

ORIGINAL ARTICLE

Evaluation of the cytotoxic activity of new jadomycin derivatives reveals the potential to improve its selectivity against tumor cells

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The jadomyces are a unique family of angucycline-derived antibiotics with interesting cytotoxic activities. In this work, six new jadomycin derivatives were produced *in vivo* by providing non-natural amino acids in fermentation media. They were further purified and identified by MS and NMR analyses. The cytotoxicities of these derivatives were evaluated against tumor cell lines MCF-7 and HCT116, as well as the normal human microvascular epithelial cells. The derivatives with alkyl side chains showed similar levels of cytotoxicity as jadomycin B and other known derivatives with nonpolar side chains, with IC₅₀ ranging from 1.3 to 10 μM; but the activities are not selective as these compounds also showed similar levels of cytotoxicity toward the normal human microvascular epithelial cells in the same concentration range. For the first time, derivatives with amino side chains (jadomycin Orn and K) were prepared and evaluated. Significantly, jadomycin Orn showed differential activity against normal and tumor cell lines. This result points to a new direction to modify jadomycin structure. The insights on the structure–activity relationship of jadomyces will guide further efforts to generate new and improved jadomycin derivatives against tumor cells.

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INTRODUCTION

Polyketides represent one of the largest families of secondary metabolites found in nature, and often possess interesting biological activities.^{1–4} Here we deal with jadomycin derivatives, which form a series of angucycline-derived molecules with a unique pentacyclic benz[b]oxazolophenanthridine skeleton,^{5,6} produced by *Streptomyces venezuelae* ISP5230 under stress conditions, such as phage infection, heat and ethanol shock.⁷ Jadomycin provides a model system to study secondary metabolism in streptomycetes, with unique biosynthetic and regulatory features. For example, five genes with regulatory functions have been characterized in the jadomycin biosynthetic gene cluster, forming one of the most sophisticated cluster-situated regulatory systems.^{8,9} In the process of jadomycin biosynthesis, an angucyclinone intermediate (UWM6) undergoes oxidative cleavage at ring B, and the resulting B-ring-opened intermediate reacts with an amino acid to generate the final pentacyclic core. Three oxygenases, JadF, JadG and JadH, have been implicated in the ring cleavage and amino-acid insertion process.^{10–13} JadH was shown to be a

FAD-dependent bifunctional hydroxylase/dehydrase *in vitro*,^{10,14} catalyzing C-12 monooxygenation and 4a,12b-dehydration of 2,3-dehydro-UWM6, whereas the functions of JadF and JadG are still not clear.

A unique feature of jadomycin biosynthesis is the promiscuity of the amino-acid incorporation step, permitting various jadomycin derivatives to be formed simply by providing different amino acids in the culture medium. Even D-amino acids and non-natural amino acids can be incorporated.^{11,15–19} Thus, a considerable range of jadomyces can be evaluated for bioactivity, notably against tumor cells. Jadomyces show cytotoxicity to several tumor cell lines, including human hepatocellular carcinoma cell line HepG2, human lymphoblast immunoglobulin-secreting cell line IM-9, non-small-cell lung cancer cell line H460²⁰ and human breast ductal carcinoma cell lines T-47D and MDA-MB-435.²¹ The observed IC₅₀ ranged from 1 to 30 μM. The stereochemistry at C1 showed little effect on the cytotoxicity of jadomycin, but the size of the side chain was critical. Jadomyces with small polar side chains (such as jadomycin S, T and DT) showed higher activity than others, whereas those with bulky

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aromatic side chains showed least activity.²¹ Jadomycins also show antimicrobial activity against several pathogenic microorganisms, including the methicillin-resistant *Staphylococcus aureus*,^{15,22} and they were also reported to have DNA-cleaving capacities.^{15,23,24}

However, the cellular targets of jadomycins are still not firmly established. Our earlier study showed jadomycin B has Aurora kinase B inhibitory activity.²⁵ As Aurora kinase B is overexpressed in a wide range of tumor cells, it was considered an attractive target for anticancer therapy.^{25,26} Jadomycin B may compete with ATP for the kinase domain of Aurora kinase B, and inhibit its kinase activity. In this study, a series of jadomycin derivatives with small side chains were prepared and isolated, and their cytotoxicity was investigated.

MATERIALS AND METHODS

General Procedures

All precursor amino acids were purchased from GL Biochem Ltd. (Shanghai, China) and used without further purification. UV-Vis spectra of purified jadomycin derivatives were recorded using a Shimadzu BioSpec-mini UV-Vis spectrophotometer. NMR spectra were recorded on a Varian Unity VNS 600 NMR spectrometer (¹H at 600 MHz, ¹³C at 150 MHz, Varian, Walnut Creek, CA, USA). Spectra were collected in CDCl₃ or dimethyl sulfoxide (DMSO)-d₆. Peak assignment of ¹H spectra was achieved using chemical shift and peak multiplicities from ¹H-NMR and ¹H-¹H COSY spectra, whereas assignment of ¹³C spectra was achieved through heteronuclear single-quantum correlation spectroscopy and heteronuclear multiple-bond correlation spectroscopy experiments. ESI-MS and MS/MS analysis were recorded on a Finnigan LCQ DecaXP ion trap mass spectrometer (Thermo Finnigan, Waltham, MA, USA). HPLC analysis of crude extract and purified derivatives were performed on a Shimadzu Prominence system with a Diamonsil C18 column (4.6 × 250 mm, Dikma, Beijing, China). The elution solvents were water with 0.1% trifluoroacetic acid (solvent A) and acetonitrile with 0.1% trifluoroacetic acid (solvent B). A 20-min linear gradient from 35 to 100% solvent B was used. Absorbances at 313 and 266 nm were monitored for jadomycin derivatives.

Production, isolation and identification of jadomycin derivatives

The conditions used for jadomycin production were similar to those reported by Jakeman *et al.*,²⁷ different amino acids being added to the production medium as the sole nitrogen source. To obtain maximum jadomycin yield for different amino acids, fermentation conditions (period of fermentation from 24 to 48 h and ethanol treatment at 3–6% v/v) were optimized for each amino acid.

After fermentation, the cultures were filtered and the supernatants were extracted with ethyl acetate. The extracts were evaporated to dryness, re-dissolved in 4 ml ethanol and analyzed by HPLC. Peaks with significant absorbance at 313 nm were collected for further ESI-MS and MS/MS analysis.

The new jadomycin derivatives were purified by HPLC. Normally, 1 l culture was filtered and the supernatant was extracted with ethyl acetate, as described previously. The purification was performed on a Waters HPLC system (Waters, Milford, MA, USA) containing a 1525 pump and a 2487 dual λ UV detector with an Agilent Extend-C18 column (4.6 × 250 mm, Agilent, Santa Clara, CA, USA). The elution solvents were water with 25 mM NH₃ (solvent A) and acetonitrile (solvent B). A 15-min linear gradient from 15 to 40% solvent B was used. The collections containing jadomycins were lyophilized and used for 1D and/or 2D NMR analysis.

To obtain sufficient samples for cytotoxicity studies, several jadomycin derivatives (including jadomycin B, L, V, S, T, Abu, Nle and Hse) were purified on a large scale by a silica gel column using a gradient of chloroform/methanol as the elution solvent, with final purification on a Superdex LH20 gel filtration column using methanol as the elution solvent. All the purified products were checked via analytical HPLC and ESI-MS before further analysis for activity.

Sulforhodamine B assay for cytotoxicity

The purified jadomycin derivatives were dissolved in DMSO and dilution series were prepared. Exponentially growing human microvascular epithelial cells, human breast cancer cells MCF-7 and human colon cancer cells HCT116

(3 × 10⁷ cells per liter) were seeded in 96-well plates (180 μl per well) and incubated for 24 h before being treated with jadomycins (20 μl) at different concentrations. After 72 h incubation, cells were fixed with cold trichloroacetic acid (50 μl, 500 g l⁻¹) for 60 min. Fixed cells were then stained with sulforhodamine B (100 μl per well) for 30 min and washed four times with acetic acid (10 ml l⁻¹). Plates were read using a multiwell spectrophotometer (VERSAmax, Molecular Devices, Sunnyvale, CA, USA) at 520 nm after adding 200 μl per well Tris buffer (10 mM, pH 10.5). All experiments were repeated twice.

RESULTS AND DISCUSSION

The production and identification of jadomycin derivatives

By adding different amino acids to the jadomycin production medium, 11 jadomycin derivatives were produced, which had incorporated L-isoleucine (B), L-leucine (L), L-valine (V), L-2-amino-butanoic acid (Abu), L-norleucine (Nle), L-serine (S), L-threonine (T), L-homoserine (Hse), L-lysine (K), L-ornithine (Orn) or L-2,4-diaminobutanoic acid (Daba) (see Figure 1). Six of them, jadomycins Abu, Nle, Hse, K, Orn, and Daba, were isolated and characterized for the first time by ESI-MS/MS and NMR. All the compounds showed the typical violet color of jadomycins, and similar UV spectra with absorbance peaks at about 522, 382, 313, 289 (shoulder),

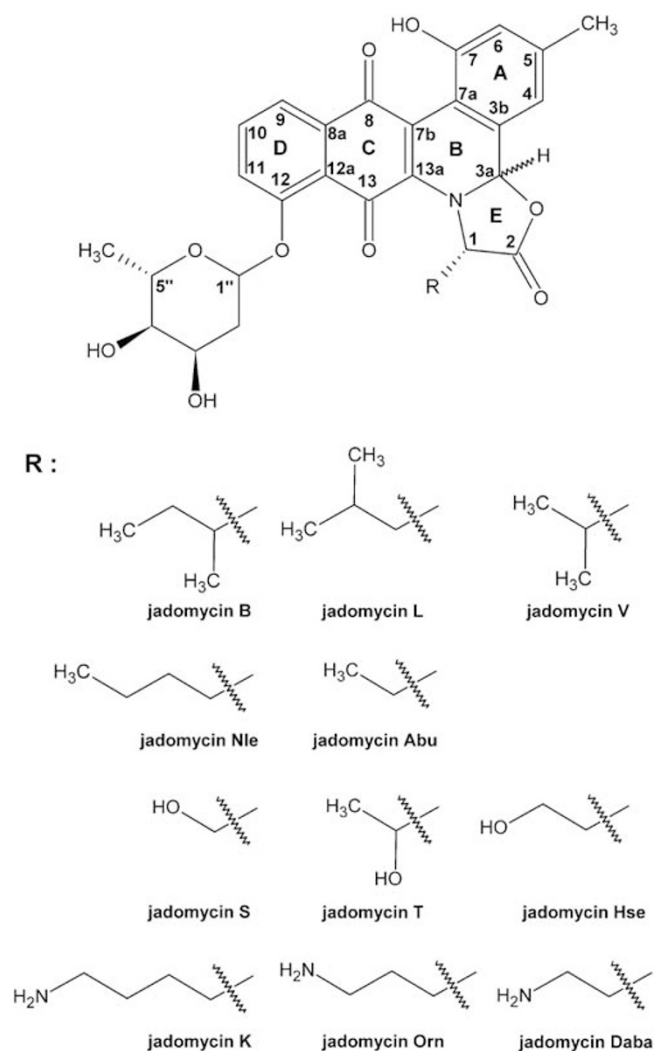


Figure 1 Structures of jadomycin derivatives prepared and tested in this study.

Table 1 Jadomycin derivatives produced by precursor-directed biosynthesis, with ESI-MS data and relative diastereomeric products

Natural product	Precursor	Yield (mg l ⁻¹)	ESI-MS [M + H] ⁺	MS/MS 3aR:3aS
Jadomycin Abu	L-2-aminobutanoic acid	5.5	522.2	392.2, 68.2:31.8 306.2
Jadomycin Nle	L-norleucine	7	550.1	420.2 41.2:58.8
Jadomycin Hse	L-homoserine	13.3	538	408.0, >95:<5 306.1
Jadomycin K	L-lysine	3	565.5	435.2, 306.1
Jadomycin Orn	L-ornithine	3	551.3	421.2 >95:<5
Jadomycin Daba	L-2,3-diaminobutanoic acid	1	537.4	406.9, 306.4

235 (shoulder) and 210 nm. The ESI-MS/MS data (Table 1) provide decisive evidence for the incorporation of the natural or non-natural amino acids and the formation of jadomycin derivatives, by the observation of the parent ion ([M + H]⁺) and the unambiguous breakdown of the parent ion to the non-glycosylated aglycone ([aglycone + H]⁺) and the phenanthroviridin ion (m/z 306.1).

The conditions for production of jadomycin derivatives were optimized by varying the fermentation strain (ISP5230 or ZY104²⁸), the concentration used for ethanol shock (3 or 6% v/v) and the fermentation times (24, 36 or 48 h). For most jadomycin derivatives, ISP5230 and lower ethanol concentration (3% v/v) gave better jadomycin yield (data not shown). Whereas the production of derivatives with alkyl side chains increased continuously in the 48-h fermentation, those containing amino side groups (such as jadomycin K and Orn) showed the highest yield at 36 h, and the yield decreased slightly thereafter (data not shown). This may be due to degradation of these basic jadomycin derivatives. In a recent study, Dupuis *et al.*¹⁵ attempted to produce jadomycin derivatives by providing L-norvaline or L-norleucine as precursors, but did not obtain the expected compounds. We also tried the experiment using L-norvaline but also failed.

The new jadomycin derivatives (including jadomycin Abu, Nle, Hse, K, Orn and Daba) identified by ESI-MS and MS/MS were purified first via HPLC on a small scale. As jadomycin B was observed to hydrolyze to jadomycin A and L-digitoxose in acid solution, we used an Agilent Extend-C18 column with broader pH tolerance, and an alkaline NH₃-containing solvent system. Finally, six jadomycin derivatives (see Table 1) were purified, and five of them were characterized via 1D and 2D NMR spectra (see Supplementary Table S1 to S6 and Supplementary Figure S1 to S16). Because of poor yield, the NMR spectra of the sixth, jadomycin Daba, were almost unreadable. In the case of jadomycin K, CDCl₃ was used as solvent for NMR experiments, but its solubility was poor and only ¹H-NMR and COSY spectra were obtained (see Supplementary Table S7 and Supplementary Figure S17 and S18). In a further experiment, we re-purified jadomycin K and used DMSO-d₆ as the NMR solvent, but it proved to be unstable in DMSO, so a 2D spectrum was still not obtained.

Overall, the production of the new jadomycin derivatives was corroborated by comparing the new NMR spectra with those of jadomycin B. Although diastereoisomers with 3aS and 3aR configuration were found for several jadomycin derivatives,^{11,21} a

Table 2 Cytotoxicity profile of jadomycin derivatives against different cell lines (IC₅₀ per μM)

Natural product	MCF-7	HCT116	HMEC
Jadomycin B	2.8	3.8	2.3
Jadomycin L	1.4	3.8	2.1
Jadomycin V	1.9	4.4	2
Jadomycin Abu	2.7	8.3	3
Jadomycin Nle	8.5	14.5	9.2
Jadomycin S	4.4	4.6	4.3
Jadomycin T	0.97	1.3	1.4
Jadomycin Hse	66.8	56.8	62.9
Jadomycin Orn	4.5	5.4	10.2

diastereoisomeric mixture was observed only for jadomycins Abu and Nle, both having alkyl side chains at position C1, whereas the other three derivatives with hydroxyl- or amino-containing side chains (jadomycin Hse, Orn and K) existed only in one diastereoisomeric form. For jadomycin Hse and Orn, no correlation peaks between H-3a and H-1 were observed in NOESY, suggesting that they were in 3aR configuration. The C-3a configuration of jadomycin K could not be determined because its NOESY spectrum was not readable. These results were consistent with previous reports about jadomycins derived from L-serine, L-histidine, L- or D-threonine, that showed mainly 3aR configuration for L-amino acid precursors and 3aS for D-amino acids. The stabilization of the 3aR configuration was caused by the formation of a hydrogen bond between the N or O atoms in the side chain with either the C-13 carbonyl group or the L-digitoxose functionality.^{11,21}

Cytotoxic activity of jadomycin derivatives

Two human cancer cell lines, MCF-7 (breast cancer cells) and HCT116 (colon cancer cells) and one normal cell line, human microvascular epithelial cells, were used to evaluate the cytotoxicity of jadomycin derivatives towards both cancer and normal cells. The cell lines were continuously exposed to a range of drug concentrations (0.01–100 μM), and cell survival monitored after 72 h using the sulforhodamine B assay. All the compounds showed inhibitory activity against MCF-7 and HCT116 with the IC₅₀ ranging from 1.4 to 66.8 μM (Table 2 and Figure 2). Jadomycins with alkyl side chains (jadomycin B, V, L, Abu and Nle) and small polar side chains (jadomycin S, T, Orn) showed higher activities. However, jadomycin Hse showed much lower activity (50–60 μM) relative to S and T (<10 μM), suggesting that not only the general physicochemical properties but also the local orientation of the side chain are important. These results were consistent with previous reports, showing that derivatives with small polar side chains, such as jadomycin T, DT and S, exhibited potent activities against T-47D and MDA-MB-435 cells,²¹ and jadomycin S against HepG2 and IM9 cells.²⁰

It is important to note that the tested jadomycins also showed cytotoxicity against the normal human microvascular epithelial cells in the same concentration range as against the cancer cell lines, thus indicating that they were not selective between tumor and the normal cells. Interestingly, jadomycin Orn showed about two-fold difference in IC₅₀ for human microvascular epithelial cells (10.2 μM) relative to two tumor cells (4.5 and 5.4 μM, respectively). This is the first time, jadomycin derivatives with amino side groups have been tested for bioactivity: among the two (K and Orn), only the latter was stable and showed selective activities against different cell lines. Compared with a

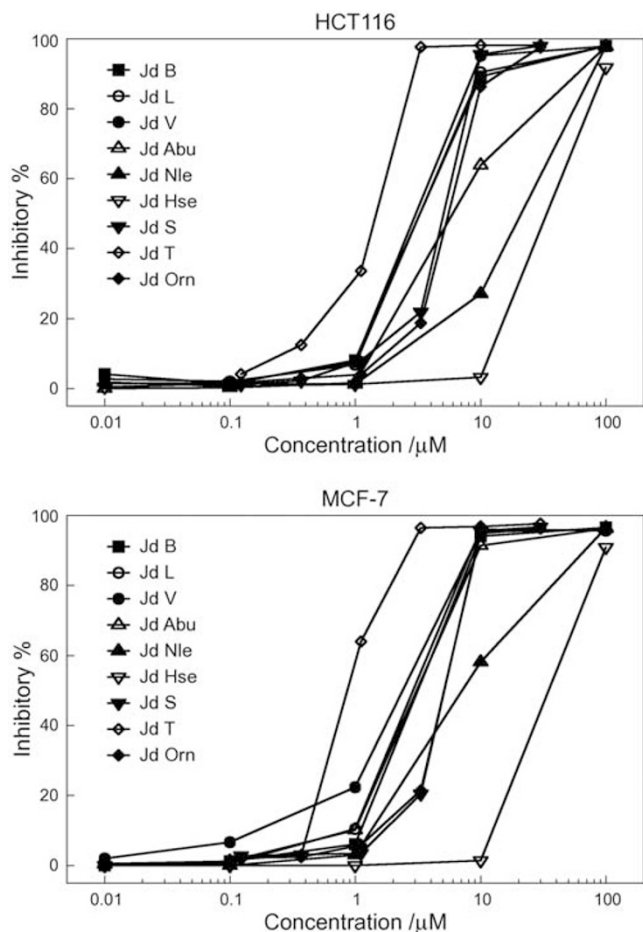


Figure 2 Cytotoxicity assessment of jadomycin derivatives against MCF-7 and HCT116 cells.

hydroxyl group, the amino group is a weaker hydrogen bond acceptor but a better donor, and might form an electrostatic interaction with a negative charge. Changing the hydroxyl functional group in the C1 side chain in jadomycin S and T to an amino group in jadomycin Orn seems to have altered the interaction of jadomycin Orn with its target in a way that promotes selectivity against different cell lines. Our results suggest that further efforts to modify jadomycin structure should focus on varying the amino-acid side chains, or sugar moiety.

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