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Copper-mediated nuclease activity of jadomycin B

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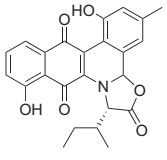
ABSTRACT

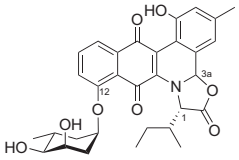
The natural product jadomycin B, isolated from *Streptomyces venezeulae* ISP5230, has been found to cleave DNA in the presence of Cu(II) ions without the requirement for an external reducing agent. The efficiency of DNA cleavage was probed using supercoiled plasmid DNA in buffered solution as a model environment. EC_{50} and $t_{1/2}$ values for cleavage were 1.7 μM and 0.75 h, respectively, and varied ±5% with the particular batch of plasmid and jadomycin employed. While UV–vis spectroscopy indicates that the cleavage event does not involve direct binding of jadomycin B to DNA, a stoichiometric Cu(II) preference for optimum cleavage suggests a weak binding interaction between jadomycin B and Cu(II) in the presence of DNA. The Cu(II)-mediated cleavage is greatly enhanced by UV light, which implicates the jadomycin B radical cation and Cu(I) as potential intermediates in DNA cleavage. Evidence in favor of this hypothesis was derived from a mechanistic assay which showed reduced cleavage as a function of added catalase and EDTA, scavengers of H_2O_2 and Cu(II), respectively. Thus, jadomycin B may serve as a source of electrons for Cu(II) reduction, producing Cu(I) which reacts with H_2O_2 to form hydroxyl radicals that cause DNA strand scission. In addition, scavengers of hydroxyl radicals and superoxide also display inhibitory effects, underscoring the ability of jadomycin B to produce a powerful arsenal of deleterious oxygen species when copper is present.

1. Introduction

The jadomycins are a family of secondary metabolites that are produced during fermentation by *Streptomyces venezeulae* ISP5230 under stress conditions such as heat shock, ethanol treatment, or phage infection.[1–3] These natural products contain an unusual pentacyclic 8*H*-benz[*b*]oxazolo[3,2-*f*]phenanthridine ring system, where the nitrogen heteroatom of the oxazolone ring is derived from the amino acid constituent of the jadomycin production medium. Growth with isoleucine as the sole nitrogen source produces jadomycins A and B, which differ by the incorporation of the relatively rare, dideoxysugar L-digitoxose at C_{12} of jadomycin B.[2]



Jadomycin A



Jadomycin B (**1**)

As a group the jadomycins are active against both Gram-positive and Gram-negative bacteria as well as certain drug-resistant cancer cells lines.[3,4] Additionally, anti-yeast activity has been observed for jadomycin B, but not jadomycin A, indicating that glycosylation plays an important role in increasing the bioactivity and (or) bioavailability of this family of antibiotics.[3] Differences in bioactivity have also been observed among a number of novel jadomycin analogues,[5,6] underscoring the importance of the identity of the substituent at C_1, the ensuing stereochemistry at C_{3a}, and both the presence and identity of the C_{12} sugar. To begin a systematic investigation of the nature and scope of biological activity exhibited by the jadomycins, we have focused our efforts to understand better the biomolecular interactions of jadomycin B and its cytotoxicity. Herein we document the DNA-damaging properties of jadomycin B in the presence of copper ions.

Jadomycin B has been screened against two human breast ductal carcinoma cell lines, T-47D and MDA-MB-435. EC_{50} values (μM) are 9.92 ± 0.96 and 6.89 ± 1.51, respectively, and are intermediate between the most potent (1.15 μM, jadomycin DT) and least potent (31.07 μM, jadomycin Y) jadomycins screened.[6] While the operative mechanism for cytotoxic activity in these cell lines has yet to be established, there is speculation that jadomycin B and its related analogues may act as selective protein kinase B (PKB)

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inhibitors, owing to their structural resemblance to lactoquinomycin A, where the mechanism of action involves opening of a lactone ring to form a quinone methide that reacts with a thiol functionality in the regulatory region of PKB.[7] Structural similarities between the jadomycins and doxorubicin and the fluoroquinolones further implicate thiol-reactive intermediates that may inhibit DNA gyrase and the topoisomerases, another possible avenue for the cytotoxic behavior characteristic of the jadomycins.[8]

In addition to inhibition of enzymes that are key to cell signaling pathways and DNA topological control, the jadomycins could exert their cytotoxic effects by directly (or indirectly) damaging the DNA backbone in a manner consistent with natural products such as prodigiosin. The cytotoxic behavior of prodigiosin, and related natural products such as tambjamine E, is thought to arise, in part, from the oxidation of the pyrrolylpyrromethene skeleton by endogenous Cu(II). The resulting Cu(I) is capable of reacting with H_2O_2 to form a deleterious Cu-oxo species that induces DNA strand scission.[9,10] Herein we disclose our initial findings regarding the interactions between jadomycin B and DNA and compare these observations to those that have been related for prodigiosenes.

2. Experimental section

2.1. Materials and methods

Jadomycin B was isolated from cultures of *S. venezeulae* ISP5230 according to improved methodology.[11,6] Purification of jadomycin B was accomplished using a Biotage SP1 HPFC purification system using both Biotage KP-C18-HS reversed phase and Biotage KP-SIL normal phase columns. ^{1}H and ^{13}C NMR spectra were recorded on Bruker AVANCE 500 or Bruker DRX 500 spectrometers in MeOD. Low resolution electrospray mass spectra were recorded by Dalhousie University Mass Spectrometry Service using a VG/Micromass Quattro triple quadrupole spectrometer. High resolution accurate mass spectra were recorded at the University of British Columbia Mass Spectrometry Service on a Water/Micromass LCT spectrometer using pentalysine as a reference compound.

Supercoiled plasmid (Form I) was a derivative of pET28a containing the *desR* gene[12] and was used in the present study due to its ready availability in-house and increased GC content (guanine is known to be particularly susceptible to oxidative damage). Larger quantities of the plasmid were prepared by transformation of NovaBlue cells (Novagen) followed by purification using the QIAprep Spin Miniprep Kit (Qiagen) to yield approximately 62 μg of plasmid DNA per 20-mL culture. The following reagents (Aldrich) and enzymes (Sigma) were obtained commercially and used without further purification: sodium azide (NaN_3), copper acetate ($Cu(OAc)_2$), 4-morpholinepropanesulfonic acid (MOPS), ethylenediaminetetraacetic acid (EDTA) and catalase and superoxide dismutase (SOD).

UV–vis measurements were recorded using Cary 50 (Varian) and Ultraspec 4000 (Pharmacia Biotech) spectrophotometers. Distilled, deionized water from a Milli-Q system was used for all aqueous solutions and manipulations and Optima-grade acetonitrile was used for all organic solutions.

2.2. DNA binding

UV–vis titration experiments were performed at 25 °C with sonicated calf thymus DNA (Sigma). The DNA concentration was determined by UV absorption at 260 nm (ϵ = 12,824 M^{-1} cm^{-1}, where concentration units refer to base pairs).[9] Samples of jadomycin (10 μM) were prepared in 50 mM MOPS buffer (pH 7.4) containing 100 mM NaCl and <1% MeCN. UV–vis spectra were collected for jadomycin alone and after each addition of calf thymus DNA (150-μM base-pair aliquots).

2.3. Copper binding

The Cu(II) binding affinity for jadomycin B was determined by UV–vis spectroscopy at 25 °C in 10 mM MOPS buffer (pH 7.4). Spectra of jadomycin B (25 μM) were acquired before and after each addition (2 μL) of $Cu(OAc)_2$ (stock solution = 15 mM). The titration was then repeated with jadomycin B pre-exposed to CT DNA (100 μM, base pairs).

2.4. DNA cleavage by jadomycin B in the presence of Cu(II)

Reaction mixtures (20 μL total volume) were prepared on ice and contained 400 ng of pDesR3 supercoiled DNA (<5% Form II). Generally, DNA (2–5 μL) was delivered to the assay tubes as a solution in 10 mM Tris–Cl (pH 8.5) and diluted with MOPS (pH 7.5, final concentration 10 mM) and NaCl (final concentration 100 mM). Solutions of jadomycin B and $Cu(OAc)_2$ were added to give the desired concentration, and the reaction mixtures were diluted to a final volume of 20 μL, when necessary, with distilled, deionized H_2O. Jadomycin and Cu(II) were added one of three ways: (i) jadomycin first followed by Cu(II), (ii) Cu(II) first followed by jadomycin, or (iii) pre-mixture of 1:1 Cu(II):jadomycin. All stock solutions and serial dilutions were carried out with distilled, deionized H_2O, with the exception of the stock solution of jadomycin B, which was prepared in MeCN (<1% MeCN in reaction mixture). Reaction mixtures were incubated at 37 °C for 2–12 h and then quenched by the addition of gel loading buffer (4 μL). Samples were loaded onto a 1% agarose gel containing ethidium bromide (0.75 μg mL^{-1}) and electrophoresed for 30 min at 10–12 V cm^{-1}. The bands were visualized with UV illumination and quantified using the Gel Doc-It Imaging system (UVP). Curve fitting to extract EC_{50} values was accomplished using the GraFit 5 data analysis software package.

2.5. Laser-enhanced DNA cleavage by jadomycin B with Cu(II)

Reaction mixtures were prepared as above and kept on ice when not irradiated. A Lambda-Physik pulsed-excimer laser system (Xe/HCl/He) with a pulse width of <10 ns at 308 nm was used (jadomycin B absorbs light strongly at this wavelength) to excite the samples individually with a 1-kHz repetition rate. The power at each sample was approximately 75 mJ, and samples were irradiated for no more than 5 min. Analysis of the reaction mixtures was carried out as noted previously.

2.6. Mechanistic aspects of DNA cleavage by jadomycin B with Cu(II)

In separate experiments, the abilities of various scavengers to suppress DNA cleavage by Cu(II)/jadomycin B were examined: EDTA (100 mM), catalase (1000 U mL^{-1}), SOD (1000 U mL^{-1}), NaN_3 (100 mM), *t*-BuOH (1 M), and DMSO (1 M). These assays were carried out as previously described for the DNA cleavage experiments except that the appropriate scavengers (5-μL delivery volumes) were added to the DNA solutions prior to the addition of Cu(II) and jadomycin. Mixtures were incubated at 37 °C for 5 h, quenched by the addition of gel loading buffer (4 μL), and analyzed by agarose gel electrophoresis (1%, 0.75 μg mL^{-1} ethidium bromide, 100 V, 30 min).

3. Results and discussion

At 10 μM, **1** produced no detectable relaxation of supercoiled (Form I) plasmid DNA, indicating that direct damage to the DNA backbone is probably not responsible for the cytotoxicity observed in vivo. However, in the presence of equimolar $Cu(OAc)_2$, **1** yielded

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significant single-strand breaks, resulting in nicked DNA (Form II) as indicated in Figure 1. The calculated EC_{50} value for single-strand scission was approximately 1.7 μM and varied ±5% across different batches of plasmid and jadomycin with $t_{1/2}$ = 0.75 h for **1**:Cu(II) (10 μM, 23 ng μL^{-1} DNA) as shown in Figure 2. At longer times, traces of linear DNA (Form III) could be discerned but did not contribute significantly to total DNA damage.

Copper-promoted DNA cleavage by jadomycin B is reminiscent of the type of DNA damage produced by prodigiosin-based natural products[9,10] and by endogenous metabolites such as bilirubin.[13] In these examples, the damage to DNA results primarily from reactive oxygen species that are generated by copper(I). In the case of prodigiosin and related derivatives, chelation of copper(II) by the 4-methoxy-2,2′-bipyrrole ring system is thought to result in a redox reaction to yield the bipyrrole-based π-radical cation and copper(I), which can react according to the pathways outlined in Eqs. 1a–1d.[9,10] Evidence for oxygen dependence in copper-mediated DNA cleavage, and in particular the involvement of H_2O_2 (Eq. 1c), is supported by the inhibitory effect of catalase, an enzyme that disproportionates H_2O_2 to afford $H_2O + O_2$.[14]

$$Cu(I) + O_2 \rightarrow Cu(II) + O_2^{\cdot-} \quad (1a)$$

$$2O_2^{\cdot-} + 2H^+ \rightarrow H_2O_2 + O_2 \quad (1b)$$

$$Cu(I) + H_2O_2 \rightarrow \text{copper-oxo species} \quad (1c)$$

$$\text{copper-oxo species} + DNA \rightarrow DNA\ \text{cleavage} \quad (1d)$$

The inability of $Cu(OAc)_2$ or jadomycin B alone to cleave DNA (Fig. 1, Lanes 8 and 9) led us to speculate that jadomycin B may exert its synergistic damaging effect with copper through a similar oxygen-dependent pathway. The requirement for copper was further confirmed by the inhibitory effect of EDTA, where no damage was observed when the cleavage assay was carried out with 100 mM EDTA, a known chelator of copper ions (Fig. 3, Lane 3). Similar to what is observed for the prodigiosin family, catalase completely inhibits cleavage (Fig. 3, Lane 4), implicating a role for H_2O_2 in DNA damage by jadomycin B.

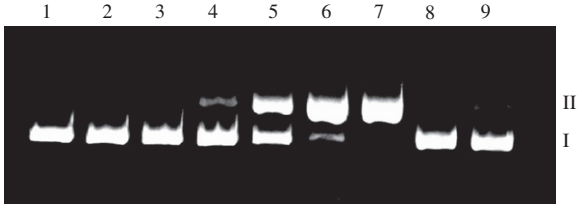

Figure 1. Concentration profile for copper-mediated nuclease activity of **1** in the presence of equimolar $Cu(OAc)_2$. Reaction mixtures (20-μL total volume) contained 400 ng Form I DNA in 10 mM MOPS buffer, pH 7.4, and were incubated at 37 °C for 4 h. Lane 1, 0.1 μM **1**:Cu(II); Lane 2, 0.2 μM **1**:Cu(II); Lane 3, 0.5 μM **1**:Cu(II); Lane 4, 1 μM **1**:Cu(II); Lane 5, 2 μM **1**:Cu(II); Lane 6, 5 μM **1**:Cu(II); Lane 7, 10 μM **1**:Cu(II); Lane 8, 10 μM **1**; Lane 9, 10 μM Cu(II).

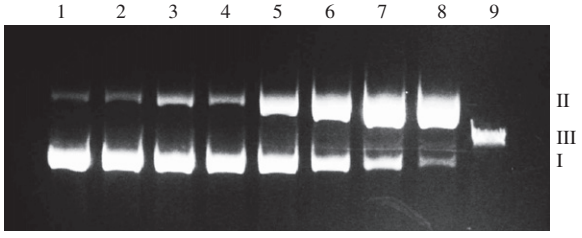

Figure 2. Time profile for copper-mediated nuclease activity of **1** in the presence of equimolar $Cu(OAc)_2$. Reaction mixtures (20-μL total volume) contained 10 μM **1**:Cu(II) with 400 ng Form I DNA in 10 mM MOPS buffer, pH 7.4. Lane 1, DNA only; Lane 2, 10 μM **1** (t = 3 h); Lane 3, 10 μM Cu(II) (t = 3 h); Lane 4, 10 μM **1**:Cu(II) at t = 0; Lane 5, t = 0.5 h; Lane 6, t = 1 h; Lane 7, t = 2 h; Lane 8, t = 3 h; Lane 9, linear marker.

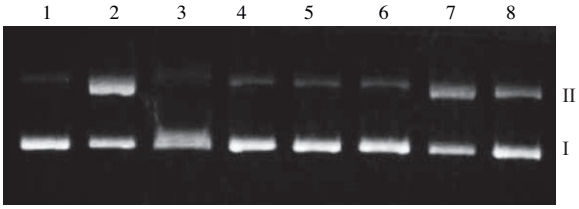

Figure 3. Effect of reactive oxygen species on copper-mediated nuclease activity of **1** in the presence of equimolar $Cu(OAc)_2$. Reaction mixtures (20-μL total volume) contained 10 μM **1**:Cu(II) with 400 ng Form I DNA in 10 mM MOPS buffer, pH 7.4, and were incubated at 37 °C for 3 h. Lane 1, DNA only; Lane 2, 10 μM **1**:Cu(II); Lane 3, 100 mM EDTA; Lane 4, 1000 U mL^{-1} catalase; Lane 5, 1000 U mL^{-1} SOD; Lane 6, 100 mM NaN_3; Lane 7, 1 M *t*-BuOH; Lane 8, 1 M DMSO.

Unlike the prodigiosenes, however, the presence of superoxide dismutase (SOD), a metalloenzyme that converts superoxide ($O_2^{\cdot-}$) to H_2O_2 and H_2O, completely protects DNA, suggesting that superoxide is an intermediate that participates in DNA damage by jadomycin B. This inhibitory effect as well as that by NaN_3, a singlet oxygen scavenger,[15,16] is shown in Figure 3 (Lanes 5 and 6). The presence of hydroxyl radical scavengers such as DMSO and *t*BuOH,[17,18] albeit to a slightly lesser extent, also inhibit DNA damage by jadomycin B. Therefore, freely diffusible hydroxyl radicals,[19,20] superoxide, singlet oxygen, and H_2O_2 together mediate the DNA cleavage initiated by jadomycin B and copper.

While the mechanistic aspects of DNA damage by jadomycin B and copper appear to involve multiple oxygen-dependent pathways, the initial reduction of Cu(II) to Cu^+ is likely a requirement. If the role of jadomycin B is to supply electrons to Cu(II), then this process will be accelerated under conditions where the formation of the angucyclinone-based π-radical cation is favored. Indeed, excitation of the phenanthridine core with 308-nm laser light reduced the time required for single-strand cleavage by ~98%. Close inspection of Lane 4 (Fig. 4) shows traces of DNA damage in the absence of copper, indicating that some strand scission may arise from direct interaction between the jadomycin radical cation and DNA. However, under the reaction time employed, its contribution to DNA damage is negligible.

Given that jadomycin B and copper propagate a powerful battery of reactive oxygen species, the absence of double-strand cleavage is curious. If one assumes a lower limit of one copper per 2000–3000 bases,[21] then jadomycin B could potentially damage DNA at a frequency of $\sim 4.5 \times 10^6/10^9$ nucleotides, resulting in detectable quantities of double-strand lesions. Under all conditions employed, no substantial amount of direct or indirect double-strand breaks were discerned. Two possible scenarios to explain the inability of jadomycin B and copper to linearize circular DNA arise: (i) the interaction between jadomycin B and Cu(II) does not involve formal chelation, and (ii) the interaction between jadomycin B and DNA is weak or nonexistent.

UV–vis titration experiments were carried out to monitor the interactions of jadomycin B with both DNA and copper. As shown in Figure 5, there is no evidence that jadomycin B interacts with calf thymus DNA under physiological conditions. Likewise, no changes in the absorption spectrum of jadomycin B occurred upon titration with $Cu(OAc)_2$, indicating that the association between copper and jadomycin, if any, is weak. Nevertheless, a stoichiometric preference for Cu(II) was observed in the DNA cleavage assays, whereby DNA cleavage increased with increasing Cu(II) and was maximal at $[Cu^{2+}]$ = [jadomycin B]. Further addition of Cu(II) had no effect over the range of stoichiometries investigated. These results suggest at least a transient interaction between Cu(II) and jadomycin B in the presence of DNA, perhaps where DNA serves as a template for the interaction. However, titrations of DNA with a mixture of copper and jadomycin B, and with sequential

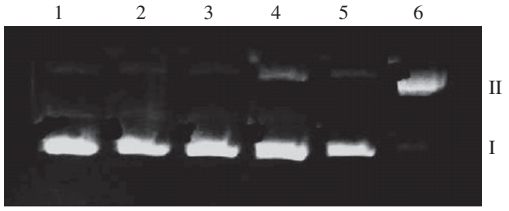

Figure 4. Laser-enhanced (λ_{ex} = 308 nm) copper-mediated nuclease activity of **1** in the presence of equimolar $Cu(OAc)_2$. Reaction mixtures (20-μL total volume) contained 400 ng Form I DNA in 10 mM MOPS buffer, pH 7.4, and were irradiated for 5 min. Lane 1, DNA only, *−hv*; Lane 2, 10 μM **1**:Cu(II), *−hv*; Lane 3, DNA only *+hv*; Lane 4, 10 μM **1** *+hv*; Lane 5, 10 μM Cu(II) *+hv*; Lane 6, 10 μM **1**:Cu(II) *+hv*.

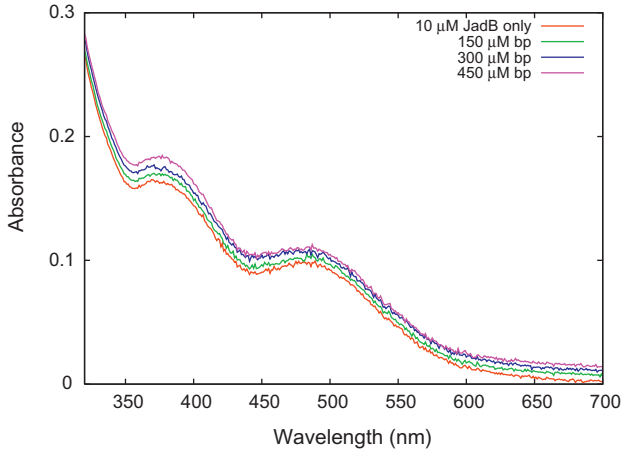

Figure 5. UV–vis titration of **1** (10 μM) with calf thymus DNA in 10 mM MOPS buffer, pH 7.4, containing 100 mM NaCl. CT DNA was added in 150 μM (base pair) aliquots.

additions of each, did not confirm this hypothesis. From these observations, we infer that the lack of affinity of the angucyclinone framework for copper or DNA precludes formation of double-strand breaks in DNA and suggests that diffusable reactive oxygen species are responsible for the observed single-strand breaks. This observation may account for the attenuated toxicity of jadomycin B relative to prodigiosin and its derivatives, which bind Cu(II) strongly and interact with DNA, if DNA damage is the primary mode of biological activity.

4. Concluding remarks

The fact that jadomycin B does not appear to interact directly with DNA or copper may reduce its toxicity relative to prodigiosin, which is known to cause double-strand scission in DNA. The attenuated toxicity of jadomycin B may lend to better toleration in vivo. The copper-dependence of jadomycin B for affecting DNA damage is advantageous for several reasons. Under normal conditions nuclear DNA has approximately one copper molecule per every 2000–3000 bases.[22,23] Further, the packaging of DNA as a nucleosome has been shown to enhance $Cu(II)/H_2O_2$-mediated DNA damage,[24] one of the pathways implicated in jadomycin B-induced DNA strand breaks. Therefore, in cells where copper levels are elevated, DNA damage will be maximized. Breast cancer cells, in particular, have significantly more copper than noncancerous breast tissue (80 μM vs 23 μM),[25,26] which may explain the efficacy of jadomycin B against the two human breast ductal carcinoma cell lines that were screened.[6] It is interesting to note that the copper-mediated DNA damage by jadomycin B can be greatly facilitated by light, which adds yet another avenue for achieving selectivity against cancer cells in vivo.

Studies are underway to explore further the nature of copper-mediated DNA cleavage by jadomycin B as well as other possible modes of biological activity. NMR experiments indicate that jadomycin B is capable of ring-opening under physiological conditions to its more electron-rich, hydrophilic counterpart, which may be the active species in DNA cleavage or other important biological interactions.[27,28] We are particularly interested in exploiting precursor-directed biosynthesis to control this ring-opening by preparing derivatives of jadomycin B that differ by the substituent at C_1, a useful handle for influencing the diastereomeric ratio at C_{3a}. Through such endeavors, we hope to optimize the pharmacokinetic properties of the jadomycins and selectively tune the mechanistic aspects of DNA damage.

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