancer results in the development of multidrug resistance, thus rendering chemotherapy unsuccessful. One mechanism by which multidrug resistance is conferred is the decreased intracellular drug accumulation due to the upregulation of the ATP-binding cassette (ABC) transporters. Jadomycins are polyketide-derived natural products produced by the soil actinomycetes *Streptomyces venezuelae*, ISP 5230. Jadomycins exhibit anticancer, antibacterial and antifungal activities. Pilot work in our laboratory demonstrated that jadomycin B exhibited similar cytotoxic effects in drug-sensitive and drug-resistant cancer cells. We hypothesize that jadomycins are poor substrates of ABCB1, ABCC1 and ABCG2 efflux transporters, and consequently will exhibit higher intracellular accumulation, which results in improved cytotoxic efficacy over existing chemotherapeutics that are rapidly effluxed by ABC transporters. Using methyltetrazolium (MTT) cell viability assays, the cytotoxic efficacy of nine jadomycin analogues (DNV, L, B, SPhG, F, W, S, T and N) in drug-sensitive and drug-resistant MCF7 breast cancer cells was evaluated. Jadomycin B, L, S and T were found to be equally toxic to drug-sensitive and drug-resistant ABCB1, ABCC1 or ABCG2-overexpressing MCF7 breast cancer cells. The inhibition of ABCB1, ABCC1 or ABCG2 efflux transporters with verapamil, MK-571 or ko143, respectively, did not significantly augment the cytotoxic effects of jadomycin DNV, L, B and S in drug-resistant MCF7 cells, suggesting that these jadomycins are poor substrates of the targeted transporter. Furthermore, all nine jadomycin analogues did not increase the intracellular accumulation of ABCB1, ABCC1 or ABCG2 probe fluorescent substrates in HEK-293 cells, indicating that these jadomycins do not inhibit the efflux function of the transporters. We conclude that jadomycins B, L and S are effective agents in the eradication of resistant breast cancer cells grown in culture, and that the ability of specific jadomycins to retain cytotoxic efficacy in resistant cells stems from their limited interactions with ABCB1, ABCC1 or ABCG2 efflux transporters.

List of Abbreviations and Symbols Used

5-FU	5-Fluorouracil
A549	Alveolar epithelial cancer cell line
ABC	ATP-binding cassette
ACF	Aberrant crypt foci
ACTB	Actin-β
ADME	Absorption, distribution, metabolism and excretion
Ala	Alanine
ALL	Acute lymphocytic leukemia
AML	Acute myeloid leukemia
ANOVA	Analysis of variance
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
B	Isoleucine
BBB	Blood brain barrier
Bcl-2	B-cell lymphoma 2
cDNA	Complementary DNA
CNS	Central nervous system
CsA	Cyclosporin A
CycA	Cyclophilin A
CYP	Cytochrome P450
DFS	Disease-free survival

DMEM	Dulbecco's modified Eagles medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNL	D-Norleucine
DNV	D-Norvaline
DOX	Doxorubicin hydrochloride
EGFR2	Epidermal growth factor receptor 2
F	Phenylalanine
FBS	Fetal bovine serum
FPGS	Folylpolyglutamyl synthetase
FTC	Fumetrimorgin C
G418	G418 sulfate
GT	Glycosyltransferase
H33342	Bisbenzimide hoechst 33342 trihydrochloride
H460	Non-small cell lung carcinoma cell line
HEK-293	Human embryonic kidney cells – 293
HEK-ABCB1	ABCB1 overexpressing HEK-293 cell line
HEK-ABCC1	ABCC1 overexpressing HEK-293 cell line
HEK-ABCG2	ABCG2 overexpressing HEK-293 cell line
HEK-CON	Control HEK-293 cell line
Hela	Cervical cancer cell line
HepG2	Human hepatocellular carcinoma cell line
HER2	Human epidermal growth factor 2

IM-9	Lymphoblastic immunoglobin-secreting multiple myeloma cell line
ISP	International Streptomyces Project
ko143	ko143 hydrate
L	Leucine
mRNA	Messenger RNA
miRNA	Micro RNA
MBC	Metastatic breast cancer
MCF7	Michigan Cancer Foundation – 7 / Breast cancer cell line
MCF7-CON	Drug sensitive MCF7 breast cancer cell line
MCF7-ETP	Etoposide-selected MCF7 breast cancer cell line
MCF7- MITX	Mitoxantrone-selected MCF7 breast cancer cell line
MCF7-TXL	Taxol-selected MCF7 breast cancer cell line
MDR	Multidrug resistance
MEM	Minimum essential medium
MeOH	Methanol
MGMT	Methylguanine-DNA methyltransferase
MK-571	MK-571 sodium salt hydrate
MTT	Methyltetrazolium
N	Asparagine
NBD	Nucleotide binding domain
NSCLC	Non-small cell lung carcinoma
OATP	Organic anion transporting polypeptide
OCT	Organic cation transporter

OS	Overall survival
p	P value
P-gp	P-glycoprotein
PCR	Polymerase chain reaction
PFS	Progression-free survival
PKS	Polyketide synthases
PPARγ	Peroxisome proliferator-activated receptor
PXR	Pregnane X receptor
qRT-PCR	Quantitative real time PCR
R123	Rhodamine 123
RFC1	Reduced folate carrier 1 gene
RNA	Ribonucleic acid
RXRα	Retinoid X receptor α
S	Serine
SAR	Structure-activity relationship
s.e.m.	Standard error of the mean
SCLC	Small cell lung carcinoma
SLC	Solute carrier transporter
SPhG	S-Phenylglycine
T	Threonine
TMD	Transmembrane domain
TMS	Transmembrane segment
TXL	Paclitaxel

V	Valine
VRP	Verapamil hydrochloride
W	Tryptophan

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CHAPTER I: INTRODUCTION

Multidrug resistance and metastatic breast cancer

Breast cancer is the second most commonly diagnosed cancer in the world, accounting for 10.9 % of all new cancer cases and 22.9 % of all female cancers [1]. It is estimated that approximately 30% of women diagnosed with breast cancer will eventually progress to metastatic breast cancer (MBC) [2], and about 70% of breast cancer related deaths demonstrate some evidence of metastasis [3]. Despite significant progress in the treatment of MBC, the estimated 5-year survival rate of women with MBC in the United States remains about 26% [4]. The exact reasons behind the failure of MBC therapies remain not fully understood. However, a large portion of the failure of MBC treatments is attributed to the problem of multidrug resistance (MDR). MDR continues to be a major obstacle in the effective treatment of MBC, and is found to impede the complete recovery of over 90% of patients with MBC [5,6]. In a MDR phenotype, the maximum tolerated dose required to eradicate tumour cells is no longer effective in achieving the desired response, and further-elevating the dose becomes clinically un-manageable [7]. MDR can occur prior to the onset of therapy, a phenomenon known as innate resistance, or following the exposure to therapy, a phenomenon known as acquired resistance [4]. Most importantly, all available therapies for MBC, especially chemotherapy are eventually affected by MDR.

Current therapeutic options for MBC

Currently, the treatment options for MBC remain limited. There are many considerations that have to be taken into account when choosing a therapy for MBC. For hormone-sensitive MBC, oestrogen deprivation appears to be one effective therapeutic strategy. Tamoxifen is a selective oestrogen receptor antagonist that competes with oestrogen for the receptor and slows down the oestrogen-induced cell proliferation [8]. Resistance to tamoxifen eventually develops and the majority of the MBC patients die from disease progression [9]. MBCs overexpressing the epidermal growth factor receptor 2 (EGFR2), also known as HER2, are treated with targeted therapy, such as the kinase inhibitor lapatinib [8,10]. Lapatinib exerts its pharmacological effects by inhibiting the tyrosine kinase activity of these EGFR2 receptors [8]. However, the majority of patients receiving lapatinib develop resistance against it within 12 months [11]. Cytotoxic chemotherapy for MBC includes anthracyclines (doxorubicin), taxanes (paclitaxel and docetaxel) and vinca alkaloids (vinorelbine) [8]. However, after prolonged exposure to these chemotherapeutic drugs, MBC patients frequently develop resistance [4].

Cellular mechanisms of MDR

Over the past four decades, intensive effort has been directed towards unravelling the mechanisms of MDR [12,13]. Two general classes of resistance to anticancer drugs have been identified: pharmacokinetic and cellular. Alteration in pharmacokinetic factors

including absorption, distribution, metabolism and excretion (ADME) result in the reduced delivery of cytotoxic drugs to cancer cells on the systemic level [7]. For instance, the inter-patient variation of etoposide plasma concentration was greatly reduced when etoposide was administered intravenously as compared to orally. This indicates that ineffective drug absorption occurs in some individuals resulting in less delivery of etoposide to the tumour and thus, pharmacokinetic resistance [14]. A second example of pharmacokinetic resistance involves tamoxifen. The growth inhibitory effects of tamoxifen on breast cancer are mediated by its metabolites 4-hydroxytamoxifen and endoxifen, which are formed by the polymorphic cytochrome P450 metabolic enzyme CYP2D6 [15]. The presence of 2 functional alleles of CYP2D6 results in greater production of the pharmacologically active tamoxifen metabolites and is associated with better clinical outcome, as compared to individuals with two non-functional CYP2D6 alleles which produce lower amounts of the active metabolites [15].

The cellular factors result from the reduced delivery of cytotoxic drugs to targets on the cellular level. The cellular factors are believed to be the major contributors to MDR phenotype [5–7,13]. Multiple cellular mechanisms by which MDR is conferred have been elucidated. These mechanisms include increased drug efflux, decreased drug influx, increased activation of DNA repair mechanisms, reduced apoptosis, drug target alteration and increased metabolic detoxification (**Figure 1**) [5–7,12,13]. These mechanisms of MDR may occur simultaneously, sequentially or may switch from one to another depending on cellular and extracellular environmental factors [7]. Experimental models that mimic MDR mechanisms were easily developed through the gradual exposure of cancer cells to increasing amounts of a specific anticancer drug, which over time resulted in the

generation of cancer cells that were simultaneously resistant to structurally and mechanistically unrelated anticancer drugs [6,7,13].

Although MDR is multifactorial, a frequently encountered mechanism whereby cancer cells circumvent chemotherapy is through reduced intracellular accumulation of cytotoxic drugs [5,6,13,16]. Decreased intracellular accumulation of cytotoxic drugs results mainly from two mechanisms, decreased transporter-mediated influx or increased transporter-mediated efflux of cytotoxic drugs [12]. The most prevalent mechanism of MDR encountered *in vitro* is the increased transporter-mediated efflux of cytotoxic drugs [16]. Since many cytotoxic drugs exert their pharmacological effects by targeting intracellular molecules (e.g. DNA, topoisomerases I and II, and tubulin), their ability to exert toxicological effects will ultimately depend on their ability to sufficiently accumulate within the cell. Therefore, in drug development it is imperative to examine in detail the factors that affect influx and efflux of cytotoxic novel drugs into cancer cells.

ABC transporters and drug efflux

ATP-binding cassette (ABC) transporters refer to a superfamily of enzymes that use ATP as their driving force to transport molecules across cellular membranes [12,13,17,18]. ABC transporters are characterized with a broad scope of substrate specificity ranging from ions to peptides, including numerous endogenous molecules such as sugars, lipids, and nucleotides (**Table 1**) [12,13,17]. The binding of a substrate to a transporter stimulates the hydrolysis of one ATP molecule. The energy produced by ATP hydrolysis induces a conformational change in the transporter that results in efflux of the substrate molecule

out of the cell. The hydrolysis of another ATP molecule restores the transporter to its initial conformation [12,13,17]. To date, 48 human ABC genes have been identified and classified into 7 subfamilies (A through G) according to their structural organization and sequence homology [13,17]. ABC transporters are expressed in a variety of normal tissues and play major physiological and pharmacological roles in the body. Dysfunctional ABC transporters can result in genetic disorders such as cystic fibrosis (ABCC7) and adrenoleukodystrophy (ABCD1) due to impaired transport of chloride and fatty acids, respectively [19]. ABC transporters also protect the body from harmful exogenous and potentially endogenous substances by mediating drug excretion in the kidney and liver, and brain to blood efflux across the blood brain barrier (BBB) (**Table 1**) [13,20,21].

ABCB1 and MDR

Juliano, RL and Ling, V (1976), at the University of Toronto, were the first to describe the role of an ABC transporter in drug resistance [22]. In their investigation, Juliano and Ling discovered that selection of chinese hamster ovary cells with colchicine resulted in cross-resistance to a variety of amphipathic drugs. The drug-resistant phenotype was due to the expression of cell surface 170 kDa glycosylated protein, which was not present in non-resistant cells [22]. This protein was originally named P-glycoprotein but is now officially termed ABCB1 according to currently accepted drug transporter nomenclature [19]. The ABCB1 transporter is encoded by the *ABCB1* gene in humans, and by *abcb1a* and *abcb1b* genes in rodents [20,23]. ABCB1 is composed of two nucleotide binding domains (NBDs) and two hydrophobic transmembrane domains (TMDs) each containing 6 transmembrane segments (TMSs)[5,13,24]. ABCB1 appears to favour the transport of neutral

and cationic hydrophobic natural products (**Table 1**) [25]. ABCB1 is expressed ubiquitously in the body, including in the intestine, liver, kidney, blood-brain and blood-cerebrospinal fluid barriers [20,21,23]. The primary function of ABCB1 in humans and abcb1a/1b in rodents is to protect the organism from exogenous toxins by regulating their intestinal absorption, tissue distribution (e.g. into the brain), and renal and biliary excretion [24]. For example, located at the apical surface of the intestinal epithelial cells, ABCB1 reduces the systemic absorption of several antineoplastic drugs, such as vinblastine and etoposide by pumping them from the intestinal enterocytes into the intestinal lumen [14,26]. Disturbing the function of abcb1, using either the *abcb1a/abcb1b* double knockout model or abcb1 inhibitors, resulted in a significant increase in the oral bioavailability of ivermectin, loperamid and tacrolimus, highlighting the functional importance of the transporter in the intestine [12,13,21]. Moreover, ABCB1 protects the central nervous system (CNS) from potentially toxic blood-born xenobiotics by regulating the permeability of the BBB [23,27–29]. Accordingly, abcb1-knockout mice displayed significantly increased CNS penetration of vinblastine (46-fold), paclitaxel (22-fold) and doxorubicin (3.8-fold) in comparison to wild type mice [30]. Furthermore, the function of ABCB1 in the BBB influences the ability of anticancer therapies to treat CNS tumours. For example, taxanes (paclitaxel and docetaxel) are efficacious chemotherapeutic drugs used primarily for the treatment of lung, ovarian and breast cancers. Clinically, paclitaxel exhibited modest but not desirable efficacy in the treatment of gliomas, an effect that was found to be due to the insufficient drug delivery to the tumor as a result of ABCB1 in the BBB [31]. Inhibition of abcb1 function by cyclosporin A, elacridar or valspodar increased the penetration of paclitaxel and docetaxel into mouse brains, indicating that the inhibition of BBB-localized ABCB1

may serve as a potential strategy to improve CNS delivery of cancer therapeutics in humans [31,32].

Chronic exposure of breast cancer cells to non-lethal doses of taxanes, vinca alkaloids or anthracyclines frequently results in an MDR phenotype characterized by the upregulation of ABCB1 expression [6,13,33]. The MDR-associated ABCB1 upregulation confers resistance to structurally and mechanistically unrelated cytotoxic drugs, including but not limited to, taxanes (docetaxel), anthracyclines (doxorubicin), epipodophyllotoxins (etoposide), and vinca alkaloids (vincristine) [5,13,24]. Increased ABCB1 protein levels results in an increased efflux and decreased intracellular accumulation of anticancer agents [5,24]. In order to address the relationship between ABCB1 and breast cancers, Leonessa and Clarke (2003) pooled the results of multiple studies that used immunohistochemistry methods to detect ABCB1 expression [24]. It was found that 40.4% of all untreated breast cancers expressed ABCB1 [24]. ABCB1 expression and breast cancer stage were not significantly associated as ABCB1 was expressed in 44.4% of operable early breast cancers and in 42.4% of locally advanced breast cancers [24]. However, Linn et. al (1995) detected a significant difference in the expression level of ABCB1 in previously untreated primary (29%) and metastatic (58%) breast cancers, indicating an association between ABCB1 expression and breast cancer metastases [34]. Metastatic recurrences were observed in 27% of ABCB1-positive patients compared to 16% in ABCB1-negative patients [24].

The effects of chemotherapy on ABCB1 expression engage both induction and selection processes in breast cancers [24]. Studies that evaluated the effects of chemotherapy with

ABCB1 substrates before and after treatment of the same cancers found that ABCB1

expression increased from 42.9% to 63.9%, and was induced in 36.8% of breast cancer

patients [24]. Additionally, the expression of ABCB1 was associated with a 3-fold increase

in the risk of failure of response to chemotherapy [35]. This risk increased to four-fold when

considering only patients that displayed a chemotherapy-induced ABCB1 expression [35].

These findings are supported by a study conducted by Atalay et. al (2008) that evaluated

the impact of ABCB1 gene induction during chemotherapy (doxorubicin and epirubicin)

on disease-free survival (DFS) and overall survival (OS) of patients with locally advanced

breast cancer. It was found that patients with and without ABCB1 induction experienced

13 and 55 months of disease-free survival (DFS) (p=0.0004), respectively, and the overall

survival (OS) was 21 and 57 months (p=0.0025), respectively [36]. These results highlight

the major impact of ABCB1 induction on DFS and OS in patients with locally advanced

breast cancers.

ABCC1 and MDR

The fact that not all cells with MDR phenotypes expressed ABCB1, led to the

identification of another ATP-dependent transporter, ABCC1 [37]. ABCC1 is a 190 kDa

protein transporter that appears to favour the transport of neutral and anionic hydrophobic

and some hydrophilic natural products [7,13,17]. ABCC1 is composed of the ABCB1-like

core linked to an additional TMD that is composed of 5 TMSs [13,24]. ABCC1 transports

multiple endogenous molecules, including organic anions, glutathione, glucuronate and

sulphate conjugates subsequent to phase I and II metabolism (**Table 1**) [38,39]. In contrast to

ABCB1, which is expressed on the apical surface and functions to pump substrates away

from the blood, ABCC1 is expressed on the basolateral surface of epithelial cells in the liver and kidneys and functions to pump substrates into the blood [21]. However, in the BBB, ABCC1 is expressed at the apical surface of endothelial cells, and contributes to the excretion of conjugated metabolites into the blood [21,40].

In vitro, ABCC1 confers resistance to anthracyclines, epipodophyllotoxins, vinca alkaloids, but not taxanes [5,13,24]. Since most cytotoxic drugs effluxed by ABCC1 are also putative substrates for ABCB1, and since ABCB1 is highly expressed in the BBB, it has been rather challenging to elucidate the involvement of ABCC1 in the distribution of cytotoxic drugs to the brain. Using *abcc1* knockout *(abcc1^{-/-})* mice, one study found that the absence of the efflux transporter did not influence the transport of etoposide, vincristine or doxorubicin across the BBB [41]. However, *abcc1^{-/-}* mice exhibited an increased sensitivity to etoposide as measured by the decreased viability of the mice and increased bone marrow toxicity in response to increasing doses of etoposide, which could indicate a functional importance in protecting bone marrow derived stem cells. ABCC1 appears to also play a major role in the elimination of glutathione-conjugated metabolites, as *abcc1^{-/-}* mice displayed elevated glutathione levels in breasts, lungs, kidneys, heart and bone marrow [42,43]. Moreover, leukotriene C_4, an endogenous metabolite of arachidonic acid, has been demonstrated to be transported mainly by ABCC1, and *abcc1^{-/-}* mice display an impaired immune response [21,39].

In humans, *ABCC1* mRNA has been detected in both normal and cancerous breast tissues [24]. Although pooled data from multiple studies indicated that *ABCC1* was detected in 49.3% of breast cancers, no correlation was observed between *ABCC1* expression and

tumour size, metastatic status, histological grade or menopausal status [24]. In relation to prognosis, however, ABCC1 has generally been associated with poorer prognostic outcome. For instance, ABCC1 expression was associated with a lower response rate to chemotherapy in previously untreated breast cancer patients, but not in the previously-treated patients [44]. Furthermore, ABCC1 expression predicted shorter OS and DFS in patients with breast carcinomas [45] and in patients with early-stage breast cancer that received cyclophosphamide, methotrexate and fluorouracil [45,46]. ABCC1 expression did not, however, predict shorter OS or DFS in patients treated with tamoxifen [46]. Taken together, these results suggest that a correlation exists between ABCC1 and poor prognosis in breast cancer in some situations but not all.

ABCG2 and MDR

Doyle et. al (1998) described a multidrug-resistant human breast cancer subline, MCF7/adrVp, that displayed an ATP-dependent resistant phenotype to anthracyclines, but in the absence of overexpression of ABCB1 and ABCC1. RNA fingerprinting led to the identification of a xenobiotic transporter, ABCG2, that conferred resistance to mitoxantrone, a poor substrate of ABCB1 and ABCC1 [47]. ABCG2 is a 72 kDa protein that is composed of one NBD and one TMD [5,21]. ABCG2 is a "half transporter" that must homodimerize in order to function [5,13,24,35]. ABCG2 is expressed and localized to the apical surface of BBB endothelial cells, placenta, intestine and colon epithelial cells [13,21,39]. Similar to ABCB1, ABCG2 plays important roles in the defence mechanisms of the body against xenobiotics; particularly in limiting the distribution of toxins to brain,

the systemic drug bioavailability following an oral dose and, the transport and bioavailability of drugs to the foetus [21,48–51].

ABCG2 has been detected in breast, lung and haematological cancer cell lines [48]. ABCG2 confers resistance to mitoxantrone, camptothecins and anthracyclines [16]. *In vitro* exposure of breast cancer cells to cytotoxic drugs resulted in the substitution of arginine for threonine or glycine at position 482. This point mutation, that was not observed in parental cell lines, enables ABCG2 to efficiently efflux anthracyclines [13,48].

Although ABCG2 was initially discovered in multidrug-resistant human breast cancer cells, its actual expression and functionality in breast tumours remain debatable [48,52]. However, clinical studies indicate that ABCG2 is detected in other tumour types, including lung, brain, liver and colon cancers [53]. In lung cancer, the leading cause of cancer-related deaths, the expression ABCG2 was detected in 48% of patients with small-cell lung carcinomas (SCLC), and was associated with both shorter OS and progression-free survival (PFS) [54]. However, in patients with non-small cell lung carcinomas (NSCLC) that received platinum-based therapy, ABCG2 was detected in 51% of the cases and was associated with shorter OS, but not with PFS [55]. Taken together, these results highlight the major impact exerted by ABCG2 on the success of chemotherapy in lung cancer patients.

Other mechanisms of MDR

Decreased Influx

Uptake of drugs into cancer cells occurs via passive diffusion, uptake transporters or endocytosis [12]. Cytotoxic drugs that rely on uptake transporters to gain access to their intracellular targets are frequently found to downregulate the uptake transporters they depend on [12,13]. This mechanism of MDR is frequently encountered with methotrexate, a folate analogue that has been used in the treatment of haematological malignancies. Methotrexate causes mutations in the reduced folate carrier 1 gene (RFC1) and renders the expression of the RFC1 transporter significantly reduced [12,56]. Confirming this mechanism of resistance, RFC1-deficient breast cancer cells transfected with human RFC1 exhibited significantly greater methotrexate uptake and were 250-fold more sensitive to methotrexate than control clones [56]. Clinically, one study examined methotrexate transport in previously untreated and relapsed patients with acute lymphocytic leukemia (ALL). It was found that only 13% of untreated patients exhibited impaired methotrexate uptake transport, and more than 70% of relapsed patients showed impaired methotrexate uptake transport. RNA analyses revealed that the expression of *RFC1* was significantly decreased in the relapsed patients, suggesting that the impaired methotrexate transport observed in relapsed ALL patients following methotrexate therapy is due to the reduced expression of *RFC1* gene [57].

Two additional families of transporters that appear to play important roles in mediating the uptake transporters-associated MDR phenotype are the solute carrier transporters SLC22 and SLCO. SLC22 and SLCO refer to organic cation and organic anion

transporting polypeptide uptake transporters, respectively. The SLC22 and SLCO

transporters are differentially expressed in a variety of cancer cell lines including, breast,

ovarian, renal and colon cancers [18]. The reduced expression of SLC22 and SLCO uptake

transporters is believed to confer resistance to methotrexate, doxorubicin, mitoxantrone

and platinum-based chemotherapeutics [18]. As an example, human embryonic kidney 293

cells (HEK-293) stably-transfected with SLC22A1 and SLC22A2 exhibited an increased

oxaplatin accumulation and cytotoxicities. The cytotoxicity of oxaplatin in colon cancer

lines was significantly reduced with cimetidine, an SLC22A1 and SLC22A2 inhibitor,

indicating that these transporters are major determinants of the anticancer effects of

oxaplatin [58].

Activation of DNA repair

Alkylating agents exert their effects by damaging the DNA molecule. O_6-

methylguanine-DNA methyltransferase (MGMT) is a ubiquitous protein involved in the

protection and repair of DNA damage that results from such agents. In response to

chronic treatments with alkylating agents, cancer cells upregulate the expression of

MGMT and other key enzymes involved in the maintenance of DNA integrity [5,7]. One

study addressed the relationship between MGMT silencing in the tumor and the survival

of glioblastoma patients found a favourable prognosis in patients that displayed impaired

MGMT-dependent DNA repair [59].

Altered apoptotic machinery

Anthracyclines exhibit their cytotoxic effects by intercalating into the DNA molecule, and by inhibiting the topoisomerases [60,61]. Such actions cause the cell to activate its apoptotic machinery and commit suicide. Continuous exposure to doxorubicin causes cancer cells to acquire changes in their apoptotic machinery in such a way that those DNA damages can no longer induce apoptosis [60]. For instance, mutations in the *p53* gene, whose product is a transcription factor that serves a crucial function in balancing cell cycle arrest and cell death, have been reported in about 50% of breast cancers and were directly associated with resistance to doxorubicin treatment and significantly early breast cancer relapse [5,6,60].

Activation of detoxifying systems

Increased metabolic deactivation of drugs is one additional mechanism that confers a MDR phenotype [12]. Folylpolyglutamyl synthetase (FPGS) is a metabolic enzyme involved in the addition of glutamate moieties to folate and antifolate derivatives. The antifolate polyglutamates are retained longer in cancer cells, and are more potent inhibitors of dihydrofolate reductase, an enzyme involved in cell growth and proliferation. Decreased expression of FPGS confers resistance to the antifolate (LY231514) in an L1210 murine leukemia cell line [62]. Furthermore, increased expression of dihydropyrimidine dehydrogenase, an enzyme involved in pyrimidine catabolism, has been associated with poor response to treatment with 5-fluorouracil (5-FU) in colorectal tumours [63].

Attempts to overcome MDR

It is evident that ABC transporters present a challenging obstacle to successful chemotherapy. Given that ABC transporters are frequently upregulated in response to chemotherapy, it was hypothesized that inhibition of relevant ABC transporters should increase the success rate of chemotherapy and avoid the problem of drug resistance [13,16,33]. The first generation of ABCB1 inhibitors verapamil, quinine and cyclosporin A (CsA) were tested in clinical trials for their potential to reverse ABCB1-dependent MDR. Initial results indicated that these compounds were weak inhibitors of ABCB1 and that the doses required to attenuate ABCB1 function were clinically unmanageable, mainly due to myelosuppression and nephrotoxicity [13,16,33]. Despite the toxicities associated with first-generation modulators, ABCB1 modulation with CsA or quinine was shown to improve responses in poor-risk cytarabine-treated acute myeloid leukemia (AML) patients and in patients with myeolodysplastic syndromes, respectively [64,65]. These results suggested that ABCB1 modulation is a plausible means for overcoming resistance and led to the development of second-generation inhibitors. The second generation of ABCB1 inhibitors such as valspodar effectively antagonized ABCB1, but demonstrated significant pharmacokinetic toxicities stemming from the blockade of metabolism and elimination of the chemotherapeutic agent [16]. These results rendered valspodar clinically toxic and stimulated further development of third generation inhibitors. Third-generation ABCB1 modulators, including elacridar, tariquidar and zosuquidar, were improved and reported to have eluded the pharmacokinetic problems associated with second-generation inhibitors;

however, the efficacy in overcoming resistance was minimal. For instance, the addition of tariquidar to doxorubicin or taxane-containing regimens resulted in only 6% response rate among patients with advanced breast carcinoma. It was concluded that in light of complex biology of MDR, the poor efficacy was attributed to alternative mechanisms of MDR that can impede the efficacy of chemotherapeutics [66]. Taken together, these results indicate that ABCB1 inhibition needs further characterization with respect to the impact of chemotherapeutic drug pharmacokinetics and pharmacodynamics on cellular and organismal levels before it can be implemented in a successful manner.

   As the first three generations of ABC drug transporters inhibitors/modulators showed very little success clinically, a fourth generation of inhibitors/modulators is currently originating from natural sources. Using natural extracts, many researchers are now dedicated to identify lead compounds that exhibit strong inhibitory/modulatory effects and selectivity for ABC transporters without the toxicological effects seen for previous inhibitors [67]. For instance, tetrahydrocurcumin, a major metabolite of curcumin; which a mixture of curcuminoids derived the Indian spice turmeric; was found to chemosensitize ABCB1, ABCC1 and ABCG2-dependent MDR phenotypes in MCF7 breast cancer cells [68]. *In vivo,* curcumin inhibits ABCG2 activity in mice, indicating the potential for *in vivo* efficacy [69]. Phase I clinical trials showed that, despite its poor bioavailability, curcumin is safe to administer to humans with up to 8 g/day when taken orally for three months in patients with high risk or pre-malignant lesions [70]. A Phase II clinical trial indicated that curcumin treatment decreased the aberrant crypt foci (ACF), the earliest identifiable neoplastic lesions in the colon, in patients with high risk of colorectal neoplasia that

showed eight or more ACF [71]. This study exemplifies the anticancer effects of the natural product curcumin in preventing the onset of malignancies.

Alternatively, I believe that drugs that poorly interact as substrates or inhibitors of ABC transporters could help manage the treatment of MDR phenotypes. If a drug can evade efflux by relevant ABC transporters without inhibiting them, it may prove to be a more effective approach in the management of drug resistance. In this scenario, the ideal drug would be efficacious in MDR tumours, but without the side effects associated with the inhibition of ABC transporters if the drug was to be given in combination with other chemotherapeutics.

CHAPTER II: JADOMYCINS

The importance of natural products as a source of novel anticancer drugs

Natural products represent the most essential source of novel leads and active ingredients of medicines [72]. In the post-genomic era, about 80% of all new drug substances were inspired from natural product sources [72]. Recently approved natural product-derived drugs originate from a variety of sources including plants, microbes and animals [72]. These products are used in the treatment of a wide variety of medical conditions including Alzheimer's disease (galantamine), bacterial infections (daptomycin) and chronic pain (ziconotide) [72]. Exploiting the novel chemical scaffolds derived from natural sources, more than 30 natural product-derived drugs are currently undergoing different phases of clinical trials as anticancer agents [73].

The filamentous soil bacteria *Streptomyces* represent the most important source of polyketides [74]. Polyketides are secondary metabolites produced in response to stressful conditions to help the organism cope with its environment [75]. *Streptomyces* produce a wide range of polyketides and are considered to be an essential source of novel chemical scaffolds [76]. Many drugs available on the market today to fight deadly diseases such as typhoid and cancer, originate from *Streptomyces*. For instance, avermectin, daunomycin and chloramphenicol are utilized to treat river blindness, cancer and typhoid, respectively; and these valuable drugs derive from *S. avermitilis*, *S. coeruleorubidus* and *S. venezuelae*, respectively [76]. *S. venezuelae* also produces an under-researched class of antibiotics called jadomycins [77].

The production of novel polyketide-derived antibiotics, jadomycins

Studies have demonstrated that extreme environmental conditions such as heat shock, ethanol treatment or phage infection can dramatically affect the production of antibiotics in the bacterial genus S*treptomyces* [77,78]. Under such environmental conditions *S. venezuelae* ISP5230 produces jadomycins by fermentation [77]. Jadomycins are pigmented angucycline-derived antibiotics with a pentacyclic 8*H*-benz[*b*]oxazolo[3,2-*f*-]-phentathridine backbone, that contains a dihydropyridine and an oxazolone ring [77–79]. Jadomycins are composed of five aromatic rings (A through E) joined together in angular manner (**Figure 2, bottom right**) [80,81]. The nitrogen heteroatom of the oxazolone ring E derives from the incorporation of an amino acid provided in the growth medium into the angucycline backbone [82,83]. In addition, a sugar, 2,6 dideoxy-L-digitoxose, is appended onto ring D of jadomycins [81]. These complex chemical structures provide jadomycins with amphipathic properties, and allow them to exhibit a variety of pharmacological effects including antibacterial and anticancer activities [80,81].

The biosynthetic pathway of jadomycins has been clearly elucidated by Vining and colleagues at Dalhousie University. Enzymes involved in the biosynthesis of jadomycins are referred to as polyketide synthases (PKSs) and glycosyltransferases (GTs) [79,84,85]. PKSs are involved in the formation of the polyaromatic backbone, and GTs are involved in the glycosylation of the final polyketide product. The biosynthetic pathway of jadomycins is described as follows (**Figure 2**). Briefly, The type II PKSs, initiate the polyketide chain by combining one acetate molecule and nine malonate molecules into an

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angucycline structure [81,86–89]. The newly produced angucycline undergoes a series of aromatization and hydrolysis reactions by the enzymes JadF, G and H, which leads to the production of an aldehyde intermediate [86,87,90]. In a non-enzymatic fashion, the aldehyde intermediate reacts with an amino acid to produce an aldimine [82,83]. The aldimine is then decarboxylated and the oxazolone ring is formed giving rise to the secondary metabolite jadomycin A [81,86,87]. The glucosyltransferase JadS catalyzes the glycosylation of the newly formed jadomycin A aglycon at position C_{12} to produce jadomycin B, if the amino acid isoleucine was provided in the medium [79,88,90–92]. Jadomycins A and B differ only by the incorporation of L-digitoxose at position C_{12} (**Figure 2**) [81,87].

The precursor-directed biosynthesis of jadomycins and its importance

One important observation noted was that the amino acid incorporation into the angucycline backbone is a spontaneous reaction and involves no enzymes. Interestingly, jadomycin B, the first glycosylated form of jadomycins to be isolated, was produced in heat-shocked *S. venezuelae* that were grown on minimal galactose-isoleucine medium [77,93]. Since isoleucine was the only nitrogen source provided in the growth medium, and since the isoleucine side chain matches exactly the R group at position C_1 of the oxazolone ring, it was evident that the bacteria had incorporated isoleucine into the polyketide angucycline backbone. This observation stimulated further research to explore whether *S. venezuelae* are capable of incorporating amino acids with different chemical properties [82,83].

Jakeman and colleagues examined the effects of altering the growth conditions by providing different amino acids in the culture medium. Using one amino acid analogue as the one and only nitrogen source, it was discovered that *S. venezuelae* cultures were able to incorporate chemically synthesized non-natural L-amino acids, D-amino acids, non-proteogenic amino acids, fluorinated amino acids or any of the 20 natural amino acids [82,83,94]. Jadomycin amounts were stoichiometrically proportionate to the amounts of amino acid analogue amounts provided in the culture medium [86,94]. Moreover, a beta-amino acid yielded a product that contains a 6-membered oxazinane ring instead of the oxazolone ring. Taken together, these observations indicate that the incorporation of the amino acid is not enzymatically-mediated. One more supporting line of evidence for the non-enzymatic incorporation of amino acids is that enzymes have the tendency to be stereospecific; which contrasts the fact that L and D amino acids can be incorporated into the jadomycin product [82,83,94]. Thus, the precursor-directed biosynthesis of jadomycins through the incorporation of multiple amino acid analogues significantly expands the reservoir of secondary metabolites produced by *S. venezuelae* ISP5230, and allows for the synthesis of a broad range of novel jadomycins that exhibit a variety of biological and pharmacological activities (**Figure 3**) [82,83,94].

The biological activities of jadomycins

Angucycline-derived polyketides, including jadomycin A and B, have been found to exhibit antibacterial, antifungal and anticancer activities [80,86]. The mechanism by which jadomycins exhibit their cytotoxic activities to cancer cells remains unclear. However, a

new generation of cell cycle regulatory proteins, Aurora kinases, is arising and are

believed to be targeted by jadomycins. A unique feature of Aurora kinases is that they are

differentially expressed in tumour tissues in comparison to normal adult tissue. Due to

this differential expression, Aurora kinases are attractive targets for novel chemotherapy

as their inhibition shows more selectivity on tumour cells versus normal dividing tissues,

and ultimately minimal toxicity to the host [95,96]. Aurora kinases are a family of

serine/threonine kinases known to play an essential role in mitosis [95,96]. Three different

Aurora kinases have been identified, A, B and C. Aurora-A kinase plays a role in the

maturation of centrosomes [95]. Aurora-B kinase is required for proper microtubule-

kinetochore attachment, alignment and segregation of chromosomes, and cytokinesis [95].

Aurora-C kinase is found to be able to complement the function of Aurora-B kinase [95,96].

Aberrant Aurora kinases have been detected in a variety of cancers, including but not

limited to, breast cancer, ovarian cancer and pancreatic cancer [95,96]. Aberrant Aurora

kinases result in chromosome alignment and segregation errors that lead to tumorigenesis.

Using structure-based virtual screening, a computerized program that identifies

interactions based on chemical structures, jadomycin B has been identified as an inhibitor

of Aurora-B kinase. Jadomycin B appears to exert its growth inhibitory effects on Aurora-

B kinase by occupying the ATP-binding pocket of the enzyme [96]. The yeast homologue of

human Aurora-B kinase, Ipl1 kinase is responsible for chromosome segregation and cell

growth. Ipl1 shares a temperature sensitive mutant, Ipl1-321 that grows well at 25°C but

not at 37°C [96]. The growth of Ipl1-321 mutant was inhibited almost completely by 10 μM

jadomycin B in comparison to the wild type Ipl1 which was affected by only 3%.

Consistent with the results in yeast, jadomycin B reduced the kinase activity of purified

human recombinant Aurora-B kinase in a competitive inhibition mode [96]. Furthermore, jadomycin B, S and T inhibited the growth of three cancer cell lines A549 (alveolar epithelial cancer), Hela (cervical cancer) and MCF7 (breast cancer). Using the A549 cancer cell line, jadomycin B induced apoptosis, and was able to inhibit the phosphorylation of histone H3, an established Aurora-B kinase substrate [96]. Taken together, these results indicate that inhibition of Aurora-B kinase is applicable to jadomycin cytotoxicity in human cancer cells. In contrast to jadomycin B, jadomycins S and T inhibited the growth of the three human cancer cell lines but did not inhibit the growth of Ipl1 yeast. This discrepancy could indicate that jadomycins more broadly interact with human Aurora-B kinase and/or that different mechanisms of action exist for different jadomycins.

The cytotoxic activities of jadomycin B and five other analogues Ala, F, V, S and T have been evaluated against four cancer cells lines, HepG2 (human hepatocellular carcinoma), H460 (non-small-cell lung carcinoma), IM-9 (lymphoblastic immunoglobin-secreting multiple myeloma) and its resistant derivative subline IM-9/Bcl-2 [80]. Results indicated that the viability of HepG2, IM-9 and IM-9/Bcl-2 cell lines were most sensitive to jadomycin S, whereas the viability of H460 cell line was most sensitive to Jadomycin F. The cytotoxic activities of jadomycins were also confirmed using the degree of apoptosis induced. It was concluded from these studies that the amino acid side chain of the oxazolone ring significantly influenced the cytotoxic activity exhibited by the corresponding jadomycin, and that jadomycins offer a novel chemical scaffold that is easily manipulated and exploited to produce varying biological/pharmacological activities [80].

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Similarly, the precursor-directed biosynthesis of jadomycins provides access to DNA-cleavage capabilities with mechanistic diversity [97,98]. The cleavage of the DNA backbone in the presence of metal ions is considered a robust strategy to screen for DNA-damaging properties of natural products including pluramycin and doxorubicin. Jadomycin B has been shown to exert DNA-damaging effects in the presence of copper ions (Cu^{2+}), and in a dose-dependent manner [97,98]. Jadomycin B alone did not produce any detectable DNA damage, indicating that jadomycin B may be able to reduce Cu^{2+} ions [97,98]. In contrast, the structural isomer of jadomycin B, jadomycin L, which derives from the incorporation of the amino acid leucine (**Figure 3**), produces DNA-damaging effects in the absence of Cu^{2+} ions, which suggests that the position of the methyl group plays an important role in copper dependence for the DNA-damaging effects [97,98]. Collectively, these results demonstrate the specificity of the pharmacological activity exhibited by individual jadomycin analogues, and also demonstrate the diverse biological activities produced by a single family of natural products [96–98].

Our group has synthesized and purified a number of jadomycins that have been tested at the National Cancer Institute (NCI) for anti-cancer efficacy in a 60-cell line screen. The 60- cell line includes: CNS, leukemia, melanoma, NSCLC, colon, ovarian, renal, prostate and breast cancer cell lines, at doses ranging 5 log units [99,100]. Through this screening, several jadomycins (e.g. DNV, DNL, L) were found to inhibit the growth of and/or kill the majority of cancer cells lines *in vitro*, with the exception of leukemia cancer cells that displayed a resistant phenotype to jadomycins. These promising results warranted further investigation of jadomycins for development as anticancer drugs [99,100]. Of particular note in the 60-cell line screen, jadomycins were effective against several breast cancer cells

including the MCF7 cell line, a widely researched, easily manipulated and readily available cell line. The MCF7 cell line offers a useful cell model to begin our evaluations of jadomycin pharmacology in breast cancer.

The aims of this project

The available evidence supports that cytotoxic effects of jadomycins are mediated through intracellular targets, namely Aurora-B kinase and DNA cleavage. Consequently, we believe it is crucial to examine the factors that affect intracellular accumulation of jadomycins in order to determine their efficacy. Our pilot study indicated that jadomycin B poorly interacted with the ABC drug efflux transporters. Thus, we hypothesize that jadomycins that are poor substrates of ABC transporter efflux mechanisms will exhibit higher intracellular accumulation and cytotoxic efficacy over existing chemotherapy drugs that are rapidly extruded from cancer cells by these ABC transporters. If we identify such jadomycins, they would warrant further development for their ability to circumvent MDR and potentially improve current MBC treatment options. Furthermore, elucidating the efflux properties of jadomycins will allow us to define their structural-pharmacokinetic relationships associated with optimal activity in MDR cancer cells leading to the rational design of additional jadomycins with improved pharmacological properties. The aims of this project are specifically:

1- To determine jadomycin cytotoxicity in drug-sensitive and ABCB1, ABCC1 and ABCG2-overexpressing drug-resistant breast cancer cells.

2- To determine if the inhibition of ABCB1, ABCC1 and ABCG2 transporters augments the cytotoxicities of jadomycins.

3- To determine if jadomycins are inhibitors of the efflux transporters ABCB1, ABCC1 and ABCG2.

4- To screen for alternative mechanisms of resistance in the drug-resistant MCF7 cells.

CHAPTER III: MATERIALS AND METHODS

Chemicals

Paclitaxel (TXL), docetaxel, mitoxantrone dihydrochloride, thiazolyl blue methyltetrazolium bromide (MTT), dimethylsulfoxide (DMSO), methanol (MeOH), rhodamine 123 (R123), doxorubicin hydrochloride (DOX), bisbenzimide hoechst 33342 trihydrochloride (H33342), verapamil hydrochloride (VRP), MK-571 sodium salt hydrate (MK-571), ko143 hydrate (ko143) and fumetrimorgin c (FTC) were all purchased from Sigma Aldrich (Oakville, ON, Canada). G418 sulfate (G418) was purchased from InvivoGen (San Diego, CA, USA).

The production of jadomycins

Dr. David Jakeman and the members of his laboratory synthesized and purified the jadomycin analogues used in this project using the following methodology. 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* bacteria were ethanol-shocked to induce secondary metabolism. The cultures were monitored spectrophotometrically for accurate measurements of cellular growth and natural product production. The crude products were collected using a reverse-phase C_{18} column. The crude extracts containing jadomycins were then purified via ion exchange column chromatography. The identity of the purified jadomycins were confirmed using UV-visible, infrared, nuclear magnetic resonance, low resolution and high resolution mass spectroscopies [94,99,100].

Cell lines

The cell lines used in this project were kindly provided by Dr. Robert Robey on behalf of Dr. Susan Bates (National Cancer Institute, NIH, Bethesda, MD). The drug-sensitive human breast carcinoma cell line MCF7 (MCF7-CON), its taxol-selected ABCB1-overexpressing derivative MCF7-TXL, etoposide-selected ABCC1-overexpressing derivative MCF7-ETP, and mitoxantrone-selected ABCG2-overexpressing derivative MCF7- MITX were cultured in Dulbecco's modified Eagles medium (DMEM, phenol red-free, Thermo Scientific, Ottawa, ON, Canada) supplemented with 10 % fetal bovine serum (FBS), 100 IU mL^{-1} penicillin, 250 μg mL^{-1} streptomycin (Invitrogen, Burlington, ON, Canada) and 1 mM sodium pyruvate (Sigma, St. Louis, MO, USA). The resistant sub-lines were established after serial passages of MCF7 cells in medium containing increasing concentrations of taxol (MCF7-TXL), etoposide (MCF7-ETP) and mitoxantrone (MCF7-MITX) [101–103]. After the establishment of the resistant phenotype, the MCF7-TXL, MCF7-ETP and MCF7-MITX cells were maintained in the presence of the selecting cytotoxic agents 400 nM of taxol, 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 standard humidified atmosphere supplemented with 5 % CO_2 at 37°C. All MCF7 cells were grown in drug-free culture medium for 1 week prior to experiments.

The human primary embryonic kidney cell line HEK-293 cells were stably transfected with either empty pcDNA3.1 vector (HEK-CON) or pcDNA3.1 vector containing ABCB1 (HEK-ABCB1), ABCC1 (HEK-ABCC1) or wild type ABCG2 (HEK-ABCG2)

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[104–106]. All of the transfected HEK cells were cultured in MEM (Invitrogen, Burlington, ON, Canada) supplemented with 10% fetal bovine serum, 100 IU/mL penicillin, 250 μg/mL streptomycin and 2 mg/mL G418. All transfected cells were confirmed by comparing the expression of their respective ABC transporter to those transfected with an empty vector (HEK-CON) [105,107]. All HEK-293 cells were maintained in a standard humidified atmosphere supplemented with 5 % CO_2 at 37°C and were grown in drug-free culture medium for 1 week before performing drug transport experiments.

RNA Isolation, Reverse Transcription and Quantitative Real-Time PCR

Total RNA was isolated from MCF7 and HEK cell lysates using the Aurum total RNA Mini Kit (Biorad, Mississauga, ON, Canada) according to the manufacturer's instructions. RNA (0.5 μg) from cells was reverse-transcribed using the Invitrogen Super Script II Reverse Transcriptase (Invitrogen, Burlington, ON, Canada) and an Eppendorff Mastercycler gradient thermocycler (Mississauga, Ontario, Canada). 1 μL of the cDNA product was amplified by quantitative PCR using 125 nM gene-specific primers (**Table 2**) in a total volume of 20 μL using SYBR Green PCR Kit (Biorad) and an Applied biosystems Step One Plus real-time PCR thermocycler (Calsbard, CA, USA). Relative gene expression was normalized to the average of Cyclophylin A (CycA), Actin-β (ACTB) and GAPDH using the ΔΔCT method [108].

MTT Cytotoxicity Assay

An MTT assay was employed to evaluate the cytotoxic activity of jadomycins and control anticancer drugs (docetaxel, etoposide, mitoxantrone and doxorubicin). Briefly,

MCF7-CON, MCF7-TXL, MCF7-ETP and MCF7-MITX cells were seeded in 96-well plates at 5000, 7500, 5000 and 10000 cells/well in 100 µL medium, so that the following day there would be about 12000 cells/well for each cell line, as each cell line replicates at a different rate. The cells were allowed to adhere for 24 hours at 37° C. The cells were then treated with increasing concentrations of jadomycins (0.1 – 100 µM) or control cytotoxic drugs for 72 hours. After 72 hours incubation, 20 µL of 5 mg/mL MTT solution was added to the cells for 2 hours. The media and MTT mixture was aspirated and the formazan-containing cells were solubilised in 100 µL of DMSO. Optical density of formazan was measured at 550 nm on the Biotek Synergy HT plate reader (Biotek, Winooski, VT, USA). The cell viability as reflected by the formazan produced in each drug treatment well was compared to vehicle-treated cells. The percentage of cell survival was calculated as absorbance in test wells divided by the average of the absorbance in vehicle control wells and then multiplied by 100. The concentration that resulted in 50% reduction (IC_{50}) in viability was calculated from the log_{10} concentration versus normalized response curves using the following equation:

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

The Fold-resistance values were obtained by dividing the mean IC_{50} value in the drug-resistant cells by that of the drug-sensitive cells [68].

To further examine if jadomycin cytotoxicities were dependent on ABCB1, ABCC1 or ABCG2 efflux function, the cytotoxicity assays were repeated by exposing drug-sensitive and drug-resistant cells to various concentrations of jadomycins with or without the specific inhibitors of the ABC transporters, VRP (ABCB1), MK-571 (ABCC1) and

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ko143 (ABCG2) [109–111]. Based on their cytotoxicity curves, VRP, MK-571 and ko143 were each used at the maximal possible concentration that did not cause cytotoxicity in either sensitive or resistant MCF7 cell lines. These concentrations were 7.5 μM for VRP, 25 μM for MK-571 and 0.5 μM for ko143. The fold-reversal of MDR was calculated by dividing the IC_{50} for the cells treated with jadomycins alone by that obtained from cells treated with jadomycins plus the inhibitor [112].

Rhodamine 123, Doxorubicin and Hoechst 33342 Accumulation/Retention Assays

To determine if jadomycins were inhibitors of ABCB1, ABCC1 or ABCG2-mediated transporter function, R123, DOX and H33342 intracellular retention assays were employed, respectively. HEK-CON, HEK-ABCB1, HEK-ABCC1 and HEK-ABCG2 cells were seeded at 200,000 cells/well in 24-well plates and allowed to reach confluence over a period of 48 hours. The cells were washed once with PBS at room temperature and incubated in 250 μL of 10 μM R123 (2 hours), 10 μM DOX (2 hours) or 10 μM H33342 (30 minutes) solution with or without increasing concentrations of jadomycins (0.5-50 μM) at 37º C [107,113,114]. VRP, MK-571 and FTC were used as positive control inhibitors for ABCB1, ABCC1 and ABCG2 transporters, respectively [109,110,115]. For the H33342 assays only, cells were then washed with PBS and incubated in substrate free media with or without jadomycins to allow efflux of H33342 for 1 hour at 37º C. Cells were then washed with ice-cold PBS and lysed in 250 μL of 0.2 μM NaOH and then neutralized with 2 M HCl. Intracellular R123 and DOX accumulation and H33342 retention levels were read on the Biotek Synergy HT fluorometer. Fluorescence levels were normalized to the protein levels of each well using the Lowry method [116]. The R123, DOX and H33342

levels in drug-treated wells were compared to vehicle-treated control wells. The percentage of R123, DOX or H33342 accumulation was calculated as the measured fluorescence in a test well divided by the average of the mean fluorescence in the vehicle-treated control wells and then multiplied by 100.

Data Analysis

For each cell culture experiment, the individual treatments were performed in triplicate or quadruplicate. Unless otherwise stated, each cell culture experiment was repeated at least three times on cells of different passages. All data are expressed as mean ± SEM. An unpaired t-test was used for statistical comparison of experiments involving two groups. A one-way analysis of variance (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 $p \leq 0.05$.

CHAPTER IV: RESULTS

Objective 1. To determine the cytotoxic efficacy of jadomycins in drug-sensitive and drug-resistant MCF7 cells

Since the drug-resistant MCF7 cells were provided by another laboratory, our first goal was to confirm the upregulation of ABC transporters prior to testing the cytotoxicity of jadomycins. MCF7-TXL (taxol-selected), MCF7-ETP (etoposide-selected) and MCF7-MITX (mitoxantrone-selected) are drug-resistant cell models that were previously reported to highly express ABCB1, ABCC1 and ABCG2, respectively [102,117,118]. Using quantitative real time PCR (qRT-PCR), the mRNA levels of *ABCB1*, *ABCC1* and *ABCG2* were determined. The basal expression of *ABCB1*, *ABCC1* and *ABCG2* were nearly undetectable in MCF7-CON cells (**Figure 4**). When compared to MCF7-CON, the MCF7-TXL, -ETP and -MITX cells exhibited approximately a 15000, 28 and 35-fold increase in *ABCB1*, *ABCC1* and *ABCG2* mRNA levels, respectively (**Figure 4A-C**).

The MTT assay is a colorimetric method that measures cell viability and proliferation [119]. MTT is a yellow compound that is reduced to purple formazan by the enzyme mitochondrial reductase present in viable and metabolically active cells [120]. The amount of formazan produced in an MTT assay, therefore, reflects the number of viable cells. MTT assays were used to evaluate the cytotoxic effects of jadomycins and control drugs on the drug-sensitive and drug-resistant MCF7 cells. The control drugs docetaxel, etoposide and mitoxantrone are cytotoxic drugs known to be effluxed by ABCB1, ABCC1 and ABCG2, respectively [47,121,122]. The MCF7-CON cells were more sensitive to

docetaxel, etoposide and mitoxantrone than the MCF7-TXL, MCF7-ETP and MCF7-

MITX cells, respectively (**Figure 5A-C**). Compared to the MCF7-CON cells, the IC_{50}

values increased 20-fold for docetaxel in MCF7-TXL cells, 3-fold for etoposide in the

MCF7-ETP cells and 79-fold for mitoxantrone in the MCF7-MITX cells (**Table 3**). These

data confirm the resistant phenotype of the MCF7-TXL, -ETP and -MITX cells. Although

the IC_{50} of etoposide increased only by 3-fold in MCF7-ETP in comparison to MCF7-

CON cells, it is important to mention that this value was based on the 4 experiments in

which the IC_{50} was computable. However, in 2 experiments the IC_{50} values in MCF7-ETP

were could not be computed as the highest concentration used did not result in enough

cytotoxicity that could be measured using the Hill plot, , indicating that the true IC_{50}

value of etoposide in the MCF7-ETP cells is likely larger than 150 µM.

Based on the structural diversity and availability of jadomycins, I chose to investigate

the analogues DNV, L, B, SPhG, F, W, S, T and N for their potential anticancer effects.

Table 3 and **figures 6-8** summarize the cytotoxic efficacies of jadomycin analogues in

drug-sensitive and drug-resistant MCF7 cells. All jadomycin analogues effectively

reduced the viability of control and resistant breast cancer cells. Jadomycins DNV, L, B,

SPhG, F, S and T exhibited IC_{50} values between 1 and 10 µM, whereas jadomycins W

and N were less potent. In comparison to the control drug-sensitive MCF7 cells, the IC_{50}s

of jadomycins B, L, S and T were similar in MCF7-TXL, -ETP and -MITX cells. The

IC_{50}s of Jadomycins DNV, SPhG and N were similar in the MCF7-TXL and -ETP cells

but significantly higher in the MCF7-MITX cells compared to the MCF7-CON cells. On

the other hand, Jadomycins F and W displayed significantly higher IC_{50}s in all three drug-

resistant cell lines compared to the control MCF7 cells. The IC_{50} data would suggest that

jadomycins B, L, S and T are not substrates for ABCB1, ABCC1 or ABCG2, whereas jadomycins F and W are substrates for all three transporters and that jadomycins DNV, SPhG and N are substrates for ABCG2 only. Notwithstanding, it is important to point out that the jadomycins that were not equipotent demonstrated a maximum of 3.7-fold increase in the IC_{50} in the taxol and mitoxantrone resistant cells, a much smaller degree than the increase in IC_{50}s for docetaxel (20-fold), mitoxantrone (79-fold).

Objective 2. To determine if the inhibition of ABCB1, ABCC1 and ABCG2 augments the cytotoxicity of jadomycins

If the increased IC_{50}s of jadomycins F and W in the MCF7-TXL, -ETP, - MITX cell lines and the increased IC_{50}s for jadomycin DNV, SPhG and N in the MCF7-MITX cells are due to enhanced drug efflux, then inhibiting the ABCB1, ABCC1 and ABCG2 transporters in the resistant cell lines should re-sensitize them to these jadomycin molecules. For instance, if an inhibitor of ABCB1 sensitizes (i.e. decreases IC_{50}) MCF7-TXL cells to a certain jadomycin analogue, it would provide evidence that the jadomycin in question is a substrate for ABCB1 and that the cytotoxicity of the jadomycin is dependent on the function of that transporter. On the other hand, if sensitization is not observed, it indicates that other mechanisms of resistance to jadomycins may be occurring.

The cytotoxicity assays were carried out as for objective 1 by exposing MCF7-CON and MCF7-TXL, -ETP and -MITX cell lines to increasing concentration of jadomycins

with or without the specific inhibitors of ABCB1 (VRP), ABCC1 (MK-571) and ABCG2 (ko143) [109–111]. We first established the effects of these inhibitors on the IC_{50}s of control substrates for ABCB1 (DOX), ABCC1 (DOX) and ABCG2 (mitoxantrone) in drug-sensitive and drug-resistant MCF7 cells [61,111,113]. All inhibitors exhibited minor effects on the IC_{50} values in the control lines, which may be a reflection of the basal expression of the targeted efflux transporter (**Figure 9** and **Tables 4-6**). In contrast, the use of VRP and MK-571 significantly reduced the IC_{50} of DOX in MCF7-TXL and -ETP cells, from 216.8 and 15.86 μM to 37.82 and 6.58 μM, respectively (**Figure 9** and **Tables 4, 5**). Similarly, ko143 significantly reduced the IC_{50} of mitoxantrone in MCF7-MITX cells from 119.6 to 3.85 μM (**Figure 9** and **Table 6**). These data indicate that the cytotoxicity of DOX depends on the function of the ABCB1 and ABCC1 efflux transporters and the mitoxantrone cytotoxicity depends on ABCG2 function.

This approach was then used to determine if jadomycin DNV, L, B and S cytotoxicities were dependent on ABCB1, ABCC1 and ABCG2 efflux function. Given that these jadomycins exhibited similar cytotoxic efficacies in drug-sensitive and drug-resistant cells (with the exception of DNV in MCF7-MITX cells), we wanted to provide a second layer of evidence and further confirm that these jadomycins were not effluxed by ABCB1, ABCC1 or ABCG2. **Tables 4-6** and **figures 10-12** summarize the findings of these experiments. In the MCF7-CON cells, VRP significantly reduced the IC_{50} of jadomycin B, but did not affect the IC_{50}s of other jadomycins in the MCF7-CON cells (**Figure 10** and **Table** 4. The ABCC1 inhibitor, MK-571, significantly reduced the IC_{50}s of jadomycins DNV, L and B in MCF7-CON cells but not that of jadomycin S (**Figure 11** and **Table 5**). Ko143 behaved similarly to VRP, in that it augmented the cytotoxicity of

jadomycin B in MCF7-CON cells without affecting other jadomycins (**Figure 12** and

Table 6). In the MCF7-TXL cells (**Figure 10** and **Table 4**), MCF7-ETP cells (**Figure 11**

and **Table 5**) and MCF7-MITX cells (**Figure 12** and **Table 6**) the respective ABCB1,

ABCC1 and ABCG2 inhibitors tended to reduce (25 to 50 %) the IC_{50} values of

jadomycins DNV, L, B and S, but none of these reductions was statistically significant. In

the respective resistant cells, the absolute reductions in the IC_{50} values of jadomycins

caused by VRP, MK-571 and ko143 were also much smaller in magnitude than the IC_{50}

reductions observed for the control drugs DOX and mitoxantrone. These results indicate

that the cytotoxicities of jadomycin DNV, L, B, and S are weakly dependent on the efflux

of ABCB1, ABCC1 and ABCG2, supporting that these jadomycins are poor substrates for

these efflux transporters. However, the fact that changes were observed for some

jadomycin IC_{50}s in control cells could indicate that other mechanisms of interactions exist

between the inhibitors and jadomycins, which could influence the cell viability in a

jadomycin-specific manner.

*Objective 3. To determine if jadomycins inhibit ABCB1, ABCC1 and ABCG2-
mediated efflux of substrate molecules*

Since anticancer drugs are frequently prescribed in combination therapies [123], coupled

with the fact that ABC transporter inhibitors failed clinically due to adverse interactions

[16], we believe that it is essential to characterize the inhibitory potential of jadomycins on

ABCB1, ABCC1 and ABCG2 transporters. To determine if jadomycins inhibit the

ABCB1 function, the intracellular accumulation of R123, an ABCB1 fluorescent

substrate, was examined in the presence or absence of jadomycins [107]. We employed

human embryonic kidney cells (HEK) that have been stably transfected with either an empty vector (HEK-CON) or the ABCB1 transporter (HEK-ABCB1). The cells were incubated with 10 μM R123 solution for 2 hours, in the presence or absence of increasing concentrations of jadomycins (0.5 – 50 μM) or the positive control inhibitor VRP [109]. The premise of the assay is that in the presence of an ABCB1 inhibitor the cellular efflux of R123 will be blocked resulting in an increase in intracellular fluorescence in the ABCB1-expressing cells but not in the control cells, which express only basal levels of ABCB1. In the absence of VRP the HEK-ABCB1 cells exhibited significantly reduced R123 intracellular accumulation in comparison to HEK-CON cells (**0 μM bars, Figure 13A**). The methanol vehicle had no effect on the R123 intracellular accumulation in HEK-CON or HEK-ABCB1 cells (**data not shown**). When the HEK-ABCB1 cells were treated with VRP, the intracellular R123 accumulation increased significantly in a dose-dependent manner, as would be expected with an inhibitor of the transporter (**Figure 13A**). VRP did not cause an increase in the accumulation of R123 in the HEK-CON cells, but rather a decrease, which could reflect an inhibition of R123 uptake (**Figure 13A**). Jadomycins had no effect on R123 intracellular accumulation in the HEK-ABCB1 cells, indicating that jadomycins do not inhibit ABCB1-mediated transport of substrates at concentration below or above the IC_{50}s of cytotoxicity observed in the MCF7 cells (**Figure 14**). However, similar to VRP, jadomycins appeared to cause a decrease in R123 intracellular accumulation in the HEK-CON cells (**Figure 14**).

 Similarly, in order to determine if jadomycins inhibit ABCC1 function, the intracellular accumulation of DOX was examined with or without jadomycins. HEK-CON cells or ABCC1-stably-transfected HEK cells (HEK-ABCC1) were incubated with 10 μM DOX

solution for 2 hours, in the presence or absence of increasing concentrations jadomycins (0.5 – 50 μM) or the positive control inhibitor of ABCC1, MK-571 [110]. The methanol vehicle had no effect on the DOX intracellular accumulation in HEK-CON or HEK-ABCC1 cells (**data not shown**). As expected, in the absence of MK-571, the HEK-ABCC1 cells exhibited significantly decreased intracellular DOX accumulation in comparison to HEK-CON cells (**0 μM bars, Figure 13B**). MK-571 treatment resulted in a significant increase in the intracellular DOX accumulation in HEK-ABCC1, but not in the HEK-CON cells, indicating that the effect of inhibition was specific to ABCC1 transport (**Figure 13B**). Jadomycins exhibited no effect on intracellular DOX accumulation in HEK-CON or HEK-ABCC1 cells (**Figure 15**), indicating that jadomycins do not inhibit ABCC1-mediated transport of substrates. The only exception was jadomycin DNV, which exhibited inhibitory effects at 50 μM, a concentration that is about 10-fold higher than their IC_{50} values in MCF7 cells (**Figure 15A and D**).

In a similar fashion, the jadomycin inhibitory potential of ABCG2-mediated transport was also examined. Here we examined the retention of H33342 rather than its accumulation over time, in the presence or absence of jadomycins [114]. Control and wild type ABCG2-stably- transfected HEK cells were used in this experiment. HEK cells were loaded with H33342 for 30 minutes with or without increasing concentrations (0.5-50 μM) of jadomycins. The medium was then aspirated and replaced with medium that contained no H33342, and the cells were then allowed to efflux the previously loaded H33342 in the presence or absence of jadomycins for 1 hour. The methanol vehicle had no effect on the H33342 retention in HEK-CON or HEK-ABCG2 cells (**data not shown**). In the absence of FTC, HEK-ABCG2 cells exhibited significantly reduced H33342

retention in comparison to HEK-CON cells (**0 μM bars, Figure 13C**). When the HEK-ABCG2 cells were treated with FTC, a positive control inhibitor of ABCG2 [115], the retention of H33342 increased significantly in a dose-dependent manner in the HEK-ABCG2 (**Figure 13C**). FTC treatment did not affect the H33342 retention in the HEK-CON cells (**Figure 13C**). Jadomycins (with the exception of DNV) had no effect on H33342 retention in HEK-ABCG2 cells (**Figure 16**), indicating that jadomycins do not inhibit ABCG2-mediated transport of substrates. However, jadomycin DNV only exhibited inhibitory effects at 50 μM, a concentration that is about 10-fold higher than the IC$_{50}$ value in MCF7 cells (**Figure 16A**). Jadomycin DNV, SPhG and S decreased the H33342 retention in HEK-CON cells, which could suggest that these jadomycins are taken into the cells by an exchange type transporter for H33342 (**Figure 16**).

Objective 4. To screen for alternative mechanisms of resistance in the resistant cell lines

The fact that jadomycins B, L, and S exhibited statistically similar potencies between the drug-sensitive and drug-resistant cells (Objective 1), but displayed a trend towards an increase, presented by an approximately 1.5 to 2 fold in the IC$_{50}$ values in all the resistant lines, suggests some degree of dependence of the toxicity on ABCB1, ABCC1 and ABCG2 functions. However, the cytotoxicity of jadomycins DNV, L, B and S in the resistant cells were poorly sensitized by ABC transporter inhibitors and jadomycins did not inhibit the ABCB1, ABCC1 and ABCG2-mediated transport of substrates. This suggests that ABC transporters may not be the limiting factor that could be impeding the cytotoxic effects of these jadomycins in drug-resistant cells, and alternative mechanisms

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of resistance may be causing a small shift in the IC_{50} values of jadomycins. To explore this, we completed a qRT-PCR screen where we compared the relative expression of genes implicated in drug-resistance in the MCF7-TXL, -ETP and -MITX cells to that in drug-sensitive MCF7-CON cells. This included other ABC transporters (*ABCC3*, *ABCC4*, *ABCC5* and *ABCC6*), uptake transporters (*SLCO3A1*, *SLCO4C1* and *SLCO5A1*), metabolic enzymes (*CYP1A1*, *CYP1A2*, *CYP2B6*, *CYP2C8*, *CYP2E1*, *BLMH*, *EPHX1* and *NAT2*) and regulatory genes (*PPARγ* and *RXRα*).

Table 7 summarizes the results obtained from the qRT-PCR studies. Briefly, in the drug efflux transporter gene category, *ABCC4* increased in all resistant cell lines. *ABCC3* decreased and *ABCC6* increased substantially in the MCF7-ETP cells. The uptake transporter *SLCO3A1* was decreased in all resistant lines. *SLCO5A1* increased in the MCF7-TXL cells, but decreased in the MCF7-ETP and -MITX cells. *SLCO2B1* increased in the MCF7-ETP cells, and *SLCO4C1* decreased in the MCF7-MITX cells. The metabolic genes *CYP1A1* and *CYP1A2* were upregulated in the MCF7-ETP cells. CYP1A2 and CYP2B6 were downregulated in the MCF7-MITX cells. Bleomycin hydroxylase (*BLMH*) was upregulated in the MCF7-ETP cells but downregulated in the MCF7-TXL and -MITX cells. N-acetyl transferase 2 (*NAT2*) was downregulated in the MCF7-TXL cells, and epoxide hydrolase 1 (*EPHX1*) was significantly upregulated in the MCF7-MITX cells. The regulatory genes *PPARγ* and *RXRα* were in general downregulated in all resistant lines, with the exception of *PPARγ* in the MCF7-MITX cells.

CHAPTER V: DISCUSSION

MDR and MBC

To date, MBC remains an incurable disease [8,124]. Despite intense efforts, current therapeutic options for MBC are limited owing to the problem of MDR [4]. MDR affects virtually all therapies available for MBC, and especially chemotherapy. Regardless of the therapy type, MBC patients are found to develop resistance shortly after the onset of therapy [4]. Depending on the pathology of MBC, first-line therapy is often selected to be a cytotoxic chemotherapy [4,8]. First-line chemotherapies for MBC include anthracyclines, taxanes and epothilones (ixabepilone analogues) [8]. If these chemotherapeutics fail, capecitabine and gemcitabine are then chosen to continue the treatment of MBC [8,123]. If resistance develops in response to these agents, then MBC patients have little or no treatment options.

The upregulation of ABC transporters, especially ABCB1, ABCC1 and ABCG2, is believed to be the most frequently encountered mechanism of MDR. This is of clinical importance since many agents developed as potential inhibitors of ABC transporters failed to restore sensitivity to chemotherapy in breast cancer. In addition, the use of these inhibitors resulted in intolerable side effects that required dose reduction of chemotherapy, which resulted in a lowered efficacy of the anticancer drug. Since most chemotherapeutics currently used for MBC treatment are substrates for ABCB1, ABCC1 or ABCG2, and since the use of inhibitors of these transporters did not prove clinically feasible, an alternative strategy is then urgently needed. One possible alternative strategy

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is to identify novel anticancer agents that are poor substrates and inhibitors of the relevant ABC transporters. Based on our data, some jadomycins B, L and S appear to be such candidates.

The present work uses the MCF7 breast adenocarcinoma cell line model to identify candidate anticancer drugs that are capable of eluding MDR phenotype. The human MCF7 breast cell line derives from a metastatic site of a mammary gland adenocarcinoma [125]. The MCF7 cancer cells were continuously cultured in the presence of sublethal doses of paclitaxel, etoposide or mitoxantrone, which resulted in the generation of resistant sublines characterized by the upregulation of ABCB1, ABCC1 and ABCG2, respectively. The IC_{50} values of docetaxel (an analogue of paclitaxel), etoposide and mitoxantrone significantly increased confirming that the expected drug-resistant phenotype was associated with increased *ABCB1*, *ABCC1* and *ABCG2* mRNA expression, as measured by qRT-PCR. Cross resistance of the MCF7-TXL, -ETP and MITX cell lines to structurally and mechanistically unrelated drugs has been confirmed by other laboratories. MCF7-TXL cells displayed a 13.5-fold resistance to doxorubicin relative to the control MCF7 cells [117]. The MCF7-ETP cells are resistant to epipodophyllotoxins (etoposide and its analogues) and anthracyclines, and the MCF7-MITX cells are resistant to mitoxantrone and camptothecins (i.e. topotecan and irinotecan) [118,126]. Taken together, these findings indicate that the MCF7 cell model along with its resistant derivatives is a useful tool to test if the cells display cross resistance to jadomycins following chronic exposure to paclitaxel, etoposide and mitoxantrone, and to conduct preliminary studies regarding the impact of drug efflux transporters on jadomycin cytotoxicity in breast cancer.

Overall, our data indicate that jadomycins effectively reduce the growth of MCF7 cancer cells along with its resistant derivatives. All jadomycin analogues eradicated drug-sensitive and drug-resistant cells with a range of potencies (1 – 10 μM), with the exception of the less potent jadomycin N in MCF7-MITX and jadomycin W in all resistant lines. These results suggest that the structural differences due to the amino acids incorporated into the jadomycin backbone influence the interactions with the intracellular targets. However, jadomycins DNV, L, B, SPhG, S and T exhibited similar IC_{50} range, indicating that there is some feasibility for a change in jadomycin structure without affecting the cytotoxic potency. This is of great value for drug development purposes. The pharmacokinetic parameters, ADME, along with the physicochemical properties of drugs, solubility and permeability, very frequently present a barrier to successful therapies. For instance, estramustine, an anticancer drug used for the treatment for prostate carcinoma, was initially not exhibiting the desired aqueous solubility. A phosphate ester functionality was added to the estramustine molecule in order to improve its aqueous solubility [127]. Given that jadomycins are amenable to precursor-directed biosynthesis, then altering the chemical structure of jadomycins without affecting the cytotoxic potency may be one effective strategy to overcome such potential pharmacokinetic problems, if any.

The results from the cytotoxicity studies in the drug-sensitive and drug-resistant MCF7 cells reveal some important patterns. First, the MCF7-MITX cells exhibited cross resistance to 5 out of 9 jadomycin analogues compared to 2 out of 9 jadomycins in the MCF7-TXL and –ETP cells, suggesting a general dependence of jadomycins cytotoxicity on ABCG2 over ABCB1 and ABCC1, in this cell model. Second, jadomycins B, L, S and

T were found to be most effective, owing to their ability to retain cytotoxic potency in the MCF7-TXL, -ETP and -MITX cell lines in comparison to control MCF7 cells. Jadomycins DNV, SPhG and N retained their cytotoxic potency in MCF7-TXL, -ETP cells but not in the MCF7-MITX cells, whereas jadomycins F and W were less potent in all resistant cell lines. This indicates that jadomycins DNV, SPhG and N may be substrates of ABCG2, and that jadomycins F and W may be effluxed by ABCB1, ABCC1 and ABCG2. The importance of these findings is that jadomycins B, L, S and T may prove more effective than jadomycins F and W in the eradication of resistant tumour cells that arise in response to treatment with taxanes, etoposide or mitoxantrone. Taxanes, etoposide and mitoxantrone are currently indicated for the treatment of a variety of cancers, including but not limited to, MBC (taxanes and mitoxantrone) [8,128], small cell lung carcinoma (SCLC) (etoposide) [129], and castrate-resistant prostate cancer (docetaxel and mitoxantrone) [130]. Since resistance may be a problem in these cancers, then it is also urgent to identify novel compounds that demonstrate efficacy in these cancers. The preliminary data of this project indicate that jadomycins B, L, S and T could be such candidates based on their insensitivity to ABCB1-, ABCC1- and ABCG2-mediated resistance mechanisms.

In a secondary analysis, I wanted to determine if the differences in jadomycin potencies could be related to the structural differences of the amino acid side chains incorporated into the jadomycin molecule. The IC_{50} values of jadomycins derived from amino acids with hydrophobic aliphatic side chains (DNV, L and B) demonstrated equipotent cytotoxic activity in MCF7-CON, -TXL and –ETP cells. This observation is attributed to

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the possibility that the aliphatic side chains present on jadomycins DNV, L and B render the jadomycins unrecognized by ABCB1 and ABCC1.

Similarly, Jadomycins derived from amino acids with hydrophilic side chains (S, T and N) demonstrated equal cytotoxic efficacies in drug-sensitive, TXL and ETP-resistant cell lines. Given that ABCB1 and ABCC1 favour the transport of hydrophobic natural products [12], the hydrophilic side chains of these jadomycins S, T and N may render them poor substrates for ABCB1 or ABCC1 transport.

In contrast, jadomycins derived from amino acids with aromatic side chains (SPhG, F and W) demonstrated reduced cytotoxic potencies in mitoxantrone-resistant cells compared to drug-sensitive cells. Careful examination of many ABCG2 substrates indicates a common chemical structure characterized by multiple aromatic rings joined in an angular manner (i.e. irinotecan, topotecan, FTC, H33342). The extra aromatic ring on the side chain of jadomycins may increase the susceptibility to recognition and transport of the jadomycins SPhG, F and W by ABCG2. An alternative possibility is that mitoxantrone-selection may have stimulated a mechanism of resistance such as reduced transporter-mediated uptake or enhanced metabolic detoxification that leads to preferentially deactivate jadomycins with aromatic side chains [6,12].

We understand that multiple mechanisms of resistance can occur simultaneously (i.e. increased efflux and increased detoxification) as shown in the results of objective 4. Therefore based only on the IC_{50} data from objective 1, we could not say with absolute certainty that jadomycin cytotoxicity is indeed dependent or independent of the function of the ABCB1, ABCC1 and ABCG2 drug efflux transporters. Thus, we decided to

generate confirmatory findings through the analysis of the ability of ABCB1 (VRP), ABCC1 (MK-571) an ABCG2 (ko143) inhibitors to sensitize the MCF7 cell lines to jadomycin cytotoxicity. Since the goal of this project is to identify jadomycins that are poor substrates of ABCB1, ABCC1 and/or ABCG2 efflux transporters, I selected jadomycins that appeared most promising with regards to lack of cross resistance (B, L and S) in the 3 resistant cell lines, along with jadomycin DNV, which showed increased IC_{50} in the MCF7-MITX cells. VRP, MK-571 and ko143 sensitized the MCF7-TXL and MCF7-ETP cells to doxorubicin and the MCF7-MITX cells to mitoxantrone as determined by the statistically significant and substantial reduction in IC_{50} values when compared to treatment with the cytotoxic drugs alone. These results demonstrated that the potency of doxorubicin and mitoxantrone is dependent on the function of ABCB1, ABCC1 and ABCG2, respectively, and confirmed the results found in objective 1. In comparison, the ABCB1, ABCC1 and ABCG2 inhibitors did not significantly reduce the IC_{50}s of jadomycins B, L, S and DNV in the respective MCF7-TXL, -ETP and MITX cell lines. These data support that the contention that potency of jadomycins B, L and S is minimally dependent on the function of the ABCB1, ABCC1 and ABCG2 efflux transporters. We did expect ko143 to sensitize the MCF7-MITX cells to jadomycin DNV, but this did not occur. This would suggest that reduced jadomycin DNV potency in the MCF7-MITX (objective 1) is independent of the ABCG2 transporter and due to an alternative mechanism of resistance. Unexpectedly, the control MCF7 cells were sensitized to jadomycin B by VRP and ko143, and to jadomycins DNV, L and B by MK-571. This further indicates that there is an ABC transporter-independent component influencing jadomycin toxicity. As for the independent mechanisms we cannot say for

sure, however, VRP, MK-571 and ko143 could be inhibiting other ABC efflux transporters, or affecting other targets, such as drug uptake transporters or metabolic enzymes. This is supported by the findings of other laboratories where VRP has been shown to not only inhibit ABCB1, but also ABCC1. VRP is a racemic mixture of two enantiomers S and R-verapamil. The R-verapamil has been established as putative inhibitor of ABCC1 [131]. Similarly, MK-571 has been demonstrated to inhibit not only ABCC1, but also ABCC2, ABCC3, ABCC4 and ABCC5 [16]. Furthermore, VRP inhibits the metabolic enzymes CYP3A4 and CYP3A5 [132]. So if jadomycins were to be substrates for CYP3A4/5, then VRP-mediated inhibition of CYP3A4/5 could somewhat augment the cytotoxicity of jadomycins in MCF7 cells.

Although the initial results of ABC transporter inhibition appeared promising in the laboratory, and in phase I and phase II clinical trials, phase III clinical trials demonstrated that this strategy is not feasible due to toxicological problems. These toxicological problems included serious drug-drug interactions between the modulator of the ABC transporter and the cytotoxic drug used, and did not result in any survival benefits [16,133,134]. Given that therapy for MBC frequently consists of combination therapy that include a chemotherapeutic agent [123,135], it is then imperative to determine if jadomycins inhibit the ABCB1, ABCC1 or ABCG2-mediated transport of substrate molecules, as this could determine the potential use for jadomycins in combination therapy. To address this possibility, the efflux of fluorescent substrates was measured in stably-transfected HEK cells with the transporter of interest. While VRP, MK-571 and ko143 significantly increased the accumulation/retention of fluorescent ABC-transported probe substrates in HEK-ABCB1, ABCC1 and ABCG2 cells, this accumulation/retention was not affected in

the presence of jadomycins, indicating that jadomycins do not inhibit the ABCB1, ABCC1 and ABCG2-mediated transport. Although ABCC1 was inhibited by jadomycins DNV and SPhG, and ABCG2 by jadomycin DNV, these observations would likely be pharmacologically irrelevant in an *in vivo* situation since the inhibitory concentration on ABCC1 and ABCG2-mediated transport was 10-fold higher than the concentration required for cytotoxicity.

Unexpectedly, all jadomycins except S and W caused a reduction in the intracellular levels of the ABCC1 substrate R123 in HEK-CON cells. This effect reveals important information about the uptake mechanism of jadomycins into the cells, as VRP has been shown to inhibit the OCT1 and OCT2-mediated uptake of R123 [136–138]. The VRP-like effects produced by jadomycins on R123 accumulation in the HEK-CON cells, coupled with the fact that HEK-293 cells do not express OCT1 and OCT2, suggest that jadomycins may also be inhibiting the uptake of R123 through an endogenous OCT that is distinct from OCT1 and OCT2 [139]. The ability of jadomycins DNV, SPhG, F and S to reduce the intracellular retention of the ABCG2 substrate H33342, in the HEK-CON cells, suggests the possibility that those jadomycins may enter the cell via an exchange transporter mechanism. In this case, the uptake of one jadomycin molecule would result in the egress of one molecule of H3342, causing a decrease in H33342 retention.

While the ABCG2 and to a lesser extent ABCC1 and ABCB1-overexpressing cell lines were weakly cross-resistant towards specific jadomycins, the lack of ability of the efflux transporter inhibitors' ability to sensitize those cell lines to jadomycins coupled with the fact that jadomycins did not inhibit the function of ABCB1, ABCC1 or ABCG2,

intriguingly suggested there were other mechanisms of cross resistance involved. This somewhat unexpected observation prompted me to perform a series of PCR screening studies that would identify other potential pathways of metabolism and transport that may been altered in the resistant cell lines. By following this line of investigation we would be able to generate further knowledge of jadomycin pharmacokinetics on the cellular level that will guide future studies. Second, the information provided would provide key information regarding alternative mechanisms of drug resistance that develop in breast cancer cells upon exposure to chemotherapeutics.

The upregulation of *ABCC4* expression in all of the resistant lines, and that of *ABCC5* and *ABCC6* in the MCF7-ETP cells, may explain the slight reduction in the cytotoxicities of jadomycins in the resistant cells as these efflux transporters could be effluxing jadomycins. Similarly, the downregulation of *SLCO3A1* in all of the resistant cells lines, and that of *SLCO4C1* (MCF7-ETP and -MITX cells) and *SLCO5A1* in the MCF7-MITX cells, could as well impede the cytotoxic efficacies of jadomycins if jadomycins depended on these uptake transporters to enter the cells. The upregulation of the metabolic enzymes *CYP1A1* and *CYP1A2* and *BLMH* in the MCF7-ETP cells, and that of *EPHX1* in the MCF7-MITX cells may significantly increase the detoxification of exogenous chemicals and therefore attenuate the cytotoxic effects of jadomycins such as DNV, F and W in these cells.

In contrast, the expression of *ABCC3* was substantially decreased in the MCF7-ETP cells, and the expression of *CYP2B6*, *CYP2C8* and *CYP2E1* (although statistical significance was lacking) was downregulated in all resistant cells, and that of NAT2 was

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decreased in MCF7-TXL cells. These results appear counterintuitive, since one might think that resistant cells should show increased metabolism and efflux to detoxify the intracellular environment. However, since the expression of *ABCC3*, *CYP2B6*, *CYP2C8* and *CYP2E1* is regulated by RXRα whose mRNA expression was downregulated in the resistant lines [140], then the observed downregulation of *RXRα* may result in the downregulation of *ABCC3*, *CYP2B6*, *CYP2C8* and *CYP2E1*. The downregulation of these genes should, in theory, increase the cytotoxic effects of jadomycins; however, further investigation is required to elucidate which enzymes may metabolize jadomycins.

Furthermore, our data show that *PPARγ* expression is reduced in the taxol and etoposide-resistant cells. PPARγ activation results in a more differentiated and less malignant state of cells [141], and inhibitory potential on hepatocellular carcinoma both *in vitro* and *in vivo* [142]. Taken together, these results suggest that decreased *PPARγ* expression may be an alternative mechanism of resistance that drives the growth of the MCF7 cells. This driving force for proliferation may confer the ability to cancer cell to sustain low to moderate doses of chemotherapy and continue to grow even in the presence of low doses of jadomycins.

Study Limitations

Although this study adequately addressed the questions asked, there remain certain limitations to the models used here. With regards to the cytotoxic efficacy of jadomycins, in this study we used only one breast cancer cell line model, MCF7, along with its derivative resistant sublines. This may present a limitation to the external validity of the effects of jadomycins in relation to other breast cancer cell models, and other cancer cell line models. Despite this limitation, the data obtained from the 60-cell line model in NCI demonstrated that jadomycins to effectively eradicate other cancer cell types [99,100]. So, the MCF7 cell model was a good starting point to begin examining jadomycin interactions with ABCB1, ABCC1 and/or ABCG2. However, animal experimentation followed by clinical trials are ultimately required in order to clearly and confidently determine the efficacies of these novel drugs in tumour regression and growth inhibition.

The use of ABC transporter inhibitors should enhance the cytotoxic effects of jadomycins if they are substrates for those efflux transporters; however, this may not be the most effective way to answer this question. The inhibitors used here may inhibit the efflux of other efflux transporters or uptake transporters, which may affect the cytotoxicity of jadomycins. In addition, these inhibitors may also inhibit metabolic enzymes that function in the detoxification of the intracellular environment. So the decrease in the observed IC_{50} if any, may not be 100% due to the inhibition of the targeted efflux transporter. This is clearly suggested by the observation that ABC transporter inhibitors enhanced the cytotoxicity of some jadomycins in the MCF7-CON cells. Ultimately, we would like to directly address the question of jadomycin transport by ABCB1, ABCC1 and ABCG2 by comparing the jadomycin intracellular concentration in

HEK cells stably-transfected with ABCB1, ABCC1 or ABCG2 relative to the HEK-CON cells. We attempted to identify fluorescent jadomycins to be used as probe substrates to directly monitor jadomycins transport using this method. It was initially believed that jadomycin SPhG would fluoresce based on its structure; however we could not detect a strong enough fluorescent signal. Alternate future approaches could include radiolabeled jadomycins or the use of HPLC to quantify intracellular jadomycin levels.

The ABCB1, ABCC1 and ABCG2-overexpressing HEK cells represent useful models in that they allowed us to address the inhibition potential of each jadomycin for each ABC transporter in isolation. However, this is also an artificial system, and may not accurately reflect the cancer microenvironment or the levels of ABC transporter expression in cancer cells. The cancer cells, especially the resistant ones, display complex mechanisms of detoxification of their intracellular environment, including increased expression of metabolic enzymes. Therefore, even if jadomycins in general appeared to be non-inhibitors of the efflux transporters studied, the translation of this observation in the tumour may be different since the ABC transporters may not even be the limiting factors in conferring drug resistance.

The MTT viability assay represents a widely-used assay to test for the inhibition of growth of cells in the presence of cytotoxic drugs. However, the MTT assay does not accurately inform us of cell death, at least not at low concentrations. For this reason, we are currently employing the "lactate dehydrogenase" (LDH) assay to verify the results of the MTT assays. LDH is an enzyme released from cells undergoing necrosis or apoptosis

[143]. The LDH assay would inform us of the cytotoxic potential of jadomycins rather than their growth inhibitory potential.

The dose-response curve of the jadomycins and cell viability is also another limitation for these jadomycins analogues. Control cytotoxic drugs showed a dose-response that required 4 to 6 log units to go from no toxicity to 75% inhibition of cell viability. This effect was only about 2 log units for jadomycins. The translation of this effect is that jadomycins in the future may show a narrow therapeutic window; however, it is hard to accurately predict that in the absence of animal or human data characterizing jadomycin pharmacodynamics *in vivo*.

Future Directions

The next step for this project should be animal experimentation. Now that we have evidence that jadomycins B, L, S and T are equally toxic to drug-sensitive and drug-resistant breast cancer cells, it is appropriate to examine the effects of jadomycins in mouse models of metastatic breast cancer and possibly other cancers. Several endpoints could be examined in response to jadomycins including, tumour regression and inhibition of metastasis. These experiments could be conducted in mouse xenograft models or in mice with intact immune systems [144,145].

Alternatively, given that we have evidence that jadomycins are poor substrates for ABCB1, ABCC1 and ABCG2, one would predict that jadomycins cross the BBB. Knock-out mouse models of ABC transporters are readily available, and would allow us to address if jadomycins cross the BBB *in vivo* and if the brain accumulation of jadomycins is independent of ABCB1, ABCC1 or ABCG2 function. If jadomycins cross the BBB, then it would be very important to test the effectiveness of jadomycins in the eradication of brain tumours *in vivo*, as brain cancers remain some of the deadliest cancers with limited therapeutic options [145]. The fact that jadomycins L and DNV demonstrated efficacy in brain cancer cell models, as observed in the NCI 60 cell line screen [99,100]. As *in vivo* studies progress and provide detail of the pharmacological behaviour of jadomycins in mouse models, the ability for precursor-directed biosynthesis of jadomycins should allow for the rational synthesis of additional jadomycins analogues to

optimize pharmacological activity and minimize adverse drug-drug interactions or host toxicity issues.

Conclusion

In summary, the data presented in this study support a novel role of jadomycins in the eradication of drug-sensitive and drug-resistant MCF7 breast cancer cells. This work also supports the hypothesis that the molecules that are poor substrates and poor inhibitors of efflux transporters may be an effective strategy in the treatment of resistant tumours. Specifically, we demonstrated that jadomycins B, L and S exhibited equal cytotoxic efficacies in drug-sensitive and drug-resistant MCF7 breast cancer cells that overexpress ABCB1, ABCC1 and ABCG2. This supports that the cytotoxic effects exerted by these jadomycins toward breast cancer cells is independent of the function of these efflux transporters. Jadomycins B, L and S appeared to be poor substrates for ABCB1, ABCC1 and ABCG2 as they did not inhibit the function of these transporters at pharmacologically active concentrations. Thus jadomycins B, L and S may be useful as chemotherapeutic agents in cancers that develop resistance through the overexpression of ABCB1, ABCC1 and ABCG2. Furthermore, the lack of inhibition of ABCB1, ABCC1 and ABCG2 by jadomycins is an advantageous pharmacokinetic property. This may potentially allow jadomycins to be safely used in combination chemotherapy regimens while being devoid of the adverse pharmacological effects that were previously observed for ABC transporter inhibitors. For these reasons, jadomycins B, L, and S should be further investigated in other cancer types and in *in vivo* models for their therapeutic potential.

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APPENDIX I: Tables

Table 1. Tissue distribution of select ABC transporters and their select substrates

ABC Transporter	Tissues [13,17,21]	Endogenous Substrates [13,17,146]	Chemotherapeutic Substrates [13,16,33]
ABCA2	Brain	cholesterol	estramustine
ABCB1	Brain, Kidneys, Intestine, Liver	neutral and cationic organic compounds	taxanes, anthracyclines, vinca alkaloids
ABCB2	Ubiquitous	peptides	
ABCC1	Lungs, Testes, Brain	neutral and anionic organic compounds, metabolic conjugates, leukotriene C4	etoposide, anthracyclines, vincristine
ABCC2	Liver, Kidney, Intestine	cyclic AMP	vinblastine, doxorubicin, cisplatin
ABCD1	Peroxisomes	fatty acids	
ABCE1	Spleen, Gonads	oligodenylate binding protein	
ABCG2	Intestine, Liver, Placenta	cyclic GMP	mitoxantrone, methotrexate, doxorubicin
ABCG5	Liver, intestine	sterols	

ABC transporters tend (with exceptions) to efflux a wide variety of compounds, and only a few have been highlighted.

Table 2. PCR Primers

Gene	Accession No.	PCR Forward and Reverse Primers	Product Size, bp
CYCA	NM_021130.3	FW: TTCATCTGCACTGCCAAGAC	138
		RV: TCGAGTTGTCCACAGTCAGC	
ACTB	NM_001101	FW: GGACTTCGAGCAAGAGATGG	234
		RV: AGCACTGTGTTGGCGTACAG	
GAPDH	NM_002046	FW: GAGTCAACGGATTTGGTCGT	238
		RV: TTGATTTTGGAGGGATCTCG	
ABCB1	NM_000927	FW: AGGCCAACATACATGCCTTC	220
		RV: CCTTCTCTGGCTTTGTCCAG	
ABCC1	NM_004996	FW: AGGTGGACCTGTTTCGTGAC	244
		RV: TCCACCAGAAGGTGATCCTC	
ABCC3	NM_003786.3	FW: GGACCCTGCGCATGAACCTG	429
		RV: AGGCAAGTCCAGCATCTCTGG	
ABCC4	NM_005845	FW: GGTTCCCCTTGGAATCATTT	193
		RV: ATCCTGGTGTGCATCAAACA	
ABCC5	NM_005688	FW: AGCTGGGTACTTCCAGAGCA	217
		RV: TCTGTCAACAGCCACTGAGG	
ABCC6	NM_001171	FW: TTGGATTCGCCCTCATAGTC	224
		RV: GGTAGCTGGCAAGACAAAGC	
ABCG2	NM_004827	FW: TTATCCGTGGTGTGTCTGGA	206
		RV: TTCCTGAGGCCAATAAGGTG	
SLCO2B1	NM_007256.4	FW: CTTCATCTCGGAGCCATACC	130
		RV: GCTTGAGCAGTTGCCATTG	
SLCO3A1	NM_013272.2	FW: GCGGTCTTCATTGACACAAG	110
		RV: GAAGAGAAGAAGAGTAAGGCACC	
SLCO4C1	NM_180991.4	FW: GATGAAACTGCTCCACCTCAGAC	147
		RV: CTCCACCTCTTGTTAGATCAGTAGTG	
SLCO5A1	NM_030958	FW: TCCCAGAAATCCTGTTCACC	236
		RV: TTTTGTCCGCTTGTTCACTG	
CYP1A1	NM_00499	FW: CTTGGACCTCTTTGGAGCTG	212
		RV: CGAAGGAAGAGTGTCGGAAG	
CYP1A2	NM_000761	FW: CAATCAGGTGGTGGTGTCAG	245
		RV: CGAAGGAAGAGTGTCGGAAG	
CYP2B6	NM_000767	FW: CCCCATACCCCATTCTCTTT	235
		RV: ACTTGGGAGCTGAGACTGGA	

CYP2C8	NM_000770	FW: CCCGCATGGAGCTATTTTA	195
		RV: TTGCAGGTGATAGCAGATCG	
CYP2E1	NM_000773	FW: ACCCGAGACACCATTTTCAG	201
		RV: TCCAGCACACACTCGTTTTC	
BLMH	NM_000386	FW: AAGTGGTGGTGGACAGGAAG	178
		RV: TTCCGTCCCTGGATCTGTCC	
EPHX1	NM_000120	FW: CACAGCTCTCTTTCCCAAGG	225
		RV: CCGGGAGATGAACCAGTAGA	
NAT2	NM_000015	FW: CCTGCCAAAGAAGAAACACC	175
		RV: GATGAAGCCCACCAAACAGT	
PPARγ	NM_138712	FW: GAGCCCAAGTTTGAGTTTGC	198
		RV: CTGTGAGGACTCAGGGTGGT	
RXRα	NM_002957	FW: GCGCCAACGAGGACATG	699
		RV: CCTAAGTCATTTGGTGCG	

Table 3. The cytotoxic effects of control drugs and jadomycins in drug-sensitive and drug-resistant MCF7 cells

$IC_{50} \pm SEM$ µM (fold-resistance)

Cytotoxic Drug	MCF7-CON	MCF7-TXL	MCF7-ETP	MCF7-MITX
Docetaxel	4.08 ± 1.46	83.52 ± 23.10 (20.49)*		
Etoposide	50.34 ± 15.57		156 ± 17.12 (3.09)*	
Mitoxantrone	1.43 ± 0.51			113.0 ± 16.64 (79.02)*
Jadomycin DNV	1.34 ± 0.44	2.00 ± 0.57 (1.49)	2.22 ± 0.19 (1.65)	4.25 ± 0.56 (3.17)*
Jadomycin L	3.79 ± 0.81	5.51 ± 0.97 (1.46)	6.84 ± 1.47 (1.81)	8.37 ± 0.89 (2.21)
Jadomycin B	4.46 ± 0.85	5.81 ± 0.6 (1.30)	8.09 ± 1.43 (1.81)	9.93 ± 2.68 (2.23)
Jadomycin SPhG	2.08 ± 0.19	5.29 ± 0.89 (2.54)	3.57 ± 0.77 (1.72)	6.56 ± 1.01 (3.16)*
Jadomycin F	0.97 ± 0.39	3.12 ± 0.36 (3.21)*	3.22 ± 0.55 (3.32)*	3.61 ± 0.39 (3.72)*
Jadomycin W	19.36 ± 4.99	73.37 ± 10.61 (3.79)*	50.28 ± 6.07 (2.60)*	75.12 ± 15.83 (3.88)*
Jadomycin S	1.97 ± 0.85	3.67 ± 1.09 (1.86)	4.33 ± 0.74 (2.20)	5.21 ± 0.86 (2.64)
Jadomycin T	2.52 ± 0.38	6.11 ± 1.30 (2.42)	4.86 ± 0.57 (1.93)	5.61 ± 0.66 (2.22)

Jadomycin N	9.46 ± 0.90	10.12 ± 1.62 (1.07)	11.77 ± 1.89 (1.24)	18.46 ± 1.68 (1.95)*

IC$_{50}$ values as determined by MTT assays after treatment with control drugs or jadomycins. Data represent the mean IC$_{50}$s $\pm$ standard error of the mean (SEM) of at least three independent experiments performed in quadruplicate. The numbers in brackets represent the fold-resistance. The fold-resistance was calculated by dividing the IC$_{50}$ of the drug in the drug-resistant cell line by the IC$_{50}$ of that drug in the drug-sensitive cells. * IC$_{50}$ values in drug-resistant cells significantly different from the corresponding IC$_{50}$ values in the drug-sensitive cells, as determined by a one-way ANOVA, followed by Bonferroni multiple comparison test ($p<0.05$).

Table 4. The effects of ABCB1 inhibition on the IC_{50}s of jadomycin DNV, L, B and S in drug-sensitive and taxol-resistant MCF7 cells

$IC_{50} \pm$ SEM µM (fold-reversal)

Cytotoxic Drug	MCF7-CON	MCF7-CON + VRP	MCF7-TXL	MCF7-TXL + VRP
Doxorubicin	1.11 ± 0.14	1.05 ± 0.09 (1.05)	216.8 ± 59.99	37.82 ± 1.34 (5.73)*
Jadomycin DNV	1.93 ± 0.06	1.79 ± 0.58 (1.07)	6.94 ± 1.11	4.71 ± 0.95 (1.47)
Jadomycin L	3.68 ± 0.99	3.70 ± 0.21 (0.99)	8.13 ± 1.81	4.55 ± 1.97 (1.78)
Jadomycin B	1.92 ± 0.12	1.18 ± 0.20 (1.62)*	5.03 ± 1.62	2.75 ± 1.25 (1.82)
Jadomycin S	2.19 ± 0.21	2.31 ± 0.42 (0.94)	5.73 ± 1.55	4.03 ± 0.38 (1.42)

IC_{50} values as determined by MTT assays after treatment with control drugs or jadomycins in the presence or absence of VRP. Data represent the mean IC_{50}s $\pm$ SEM of at least three independent experiments performed in quadruplicate. The numbers in brackets represent the fold-reversal. The fold-reversal was calculated by dividing the IC_{50} values for the group without VRP by the IC_{50} values of the group with VRP. * IC_{50} values in the VRP-treated group were significantly different from IC_{50} values of the no VRP-treated group, as determined by t-test (p<0.05).

Table 5. The effects of ABCC1 inhibition on the IC_{50}s of jadomycin DNV, L, B and S in drug-sensitive and etoposide-resistant MCF7 cells

$IC_{50} \pm$ SEM μM (fold-reversal)

Cytotoxic Drug	MCF7-CON	MCF7-CON + MK-571	MCF7-ETP	MCF7-ETP + MK571
Doxorubicin	0.11 ± 0.0085	0.47 ± 0.083 (0.23)*	15.86 ± 2.05	6.58 ± 3.07 (2.41)*
Jadomycin DNV	1.93 ± 0.06	1.15 ± 0.26 (1.67)*	4.53 ± 1.22	2.20 ± 0.54 (2.05)
Jadomycin L	3.81 ± 0.87	0.90 ± 0.19 (4.23)*	6.77 ± 0.84	3.08 ± 1.41 (2.19)
Jadomycin B	1.92 ± 0.12	0.63 ± 0.24 (3.04)*	5.11 ± 1.45	2.57 ± 1.39 (1.98)
Jadomycin S	2.19 ± 0.21	1.56 ± 0.09 (1.40)	3.62 ± 0.82	2.34 ± 0.74 (1.54)

IC_{50} values as determined by MTT assays after treatment with control drugs or jadomycins in the presence or absence of MK-571. Data represent the mean IC_{50}s $\pm$ SEM of at least three independent experiments performed in quadruplicate. The numbers in brackets represent the fold-reversal. The fold-reversal was calculated by dividing the IC_{50} values for the group without MK-571 by the IC_{50} values of the group with MK-571. * IC_{50} values in the MK-571-treated group were significantly different from IC_{50} values in the no MK-571-treated group, as determined by t-test ($p<0.05$).

Table 6. The effects of ABCG2 inhibition on the IC_{50}s of jadomycin DNV, L, B and S in drug-sensitive and mitoxantrone-resistant MCF7 cells

$IC_{50} \pm$ SEM µM (fold-reversal)

Cytotoxic Drug	MCF7-CON	MCF7-CON + ko143	MCF7-MITX	MCF7-MITX + ko143
Mitoxantrone	7.96 ± 0.89	1.17 ± 0.25 (6.8)*	119.6 ± 21.99	3.85 ± 0.60 (31.06)*
Jadomycin DNV	1.93 ± 0.06	1.74 ± 0.88 (1.10)	5.36 ± 1.06	4.06 ± 0.54 (1.32)
Jadomycin L	3.68 ± 0.99	2.22 ± 0.29 (1.65)	9.81 ± 0.61	6.23 ± 1.54 (1.57)
Jadomycin B	1.92 ± 0.12	0.86 ± 0.18 (2.23)*	5.11 ± 1.69	3.77 ± 1.45 (1.35)
Jadomycin S	2.19 ± 0.21	2.09 ± 0.24 (1.04)	4.42 ± 1.35	3.05 ± 0.97 (1.44)

IC_{50} values as determined by MTT assays after treatment with control drugs or jadomycins in the presence or absence of ko143. Data represent the mean IC_{50}s $\pm$ SEM of at least three independent experiments performed in quadruplicate. The numbers in brackets represent the fold-reversal. The fold-reversal was calculated by dividing the IC_{50} values for the group without ko143 by the IC_{50} values of the group with ko143. * IC_{50} values in the ko143-treated group significantly different from IC_{50} values of the no ko143-treated group, as determined by t-test (p<0.05).

Table 7. The mRNA expression of genes implicated in drug-resistance

Gene	MCF7-CON	MCF7-TXL	MCF7-ETP	MCF7-MITX
Efflux Transporters				
ABCC3	1.00	1.53	**-208.10**	6.52
ABCC4	1.00	**5.92**	**2.19**	**6.17**
ABCC5	1.00	1.20	**2.33**	1.47
ABCC6	1.00	-1.82	**485.00**	-1.39
Uptake Transporters				
SLCO2B1	1.00	-1.64	**7.07**	5.53
SLCO3A1	1.00	**-1.51**	-4.28	**-1.74**
SLCO4C1	1.00	1.44	0.99	**-7.89**
SLCO5A1	1.00	**5.43**	**-5.11**	**-10.87**
Metabolic Enzymes				
CYP1A1	1.00	2.95	**11.28**	-3.32
CYP1A2	1.00	-2.47	**47.82**	**-10.57**
CYP2B6	1.00	-14.41	-3.57	**-71.03**
CYP2C8	1.00	-7.19	-3.79	-102.50
CYP2E1	1.00	-3.81	-3.53	-37.54
BLMH	1.00	**-1.30**	**2.62**	**-1.30**
EPHX1	1.00	2.63	2.24	**9.07**
NAT2	1.00	**-3.60**	1.90	-1.28
Regulatory Genes				
PPARg	1.00	**-17.78**	**-29.53**	-1.84
RXRa	1.00	**-2.80**	**-2.19**	**-2.81**

The mRNA expression of genes implicated in drug resistance in drug-resistant MCF7 cells. The difference in RNA levels is expressed as fold-change of expression relative to control MCF7 cells. Values shown are the average of 3 independent experiments performed in quadruplicate. Numbers in boldface indicate a significant change in expression in comparison to MCF7-CON cells; those with a negative sign indicate a

decrease, as measured by one-way ANOVA, followed by multiple comparison test

(p<0.05).

APPENDIX II: Figures
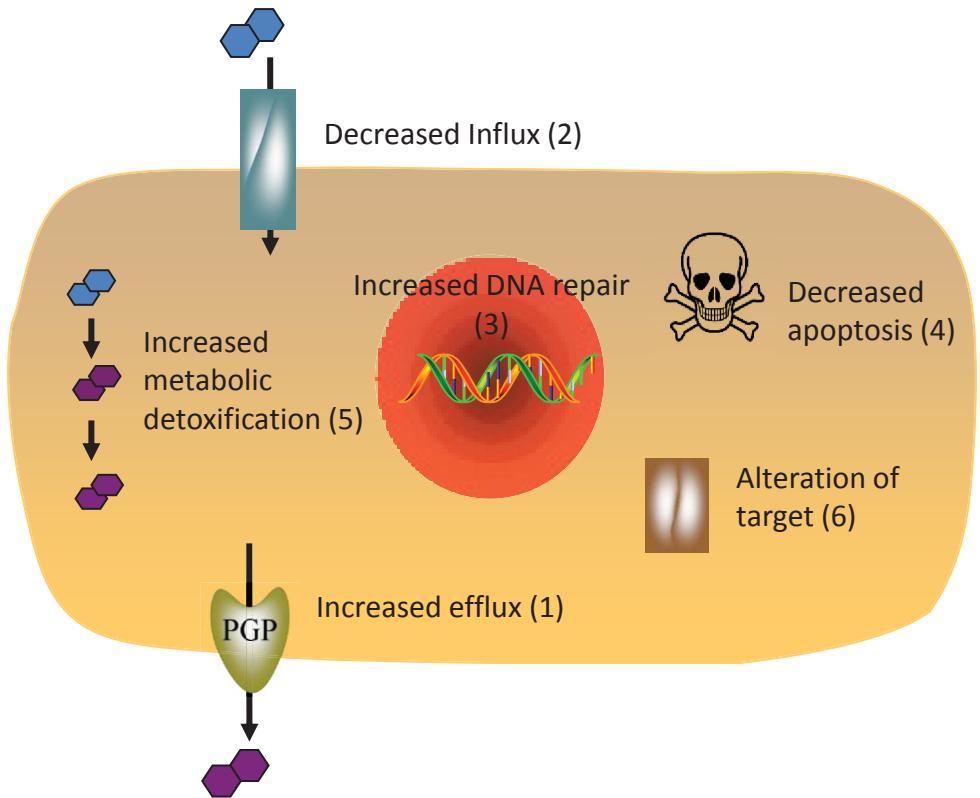
Figure 1. The cellular mechanisms of drug resistance. Cancer cells can acquire resistance to anticancer drugs by several mechanisms: increased efflux of drugs to the extracellular space (**1**), decreased uptake of anticancer drugs into the cytoplasm (**2**), increased DNA repair in response to DNA damage caused by DNA poisons (**3**), decreased apoptosis in response to cytotoxic drugs (**4**), increased metabolic detoxification of drugs (**5**) and alteration in the target of the anticancer drug (**6**).
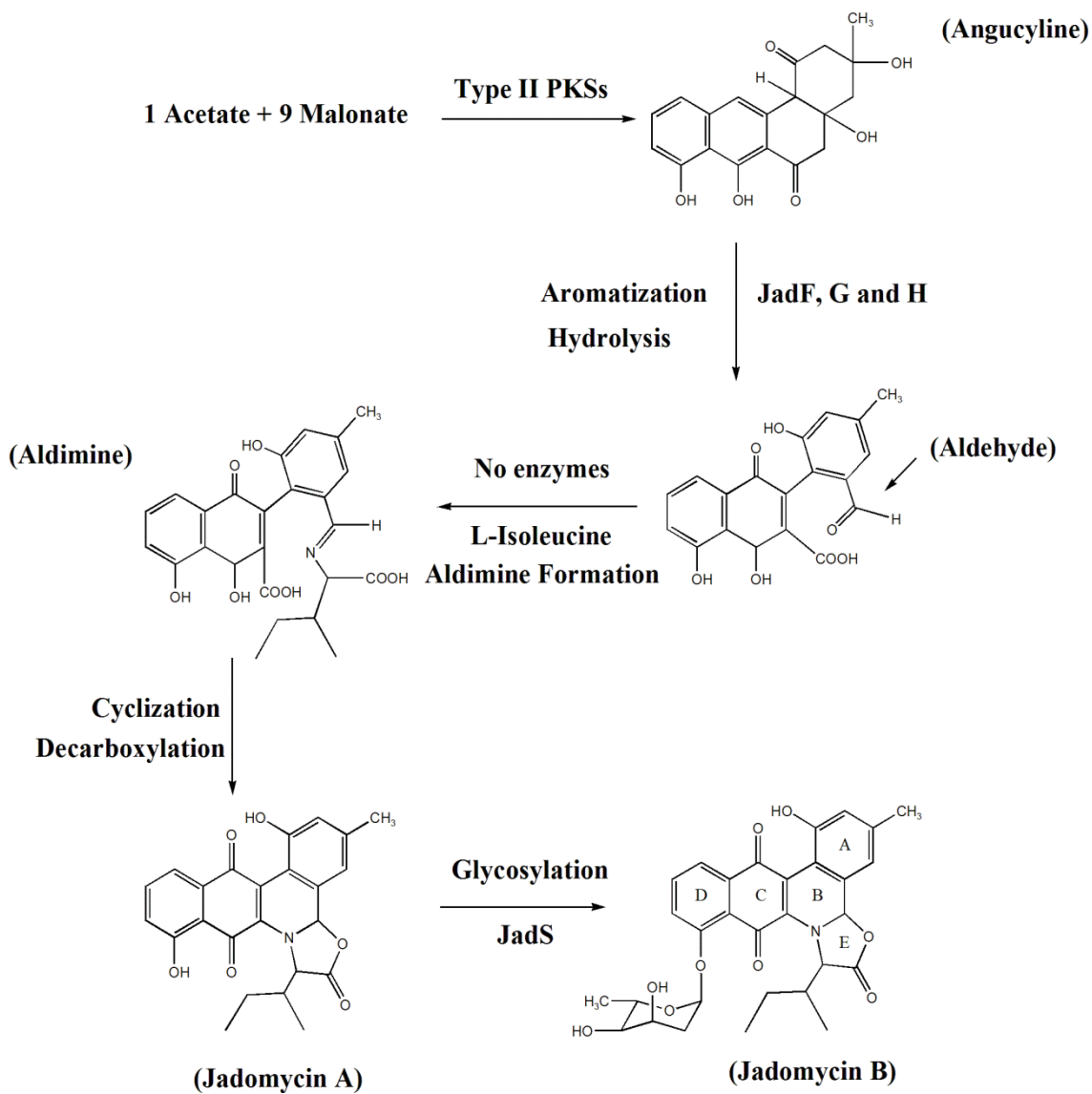
Figure 2. The biosynthetic pathway of jadomycin B.
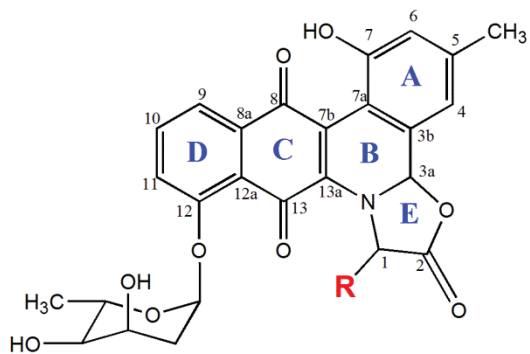
Jadomycin Skeleton
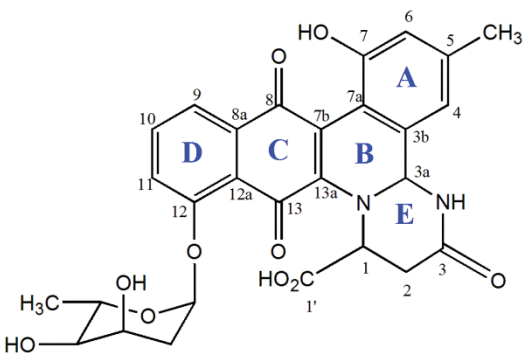
Jadomycin N

1 D-Norvaline (DNV) R=
2 Leucine (L) R=
3 Isoleucine (I or B) R=
4 S-Phenylglycine (SPhG) R=
5 Phenylalanine (F) R=
6 Tryptophan (W) R=
7 Serine (S) R=
8 Threonine (T) R=
9 Asparagine (N) R=

Figure 3. Structures of jadomycin analogues used in this study.
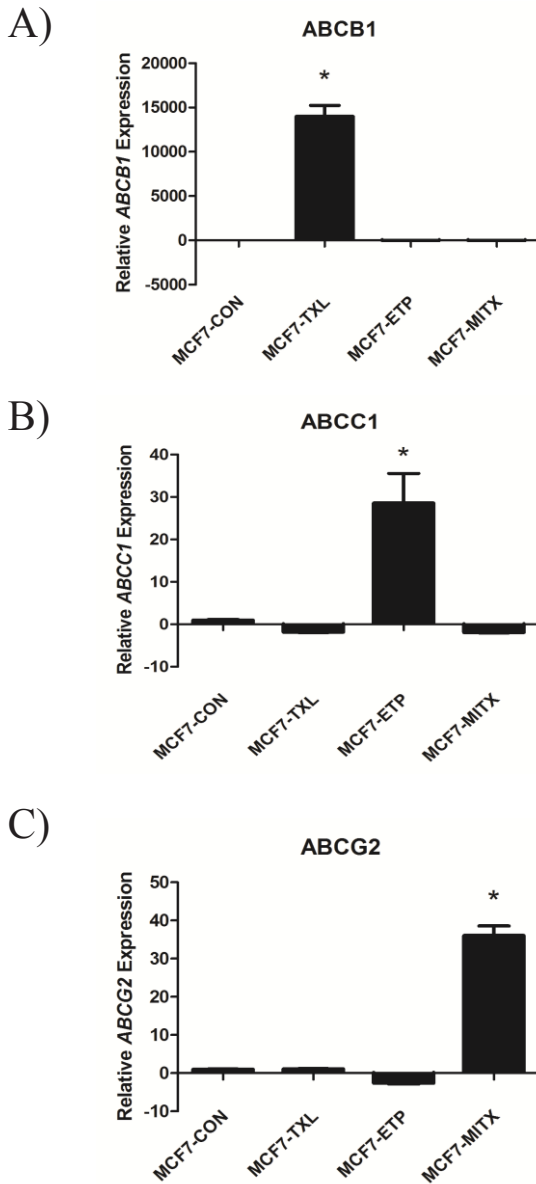
Figure 4. The mRNA expression of *ABCB1*, *ABCC1* and *ABCG2* transporters in drug-sensitive, taxol, etoposide and mitoxantrone-resistant cells. Relative mRNA expression of *ABCB1* (**A**)*, ABCC1* (**B**) and *ABCG2* (**C**) in drug-resistant MCF7 cells relative to MCF7-CON cells. Each bar represents the mean ± SEM of 9 samples pooled from of 3 independent experiments. * indicates values that were significantly different from MCF7-CON, as measured by one-way ANOVA followed by Bonferroni multiple comparison test (p<0.05).
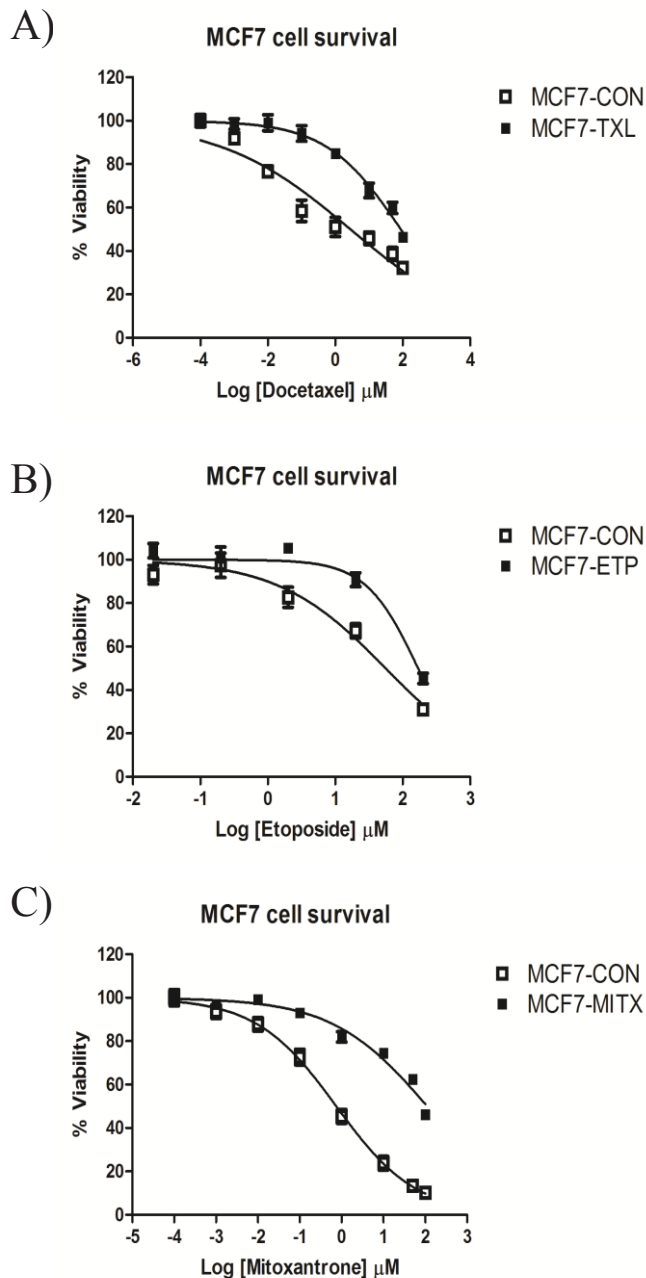
Figure 5. The cytotoxicity of control cytotoxic drugs in drug-sensitive and drug-resistant MCF7 cells. MTT cytotoxicity assays were used to determine the viability of MCF7-CON and MCF7-TXL cells in response to docetaxel (**A**), MCF7-CON and MCF7-ETP cells in response to etoposide (**B**) and in MCF7-CON and MCF7-MITX cells in response to mitoxantrone (**C**). Data points represent the means ± SEM of at least 4 independent experiments performed in quadruplicate.
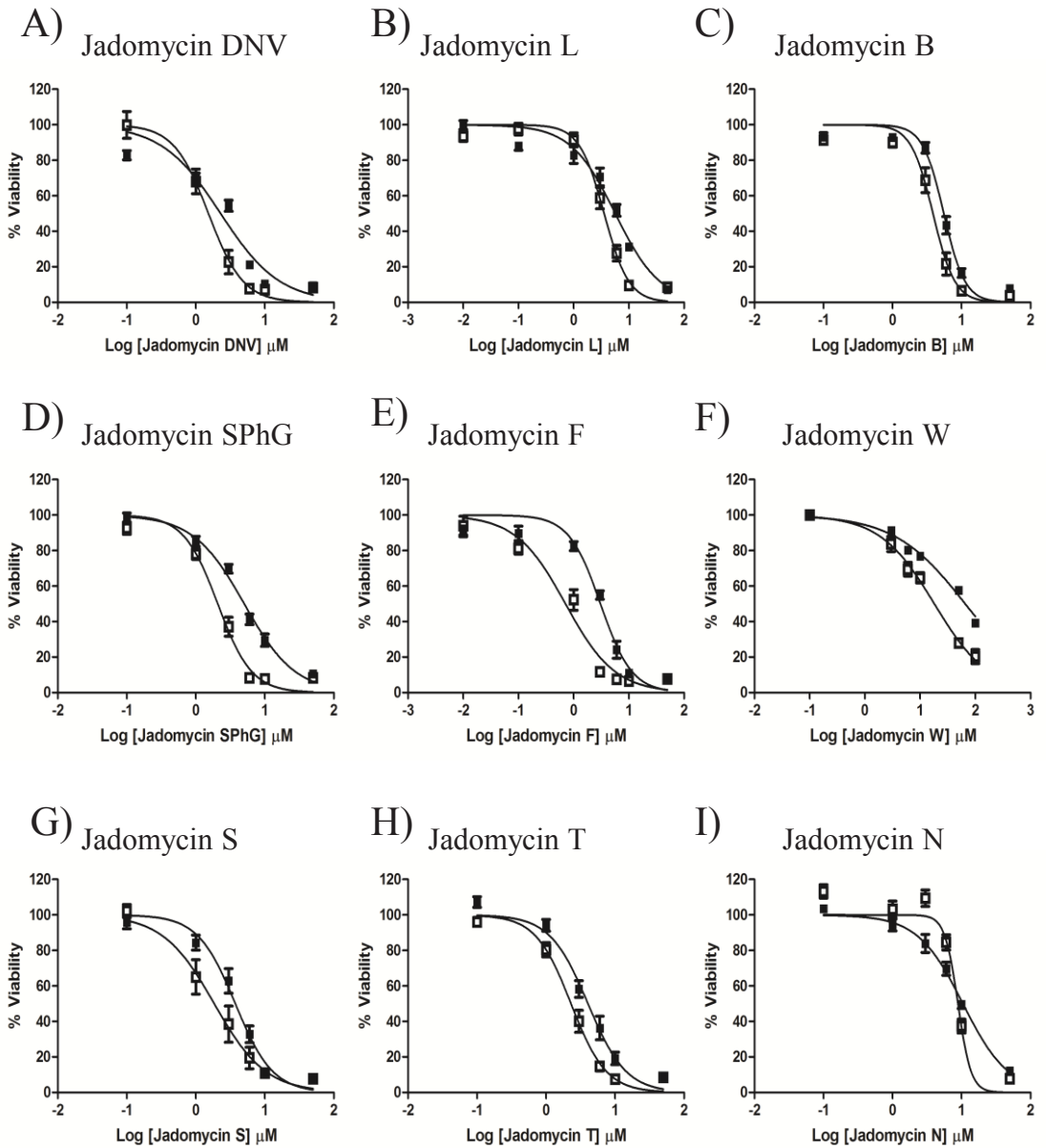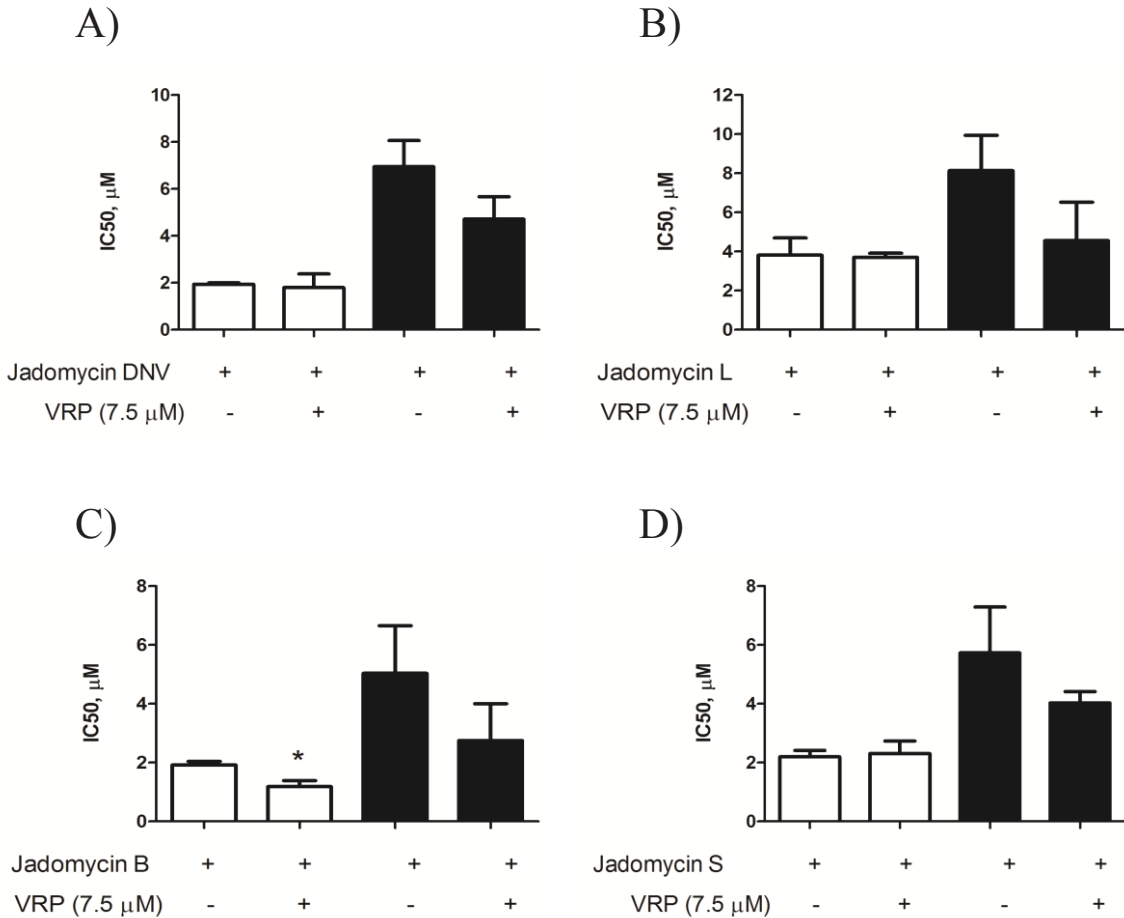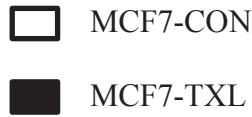
Figure 6. The cytotoxicity of jadomycins in drug-sensitive and taxol-resistant ABCB1-overexpressing MCF7 cells. MTT cytotoxicity assays were used to determine the viability of MCF7-CON versus MCF7-TXL cells in response to jadomycins. Data points represent the means ± SEM of at least 3 independent experiments performed in quadruplicate.
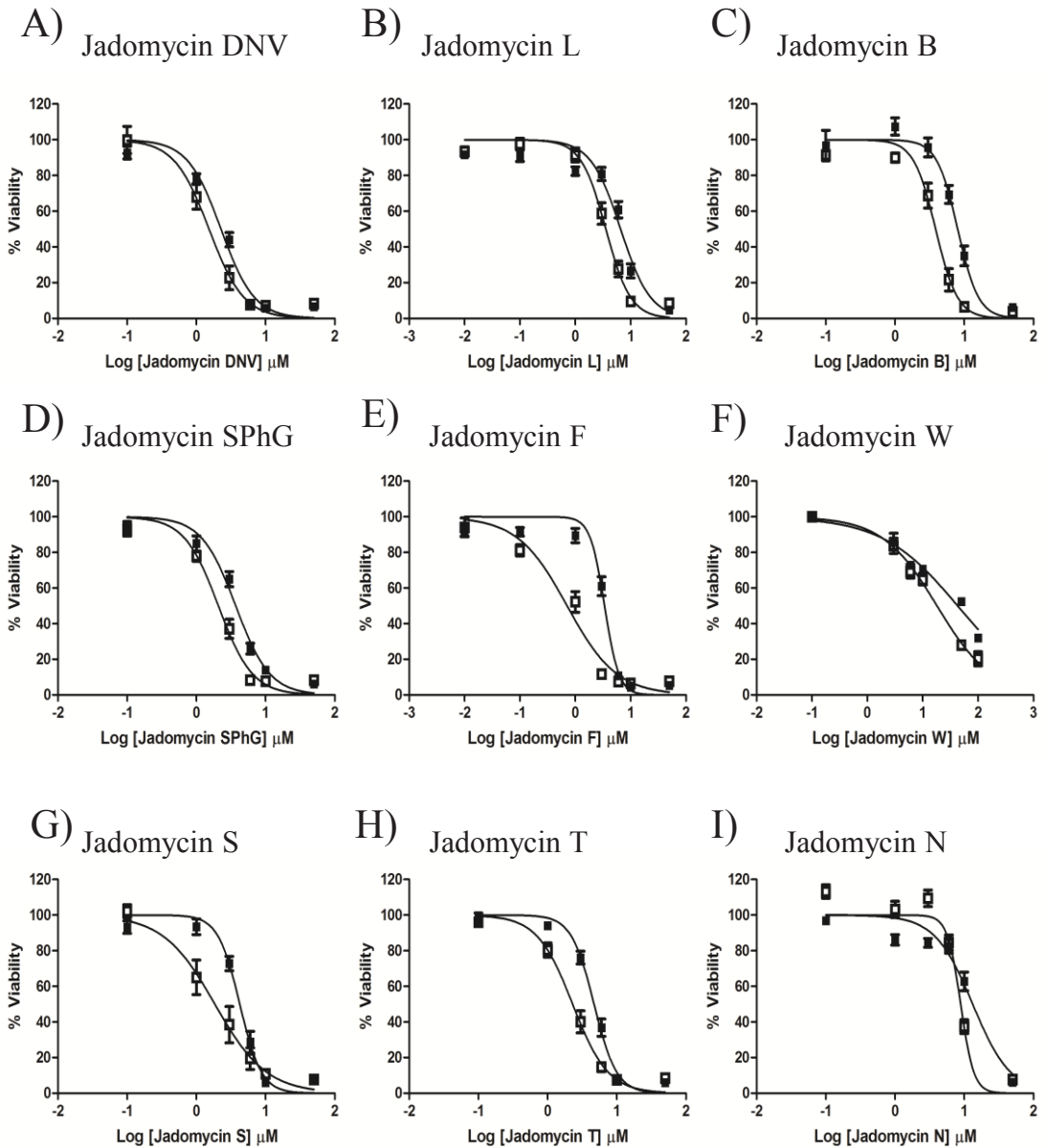
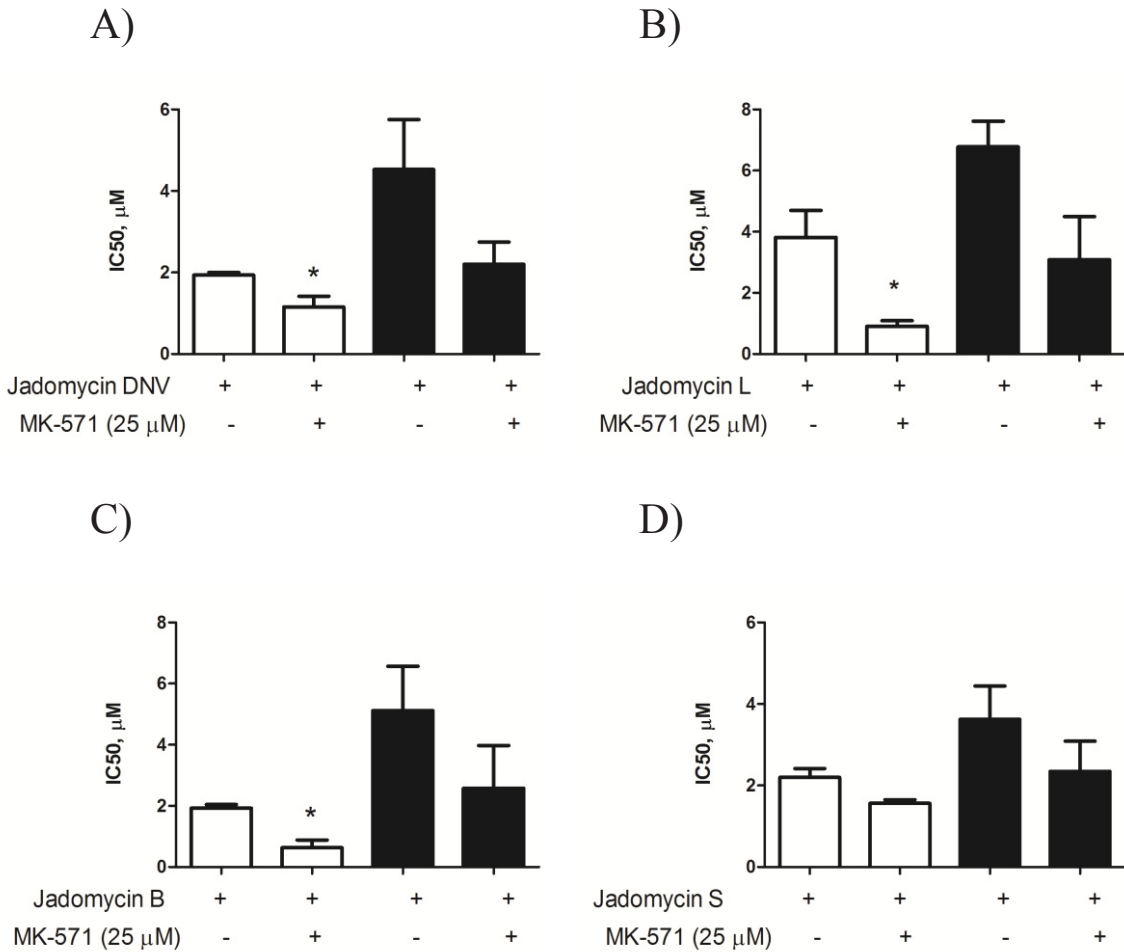**Figure 7. The cytotoxicity of jadomycins in drug-sensitive and etoposide-resistant ABCC1-overexpressing MCF7 cells.** MTT cytotoxicity assays were used to determine the viability of MCF7-CON versus MCF7-ETP cells in response to jadomycins. Data points represent the means ± SEM of at least 3 independent experiments performed in quadruplicate.
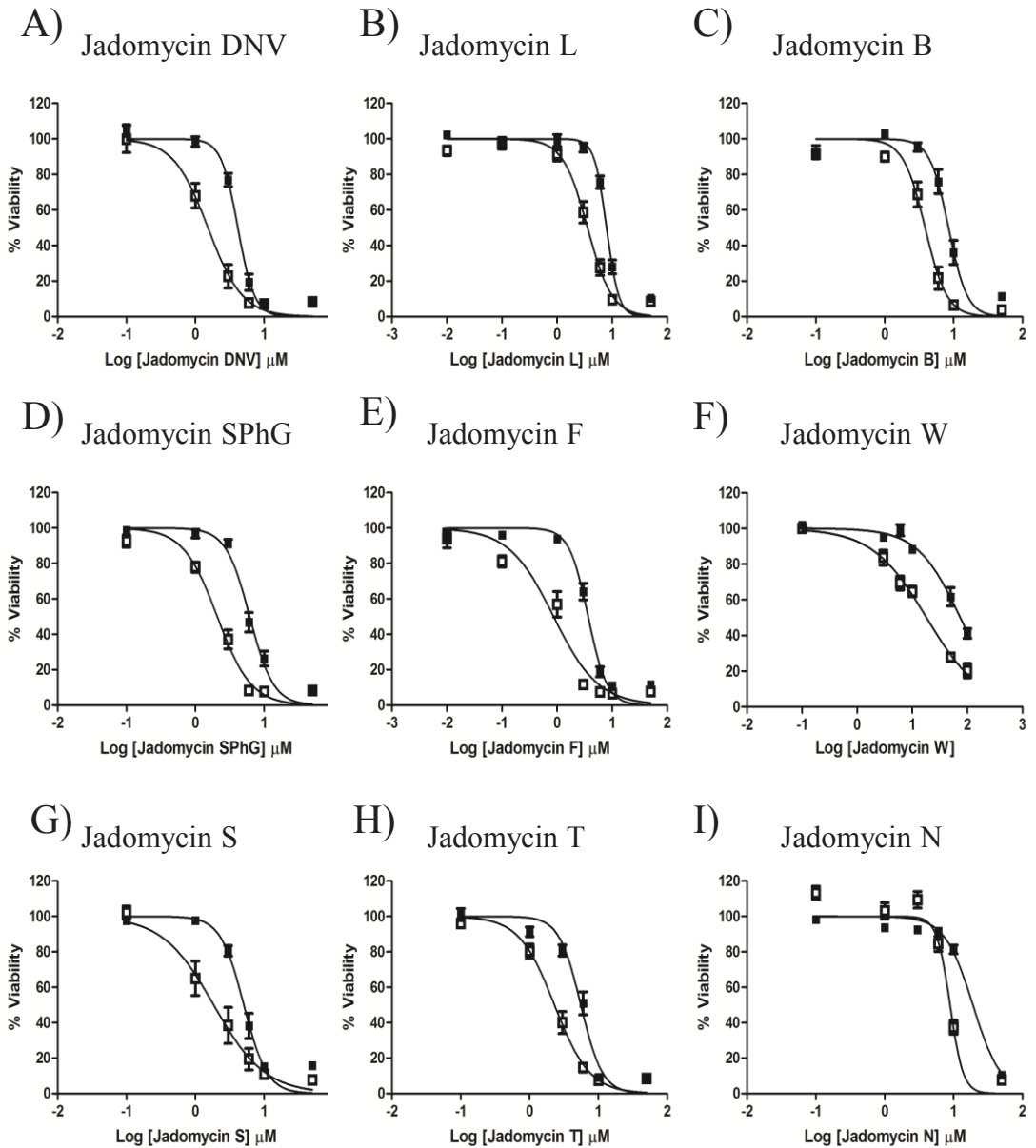
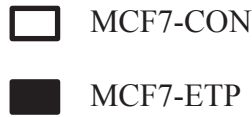**Figure 8. The cytotoxicity of jadomycins in drug-sensitive and mitoxantrone-resistant ABCG2-overexpressing MCF7 cells.** MTT cytotoxicity assays were used to determine the viability of MCF7-CON versus MCF7-MITX cells in response to jadomycins. Data points represent the means ± SEM of at least 3 independent experiments performed in quadruplicate.
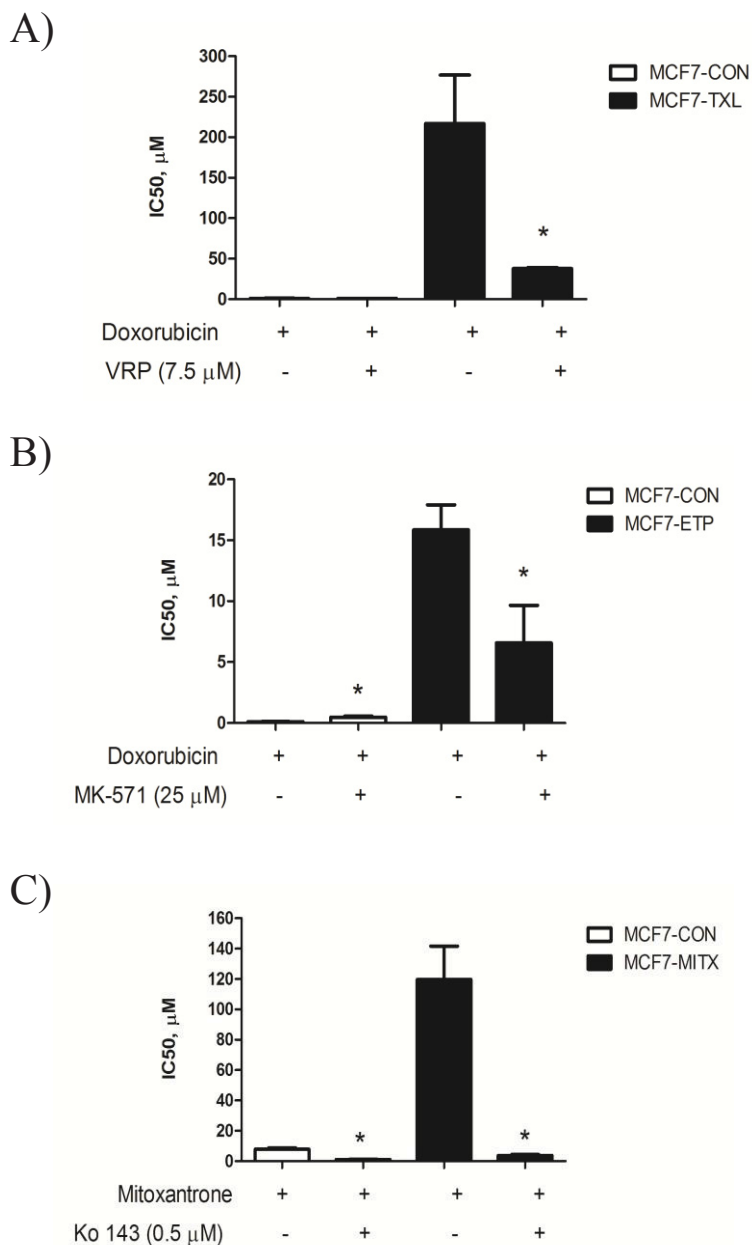
Figure 9. The effect of the inhibition of ABC transporters on the cytotoxicity of control cytotoxic drugs in drug-sensitive and drug-resistant MCF7 cells. Cell survival was determined by MTT assays as described in "Material and Methods". Data represent the mean IC_{50}s ± SEM of 4 independent experiments performed in quadruplicate. * indicates that the IC_{50} value was significantly different in response to the inhibition of ABCB1 (**A**), ABCC1 (**B**) and ABCG2 (**C**) as compared to the respective controls without inhibition, as determined by t-test ($p<0.05$).
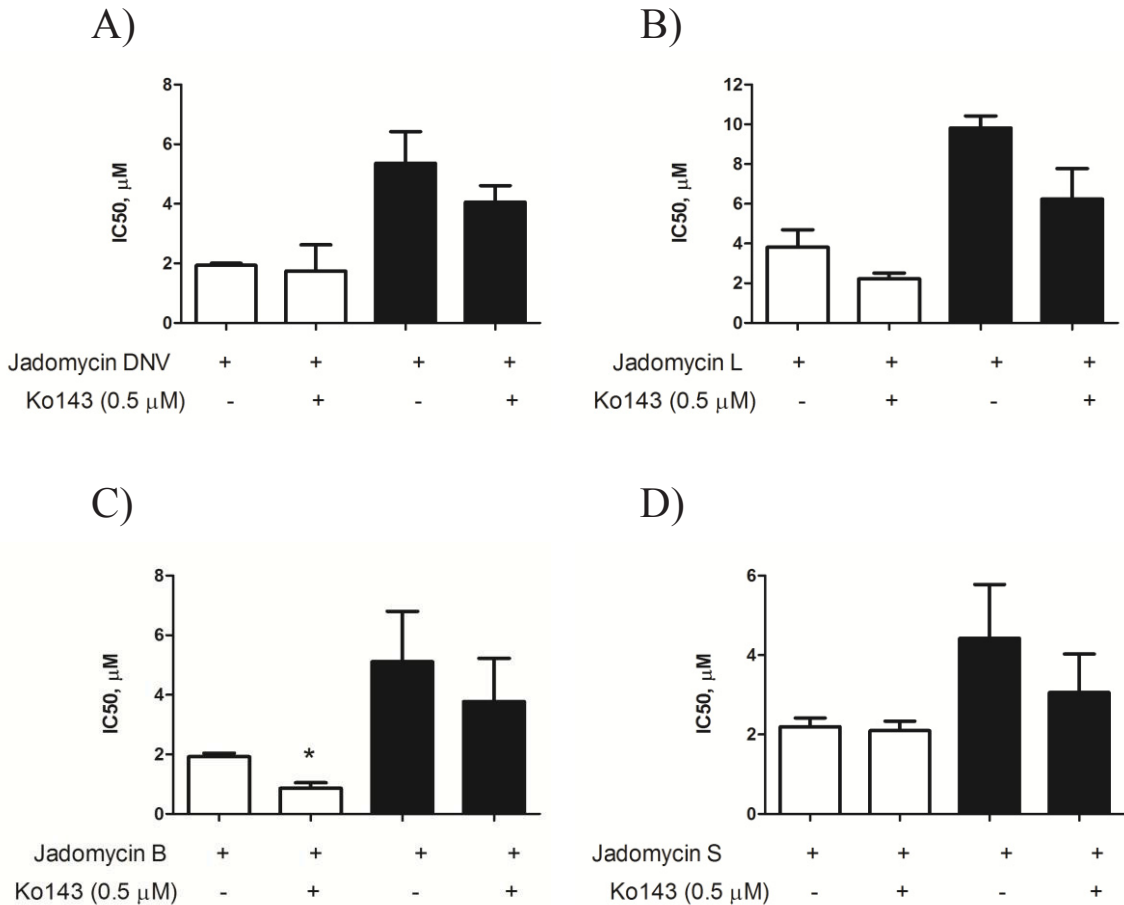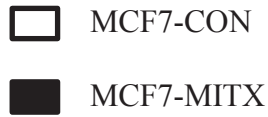
Figure 10. The effect of the inhibition of ABCB1 on the cytotoxicity of jadomycin DNV, L, B and S in drug-sensitive and taxol-resistant MCF7 cells. Cell survival was determined by MTT assays as described in "Material and Methods". Data represent the mean $IC_{50}s \pm$ SEM of at least 3 independent experiments performed in quadruplicate. * indicates that the IC_{50} value was significantly different in response to the inhibition of the ABCB1 transporter compared to the respective cell group without inhibition, as determined by t-test ($p<0.05$).

Figure 11. The effect of the inhibition of ABCC1 on the cytotoxicity of jadomycin DNV, L, B and S in drug-sensitive and etoposide-resistant MCF7 cells. Cell survival was determined by MTT assays as described in "Material and Methods". Data represent the mean IC_{50}s ± SEM of at least 3 independent experiments performed in quadruplicate. * indicates that the IC_{50} value was significantly different in response to the inhibition of the ABCC1 transporter compared to the respective cell group without inhibition, as determined by t-test ($p<0.05$).

Figure 12. The effect of the inhibition of ABCG2 on the cytotoxicity of jadomycin DNV, L, B and S in drug-sensitive and mitoxantrone-resistant MCF7 cells. Cell survival was determined by MTT assays as described in "Material and Methods". Data represent the mean IC_{50}s $\pm$ SEM of at least 3 independent experiments performed in quadruplicate. * indicates that the IC_{50} value was significantly different in response to the inhibition of the ABCG2 transporter compared to the respective cell group without inhibition, as determined by t-test ($p<0.05$).
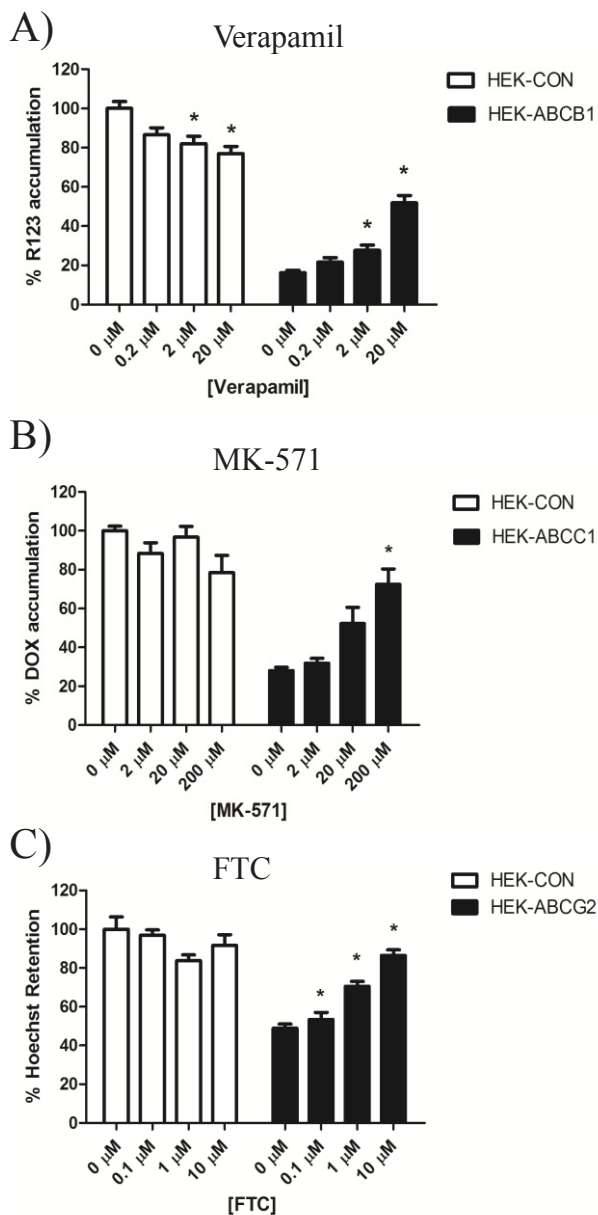
Figure 13. The effects of the control ABC transporter inhibitors VRP, MK-571 and ko143 on ABCB1, ABCC1 and ABCG2 function HEK-293 cells. The R123, DOX and H33342 intracellular accumulation/efflux assays were conducted as described in "Material and Methods. Each bar represents the percent R123 and DOX accumulation and H33342 retention in the treated cells relative to the corresponding vehicle-treated (0 μM bar) HEK-CON cells. Data are shown as means ± SEM of 3 experiments performed in triplicate. * indicates significantly different when compared to the vehicle control treatment (0 μM bar) of the corresponding cell line, as determined by a one-way ANOVA, followed by Bonferroni multiple comparison test (p<0.05).
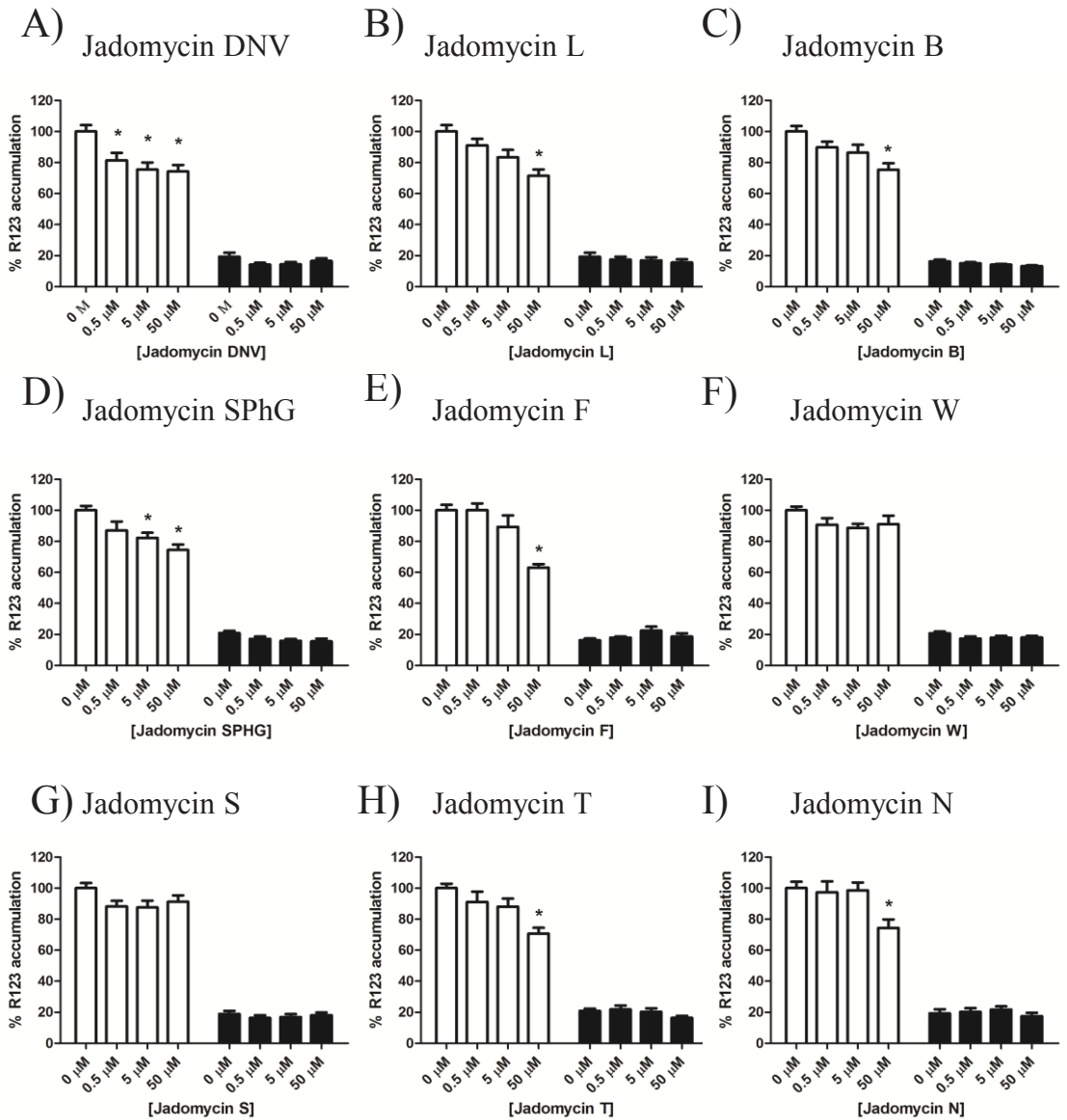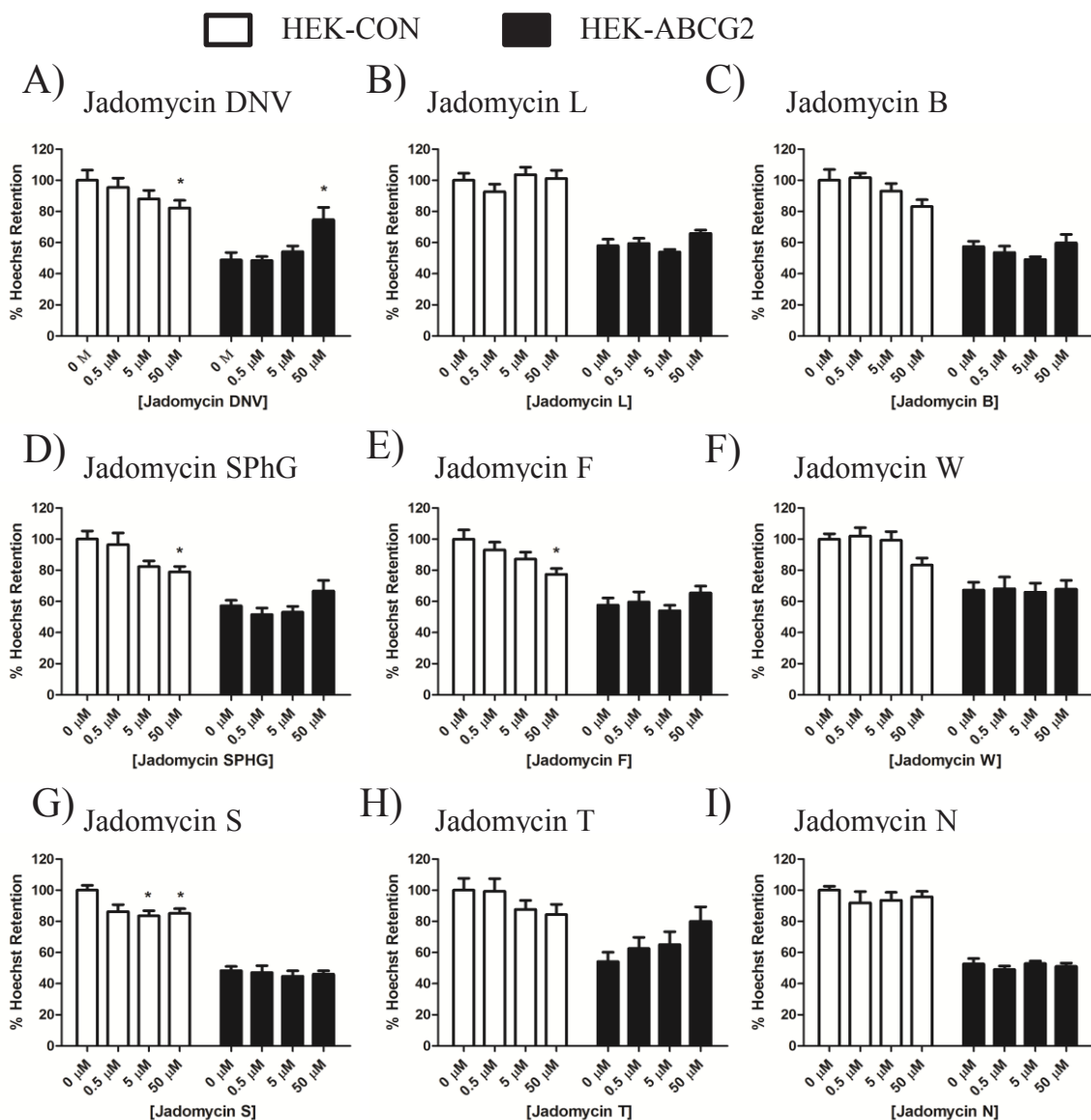
Figure 14. The effects of jadomycins on the accumulation of R123 in HEK-CON and HEK-ABCB1 cells. The intracellular accumulation of R123 in HEK-CON and HEK-ABCB1 cells was measured by fluorescence spectroscopy following incubation in 10 μM R123 solution with or without jadomycins for 2 hours at 37°C. R123 levels are expressed as the percentage of R123 accumulation in vehicle-treated HEK-CON cells. Data are shown as means ± SEM of 3 experiments performed in triplicate. * indicates significantly different when compared to the vehicle control treatment of the corresponding cell line, as determined by a one-way ANOVA, followed by Bonferroni multiple comparison test (p<0.05).
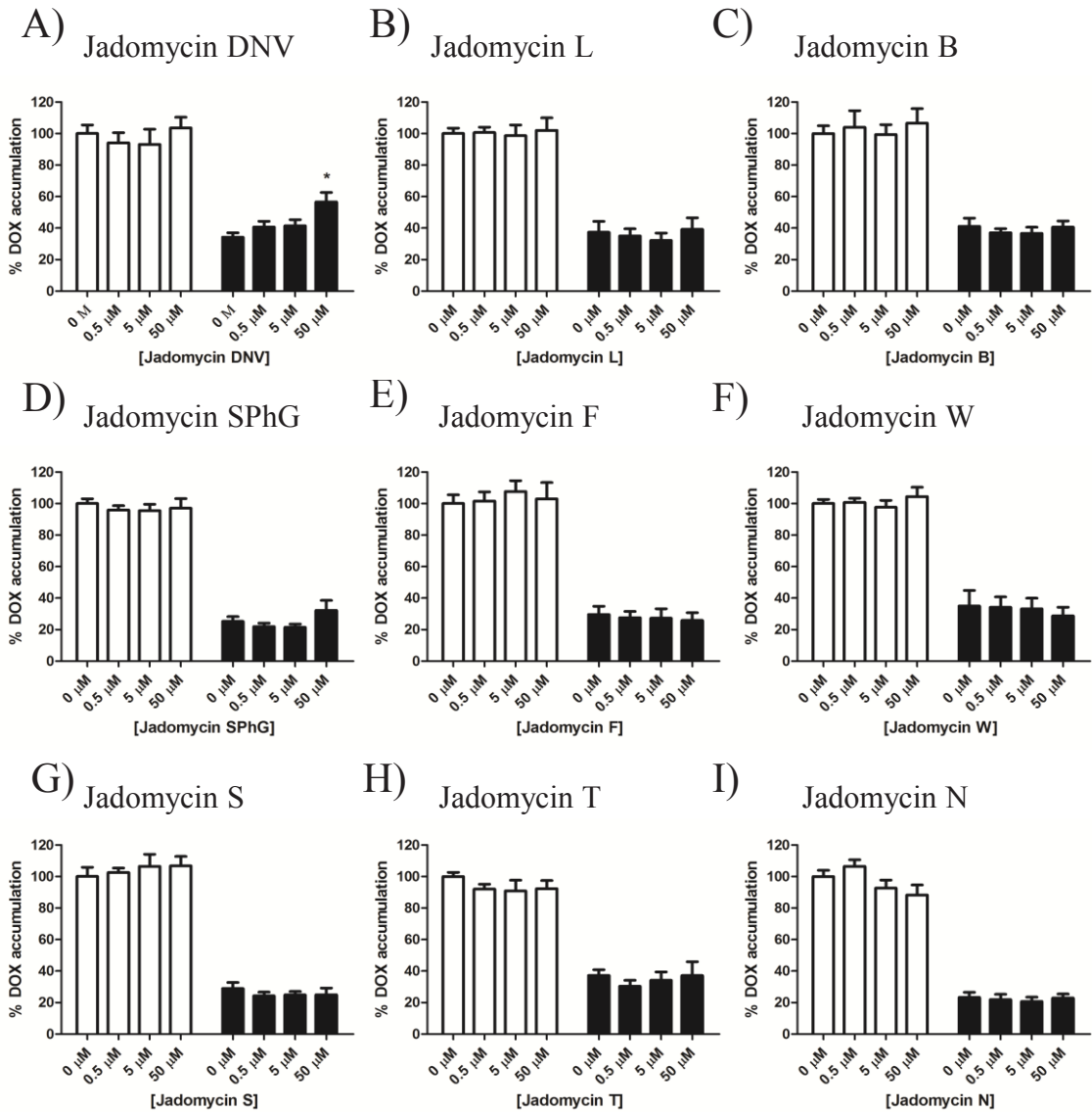
Figure 15. The effects of jadomycins on the accumulation of DOX in HEK-CON and HEK-ABCC1 cells. The intracellular accumulation of DOX in HEK-CON and HEK-ABCC1 was measured by fluorescence spectroscopy following incubation in 10 μM DOX solution with or without jadomycins for 2 hours at 37°C. DOX levels are expressed as the percentage of DOX accumulation in vehicle-treated HEK-CON cells. Data are shown as means ± SEM of 3 experiments performed in triplicate. * indicates significantly different when compared to the vehicle control treatment of the corresponding cell line, as determined by a one-way ANOVA, followed by Bonferroni multiple comparison test ($p < 0.05$).

Figure 16. The effects of jadomycins on the retention of H33342 in HEK-CON and HEK-ABCG2 cells. HEK-CON and HEK-ABCG2 cells were incubated in 10 μM H33342 solution with or without jadomycins for 0.5 hours. The cells were allowed to efflux H33342 in the presence or absence of jadomycins for 1 hour. The intracellular retention of H33342 in was then measured by fluorescence spectroscopy. H33342 retention levels are expressed as the percentage of H33342 retention in vehicle-treated HEK-CON cells. Data are shown as means ± SEM of 3 experiments performed in triplicate. * indicates significantly different when compared to the vehicle control treatment of the corresponding cell line, as determined by a one-way ANOVA, followed by Bonferroni multiple comparison test ($p < 0.05$).