Binding of Actinomycin D to Single-Stranded DNA of Sequence Motifs d(TGTCT$_n$G) and d(TGT$_n$GTCT)

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ABSTRACT Our recent binding studies with oligomers derived from base replacements on d(CGTCGTCG) had led to the finding that actinomycin D (ACTD) binds strongly to d(TGTCATTG) of apparent single-stranded conformation without GpC sequence. A fold-back binding model was speculated in which the planar phenoxazone inserts at the GTC site with a loop-out T base whereas the G base at the 3′-terminus folds back to form a basepair with the internal C and stacks on the opposite face of the chromophore. To provide a more concrete support for such a model, ACTD equilibrium binding studies were carried out and the results are reported herein on oligomers of sequence motifs d(TGTCT$_n$G) and d(TGT$_n$GTC). These oligomers are not expected to form dimeric duplexes and contain no canonical GpC sequences. It was found that ACTD binds strongly to d(TGTC*TTTT*G), d(TG*TTTT*GTC), and d(TG*TTTTT*GTC), all exhibiting 1:1 drug/strand binding stoichiometry. The fold-back binding model with displaced T base is further supported by the finding that appending TC and TCA at the 3′-terminus of d(TGTC*TTTT*G) results in oligomers that exhibit enhanced ACTD affinities, consequence of the added basepairing to facilitate the hairpin formation of d(TGTC*TTTT*G**TC**) and d(TGTC*TTTT*G**TCA**) in stabilizing the GTC/GTC binding site for juxtaposing the two G bases for easy stacking on both faces of the phenoxazone chromophore. Further support comes from the observation of considerable reduction in ACTD affinity when GTC is replaced by GTTC in an oligomer, in line with the reasoning that displacing two T bases to form a bulge for ACTD binding is more difficult than displacing a single base. Based on the elucidated binding principle of phenoxazone ring requiring its opposite faces to be stacked by the 3′-sides of two G bases for tight ACTD binding, several oligonucleotide sequences have been designed and found to bind well.

INTRODUCTION

Actinomycin D (ACTD) is an extensively studied antitumor agent that contains a planar 2-aminophenoxazin-3-one chromophore and two bulky cyclic pentapeptide lactones (Fig. 1). It binds to duplex DNA with high affinity and dissociates slowly, which led to a suggested mechanism whereby the drug blocks the progression of RNA polymerase along the template DNA and terminates the transcription (Muller and Crothers, 1968). The DNA binding of ACTD is quite sequence specific and has been shown to prefer greatly the duplex GpC site. This base-sequence specificity derives mainly from the formation of strong hydrogen bonds in the minor groove, between the N-2 amino group and N-3 ring nitrogen of the two guanine-residues with the carbonyl oxygen atoms and amide groups of threonine residues of the cyclic peptapeptides, respectively (Sobell and Jain, 1972; Kamitori and Takusagawa, 1992). These essential drug-DNA hydrogen bonds are protected by the cyclic pentapeptides, which effectively shield them from solvent exposure. It is somewhat ironic that the notion of GpC sequence preference of ACTD has by now been so well established that it tends to blind-sight the historical fact that this preference was, in fact, originally proposed based on the crystal structure of an ACTD-deoxyguanosine complex (Sobell and Jain, 1972), with both faces of the ACTD chromophore being stacked by the G bases. The validity of such a model was subsequently confirmed by optical (Krugh, 1972) as well as NMR (Krugh and Neely, 1973) studies on dinucleotides in solutions.

Although the GpC sequence-specificity and the effects of adjacent bases on the ACTD binding appear to be well characterized (Chen, 1988; 1992), there have been recent reports to indicate that this drug may also bind strongly to some non-GpC-containing sequences (Snyder et al., 1989; Rill et al., 1989; Bailey et al., 1994) and even to some single-stranded DNA (Wadkins and Jovin, 1991; Hsieh et al., 1994; Wadkins et al., 1996; Rill and Hecker, 1996; Wadkins et al., 1998). In particular, it has been reported that ACTD binds strongly and cooperatively to a non-GpC-containing but self-complementary octamer d(CGTCGACG) with a 2:1 drug/duplex ratio (Snyder et al., 1989). In subsequent studies, our laboratory found that d(CGTCGTCG) and d(CGACGACG) also bind strongly to ACTD despite the lack of self-complementarity (Sha and Chen, 2000). Further studies with base replacements on d(CGTCGTCG) led us to uncover that ACTD binds strongly to the sequence d(TGTCATTG) of apparent single-stranded conformation without GpC sites (Chen and Sha, 2001). A fold-back binding mode was speculated for such a binding, wherein the planar phenoxazone inserts at the GTC site with a loop-out T base whereas the 3′-terminus G folds back to form a basepair with the internal C and stacks on the opposite face of the chromophore. Such a finding is significant and may have relevance in ACTD binding to single-stranded DNA in general. The purpose of this report is to present evidence to further support such a

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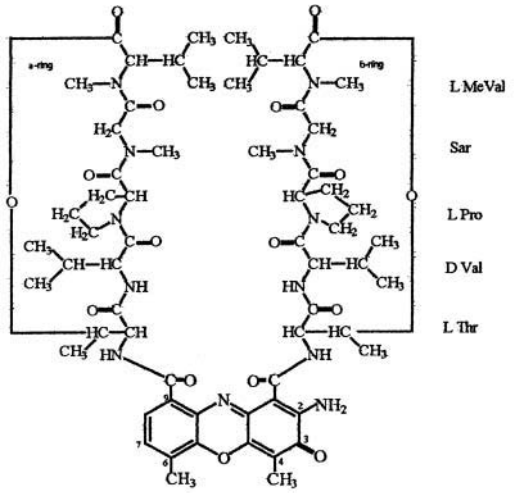

FIGURE 1 The chemical structure of actinomycin D.

binding model and from which to design other strong ACTD binding sequences using the delineated binding principle. Detailed elucidation of ACTD binding to DNA was made with oligomers of sequence motifs d(TGTCT$_n$G) and d(TGT$_n$GTCT) that are devoid of GpC sequences and exist predominantly in single-stranded conformations.

Hairpin model for single-stranded ACTD binding has been suggested earlier but in quite distinct sequence contexts (Rill and Hecker, 1996; Wadkins et al., 1998). For example, systematic binding studies of 7-amino-actinomycin D (7-AM-ACTD) with single-stranded oligomers containing -TAGT- sequences have led to the suggestion that the drug binds to metastable hairpins. The hairpins bound most tightly by ACTD contain non-Watson-Crick basepairs, including an A/G and two T/T mismatches (Wadkins et al., 1998). Understanding the ACTD binding to sequences that do not contain the classic GpC sites should provide considerable insights into its modes of binding to DNA and assist in uncovering other new strong binding sequences. Furthermore, studying ligands that bind to single-stranded DNA with particular sequence preferences may be of importance in the regulation of processes that require single-stranded DNA as intermediates.

MATERIALS AND METHODS

Synthetic oligonucleotides were purchased from Research Genetics (Huntsville, AL) and used without further purification. These oligomers were purified by the vendor via reverse-phase oligonucleotide purification cartridges and exhibited single-band electrophoretic mobility in denaturing polyacrylamide gel electrophoresis with stated purity of ≥95%. Concentrations of the DNA solutions (in nucleotide) were determined by measuring the absorbances at 260 nm after melting (at 95°C). The extinction coefficients of DNA oligomers were obtained via nearest-neighbor approximation using mono- and dinucleotide values tabulated in Fasman (1975). ACTD and 7-AM-ACTD were purchased from Sigma (St. Louis, MO). Concentrations of the drug solutions were determined by measuring the absorbances at 440 nm (for ACTD) and 528 nm (for 7-AM-ACTD), using extinction coefficients of 24,500 and 23,600 cm^{-1} M^{-1}, respectively. Stock solutions for oligonucleotides and drugs were prepared by dissolving in 10 mM Tris-borate buffer solution of pH 8 containing 0.1 M NaCl and 1 mM $MgCl_2$. Absorption spectra were measured with a Cary 1E spectrophotometric system (Varian, Palo Alto, CA). Absorption spectral titrations were carried out by starting with a 5 μM ACTD solution of 2 mL followed by progressive additions of the oligomer stock at equal time intervals. Absorbance differences between 427 and 480 nm during absorption spectral titrations were used to obtain the binding isotherms. The choice of 427 nm is based on the fact that the absorbance change at this wavelength is slightly larger than that of 440 nm upon DNA binding and that of the 480 nm is to mimic the baseline value such that the absorbance differences of two wavelengths should minimize the errors due to baseline fluctuations during titrations.

Association binding constants (K_1) were extracted via nonlinear least-squares fits on the experimental binding isotherms using 1:1 drug/strand binding model: $D + S = DS$, where D, S, and DS are free drug, free single-stranded DNA and drug-DNA complex, respectively. By means of equations for mass balances of drug and DNA (in strand), the following equations can be derived:

$$D^2K_1 + D[K_1D_t(X-1)+1] = D_t$$
$$S = D_t(X-1) + D$$
$$Y = (\varepsilon_D + \varepsilon_1K_1S)D/D_t,$$

where $X \equiv S_t/D_t$, ε_D, and ε_1 are the extinction coefficients of the free and bound drugs, whereas D_t and S_t are the total drug and DNA strand concentrations, respectively, at each point of the titration. Experimental binding isotherms were plotted as the apparent extinction coefficient (Y) vs. X and nonlinear least-squares fits with the above equations were made to extract the binding constant K_1 and other parameters ε_D and ε_1. Nonlinear least-squares fit program of MicroMath (Salt Lake City, Utah) was used for our fitting purposes.

RESULTS AND DISCUSSION

ACTD binds strongly to d(TGTCTTTTG), d(TGTTTTGTCT), and d(TGTTTTTGTCT) with 1:1 drug/strand binding stoichiometry

Equilibrium binding studies of ACTD were carried out with oligomers of sequence motifs d(TGT$_n$GTCT) and d(TGTCT$_n$G). The absence of A base in these oligomers should reduce possible contributions from dimeric duplex conformations. Equilibrium binding isotherms for these two series along with their nonlinear least-squares fitted curves are shown in Fig. 2. As is apparent from panel *A*, the ACTD binding affinity for the d(TGT$_n$GTCT) oligomers increases progressively as the T-run is increased from 2 to 5. Although d(TG*T*GTCT) also exhibits weak ACTD affinity, it is slightly higher than that of d(TG*TT*GTCT). It is also quite evident that ACTD binds avidly to d(TG*TTTTT*GTCT) with a binding stoichiometry of 1:1 drug/DNA strand, as evidenced by the abrupt saturation of the titration curve at this ratio. Thus, the binding isotherms were fitted with a 1:1 binding model, using nonlinear least-squares method. As can be seen from the plots, the fits for this series are excellent. The binding constants extracted from these fits are summarized in Table 1 for oligomers of both sequence motifs. The shorter oligomers d(TG*T*GTCT) and d(TG*TT*GTCT) exhibit binding constants of 0.027×10^6

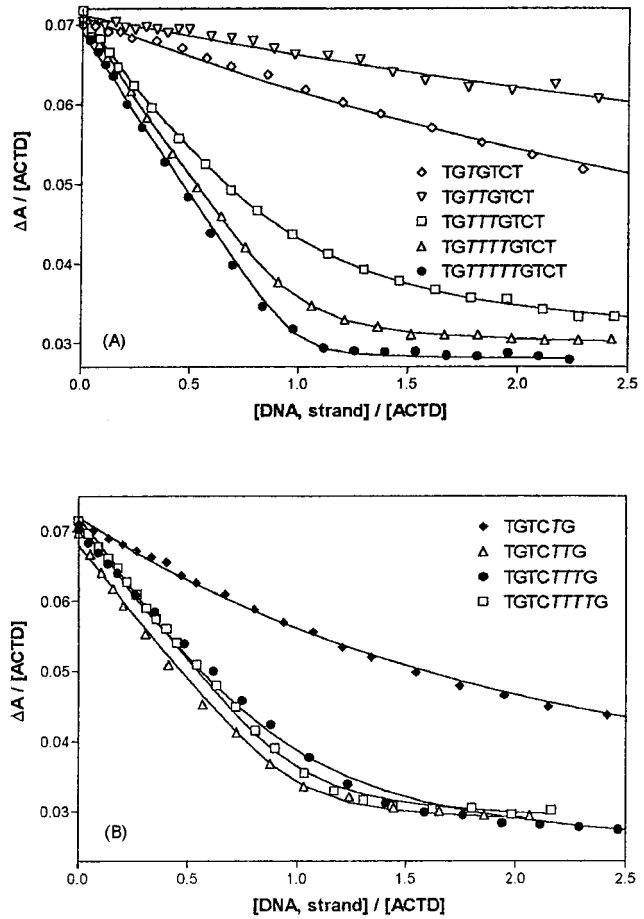

FIGURE 2 Equilibrium binding isotherms for ACTD binding to oligomers of d(TGT$_n$GTCT) sequence motif (*A*) and oligomers of d(TGTCT$_n$G) sequence motif (*B*). Absorbance spectral titrations were carried out at 25°C and the absorbance difference ΔA is that between 427 and 480 nm. Concentrations of both ACTD and DNA (in strand) are expressed in μM. Solid curves are those of nonlinear least-squares fits of the experimental isotherms using the 1:1 drug/strand binding model.

and $0.013 \times 10^6\ M^{-1}$, respectively. The longer oligomers exhibit considerably stronger ACTD affinities with extracted binding constants of 1.1×10^6, 7.5×10^6, and $19 \times 10^6\ M^{-1}$ for d(TG*TTT*GTCT), d(TG*TTTT*GTCT), and d(TG*TTTTT*GTCT), respectively.

The ACTD binding isotherms for oligomers of the d(TGTCT$_n$G) series are shown in Fig. 2 *B*. It is immediately apparent that d(TGTC*T*G) binds ACTD considerably weaker than the longer ones. The binding constant for this oligomer was found to be around $0.01 \times 10^6\ M^{-1}$, whereas the binding constants for d(TGTC*TT*G), d(TGTC*TTT*G), and d(TGTC*TTTT*G) were found to be 2.8×10^6, 1.1×10^6, and $4.7 \times 10^6\ M^{-1}$, respectively. Except for d(TGTC*TT*G), its unusually strong ACTD affinity requires further investigation, the ACTD affinity of this series also increases as the number of bases in the T-run is increased. The severalfold enhancement in ACTD affinity of d(TGTC*TTTT*G) as compared to that of d(TGTC*TTT*G), which in turn is two

TABLE 1 Comparison of binding and melting parameters for oligomers of sequence motifs d(TGT$_n$GTCT) and d(TGT$_n$G)

Oligonucleotides	K_1 (μM^{-1})	T_m^o (°C)	T_m (°C)
d(TG*T*GTCT)	0.027 ± 0.07		24
d(TG*TT*GTCT)	0.013 ± 0.01		25
d(TG*TTT*GTCT)	1.09 ± 0.08		41
d(TG*TTTT*GTCT)	7.5 ± 0.6		55
d(TG*TTTTT*GTCT)	19 ± 6		56
d(TGTC*T*G)	0.012 ± 0.02		27
d(TGTC*TT*G)	2.8 ± 0.2		55
d(TGTC*TTT*G)	1.1 ± 0.2		54
d(TGTC*TTTT*G)	4.7 ± 0.9		55

Equilibrium binding titrations were carried out at 25°C and the equilibrium binding constant K_1 is extracted from a nonlinear least-squares fit of the experimental binding isotherm using the 1:1 drug/strand binding model. T_m^o and T_m are melting temperatures of 40 μM of DNA/0.1 M NaCl (in nucleotide) in the absence and in the presence of 7 μM ACTD. Most of the melting profiles for DNA alone are too broad and diffuse for accurate melting temperature determinations.

orders of magnitude higher than that of d(TGTC*T*G), is again consistent with the monomeric fold-back binding model. Although significant ACTD binding strengths exhibited by d(TGTC*TT*G) is not expected, they may have their origin in major contributions from sources other than monomeric hairpin formation.

The stronger ACTD binding exhibited by the oligomers with longer T-run appears to be in line with our speculated fold-back binding model, as the propensity for dimeric duplex formation is low and hairpin formation with 3- to 5-base loops can easily be formed. A schematic drawing of our proposed hairpin-binding model as represented by d(TGTC*TTT*G) is shown in Fig. 3. The phenoxazone plane inserts at the GTC site by pushing out the T base and induces the oligomer to fold back to form a G • C basepair so as to facilitate stacking of the 3′-sides of the two G bases on the opposite faces of the drug chromophore to form a tight complex. The weaker ACTD affinities for the shorter oligomers are, thus, partly the consequence of their inability to form stable drug-induced fold-back complexes.

Binding studies with oligomers derived from appending bases at the 3′-end of d(TGTCTTTTG)

If the drug induced fold-back to form a G • C basepair is indeed the mechanism for ACTD binding to d(TGTC*TTTT*G), its binding may be affected by base additions at the 3′-terminus, especially by those that can facilitate the hairpin formation. Binding studies were thus made with oligomers derived from appending C, AC, and TC at the 3′-end of d(TGTC*TTTT*G) and their binding isotherms are compared in Fig. 4 *A*. The enhanced ACTD binding affinity due to these additions can clearly be seen, with the order of enhancement being: d(TGTC*TTTT*G**C**) > d(TGTC*TTTT*G**TC**) > d(TGTC*TTTT*G**AC**) > d(TGTC-*TTTT*G). The extracted binding parameters via nonlinear least-squares fits with the 1:1 drug/strand binding model are

summarized in Table 2 and the results appear to strongly support the hairpin-binding model. If the ACTD-induced fold-back of the oligomer is the mechanism for binding, stronger ACTD binding would be anticipated for d(TGTC*TTTT*G**C**), where one additional G • C basepair can be formed at the GTC/GC stem of the hairpin to facilitate the binding site formation as well as to assist in the displacement of the T base for the ACTD binding. Indeed, a binding constant of around 100 $\times$ 10^6 M^{-1} was found, a more than 10-fold increase in the ACTD affinity as compared to that of d(TGTC*TTTT*G), with the same 1:1 drug/strand stoichiometry (Fig. 4 *A*). By the same reasoning, one would also expect d(TGTC*TTTT*G**TC**) to bind strongly inasmuch as a GTC/GTC site will also have two G • C basepairs and the T/T mismatch should facilitate T-base displacements for ACTD binding. Indeed, a binding constant of 20 $\times$ 10^6 M^{-1} was found with the same binding stoichiometry. This value is somewhat lower than that of d(TGTC*TTTT*G**C**), inasmuch as the GTC/GC site requires only a single T-base displacement whereas GTC/GTC requires two single-base displacements. If base loop-outs are in fact required for ACTD binding to the GTC/GTC site, one would anticipate a weaker affinity at the GTC/GAC site because of the T • A basepair formation, which would somewhat hinder such displacements. Indeed, the ACTD binding strength for d(TGTC*TTTT*G**AC**) is found to be 9 $\times$ 10^6 M^{-1}, still a quite sizable value but is about twofold less than that of the GTC/GTC counterpart. It is also interesting to note that the ACTD binding affinity for d(TGTC*TTTT*G**TCA**) is around 100 $\times$ 10^6 M^{-1} (Table 2), severalfold greater than that of d(TGTC*TTTT*G**TC**). This can be attributed to the added A • T basepair formation at the duplex ends to further stabilize the GTC/GTC binding sites.

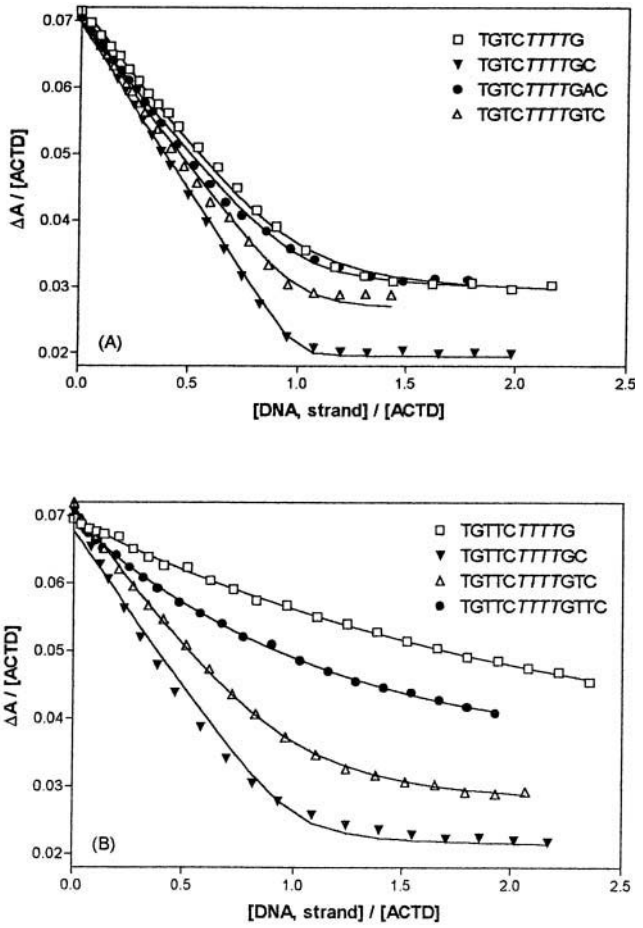

FIGURE 4 ACTD equilibrium binding isotherms at 25°C for oligomers derived by appending 3′-terminus of d(TG**T**C*TTTT*G) with C, AC, and TC (*A*); and for oligomers derived from appending 3′-end of d(TG**TT**C*TTTT*G) with C, TC, and TTC (*B*).

Studies with the corresponding GTTC-containing oligomers

If binding at the GTC site requires T-base displacements, one would expect a weaker binding at the GTTC site because of the need to displace two T bases in this case, which should be more difficult than a single-base displacement. Thus, studies were also made with the corresponding oligomers containing GTTC instead of GTC and the resulting isotherms are shown in Fig. 4 *B*. It is immediately apparent from the profiles that the ACTD affinities are considerably weaker than their GTC counterparts (compare with Fig. 4 *A*) but the trend in binding affinities are similar: d(TG**TT**C*TTTT*G***C**) > d(TG**TT**C*TTTT*G***TC**) > d(TG**TT**C*TTTT*G***TTC**) > d(TG**TT**C*TTTT*G). The model-fitted binding parameters are also included in Table 2 for comparison. ACTD binding constant for d(TG**TT**C*TTTT*G) is found to be 0.07 $\times$ 10^6 M^{-1}, ~70-fold weaker than that of d(TG**T**C*TTTT*G). However, the addition of a C at the 3′-end may result in the formation of two G • C basepairs with TT-bulge formation, which should facilitate ACTD binding. Indeed, a nearly two orders of magnitude enhancement of ACTD binding affinity is observed with a binding constant of 17 $\times$ 10^6 $M^{-1}$for d(TG**TT**C*TTTT*G***C**). This value, however, is considerably lower than that of the corresponding oligomer d(TG**T**C*TTTT*G***C**), illustrating the added difficulty

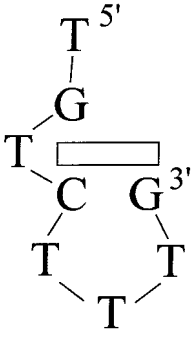

FIGURE 3 Schematic drawing of the speculated hairpin-binding model as represented by d(TGTC*TTT*G). The rectangle represents the phenoxazone ring of ACTD.

TABLE 2 Comparison of binding and melting parameters for oligomers of various sequences

Oligonucleotides	K_1 (μM^{-1})	T_m^o (°C)	T_m (°C)
d(TGTC*TTTT*G)	4.7 ± 0.9		55
d(TGTC*TTTT*GC)	>100	30	70
d(TGTC*TTTT*GAC)	9.0 ± 3.5	41	57
d(TGTC*TTTT*GTC)	20 ± 16		65
d(TGTC*TTTT*GTCA)	>100		70
d(TGTTC*TTTT*G)	0.07 ± 0.01		32
d(TGTTC*TTTT*GC)	17 ± 12		47
d(TGTTC*TTTT*GTC)	3.1 ± 0.3		38
d(TGTTC*TTTT*GTTC)	0.47 ± 0.07		29
d(CGT*TTT*GTG)	1.18 ± 0.08		39
d(CCGT*TTT*GTGG)	10 ± 5		47
d(CGTGT*TTT*GTACG)	18 ± 14	34	50

in forming a two-base bulge. It is also interesting to note that a binding constant of 3.1 × 10^6 M^{-1} is observed for d(TG**TT**C*TTTT*G***TC***), severalfold weaker than d(TG**TT**C*TTTT*G***C***) as well as d(TG**T**C*TTTT*G***TC***). A binding constant of 0.47 × 10^6 M^{-1} is observed for d(TG**TT**C*TTTT*G***TTC***), which is nearly sevenfold weaker than that of d(TG**TT**C*TTTT*G***TC***), demonstrating the less favorable nature of the GTTC/GTTC site as compared to that of GTTC/GTC GTTC/GC sites. Similar measurements were also made for oligomers containing -*TTT*- loops (not shown) and the results are found to be similar in trend to those of -*TTTT*- containing oligomers.

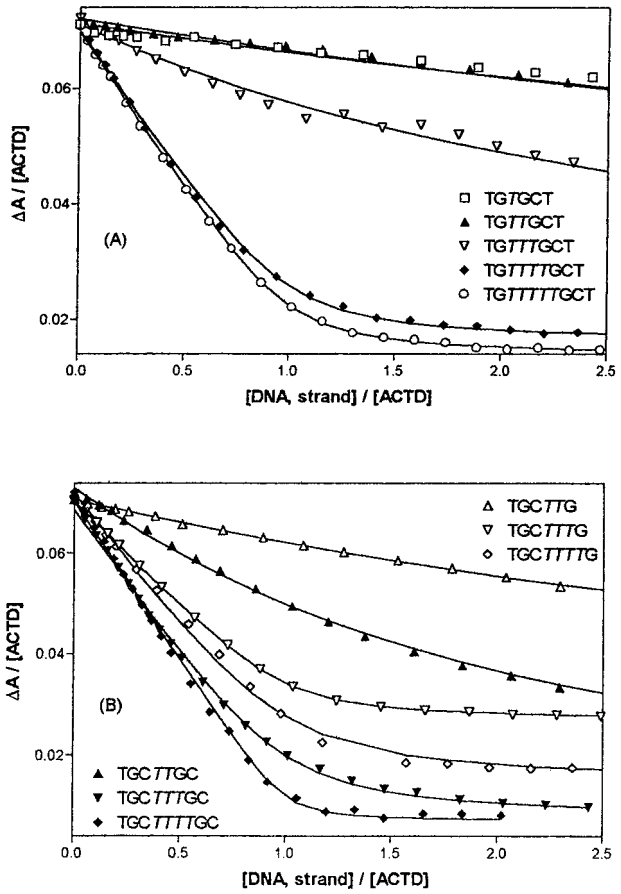

FIGURE 5 ACTD equilibrium binding isotherms at 25°C for oligomers of d(TGT_nGCT) motif (*A*) and for oligomers of d($TGCT_nG$) and d($TGCT_nG$**C**) motifs (*B*).

Studies with GC-containing counterparts, d(TGT_nGCT) and d($TGCT_nG$)

If the speculated binding model is valid for the GTC-containing oligomers, it should also be applicable to the corresponding GC-containing sequences because no T-base displacements will be needed. ACTD binding studies were, thus, also made with d(TGT_nGCT) and d($TGCT_nG$) sequence motifs and the profiles of binding isotherms are shown in Fig. 5 and the extracted binding parameters are summarized in Table 3. For the d(TGT_nGCT) series (Fig. 5 *A*), the results indicate that ACTD binds weakly to d(TG*T*GCT), d(TG*TT*GCT), and d(TG*TTT*GCT) with respective binding constants of 0.013 × 10^6, 0.014 × 10^6, and 0.06 × 10^6 M^{-1}, but quite well to d(TG*TTTT*GCT) and d(TG*TTTTT*GCT) with binding constants of 4.7 × 10^6 and 6.2 × 10^6, respectively. These results support the drug-induced fold-back binding model with G • C basepair formation and two G bases stacking on the opposite faces of the ACTD chromophore.

For the d($TGCT_nG$) series, the binding order is found to be: d(TGC*T*G) ≈ d(TGC*TT*G) ≪ d(TGC*TTT*G) ≈ d(TGC*TTTT*G), as expected. The extracted binding constants for d(TGC*TTT*G) and d(TGC*TTTT*G) are 5.5 × 10^6 and 3.0 × 10^6 M^{-1}, respectively, an order of magnitude higher than their shorter counterparts d(TGC*T*G) and d(TGC*TT*G), which exhibit binding constants of 0.33 × 10^6 and 0.27 × 10^6 M^{-1}, respectively. It should be pointed out that whereas ACTD binds reasonably well to d(TGC-*TTT*G), it binds quite weakly to d(TG*TTT*GCT) despite the same number of intervening T bases between GC and G. This may be rationalized by the fact that the formation of a G • C basepair via folding-back results in a 3-base T-loop for the former, whereas it will result in only a 2-base-loop (with GCT/TGT stem) for the latter, which will likely suffer considerable strain. The addition of a C base at the 3′-terminus of d($TGCT_nG$) will result in d($TGCT_nG$**C**) which should considerably facilitate the hairpin formation and will in turn enhance the ACTD binding. This is indeed the case, as can be seen from Fig. 5 *B*, where the binding isotherms for the d($TGCT_nG$**C**) series are also included for comparison. It should also be pointed out in passing that whereas ACTD binds d(TG*TTT*GCT) weakly, it binds to d(TG*TTT*G**T**CT) an order of magnitude stronger. The basis for such a difference, however, is not clear.

TABLE 3 Comparison of binding and melting parameters for oligomers with GTC replaced by GC

Oligonucleotides	K_1 (μM^{-1})	T_m^o (°C)	T_m (°C)
d(TG*T*GCT)	0.013 ± 0.008		28
d(TG*TT*GCT)	0.014 ± 0.009		31
d(TG*TTT*GCT)	0.06 ± 0.01		30
d(TG*TTTT*GCT)	4.7 ± 0.8		50
d(TG*TTTTT*GCT)	6.2 ± 0.5		56
d(TGC*T*G)	0.33 ± 0.01		36
d(TGC*TT*G)	0.27 ± 0.03		25
d(TGC*TTT*G)	5.5 ± 0.1		30
d(TGC*TTTT*G)	3.0 ± 0.5		55
d(TGC*TT*GC)	0.14 ± 0.01		37
d(TGC*TTT*GC)	4.1 ± 0.6	26	41
d(TGC*TTTT*GC)	11.7 ± 1.0	33	64

Designing other strong-binding sequence motifs

To further test and to make use of the binding principle, ACTD binding studies were also made with d(**C**GT*TTT*GT**G**), d(**CC**GT*TTT*GT**GG**), and d(**CGT**GT*TTT*GT**ACG**). These oligomers were designed based on the delineated binding principle. The increasing presence of the bold-faced complementary bases at both ends will presumably facilitate the drug-induced hairpin formation. This should bring the GT/GT in close proximity to form G • T and T • G mismatches to create a binding site such that the ACTD chromophore can be stacked by the 3′-sides of the two G bases. A binding stoichiometry of 1:1 drug/strand would be predicted for these oligomers, if no additional sites were bound by ACTD. The binding isotherms of these three oligomers along with that of d(TGTC*TTT*G) are compared in Fig. 6. As is apparent, the ACTD binding affinity of d(**C**GT*TTT*GT**G**) is seen not to be greatly different from that of d(TGTC*TTT*G), whereas d(**CC**GT*TTT*GT**GG**) and d(**CGT**GT*TTT*GT**ACG**) exhibit considerably higher binding propensities with an apparent 1:1 drug/strand binding stoichiometry. Binding constants for d(**C**GT*TTT*GT**G**), d(**CC**GT*TTT*GT**GG**), and d(**CGT**-GT*TTT*GT**ACG**) were found to be 1.2×10^6, 10×10^6, and $18 \times 10^6\ M^{-1}$, respectively, which are to be compared with that of $1.1 \times 10^6\ M^{-1}$ found for d(TGTC*TTT*G). The ability to form a 2- or 3-basepaired stem for the stronger binding oligomers is consistent with the drug-induced hairpin-binding model.

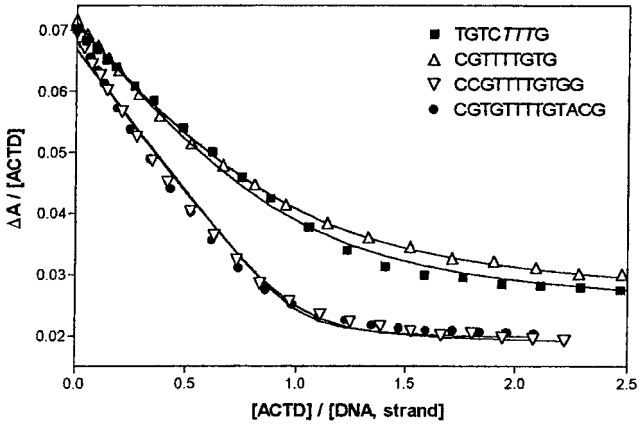

FIGURE 6 ACTD equilibrium isotherms at 25°C for d(CGT*TTT*GTG), d(CCGT*TTT*GTGG), and d(CGTGT*TTT*GTACG). Binding isotherm for d(TGTC*TTT*G) is also included for comparison.

CONCLUSION

Equilibrium binding studies with oligomers of sequence motifs d(TGTCT$_n$G) and d(TGT$_n$GTC) have led to the finding that ACTD binds strongly to d(TGTC*TTTT*G), d(TG*TTTT*GTC), and d(TG*TTTTT*GTC), all exhibiting 1:1 drug/strand binding stoichiometry. These oligomers contain no canonical GpC sequences and are not expected to form dimeric duplexes. A monomeric binding model was proposed to account for the strong ACTD affinities of these oligomers, in which the drug molecule inserts at the GTC site with a displaced T base while inducing the G base at the other end to fold back and form a G • C basepair so that the 3′-sides of the two G bases can stack on the opposite faces of the planar phenoxazone chromophore (Fig. 3).

The fold-back binding model with displaced T base is further supported by the finding that appending TC or TCA at the 3′-terminus of d(TGTC*TTTT*G) results in oligomers that exhibit enhanced ACTD affinity. This may be attributed to the facilitated hairpin formation of d(TGTC*TTTT*G**TC**) and d(TGTC*TTTT*G**TCA**) via extra basepair(s) at the duplex stem to form the GTC/GTC binding site such that the two G bases are juxtaposed for easy stacking on both faces of the phenoxazone chromophore. The identification of GTC/GTC as the binding site and the need for T-base displacements during ACTD binding are consistent with the finding that although the oligomer resulted from adding AC at the 3′-end also exhibits enhanced ACTD binding, its effect is somewhat less than that of the TC addition. This can be attributed to the fact that even though the formation of the GTC/GAC site is facilitated via the hairpin formation of d(TGTC*TTTT*G**AC**), the ACTD binding requires the disruption of the **T • A** basepair with subsequent base displacements from the duplex stem, which is expected to be somewhat more difficult than that of the GTC/GTC counterpart.

The hairpin-binding model with displaced bases is further supported by the considerable reduction on ACTD affinity when GTC in an oligomer is replaced by GTTC. This is in line with the reasoning that displacing two T bases to form a bulge for ACTD binding is more difficult than that of a single-base bulge. The ACTD binding can, however, be greatly enhanced if GC or GTC is placed at the other end of the oligomer to facilitate the hairpin formation and to induce base displacements. Comparative studies with the corre-

sponding GC-containing oligomers revealed similar binding stoichiometries to those of their GTC counterparts, further supporting the proposed hairpin-binding model.

Some interesting comparisons on the binding energetics are worthy of mention. For example, the formation of a bulged T upon ACTD binding to d(TG**T**CTTTTGC) ($\geq$100 μM^{-1}) resulted in a gain of binding free energy by ~5.3 kJ/mol from that of d(TGCTTTTGC) (11.7 μM^{-1}), suggesting favorable contributions from the bulged base on binding. Addition of a T base to the other GC site resulted in d(TG**T**C*TTTT*G**T**C) (20 μM^{-1}) with the loss of the binding free energy by ~4 kJ/mol from d(TG**T**CTTTTGC), partly due to the somewhat less favorable G • C basepair formation in GTC/GTC as compared to GTC/GC despite the additional T-bulge formation for binding facilitation. This energy loss, however, can be regained by appending an A base at the 3′-end to form d(TG**T**C*TTTT*G**T**CA) ($\geq$100 μM^{-1}) to stabilize the GTC/GTC site via additional basepair formation. Thus it is clear that a T-bulge significantly enhances ACTD binding, likely through favorable interactions with the drug. This is further confirmed by the observation of an avid binding of ACTD to d(TGCTTTTGTC) ($\geq$100 μM^{-1}) (data not shown), where the bulged T is now on the 3′- rather than the 5′-side. As to whether the bulged base would be more important for the quinoid or the benzenoid portion of the chromophore requires further studies.

The strongest evidence in support of the proposed hairpin-binding model with base displacements and favorable contributions from the bulged bases comes from our most recent NMR study on the ACTD binding to d(GTC*ACC*GAC) and d(GGC*ACC*GCC) (Chou et al., 2002). In the absence of the drug, these oligomers exist as stable hairpins with *ACC* loop and 5′-GTC/CAG-5′ and 5′-GGC/CCG-5′ duplex stems. In the presence of ACTD, however, they form DNA-drug complexes with a rather novel intercalation mode. The central Watson-Crick T/A and G/C basepairs are looped out and displaced by the ACTD chromophores. The looped-out bases are not disordered but interact perpendicularly with the base/chromophore and form specific hydrogen bonds with DNA. Such a novel complex structure provides intriguing insights into how ligands interact with DNA and enlarges the repertoires for sequence-specific DNA recognition.

Based on the binding principle of requiring the simultaneous stacking of 3′-sides of both G bases on the opposite faces of the planar phenoxazone chromophore, sequences such as d(**C**GT*TTT*GT**G**), d(**CC**GT*TTT*GT**GG**), and d(**CGT**GT*TTT*GT**ACG**) were designed and tested for their ACTD binding propensity. Folding back of these oligomers should result in hairpin formation with duplex stems of 1, 2, and 3 basepairs, and ACTD molecule is expected to bind at the GT/GT site with the 3′-sides of the two G bases stacking on the opposite faces of the phenoxazone chromophore. Stronger ACTD affinities will be expected for those oligomers with easier hairpin-forming propensity. Indeed, d(**C**GT-*TTT*GT**G**) exhibits only moderately strong ACTD binding affinity whereas d(**CC**GT*TTT*GT**GG**) and d(**CGT**GT*TTT*-GT**ACG**) exhibit an order of magnitude stronger binding.

Finally, it should also be noted that the strong ACTD binding to GXC site with bulged X base is not restricted simply to hairpin motifs, as we have already demonstrated earlier for some dimeric duplex forming sequences (Chen and Sha, 2002). By regarding ACTD to prefer having the 3′-sides of two G bases stacked on the opposite faces of its planar phenoxazone, one has greatly expanded the repertoire of this drug. The classic duplex GpC sequence preference of ACTD can, thus, be simply viewed as the consequence of accomplishing such a juxtaposition of the G bases via G • C basepair formation.

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