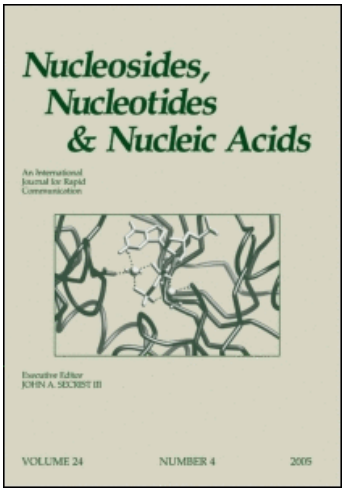

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SHAPE-SELECTIVE BINDING OF GEOMETRICALLY-CONSTRAINED BIS-DISTAMYCINS TO A DNA DUPLEX AND A MODEL OKAZAKI FRAGMENT OF IDENTICAL SEQUENCE

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Abstract. The binding of ligands to nucleic acids is of great interest for the control of gene expression and other nucleic acid mediated processes. We have evaluated the binding of several geometrically-constrained bis-distamycins to a model Okazaki fragment [OKA], or a DNA duplex having identical base sequence [DD], using gel-shift assays, optical spectroscopy and differential scanning calorimetry. In the case of covalent attachment of two distamycins to a central benzene ring, a similar binding profile was observed for [DD] as was observed for [OKA] (*para* binds [$K_{app} > 10^6$ M^{-1}], *meta* binds only weakly). For a central pyridyl ring, however, clear distinction between the binding to [DD] and binding to [OKA] was observed. While none of the three *meta* isomers having a central pyridyl ring bound [OKA], two of them (MT-17 and MT-12) bound [DD] [$K_{app} > 10^6$ M^{-1}]. These results demonstrate subtle differences in lexitropsin shape and placement of electronegative atoms may result in selective binding to a nucleic acid duplex based both on base sequence and chemical composition. Selective binding to DNA duplexes may be useful for designing ligands that regulate transcription, but do not interfere in other nucleic acid mediated processes.

Nucleic acids mediate the essential cellular processes of replication, transcription and translation.[1] In various pathological conditions, nucleic acid structure and function deviates from that characteristic of normal cells. For example, the rate of DNA replication is often elevated in malignant cells,[2] particularly in aggressive tumors, while levels of gene expression are elevated in numerous disease states.[3] These alterations in nucleic acid sequence, structure, and level of expression in diseased cells provide an

opportunity for the design of drugs to eradicate aberrant cells without being toxic to normal cells. In this regard, the essential role of DNA for information storage has resulted in investigations by numerous researchers into the design of small molecules that would bind to duplex DNA, and inhibit DNA-mediated processes, particularly transcription.[4,5]

In the last several years, the research team of Dervan has described the chemical synthesis of relatively low molecular weight oligopeptidic molecules that bind to pre-determined DNA sequences of less than nine base pairs.[6-8] These molecules were designed based on rational modification of the naturally occurring oligopeptidic molecules netropsin and distamycin which bind to A/T-rich regions of duplex DNA, displacing the spine of hydration in the minor groove.[9] The chemical modification of N-methyl pyrrole rings present in both distamycin (three rings) and netropsin (two rings) resulted in modified pyrroles that can be stacked edgewise in the DNA minor groove to recognize exclusively one type of base pair (e.g. distinguish A/T from G/C, C/G and T/A). Two observations were central to these design principles. The first was made by the groups of Dickerson and Lown who demonstrated that substitution of an imidazole for a pyrrole ring permitted hydrogen bonds to form to G/C base pairs in the minor groove.[10,11] The second was made by Wemmer and colleagues who showed that two distamycins could bind side-by-side in antiparallel fashion in the minor groove of a DNA duplex.[12] Further chemical modification has resulted in the preparation of ligands that can bind to pre-determined sequences of DNA with very high specificity and affinity. Recently, Gottesfeld and co-workers have shown that these engineered linked-polyamides can regulate gene expression in living cells.[13]

Over the last several years, our laboratory has investigated Okazaki fragments as targets for anticancer drugs.[14-16] Okazaki fragments occur selectively in replicating cells, and drugs that interfere with Okazaki fragment synthesis or processing would be expected to display anticancer activity. In this regard, it has been shown that the anti-leukemic drug cytarabine interferes with lagging strand replication.[17] Our laboratory recently reported the NMR structure of a model Okazaki fragment, [OKA], with and without cytarabine substitution, and found that cytarabine induced an increased bend in the helical axis.[15] The three dimensional structures of all Okazaki fragments determined to date have shown a significant (~20°) bend in the helical axis between the DNA duplex

region (DDR) and the RNA-DNA hybrid duplex region (HDR).[18,19] The increased helical bend of Okazaki fragments is one of several structural differences that cause Okazaki fragments to be structurally distinct from duplex DNA having identical sequence. These structural differences can, in principle, be used to design ligands that bind preferentially to an Okazaki fragment without binding to a DNA duplex of identical sequence.[16] Such selectivity may be useful for the design of anticancer drugs with a large therapeutic index. Conversely, ligands that bind to duplex DNA, but not to Okazaki fragments or other nucleic acids having identical base sequence, may be selective inhibitors of transcription.

We recently reported the shape-selective binding of geometrically constrained bis-distamycins to [OKA].[16] The two distamycins in each bis-distamycin were geometrically constrained by covalent attachment to either a benzene or pyridine ring with *para* or *meta* configurations.[20] For both the benzene and pyridine linker, the *para* bis-distamycins bound tightly to [OKA] ($K_d \sim 10^{-6}$ M from gel-shift assays; $K_d \sim 10^{-8}$ M from ΔT_M) while the *meta* bis-distamycins did not bind. In the present manuscript, we extend these studies to investigate the binding of these geometrically-constrained bis-distamycins to duplex DNA. Specifically, we assess the propensity for this class of ligands to selectively bind either to [OKA] or to a DNA duplex having an identical base sequence, [DD] (FIG. 1). In the case of covalent attachment of the two distamycins to a central benzene ring, a similar binding profile was observed for [DD] as was observed for [OKA] (*para* binds, *meta* binds only weakly). For a central pyridyl ring, however, clear distinction between the binding to [DD] and binding to [OKA] was observed. While none of the three *meta* isomers having a central pyridyl ring bound [OKA], two of them (MT-17 and MT-12; FIG. 2) bound [DD] strongly. These results indicate subtle differences in lexitropsin shape and placement of electronegative atoms can result in selective binding to a nucleic acid duplex based both on base sequence and chemical composition.

EXPERIMENTAL METHODS

Synthesis of Nucleic Acids. The base sequence for the model Okazaki fragment, [OKA] considered in the present study is identical to that for the DNA duplex, [DD], and is shown in FIG. 1. The RNA:DNA hybrid strand of [OKA] consists of five ribo- and

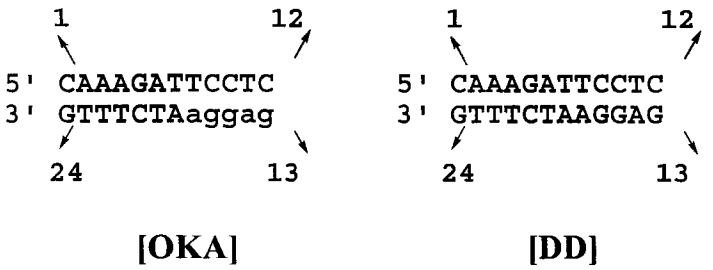

FIG. 1. The sequences of the model Okazaki fragment [OKA] and the duplex DNA [DD] considered in the present study. Ribonucleotides are indicated in lower case and deoxyribonucleotides in upper case in the figure and throughout the text. The base sequences for the two nucleic acid duplexes are identical, but the duplexes differ in chemical composition.

seven deoxyribonucleotides, while the equivalent strand for [DD] consists of 12 deoxyribonucleotides. The complementary strand is identical for both [OKA] and [DD]. Both the hybrid and the DNA strands of [OKA], as well as both DNA strands of [DD], were prepared by automated synthesis using a Perkin-Elmer/Applied Biosystems Division 394 DNA synthesizer. Phosphoramidites were used as 0.1 M acetonitrile solutions with the rA and rG reagents installed on the extra base positions of the DNA synthesizer for the hybrid strand. Preparation of the DNA strand for gel-shift assays was done as previously described.[16] [OKA] and [DD] were formed from their component strands by mixing equimolar ratios of the two strands as determined from the sequence-specific extinction coefficients.[14]

Synthesis of Bis-Distamycins. The structures of the bis- and mono-distamycins used in these studies are shown in FIG. 2. The procedures for synthesis and purification of these compounds, and their chemical characterization, were described previously.[20] The distamycin moiety in all cases contained an *N,N*-dimethyl end-group in place of the terminal amidinium group of distamycin. For convenience in the present manuscript the bis-distamycins are separated into two classes: those with a *para* arrangement of the two distamycins about the central ring (e.g. MT-9) and those with a *meta* arrangement of the two distamycins (e.g. MT-11) (FIG. 2). Thus, MT-15 is a 2,5-*para*-pyridyl bis-distamycin and is referred to as a *para* bis-distamycins throughout the text. Similarly, MT-10 is a 2,6-*meta*-pyridyl bis-distamycin, MT-12 is a 3,5-*meta*-pyridyl, and MT-17 is a 2,4-*meta*-pyridyl bis-distamycin and each is referred to as a *meta* bis-distamycin.

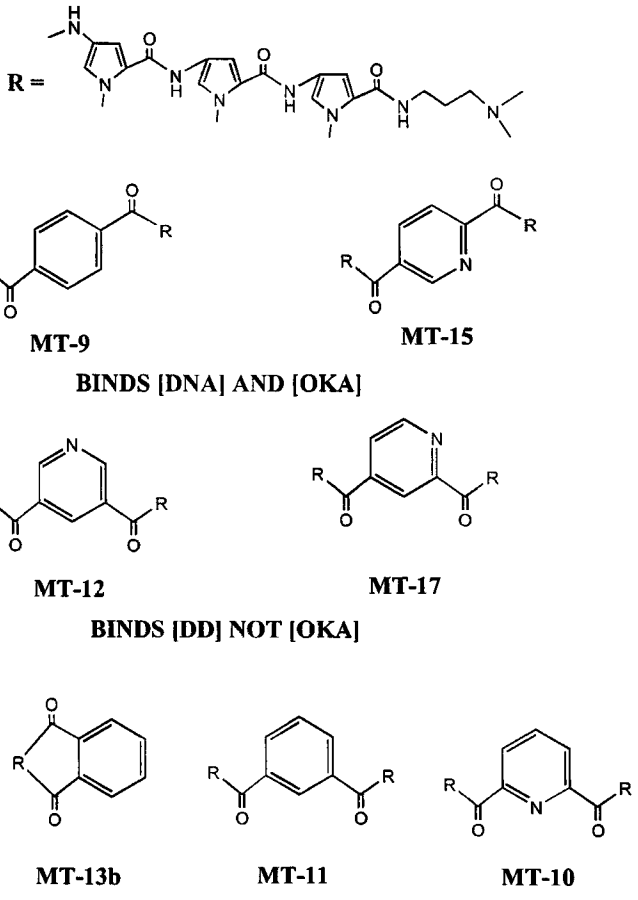

FIG. 2. Structures of the bis-distamycins (MT-9, MT-10, MT-11, MT-12, MT-15 and MT-17) and the mono-distamycins (MT-13a and MT-13b) considered in the present study. The R-group in each is a distamycin with a modified end group. The lexitropsins are classified into three groups based on their binding to [DD] and/or [OKA]. MT-9 and MT-15 bind (K_{app} > 10^6 M^{-1}) both [DD] and [OKA]. MT-12 and MT-17 bind [DD], but do not bind [OKA]. MT13a, MT-13b, MT-11 and MT-10 bind neither [DD] nor [OKA] (K_{app} > 10^6 M^{-1}).

Gel-Shift Analyses. Detection of mobility-shifted bands of [OKA] and [DD] due to complexation with one of the six bis-distamycins, or two mono-distamycins, was accomplished by preparing [OKA] with the DNA strand fluorescently labeled or [DD] with the same strand labeled. The fluorescent labeled DNA strand (100 pmol) was mixed together with the hybrid strand (100 pmol) in 10 μL of 10x annealing buffer (200 mM

Tris-HCl pH 7.6, 50 mM $MgCl_2$, 1 mM DTT, 0.1 mM EDTA). The reaction was diluted to a final volume of 100 μL with dH_2O, heated for 10 min at 70 °C, and then slowly cooled to room temperature over 30 min. Annealed [OKA] and [DD] were kept at 0 °C until used in drug binding reactions. Each drug binding reaction included 5 pmol of [OKA] or [DD] in annealing buffer, and from 10-100 pmol of one of the drugs, also in annealing buffer. The final volume of the binding reactions was 10 μL. The binding reactions were incubated for one hour at room temperature. The binding reactions were diluted with 4 μL of 25% glycerol followed by gentle mixing, and analyzed by gel electrophoresis on 5% non-denaturing polyacrylamide gels. Tracking dye was loaded in a separate lane. The reservoirs were filled with 0.25 x TBE, and the gel was electrophoresed at 120 V for 2 h at 4 °C. Visualization of the fluorescent labeled bands, and integration of their intensities, was accomplished using a Storm FluorImager system. The fractional saturation, θ, of the duplex in each gel lane was then calculated using equation [1][21]:

$$\theta = S_{complex} / (S_{complex} + S_{duplex}) \qquad [1]$$

Where $S_{complex}$ and S_{duplex} are the fluorescent signals associated with the drug:[OKA] (or drug:[DD]) complex and free [OKA] (or [DD]), respectively. Values of the apparent drug:[OKA] (drug:[DD]) dissociation constant, K_d, were then obtained by fitting of the data using equation [2]:

$$\theta = ([OKA]/K_d)/(1+ [OKA]/K_d) \qquad [2]$$

Temperature Dependent UV-Spectroscopy. Absorbance *versus* temperature profiles (melting curves) in appropriate solution conditions were measured at 260 nm with a thermoelectrically controlled Perkin-Elmer Lambda-10 spectrophotometer. The temperature was scanned at a constant heating rate of 1 °C/min. Melting curves were carried out for free and 1:1, 2:1 ligand-bound [OKA] or [DD]. The analysis of the melting curves, using standard procedures described earlier[22], allowed measurement of the helix-coil transition temperatures, T_M. Binding affinities resulting from stabilization

of the helix due to drug binding were calculated from the change in T_M using equation [3][23]:

$$1/T°_M - 1/T_M = (BR/\Delta H_{DSC}) \ln [1 + K_{app}D_f] \qquad [3]$$

where $T°_M$ represents the melting temperature of free duplex, T_M is the melting temperature of the drug-nucleic acid complex, B is the number of ligands bound per duplex molecule, R is the gas constant, ΔH_{DSC} is the enthalpy for the unfolding of the number of base-pair stacks involved in a binding site, D_f is the concentration of free ligand at T_M, and is usually taken as half of the total concentration of ligand, and K_{app} is the binding affinity at a temperature equal to T_M.

RESULTS

Benzene Scaffold Bis-Distamycins. The bis-distamycins MT-9 and MT-11 are the two possible regioisomers with two distamycins covalently attached to a central benzene ring. MT-9 has the distamycins in a 1,4-*para* configuration while for MT-11 the distamycins are in a 1,3-*meta* arrangement (FIG. 2). The binding of these two ligands to [OKA] and [DD] was studied using gel-shift assays and melting techniques. The results are shown in FIG. 3 and FIG. 4. The binding of MT-9 to both [OKA] and [DD] was readily detected using gel-shift assays. The results are shown in FIG. 3. Upon addition of an excess of MT-9 to either [OKA] or [DD], a single, mobility-shifted band consistent with formation of a stable complex between MT-9 and the nucleic acid duplex was apparent in the gel. The K_d calculated from the fraction of [DD] mobility-shifted as a function of MT-9 concentration was 1×10^{-6} M, a value similar to that for binding of MT-9 to [OKA] ($K_d = 4 \times 10^{-6}$ M). The two duplexes differed somewhat in their affinity for MT-11, the *meta* bis-distamycin with a central benzene ring. Although no evidence of a mobility-shifted band of [OKA] was observed even upon addition of a large excess of MT-11 to [OKA], similar ratios of MT-11 did induce a mobility-shifted band of [DD]. The K_d calculated from the fraction of [DD] mobility-shifted as a function of MT-11 concentration was 2×10^{-4} M. Although MT-11 could induce a mobility-shifted band of

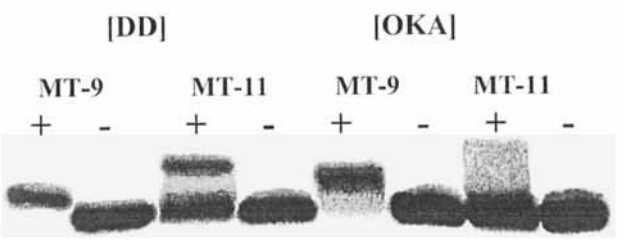

FIG. 3. Gel mobility shift assay of [DD] or [OKA] with the bis-distamycins MT-9 and MT-11. The addition of excess MT-9 (40x) to both [DD] and [OKA] resulted in a single mobility shifted band consistent with complex formation and the complete disappearance of free nucleic acid duplex. Upon addition of excess MT-11 (40x) to [DD] and [OKA], the majority the nucleic acid duplex remained in free form.

[DD], the binding affinity was significantly weaker than that of MT-9 to either [DD] or [OKA].

The binding of two mono-distamycin lexitropsins MT-13a and MT-13b to [OKA] and [DD] was also explored using gel-shift analysis and melting techniques. MT-13a and MT-13b have a single distamycin covalently attached to a benzene scaffold. The structures of MT-13a and MT-13b are shown in FIG. 2. Neither of the mono-distamycins caused any detectable mobility-shifted complex of either [OKA] or [DD] at drug concentrations as large as 20:1.

Binding of MT-9 to both [OKA] and [DD] resulted in stabilization of the duplex as evidenced by an increased melting temperature of the complex relative to the free duplex of 19.4 °C and 29.7 °C, respectively (FIG. 4). Using the enthalpy of the helix-coil transition determined by differential scanning calorimetry (83.0 kcal/mol [OKA] and 85.3 kcal/mol [DD])[24,25], the increase in the T_Ms corresponds to K_{app} values of 1.3 x 10^9 M^{-1} (at 61.6 °C) and 9.3 x 10^{10} M^{-1} (at 68.7 °C), respectively. However, these values are overestimated because not all the base pairs in each duplex participate in the interaction with MT-9. Taking advantage of the two-state transition behavior of each duplex and the good agreement with the enthalpy values from nearest-neighbor parameters, i.e. the calorimetric, van't Hoff and predicted enthalpies are similar[24,25], we calculated the enthalpy corresponding to the helix-coil transition of the base pairs involved in the interaction. Using the predicted value for ΔH obtained from the nearest neighbor model (48.7 kcal/mol [OKA] and 49.3 kcal/mol [DD]) [24,25], we obtained K_{app}s of 5.4 x 10^7 M^{-1}

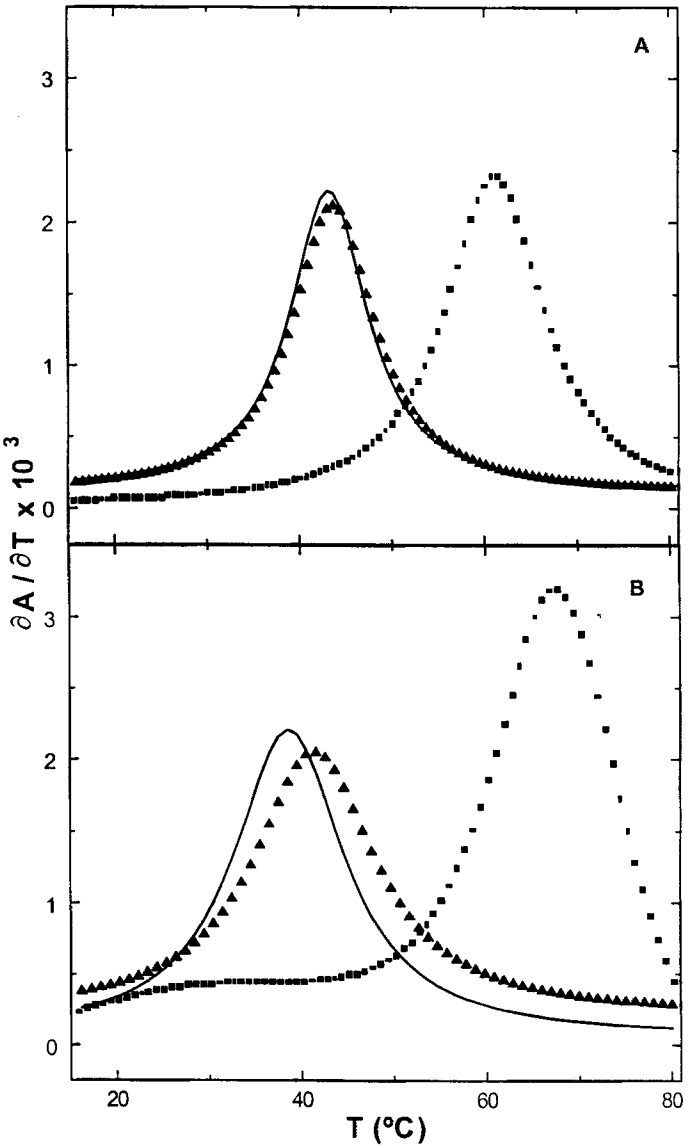

FIG. 4. Differential UV-Melts for the thermal unfolding of duplexes (~4 μM in total strands) and its saturated complexes with bis-distamycins in 10 mM sodium cacodylate buffer, 100 mM Sodium Chloride at pH 7. Panel A: [OKA] (solid line) and its complexes with MT-9 (squares) and MT-11 (triangles). Panel B: [DD] (solid line) and its complexes with MT-9 (squares) and MT-11 (triangles). The peak of each curve represents the T_M for the duplex or complex. MT-9 significantly stabilizes the complex while MT-11 does not.

(K_d = 1.9 x 10^{-8} M) and 6.0 x 10^8 M^{-1} (K_d = 1.7 x 10^{-9} M), respectively. A graph of the change in T_M for [OKA] and [DD] upon complex formation with MT-9 is shown in FIG. 4. Furthermore, the addition of MT-11 to [OKA] did not result in a shift in T_M, therefore, K_{app} for this interaction is estimated to be below 1 x 10^4 M^{-1}. On the other hand, addition of MT-11 to [DD] results in a thermal stabilization of 2.4 °C that corresponds to a K_{app} of 4.7 x 10^5 M^{-1}, significantly weaker binding than observed for MT-9.

Pyridine Scaffold Bis-Distamycins. MT-9 contains two distamycins with a 1,4-*para* orientation about a benzene ring scaffold. MT-15 is analogous to MT-9, but with the two distamycins in a 2,5-*para* arrangement relative to a pyridyl ring (FIG. 2). Similarly, the bis-distamycins MT-10, MT-12 and MT-17 have two distamycins in *meta* configurations, similar to MT-11. The binding of MT-15, MT-10, MT-12, and MT-17 to [OKA] and [DD] was also investigated using gel-shift assays and thermodynamic techniques. As was observed for MT-9, addition of a two-fold excess of the *para* pyridyl bis-distamycin MT-15 to either [OKA] or [DD] resulted in observation of a single, mobility-shifted band consistent with formation of a stable complex between MT-15 and [OKA]. No evidence of a distinct mobility-shifted band of [OKA] was observed upon addition of similar ratios of MT-10, MT-12 or MT-17 to [OKA], nor was any evidence for complex formation observed between MT-10 and [DD] (FIG. 5). However, addition of two-fold or greater excesses of MT-12 or MT-17 to [DD] resulted in mobility shifted bands due to complex formation of the DNA duplex with these ligands (FIG. 5). Thus, the *meta* pyridyl bis-distamycins MT-12 and MT-17 were able to preferentially bind duplex DNA without displaying significant binding affinity for a model Okazaki fragment of identical base composition (FIG. 5).

Binding of MT-17 to [DD] resulted in stabilization of the DNA duplex as evidenced by an increased melting temperature of the complex (45.6 °C) relative to the free duplex of 7.3 °C (FIG. 6). Using a ΔH_{DSC} value 85.3 kcal/mol[24,25], this increase in T_M corresponds to a K_{app} of 1.2 x 10^7 M^{-1} for 1:1 stoichiometry (at 45.6 °C), or a K_{app} of 2.0 x 10^6 M^{-1} (per ligand) for 2:1 stoichiometry at the same temperature. Gel-shift assays showed only a single mobility-shifted band consistent with formation of a 1:1 complex between MT-17 and [DD], although detection of the 2:1 complex may not be possible under the electrophoretic conditions used due to complex instability, or because the 2:1 and 1:1 complexes have similar mobilities. The K_{app} values probably overestimate the

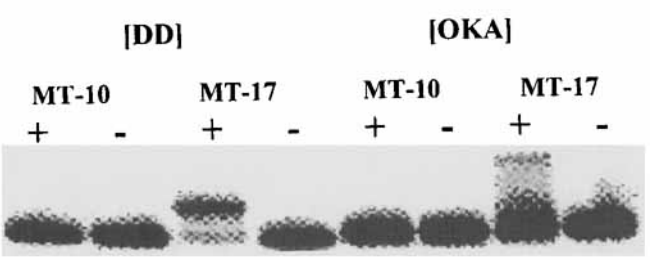

FIG. 5. Gel mobility shift assay of [DD] or [OKA] with the bis-distamycins MT-10 and MT-17. The addition of excess MT-17 (40x) to [DD], but NOT to [OKA], resulted in a single mobility shifted band consistent with complex formation and the complete disappearance of free nucleic acid duplex. Upon addition of excess MT-10 (40x) to [DD] and [OKA], the majority the nucleic acid duplex remained in free form.

true association constant because not all of the base pairs in the duplex are involved in complex formation. A reasonable estimate of the number of base pairs involved in complex formation is eight. Using the predicted value for ΔH obtained from the nearest neighbor model (49.3 kcal/mol)[24,25], we obtained a K_{app} of 2.7 x 10^6 M^{-1} for 1:1 stoichiometry or a K_{app} of 7.8 x 10^5 M^{-1} for 2:1 stoichiometry, both at 45.6 °C.

Addition of MT-17 to [OKA] increased the melting temperature to 43.7 °C, a change in T_M of only 1.0 °C. This change in T_M corresponds to an apparent association constant two orders of magnitude lower for the binding of MT-17 to [OKA] compared to that of MT-17 with [DD]. Thus, both gel-shift assays and thermodynamic measurements clearly indicate that MT-17 binds tightly and preferentially to [DD] and differentiates between binding to a DNA duplex and a model Okazaki fragment of identical base composition. A graph of the change in T_M for [DD] and [OKA] upon complex formation with MT-17 is shown in FIG. 6.

DISCUSSION

The regulation of gene expression by drugs promises to provide an era in which the biological function of each gene can be clearly studied and nearly all diseases can be controlled by therapeutic intervention. Among the most effective approaches to the control of gene expression described to date are lexitropsins, or information reading

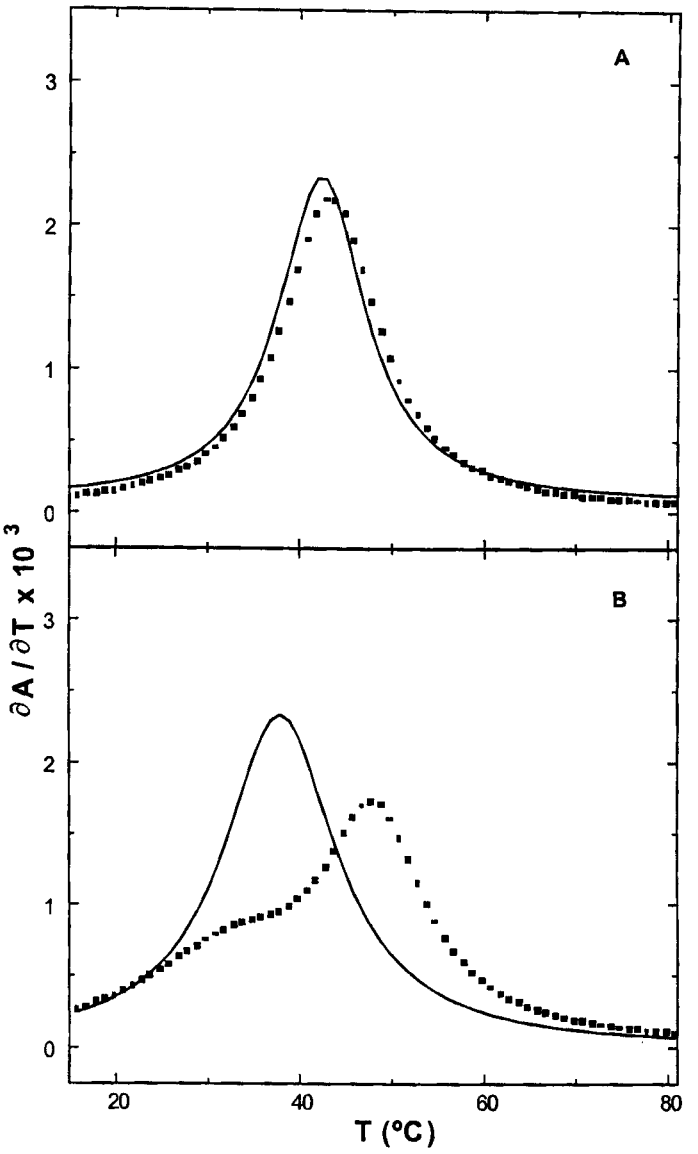

FIG. 6. Differential UV-Melts for the thermal unfolding of duplexes (~4 μM in total strands) and its saturated complexes with bis-distamycins in 10 mM sodium cacodylate buffer, 100 mM Sodium Chloride at pH 7. Panel A: [OKA] (solid line) and its complex with MT-17 (squares). Panel B: [DD] (solid line) and its complex with MT-17 (squares). MT-17 significantly stabilizes the complex with [DD] but not with [OKA].

oligopeptides. The work of Dervan and Gottesfeld[7,13] has shown that linked polyamide lexitropsins can bind pre-determined sequences of DNA *in vitro* and inhibit gene expression *in vivo*. Duplex DNA is only one of several types of nucleic acid present in living cells, and an important design consideration of lexitropsins is their propensity to bind to duplex DNA rather than to folded RNA, DNA-RNA hybrids or other nucleic acid structures. Conversely, the design of molecules that preferentially bind unusual nucleic acid structures, such as Okazaki fragments, may be useful for controlling biological function.

The present studies have described the binding of several geometrically-constrained bis-distamycins to a DNA duplex and to a model Okazaki fragment of identical sequence ([DD] and [OKA]; FIG. 1). We have found that two of the three *meta* pyridyl bis-distamycins bind [DD] tightly (K_{app} 2.7 x 10^6 M^{-1} for 1:1 stoichiometry for MT-17), but have considerably reduced affinity for [OKA]. In the case of a benzene scaffold, the bis-distamycin with a *meta* configuration (MT-11) bound only weakly to [DD] (K_{app} 4.7 x 10^5 M^{-1} for 1:1 stoichiometry), and did not bind [OKA]. The relatively increased affinity of *meta* bis-distamycins for [DD] compared to [OKA] may be a consequence of the decreased stability of [DD] (T_M = 38.3 °C) relative to [OKA] (T_M = 41.7 °C) that permits the DNA duplex to be slightly more flexible or more easily deformed. Alternatively, the three dimensional structure of [DD] may provide a more complementary shape for *meta* bis-distamycins than the structure of [OKA]. Although dissociation constants calculated from gel mobility-shift assays resulted in systematically lower values of the dissociation constants, the same trends apparent in the thermodynamic analyses were also present in the gel mobility-shift assays.

The relatively tight binding of the *meta* pyridyl bis-distamycins MT-17 and MT-12 preferentially to [DD] probably results from hydrogen bond formation, or other electrostatic attraction, between the pyridyl nitrogen of MT-17 and MT-12 and one or more electropositive atoms of [DD]. Formation of this contact must be disfavored for the interaction of these ligands with [OKA]. Although no high resolution structural data are currently available that would clarify the nature of the interacting group with the pyridyl nitrogen, previous docking studies have placed both distamycins of MT-9 in the minor groove of [OKA].[16] A similar docking motif of MT-17 in the minor groove of [DD] might permit the pyridyl nitrogen to accept a hydrogen bond from the N2 amino group of G5 of the G5-C20 base pair (FIG. 1).

In summary, we have shown that geometrically-constrained bis-distamycins can bind both to a DNA duplex and a model Okazaki fragment. The relative configuration of the two distamycins about the central aromatic ring dramatically affects binding affinity towards both types of nucleic acid duplex. We identified two *meta* pyridyl bis-distamycins, MT-12 and MT-17 that bound tightly and preferentially to a DNA duplex relative to the model Okazaki fragment of identical sequence. These results indicate that ligands can be designed that bind nucleic acid duplexes based on both chemical composition and base sequence.

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