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Kinamycin-mediated DNA cleavage under biomimetic conditions

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Abstract

The kinamycins are biologically active secondary metabolites characterized by an uncommon diazobenzo[*b*]fluorene skeleton. Kinamycin D has been shown to potently cleave DNA under mild biomimetic conditions. Use of the endogenously abundant reductant glutathione at 570 μM, kinamycin D effectively cleaved DNA in a concentration, temperature, and time-dependent fashion. Dithiothreitol also proved effective at low concentration while other reductants failed to induce DNA cleavage. Mechanistic consequences of the DNA cleavage results are described.

1. Introduction

The kinamycins are a family of complex natural products that are characterized by an uncommon diazo subunit within a benzo[*b*]fluorenone skeleton (Fig. 1).[1–4] These bacteria-derived metabolites are biologically active and have been reported to have both anti-tumor and antibiotic activity.[2,4,5] Disclosure of lomaiviticin A and B by Wyeth–Ayerst have further accentuated interest in this class of natural products (Fig. 1).[6] The lomaiviticins are glycosylated dimeric analogues of the kinamycins and were reported to have potent anti-cancer/antibiotic activity.[6] Furthermore, lomaiviticin A was reported to cleave DNA under reducing conditions; however, no experimental details were disclosed.[6]

As part of our research program directed toward understanding the mechanism by which the kinamycins/lomaiviticins cleave DNA, and subsequently harnessing this information toward the design of simple analogues for the development of potential anti-cancer compounds, we recently reported that kinamycin D cleaved DNA in the presence of dithiothreitol (DTT).[7] Furthermore, we were able to recapitulate this activity with simple, electronically tuned diazofluorene analogues, and were able to demonstrate that these simple analogues had anti-proliferative activity against HeLa cells. Herein, we delineate the effect that temperature, thiol concentration, and thiol identity have upon the extent of kinamycin-mediated DNA cleavage. From these studies, we demonstrate that kinamycin D displays potent DNA damaging capability under biomimetic conditions.

In our previous report, modest kinamycin-mediated DNA cleavage was observed after 48 h when the reaction was initiated in the presence of a large excess of thiol ([DTT] = 1.0 M).[7] Although these conditions successfully promoted kinamycin-dependent DNA cleavage, the large excess of DTT employed was not consummate with typical intracellular thiol concentrations. Glutathione (GSH) is the most abundant intracellular thiol with concentrations ranging from 0.1 to 10 mM.[8–10] Given this disparity, it was uncertain if our previous work was directly applicable to potential models of in vivo activity.

To address this discrepancy, we first performed a room temperature kinamycin D-mediated DNA cleavage study in which we varied the thiol identity (DTT or GSH), thiol concentration (data not shown), and kinamycin D concentration. The extent of DNA cleavage was assayed using a plasmid relaxation assay in which type I supercoiled plasmid was converted into type II nicked or type III linear DNA followed by gel electrophoresis analysis. It was found that moderate DNA cleavage was observed at low mM

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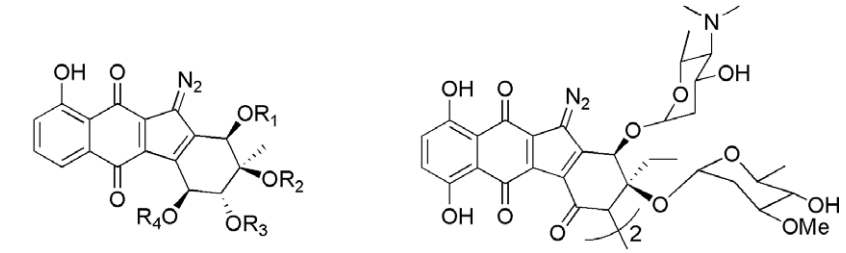


Fig. 1. Kinamycins A–D, and lomaiviticin A.

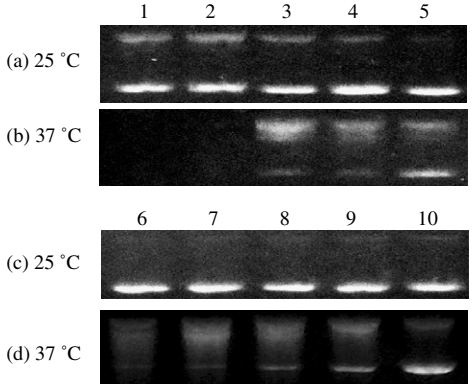


Fig. 2. Temperature-dependent plasmid cleavage assay, top band is type II nicked DNA, middle band is type III linear DNA, bottom band is type I supercoiled DNA. Conditions: all lanes contain 714 ng pBR322 DNA (37 °C GSH expeiment required 1.4 μg DNA), lanes 1–5, final [DTT] = 5.7 mM, lanes 6–10, final [GSH] = 0.57 mM, assay run for 48 h at designated temperature. Lane 1, [KinD] = 1.0 mM; lane 2, [KinD] = 750 μM; lane 3, [KinD] = 500 μM; lane 4, [KinD] = 250 μM; lane 5, [KinD] = 0 mM; lane 6, [KinD] = 1000 mM; lane 7, [KinD] = 750 μM; lane 8, [KinD] = 500 μM; lane 9, [KinD] = 250 μM; lane 10, [KinD] = 0 mM.

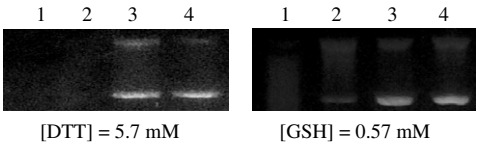


Fig. 3. Time-dependent plasmid cleavage assay, top band is type II nicked DNA, middle band is type III linear DNA, bottom band is type I supercoiled DNA. Conditions: all lanes contain 714 ng pBR322 DNA (37 °C GSH expeiment required 1.4 μg DNA), lanes 1–3 final [KinD] = 1.0 mM, assay run at 37 °C. Lane 1, 48 h; lane 2, 24 h; lane 3, 12 h; lane 4, [KinD] = 0 mM for 48 h.

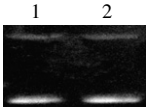
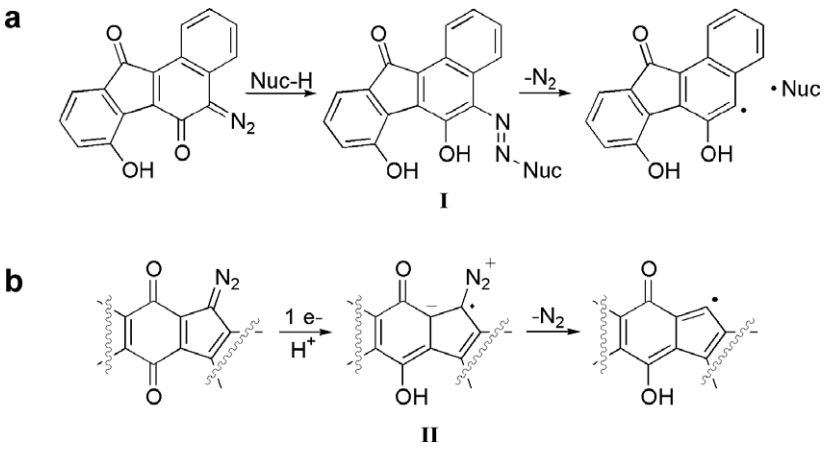


Fig. 4. Plasmid cleavage assay, top band is type II nicked DNA, bottom band is type I supercoiled DNA. Conditions: all lanes contain 714 ng pBR322 DNA and final [NADPH] = 5.7 mM, assay run at 37 °C for 48 h. Lane 1, [KinD] = 1.0 mM; lane 2, [KinD] = 0 mM.

concentrations of DTT (Fig. 2a) and no cleavage with μM concentrations of GSH (Fig. 2c) at room temperature. The extent of DTT-mediated DNA cleavage under these conditions was similar to what was observed under 1.0 M DTT conditions.

Next, the effect that the temperature had upon the extent of DNA cleavage was examined. DNA cleavage experiments with kinamycin D at physiological temperature (37 °C) showed potent DNA cleavage activity far surpassing the cleavage results at room temperature (Fig. 2b and d). The result with GSH is particularly significant because it represents a molecular ratio of 5:1/GSH:kinamycin D when [KinD] = 250 μM (Fig. 2d, lane 9). Kinamycin D was also shown to display time-dependent DNA cleavage activity at 1 mM at 37 °C in both DTT and GSH (Fig. 3). The plasmid was completely degraded within 24 h in DTT while a minimal amount of DNA remained at 24 in GSH (Fig. 3). Alternative biological reductants, such as NADPH, failed to induce kinamycin D-mediated DNA cleavage (Fig. 4).

Given that this is the first report of kinamycin-mediated DNA cleavage under biomimetic conditions, we considered the potential mechanism of DNA cleavage under these experimental conditions. There have been a limited number of reports that address the mechanism of action (MOA) of the diazobenzo[*b*]fluorenes. Using the diazobenzo[*a*]fluorene isoprekinamycin as a model substrate, Laufer and Dmitrienko postulated that the electrophilicity of the diazo group would mediate the formation of a nucleophilic adduct **I** that would subsequently decompose to form a diradical and induce DNA damage (Scheme 1a).[11] By using prekinamycin as their model substrate and guided by the established biological chemistry of other quinone natural products, Feldman and Eastman have postulated that the bioreduction of the quinone to a semiquinone **II** would destabilize the diazo moiety, resulting in decomposition to a carbon-based radical with concomitant release of N_2 (Scheme 1b).[12]



Scheme 1. Proposals for kinamycin mechanism of action.

The extrapolation of the reported reaction conditions to a cellular environment remains ambiguous. Dmitrienko utilized β-naphthol as a nucleophile in THF, while Feldman employed AIBN/Bu_3SnH in refluxing benzene as the source of a one-electron reductant. A subsequent report from Feldman delineated that the reactivity of prekinamycin under AIBN/Bu_3SnH was limited to aromatic organic solvents.[13]

Given that sulfides are a well-established source of $2e^-$ reductions it is unlikely that, under these biomimetic conditions, a $1e^-$ reduction is occurring. We have previously documented that simple nucleophiles will not promote kinamycin D-mediated DNA cleavage;[7] therefore, simple nucleophilic activation is also unlikely. To resolve this ambiguity, the LUMO of kinamycin D (without substituted D-ring) was modeled with Spartan (Fig. 5). This model predicts that the LUMO of kinamycin D resides within the benzoquinone portion of the molecule. Therefore, it is reasonable to predict that under the mild reducing conditions employed in this study a $2H^+/2e^-$ reduction occurs to reduce the benzoquinone to hydroquinone **III** (Scheme 2).

Once the hydroquinone is formed, there are two potential routes for DNA cleavage that parallel the previously proposed mechanisms (Scheme 3). The first is the nucleophilic attack on the distal diazo nitrogen, which would subsequently undergo homolytic cleavage to generate a carbon-based radical **IV** that would mediate DNA strand scission (Route A; Scheme 3). The construction of the LUMO map of the hydroquinone clearly shows the LUMO resides on the diazo group (Fig. 5). This route proves viable based on the already precedented carbon-based radical hypotheses (Scheme 1) and the LUMO map of the hydroquinone (Fig. 6). The second route is protonation followed by the spontaneous decomposition of the diazo group to generate an orthoquinone methide **V** (Route B; Scheme 3).

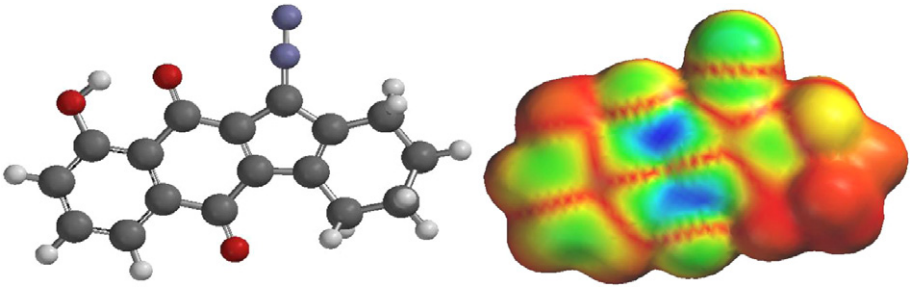
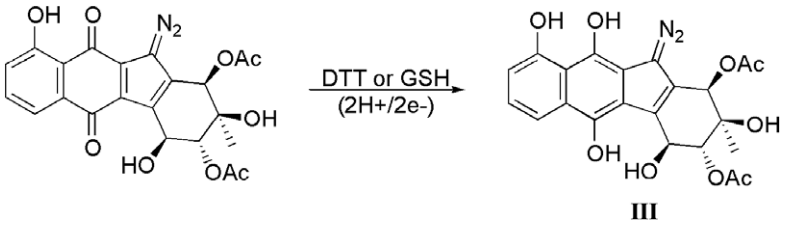
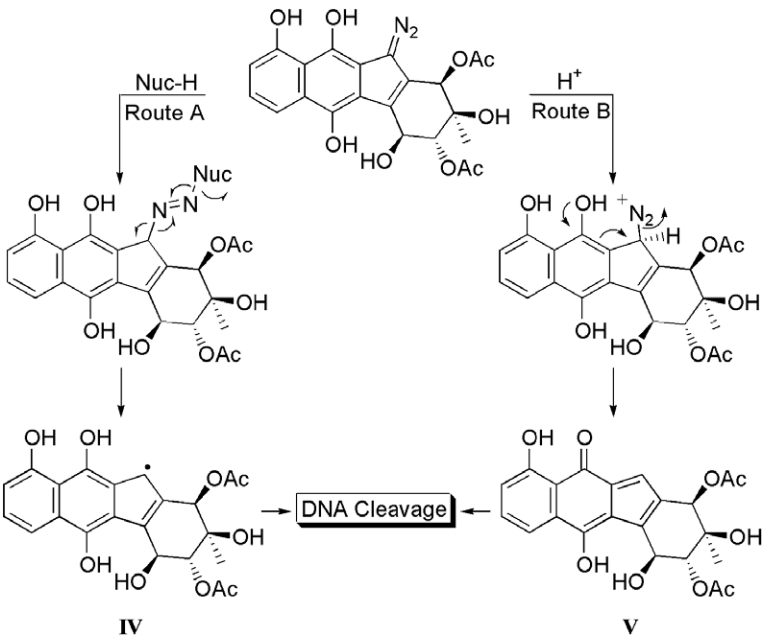


Fig. 5. Kinamycin D (model) LUMO.



Scheme 2. The reduction of kinamycin D.



Scheme 3. Potential routes for DNA cleavage activity.

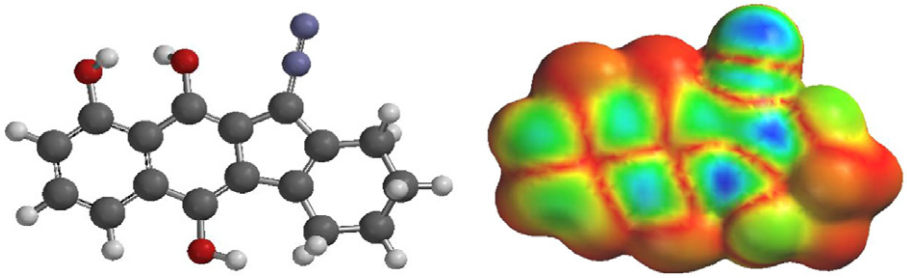


Fig. 6. Reduced kinamycin D (model) hydroquinone LUMO.

Although to the best of our knowledge, there have been no published accounts of an orthoquinone methide-mediating DNA cleavage under the conditions that were employed in this study. Orthoquinone methides have been proposed as the active alkylating species for the structurally similar anthracycline drugs but this has also been brought into question and alkylation does not, in itself, cause DNA cleavage.[14,15] The hydroquinone LUMO map, coupled with the extensive DNA cleavage observed in this study suggests that once kinamycin D has been reduced, nucleophilic activation resulting in a carbon-based radical is necessary to initiate DNA cleavage; however, further mechanistic studies will be required to deconvolute these two pathways.

In conclusion, we have demonstrated that kinamycin D has potent DNA cleavage activity under biomimetic conditions. Since the submission of this article, it has also shown that Kinamycin F cleaves DNA at 37 °C using 5 mM GSH.[16] Based upon the mild conditions that were employed in this study in conjunction with our LUMO calculations, it is probable that the first step of kinamycin D-mediated DNA cleavage is a $2H^+/2e^-$ reduction to the corresponding hydroquinone. LUMO calculations and previous results from our research group suggest that that nucleophilic activation is subsequently required to mediate DNA cleavage; however, further experiments are necessary to substantiate this mechanism.

2. Experimental

2.1. General procedure for DNA cleavage assay

Kinamycin D was isolated as previously described from *S. murayamaensis* (ATCC 21414) and dissolved in DMF.[17] Covalently closed, supercoiled DNA (pBR322) was used as supplied by the New England Biolabs in a 10 mM Tris–HCl buffer (pH 8.0) containing 1.0 mM EDTA. DL-Dithiothreitol (DTT), glutathione (GSH) and β-nicotinamide adenine dinucleotide 2′-phosphate reduced tetrasodium salt (NADPH) were dissolved in dH_2O. Incubations with DNA were carried out in a microfuge tube with the total volume not exceeding 4 μL. The reactions were conducted in the dark at room temperature (25 °C) or 37 °C for 48 h. DNA samples were then mixed with 8 μL of 10X Ficoll®

loading buffer (0.25% xylene cyanol FF, 0.25% bromophenol blue, 15% w/v Ficoll® in water) and loaded on a 0.7% agarose gel (containing 50 μL ethidium bromide) and run at 50 V in a TBE buffer (pH 7.6), until bromophenol blue reached the bottom of the gel (2 h). The gel was photographed on a UV (302 nm) transilluminator with a Kodak EasyShare digital camera. DNA cleavage was quantitated with ImageQuant TL.

2.2. Dose response DNA cleavage assay

Followed general procedure. Cleavage assay with 0.57 mM GSH at 37 °C required the total volume to be increased to 8 μL while all concentrations of components were adjusted accordingly.

2.3. Time-dependent DNA cleavage assay

Followed general procedure. Forty-eight hours time point setup first, followed by 24 h and 12 h time points, respectively. Cleavage assay with 0.57 mM GSH required the total volume to be increased to 8 μL while all concentrations of components were adjusted accordingly.

Acknowledgments

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