ARTICLE

Isolation of a jadomycin incorporating L-ornithine, analysis of antimicrobial activity and jadomycin reactive oxygen species (ROS) generation in MDA-MB-231 breast cancer cells

Stephanie M. Forget[1] · Andrew W. Robertson[1] · Steven R. Hall[2] · Jeanna M. MacLeod[2] · David P. Overy[3] ·
Russell G. Kerr[3] · Kerry B. Goralski[2] · David L. Jakeman[1,2]

Abstract
Herein, we report the characterization and antimicrobial activity of a previously unreported jadomycin (**1**) obtained from a culture of *S. venezuelae* ISP5230 with L-ornithine (Orn). **1** arises from the rearrangement of a putative five-membered ring containing jadomycin incorporating Orn, whereby intramolecular attack of the E-ring carbonyl from the δ-NH$_2$ group of the Orn side chain results in collapse of the oxazolone ring and formation of a stable six-membered lactam. This rearrangement produces a jadomycin with a 3a hemiaminal position that is susceptible to solvolysis. A structure–activity relationship is discussed based on the antimicrobial activity of **1** compared to previously reported jadomycins, providing evidence that the presence of a 3a hemiaminal enhances activity against Gram-positive bacteria. Additionally, assays to quantify reactive oxygen species (ROS) generation and cell viability were performed using a series of nine jadomycins. Compound **1** was found to produce the highest ROS activity and to possess the greatest cytotoxicity against MDA-MB-231 breast cancer cells.

Introduction

The jadomycins are pigmented angucyclic secondary metabolites isolated from the soil bacterium *Streptomyces venezuelae* ISP5230 (ATCC 10217) [1, 2]. Antimicrobial and cytotoxic properties in breast cancer cells have been attributed to jadomycin secondary metabolites [3–5]. Jadomycins evade transport by ATP-binding cassette drug efflux transporters allowing them to retain their potency in certain

multidrug-resistant cancer cell lines [3]. Jadomycins also appear to act polypharmacologically through a number of putative mechanisms, including DNA cleavage mediated by copper-dependant reactive oxygen species (ROS) activity [6, 7], inhibition of aurora-B kinase [8] and type-II topoisomerases [9], which may contribute to their cytotoxic efficacy in a variety of cancer cell lines. Jadomycin biosynthesis generates an aldehyde intermediate that is trapped non-enzymatically by nucleophiles, often the α-amine of an amino acid (Fig. 1). Exploitation of this step has provided a platform for precursor-directed derivatization leading to the formation of more than 70 jadomycin analogs to date [10]. Trapping of D-amino acids or L-amino acids usually results in the formation of a five-membered oxazolone ring (E-ring) [11–13]. Different modes of incorporation have been reported depending on the precursor which can extend beyond proteinogenic amino acids [13–15]. Jadomycin production with L-ornithine (Orn) was shown to produce a jadomycin bearing an eight-membered E-ring (Jd Oct). In the mechanism that gives rise to Jd Oct, condensation of the aldehyde-containing biosynthetic intermediate with the side chain δ-NH$_2$ group of Orn results in cyclization to form an eight-membered ring (Fig. 1) [16]. Analogous condensation with the α-NH$_2$ group would generate the five-membered oxazolone Orn derivative, Jd Orn. Herein we report a

These authors contributed equally: Stephanie M. Forget, Andrew W. Robertson.

Electronic supplementary material The online version of this article (https://doi.org/10.1038/s41429-018-0060-0) contains supplementary material, which is available to authorized users.

✉ David L. Jakeman
david.jakeman@dal.ca

1   Department of Chemistry, Dalhousie University, Halifax, NS B3H 4R2, Canada

2   College of Pharmacy, Dalhousie University, Halifax, NS B3H 4R2, Canada

3   Department of Chemistry, University of Prince Edward Island, Charlottetown, PE C1A 4P3, Canada

Fig. 1 Biosynthetic incorporation of Orn into jadomycins; Jd Oct [16], Jd Orn (not isolated) and **1**
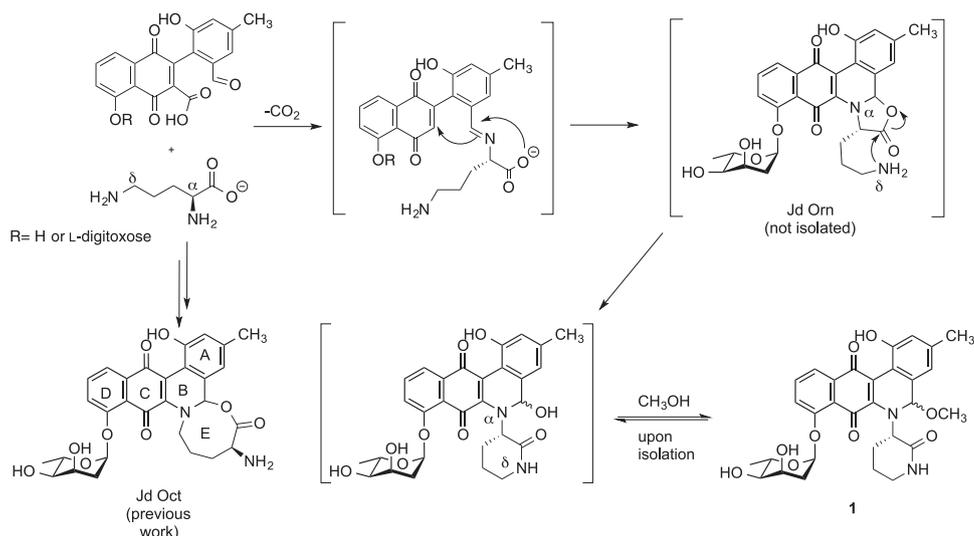
second isolate, compound **1**, produced from *S. venezuelae* culture in the presence of Orn. We propose that **1** is derived from spontaneous rearrangement of the five-membered ring product Jd Orn. We report biological activity against an antimicrobial and antifungal panel. An assay to quantify ROS generation using a series of nine jadomycins with variable amino acid and/or variable B and E ring assembly, including **1**, five additional jadomycins, and three previously tested jadomycins [7], was performed in order to gain insight into the potential role of the chemical structure in ROS generation as it related to jadomycin cytotoxicity. Results from the ROS assay were correlated to jadomycin cytotoxicity in MDA-MB-231 breast cancer cells.

Experimental procedures

General methods

All reagents were purchased from commercial sources and used without further purification unless otherwise stated. Solvents used for all chromatographic methods were high-performance liquid chromatography (HPLC) grade. Glass-backed thin-layer chromatography (TLC) plates (SiliCycle®) layered with 250-μm silica were used to assess purity of compounds. All reported R_f values were determined using 250-μm silica TLC plates. All compounds were characterized by liquid chromatography tandem-mass spectrometry (LC-MS/MS), high-resolution mass spectrometry (HRMS), and 1D-nuclear magnetic resonance (NMR) and 2D-NMR spectroscopy. Low-resolution LC-MS/MS spectra were obtained on an Applied Biosystems hybrid triple quadrupole linear ion trap (2000Qtrap) mass spectrometer using an electrospray ionization (ESI) source. This was coupled with an Agilent 1100 HPLC instrument with a Phenomenex

Kinetex 2.6-μm Hilic column (150 mm × 2.10 mm). Samples were prepared in methanol and 5 μL aliquots were injected onto the column. Elution of compounds was accomplished using an isocratic solvent system of (7:3) CH_3CN: 2 mM ammonium acetate in water (pH 5.5) with a flow rate of 120 μL min^{-1} for 10 min. The instrument was used in positive mode (ESI+). Enhanced product ionization (EPI) was performed with a capillary voltage of +4500 kV, declustering potential +80 V, and curtain gas 10 arbitrary units. EPI scans were conducted over a range of 300-900 m/z scanning for $[M + H]^+$ and the appropriate jadomycin fragmentation. Scans were conducted using two steps, 300.0 amu to 320.0 amu (0.005 s) and 300.0–900.0 amu (0.150 s). Spectra were analyzed using Analyst software version 1.4.1 (Applied Biosystems). HRMS traces of compound **1** was recorded on a Bruker Daltonics MicroTOF Focus Mass Spectrometer using an ESI + source. NMR spectra of **1** were recorded using a Bruker AV-III 700 MHz Spectrometer (^{1}H: 700 MHz, ^{13}C: 176 MHz) equipped with an ATMA 5 mm TCI cryoprobe located at the Canadian National Research Council Institute for Marine Biosciences (NRC-IMB) in Halifax, Nova Scotia. All spectra were recorded in CD_2Cl_2. Chemical shifts (δ) were given in ppm, and calibrated to residual solvent peaks (CD_2Cl_2: 5.32 ppm). Structural characterization and signal assignments were accomplished using ^{1}H-NMR chemical shifts and multiplicities, and ^{13}C-NMR chemical shifts. In addition, ^{1}H–^{1}H correlated spectroscopy, ^{1}H–^{13}C heteronuclear single-quantum coherence NMR, and ^{1}H–^{13}C heteronuclear multiple bond correlation (HMBC) NMR experiments were used to support assignments where appropriate. HPLC analysis of **1** was performed on a Hewlett Packard Series 1050 instrument with an Agilent Zorbax 5 μm Rx-C18 column (4.6 × 150 mm). Elution of the compounds was monitored at an absorbance of 254 nm using an isocratic

Table 1 NMR chemical shifts and correlations for compound 1

pos.	δ_C, type		δ_H (J in Hz)	HMBC	Pos.	δ_C, type[b]		δ_H (J in Hz)
Jadomycin Orn-Lactam 3a major stereoisomer					Jadomycin Orn-Lactam 3a minor stereoisomer			
C1	64.2, CH		4.51, bs	None	C1	63.8, CH		4.51, bs
C2	169.6, C				C2	C		
C3a	91.1, CH		5.55, s	1, 3b, 4, 7a, 13a, 3a-OCH$_3$	C3a	89.9, CH		5.61, s
OCH$_3$	53.2, CH$_3$		3.16, s	3a	OCH$_3$	CH$_3$		3.07, s
C3b	128.7, C				C3b	C		
C4	119.9, CH		6.65, s	3a, 3b, 5-CH$_3$, 6, 7, 7a	C4	119.3, CH		6.66, s
C5	140.7, C				C5	C		
C5-CH$_3$	21.2, CH$_3$		2.35, bs	4, 5, 6	C5-CH$_3$	CH$_3$		2.36, bs
C6	120.6, CH		6.86, bs	4, 5-CH$_3$, 7, 7a	C6	CH		6.88, bs
C7	154.4, C				C7	C		
7-OH			11.08, s		7-OH			10.62, s
C7a	114.6, C				C7a	C		
C7b	[a], C				C7b	CH		
C8	182.5, C				C8	183.6, C		
C8a	135.7, C				C8a	C		
C9	120.9, CH		7.81, d (8.0)	8, 10, 11, 12	C9	120.1, CH		7.85, d (8.0)
C10	135.7, CH		7.62, t (8.0)	8a, 12	C10	135.7, CH		7.65, t (8.0)
C11	119.5, CH		7.42, d (8.1)	9, 10, 12, 12a, 13	C11	119.6, CH		7.42, d (8.1)
C12	154.4, C				C12	C		
C12a	119.8, C				C12a	C		
C13	185.3, C				C13	182.3, C		
C13a	156.1, C				C13a	C		
C1′	29.3, CH$_2$	a:	2.55, m		C1′	CH$_2$	a:	2.55, m
		b:	2.26, m				b:	2.26, m
C2′	22.6, CH$_2$	a:	2.03, m		C2′	CH$_2$	a:	2.13, m
		b:	1.85, m				b:	1.92, m
C3′	42.7, CH$_2$	a:	3.84, m	2	C3′	CH$_2$	a:	3.73, m
		b:	3.24, m	2			b:	3.29, m
3′-NH			5.94, m	1, 2′	3′-NH			5.94, m
C1″	95.3, CH		5.85, d (3.0)	3″	C1″	95.3, CH		5.88, d (3.0)
C2″	35.6, CH$_2$	a:	2.39, dd (15.2, 2.3)	1″, 3″, 4″	C2″	CH$_2$	a:	2.39, dd (15.2, 2.3)
		b:	2.16, dt (15.2, 3.5)	1″, 3″, 4″			b:	2.16, dt (15.2, 3.5)
C3″	66.8, CH		4.00, m	1″	C3″	CH		4.00, m
3″-OH			4.64, d (10.8)	2″, 4″	3″-OH			4.64, d (10.8)
C4″	72.8, CH		3.19, m	5″-CH$_3$	C4″	CH		3.19, m
4″-OH			2.68, d (10.8)	2″, 4″	4″-OH			2.68, d (10.8)
C5″	66.2, CH		3.72, m	4″	C5″	CH		3.72, m
C5″-CH$_3$	16.8, CH$_3$		1.20, d (6.2)	4″, 5″	C5″-CH$_3$	16.8, CH$_3$		1.20, d (6.2)

[a]Signal not observed

[b]^{13}C chemical shifts for the minor compound were determined from 2D spectra; missing signals could not be assigned with confidence either due to low signal or signal overlap with the major species

Table 2 Antimicrobial screening results for compound 1

	MRSA	*S. warneri*			VRE	
	90% growth inhibition (µg/ml)	IC$_{50}$ (µg/ml)	90% growth inhibition (µg/ml)	IC$_{50}$ (µg/ml)	90% growth inhibition (µg/ml)	IC$_{50}$ (µg/ml)
1	3.13	1.70 ± 0.14	3.13	2.51 ± 0.52	100	10.3 ± 1.3
Vancomycin	0.78	0.51 ± 0.08	0.39	0.24 ± 0.05		
Rifampicin					3.13	1.40 ± 0.02
	P. aeruginosa		*P. vulgaris*		*C. albicans*	
	90% growth inhibition $_0$ (µg/ml)	IC$_{50}$ (µg/ml)	90% growth inhibition (µg/ml)	IC$_{50}$ (µg/ml)	90% growth inhibition (µg/ml)	IC$_{50}$ (µg/ml)
1	>100	>100	>100	>100	>100	>100
Gentamicin	1.56	1.04 ± 0.31				
Ciprofloxacin			0.004	0.0037 ± 0.0001		
Nystatin					1.56	0.87 ± 0.03

solvent system of 9:1 (A:B) over 0.5 min followed by an increasing linear gradiet from 9:1 (A:B) to 4:6 (A:B) over 7.5 min, followed by an isocratic solvent system of 4:6 (A:B) for an additional 2 min. This was then followed by a decreasing linear gradient from 4:6 (A:B) to 9:1 (A:B) over 1 min, ending with an isocratic solvent composition of 9:1 (A:B) over 4 min (total time 15 min; flow rate of 1 ml/min). Buffer A was an aqueous buffer comprised of 12 mM Bu$_4$NBr, 10 mM KH$_2$PO$_4$, and 5% HPLC grade CH$_3$CN (pH 4.0) and B was HPLC grade CH$_3$CN. Preparatory scale HPLC was performed using a C-18 reversed phased column (Ultrasphere ODS, 5 µm particle size, 10 mm × 25 cm) with degassed methanol (A) and water (B) using the following method: a linear gradient from 5:95 A:B to 95:5: A:B over 30 min, followed by a hold at 95:5 A:B for 30 min at 5 mL min^{-1}.

Media and growth conditions

All media were prepared with deionized and distilled water unless otherwise stated. MYM broth [maltose 4 g/L, yeast extract 4 g/L, malt extract 10 g/L]; MYM agar [maltose (4 g/L), yeast extract (4 g/L), malt extract (10 g/L), agar 15 (g/L), pH 7.0]; MSM media [MgSO$_4$ (0.4 g/L), MOPS (3.77 g/L), salt solution (9 mL 1% w/v NaCl, 1% w/v CaCl$_2$), FeSO$_4$·7H$_2$O (4.5 mL 0.2% w/v), trace mineral solution (4.5 mL), pH 7.5]. Trace mineral solution [ZnSO$_4$·7H$_2$O (880 mg/L), CuSO$_4$·5H$_2$O (39 mg/L), MnSO$_4$·4H$_2$O (6.1 mg/L), H$_3$BO$_3$ (5.7 mg/L), (NH$_4$)$_6$Mo$_7$O$_{24}$·4H$_2$O (3.7 mg/L). MYM or MSM broth (250 mL) were prepared in 1 L glass Erlenmeyer flasks. MYM agar (125 mL) was prepared in 250-mL glass Erlenmeyer flasks. Agar solutions were supplemented with 50 µg mL^{-1} apramycin sulfate before being poured into standard petri dishes while molten. All media was adjusted to pH 7 or 7.5 with 5 M NaOH or 5 M HCl as required. All solutions were autoclaved at 120 °C for 20 min prior to use.

Jadomycin Orn-lactam (1)

Streptomyces venezuelae ISP5230 VS1099 fermentations containing L-ornithine as the sole nitrogen source were carried out as previously described on a 5-L scale [16]. Bacterial cells were removed by suction filtration through Whatman No. 5 filter paper, followed by 0.45 µm then 0.22 µm Millipore Durapore® membrane filters. The clear media (5 L) was passed through a reversed-phase SiliCycle® phenyl column (70 g) and washed with distilled water (8 L) to remove water soluble material. The remaining material was eluted with 100% methanol (750 mL) and dried in vacuo to yield a crude extract (468.4 mg) containing **1**. The extract was dissolved in H$_2$O (500 mL) and the remaining H$_2$O insoluble material was dissolved in ethyl acetate EtOAc (500 mL). The aqueous layer and the EtOAc layer were combined, mixed vigorously and allowed to separate from one another. The EtOAc layer was removed and set aside. The aqueous layer was further extracted with EtOAc (3 × 500 mL). The organic fractions were combined and dried in vacuo yielding 97.9 mg of EtOAc extract containing impure **1**. The EtOAc extract (97.9 mg) was further purified in small batches using the preparatory HPLC method outlined in the general methods. Colored fractions were combined and concentrated to yield 4.4 mg of the red product as a mixture of diastereomers (ratio of major/minor stereoisomer = 100/45) as determined by NMR integrations. TLC R_f: 0.54 (9:1 CH$_2$Cl$_2$: CH$_3$OH), HPLC R_t = 8.1 min, NMR data see Table 1, HRMS (ESI$^+$): 587.1984 found, 587.2000 calculated for C$_{30}$H$_{32}$N$_2$NaO$_9$.

Table 3 Cytotoxicity screening results for compound 1

	Fibroblast		Kidney	
	90% growth inhibition (µg/ml)	IC$_{50}$ (µg/ml)	90% growth inhibition (µg/ml)	IC$_{50}$ (µg/ml)
1	32	27.3 ± 0.5	64	42.2 ± 0.9
Zinc pyrithione	16	7.8 ± 0.4		
Phenoxyethanol			0.63[a]	0.32 ± 0.01[a]

[a]units are a% based on total well volume

Antimicrobial and cytotoxicity assays

Antimicrobial and cytotoxicity assays were carried out as described previously [14]. The concentration where half the growth inhibition was observed (IC$_{50}$), as well as the minimal concentrations where complete inhibition (≥90% inhibition) of growth occurred, were determined using optical density measurements after 22 h incubation at 37 °C. Control experiments against each strain were carried out with known antimicrobial agents: vancomycin for methicillin-resistant *Staphylococcus aureus*(MRSA) and *S. warneri*; rifampicin for vancomycin-resistant *Entrococcus faecium*(VRE); gentamicin for *P. aeruginosa*; ciproflaxin for *P. vulgaris*; and nystatin for *C. albicans*. Cytotoxicity assay were performed using human foreskin BJ fibroblast cells (ATCC CRL-2522) and *Cercopithecus aethiops* kidney epithelial cells (Vero, ATCC CCL-81). Positive cytotoxin controls used were zinc pyrithione for fibroblast cells and phenoxyethanol for kidney cells. Results are summarized in Tables 2 and 3.

ROS detection assays

Jadomycins used in these assays were isolated from *S. venezuelae* cultures as described elsewhere [14, 17, 18]. MDA-MB-231 triple-negative breast cancer cells were treated with jadomycins (40 µM) for 24 h, after which the ROS activity was measured. ROS assays followed previously described procedures [7, 9].

Percent cell viability determination

A 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to measure cell viability after 24 h treatments with 40 µM of each jadomycin, to quantify cell viability at the same time point that ROS activity was measured. The protocol used is the same as previously published [3]. This concentration allowed for a measurable cytotoxic effect after 24 h, but was insufficiently toxic to kill all of the cells during that time frame. The vehicle control was 1:7 methanol: water.

Statistical analysis

All ROS and cell viability data are presented as the mean value of at least four separate replicate trials with each trial's values displayed in scatter plots. A one-way analysis of variance (ANOVA) was performed for multiple comparisons in experiments with one independent variable. A Bonferroni's multiple comparison test was used for post hoc analysis of the significant ANOVA. A non-parametric Spearman correlation test was used to quantify the correlation between intracellular ROS activity and cell viability after jadomycin treatments. A difference between mean values between groups was considered significant if $P \leq 0.05$.

Results

Isolation and characterization of 1

Following well-established jadomycin production and work-up protocols, previously described for cultures with Orn, analysis by TLC of the extracted materials from a silica–phenyl column revealed a new compound [16, 19]. Characterization data for **1** confirmed that a previously uncharacterized molecule was obtained in comparison to jadomycins Jd Oct and Jd Orn reported by Robertson et al. and Fan et al., respectively [16, 20]. These crude materials were subject to ethyl acetate extraction from water, and the organic soluble material was further separated by preparatory HPLC to afford **1**. The material obtained was characterized by HRMS, identifying a molecular formula ($C_{30}H_{32}N_2O_9$) consistent with Jd Orn with an appended methoxy group. 1D NMR experiments allowed for the identification of key jadomycin features consistent with the proposed structure. Complete NMR assignments are found in Table 1. The ^{1}H NMR spectrum revealed two sets of signals, with a major and minor compound in a 1:2.5 ratio, as determined by integration. The doubling of resonances arises from the two equilibrating stereoisomers arising from the configuration at C-3a. The following discussion examines the chemical shifts of the major compound only,
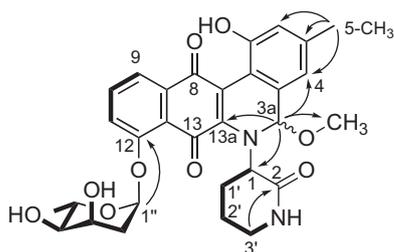
Fig. 2 Key NMR Correlations for compound **1** and numbering

although analogous observations can be stated for the minor compound. By proton NMR, characteristic features for jadomycins were observed, including the H-9, H-10, H-11 (d,t,d) aromatic spin system, and singlets corresponding to H-4 and H-6 protons in the anticipated region, between $\delta6.5$–7.0. Signals corresponding to the L-digitoxose spin system were present indicating glycosylation as expected. An HMBC correlation between H-3′ protons (δ 3.20 and 3.84) and C-2 (δ 169.6) demonstrated the appropriate correlation for a cyclized lactam (Fig. 2). By HMBC, H-3a (δ 5.55) was observed to correlate to C-1 and to a methyl group (δ 3.16), indicating methoxylation at the 3a position.

Antimicrobial and cytotoxicity screening

Compound **1** demonstrated antimicrobial activity against Gram-positive methicillin-resistant MRSA and *Staphylococcus warneri* but not Gram-positive VRE or Gram-negative *Proteus vulgaris, Pseudomonas aeruginosa* and a yeast strain (*Candida albicans*) (Table 2). In MRSA the IC$_{50}$ of compound **1** was similar in magnitude to the positive control vancomycin. In *S. warneri* the potency of compound **1** was about 10-fold lower than vancomycin. Cytotoxicity assays against healthy human fibroblast and monkey kidney cell lines were also performed (Table 3). Compound **1** was found to be cytotoxic to both, albeit with low potency. The IC50$_s$ of compound **1** were significantly higher than the control cytotoxin zinc pyrithione in fibroblasts and phenoxyethanol in kidney cells. Furthermore, the IC50$_s$ of compound **1** in fibroblast and kidney cells were between 10-25 fold higher than the bacterial strains MRSA and *S. warneri*, which were sensitive to compound 1.

ROS detection and cell viability assays

A series of nine jadomycins was tested for ROS generating activity in MDA-MB-231 breast cancer cells using previously established methodology. These included four jadomycins with a cyclic, hemiaminal-containing E-ring scaffold (group 1) including jadomycin L-isoleucine (Jd B), jadomycin L-serine (Jd S), jadomycin L-phenylalanine (Jd F), and jadomycin Nε-trifluoroacetyl-L-lysine (Jd TFAL);

two C-branched acetyl analogs (group 2) including jadomycins 3-AMBA and 4-AMBA acetoxy (Jd 3/4AMBA-Ac); two open chain methoxy derivatives (group 3) including **1** and jadomycin 4AMBA-methoxy (Jd 4AMBA-OMe) and one acyclic lactam-containing jadomycin (group 4), Jd TFAL-O. All of the jadomycins induced significant cell death in comparison to the vehicle control, with 4AMBA-Ac being the least effective in the assay, and **1** being statistically more efficacious than Jd 4AMBA-Ac, Jd 3AMBA-Ac, Jd TFAL-O, Jd-4AMBA-OMe, and Jd B (Fig. 3a). **1** was found to significantly increase ROS activity versus the vehicle control, as well as versus Jd 4AMBA-Ac, Jd 4AMBA-OMe, Jd B, and Jd TFAL (Fig. 3b). An analysis of the data with the jadomycins arranged into groups 1-4 (as described above) showed group two jadomycins, with C-linked acetate at the 3a position, to be less cytotoxic than groups 1 and 3 (Fig. 3c). No significant differences in ROS activity were observed between the groups (Fig. 3d). A dot plot analysis comparing %-cell viability versus fold-change in ROS activity demonstrated a correlation, measured using a non-parametric Spearman correlation test, between the two dimensions with a statistically significant *r*-value of −0.5405, evincing a negative correlation (Fig. 3e). The Spearman correlation test was repeated when the outlying Jd 4AMBA-Ac data was excluded and was found to maintain a significant *r*-value of -0.4562.

Discussion

The NMR characterization demonstrated features consistent with the proposed structure of **1** (Fig. 2). In previous studies, incorporation and cyclization of Orn into the jadomycin structure was probed. Incorporation (imine formation) and subsequent cyclization could be facilitated by either the α or δ-amino groups providing both the five-membered and eight-membered structural isomers, respectively. Interestingly, upon workup, only the eight-membered ring containing Jd Oct was observed by MS ion-guided fractionation [16]. The isolation of **1** provides indirect evidence for the formation of the five-membered E-ring containing, Jd Orn, that was not observed directly in our studies. This contrasts a previous study, where fragmentation experiments with ^{15}N-labeled L-lysine suggested that the five membered-ring analog was the predominant product in jadomycin cultures with L-lysine, which possesses an extra methylene unit in the side chain, and not the nine-membered ring containing structural isomer, although the instability of the molecule prevented its isolation and characterization [14]. In the case of **1**, following formation of the five-membered E-ring, attack of the δ-amino acid on the side chain to the carbonyl of the oxazolone ring would produce the six-membered lactam. Spontaneous
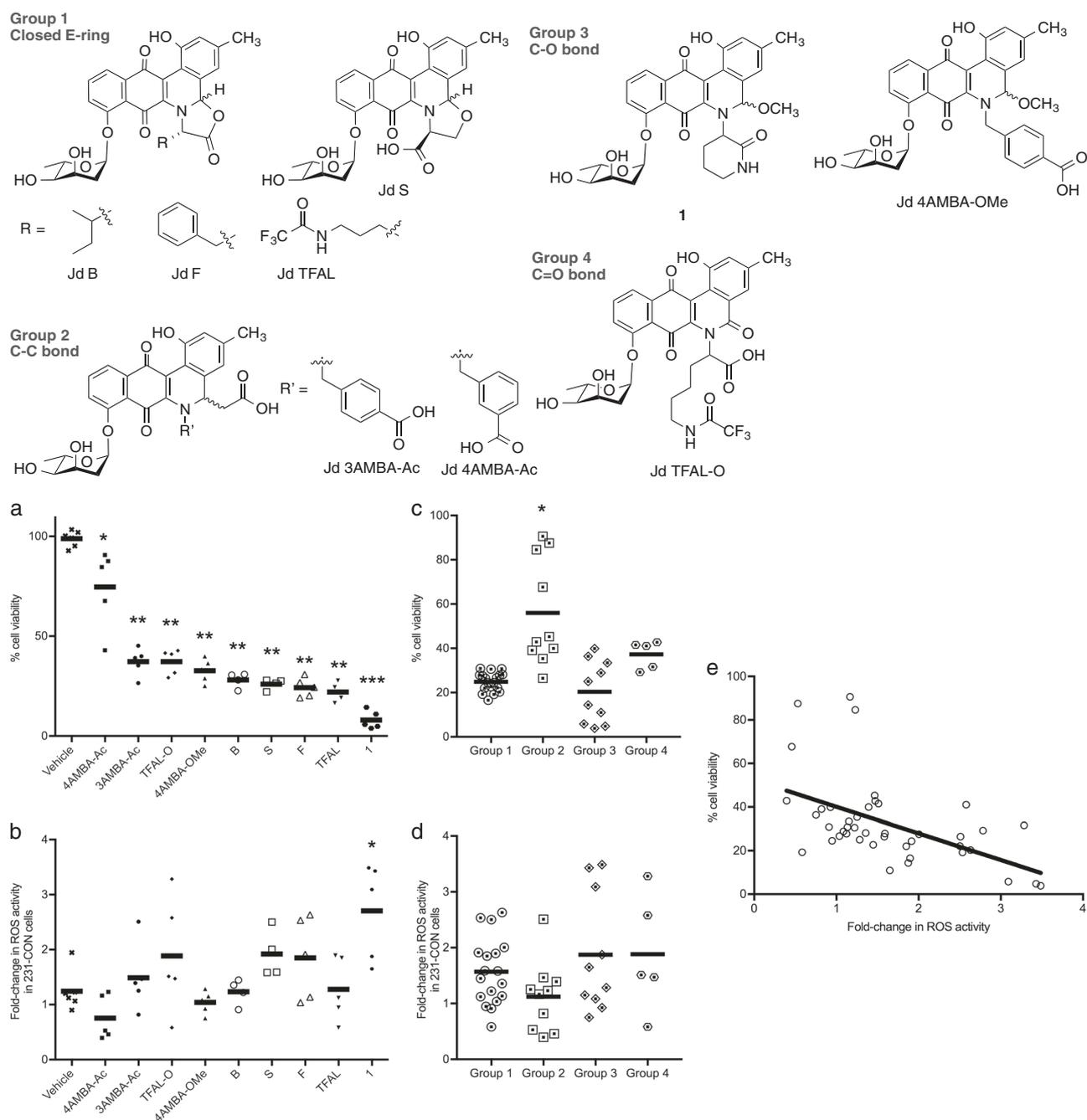
Fig. 3 The jadomycins evaluated in ROS and MTT cell viability assays in MDA-MB-231 breast cancer cells after 24 h treatments. **a** Percent-cell viability in order of least to most potent; $P \leq 0.05$, * signifies value is significantly different from vehicle, ** signifies value is significantly different from Jd 4AMBA-Ac, and *** signifies value is significantly different from Jd 4AMBA-Ac, Jd 3AMBA-Ac, Jd TFAL-O, Jd-4AMBA-OMe, and Jd B. **b** ROS Activity induced by jadomycins; $P \leq 0.05$, * signifies value is significantly different from vehicle, Jd 4AMBA-Ac, Jd 4AMBA-OMe, Jd B, and Jd TFAL; **c** Percent cell-viability analyzed by comparing jadomycin structural groups; $P \leq 0.05$, * signifies value is significantly different from Groups 1 and 2. **d** Fold-change in ROS activity by jadomycin structural groups. **e** Non-parametric Spearman correlation test was used to quantify the correlation between intracellular ROS activity (*x*) and cell viability after jadomycin treatments (*y*) of each trial of each jadomycin

lactamization of Orn is well documented within the peptide literature [21–23]. The resulting heminaminal position (3a) was susceptible to solvolysis, thus furnishing the 3a methoxylated derivative **1** as a result of the presence of methanol

in the isolation protocol. Solvolysis at the 3a position, evidenced by the detection of water, methanol, and ethanol adducts, has been described in jadomycins where E-ring formation is obfuscated [17].

Antimicrobial activity for **1** against Gram-positive bacteria was observed. This has been often observed with jadomycin family analogs [4, 14]. Compound **1** was found to be most active against MRSA and *S. warneri* with IC_{50} values of 1.7 and 2.5 µgmL^{-1}, respectively. These values are comparable to those reported for the jadomycins derived from *Nε*-trifluoroacetic acid (Jd TFAL, values 2.1 and 2.8 µgmL^{-1}, respectively) [10] and 4-aminobenzoic acid (Jd 4AMBA-OMe, values of 5.9 and 10.0 µgmL^{-1}, respectively) [17]. All three of these compounds possess a hemiaminal at the 3a-position, either incorporated into the oxazolone ring, as is the case with Jd TFAL, or in an acyclic form prone to solvolysis such as in the case of both **1** and Jd 4AMBA-OMe. By contrast jadomycins lacking this reactive centre (C-3a) are comparatively less active against Gram-positive strains [17]. In terms of cytotoxicity, **1** was found to inhibit the growth of human fibroblast and monkey kidney cell lines. This cytotoxicity was not observed with Jd TFAL, however, the Jd 4AMBA-OMe derivative also possessed increased cytotoxicity towards these two cell lines [14, 17]. While further examples are required to validate these trends, the above analysis suggests that modification of the 3a position by incorporation into an oxazolone ring reduces, by an intramolecular addition, overall cytotoxicity while maintaining Gram-positive antimicrobial activity, as was observed in assays with Jd TFAL [14].

In order to determine if differences in jadomycin structure affect their ROS generation and cytotoxicity, ROS generation versus cytotoxicity was measured using nine different analogs. The jadomycins evaluated each possessed a quinone functionality as a component of the core scaffold, with variations in structure arising from amino acid incorporation (B, S, F, TFAL, 3AMBA, 4AMBA or Orn) or to minor alterations in scaffold, such as jadomycins lacking cyclic amino acid incorporation (E-ring), and those with either solvolysis prone or C-branched acetate derivatization at the 3a positions. One question was whether ROS generation would be affected by the amino acid incorporation, by the arrangement of the B and E-rings, or whether the same ROS activity would be observed for all jadomycins, given that they all possess a common anthraquinone architecture. While some ROS activity may function as a component of the cytotoxicity induced by jadomycins, ROS cytotoxicity as a primary mode of toxicity is undesirable due to the increased likelihood of off-target effects; ROS activity has been implicated as one reason for anthracycline cardiotoxicity, with reactivity arising from the reducing properties of the quinone [24, 25]. In addition, while it has been shown jadomycins induce ROS activity in breast cancer cells [7] a more recent study confirmed that the major routes of jadomycin-induced cancer cell death occur independently of ROS-induction [9]. In the present study, compound **1** was found to produce greater ROS activity

compared to the other jadomycins, and this correlated with higher toxicity against MDA-MB-231 breast cancer cells. Alternatively, Jd TFAL was found to be the second most potent cytotoxic analog evaluated, despite being one of the lowest ROS-producing jadomycins, suggesting that selection of amino acids may enable the design of potent jadomycins with minimal ROS-induction activity. Despite these somewhat counterintuitive results, a dot-plot analysis using the data from all nine jadomycins demonstrated a minor but significant negative correlation between ROS and cell viability. To further examine the potential correlations between ROS activity, cytotoxicity, and jadomycin structure, the data were visualized according to groups 1–4. Jadomycins in group 1 possess cyclized E-rings, while those in groups 2, 3, and 4 feature acyclic amino acid incorporation. The 3a-C position is subject to solvolysis in group 3, whereas the equivalent position is unreactive in group 2 due to the addition of the C-linked acetate. Group 4 comprises a single compound possessing an oxidized 3a-C. Overall, while the ROS generating properties do not vary greatly between the different jadomycin groupings, some group differences are observed, although it is unclear whether the amino acid or the scaffold (as reflected in the groups) is more significant. Based on the four groupings, there appears to be some variance in jadomycin cytotoxicity. Group two was significantly less cytotoxic in comparison to groups 1 and 3, signifying that carbon branching at the 3a position the on these compounds decreases jadomycin cytotoxicity. Jadomycins bearing an unreactive 3a position (group 2), were the least cytotoxic but were not statistically different in ROS compared to other jadomycins, providing additional evidence that the reactivity at this position is important to cytotoxicity, and that ROS generation is not the primary determinant in cytotoxicity.

Conclusions

Jadomycin derivative **1**, from a previously studied culture of *S. venezuelae* with Orn, was isolated. This serves as the second report of jadomycins bearing an un-cyclized hemiaminal at the 3a position. Bioassays revealed that **1** possessed inhibitory activity against resistant Gram-positive strains, while no activity was observed against either Gram-negative strains or yeast. We propose that the reactive 3a position is important for antibacterial activity. Cytotoxic activity against fibroblast and kidney cells lines was also observed, a property that was not observed for jadomycins containing a cyclized E-ring, such as Jd TFAL [14]. The cytotoxic activity of **1** is likely partially explained by its ROS generating activity, which was found to be significantly higher in comparison to other jadomycins. All other jadomycins in the study were comparable in terms of

their ROS generating activity with ROS activity being weakly correlated to cytotoxicity. Based on these results we attribute differences in cytotoxicity to variations in chemical structure, particularly the functionality at the 3a position. This study has provided additional insight into structure–activity relationships of jadomycin congeners. These data will guide future derivatization efforts that will aim to improve jadomycin cytotoxicity without increasing ROS activity (Supplementary information).

References

1. Ayer SW, McInnes AG, Thibault P, Wang L, Doull JL, Parnell T, Vining LC. Jadomycin, a novel 8H-benz[b]oxazolo[3,2-f]phenanthridine antibiotic from *Streptomyces venezuelae* ISP5230. Tetrahedron Lett. 1991;32:6301–4.

2. Doull JL, Ayer SW, Singh AK, Thibault P. Production of a novel polyketide antibiotic, jadomycin B, by *Streptomyces venezuelae* following heat shock. J Antibiot. 1993;46:869–71.

3. Issa ME, Hall SR, Dupuis SN, Graham CL, Jakeman DL, Goralski KB. Jadomycins are cytotoxic to ABCB1-, ABCC1-, and ABCG2-overexpressing MCF7 breast cancer cells. Anti-Cancer Drugs. 2014;25:255–69.

4. Jakeman DL, Bandi S, Graham CL, Reid TR, Wentzell JR, Douglas SE. Antimicrobial activities of jadomycin B and structurally related analogues. Antimicrob Agents Chemother. 2009;53:1245–7.

5. Zheng JT, Rix U, Zhao L, Mattingly C, Adams V, Quan C, Rohr J, Yang KQ. Cytotoxic activities of new jadomycin derivatives. J Antibiot. 2005;58:405–8.

6. Monro SMA, Cottreau KM, Spencer C, Wentzell JR, Graham CL, Borissow CN, Jakeman DL, McFarland SA. Copper-mediated nuclease activity of jadomycin B. Bioorg Med Chem. 2011;19:3357–60.

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

8. Fu DH, Jiang W, Zheng JT, Zhao GY, Li Y, Yi H, Li ZR, Jiang JD, Yang KQ, Wang Y, Si SY. Jadomycin B, an Aurora-B kinase inhibitor discovered through virtual screening. Mol Cancer Ther. 2008;7:2386–93.

9. Hall SR, Toulany J, Bennett LG, Martinez-Farina CF, Robertson AW, Jakeman DL, Goralski KB. Jadomycins inhibit type II

10. topoisomerases and promote DNA damage and apoptosis in multidrug resistant triple negative breast cancer cells. J Pharmacol Exp Ther. 2017;363:196–10.

10. MacLeod JM, Forget SF & Jakeman DL The expansive library of jadomycins. Can J Chem (in press). https://doi.org/10.1139/cjc-2017-0573

11. Doull JL, Singh AK, Hoare M, Ayer SW. Conditions for the production of jadomycin B by *Streptomyces venezuelae* ISP5230: effects of heat shock, ethanol treatment and phage infection. J Ind Microbiol. 1994;13:120–5.

12. Rix U, Zheng J, Remsing Rix LL, Greenwell L, Yang K, Rohr J. The dynamic structure of jadomycin B and the amino acid incorporation step of its biosynthesis. J Am Chem Soc. 2004;126:4496–7.

13. Jakeman DL, Farrell S, Young W, Doucet RJ, Timmons SC. Novel jadomycins: incorporation of non-natural and natural amino acids. Bioorg Med Chem Lett. 2005;15:1447–9.

14. Forget SM, Robertson AW, Overy DP, Kerr RG, Jakeman DL. Furan and lactam jadomycin biosynthetic congeners isolated from *Streptomyces venezuelae* ISP5230 cultured with Nε-trifluoroacetyl-l-lysine. J Nat Prod. 2017;80:1860–6.

15. Martinez-Farina CF, Jakeman DL. Jadomycins, put a bigger ring in it: isolation of seven- to ten-membered ring analogues. Chem Commun. 2015;51:14617–9.

16. Robertson AW, Martinez-Farina C, Smithen DA, Yin H, Monro S, Thompson A, Mcfarland SA, Syvitski RT, Jakeman DL. Eight-membered ring-containing jadomycins: implications for non-enzymatic natural products biosynthesis. J Am Chem Soc. 2015;137:3271–5.

17. Robertson AW, MacLeod JM, MacIntyre LW, Forget SM, Hall SR, Bennett LG, Correa H, Kerr RG, Goralski KB, Jakeman DL. Post polyketide synthase carbon-carbon bond formation in type-II PKS derived natural products from Streptomyces venezuelae. J Org Chem. 2018;83:1876–90. https://doi.org/10.1021/acs.joc.7b02823.

18. Borissow CN, Graham CL, Syvitski RT, Reid TR, Blay J, Jakeman DL. Stereochemical integrity of oxazolone ring-containing jadomycins. Chembiochem. 2007;8:1198–203.

19. Jakeman DL, Graham CL, Young W, Vining LC. Culture conditions improving the production of jadomycin B. J Ind Microbiol Biotechnol. 2006;33:767–72.

20. Fan K, Zhang X, Liu H, Han H, Luo Y, Wang Q, Geng M, Yang K. Evaluation of the cytotoxic activity of new jadomycin derivatives reveals the potential to improve its selectivity against tumor cells. J Antibiot. 2012;65:449–52.

21. de Gracia Lux C, Olejniczak J, Fomina N, Viger ML, Almutairi A. Intramolecular cyclization assistance for fast degradation of ornithine-based poly(ester amide)s. J Polym Sci A Polym Chem. 2013;51:3783–90.

22. McGee WM, McLuckey SA. The ornithine effect in peptide cation dissociation. J Mass Spectrom. 2013;48:856–61.

23. Weber AL, Miller SL. Reasons for the occurrence of the twenty coded protein amino acids. J Mol Evol. 1981;17:273–84.

24. Minotti G, Menna P, Salvatorelli E, Cairo G, Gianni L. Anthracyclines: molecular advances and pharmacologic developments in antitumor activity and cardiotoxicity. Pharmacol Rev. 2004;56:185–229.

25. Kim S, Kim S, Kim B, Rah S, Chung SM, Im M, Kim U. Doxorubicin-induced reactive oxygen species generation and intracellular Ca2 increase are reciprocally modulated in rat cardiomyocytes. Exp Mol Med. 2006;38:535–45.