Differential Rates of Reversibility of Ecteinascidin 743−DNA Covalent Adducts from Different Sequences Lead to Migration to Favored Bonding Sites

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Abstract: Ecteinascidin 743 (Et 743), one of a series of structurally related antitumor antibiotics isolated from a marine tunicate, is currently in phase II clinical trials. Et 743 alkylates guanine N2 through the minor groove of DNA. Hydrogen-bonding networks that associate the drug with a three base pair DNA recognition site have been proposed to contribute to both the reactivity and the stability of the Et 743−DNA adduct. Here, we report that the reaction of Et 743 with DNA is reversible under nondenaturing conditions and that the rate of this reverse reaction depends critically upon the DNA-modified sequence. Quite unexpectedly, it was found that although the rates of alkylation are similar for the 5′-AGT and 5′-AGC sequences, reversal from the 5′-AGT sequence occurs faster than from the 5′-AGC sequence. Consequently, it is the differences in the rate of the reverse reaction that dictate the sequence selectivity of Et 743 toward its favored target sequence. As a direct consequence of the reversible nature of Et 743 with DNA, Et 743 can migrate from the nonfavored bonding sequence (e.g., 5′-AGT) to the favored DNA target site (e.g., 5′-AGC). The data suggest that the observed differences in the rate of reversibility arise from differences in the stability of the Et 743−DNA adduct at the 5′-AGT and 5′-AGC target sequences. On the basis of gel electrophoresis and ^{1}H NMR experiments, the Et 743−AGT adduct is less stable, has more dynamic motion, and produces different conformational changes in the DNA than the more stable Et 743−AGC adduct. The shuffling of Et 743−DNA adducts to the more stable alkylation sites has important implications for understanding the underlying relationship between the structural modification of DNA by Et 743 and its biological potency and efficacy in tumor cells.

Introduction

Ecteinascidin 743 (Et 743), a natural product derived from the marine tunicate *Ecteinascidia turbinata*, is a potent antitumor agent currently in phase II clinical trials following demonstrated antitumor activity in phase I trials.[1−4] The mode of action of Et 743 is believed to be related to its covalent reaction with DNA through the exocyclic 2-amino group of guanine. Et 743 (Figure 1) is composed of three subunits (A, B, and C) and occupies and bonds covalently in the minor groove of DNA, concomitantly bending the duplex toward the major groove.[5] The A-subunit, which is positioned 5′ to the site of covalent attachment to DNA, and the B-subunit of Et 743 provide the scaffold for DNA recognition and covalent bonding. The C-subunit protrudes out of the minor groove, making quite limited contacts with the DNA.[6] DNA covalent adduct formation is believed to be mediated through an intramolecular acid-

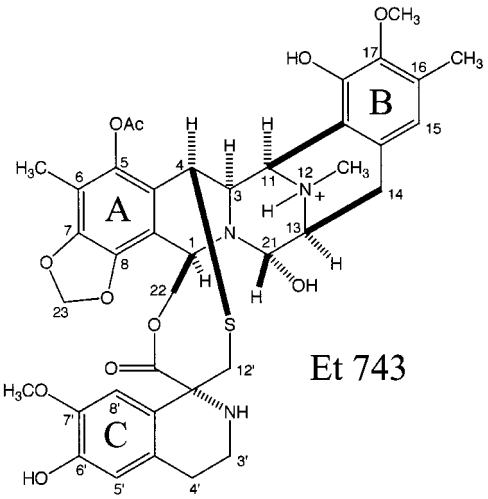

Figure 1. Structure of ecteinascidin 743 (Et 743) showing the A-, B-, and C-subunits.

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catalyzed dehydration of the carbinolamine moiety, resulting in a cyclic iminium ion intermediate, which alkylates N2 of guanine.[7]

The site selectivity of Et 743 is believed to be governed by a three base pair recognition sequence where the flanking base pairs immediately 5′ and 3′ to the modified guanine are involved in stabilizing the drug−DNA adduct.[8] By oligonucleotide shift assays and high-field NMR spectroscopy, it has been determined that the favored base 3′ to the covalent attachment site is either a guanine or a cytosine.[8,9] Additionally, a pyrimidine is favored for the base 5′ to the target guanine when the base to the 3′-side is a guanine, and a purine is favored for the base 5′ to the covalent attachment site when the base to the 3′-side is a cytosine. There are a total of three hydrogen bond contacts between the A- and B-subunits of Et 743 and the three base pair recognition sequence, the most critical being the interaction of the B-subunit with the base located 3′ to the modification site. When this base is adenine or thymine, the site becomes a nonfavored sequence toward Et 743 due to the loss of an amino group hydrogen bond donor. This hydrogen bond network between Et 743 and the triplet target sequence stabilizes the covalent adduct and has been proposed to direct the course of the sequence recognition by a direct readout mechanism.[8] A recently published molecular modeling study[10] has provided additional support for this proposal. Since the designated favored sequences (5′-PuGC and 5′-PyGG) form adducts that are stabilized by hydrogen bonds, then adducts at the apparent nonfavored sequence 5′-NG(A/T), which cannot form the hydrogen bond with the base 3′ to the modified guanine, are predicted to be much less stable. These previously reported studies provided a possible molecular basis for the recognition of, and reactivity toward, different DNA sequences by Et 743.[8−10] Experiments that indicated differential stability of the high and low reactivity sequences prompted us to further explore the premise behind the apparent reactivity of Et 743.

In this contribution, we have compared the covalent reactivity and reversibility, stability, and conformational differences of Et 743 at two different target sequences, the favored 5′-AGC and the nonfavored 5′-AGT. Our results demonstrate that the alkylation reaction of Et 743 with both favored and nonfavored sequences is reversible under nondenaturing conditions. More importantly, the reversibility of Et 743 from the nonfavored sequence occurs at an enhanced rate. In contrast, we find that the reaction of Et 743 with DNA occurs at the same rate at both favored and nonfavored target sequences. Therefore, we propose that it is the rate of covalent reversibility, and not the covalent reaction rate, that governs the observed specificity of Et 743 toward the sequences previously reported. While the migration of a noncovalently bound DNA-interactive compound from nonspecific to specific sites has been demonstrated,[11−13] the present study provides a novel mechanism for how sequence selectivity of covalent adducts can be achieved.

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Experimental Section

Preparation and End-Labeling of Oligonucleotides. Oligonucleotides were synthesized on an Expedite 8900 nucleic acid synthesis system (PerSeptive Biosystems), eluted out of the column with saturated ammonium hydroxide, and deprotected by heating at 75 °C for 1 h. The oligomers were dried under vacuum and redissolved in formamide loading buffer, and the individual strands of oligomers were then purified on a 20% denaturing polyacrylamide gel. To construct the 5′-end-labeled duplex DNA, the DNA strands (500 ng) were incubated in a 20 μL solution containing 70 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 5 mM dithiothreitol, 40 μCi of [γ-^{32}P]ATP (Amersham), and 10 units of T4 polynucleotide kinase (New England Biolabs) for 2 h at 37 °C. The resulting labeled oligonucleotides were annealed to their complementary strand in annealing buffer [10 mM Tris-HCl (pH 7.5) and 0.1 M NaCl] by heating the reaction mixtures to 90 °C and allowing them to cool slowly to 4 °C overnight. The resulting duplex DNA was then passed through a Bio-Rad chromatography column to remove free ATP and single-stranded DNA. For the helicase assay, the labeled duplex DNA was further purified on a 16% native polyacrylamide gel. The DNA band was excised from the gel and extracted by shaking overnight with annealing buffer.

Translocation Experiments. The radiolabeled 22-mer was incubated with 20 mM Et 743 in buffer containing 10 mM Tris-HCl (pH 7.5) and 20 mM NaCl for 2 h at room temperature and then passed through a Bio-spin column (Bio-Rad) twice and ethanol precipitated to remove free drug. The resulting pellet was incubated with the 30-mer duplex in 10 mM Tris-HCl (pH 7.5) and 20 mM NaCl. An aliquot was removed at the times indicated and frozen at −70 °C. The aliquots were then run on a native 16% polyacrylamide gel at 4 °C.

Temperature Stability Experiments. The labeled 22-mer was incubated with 20 mM Et 743 at room temperature for 2 h. The reaction was stopped with 0.5% SDS and ethanol precipitated to remove unbound drug. The resulting pellet was dried under vacuum, resuspended in 15 μL of deionized water, and immediately loaded on a 20% native gel at 4 °C. The gel was exposed to X-ray film, and the DNA band corresponding to the completely modified DNA was excised from the gel and extracted with 400 μL of annealing buffer at 4 °C. The eluted DNA was then incubated at 22, 37, 42, 55, and 90 °C for 10 min and immediately loaded on a 16% polyacrylamide gel run at 4 °C.

Proton NMR Experiments. 5′-CATAAGTTAAG-3′ and its complementary sequence were synthesized on a DNA synthesizer (PerSeptive Biosystems Expedite 8909), purified by reverse-phase HPLC, and dialyzed extensively against deionized water. Preparation of the Et 743 adduct involved the reaction of 15 mg of 5′-AGT 11-mer in buffer [800 μL of 100 mM KCl, 50 mM potassium phosphate (pH 7.0), and 1 mM EDTA] with 3.0 mg of Et 743 in 150 μL of DMSO. Unreacted drug was removed by centrifugation, and the DMSO was removed through repeated evaporations under vacuum using multiple additions of D_2O. One- and two-dimensional 500 MHz ^{1}H NMR data sets in H_2O- and D_2O-buffered solution were recorded on Bruker AMX 500 and Varian UNITYplus 500 FT NMR spectrometers.

Results

The Rate of Reversal of Et 743 from Drug-Modified 5′-AGT Is Faster than That from Drug-Modified 5′-AGC Sequences. The first indication that the Et 743 alkylation reaction is reversible came from initial endeavors to create a 60-mer oligonucleotide containing an Et 743 site-directed adduct at either a 5′-AGT or 5′-AGC sequence. The substrates were constructed by ligating a duplex oligonucleotide modified with Et 743 at a single guanine to flanking duplex oligonucleotides on both the 5′- and 3′-sides. Although we succeeded at creating a site-directed adduct at a 5′-AGC sequence, attempts at generating a site-directed adduct at the 5′-AGT sequence failed. After several ligation and purification steps, it was observed that the ligated 60-mer no longer contained Et 743 covalently

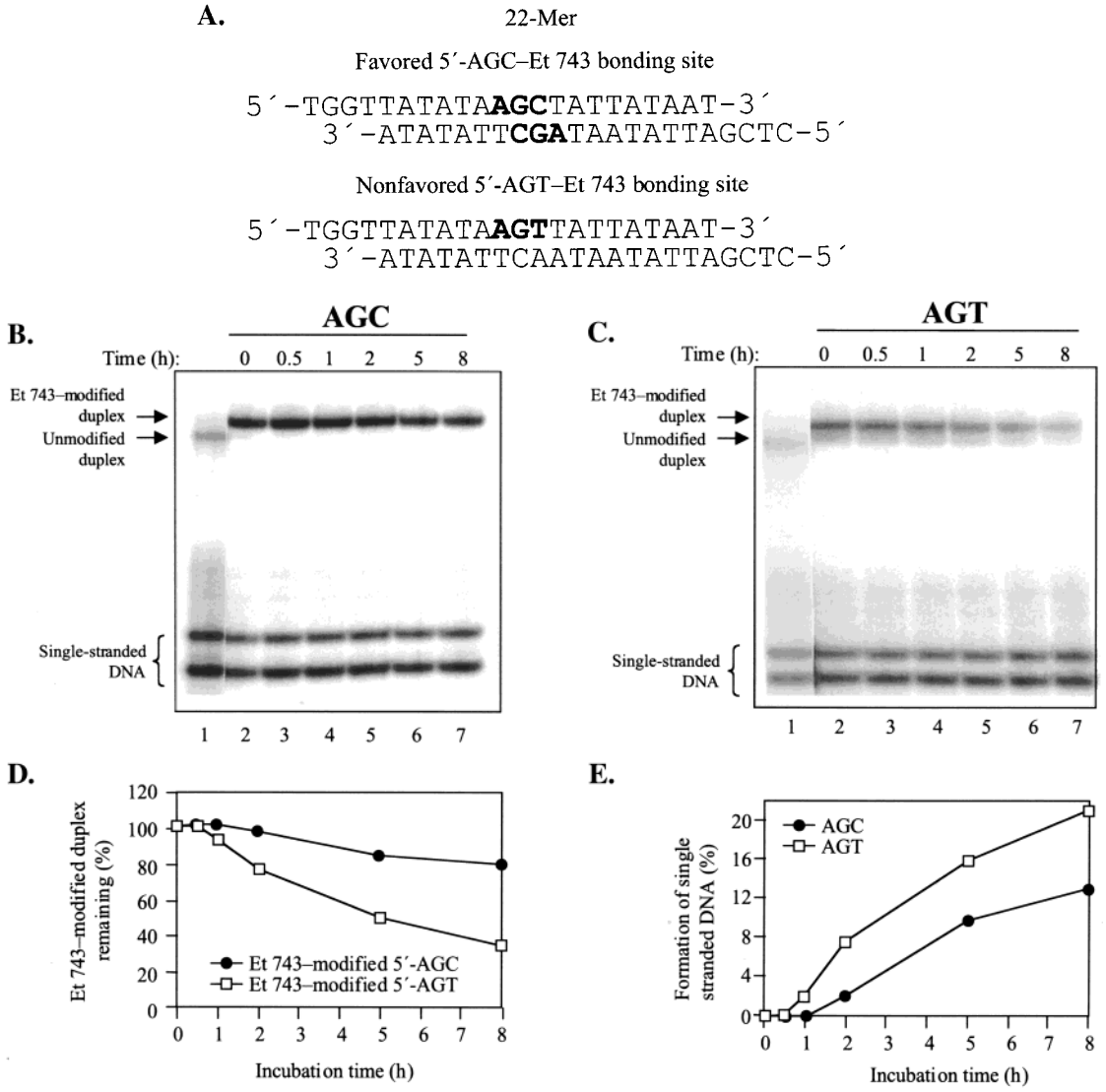

Figure 2. Time dependency of the reduction of Et 743 adducts at different target sequences. (A) Sequence of the 22-mer duplex DNA containing either the favored 5′-AGC or the nonfavored 5′-AGT Et 743 target sequence. These oligonucleotides, containing either a single 5′-AGC (B) or 5′-AGT (C), were incubated with 20 μM Et 743. The resulting covalent adduct was incubated for the indicated amount of time at room temperature and loaded on a native gel. (D) Plot of the amount of each drug-modified duplex, expressed as a percent, over time. The intensity of the band corresponding to Et 743-modified DNA at the designated times is normalized to the intensity at time zero, which is expressed as 100%. (E) Plot of the relative amount of single-stranded DNA being formed over time. The amount of single-stranded DNA is expressed as the percent increase in the formation of single-stranded DNA over time with respect to the amount of single-stranded DNA at time zero.

bound at the 5′-AGT sequence. This result prompted us to compare the stability of the Et 743−DNA adduct at the 5′-AGC versus 5′-AGT sequences.

Previous reports indicate that drug modification produces a gel shift during electrophoresis;[5,9] hence, by quantification of the intensity of the retarded band, the level of Et 743 modification can be determined. This band shift assay was used to monitor the amount of drug-modified duplex in order to determine if the covalent adduct is stable over time. A 22-mer oligonucleotide containing a single modification site, either 5′-AGT or 5′-AGC, surrounded by a common sequence (Figure 2A), was modified completely with Et 743. Because the 22-mer is largely made up of the alternating dinucleotide AT, it is inherently unstable. This results in the single-stranded DNA found in the gel. The Et 743−DNA adducts were then monitored at room temperature for the indicated amounts of time (Figure 2B,C), and the intensity of the Et 743−DNA complex was quantified (Figure 2D). The level of Et 743-modified DNA decreases over time for both Et 743 target sequences, and the level of single-stranded DNA increases (Figure 2E), suggesting that the modified duplex melts to single-stranded DNA. At the end of the 8 h time period, the amount of the Et 743−DNA adduct at the 5′-AGC sequence was reduced by approximately 20%, whereas the amount of the Et 743−DNA adduct at the 5′-AGT sequence decreased by over 60% (compare lanes 7 in panels B and C of Figure 2). This 3-fold difference indicates that the Et 743 adduct at the 5′-AGC sequence is more stable than the Et 743 adduct at the 5′-AGT sequence under the conditions used here. Furthermore, the results suggest that Et 743 can reverse from DNA and that the rate of reversal depends on the base pair immediately 3′ to the site of covalent attachment of Et 743 (5′-AGC versus 5′-AGT). The observed reduction in the amount of Et 743−DNA adducts with time prompted us to look at the possibility that the released Et 743 might be able to "walk" the DNA, migrating from one sequence to another.

Et 743 Can Reverse from Its Initial Covalent Adduct Site and Bond to an Unmodified Target Sequence. In principle, once Et 743 is released from the covalent modification sites, it could alkylate other available target sites. To determine if Et 743 can rebond at other recognition sequences, Et 743-modified oligonucleotides (22-mer) containing the site-directed adducts at either the 5′-AGC or 5′-AGT target sequence were incubated with a different size oligonucleotide (30-mer) (Figure 3A) containing either the favored or the nonfavored Et 743 target sequence, respectively. Figure 3C shows that as the level of the Et 743-modified 5′-AGT adduct decreases over time, the other duplex (30-mer) containing the favored drug target sites becomes modified with Et 743. After 10 h, approximately 20% of the 30-mer is modified by Et 743, suggesting that as Et 743 reverses from the nonfavored 5′-AGT target sequence, it can rebind another DNA strand containing the favored target sites (Figure 3D). The same profile can be seen when the 5′-AGC sequence is modified, but to a much lesser extent (Figure 3B). Here, less than 10% of the 30-mer becomes modified with Et 743 after 10 h (Figure 3D). These results support the notion that the reaction of Et 743 with duplex DNA is reversible and that, in the presence of competitor DNA, free drug released from the covalent DNA adducts can alkylate other available DNA sites, particularly when the initially alkylated sequence is a nonfavored sequence. Just as in the case of experiments shown in Figure 2, the duplexes melt after the release of drug and appear as single-stranded DNA. No evidence of mixed oligomers (i.e., 22- and 30-mer) is found.

Upon dealkylation, the accumulation of duplex DNA is not observed under the described conditions (Figure 3B,C). There are two possible explanations for this result. First, Et 743 may reverse, leaving duplex DNA, which then proceeds to melt due to the decrease in melting temperature brought about by reduced hydrogen bonding. Second, covalently bound Et 743 could induce transient melting in the DNA, nucleating the duplex to melt. It has been reported that Et 743 reacts only with duplex DNA and not single-stranded DNA; hence, Et 743 could not realkylate the DNA once the duplex has melted.[9]

Et 743 Reversal Is Independent of the Inherent Duplex Stability of the Oligonucleotide. To determine if the observed reversibility is due to the instability of the duplex DNA, the same time−course experiment was repeated, but rather than using site-directed adducts, a more stable 30-mer duplex containing either multiple favored or nonfavored target sequences was modified with Et 743. This duplex has a substantial increase in melting temperature due to the increase in the number of GC base pairs. A radiolabeled 30-mer DNA containing either nonfavored or favored target sequences was incubated with an unlabeled Et 743-modified 30-mer containing either favored or nonfavored target sites. A shift in mobility occurs for the radiolabeled 30-mer corresponding to Et 743-modified DNA. The oligonucleotide containing the favored sequences becomes preferentially modified with Et 743 when it is incubated with the Et 743-modified DNA containing the nonfavored target sequences (panel A in Figure S1; Supporting Information). The multiple bands are most likely indicative of Et 743-modified DNA containing more than one Et 743−DNA adduct, and therefore the data represent a summation of adducts. By 10 h, the radiolabeled 30-mer is near complete modification. In the converse experiment, the Et 743-modified DNA containing the favored sequences is incubated with the radiolabeled, unmodified DNA containing the nonfavored sequences (panel B in Figure S1; Supporting Information). Here, about 40% of the unmodified 30-mer containing the nonfavored Et 743 bonding

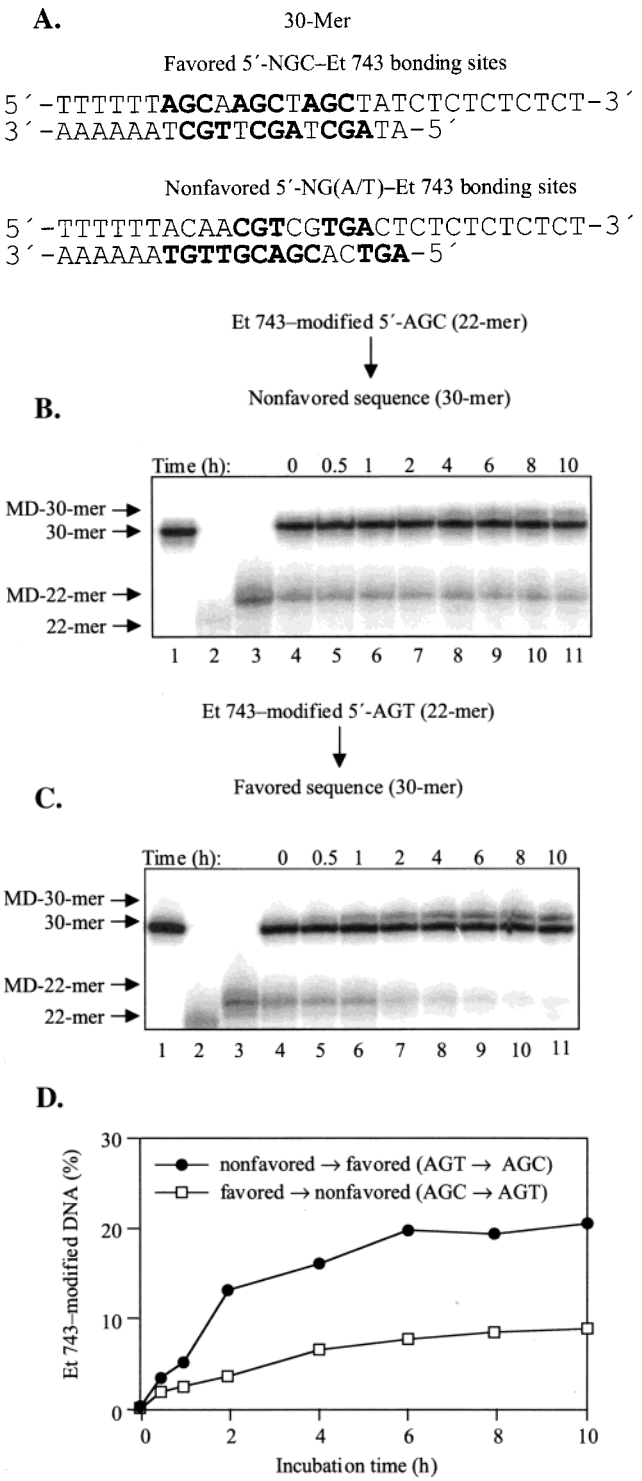

Figure 3. Translocation of covalently bound Et 743 from its DNA adduct to another, unmodified target sequence. (A) Sequence of the 30-mer containing either the favored or nonfavored Et 743 target sites. (B) A 30-mer containing only favored Et 743 target sequences was incubated for the indicated amount of time with an Et 743-modified 5′-AGC site-directed adduct (22-mer). The resulting mixture was run on a 16% native gel. Arrows point to the sites of the unmodified duplex and the Et 743-modified duplex (MD). Lanes 1−3 contain the unmodified 30-mer, the unmodified 22-mer (AGT), and the Et 743-modified 22-mer (AGC), respectively. Lanes 4−11 contain the incubation mixture at various times after the reaction was started. (C) As in (B) but with AGT rather than AGC. (D) The amount of drug-modified duplex was quantified and plotted as a function of incubation time.

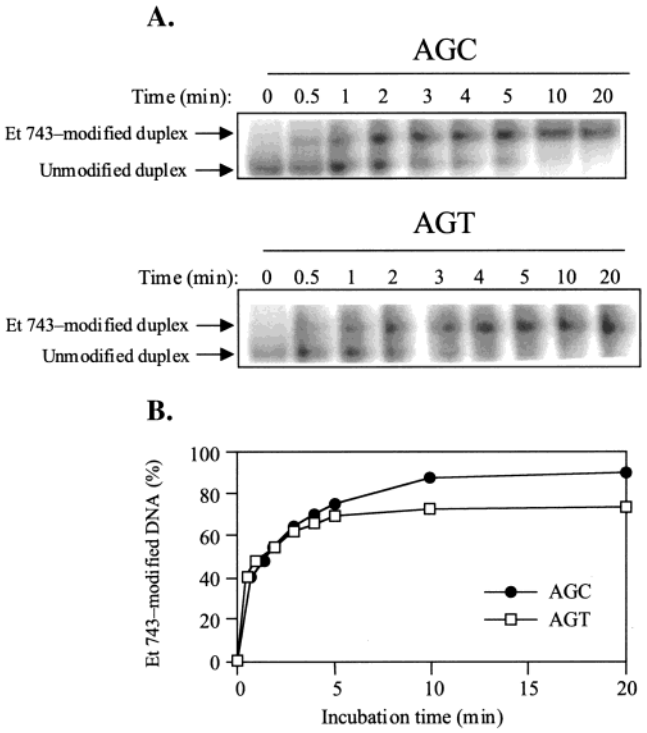

Figure 4. Time−course experiment showing the reaction of Et 743 with DNA. (A) An oligonucleotide containing either the 5′-AGC or 5′-AGT sequence was reacted with 20 μM Et 743 for the indicated amount of time. The reaction was immediately loaded on a 16% native gel. (B) The amount of Et 743-modified duplex DNA was quantified and plotted as a function of time.

sites is modified with Et 743 compared to 95% observed for the DNA containing the favored Et 743 target sites (panel C in Figure S1; Supporting Information). These results suggest that Et 743 is even able to reverse from the more stable oligonucleotide and that the observed reversal is independent of the stability of the DNA duplex, and therefore is most likely due to local breathing effects. Furthermore, the results support the previous data (Figure 3) where Et 743 reverses more readily from the nonfavored sequence and rebinds the favored sequence.

The Kinetics of the Covalent Modification of 5′-AGT and 5′-AGC Sequences by Et 743 Are Similar. In addition to differences in the rate of the reverse reaction, there could also be significant differences in the rate of DNA alkylation at different bonding sequences. To measure the relative kinetics of the forward reaction in different Et 743 target sequences, the 22-mer duplex DNA containing either the 5′-AGC or 5′-AGT sequence (Figure 2A) was used to monitor the formation of Et 743-modified DNA as a function of time. Although the reaction of Et 743 with both target sequences is equally fast up to 3 min, the final extent of modification is slightly less for the 5′-AGT sequence (Figure 4A,B). In the reaction of Et 743 with the 5′-AGC sequence, 75% of the duplex DNA becomes modified after 5 min, whereas with the 5′-AGT sequence, about 65% of the DNA is modified in the same time period. This small variation probably results from differences in equilibrium between covalently bound and unbound Et 743 at favored and nonfavored sequences.

DNA Flanking Sequences Can Affect the Rate of DNA Alkylation and the Rate of Reversibility. The rate of alkylation of DNA by alkylating drugs such as the anthramycins and pluramycins is sensitive to flanking sequence-dependent variations in DNA helical structure.[14,15] For this reason, we wanted to determine if the rates of both the forward and reverse reactions of Et 743 with DNA are also affected by structural variations outside the target sequence. More specifically, we introduced a minor groove bend in the DNA by incorporating an A-tract into the DNA sequence. Hence, by altering the flanking sequence surrounding the Et 743 alkylation site, the overall structure and dynamics of the DNA are modified as well. The Et 743 alkylation site was positioned either five or ten base pairs away from the center of the A-tract (Figure 5A). First, the reactivity of Et 743 was measured using gel electrophoresis, as described before. Regardless of the three base pair Et 743 target recognition sequence, Et 743 is initially more reactive at the guanine located ten base pairs from the center of the A-tract bend (Figure 5B,C). However, after 5 min, both sequences are fully modified. The initial slower reactivity of the sequence nearest the A-tract may be explained by the narrowing of the minor groove induced by the A-tract. With this more proximal change in width of the minor groove, Et 743 might be unable to achieve the optimum juxtaposition required to promote catalytic activation of the drug, and as a result, the initial rate of reaction would be slower.

When the stability of the Et 743−DNA adducts with the A-tract was compared, again using the gel shift assay with the duplex DNA shown in Figure 5A, the Et 743−DNA adducts were surprisingly stable, regardless of the DNA target sequence or the distance from the A-tract (Figure 6). Since Et 743 bends DNA toward the major groove and the A-tract is toward the minor groove, the overall structure of the DNA is varied, depending on the distance of the site of alkylation from the A-tract.[16−18] When Et 743 is covalently bound five base pairs away from the center of the A-tract, the result is an overall bent DNA structure. In contrast, when the Et 743-induced bend is positioned ten base pairs (about one helical turn) away from the A-tract-induced bend, the overall structure of the DNA is straight. Both DNA structures (straight and bent) appear to stabilize the Et 743−DNA adduct independent of the Et 743 target sequence. This result is in sharp contrast to the previous results where, with a different flanking sequence, Et 743 could reverse from the target sequence, and the rate was dependent on the bonding sequence.

Differential Stability of Et 743−AGC and Et 743−AGT Adducts. (A) Covalent Bonding of Et 743 at the 5′-AGC Sequence Confers More Stability on the Duplex DNA than Bonding at the 5′-AGT Sequence. The reason for the differences in the rate of the reverse reaction may be derived from structural and/or dynamic differences at the two target sequences. To validate our assumption about the stability of the Et 743−AGC adduct versus the instability of the Et 743−AGT adduct, the relative stability of the two DNA adducts was monitored as a function of temperature. An Et 743-modified oligonucleotide containing a single alkylation site (either 5′-AGC or 5′-AGT) was gel purified. The presumably completely modified DNA was then excised, eluted from the gel piece, and incubated at the indicated temperatures. The results show that, under these conditions, most of the unmodified duplex containing a 5′-AGC sequence melts at a temperature slightly under 32 °C (panel A in Figure S2; Supporting Information). The

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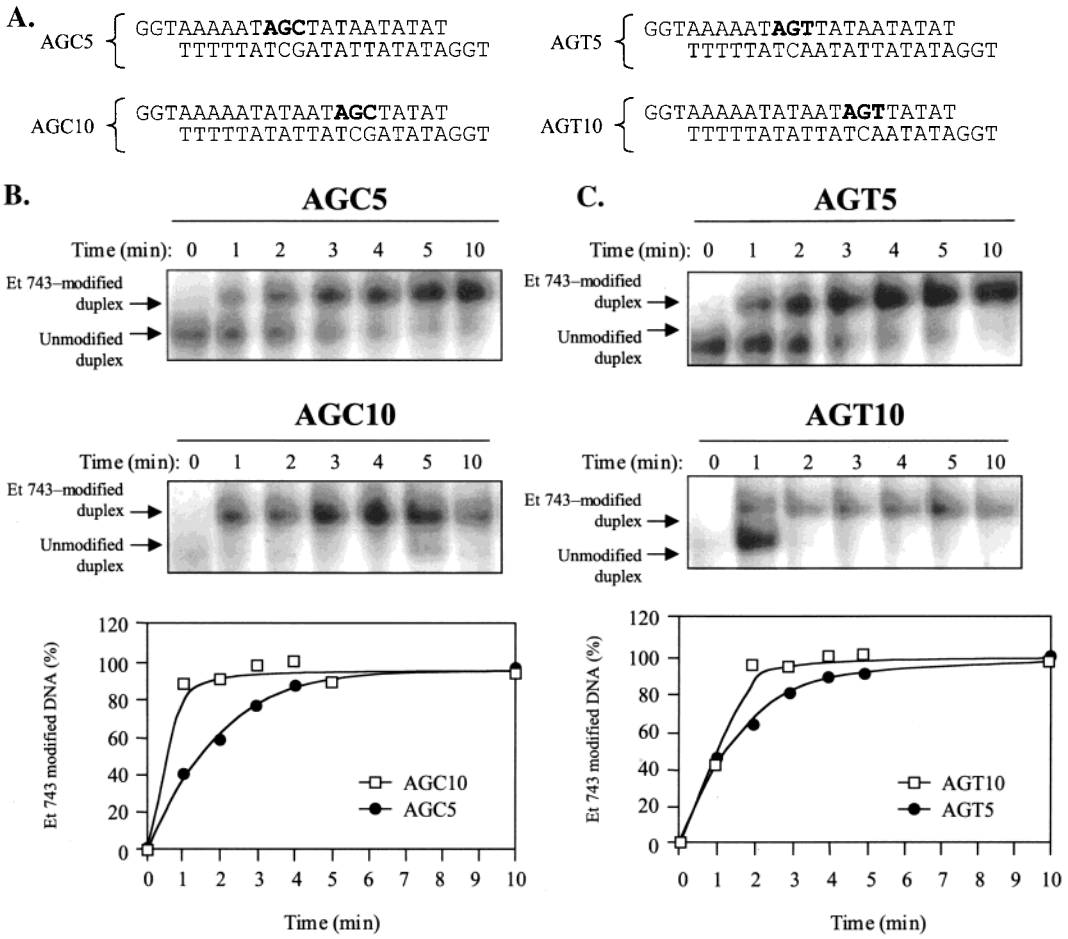


Figure 5. Intrinsic bending induced by an A-tract modulates the rate of alkylation of Et 743. (A) DNA sequences used in this study. The sequences AGC5 and AGT5 contain either an AGC or AGT alkylation site five base pairs away from the center of an A-tract bend. AGC10 and AGT10 represent the sequence when the Et 743 target site (AGC or AGT) is positioned ten base pairs away from the center of the A-tract-induced bend. (B) Oligonucleotides AGC5 (top panel) and AGC10 (middle panel) were incubated with 20 μM Et 743 for the times indicated. The reactions were stopped and immediately loaded on a 16% polyacrylamide gel. (C) Oligonucleotides AGT5 and AGT10 were reacted with Et 743 for the times indicated. The lower panels show a graphical representation of the results.

continued presence of a small amount of duplex at the higher temperatures is indicative of reannealed DNA. Upon modification with Et 743, the DNA adduct is much more stable than the duplex, as evidenced by the increased amount of duplex DNA that occurs in the presence of Et 743 at 22, 37, and 42 °C. The majority of the modified DNA at the 5′-AGC sequence melts between 42 and 54 °C.

In the case of the 5′-AGT sequence, most of the unmodified duplex also melts at a temperature slightly under 32 °C (panel B in Figure S2; Supporting Information). Upon DNA alkylation, about 30% of the modified DNA is stable up to 42 °C. Although both adducts stabilize the duplex DNA, the stability of the 5′-AGC adduct is greater, presumably due to the enhanced ability of Et 743 to form hydrogen bonds with the favored DNA sequence. At 42 °C, there is over twice as much Et 743-modified duplex DNA at the 5′-AGC versus the 5′-AGT sequence. In addition, the change in melting temperature between the unmodified and modified duplex is calculated to be approximately 19 °C for the 5′-AGC adduct and 13 °C for the 5′-AGT adduct, representing a ΔT_m of 6 °C between the two Et 743−DNA adducts, which correlates with the more facile reversal from 5′-AGT sequences.

In addition, it is interesting to note that, in lane 6 of panel B in Figure S2 (Supporting Information), both unmodified and Et 743-modified 5′-AGT DNA are present, which does not occur with the Et 743-modified 5′-AGC sequence (panel A in Figure S2; Supporting Information). These results indicate that Et 743 partially reverses from the 5′-AGT sequence during the purification process, forming unmodified duplex DNA. This formation of unmodified DNA substantiates our previous results that demonstrate the enhanced reversibility of the reaction of Et 743 with DNA, notably from the less stable 5′-AGT DNA sequence.

(B) Et 743−DNA Adducts at the Favored Target Sequences Inhibit T-Antigen Helicase Unwinding, While Adducts at the Nonfavored Sequences Can Be Unwound by T-Antigen. The SV40 large tumor antigen (T-antigen), a well-characterized DNA helicase that translocates in the 3′ → 5′ direction, was used as a probe to explore further the stability differences of Et 743−DNA adducts at different target sequences. The helicase substrates used in these reactions consist of two oligonucleotides (30-mer and 22-mer) annealed together to form a partial duplex with a 3′ single-stranded tail. Et 743 was able to inhibit 50% of the dissociation of the complex by helicase in DNA containing nonfavored sites (5′-AGT) in comparison to the unmodified DNA (unpublished results). However, whenever Et 743 was bonded to the favored DNA target sequence, there was no helicase-catalyzed formation of single-stranded DNA from duplex DNA, indicating that Et 743−5′-AGC adducts completely block helicase translocation. In addition, the formation of the unmodified duplex is not detected

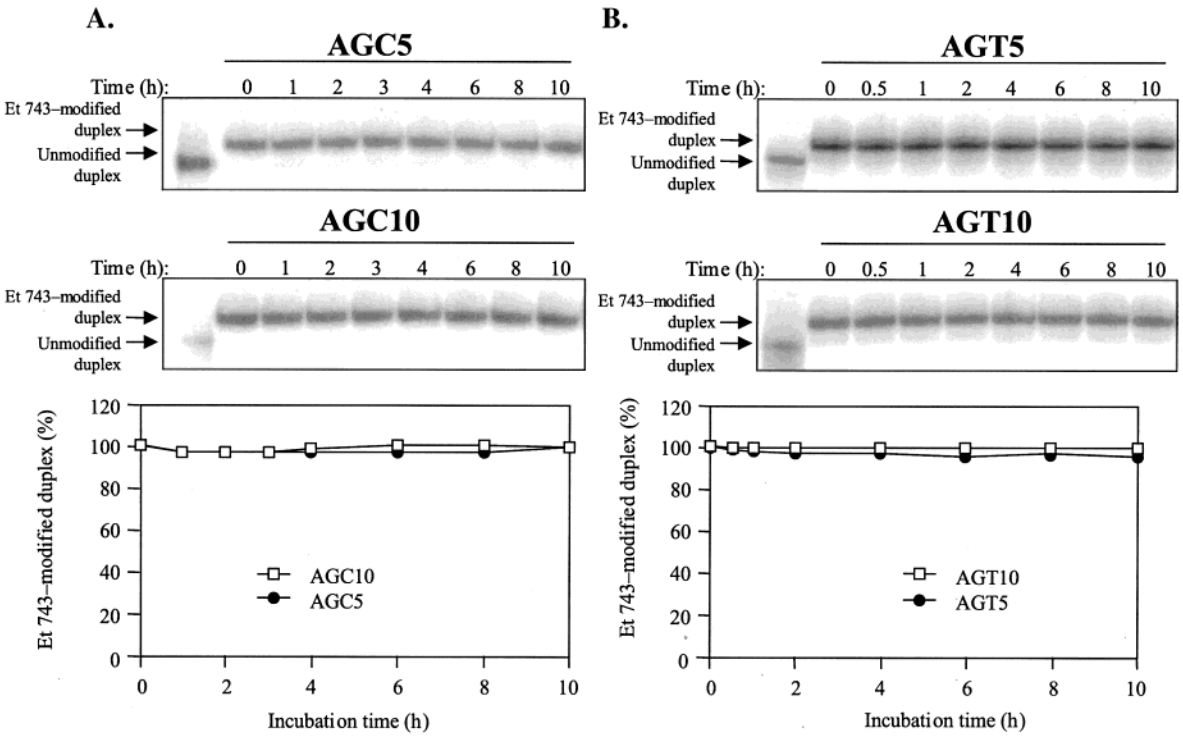


Figure 6. Intrinsic bending induced by an A-tract modulates the rate of dealkylation of Et 743. (A) Duplexes AGC5 and AGC10 and (B) duplexes AGT5 and AGT10 were mixed with 20 μM Et 743 and allowed to incubate at room temperature for the times indicated. The reactions were stopped and immediately loaded on a 16% polyacrylamide gel. The lower panels show a graphical representation of the results.

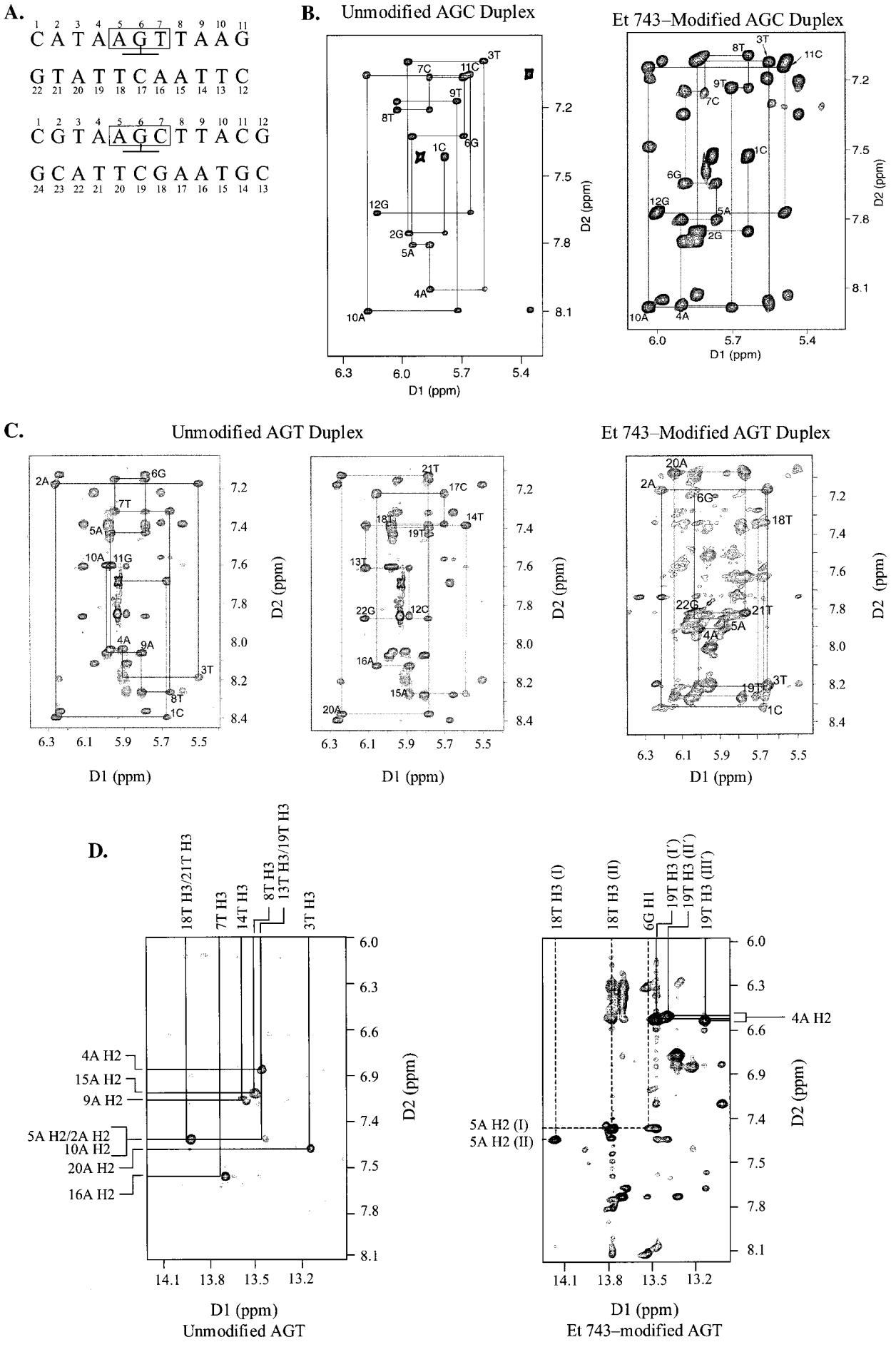
(unpublished results). These results show a clear difference between the helicase-catalyzed instability of the Et 743−AGT adduct and the corresponding stability of the Et 743−AGC adducts. It is important to investigate the effect of DNA processing by helicases, because in cells this is a likely factor in Et 743 reversal.

Two-Dimensional ^{1}H NMR Analysis Reveals Multiple Conformers for the Et 743−AGT Adduct in Which Watson−Crick Base Pairing Is Disrupted to the 5′-Side of the Drug Attachment Site. A comparison of the two-dimensional ^{1}H NMR spectra of the 5′-AGT adduct and the 5′-AGC adduct might offer an explanation for the stability differences between the two Et 743−DNA adducts. On the basis of the premise that formation of the hydrogen bond between the amino guanine and C8−O−C23 oxygen of Et 743 is critical in terms of maintaining a stable adduct,[8] it is expected that by removing the hydrogen bond acceptor, which is normally located 3′ to the target guanine (as is the case for 5′-AGT), the resulting Et 743 adduct would be less stable. Hence, the lack of this H-bond may confer the instability seen in the 5′-AGT adduct by modulating the observed rate of dealkylation for the 5′-AGT covalent adducts. Using high-field NMR spectroscopy, we attempted to determine the structural basis for the lower stability, and therefore the increased rate of dealkylation, of the Et 743−AGT adduct.

^{1}H NMR studies were conducted on an Et 743−(N2-guanine) DNA adduct using the 11-mer duplex containing the nonfavored 5′-AGT sequence (Figure 7A). In comparison to the 5′-AGC adduct, which maintains a stable hydrogen bond network and induces no significant distortion in the DNA,[8] the structure of the Et 743−AGT adduct is distinctly different. In the 5′-AGC adduct, virtually all the exchangeable and nonexchangeable ^{1}H NMR signals were assigned.[8] The contour plots of the two-dimensional NOESY of unmodified AGC and Et 743-modified AGC duplexes are shown in Figure 7B, displaying connectivities between aromatic base (H8/H6) protons and sugar (H1′) protons of neighboring bases. Likewise, in the two-dimensional NOESY spectra of the unmodified duplex containing the 5′-AGT sequence, all of the sequential aromatic H8/H6 to deoxyribose H1′ connectivities could be identified (Figure 7C). However, with the Et 743−AGT adduct, the sequential connectivities are broken because of the presence of very broadened NOE cross-peaks or the absence of cross-peaks that were detectable in the unmodified duplex (Figure 7C). Such disruption of NOESY connectivities is probably associated with multiple conformations of Et 743 within the minor groove. This signal-broadening phenomenon led to the predictable absence of cross-peaks from the COSY spectrum (unpublished results). The dynamic nature of the covalent adduct presumably arises from the inability to form the essential hydrogen bonds. Somewhat analogous effects have been observed with benzylpyrene diol epoxide, which also alkylates N2 of guanine, and when the flanking base pairs are AT rather than GC, this results in conformational heterogeneity.[19]

The D_2O and H_2O/D_2O NOESY cross-peaks generated by the signals from the Et 743−AGT adduct reveal that Et 743 disrupts the base pairing immediately 5′ to the site of covalent attachment. The NOESY spectrum of exchangeable resonance shows multiple imino-to-imino and imino-to-AH2 cross-peaks for the base adjacent to the drug on the noncovalently modified strand (Figure 7D, right panel). Multiple sets of NOESY connectivities link base protons for which there is only one set in the unmodified duplex spectrum (Figure 7D, left panel). Specifically, there appear to be two sets of cross-peaks (I and II) showing connectivity between the exchangeable 18T H3 and its pairing adenine's H2 (5A H2) (Figure 7D, right panel). A parallel set of connectivities (I′−III′) links 19T H3 and 4A H2. Likewise, there are multiple imino-to-imino cross-peaks linking

(19) Fountain, M. A.; Krugh, T. R. *Biochemistry* **1995**, *34*, 3152−3161.


A.
1 2 3 4 5 6 7 8 9 10 11
C A T A A G T T A A G
G T A T T C A A T T C
22 21 20 19 18 17 16 15 14 13 12
1 2 3 4 5 6 7 8 9 10 11 12
C G T A A G C T T A C G
G C A T T C G A A T G C
24 23 22 21 20 19 18 17 16 15 14 13
B.
Unmodified AGC Duplex
Et 743–Modified AGC Duplex
D1 (ppm)
D2 (ppm)
C.
Unmodified AGT Duplex
Et 743–Modified AGT Duplex
D1 (ppm)
D2 (ppm)
D.
18T H3/21T H3
7T H3
14T H3
8T H3
13T H3/19T H3
3T H3
4A H2
15A H2
9A H2
5A H2/2A H2
10A H2
20A H2
16A H2
18T H3 (I)
18T H3 (II)
6G H1
19T H3 (I′)
19T H3 (II′)
19T H3 (III′)
4A H2
5A H2 (I)
5A H2 (II)
D1 (ppm)
Unmodified AGT
D1 (ppm)
Et 743–modified AGT

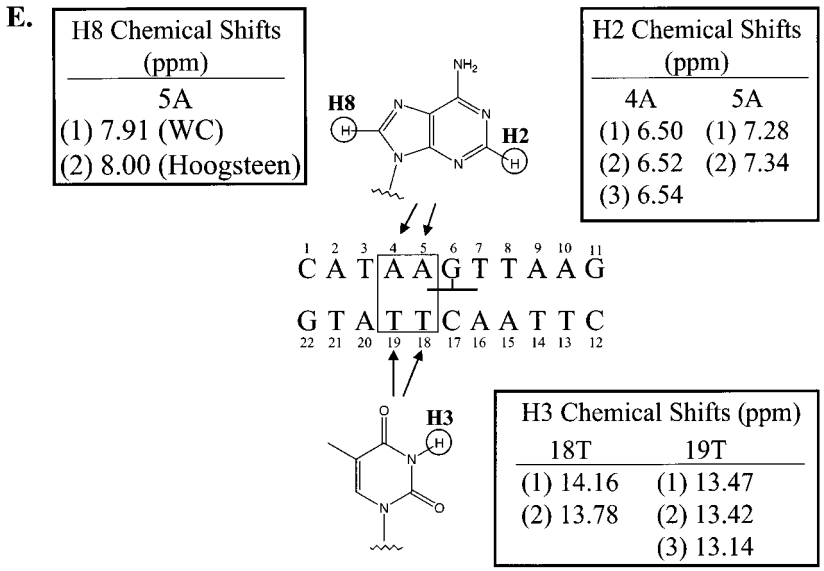


Figure 7. Evidence for multiple conformers of an Et 743−6G adduct in which Watson−Crick base pairing is disrupted to the 5′-side of drug attachment. (A) Sequence of the 12-mer duplex containing the 5′-AGT sequence and the 11-mer duplex containing the 5′-AGC sequence. (B) ^{1}H NMR signals of the 5′-AGC duplex generated H_2O and D_2O NOESY cross-peaks with well-defined connectivities between aromatic base protons and sugar protons of neighboring bases (left panel). The Et 743−(N2-guanine) DNA adduct indicates that H8/H6 to H1′ cross-connectivity patterns are still observed (right panel). (C) ^{1}H NMR signals of the 5′-AGT duplex generated H_2O and D_2O NOESY cross-peaks of the top and bottom strands (left and middle panel) and an attempted contour plot (right panel) showing the loss of connectivities of the Et 743−AGT adduct. (D) Contour plot of the two-dimensional NOESY of the Et 743−AGT adduct showing connectivities from the imino H3 of 18T and 19T into the adenine H2 of 4A and 5A. The connectivities of the 5A H2 are indicated by dashed lines and the 4A H2 connectivities are shown by solid lines. (E) Summary of the multiplicity of imino-to-imino and imino-to-aromatic cross-peaks in the Et 743−AGT adduct sequence.

18T H3 and 19T H3 (spectra not shown). The multiplicity of chemical shifts for 4A H2, 5A H2, 18T H3, and 19T H3 strongly suggests the presence of a mixture of conformers for the Et 743 adduct (Figure 7D, right panel). Data from the D_2O NOESY spectrum (5A H8 and 5A H1′ cross-peak intensity) suggest that one conformer may incorporate a Hoogsteen-paired 18T-5A base pair (unpublished results). However, the incomplete and confusing system of connectivities does not permit confirmation of this interesting possibility. A summary of the data from the imino-to-imino and imino-to-aromatic connectivities is shown in Figure 7E.

Discussion

This study provides evidence that the reaction of Et 743 with DNA is reversible under nondenaturing conditions. The data further suggest that the observed DNA sequence preference of Et 743 relies on the *differential rates of the reverse reaction of the Et 743 from different sequences* (i.e., 5′-AGC versus 5′-AGT). In contrast, the rate of alkylation appears to be independent of the DNA target sequence. Hence, it is the differences in the rate of reversibility that led to the apparent reactivity preference previously reported.[9] This report therefore defines a new mechanism for sequence specificity for molecules that covalently bond to DNA.

On the basis of these studies, two insights into the chemical reaction of Et 743 with DNA can be proposed. The first insight is an important modification of the previously reported catalytic mechanism for the reaction of Et 743 with DNA,[7] which shows the reversible reaction (Figure 8). The second insight provides an explanation for the apparent kinetics involved in the reaction of Et 743 with DNA. Assuming that the reaction takes place in two stages—a noncovalent association with DNA (noncov) followed by the formation of a covalent adduct (cov), as suggested previously[8]—the following kinetic model is proposed for the two different sequences:

$$\mathrm{AGC} + \text{Et 743} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} [\text{Et 743}\cdot\mathrm{AGC}]_{\mathrm{noncov}} \underset{k_{r_{-1}}}{\overset{k_{r_1}}{\rightleftharpoons}} \text{Et 743}-\mathrm{AGC}_{\mathrm{cov}} \quad (1)$$

$$\mathrm{AGT} + \text{Et 743} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} [\text{Et 743}\cdot\mathrm{AGT}]_{\mathrm{noncov}} \underset{k_{r_{-2}}}{\overset{k_{r_2}}{\rightleftharpoons}} \text{Et 743}-\mathrm{AGT}_{\mathrm{cov}} \quad (2)$$

The forward reaction is equally fast for both DNA sequences (i.e., $k_{r_1} \cong k_{r_2}$) and appears to be independent of the DNA target sequence. Indeed, the catalytic mechanism involving a proton shuttle does not involve the sequence-specific hydrogen bonding unique to either sequence, and it is therefore not surprising that the initial rate is independent of sequence. In contrast, the rates of the reverse reaction for 5′-AGC and 5′-AGT ($k_{r_{-1}}$and $k_{r_{-2}}$) do depend on the triplet target sequence and show an approximate first-order rate constant of 2.8×10^{-4} s^{-1} for 5′-AGC and 1.2×10^{-3} s^{-1} for 5′-AGT (Figure 2D), with $k_{-2} > k_{-1}$.

The Differences in the Rate of the Reverse Reaction May Be Derived from Structural Differences between Et 743−DNA Adducts at the 5′-AGC and 5′-AGT Sequences. The observed differences in the rate of dealkylation between the different sequences can be explained in terms of differential hydrogen bonding and the resulting stability of the covalent adducts. The results of previous studies from our laboratory led us to postulate that the ability to form the hydrogen-bonding network between Et 743 and the recognition sequence determines the relative drug-binding stability and reactivity.[8] In the Et 743−AGC adduct, Et 743 is positioned within the minor groove such that the coordination of hydrogen bonds is

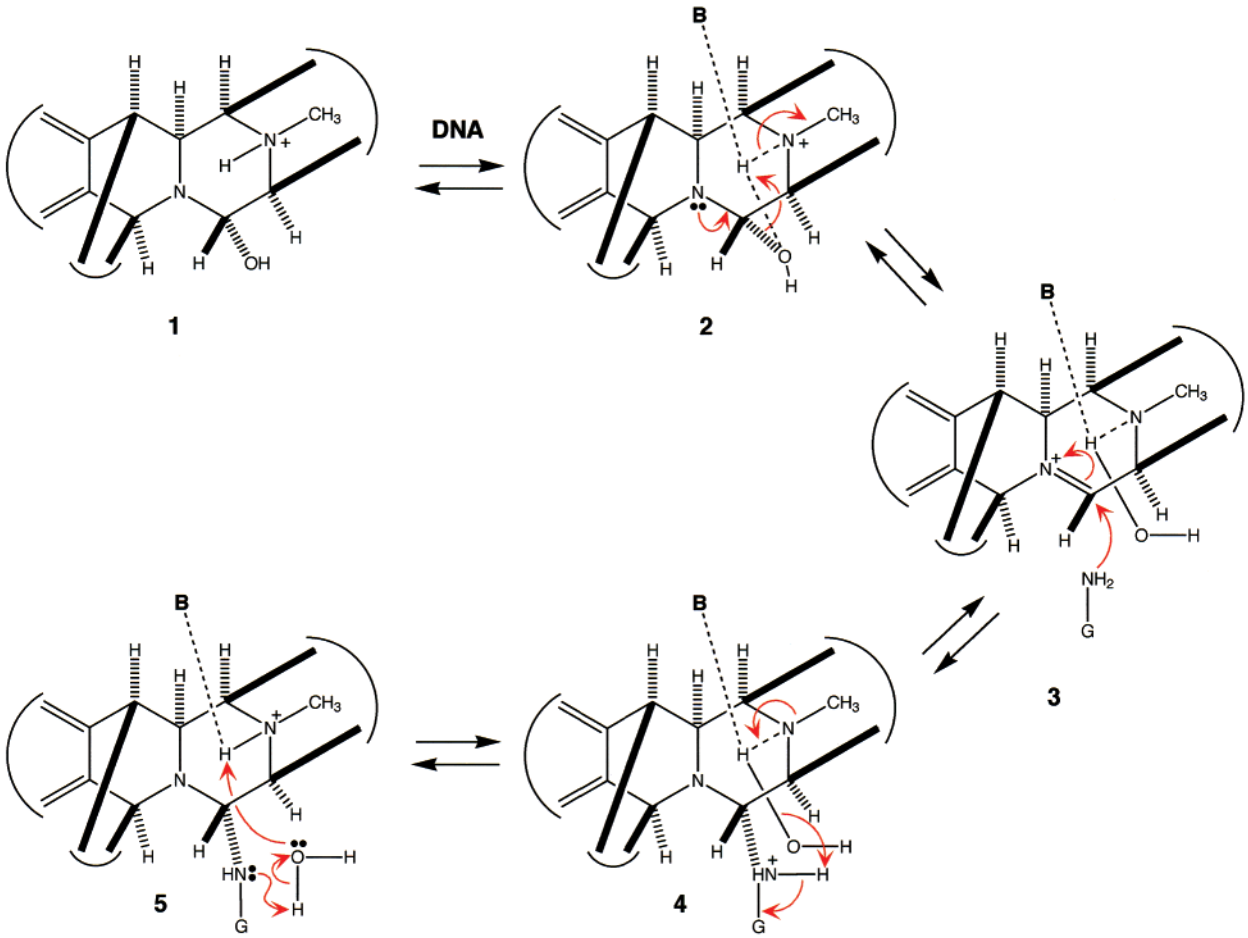


Figure 8. Reaction of Et 743 with DNA, showing the reverse mechanism.

maximized, which results in maximum stabilization of the covalent adduct. The NMR data presented here demonstrate that changing the target sequence from 5′-AGC to 5′-AGT yields an Et 743 adduct in which Watson−Crick base pairing is disrupted at least to the 5′-side of the target guanine. As a result, the thermal stability of the covalent adduct at the 5′-AGT sequence is reduced. These results demonstrate the importance of the hydrogen bond between the 2-amino group of guanine on the 3′-side of the covalently modified guanine and the C8−C23 oxygen of Et 743 as a major contributor to the stability of Et 743−DNA adducts.

This loss of structural integrity of the Et 743−AGT adduct could account for the differential rate of reversibility that is observed between the two target sequences. For example, this could lead to a change in the microenvironment surrounding the covalent linkage between DNA and Et 743. At the 5′-AGC target sequence, where Et 743 forms a stable, tight complex, the linkage is less accessible to attack by a water molecule (5 → 4 in Figure 8). This is in direct contrast to the Et 743−AGT adduct, where the minor groove site of covalent attachment is more accessible to solvation due to the dynamic nature of the DNA adduct forming an open complex. Hence, this increased solvent accessibility could increase the rate of the reverse reaction of Et 743 with DNA, whereas stable adducts (e.g., AGC) that are excluded from attack by the water molecule would have a lower rate of reversibility.

Implications for the Differential Reversibility of Et 743−DNA Adducts. When Et 743 reverses from the DNA, as shown in Figure 8, the resulting unbound drug can either modify its initial DNA target sequence or translocate to alkylate another unmodified site. The results presented here demonstrate that Et 743 can walk the DNA, binding to unmodified target sequences. However, since the reaction of Et 743 with DNA to form the covalent adduct is faster than the reverse reaction, it is not possible from these experiments to observe remodification of the initial DNA target sequence. Thus, remodification is entirely feasible as a possible consequence of Et 743 reversal.

Though the three base pair target sequence modulates stability and reversibility of the covalent adduct, the flanking sequence beyond the site of covalent attachment can also play a role in dictating both the rate of alkylation and the rate of the reverse reaction at a defined sequence. This is evidenced by the results that show that the forward reaction rates are modulated by the distance of an A-tract from the Et 743 alkylation site. More strikingly, incorporation of an A-tract, either five or ten base pairs away from the Et 743 alkylation site, stabilizes the adduct at both 5′-AGC and 5′-AGT sequences, preventing the reverse reaction from occurring. These observations may be explained in terms of the overall structure that is formed in the presence of the A-tract. Since the intrinsic A-tract bending exists in equilibrium between straight and bent conformations,[20] Et 743 could freeze out a defined conformation that is rigid, preventing the accessibility of water and thereby decreasing the rate of the reverse reaction. The A-tract is able to define a structure such that adducts at both 5′-AGC and 5′-AGT are stable. In a previous paper, we observed “multiple” bands when the A-tract was out of phase (i.e., 5, 7, or 13 base pairs away) with the Et 743 5′-AGC bonding site in ligation ladders, and when the A-tract was

(20) Thompson, A. S.; Sun, D.; Hurley, L. H. *J. Am. Chem. Soc.* **1995**, *117*, 2371−2372.

in phase (11 base pairs away), the multiplicity was lost.[5] A possible explanation for this is that Et 743 selectively freezes out just one conformation when the A-tract is in phase, but when the A-tract is out of phase with Et 743, either conformation can be frozen out, and this leads to mixtures of conformationally restrained oligomers when these molecules are ligated into polymers. A second possibility is that reversal results in loss of Et 743, but only in oligomers where the A-tract is out of phase.

Regardless of the three base pair recognition sequence, Et 743 will react with a wide range of different target sequences, initially forming both stable and unstable adducts. When Et 743 bonds at sites that result in covalent adducts of low stability, the reaction is readily reversible, allowing Et 743 to be preferentially released from these target sequences. The resulting released drug could migrate along the DNA, translocating from one sequence to another and concomitantly interfering with various biological processes until arresting at a thermodynamically stable site. As this thermodynamically driven process continues, whereby Et 743 bonds, reverses, and rebonds at different target sequences, the drug will be ultimately funneled to specific target sites, which results in stable, irreversible covalent adducts. This funneling process could be catalyzed chemically (i.e., 5 → 4 in Figure 8) or by helicase-catalyzed unwinding at or near the site of covalent attachment, which could increase the rate of the reverse reaction. As the number of Et 743−DNA adducts at the thermodynamically favored sites increases over time, the overall character of the population of Et 743−DNA adducts will change as well. These stable adducts could ultimately cause lethality by, for example, evading DNA repair. Indeed, antitumor activity of Et 743 has been observed to be time dependent.[21]

Comparison of Et 743−DNA Adducts with Other N2-Guanine Adducts. Although Et 743 is structurally similar to the saframycins, and both antibiotics react with the N2 position of guanine, only Et 743 has demonstrated sufficient efficacy in vitro and in vivo as an antitumor agent.[1,22] These differences in antitumor efficacy, despite structural similarities, suggest a unique mechanism of action of Et 743. One unique feature of Et 743 that we have previously reported is that it bends DNA toward the major groove while occupying the minor groove.[5] Another novel characteristic, reported here, is that Et 743−DNA adducts may be reversible under nondenaturing conditions. Although both saframycin A and anthramycin are also reported to be reversible under conditions that denature duplex DNA, such as acidic pH and elevated temperature,[22,23] only the Et 743−DNA adduct reverses under physiological conditions. The main structural difference between these DNA alkylators and Et 743 is that while both saframycin A and anthramycin fit snugly in the minor groove and do not dramatically distort the DNA, the more bulky Et 743 protrudes out of the minor groove *and* requires the bending of DNA in order to accommodate the drug within the minor groove. Furthermore, at the less stable sequences this results in base pair opening and associated covalent reversibility. Similarly, (+)-CC-1065 analogues, such as adozelesin, that lack the strong hydrophobic interactions of the parent compound but bend DNA are also reversible.[24] Hence, in both cases the driving force for the reversible reaction may arise from the instability that occurs in the duplex due to unfavorable steric interactions between the drug and the minor groove of DNA.

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Supporting Information Available: Two figures showing the reversal of covalently bound Et 743 from its target sequence and an autoradiogram demonstrating the temperature dependency of the stability of the site-directed adducts (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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