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

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Abstract

Jadomycins are natural products biosynthesized by the bacteria Streptomyces venezuelae which kill drug-sensitive and multidrug-resistant breast cancer cells in culture. Currently, the mechanisms of jadomycin cytotoxicity are poorly understood; however, reactive oxygen species (ROS)–induced DNA cleavage is suggested based on bacterial plasmid DNA cleavage studies. The objective of this study was to determine if and how ROS contribute to jadomycin cytotoxicity in drug-sensitive MCF7 (MCF7-CON) and taxol-resistant MCF7 (MCF7-TXL) breast cancer cells. As determined using an intracellular, fluorescent, ROS-detecting probe, jadomycins B, S, SPhG, and F dose dependently increased intracellular ROS activity 2.5- to 5.9-fold. Cotreatment with the antioxidant N-acetyl cysteine lowered ROS concentrations to below baseline levels and decreased the corresponding cytotoxic potency of the four jadomycins 1.9- to 3.3-fold, confirming a ROS-mediated mechanism. Addition of CuSO4 enhanced, whereas addition of the Cu(II)-chelator β-penicillamine reduced, the ROS generation and cytotoxicity of each jadomycin. Specific inhibitors of the antioxidant enzymes, superoxide dismutase 1, glutathione S-transferase, and thioredoxin reductase, but not catalase, enhanced jadomycin-mediated ROS generation and anticancer activity. In conclusion, the results indicate that jadomycin cytotoxicity involves the generation of cytosolic superoxide via a Cu(II)-jadomycin reaction, a mechanism common to all jadomycins tested and observed in MCF7-CON and drug-resistant MCF7-TXL cells. The superoxide dismutase 1, glutathione, and peroxiredoxin/thioredoxin cellular antioxidant enzyme pathways scavenged intracellular ROS generated by jadomycin treatment. Blocking these antioxidant pathways could serve as a strategy to enhance jadomycin cytotoxic potency in drug-sensitive and multidrug-resistant breast cancers.

Keywords
Antioxidants, breast cancer, jadomycins, multidrug resistance, natural products, reactive oxygen species

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Introduction

Breast cancer remains one of the most common cancers affecting women globally (Yu et al. 2013) with up to 30% of all breast cancers estimated to ultimately metastasize (Rivera 2010). Metastatic breast cancer remains essentially incurable with a median survival rate of 2–3 years (Morris et al. 2009; Lluch et al. 2014). This is largely due to the development of multidrug resistance which significantly reduces the effectiveness of almost all therapies available for metastatic breast cancer (Morris et al. 2009; Rivera 2010).

Jadomycins are a class of naturally biosynthesized, polyketide-derived compounds produced by the soil bacteria *Streptomyces venezuelae* (Jakeman et al. 2006). Jadomycin analogs with distinct functional groups on the oxazolone ring can be created by adding different amino acids to the *Streptomyces* growth media as the sole nitrogen source (Jakeman et al. 2005a,b). Current data support a broad spectrum of jadomycin anticancer activity, including activity against multiple breast cancer cell subtypes. Jadomycin cytotoxic potencies, measured by the half maximal inhibitory concentration (IC50), typically range between 1 and 30 μmol/L and depend on the structure of the oxazolone ring side chain, the presence of the sugar moiety, and the type of cancer cell treated (Borissow et al. 2007; Fu et al. 2008; Jakeman et al. 2009a; Cottreau et al. 2010; Dupuis et al. 2011; Dupuis et al. 2012). Our previous work identified that jadomycins with hydrophobic aliphatic functional groups – isoleucine (B) and leucine (L), hydrophobic aromatic functional groups – phenylalanine (F) and S-phenylglycine (SPhG), and hydrophilic functional groups – serine (S) and threonine (T), are effective cytotoxic agents against MCF7 breast cancer cells in vitro (Jakeman et al. 2009a; Cottreau et al. 2010; Issa et al. 2014). Furthermore, jadomycin potency was minimally affected by overexpression of the ATP-binding cassette (ABC) drug efflux transporters ABCB1, ABCC1, or ABCG2 (Issa et al. 2014). Jadomycins therefore warrant additional pharmacological characterization in breast cancer cell models, including those with a multidrug-resistant phenotype.

Only a basic understanding of the mechanisms behind jadomycin anticancer activity is currently known (Borissow et al. 2007; Fu et al. 2008; Cottreau et al. 2010; Issa et al. 2014). One possible mechanism for jadomycin cytotoxicity is indirect DNA cleavage, resulting from the generation of the reactive oxygen species (ROS) superoxide, singlet oxygen, hydroxyl radical, and H2O2, though this has only been tested in extracellular models using bacterial plasmids (Monro et al. 2011). Additionally, through virtual screening, jadomycin B was proposed to act as an aurora B kinase (ABK) inhibitor (Fu et al. 2008). The
ability of jadomycin B to block the activity of purified ABK and the phosphorylation of an ABK target protein in cells further supports this proposed function (Fu et al. 2008; Issa et al. 2014).

Many currently available anticancer drugs are cytotoxic to cancer cells through the generation of ROS (Wondrak 2009). Cancer cells have innate levels of ROS higher than those typically observed in healthy cells, and are therefore already heavily dependent on their cellular antioxidant systems. Certain anticancer drugs increase ROS activity in the cancer cells past their tolerability threshold, inducing cell death. This is known as the “threshold concept for cancer therapy” (Kong et al. 2000; Schumacker 2006). Since healthy cells have lower innate levels of ROS, they have a higher capacity to cope with the increased oxidative stress induced by ROS-inducing anticancer drugs in comparison to tumor cells (Schumacker 2006), and therefore selectivity toward cancer cells versus healthy cells can be achieved (Kong et al. 2000). Thus, the hypothesis that jadomycin breast cancer toxicity could be achieved through ROS activity modification is a viable option that needs to be tested experimentally.

Four jadomycins, B, S, SPhG, and F, which together represent three structural classes of these compounds, were tested in BT474, SKBR3, MDA-MB-231, and drug-sensitive control (CON) and taxol (TXL)-resistant MCF7 breast cancer cells. The objectives were (1) to determine if jadomycins alter the activity of ROS within breast cancer cells and if jadomycin cytotoxicity is dependent on this ROS activity, and (2) to determine the specific ROS induced by jadomycin treatment and the antioxidant pathways involved in their elimination using pharmacological modulators of ROS homeostasis.

Materials and Methods

Chemical and biological materials

Thiazolyl blue methyltetrazolium bromide (MTT), N-acetyl cysteine (NAC), Triton X-100, H2O2 30% (w/w) in water, paclitaxel, dimethylsulfoxide, methanol, sodium lactate, phenazine methosulfate, β-nicotinamide adenine dinucleotide, iodonitrotetrazolium chloride, copper (II) sulfate (CuSO4), d-penicillamine (d-Pen), MitoTEMPO, sodium diethyldithiocarbamate (DDC), ellagic acid (EA), and polymerase chain reaction (PCR) primers were purchased from Sigma Aldrich (Oakville, Ontario, Canada). Aurum total RNA Mini Kit and SYBR Green were purchased from Bio-Rad (Mississauga, Ontario, Canada). Aurum total RNA Mini Kit and SYBR Green were purchased from Bio-Rad (Mississauga, Ontario, Canada). Aurum total RNA Mini Kit and SYBR Green were purchased from Bio-Rad (Mississauga, Ontario, Canada). Aurum total RNA Mini Kit and SYBR Green were purchased from Bio-Rad (Mississauga, Ontario, Canada). Aurum total RNA Mini Kit and SYBR Green were purchased from Bio-Rad (Mississauga, Ontario, Canada). 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ROS measuring assays

To quantify the presence of intracellular ROS in MCF7 cells, a fluorescent assay utilizing the ROS-reactive CM-DCFH$_2$-DA was used. CM-DCFH$_2$-DA passively diffuses through cell membranes where it is de-esterified to CM-DCF, confining it within the cell. In the presence of ROS, CM-DCF is oxidized yielding a fluorescent compound that can be quantified as a general oxidative stress indicator (Rhee et al. 2010). MCF7 cells were seeded in black-sided, clear-bottomed 96-well plates at 40,000 cells/well in 100 µL of 10% FBS standard assay media. Cells were left to adhere for 24 h under standard conditions, at which time they were ~90% confluent. After 24 h, the media was removed and 50 µL of 7.5 µmol/L CM-DCFH$_2$-DA in Dulbecco’s modified Eagle’s media supplemented with 1% FBS, 1% sodium pyruvate, and 1% penicillin/streptomycin (1% FBS standard assay media) was added to each well, excluding blanks, for 1 h under standard conditions. The CM-DCFH$_2$-DA-containing media was removed and cells were treated with 100 µL of jadomycin B, S, SPhG, or F (2.5–30 µmol/L), H$_2$O$_2$ (125–2,000 µmol/L), or vehicle in 1% FBS standard assay media for 24 h in quintuplicate. After 24 h fluorescence was read at excitation 485/20 nm and emission 528/20 nm using a Biotek Synergy HT plate reader (BioTek, Winooski, VT, USA). Background fluorescence from blanks was subtracted and the fold change in fluorescence was calculated via the following equation:

\[
\text{Fold increase} = \frac{F_{\text{avg,treatment}}}{F_{\text{avg,vehicle}}}
\]

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

Effect of NAC on jadomycin IC$_{50}$ values

MCF7-CON cells were cotreated with jadomycins and the antioxidant NAC to examine the role of intracellular ROS as a mechanism of jadomycin-mediated cytotoxicity (Rogalska et al. 2013). MCF7-CON cells were seeded in 96-well plates at 20,000 cells/well in 100 µL of 1% FBS standard assay media and grown for 24 h under standard conditions. The media was removed and 80 µL of 1% FBS standard assay media containing NAC at a final concentration of 0.3–15 mmol/L or H$_2$O vehicle was added to each well and left at standard conditions for 1 h, followed by the addition of 20 µL of 1% FBS standard assay media containing jadomycin B, S, SPhG, or F at final concentrations of 1.25–35.0 µmol/L or the 1:7 MeOH: H$_2$O vehicle control (jadomycin vehicle) in quintuplicate for 2 h under standard conditions. Media was aspirated from each well, 50 µL of phosphate-buffered saline added and aspirated to remove residual dead cells, and 100 µL of 0.1% (v/v) Triton X-100 in water added to kill the remaining viable cells followed by plate shaking at 500 rpm for 5 min. Following cell lysis with 0.1% Triton X-100, lactate dehydrogenase (LDH) activity was measured by treating each well with 100 µL of in-house LDH reaction solution (25 mmol/L sodium lactate, 147 µmol/L phenazine methosulfate, 644 µmol/L β-nicotinamide adenine dinucleotide, and 326 µmol/L iodonitrotetrazolium chloride in 200 mmol/L Tris buffer, pH = 8), the plate shaken at 500 rpm for 1 min, and absorbance (490 nm) quantified using a Biotek Synergy HT plate reader as a measure of cell viability post-drug treatment (Weidmann et al. 1995; Sepp et al. 1996; Swart and Pool 2009). The % cell viability for each jadomycin or H$_2$O$_2$ treatment concentration was calculated as follows:

\[
\% \text{Cell viability} = \frac{A_{490,\text{avg,treatment}}}{A_{490,\text{veh,avg}}} \times 100\% \tag{2}
\]

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

Western blot analysis

Western blot analyses were performed as published previously (Issa et al. 2014) with the following modifications. MCF7-CON cells seeded in clear, six-well plates were treated with 800 µL of 10% FBS standard assay media containing NAC (final concentration of 15 mmol/L) or H$_2$O vehicle under standard conditions for 1 h. This was followed by the addition of 200 µL of 10% FBS standard assay media containing fivefold concentrated jadomycin B, S, SPhG, or F (final concentration of 10 µmol/L), H$_2$O$_2$ (final concentration of 500 µmol/L), or jadomycin vehicle in triplicate for 24 h under standard conditions. A 20 µg aliquot of protein from each pooled sample was separated using a 12.5% SDS-PAGE and transferred to nitrocellulose membranes. Membranes were incubated overnight in a combination 1:500 dilution of monoclonal mouse anti-human P-His3(Ser10) antibody and 1:2000 dilution of a polyclonal rabbit anti-human His3 antibody at 4°C. Membranes were then incubated in a combination 1:15,000 dilution of IRDye 680RD-conjugated donkey anti-mouse and 1:15,000 dilution of IRDye 800CW-conjugated goat anti-rabbit secondary antibodies. Membranes were scanned at 700 and 800 nm infrared wavelengths using a Licor Odyssey (Mandel Scientific). The integrated intensity and area of P-His3(Ser10) were measured and normalized to those of His3 for each protein sample. These ratios were then compared with those of the vehicle controls and calculated as a % change in protein expression.
Effects of pro- and antioxidant cotreatments on jadomycin-dependent ROS activity and cytotoxicity

To determine how jadomycin-induced ROS activity is linked to jadomycin cytotoxicity in MCF7-CON or -TXL cells, we developed an assay where the change in ROS activity and LDH activity were sequentially measured in the same cells following jadomycin treatment in the presence or absence of pharmacological modulators of ROS generation or deactivation. MCF7-CON and -TXL cells were plated and pretreated with CM-DCFH2-DA as described earlier for the ROS assays. This was followed by a 1 h preincubation with the following compounds based on published effective final concentrations with some modifications for ideal dosing: 0.3–15 mmol/L NAC (Gundelach et al. 2012), 10 μmol/L CuSO4, 1 mmol/L d-Pen (Gupte and Mumper 2007), 0.1–10 nmol/L Mito-TEMPO (Liang et al. 2009), 1 mmol/L DDC (Tatrai et al. 2001), 10 mmol/L 3-AT (Kimani et al. 2012), 20 μmol/L EA (Hayeshi et al. 2007), or 1 μmol/L auranozin (Liu et al. 2013), followed by a 24 h treatment in quadruplicate or quintuplicate with jadomycin B (7.5–25 μmol/L), S (12–35 μmol/L), SPhG (7.5–20 μmol/L), or F (7.5–20 μmol/L) or jadomycin vehicle. Sequentially, ROS activity and LDH activity assays were performed for each treatment as described in the earlier methods.

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

Total RNA was isolated from lysates of MCF7-CON cells treated with jadomycin B (10 μmol/L) or jadomycin vehicle for 24 h under standard conditions using the Aurum total RNA Mini Kit according to the manufacturer’s instructions. Isolated RNA (0.5 μg) was reverse transcribed to complementary DNA using Super Script II Reverse Transcriptase (Life Technologies). The complementary DNA was amplified via quantitative PCR using 125 nmol/L gene-specific primers in a total volume of 20 μL using a SYBR Green PCR Kit and a Step One Plus real-time PCR thermocycler (Life Technologies) 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 (also known as cyclophilin A) via the ΔΔCt method (Livak and Schmittgen 2001).

Statistical analysis

All data are presented as mean ± standard error of the mean (SEM). 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. In the case of nonparametric data involving one independent variable, a Kruskal–Wallis one-way ANOVA followed by Dunn’s multiple comparison test was used. A difference in mean values between groups was considered significant if P ≤ 0.05.

Results

Jadomycins are equally cytotoxic against MCF7-CON, BT474, SKBR3, and MDA-MB-231 breast cancer cells

MTT cell viability measuring assays quantified the % cell viability of MCF7-CON, BT474, SKBR3, and MDA-MB-231 breast cancer cells after being treated with various concentrations of jadomycins B, S, or F. The IC50 of each jadomycin in each cell line was calculated and used as a measure of drug potency (Table 1). The IC50 values of jadomycins B, S, and F were equal in each of the four breast cancer cell lines tested. Given the similar jadomycin cytotoxicity profiles across several breast cancer cells we chose a single cell line (MCF7) to investigate their mechanisms of action.

Jadomycins increase intracellular ROS activity in MCF7-CON cells

Using the general intracellular ROS-detecting probe CM-DCHF2-DA, jadomycin treatments in MCF7-CON breast cancer cells showed a dose-dependent increase in ROS activity.

Table 1. IC50 values (μmol/L) as determined by MTT assays after treatment with jadomycins.

| Cell line     | Jadomycin B | Jadomycin S | Jadomycin F |
|---------------|-------------|-------------|-------------|
| MCF7-CON      | 2.58 ± 0.39 | 3.38 ± 0.09 | 3.59 ± 0.52 |
| BT474         | 4.16 ± 0.54 | 3.09 ± 0.54 | 5.05 ± 1.62 |
| SKBR3         | 3.82 ± 0.84 | 3.08 ± 0.73 | 4.70 ± 0.90 |
| MDA-MB-231    | 1.76 ± 0.33 | 2.79 ± 0.48 | 3.25 ± 0.29 |

Data show the mean IC50 ± SEM of at least three independent experiments performed in quadruplicate. The IC50 values for each jadomycin were equal in each of the four breast cancer cell lines tested as determined by one-way ANOVAs, followed by Bonferroni’s multiple comparison tests (P ≤ 0.05). IC50, half maximal inhibitory concentration; MTT, thiazolyl blue methyltetrazolium bromide; ANOVA, analysis of variance.
activity (Fig. 1). Jadomycin B and F concentrations of 20 or 30 μmol/L and jadomycin S and SPhG concentrations of 30 μmol/L significantly increased intracellular ROS activity in MCF7-CON in comparison to the jadomycin vehicle control. To verify the increase in fluorescence was solely due to increased intracellular ROS activity; the autofluorescence of the four jadomycins and their ability to react directly with CM-DCFH-DA were tested, which showed no autofluorescence or reactivity (Fig. S1).

NAC cotreatment decreases the cytotoxic potency of jadomycins in MCF7 cells

The antioxidant NAC is a cysteine precursor which is taken up into cells and converted to cysteine, the rate-limiting molecule in the synthesis of glutathione (GSH), an integral component in a cell’s antioxidant defense system (Dodd et al. 2008). In the absence of NAC, jadomycins B, S, F, and SPhG had equal mean IC₅₀ values (4.0–5.3 μmol/L) in MCF7-CON cells. In the presence of increasing concentrations of NAC, the concentration–cytotoxicity response curves, as measured by LDH assays, were right-shifted to varying degrees for jadomycins B, S, SPhG, and F (Fig. 2A–D, respectively). The treatment of MCF7-CON cells with 3–15 mmol/L NAC alone resulted in a small reduction (17–25%) in cell viability (Fig. 2E). The shift in jadomycin potency was quantified by a progressive yet saturable increase in jadomycin IC₅₀ values with increasing NAC concentration (Fig. 2F). Overall jadomycin SPhG was least affected by NAC as demonstrated by the IC₅₀ values that were lower than those of jadomycins B, S, or F when cotreated with ≥3 mmol/L NAC. While NAC reduced the potency of each jadomycin, greater than 95% loss in cell viability could still be attained using higher doses of jadomycin in the presence of NAC.

NAC dose dependently decreases jadomycin-mediated ROS activity while simultaneously increasing the viability of MCF7-CON and MCF7-TXL cells

The results of our initial experiments suggested that ROS generation is a mechanism of jadomycin toxicity. The next experiments were designed to confirm the mechanistic link between ROS generation and jadomycin toxicity by performing ROS assays and LDH cytotoxicity assays sequentially in the same cells. In MCF7-CON cells, jadomycins B, S, SPhG, and F produced between a 1.4- and 2.6-fold increase in intracellular ROS which was associated with a 61% to 78% loss in viable cells compared to vehicle-treated cells in the absence of NAC. When cotreated with increasing concentrations of NAC, similar dose-dependent reductions in intracellular ROS activity and increases in % cell viability were observed for each of the four jadomycins in MCF7-CON cells (Fig. 3A and B). Comparable results were observed in MCF7-TXL cells, albeit using marginally higher concentrations of each jadomycin (Fig. 3C and D).
Inhibition of ABK by jadomycins is maintained when ROS are inhibited

To assess if the second putative mechanism of jadomycin cytotoxicity (ABK inhibition) is dependent or independent of ROS generation, we examined jadomycin inhibition of ABK activity with and without ROS neutralization using 15 mmol/L NAC. ABK activity was quantified by measuring the phosphorylation of the ABK downstream target serine 10 in Histone H3 [P-His3(Ser10)] (Fu et al. 2008; Issa et al. 2014) by western blotting (Fig. 4A and B). Jadomycins B, S, SPhG, and F all inhibited phosphorylation of His3(Ser10) compared to the jadomycin vehicle control. A similar level of inhibition was maintained for all four jadomycins when ROS activity was inhibited with NAC. The H$_2$O$_2$ positive control had no effect on P-His3(Ser10) in the presence or absence of NAC. The NAC treatment did not alter P-His3(Ser10) compared to the NAC vehicle in the absence of jadomycin treatments.

Jadomycin-induced ROS activity and corresponding cytotoxicity are copper dependent

MCF7-CON cells cotreated with CuSO$_4$ and jadomycin S, SPhG, or F demonstrated 2.2- to 3.1-fold greater...
intracellular ROS activity compared to cells treated with those jadomycins alone, with a similar trend observed for jadomycin B (Fig. 5A). The increased ROS activity was correlated with a further 34.5% to 51.6% decrease in cell viability for all four jadomycin treatments relative to the vehicle-treated control cells. While 10 μmol/L CuSO₄ increased ROS activity by 2.1-fold in the absence of jadomycins, this was not associated with any change in cell viability indicating the cytotoxicity was jadomycin dependent (Fig. 5B). The ROS activity in cells treated with jadomycin B, S, or F and the copper chelator D-Pen decreased 1.6- to 2.3-fold compared to the respective controls. A similar trend was observed with jadomycin SPhG. These reductions in intracellular ROS were associated with 41.1% to 57.9% increases in % cell viability for jadomycin B, SPhG, or F treated cells. D-pen treatment in the absence of jadomycins had no effect on ROS activity or % cell viability (Fig. 5C and D).

Pro- and antioxidant cotreatments alter jadomycin-induced ROS activity and cytotoxicity

The thioredoxin reductase (TrxR) inhibitor auranofin significantly increased ROS activity 2.5- to 3.8-fold and decreased MCF7-CON cell viability 43.7% to 76.8% when cotreated with each of the four jadomycins in comparison to jadomycin alone (Fig. 6). The superoxide dismutase 1 (SOD1) inhibitor DDC increased ROS activity in all but the jadomycin B cotreated cells 1.7- to 2.0-fold, though it consistently decreased cell viability for each jadomycin 41.2% to 68.2%. The glutathione S-transferase (GST) inhibitor EA did not alter ROS activity, but decreased cell viability in cells cotreated with jadomycins S or SPhG by 40.5% or 28.6%, respectively. The catalase inhibitor 3-AT had no effect on ROS activity or cell viability when cotreated with any of the jadomycin analogs used. None of these inhibitors affected ROS activity or % cell viability in the
Figure 4. Jadomycins S, SPhG, and F inhibit phosphorylation of the ABK target His3(Ser10), independent of ROS activity. MCF7-CON cells were treated for 24 h with jadomycins B, S, SPhG, or F (10 μmol/L), H₂O₂ (500 μmol/L) or jadomycin vehicle control and cotreated with NAC (15 mmol/L) or H₂O vehicle control, after which total protein was collected. Levels of P-His3(Ser10) and His3 control were determined via western blotting (A). Band intensities were measured and the fold changes in P-His3(Ser10)/His3 ratios calculated relative to either jadomycin vehicle and H₂O vehicle controls or jadomycin vehicle and NAC (15 mmol/L) control (B). *P ≤ 0.05, the fold change in protein band intensity was significantly different compared to the jadomycin vehicle and H₂O vehicle control (left side, clear bar). †P ≤ 0.05, the fold change in protein band intensity was significantly different compared to the jadomycin vehicle and NAC (15 mmol/L) control (right side, clear bar). A two-way ANOVA, followed by the Bonferroni’s multiple comparison test, were used for the analyses.

Figure 5. Jadomycin-induced ROS activity and corresponding cytotoxicity are copper dependent. MCF7-CON cells were treated for 24 h with jadomycins B, S, SPhG, or F (7.5–18 μmol/L) or the jadomycin vehicle control (labeled No Jadomycin) with (+) CuSO₄ (10 μmol/L) or Δ-Pen (1 mmol/L), or an H₂O vehicle (−). ROS activity is expressed as fold change (A, C) and cell viability as a percentage (B, D) relative to the No Jadomycin control in the absence of CuSO₄ or Δ-Pen. Each bar represents the mean ± SEM of at least three independent experiments. *P ≤ 0.05, for the indicated jadomycin, the fold change in ROS activity or % cell viability was significantly different in the presence versus absence of CuSO₄ or Δ-Pen as determined by an unpaired t test.

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The absence of jadomycin. The antioxidant, superoxide dismutase 2 (SOD2) mimetic MitoTEMPO at multiple concentrations did not affect ROS activity or cell viability when cotreated with any of the jadomycins (Fig. S2).

The three cotreatments, which resulted in the largest ROS and cell viability changes, CuSO₄, auranofin, and DDC, all produced similar results in MCF7-TXL cells (Fig. 7). CuSO₄ consistently increased ROS activity for
each jadomycin treatment as well as for the no jadomycin control 1.9- to 2.8-fold, auranofin increased ROS activity 2.3- and 2.0-fold when cotreated with jadomycins S and SPhG, respectively, while DDC had no effect on ROS. CuSO₄ decreased cell viability for each jadomycin cotreatment, except SPhG, by 32.6% to 40.0%. Auranofin and DDC decreased cell viability for each jadomycin treatment by 51.5% to 66.8% and 37.9% to 55.3%, respectively.

Jadomycin B treatment induces the expression of TrxR1

Quantitative PCR was completed to determine if jadomycin B treatment altered the expression of antioxidant encoding genes. A 24 h treatment of jadomycin B versus jadomycin vehicle in MCF7-CON cells at standard conditions resulted in no change in SOD1, SOD2, thioredoxin (Trx), catalase, or nuclear factor (erythroid-derived 2)-like 2 (Nrf2) mRNA levels when compared with the housekeeping gene GAPDH. A significant, 10.2-fold increase in TrxR1 expression was observed (Fig. 8). The quantitative PCR primer sequences used are found in Table 2.

Discussion

Many anticancer drugs increase ROS activity within cancer cells, including vinblastine (Wersinger and Sidhu 2005), paclitaxel (Taxol) (Rogalska et al. 2013), and doxorubicin (Kotamraju et al. 2000). While ROS are often considered oncogenic and certain ROS-inducing drugs are known to induce toxic side effects related to ROS generation, ROS production remains a vital mechanism shared by all nonsurgical methods of cancer treatment due to their effectiveness in inducing cancer cell death (Wang and Yi 2008; Deavall et al. 2012). Monro et al. (2011) determined that jadomycin B, in the presence of Cu(II), caused single strand cleavage of supercoiled bacterial plasmid DNA. This effect was blocked using the antioxidants catalase, superoxide dismutase, or hydroxyl radical and singlet oxygen scavengers, suggesting ROS were responsible for the DNA cleavage. Using the rapidly proliferating MCF7 cell line, to which jadomycins are equally toxic in comparison to the three other breast cancer cell lines tested, we demonstrated that multiple jadomycin-amino acid derivatives, B, S, F, and SPhG, increased intracellular ROS activity, supporting the findings of Monro et al. Consistent with a ROS-mediated mechanism of action, we then determined that the antioxidant NAC dose dependently blocked jadomycin-generated ROS activity, which correlated with increased cell viability and reduced jadomycin potency. Despite this, higher concentrations of jadomycins were still able to attain close to 100% efficacy in the presence of NAC.

Table 2. PCR primers used to determine the expression of antioxidant and housekeeping genes in MCF7-CON cells treated with either jadomycin B (10 µmol/L) or vehicle control for 24 h.

| Gene   | PCR forward primers (5′–3′)                  | PCR reverse primers (5′–3′)                  |
|--------|---------------------------------------------|---------------------------------------------|
| SOD1   | GAGAAGCTTGGGCAATGTGAC                      | CACAAGCACAACGACTCCA                       |
| SOD2   | AACCTCGGAGCCCTACAGGCG                      | CCCACACTAATCCCAAGCA                       |
| TrxR1  | CCACGTGTGAAAGACCACCGTT                    | AGGAGAAAGAGCATCAGTCGC                     |
| Trx    | GGTGAGAAGAAGATGAGAGC                      | CCACGTGGCTGAGAAGCTCA                       |
| Catalase| ACTTTGGAGCTCCAGGCC                                 | AAGCTTGGGCGGCATCCTCA                       |
| Nrf2   | ACACGGTCAACAAGATGGGTGTGT                   | TGCTTTCCAAATCCGATGTGTGT                   |
| GAPDH  | GAATTCAGGGGGATTTGGGTGT                    | TTTGATTGGGAGGATCTCG                       |
| β-actin| GGATTCAGGGGGATTTGGGTGT                   | AGCTGTAATCCGAGATGTGTGT                   |
| PPIA   | ACCGCGAGGGAAAACCGGT                       | CGTCTTTGGAGACCTTCGG                       |
One explanation for this efficacy preservation is that the ROS generated by the higher concentrations of jadomycins exceeded the GSH-generating capacity of the maximum tolerated dose of NAC. A second possibility is that a ROS-independent mechanism of cytotoxicity is triggered by higher concentrations of jadomycin. This second putative mechanism could involve the inhibition of ABK, a protein often overexpressed in cancers and linked to tumor formation and progression (Fu et al. 2008; Katayama and Sen 2010) and for which jadomycin B has been previously shown to be an inhibitor (Fu et al. 2008; Issa et al. 2014). Supporting this hypothesis, we showed that jadomycins similarly reduced the phosphorylation of the downstream ABK target His3(Ser10) both in the presence of elevated ROS and after ROS neutralization to baseline levels using NAC (15 mmol/L). This observation, coupled with the fact that the ROS positive control H2O2 did not inhibit His3(Ser10) phosphorylation, supports that ABK inhibition by jadomycins could proceed independently of ROS generation. However, the exact role of the ABK pathway in jadomycin-mediated cytotoxicity remains to be determined.

Next, a pharmacological approach was used to determine which ROS are involved in jadomycin cytotoxicity and the potential mechanisms involved in ROS detoxification following jadomycin treatment. Monro et al. (2011) proposed that jadomycin B serves as a source of electrons for the reduction of Cu(II) to Cu(I), which reacts further to form multiple ROS including superoxide, hydroxyl radicals, and H2O2, leading to bacterial plasmid DNA cleavage (Monro et al. 2011). The observation that the copper-chelating agent D-Pen (Gupte and Mumper 2007) reduced whereas added CuSO4 increased ROS production and cytotoxicity of jadomycins B, S, SPbG, and F indicated that jadomycin cytotoxicity in MCF7-CON and drug-resistant MCF7-TXL cells is Cu(II) dependent. This Cu(II) dependency varied slightly between jadomycins, suggesting the cytotoxic mechanisms of each analog are not identical. Differences in jadomycin Cu(II) dependency have also been previously observed, with jadomycin B being Cu(II) dependent and jadomycin L being Cu(II) independent (Cottreau et al. 2010). Endogenous copper in MCF7 cells was sufficient to mediate jadomycin cytotoxicity and increasing the media copper concentration enhanced jadomycin cytotoxicity, and vice versa. Since serum and tumor copper levels are elevated in various cancers (Gupte and Mumper 2007), this Cu(II) dependency of jadomycins indicates a possible exploitable route of cancer cell selectivity over healthy cells. Interestingly, CuSO4 treatment alone produced a similar increase in intracellular ROS compared to CuSO4 plus jadomycin co-treatments, with no effect on cell viability. Cu(II) is widely known to be involved in the production of ROS, particularly superoxide, hydroxyl radicals, and H2O2 (Houghton and Nicholas 2009). This could indicate that the type of ROS being produced are different and more damaging in the presence of Cu(II) and jadomycin versus Cu(II) alone, such as higher levels of superoxide and less H2O2.

Our experiments showed how the cytosolic SOD1 inhibitor DDC (Tatrai et al. 2001) enhanced jadomycin-mediated ROS production and cytotoxicity, whereas the mitochondrial antioxidant MitoTEMPO (Liang et al. 2009), a SOD2 mimetic, had no effect. This supports increased cytosolic, but not mitochondrial, concentrations of superoxide as a primary mechanism of jadomycin-mediated killing of MCF7-CON and MCF7-TXL breast cancer cells. Furthermore, the augmenting effect of DDC on jadomycin-induced ROS generation and cell death indicates that SOD1 conversion of superoxide to H2O2 is an important cellular mechanism for jadomycin detoxification. The ROS-inducing anthracycline, doxorubicin, is known to have a high affinity for mitochondria. Since cardiac tissue is rich in mitochondria, doxorubicin is quite cardiotoxic (Deavall et al. 2012). By increasing only cytosolic superoxide it will be interesting to determine if jadomycins do not induce similar cardiotoxic side effects.

The reduction of H2O2 to H2O can be completed through multiple antioxidant pathways. One is the peroxiredoxin (Prx)/Trx pathway, in which Prx reduces H2O2 to water, Trx reduces Prx, TrxR reduces Trx, and NAPDH reduces TrxR, thus reactivating the pathway (Arner and Holmgren 2006; Yoshioka et al. 2006; Lopert et al. 2012). The TrxR inhibitor auranofin (Liu et al. 2013) increased ROS activity and decreased cell viability when cotreated with each jadomycin tested in MCF7-CON and -TXL cells, indicating that conversion of H2O2 to H2O by the Prx/Trx pathway is vital for the detoxification of jadomycin-induced ROS. The importance of this pathway is further exemplified by the increased TrxR expression in MCF7-CON cells treated with jadomycin B, a gene expression change that suggests an attempt by the breast cancer cells to increase Prx/Trx pathway activity to survive the treatment.

EA is an inhibitor of the cytosolic glutathione S-transferases (GST) P1-1, A1-1, A2-2, M1-1, and M2-2, which are multifunctional detoxifying enzymes involved in a second pathway of H2O2 reduction (Hayeshi et al. 2007). GSTs catalyze the conjugation of GSH to toxins, such as H2O2, after which glutathione peroxidases (GPx) reduce the GSH-H2O2 complex into two water molecules and glutathione disulfide (GS-GS) (McLellan and Wolff 1999; Hayeshi et al. 2007; Bhabak and Mugesh 2010). Despite reports of low expression of GST and GPx1 enzymes in MCF7 cells (Morrow et al. 1998; Hu and Diamond 2003), the observed abilities of EA to enhance and NAC to
inhibit jadomycin cytotoxicity supports that the GST/GPx system of H$_2$O$_2$ reduction is active in vitro in the detoxification of some jadomycins. Differences in the mechanisms of cytotoxicity between jadomycins are also further evidenced here as EA did not consistently alter the potency for each jadomycin tested. The third tested inhibitor, 3-AT, which inhibits H$_2$O$_2$ reducing catalase (Kimani et al. 2012) showed no activity in MCF7-CON cells when cotreated with any of the four tested jadomycins. This is inconsistent with Monro et al. (2011) who found the addition of catalase inhibited bacterial plasmid DNA cleavage induced by jadomycin B. We therefore hypothesize that the Prx/Trx and GST/GPx antioxidant systems are more heavily involved in the reduction of jadomycin-induced H$_2$O$_2$ within MCF7 cells than catalase. However, the results of Monro et al. suggest that if the Prx/Trx and GST/GPx systems were inhibited, catalase could become involved.

A limitation of our study is that we only assessed the mechanism of jadomycin cytotoxicity in the MCF7 breast cancer cell line. We feel that this approach was justified given that none of the jadomycin treatments yielded a significant difference in cytotoxicity between the MCF7 (+ estrogen receptor (ER) positive (×), progesterone receptor (PR) +, and human epidermal growth factor receptor (HER2) negative (−)], BT474 (ER−, PR+, HER2+), SKBR3 (ER−, PR−, HER2+), and MDA-MB-231 (ER+, PR−, HER2−) cell lines (Subik et al. 2010). Furthermore, since cell viability was reduced independently of ER, PR, or HER2 status, it is likely the mechanism(s) through which jadomycins reduce cell viability is unrelated to those targets.

In conclusion, jadomycins demonstrate potential as anticancer agents due to their ability to retain cytotoxicity in multidrug-resistant breast cancer cells that overexpress drug efflux ABC-transporters (Issa et al. 2014). On the basis of our results, we have proposed a working model (Fig. 9) describing how jadomycins induce breast cancer cell death in vitro by increasing cytosolic superoxide and H$_2$O$_2$ in a Cu(II)-dependent reaction, and that these ROS are reduced in the cytosol by SOD1 and the Prx/Trx and GST/GPx antioxidant pathways. Inhibition of these pathways presents viable cotreatment options that should be further tested for their ability to improve jadomycin potency and cancer cell selectivity in preclinical models.

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Author Contributions

Hall and Goralski participated in research design. Hall, Blundon, and Ladda conducted experiments. Robertson, Martinez-Farina, and Jakeman contributed new reagents or analytical tools. Hall, Blundon, Ladda, and Goralski performed data analysis. Hall, Blundon, Ladda, Robertson, Martinez-Farina, Jakeman, and Goralski wrote or contributed to the writing of the manuscript.

Disclosures

None declared.

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Supporting Information
Additional Supporting Information may be found in the online version of this article:

Figure S1. Jadomycins B, S, SPhG, and F do not autofluoresce at the wavelengths used to detect CM-DCF fluorescence and they do not react with CM-DCF fluorescence (excitation 485/20 nm and emission 528/20 nm) of jadomycins (30 μmol/L) and the H2O2 ROS control (2 mmol/L) alone (A) and with CM-DCF (7.5 μmol/L) (B) was measured in MCF7 cell media. Each bar represents the mean ± SEM of three independent experiments. *P ≤ 0.05, the fold change in fluorescence was significantly different when compared with the H2O2 control as determined by a one-way ANOVA, followed by Bonferroni’s multiple comparison test.
Figure S2. MitoTEMPO cotreatments do not alter jadomycin-induced ROS activity and cytotoxicity in MCF7-CON cells. After MCF7-CON cells were treated for 24 h with jadomycins B, S, SPhG, or F (7.5–18 μmol/L) or the jadomycin vehicle control (labeled no jadomycin) and co-treated with MitoTEMPO (0.1–10 nmol/L) or a H2O vehicle control (bar labeled 0 nmol/L), ROS activity was quantified by measuring the fluorescence of CM-DCF and cell viability was measured using LDH assays. Data are expressed as a fold change in ROS activity (A) and %-cell viability (B), respectively. Each bar represents the mean ± SEM of three independent experiments. The fold change in ROS activity and %-cell death were not significantly different when compared with the H2O vehicle control as determined by a one-way ANOVA, followed by Bonferroni’s multiple comparison test.