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Analysis of kinamycin D-mediated DNA cleavage

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ABSTRACT

Kinamycin D is a potent antitumor antibiotic; however the biological mode of action is poorly understood. Recent efforts suggest the natural product is capable of generating reactive oxygen species under acidic pH to induce DNA damage in the presence of a reducing agent.

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The kinamycins (Fig. 1) are a complex class of natural products characterized by an uncommon diazobenzo[*b*]fluorene skeleton. These biological metabolites, first isolated from *Streptomyces murayamaensis* in 1970, have been reported to possess both antitumor and antibiotic activities.[1–3] Originally reported as *N*-cyanobenzo[*b*]carbazoles and later characterized as diazobenzo[*b*]fluorenes,[4,5] the kinamycin family has received renewed interest for their potent biological activities believed to be effected by the diazo moiety. Recent reports have shown that the lomaiviticins, glycosylated diazobenzo[*b*]fluorene dimers, cleave DNA under reducing conditions and have potent anticancer and antibacterial activities.[6] This has lead to an intensified interest in the total synthesis and mechanism by which these classes of natural products elicit their biological activity.

There are few reports detailing the mode of action for the antitumor activities that these diazoparaquinones possess. Dmitrienko, using isoprekinamycin as a model, originally postulated that the electrophilicity of the diazo group could facilitate the formation of a nucleophilic adduct on the terminal diazo nitrogen. This adduct would then decompose, providing a carbon-based radical that could then mediate both the antibiotic and anticancer activities reported.[7] Subsequent studies by Feldman and Eastman on prekinamycin have shown the radical initiators AIBN and Bu_3SnH in refluxing benzene can generate radical trapping arene products and a concomitant loss of N_2. This led to the proposal of a single electron reduction to a cyclopentenyl radical and subsequently to an orthoquinonemethide electrophile to account for the observed reaction products.[8]

Recently we demonstrated the ability of kinamycin D to cleave DNA in the presence of dithiothriotol (DTT) and that this activity could be recapitulated with simple diazofluorene analogues.[9] This led us to propose that a $2H^+/2e^-$ reduction activated kinamycin D for subsequent nucleophile-promoted DNA cleavage as originally postulated by Dmitrienko. In an extensive in vivo study, Hassinoff subsequently determined that kinamycin F: (1) induces DNA damage in an iron-dependent manner, (2) induces DNA strand cleavage in cell culture, and (3) generates air-stable free radicals upon reaction with glutathione (GSH).[10] Further studies from our group have shown that under biomimetic conditions (37 °C and physiological thiol concentrations) kinamycin D is able to potently cleave DNA.[11] These results appear consistent with a $2H^+/2e^-$ reduction followed by the nucleophilic activation mechanism initially proposed (Fig. 2). However, we also discussed an alternative mechanism based upon protonation driving the formation of an orthoquinone methide, which would subsequently lead to DNA cleavage. The latter mechanism was not favored due to the lack of literature reports of orthquinone methides mediating DNA cleavage under these conditions.

Recently, Skibo provided an extensive analysis on the behavior of prekinamycin, which clearly demonstrated that under acidic and reducing conditions (H_2, Pd/C), that prekinamycin forms an orthoquinone methide.[12] This provides evidence for the alternative mechanism we had proposed. However, the extrapolation of these results to a biological setting remains ambiguous. Although cellular cytotoxicity data was referenced, no direct evidence in the context of a biological target was presented. Additionally, the interpretation of these results did not account for the extensive in vitro work done by Hasinoff, who clearly demonstrated that kinamycin F generates a free radical upon treatment with GSH.

In order to further elucidate the mechanism by which the kinamycins mediate DNA damage, we employed manual DNA sequencing gel electrophoresis to analyze the pattern of kinamycin D-mediated DNA cleavage under a variety of conditions. Given that proton transfer is involved in both mechanisms that we recently proposed, we first chose to confirm the effect of pH on the ability of kinamycin D to cleave DNA under reducing conditions. Phosphate buffered solutions containing 5.7 mM DTT were incubated with 100 μM kinamycin D and a 175-mer radiolabeled DNA (10,000 cpm/reaction, ca. 10 pM) at a pH range of 5.5–8.0 at 37 °C overnight. Previous results from our group have demonstrated that both DTT and GSH promote kinamycin D-mediated

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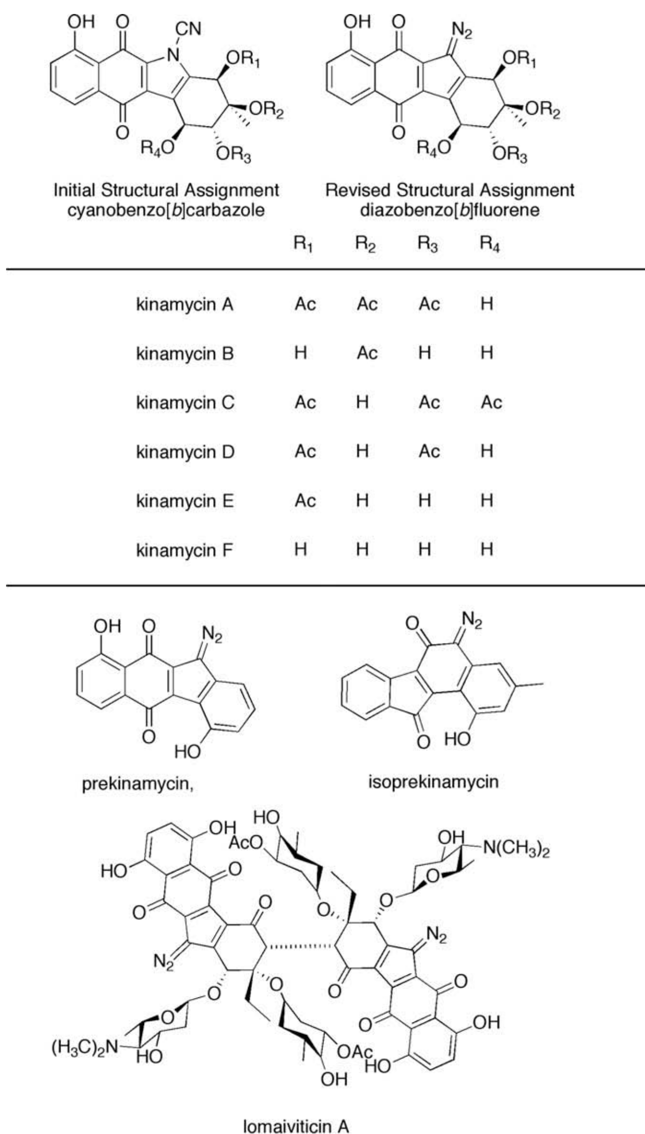

	R_1	R_2	R_3	R_4
kinamycin A	Ac	Ac	Ac	H
kinamycin B	H	Ac	H	H
kinamycin C	Ac	H	Ac	Ac
kinamycin D	Ac	H	Ac	H
kinamycin E	Ac	H	H	H
kinamycin F	H	H	H	H

Figure 1.

DNA cleavage in an identical manner; therefore we chose to simply employ DTT in these studies. Reactions were also performed at room temperature, as extensive DNA damage was observed at 37 °C (as expected),[11] making analysis by gel electrophoresis difficult given that analysis relies upon introducing a single strand break into a subpopulation of DNA strands. The results of this assay (Fig. 3A) clearly illustrate that the reaction is favored in acidic media. At the two lowest pH's studied (5.5 and 6.0), the extent of DNA cleavage was so severe that only a minimal amount of the full length DNA template remained intact, with the majority of the DNA cleaved multiple times into small fragments which ran at the bottom/off the gel.

After confirming that DNA cleavage preferentially occurred at acidic pH, we sought to investigate the lower limit of kinamycin D concentration that could elicit a measurable cleavage response. Incubations were performed in 5 mM phosphate buffer, 50 mM DTT at pH 6.0 with kinamycin D concentrations ranging from 50–1 μM. Significant DNA cleavage is observed when treated with as little as 10 μM kinamycin D and a minimal observable cleavage is promoted with 1 μM kinamycin D (Supplementary data). In addition to exploring the effects of kinamycin D concentration we wanted to reconfirm the effect DTT concentration had upon the cleavage reactions. Therefore, we chose to investigate a wide range of thiol concentrations from 500 mM–500 μM. As previously noted,[11] DTT concentrations as low as 5 mM are effective at promoting cleavage of the majority of DNA template when treated with 50 μM kinamycin D. However, the upper limit of 500 mM DTT is actually shown to inhibit DNA cleavage (Supplementary data) as demonstrated by our previous results in vitro. This result also parallels the recent work of Hassinoff, which indicated that reduced intracellular GSH levels increased the cytotoxicity of kinamycin F and increased GSH levels decreased cytotoxicity.[10] This concentration-dependent cleavage, coupled with Hasinoff's EPR studies, can be explained as kinamycin D mediates DNA cleavage via a radical-based mechanism, where low concentrations of DTT can promote the formation of a reactive radical intermediate, whereas at higher concentrations DTT is capable of quenching the activity of the reactive intermediates capable of inducing DNA damage.

The radical-based mechanism postulated is also supported by the lack of sequence specific DNA cleavage products. Although the electrophoretic radiograms indicate general DNA cleavage at

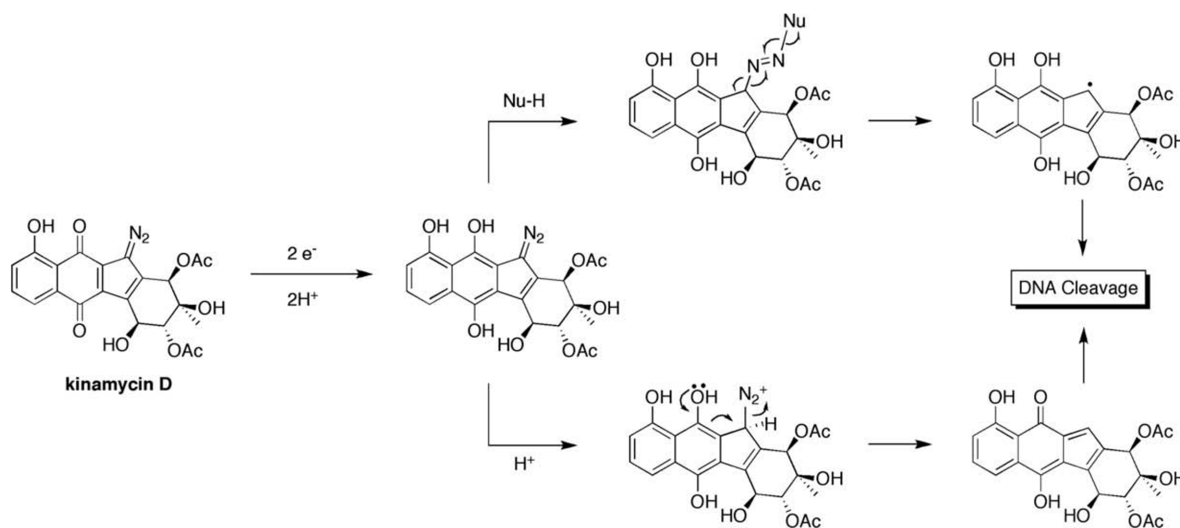

Figure 2. Potential routes to DNA cleavage.

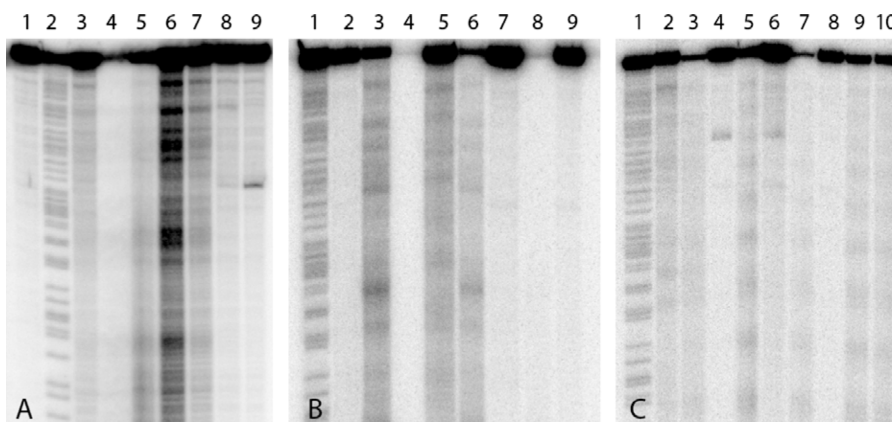

Figure 3. Electrophoretic DNA Sequencing Gels. **A**. pH dependence of DNA cleavage. Lane 1: DNA, Lane 2: G + A ladder, Lane 3: DNA + 5.7 mM DTT, Lane 4: DNA + 5.7 mM DTT + 100 μM Kin D (standard rxn) pH 5.5, Lane 5: standard rxn pH 6.0, Lane 6: standard rxn pH 6.5, Lane 7: standard rxn pH 7.0, Lane 8: standard rxn pH 7.5, Lane 9: standard rxn pH 8.0. **B**. Hydroxyl Radical Scavenger Assay. Lane 1: G + A ladder, Lane 2: DNA + 100 μM Kin D + 50 mM DTT (standard rxn pH 6.0) + 100 mM NaN_3, Lane 3: standard rxn + 100 mM MeOH, Lane 4: standard rxn + 100 mM glycerol, Lane 5: standard rxn + 100 mM thiourea, Lane 6: standard rxn + 100 mM DMSO, Lane 7: standard rxn + 100 μM desferoxamine, Lane 8: standard rxn, Lane 9: DNA + DTT. **C**. ROS Assay. Lane 1: G + A ladder, Lane 2: DNA + DTT, Lane 3: DNA + 100 μM Kin D + 50 mM DTT (standard rxn), Lane 4: standard rxn + 1 U CAT, Lane 5: standard rxn + 3 U SOD, Lane 6: standard rxn + CAT (95 °C, 10 m), Lane 7: standard rxn + SOD (95 °C 10 m), Lane 8: standard rxn + CAT-trypsin, Lane 9: standard rxn + SOD-trypsin, Lane 10: standard rxn + trypsin.

every position in the oligonucleotide, these observations could not rule out the possibility that kinamycin D could also alkylate DNA as well. Therefore, we subjected the reaction products to heat lability and hot piperidine treatment to generate strand breaks at DNA bases modified through alkylation as shown previously with quinone methides.[13] These experiments did not promote the formation of any additional DNA cleavage products.

Given the generic cleavage pattern, and previous results that indicate trace iron is important for kinamycin F DNA cleavage and catalase suppresses kinamycin F-mediated DNA cleavage, we hypothesized that the general DNA cleavage promoted by kinamycin D under reducing conditions was symptomatic of a general diffusible reactive oxygen species (ROS). To test this hypothesis, we first explored the effects of introducing EDTA, a generic metal chelator, to the reaction system. At the lowest concentration tested, 20 μM EDTA was able to completely suppress DNA degradation when treated with 100 μM kinamycin D and 50 mM DTT at pH 6.0 incubated for 14 h at 37 °C (data not shown). The ability of EDTA to suppress this reaction through chelation seemed promising; however, it did not reveal which trace metal was responsible for catalyzing this behavior. Therefore we introduced desferoxamine, an Fe^{3+} chelator to our reaction system to indicate whether iron has a specific role in promoting DNA damage (Fig. 3B). Paralleling the results with kinamycin F, kinamycin D did not induce any amount of DNA cleavage when treated with 50 μM desferoxamine, highlighting the importance of trace iron and its catalytic role in kinamycin D-mediated DNA cleavage. A variety of hydroxyl radical scavengers (100 mM) were also employed to further clarify the reaction mechanism.[14] Glycerol was the only scavenger shown to have no inhibitory effect while DMSO had modest inhibitory activity; however sodium azide prevented nearly all DNA degradation and thiourea and methanol, prevented a majority of degradation as well.

Given that: (1) the ROS in our reaction is most likely either superoxide and/or hydrogen peroxide, and (2) kinamycin F DNA cleavage is inhibited by catalase (CAT), we probed for the necessity of either species to mediate DNA cleavage. The enzymes catalase (CAT) and superoxide dismutase (SOD) catalyze the dismutation of hydrogen peroxide (1) and superoxide (2), respectively.

(1) $H_2O_2 \rightarrow H_2O + O_2$
(2) $O_2^{-} \rightarrow H_2O_2 + O_2$

As with kinamycin F, catalase completely inhibited DNA cleavage when treated with 100 μM kinamycin D and 50 mM DTT at pH 6.0 emphasizing that the production of hydrogen peroxide is crucial in generating DNA damage (Fig. 3C). Superoxide dismutase was shown to slightly inhibit the cleavage reaction; however we note that the optimum pH for SOD activity is 8.0, not 6.0 as in our cleavage assay. When each enzyme was heated to 95 °C for 10 min, unlike kinamycin F, CAT retained the ability to prevent DNA cleavage. Heat treated SOD showed enhanced DNA cleavage indicating a role for superoxide under the tested conditions. We investigated the possibility of a non-specific interaction between CAT and the DNA template by performing a trypsin digest of CAT prior to its addition into the DNA cleavage reaction to cleave CAT into multiple fragments and suppress any potential non-specific DNA binding by denatured CAT protein. However, trypsin itself inhibited the reaction, raising the possibility of other biological targets for kinamycin D in addition to DNA.

In conclusion, we have been able to demonstrate the important role pH plays in reaction progress, highlighting the necessity of acidic media to facilitate kinamycin D-mediated DNA cleavage under reducing conditions. Furthermore, paralleling the results of kinamycin F, we have shown that free trace iron and ROS are critical for DNA cleavage. We have further shown that superoxide may also play a role in DNA cleavage. In light of previous results, it appears kinamycins may mediate DNA cleavage through similar mechanisms even though their D-rings have different substitution patterns. Our results also raise the possibility that there are other biological targets that are D-ring specific; however, whether these targets are biologically relevant and/or occur in in vivo remains to be validated.

Acknowledgment

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Supplementary data

DNA sequencing gels, PCR primers, reaction conditions, and stop solution/precipitation methods are available. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tetlet.2009.12.142.

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