DNA Binding by Analogues of the Bifunctional Intercalator TANDEM

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ABSTRACT: We have used DNase I footprinting to study the binding strength and DNA sequence selectivity of novel derivatives of the quinoxaline bis-intercalator TANDEM. Replacing the valine residues in the cyclic octadepsipeptide with lysines does not affect the selectivity for TpA but leads to a 50-fold increase in affinity. In contrast, replacing both of the quinoxaline chromophores with naphthalene rings abolishes binding, while changing a single ring decreases the affinity, and footprints are observed at only the best binding sites (especially TATATA). By using fragments with different lengths of $[(AT)_n]$, we demonstrate that these ligands bind best to the center of the longer $(AT)_n$ tracts.

The quinoxaline group of antibiotics consists of an octadepsipeptide ring to which two quinoxaline chromophores are attached (*1*). These compounds have long been known to bind to DNA by bifunctional intercalation (*2, 3*), sandwiching two base pairs between the quinoxaline rings. The natural antibiotics echinomycin and triostin A (containing a hemithioacetal and a disulfide cross bridge, respectively) bind with micromolar dissociation constants to GC-rich DNAs (*2, 4*) and are selective for the dinucleotide CpG (*5, 6*). Their sequence selectivity has been explained with several high-resolution structures of the ligands bound to short oligonucleotides (*7–12*) and arises from the formation of hydrogen bonds between the alanine carbonyls and the 2-amino groups of the guanines. In contrast, the synthetic derivative TANDEM (*13*) (Figure 1), which lacks the four *N*-methyl groups, binds best to AT-rich DNA (*13*) and is selective for the dinucleotide TpA (*14–18*). The very different sequence selectivity of this derivative arises because removal of the *N*-methyl groups enables the formation of intramolecular hydrogen bonds between the alanine carbonyls and the valine NH groups (*19*), and several NMR studies have shown the details of its interaction with the dinucleotide TpA (*20–23*). The derivative containing *N*-methylcysteine and valine is also AT-selective (*14, 15, 17*). These synthetic derivatives bind with slightly lower affinities, though they display highly cooperative interactions with poly(dA-dT). The interaction with TpA depends on the flanking sequences, and the ligand binds best to ATAT and not to TTAA (*14, 15, 24*).

The properties of several natural and synthetic derivatives of these compounds have been investigated. The cross bridge does not affect the sequence selectivity, though its removal causes a dramatic decrease in affinity (*13, 25*). The quinoxaline chromophores alone do not bind to DNA, though derivatives lacking these intercalating groups do not bind (*13*). Replacing the quinoxaline moieties of echinomycin with quinolines increases the affinity for AT-rich DNAs (*26, 27*), suggesting that the aromatic rings are more than simple inert wedges. Other modifications of the amino acids in the octadepsipeptide ring are found in the natural derivatives quinomycin C and triostin C, which contain *N*,γ-dimethyl-L-alloisoleucine in place of *N*-methylvaline; these derivatives exhibit the same selectivity for CpG, are less soluble, and bind with higher affinity (*13*). Several other natural derivatives of these compounds, including luzopeptin and thiocoraline, contain decadepsipeptide rings and bind with high affinity but reduced sequence selectivity (*28, 29*).

In this paper, we have used DNase I footprinting to study the DNA binding and sequence selectivity of novel synthetic derivatives, accessible through the recently described solid-phase synthesis (*30*), in which the valine residues have been replaced with lysines ([Lys^4,Lys^8]TANDEM) or in which one or both quinoxaline rings have been replaced with naphthalenes (mononaphthoyl-TANDEM and bisnaphthoyl-TANDEM). For these studies, we have used a footprinting fragment that contains all 64 symmetrical hexanucleotide sequences (*24*), which we have previously used to explore the sequence selectivity of TANDEM. We have also explored the cooperativity by examining the interaction of these ligands with fragments that contain different length $(AT)_n$ tracts.

MATERIALS AND METHODS

TANDEM Analogues. The chemical structures of the TANDEM analogues used in this work are shown in Figure 1a; the synthesis of these compounds will be reported elsewhere. As a result of the known low aqueous solubility of the parent compounds, stock solutions (2 mM) were prepared in DMSO and stored at −20 °C. These were diluted to the working concentration in 10 mM Tris-HCl (pH 7.4) immediately before being used.

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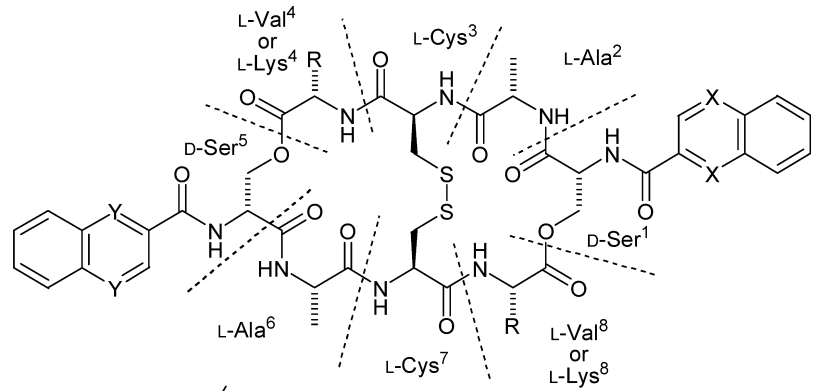
a)



TANDEM R = X = Y = N

[Lys^4,Lys^8]TANDEM R = NH_2 X = Y = N

bis-Naphthoyl-TANDEM R = X = Y = C

mono-Naphthoyl-TANDEM R = X = N; Y = C

b)

HexA

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5′-GGATCCCGGGATATCGATATATGGCGCCAAATTTAGCTATAGATCTAGAATTCCGGACCGCGGTTTAAAC
GTTAACCGGTACCTAGGCCTGCAGCTGCGCATGCTAGCGCTTAAGTACTAGTGCACGTGGCCATGGATCC-3′
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HexB

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5′-GGATCCGGCCGATCGCGAGCTCGAGGGCCCTAATTAGCCGGCAATTGCAAGCTTATAAGCGCGCTACGTA
TACGCGTACGCGCGTATATACATATGTACATGTCGACGTCATGATCAATATTCGAATTAATGCATGGATCC-3′
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FIGURE 1: (a) Chemical structure of the TANDEM analogues. (b) Sequences of the HexAfor and HexBfor fragments. HexArev and HexBrev are visualized by labeling the complementary strands.

DNA Fragments. The HexA and HexB DNA fragments (Figure 1b) contain all 64 symmetrical hexanucleotide sequences. These were prepared as previously described (*24*). Briefly, HexAfor and HexArev, which contain the same sequence cloned in opposite orientations, were obtained by cutting the parent plasmid with *Hin*dIII and *Sac*I, while HexBfor and HexBrev were obtained by cutting with *Eco*RI and *Pst*I. These were labeled at the 3′-end of the *Hin*dIII or *Eco*RI site with [α-^{32}P]dATP using reverse transcriptase. Each of the fragments is cloned in both orientations as the "forward" and "reverse" sequences so that regions toward the top of the gel with the forward sequences are located toward the bottom of the reverse sequences. A synthetic fragment was also prepared for the examination of the interaction of these compounds with long $(AT)_n$ tracts. Synthetic oligonucleotides (obtained from ATD Biosciences, Southampton, U.K.) corresponding to the sequence in Figure 5 were cloned into the *Bam*HI site of pUC19. These contain $(AT)_2$, $(AT)_4$, and $(AT)_8$ tracts, separated by the GTTC sequence to which TANDEM is not expected to bind. The clone that was used contained a dimeric insert of this sequence.

DNase I Footprinting. DNase I footprinting was performed as previously described (*31, 32*); 1.5 μL of radiolabeled DNA [dissolved in 10 mM Tris-HCl (pH 7.5) containing 0.1 mM EDTA at a concentration of approximately 10 cps/μL] was mixed with 1.5 μL of ligand [dissolved in 10 mM Tris-HCl (pH 7.5) containing 10 mM NaCl]. The mixture was equilibrated for 30 min at room temperature before digestion with 2 μL of DNase I at a concentration of approximately 0.01 unit/mL (dissolved in 20 mM NaCl, 2 mM $MgCl_2$, and 2 mM $MnCl_2$). The reaction was stopped after 1 min by adding 4 μL of formamide containing 10 mM EDTA and bromophenol blue. The digestion products were resolved on 8% (w/v) denaturing polyacrylamide gels. After electrophoresis, the gels were fixed, dried, and exposed to a phosphorimager screen (Kodak).

Quantitative Analysis. The intensity of each of the bands within the footprints obtained with the long $(AT)_n$ tracts was estimated using ImageQuant. C_{50} values (*33*), representing the ligand concentration that reduced the intensity of bands in the footprint by 50%, were estimated from this by fitting the data with a simple binding curve. Since the DNA concentration (approximately nanomolar) is much lower than the ligand dissociation constants (typically micromolar), the C_{50} values approximate the ligand dissociation constants.

RESULTS

TANDEM Analogues. Figure 2 shows the results of DNase I footprinting experiments with [Lys^4,Lys^8]TANDEM, mononaphthoyl-TANDEM, and bisnaphthoyl-TANDEM on the HexBfor fragment, alongside a similar experiment with TANDEM for comparison. It can be seen that the footprinting patterns for [Lys^4,Lys^8]TANDEM and TANDEM are very similar, suggesting that they bind to the same TpA sequences.

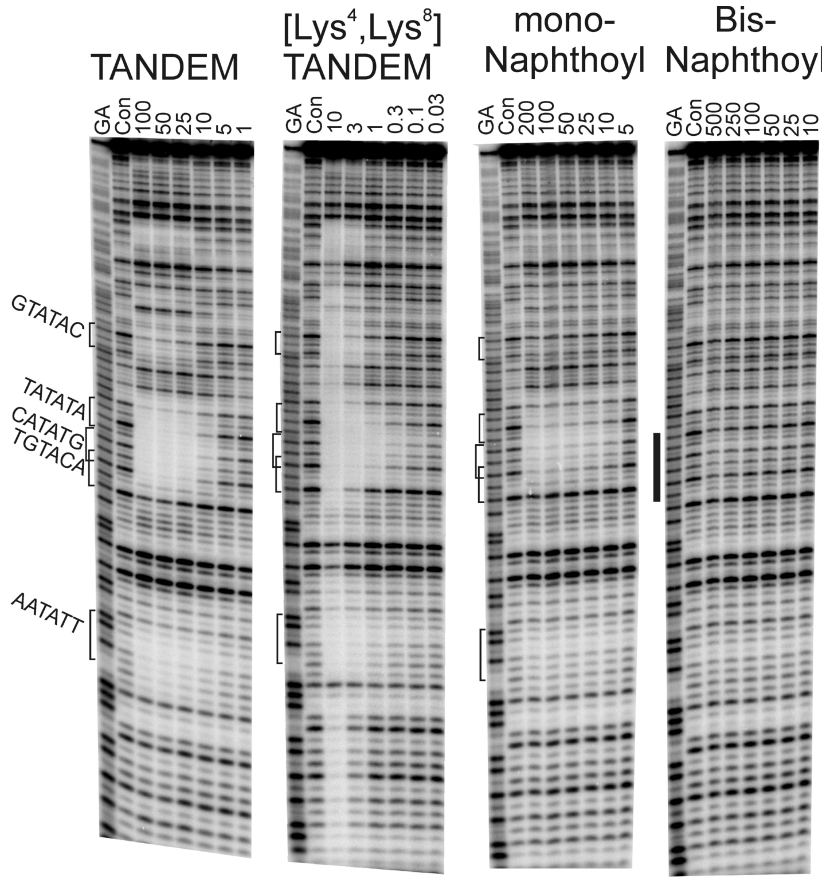

FIGURE 2: DNase I footprints for the TANDEM analogues with the HexBfor fragment. Ligand concentrations (micromolar) are shown at the top of each gel lane. Tracks labeled GA are markers specific for purines. The locations of some of the best TpA sites that are contained within symmetrical hexanucleotide sequences are indicated by the brackets. The footprint toward the top of the gel corresponds to the CTAATTAG sequence. The bar shown alongside the gel for bisnaphthoyl-TANDEM indicates the single region of attenuated cleavage at TATATA.

However, it is also apparent that [Lys^4,Lys^8]TANDEM binds with higher affinity, producing footprints at approximately 0.3 μM, compared with a value of 10 μM for TANDEM. Replacing valine with lysine appears to have dramatically enhanced the binding affinity, without altering the sequence selectivity. In contrast, mononaphthoyl-TANDEM binds more weakly and requires concentrations of $\geq$50 μM to attenuate the cleavage pattern. In this case, footprints are observed around only the strongest TANDEM binding sites, most notably at those with a central ATAT sequence (TATATA and CATATG). Replacing both quinoxaline rings with naphthalene (bisnaphthoyl-TANDEM) further reduces the affinity, and a single region of attenuated cleavage at ATAT is apparent only at the highest ligand concentrations (500 μM) with no evidence of binding at any of the other TpA-containing sites. Other analogues in which one or both of the quinoxaline chromophores had been replaced with methyl groups failed to show any interaction with DNA at concentrations of up to 500 μM (not shown).

The results were extended by performing similar experiments on the other Hex fragments, and the results are shown in Figure 3. It can be seen that [Lys^4,Lys^8]TANDEM produces clear footprints at concentrations of $<$1 μM, which are similar to those produced by TANDEM on these fragments (*24*). Mononaphthoyl-TANDEM generated much weaker footprints on HexAfor (fourth panel in Figure 3) in the same locations as TANDEM, which are evident only at higher ligand concentrations. These footprints are weaker than those in Figure 2, presumably since none of these contain a central ATAT sequence. Bisnaphthoyl-TANDEM failed to produce any footprints at concentrations up to 500 μM (not shown). C_{50} values, corresponding to the ligand concentration that reduces the intensity of bands within the footprints by 50%, were estimated from quantitative analysis of these footprinting patterns, and these are presented in Table 1, alongside those previously determined for TANDEM (*24*). The rank order of the binding sites is the same for [Lys^4,Lys^8]TANDEM and TANDEM, and the best sites contain the central ATAT tetranucleotide, especially TATATA and CATATG. TpA-containing sequences with a central TTAA sequence are the weakest ligand binding sites, as observed with TANDEM, though in this instance even TTTAAA is protected at micromolar concentrations of [Lys^4,Lys^8]TANDEM. Substitution of lysine for valine has clearly increased the binding affinity between 15- and 50-fold. In contrast, the C_{50} values for mononaphthoyl-TANDEM show a much weaker interaction and are between 2.5- and 20-fold greater. Once again, the selectivity remains the same, with the best binding observed at TATATA and CATATG sequences. However, no interaction is seen with many of the weaker sites, especially all those with a central TTAA sequence. Surprisingly, no footprint is evident at AATATT, though this is among the better sites for TANDEM and [Lys^4,Lys^8]TANDEM. It appears that substitution of one of the quinoxaline chromophores with a naphthalene ring causes a large decrease in affinity so that the ligand

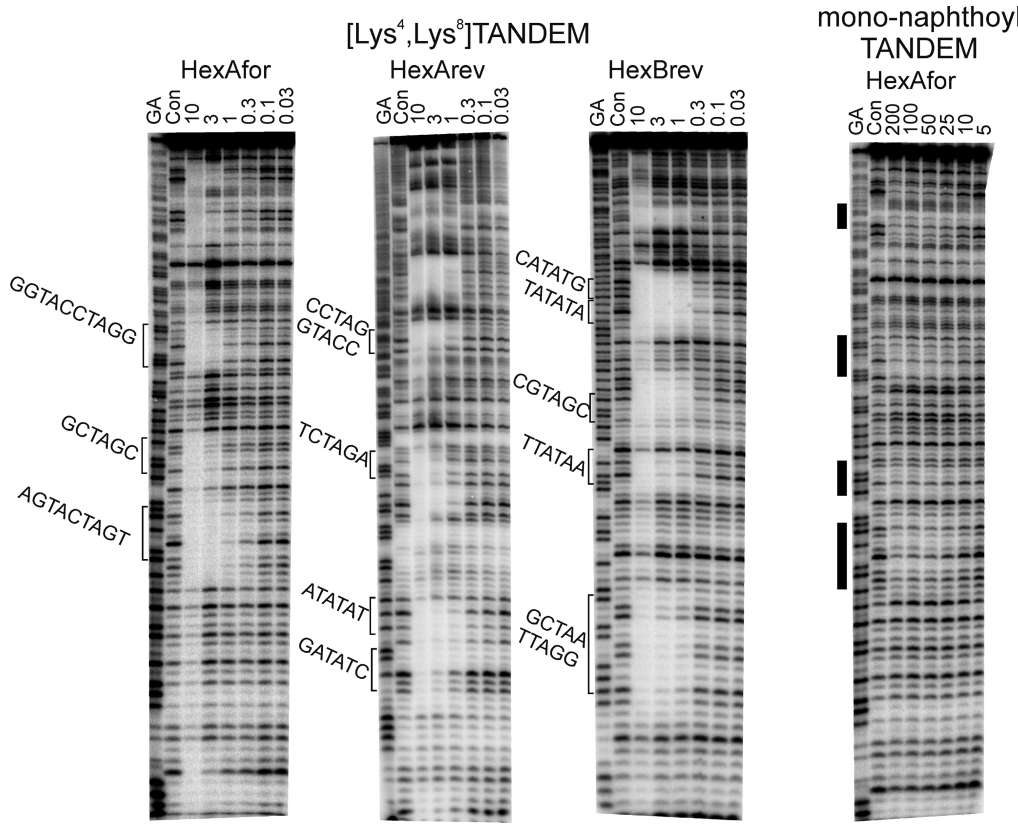

FIGURE 3: DNase I footprints for [Lys^4,Lys^8]TANDEM and mononaphthoyl-TANDEM. The first three panels show the interaction of [Lys^4,Lys^8]TANDEM with the HexAfor, HexArev, and HexBrev fragments, while the final panel shows the interaction of mononaphthoyl-TANDEM with the HexAfor fragment. Ligand concentrations (micromolar) are shown at the top of each gel lane. Tracks labeled GA are markers specific for purines. The locations of some of the best TpA-containing sites are indicated. The bars shown alongside the gel for mononaphthoyl-TANDEM indicate the regions of attenuated cleavage.

Table 1: C_{50} Values (micromolar) for the Interaction of [Lys^4,Lys^8]TANDEM, TANDEM, and Mononaphthoyl-TANDEM with the Symmetrical Hexanucleotide Sites That Contain a Central TpA

	C_{50} (μM)		
sequence	[Lys^4,Lys^8]TANDEM	TANDEM[a]	mononaphthoyl-TANDEM
GGTACC	0.85 ± 0.30	14 ± 4	105 ± 30
AGTACT	0.22 ± 0.07	10 ± 3	24 ± 8
CGTACG	3.0 ± 1.5	>100	>200
TGTACA	0.12 ± 0.03	23 ± 5	>200
GATATC	0.4 ± 0.3	11 ± 4	76 ± 52
AATATT	0.34 ± 0.04	8 ± 2	>200
CATATG	0.060 ± 0.015	4 ± 1	22 ± 10
TATATA	0.09 ± 0.02	4 ± 1	11 ± 4
GTTAAC	3.0 ± 1.1	75 ± 17	>200
ATTAAT	2.4 ± 1.2	59 ± 14	>200
TTTAAA	4.5 ± 2.5	>100	>200
CTTAAG	1.2 ± 0.7	41 ± 14	>200
GCTAGC	1.2 ± 0.6	14 ± 4	>200
ACTAGT	0.17 ± 0.05	10 ± 3	57 ± 18
TCTAGA	2.2 ± 1.9	30 ± 6	>200
CCTAGG	0.6 ± 0.2	10 ± 3	125 ± 25

[a] Values taken from ref (*24*).

interacts with only a subset of the better binding sites. Replacing both quinoxalines causes a dramatic reduction in affinity, and even the best binding site (TATATA) is only attenuated at the highest ligand concentration.

Interaction with (AT)$_n$ Tracts. Several studies, in addition to those presented above, have shown that TANDEM binds best to TpA steps that are flanked by AT residues (*14, 15, 24*) and that it binds cooperatively to poly(dA-dT) (*13*). We therefore examined the interaction of TANDEM and [Lys^4,Lys^8]TANDEM with a DNA fragment that contains

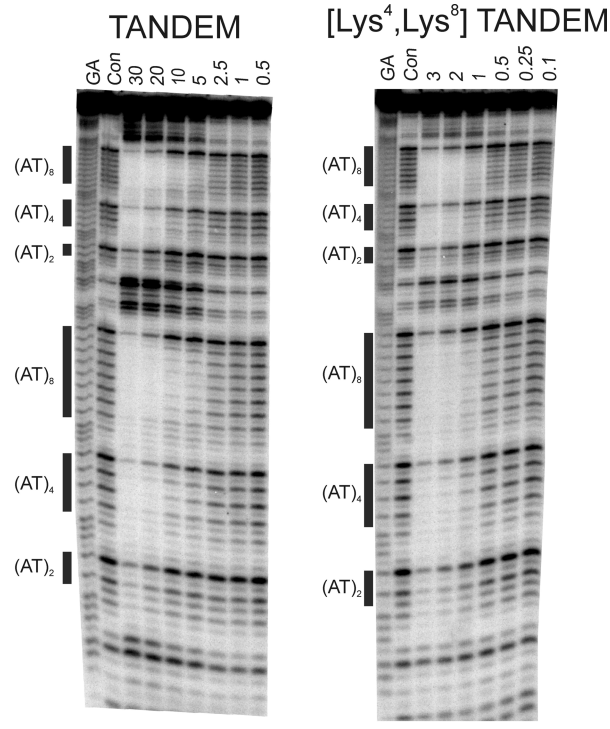

FIGURE 4: DNase I footprints for TANDEM and [Lys^4,Lys^8]-TANDEM with a DNA fragment containing three different length (AT)$_n$ tracts. Ligand concentrations (micromolar) are shown at the top of each gel lane. Tracks labeled GA are markers specific for purines. The locations of each of the (AT)$_n$ tracts are indicated by the bars.

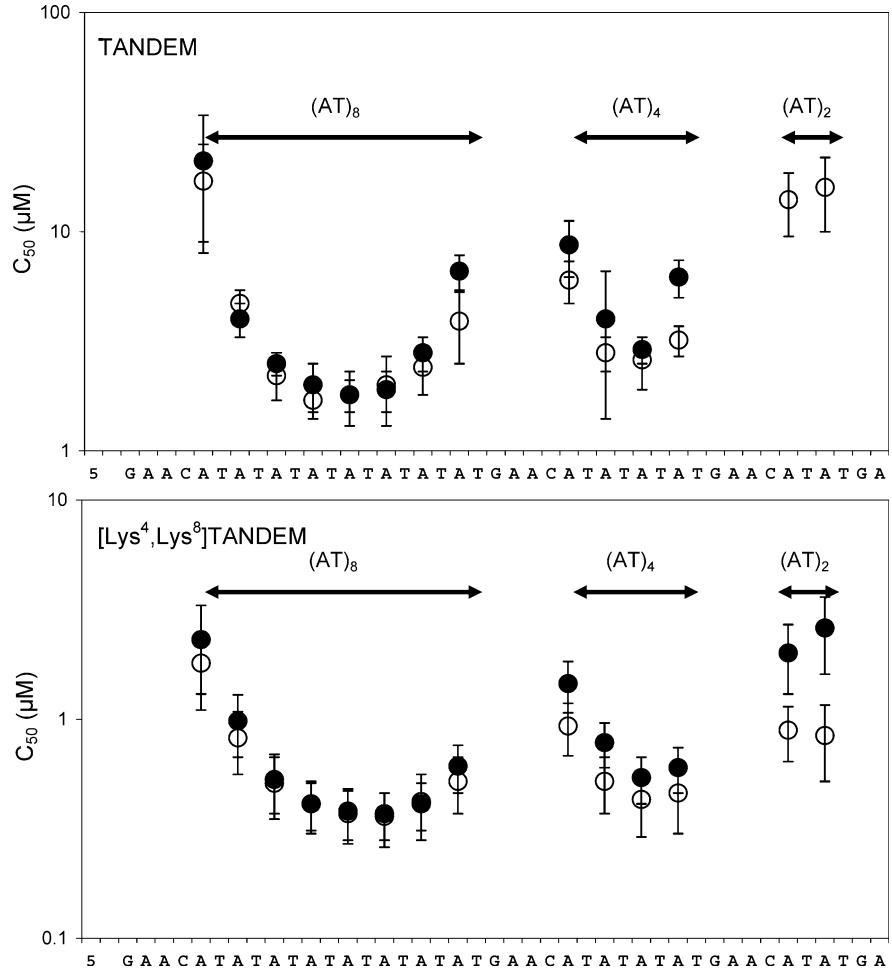

FIGURE 5: C_{50} values for TANDEM and [Lys^4,Lys^8]TANDEM at different positions in the $(AT)_n$ tracts. White symbols depict data for the lower half of the dimer while black symbols data for the upper half of the dimer. The *y*-axis shows the C_{50} values (micromolar) plotted on a logarithmic scale.

several $(AT)_n$ tracts of different lengths. The results of these footprinting experiments are shown in Figure 4. It can be seen that, as expected, both ligands inhibit DNase I cleavage within the $(AT)_n$ tracts. Visual inspection of these gels shows that the footprints at the center of $(AT)_8$ persist to lower ligand concentrations than at the center of $(AT)_4$ and $(AT)_2$. We performed quantitative analysis of the cleavage at each ApT step throughout the fragments (note that DNase I cuts ApT much better than TpA), yielding apparent C_{50} values at each position, and these values are presented in Figure 5. These confirm that both ligands bind best at the center of the $(AT)_n$ tracts, with the weakest binding at $(AT)_2$, which possesses only a single TpA step.

DISCUSSION

[Lys^4,Lys^8]TANDEM. This derivative has the same sequence selectivity as the parent compound TANDEM, as expected since the valine residues are not thought to be involved in contacts with the DNA bases, but it binds with much higher affinity. This is presumably due to ionic interactions between the positive charges on the ligand and the negatively charged phosphodiester backbone. However, this contrasts with studies on other derivatives which have demonstrated that hydrophobic interactions play a large part in the binding process (*34*). Triostin A and echinomycin (with *N*-methylvaline at this position) bind less well than triostin C and quinomycin C (with *N*-γ-dimethyl-L-alloisoleucine), and these all have a higher affinity than TANDEM (with valine) (*4, 13*). In these previous studies, there has been a general correlation between low aqueous solubility and high affinity for DNA. [Lys^4,Lys^8]TANDEM is the first derivative to break this trend, generating an AT-selective ligand with an affinity higher than those of any of the parent compounds.

It has not escaped our notice that the amino groups in this lysine-containing analogue may permit the addition of further functional groups to this position. This might be used to tether other functional groups or DNA binding agents. We speculate that the affinity might be further enhanced also via replacement of the alanine residues with lysine.

Although echinomycin and triostin A are potent antibiotics, TANDEM is devoid of any biological activity (*35*). This could be due to its lower affinity or altered sequence selectivity or because removal of the *N*-methyl groups makes it susceptible to degradation. We therefore tested [Lys^4,Lys^8]TANDEM in a simple antibacterial assay against *Staphylococcus aureus* and found that it too has no antibacterial activity (at concentrations up to 25 μM), compared with echinomycin which inhibited growth at concentrations of <50 nM. The lack of activity of TANDEM is therefore unlikely to result from its lower DNA binding affinity.

Role of the Chromophores. The naphthalene derivatives presented in this paper suggest that the chromophores play

an important part in the binding process and are not merely inert aromatic wedges. 2QN, the semibiosynthetic derivative of echinomycin, possesses quinolines in place of quinoxalines (*27*), and although it is still selective for CpG (*26*), it has enhanced affinity for poly(dA-dT) (*27*) and exhibits particularly strong binding to DTDT (D is diaminopurine) (*26*). Moreover TANDEM does not bind to CpI (I is inosine), even though this has the same minor groove hydrogen bonding possibilities as TpA (*36*). It is clear that subtle differences in the stacking geometries between the intercalating rings and the DNA base pairs can modulate the sequence binding properties (*37*). It should be noted that, unlike echinomycin, the alanine carbonyls of TANDEM are involved in intramolecular hydrogen bonds rather than interacting with the DNA minor groove. Instead, hydrogen bonds are formed between the NH group of alanine and N3 of adenine, a group which is also present in guanine, to which it does not bind. The binding may therefore be more sensitive to changes in the hydrophobic stacking interactions. It is therefore surprising to find that the derivative with two naphthalene chromophores does not bind to DNA, even at concentrations as high as 500 μM. It is interesting to note that, although mononaphthoyl-TANDEM still binds to most of the best sites seen with TANDEM and [Lys^4,Lys^8]TANDEM, albeit with lower affinity, it shows little interaction with some of the good sites, most notably AATATT. We can offer no molecular explanation for this difference, but it serves to emphasize the subtle effects of neighboring sequences on binding affinity.

(AT)$_n$ Tracts. The results demonstrate that TANDEM and [Lys^4,Lys^8]TANDEM bind best to TpA steps that are located in long (AT)$_n$ tracts (*13, 14, 18, 24*). The C_{50} values are lower at the center of the (AT)$_8$ tracts than at the ends, and the affinity is especially lower toward the 5′-end. The results with (AT)$_4$ are interesting as the ligands can bind to this sequence in two different arrangements. A single ligand bound to the central TpA step (ATATATAT) will occlude further binding to the proximal and distal TpA steps, while two ligands can bind to both the outermost TpA steps (ATATATAT). The observation that the apparent size of the footprinting site does not change with ligand concentration suggests that binding does not switch between these two arrangements. The observation that binding is strongest toward the center of long (AT)$_n$ tracts is consistent with the observation that YATATR is a better binding site than RATATY (Table 1) and confirms that neighboring sequences affect the structure and/or dynamics of the central ATAT, influencing either intercalation at the ApT steps or interaction with the central TpA.

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