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Jadomycins inhibit type II topoisomerases and promote DNA damage and apoptosis in multidrug resistant triple negative breast cancer cells

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Running Title Page

a) **Running Title:** Jadomycins are DNA-damaging type-II topoisomerase inhibitors

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d) **Abbreviations:**

231-CON	Control MDA-MB-231 breast cancer cells
231-TXL	Paclitaxel-resistant MDA-MB-231 breast cancer cells
ABC	ATP-binding cassette
CM-DCFH$_2$-DA	5-(and 6-)chloromethyl-2’7’-dichlorodihydrofluorescein diacetate
ER	Estrogen receptor
FBS	Fetal bovine serum
HER2	Human epidermal growth factor receptor 2
IC_{50}	Half-maximal inhibitory concentration
kDNA	Kinetoplast DNA
MDR	Multidrug resistance
MTT	Thiazolyl blue methyltetrazolium bromide
NAC	N-acetyl cysteine
PARP	Poly(ADP-ribose) polymerases
PR	Progesterone receptor
qPCR	Quantitative polymerase chain reaction
ROS	Reactive oxygen species
TXL	Paclitaxel (Taxol)
Z-VAD	N-Benzyloxycarbonyl-Val-Ala-Asp(O-Me) fluoromethyl ketone (Z-VAD[OMe]-FMK)

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Abstract

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

Introduction

Breast cancers are among the most common types of cancer to affect women worldwide (Yu et al., 2013). Despite progress in its treatment as a local disease, metastatic breast cancer remains essentially incurable with a median survival time of 2-3 years (Morris et al., 2009; Lluch et al., 2014). Its incurability is primarily due to the development of multidrug resistance (MDR) within the cancerous cells, reducing the effectiveness of available therapies (Morris et al., 2009; Rivera, 2010). The most commonly observed mechanism of MDR is the overexpression of ATP-binding cassette (ABC) efflux transporters, which expel chemotherapeutics from within the cell, rendering the treatments ineffective. In cell culture, the ABCB1, ABCC1, and ABCG2 transporters are most likely to be overexpressed in MDR tissue samples (Szakacs et al., 2006).

Additionally, certain categories of breast cancer are innately more difficult to treat than others. Breast tumour cells that lack or have little expression of estrogen receptor (ER) and progesterone receptor (PR) and do not overexpress human epidermal growth factor receptor 2 (HER2) are known as triple-negative breast cancers. Triple-negative breast cancers are typically of a larger size and higher grade than non-triple-negative breast cancers, with a higher rate of metastasis development and a lower overall survival rate. About 15% of all breast cancers are triple-negative, and they disproportionally affect women under the age of forty. Treatment options for triple-negative breast cancer are limited as hormone-receptor or HER2-targeted therapies are ineffective, and for advanced cases the only treatments available are cytotoxic chemotherapies (Bauer et al., 2007; Rakha et al., 2007; Elias, 2010). With up to 30% of all cases of breast cancer ultimately metastasizing, and the high prevalence of MDR and triple-negative breast cancers (Elias, 2010; Rivera, 2010), new and more effective treatments are needed.

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Jadomycins (**Figure 1a-d**) are a class of naturally biosynthesized, polyketide-derived compounds produced by the soil bacteria *Streptomyces venezuelae* ISP5230 (Jakeman et al., 2006). Jadomycin analogues with distinct functional groups on the oxazolone ring can be biosynthesized by using different amino acids as the sole nitrogen source in the bacterial growth medium (Dupuis et al., 2012; Martinez-Farina and Jakeman, 2015; Robertson et al., 2015). We have shown that many jadomycin analogues are effective cytotoxic agents against ER-positive, PR-positive, HER2-negative MCF7 breast cancer cells and that they largely retain their potency in MDR MCF7 breast cancer cells that overexpress the ABC drug efflux transporters *ABCB1*, *ABCC1*, or *ABCG2*. In comparison, drugs that are known ABC-transporter substrates lose their cytotoxic potency, such as doxorubicin, etoposide, or mitoxantrone (**Figure 1e-f**) (Issa et al., 2014). We have also determined that jadomycins are equally cytotoxic in triple-negative MDA-MB-231 versus non-triple-negative MCF7, BT474, and SKBR3 breast cancer cells (Hall et al., 2015). Jadomycins are therefore attractive compounds for the treatment of drug resistant and triple-negative breast cancers.

Currently we only have a basic understanding of how jadomycins exert their anticancer activity. We initially determined that these compounds induce intracellular ROS activity through a Cu(II)-dependent mechanism in drug-sensitive and MDR *ABCB1*-overexpressing MCF7 breast cancer cells, and that jadomycin potency can be altered when co-treated with anti- or pro-oxidants, suggesting that jadomycin anticancer activity is at least partially dependent on ROS. Interestingly, it was also found that when ROS were inhibited jadomycins still retained 100% cytotoxic efficacy in the breast cancer cells (albeit with lower potency), evidencing that jadomycins are also acting through ROS-independent mechanisms (Hall et al., 2015). One such alternate mechanism is the inhibition of aurora B kinase, an important mitotic protein, which can

lead to cancer cell death (Fu et al., 2008; Issa et al., 2014; Hall et al., 2015). Jadomycins may also interact with topoisomerase IIβ, an enzyme that reduces DNA tension during replication, to which jadomycin DS was recently discovered to bond (Martinez-Farina et al., 2015). The polypharmacological nature of jadomycins' anticancer activity could help explain how these compounds evade drug resistance.

To date we have only tested the mechanisms of jadomycin anticancer activity in MCF7 breast cancer cells (Issa et al., 2014; Hall et al., 2015). To advance our previous work a more aggressive triple-negative cell line, MDA-MB-231, was chosen for this study. Additionally, while we determined that jadomycins kill MDR and drug-sensitive breast cancer cells (Issa et al., 2014), a better understanding of their intracellular cytotoxic targets and mechanisms of action are still needed. Building on our past experiments that determined jadomycins induce ROS and because oxidative stress can cause DNA damage and apoptosis (Bertram and Hass, 2008), we hypothesized that jadomycins damage DNA leading to breast cancer cell apoptosis.

Materials and Methods

Chemical and Biological Materials

Thiazolyl blue methyltetrazolium bromide (MTT), NAC, methanol, propidium iodide, mitoxantrone, paclitaxel (TXL), doxorubicin, agarose, benzamide, $CaCl_2$, NaCl, Tris-HCl, Tris base, HEPES, KCl, $MgCl_2$, Na-EDTA, SDS, DMSO, glycerol, sucrose, adenosine triphosphate (ATP), bovine serum albumin, ethidium bromide, proteinase K, bromophenol blue, and phosphate buffered saline (PBS) were purchased from Sigma Aldrich (Oakville, Ontario, Canada). Auranofin was purchased from Santa Cruz Biotechnology Inc. (Dallas, Texas, USA). Molecular biology grade water, Dulbecco's modified Eagle's medium, fetal bovine serum (FBS), penicillin and streptomycin, sodium pyruvate, 5-(and 6-)chloromethyl-2’7’-dichlorodihydrofluorescein diacetate (CM-$DCFH_2$-DA), Super Script II Reverse Transcriptase, dithiothreitol, and TrypLE Express were purchased from Thermo Fisher Scientific (Burlington, Ontario, Canada). Annexin-V-FLUOS was purchased from Roche Diagnostics (Indianapolis, Indiana, USA). The cell fractionation kit, Z-VAD(OMe)-FMK, mouse monoclonal to γH2AX (phospho S139) antibody, mouse monoclonal to topoisomerase IIα antibody, and rabbit polyclonal to Histone H3 antibody were purchased from Abcam Inc. (Toronto, Ontario, Canada). Blocking buffer, IRDye 680RD-conjugated donkey anti-mouse antibody, and IRDye 800CW conjugated goat anti-rabbit antibody were purchased from Mandel Scientific (Guelph, Ontario, Canada). SsoAdvanced Universal SYBR Green Supermix was purchased from Bio-Rad (Mississauga, Ontario, Canada). Kinetoplast DNA, pHOT1 DNA, purified topoisomerase IIα, and 5x stop buffer were purchased from TopoGEN, Inc. (Buena Vista, Colorado, USA). Purified topoisomerase IIβ was kindly provided by Dr. Neil Osheroff and Jo Ann Byl (Vanderbilt University, Nashville, Tennessee, USA).

Production of Jadomycins

Jadomycins B, S, and F were isolated and characterized as previously described (Jakeman et al., 2009; Dupuis, 2011; Dupuis et al., 2012; Issa et al., 2014).

Cell Lines

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

RNA isolation, reverse transcription, and quantitative real-time PCR

Total RNA was isolated from lysates of 231-CON and 231-TXL cells (a) with no drug treatment, or (b) treated with jadomycin B, S, or F (20 µM), mitoxantrone (1 µM), or 1:7 methanol:H_2O vehicle control (jadomycin vehicle) for 36 hours under standard conditions using the Aurum total RNA Mini Kit according to the manufacturer's instructions. Isolated RNA (0.5

µg) was reverse-transcribed to complementary DNA using Super Script II Reverse Transcriptase (Thermo Fisher Scientific). The complementary DNA was amplified *via* quantitative polymerase chain reaction (qPCR) using 125 nM gene-specific primers (Table 1) in a total volume of 20 µL using a SYBR Green PCR Supermix (Bio-Rad), and a Step One Plus real-time PCR thermocycler (Applied Biosystems, Foster City, California, USA) in duplicate for each primer set. Gene expression was normalized using the average of the three housekeeping genes *glyceraldehyde phosphate dehydrogenase* (*GAPDH*), *beta-actin*, and *peptidylprolyl isomerase A* (*PPIA*, also known as *cyclophilin A*) *via* the $\Delta\Delta C_t$ method (Livak and Schmittgen, 2001).

MTT viability assays

MTT assays were used to evaluate the anticancer activity of jadomycins B, S, and F (0.1 – 20 µM) and the ABCB1 substrates mitoxantrone (0.1 nM – 50 µM) and doxorubicin (0.5 nM – 100 µM) in 231-CON and 231-TXL breast cancer cells and completed according to our previously described methods (Issa et al., 2014).

ROS measuring assays

To quantify the presence of intracellular ROS in 231-CON and 231-TXL cells, a fluorescent assay utilizing the ROS-reactive CM-$DCFH_2$-DA (Thermo Fisher Scientific) was used as previously described (Hall et al., 2015) with minor alterations. Briefly, on day one, 20,000 cells were seeded in each well of a black-sided, clear bottomed 96-well plate. On day two, the medium was removed and replaced with 100 µL of 1%-FBS standard assay medium that contained 7.5 µM CM-$DCFH_2$-DA for 1 h under standard conditions. The CM-$DCFH_2$-DA

containing medium was then removed, wells washed with PBS, and the cells were either (a) treated with 100 µL of jadomycin B, S, or F (2.5-40 µM) or vehicle in 1%-FBS standard assay medium for 24 hours in triplicate, (b) treated with 100 µL of jadomycin B, S, or F (40 µM), doxorubicin (40 µM), H_2O_2 (40 mM), or vehicle in 1%-FBS standard assay medium for 0, 1, 2, 4, 6, 8, 12, or 24 hours in triplicate, or (c) pre-treated with 80 µL of medium control, NAC or auranofin (final concentrations of 2.5 mM and 1 µM, respectively) for 1 hour and then treated with 20 µL of jadomycin B, S, or F (final concentrations of 5-20µM) in 1%-FBS standard assay medium for 24 hours in triplicate.

Western Blot Analysis

231-CON or 231-TXL breast cancer cells were seeded in 6-well plates at 400,000 cells/well and left to adhere overnight in standard assay medium at standard conditions. They were then either (a) treated in triplicate for 24 h with medium control, jadomycin vehicle, jadomycin B, S, or F (15 µM), or mitoxantrone (1 µM), or (b) pre-treated in triplicate for 1 h with NAC, auranofin, or benzamide (2.5 mM, 1 µM, and 5 mM, respectively) then treated with jadomycin S (15 µM) or jadomycin vehicle for 24 h. The triplicate samples for each treatment were pooled, and the cytosolic, mitochondrial, and nucleic protein was then fractionated and collected using a Cell Fractionation Kit (Abcam Inc.; ab109719) as per the manufacturer's instructions. Protein content in each fraction was measured using the Lowry method (Lowry et al., 1951). Nucleic protein was separated on a 15% or 6% sodium dodecyl sulfate polyacrylamide gel electrophoresis (for γH2AX and topoisomerase IIα western blots, respectively) and transferred to a nitrocellulose membrane. Membranes were incubated overnight in either (a) a 1:1,000 dilution of a mouse monoclonal γH2AX (phospho S139) antibody (Abcam Inc.; ab26350) and a rabbit

polyclonal Histone H3 antibody (ab1791), or (b) a 1:500 dilution of a mouse monoclonal topoisomerase IIα antibody (ab180393) and a 1:20,000 dilution of rabbit polyclonal to Histone H3 antibody, at 4 °C. Following washing, membranes were incubated in 1:10,000 dilutions of IRDye 680RD conjugated donkey anti-mouse and IRDye 800CW conjugated goat anti-rabbit secondary antibodies (Mandel Scientific; 926-68072 and 926-32211, respectively) for 1 h at room temperature. For visualization of γH2AX and Histone H3, membranes were scanned at 700 and 800 nm infrared wavelengths, using a LI-COR Odyssey scanner (Mandel Scientific). Pixel intensity of each γH2AX band was normalized to the intensity of the respective Histone H3 bands using ImageJ, and these ratios expressed as a fold-change versus the medium-control treated MDA-MB-231 cells.

Flow Cytometric Analysis of Apoptosis

Flow cytometric analysis of 231-CON and 231-TXL cells stained with annexin-V-FLUOS and propidium iodide was used to determine if jadomycins induced apoptosis. On day one, cells were seeded at 50,000 cells/well into 12-well flat-bottomed plates and left to adhere overnight. On day two, cells were treated with jadomycin B, S, or F (1.25 – 30 μM) or the positive control mitoxantrone (0.1 – 1 μM), with or without a 1-h pre-treatment of auranofin, benzamide, Z-VAD, or NAC (1 μM, 5 mM, 100 μM, and 2.5 mM, respectively) or vehicle control, in 500 μL of standard assay medium for 24 – 48 h, depending on which time point best exemplified the effects of the co-treatment. Nonadherent and adherent cells were combined in 5 mL round bottom tubes (Corning; Corning, New York, USA), which were harvested using TrypLE Express (Thermo Fisher Scientific). Cells were washed with PBS and labeled with annexin-V-FLUOS (Roche Diagnostics) diluted as per the manufacturer's instructions and propidium iodide (1

μg/mL; Sigma Aldrich) in detection buffer (10 mM HEPES, 140 mM NaCl, and 5 mM $CaCl_2$) for 15 minutes at room temperature. Each sample was then diluted with 300 μL of cold detection buffer and analyzed by flow cytometry using a FACSCalibur flow cytometer (BD Biosciences; Mississauga, Ontario, Canada). Percentage of healthy, early apoptotic, and late apoptotic/necrotic cells were analyzed using FCS Express 5 (De Novo Software, Glendale, California, USA).

DNA Decatenation Assays

The inhibition of topoisomerase IIα or IIβ activity was measured using the ATP- and type II topoisomerase-dependent decatenation reaction of kinetoplast DNA (kDNA) catenanes to open and closed circular decatenated kDNA (Sahai and Kaplan, 1986). Methods were based on those of Hasinoff, *et al* (Hasinoff et al., 1997). Individual reactions took place in 10 μL of 50 mM Tris HCl (pH = 8) buffer that contained 120 mM KCl, 10 mM $MgCl_2$, 0.5 mM ATP, 0.5 mM dithiothreitol, 30 μg/mL bovine serum albumin, 250-500 ng kDNA, 0.5 units of purified topoisomerase IIα or 20 ng/mL of purified topoisomerase IIβ enzyme, and jadomycins B, S, and F (10 – 640 μM), positive control doxorubicin (0.31 – 10 μM), or jadomycin vehicle. Reactions were incubated for 30 minutes at 37 °C and stopped using 5x stop buffer containing 5% sarkosyl, 0.125% bromophenol blue, and 25% glycerol. The reaction products were separated by agarose gel (1% w/v) electrophoresis using TAE buffer. Both the agarose gel and the running TAE buffer contained 0.5 μg/mL of ethidium bromide. Gels were run at 135 V for 15 minutes, then destained in water for 10 minutes. Gels were photographed using an Olympus C-4000 Zoom camera under UV transillumination. Decatenated kDNA (TopoGEN) was run as a control, along with kDNA untreated with topoisomerase IIα. The presence and brightness of the open circular and closed

circular kDNA bands was used as a measure of topoisomerase IIα or IIβ activity, with intensity of these bands measured using ImageJ. The intensity of these bands for each given treatment was compared to that of the jadomycin vehicle (labelled 0 μM) for which there was no topoisomerase inhibition, and relative topoisomerase IIα or IIβ inhibition was calculated.

DNA Cleavage Assays

The transient covalent complex that occurs between type II topoisomerases and DNA can be stabilized by topoisomerase II poisons (Liu, 1989), also known as interfacial inhibitors (Marchand and Pommier, 2012), and the presence of these poison-DNA-topoisomerase complexes can be measured by quantifying the formation of linear DNA cleavage products from supercoiled plasmid DNA (Burden et al., 2001). The following protocol is based on that of Burden, *et al* with minor alterations (Burden et al., 2001). Individual reactions were conducted in 20 μL of 10 mM Tris HCl (pH = 7.9) buffer that contained 100 mM KCl, 0.1 mM Na-EDTA, 5 mM $MgCl_2$, 2.5% glycerol, 250 ng pHOT1 DNA, 8 units of purified topoisomerase IIα or 50 μg/mL of purified topoisomerase IIβ, and jadomycins B, S, and F (10 – 640 μM), positive control etoposide (100 μM), or jadomycin vehicle. Reactions were incubated for 6 minutes at 37 °C and stopped by adding 2 μL of 5% SDS, followed by treatment with 1 μL of 375 mM Na-EDTA and 2 μL of 0.8 mg/mL proteinase K, respectively. Reactions were further incubated for 30 minutes at 45 °C, and then treated with 2 μL of loading dye (0.6 g/mL sucrose, 10 mM Tris HCl [pH = 7.9], and 2.5 mg/mL bromophenol blue). The reaction products were separated by agarose gel (1% w/v) electrophoresis in TAE buffer that contained 0.5 μg/mL of ethidium bromide. Gels were run at 100 V for ~30 minutes, destained in water for 15 minutes, and photographed using an Olympus C-4000 Zoom camera under UV transillumination. The

formation of linear pHOT1 DNA normalized to the remaining supercoiled DNA was used as a measure of topoisomerase IIα or IIβ poisoning for each given drug treatment, with intensity of these bands measured using ImageJ. The normalized intensity of each band was then compared to that of the jadomycin vehicle (labelled 0 μM) in order to calculate the relative topoisomerase IIα or IIβ poisoning.

Statistical Analysis

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

Results

231-TXL cells overexpress *ABCB1* and jadomycins are equipotent in 231-TXL versus 231-CON cells

A 95,000-fold increase in the mRNA level of *ABCB1* was observed in the 231-TXL versus 231-CON cells, while no difference was seen in the expression of *ABCC1* or *ABCG2* (**Figure 2a**). Using MTT assays the half-maximal inhibitory concentration (IC_{50}) values of jadomycins B, S, and F were determined to be equal in both the drug-sensitive 231-CON and MDR 231-TXL breast cancer cells, while the IC_{50} values of the ABCB1 substrates mitoxantrone and doxorubicin were significantly higher in the 231-TXL versus 231-CON cells (**Figure 2b**).

Jadomycins induce intracellular ROS activity in 231-CON and 231-TXL cells, which can be altered using anti- or pro-oxidant co-treatments

Jadomycins B (40 μM), S (30 and 40 μM), and F (40 μM) significantly increased ROS in 231-CON cells in comparison to the jadomycin vehicle (**Figure 3a**). This ROS activity increases linearly with time up to 24 h (**Supplemental Figure 1**). The antioxidant and glutathione precursor NAC and the pro-oxidant and thioredoxin reductase inhibitor auranofin were used to inhibit or enhance ROS levels in the cells following jadomycin treatments (Hall et al., 2015). NAC (2.5 mM) and auranofin (1 μM) significantly decreased and increased, respectively, ROS activity in the 231-CON cells when co-treated with jadomycins S or F (40 μM), though not when co-treated with jadomycin B (40 μM) (**Figure 3b**). Since all jadomycins induced ROS, jadomycin S was chosen as a representative jadomycin for this and all following replicative experiments involving 231-TXL cells. Jadomycin S was chosen due to greater water solubility

and biosynthetic yields versus jadomycins B and F. Jadomycin S (20 and 40 μM) significantly increased ROS activity in the 231-TXL cells, and while NAC significantly decreased jadomycin S (40 μM) induced ROS activity, auranofin had no effect (**Figure 3c**).

Jadomycins induce DNA double strand breaks in 231-CON and 231-TXL cells

When double strand breaks occur within DNA it is always followed by the phosphorylation of histone H2AX; the amount of phosphorylated histone H2AX (γH2AX) in cells treated with cytotoxic agents can therefore be used as a measure of DNA double strand breaks (Kuo and Yang, 2008). In 231-CON cells, jadomycins B, S, and F (15 μM) and the control mitoxantrone (1 μM) significantly increased γH2AX protein levels versus the vehicle control, as measured using western blotting (**Figure 4a**). Jadomycin S (15 μM) significantly increased γH2AX protein expression in 231-TXL cells whereas mitoxantrone did not (**Figure 4b**). The induction of γH2AX protein expression in 231-CON cells by jadomycin S (15 μM) was not altered by co-treatment with the antioxidant NAC (2.5 mM) or pro-oxidant auranofin (1 μM), while co-treatment with benzamide (100 μM), an inhibitor of DNA repair poly(ADP-ribose) polymerases (PARPs) (Steffen et al., 2011), significantly increased γH2AX protein expression. None of the co-treatments affected γH2AX levels on their own (**Figure 4c**).

Jadomycins induce apoptosis in 231-CON and 231-TXL cells

Apoptosis induced by cytotoxic drugs can be measured using annexin V affinity assays which differentiate healthy, early apoptotic, and dead (also labelled late apoptotic/necrotic) cells using fluorescently labelled annexin V and propidium iodide followed by fluorescence-activated cell

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sorting (FACS) analysis (van Engeland et al., 1998; Greenshields et al., 2015). Two examples of annexin V affinity assays can be seen in **Figure 5a**, depicting 231-CON cells treated with either the vehicle control (left hand side) or jadomycin S (20 μM; right hand side) for 36 hours. Healthy cells are in the bottom-left quadrant (no fluorescence), early apoptotic in the lower-right (annexin V fluorescence), and late apoptotic/necrotic cells in the upper-right (annexin V and propidium iodide fluorescence).

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

Jadomycin cytotoxicity is enhanced by auranofin and benzamide and reduced by Z-VAD

Jadomycins B (30 μM), S (20 μM), and F (30 μM) and mitoxantrone (1 μM) induced equal amounts of early apoptosis and late apoptosis/necrosis with or without the antioxidant NAC (2.5 mM) co-treatment after 36 hours (**Figure 6a**). The pro-oxidant auranofin (1 μM) had no effect on the amount of early apoptosis induced by jadomycins B, S, or F (5 μM), however it did significantly increase the number of late apoptotic/necrotic cells. Auranofin did not affect the cytotoxicity of mitoxantrone (0.1 μM) (**Figure 6b**). The PARP inhibitor benzamide (5 mM),

while having no effect on late apoptosis/necrosis when co-treated with any of the jadomycin (5 μM) or mitoxantrone (0.1 μM) treatments, did significantly increase the amount of early apoptosis induced by jadomycin S after a 48 h treatment (**Figure 6c**). The cell permeable, irreversible pan-caspase inhibitor Z-VAD (100 μM) (Cohen, 1997) had no effect on late apoptosis/necrosis, though it did significantly reduce the number of early apoptotic cells when co-treated with jadomycins B (30 μM), S (20 μM), and F (30 μM) or mitoxantrone (1 μM) for 36 h (**Figure 6d**). After 36 h co-treatments in the 231-TXL cells, Z-VAD significantly decreased jadomycin S (20 μM)-induced early apoptosis, while NAC, auranofin, and benzamide had no effect. Auranofin and benzamide both significantly increased the amount of late apoptosis/necrosis measured in the 231-TXL cells when co-treated with jadomycin S, while NAC and Z-VAD had no noticeable effect. No significant differences in early apoptosis or late apoptosis/necrosis were observed with any of the co-treatments when used with mitoxantrone (1 μM) (**Figure 6e**). None of these co-treatments had any effect on cell death on their own at the concentrations indicated.

Jadomycins are type II topoisomerase inhibitors

The lack of effect of NAC and auranofin on jadomycin-induced DNA damage and early apoptosis suggested a ROS-independent mechanism. Martinez-Farina *et al* recently determined that the jadomycin analogue DS bonds to human topoisomerase IIβ protein (Martinez-Farina et al., 2015), and a preliminary cancer gene target array we completed in MCF7 cells showed decreased expression of topoisomerase genes when treated with 10 μM jadomycin S (data not shown). Therefore we chose to probe the possible involvement of topoisomerase inhibition by jadomycins as a ROS-independent mechanism of DNA damage and apoptosis.

Jadomycins B, S, and F (20 μM, 36 h treatments) significantly reduced the expression of *TOP2A* and *TOP2B*, the genes that encode for topoisomerases IIα and IIβ, respectively, in 231-CON cells versus the vehicle control (**Figure 7a**). A smaller but statistically significant decrease in *TOP1*, the gene that encodes topoisomerase I, was observed for jadomycin S with no significant changes for jadomycins B or F. The mitoxantrone control had no effect on *TOP1* expression, though it did cause a small *TOP2A* increase and *TOP2B* decrease versus the vehicle. Jadomycin S (20 μM, 36 h) caused similar significant decreases in *TOP1*, *TOP2A*, and *TOP2B* expression in the 231-TXL cells while mitoxantrone had no effect (**Figure 7b**). The PCR primers used are listed in **Table 1**.

Jadomycins B, S, and F (15 μM, 24 h) and mitoxantrone (1 μM, 24 h) significantly lowered the levels of topoisomerase IIα protein versus the vehicle control (**Figure 7c**). Jadomycin S (15 μM, 24 h) but not mitoxantrone (1 μM, 24 h) decreased topoisomerase IIα in the 231-TXL cells (**Figure 7d**).

Using a protocol adapted from Topogen (Colorado, USA) and Hasinoff, *et al* (Hasinoff et al., 1997), the ability of jadomycins and the known topoisomerase poison doxorubicin (Hasinoff et al., 2016) to directly inhibit topoisomerases IIα and IIβ was measured. Jadomycins B, S, and F (10 – 640 μM) and doxorubicin (0.3125 – 10 μM) all concentration-dependently and directly inhibited both topoisomerase isoforms (**Figure 8**). The topoisomerase IIα IC_{50} values of jadomycins S, F, and doxorubicin were significantly lower than that of jadomycin B, and the topoisomerase IIβ IC_{50} value for DOX was lower than that of jadomycin B. No drug was differentially potent in its inhibition of topoisomerase IIα versus IIβ (**Table 2**).

DNA cleavage assays were completed to determine if jadomycins are type II topoisomerase poisons or catalytic inhibitors (Burden et al., 2001). The positive control etoposide (Hasinoff et

al., 2016) increased the amount of DNA cleavage induced by topoisomerases IIα and IIβ at 100 μM, while Jadomycins B and F selectively increased DNA cleavage induced by topoisomerase IIβ at 640 and 320 μM, respectively, with no effect on topoisomerase IIα. Jadomycin S did not affect either topoisomerase isoform at any concentration used (**Figure 9**). Bands representing nicked open circular plasmid DNA in the agarose gels, which can be used to quantify topoisomerase II-generated single-strand DNA breaks (Bandele and Osheroff, 2009), were not visible or were too dim to be quantified, and were therefore not included in the analysis.

Discussion

By exposing triple-negative MDA-MB-231 breast cancer cells to gradually increasing concentrations of paclitaxel (TXL), we successfully created a polyclonal MDR cell line that overexpressed *ABCB1* and was resistant to the ABCB1 substrates mitoxantrone and doxorubicin (Consoli et al., 1997; Shen et al., 2008), but not to jadomycins. This corroborates our earlier results describing how jadomycin potency is largely unaffected by ABC-transporter overexpression in MCF7 cells (Issa et al., 2014), providing further evidence of jadomycins' potential in ABC-transporter overexpressing MDR cancers.

We verified that jadomycins retained their ROS-inducing properties in 231-CON and 231-TXL triple-negative breast cancer cells, as previously observed in hormone receptor-positive MCF7 cells (Hall et al., 2015), evidencing that jadomycin ROS induction is independent of hormone receptor expression. While the antioxidant effects of NAC (Dodd et al., 2008) were retained in jadomycin-treated resistant 231-TXL cells, the pro-oxidant effects of auranofin (Liu et al., 2013) were not, suggesting these cells developed resistance to auranofin's ROS-inducing properties.

Since ROS can induce DNA double strand breaks (Khanna and Jackson, 2001), we hypothesized that jadomycins would cause double strand breaks in MDA-MB-231 cells. The increases in γH2AX observed when 231-CON and 231-TXL cells were treated with jadomycins support this hypothesis. The ability of jadomycin S (but not the ABCB1 substrate mitoxantrone) to retain its γH2AX inducing effect in 231-TXL cells is consistent with the ability of jadomycins to evade the ABCB1 drug efflux mechanism of MDR (Issa et al., 2014). Interestingly, when 231-CON cells were treated with NAC or auranofin with jadomycin S there was no additional change in γH2AX levels, while co-treatment with the DNA repair PARP-inhibitor benzamide (Steffen et

al., 2011) significantly increased γH2AX levels. This confirms that jadomycins cause DNA double strand breaks, but also suggests they occur independently of ROS.

Increased ROS activity and double strand breaks within cells are common triggers of apoptosis (Kaina, 2003; Bertram and Hass, 2008). Additionally, using chromatin condensation assays Fu, *et al* provided evidence that jadomycin B induces apoptosis in lung carcinoma A549 cells (Fu et al., 2008). Therefore we suspected jadomycins would also induce apoptosis in breast cancer cells. Our annexin V affinity assays support this idea, showing significantly more early apoptotic 231-CON when treated with jadomycins B, S, or F versus the vehicle control. Consistent with the results of the γH2AX assays, the effect of jadomycin S on apoptosis was not impacted by ABCB1 overexpression, providing further evidence that jadomycins evade ABCB1 efflux. Additionally, jadomycins increased the number of annexin V and propidium iodide dual-stained cells, signifying cells killed through either apoptosis *or* necrosis (Greenshields et al., 2015). Therefore, while we can conclude that jadomycins induce apoptosis, we cannot determine if cell death occurs solely through apoptosis or a combination of cell-death mechanisms.

To determine the importance of jadomycin-induced ROS in eliciting apoptosis, annexin V affinity assays were completed in cells co-treated with NAC or auranofin along with jadomycins. The antioxidant NAC had no effect on jadomycin-induced early apoptosis or late apoptosis/necrosis in 231-CON and 231-TXL cells, suggesting jadomycins induce apoptosis and cell death independently of ROS. Conversely, when the cells were co-treated with auranofin, an increase in late apoptosis/necrosis was observed. However, since auranofin did not increase ROS in 231-TXL cells, this suggests it augmented jadomycin-mediated cell death independently of ROS (perhaps through its inhibition of the DNA repair ubiquitin-proteasome system) (Roder and Thomson, 2015). This contrasts with our previous study, which showed NAC decreased and

auranofin increased jadomycin potency in MCF7 breast cancer cells (Hall et al., 2015). This suggests ROS may still play a role in jadomycin cytotoxic potency, however as MCF7 cells are more sensitive to ROS-inducing drugs than MDA-MB-231 cells (Kang et al., 2010), their effects may depend on the cell line used.

The greater induction of early apoptosis by jadomycin S in 231-CON cells and late apoptosis/necrosis in 231-TXL cells when co-treated with benzamide, which inhibits DNA repair PARP proteins (Steffen et al., 2011), further evidences that jadomycin-induced damage DNA is linked to apoptosis. Additionally, the observation that benzamide altered the potency of jadomycin S but not of jadomycins B or F in 231-CON cells supports that the apoptotic mechanisms of jadomycins are dependent on their structures. The pan-inhibitor of the apoptotic family of caspases, Z-VAD (Cohen, 1997), lessened jadomycin-induced early apoptosis, suggesting jadomycins induce caspase-dependent apoptosis. The fact that similar results were seen in 231-TXL versus 231-CON cells with and without the co-treatments indicates that the mechanisms behind jadomycin cytotoxicity are largely preserved in the MDR cell line.

Our γH2AX and annexin-V affinity assays suggest jadomycins induce DNA damage and apoptosis in 231-CON and 231-TXL cells through a ROS-independent mechanism. Jadomycins inhibit aurora B kinase independently of ROS (Hall et al., 2015); however, this mechanism is not likely to induce DNA damage since the opposite occurs: DNA damage inhibits aurora B kinase (Monaco et al., 2005). Alternatively, we hypothesized that jadomycins could inhibit topoisomerases. Topoisomerases prevent DNA supercoiling by regulating over- and under-winding during cellular processes such as replication and transcription (Pommier et al., 2010), and their inhibition can cause DNA damage and apoptosis (Sordet et al., 2003).

The large decreases in *TOP2A* and *TOP2B* gene expression caused by jadomycins B, S, and F, with only a small *TOP1* decrease observed with jadomycin S, suggest jadomycins preferentially inhibit type II versus type I topoisomerase gene expression. The known topoisomerase II inhibitor, mitoxantrone (Pommier et al., 2010), slightly increased and decreased *TOP2A* and *TOP2B*, respectively, while having no effect on *TOP1*, evidencing that jadomycins more potently inhibit topoisomerase II gene expression than mitoxantrone. The decreased topoisomerase IIα enzyme levels, measured through western blotting, caused by jadomycins in 231-CON and 231-TXL cells suggest that the inhibition of topoisomerase II gene expression decreased protein synthesis, and that this mechanism is retained in ABCB1-overexpressing MDR cells, while the ABCB1-substrate mitoxantrone (Consoli et al., 1997) lost its inhibitory properties.

The DNA decatenation assays showed that jadomycins B, S, and F and the topoisomerase II poison, doxorubicin (Hasinoff et al., 2016), concentration-dependently inhibited both topoisomerases IIα and IIβ with 100% inhibitory efficacy. The higher IC_{50} value of jadomycin B versus those of jadomycins S and F for topoisomerase IIα suggests the structural differences of jadomycin analogues can alter their inhibitory potency. The topoisomerase II-inhibition IC_{50} values were higher than the concentrations required to inhibit topoisomerase II gene and IIα protein levels in cellular assays, and higher than the IC_{50} values measured through MTT cell viability assays. This suggests the reduction of topoisomerase II gene and protein expression would be more likely to occur in breast cancer cells exposed to jadomycins, versus direct enzyme inhibition. However, depending on the level of jadomycin accumulation within cells, direct topoisomerase II inhibition is possible.

To determine if jadomycins are catalytic inhibitors or interfacial poisons of type II topoisomerases, DNA cleavage assays were completed (Burden et al., 2001). Type II topoisomerases covalently bind with cleaved DNA, forming cleavage complexes, which are normally short-lived intermediates. Topoisomerase poisons, like etoposide, trap topoisomerases in these topoisomerase II-DNA cleavage complexes, causing cell-lethal DNA strand breaks and converting these essential enzymes into potent toxins (Lindsey et al., 2004). High concentrations of jadomycins B and F increased the formation of linear DNA from supercoiled pHOT1 DNA when incubated with topoisomerase IIβ, but not IIα, suggesting they are selective topoisomerase IIβ poisons. In contrast, jadomycin S did not increase the formation of cleaved plasmid DNA by either isoenzyme, suggesting it does not poison type II topoisomerases. These results raise some concern for jadomycins B and F, since despite the potent anticancer activity of topoisomerase II poisons, their use in cancer patients has been linked to the development of secondary malignancies, like acute myeloid leukemia (Nitiss, 2009), and topoisomerase IIβ-poisons have been correlated with significant cardiotoxicity, as observed with anthracyclines (Lyu et al., 2007; Sawyer, 2013). Alternatively, the lack of topoisomerase II poisoning by jadomycin S could be advantageous from an adverse drug reaction perspective, especially considering it is equally potent against breast cancer cells versus other jadomycins (Issa et al., 2014; Hall et al., 2015). However, this remains to be determined in future *in vivo* studies.

In conclusion, jadomycins demonstrate potential as novel treatments for drug resistant breast cancer by retaining their cytotoxic potency in MDR, triple-negative 231-TXL cells and as previously described in MDR MCF7 cells (Issa et al., 2014). We have also demonstrated that jadomycins exert their anticancer activity in 231-CON and 231-TXL cells through a novel ROS-independent mechanism that leads to DNA double strand breaks and apoptosis. Through further

investigation, we discovered that jadomycins inhibit the gene and protein expression of the validated anticancer targets, topoisomerases IIα and IIβ (Pommier et al., 2010), and act as type II topoisomerase catalytic inhibitors and, in some cases, interfacial poisons, thus advancing our understanding of jadomycins' mechanisms of action. However, further studies are needed to establish the functional link between jadomycins' inhibition of type II topoisomerases and ROS-independent DNA damage and apoptosis in breast cancer cells, and their safety and effectiveness in the treatment of MDR breast cancer in animal models.

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Authorship Contributions

Participated in research design: Hall, Goralski

Conducted experiments: Hall, Toulany, Bennett

Contributed new reagents or analytical tools: Martinez-Farina, Robertson, Jakeman

Performed data analysis: Hall, Goralski

Wrote or contributed to the writing of the manuscript: Hall, Goralski

References

Bandele OJ and Osheroff N (2009) Cleavage of plasmid DNA by eukaryotic topoisomerase II. *Methods Mol Biol* **582**:39-47.

Bauer KR, Brown M, Cress RD, Parise CA and Caggiano V (2007) Descriptive analysis of estrogen receptor (ER)-negative, progesterone receptor (PR)-negative, and HER2-negative invasive breast cancer, the so-called triple-negative phenotype: a population-based study from the California cancer Registry. *Cancer* **109**:1721-1728.

Bertram C and Hass R (2008) Cellular responses to reactive oxygen species-induced DNA damage and aging. *Biol Chem* **389**:211-220.

Burden DA, Froelich-Ammon SJ and Osheroff N (2001) Topoisomerase II-mediated cleavage of plasmid DNA. *Methods Mol Biol* **95**:283-289.

Cohen GM (1997) Caspases: the executioners of apoptosis. *Biochem J* **326 (Pt 1)**:1-16.

Consoli U, Van NT, Neamati N, Mahadevia R, Beran M, Zhao S and Andreeff M (1997) Cellular pharmacology of mitoxantrone in p-glycoprotein-positive and -negative human myeloid leukemic cell lines. *Leukemia* **11**:2066-2074.

Dodd S, Dean O, Copolov DL, Malhi GS and Berk M (2008) N-acetylcysteine for antioxidant therapy: pharmacology and clinical utility. *Expert Opin Biol Th* **8**:1955-1962.

Dupuis SN, Robertson AW, Veinot T, Monro SMA, Douglas SE, Syvitski RT, Goralski KB, McFarland SA and Jakeman DL (2012) Synthetic diversification of natural products: semi-synthesis and evaluation of triazole jadomycins. *Chem Sci* **3**:1640-1644.

Dupuis SN, Veinot, T., Monro, S. M., Douglas, S. E., Syvitski, R. T., Goralski, K. B., McFarland, S. A., Jakeman, D. L. (2011) Jadomycins derived from the assimilation and incorporation of norvaline and norleucine. *J Nat Prod* **74**:2420-2424.

Elias AD (2010) Triple-negative breast cancer: a short review. *Am J Clin Oncol* **33**:637-645.

Fu DH, Jiang W, Zheng JT, Zhao GY, Li Y, Yi H, Li ZR, Jiang JD, Yang KQ, Wang Y and Si SY (2008) Jadomycin B, an Aurora-B kinase inhibitor discovered through virtual screening. *Mol Cancer Ther* **7**:2386-2393.

Greenshields AL, Doucette CD, Sutton KM, Madera L, Annan H, Yaffe PB, Knickle AF, Dong Z and Hoskin DW (2015) Piperine inhibits the growth and motility of triple-negative breast cancer cells. *Cancer Lett* **357**:129-140.

Hall SR, Blundon HL, Ladda MA, Robertson AW, Martinez-Farina CF, Jakeman DL and Goralski KB (2015) Jadomycin breast cancer cytotoxicity is mediated by a copper-dependent, reactive oxygen species-inducing mechanism. *Pharmacol Res Perspect* **3**:e00110.

Hasinoff BB, Creighton AM, Kozlowska H, Thampatty P, Allan WP and Yalowich JC (1997) Mitindomide is a catalytic inhibitor of DNA topoisomerase II that acts at the bisdioxopiperazine binding site. *Mol Pharmacol* **52**:839-845.

Hasinoff BB, Wu X, Patel D, Kanagasabai R, Karmahapatra S and Yalowich JC (2016) Mechanisms of Action and Reduced Cardiotoxicity of Pixantrone; a Topoisomerase II Targeting Agent with Cellular Selectivity for the Topoisomerase IIalpha Isoform. *J Pharmacol Exp Ther* **356**:397-409.

Issa ME, Hall SR, Dupuis SN, Graham CL, Jakeman DL and Goralski KB (2014) Jadomycins are cytotoxic to ABCB1-, ABCC1-, and ABCG2-overexpressing MCF7 breast cancer cells. *Anti-Cancer Drug* **25**:255-269.

Jakeman DL, Dupuis SN and Graham CL (2009) Isolation and characterization of jadomycin L from Streptomyces venezuelae ISP5230 for solid tumor efficacy studies. *Pure Appl Chem* **81**:1041-1049.

Jakeman DL, Graham CL, Young W and Vining LC (2006) Culture conditions improving the production of jadomycin B. *J Ind Microbiol Biotechnol* **33**:767-772.

Kaina B (2003) DNA damage-triggered apoptosis: critical role of DNA repair, double-strand breaks, cell proliferation and signaling. *Biochem Pharmacol* **66**:1547-1554.

Kang KS, Wang P, Yamabe N, Fukui M, Jay T and Zhu BT (2010) Docosahexaenoic acid induces apoptosis in MCF-7 cells in vitro and in vivo via reactive oxygen species formation and caspase 8 activation. *PLoS One* **5**:e10296.

Khanna KK and Jackson SP (2001) DNA double-strand breaks: signaling, repair and the cancer connection. *Nat Genet* **27**:247-254.

Kuo LJ and Yang LX (2008) Gamma-H2AX - a novel biomarker for DNA double-strand breaks. *In Vivo* **22**:305-309.

Lindsey RH, Jr., Bromberg KD, Felix CA and Osheroff N (2004) 1,4-Benzoquinone is a topoisomerase II poison. *Biochemistry (US)* **43**:7563-7574.

Liu C, Liu Z, Li M, Li X, Wong YS, Ngai SM, Zheng W, Zhang Y and Chen T (2013) Enhancement of auranofin-induced apoptosis in MCF-7 human breast cells by selenocystine, a synergistic inhibitor of thioredoxin reductase. *PLoS One* **8**:e53945.

Liu LF (1989) DNA topoisomerase poisons as antitumor drugs. *Annu Rev Biochem* **58**:351-375.

Livak KJ and Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* **25**:402-408.

Lluch A, Alvarez I, Munoz M, Segui MA, Tusquets I and Garcia-Estevez L (2014) Treatment innovations for metastatic breast cancer: nanoparticle albumin-bound (NAB) technology targeted to tumors. *Crit Rev Oncol Hematol* **89**:62-72.

Lowry OH, Rosebrough NJ, Farr AL and Randall RJ (1951) Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**:265-275.

Lyu YL, Kerrigan JE, Lin CP, Azarova AM, Tsai YC, Ban Y and Liu LF (2007) Topoisomerase IIbeta mediated DNA double-strand breaks: implications in doxorubicin cardiotoxicity and prevention by dexrazoxane. *Cancer Res* **67**:8839-8846.

Marchand C and Pommier Y (2012) Topoisomerase Inhibitors: A Paradigm for Interfacial Inhibition, in *DNA Topoisomerases and Cancer* pp 175-184.

Martinez-Farina CF and Jakeman DL (2015) Jadomycins, put a bigger ring in it: isolation of seven- to ten-membered ring analogues. *Chem Commun (Camb)* **51**:14617-14619.

Martinez-Farina CF, McCormick N, Robertson AW, Clement H, Jee A, Ampaw A, Chan NL, Syvitski RT and Jakeman DL (2015) Investigations into the binding of jadomycin DS to human topoisomerase IIbeta by WaterLOGSY NMR spectroscopy. *Org Biomol Chem* **13**:10324-10327.

Monaco L, Kolthur-Seetharam U, Loury R, Murcia JM, de Murcia G and Sassone-Corsi P (2005) Inhibition of Aurora-B kinase activity by poly(ADP-ribosyl)ation in response to DNA damage. *Proc Natl Acad Sci U S A* **102**:14244-14248.

Morris PG, McArthur HL and Hudis CA (2009) Therapeutic options for metastatic breast cancer. *Expert Opin Pharmaco* **10**:967-981.

National Center for Biotechnology Information PubChem Compound Database; SIDs = 0023214928, 0033419420, & 0065271809, https://chem.nlm.nih.gov/chemidplus/sid, (accessed July 15, 2017), in.

Nitiss JL (2009) Targeting DNA topoisomerase II in cancer chemotherapy. *Nat Rev Cancer* **9**:338-350.

Pommier Y, Leo E, Zhang H and Marchand C (2010) DNA topoisomerases and their poisoning by anticancer and antibacterial drugs. *Chem Biol* **17**:421-433.

Rakha EA, El-Sayed ME, Green AR, Lee AH, Robertson JF and Ellis IO (2007) Prognostic markers in triple-negative breast cancer. *Cancer* **109**:25-32.

Rivera E (2010) Implications of anthracycline-resistant and taxane-resistant metastatic breast cancer and new therapeutic options. *Breast J* **16**:252-263.

Robertson AW, Martinez-Farina CF, Smithen DA, Yin H, Monro S, Thompson A, McFarland SA, Syvitski RT and Jakeman DL (2015) Eight-membered ring-containing jadomycins: implications for non-enzymatic natural products biosynthesis. *J Am Chem Soc* **137**:3271-3275.

Roder C and Thomson MJ (2015) Auranofin: repurposing an old drug for a golden new age. *Drugs R&D* **15**:13-20.

Sahai BM and Kaplan JG (1986) A quantitative decatenation assay for type II topoisomerases. *Anal Biochem* **156**:364-379.

Sawyer DB (2013) Anthracyclines and heart failure. *N Engl J Med* **368**:1154-1156.

Shen F, Chu S, Bence AK, Bailey B, Xue X, Erickson PA, Montrose MH, Beck WT and Erickson LC (2008) Quantitation of doxorubicin uptake, efflux, and modulation of multidrug resistance (MDR) in MDR human cancer cells. *J Pharmacol Exp Ther* **324**:95-102.

Sordet O, Khan QA, Kohn KW and Pommier Y (2003) Apoptosis induced by topoisomerase inhibitors. *Curr Med Chem: Anti-Cancer Agents* **3**:271-290.

Steffen JD, Coyle DL, Damodaran K, Beroza P and Jacobson MK (2011) Discovery and structure-activity relationships of modified salicylanilides as cell permeable inhibitors of poly(ADP-ribose) glycohydrolase (PARG). *J Med Chem* **54**:5403-5413.

Szakacs G, Paterson JK, Ludwig JA, Booth-Genthe C and Gottesman MM (2006) Targeting multidrug resistance in cancer. *Nat Rev Drug Discov* **5**:219-234.

van Engeland M, Nieland LJ, Ramaekers FC, Schutte B and Reutelingsperger CP (1998) Annexin V-affinity assay: a review on an apoptosis detection system based on phosphatidylserine exposure. *Cytometry* **31**:1-9.

Yu KD, Huang S, Zhang JX, Liu GY and Shao ZM (2013) Association between delayed initiation of adjuvant CMF or anthracycline-based chemotherapy and survival in breast cancer: a systematic review and meta-analysis. *BMC cancer* **13**:240.

Footnotes

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Figure Legends

Figure 1. Chemical structures of drug treatments used. **(a)** Jadomycin backbone; alternate jadomycin analogues differ at the indicated "R"-group, such as with jadomycins **(b)** B, **(c)** S, and **(d)** F (Issa et al., 2014). Control chemotherapeutic drugs used were **(e)** doxorubicin, **(f)** etoposide, and **(g)** mitoxantrone (National Center for Biotechnology Information).

Figure 2. (a) Growth of MDA-MB-231 cells in paclitaxel selection medium for seven months generated the MDR breast cancer cell line 231-TXL that specifically overexpressed *ABCB1* versus drug sensitive 231-CON cells, as measured using qPCR. **(b)** The IC_{50} values of Jadomycins (Jads) B, S, and F (72 h treatments) in MTT assays were equal in 231-TXL cells versus 231-CON. The IC_{50} values of the control drugs mitoxantrone (MITX) and doxorubicin (DOX) were significantly higher in the 231-TXL cells versus 231-CON cells. Each bar represents the mean of at least three independent experiments. * $P \leq 0.05$, (a) the indicated gene's expression was significantly different from that of the *GAPDH* housekeeping control as determined by a one-way ANOVA followed by Bonferroni's multiple comparison test, or (b) the average IC_{50} value of the indicated drug treatment in 231-TXL cells was significantly different from that measured in the 231-CON cells as determined by an unpaired *t* test.

Figure 3. (a) Jadomycins (Jads) B, S, and F (2.5 - 40 µM) concentration-dependently increased ROS activity in 231-CON cells. **(b)** The anti-oxidant N-acetyl cysteine (NAC, 2.5 mM) decreased and the pro-oxidant auranofin (Aur; 1 µM) increased intracellular ROS activity in Jad S and F treated 231-CON cells. **(c)** Jad S (20 - 40 µM) concentration-dependently increased ROS

activity in 231-TXL cells. NAC significantly decreased ROS activity induced by Jad S (40 μM) in 231-TXL cells, while Aur had no effect. ROS activity was expressed as a fold-change relative to the medium-treated control cells. Each bar represents the mean of at least three independent experiments. * $P \leq 0.05$, (a and c) the fold-change in ROS activity was significantly different compared with the vehicle control (0 μM or S0), or (b) compared with the no co-treatment control for that specific jadomycin and † $P < 0.05$ compared to the S40 treatment (c) as determined by one-way ANOVAs, followed by Bonferroni's multiple comparison tests.

Figure 4. (a) Jadomycins (Jads) B, S, and F (15 μM, 24 h) and mitoxantrone (MITX; 1 μM) increased the phosphorylation of histone H2AX (γH2AX; a marker of double strand DNA breaks) versus vehicle control in 231-CON cells. **(b)** In 231-TXL cells Jad S (15 μM, 24 h) but not MITX (1 μM) increased γH2AX protein expression versus vehicle control. **(c)** The PARP-inhibitor benzamide (Benz; 5mM), but not N-acetyl cysteine (NAC; 2.5 mM) or auranofin (Aur; 1 μM), further increased γH2AX in Jad S-treated (15 μM, 24 h) 231-CON cells. When administered as single treatments NAC, Aur, and Benz did not affect γH2AX levels. γH2AX protein expression was depicted as a fold-change relative to the medium-treated control cells. Each bar represents the mean of at least four independent experiments. * $P \leq 0.05$, (a and b) the fold-change in γH2AX protein expression was significantly different when compared with the vehicle or (c) when compared to 15 μM Jad S alone as determined by one-way ANOVAs, followed by Bonferroni's multiple comparison tests.

Figure 5. (a) Right-hand side (RHS) representative FACS figure shows how jadomycin S (20 μM; 36 h) induced more 231-CON cell death than jadomycin vehicle on the left-hand side (LHS). Lower LHS quadrants show the percentage of healthy cells, lower RHS quadrants show early apoptotic cells, and upper RHS quadrant shows late apoptotic/necrotic cells. Jadomycins B, S, or F (1.25-20 μM) or mitoxantrone (0.1-1 μM) treatments for 36 h induced significantly greater **(b)** early apoptosis and **(c)** late apoptosis/necrosis versus vehicle (labelled 0 μM) in drug-sensitive 231-CON cells. **(d)** Jadomycin (Jad) S (20 μM) and mitoxantrone (MITX; 1 μM) significantly increased early apoptosis in multidrug-resistant 231-TXL cells versus the vehicle control after 36 h treatments, and **(e)** Jad S also increased late apoptosis/necrosis. Each bar represents the mean of at least three independent experiments. * $P \leq 0.05$, the %-early apoptosis or %-late apoptosis/necrosis was significantly different compared with the vehicle treatment controls as determined by one-way ANOVAs, followed by Bonferroni's multiple comparison tests.

Figure 6. (a) NAC (2.5 mM) did not affect jadomycin (Jad) B, S, or F (30, 20, and 30 μM, respectively) or mitoxantrone (MITX; 1 μM) induced early apoptosis or late apoptosis/necrosis after 36 h in 231-CON cells. **(b)** Auranofin (Aur; 1 μM) did not affect early apoptosis with Jads B, S, or F (5 μM) or MITX (0.1 μM) after 24 h in 231-CON cells. It did increase late apoptosis/necrosis when co-treated with each Jad though not with MITX. **(c)** Benzamide (Benz; 5 mM) increased early apoptosis induced by Jad S (5 μM) after 48 h in 231-CON cells, with no effect on late apoptosis/necrosis. It had no significant effect with Jads B and F (5 μM) or MITX (0.1 μM). **(d)** Z-VAD (100 μM) significantly reduced early apoptosis induced by Jads B, S, and F and MITX (30, 20, 30, and 1 μM, respectively) after 36 h in 231-CON cells, while having no

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

Figure 7. (a) Jadomycins (Jads) B, S, and F (20 μM) all significantly reduced the expression of *TOP2A* and *TOP2B* genes in 231-CON cells after 36 h. A small *TOP1* decrease was also observed with Jad S. The mitoxantrone (MITX) control (1 μM) did not alter *TOP1,* though it did increase *TOP2A* and decrease *TOP2B* expression. **(b)** Jad S (20 μM) significantly decreased *TOP1, TOP2A,* and *TOP2B* expression in 231-TXL cells after 36 h. MITX (1 μM) had no effect. **(c)** Jads B, S, and F (15 μM) and MITX (1 μM) significantly lowered the histone H3 normalized topoisomerase (Topo) IIα protein expression after 24 h in 231-CON cells relative to the Veh

control. **(d)** Jad S (15 μM, 24 h) significantly lowered Topo IIα protein detected while MITX (1 μM) did not after 24 h in 231-TXL cells. Each bar represents the mean of at least three independent experiments. * $P \leq 0.05$, the value is significantly different from the vehicle control (Veh) as determined by a one-way ANOVA, followed by Bonferroni's multiple comparison test.

Figure 8. The conversion of catenated kDNA circles (CK) to open circular (OC) and closed circular (CC) decatenated kDNA by purified **(a)** topoisomerase IIα or **(c)** IIβ enzyme was concentration-dependently inhibited by jadomycins (Jads) B, S, and F and doxorubicin (DOX). The size of the OC and CC bands for each treatment were calculated for each Jad and DOX treatment from which the % inhibition curves for topoisomerase IIα (**b**) or IIβ (**d**) were generated. Each point represents the mean of at least three independent experiments. * $P \leq 0.05$, the value is significantly different from the vehicle control (Veh) as determined by a one-way ANOVA, followed by Bonferroni's multiple comparison test.

Figure 9. Inverse-colour representative gels show **(a)** jadomycins (Jad) B, S, and F did not alter linear DNA formed from supercoiled pHOT1 DNA in the presence of topoisomerase IIα versus vehicle. **(c)** Jadomycins B and F (at 640 and 320 μM, respectively) increased linear DNA formed by topoisomerase IIβ versus vehicle, while jadomycin S had no effect. The positive control etoposide (Etop; 100 μM) increased linear DNA formed by both isoenzymes. Fold-changes in linear DNA band size were calculated for each treatment versus vehicle control for topoisomerases **(b)** IIα and **(d)** IIβ. * $P \leq 0.05$, the value is significantly different from the

vehicle control (0 µM) as determined by an unpaired *t*-test (etoposide) or one-way ANOVAs (Jads), followed by Bonferroni's multiple comparison test.

Tables

Table 1. PCR primers used to determine the expression of relevant genes in 231-CON and 231-TXL cells.

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

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Table 2: IC_{50} values of jadomycins (Jad) B, S, and F and doxorubicin (DOX) for the inhibition of topoisomerases (Topo) IIα and IIβ, as measured with kDNA decatenation assays.

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

Each value represents the mean of at least four independent experiments. $P < 0.05$, the IC_{50} value is significantly different from that of * Jad S, Jad F, and DOX, or † only DOX, for the given topoisomerase. No drug treatment was significantly more potent at inhibiting one topoisomerase versus the other, as determined by a two-way ANOVA followed by Bonferroni's multiple comparison test.

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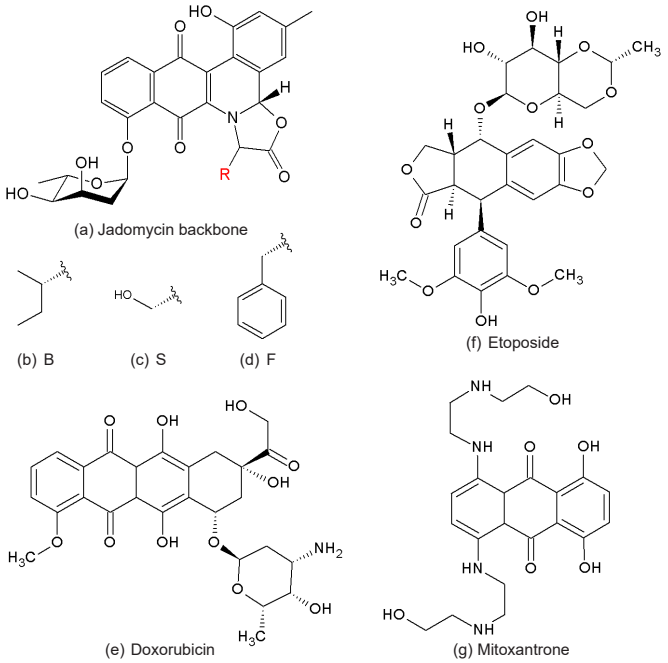

Figure 1

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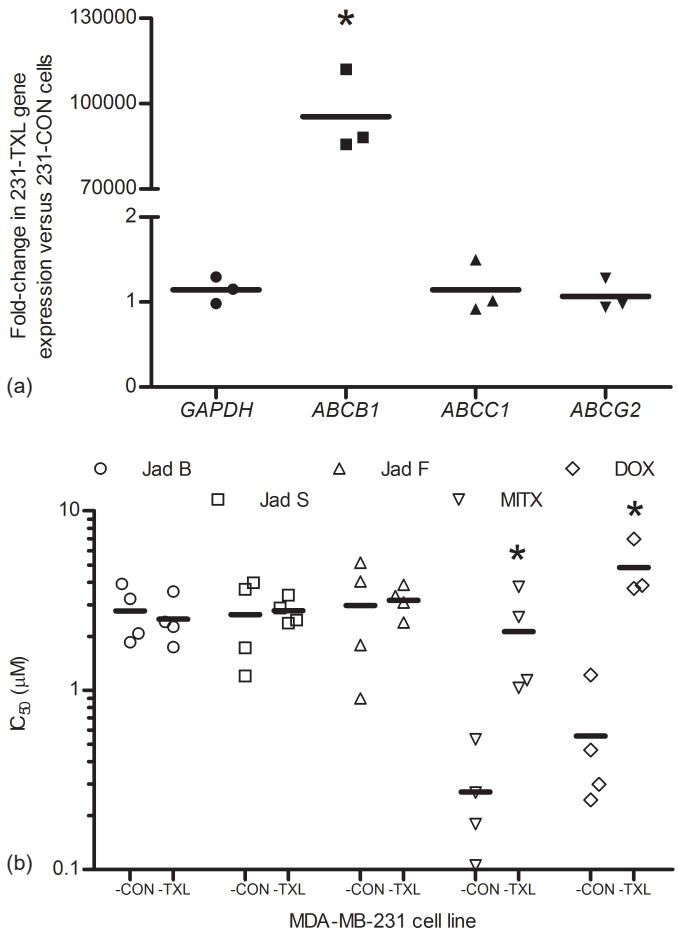

Figure 2

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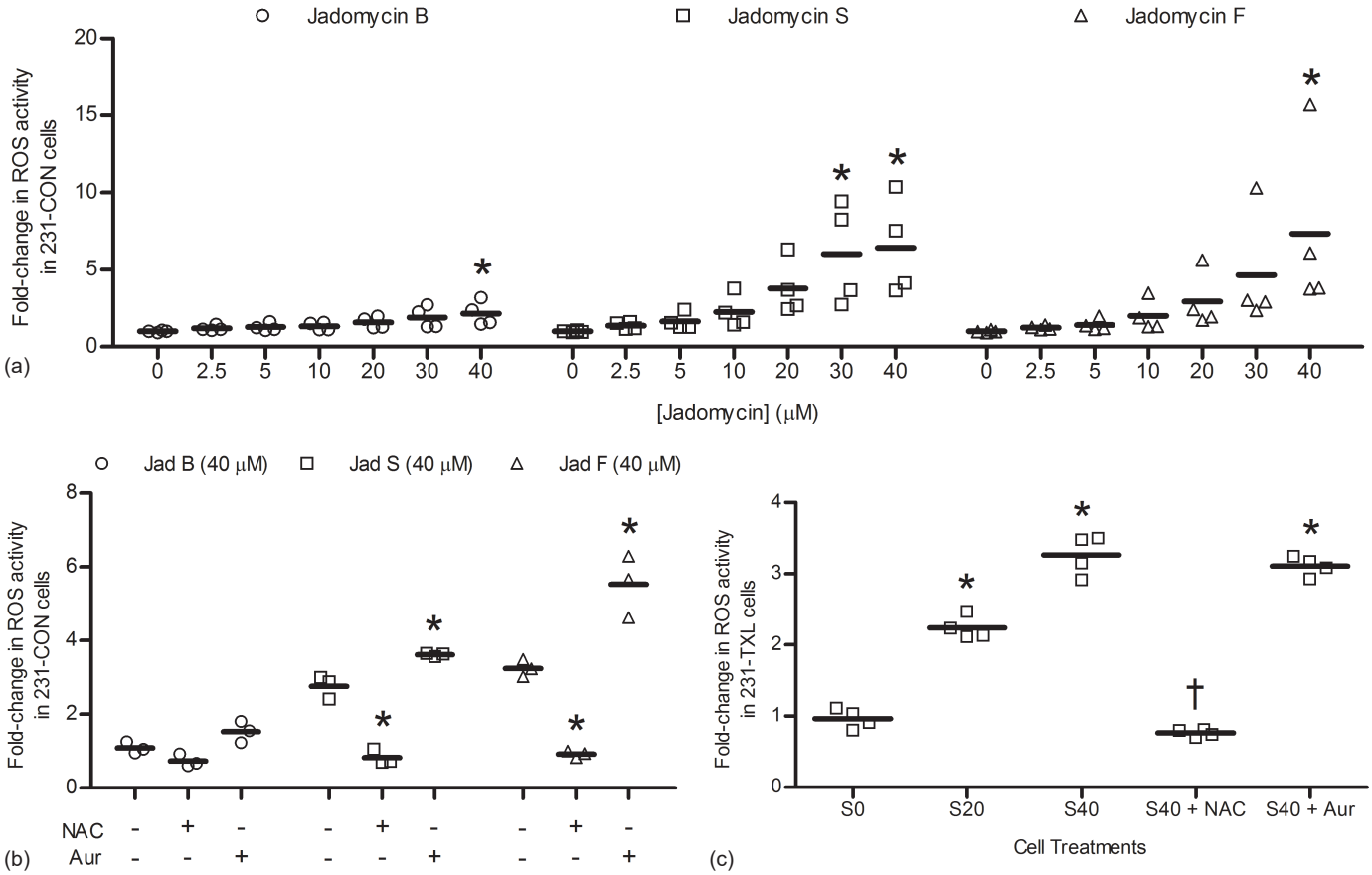

Figure 3

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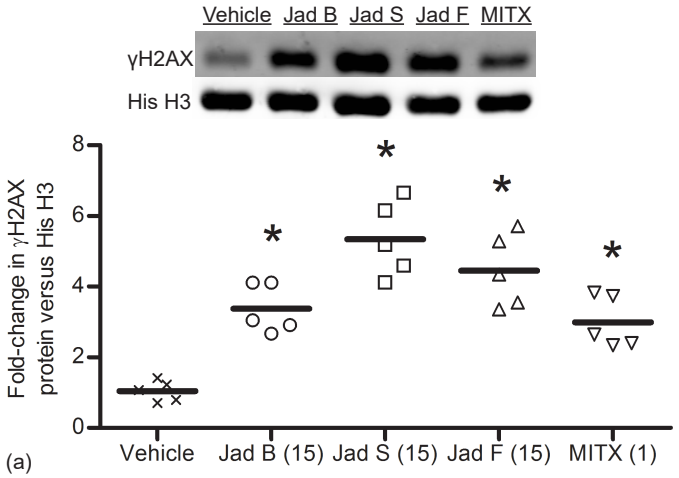

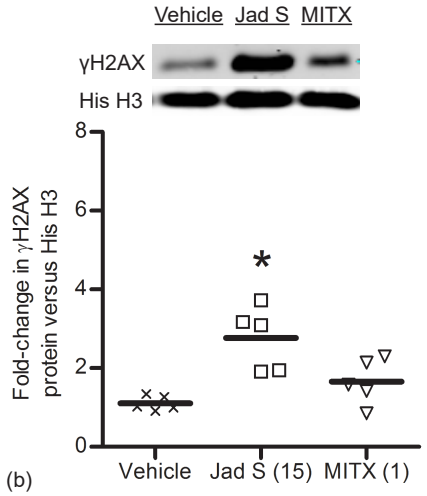

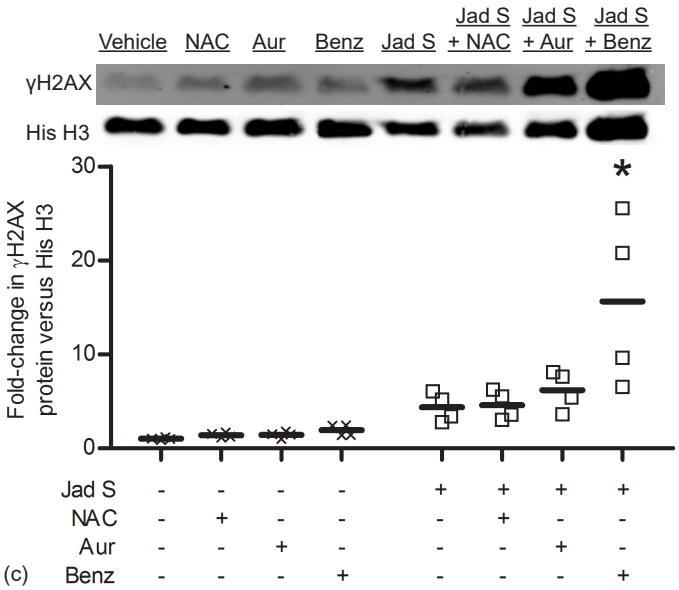

Figure 4

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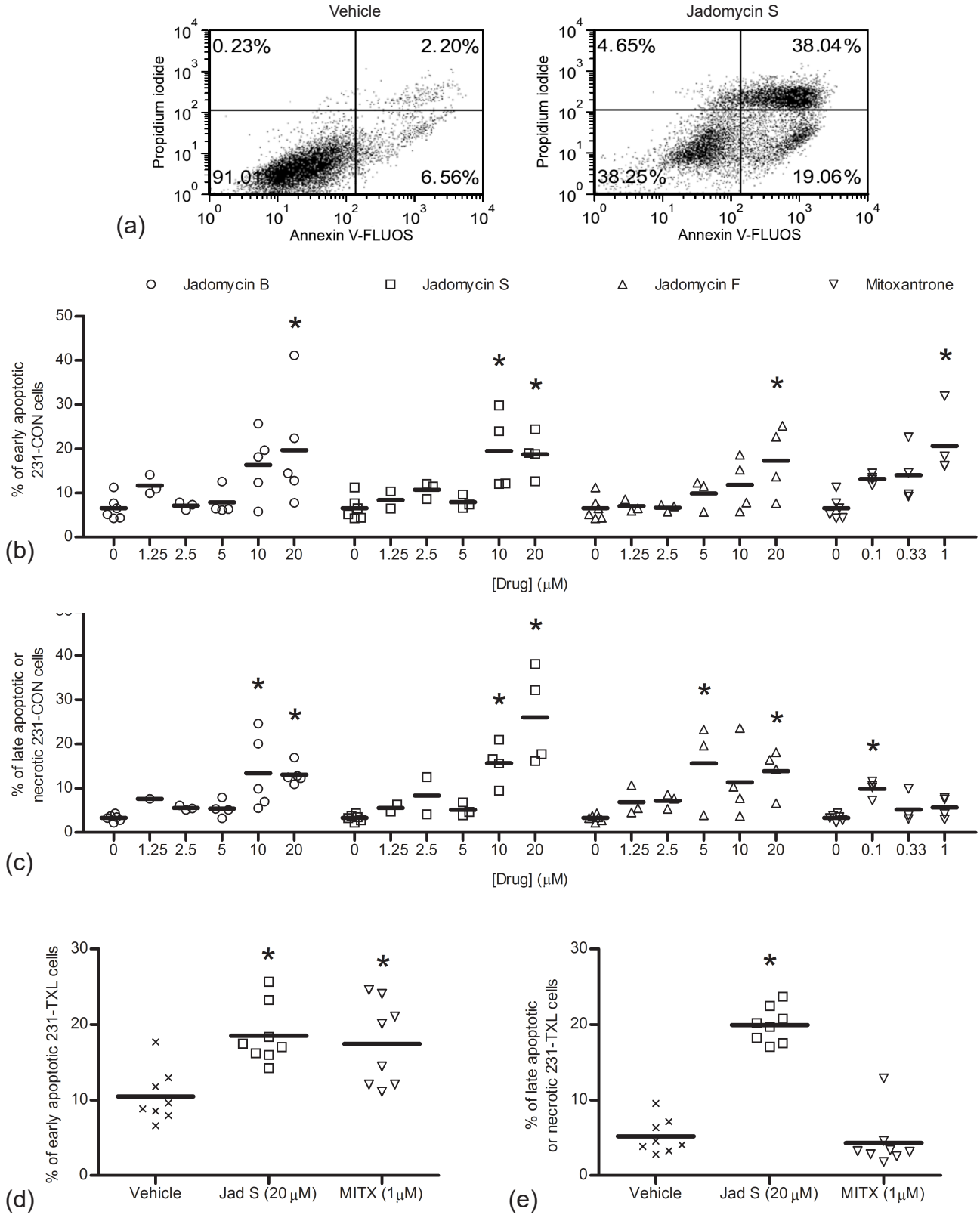

Figure 5

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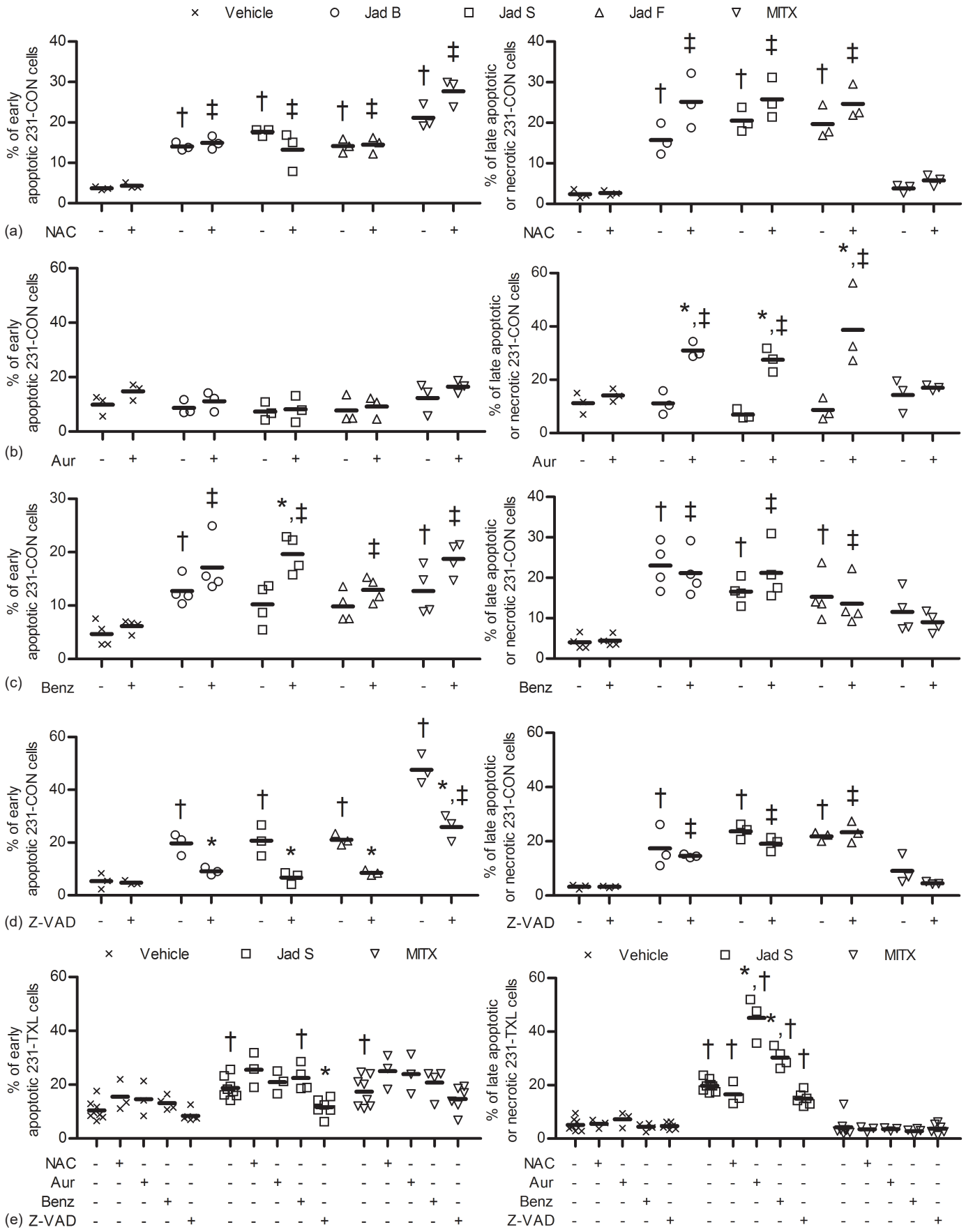

Figure 6

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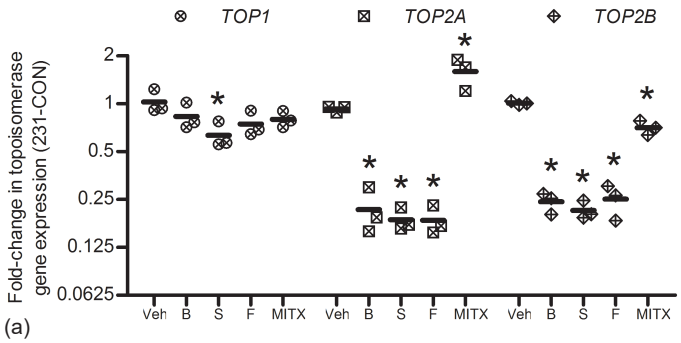

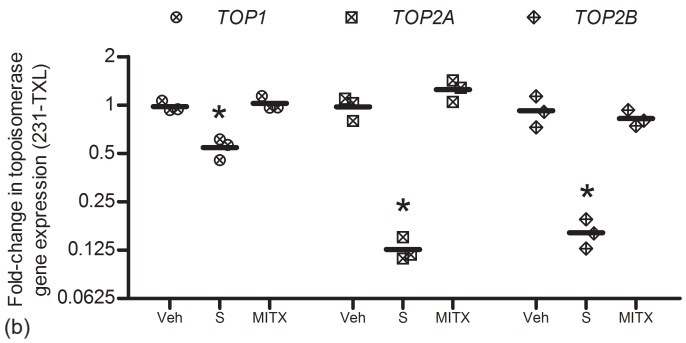

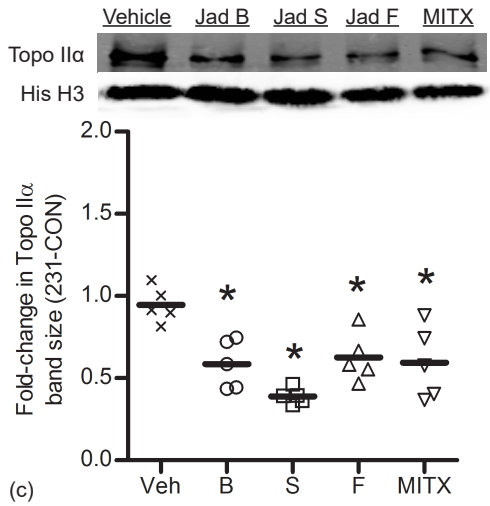

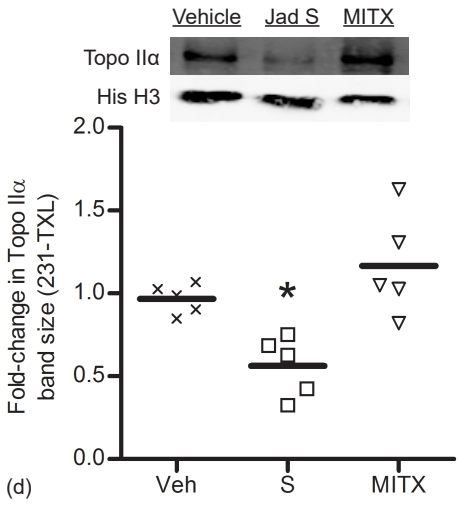

Figure 7

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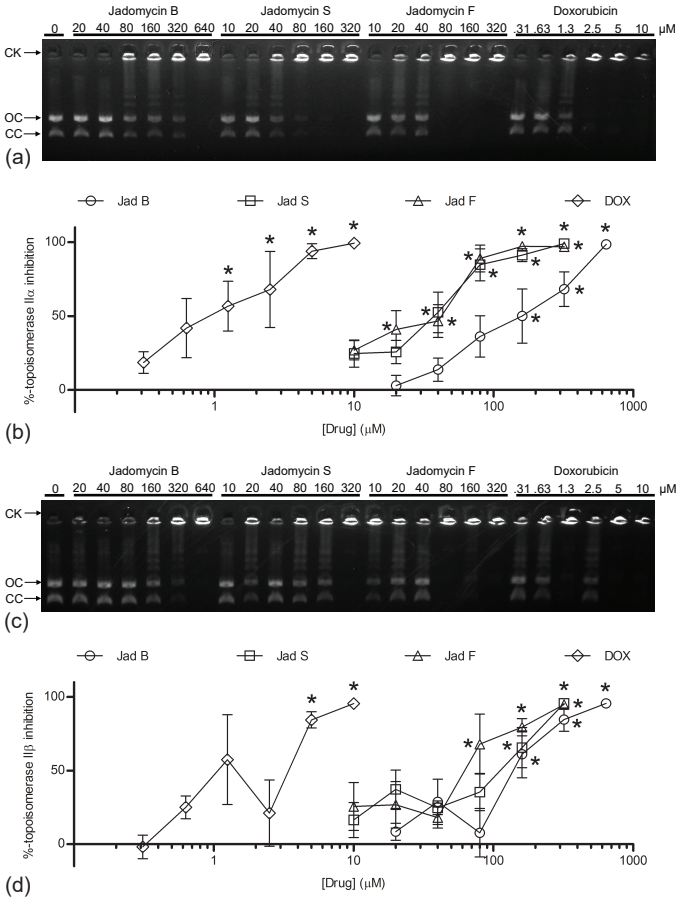

Figure 8

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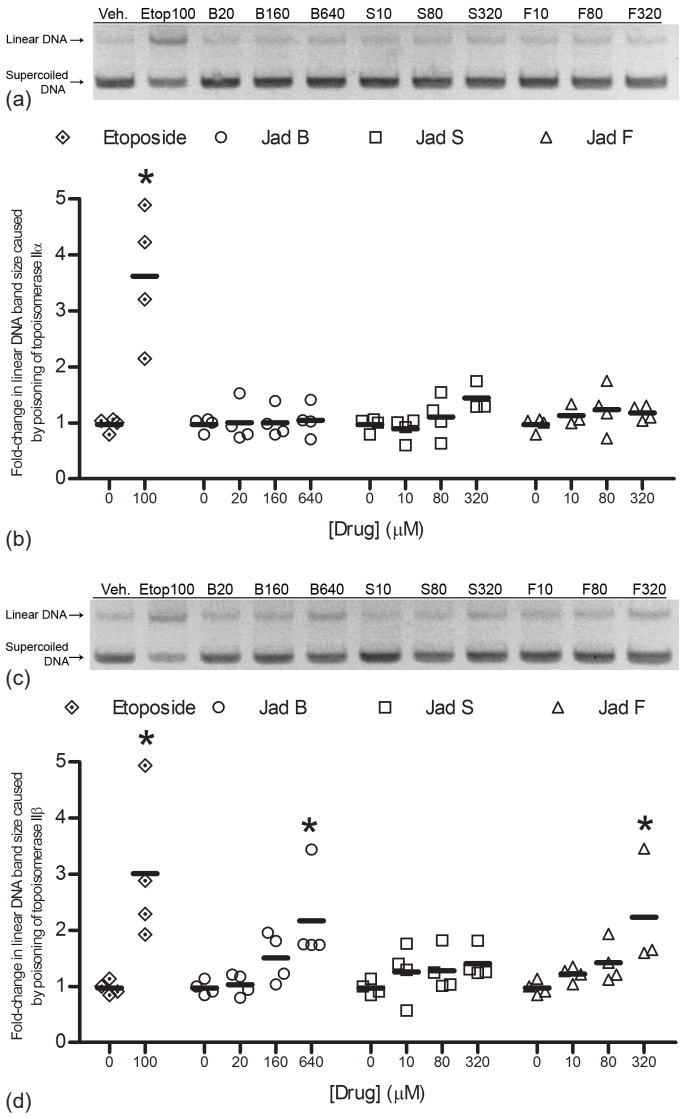

Figure 9