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Analysis of Diazofluorene DNA Binding and Damaging Activity: DNA Cleavage by a Synthetic Monomeric Diazofluorene**

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Abstract: *The lomaiviticins and kinamycins are complex DNA damaging natural products that contain a diazofluorene functional group. Herein, we elucidate the influence of skeleton structure, ring and chain isomerization, D-ring oxidation state, and naphthoquinone substitution on DNA binding and damaging activity. We show that the electrophilicity of the diazofluorene appears to be a significant determinant of DNA damaging activity. These studies identify the monomeric diazofluorene* ***11*** *as a potent DNA cleavage agent in tissue culture. The simpler structure of* ***11*** *relative to the natural products establishes it as a useful lead for translational studies.*

Lomaiviticins A–E (**1**–**5**)[1] and kinamycins (**6**–**8**)[2] are complex antiproliferative agents that contain one (for **3**–**8**) or two (for **1** and **2**) diazotetrahydrobenzo[*b*]fluorene (diazofluorene) functional groups (Figure 1).[3] (−)-Kinamycin C (**6**) and lomaiviticins C–E (**3**–**5**) display half maximal inhibitory potencies in the 300 nM range against various cultured human cancer cell lines, while (−)-lomaiviticin A (**1**) is two to five orders of magnitude more cytotoxic, with IC_{50} values in the low nano- to picomolar range.[1a] We recently established that the superior potency of **1** derives from the production of DNA double-strand breaks (dsbs) that are induced by vinyl radicals[4] formed from each diazofluorene.[5,6] This mode of DNA damage is not recapitulated by (−)-lomaiviticin C (**3**) or (−)-kinamycin C (**6**).[5] The laboratories of Melander and Hasinoff-Dmitrienko have demonstrated that kinamycins D (**7**), F (**8**), and synthetic analogs nick DNA in vitro and in tissue culture,[6d–f,h] but DNA cleavage has not been detected, in accord with our observations using **6**. DNA dsbs are the most cytotoxic of all DNA lesions,[7] and these data provide an explanation for the superior potency of **1**.

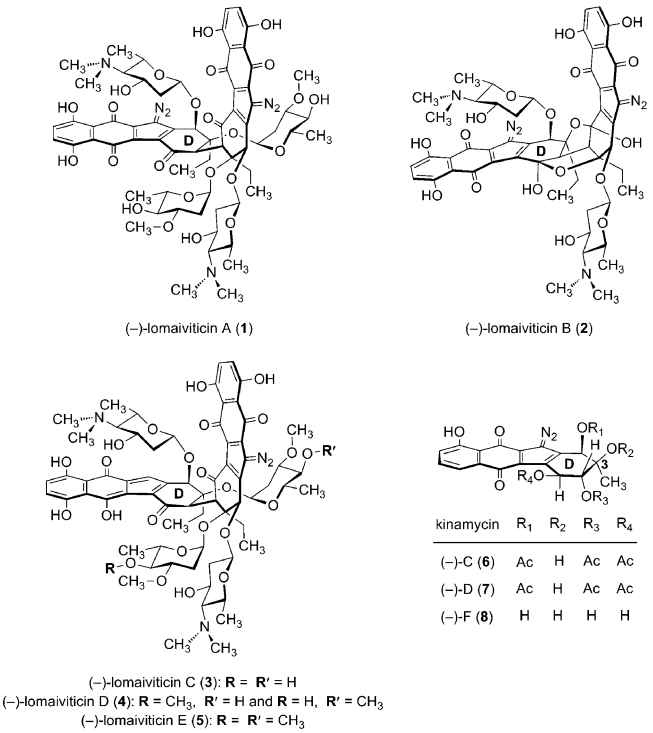

Figure 1. Structures of (−)-lomaiviticins A–E (**1**–**5**) and (−)-kinamycins C, D, and F (**6**–**8**, respectively).

Thermal denaturation and fluorescence intercalator displacement studies using calf thymus DNA and various kinamycins[6c,e] have established their DNA-binding ability, but nothing is known about the sequence selectivity of binding or the structural features that enhance or inhibit DNA damaging activity. Such information is central to an understanding of the mechanism of action of these metabolites and the preclinical development of synthetic diazofluorene-based anticancer agents. Accordingly, we report a comprehensive evaluation of the DNA-binding and cleavage activities of a panel of diazofluorenes embodying the essential structural features of the lomaiviticins and kinamycins. We demonstrate that certain synthetic diazofluorenes induce formation of DNA dsbs in tissue culture, including drug-resistant cell lines.[8] We employed kinamycins C (**6**), F (**8**), and the synthetic diazofluorenes **9**–**13**[9] in this study (Figure 2). These compounds were chosen because they allow for evaluation of the influence of dimerization, ring and chain isomerization, D-ring oxidation state, and naphthoquinone substitution on activity.

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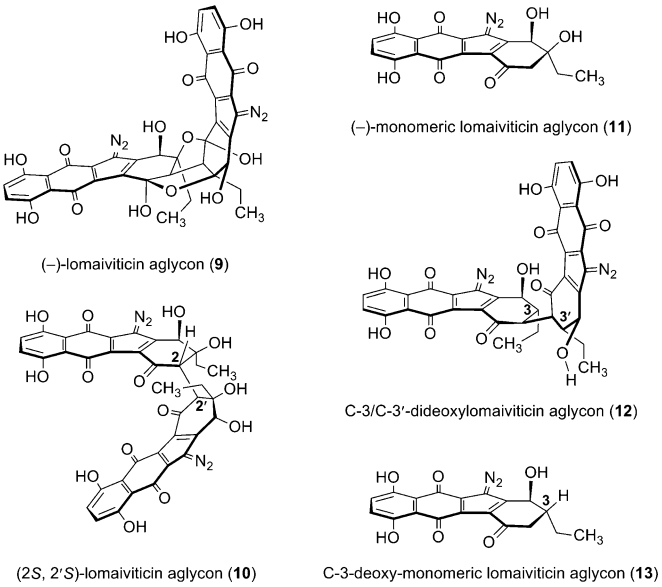

Figure 2. Structures of synthetic dimeric and monomeric diazofluorenes employed in this work.

Our studies began by determining the relative affinities of these diazofluorenes for DNA by a fluorescent intercalator displacement (FID) assay, employing thiazole orange (TO) as the intercalator probe.[10] Among all of the diazofluorenes examined, (−)-lomaiviticin aglycon (**9**) displayed the highest affinity for dsDNA (30% ± 1.2% decrease in fluorescence, Figure 3) and in general, dimeric diazofluorenes bound DNA

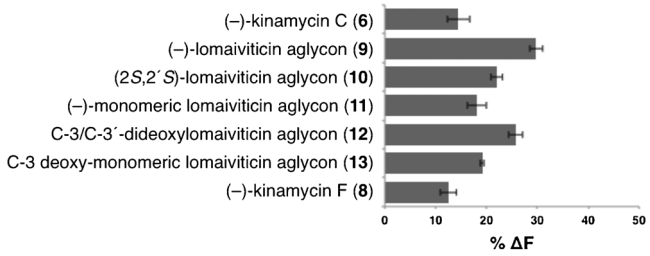

Figure 3. FID assays of equimolar concentrations of diazofluorenes (0.88 μM) against thiazole orange (TO, 1.25 μM) using calf thymus DNA as substrate (0.88 μM in base pairs).

with higher affinity than monomeric diazofluorenes (29–22% and 19–12% decrease in fluorescence for dimeric and monomeric diazofluorenes, respectively). We performed FID titration experiments to determine DC_{50} values (where DC_{50} corresponds to the amount of ligand required to displace 50% of the bound intercalator).[11] These studies showed that dimeric diazofluorenes bind polynucleotides with low micromolar affinity (Table 1). (−)-Lomaiviticin aglycon (**9**) displayed a modest preference for GC-rich sequences, while the C-3/C-3′-dideoxy aglycon (**12**) bound AT-rich sequences with highest affinity. The DC_{50} values of monomeric diazofluorenes were much higher (>100 μM) than dimeric diazofluorenes, in agreement with the TO displacement assays above. In both FID assays, the C-3/C-3′-dideoxy aglycon (**12**) bound

Table 1: FID-based determination of DC_{50} values [μM] of dimeric diazofluorenes against polynucleotides of increasing GC content.

polynucleotide (%GC content)	*C. perfringens* (32%)	Calf thymus (42%)	*M. lysodeiktius* (75%)
(−)-lomaiviticin aglycon (**9**)	14.4	7.47	9.98
(2*S*,2′*S*)-lomaiviticin aglycon (**10**)	56.2	40.7	63.2
C-3/C-3′-dideoxy-lomaiviticin aglycon (**12**)	18.6	19.8	59.7
(−)-monomeric lomaiviticin aglycon (**11**)	>100	>100	>100
(−)-kinamycin C (**6**)	>100	>100	>100
C-3-deoxy-monomeric lomaiviticin aglycon (**13**)	>100	>100	>100

with higher affinity than the (2*S*, 2′*S*)-lomaiviticin aglycon (**10**), suggesting the (2*R*, 2′*R*)-configuration found in **12** and the natural lomaiviticins may be stereochemically matched with the absolute configuration of DNA.

FID titration plots were utilized to determine the binding site size (Figure S1 in the Supporting Information). (−)-Lomaiviticin aglycon (**9**), the (2*S*, 2′*S*)-lomaiviticin aglycon (**10**), and the C-3/C-3′-dideoxy aglycon (**12**) showed a binding site size of approximately 2 base pairs per molecule (1.8, 2.0, 2.0 base pairs per ligand for **9**, **10**, and **12**, respectively). This binding site size is similar to that of well-known intercalators, such as ethidium bromide.[12] Circular dichroism (CD) and linear dichroism (LD) titration experiments using (−)-lomaiviticin aglycon (**9**) or (−)-kinamycin C (**6**), and calf thymus DNA, established an intercalative mode of binding. Sequential additions of (−)-lomaiviticin aglycon (**9**) to DNA led to small changes in the CD signal at 303 nm and a small positive induced CD at 553 nm (Figure S2A). Serial additions of (−)-kinamycin C (**6**) resulted in an induced CD at 313 nm and 406 nm, and changes in the DNA absorption region at 280 nm were also discernable (Figure S2B). The small induced CD observed in our studies suggests intercalation of the diazofluorenes into the base stack.[13] An LD titration using (−)-lomaiviticin aglycon (**9**, ratio of base pairs to drug 8:1) led to changes in the intensity of LD signal at 221 nm and 258 nm (Figure 4a). The enhancement in the negative induced LD of DNA bases arises from lengthening of the DNA helix,[14] and is consistent with the known intercalator ethidium bromide (Figure 4b). LD spectra of (−)-kinamycin C (**6**) additionally supported intercalation as the primary mode of binding (Figure S3).

To evaluate the DNA cleavage ability of these diazofluorenes, we performed a plasmid cleavage assay in the presence of the reducing cofactor dithiothreitol (DTT, Figure 5).[15] (−)-Kinamycin C (**6**) was inactive at concentrations up to 500 μM (lane 2). Surprisingly, (−)-lomaiviticin aglycon (**9**), which bound DNA with the highest affinity (vide supra), was also an ineffective cleavage agent at concentrations up to 500 μM (lanes 3–5). In contrast, the (2*S*, 2′*S*)-lomaiviticin

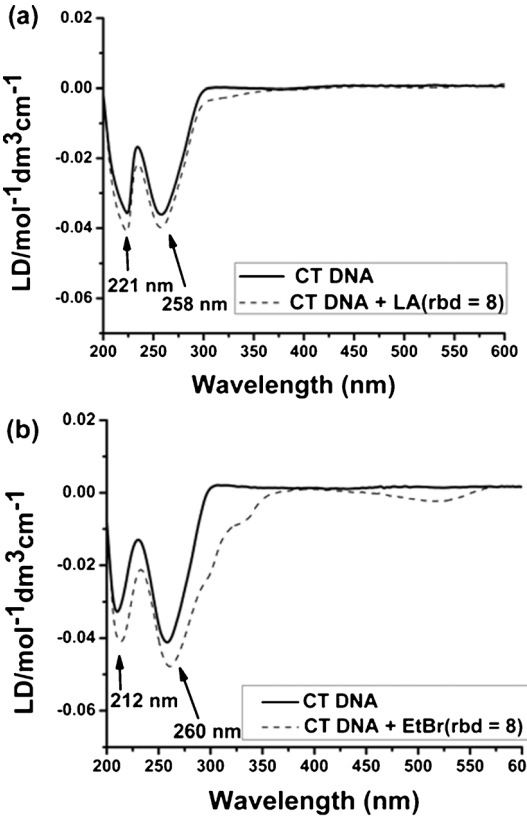

Figure 4. Linear dichroism spectra of a) (−)-lomaiviticin aglycon (**9**) and b) ethidium bromide at r_{bd} 8. Buffer conditions: 10 mM sodium cacodylate, 0.5 mM EDTA, 100 mM KCl at pH 6.8 (T = 22–23 °C). r_{bd} = ratio of base pairs to drug.

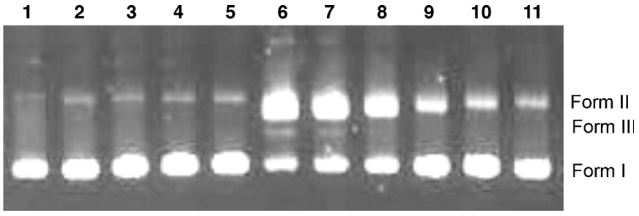

Figure 5. Agarose gel electrophoresis of the DNA cleavage of pBr322 DNA (800 ng) treated with diazofluorenes and DTT (5 mM) cofactor (24 h, 37 °C). Lane 1: pBr322 DNA. Lane 2: [**6**] = 500 μM. Lane 3: [**9**] = 500 μM. Lane 4: [**9**] = 250 μM. Lane 5: [**9**] = 125 μM. Lane 6: [**10**] = 500 μM. Lane 7: [**10**] = 250 μM. Lane 8: [**10**] = 125 μM. Lane 9: [**11**] = 500 μM. Lane 10: [**11**] = 250 μM. Lane 11: [**11**] = 125 μM. Form I = supercoiled DNA; Form II = nicked DNA; Form III = linearized DNA.

aglycon (**10**) displayed potent levels of DNA nicking (lanes 6–8). Minor amounts of DNA dsbs were observed at 500–250 μM **10** (lanes 6 and 7). The (−)-monomeric lomaiviticin aglycon (**11**) also nicked DNA in a concentration-dependent fashion (lanes 9–11). Identical cleavage activities were observed with NADPH or GSH as reductant (Figure S4).

Surprisingly, we observed that the (2*S*, 2′*S*)-lomaiviticin aglycon (**10**) and, to a lesser extent the (−)-monomeric lomaiviticin aglycon (**11**), nick plasmid DNA in the absence of added reductant (Figure 6). At concentrations of 250 μM,

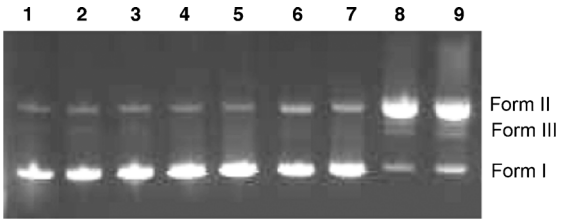

Figure 6. Agarose gel electrophoresis of the DNA cleavage of pBr322 DNA (800 ng) treated with diazofluorenes (24 h, 37 °C). Lane 1: pBr322. Lane 2: [**6**] = 500 μM. Lane 3: [**6**] = 250 μM. Lane 4: [**9**] = 500 μM. Lane 5: [**9**] = 250 μM. Lane 6: [**11**] = 500 μM. Lane 7: [**11**] = 250 μM. Lane 8: [**10**] = 500 μM. Lane 9: [**10**] = 250 μM. Form I = supercoiled DNA; Form II = nicked DNA; Form III = linearized DNA.

substantial amounts of DNA nicking were observed with **10** (lane 9). Although DNA nicking activity by **11** was considerably lower, an increase in Form II DNA was observed at 500–250 μM **11** (compare lanes 1, 6, and 7). (−)-Kinamycin C (**6**) and (−)-lomaiviticin aglycon (**9**) were inactive in this assay, as expected (lanes 2–5).

We sought to determine if the in vitro DNA cleavage activity by the (2*S*, 2′*S*)-lomaiviticin aglycon (**10**) was recapitulated in tissue culture. Production of phospho-SER139-H2AX (γH2AX) is a widely-employed marker for detection of DNA dsbs.[16] We evaluated the ability of **10**, (−)-kinamycin C (**6**), the (−)-monomeric lomaiviticin aglycon (**11**), and the C-3/C-3′-dideoxy lomaiviticin aglycon (**12**), to induce production of γH2AX in K562 cells. The cells were incubated with the diazofluorenes (1 μM) for 4 h at 37 °C, treated with an anti-γH2AX antibody conjugated to fluorescein isothiocyanate (FITC), and counted by flow cytometry (Table 2). Surprisingly, the (−)-monomeric lomaiviticin aglycon (**11**) was significantly more potent than any other compound investigated, and upregulated the production of γH2AX by 600 % relative to the control. These results point to the existence of an optimal balance of uptake and reactivity within the studied diazofluorenes that is captured by **11**. Serial dilutions of the (−)-monomeric lomaiviticin aglycon (**11**) revealed a lower limit of 50 nM for DNA dsbs production (23 % upregulation of γH2AX, Figure S5). Consistent with this finding, **11** displayed an IC_{50} = 530 nM against this cell

Table 2: H2A.X phosphorylation assay of human leukemia cells (K562) treated with diazofluorenes (1 μM) for 4 h at 37 °C.[a]

compound	geometric mean	% increase
(−)-kinamycin C (**6**)	1.41×10^4	82 %
(−)-lomaiviticin aglycon (**9**)	8.95×10^3	15 %
(2*S*,2′*S*)-lomaiviticin aglycon (**10**)	9.83×10^3	27 %
C-3/C-3′-dideoxy lomaiviticin aglycon (**12**)	1.03×10^4	33 %
*(−)-monomeric lomaiviticin aglycon (**11**)*	*5.43×10^4*	*600 %*
control	7.76×10^3	–

[a] Cells were stained for γ-H2AX. Immunological detection was performed by labeling with anti-γH2AX (Ser139) AB-fluorescein isothiocyanate conjugate. Sample analysis was performed on an Accuri flow cytometer using an 488 nm excitation laser. Emission detected with the filter/bandpass: 530/30 for FITC.

line. The other analogs **9**, **10**, and **12** were 3- to 5-fold less potent. By comparison, (−)-lomaiviticin A (**1**) is still significantly more potent ($IC_{50} = 8$ nM). The increased potency of **1** likely arises from an increased efficiency in production of DNA dsbs and the higher solubility of the natural metabolite.[5] Additionally, DNA dsb production by **11** was observed in both a cisplatin-sensitive ovarian cancer cell line (PEO1) and a cisplatin-resistant ovarian cancer cell line (PEO4, Table S1).[8]

Taken together, several important conclusions emerge from these studies. First, dimeric diazofluorenes bind DNA with higher affinity than monomeric diazofluorenes, and the primary mode of binding appears to be intercalation into the double helix. However, while the dimeric structure of the lomaiviticins increases affinity for dsDNA, this structure is not sufficient for DNA cleavage activity. Instead, our studies suggest that the D-ring carbonyl of the diazofluorenes is critical for DNA damaging activity. We have previously shown[9b] that the (2*S*, 2′*S*)-aglycon **10** and the (−)-monomeric lomaiviticin aglycon (**11**) undergo hydrodediazotization under conditions (1 equiv DTT, methanol, 37 °C) where (−)-kinamycin C (**6**) and (−)-lomaiviticin aglycon (**9**) are inert. It is likely that the D-ring carbonyl raises the oxidation potential of the diazofluorene functional group, facilitating nucleophilic addition to the diazo group and the production of vinyl radical intermediates.[5] Interestingly, the activity of the (2*S*, 2′*S*)-lomaiviticin aglycon (**10**), which is the most potent DNA damaging agent in vitro, is not recapitulated in tissue culture. Instead, the (−)-monomeric lomaiviticin aglycon (**11**) appears to present the optimal balance of reactivity, stability, and cellular uptake. As this compound is readily prepared in ten steps from 3-ethylphenol,[9b] it provides a useful starting point for translational development. Future studies will focus on increasing the potency of **11** by increasing its solubility and affinity for DNA. Given the structural dissimilarities of **1** and **11**, it seems plausible that the nature of the DNA breaks produced by the two compounds are distinct.

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