A 3·(ET743)-DNA Complex That Both Resembles an RNA-DNA Hybrid and Mimicks Zinc Finger-Induced DNA Structural Distortions

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The antitumor ecteinascidin ET743 has been shown to inhibit the transcriptional activation of a number of genes at nanomolar concentrations. Cell sensitivity to subnanomolar concentrations of the drug has also been shown to specifically depend on the transcription-coupled nucleotide excision repair system. ET743 is known to bind covalently to the minor groove of a DNA double helix in regions comprising selected sets of three consecutive base pairs. Following alkylation of a central guanine, the minor groove is widened and the DNA is bent toward the major groove. We have previously shown that in the resulting adduct the DNA triplet containing the covalently modified guanine bears a strong resemblance to a DNA triplet recognized by a C_2H_2 zinc finger. We now expand this earlier finding and use simulation methods to show that head-to-tail binding of three ET743 molecules to three adjacent optimal binding sites stabilizes a DNA structure whose conformation is intermediate between A- and B-form DNA. Furthermore, despite the increase in roll at the sites of covalent attachment, no net curvature is apparent in this complex due to cancellation of the localized bends over virtually one turn of the helix. Both observations are in good analogy to findings in zinc finger-DNA complexes. Triplets are virtually superimposable both directly and upon shifting the register one base pair. In this latter case, the central guanine in a triplet alkylated by ET743 corresponds to the third nucleic base in the triplet recognized by a zinc finger of transcription factors such as EGR1 or Sp-1. The DNA conformation found in the ET743-DNA complex is also strongly reminiscent of an RNA-DNA hybrid, as found in the RNA polymerase II elongation complex. The possible biological implications of these findings in relation to the antitumor action of ET743 are discussed.

Introduction

Transcription of protein-encoding genes is a finely tuned and highly controlled process. In eukarytic chromosomes, the activity of RNA polymerase II at specific promoters is regulated by the concerted action of a large array of positive and negative regulatory factors, including site-specific activators, repressors, co-activators and co-repressors, and chromatin-associated proteins.[1] Transcription factors are composed of both a DNA-binding domain (DBD) that localizes the protein to a specific site within the genome[2] and accessory effector domains that are utilized to activate or repress transcription at or near that site.[3] Disregulation of many transcription factors is associated with the development of human neoplasia, and the proteins encoded by some upregulated genes are known to play a role in the maintenance, progression, or failure of therapy of the tumor.[4]

Mammalian transcription factors are usually members of large protein families that bind to similar DNA sequences. Members of one of these families are characterized by having a tandem array of three or more Cys_2-His_2 (C_2H_2) zinc finger modules in their DBD. Structurally, a C_2H_2 zinc finger is a $\beta\beta\alpha$ motif stabilized by chelation of a zinc ion between a pair of cysteine residues from the β-sheet and the imidazole rings of a pair of histidines from the α-helix.[5] The α-helical portion of each finger is capable of fitting in the DNA major groove in such a way that binding of successive fingers causes the protein to wrap around the DNA. Most of the binding specificity is dictated by a set of 1:1 interactions between residues in four positions of each zinc finger α-helix (−1, 2, 3, and 6) and the functional groups present in the major groove.[6,7]

By fusing polydactyl zinc finger proteins[8] with suitable repressor and activator domains it has been possible to regulate endogenous genes within living human cells,[9] and application of this strategy to repress oncogene expression in tumor cells has been shown to result in inhibition of cell-cycle progression.[10] Other approaches aimed at interfering with the transcriptional machinery include formation of triple helix structures, both with short oligonucleotides and with polyamide nucleic acids,[11] and use of minor groove-reading distamycin-like hairpin polyamides.[12] One recognized problem with all of these methods, however, is ensuring that physiologically useful amounts of these molecules enter the cell. For this reason, in the search for antitranscription reagents,[13] much effort is being directed at the identification of small molecules that readily diffuse into cells.

Ecteinascidin 743, ET743 (Figure 1; for a review see ref 14), a natural product originally isolated from the sea squirt *Ecteinascidia turbinata*,[15] is highly active

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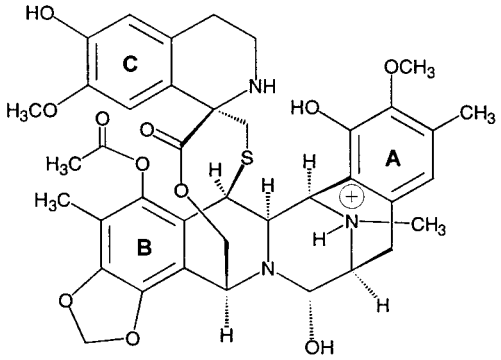

Figure 1. Chemical structures of ecteinascidin ET743 with three main subunits labeled A, B, and C.

against a variety of human tumor cells and is presently being evaluated in phase II clinical trials.[16] ET743 induces cell death at nanomolar concentrations[15,17] and, at similarly low doses, is able to abrogate the transcriptional activation of a number of endogenous genes, including *HSP70*, *c-fos*, *c-jun*, *E2F1*, *H2B*, *H4*, and *mdr1*.[18−21] The promoter regions of these genes contain GC-rich sites ('GC boxes') that harbor recognition sequences in the major groove for C_2H_2 zinc finger-containing transcription factors (e.g., EGR1 and Sp1). Coincidentally, some of these sequences are also target sites for ET743, which is known to bind in the minor groove of double-stranded DNA triplets of the type 5′-XGY, where Y is preferentially a G or a C. If it is a G, the preferred base for X is a pyrimidine, be it T or C. If Y is a C, then a purine (either A or G) is preferred for X.[22,23] Thus, examples of favored triplets are AGC, CGG, GGC, and TGG, in which ET743 alkylates the 2-amino group of the underlined guanine.[24] Different combinations of these triplets are present in GC and GT boxes, which are widely distributed in promoters, enhancers, and locus control regions of housekeeping as well as tissue-specific genes.[25]

NMR-based molecular dynamics simulations in aqueous solution of the covalent complexes between ET743 and the non-self-complementary nonamers d(TAAAGCTTA)$_2$ and d(TAACGGTTA)$_2$, carried out in our laboratory,[26] revealed that adduct formation brings about significant widening of the minor groove and an increase in positive roll at the base pair step involved in covalent bond formation. As a result, the DNA appeared bent toward the major groove, and the magnitude of the bend was found to be in very good agreement with estimates from previous gel electrophoresis experiments.[27]

Despite progress in our understanding of the sequence preferences for ET743 binding and the subsequent structural distortions inflicted on the DNA molecule, the molecular details of the mechanism(s) by which this drug exerts its potent antiproliferative activity are still unknown. Some anticancer drugs (e.g., nogalamycin, chromomycin A_3, and the bisanthracycline WP631) are known to interfere with binding of some of these transcription factors to their respective cognate sites.[28,29] However, in the case of ET743, concomitant binding of the drug and the protein to the same DNA stretch is possible as protein and drug occupy opposite faces of the DNA by filling either the major or the minor groove, respectively. Furthermore, we recently noted a remarkable similarity in the manner that a C_2H_2 zinc finger from the early growth response protein 1 (EGR1/

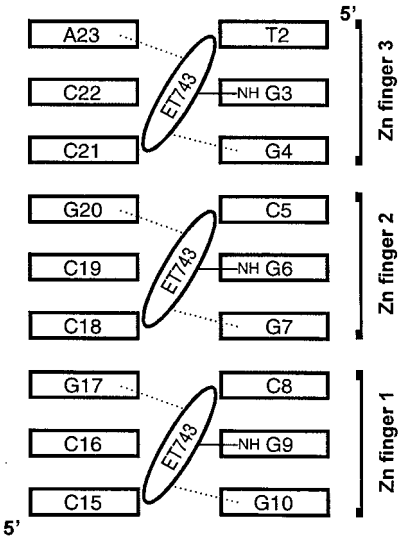

Figure 2. Schematic representation of the central part of the oligonucleotide studied showing sequence composition and numbering. The covalent bonds as well as the hydrogen bonds (broken lines) between each ET743 and the DNA are displayed. The region covered by individual zinc fingers from a hypothetical transcription factor is shown on the right. Note that the N → C direction of the bound protein runs antiparallel to the 5′ → 3′ direction of the DNA strand.

Zif268),[30] or the ubiquitous transcription factor Sp1,[25,31] and an ET743 molecule induce DNA structural distortions.[32] In our view, this observation raises the possibility that binding of ET743 to a binary complex made up of a suitable DNA GC-rich region with a bound EGR1-like DBD should be favored, due to the protein-induced DNA structural preorganization, over binding to a naked DNA site.[32]

Since EGR1 and Sp1-like transcription factors contain three consecutive zinc fingers in their DBDs, we decided to study the covalent complex formed between three ET743 molecules and an oligonucleotide containing three adjacent target sites. When we searched for genes containing putative Sp1- and/or EGR1-binding sites in their promoters that could provide contiguous binding sites to three ET743 molecules, we found one such sequence, TGGCGGCGG, in the 5′-upstream promoter region of the human *p73* gene, which encodes a protein with structural and functional homologies with the p53 tumor suppressor protein.[33]

We have studied this representative sequence embedded in the dodecanucleotide d(GTGGCGGCGGCC)$_2$ in the presence and in the absence of three bonded ET743 molecules (Figure 2) by means of 2 ns molecular dynamics simulations in aqueous solution. The specific objectives were (i) to ascertain the feasibility of having three ET743 molecules bonded to three consecutive target sites, (ii) to examine the conformational preferences of this GC-rich target oligonucleotide checking for any possible starting conformation dependencies, (iii) to establish whether the previously reported similarity in DNA structure between an EGR1-DNA complex and an ET743-DNA adduct[32] extends beyond a single binding site, and (iv) to probe major and minor groove accessibilities in these complexes to evaluate the compatibility of simultaneous binding of ET743 and the DBD of a C_2H_2 zinc finger-containing transcription factor.[34]

Table 1. Intermolecular Hydrogen Bonding Donor−Acceptor Distances (Å) Observed in the 3·(ET743)-d(GTGGCGGCGGCC)$_2$ Complex[a]

	mean distance	standard deviation
N12−N3(A23)	3.0	0.1
N12−N3(G20)	3.2	0.1
N12−N3(G17)	3.2	0.1
OM−N2(G4)	2.9	0.1
OM−N2(G7)	3.0	0.2
OM−N2(G10)	2.9	0.1
OHC−O1P(G6)	2.8	0.5
OHC−O1P(G9)	2.9	0.6
OHC−O1P(C12)	3.6	1.3

[a] Averaged over the last nanosecond of the simulation. OM and OHC are the methylenedioxy and phenolic oxygens from subunit C, respectively.

Results

Structure of the 3·(ET743)-d(GTGGCGGCGGCC)$_2$ Complex. Model building based on our previous work[26,32] initially suggested that tandem binding of several ET743 molecules to suitable adjacent DNA sites was sterically feasible. Monitoring of the root-mean-square deviation (rmsd) from the initial structure along the trajectory (Figure S1, Supporting Information) showed that the present complex with three bonded drug molecules is very stable (1.0 Å ≤ rmsd ≤ 1.5 Å), in agreement with the fact that the TGG and CGG triplets represent equally optimal binding sites for ET743.[22] The expected intermolecular hydrogen bonding scheme between ET743 and the Pyr-G-G sites[22,26] (Figure 2) is maintained during the whole length of the simulation (Table 1) and, for each individual binding site, is essentially identical to that reported previously for the ET743-d(TAACGGTTA)$_2$ covalent complex.[26] In the complex with d(GTGGCGGCGGCC)$_2$, the protonated N12 of ET743 is engaged in a hydrogen bond with the N3 acceptor atom of A23 in the first triplet, G20 in the second triplet, and G17 in the third triplet; the methylendioxy oxygen facing the minor groove is involved in a hydrogen bond with the amino N2 of G4, G7, and G10 (the bases 3′ to the guanines that are alkylated by each ET743 molecule), and the OH on subunit C is hydrogen bonded to the O1P of the phosphate linking C5−G6, C8−G9, and C11−C12 (Table 1). Of all these hydrogen bonds, only the last one is seen to be disrupted, after ∼1.6 ns (hence the larger standard deviation), due to end-effects on the 3′-side, most likely due to the poor stacking between C11 and C12.

As a result of drug binding, the DNA minor groove is notably wider than in the free oligonucleotide (Table 2). Minor groove width is largest at P5−P24, P8−P21, and P11−P18, reflecting the positioning of the bulky drug in the minor groove. The ensuing compression of the major groove is comparable to that found previously in the ET743-d(TAACGGTTA)$_2$ complex:[26] local bending in the direction of the major groove is apparent by an increase in positive roll at successive steps (Table 3), which is manifested by dot clustering on the upper hemisphere of the bending dial (Figure 3), and is interrupted only at G4/C5, G7/C8, and G10/C11. A common pattern then arises in which the first and second steps of each triplet are bent toward the major groove and no bending is detected at the 3′-end of each target site. In addition, the CpG and TpG steps display negative tilt values (apparent as dots on the left hemisphere of the dial), indicative of bending into the sugar phosphate backbone of their complementary strand. On the contrary, tilt is slightly positive for all the GpG steps.

Both roll and tilt components contribute to bending of the double helix[35,36] but, in the present complex, the alternation just described of positive roll at Pyr/Pur and Pur/Pur steps with zero roll at G/C steps on the 3′ side leads to a continuous gentle writhe that does not change the overall direction of the helix. Since binding of the three ET743 molecules takes place within a full turn of the helix, any local curvature induced by just one molecule, as reported earlier,[26,27] is canceled by binding of the others.

The average conformational features of the DNA in the complex with three ET743 molecules are best described as intermediate between those of A- and B-form DNA (Table 4). The negative sign of the average X-displacement means[37] that the base pairs have been displaced from the helical axis in the direction of the minor groove to create a deeper major groove. Average values for roll, slide, and minor groove width are closer to those of canonical A-DNA whereas two different situations are observed with respect to sugar puckering and backbone conformation (Table 5). The first one corresponds to deoxyriboses whose phase angles oscillate during the trajectory from south to north conformations (C2′-endo to C3′-endo), as those attached to the cytosines (C22, C19, and C16) that are complementary to the ET743-alkylated guanines (G3, G6, and G9), and also the pyrimidines 3′ to these guanines in the same strand. This behavior differs from that expected for a B-DNA conformation whose values are distributed between C2′-endo and C1′-exo, as seen in most nucleotides of the free oligo. The second situation corresponds to deoxyriboses with low standard deviation in their phase angles, as seen in those linked to the ET743-alkylated guanines and the purines in the complementary strand. These differences in phase angles appear correlated with distinct values in the glycosyl torsional angle, χ, such that nucleosides with high standard deviations in sugar puckering have average χ values midway between those corresponding to A- ($\chi = -154°$) and B-DNA ($\chi = -90°$) conformations whereas typical B-DNA values are found in nucleosides with low standard deviation in phase angles. This effectively means

Table 2. Width of the Minor Groove[a] (Å) in the Free d(GTGGCGGCGGCC)$_2$ Dodecanucleotide and in the Complex with Three ET743 Molecules

	P5−P24	P6−P23	P7−P22	P8−P21	P9−P20	P10−P19	P11−P18	P12−P17
free	8.4 (1.55)	6.7 (1.3)	7.9 (1.2)	7.2 (1.2)	7.5 (1.4)	7.1 (1.4)	7.4 (1.8)	6.2 (1.6)
ET743	9.4 (0.5)	8.8 (0.6)	8.4 (0.9)	9.5 (0.4)	8.5 (0.7)	8.1 (0.9)	9.7 (0.4)	8.3 (1.1)

[a] Measured as the shortest interphosphate distances across the groove (P−P distance minus 5.8 Å). The P−P distance in canonical B-DNA is 5.9 Å. Mean values obtained from the 2000 ps of the MD simulations are shown, with standard deviations in parentheses. Measurement of O1′−O1′ distances provides similar relative differences.

Table 3. Comparison of Local Helix Parameters for the Base Pair Steps Involved in Binding of ET743[a]

	roll (°)		twist (°)		slide (Å)	
	complex	free	complex	free	complex	free
T2−G3	13.4 (7.7)	18.8 (8.6)	30.3 (4.1)	27.5 (5.7)	−0.5 (0.4)	−0.9 (0.5)
G3−G4	10.3 (8.2)	3.1 (8.4)	30.7 (5.6)	22.0 (4.7)	−1.3 (0.4)	−1.7 (0.5)
G4−C5	−4.4 (6.9)	−4.8 (8.1)	34.2 (4.1)	35.9 (4.5)	−0.8 (0.4)	−1.4 (0.6)
C5−G6	10.3 (7.5)	15.8 (9.0)	33.9 (4.1)	27.5 (6.8)	−0.5 (0.3)	−0.3 (0.5)
G6−G7	11.9 (8.0)	−2.1 (8.3)	31.1 (5.1)	36.5 (4.2)	−1.3 (0.4)	−0.3 (0.5)
G7−C8	−2.4 (7.2)	−5.7 (8.1)	34.8 (4.1)	34.3 (4.3)	−1.1 (0.4)	−1.0 (0.5)
C8−G9	12.8 (8.0)	13.9 (8.7)	31.5 (3.9)	28.7 (5.9)	−0.7 (0.4)	−0.9 (0.6)
G9−G10	10.2 (8.2)	0.8 (7.5)	31.9 (5.5)	30.9 (4.8)	−1.3 (0.4)	−1.7 (0.6)
G10−C11	0.4 (6.8)	0.2 (7.9)	33.5 (4.6)	31.8 (5.2)	−1.1 (0.5)	−1.4 (0.5)

[a] Mean values obtained from the 2000 ps of the MD simulations are shown, with standard deviations in parentheses.

Table 4. Conformational Parameters of the DNA in the 3·(ET743)-d(GTGGCGGCGGCC)$_2$[a] and EGR1-DNA Complexes;[b] Values for A-DNA and B-DNA [c]Are Shown for Comparison

	A-DNA	ET743-DNA	EGR1-DNA	B-DNA
inclination, Å	20.1	5.3	6.7	3.4
twist, deg	30.4	33.0	31.0	35.6
roll, deg	10.0	6.4	4.8	1.6
X displacement, Å	−4.5	−2.0	−1.7	−0.1
slide, deg	−1.7	−0.9	−0.7	0.4
minor groove width, Å	10.0	9.2	8.1	6.2

[a] Energy-minimized average structure for TGGCGGCGG obtained from the last 1000 ps of the MD simulations. [b] Measured for the GCGTGGGCG sequence in the X-ray crystal structure (PDB entry 1aay). [c] Mean values for A- and B-DNA from X-ray crystal structures (ref 42).

Table 5. Pseudorotation Phase Angles for the Sugar Rings (P) and Glycosidic Torsion Angle (χ) in the Central Segments of Both the Complexed and Free Forms of the Dodecanucleotide d(GTGGCGGCGGCC)$_2$

	complex		free	
	phase[a]	χ[a]	phase[a]	χ[a]
T2	106.3 ± 57.3	−130.0 ± 17.5	133.3 ± 29.4	−124.3 ± 14.8
G3	157.5 ± 17.0	−107.0 ± 11.8	139.8 ± 14.2	−97.0 ± 16.0
G4	141.7 ± 15.4	−104.9 ± 10.9	117.3 ± 49.2	−89.31 ± 33.7
C5	115.5 ± 38.6	−134.9 ± 13.5	136.2 ± 27.6	−121.6 ± 15.1
G6	165.4 ± 16.1	−103.8 ± 10.4	143.2 ± 11.6	−88.1 ± 12.1
G7	135.6 ± 16.5	−108.0 ± 11.3	165.5 ± 20.8	−111.9 ± 11.8
C8	110.0 ± 50.6	−136.6 ± 13.5	94.1 ± 34.7	−129.9 ± 15.6
G9	161.1 ± 16.4	−107.3 ± 11.3	134.2 ± 38.7	−115.7 ± 19.3
G10	141.5 ± 14.4	−106.0 ± 11.4	111.9 ± 44.7	−138.1 ± 21.2
C15	117.4 ± 47.8	−121.7 ± 18.2	124.7 ± 24.7	−125.3 ± 15.7
C16	81.4 ± 70.8	−139.8 ± 12.9	107.6 ± 30.7	−135.7 ± 14.8
G17	166.4 ± 14.9	−109.2 ± 9.0	140.3 ± 23.1	−108.6 ± 21.6
C18	123.7 ± 55.4	−123.9 ± 17,1	138.9 ± 31.3	−124.2 ± 14.8
C19	101.2 ± 53.3	−134.6 ± 13.1	110.9 ± 42.4	−116.5 ± 23.2
G20	168.0 ± 16.0	−105.6 ± 8.8	155.7 ± 19.6	−105.2 ± 14.2
C21	117.8 ± 52.7	−121.9 ± 16.3	104.2 ± 37.0	−134.3 ± 15.0
C22	69.8 ± 66.9	−140.5 ± 11.4	98.4 ± 33.7	−136.4 ± 13.6
A23	173.7 ± 15.8	−104.4 ± 8.8	138.0 ± 31.7	−109.9 ± 19.5

[a] Averaged from the 2000 ps of the MD simulation.

that B-type nucleotides in the alkylated strand are paired with A/B-type nucleotides in the complemtary strand. In order words, the double helix resembles an RNA-DNA hybrid, as seen in the elongation complex with RNA polymerase II.[38]

Conformational Preferences of d(GTGGCGGCGGCC)$_2$. Comparing the above tendencies for the complex with those of the free dodecanucleotide (Figure 3), it can be noted that G3/G4, G6/G7, and G9/G10 steps bend toward the major groove as a consequence of drug binding whereas T2/G3, C5/G6, and C8/G9 steps appear to be intrinsically bent as their preferences are the same irrespective of the presence of bonded drug. These observations are in good agreement with results of surveys of X-ray crystal structures demonstrating a significant bias of CpG and TpG steps toward bending into the major groove[39] and also with results from molecular dynamics simulations.[40]

To assess the possible dependency of the observed conformation on the starting geometry, the free dodecamer was built in both an A-form and a B-form,[41] and each was simulated under identical conditions (see Methodology). The A-form dodecanucleotide evolved toward a conformation remarkably similar to that obtained when the simulation started from B-DNA (Figure S2, Supporting Information) and intermediate between A- and B-type DNA.[42]

Structural Similarity between the 3·(ET743)-d(GTGGCGGCGGCC)$_2$ and the EGR1-DNA Complexes. The groove characteristics, base pair geometries, and helical parameters calculated for the 3·(ET743)-d(GTGGCGGCGGCC)$_2$ complex (Table 4) are strongly reminiscent of those reported for zinc finger-DNA complexes.[43] Particularly relevant, and directly related to transcription regulation in some of the genes affected by ET743,[21] is the complex containing the three zinc fingers from the DBD of the prototypical transcription factor EGR1/Zif268 and a consensus DNA binding site (PDB entry 1aay).[44] Direct superposition of the DNA triplets recognized by ET743 (i.e., TGG, CGG, and CGG) onto the corresponding DNA triplets recognized by each EGR1 zinc finger (i.e., GCG, GGG, and GCG) shows a good match (rmsd = 1.1 Å, using either the C1′ or the O1′ atoms, or rmsd = 2.1 Å, using all sugar-phosphate backbone atoms) for the three triplets (Figure 4A). Moreover, equally good structural similarity (rmsd = 0.9−1.0 and 2.0 Å, respectively) can be obtained if one complex is shifted one base pair upstream or downstream for the superposition (Figure 4B). In addition, comparable rmsd values were obtained when all sugar-phosphate backbone atoms of the 3·(ET743)-DNA and EGR1(DBD)-DNA complexes were superimposed onto canonical[41] B-DNA (3.6 and 3.5 Å, respectively) and A-DNA (4.3 and 4.4 Å, respectively). The reported change in register, however, affects the number of identical base pairs that are superimposed, as well as the number of pyrimidine-purine and purine-purine steps that find exact correspondence between the two complexes. For this reason, the best match will ultimately depend on the particular sequences that can be recognized by both ET743 and the DBD of different zinc fingers, including Sp1 (manuscript in preparation), but our results clearly show that, in any case, the geometries of the grooves will be such that concomitant

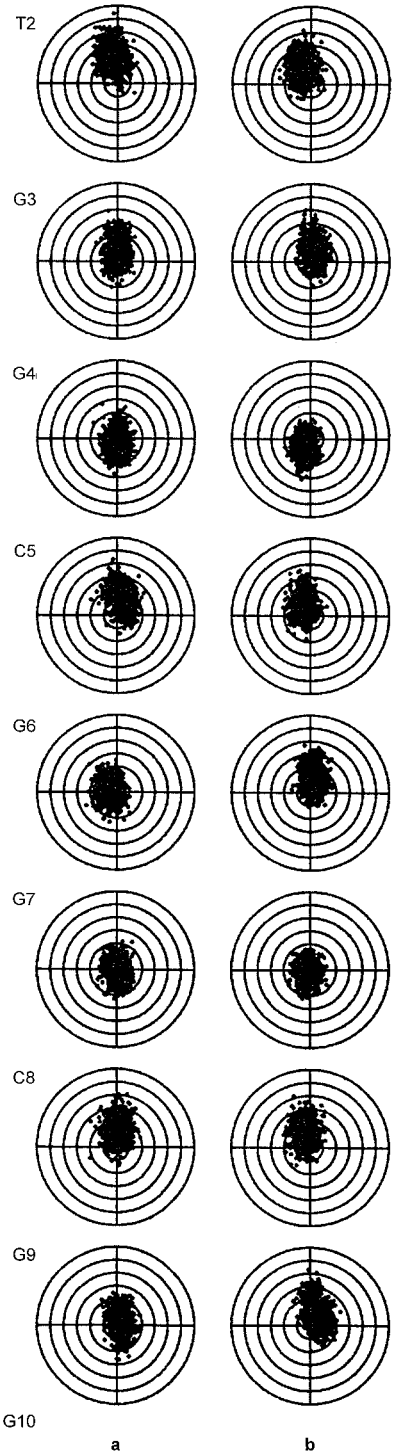

Figure 3. Polar plots ("bending dials") for the eight consecutive steps involved in ET743 binding at the central DNA segment in the (a) absence and (b) presence of three bonded ET743 molecules. Individual points are for structures separated by 1 ps throughout the course of the trajectory and when viewed together constitute a probability density. Bending compressing the major groove is plotted on the northern hemisphere of the dial.

binding of three ET743 molecules (in the minor groove) and three zinc fingers to the same DNA stretch is sterically and energetically feasible (Figure 5). This finding lends further support to the proposal of a ternary protein-DNA-ET743 complex[14,32] and allows for more detailed exploration of intermolecular contacts.

Discussion

The ability of many transcription factors to bind to their specific target sites depends not only on a direct read-out of sequence information but also on the intrinsic flexibility and bendability of the DNA stretch to which they bind. Protein-induced DNA distortions enhance the recruitment of proteins to their specific DNA targets[45] and/or favor protein-protein interactions.

ET743 is known to produce localized DNA bends[26,27] and also to decrease induced activation of expression of a number of endogenous genes.[18,19] However, it is presently unknown whether these two observations are directly related. Optimal target sites for ET743, consisting of DNA triplets, are scattered all over the genome, but the effects of the drug on transcription, cell cycle progression, and apoptosis are achieved at extremely low concentrations.[14,17−19] This can be taken as an indication of some kind of preferential binding. In light of the present results, we propose that the demonstrated effect of ET743 on DNA bending,[27] which reflects an influence on the energetics of DNA conformation, could be accompanied by a reciprocal effect of DNA conformation on drug binding. Thus, if ET743 widens the minor groove and bends DNA toward the major groove, ET743 can be expected to bind more tightly to a locally bent DNA-protein complex displaying these structural characteristics than to linear B-DNA. Such quasi-thermodynamic linkage has been demonstrated for catabolite activator protein (CAP) binding to its DNA target site, which is slightly bent per se,[46] and for the association of TATA-binding protein (TBP) to the minor groove of the TATA element, which is enhanced when the DNA is slightly pre-bent in the direction of the major groove.[47]

The conformational similarities reported above have been advanced for ET743 binding to a single site,[32] but little was known about the effects of the drug when more than one molecule bind to closely spaced target sites. In this case, it can be anticipated that the overall effect will depend on the phasing of the multiple drug binding sites. Thus, if the alkylated guanines are spaced by a full turn of the helix, cummulative bending can be expected, but if the sites are comprised within one complete turn they will likely cancel out. This is precisely what we observe in the present complex in which three ET743 molecules bind to three adjacent sites and appears to be the same reason for which curvature is absent from the complexes of DNA with the multiple C2H2 zinc fingers that make up the DBD of many transcription factors, as exemplified here by EGR1/Zif268. The fact that three optimal binding sites for ET743 are adjacent in sequence brings about the possibility of cooperative binding. In this respect, it has recently been reported that the reaction of ET743 with DNA can be reversible under nondenaturing conditions and that the rate of the reverse reaction depends critically upon the stability of the different ET743-DNA adducts.[48] This means that in a cellular context the population of these adducts is likely to change over time as ET743 will be able to migrate from some sequences to others until most of the drug becomes covalently trapped at the most thermodynamically favored sites, which can likely be protein-bound sites, as recently proposed for actinomycin D and the complex of DNA with RNA polymerase.[49] Interestingly, for the sequence,

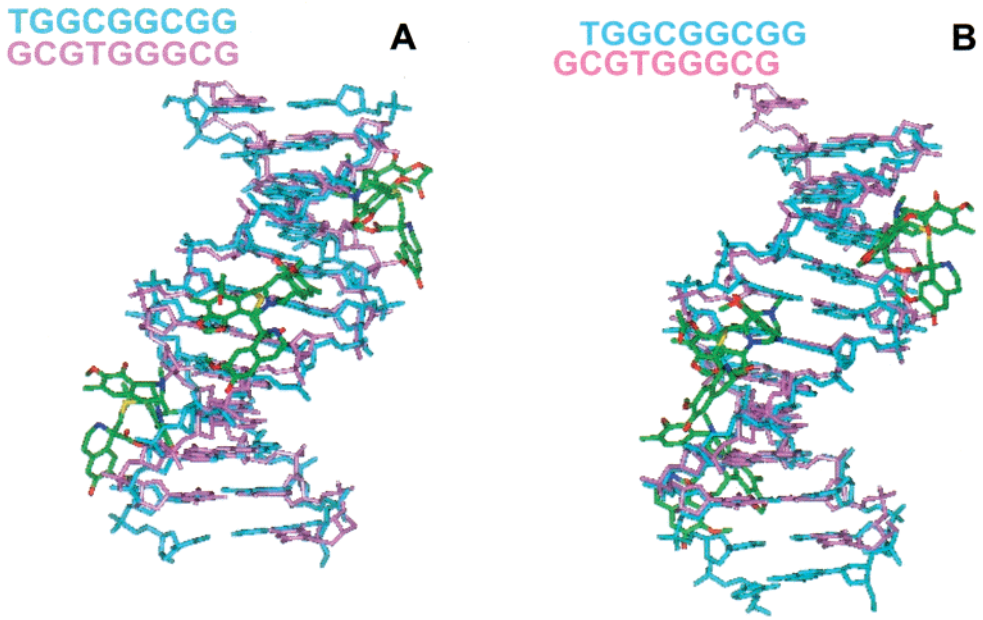

Figure 4. Stick representation showing two possible modes of superposition for the DNA (blue) from the present ET743-DNA complex onto the DNA (pink) from an EGR1-DNA complex (PDB entry 1aay). Coordinates for the covalent 3•(ET743)-d(GTGGCGGCGGCC)$_2$ complex are being deposited with the Research Collaboratory for Structural Bioinformatics with PDB identification code 1KML for immediate release.

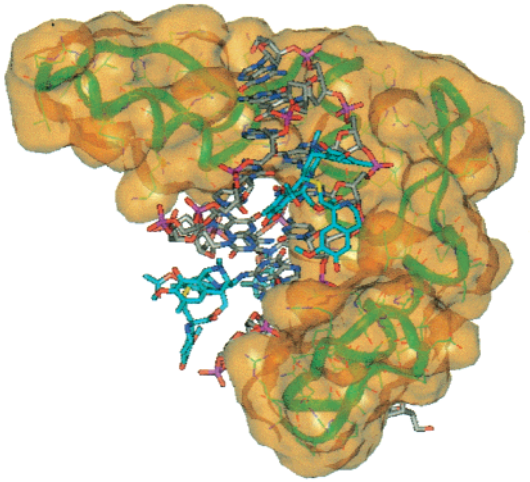

Figure 5. Composite model from Figure 4B showing three ET743 molecules (carbon atoms in cyan) bonded to the minor groove of a DNA oligo (carbon atoms in gray) to which three zinc fingers (green ribbon) are bound in the major groove. Protein residues are enveloped by a solvent-accessible surface colored in orange. Note that the ET743 molecule closest to any protein atom is the central one, whose C subunit is adjacent to the C-terminus of the recognition helix from finger 1.

5′-TTT**AGC**A**AGC**T**AGC**TAT-3′, containing three favored AGC triplets (in bold) in one strand separated by one additional base, progressive drug saturation has been shown (Figure S1 in ref 48) until all three binding sites become occupied. In this particular case, data interpretation can be complicated by the fact that another set of two overlapping and competing binding sites (underlined) are present in the complementary strand. On the contrary, in sequences such as the one presented herein, binding of ET743 can take place only on one of the strands.

Despite the absence of overall curvature, in both the simulated 3•(ET743)-DNA complex and several experimental EGR1(DBD)-DNA complexes, the local helical axes of the base pairs are writhed around an unbent global axis, resembling A-DNA.[36] This and other distinctive A-type geometrical features (Table 4)[43] are common motifs in a number of protein-bound DNA structures[50] and, together with the fact that the zinc fingers and the drug studied here bind to different grooves of the helix, appear to lend further credence to the possibility of favored simultaneous binding of a protein and ET743 to the same DNA stretch.

Triplets recognized by the zinc fingers of EGR1 and the related Sp1-like transcription factors (manuscript in preparation) include GCG, GAG, GGG, and TGG.[5,25] Favored sites for ET743 binding are CGG, TGG, AGC, and GGC.[22,23] It is perhaps no coincidence that CpG has an increased positive roll relative to other dinucleotide steps, which correlates with lower average twist, and that TpG is the step that can potentially occupy the broadest range of conformational space.[39] It is seen that different combinations of these sites, as present in many of the GC-rich regulatory regions of a variety of gene promoters,[51] afford binding sites not only to different transcription factor family members but also to ET743. Notably, the majority of optimal sites for ET743 binding would be located at the junctions between triplets recognized by successive zinc fingers rather than at the triplets themselves (TGG would be the most obvious exception). Remarkably, this observation is in nice correspondence with our results from superimposition of DNA structures in the respective complexes, which show a very good match when the sites are shifted in register by one base pair (Figure 4B). Furthermore, GC-boxes usually contain partially overlapping binding sequences for factors with opposite actions at the level of transcription so that activation or repression at any given time will be influenced by the relative abundance of each factor within the cell nucleus. Taking the EGR1-Sp1 pair and the *mdr1* promoter as an example,[52] the

resulting balance in such competition could be influenced by the presence of minor-groove bonded ET743 at the site occupied by the major-groove binding zinc finger or at a junction between adjacent sites. As the binding of the two factors is mutually exclusive, the presence of covalently bonded ET743 could be differentially affecting the DNA association and dissociation rates of one or several zinc fingers from the same or different transcription factors. The presence of subunit C of ET743 protruding out of the minor groove could also affect binding of additional regulatory proteins. Thus, the protein region closest to the protruding C subunit is the C-terminus of the recognition α-helix from the first zinc finger. Position 10 in this α-helix, immediately before one of the zinc-coordinating histidines, is an isoleucine in EGR1 and a tryptophan in Sp1 and is precisely one of the nonconserved residues in the Sp1-like family of transcription factors.[25] The fact that chemical modification in this ET743 subunit is accompanied by modulation of activity[15,17] is suggestive of additional interactions possibly involving a transcription factor (e.g., E2F1-DP) or other proteins from the transcriptional machinery. These hypotheses, which are amenable to experimental verification, may account for the variety of effects found in a number of genes upon exposure of cells to ET743 ranging from down-regulation to up-regulation.[21] Besides, the proposed enhanced DNA-binding affinity of single or multiple ET743 molecules toward protein-DNA complexes may not necessarily be restricted to complexes of DNA with C_2H_2-containing DBDs of transcription factors. While it is true that Sp1 plays an important role in cell proliferation[25,31] and that recent studies have revealed that EGR1, originally cloned as an "immediate-early gene", is also a cell growth regulator and suppressor of transformation,[30] a wide variety of DNA-binding factors are key components in tumor development and progression, including those encoded by novel chimeric fusion genes arising from chromosomal translocations.[53]

It is still the subject of intense investigation how ET743's potent inhibitory effects on transcriptional activation[18−20] are translated into growth arrest and induction of apoptosis. Very recently, in a series of elegant experiments, Pommier and collaborators have provided conclusive evidence that ET743 cytotoxicity is, at least at subnanomolar concentrations, mediated by the transcription-coupled (TC) pathway of nucleotide-excision repair (NER).[54] Thus, SW480 and HCT116 cells deficient in XPA, XPD, XPF, XPG, CSA, or CSB are between 10- and 23-fold more resistant to this drug compared with their isogenic parental cells, and similar findings have been independently reported by other laboratories for other cell lines.[55,56] Cell death was then closely correlated with a dose-dependent increase of ET743-induced DNA single-strand breaks.[54] In the model presented, the ET743-modified double-strand DNA in transcribed genes would be recognized as damage by the TC-NER machinery and the resulting complex would be trapped following DNA cleavage. However, since the efficiency of ET743 alkylation using purified DNA appears to be relatively low[22] and there are multiple target triplets in most genes, the actual target site for ET743 binding is not clear. In light of the results presented herein and further work in progress, ET743 binding to a DNA-RNA hybrid could be favored over binding to double-stranded DNA (Tables 4 and 5). For Pyr-G-G triplets of the type reported in the present work, a covalently bonded ET743 in one strand establishes just one hydrogen bond with a base in the opposite strand (Figure 2), but with Pur-G-C triplets of the type described earlier[23,26] two such hydrogen bonds are possible. If the opposite strand is RNA, the presence of the extra 2′-hydroxyl group on the ribose might stabilize the adduct still further so as to hamper strand separation. If the DNA-RNA hybrid happens to be that under construction at or near the active site of RNA polymerase II (Pol II), for example, binding of one or more ET743 molecules to the appropriate sequence (e.g., one or more GGC triplets) could mean blockade of transcription and recruitment of the TC-NER system[57] that could then become engaged in futile repair. This suggestion does not appear to be in contradiction with available experimental evidence and provides a basis for the design of new experiments. In this respect, it is worth remembering that the surface charge of Pol II is almost entirely negative[58] and that ET743 is positively charged.

Conclusions

We have shown that binding of multiple ET743 molecules to adjacent optimal sites, such as those present in the proximal promoter of the human *p73* gene, is structurally and energetically possible and is probably favored over binding to individual sites.

The similarity in DNA structure detected earlier between a C_2H_2 zinc finger-DNA complex and an ET743-DNA complex is now shown to extend beyond one single binding site. The present results support our earlier proposal that the DNA structure in the complexes with C_2H_2 zinc fingers from transcription factors such as EGR1 and Sp1 is highly preorganized for ET743 binding, which we now show that can take place in a tandem fashion. The sequences recognized by this large family of transcription factors are globally referred to as GC-boxes and contain high-affinity sites for ET743, especially at the junctions between individual triplet sites.

Competition for DNA binding sites among proteins of the transcriptional machinery is known to exist not only between repressors and activators but also between different activators. If the respective promoter elements are overlapped, as is often the case, this competition can lead to mutually exclusive processes (e.g., terminal differentiation or cellular proliferation).[3] As a result of this complex pattern of interactions, gene expression is subject to combinatorial control and the response of a particular gene will depend on the relative proportions of competing activators and repressors, their relative DNA-binding affinities, and possible contributions from other proteins binding at other sites. The resulting balance can be modified by specific extracellular stimuli including drugs, but the specific outcome is extremely difficult to predict, as shown by results from studies on ET743-induced changes in gene expression using microarrays.[21]

The present results also reveal another notable feature of the ET743-DNA complex, namely the adoption of A-DNA characteristics in the strand complemen-

tary to the one alkylated by ET743. The overall conformation, intermediate between canonical A- and B-DNA, is reminiscent of that found in DNA-RNA hybrids, including the double helix of template DNA paired to nascent RNA found in the active site of RNA Pol II. We are currently exploring by simulation methods the feasibility of ET743 binding to DNA-RNA hybrids in the presence and the absence of bound RNA Pol II.

Despite continuing efforts and notable advances, it has not been easy to dissect, at the molecular level, the elements of the transcriptional machinery that are most affected by ET743. We hope that the observations reported here will help to point the experimentalists in the right direction to achieve this goal.

Computational Methods

Force Field and Charges. The AMBER force field,[59] updated with new parameters for improved sugar pucker phases and helical repeat,[60] was used. RESP charges[61] and additional parameters for the adduct between ET743 and guanine were as described previously.[26]

Construction of the Starting Structures. Models of the free oligonucleotides were built using optimized parameters for B-DNA and A-DNA.[41] To model the complex between d(GTGGCGGCGGCC)$_2$ and three ET743 molecules (covalently modified guanines underlined), three copies of the ET743-bound central CGG triplet in the ET743-d(TAACGGTTA)$_2$ complex[26] (PDB entry 1ezh) were merged, using the flanking base pairs for fragment superposition, followed by appropriate modifications of base composition. The complex was then gradually refined using energy minimization techniques essentially as described.[26]

Molecular Dynamics of the Free and ET743-Bonded Dodecanucleotides in Water. Each molecular system was neutralized by addition of the appropriate number of sodium ions[62] placed along the phosphate bisectors, and immersed in a rectangular box of ~3000 TIP3P water molecules.[63] Each water box extended 7 Å away from any solute atom, and the cutoff distance for the nonbonded interactions was 9 Å. Periodic boundary conditions were applied and electrostatic interactions were represented using the smooth particle mesh Ewald method[64] with a grid spacing of ~1 Å. Unrestrained molecular dynamics (MD) simulations (lasting for 2 ns) at 300 K and 1 atm were then run for the free oligos and the covalent adduct using the SANDER module in AMBER.[65] SHAKE[66] was applied to all bonds involving hydrogens and an integration step of 2 fs was used throughout. The simulation protocol was the same as previously described, involving a series of progressive energy minimizations followed by a 20 ps heating phase and a 70 ps equilibration period before data collection.[26] System coordinates were saved every picosecond which yielded 2000 sets of structures in each case for further analysis.

Analysis of the Molecular Dynamics Trajectories. Three-dimensional structures and trajectories were visually inspected using the computer graphics program InsightII.[67] Root-mean-square (rms) deviations from the initial structures and interatomic distances were monitored using the CARNAL module in AMBER. The conformational and helical parameters of the DNA dodecamers were analyzed by means of program CURVES.[68] The magnitude and directionality of bending was related to the local helicoidal parameters roll and tilt and quantified in terms of the angle of axis deflection (θ) and its orientation relative to the major groove (ϕ). Graphical display of these quantities for individual base pair steps was performed using polar plots that allow both the magnitude and the direction of the helical bend to be represented in the form of "bending dials" in which θ and ϕ are the radial and angular coordinates, respectively. Points on the northern hemisphere of the dial reflect positive roll and compression of the major groove whereas bending into the minor groove is plotted on the southern hemisphere of the dial. Each ring on our bending dials indicates a 10° deflection of the helical axis, and ϕ runs clockwise from the top.

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Note Added in Proof

The subscript 2 following the brackets that enclose the sequence of the non-self-complementary oligonucleotides refers to the fact that they are double-stranded DNA, but it must be noted that the sequence of the complementary strand differs from the one shown.

Supporting Information Available: Two figures showing the time evolution of the rms deviation along the molecular dynamics trajectories for (1) the covalent 3·(ET743)-d(GTGGCGGCGGCC)$_2$ complex and (2) the d(GTGGCGGCGGCC)$_2$ oligonucleotide starting from A-DNA and B-DNA conformations. This material is available free of charge via the Internet at http://pubs.acs.org.

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