Insights into the Mechanism of DNA Cleavage by Dynemicin A as Revealed by DNA-Binding and -Cleavage Studies of Synthetic Analogs

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Insights into the Mechanism of DNA Cleavage by Dynemicin A As Revealed by DNA-Binding and -Cleavage Studies of Synthetic Analogs

Andrew G. Myers,* Scott B. Cohen, Norma J. Tom, David J. Madar, and Mark E. Fraley

Contribution No. 9059, Arnold and Mabel Beckman Laboratories of Chemical Synthesis California Institute of Technology Pasadena, California 91125

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Dynemicin A (**1**) is a potent natural antitumor agent that is capable of cleaving double-stranded DNA in the presence of a reducing cofactor such as glutathione (GSH) or NADPH. The first step in the proposed cleavage mechanism involves reduction of the anthraquinone,[1] a functional group that is unique to **1** among the enediyne antibiotics.[2] The anthraquinone is also believed to serve as a DNA-binding element by intercalation into the base stack, while the (*Z*)-enediyne bridge is positioned in the minor groove.[1a,3] Another unusual feature of **1**, as a DNA-binding molecule, is that it bears a negative charge at physiological pH, by virtue of its carboxylate group. All other members of the enediyne antibiotic family are positively charged at neutral pH, as is common for molecules that bind to the DNA polyanion.[4] By studying the DNA-binding and -cleaving properties of **1** and synthetic analogs of **1** we show that the E-ring hydroxyl groups are beneficial for binding and propose that the carboxylate group of **1** plays a critical role in the DNA-cleavage process by destabilizing the DNA−drug complex. A rationale for this finding and mechanistic detail for the dynamic process of DNA cleavage by dynemicin A (**1**) and analogs are presented.

We have recently developed a convergent synthetic route to enantiomerically pure dynemicin A (**1**) that has also provided access to dynemicin A methyl ester (**3**), dynemicin A-ring analog **5**, and the corresponding dideoxy compounds **2**, **4**, and **6**.[5] Each of the molecular pairs **1** and **2**, **3** and **4**, and **5** and **6** varies only in the presence or absence of the E-ring hydroxyl groups, whereas pair **1** and **3** and pair **2** and **4** are related as carboxylate and (charge-neutral) methyl ester. Compounds **5** and **6** may also be viewed simply as charge-neutral A-ring analogs of **1** and **2**, respectively.

Equilibrium constants for the binding of compounds **1**−**6** to double-stranded calf thymus DNA were determined by equilibrium dialysis in aqueous tris buffer solution (30 mM, pH 7.5; NaCl, 50 mM) at 25 °C using a dialysis membrane with molecular weight cutoff 12 000−14 000. The binding constants span a range of 4 orders of magnitude, from 6×10^2 M^{-1} for dideoxydynemicin A (**2**, weakest binding) to 8×10^6 M^{-1} for dynemicin A methyl ester (**3**, tightest binding, Figure 1). Comparison of binding constants for structure pairs **1** and **2**, **3** and **4**, and **5** and **6** shows that the two hydroxyl groups of the anthraquinone E-ring contribute approximately 2.7 kcal/mol in binding energy. According to models of the intercalative binding of dynemicin A to DNA, these hydroxyls penetrate into the major groove of DNA.[3] It is not unreasonable to propose that one or both of the E-ring hydroxyl groups of **1** are engaged in hydrogen bonding in the major groove of DNA.[6]

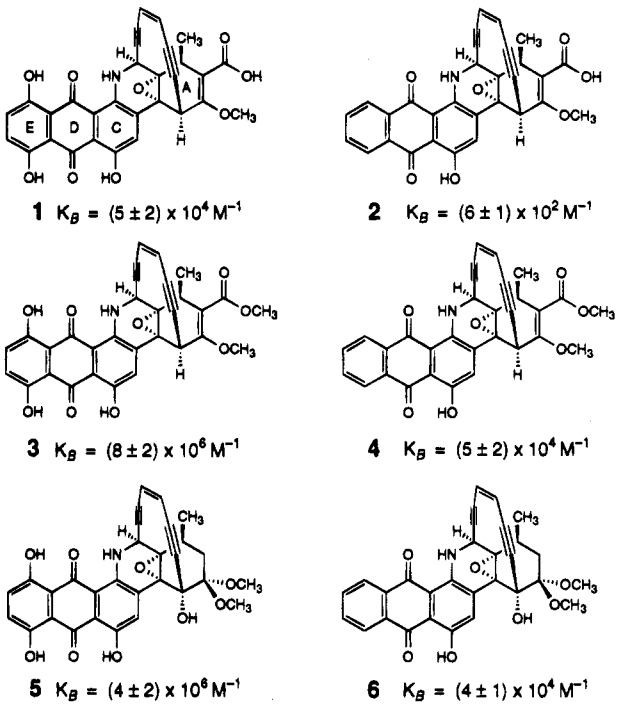

Figure 1.

Comparing binding constants of dynemicin A with its methyl ester (**1** vs **3**) and dideoxydynemicin A with its methyl ester (**2** vs **4**) reveals that neutralization of the charged carboxylate residue by methyl ester formation results in greatly increased DNA binding. This charge neutralization produces ~3 kcal/mol of stabilization of the DNA−drug complex. Binding constants for analogs **5** and **6** provide further support for this conclusion. Given the opportunity for stabilization of this magnitude by such a modest structural change, and one easily accommodated in nature (e.g., by esterification), it is reasonable to ask why nature has not chosen to modify dynemicin A (**1**) in this way. The answer to this question is apparent upon consideration of DNA-cleavage data for **1**−**6**.

DNA-cleavage reactions of a 193-base-pair restriction fragment were carried out with compounds **1**−**6** using GSH or NADPH as activating factors and were analyzed by gel electrophoresis (Figure 2). Comparison of GSH- and NADPH-induced cleavage reactions for a given compound shows that both methods of activation produce identical cleavage patterns, albeit with varying efficiencies, suggesting that both reductants produce a common intermediate in the cleavage reaction.[7] The most striking outcome of the DNA-cleavage experiments is that dynemicin methyl ester (**3**) and dynemicin analog **5** produce no detectable DNA cleavage after a 12-h reaction period. This is a surprising result considering that **3** and **5** are the strongest DNA binders ($K_B > 10^6$ M^{-1}). Monitoring of these reactions by rp-HPLC showed that **3** and **5** were unchanged in the reaction mixture. In contrast, treatment of **3** or **5** (0.1 mM) with GSH

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(2) Nicolaou, K. C.; Dai, W. M. *Angew. Chem., Int. Ed. Engl.* **1991**, *30*, 1387.

(3) (a) Langley, D. R.; Doyle, T. W.; Beveridge, D. L. *J. Am. Chem. Soc.* **1991**, *113*, 4395. (b) Cardozo, M. G.; Hopfinger, A. *J. Mol. Pharmacol.* **1991**, *40*, 1023. (c) Wender, P. A.; Kelly, R. C.; Beckham, S.; Miller, B. L. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 8835.

(4) von Hippel, V. H.; McGhee, J. D. *Annu. Rev. Biochem.* **1972**, *41*, 231.

(5) (a) Myers, A. G.; Fraley, M. E.; Tom, N. J. *J. Am. Chem. Soc.* **1994**, *116*, 11556. (b) Myers, A. G.; Fraley, M. E.; Tom, N. J.; Cohen, S. B.; Madar, D. J. *Chem. Biol.* **1995**, *2*, 33.

(6) For an example, see: Smith, C. K.; Davies, G. J.; Dodson, E. J.; Moore, M. H. *Biochemistry* **1995**, *34*, 415.

(7) The possible role of trace metals in mediating anthraquinone reduction by NADPH in the present case is under investigation.

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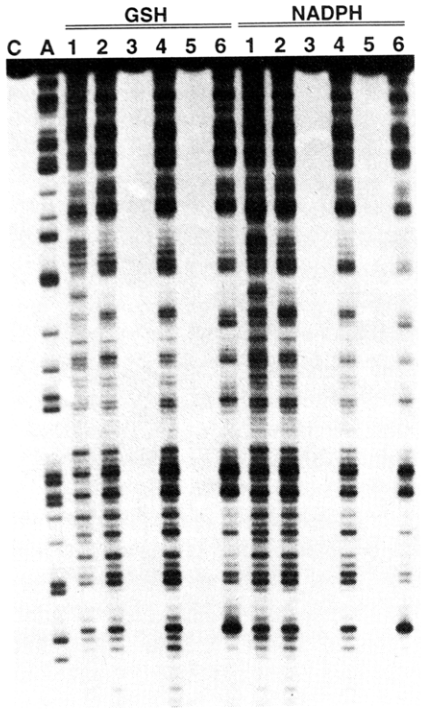
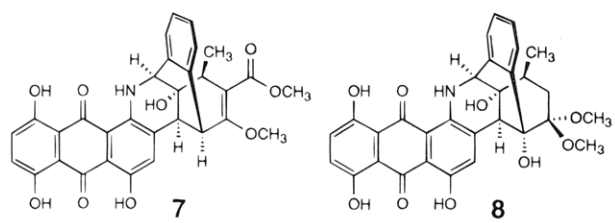


Figure 2. Cleavage of a 5′-^{32}P-labeled 193-base-pair restriction fragment of pBR322 (*Eco*RI/*Ssp*I digests) by **1**−**6** and GSH or NADPH [calf thymus DNA (1.0 mM bp), restriction fragment (~10^5 cpm), tris-HCl buffer (30 mM, pH 7.5), sodium chloride (50 mM), dynemicin A or synthetic anthraquinone (0.05 mM), 37 °C, 12 h]. Reactions initiated by addition of GSH (20 mM) or NADPH (20 mM), as indicated. Lane C: 193-bp restriction fragment alone. Lane A: products from an adenine-specific cleavage reaction (Iverson, B. L.; Dervan, P. B. *Nucleic Acids Res.* **1987**, *15*, 7823).

(5 mM) in methanol[8] (1,4-cyclohexadiene, 0.5 M; Et_3N, 0.2 M; 37 °C) in the absence of DNA led rapidly ($t_{1/2} \leq 5$ min for **3**) to the formation of aromatized products **7** and **8**, respectively, as the major reaction products.[1c,9] These results demonstrate that **3** and **5** have the capacity to form biradical intermediates in the presence of GSH, in analogy to **1**, but are prevented from doing so in the presence of DNA.



We propose that **1** and analogs **2**−**6** must dissociate from the DNA-binding complex before reductive activation can take place. Support for this proposal is obtained by analysis of the kinetics of DNA cleavage when the ratio of free to bound drug is varied ([DNA]/[drug] and [^{32}P-DNA]/[carrier DNA] held constant, for **1**: see Figure 3); DNA is observed to inhibit DNA cleavage. Together, our experiments support the idea that the intercalated anthraquinone of **1** is not reduced by GSH or NADPH at any appreciable rate.

(8) The use of methanol as a solvent is required due to the insolubility of these compounds in aqueous solution in the absence of DNA.

(9) Aromatized products **7** and **8**, as well as the analogous products derived from **2**, **4**, and **6**, were isolated (30−40% yield) and fully characterized.

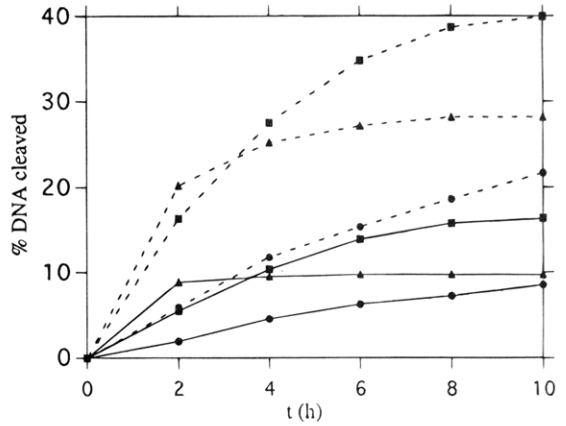


Figure 3. Reaction of **1** with GSH or NADPH at varying concentrations of DNA. Reactions were performed at 37 °C in tris-HCl buffer (30 mM, pH 7.5) containing sodium chloride (50 mM) with a constant ratio of drug to base pair DNA (1:20): (●) **1** (0.25 mM), DNA (5.0 mM bp); (■) **1** (0.05 mM), DNA (1.0 mM bp); (▲) **1** (0.01 mM), DNA (0.2 mM bp); (—) GSH (10 mM); (- - -) NADPH (5 mM).

These findings reveal the dynamic nature of DNA cleavage by **1** and its analogs. Although **1** binds to DNA with high affinity, it must dissociate prior to reductive activation. The activated intermediate(s) must then bind to DNA in order to induce DNA cleavage.[10] If the DNA-cleaving agent binds too tightly to DNA, as with compounds **3** and **5**, activation of the drug is prohibitively slow.[11] It follows that the weakest binder (**2**) should be the most reactive. This was confirmed experimentally. Monitoring the pseudo-first-order reaction of **1** and **2** with GSH in the presence of DNA by rp-HPLC revealed that **2** was some 50-fold more reactive toward GSH than **1** ($k = 5.0 \times 10^{-1}$ M^{-1} s^{-1} and 1.1×10^{-2} M^{-1} s^{-1}, respectively). The rate of DNA cleavage by **2** versus **1** in the presence of GSH was found to be correspondingly accelerated. Comparisons of the reactivity of **2** and **4** revealed similar behavior: binding affinity and reactivity are inversely related.

Although **2** is considerably more reactive than **1**, it is not necessarily a superior DNA-cleaving agent. The efficiency of DNA cleavage by **2** is found to decrease markedly with decreasing concentrations of DNA, reflecting the modest binding affinity of the corresponding activated intermediate(s) in this case. The efficiency of DNA cleavage by **1** also decreases with decreasing concentrations of DNA (compare 1.0 and 0.2 mM curves in Figure 3), but not nearly so rapidly as with **2**. For example, the efficiency of DNA cleavage using 0.2 and 1.0 mM bp DNA is 7% and 10%, respectively, for cleavage by **1** (NADPH activation, [DNA]/[drug] constant), versus ~0.5% and 5%, respectively, for cleavage by **2**. Thus, dynemicin A (**1**) appears to strike a near-optimum balance between reaction rate, where weak binding is advantageous, and cleavage efficiency, where tight binding is advantageous. If the microorganism that manufactures **1** should utilize it as a DNA-cleaving agent (an important and unresolved question), our results suggest that **1** may be highly evolved for this task.[12]

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(10) We have previously observed similar behavior for the calicheamicin−glutathione disulfide: Myers, A. G.; Cohen, S. B.; Kwon, B. M. *J. Am. Chem. Soc.* **1994**, *116*, 1255.

(11) Under forcing conditions, we were able to observe DNA cleavage by dynemicin methyl ester (**3**). These conditions involved the use of dilute solutions of DNA (1−25 μM), an increased ratio of drug to base pairs of DNA (1:10), and an increased concentration of reductant (50 mM).

(12) Generous financial support from the National Institutes of Health is gratefully acknowledged.