

STUDY OF JADOMYCIN PHARMACOKINETICS AND ANTI-BREAST CANCER  
ACTIVITY IN BALB/C MICE

by

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## ABSTRACT

Breast cancer is the most common form of cancer in Canadian women. Despite numerous therapeutic options, multidrug resistance (MDR) remains an obstacle to successful therapy.

Jadomycins are natural products derived from the soil bacteria *Streptomyces venezuelae* ISP5230 and maintain cytotoxicity in multidrug resistant human breast cancer cell lines. The objectives were to determine the single dose pharmacokinetics and safety of jadomycins and their effects on 4T1 breast primary tumors and lung metastasis in mice.

In the pharmacokinetic studies, intraperitoneal-administered jadomycins B, S, and F were rapidly absorbed, achieved serum concentration in the predicted therapeutic range and had a biphasic distribution and elimination profile. In tumor studies jadomycin B partially reduced tumor volumes and lung metastasis.

In conclusion, jadomycins are safe to administer chronically to mice at doses up to 13.8 mg/kg twice daily. The partial anti-tumor and anti-metastatic effects of jadomycin B were encouraging results and require further study.

## LIST OF ABBREVIATIONS USED

4T1	Mouse mammary carcinoma cell line
5-FU	5-fluorouracil
6-TG	6-thioguanine
ABC	ATP-binding cassette
ADME	Absorption, distribution, metabolism and excretion
ADP	Adenosine diphosphate
AIs	Aromatase inhibitors
AKT	Protein kinase B
B	Isoleucine
BBB	Blood-brain barrier
BCL-2	B cell lymphoma 2
BCRP	Breast cancer resistance protein
<i>BRCA1/2</i>	Breast cancer susceptibility genes 1/2
CCAC	Canadian Council of Animal Care
$C_{max}$	Maximum serum concentration
CML	Chronic myeloid leukemia
CYP3A4	Cytochrome P450 3A4
CYP450s	Cytochrome P450 metabolism enzymes
DCIS	Ductal carcinoma in situ
DMSO	Dimethylsulfoxide
DNL	D-norleucine



DNV	D-norvaline
DSB	Double-stranded break
ECGs	Electrocardiograms
EGFR2	Epidermal growth factor receptor 2
ELISA	Enzyme-linked immunosorbent assay
EphA2	Eph receptor A2
EpoR	Activated receptor for erythropoietin
ER	Estrogen receptor
F	Phenylalanine
FBS	Fetal bovine serum
FDA	Food and drug administration
<i>GAPDH</i>	Glyceraldehyde phosphate dehydrogenase
GI <sub>50</sub>	Concentration at which growth inhibition is half its maximal value
HER2	Human epidermal growth factor receptor 2
hOCT1	Human organic cation transporter 1
HSV	Herpes simplex virus
IC <sub>50</sub>	Half maximal inhibitory concentration
IGF1R	Insulin-like growth factor 1 receptor
IP	Intraperitoneal
ISP	International Streptomyces project
IT	Intratumoral
IV	Intravenous
LCMS	Liquid chromatography-mass spectrometry

MBC	Metastatic breast cancer
MCF7	Michigan Cancer Foundation – 7 / Human invasive ductal carcinoma cell line
MCF7-CON	Control MCF7 cell line
MCF7-ETP	Etoposide-resistant MCF7 cell line
MCF7-MITX	Mitoxantrone-resistant MCF7 cell line
MCF7-TXL	Paclitaxel-resistant MCF7 cell line
MDR	Multidrug resistance
MDSC	Myeloid-derived suppressor cell
MeCN	Acetonitrile
MeOH	Methanol
MM	Metastatic melanoma
MTOR	Mammalian target of rapamycin
MTT	Thiazolyl blue methyltetrazolium bromide
NAC	N-acetylcysteine
NOD/SCID	Nonobese diabetic/severe combined immunodeficient
OS	Overall survival
PARP	Poly adp-ribose polymerase
P <sub>i</sub>	Phosphate
PI3K	Phosphoinositide 3-kinase
PR	Progesterone receptor
QC	Quality control
RFS	Relapse-free survival

ROS	Reactive oxygen species
RTK	Receptor tyrosine kinase
S	Serine
SN-38	7-ethyl-10-hydroxycamptothecin
SNPs	Single nucleotide polymorphisms
SOD	Superoxide dismutase
SXR	Nuclear steroid and xenobiotic receptor
TGF- $\beta$	Transforming growth factor- $\beta$
TGI	Total growth inhibition
TK	Tyrosine kinase
TKIs	Tyrosine kinase inhibitors
$T_{\max}$	Time when the maximum serum concentration is reached
TNBC	Triple-negative breast cancer
TP53	Tumour protein p53
TrxR	Thioredoxin reductase
TXL	Paclitaxel (Taxol)
$V_d$	Volume of distribution

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## CHAPTER 1.00.00: INTRODUCTION

### *1.01.00: Cancer statistics, metastatic breast cancer, and multidrug resistance*

Breast cancer is the most common cancer diagnosis in Canadian women and accounts for about 1 in 4 new cancer diagnoses.<sup>1</sup> Approximately 20-30% of the women who are diagnosed with breast cancer will ultimately develop metastatic breast cancer (MBC).<sup>1</sup> MBC, also known as late-stage or stage IV breast cancer, has a 5-year relative survival rate of approximately 22%.<sup>2</sup> This low survival rate is largely due to the development of multidrug resistance (MDR), which is the development of cancer cell resistance to multiple classes of drugs that are structurally and mechanistically unrelated.<sup>3</sup> MDR breast cancers are less affected by many anticancer treatments, thereby requiring increased doses to elicit the same therapeutic effect, which can be impractical or impossible due to toxicity and safety concerns.<sup>4</sup> MDR can occur before the start of therapy, termed innate resistance, and results from the natural selection of cancer cells that happen to display a drug-resistant phenotype.<sup>5</sup> Alternatively, acquired MDR develops when cancer cells are exposed to anti-cancer drugs that fails to eliminate all cancer cells in the patient, giving a survival-advantage to the remaining cells that express the MDR phenotype.<sup>6</sup> MDR remains a major problem in the treatment of cancer, warranting further research.

### *1.02.00: Current breast cancer therapies*

Selecting the appropriate type of breast cancer treatment depends on the associated biology and behavior of the cancer. Factors to consider when selecting a breast cancer treatment include the tumor stage and subtype, availability of genomic markers, and nature of the genetic mutations.

Patient factors such as age, health, and menopausal status are also considered.

Oftentimes, treatment plans are highly personalized due to the complex heterogeneity of breast cancers.<sup>7</sup>

#### *1.02.01: Surgery*

If a breast tumor is sufficiently small and accessible, surgery may be used for its removal.<sup>8</sup> A lumpectomy will remove the tumor as well as some healthy surrounding tissue of the breast whereas a mastectomy is the removal of the entire breast.<sup>9</sup> Another common surgery performed in breast cancer patients is sentinel lymph node biopsy or axillary lymph node dissection in order to help determine if and to what extent the breast cancer has spread.<sup>10</sup> If there are no cancerous cells present in the sentinel lymph node, then it is more likely the cancer has not metastasized. On the other hand, if cancerous cells are present, then this may warrant further surgery and tests to search for metastases.<sup>11</sup>

#### *1.02.02: Radiation*

Radiation therapy, or radiotherapy, is a targeted method for killing breast cancer cells that may have remained after surgery.<sup>12,13</sup> Radiation therapy can reduce the risk of recurrence after surgery and uses high-energy beams such as x-rays that damage cell DNA.<sup>12</sup> Although radiation therapy damages both healthy and cancerous cells, it affects cancer cells more due to how quickly they grow and divide.<sup>13</sup> Radiation may be delivered from outside the body by a machine or from within by seeds or pellets of material that give off radiation, which can improve long-term patient outcomes.<sup>12</sup>

### *1.02.03: Hormone therapy*

Hormone therapy, also known as endocrine therapy, can be effective when tumors test positive for estrogen or progesterone receptors (ER-positive or PR-positive). Tumors that have these receptors can use these hormones to promote proliferation. By blocking these hormones or their receptors with drugs, like tamoxifen or aromatase inhibitors, it is possible to shrink a patient's tumor.<sup>14,15</sup>

Tamoxifen blocks the binding of estrogen to breast cancer cells through competitive inhibition of the estrogen receptor (ER).<sup>16</sup> It has a low affinity for its intended receptor and is itself a prodrug which gains a much higher affinity when metabolized to endoxifen by cytochrome P450 isoforms CYP2D6 and CYP3A4.<sup>17</sup> While treatment with tamoxifen can be effective, development of tamoxifen resistance eventually takes place resulting in cancer progression.<sup>18</sup> Aromatase inhibitors (AIs) work by decreasing the amount of estrogen being produced by tissues other than the ovaries in postmenopausal women.<sup>19</sup> They inhibit the enzyme aromatase, which converts androgens into estrogens.<sup>19</sup>

### *1.02.04: Targeted therapy*

Targeted therapy treats MBC by targeting specific genes and proteins involved in cancer growth and survival.<sup>20,21</sup> It does so by addressing the issue of overexpression of human epidermal growth factor receptor 2 (EGFR2), also known as HER2 or erbB-2. The human epidermal growth factor (HER) family is made up of transmembrane receptors that are mediators of cell growth and development.<sup>22,23</sup> When overexpressed, receptors from this family such as HER2 can play a role in the development of breast cancers. HER2 overexpression is seen in ~15-20% of breast cancers and is associated with

aggressive disease as well as decreased survival.<sup>24,25</sup> Therapies that have been developed against HER2 overexpressing breast cancers include monoclonal antibodies as well as small-molecule tyrosine kinase inhibitors (TKIs). Trastuzumab is a monoclonal antibody that interferes with the HER2 receptor, and Lapatinib is a small molecule TKI of HER1 and HER2, either of which may be used in patients with advanced or metastatic breast cancer who have HER2 overexpressing tumors.<sup>21,26</sup>

#### *1.02.05: Immunotherapy*

Immunotherapy, also called biologic therapy, is a treatment that uses the body's immune system in order combat infection or disease and is increasingly used for cancer treatment in conjunction with other standards of care.<sup>27</sup> It has great potential in terms of being highly personalized and being able to target and eliminate metastases based on the expression of specific proteins.<sup>28</sup> An example of immunotherapy is the targeting of PD-1 and PD-L1. PD-1 is a checkpoint protein expressed on activated T-cells, natural killer cells, and B-cells.<sup>29,30</sup> It normally keeps T cells from attacking other cells by attaching to the protein PD-L1. Some cancer cells have high levels of PD-L1, which enables them to evade immune destruction. PD-1 and PD-L1 inhibitors are monoclonal antibodies that prevent their binding and can help boost immune response against cancerous cells.

Immunotherapy also includes oncolytic virus therapy, which is the use of viruses that are able to selectively replicate in cancer cells to exert their anti-tumor action. In 2015, the United States Food and Drug Administration (FDA) approved a genetically modified version of the herpes simplex virus (HSV) as the first oncolytic virus therapy for melanoma.<sup>31</sup>



Cancer vaccines are another form of immunotherapy and are administered to either healthy or cancerous patients with the intent of killing cancer cells by bolstering the patient's own immune response or preventing cancer from developing. In 2010, the FDA approved the first cancer vaccine, which was approved for treatment of metastatic prostate cancer. The use of this vaccine in clinical trials increased the overall survival time of men with metastatic castration-resistant prostate cancer by approximately 4 months.<sup>32</sup> There are numerous other types of sub-therapies within immunotherapy, making it a diverse and potentially effective form of treatment.

#### *1.02.06: Chemotherapy*

Chemotherapy can be used in all stages of breast cancer, can be used as an adjuvant or neoadjuvant therapy, and is a common option for the treatment of metastatic disease. Chemotherapy is the use of cytotoxic drugs to damage and kill cancer cells so they can't grow and replicate. This treatment uses drug such as taxanes (taxol or paclitaxel), anthracyclines (doxorubicin), vinca alkaloids (vinblastine), and topoisomerase inhibitors (irinotecan), among many others.<sup>33,34</sup> In some cases, such as in patients who have ductal carcinoma in situ (DCIS), the most common type of non-invasive breast cancer originating from the milk ducts, there is a relatively low risk of cancer spreading to other areas of the body so chemotherapy may not be recommended.<sup>35</sup>

First-line chemotherapy for breast cancer depends on its stage and hormone receptor and HER2. For patients with MBC that is HER2-negative, single-agent chemotherapy is the standard and is usually given in the form of anthracyclines or taxanes instead of combination treatments which may add to the toxicity associated with treatment.<sup>36</sup>

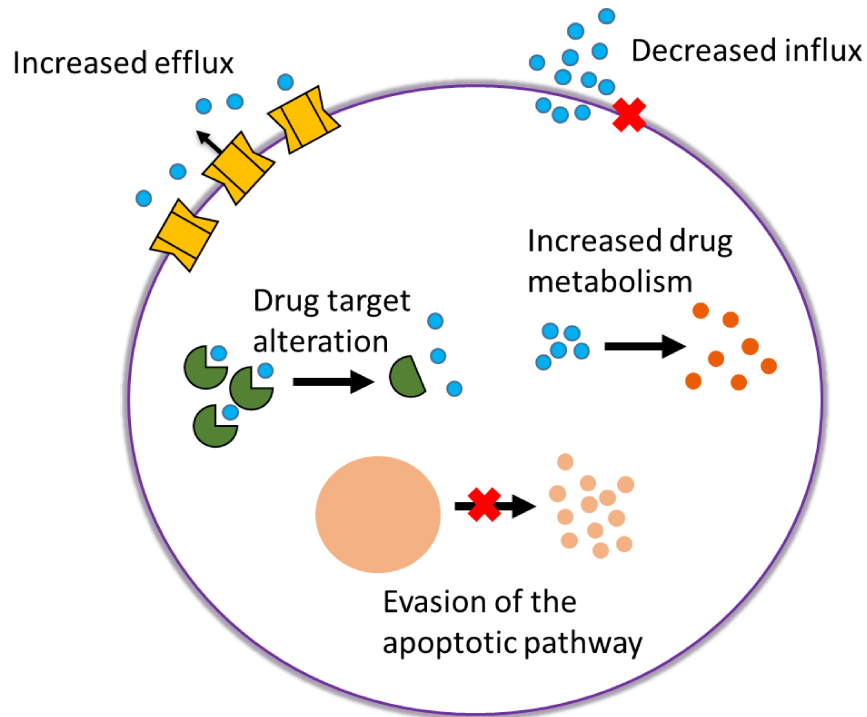
Subsequent treatments may include drugs such as gemcitabine, platinum agents, and vinca alkaloids.<sup>37,38</sup> Different chemotherapy drugs are administered because of their unique mechanisms of action, which increases the chances of killing all cancer cells and preventing the formation of MDR cells. For example, paclitaxel promotes microtubule polymerization and stabilization in living cells.<sup>39</sup> It arrests cells in mitosis, which eventually leads to cell death.<sup>40</sup> Anthracyclines on the other hand, can interact with the DNA topoisomerase II complex or intercalate the DNA directly, which disrupts DNA replication and repair and ultimately induces apoptosis.<sup>41</sup>

#### *1.02.07: Therapy for triple-negative breast cancer (TNBC)*

The specific focus of my project is TNBC, which is negative for ER, PR, and HER2.<sup>42</sup> TNBC accounts for approximately 15% to 20% of invasive breast cancer cases and is usually aggressive, exhibiting higher tumor grades, as well as frequent metastasis to lymph nodes, and occurs more often in young patients.<sup>43,44</sup> Since TNBCs can be quite resistant to chemotherapy, this severely limits the treatment options for TNBCs as surgery and chemotherapy, alone or in combination, are the mainstay of treatment.<sup>42,45</sup> Both anthracyclines and taxanes are commonly used as neoadjuvant treatments of TNBC, but their efficacy as well as that of other chemotherapeutic drugs often proves to be suboptimal and eventually results in disease progression.<sup>46</sup> To date, there has not been a single targeted therapy approved for the treatment of TNBC, which may be due to treatment challenges associated with tumor heterogeneity.<sup>46</sup> Overall, TNBC still lacks clear prognostic markers and effective treatment options, therefore warranting further research into novel drugs that are able to effectively kill this subset of MDR cancers.

### 1.03.00: Mechanisms of MDR

There are a number of MDR mechanisms that are well-understood, and which are summarized in **Figure 1**. These include cellular as well as pharmacokinetic mechanisms, and their relation to breast cancer will be discussed in the following sections.



**Figure 1: Cellular mechanisms of MDR.**

### 1.03.01: ATP-binding cassette (ABC) transporter overexpression

ABC transporters are a superfamily of transmembrane proteins that utilize ATP in order to facilitate the transport of molecules across cell membranes.<sup>47</sup> ABC transporters are involved in the cellular efflux of metabolites, drugs, and lipids, among other substances<sup>47,48</sup>. The human genome contains 49 ABC genes arranged into seven subfamilies (A-G).<sup>4,47</sup> In particular, the subfamilies ABCB, ABCC, and ABCG are

important in chemotherapy drug efflux transport and MDR.<sup>49</sup> ABC transporters are mostly expressed in the liver, intestine, blood-brain barrier, and kidneys.<sup>47</sup> When a substrate molecule binds to the ABC transporter, it induces hydrolysis of an ATP molecule and a conformational change in the transporter itself. The result is efflux of the substrate molecule, after which hydrolysis of another ATP molecule will restore the transporter to its original conformation through the release of a phosphate (P<sub>i</sub>) and adenosine diphosphate (ADP).<sup>50</sup> While overexpression of ABC transporters can lead to MDR in cancer, they also remove many toxins from within healthy cells and play an important role in drug detoxification.<sup>51</sup>

**ABCB1**, also known as multidrug resistance gene (MDR1), can be overexpressed in cancer cells where it effluxes chemotherapy drugs such as vinblastine, doxorubicin and paclitaxel (**Table 1**)<sup>52-54</sup>. Multiple studies have shown that its upregulation is one potential cause of MDR.<sup>55,56</sup> In breast cancer, an increase in ABCB1 expression occurs following chemotherapy with taxanes or anthracyclines, and has been associated with higher rates of treatment failure.<sup>57,58</sup> In addition to cancer cells, ABCB1 is present in the luminal membrane of enterocytes in the small intestine and colon, the canalicular membrane of hepatocytes and the luminal membrane of renal proximal tubule cells, which contributes to intestinal, biliary and urinary elimination of drugs, respectively.<sup>59</sup> The ability to eliminate drugs from the body via the kidney, bile and intestine can contribute to pharmacokinetic MDR when it is overexpressed.<sup>52,53</sup> It is also present in the brain capillary endothelium where it enhances the function of the blood-brain barrier (BBB) function by restricting drug access to the brain.<sup>59</sup> On one hand, ABCB1 serves as a protective function for the brain but, on the other hand, it is also a barrier for the

treatment of brain cancers. Thus, in the treatment of MDR breast cancer it is important to consider ABCB1 expression in tissues as this can have an impact on responses or adverse effects of drug therapies.

**Table 1: Representative anticancer drugs that are ABCB1, ABCC1 or ABCG2 substrates.**

ABCB1 substrates	ABCC1 substrates	ABCG2 substrates
irinotecan, topotecan, SN-38, etoposide, teniposide, paclitaxel, docetaxel imatanib, masitinib, nilotinib, toceranib, vinblastine, vincristine, vinorelbine actinomycin D, daunorubicin, doxorubicin	mitoxantrone, methotrexate, topotecan, irinotecan, SN-38, etoposide (VP-16), teniposide, vinblastine, vincristine	mitoxantrone, topotecan, SN-38, irinotecan, methotrexate, AZT, daunorubicin, doxorubicin, 5-fluorouracil

**ABCC1**, also known as multidrug resistance-associated protein 1 (MRP1),<sup>1</sup> has also been linked to the development in MDR in several different types of cancer.<sup>60</sup> ABCC1 is mostly involved in the transport of bulky hydrophobic molecules and transports a broad range of xenobiotics (**Table 1**), which include anthracyclines, *vinca*-alkaloids, methotrexate, and heavy metals.<sup>61,62</sup> The overexpression of ABCC1 has been implicated

as a negative prognostic marker for early-stage breast cancer.<sup>63</sup> There have also been studies that show the expression level of ABCC1 is correlated to relapse time and overall survival rate.<sup>63</sup> In one study, biopsies of 64 primary tumors from patients with operable breast cancer were analyzed for ABCC1 expression.<sup>64</sup> Patients with ABCC1-positive tumors who received first-line chemotherapy treatment showed a shorter time to disease progression than those with ABCC1-negative tumors.<sup>64</sup> The role of ABCC1 in MDR and breast cancer has been shown to correlate with chemoresistance in some clinical studies but not in others, suggesting further studies will likely be necessary to determine the exact involvement of ABCC1 in MDR and breast cancer.

**ABCG2**, also known as the breast cancer resistance protein (BCRP), is expressed on the apical membrane of the intestinal epithelium, liver hepatocytes, renal proximal tubular cells and endothelial cells of brain microvessels.<sup>65</sup> Like ABCB1 and ABCC1, ABCG2 expression is responsible for the elimination of toxins into the intestinal lumen, bile and urine as well as prevention of drug entry into the brain. ABCG2 transports substrates such as mitoxantrone, camptothecins, anthracyclines, flavopridol as well as antifolates, but not cisplatin, paclitaxel, or etoposide (**Table 1**).<sup>66</sup> As a result, cancer cells that express ABCG2 can become resistant to these agents. For example, in one study, the correlation between mRNA expression of various transporters in peripheral blood leukocytes and clinical outcomes was assessed in CML patients who received the TKI imatinib mesylate.<sup>67</sup> ABCG2 mRNA expression in non-responders was found to be higher than in responders before as well as during imatinib mesylate therapy.<sup>67</sup> As well, ABCG2 was overexpressed in those who did not achieve a major molecular response. This is suggestive of the fact that ABCG2 overexpression could play a part in imatinib

resistance in CML patients and could be an indication of effectiveness of drug therapy.<sup>67</sup>

In another study involving breast cancer, ABCG2 expression and resistance to 5-fluorouracil, an ABCG2 substrate, was investigated in 140 breast cancer tissue samples, which showed that resistance to 5-fluorouracil was correlated to levels of ABCG2 expression.<sup>68</sup> In another study looking at ABCG2 expression in pancreatic ductal adenocarcinoma samples, it was found that ABCG2 expression was a prognostic factor in determining early tumor recurrence and poor survival, depicting how ABCG2 can induce MDR in cancer cells.<sup>69</sup>

#### *1.03.02: Decreased drug activation and increased drug inactivation*

Anti-cancer drugs may be administered as inactive pro-drugs that must be converted to active metabolites in order to produce the cytotoxic effects necessary to kill cancer cells. One example is cytarabine or AraC which is a nucleoside drug used to treat acute myelogenous leukemia.<sup>70</sup> This drug is phosphorylated in a two-step process in order to achieve the active form known as cytarabine triphosphate.<sup>70</sup> Cancer cells can become resistant to these drugs by decreasing their activation, such as through gene downregulation or mutation of certain metabolic enzymes.<sup>70</sup> Another example is gemcitabine, an antimetabolite chemotherapeutic, which can be rendered ineffective by decreased drug activation.<sup>71</sup>

For drugs that are not prodrugs, resistance can develop due to increased drug metabolism and detoxification. An example of this is conjugation of a drug to glutathione (GSH), which is an antioxidant that is capable of preventing damage to cells by reactive oxygen species.<sup>72</sup> GSH conjugation with drugs such as cisplatin or oxaliplatin makes

these drugs good substrates for ABC transporters and as such they can be more easily effluxed from the cell, resulting in resistance.<sup>73</sup> Another example of drug inactivation is of the topoisomerase I inhibitor irinotecan, a prodrug which is converted to the active form 7-ethyl-10-hydroxycamptothecin (SN-38) by carboxylesterase enzymes while the inactive metabolite, aminopentancarboxylic acid (APC), is produced by cytochrome P450 3A4 (CYP3A4). In one study it was observed that patients receiving enzyme-inducing antiepileptic drugs such as phenytoin, carbamazepine and phenobarbital led to the induction of CYP3A4 and increased conversion of irinotecan to the inactive APC form rather than the active SN-38 form. By preferentially altering the body's metabolism of the prodrug to the inactive metabolite, the cancer cells were better able to survive the irinotecan treatment.<sup>74</sup>

### *1.03.03: Oncogene addiction*

When cancer cells become dependent on an oncogene or gene that can transform normal cells into tumor cells, this is known as oncogene addiction.<sup>75</sup> Oncogene addiction can aid in maintenance of malignant cancer cell phenotypes and survival.<sup>75</sup> For example, cancer cells may become highly dependent on an oncogene due to a loss of function mutation on a gene that normally performed a similar function during development. Therefore, a drug that is able to target and inhibit the activity of a specific oncogene would be able to selectively target cancer cells instead of healthy cells. One of the earliest examples of oncogene addiction was seen with the use of the antibody trastuzumab, a target for the receptor tyrosine kinase (RTK) HER2. The act of targeting an oncogene as a means of therapy is the basis of targeted therapy; however, drugs that can target HER2



such as trastuzumab and lapatinib are prone to drug resistance through mechanisms such as alteration in the binding sites or tyrosine kinase (TK) receptor domains as well as activation of by-pass signaling pathways.<sup>76</sup> An example of some of these pathways includes the Eph receptor A2 (EphA2), which is a RTK that functions in tumor progression and metastasis as well as the activated receptor for erythropoietin (EpoR) which was found to be coexpressed with HER2 in a significant percentage of human breast tumors.<sup>77-80</sup> As such, oncogene addiction remains a mechanism by which cancer cells can promote their survival and resist treatments.

#### *1.03.04: Altered cell signaling pathways*

Another way by which cancer cells can evade cell death is by seeking out alternative pathways that enhance their survival. An example of this is the PI3K phosphatidylinositol 3-kinase (PI3K)/ protein kinase B (AKT)/ mammalian target of rapamycin (mTOR) pathway (PAM), an intracellular pathway that regulates the cell cycle.<sup>81,82</sup> When activated, this pathway has been associated with resistance to hormone therapy, HER2 therapy, as well as cytotoxic therapy in breast cancer.<sup>83</sup> Activation of the PAM pathway has been estimated to occur in up to 70% of all breast cancers as well studies suggest that the PI3K pathway is the most frequently altered pathway in human cancers, with *PIK3CA* and *PTEN* being some of the most frequently altered oncogenes and tumor suppressor genes, respectively.<sup>84-86</sup> In early clinical studies, PI3K inhibitors buparlisib and alpelisib showed modest clinical efficacy as single-agent treatments while the trials for combination treatments of PI3K inhibitors and other agents is ongoing.<sup>87</sup> Cancer cells often use more than one pathway in order to enhance their chance of proliferating so

when one pathway is targeted using a particular anticancer therapy, another alternative pathway may be used to ensure survival.<sup>88</sup> This phenomenon is evidenced in the activation of escape pathways as a method of endocrine resistance in breast cancer and the ER pathway.<sup>89</sup> This is why oftentimes therapies may need to target multiple pathways in order to be effective and compounds that can circumvent or target these alternative pathways must be explored.

#### *1.03.05: Evasion of apoptotic pathways*

Evasion of apoptotic pathways can occur through mutations that either have an activating or inactivating effect. An example of mutations having an inactivating effect would be mutations that cause a decrease in the activity of the TP53 gene, thus preventing the cells from undergoing apoptosis. This reduction in gene activity has been correlated with resistance to doxorubicin treatments in patients with late-stage breast cancer.<sup>90</sup> Activating mutations that aid in cancer cell death evasion include those that increase the activity of B cell lymphoma 2 (Bcl-2), an anti-apoptotic protein.<sup>91</sup> Other studies have shown that microRNAs inhibit Bcl-2 mRNA translation, which suggest microRNAs can play a role and provide a possible mechanism for apoptosis evasion.<sup>92</sup>

DNA constantly experiences damage through various chemical and physical entities and so it has intrinsic mechanisms of surveillance in order to monitor DNA throughout transcription and cell cycle checkpoints. In the presence of DNA damage, pathways that lead to DNA repair or apoptosis can be activated.<sup>93</sup> The cell cycle is made up of the G<sub>0</sub>, G<sub>1</sub>, G<sub>2</sub>, S and M phases which together are responsible for growth, replication, and division of cells.<sup>93</sup> Restriction checkpoints exist between the G<sub>1</sub> and S phase, the G<sub>2</sub> and

M phase as well as during metaphase of mitosis. In order for cells to proceed past these checkpoints, certain criteria must be met such as proper duplication of chromosomes during S phase.<sup>94</sup> Proteins such as TP53, which act at cell cycle checkpoints, can stop the cell from progressing and signal enzymes to repair DNA or trigger apoptosis. When mutations arise in these checkpoint-controlling molecules, cells have the ability to replicate uncontrollably and can become cancerous. In breast cancer, TP53 mutations are associated with worse overall survival and more aggressive disease.<sup>95</sup> The ABCB1 gene has also been shown to be influenced by mutated TP53 and can stimulate the promoter of the ABCB1 gene. As such, dysregulation in the TP53 pathway can be a mechanism of cytotoxic drug resistance and MDR.<sup>96</sup> Novel therapies that are able to target and restore TP53 would be of great clinical value.

#### *1.03.06: Pharmacokinetic resistance*

Pharmacokinetic resistance refers to body-related factors that change the effectiveness of a drug that is being administered, rather than the cancer cells developing ways to avoid the cytotoxic action of these drugs. When thinking about pharmacokinetics and the body's response to drugs, this concept is very often divided into the categories of absorption, distribution, metabolism, and excretion (ADME).<sup>97</sup>

Absorption refers to a compound or molecule being taken up by the body before it is transported to its intended site of action. There are many factors responsible for drug absorption that determine the bioavailability of a drug.<sup>98</sup> The preferred route of cancer chemotherapy is switching to oral from intravenous (IV). This means that bioavailability needs special consideration and that factors such as food intake and first-pass metabolism

must be considered.<sup>98,99</sup> It has been shown that ABCB1 can reduce the oral bioavailability of chemotherapeutic drugs because of its expression across the gastrointestinal tract and ability to efflux drugs.<sup>100</sup> Also, some foods can decrease the oral bioavailability of a drug so it is imperative to maintain an appropriate diet and dosing regimen to maintain therapeutic and subtoxic drug levels.<sup>101</sup>

Metabolism of many chemotherapeutics occurs in the liver and is facilitated by cytochrome P450 enzymes.<sup>102</sup> The drugs are usually broken down into more polar and water soluble metabolites to facilitate excretion. The CYP enzymes are therefore important to drug response because they metabolize and determine the clearance rate of many drugs. Genetic single nucleotide polymorphisms (SNPs) that result in dysfunctional CYPs can significantly impact their effectiveness to metabolize chemotherapeutic drugs.<sup>103</sup> An example is of patients who receive tamoxifen, which is metabolized to N-desmethyltamoxifen and endoxifen. Endoxifen has an affinity approximately 100 times greater than tamoxifen or N-desmethyltamoxifen for the ER.<sup>104,105</sup> Based on the fact that endoxifen is mostly formed by CYP2D6 metabolism, patients who have defective CYP2D6 alleles may get less benefit from tamoxifen therapy than those who have functional copies of CYP2D6. In a study of 80 women with breast cancer who were starting tamoxifen adjuvant therapy, concentrations of endoxifen in the plasma after 4 months of therapy were significantly lower in those patients who were homozygous or heterozygous for carrying defective CYP2D6 genes in comparison to those who had two functional alleles.<sup>104</sup> However, not all studies have shown a relationship between CYP2D6 polymorphisms and tamoxifen effectiveness.<sup>106,107</sup> Genotyping and knowledge

of how certain mutations can affect CYP activity can be used as a means to optimize cancer chemotherapy.

Excretion refers to removal of drugs and their metabolites from the body. ABC transporters are involved in the process of biliary and renal excretion and their overexpression or induction in the liver and kidneys can contribute to resistance to chemotherapy.<sup>108,109</sup> Differences in inter-individual renal excretion as well as whether or not a drug is excreted through bile are factors to consider when predicting a drug's overall bioavailability, systemic exposure and therapeutic effects.<sup>61,62</sup>

#### *1.04.00: Mechanisms and strategies to overcome MDR in cancer*

When attempting to overcome MDR in cancer there are generally a couple of ways to approach this very complex issue. One is to develop drugs or other therapies that will inhibit specific MDR mechanisms while the other route is to design drugs that evade these mechanisms. An example of inhibiting a mechanism involved in MDR can be seen when stimulating ABCB1 and the expression of the CYP3A4 gene by activation of the nuclear steroid and xenobiotic receptor (SXR) after taxol treatment of tumor cells.<sup>110</sup> This leads to an increase in the cells' drug resistant phenotype and faster drug clearance. In order to combat these effects, inhibitors of SXR in combination with cancer drugs can be used in order to decrease gene expression of ABCB1 and CYP3A4.<sup>110</sup> In order to treat MDR in ABCB1-positive cancer cells, ABCB1 inhibitors such as verapamil (first generation ABCB1 inhibitor) have been used in combination with antineoplastic agents like doxorubicin or paclitaxel.<sup>111</sup> First generation inhibitors have long since been abandoned due to toxicity issues and since then the use of second and third generation

inhibitors has been documented. Second generation inhibitor drugs such as valspodar have been used but were also unfavorable due to adverse drug-drug interactions with chemotherapeutics that were concurrently administered, resulting in unacceptable plasma concentrations of these drugs.<sup>112</sup>

The third generation inhibitors didn't fare much better as drugs such as tariquidar only showed a partial response in 1 patient among 17 ( 6% response rate) overexpressing ABCB1 MDR breast cancer and receiving combination treatment with anthracyclines or taxanes in phase II trials.<sup>113</sup> 4<sup>th</sup> generation inhibitors are currently being developed from natural products and based on some promising results from *in vitro* and *in vivo* trials, will have better success than the previous generation of inhibitors.<sup>114</sup> As a result, inhibition of ABCB1 is not yet a promising co-therapy strategy but a field worth exploring.

Alternatively, some novel drug therapies may be able to avoid the issue of overexpressed drug-efflux transporters altogether by being poor substrates for these transporters. Such drugs would be able to remain within MDR cells and retain their anticancer effects.<sup>115</sup> One such compound is DJ-927, which has been shown to be a poorer substrate of ABCB1 than docetaxel or paclitaxel and overcame ABCB1 MDR in different cancer cell lines both *in vitro* and *in vivo*.<sup>116</sup> Another such example is MAC-321, a novel chemically modified docetaxel and third-generation taxane, which has minimal affinity for the ABCB1 transporter and was able to overcome paclitaxel and docetaxel resistance mediated by ABCB1.<sup>117</sup> A third example is thaspine, a natural product and dual-inhibitor of topoisomerase I/II of which cytotoxicity is only marginally affected by overexpression of ABCB1.<sup>34</sup> Therefore, finding new drugs that are able to evade or circumvent ABC transporters is a possible method to improve cancer therapies.

### 1.05.00: Natural products

Natural products are compounds that are produced by living organisms, which include plants, marine organisms and microbes. The use of natural products as therapeutics to treat human disease has been documented since 2600 BCE.<sup>118</sup> Recently, an analysis of new medicines that were approved by the US Food and Drug Administration (FDA) showed that between 1981 and 2010 approximately 34% of these new drugs that were based on small molecules came from natural products or their derivatives, which is a testament to their utility.<sup>119,120</sup> Several anticancer drugs used today are natural products or modified derivatives.

Plants are a very important source of natural products for many ailments and diseases. The World Health Organization (WHO) estimates that in 1985, approximately 65% of the world's population were reliant on medicines that came from plants as a means of primary healthcare.<sup>121</sup> An example of a plant source for natural product drug development includes *Ammi visnaga*, which was used in the development of chromolyn, a mast cell stabilizer as well as a bronchodilator.<sup>122</sup> Importantly, plant-derived compounds have also been used to treat cancer. Vinblastine and vincristine are plant-derived drugs used to treat cancer and are termed vinca alkaloids. Paclitaxel (Taxol) is another anti-cancer drug that was discovered from the bark of the Pacific yew tree *Taxus brevifolia* and is now used extensively in the treatment of breast cancer.<sup>123</sup>

Marine organisms are also a valuable source for drug discovery with approximately 70% of the earth's surface being covered by water. The first marine-derived natural product that was approved as a drug is Ziconotide, an analgesic used in treating severe and chronic pain.<sup>124</sup> A recent increase in natural product research has focused on

discovering new anticancer agents. Ecteinascidin, an alkaloid that was isolated from a colonial tunicate, has been used to treat soft tissue sarcomas and ovarian cancers.<sup>125</sup>

Another marine source of anti-cancer drugs is from a species of bryozoans, which were used to isolate a group of macrolide lactones known as bryostatins. These bryostatins were efficacious against cancer cells because they were able to downregulate protein kinase C (PKC) isozymes<sup>126,127</sup>. Bryostatins are currently in clinical trials.<sup>126</sup>

Microorganisms have been used as a source for drug discovery since the discovery of penicillin from the fungus, *Penicillium notatum*. Many chemotherapeutic drugs such as the anthracyclines, enediynes, staurosporines and in recent years the epothilones have come from microbial sources.<sup>128</sup> The epitholones were isolated from myxobacteria and are macrolides which have a mechanism of action similar to paclitaxel.<sup>128</sup> In particular, the *Streptomyces* genus of Actinobacteria has been used to produce a significant number of antibiotics which includes jadomycins, the natural products we have been researching as putative anti-cancer agents against breast cancer expressing MDR phenotypes.<sup>129</sup>

#### 1.06.00: Jadomycins

*Streptomyces* is a filamentous bacteria that produces polyketides, molecules that are assembled by head to tail condensation of thioesters from short chain carboxylic acids, which can be a result of stressful conditions.<sup>130</sup> Jadomycins are polyketide antibiotics produced by *Streptomyces venezuelae* ISP5230. Jadomycins are pigmented and glycosylated secondary metabolites that are angucycline-derived antibiotics with a pentacyclic 8*H*-benz[*b*]oxazolo[3,2-*f*]-phenanthridine backbone, which contain five aromatic rings (**A-E, Figure 2B**) and include a dihydropyridine (**B, Figure 2B**) and five-

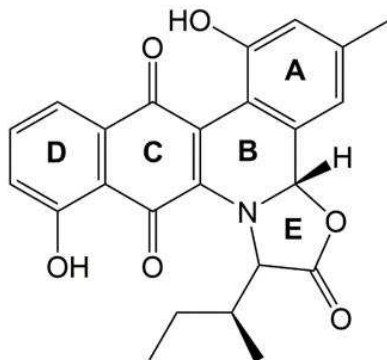


membered oxazolone ring (**E, Figure 2B**).<sup>131</sup> The R-group of the oxazolone ring (**E, Figure 2B**) is derived from the addition of an amino acid into the growth media, which is incorporated into the jadomycin backbone during biosynthesis.<sup>132,133</sup> Jadomycins also have a 2,6-dideoxysugar, L-digitoxose that is appended onto ring D (**Figure 2**) by an enzyme called JadS.<sup>134,135</sup> With the exception of jadomycin B (isoleucine), all other jadomycins that incorporate natural amino acids are identified using single-letter amino acid nomenclature such as jadomycin F for phenylalanine and jadomycin S for serine. Jadomycins that incorporate non-natural amino acids use an abbreviation specific to the non-natural amino acid such as jadomycin SphG for S-phenylglycine.

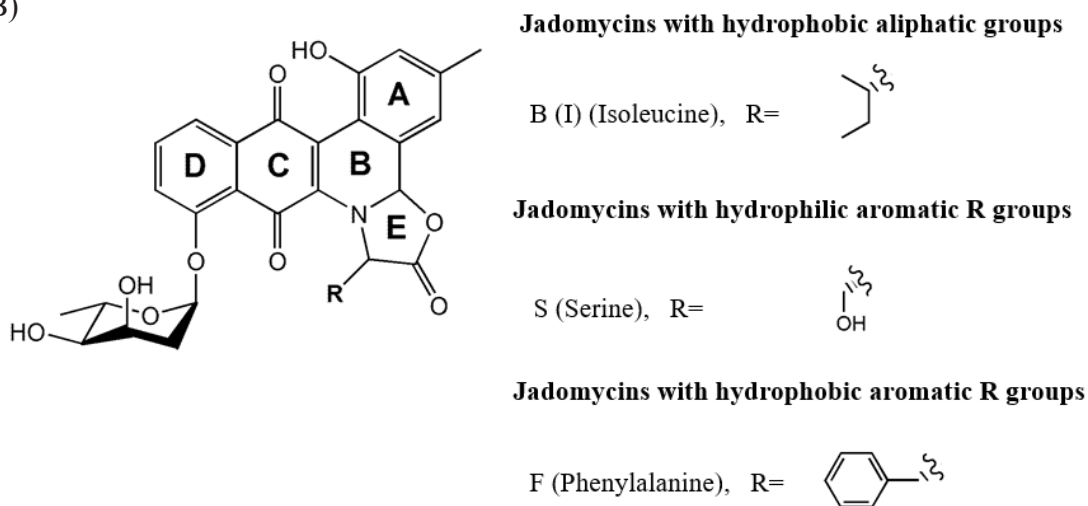
The complex structure of jadomycins is likely part of the reason why they are able to show a wide range of properties, which include their anti-cancer activity within different cancer cell lines.<sup>136</sup> The first jadomycin analogue, jadomycin A (**Figure 2A**), was synthesized by a research team at Saint Mary's University, Mount Saint Vincent University, and the National Research Council in Halifax in 1991.<sup>137</sup> The second jadomycin, jadomycin B, was synthesized by streptomycin bacteria grown in media where the sole nitrogen source was an amino acid, isoleucine.<sup>138</sup> Doull, *et al* observed that isoleucine had become a part of the amino side chain of the jadomycin oxazolone ring, which meant the bacteria might be metabolizing the amino acid in order for this incorporation to occur. By increasing the concentration of L-isoleucine and L-galactose present within the growth medium, higher amounts of jadomycin B could be obtained.<sup>139</sup> They also observed the formation of different colored pigments, which were presumed to be analogs of jadomycin B that had formed when isoleucine was replaced and other amino acids were used as the sole nitrogen source. All of this research led to further

interest into the biosynthesis of these compounds, resulting in the successful preparation and isolation of more than 25 jadomycins.<sup>140,141</sup>

A)



B)



**Figure 2: Structure of jadomycin A and jadomycins used in experimental work.** A)

The first biosynthesized jadomycin analogue was named jadomycin A simply because it was the first discovered and is shown above. Jadomycin A does not have the 2,6-dideoxy-L-digitoxose sugar incorporated into ring D like in some other jadomycins. B) Structure of various jadomycin analogues B, S, and F. These jadomycins were used in pharmacokinetic and tumor studies in balb/C mice. A specific amino acid can be used as

the sole nitrogen source during bacterial growth in order to produce a variety of analogues as shown by the R-group.

#### *1.06.01: Jadomycin Bioactivity*

The potential of jadomycins as anticancer agents was first evaluated in 2005 by Jian-Ting Zheng *et al.*, in which the cytotoxic activity of jadomycin B and five new jadomycin analogues was tested against four human cancer cell lines: HepG2 (human hepatocellular carcinoma), IM-9 and IM-9/Bcl-2 (human lymphoblast, immunoglobulin-secreting cell lines derived from a multiple myeloma), and H460 (non-small-cell lung cancer).<sup>136</sup> All of the jadomycin analogues tested had the ability to suppress the proliferation of tumor cells; however, they also exhibited a different range of cytotoxic potency. Jadomycin S was most potent against HepG2 and IM-9 (IC<sub>50</sub> 9.8  $\mu$ M and 6.3  $\mu$ M respectively) while jadomycin F was most potent against H460 (IC<sub>50</sub> 12.4  $\mu$ M). Structure-activity-relationship analyses were conducted and confirmed that the amino side chains of the oxazolone ring derived from amino acid incorporation had a significant impact on biological activity.<sup>136</sup>

Cytotoxicity testing of jadomycins was also conducted in another study to look at the potential effects of different substituents in the oxazolone ring.<sup>140</sup> There were 19 jadomycin compounds tested against two human breast ductal carcinoma cell lines (T47D and MDA-MD435). The EC<sub>50</sub> for both cell lines were reported and ranged from approximately 1-30  $\mu$ M. The jadomycins proved to be more potent against the MDA-MD-435 cell line as opposed to the T-47D cell line. Jadomycins S, DT, and T were the most potent with EC<sub>50</sub>'s of approximately 1-3 $\mu$ M while jadomycins Y, H and  $\beta$ ala were

the least potent with EC<sub>50</sub>'s of approximately 20-30µM. The jadomycin analogues with small polar side chains were most bioactive.<sup>140</sup>

Further testing of jadomycins as anticancer agents was done by Dupuis *et al.* in which jadomycins D-norvaline and D-norleucine (DNL and DNV, respectively) were evaluated in the National Cancer Institute cell line cancer growth inhibition and cytotoxicity screens.<sup>142</sup> Jadomycins DNL and DNV were tested by the NCI against six leukemia, nine non-small-cell lung carcinoma, six colon, six central nervous system, nine melanoma, seven ovarian, eight renal, two prostate, and six breast cancer cell lines. Both compounds were found to inhibit the growth of the majority of the 59 different cancer cell lines that were tested. The values for the concentration at which growth inhibition is half its maximal value (GI<sub>50</sub>) and the concentration of test drug that caused total growth inhibition (TGI) were both in the low micromolar range. This indicated that the DNL and DNV had very similar growth-inhibition potencies across different types of cancer cell lines. The one exception to this were the leukemia cell lines, which were all resistant to cytotoxicity of jadomycin DNL and DNV.<sup>142</sup>

#### *1.06.02: Jadomycins as treatments for MDR breast cancer*

In our laboratory, we have studied jadomycins' effects in drug-sensitive control MCF7 breast cancer cells in comparison to three different multidrug resistant MCF7 cell lines that overexpress the ABC-transporters ABCB1 (MCF7-TXL), ABCC1 (MCF7-ETP) and ABCG2 (MCF7-MITX). Issa *et al.* showed that IC<sub>50</sub> values for the ABCB1 substrates taxol and doxorubicin were 20- and 58-fold higher, in the MCF7-TXL versus MCF7-CON cells.<sup>143</sup> Similarly, the IC<sub>50</sub> values for the ABCC1 substrates etoposide and

doxorubicin were 3.7- and 17-fold higher, in the MCF7-ETP versus MCF7-CON cells and the IC<sub>50</sub> values for the ABCG2 substrate mitoxantrone was 70-fold higher, in the MCF7-MITX versus MCF7-CON cells.<sup>143</sup> These results show that the control drugs are less potent in the ABC transporter overexpressing cells versus the control cells.

Jadomycins DNV, B, L, SPhG, F, S, and T, on the other hand, were able to kill the MCF7-TXL, -ETP and MITX MDR cells and bring cell viability close to 0% with smaller and largely non-significant decreases in potency versus the control cells (1.3- to 3.8-fold) as measured by IC<sub>50</sub> values ranging from 1.3 to 4.4 μM. Thus suggesting jadomycins largely retain their cytotoxic effects within MDR ABC-transporter overexpressing breast cancer cells. These findings have been recently replicated in ABCB1-expressing MDA-MB-231 breast cancer cells.<sup>144</sup>

#### *1.07.00: Jadomycins' mechanisms of action*

Although we do not fully understand how jadomycins are able to kill cancer cells, there have been some studies performed by our lab and others, which give us a better understanding of how these compounds exert their cytotoxic effects. A general outline of these mechanisms is shown below.

#### *1.07.01: Induction of DNA Cleavage and Reactive Oxygen Species*

The existence of reactive oxygen species (ROS), was discovered in biological systems as early as 1954.<sup>145</sup> Shortly thereafter, they were claimed to have a role in the process of aging.<sup>146</sup> Further investigation about the role of ROS resulted in the isolation of the enzyme superoxide dismutase (SOD), which gave rise to numerous other studies.<sup>147</sup>

Some pathologies where ROS were categorized as contributing factors included cardiovascular disease, rheumatoid arthritis, and cancer.<sup>148</sup> ROS have also been shown to regulate growth, apoptosis, and signaling at the cellular level as well as being involved in cognitive and immune functions.<sup>149</sup> When ROS levels are elevated above normal levels within the cell, they can cause damage to DNA in the form of double-stranded breaks, which is also known as oxidative stress and has been shown to be involved in cancer progression.<sup>150</sup> When ROS levels surpass a certain threshold within the cell they can ultimately cause cell death. This method of cell death is exploited by certain chemotherapies and functions on the premise that cancer cells have higher innate levels of ROS compared to normal healthy cells so they will undergo cell death whereas the healthy cells will still be able to use antioxidant systems to cope with the increase in oxidative stress.<sup>151,152</sup>

In a study done by Monro *et al.*, it was shown that jadomycin B could cleave bacteria DNA in the presence of Cu(II) ions *in vitro*.<sup>153</sup> It was also elucidated that cleavage did not involve the direct binding of jadomycin B to DNA but rather a weak binding interaction between jadomycin B and Cu(II) with DNA present. The cleavage was also shown to be enhanced by UV light, which means that the jadomycin B radical cation and Cu(I) may be potential intermediates in this process. Ultimately, it was found that jadomycin B may be a source of electrons for the reduction of Cu(II), producing Cu(I) which can react with hydrogen peroxide in order to form radicals and eventually causing DNA cleavage.<sup>153</sup> In another study by Cottreau *et al.*, gel mobility assays were performed which showed that some jadomycins could act as DNA cleaving agents. Small changes in

jadomycin structure created through precursor-directed biosynthesis of the substituent at the C1 carbon led to changes in the ability of jadomylicins to damage DNA.<sup>154</sup>

Work by Hall *et al.* supports a mechanism of jadomycin cytotoxicity in the MCF7 breast cancer cell line whereby jadomylicins induce breast cancer cell death *in vitro* by increasing cytosolic superoxide and H<sub>2</sub>O<sub>2</sub> in a Cu(II)-dependent reaction, and that the ROS are then reduced in the cytosol by SOD1, Prx/Trx and GST/GPx antioxidant pathways.<sup>155</sup> However, using annexin V affinity assays it was also shown that the human breast cancer cell lines (231-CON and 231-TXL) co-treated with N-acetylcysteine (NAC), an antioxidant, had no effect on jadomycin-induced apoptosis or late apoptosis/necrosis, which suggests that jadomylicins are also capable of inducing apoptosis and cell death in a manner independent of ROS.<sup>144</sup> In support of this Jadomycin B was shown to cause apoptosis with a 5 µg/mL dose in A549 human adenocarcinoma alveolar basal epithelial cells.<sup>129</sup> We have also recently confirmed that jadomylicins B, S, and F cause apoptosis in triple-negative MDA-MB-231 cells. This effect is also maintained within drug-resistant 231-TXL cells.<sup>144</sup>

#### *1.07.02: Topoisomerase II inhibition*

Topoisomerase II is an enzyme that is responsible for the control of DNA topology by relaxing the supercoiling of DNA and decatenation of sister chromatids. During the process of replication, topoisomerase II cleaves the phosphodiester backbone of DNA to generate a double-stranded break (DSB) through which another DNA duplex can pass through before ligation.<sup>157</sup> Topoisomerase inhibitors cause DNA damage by interfering with the enzyme-mediated breaks and ligation during replication, which often leads to

apoptosis.<sup>158</sup> Martinez-Farina *et al.* recently found that there is direct binding between human topoisomerase II $\beta$  and jadomycin DS through the use of Water-LOGSY NMR spectroscopy. They also showed that jadomycin LN did not appear to bind human topoisomerase II $\beta$ , which means differences between jadomycin analogues may play a role in interaction with proteins.<sup>159</sup> Our lab has also confirmed that jadomycins are catalytic inhibitors of topoisomerases II $\alpha$  and II $\beta$ , and that jadomycins B and F are topoisomerase II $\beta$  interfacial poisons.<sup>144</sup>

### *1.07.03: Inhibition of Aurora B Kinase*

Aurora B kinase is an enzyme that regulates the attachment of spindle microtubules to kinetochores, sister chromatid cohesion, and cytokinesis.<sup>160</sup> Elevated expression of Aurora A and B kinase is commonly seen in many different cancers, which signals that the expression of these kinases may be linked to the development and proliferation of cancer cells.<sup>160</sup> There is also evidence which suggests that high levels of aurora B kinase expression is oncogenic *in vivo*.<sup>161</sup> In one study, jadomycin B was found to be an aurora B kinase inhibitor by method of virtual screening.<sup>156</sup> Utilizing an *in vitro* assay that uses purified recombinant human aurora B kinase, jadomycin B was shown to inhibit the activity of aurora B kinase dose-dependently. It was also shown that jadomycin B was able to enter the ATP-binding pocket of aurora B kinase by formation of hydrogen bonds with amino acid residues.<sup>156</sup> In our laboratory we have shown that Jadomycin B blocks the phosphorylation of histone H3 on Ser10, a downstream target and marker of aurora B kinase function, in MCF7 drug sensitive and drug resistant tumor cells, providing additional evidence for jadomycins' inhibition of aurora B kinase.<sup>143,155</sup>



Jadomycins are a novel and diverse group of compounds that have shown promising anti-cancer activity. They retain their cytotoxic potency in MDR, ABC-transporter overexpressing breast cancer cells, and act through multiple intracellular mechanisms, making them an interesting class of anti-cancer drugs for further study.

#### *1.08.00: 4T1 mouse breast tumor model*

With the advent of new immunotherapeutic strategies and the discovery of new medical techniques, there has been much progress in the field of breast cancer research. One such model that has helped better understand breast cancer is the mouse 4T1 breast tumor model. The 4T1 cell line is useful for modeling stage IV human breast cancer for a number of reasons. The 4T1 tumor model exhibits a similar aggressive phenotype to human TNBC, which is a highly metastatic disease associated with poor patient prognoses.<sup>42</sup> The primary tumor growth and metastasis of 4T1 cells in balb/C mice very closely resembles what is seen in late-stage human breast cancer.<sup>162</sup> Unique to the 4T1 model, mouse mammary tumor cells spontaneously metastasize to many sites within the body including the lungs, liver, brain and bone, which is similar to what is seen in women with metastatic breast cancer.<sup>162,163</sup> Like human mammary cancer, metastasis in the 4T1 model will begin while the primary tumor is in place. The 4T1 model is a syngeneic or allograft transplantation model, meaning the donor tumor cells have the same genetic background as the recipient balb/C mouse strain. This is advantageous in that it allows for use of immunocompetent mice and modeling of tumor-immune system interactions, as would be present in a human host tumor.<sup>164</sup> The 4T1 tumor cells are like human breast tumors and are poorly immunogenic, meaning they will produce a poor immune response

and are more likely to be accepted instead of eradicated by the host's immune system.<sup>162</sup> In addition, the 4T1 cell line is resistant to 6-thioguanine, which allows for the detection of micro-metastatic cells in distant organs through the use of a 6-thioguanine colony assay, where non-cancerous cells are killed but cancer cells continue to grow and can be subsequently stained and then quantified.<sup>42</sup>

The 4T1 cell line is also highly tumorigenic. One-hundred percent of balb/C mice will develop tumors when injected with as little as  $5 \times 10^3$  4T1 cells.<sup>162</sup> After injection, metastatic disease will develop quite quickly and will progress over approximately 2 weeks, which allows for study of early or advanced stages of the disease. It is also possible to model surgical removal of the primary tumor in humans using the 4T1 model.<sup>163,165</sup> Therefore, it is also possible to then look at metastatic disease by itself after tumor removal, which more closely mimics breast cancer discovery and course of action in human breast cancer patients. For our initial proof of principal experiments *in vivo*, we chose to focus on the primary tumor model instead of the resection model because it is a less invasive and less technically challenging model. If treatment efficacy is seen against the primary tumor and/or lung metastasis in the primary tumor model, then future experiments will be conducted in the more clinically relevant resection model.

Despite the 4T1 model being an excellent working model, there are also limitations. In order for the 4T1 model to be performed optimally, it is recommended to mix the cell suspension before preparing the syringe in order to avoid cell clumping that could produce variability from mouse to mouse.<sup>162</sup> Since it is well known that there can be significant variability in the number of metastatic cells in each individual mouse, it also

recommended to use larger mouse groups of 8-10 mice per group in order to account for this natural variability.<sup>162</sup>

There are also other types of mouse models such as xenograft models, in which human cancer cells are implanted into the mouse. In order for the mouse to accept these foreign cells without rejection, an immune compromised mouse strain such as nonobese diabetic/severe combined immunodeficient or NOD/SCID mice are used.<sup>166</sup> This model is useful because it uses actual human breast cancer cells, but it is limited as well due to the potential complication presented by an immunocompromised host organism.<sup>166</sup>

Although we are currently not using any xenograft models, we may explore this option in the future to tell us more about how and/or if our treatments are affecting human breast cancer tumors and metastasis.

*1.09.00: Project Hypothesis and Objectives*

My **hypothesis** is that the intraperitoneal (IP) administration of jadomycins will achieve cytotoxic concentrations that are able to prevent the growth of 4T1 breast cancer and formation of lung metastases in balb/C mice.

While jadomycins have shown promising activity in MDR breast cancer cells *in vitro*, *in vivo* experiments have yet to be conducted. Therefore, the **objectives** of my project are to:

- 1) Conduct the first *in vivo* pharmacokinetic and safety studies of jadomycins B, S, and F in balb/C mice, which will guide drug dosing for objective 2.
- 2) Determine if IP-dosed jadomycin B, S and F inhibit the growth of control and drug resistant 4T1-breast cancer primary tumors and the formation of lung metastases following their implantation into mammary fat pads of female balb/C mice.

## CHAPTER 2.00.00: MATERIALS AND METHODS

### 2.01.00: Chemicals

Thiazolyl blue methyltetrazolium bromide (MTT), dimethylsulfoxide (DMSO), methanol (MeOH), doxorubicin (DOX), and acetonitrile (MeCN) were all purchased from Sigma Aldrich (Oakville, ON, Canada). Liver and kidney toxicity assays were purchased from Cayman Chemicals to measure alanine aminotransferase (ALT) and creatinine biomarkers, respectively. The heart enzyme-linked immunosorbent assay (ELISA) was purchased from Lifespan BioSciences, Inc. to measure troponin-t levels.

### 2.02.00: Cell culture

Dr. Brent Johnston (Dalhousie University, Halifax, NS, Canada) kindly provided the 4T1 control (4T1-CON) breast cancer cells that were used for this project. Taxol-resistant 4T1 cells, which overexpresses ABCB1 (4T1-TXL), were developed in our laboratory by growing the 4T1-CON cells in increasing concentrations of taxol selection medium. The 4T1-CON and 4T1-TXL cells were cultured in Dulbecco's modified Eagles medium (DMEM, phenol red-free, ThermoFisher Scientific, Ottawa, ON, Canada) that contained 10 % fetal bovine serum (FBS), 100 IU mL<sup>-1</sup> penicillin, 250 µg mL<sup>-1</sup> streptomycin (Invitrogen, Burlington, ON, Canada) and 1 mM sodium pyruvate (Sigma, St. Louis, MO, USA). In addition, the resistance phenotype of 4T1-TXL cell was maintained by a concentration of 1.17 µmol/L of paclitaxel in the growth media. Growth media was changed every 3 days for both the 4T1-CON and -TXL cell lines and the cells were incubated in a humidified atmosphere supplemented with 5 % CO<sub>2</sub> at 37°C.

### *2.03.00: Mice*

Adult female Balb/C mice aged 6-8 weeks were purchased (Charles River Laboratories, Halifax, NS, Canada) and were housed in Dalhousie's animal care facility for one week to allow for acclimation prior to any experiments. All experiments were done with strict adherence to the Canadian Council of Animal Care (CCAC) guidelines.

### *2.04.00: MTT assays*

MTT assays were performed in the 4T1-CON and -TXL cell lines to establish the cytotoxic potencies of jadomycins B, S, and F. Cells were seeded at 5000 cells/well in 96-well plates in 100  $\mu$ l of media. The cells were then placed in an incubator for 24 hours at 37° C in order for them to adhere and enter the growth phase. After the initial 24 hour period, cells were treated with jadomycin concentrations ranging from 3.75  $\mu$ M – 50  $\mu$ M. Doxorubicin control concentrations ranged from 0.016  $\mu$ M – 10  $\mu$ M in –CON cells to 0.16  $\mu$ M – 100  $\mu$ M in –TXL cells and all cells were then incubated for a period of 72 hours. Once 72 hours elapsed, 20  $\mu$ l of 5 mg/mL MTT solution was added to the cells in the absence of light and they were then incubated for 2 hours. The wells were then aspirated and 100  $\mu$ L of DMSO was added to each well. The 96-well plates were then shaken for a period of 5 minutes using the Orbit P4 Digital Shaker (Labnet, Edison, NJ, USA) at which point optical density readings were then measured at the 550 nm wavelength using the Biotek Synergy HT plate reader (Biotek, Winooski, VT, USA). Cell viability was then analyzed by comparing the amount of formazan

produced in each treatment well compared to the amount produced in vehicle control wells. The percent cell viability was calculated by dividing the average absorbance of each set of treatment wells by the average absorbance of the vehicle control wells and multiplied by 100. IC<sub>50</sub> values were then calculated using log (inhibitor) vs. normalized response--variable slope using the following equation:

$$Y=100/(1+10^{((\text{LogIC}_{50}-X)*\text{HillSlope}))})$$

#### *2.05.00: Pharmacokinetics study*

For the initial pilot *in vivo* pharmacokinetic study, jadomycin B was injected into male Balb/C mice at a 0.6 mg/kg dose IP. A terminal blood sample was collected by cardiac puncture using a 27 gauge needle at 30 mins, 1 hour, 2 hours, 4 hours and 8 hours after the IP jadomycin injection was given (n = 6 per time). Blood was collected into 1.5 mL microfuge tubes and allowed to clot for 30 mins and was then centrifuged at 1,000 x g for 10 mins in a Sorvall Legend Micro 21 centrifuge (ThermoFisher, Ottawa, ON, Canada). After centrifugation, the serum samples were collected, placed in clean separate tubes and were stored at -80°C until extraction for quantification of jadomycin concentrations.

For subsequent experiments conducted in male mice, a 10x or 6 mg/kg dose of jadomycin B and S (n = 10 per treatment) was injected IP and the saphenous vein blood collection technique was utilized. A saphenous vein sample was collected at 15 mins, 30 mins, 1 hour, 1.5 hours, 2 hours, 4 hours, 6 hours, 8 hours, and 10 hours in

male mice with a max of 4 blood collections (40  $\mu$ L each) per mouse and one terminal collection occurring at either 2 hours or 10 hours. Similar pharmacokinetic studies were completed for jadomycin B, S, and F (n = 10 per treatment) in female mice using the saphenous technique and experimental outline as described above. However, due to their smaller size the blood collections occurred at 15 mins, 30 min, 1 hour, 2 hours, 4 hours, 6 hours, and 8 hours with a max of 3 blood collections (40  $\mu$ L each) per mouse and one terminal collection occurring at either 2 hours or 8 hours. The Mice had their left hind leg shaved and were pricked with a 27 gauge needle; droplets of blood were then collected using microhematocrit tubes and closed using sealing compound. They were then centrifuged using a microcentrifuge in order to isolate serum. The microhematocrit tubes were then cut at the serum-end using scissors and a 27 gauge tuberculin syringe was used in order to draw up serum for transfer into a 1.5 ml microfuge tube. The serum was then stored at -80°C.

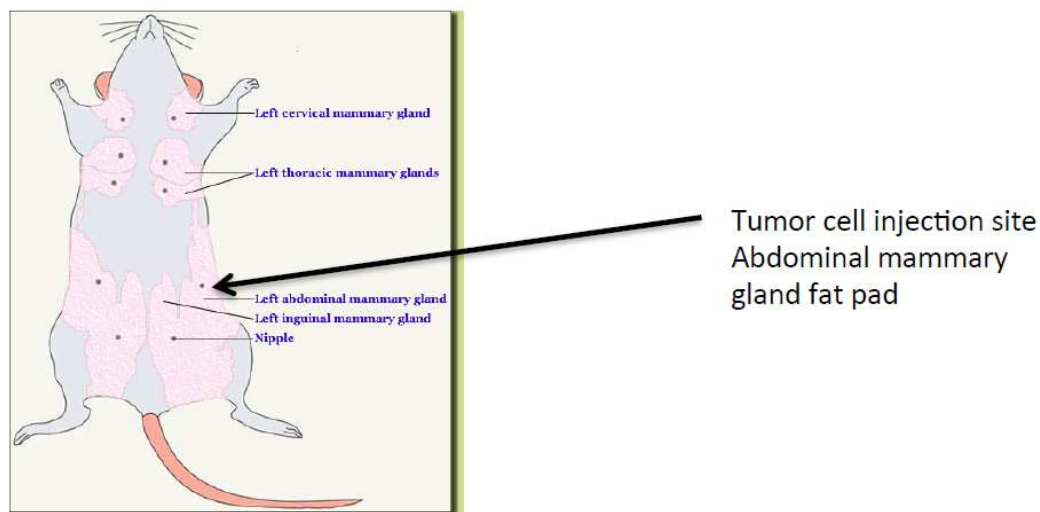
#### *2.06.00: 4T1 tumor model*

One week prior to the planned injection date, 4T1-CON cells were thawed from cryostorage by heating in a 37°C water bath. Cells were then seeded into a 75 cm<sup>2</sup> flask and were incubated until they were split 2 days prior to injection. In order to prepare cells for injection, media was aspirated from the flask and then cells were washed with 5 ml of PBS. They were then rinsed with 2.5 mL of trypsin and incubated at 37°C for 5 mins. 12.5 ml of media was added to neutralize trypsin activity and the cells were transferred to a clean 15 mL falcon tube to be centrifuged at 500 x g for 5 mins. The supernatant was



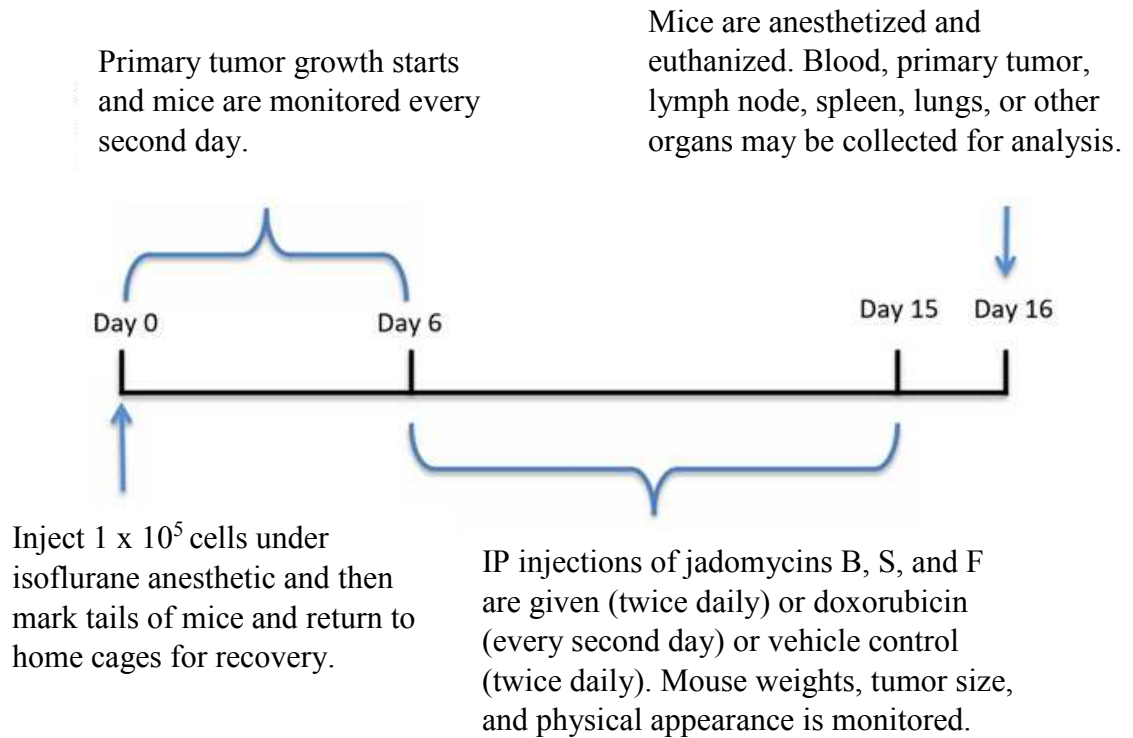
aspirated and the pellet was resuspended in 10 mL of sterile PBS to be centrifuged at 500 x g for another 5 mins. Aspiration of the supernatant was repeated and the pellet was resuspended in 2 mL of PBS. A hemocytometer was used to count cells and to dilute them to  $2 \times 10^6$  cells/ml or 100,000 cells/50  $\mu$ L in sterile PBS. Cells were placed on ice and held there between injections.

In order to prepare mice for injection, mice were anesthetized with 2-5% isoflurane and their dorsal surface was shaved (**Figure 3**). The 4th mammary fat pad was swabbed with 70% alcohol. Syringes with no needle (1 mL) were used to load cells into the syringe and were capped with a 30 gauge needle. The mouse abdomen was tented using a pair of forceps and the skin was penetrated 3-4mm below the nipple, with cells being injected once the needle was under the nipple. 100,000 4T1 cells were injected subcutaneously into each mouse in an injection volume of 50  $\mu$ L of sterile PBS. Following the injections the mice were then monitored until they woke up and were then placed on heating pads where they were monitored every 30 minutes for the next two hours. Mice were randomized and afterwards their tails were marked for identification during the treatment period.



**Figure 3: Tumor cell injection site.**

During day 0-6 the tumor began to grow and mice were checked every second day in case of any adverse health effects (**Figure 4**). From day 6-16 mice received IP injections of 10-13.8 mg/kg jadomycin B, S or F or 5 mg/kg doxorubicin or vehicle control (sterile phosphate buffered saline with 10% v/v ethanol). Jadomycins were injected IP twice per day or every twelve hours and doxorubicin was injected every second day. The jadomycin injections were administered in a 200  $\mu$ L volume using a 27 gauge needle while the doxorubicin injection was given in a 100  $\mu$ L volume. Each mouse received a total of 20 jadomycin injections or 5 doxorubicin injections. Mice were weighed and their tumor volumes were measured using Vernier calipers every second day.



**Figure 4: 4T1 mouse mammary tumor experimental design.**

For humane endpoints, the experiment was stopped and animals were euthanized if tumor size was greater than 10% of the body weight of the mouse, weight loss of more than 15 % of body weight occurred, abdominal distension approximately equal to that of a near-term female was seen, there was clear ulceration or necrosis of the tumor, there was impaired mobility of the mouse due to the tumor, respiratory distress was seen, blood stained or mucopurulent discharge was seen, or there were signs of morbidity such as depression, anorexia, marked change in fecal or urine output, ruffled fur, dehydration and hunched posture.

For sample collection the predetermined endpoint was day 16; mice were anesthetized with isoflurane and a facial bleed was used to collect blood and then serum was isolated

as described above. Blood collection technique was switched from saphenous to facial bleed in order to quickly obtain blood without having to shave the mice. Mice were then euthanized using cervical dislocation while under anesthesia and immediately dissected. The following organs were collected: liver, kidneys, and brain. They were placed in tubes and flash frozen in liquid nitrogen immediately after collection. For immunology analysis the lymph nodes, spleen, lungs, and tumor were collected. The tumor was weighed after removal from each mouse and the lungs were specifically prepared for further analysis in an assay for lung metastasis.

#### *2.07.00: Lung metastasis assay*

Using a syringe plunger, lung tissues were pushed through a 70 micron wire mesh into a 15 mL falcon tube for each mouse in order to prepare a single cell suspension. The cells were then centrifuged at 300 x g for 5 mins and the supernatant was decanted. Red blood cell lysis was then performed by adding 3 mL of ammonium chloride to each tube, followed by a rapid vortex and 5 min incubation. An equal amount of media was then added and the cells were centrifuged at the same settings. Again, the supernatant was decanted and the cells were resuspended in 5 mL of 60  $\mu$ M 6-thioguanine media (6-TG). At this point the cell-media suspension from each tube was plated onto separate petri plates and placed in an incubator for a period of 14 days. Each plate was topped off with media as required, but only after the first 3 days of incubation to allow for cells to settle and adhere. Once 14 days had passed the lung colonies were stained and fixed using the following procedure: Media was aspirated and 5 mL of methanol was added to each plate so all colonies would be covered. Plates were swirled and then cells were allowed to fix at room temperature for 5 mins. Methanol was then aspirated and plates were rinsed with 5 mL of

distilled water. Five mL 0.03% (w/v) methylene blue solution was added to each plate and the plates were gently swirled and allowed to stain for 5 minutes. The dye was aspirated, plates were rinsed with distilled water and air dried for 24 hr before counting blue lung tumor colonies.

#### *2.08.00: Jadomycin analysis in mouse serum samples*

In order to extract jadomyccins from serum samples for quantification, the following procedure was developed in collaboration with Dr. Lekha Sleno, Department of Chemistry, Université du Québec à Montréal, Montréal, QC, Canada. To prepare stock jadomycin standard curves, stock solutions of jadomyccins (0, 0.01, 0.05, 0.1, 0.2, 0.5, 1, 2, 5, and 10  $\mu\text{M}$ ) were prepared in 100% MeCN. These were subsequently diluted 1:10 in blank mouse serum to obtain final concentrations of (0, 0.001, etc). Each standard and blank was prepared in duplicate.

For quality control (QC) samples, three separately created QC's were prepared (as above) using different original jadomycin stocks and were labelled QC A, B, and C with final concentrations 0.0015  $\mu\text{M}$ , 0.05  $\mu\text{M}$ , and 0.75  $\mu\text{M}$  respectively.

To extract jadomyccins, 150  $\mu\text{L}$  of 0.1  $\mu\text{M}$  internal standard working solution (ISWS) in MeCN was added to 50  $\mu\text{L}$  of each standard, QC, or test mouse serum sample. The internal standard was jadomycin F for S or B and jadomycin B for jadomycin S. All standard, QC, or mouse serum sample were then vortexed for 10 seconds before centrifugation at 14000 rpm for 10 mins. 140  $\mu\text{L}$  of supernatant was then removed and transferred into a new tube. Supernatants were then dried in a genevac for 2 hours. Dried extracts were then sent to Dr. Lekha Sleno's laboratory at the Université du Québec à Montréal, Montréal, QC, Canada for analysis using liquid chromatography mass spectrometry.

### *2.09.00: Jadomycin Analysis in tissue samples*

Standard curves and QC's for tissue samples were prepared as mentioned above. The following organs were homogenized and diluted as such: brain (100mg) in 500  $\mu$ L PBS (5:1 v/w tissue), lung (50mg) in 500  $\mu$ L PBS (10:1 v/w tissue), liver (20 mg) in 1.5 ml of PBS (75:1 v/w tissue), and kidney (20mg) in 500  $\mu$ L PBS (25:1 v/w tissue). Tissues were placed in 1.5 ml microfuge tubes and were homogenized with the PowerGen 700 Homogenizer. 50  $\mu$ L aliquots were taken of the homogenate in duplicate and put in separate clean microfuge tubes to be kept on ice. ISWS was then added to these tubes and jadomyicins were extracted by the same process as described for mouse serum samples.

### *2.10.00: Mass spectrometry*

Samples (10  $\mu$ L) were injected onto an XDB C18 4.6 x 50 mm column with 1.8  $\mu$ m particles (Agilent, La Jolla, CA) fitted with a SecurityGuard C18-peptide guard column (Phenomenex, Torrance, CA) using a Nexera UHPLC (Shimadzu, Columbia, MD). Mobile phases were water (A) and ACN (B), both containing 0.1% formic acid. The gradient included a 2 min hold at 40% B with a linear increase to 90% B until 5 at 400  $\mu$ L/min flow rate and column temperature of 40  $^{\circ}$ C. Multiple reaction monitoring (MRM) detection in positive mode was performed using a hybrid quadrupole linear ion trap (QqLIT) 5500 QTRAP mass spectrometer (SCIEX, Concord, ON) with a TurboIonSpray ion source, using the following source conditions: ion spray voltage (IS V), 5000 V; temperature, 450  $^{\circ}$ C; nebulizer and drying gases (GS1 and GS2), 50 psi; and curtain gas, 30 psi. The MRM transitions used for quantitation of analytes were  $m/z$  550.0  $\rightarrow$  306.0 (JadB), 524.0  $\rightarrow$  394.0 (Jad S), and 584.0  $\rightarrow$  454.0 (Jad F). Two additional transitions per analyte were used for quantification: 550 $\rightarrow$  420 and 550  $\rightarrow$  263 (JadB), 524  $\rightarrow$  306 and 524  $\rightarrow$

350 (Jad S), and 584 → 306 and 584 → 131 (JadF). The dwell time for each transition was 50 ms.

To reduce source contamination from the sample matrix, an integrated switch valve was used to send the eluent to waste for the first 1.5 min of each LC run. The remaining acquisition window from 1.5 to 5 min ensured monitoring of analyte and internal standard eluting from the column. Data Analyst software version 1.6 was used for data acquisition, and raw data were processed using MultiQuant version 3.0 (SCIEX, Concord, ON).

#### *2.11.00: Preparation of tissues for fluorescence activated cell sorting (FACS) of immune cells*

Tumor lymphocytes were isolated by centrifugation at 700 x g for 16 mins in 20 mL of a 33% Percoll gradient (GE Healthcare, Baie d'Urfe, QC). Red blood cells in the pellet were then lysed by immersion in ammonium chloride for 5 mins, and then neutralized with 1X PBS 2% FBS solution. The resulting suspension was centrifuged at 300 x g for 10 mins. The pellet was resuspended in 1X PBS containing 2% FBS.

To isolate the spleen cells, the spleen was removed and cut into small pieces. A wire mesh was placed over a 15 ml conical tube and two drops of PBS supplemented with 2% FBS were added. The spleen fragments were pushed through the wire mesh using the rubber end of a 1 ml syringe, while continuously adding a few drops of media at a time. The volume was brought up to 12-15 ml with media and the tube was centrifuged at 300 x g for 10 mins. The supernatant was then aspirated and an erythrocyte lysis and collection of cells was performed as described for the tumor lymphocytes.

### *Antibody Staining*

The following antibodies were obtained from eBioscience (San Diego, CA), BioLegend (San Diego, CA), or BD Biosciences (Mississauga, ON): Fluorescein isothiocyanate (FITC)-labeled CD11b (clone M1/70) for macrophage detection, FITC-anti-CD8 $\alpha$  (clone 53-6.7) for t-cytotoxic cell detection, allophycocyanin (APC)-conjugated anti-CD4 (clone RM4-5) for t-helper cell detection, peridinin chlorophyll protein (PerCP)-anti-TCR $\beta$  (clone H57-597) to detect the beta component of the T-cell receptor, PERCP-anti-F4/80 (clone BM8) for macrophage detection, phycoerythrin (PE)-anti-CD69 (clone H1.2F3) to detect the CD69 antigen indicative of immune activation, PE-anti-Gr1 (clone RB6-8C5) for detection of MDSCs. Dilution of antibodies and isotype controls was done using 1X PBS at the suggested  $\sim 0.15 \mu\text{g}$  per  $10^6$  cells in 100  $\mu\text{L}$  volume. Samples were centrifuged at 300 x g for 10 minutes, supernatant was discarded, and then the pellet was resuspended in 2-5 mL buffer.  $10^6$  cells were stained with 10  $\mu\text{L}$  antibody mastermix and incubated in a refrigerator for 20-30 mins. Two different antibody stains were done using two separate mastermixes. The first stain contained FITC-anti-CD8 $\alpha$ , PE-anti-CD69, PerCP-anti-TCR $\beta$ , and APC-conjugated anti-CD4 while the second contained FITC-labeled CD11b, PE-anti-Gr1 and PERCP-anti-F4/80. Cells were washed in approximately 3 mL 1X PBS and then centrifuged at 300 x g for 10 mins and supernatant was discarded. Then, the cells were washed in approximately 2 mL 1X PBS, centrifuged at 300 x g for 10 mins and supernatant was discarded, and cells were fixed in 200-300  $\mu\text{L}$  paraformaldehyde for FACS analysis. Tumor, spleen, and lymph node samples were stained for fluorescent-activated cell sorting (FACS) using the same procedure.



## *FACS*

Cells were analyzed using a BD FACSCalibur flow cytometer (BD Biosciences) and Cell Quest Pro software with four-color live stain. 250,000 spleen cells or 100,000 lymph node or tumor cells were gated. The compensation strategy used accounted for overlap in absorption spectrums between side-by-side detectors. This was accomplished by looking at a dot plot of two separate channels such as FL1 and FL2 (FITC and PE), which was established by an isotype control (arbitrary spleen sample) that was positive for those particular detection molecules and then moving the populations of those fluorochromes into the proper quadrants. The FLOWJO software was used for flow cytometry analysis in order to characterize percent populations of the lymphocytes referred to in the antibody section directly above.

### *2.12.00: Data analysis and statistics*

For cell culture experiments, all treatments were performed in quadruplicate and each experiment was then repeated three times. All data are expressed as the mean  $\pm$  SEM. A one-way ANOVA was used for multiple comparisons in experiments with only one variable and the Bonferroni test was used for *post-hoc* analysis. For all statistical analyses, a p value  $\leq$  0.05 was considered to be significant.

*In vivo* data were expressed as the mean  $\pm$  SEM for pharmacokinetic curves and parameters, tissue concentrations, tumor weights, volumes, immunology analysis, toxicology. The % of control was used for metastases present in lung tissue. N values for individual experiments are described in the corresponding figure legends. The  $k_d$  and  $k_{el}$  parameters were determined by multiplying 2.303 by the slope from the appropriate phase (distribution or elimination) in order

to convert from  $\text{Log}_{10}$  to the natural log.  $T_{1/2d}$  and  $t_{1/2el}$  were subsequently determined by the formulas  $0.693/k_d$  and  $0.693/k_{el}$ , respectively. AUC was calculated using the trapezoidal method using Graphpad Prism 5 (Graphpad Software Inc., San Diego, CA, USA). Clearance ( $CL_{IP}$ ) and  $V_d$  following IP administration was calculated using the equation:  $CL_{IP} = \text{Dose}_{IP}/\text{AUC}$  and  $V_d$   $_{IP} = CL_{IP}/k_{el}$ . Graphpad Prism 5 was also used for graphs and statistical analysis while the FLOWJO software was used to analyze flow cytometry data obtained from FACS. One-way ANOVA was used to compare the means in experiments with one independent variable. For non-parametric data, the Kruskal-Wallis one-way ANOVA followed by Dunn's multiple comparison test was used. To compare all pairs of columns, a one-way ANOVA using Tukey's test was used. An unpaired t-test was used to compare the means of two independent groups in order to determine if they were significantly different. Two-way ANOVA was used for multiple comparisons in experiments with two independent variables. For all statistical analyses, a p value  $\leq 0.05$  was considered to be significant.

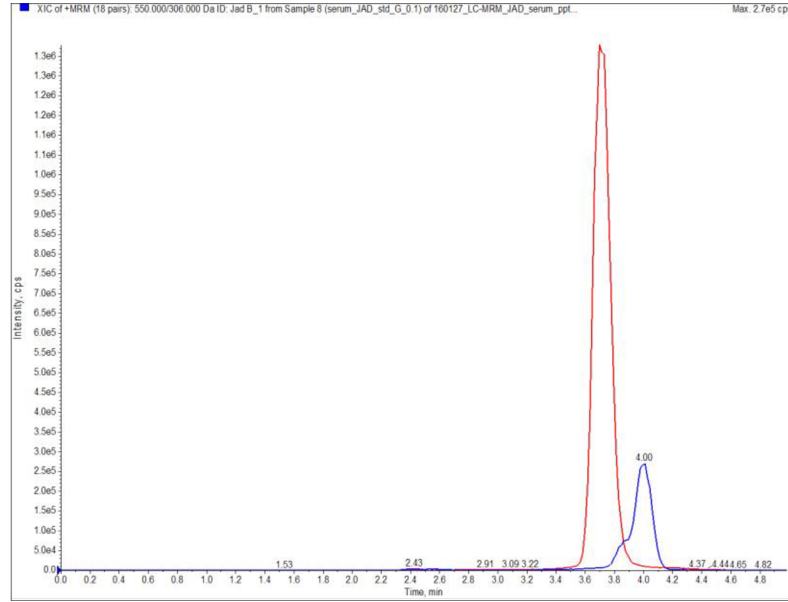
## CHAPTER 3.00.00: RESULTS

**Objective 1)** Pharmacokinetic study of jadomycins B, S and F in balb/C mice

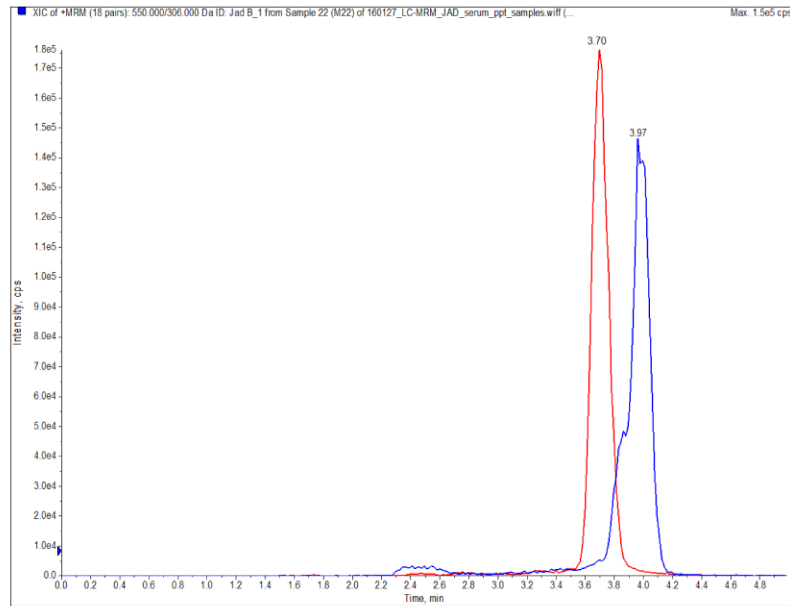
*3.01.00: Pilot pharmacokinetic study of jadomycin B in male mice*

An initial pilot study was performed with thirty 6-8 week-old male balb/C mice. Male mice were chosen, as their larger size would allow for larger samples in which to develop our analytical methodology. Ultimately the experimental plan used both male and female mice to determine if there were sex differences in pharmacokinetics. We initially chose jadomycin B as the starting compound for our studies due to it being the most well characterized jadomycin with a reasonable solubility and it was administered as a single 0.6 mg/kg IP dose. This dose was chosen to produce a serum concentration of 1  $\mu\text{M}$  assuming rapid and even distribution throughout the mouse body and a  $V_d$  of 1 mL/g body weight ( $0.6 \text{ mg/kg} * 0.025 \text{ kg male mouse} / 549 \text{ mg/mmol jadomycin B}/0.025 \text{ L} = 1.09 \mu\text{M}$ ). The 1  $\mu\text{M}$  was estimated to be at the lower end of the desired therapeutic range (1-10  $\mu\text{M}$ ) and would thus be safe while still allowing for a high enough concentration to develop analytical methods. There were no acute adverse effects observed from treatment of jadomycin B. The behaviors used to assess if mice may be experiencing pain/distress were failure to groom, changes in posture or gait, decrease in food or water intake, lethargy or reluctance to move, vocalization, failure to interact with other mice, guarding, avoidance or resentment of handling, and scratching or biting. The assessment of these parameters was qualitative as the primary focus was to see if these behaviors were occurring rather than to quantify them. By using LCMS and internal standards or jadomycins with a known concentration, samples with unknown concentration of jadomycin could be quantified by comparing the areas under the two peaks (**Figure 5**).

A)



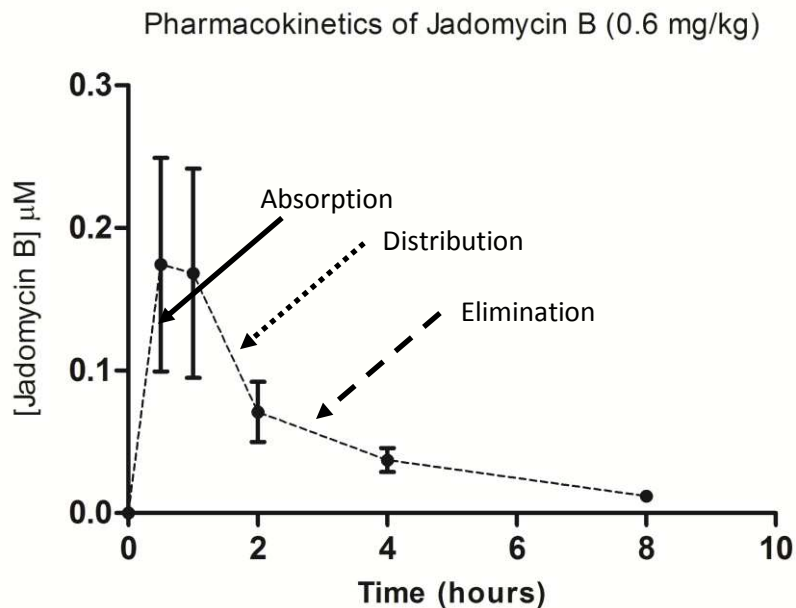
B)



**Figure 5: Liquid Chromatography Mass Spectrometry of Jadomycins.** A) A representative chromatogram showing the peaks and retention times of 0.1  $\mu$ M jadomycin B standard relative to the internal standard jadomycin F. B) A representative chromatogram showing detection of jadomycin B in mouse serum (2 hour time point)

relative to the internal standard jadomycin F. Blue = standard (A) or test sample (B) and red = internal standard (A and B).

Results of the pilot experiment showed that jadomycin B was quickly absorbed after IP injection into the circulation and had a mean peak serum concentration of 0.17  $\mu\text{M}$  at 0.5 h. The absorption phase (**solid arrow Figure 6**) was followed by a fairly rapid distribution phase (**dotted arrow Figure 6**), with a distribution constant of  $0.44 \text{ h}^{-1}$  ( $k_d$ ) and distribution half-life of 1.57 h ( $t_{1/2d}$ ). The also rapid but slower terminal elimination phase (**dashed arrow, Figure 6**) had an elimination constant of  $0.27 \text{ h}^{-1}$  ( $k_{el}$ ) and a terminal elimination half-life of 2.57 h ( $t_{1/2el}$ ). Clearance (CL) was assessed to be 6.08 ml/min by using the calculated area under the curve from 0 to infinity ( $\text{AUC}_{0-\infty}$ ) of  $0.41 \mu\text{mol/L} \times \text{h}$ . Since only a single point was determined for each mouse, the kinetic constants could only be determined from the compiled average data from all 30 mice. Therefore, only a single value without standard error is reported for the pilot experiment.

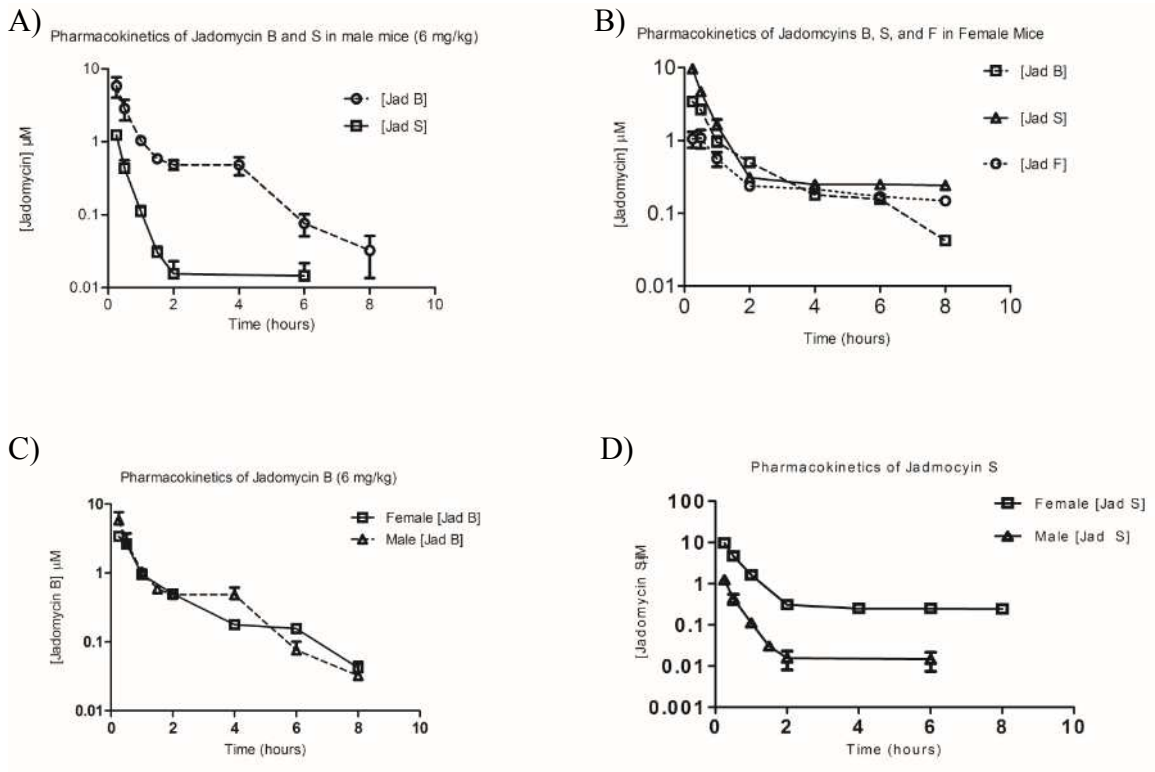


**Figure 6: Single dose pharmacokinetics of 0.6 mg/kg jadomycin B administered to male balb/C mice.** A terminal blood sample was collected by cardiac puncture at each of the indicated times (n=6 per time). Serum was prepared from whole blood, followed by liquid-liquid extraction of jadomyicins and analysis by liquid chromatography mass-spectrometry. Values represent the mean serum concentration  $\pm$  SEM.

### *3.01.01: Pharmacokinetics of 6 mg/kg jadomycin B and S in male mice*

Due to the mean peak serum concentration being approximately 10-fold lower than the predicted 1  $\mu$ M target, the jadomycin B dose was increased 10-fold to 6 mg/kg for subsequent pharmacokinetic studies and jadomycin S was introduced at the 6 mg/kg dose. For this experiment 20 male balb/C mice aged 6-8 weeks were used (n = 10 per treatment group). The sample collection technique was refined and the number of animals reduced by switching to multiple saphenous vein blood collections. Both jadomycin B and S were rapidly absorbed with a  $t_{max}$  of 15 mins but jadomycin B had a 4.7 times higher mean peak concentration compared to the same dose of jadomycin S (5.84  $\mu$ M versus 1.24  $\mu$ M) (**Figure 7A and Table 2**). Importantly, the mean peak serum concentrations for jadomycin B and S were also reaching values in the cytotoxic range of 1-10  $\mu$ M in reference to our *in vitro* studies with human breast cancer cells.<sup>143,155</sup> The rate of distribution as measured by  $k_d$  and  $t_{1/2d}$  was similar for jadomycin B and S at the 6 mg/kg dose. The terminal elimination phases for jadomycin B and jadomycin S were also similar based on comparison of the elimination rate ( $k_{el}$ ) and terminal elimination half-life ( $t_{1/2}$ ). The overall systemic exposure as measured by  $AUC_{0-\infty}$  was significantly increased (10-fold) for jadomycin B compared to jadomycin S. Correspondingly, the

CL<sub>IP</sub> was significantly lower for jadomycin B compared to S and V<sub>d</sub> was approximately 15-times smaller for Jadomycin B compared to jadomycin S. Jadomycin B and S concentrations were below the lower limit of quantification (0.001 μM) after 8 or 10 hours, respectively.



**Figure 7: Pharmacokinetics of jadomycin B, S (6 mg/kg) in male and jadomyocins B, S, and F (6 mg/kg) in female balb/C mice.** A) The pharmacokinetics of jadomycin B and S in male mice. B) The pharmacokinetics of jadomycin B, S, and F in female mice. The data for in panels A and B were re-plotted to better illustrate the male versus female comparisons for jadomycin B (C) and jadomycin S (D). For these experiments male and female balb/C mice were administered a single dose of jadomycin B, S or F (6 mg/kg) by IP administration. A saphenous vein sample was collected at each time point with a max

of 4 blood collections (40µl each) per mouse and one terminal collection occurring at either 2 hours or 10 hours (n = 5 per time) for males and a max of 3 blood collections (40µl each) per mouse for females with terminal collection at either 2 hours or 8 hours (n = 5 per time). Serum was prepared from whole blood, followed by liquid-liquid extraction of jadomycins and analysis by liquid chromatography mass-spectrometry for all mice. Values represent the mean serum concentration ± SEM.

**Table 2: Pharmacokinetic parameters of jadomycin B and S measured experimentally in male balb/C mice.**

Pharmacokinetic parameters	6 mg/kg (B)	6 mg/kg (S)
T <sub>max</sub>	15 min	15 min
C <sub>max</sub>	5.84 ± 1.80 µM *	1.24 ± 0.05 µM
K <sub>d</sub>	1.27 ± 0.25 h <sup>-1</sup>	2.78 ± 0.22 h <sup>-1</sup>
T <sub>1/2d</sub>	0.66 ± 0.14 h	0.25 ± 0.02 h
K <sub>el</sub>	0.35 ± 0.06 h <sup>-1</sup>	0.51 ± 0.33 h <sup>-1</sup>
T <sub>1/2el</sub>	2.21 ± 0.32 h	2.91 ± 1.29 h
AUC <sub>0-∞</sub>	4.20 µmol/L x h * (n = 10)	0.45 µmol/L x h (n = 10)
CL <sub>IP</sub>	.0042 ± 0.00096 L/h *	.0912 ± 0.041 L/h
Vd <sub>IP</sub>	0.012 L *	0.18 L

For all parameters measured in the 6 mg/kg (B) and (S) doses, the values represent the mean or mean ± SEM (n = 5 per time point) unless otherwise noted. The 0.6 mg/kg (B)



pilot data is described in the text.  $T_{\max}$  = time of maximum serum concentration,  $C_{\max}$  = maximum serum concentration,  $K_d$  = distribution constant,  $T_{1/2d}$  = distribution half-life,  $K_{el}$  = elimination constant,  $T_{1/2el}$  = elimination half-life,  $AUC_{0-\infty}$  = area under the curve from time 0 to  $\infty$ ,  $CL_{IP}$  = clearance IP, and  $V_{dIP}$  = volume of distribution IP. \*  $P < 0.05$  significantly different compared to jadomyocins S by unpaired t-test.

### *3.01.02: Pharmacokinetics of 6 mg/kg Jadomyocin B, S and F in female mice*

Ultimately, female mice were used because the 4T1 breast cancer studies would be conducted in female mice and pharmacokinetic can differ between male and females.<sup>167</sup> With the baseline pharmacokinetics established for jadomyocin B and S in male mice, the next step was to complete and compare the pharmacokinetic studies of jadomyocin B, S and F in female mice using a one-way ANOVA. When testing the pharmacokinetics of jadomyocins B, S, and F in females, the mean peak serum concentrations were significantly different. Jadomyocins B, S and F were quickly absorbed into the circulation following IP administration with  $t_{\max}$  values of 15-30 min. The mean peak concentration of jadomyocin S was 3-fold and 10-fold higher than jadomyocin B and jadomyocin F, respectively (**Figure 7B and Table 3**). The distribution phase was more rapid for jadomyocin S compared to jadomyocins B and F. The terminal elimination was similar for jadomyocin B and F and approximately 10-fold more rapid than observed for jadomyocin S as measured by the  $k_{el}$  or  $t_{1/2el}$  values.  $AUC_{0-\infty}$  was significantly higher for jadomyocin S compared to jadomyocins B and F while  $CL_{IP}$  was significantly slower for jadomyocin S compared to F with jadomyocin B in between the two.  $V_d$  values of jadomyocins were within approximately 7-fold of each other.

The second analysis was to compare jadomycin B and S pharmacokinetics between male and female mice (**Figure 7C and 7D**). The mean peak serum concentrations, the rate of distribution and terminal elimination phases were similar for jadomycin B across both sexes. The calculated  $AUC_{0-\infty}$ ,  $CL_{IP}$ , and  $V_d$  values were also similar between males and females. Comparatively different trends were observed for jadomycin S in male versus female mice. Female mice had a 7.8-fold higher ( $P < 0.05$ ) mean peak serum concentration compared to males. While the distribution rates were similar for both males and females,  $AUC_{0-\infty}$  was significantly higher for female mice and this was reflected by a lower  $CL_{IP}$  in females than males ( $P < 0.05$ ). The elimination half-life of jadomycin S was approximately 10-fold longer in females versus males.  $V_d$  values were not significantly different for jadomycin S between male and females.

**Table 3: Pharmacokinetic parameters of jadomycin B, S, and F measured experimentally in female balb/C mice.**

Pharmacokinetic parameters	Jadomycin B	Jadomycin S	Jadomycin F
$T_{max}$	15 min	15 min	30 min
$C_{max}$	$3.42 \pm 0.27 \mu\text{M}^*$	$9.65 \pm 1.0 \mu\text{M}^\ddagger$	$1.08 \pm 0.30 \mu\text{M}$
$K_d$	$1.12 \pm 0.069 \text{ h}^{-1}^*$	$1.92 \pm 0.07 \text{ h}^{-1}^\ddagger$	$0.81 \pm 0.19 \text{ h}^{-1}$
$T_{1/2d}$	$0.63 \pm 0.039 \text{ h}^*$	$0.36 \pm 0.01 \text{ h}^\ddagger$	$1.55 \pm 0.80 \text{ h}$
$K_{el}$	$0.31 \pm 0.052 \text{ h}^{-1}$	$0.03 \pm 0.006 \text{ h}^{-1}$	$0.36 \pm 0.25 \text{ h}^{-1}$
$T_{1/2el}$	$2.75 \pm 0.79 \text{ h}$	$24.8 \pm 6.31 \text{ h}$	$5.06 \pm 1.61 \text{ h}$

AUC <sub>0-∞</sub>	3.64 μmol/L x h * (n = 10)	5.89 μmol/L x h ‡ (n = 10)	2.23 μmol/L x h (n = 10)
CL	0.0043 ± 0.00052 L/hr	0.0031 ± 0.00048 L/hr ‡	0.0070 ± 0.0017 L/hr
Vd <sub>IP</sub>	0.014 L	0.10 L	0.019 L

For all parameters measured, the values represent the mean ± SEM (n = 5 per time point) unless otherwise noted. T<sub>max</sub> = time of maximum serum concentration, C<sub>max</sub> = maximum serum concentration, K<sub>d</sub> = distribution constant, T<sub>1/2d</sub> = distribution half-life, K<sub>el</sub> = elimination constant, T<sub>1/2el</sub> = elimination half-life, AUC<sub>0-∞</sub> = area under the curve from time 0 to ∞, CL<sub>IP</sub> = clearance IP, and Vd<sub>IP</sub> = volume of distribution IP. \* P < 0.05, jadomycin B was significantly different than jadomycin S; and ‡ P < 0.05, jadomycin S was significantly different than jadomycin F, one-way ANOVA followed by Tukey's post hoc comparison of the significant ANOVA.

### 3.02.00: Jadomycin tissue distribution

At the terminal points of our studies in male mice (2 hours for distribution phase or 10 hours for elimination phase) the tissue to serum ratios of jadomycin B were determined in order to better understand if jadomycins were penetrating different tissues and to what extent. As evidenced in table 4, we saw the highest accumulation of jadomycin B in the liver followed by kidney, and lung respectively. Jadomycin in the brain was not detected at the LLQ of 0.001 μM. Tissue to serum ratios also decreased from the 2 hr to 10 hr time

points as expected and the same trend was evident for jadomycin B concentration in nmol/g of tissue (**Table 4**).

**Table 4: The tissue concentrations and tissue to serum ratios of jadomycin B following a 6mg/kg dose in male balb/C mice.**

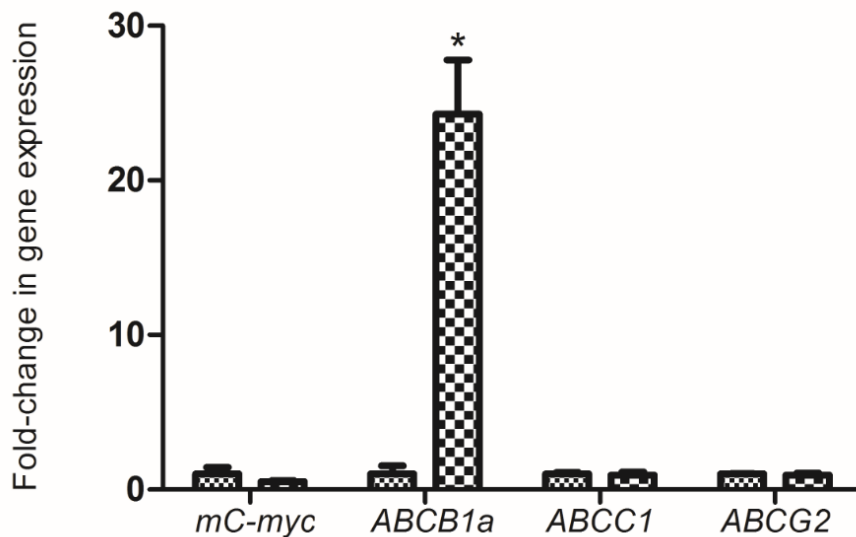
Tissue	Jadomycin B nmol/g of tissue	Average tissue/serum ratio at 2 h	Jadomycin B nmol/g of tissue	Average tissue/serum ratio at 10 h
Liver	65.66 ± 4.21	154.9 ± 24.63	4.27 ± 0.66	10.1 ± 2.46 *
Kidney	4.86 ± 0.42	11.7 ± 2.44	0.41 ± 0.067	1.0 ± 0.23 *
Lung	1.49 ± 0.092	3.4 ± 0.45	0.36 ± 0.13	0.9 ± 0.36 *
Brain	Low <sup>#</sup>	Low <sup>#</sup>	Low <sup>#</sup>	Low <sup>#</sup>

All values for amounts of jadomycin B present per mg of tissue and tissue to serum ratios represent the mean ± SEM. Low<sup>#</sup> indicates that values were below the LLQ of 0.001 µM.

\* P <0.05 significantly different from 2 h to 10h tissue/serum ratio by unpaired t-test.

**Objective 2)** Testing the anti-tumor and anti-metastatic efficacy of jadomycins B, S, and F within the 4T1 tumor model

Prior to any experiments, the 4T1-TXL cells that were originally provided as 4T1-CON cells and made to be taxol resistant in-house were first tested for confirmation of upregulation of ABC transporters. By using quantitative real time PCR (qRT-PCR), we confirmed that there was a significant 48-fold upregulation of *abcb1a* (murine *abcb1* transporter) in the 4T1-TXL cell line relative to the 4T1 control cells. In comparison *abcc1* and *abcg2*, were not significantly increased in the 4T1-TXL cells compared to the 4T1-CON (**Figure 8**). Before the *in vivo* studies were started, the 4T1-CON and -TXL cell lines were screened for mouse pathogens at Charles River using the mouse essential clear PCR panel, which tests for 11 different pathogens including mycoplasma. The cells tested negative for all pathogens (**Appendix 1**). Once the 4T1 cells were shown to be non-infected and qRT-PCR confirmed upregulation of ABCB1 we proceeded with our initial 4T1 tumor studies.



**Figure 8: Fold-change in ABC-transporter gene expression in 4T1-CON cells versus 4T1-TXL cells** (shown left to right, respectively, per gene). The expression of the housekeeping gene *mC-myc* as well as the ABC transporters *abcb1a*, *abcc1*, and *abcg2* were measured *via* qPCR. Calculation of fold-change was done using the  $\Delta\Delta C_t$  method and normalized to the average of the each gene. Each bar represents the mean  $\pm$  SEM of three independent experiments. A one-way ANOVA, followed by Bonferroni's multiple comparison test was done with \*  $p \leq 0.05$ , showing that the fold-change in gene expression was significantly different from the 4T1-TXL to -CON cell lines.

MTT assays were performed to confirm that the 4T1-TXL cells were functionally resistant to doxorubicin a known ABCB1 substrate and to establish the cytotoxic potency of 3 structurally unique jadomycins, jadomycin B, S, and F. As expected based on the overexpression of *abcb1* determined by qRT-PCR, the 4T1-TXL cells were significantly less sensitive to doxorubicin than the 4T1-CON cells. Comparing  $IC_{50}$  values, it was shown that the  $IC_{50}$  value for the 4T1-CON cells was in the single digit micromolar range whereas it was  $>100 \mu\text{M}$  in the 4T1-TXL cell line. As we previously observed in comparisons of drug-resistant and drug-sensitive human breast cancer cells, the  $IC_{50}$  values for jadomycins B, S and F remained similar in the 4T1-CON and 4T1-TXL cell lines (**Table 5**). This data shows that jadomycins are not affected or minimally affected by the ABCB1-overexpressing-4T1-TXL cell line. However, the jadomycins were slightly less potent in the 4T1 cell lines ( $IC_{50s} = 10-18 \mu\text{M}$ ) compared to previously published results in human breast cancer cell lines (1-10  $\mu\text{M}$ ).<sup>143</sup>

**Table 5: IC<sub>50</sub>'s of jadomycins and doxorubicin in 4T1-CON and 4T1-TXL cell lines as measured by MTT assay.**

	IC <sub>50</sub> ± SEM μM	
	4T1-CON	4T1-TXL
<b>Jadomyacin</b>		
Jadomyacin B	10.1 ± 2.2 ‡	9.7 ± 2.0 ‡
Jadomyacin S	11.0 ± 0.9 ‡	9.8 ± 1.1 ‡
Jadomyacin F	18.0 ± 1.6	18.5 ± 1.8
<b>Control drug</b>		
Doxorubicin	2.8 ± 0.3	159.2 ± 23.9 *

IC<sub>50</sub> values (μM) were determined by MTT assays after treatment with doxorubicin or jadomycins. The data represents the mean IC<sub>50</sub>s ± SEM of three individual replicates performed in treatment of quadruplicate. Two-way ANOVA, followed by a Bonferroni post-test was used to confirm any significant difference between jadomycins within and across the 4T1-CON and –TXL cell lines (‡ p < 0.05), significantly different from jadomyacin F. \* p < 0.05, doxorubicin significantly different between 4T1-CON and –TXL cell lines.

### *3.03.00: Jadomyacin toxicity profiles in mouse 4T1 breast cancer cells*

Based on the mean peak serum concentrations with the 6 mg/kg dose and the slightly higher IC<sub>50</sub>'s for jadomycins in 4T1 cells versus human breast cancer cells, it was decided that 10 mg/kg would be used to ensure target concentrations were reached. Twice

daily dosing was also adapted because of the rapid elimination profile. The first experiment was conducted as a small pilot study with a small number of mice ( $n = 6$ ) to become acquainted with the methodology and to ensure that chronic IP dosing of jadomycins was not causing any significant harm to the animals. Based on what was seen, chronic dosing of jadomycins B, S, and F for a total of 20 cumulative doses per animal did not cause any physical signs of distress as mentioned earlier in the methods sections. Organ-specific toxicology data will be discussed in a separate section later in the results.

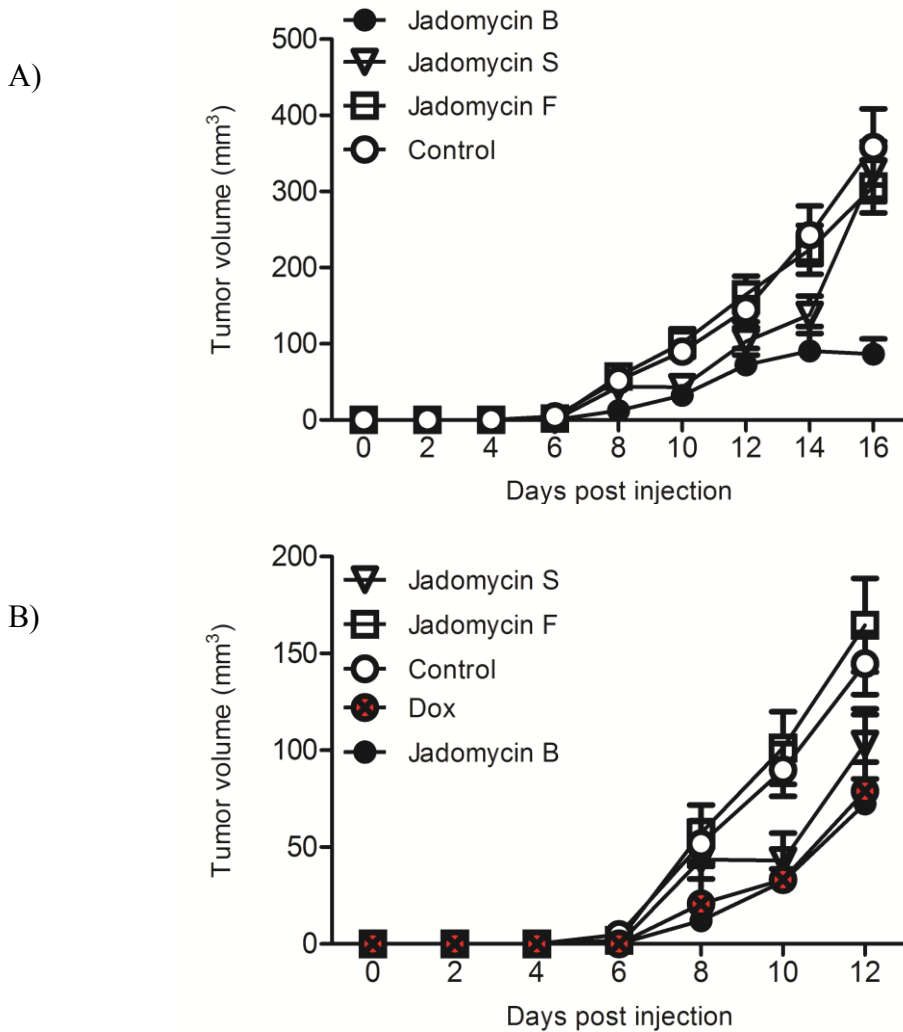
#### *3.04.00: Effect of jadomycins on tumor weights and volumes*

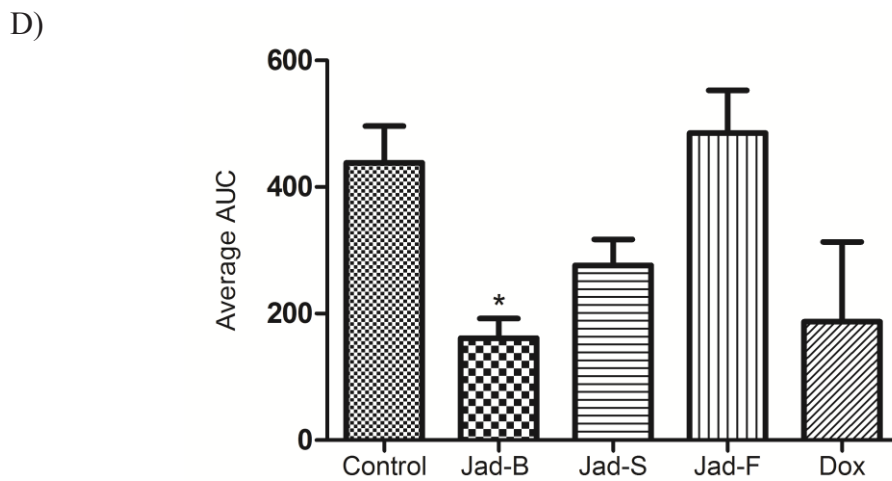
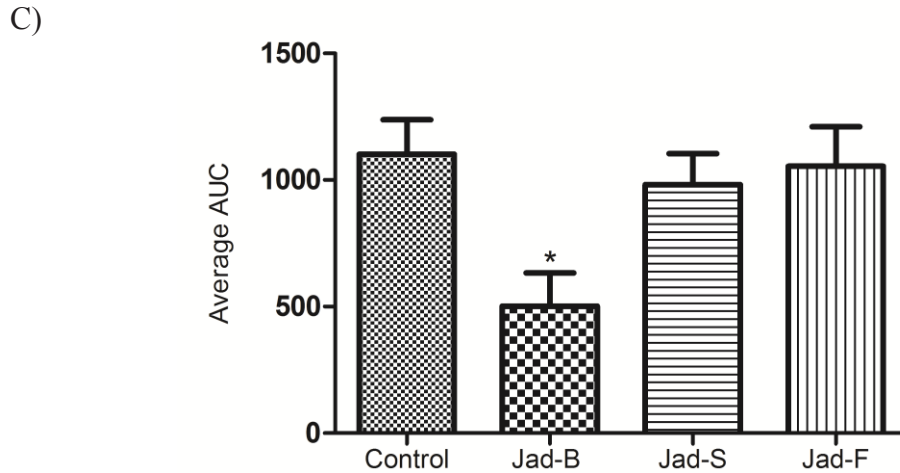
From day 0 to day 6 of the 4T1 tumor model, there was minimal growth of the tumor across all groups as measured by tumor volume. Day 6 was the first day at which tumor growth acceleration occurred and a very small tumor was palpable. The corresponding tumor volumes for day 0-16 and day 0-12 and their treatments can be found in **Figure 9A, 9B**. The rate of tumor growth was similar in the control and in the jadomycin S and F treatment groups but was reduced for jadomycin B. Doxorubicin also appeared to have slowed tumor growth but the mice had to be humanely euthanized on day 12 due to the adverse effects associated with this drug. The mice were able to tolerate jadomycins with no observable behavioral or physical abnormalities up until the endpoint at 16 days post-tumor injection. Average AUC's calculated from tumor volumes (day 0-16) were compared for jadomycins versus control (**Figure 9C**). Jadomycin B had a significantly lower AUC compared to control and was also significantly lower on analysis of day 0-12, with a lower AUC than a common chemotherapeutic doxorubicin (**Figure 9D**). At the 16



day endpoint, the primary tumor was collected and its weight was recorded. It was found that there was no significant difference between the control group and jadomycin B, S, and F treatment groups. An n of at least n = 8 was done for each jadomycin treatment group, which is common for the 4T1 model due to variability seen within the model. There was however, a trend in decreased tumor weight with jadomycin B treatment, reducing the tumor size by as much as 23% compared to control (**Figure 10**).

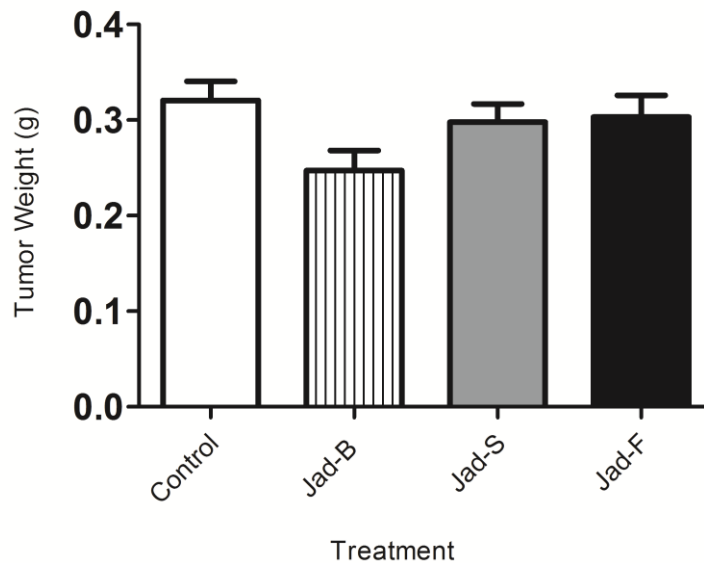
Doxorubicin reduced the average tumor volume over time (as measured by AUC) by up to 57% compared to control when harvested on day 12 due to adverse effects associated with toxicity.





**Figure 9: Effect of IP-dosed jadomyjcins B, S and F on tumor volumes.** A) Tumor volumes from which AUC were calculated are shown for jadomyjcins B, S, F and saline control (day 0-16). B) Tumor volumes of jadomyjcins B, S, F as well as saline control and doxorubicin (day 0-12). C) Average AUC calculated from tumor volumes of female balb/C mice for jadomyjcins B, S, and F as well as saline control (day 0-16). D) Average AUC calculated from tumor volumes of female balb/C mice for jadomyjcins B, S, and F as well as saline control and doxorubicin at the early endpoint of doxorubicin (day 0-12).

All treatments were started on day six and the following dosing regimen was used for treatments: Jadomycin B, S, and F (10 or 13.8 mg/kg) twice daily (n = 7, n = 10, n = 14 respectively), doxorubicin (5 mg/kg) once every two days (n = 5), and saline control twice daily (n = 26). Data shown for jadomyjcins is pooled data of the two doses. Dox = doxorubicin. A one-way ANOVA was done using Dunn's multiple comparison test with \* p < 0.05.

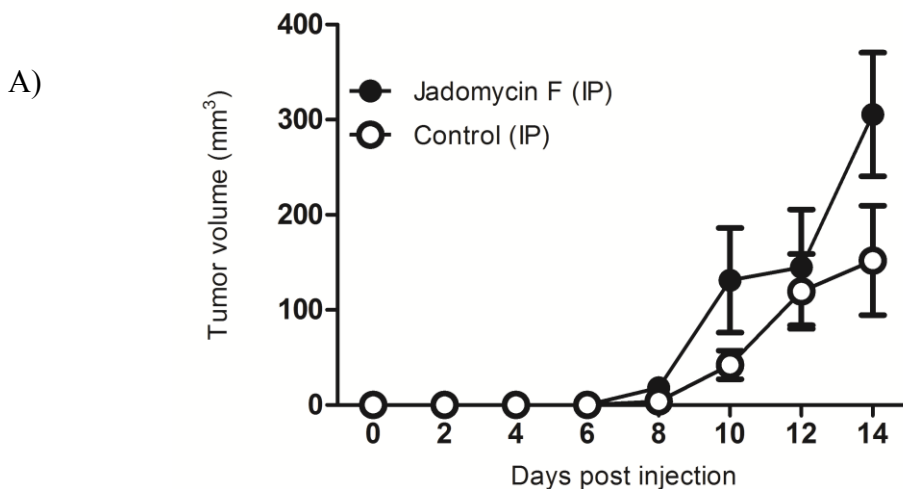


**Figure 10: Effect of IP-dosed jadomyjcins B, S and F on tumor weights.** Final tumor weights of female balb/C mice treated with jadomyjcins or saline control. A minimum n value of n = 10 is shown for saline control, jadomyjcins S, and jadomyjcins F treated mice. Jadomyjcins B treatment had an n value of n = 7 due to it being part of the last 4T1 tumor model experiments. Jadomyjcins were given twice daily at a dose of 10 or 13.8 mg/kg. Data shown is pooled data of these doses. A one-way ANOVA was done using Dunn's

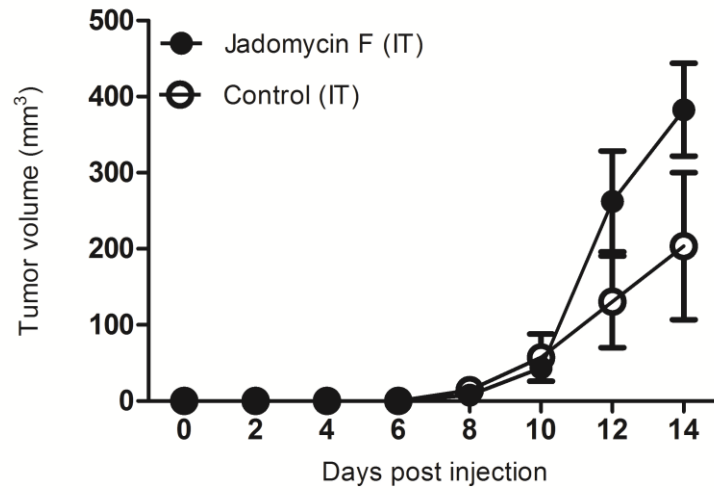
multiple comparison test with  $p < 0.05$ . Jad-B = jadomycin B, Jad-S = jadomycin S, and Jad-F = jadomycin F.

### 3.04.01: Effect of IP vs IT jadomycin F on tumor weights and volumes

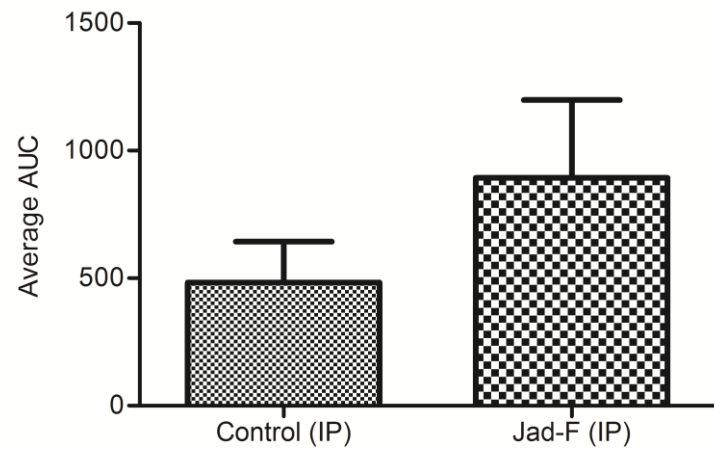
Given the minimal effects observed following IP dosing of jadomycin F and S, IT dosing was attempted to determine if delivering the drug directly to the tumor would provide an improved effect. Jadomycin F was chosen for these experiments due to greater availability of this compound. After IT injection of jadomycin F, the change in tumor volume was monitored and measured over time and the primary tumor was collected for tumor weight measurements. The rate of tumor growth was not reduced for IT and IP jadomycin F treatments compared to control (Figure 11A and Figure 11B). The same trend was seen when looking at the Average AUC's calculated for IP vs. IT jadomycin F treatments (Figure 11C and Figure 11D). Jadomycin F showed no significance in efficacy against primary tumor weights between treatment and control groups, whether IP or IT (Figure 12).



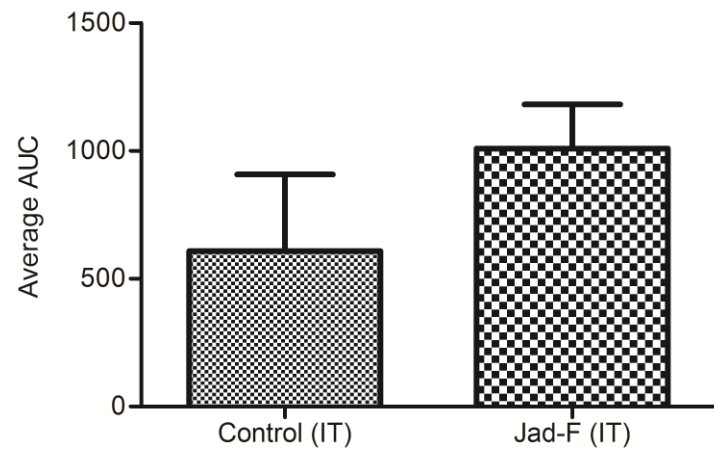
B)



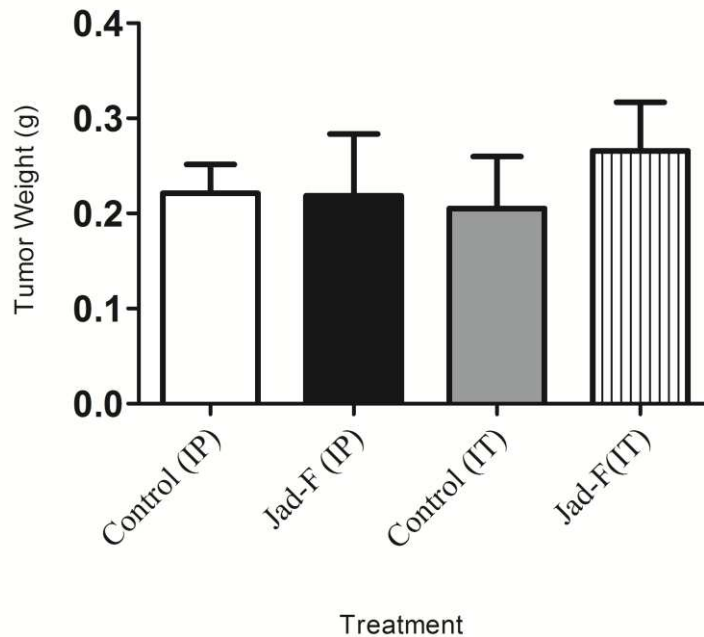
C)



D)



**Figure 11: Effect of IP versus IT-dosed jadomycin F on tumor volumes.** Tumor volumes of female balb/C mice treated with (A) jadomycin F IP, (B) jadomycin F IT or saline control. C) Average AUC calculated from tumor volumes of female balb/C mice for jadomycin F (IP) as well as saline control and D) Average AUC of female balb/C mice for jadomycin F (IT). All treatments were started on day six and the following dosing regimen was used for treatments: Jadomycin F (13.8 mg/kg) twice daily (n = 5 IP and IT) and saline control twice daily (n = 4 IP, n = 5 IT). The endpoint volume was measured on day 14 due to lack of data for day 16. Jad-F = jadomycin F.



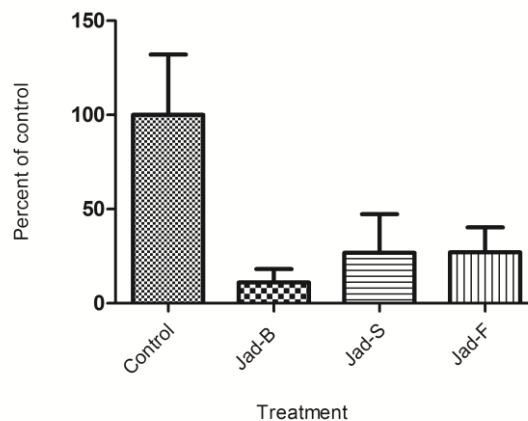
**Figure 12: Effect of IT-dosed jadomycin F on tumor weights.** Final tumor weights of female balb/C mice treated with IT jadomycin F or saline control. A value of n = 5 is shown for all groups with the exception of the control (IP) group which was n = 4. Jad-F (IP) was given at a 13.8 mg/kg dose twice daily for a period of 10 days while Jad-F (IT) was given once daily at 13.8 mg/kg for 10 days. A one-way ANOVA was done using

Dunn's multiple comparison test with \*  $p < 0.05$ . Jad-F = jadomycin F, (IP) = intraperitoneal and (IT) = intratumoral.

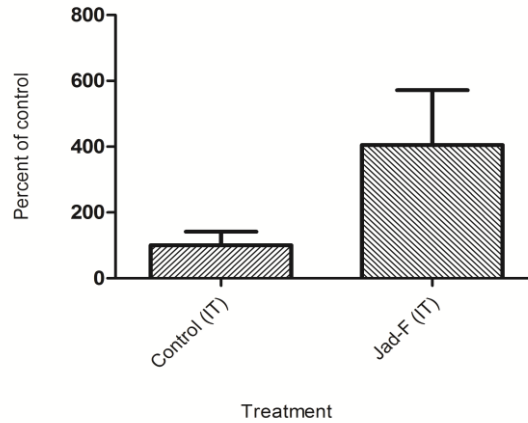
### 3.05.00: Effect of jadomycins on lung metastasis

At the study endpoint the impact of jadomycin treatments on the formation of lung metastasis was examined. Since metastasis is what ends up causing most of breast cancer fatalities, this endpoint was very important to include. The lungs were collected from each mouse, and the 6-thioguanine lung-metastasis assay was used to quantify the formation of 4T1 tumorigenic lung colonies. Results from the lung metastasis assays showed that metastases were present in the lungs of control and treatment mice 16 days after tumor cells were first implanted. There was also a trend for reduction in the amount of metastases for all IP-dosed jadomycin treatments. Jadomycins B, S, and F reduced the amount of metastases seen in the lungs to approximately 11%, 27%, and 27% of the control metastases (**Figure 13A**). Interestingly in IT-dosed jadomycin F treatment mice, there was roughly 4 times as many metastases compared to control (**Figure 13B**). When excluded all zero values for the number of metastases, jadomycin B showed a significant reduction of lung metastases compared to control (**Figure 13C**).

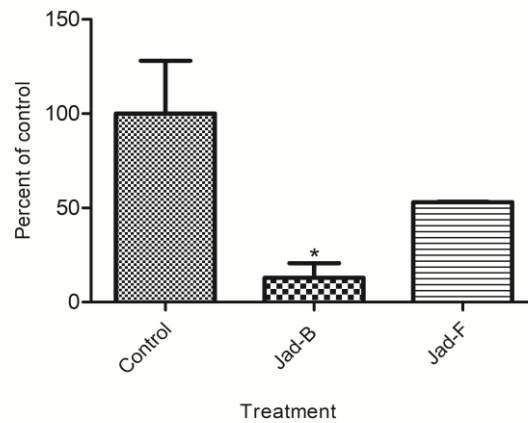
A)



B)



C)



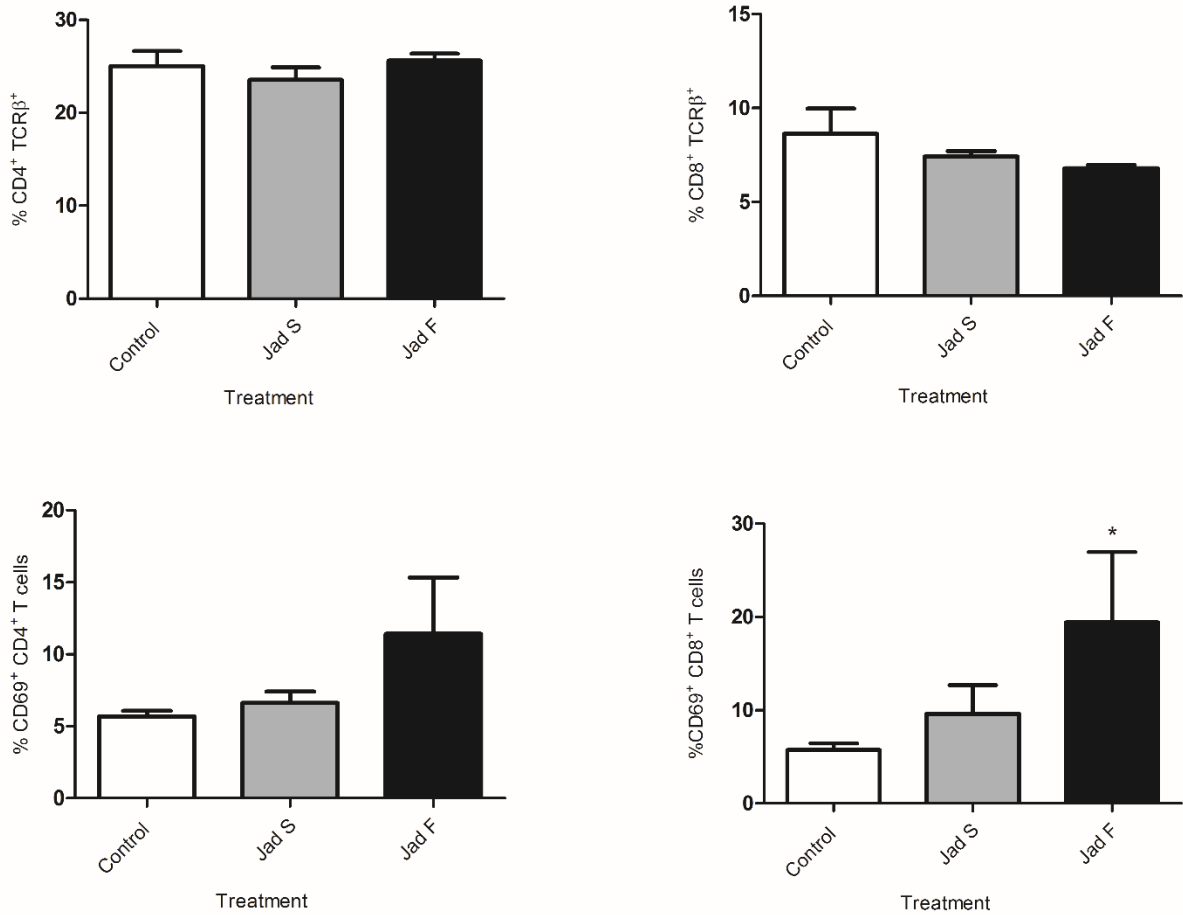
**Figure 13: Effect of jadomyicins B, S and F on lung metastases.** Amount of metastases present in lungs of balb/C mice after jadomyicin treatment and expressed as a percent of control. Each treatment had an n value of n = 8 with the exception of jadomyicin S which was n = 3 due to some lung assays being unsuccessful. A) Comparison of IP Jad-B, S, and F to IP vehicle control. B) Comparison of IT Jad-F to IT vehicle control. C) The data from panel A were reevaluated after the exclusion of those samples with zero visible colonies. Jadomyicins were dosed at 13.8 mg/kg twice daily (IP) and once daily (IT) for a period of 10 days. A one-way ANOVA was done using the Kruskal-Wallis test with  $p <$



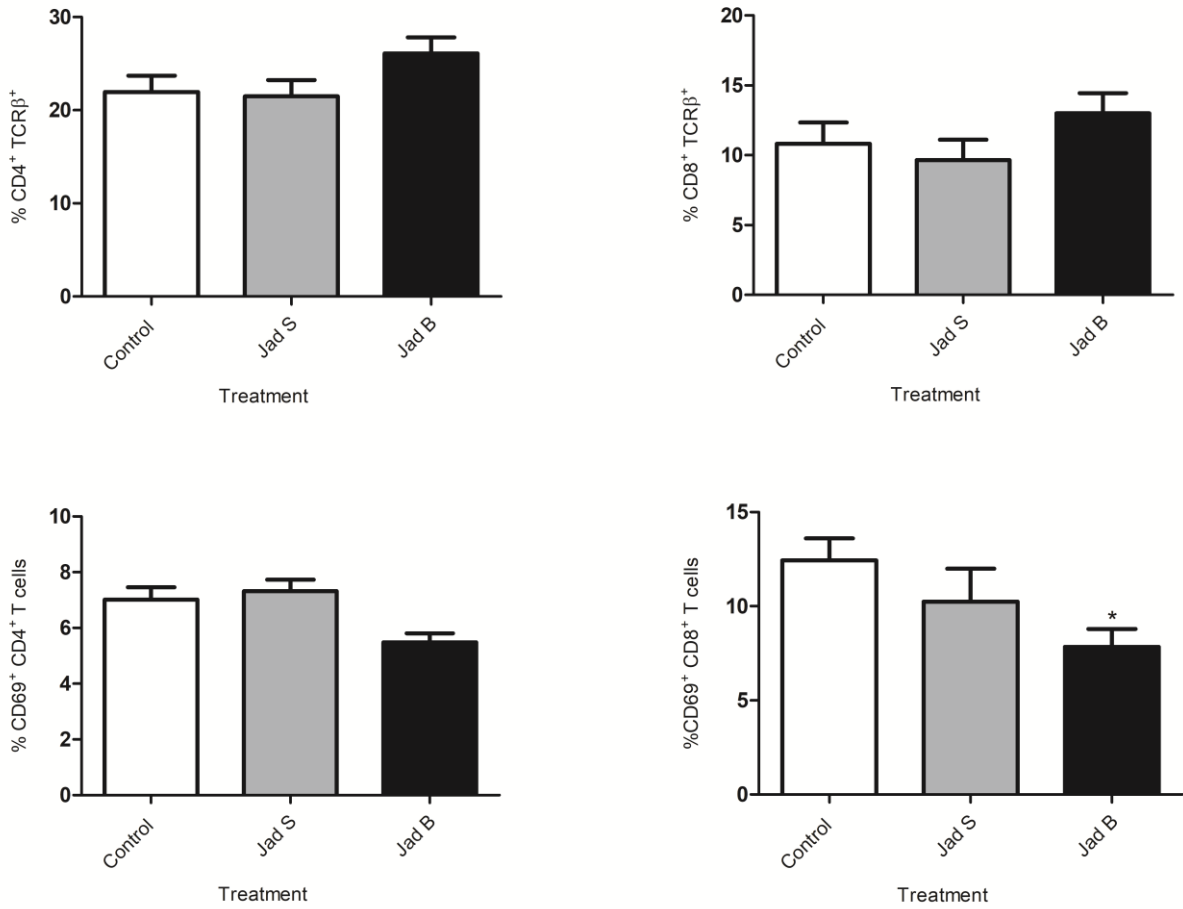
0.05 for significance. Jad-B = jadomycin B, Jad-S = jadomycin S, Jad-F = jadomycin F, and IT = intratumoral.

### *3.06.00: Effect of jadomycins on immune cell markers/FACS*

To assess if jadomycins impact immune responses in breast cancer we examined the impact of jadomycin treatments on immune cell profiles in spleen, lymph node, and tumor samples that were collected on the final study day (day 16). CD4<sup>+</sup> (T-helper cells), CD8<sup>+</sup> (T-cytotoxic cells), and the CD69<sup>+</sup> immune activated version of these T cells were examined as well as macrophages using the F4/80 marker and the Gr-1 marker for myeloid-derived suppressor cells (MDSC). The significant differences seen in immune populations involved changes in either the CD69<sup>+</sup> CD8<sup>+</sup> T cells or CD69<sup>+</sup> CD4<sup>+</sup> T cells within the spleen. The % of CD69<sup>+</sup> CD8<sup>+</sup> T cells in the spleen was significantly higher in the jadomycin F treatment group (**Figure 14**) and was significantly lower in the jadomycin B treatment group (**Figure 15**) compared to control, which may indicate that jadomycin analogues moderated the immune system in different ways. A significant difference was also seen between jadomycins S and B when comparing the % of CD69<sup>+</sup> CD4<sup>+</sup> T cells in the spleen, with jadomycin S showing a higher percentage, reiterating that jadomycins may display differences in immune phenotype (**Figure 15**).

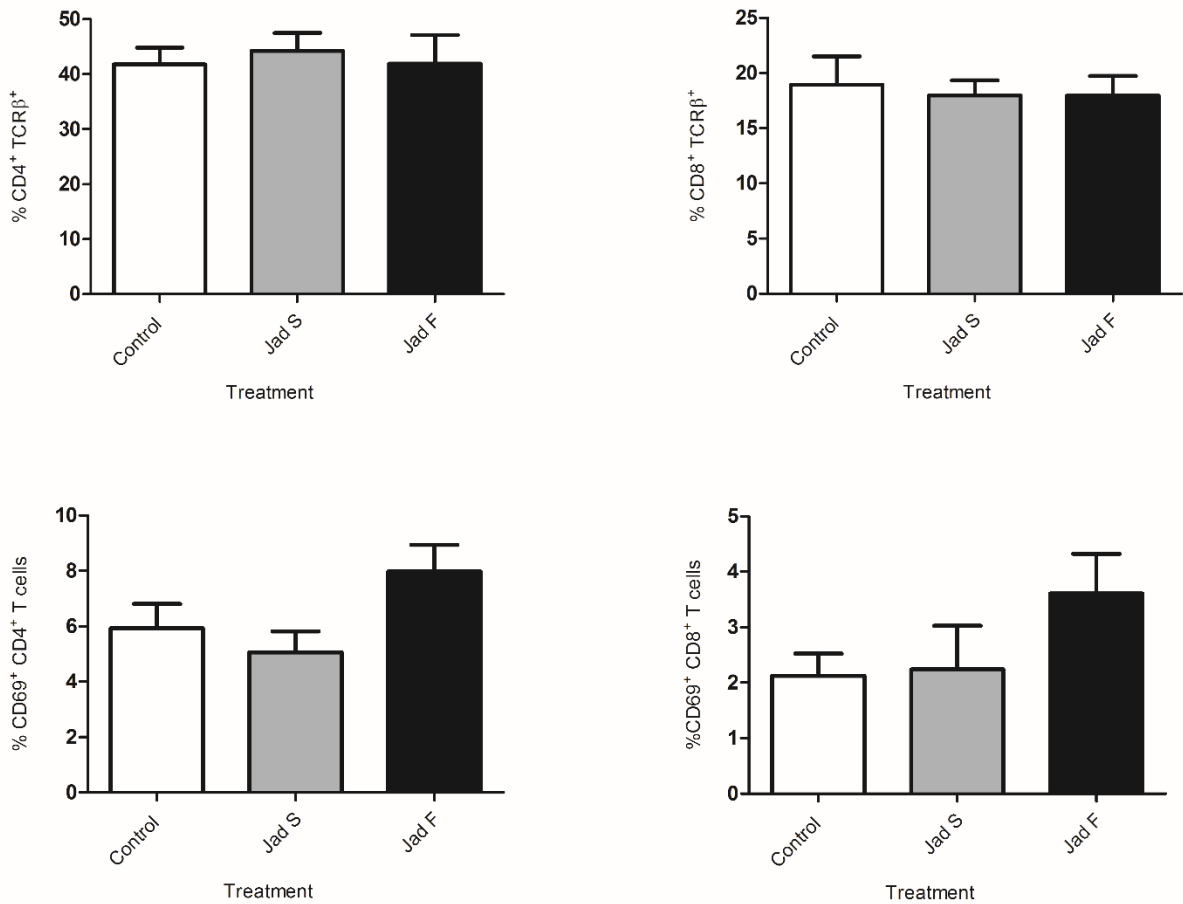


**Figure 14: Effect of jadomyicins S and F on splenic immune cells.** Percentage of splenic CD4<sup>+</sup> TCRβ<sup>+</sup> T cells (A), CD8<sup>+</sup> TCRβ<sup>+</sup> T cells (B), immune activated CD69<sup>+</sup> CD4<sup>+</sup> T cells (C) and immune activated CD69<sup>+</sup> CD8<sup>+</sup> T cells (D) found in female balb/c mice treated with jadomycin S or F or saline control. Jadomyicins were given over a period of 10 days at a dose of 10 mg/kg twice daily. A one-way ANOVA was done using the Kruskal-Wallis test with  $p < 0.05$  for significance. Jad S = jadomycin S, Jad F = jadomycin F.

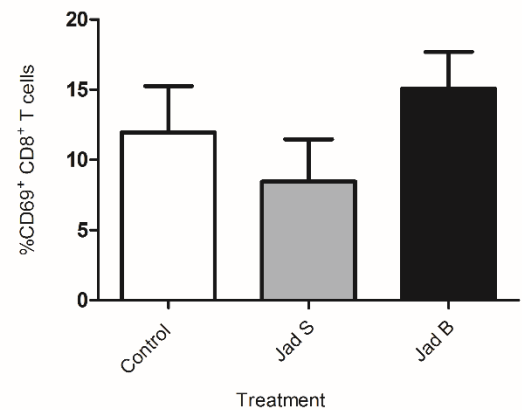
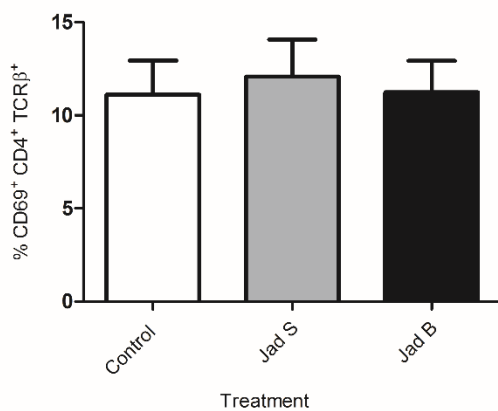
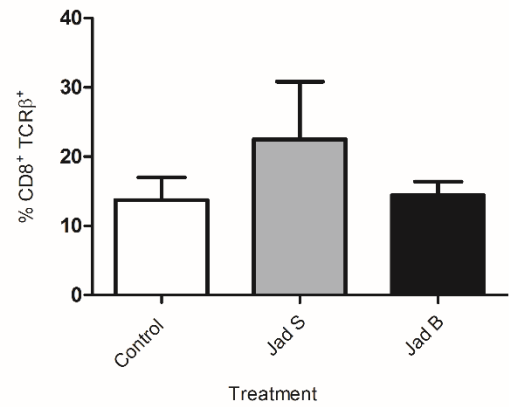
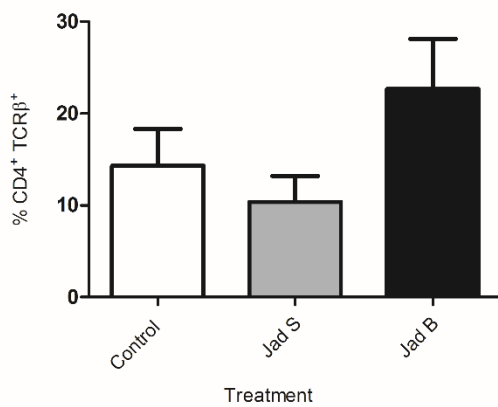


**Figure 15: Effect of jadomyicins S and B on splenic immune cells.** Percentage of splenic CD4<sup>+</sup> TCRβ<sup>+</sup> T cells (A), CD8<sup>+</sup> TCRβ<sup>+</sup> T cells (B), immune activated CD69<sup>+</sup> CD4<sup>+</sup> T cells (C) and immune activated CD69<sup>+</sup> CD8<sup>+</sup> T cells (D) found in female balb/c mice treated with jadomycin S or B or saline control. Jadomyicins were given over a period of 10 days at a dose of 13.8 mg/kg twice daily. A one-way ANOVA was done using the Kruskal-Wallis test with  $p < 0.05$  for significance. Jad S = jadomycin S, Jad B = jadomycin B.

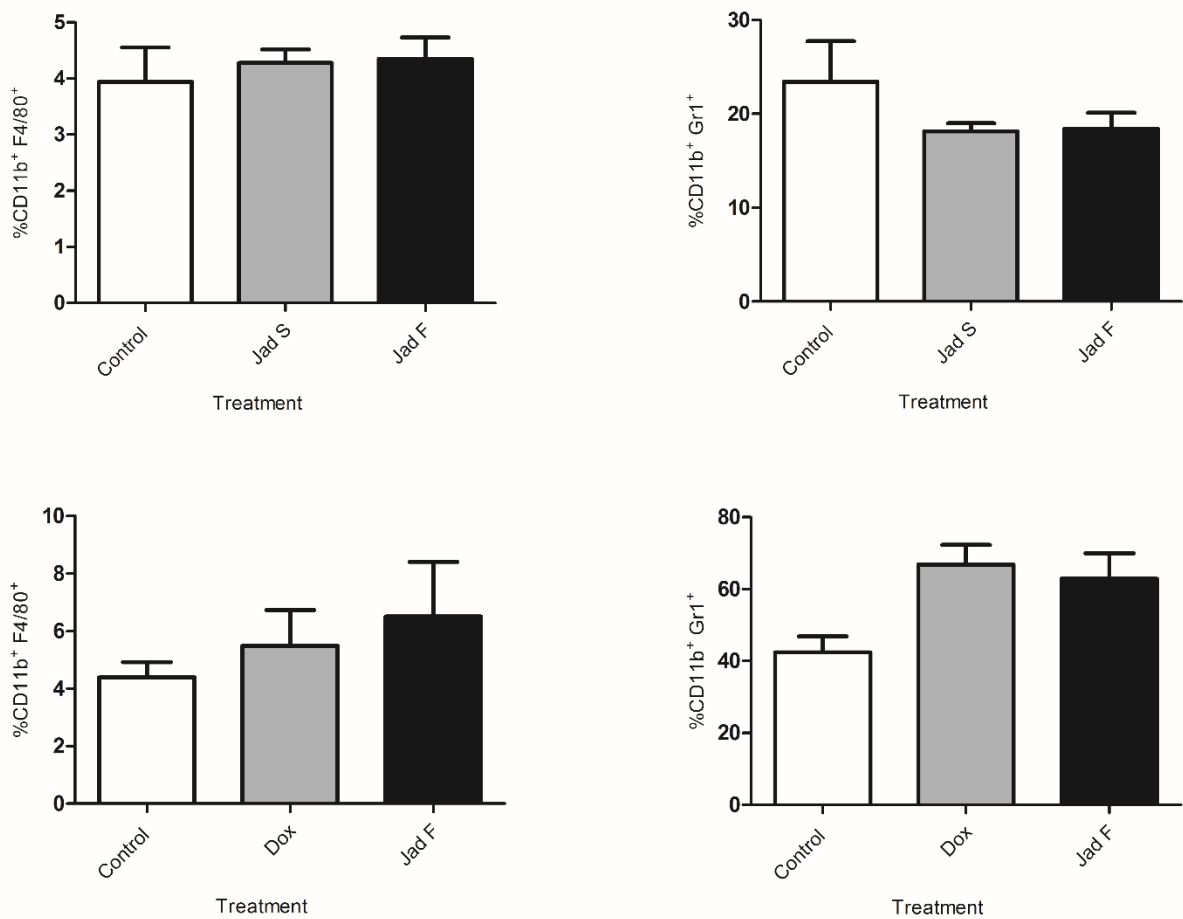
No significant differences in the percentage of T cell populations was seen within the lymph nodes or tumor samples (**Figure 16 and 17**). When examining the F4/80 marker for macrophages and the Gr-1 marker for MDSC's, there was no significant difference seen regardless of sample type (**Figure 18**). As individual experiments could not include all jadomycins and compound availability was a limiting factor, spleen, lymph node, and tumor samples were not analyzed for every experiment. Tumor samples were included for analysis after initial experiments and macrophage and MDSC markers were not followed up after showing no significance in initial experiments.



**Figure 16: Effect of jadomycins S and F on immune cells in lymph nodes.** Percentage of lymph node CD4<sup>+</sup> TCRβ<sup>+</sup> T cells (A), CD8<sup>+</sup> TCRβ<sup>+</sup> T cells (B), immune activated CD69<sup>+</sup> CD4<sup>+</sup> T cells (C) and immune activated CD69<sup>+</sup> CD8<sup>+</sup> T cells (D) found in female balb/c mice treated with jadomycin S or F or saline control. Jadomycins were given over a period of 10 days at a dose of 13.8 mg/kg twice daily. A one-way ANOVA was done using the Kruskal-Wallis test with p < 0.05 for significance. Jad S = jadomycin S, Jad F = jadomycin F.



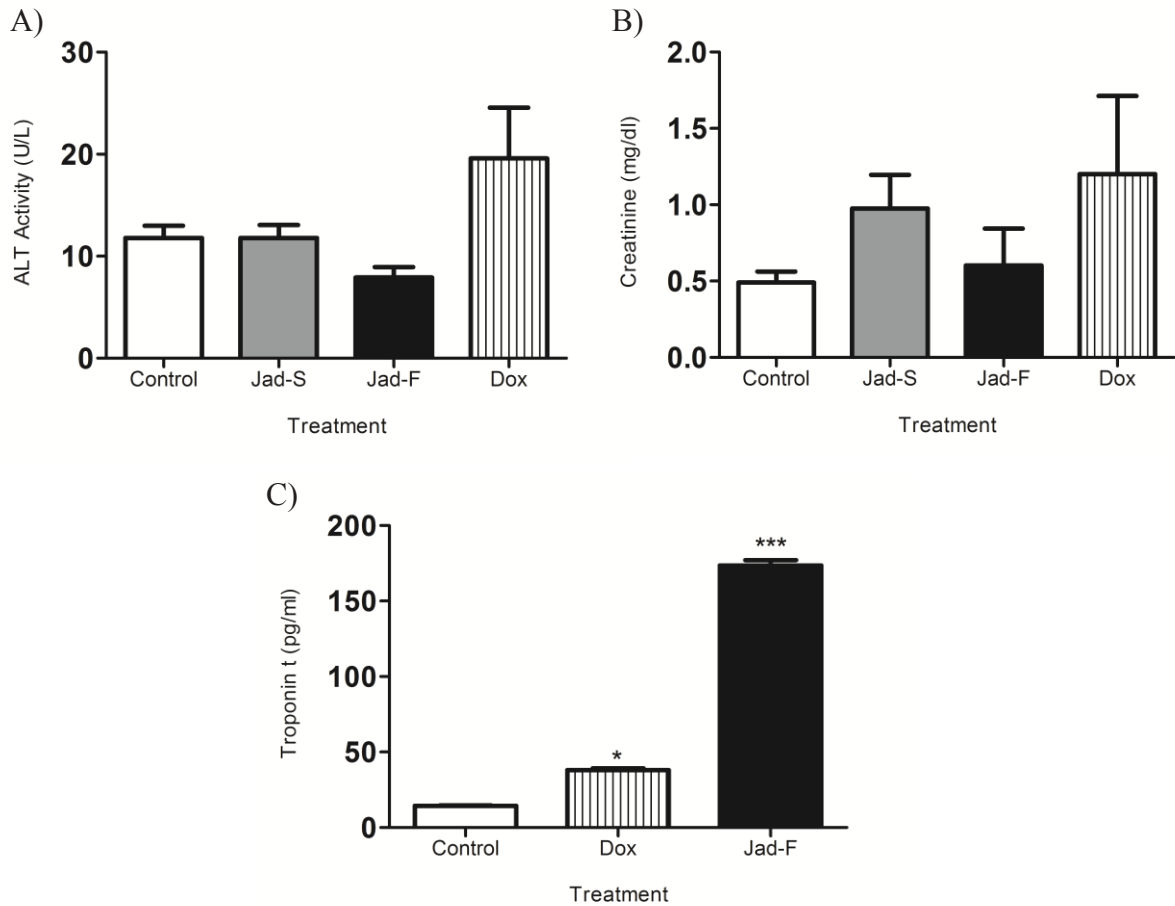
**Figure 17: Effect of jadomycins S and B on immune cells in tumors.** Percentage of tumor CD4<sup>+</sup> TCRβ<sup>+</sup> T cells (A), CD8<sup>+</sup> TCRβ<sup>+</sup> T cells (B), immune activated CD69<sup>+</sup> CD4<sup>+</sup> T cells (C) and immune activated CD69<sup>+</sup> CD8<sup>+</sup> T cells (D) found in female balb/c mice treated with jadomycin S or B or saline control. Jadomycins were given over a period of 10 days at a dose of 13.8 mg/kg twice daily A one-way ANOVA was done using the Kruskal-Wallis test with p < 0.05 for significance. Jad S = jadomycin S, Jad B = jadomycin B.



**Figure 18: Effect of jadomycin F on splenic macrophages and MDSC's.** Percentage of splenic CD11b<sup>+</sup> F4/80<sup>+</sup> cells (A), CD11b<sup>+</sup> Gr1<sup>+</sup> (B), CD11b<sup>+</sup> F4/80<sup>+</sup> (C) and CD11b<sup>+</sup> Gr1<sup>+</sup> cells (D) found in female balb/c mice treated with jadomycin, doxorubicin, or saline control. Jadomyccins were given over a period of 10 days at a dose of 13.8 mg/kg twice daily while doxorubicin was given once every two days at 5 mg/kg. A one-way ANOVA was done using the Kruskal-Wallis test with  $p < 0.05$  for significance. Jad S = jadomycin S, Jad F = jadomycin F, and Dox = doxorubicin.

### *3.07.00: Evaluation of jadomycin safety and toxicity in mice*

In order to see if jadomyccins were having any toxicological effects that were unobservable just by routine monitoring or physical signs, three biomarkers of organ damage were assessed: ALT for liver damage, creatinine for kidney damage, and troponin-t for heart damage. As compared to control mice, we did not see significant differences in the levels of ALT activity (n = 5 per treatment) or creatinine levels (n = 12) in jadomycin treated mice, which means these treatments likely do not incur damage to the liver or kidneys with the multiple dosing at the 10 mg/kg dose. The troponin-t assay was run in duplicate due to serum volume requirements of this assay and provided initial results that appear to indicate that jadomycin F significantly raises the level of troponin-t as measured in the blood by approximately 1200% (**Figure 19**).



**Figure 19: Biomarkers of liver, kidney and heart toxicity.** A) ALT biomarker of liver damage, B) creatinine biomarker of kidney damage, and C) troponin t biomarker of heart damage for jadomycin S and F or doxorubicin as compared to control. Jadomycin B was not analyzed due to the serum samples being collected after biomarker tests were performed. A one-way ANOVA using Tukey's multiple comparison test was used with  $p < 0.05$  for significance. Jad-S = jadomycin S, Jad-F = jadomycin F, and Dox = doxorubicin.



## CHAPTER 4.00.00: DISCUSSION

### 4.01.00: MDR and MBC

Presently, MBC is still considered to be an incurable disease despite the development of new therapies and earlier detection.<sup>168</sup> ABC transporters, specifically ABCB1, ABCC1, and ABCG2 have been shown to be largely responsible for the development of MDR when they are upregulated.<sup>169</sup> The potential of ABC transport inhibitors has also been assessed for their ability to make chemotherapy effective again, but results have shown that they are not effective and in addition cause a variety of unwanted side effects.<sup>170</sup> In previous studies, it was shown that the inhibition of ABCB1, ABCC1, and ABCG2 efflux transporters with verapamil, MK-571, or ko143, respectively, did not significantly affect jadomycin cytotoxicity in drug-resistant MCF7 cells, which suggest jadomycins are poor substrates of efflux transporters.<sup>143</sup> On that note, cytotoxic compounds that are poor substrates of ABC transporters could be particularly useful. The first ever pharmacokinetic studies of jadomycins in mice has taught us more about their activity *in vivo*.

### 4.02.00: Pharmacokinetics

In our current study of jadomycins *in vivo*, we determined the serum concentrations and pharmacokinetics of jadomycins B, S, and F after IP injection in balb/C mice. After IP administration of jadomycins, all jadomycins were rapidly absorbed into circulation. This was expected based on the high rate of blood perfusion of the peritoneal membrane. While the exact mechanism is not known, jadomycins appear to show sex dependent pharmacokinetics. This is evidenced by the higher  $C_{max}$  of jadomycin S in females than males, suggesting that jadomycin S is better absorbed or less subject to first-pass elimination by the liver in females. In terms of the

distribution phase, the rapid decline in the concentration of jadomycins, which was then followed by a more steady or slower elimination phase, is characteristic of many drugs following multi-compartment pharmacokinetic profiles. The differences seen in the serum concentrations of jadomycins may be due to differences in chemical structure, especially if the different amino side groups (R-groups) determines their ability to enter tissues or be eliminated. The serine R-group is a small hydrophilic OH group so it may have more trouble in penetrating membranes. On the other hand the R-groups of jadomycin B and F, which are isoleucine and phenylalanine, respectively, may have better membrane permeability due to their more hydrophobic nature. However, since there was no clear link between the R-group and serum concentration and opposite effects were seen in female versus male mice in this regard, any effect of the R-group on jadomycin concentration may be minimal compared to sex differences. Another potential reason for this difference in concentrations could be due to differences in drug transport associated directly with analogue structure.

We have no data to support that jadomycins are being metabolized. No metabolites were detected in serum or liver samples of jadomycin B suggesting jadomycins are not metabolized but rather eliminated unchanged. This combined with the high levels of jadomycin B accumulation in the liver and kidney suggest biliary and urinary elimination of the unchanged drug would be the primary routes. However, this remains to be confirmed by a future analysis of jadomycins in mouse fecal and urine samples. While our assessment of metabolism was not exhaustive, the results suggest that drug interactions involving common CYP enzymes would be unlikely and this would be a favorable property.

It is also not certain how jadomycins will interact with other drugs *in vivo* but we have seen that their cytotoxic activity can be potentiated or hindered *in vitro* with the combination of other

compounds such as the anti- and pro-oxidants NAC and auranofin, respectively.<sup>144</sup> By testing these types of compounds in combination treatment with jadomycins, more could be gleaned on the mechanism of action of jadomycins and how they compare to other combination chemotherapies that use the same compounds and administration strategies.

Combining all of our pharmacokinetic studies performed in balb/C mice with jadomycins B, S, and F, we saw that the animals tolerated the single dose of jadomycin at 6 mg/kg very well. There were no adverse events that we could report and there were no physical signs of distress that manifested. We have since followed up with biomarker tests for organ damage in our chronic tumor studies and have showed that there is likely no damage occurring in the liver and kidneys by way of measuring ALT and creatinine but there may be elevated levels of troponin-t to indicate that heart damage is a possibility of our treatments. Notwithstanding the observation of increased troponin-t is an endpoint of concern and will require confirmation and follow-up in future studies.

While the peak serum concentrations after the 6 mg/kg dose achieved concentrations required for *in vitro* cytotoxicity, we found that mouse 4T1 breast cancer cells required slightly higher IC<sub>50</sub>s so the dosage was increased to 10 mg/kg. A 13.8 mg/kg maximum dose was then used for later studies because we wanted to see if a higher dose of jadomycins would have increased efficacy in reducing tumor size and volume but we ran into solubility issues due to the fact that the compounds were not dissolving at higher concentrations and the maximum IP volume was already administered. An additional reason for the dose increase as well as adopting a twice daily dosing was that plasma concentrations of jadomycins rapidly declined following administration. In order to maintain jadomycin concentrations within the cytotoxic range, multiple daily dosing was required. Although we also considered other dosing strategies, this option was the most

feasible and appeared to be tolerated by the animals. Importantly, we are also aware that different jadomycin analogues exhibit different pharmacokinetic profiles and that this may play a role in how effective they will be in tumor studies. Jadomycin pharmacokinetics exhibit a faster clearance rate and short  $t_{1/2el}$ , which could be a potential positive aspect of these compounds as a quick  $t_{1/2el}$  could lessen the potential adverse effects that could arise due to excess drug accumulation. The  $t_{1/2el}$  of jadomycons is also relatively short compared to some other chemotherapeutic drugs such as doxorubicin, which has been shown to be quite toxic and remains in the body for longer periods of time. However, if jadomycons are being cleared too quickly and have a relatively short half-life, this could prove to be a challenge in maintaining efficacious levels of treatment. A drug delivery approach could help address issues such as these if they were indeed preventing adequate concentrations of jadomycons. Some drug delivery approaches that could be explored are the use of liposomes and nanoparticles which help reduce toxic and side effects of drugs as well as help to overcome challenges associated with drug uptake in cells and tissues.<sup>171,172</sup>

#### *4.03.00: 4T1 model and TNBC*

The 4T1 mammary adenocarcinoma mouse cell line has proved to be a good model system in that it closely mimics human TNBC. It is also a useful system to analyze because the host organisms are left immunocompetent to better resemble a true biological response to development and on-going disease process of a tumor.<sup>173</sup> Current options for the treatment of TNBC may include surgery, radiotherapy, or other chemotherapeutics. However, despite all these new therapies, TNBC remains extremely hard to treat due to development of MDR and so there is a need for continuous research into novel anti-breast cancer therapies. With further

development Jadomycins may have possible applications as they have been shown to be quite effective at killing MDR triple-negative breast cancers cell *in vitro*.

Results of gene expression in the 4T1 cell line by way of qPCR helped to confirm upregulation of the ABCB1 transporter and results from MTT assays confirm jadomycin toxicity was retained between the 4T1-CON and 4T1-TXL cell lines as demonstrated for human breast cancer cell types. This confirms that although slightly less potent in the 4T1 cells, jadomycins appear to behave similarly in mouse versus human control and MDR breast cancer cells and that this an encouraging result as far as potential translation of our findings with the 4T1 mouse model to human breast cancer. Originally, we had planned to assess both control and 4T1-TXL cells *in vivo* to expand on the work done in cell culture; however, based on lower than expected efficacy of jadomycin in 4T1 control cells *in vivo*, the goal was switched to first optimize our experiments in 4T1 control cells before testing the 4T1 MDR cells.

#### 4.04.00: Tumors

We observed a differential effect of jadomycins on the primary 4T1 breast tumors. Specifically jadomycin-B IP treated mice demonstrate reduced tumor volumes as measured by AUC values and a trend for a smaller primary tumor weights on day 16 compared to controls. In comparison, Jadomycin S and F did not affect tumor volume or weight compared to controls. The lack of effect of jadomycin F could relate to its lower potency in the 4T1-CON cells compared to jadomycin B. On the other hand, while jadomycin S and B had similar potencies in the 4T1-CON cells in culture, different pharmacokinetic characteristics may result in less jadomycin S than B reaching the tumor in adequate levels. Although the serum concentrations of jadomycin B in our PK studies were within cytotoxic range seen *in vitro*, they did not remain in that range for

more than approximately an hour based on the single dose pharmacokinetic study. This could explain why only a partial reduction in tumor volume and weight was observed for jadomycin B. In addition, even if serum levels of jadomycins remained high, this still does not mean that the jadomycins are necessarily reaching and penetrating the tumor to a sufficient degree.

It is possible that higher doses of jadomycins would result in more robust effects on the primary tumor; however, we were limited by the amount of compound we possessed as it was timely and difficult to produce. Mindful of the fact that jadomycins had to be prepared in a non-toxic vehicle for administration in balb/C mice, we were not able to solubilize jadomycins at a much higher concentration than 1mg/ml in our vehicle control. We did not want to change the composition of our vehicle control midway through our experiments in order to avoid introducing a new variable but a vehicle control other than 10% volume ethanol in PBS may need to be assessed in the future. Since we were already administering the maximum IP volume of approximately 10 ml/kg twice a day, we could not simply raise the dose by giving a larger volume in injection.<sup>174</sup>

The doxorubicin treatments we administered have shown that the 4T1 model is working properly but in order for us to see greater anti-tumor effects with jadomycin treatments we likely need to find a solution to increase the doses and thus, the amount of active compounds reaching the primary tumors. The method of intravenous (IV) jadomycin administration has yet to be tried and could prove effective if jadomycins are being quickly rendered ineffective by first-pass elimination of the liver. One other option would be to try subcutaneous administration, which could result in a slower rate of absorption over time compared to IP injection, allowing for higher drug concentrations. Combination treatments can also be explored in order to see if treatments have a potential to be additive or synergistic in their cytotoxicity and more effective

than individual treatment alone, which has already been seen in some treatments against MBC.<sup>175</sup> Of particular interest is the TrxR inhibitor auranofin, which exerts its mechanism of action by inhibiting reduction/oxidation enzymes, ultimately causing oxidative stress and apoptosis.<sup>176</sup> We have previously shown that auranofin significantly increased ROS activity of jadomycins 2.5 to 3.8-fold and decreased MCF7-CON human breast cancer cell viability 43.7 to 76.8 % when co-treated with jadomycins B, S, F or SPhG in comparison to auranofin alone, which had no effect. By blocking antioxidant pathways, auranofin prevented the neutralization of jadomycin-produced ROS.<sup>155</sup> Also, auranofin is already approved for rheumatoid arthritis in humans and is being investigated in cancer treatment so this makes it a good combination treatment for follow-up.<sup>177,178</sup> An option that we have considered but not yet explored is switching to the human MDA-MB-231 cell xenograft model in NOD/SCID mice. Since jadomycins have higher potencies in MDA-MB-231 cells (1.76 – 3.25  $\mu$ M for jadomycins B, S, and F) versus 4T1 cells, this may translate to improved anti-cancer effects *in vivo* considering dose limitations we are faced with. Other factors that may be involved in the lack of tumor shrinkage after jadomycin administration include drug inactivation *in vivo* of which we are not aware of as well as cancer cell and tumor heterogeneity, which can result in cells that have stem cell properties and are drug resistant.<sup>179</sup>

We have found reduction in primary tumor volume following jadomycin B treatment did not translate to an equivalent and significant reduction in tumor weight. While the exact reasons for this are unknown it is possible that due to the fact that tumor volume measurement does not take into account the irregular shape of tumors and that the depth of the tumor is estimated using the tumor volume equation.  $V = (L \times W \times W)/2$ , where V is tumor volume, W is tumor width, L is tumor length. It is also possible that there could be differences in tumor compositions such as

different types of cells, fluid, and extracellular matrix that would contribute to tumor heterogeneity resulting in different densities.<sup>180,181</sup>

Another area of jadomycin research that has yet to be explored is the effect of jadomyicins on chemotherapy-resistant cancer stem cells as this specific cell type could be targeted by jadomyicins in order to exert their anti-tumor activity. Cancer stem cells are a select, small portion of cancer cells that can self-renew and promote tumorigenesis by giving rise to many cell types that constitute a tumor.<sup>182</sup> They are also implicated in the development of metastasis in cancers.<sup>182</sup> Testing whether or not jadomyicins are able to kill these cells would add to the knowledge of how jadomyicins are able to kill MDR cells and retain efficacy in drug-resistant cell lines as well as reduce metastasis. In particular, jadomyicins could be exploiting the fact that the drug efflux transporter ABCG2 is a stem cell marker of MDR since they themselves are not substrates of this transporter.<sup>183</sup> This means they would be able to accumulate to therapeutic concentrations and kill MDR cancer stem cells because they are not subject to efflux by ABCG2 like other drugs.

#### *4.05.00: Lung assay*

Using the 6-TG colony assay, we were able to measure the extent of metastasis to the lungs. Our primary concern is metastatic disease as this is what eventually causes cancer to become lethal. Initially, we were met with some technical challenges including incomplete dissociation of cells from lung tissue, excess times to plate cells and poor cell adherence, resulting in a lack of detection of metastatic colonies. With some optimization of procedures, these initial challenges were overcome and we were able to detect and quantify metastatic colonies in subsequent assays. Positive results were seen with evidence of metastatic colony formation across control and



treatment groups as well as a trend for reduced amounts of metastases across all jadomycin treatment groups compared to control. When excluding all zero values for metastases, jadomycin B significantly reduced the amount of metastatic colonies compared to control. Since it is not clear if the zero values were due to the treatment working or failure of the assay, we chose to represent the data in this fashion as well and believe it is a significant observation. The, significant effect of jadomycin B (but not jadomycin F) on lung metastasis is consistent with the relative effects of these jadomycins on the primary tumors. While these initial results are promising there are some limitations in the colony assay that should be noted. First was the relatively early endpoint (day 16) compared to other studies that have used longer endpoints (day 35), which resulted in a lower number of metastatic colonies.<sup>184</sup> Our clonogenic metastasis data also showed considerable variation in the amount of colonies forming all the way from a few colonies to hundreds, which has been reported by other groups using this assay in the same tumor model.<sup>163,185</sup> One way to address the limitations would be to extend the study endpoint beyond 16 days. A second mechanism would be to utilize a green-fluorescent protein expressing 4T1 cell line, that may improve assay sensitivity.<sup>186</sup> A final approach could be to assess liver metastasis as there is generally a higher number of metastatic colonies formed there. Since jadomycins accumulated more in the liver than in the lung, they might be more effective at reducing metastasis in that particular organ. This could help in addressing a low number of metastatic colonies while validating the effects of jadomycins on metastasis.

#### *4.06.00: Immunology*

Tumor composition is not only made up of cancerous cells but many other cells as well. These cells include fibroblasts, endothelial cells, and leukocytes which include macrophages,

neutrophils, mast cells, and also B or T lymphocytes.<sup>187,188</sup> Leukocyte infiltration of the tumor has been shown to be antagonistic to tumor growth as well as to promote tumor formation and angiogenesis depending on the type of leukocyte.<sup>189,190</sup> For example, while CD8<sup>+</sup> T lymphocytes are effective at killing transformed cancer cells, a subset of CD4<sup>+</sup> T helper cells known as Th17 cells can produce interleukin-17, which has a pro-inflammatory role and can promote tumor growth.<sup>191</sup> Clinical data for solid tumors has also shown a correlation between high-density leukocyte infiltration in tumors and poor outcome of patients.<sup>188</sup> Although the innate immune system is able to stimulate acquired immune responses, there is also evidence that suggests innate immune cell infiltration can actually be tumor-promoting.<sup>188,192,193</sup> Macrophages have also been implicated in tumorigenesis.<sup>194</sup>

From our results of processing spleen, lymph node, and tumor samples for jadomycins B, S, and F, there was no significant changes in the immune populations of CD4<sup>+</sup> TCR $\beta$ <sup>+</sup> (T-helper cells), CD8<sup>+</sup> TCR $\beta$ <sup>+</sup> (T-cytotoxic cells), CD69<sup>+</sup> CD4<sup>+</sup> T cells, CD11b<sup>+</sup> F4/80<sup>+</sup> (macrophages), as well as CD11b<sup>+</sup> Gr-1<sup>+</sup> (MDSC's) cells in our jadomycin treatments compared to control. The one exception was the % of CD69<sup>+</sup> CD8<sup>+</sup> T cells in the spleen, which were significantly higher in the jadomycin F treatment group and was significantly lower in the jadomycin B treatment group compared to control, indicating that jadomycin analogues may affect the immune system in different ways. CD69 is one of the earliest antigen activation markers to appear on the surface of activated lymphocytes.<sup>195</sup> Studies of CD69 have shown that it is a negative regulator of the antitumor immune response partly due to production of transforming growth factor- $\beta$  (TGF- $\beta$ ), a cytokine involved in controlling cell proliferation and differentiation.<sup>196</sup> A deficiency in CD69 promotes autoimmune and antitumor response as observed by *in vivo* experimentation with CD69<sup>-/-</sup> mice.<sup>197</sup> In agreement with this are the results from jadomycin B, which show CD69<sup>+</sup>

CD8<sup>+</sup> T cells are decreased, which could be linked to the trend in reduction of primary tumor size for that treatment but not for jadomycin F in which CD69<sup>+</sup> CD8<sup>+</sup> T cells increased. The fact that these activated T-cells are significantly changed in the spleen could indicate that there was a systemic immune response to treatment, which may include inflammation. The relatively modest change in the amount of CD69<sup>+</sup> CD8<sup>+</sup> T cells in the tumor samples could be due to the fact that these cells were not infiltrating into the tumor well. This would mean these cells were also not subsequently being drained out from the tumor into lymph and that the levels of these cells in lymph would thereby not change significantly. Ultimately, this type of information could be important in selecting which jadomycins would be most useful for further *in vivo* testing. A significant difference was also seen between jadomycins S and B when comparing the % of CD69<sup>+</sup> CD4<sup>+</sup> T cells in the spleen with jadomycin S showing a higher percentage, reiterating that each jadomycin may exhibit unique immune profiles. The potential anti-tumor effects of jadomycins may stem from immunomodulatory effects but it is also possible that it is through direct cytotoxic effects as well. Since we have yet to test other analogues as well as higher doses, this is not yet clear but will hopefully be elucidated in future experiments. Also, we do not see significance differences in macrophage or myeloid derived suppressor cell populations, which may mean that jadomycins B, S, and F are not causing myelosuppression at least at the current dose with IP administration. Overall, the immune data suggests that jadomycins exhibit different effects on immune cell profiles and that further investigation is required to determine how this might influence their anti-tumor effects.

Future investigation into the effect of jadomycins on the immune system could involve examining how they may affect regulatory T cells or natural killer cell populations. Regulatory T cells have been found to promote primary tumor progression as well as

metastatic tumor growth.<sup>198</sup> Since jadomymins have been shown to decrease the amounts of metastases in balb/C mice compared to control, any changes in regulatory T cell populations following jadomymin treatment could relate to how jadomymins are able to reduce lung metastases. In addition, T regulatory cells are also necessary for metastasis to occur within the 4T1 model and so this makes this population especially important to study.<sup>199</sup> Natural killer cells on the other hand, have been shown to be able to kill tumor cells and play a critical role in controlling tumor growth and metastasis.<sup>200</sup> If jadomymins were able to somehow increase or bolster natural killer cell populations, this could provide additional evidence for their anti-tumor and anti-metastatic effects.

#### *4.07.00: Toxicology*

In general, the animals tolerated the single dose of jadomymin at 6 mg/kg very well. There were no apparent adverse events we could report and there were no physical signs of distress. We have since followed up with biomarker tests for organ damage in our chronic tumor studies and have showed there is likely no damage occurring in the liver and kidneys by way of measuring ALT and creatinine as these levels were normal in the jadomymin F treatment groups compared to control. However, there were elevated concentrations of troponin-t, suggesting possible cardiotoxicity with jadomymin F treatment. This could be a potential consequence of the fact that jadomymins contain a ROS-generating quinone group similar to doxorubicin, a known cardiotoxin.<sup>201,202</sup> Thus, it is entirely possible that a similar effect could occur with jadomymins. However, we are careful not to make this link as we used a ‘serum cocktail’ or pool of individual serum samples from jadomymin F in order to meet the minimum amount of serum required by the assay we used, meaning that only some of the mice may have been experiencing elevated levels

of troponin-t. Another possibility is that since jadomycins are pigmented molecules, there could be interference with the troponin assay, which would cause a false positive. Notwithstanding the observation of increased troponin-t is an endpoint of concern and will require confirmation and follow-up in future studies. One way to do this would be to perform an *in vivo* electrophysiology study to record electrocardiograms (ECGs).<sup>203</sup> This would shed light on whether or not the heart was functioning properly after jadomycin administration and could be done with a variety of doses or timings. It is also possible that observation of elevated troponin T following jadomycin F is not representative of the other jadomycin analogues. Therefore, it will be important to complete cardiotoxicity analysis with other jadomycins to determine if they have different impacts on biomarkers of cardiovascular toxicity. Ultimately, this would help identify the jadomycins with the best safety/toxicity profiles.

#### *Limitations and future experiments*

Further pharmacokinetic studies of additional jadomycins analogues will provide a better understanding of how structure influences jadomycin pharmacokinetics and could give a better picture of how these compounds distribute within the mouse. Tests with species other than mice could also show if there are any specific interspecies pharmacokinetic differences. For example, mice tend to have faster elimination rates than humans and it has been reported that there are differences in expression levels of certain drug transporters.<sup>204</sup> Although we don't know if the serum concentration of jadomycins are representative of those found within an identical but tumor-bearing model, future work could look into how an active tumor or metastases affect their pharmacokinetics. The presence of a tumor can change the pharmacokinetics of drugs in the body and so at times it may not be possible to accurately extrapolate the pharmacokinetics of anticancer drugs in healthy individuals compared to those with cancer.<sup>205</sup> In general, the

concentrations of anticancer drugs in normal tissues will be higher than in the primary tumor itself.<sup>205</sup> One study found that histamine dihydrochloride, a drug used to treat metastatic melanoma (MM), had significantly altered pharmacokinetics between healthy patients and those with MM. In patients with MM, the elimination rate constant was significantly lower while the AUC was significantly higher compared to healthy individuals.<sup>206</sup> Another limitation is that we only studied 3 jadomyocins and while they are representative of different structure groups, further pharmacokinetic studies are required to fully evaluate how structure translates to pharmacokinetics. A second limitation was that we were unable to test higher doses due to limited amounts of compound, which is not available for purchase commercially. Tied to this issue, we encountered a problem with solubilizing jadomyocins in our vehicle at a dose higher than 13.8 mg/kg in the maximum injection volume. Future experiments could look at different vehicles for administration *in vivo*. With the current limitations and compound availability, the mouse remains a feasible model for further studies.

### *Conclusion*

In conclusion we have conducted the very first in animal pharmacokinetic/pharmacodynamics studies of jadomyocins B, S and F. The results have significantly advanced our knowledge of these compounds and will guide how they can be used for future *in vivo* pharmacological studies. Jadomyocin pharmacokinetics were dependent on sex and the specific jadomyocin compound. Due to their rapid elimination frequent dosing was a requirement for pharmacological studies in mice. Following chronic dosing in the 4T1 breast cancer model we did not observe significant effects with jadomyocin S and F, but were encouraged by the significance and trends observed for jadomyocin B involving reduced tumor volumes, primary tumor weight, lung metastasis, and reduced splenic CD69<sup>+</sup> CD8<sup>+</sup> T cells. Our results support that jadomyocins may induce different

immune responses. The extent to which this may contribute to anti-tumor or metastatic effects of jadomycins cannot be discerned from our data and will require evaluation in future studies. The observation that jadomycins B, S, and F imparted no physical signs to indicate that the health status of the animals is being compromised was a promising result supporting further *in vivo* testing of these compounds is possible. However, elevated troponin-t levels from chronic jadomycin F treatment indicates cardiotoxicity as a potential concern that requires further follow-up. An additional priority for future studies will be to test alternative dosing or formulation strategies and human xenograft tumor models in order to fully elucidate the anti-tumor effects of jadomycins *in vivo*. We are hopeful that future experiments on jadomycins will add to the understanding of how these compounds could be used in the treatment of MDR breast cancer.

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## **APPENDIX I: Tables**

**Mouse Essential CLEAR Panel**

	<u>1</u> 4T1-CON	<u>2</u> 4T1-TXL
<i>HANT (Hantavirus Hantaan) PCR</i>	-	-
<i>LCMV PCR</i>	-	-
<i>LDV PCR</i>	-	-
<i>MAV 1 &amp; 2 PCR</i>	-	-
<i>MHV PCR</i>	-	-
<i>MNV PCR</i>	-	-
<i>Mouse Parvovirus (MPV/MVM) P</i>	-	-
<i>Mousepox (Ectromelia) PCR</i>	-	-
<i>MRV (EDIM) PCR</i>	-	-
<i>POLY PCR</i>	-	-
<i>REO PCR</i>	-	-
<i>SEND PCR</i>	-	-
<i>TMEV/GDVII PCR</i>	-	-
<i>Vesivirus</i>	-	-
<i>Mycoplasma Genus PCR</i>	-	-
<i>M. pulmonis PCR</i>	-	-

*Remarks*

- = Negative, +/- = Equivocal; + = Positive; I = Inconclusive.

An equivocal result indicates inconsistent amplification detected by real-time PCR.

Inconclusive indicates failure of control result.