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Expedited Articles

Ecteinascidin 743: A Minor Groove Alkylator That Bends DNA toward the Major Groove

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The ecteinascidins (Ets), which are natural products derived from marine tunicates, exhibit potent antitumor activity. Of the numerous Ets isolated, Et 743 is presently being evaluated in phase II clinical trials. Et 743 binds in the minor groove of DNA and alkylates N2 of guanine. Although structurally similar to saframycin, which exhibits poor activity in cellular assays, Et 743 has shown good efficacy as an antitumor agent. In this study, DNA structural distortions induced by Et 743 were examined to provide insight into the molecular basis for the antitumor activity of Et 743. Electrophoretic mobility shifts of ligated oligomers containing site-directed adducts were used to examine the extent and direction of the Et 743-induced bend. Surprisingly, we find that Et 743 bends DNA toward the major groove, which is a unique feature among DNA-interactive agents that occupy the minor groove.

Introduction

The ecteinascidins (Ets) are natural products derived from the marine tunicate *Ecteinascidia turbinata* and exhibit potent antitumor activity.[1] Of the Et analogues that have been isolated, Et 743 has gained considerable attention due to its efficacy as an antitumor agent, which prompted the total synthesis of Et 743.[2] Et 743 demonstrated 1−100 pM potencies against various NCI cell lines including colon, CNS, melanoma, renal, and breast.[3] Furthermore, mice with early-stage MX-1 xenografts were all found to be tumor-free following treatment with Et 743.[4] Currently, Et 743 is being evaluated in phase II clinical trials after showing impressive activity in phase I clinical studies with responses in breast cancer and melanoma.[5−7]

Et 743 is a carbinolamine-containing antitumor antibiotic composed of three fused tetrahydroisoquinolone subunits (A−C) and is structurally related to naphthyridinomycin and the saframycin family of antibiotics (Figure 1). However, Et 743 has good efficacy as an anticancer agent, while structurally related compounds such as saframycin A have shown lower efficacy.[4,8] The main structural difference between Et 743 and saframycin is a tetrahydroisoquinolone C-subunit which is absent in saframycin A. Modification of the C-subunit affects the potency and antitumor selectivity of this class of compounds. For example, changing the C-subunit of Et 743 to a tetrahydro-β-carboline reduces the biological activity, suggesting that the C-subunit plays an important role in the cytotoxicity.[4] Recent work has shown that Et 743 and related synthetic compounds induce DNA−topoisomerase I cross-linking; however, the high dose levels required to produce this effect suggest that this may not be the primary mode of action.[9,10] The exact mechanism of antitumor activity remains to be determined.

Et 743 binds in the minor groove of DNA and alkylates the N2 position of guanine with a sequence

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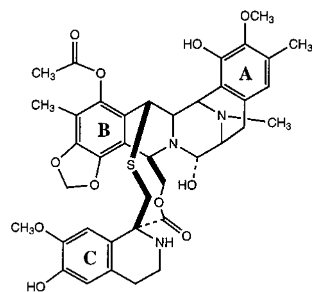
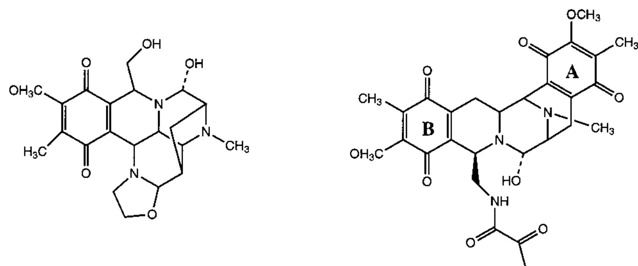


Et 743



naphthyridinomycin **saframycin A**

Figure 1. Structures of Et 743, naphthyridinomycin, and saframycin A, showing the A-, B-, and C-subunits.

A	ET21	GGTTATATAAGCTTATATAAT ATATATTCIAATATATTACCA
B	P5	AAAAATAGCTTATATTATATT TTATCIAATATAATATAATTT
	P7	AAAAATATAGCTTATATTATT TTATATCIAATATAATAATTT
	P11	AAAAATAATATAAGCTATATT TTATTATATTCIATATAATTT
	P13	AAAAATAATATATTAGCTATT TTATTATATAATCIATAATTT

Figure 2. Sequences of the oligonucleotides used in this study. Each oligonucleotide contains one Et 743 binding site (5′-AGC): (A) 21-base-pair oligonucleotide (ET21) used to determine if Et 743 bends DNA; (B) 21-base-pair oligonucleotides used to determine the direction of Et 743-induced bending. The Et 743 modification site is positioned 5, 7, 11, or 13 base pairs away from the center of an A-tract.

preference for 5′-AGC, 5′-GGC, and 5′-GGG.[11,12] The mechanism of covalent adduct formation involves the reaction of Et 743 with DNA via an iminium intermediate caused by the intramolecular acid-catalyzed dehydration of the carbinolamine functional group.[13] Furthermore, an NMR-based model of Et 743 with duplex DNA indicates that the A- and B-subunits are responsible for DNA recognition and bonding, while the C-subunit is projected out of the minor groove and makes limited contacts with the DNA.[12] Et 743 and the related Et 736 recognize DNA sequence information through a direct readout mechanism involving specific donor–acceptor pairs between the B-subunit of the drug and the minor groove.[14]

To provide further insight into the structural basis for the antitumor activity of Et 743, we studied the effect of the covalent bonding of Et 743 on DNA structure. We find that the reaction of Et 743 with DNA induces a bend in the DNA helix and that the bending directionality is toward the major groove. This is the first example of a DNA minor groove alkylator that bends DNA toward the major groove, and this property may differentiate this compound from other structurally or mechanistically similar drugs.

Results

Et 743 Bends DNA When Covalently Bound at the 5′-AGC Sequence. Gel electrophoresis is a common method used to determine alterations in DNA curvature induced by covalent adducts.[15-17] In this study, possible DNA helical distortions induced by Et 743 were examined to provide structural insight into the antitumor activity of Et 743. A 21-base-pair oligonucleotide (ET21) was designed to contain only one Et 743 alkylation site (5′-AGC) by substituting inosine for guanine on the noncovalently modified strand (Figure 2A). The oligonucleotides also contain a 3-base overhang to ensure proper head-to-tail ligation to each other. The oligonucleotide treated with Et 743 was separated from unmodified DNA by gel electrophoresis.

Nondenaturing gel analysis of the ligated 21-mers containing the Et 743–DNA adduct shows a retardation in electrophoretic mobility of the visible bands corresponding to linear DNA (L2–L6) compared to the unmodified linear multimers (compare lanes 1 and 2, Figure 3A). The ratios of the apparent length to the true length (R_L) were calculated for each ligation product (Figure 3B) based on the mobility of a reference oligonucleotide (lane 3, Figure 3A). The increase in R_L with molecular weight suggests that Et 743 bends DNA. The angle of absolute curvature was calculated based on the empirical relation described by Koo and Crothers and was found to be $17 \pm 3°$.[18] In addition, it should be noted that in the presence of Et 743 duplicity of bands occurs at the higher molecular weight DNA species (lane 2). Explanations as to why this phenomenon occurs will be addressed in the next section.

Covalent Bonding of Et 743 to the 5′-AGC Sequence Bends DNA into the Major Groove. Phasing analysis was used to confirm that Et 743 induces bending and to determine the directionality of the induced bend. It has been well-established that consecutive adenines (A-tracts) bend DNA toward the minor groove.[19-21] To determine if the Et 743-induced bending is toward the major or minor groove, the A-tract bend was used as a reference point. Oligonucleotides containing a single Et 743 alkylation site were positioned 5, 7, 11, and 13 base pairs away from the center of an A-tract (Figure 2B). These constructs place the A-tract either "in phase" or "out of phase" with the Et 743 alkylation site. Anomalous mobility was maximum when the Et 743 alkylation site is positioned 5 base pairs away from the A-tract-induced bend (Figure 4). This indicates that there is an overall bend of the DNA helix and that the bends caused by both the A-tract and Et 743 are constructive when the A-tract is out of phase with the Et 743 alkylation site. However, when the A-tract-induced bend is positioned one helical turn away from the drug binding site, the anomalous migration is at a minimum, indicating that the two bends are destructive,

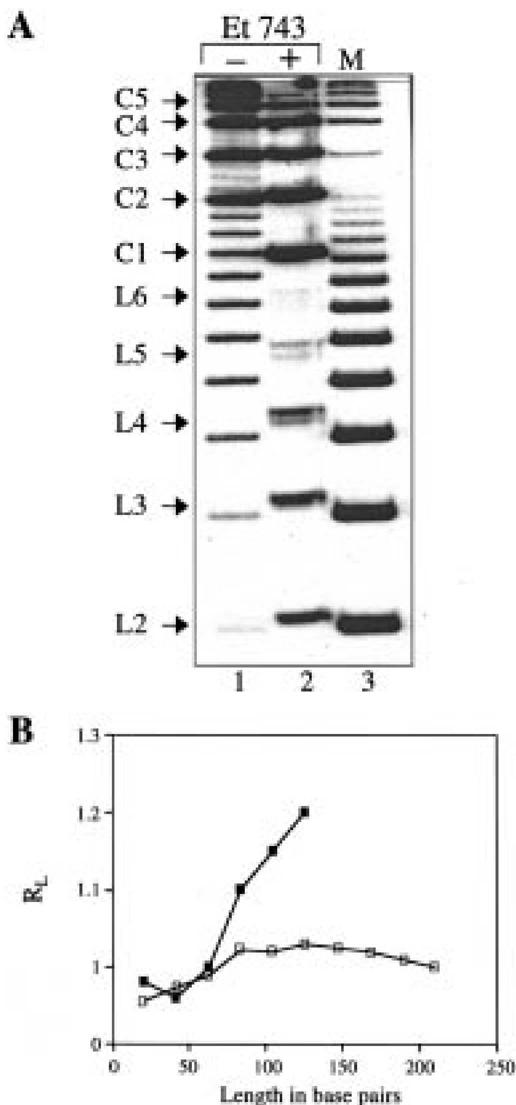


Figure 3. (A) Autoradiogram of the ligation products generated from the ET21 oligonucleotide: lane 1, ligation products of the unmodified DNA; lane 2, ligation products of Et 743-modified DNA; lane 3, marker DNA, which was used as a reference to calculate the R_L values. L2–L6 indicate the positions of the linear multimers of the Et 743-modified DNA, where L2 corresponds to the dimer and C1–C5 refer to the different sized circular DNA. (B) Plot of the relative length, R_L, as a function of total length in base pairs: (■) Et 743-modified DNA and (□) unmodified DNA. The R_L values for the higher molecular weight species that exhibit double bands were calculated for the slower migration product. The experiment was performed three times, and the R_L values were reproducible.

forming a more straight DNA structure. The R_L values were determined for each Et 743-modified oligonucleotide (Figure 4B). The R_L value is at a minimum when Et 743 is positioned one helical turn away from the A-tract, and the R_L is at a maximum when Et 743 is located one-half helical turn from the center of an A-tract. These results indicate that Et 743 bends the duplex DNA in the opposite direction of the A-tract. Hence, Et 743 bends DNA toward the major groove, which is a novel feature among minor groove DNA-interactive agents.

In addition, it is important to note that there is a greater tendency to form closed circular DNA with the ET21 oligonucleotide (C1–C4, Figure 3A) and when the

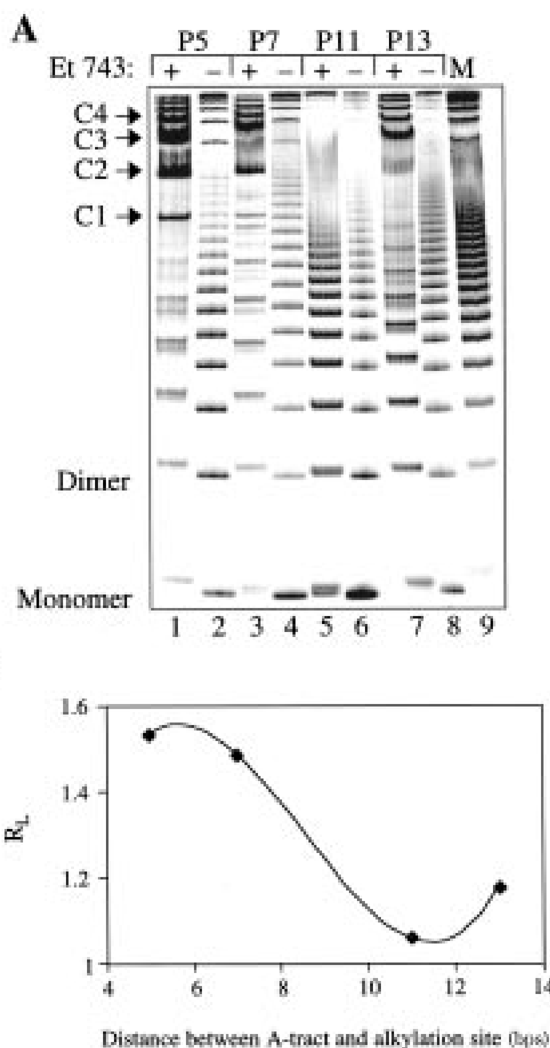


Figure 4. (A) Autoradiogram of the ligation products of the 21-base-pair oligonucleotides containing an A-tract that is variably spaced from the Et 743 modification site. Lanes 2, 4, 6, and 8 are the ligated products for the P5, P7, P11, and P13 oligonucleotides, respectively. Lanes 1, 3, 5, and 7 are the ligation products for Et 743-modified DNA. Lane 9 is marker DNA. C1–C4 refer to the circular DNA species formed in lane 1. C1 represents circular DNA 126 base pairs in size (six ligated monomer units). (B) Effect of distances between the center of an A-tract and the Et 743 alkylation site on R_L values. The R_L values were calculated based on the mobilities of the higher mobility bands for the 105-mer. The cause of the multiple bands that are seen with some of the drug-modified DNA will be addressed in future studies.

Et 743 alkylation site is 5 base pairs away from the A-tract (lane 1, Figure 4A). The circular species were confirmed using two-dimensional electrophoresis where, in the second dimension, the gel was run in the presence of chloroquine, an intercalating agent that separates circular DNA from linear DNA (data not shown). Et 743 could cause a directional bend with some flexibility, which would ease the formation of closed circular DNA.

Also, "multiple" bands occur at high molecular weight DNA when the A-tract is positioned out of phase with the Et 743 alkylation site. However, the multiplicity of the bands is lost when the A-tract is positioned one helical turn from the Et 743 binding site. There are two possible explanations for this phenomenon. One is incomplete ligation by the ligase. When Et 743 is close to the ligation site, multiple bands occur, whereas when

Et 743 is located farther from the site, the multiplicity of the bands is lost. However, if this were truly a ligation problem, then it would be expected that nicked circular DNA would form in addition to the closed circular DNA. This can be ruled out since only closed circular DNA and not nicked DNA was formed in both the absence and presence of Et 743 (data not shown). Another possible explanation involves the combination of an A-tract, which can alternate between straight and bent structures,[22] and a DNA lesion with dynamic features, both bending on the same side of the helix. It is quite striking that the duplicity of bands is maximum where the bends are on the same side of the helix (lanes 1, 3, and 7) and minimum where they are on opposite sides (lane 5).

Discussion

Et 743 is one of a group of closely related marine natural products that have potent antitumor activity and show promising activity in phase I clinical trials without the severe myelosuppression commonly shown with other minor groove DNA alkylating agents. This clinical potential sets them apart from other minor groove monoalkylating agents, such as the (+)-CC-1065 analogues Adozelesin and Carzelesin and the pyrrolo-[1,4]benzodiazepines (P[1,4]Bs) anthramycin and tomaymycin. The results described in this contribution provide structural evidence for how Et 743 differs from the other minor groove alkylating agents and thus may provide a starting point to rationalize the improved clinical efficacy of this group of drugs. The most important finding described here is that this is the first example of a *minor groove occupancy* drug that *bends DNA* into the *major groove*. In addition, there are other factors such as duplex stabilization and extrahelical protrusion of the C-subunit, which has conformational flexibility, that may also contribute to the clinical effectiveness of the Ets.

Structural Comparison of Et 743 with Other Minor Groove Monoalkylating Drugs. While the major groove, and in particular N7 of guanine, is the favored site for DNA alkylation, a number of monoalkylating compounds such as Adozelesin and Carzelesin (derived from (+)-CC-1065) and the carbinolamine-containing antitumor antibiotics such as saframycin, naphthyridinomycin, and the P[1,4]Bs specifically modify N3 of adenine or N2 of guanine and are thus minor groove alkylating agents. (We restrict our discussion to monoalkylating compounds although there are a number of clinically useful compounds, such as mitomycin C and cisplatin, that cross-link DNA in the minor and major grooves, respectively.) In comparison to (+)-CC-1065 and its related compounds, which covalently modify N3 of adenine in AT-rich regions and bend DNA into the minor groove,[17] Et 743 reacts in GC-rich regions and bends into the major groove. Thus, although both drugs have minor groove occupancy, they bend DNA in opposite directions. For the P[1,4]Bs, the picture is more complex, with a modest amount of bending (0−13°) dependent upon both drug and bonding sequence.[23]

In comparison to other carbinolamine antitumor antibiotics that also alkylate N2 of guanine in GC-rich regions, the Ets are more wedge-shaped than the helical-twisted shape of the P[1,4]Bs and the planar saframycin and naphthyridinomycin.[24−26] This wedge shape is due to the C-subunit, which imparts rigidity to the drug molecule and for covalent adduct formation forces the minor groove to widen and concomitantly bends DNA into the major groove. Also, in contrast to other carbinolamine antibiotics, Et 743 and related drugs interact with the minor groove of DNA through an extensive network of hydrogen bonds, which presumably results in enhanced stability of the duplex DNA.[14]

How Might Et 743 Produce Different Biological Consequences Than Other Minor Groove Alkylating Agents? It is the structural and perhaps dynamic effects of the DNA modification by DNA-reactive drugs that give rise to the biochemical and biological consequences. Clearly, Et 743 is quite likely to be different from the other minor groove alkylating compounds in these regards. First, the bending of DNA and its directionality are quite distinctive. As described before, Ets occupy the minor groove but bend into the major groove. Many if not all DNA-interactive compounds produce their biological consequences through interaction with DNA−protein complexes such as topoisomerases or by hijacking other proteins that may hinder repair. There is evidence that Et 743 may produce protein−DNA cross-links in cells due to targeting the topoisomerase I−DNA complex,[10] and there are suggestions that the extrahelical protrusion of the C-subunit might be involved in hijacking.[27] Indeed, we have evidence for the stabilization of protein complexes on DNA at the site of Et 743 adducts (unpublished results, M. Zewail-Foote and L. H. Hurley). If the bending and minor groove occupancy are recognized by the excision repair system, the effective repair may be hindered by the duplex stabilization of the Et 743−DNA lesions.

In conclusion, the structural consequence of the additional C-subunit found uniquely in the Ets is a rigid, wedge-shaped molecule that occupies the minor groove, forcing a widening of this groove and causing bending into the major groove. We propose that this novel occupancy and groove bending lead to interaction with specific protein−DNA complexes, and this may in part be responsible for the promising clinical activity of these compounds.

Experimental Section

Preparation and End-Labeling of Oligonucleotides. Oligonucleotides were synthesized on an Expedite 8900 nucleic acid synthesis system (Perseptive Biosystem). The oligonucleotides were eluted out of the column with aqueous ammonia and deprotected by heating at 75 °C for 1 h followed by polyacrylamide purification. The 5′-end-labeled oligonucleotides were obtained using T4 polynucleotide kinase (New England Biolabs), [γ-^{32}P]ATP (Amersham), and cold ATP. The single-stranded oligonucleotides were annealed to their complementary strand, and the annealed duplex DNA was passed through a Bio-Rad chromatography column to remove free ATP.

Drug Reactions and Ligation Experiments. The labeled double-stranded oligonucleotides were incubated with 50 μM Et 743 in buffer containing 10 mM Tris-HCl (pH 7.5) and 25 mM NaCl for 2 h at room temperature. The reactions were stopped by the addition of 0.05% SDS followed by ethanol precipitation. The Et 743-modified DNA was separated from unmodified DNA by gel electrophoresis. After eluting the DNA from the gel, the modified and unmodified DNA were then self-ligated with 200 U of T4 DNA ligase (New England Biolabs)

overnight at 16 °C. The ligation mixtures were separated on an 8% nondenaturing polyacrylamide gel at 4 °C.

Calculation of Band Mobilities. The electrophoretic mobilities of each band were converted to relative length (R_L) by the following equation: $R_L = L_a/L_r$, where L_a is the apparent length of the multimer and L_r is the real length of the multimer. The gel mobilities of each multimer were compared to the mobilities of the ligated products of a reference oligonucleotide. L_a is determined from the size of the reference oligonucleotide multimer whose mobility corresponds to the multimer of interest.

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