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DNA Binding Properties of Key Sandramycin Analogues: Systematic Examination of the Intercalation Chromophore

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Abstract—The examination of a key series of chromophore analogues of sandramycin (**1**) is detailed employing surface plasmon resonance to establish binding constants within a single high affinity bis-intercalation binding site 5′-d(GCATGC)$_2$, and to establish the preference for sandramycin binding to 5′-d(GCXXGC)$_2$ where XX = AT, TA, GC, and CG. From the latter studies, sandramycin was found to exhibit a preference that follows the order: 5′-d(GCATGC)$_2$ > 5′-d(GCGCGC)$_2$, $\Delta\Delta G^\circ = 0.4$ kcal/mol > 5′-d(GCTAGC)$_2$, $\Delta\Delta G^\circ = 0.9$ kcal/mol$\geq$5′-d(GCCGGC)$_2$, $\Delta\Delta G^\circ = 1.0$ kcal/mol although it binds with high affinity to all four deoxyoligonucleotides. The two highest affinity sequences constitute repeating 5′-PuPy motifs with each intercalation event occurring at a 5′-PyPu step. The most effective sequence constitutes the least stable duplex, contains the sterically most accessible minor groove central to the bis-intercalation site, and the ability to accept two gly-NH/T C2 carbonyl H-bonds identified in prior NMR studies. Similarly, the contribution of the individual structural features of the chromophore were assessed with the high affinity duplex sequence 5′-d(GCATGC)$_2$. In addition to the modest affinity differences, one of the most distinguishing features of the high affinity versus lower affinity bis-intercalation or mono-intercalation directly observable by surface plasmon resonance was the temporal stability of the complexes characterized by the exceptionally slow off-rates.

Introduction

Sandramycin (**1**), a potent antitumor antibiotic[1] structurally identified in spectroscopic and chemical degradation studies,[2] constitutes one of the newest members of a growing class of cyclic decadepsipeptides including luzopeptins A–C and E_2,[3] quinaldopeptin[4] and quinoxapeptins A and B[5] which possess potent antitumor, antiviral, and antimicrobial activity (Fig. 1).[3–5] Characteristic of this class of agents, sandramycin possesses a two-fold axis of symmetry and two heteroaromatic chromophores that results in sequence-selective DNA bis-intercalation spanning two base-pairs preferentially at 5′-AT sites.[6–9] In this respect, the agents are functionally related to the quinoxaline antitumor antibiotics[10] including echinomycin and triostin A which also bind to DNA by bis-intercalation but with a substantially different sequence selectivity (5′-CG versus 5′-AT).[11,12]

We recently reported the synthesis and preliminary examination of a series of sandramycin analogues in which modifications to the heteroaromatic chromophore were introduced in the final stages (Fig. 2).[13] Using this approach, incremental changes in the chromophore were used to assess the role of each of its structural components.

Herein, we report the extension of these studies to the examination of the DNA binding properties of **1–22** and **23–24** (Fig. 3) employing surface plasmon resonance and their comparison with results obtained by fluorescence quenching techniques.[13]

DNA Binding Affinity: Surface Plasmon Resonance

In efforts to permit the binding constant measurements of the analogues not addressable by fluorescence quenching,[13] we examined the use of surface plasmon resonance detection.[14] Not only has this technique allowed the extension of the studies to analogues that were unable (i.e., **23**) or failed to provide an effective fluorescence, but it also allowed us to address the ambiguities observed with the fluorescence measurements. The technique is experimentally more reliable and general than our experience with fluorescence quenching measurements. It does not require a UV or fluorescence active chromophore or a radioactive label and could prove generally applicable to a wide range of related studies. More fundamentally, the technique allows the direct observation of association and dissociation rate constants that contribute to the overall binding affinity. This was expected to vary widely for agents that bind by bis-intercalation and could be important to the expression of the agents' properties.

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Hairpin loops[15] 5′-GCXXGCTTTTGCXXGC where XX = AT, GC, TA, or CG 5′-linked to biotin were immobilized onto gold coated sensor chips in a dextran matrix containing covalently bound streptavidin.[16] The gold surface forms one wall of a flow cell through which an analogue in buffer solution is passed and binding is measured in real time by surface plasmon resonance detection of changes in the surface bound mass. The stoichiometry of binding can be determined, on and off rates of binding are directly observable, and the affinity constants may be calculated by two methods. Under pseudo first order conditions where the free analogue concentration is held constant in the flow cell, the binding is described by eq (1):[17]

$$dR_A/d_t = k_a c(R_{max} - R_A) - k_d R_A \quad (1)$$

where R_A and R_{max} are the measured and maximum response signal measured with binding, c is the injected concentration of the analogue (M), k_a is the association rate constant or on-rate ($M^{-1}s^{-1}$) and k_d is the dissociation rate or off-rate (s^{-1}). The association constant may be calculated as $K_b = k_a/k_d(M^{-1})$. At equilibrium, $dR_A/dt = 0$ and eq (1) can be rewritten:

$$R_A/c = K_b R_{max} - K_b R_A \quad (2)$$

Therefore, the steady state association constant can be obtained from a plot of R_A/c versus R_A. For analysis of the on and off rates, a plot of the change in total detector response (dR/dt) versus R gives a value k_s as the slope which relates the on and off rates as follows:

$$k_s = k_a c + k_d \quad (3)$$

A plot of k_s against the injected analogue concentration gives k_a as the slope and k_d as the y-intercept. Since the

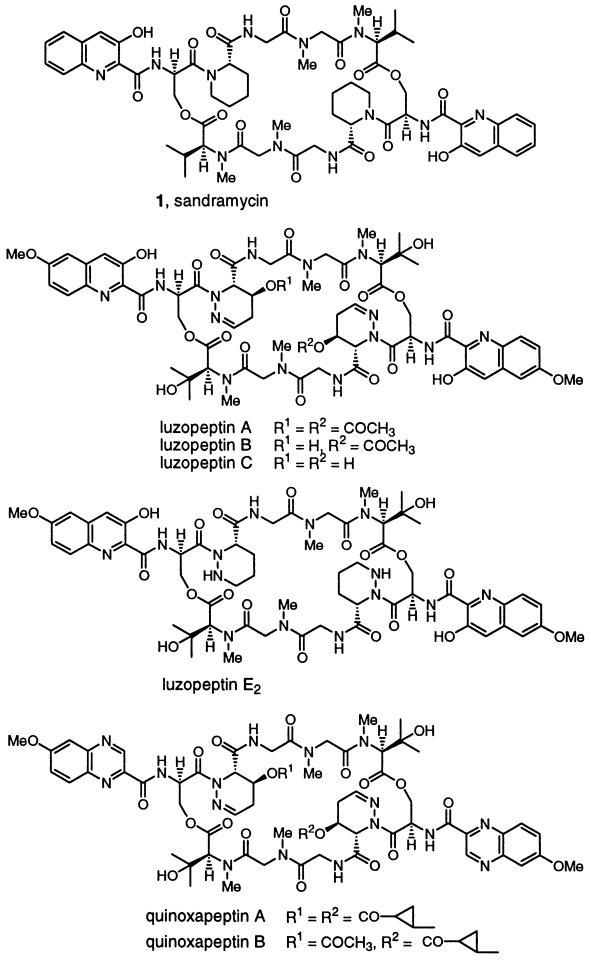

Figure 1.

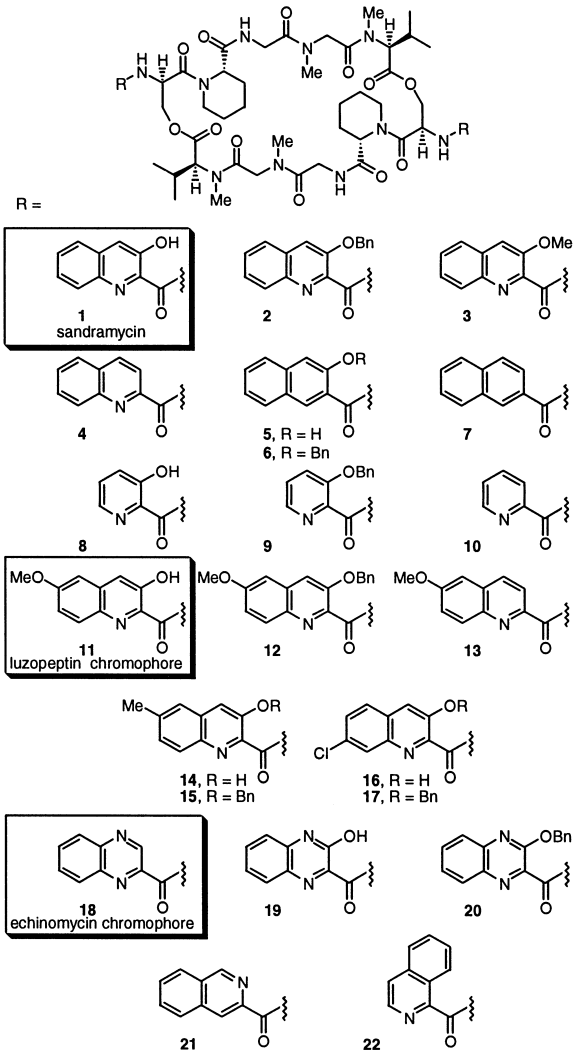

Figure 2.

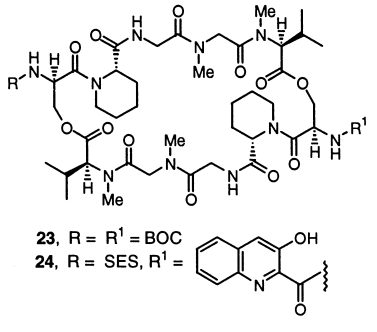

Figure 3.

y-intercept cannot be determined accurately when k_d is small, a more accurate way to obtain this value is by direct measurement of the dissociation from saturated binding sites into a buffer solution flow that contains no analogue and the dissociation is quantified by:

$$\ln(R_{A1}/R_n) = k_d(t_n - t_1) \quad (4)$$

where R_{A1} is the initial response level at t_1 and R_n and t_n represent values obtained along the dissociation curve.

The matrix of a Pharmacia[18] carboxymethylated dextran sensor chip was loaded with streptavidin to provide 3500, 2300, 1300 and 250 response units (RU) of immobilized streptavidin corresponding to a surface concentration of 3.5, 2.3, 1.3 and 0.25 ng/mm^2 and a volume concentration of 35, 23, 13 and 2.5 mg/mL, respectively. The 5′-biotinylated form of the deoxyoligonucleotides, 5′ biotin-GCXXGCTTTTGCXXGC, were dissolved in 10 mM Tris–HCl (pH 7.4) buffer solution containing 75 mM NaCl, and were immobilized on the sensor chip surface in a 2:1 DNA:streptavidin ratio as determined by RU:mass ratios. Since the sensor chips have four flow cells, the binding to each of the four sequences was conducted and monitored simultaneously with each flow cell loaded with 2300 RU of streptavidin. Analogue solutions in DMSO were diluted with the same buffer at 8 different concentrations from 1 μM to 10 nM. A flow rate of 10 μL/min was used to introduce the agent to the sensor over a period of 30 min to 2.5 h to obtain equilibrium by stacking contiguous 250 μL injections in the automated method protocol. The RU:mass ratios of oligomer to analyte supported a 1:1 stoichiometry (±30%) as the major binding mode. The analogue solution was replaced with a flow of buffer solution in which dissociation of the most concentrated run was followed for a duration of 3 h. To remove any bound analogue from the DNA, a 1 min pulse of 25 mM HCl was used to regenerate the DNA–streptavidin surface with loss of less than 5% activity per run.

Since the runs were allowed to reach equilibrium, on rates (k_a) and k_s values as well as R_{max} values could be obtained from each. This allowed comparison of on rates at a variety of concentrations and their determination by a plot of k_s versus c according to eq (3). The off rates were determined according to eq (4) and also compared to the y-intercept of the plot of eq (3). The association constant could be obtained from the ratio k_a/k_d and these values are presented in Tables 1 and 2.

Table 1. Sandramycin rate and binding constants for 5′-d(GCXXGCTTTTGCXXGC): surface plasmon resonance

XX	k_a ($M^{-1}s^{-1}$)[a]	$k_b(s^{-1})$[b]	K_b (M^{-1})[c]	ΔG° (kcal/mol)	K_b (M^{-1})[d]	ΔG° (kcal/mol)	n[e]
AT	5200	5.8×10^{-5}	9.0×10^{7}	−10.8	7.8×10^{7}	−10.8	1.0
GC	3100	8.1×10^{-5}	3.8×10^{7}	−10.3	4.0×10^{7}	−10.4	1.0
CG	2800	1.1×10^{-4}	2.5×10^{7}	−10.1	1.5×10^{7}	−9.8	0.83
TA	2500	1.1×10^{-4}	2.3×10^{7}	−10.0	1.8×10^{7}	−9.9	0.83

[a]Association rate constant.
[b]Dissociation rate constant.
[c]Binding constant calculated as k_a/k_d.
[d]Steady state binding constant calculated as $R_A/c = K_bR_{max} - K_bR_A$.
[e]Stoichiometry of binding.

Table 2. Rate and affinity constants determined for matrix bound 5′-d(GCATGCTTTTGCATGC) binding: surface plasmon resonance

Analogue	k_a[a] ($M^{-1}s^{-1}$)	k_d[b] (s^{-1})	K_b[c] (M^{-1})	ΔG° (kcal/mol)	K_b[d] (M^{-1})	ΔG° (kcal/mol)
1	5200	5.8×10^{-5}	9.0×10^{7}	−10.8	7.8×10^{7}	−10.8
2	170	1.6×10^{-4}	1.1×10^{6}	−8.2	5.7×10^{6}	−9.2
3	3000	6.6×10^{-5}	4.6×10^{7}	−10.4	3.9×10^{6}	−9.0
4	7300	1.0×10^{-4}	7.3×10^{7}	−10.7	2.4×10^{7}	−10.1
5	2600	2.6×10^{-4}	1.0×10^{7}	−9.5	—	—
7	—	—	—	—	$\leq7.1\times10^{5}$	≤8.0
8	280	9.4×10^{-5}	3.0×10^{6}	−8.8	6.4×10^{6}	−9.3
10	95	1.2×10^{-4}	8.3×10^{5}	−8.1	2.0×10^{4}	−5.9
11	2600	5.7×10^{-5}	4.5×10^{7}	−10.4	3.8×10^{7}	−10.3
13	2100	2.4×10^{-5}	8.8×10^{7}	−10.8	1.1×10^{7}	−9.6
14	630	5.1×10^{-5}	1.2×10^{7}	−9.7	5.6×10^{7}	−10.6
16	6300	3.2×10^{-5}	2.0×10^{8}	−11.3	5.6×10^{7}	−10.6
17	230	1.5×10^{-3}	1.5×10^{5}	−7.1	2.0×10^{5}	−7.2
18	12400	1.0×10^{-4}	1.2×10^{8}	−11.0	2.0×10^{8}	−11.3
21	11000	1.7×10^{-3}	6.5×10^{6}	−9.3	2.1×10^{6}	−8.6
22	820	1.2×10^{-4}	6.8×10^{6}	−9.3	6.5×10^{6}	−9.3
23					2.0×10^{4}	−5.9
24		$>5\times10^{-4}$				
Luzopeptin A	2000	5.5×10^{-5}	3.6×10^{7}	−10.3	4.4×10^{7}	−10.4

[a]Rate constants determined from plots of k_s versus analogue concentration (c) according to the following equation: $k_s = k_ac + k_d$.
[b]Dissociation rate constants determined from dissociation of bound analogues in buffer flow according to the following equation: $\ln(R_{A1}/R_n) = k_d(t_n - t_1)$.
[c]Binding constants calculated as k_a/k_d.
[d]Binding constants determined from steady state binding according to the following equation: $R_A/c = K_bR_{max} - K_bR_A$.

A second method of determining K_b is by a plot of R_A/c against R_A according to eq (2) and this is also presented in Tables 1 and 2 (Fig. 4). Given the exceptionally slow off rates for many of the agents, the latter approach to establishing K_b was presumed to provide the more reliable estimates.[19] Finally, each of the determinations in Tables 1 and 2 is an average of runs in three of the four flow cells of the sensor chip conducted at different concentrations of DNA with the fourth flow cell as a control.

Several important conclusions could be drawn from these studies. First, there was good agreement between the two techniques of fluorescence quenching[13] and surface plasmon resonance. For example, sandramycin exhibited a $\Delta G°$ of 10.6–11.4 kcal/mol for 5′-d(GCATGC)$_2$ measured by fluorescence quenching and 10.8 kcal/mol for the hairpin loop containing the duplex 5′-d(GCATGC)$_2$. In addition, both estimates of the K_b derived from the surface plasmon resonance qualitatively provided the same trends. However, that derived from steady state binding is assumed to be more reliable than the simpler $K_b = k_a/k_d$ due to the intrinsic error in measuring extremely slow off rates. Using the steady state binding, several important trends are clear and followed trends established in the fluorescence quenching studies.

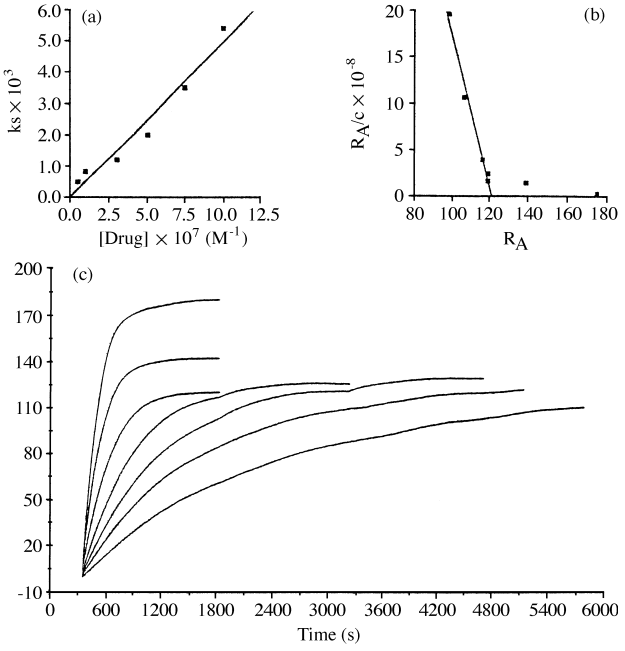

Figure 4. All data in this figure was obtained from one of the four flow cells of a Pharmacia Biosensor chip containing immobilized streptavidin at a volume concentration of 23 mg/mL with a 2:1 ratio of bound 5′-biotinylated 5′-d(GCATGCTTTTGCATGC) to streptavidin. Sandramycin (**1**), dissolved in a 10 mM Tris–HCl (pH 7.4) buffer solution containing 75 mM NaCl was passed through the flow cell at a rate of 10 μL/min at 25 °C. (a) Plot of slope value k_s versus sandramycin concentration c according to eq (3). (b) Analysis of steady state binding according to eq (2). (c) Sensorgram illustrating affinity and kinetic measurements of sandramycin. The concentrations of the agent, c, are as follows, beginning with the maximum response signal: 5×10^{-6}, 1×10^{-6}, 7.5×10^{-7}, 5×10^{-7}, 3×10^{-7}, 1×10^{-7}, and 5×10^{-8} M corresponding to the lowest response signal. (Instrument noise at each 250 μL injection was removed and the resulting curves were spliced together.)

Among the four hairpin loops containing 5′-d(GCXXGC) where XX = AT, GC, CG, or TA, the binding was greatest with 5′-d(GCATGC)$_2$, Table 1. This proved to be approximately 0.4 kcal/mol greater than 5′-d(GCGCGC)$_2$ and 1.0 kcal/mol greater than either 5′-d(GCCGGC)$_2$ or 5′-d(GCTAGC)$_2$. These relative trends proved analogous to those observed in the fluorescence quenching studies[13] and, in our assessment, provide a more reliable measurement of the absolute binding constants. Moreover, the results confirm the expectations based on prior footprinting studies where sandramycin exhibited a perceptible preference for 5′-CAT and provide further insights into the extent of this selectivity and its potential origin. The two highest affinity sequences, 5′-(GCATGC)$_2$ and 5′-d(GCGCGC)$_2$, constitute repeating 5′-PuPy sequences such that each intercalation event occurs at a 5′-PyPu step. This preference for intercalation binding at a 5′-PuPy step is analogous to that observed with simple monointercalations.[20] The highest affinity sequence of the pair constitutes the less stable duplex, contains the sterically most accessible minor groove central to the bis-intercalation site, and the ability to accept the two gly-NH/T C2 carbonyl H-bonds identified in NMR studies.[6] The two lower affinity sequences involve intercalation at both a 5′-PyPy step and 5′-PuPu step if it occurs about the central two base-pairs. The intercalation event interrupting the 5′-PuPu step would seem energetically more costly while that interrupting the 5′-PyPy step would provide less stabilization.[20]

Given the highest affinity binding for the hairpin loop containing the duplex 5′-d(GCATGC)$_2$, the key set of chromophore analogues were examined using this sequence (Table 2). Several important conclusions can be drawn from these studies (Fig. 5). Removal of the chromophore phenol reduced the binding affinity by 0.7 kcal/mol (**1** versus **4** and **11** versus **13**). Thus, each phenol contributes nearly 0.4 kcal/mol toward the binding affinity of sandramycin for 5′-GCATGC and this value is in agreement with that established by fluorescence quenching (0.4–0.5 kcal/mol per chromophore).[13] Both *O*-methylation and *O*-benzylation of the chromophore phenol substantially reduced the binding affinity. *O*-Methylation of **1** reduced binding by 1.8 kcal/mol (**1** versus **3**) and *O*-benzylation reduced binding by 1.6–3.4 kcal/mol (**1** versus **2** and **16** versus **17**). Removal of the fused benzene ring by substitution with the 3-hydroxypyridine-2-carboxylate chromophore in **8** resulted in a substantial 1.5–2.0 kcal/mol (0.7–1.0 kcal/mol per chromophore) reduction in binding which compares favorably to the 2.3–2.6 kcal/mol (1.1–1.3 kcal/mol per

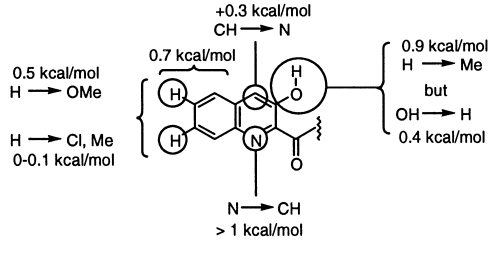

Figure 5.

chromophore) established by fluorescence quenching.[13] Further removal of the phenol from this chromophore (**10** versus **8**) resulted in a further diminished binding. Incorporation of the luzopeptin chromophore (**11** versus **1**) bearing a C6 methoxy group had little or no impact on the binding affinity although a small and reproducible 0.5 kcal/mol reduction was observed. An identical 0.5 kcal/mol reduction was observed in comparing **13** with **4** and in the comparison of **1** with luzopeptin A suggesting that the C6 methoxy substituent of the luzopeptins may in fact slightly diminish the binding affinity for 5′-GCATGC. Introduction of a C6 methyl group (**14** versus **1**) or a C7 chlorine substituent (**16** versus **1**) had a similar 0.2 kcal/mol reduction effect. Thus, the C6 methoxy group of the luzopeptins is not contributing productively to the DNA binding affinity of the agents. In addition, there appears to be a tolerance for significant substitution at both C6 and C7 of the chromophore without detrimental effects on binding, although none to date have been found that improve the affinity. Finally, the comparisons of **1** with **21** and **22** proved interesting. Both exhibited respectable binding affinities of $\Delta G^\circ = -8.6$ to 9.3 kcal/mol although they were 1.5–2.2 kcal/mol lower than **1**. What distinguished both **21** and **22** from **1** and the tighter binding analogues was their relatively rapid dissociation kinetics (off rates). Both **21** and **22** exhibited relatively fast dissociation rates (1.7×10^{-3} and $1.2\times10^{-4}\,s^{-1}$) analogous to that observed with **24** ($>5\times10^{-4}\,s^{-1}$) and 10–100× faster than **1** and related agents ($2.4–6.2\times10^{-5}\,s^{-1}$). We have interpreted this to suggest that both **21** and **22** bind to 5′-d(GCATGC)$_2$ by mono-intercalation and both exhibit free energies of binding consistent with this (cf. **24**, $\Delta G^\circ = -9.2$ kcal/mol). The much slower dissociation for **1** and related agents ($2.4–6.6\times10^{-5}\,s^{-1}$) may be a characteristic that is diagnostic of bis-intercalation and may be the most important distinguishing feature contributing to the productive properties of the agents.[21]

Using surface plasmon resonance, we were also able to establish a binding constant for **23** for duplex 5′-d(GCATGC)$_2$, Table 2. Previously, we had established a tentative value of −6.0 kcal/mol for calf thymus DNA by an indirect method involving the displacement binding of ethidium bromide and the value of −5.9 kcal/mol established herein for 5′-d(GCATGC)$_2$ compares well with this prior estimate.[6] The dissociation of the mono-intercalators in this series (i.e., **24**) is slow compared to simple intercalators ($20–0.25\,s^{-1}$)[21] or even the most effective mono-intercalators that benefit from extensive additional groove binding (actinomycin: 1×10^{-3} to $3.4\times10^{-4}\,s^{-1}$).[21] This is consistent with the observed binding affinity of **23** which suggests that the cyclic decadepsipeptide itself contributes very substantially to the properties of the agents.

Thus, the addition of a single chromophore to **23** ($\Delta G^\circ = -5.9$ kcal/mol) increases binding by 2.7–3.4 kcal/mol (i.e., even **21–22**) and the addition of a second chromophore further increases binding by 2.1–1.4 kcal/mol in good agreement with our prior estimates of 3.2 and 1.0 kcal/mol, respectively.[6] The differences that distinguish mono-intercalation and bis-intercalation within this class of agents is not so much the incremental increase in binding affinity, but rather the temporal stability of the complex. The bis-intercalators proved to exhibit off rates that were exceptionally slow being approximately 10–100× slower than the mono-intercalator, and are the slowest off rates recorded to date for natural[22] or synthetic[23] bis-intercalators.

Conclusions

Both fluorescence quenching[13] and surface plasmon resonance were employed to establish the DNA binding affinity of sandramycin and a key series of chromophore analogues within 5′-d(GCXXGC)$_2$ where XX = AT, TA, GC, and CG. With the studies, the determination of absolute binding constants within a single high affinity bis-intercalation site permitted a quantitative assessment of the sequence selectivity of sandramycin (**1**) as 5′-d(GCATGC)$_2$ > 5′-d(GCGCGC)$_2$, $\Delta\Delta G^\circ = 0.4$ kcal/mol ≥ 5′-d(GCTAGC)$_2$, $\Delta\Delta G^\circ = 0.9$ kcal/mol ≥ 5′-d(GCCGGC), $\Delta\Delta G^\circ = 1.0$ kcal/mol and a quantitative assessment of the chromophore structural features contributing to binding at a single high affinity bis-intercalation site. The results obtained by surface plasmon resonance parallel those derived from fluorescence quenching studies.[13] For example, the selectivity studies for the four deoxyoligonucleotides provided $\Delta\Delta G^\circ$ values of 0.4, 0.9 and 1.0 kcal/mol versus 0.3, 0.6, and 0.6 kcal/mol. The two highest affinity sequences constitute repeating 5′-PuPy motifs with each intercalation event occurring at a 5′-PyPu step. The higher affinity sequence of the pair constitutes the less stable duplex, possesses the sterically most accessible minor groove central to the bis-intercalation site, and the ability to accept the two gly-NH/T C2 carbonyl H-bonds identified in NMR studies.[6] Whether these features, or more subtle features, are responsible for the binding preference will be the subject of continued examination. In addition to not requiring a fluorescent chromophore or related spectroscopic/radioactive probe, surface plasmon resonance allows the direct measurement of association and dissociation rate constants that contribute to the overall affinities. This revealed that the high affinity binding agents and those that possess the most potent cytotoxic activity exhibit the slowest off rates.[13] This temporal stability of the complexes, which also proved characteristic of the distinction between bis and mono intercalation, may prove important to the expression of the agents' properties. Moreover, the cyclic decadepsipeptide **23** lacking both chromophores exhibits a significant binding affinity in its own right ($\Delta G^\circ = -5.9$ kcal/mol). This suggests that sandramycin is best represented as a DNA minor groove binding cyclic decadepsipeptide incrementally stabilized by mono- and bis-intercalation. The chromophore nitrogen inherent in the quinoline-2-carboxylate structure is essential for binding affinity (>1 kcal/mol per chromophore), the fused benzene ring contributes substantially (0.7–1.1 kcal/mol per chromophore) while the C3 phenol only slightly enhances binding (0.4 kcal/mol per chromophore). The addition of C6 or C7 substituents only slightly diminishes binding affinity and the luzopeptin

chromophore incorporating a C6 methoxy substituent was established to be slightly less effective than the sandramycin chromophore. These studies suggest substantial modifications may be made at both the C6 and C7 positions without adversely affecting binding affinity but none to date have been observed to enhance binding. A prominent distinction found with the effective agents and characteristic of the high affinity bis-intercalation binding was the unusually slow off rates required for binding dissociation.

Experimental

DNA binding studies. Analogue solution preparation

The analogues were dissolved in DMSO to a concentration of 1×10^{-3} M. These solutions were stored under Ar at $-78\,^{\circ}C$ and the integrity of the agents was checked periodically by ^1H NMR in 10% DMSO-d_6/$CDCl_3$. In most cases, a final cuvette concentration of 1×10^{-5} M in a 2 mL aqueous buffer containing 10 mM NaCl, 75 mM Tris–HCl (pH 7.4) was achieved by adding 20 μL of the analogue solution to the buffer. An additional 20 μL DMSO was added to promote dissolution of the analogues in the aqueous buffer.

DNA binding constants: surface plasmon resonance

Surface plasmon resonance measurements were performed on a Pharmacia Biosensor BIAcore 2000™ instrument using CM5 (carboxymethylated dextran) research grade sensor chips.[18,24] Immobilization of streptavidin on the dextran matrix via primary amine groups[16] was accomplished as follows: a continuous flow of 0.01 M HEPES pH 7.4, 0.15 M NaCl, 3.4 mM EDTA, 0.0005% Surfactant P20 at 10 μL/min in all four of the individual flow cells of the sensor chip was maintained. The sensor chip surface was activated by injection of 70 μL of a mixture of 0.2 M 1-ethyl-3-[(3-dimethylamino)propyl]carbodiimide (EDCI) and 0.05 M *N*-hydroxysuccinimide in water.

A 1 mg/mL solution of streptavidin (Sigma) in H_2O diluted to 50 μg/mL with an aqueous buffer of 10 mM sodium acetate (pH 5.0) was used to immobilize the enzyme. Four different amounts (75 μL, 60 μL, 40 μL and 5 μL) were simultaneously injected into the four flow cells for the studies with **1–24**. This method consistently resulted in 3500, 2300, 1300, and 250 ± 10% RU of streptavidin for a final volume concentration of 35, 23, 13, and 2.5 mg/mL in the four flow cells of the sensor chip. For the studies of **1** with the four deoxyoligonucleotides, 60 μL of the streptavidin solution was injected into each of the four flow cells for a final volume concentration of 23 mg/mL. Remaining active esters were blocked with 70 μL of a 1.0 M aqueous solution of ethanolamine.

The 5′-GCXXGCTTTTGCXXGC biotinylated at the 5′ end were obtained commercially (Operon Technologies Inc., Alameda, CA) in 2–5 OD quantities and dissolved in 10 mM Tris–HCl (pH 7.4) buffer solution containing 75 mM NaCl to a final concentration of 0.3 μg/mL. For the analogue studies, 250 μL of the XX = AT solution was injected into all four flow cells which was sufficient for saturation binding of the streptavidin and resulted in a ratio of 2:1 DNA:enzyme ratio by RU:mass ratios. For the sequence studies, 295 μL of each of the four deoxyoligonucleotides (XX = GC, AT, CG, and TA) was injected and allowed to reach equilibrium and also reach a stoichiometry of 2:1 DNA:enzyme in each of the four flow cells.

For the kinetic runs, performed at 25 °C, 1 mM concentrations of the analogues in DMSO were diluted with an aqueous buffer of 10 mM Tris–HCl (pH 7.4) containing 75 mM NaCl to concentrations ranging from 1 μM to 10 nM. Each solution was injected for 30 min to 2.5 h (necessary to reach equilibrium) by stacking contiguous 250 μL injections (maximum kinetic injection possible on BIAcore 2000). Each analogue run was followed by a 1 min pulse of 25 mM aqueous HCl, and finally 30 min of buffer flow before the next analogue injection was started. The regeneration protocol returned the response level to within 5% of the baseline level and was found to have little effect on the response level of the streptavidin alone or the DNA bound sensor chip. The injections were automated with the most concentrated solution being injected first and this initial run was followed by a 3 h buffer flow to obtain a dissociation rate before regeneration. Data was processed using BIAcore software and as described in the present text.

The data for **10, 23** and **24** were determined based on estimates of concentrations necessary to give 50% R_{max} value which can be related back to an overall affinity constant based on eq (2).

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