Interactions of Antitumor Drugs with Natural DNA: ^{1}H NMR Study of Binding Mode and Kinetics

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^{1}H NMR has been used to study the interactions of over 70 clinical and experimental antitumor drugs with DNA. Spectra of the low-field (H-bonded imino proton) resonances of DNA were studied as a function of drug per base pair ratio. From the spectral changes observed, it was possible to distinguish three modes of drug binding (intercalation, groove binding, and nonspecific outside binding), to determine the kinetics of drug binding (approximate lifetime of the bound drug), and, in favorable cases, to determine the specificity of the drugs for A·T or G·C base pairs. This method is a useful assay for general drug-binding characteristics. For the intercalating compounds there appears to be a correlation between drug-binding kinetics and useful antitumor activity.

The binding of small ligands to DNA has attracted an enormous amount of study, not only as a topic in physical chemistry, but also in molecular pharmacology,[1] medicinal chemistry,[2,3] and carcinogenesis.[4] Given the central role of DNA in the regulation of biochemical processes, it is not surprising that compounds capable of interacting with it exhibit a wide spectrum of antibacterial, antiprotozoal, antiviral, and antitumor activity. Virtually all of the current clinical and experimental antitumor drugs are thought to act by disruption of nucleic acid metabolism at some level.[5] The class of antitumor compounds that has received most development recently is the DNA-binding agents, or "DNA adductors", that bind tightly, but reversibly, to DNA by a combination of hydrophobic, electrostatic, hydrogen-bonding, and dipolar forces.

At least three aspects of the binding of drugs to DNA could be expected to influence biological activity; (1) mode of binding to the DNA, (2) selectivity for A·T or G·C base pairs, or specific sequences, and (3) the kinetics of binding. The most well-studied ligands are those that possess conjugated π-electron systems and bind by intercalation of the chromophore between the base pairs of the DNA duplex.[1,6-13] A second well-defined mode of binding is exhibited by a large number of compounds that typically possess charged groups at each end of a rigid linear framework.[14] Binding of these drugs elevates the denaturation temperature of the DNA, without unwinding the helix. These compounds show selectivity for A·T-containing sequences and have a large DNA site size, approximately proportional to their linear dimension. It has, therefore, been proposed that these drugs bind in the minor groove of the DNA duplex.[2,14-18] Other modes have been proposed for binding at high ligand/DNA ratios (cooperative stacking on the outside of the DNA) and for particular compounds (e.g., the binding of irehediamine to the "kink" sites),[19] but these are of lesser general importance.

Selectivity for specific sequences in the DNA may be of particular importance in the effectiveness of a drug, since, depending upon their sequence specificity, drugs could affect the activity of certain genes. Furthermore, it is known that certain drugs tend to disrupt chromatin structure.[20-22] Specific sequences are known to have profound effects on DNA conformation,[23,24] and this, in turn, can effect drug-binding properties.[25]

The kinetics of the binding are another aspect of drug–DNA interactions of some importance from a biomedical viewpoint. There is evidence to suggest that where biological activity of drugs is a consequence of their ability to interact reversibly with DNA, slow rates of dissociation from the DNA correlate with greater levels of in vitro inhibition of DNA polymerase and possibly with enhanced in vivo activity.[26] Drug-binding kinetics have

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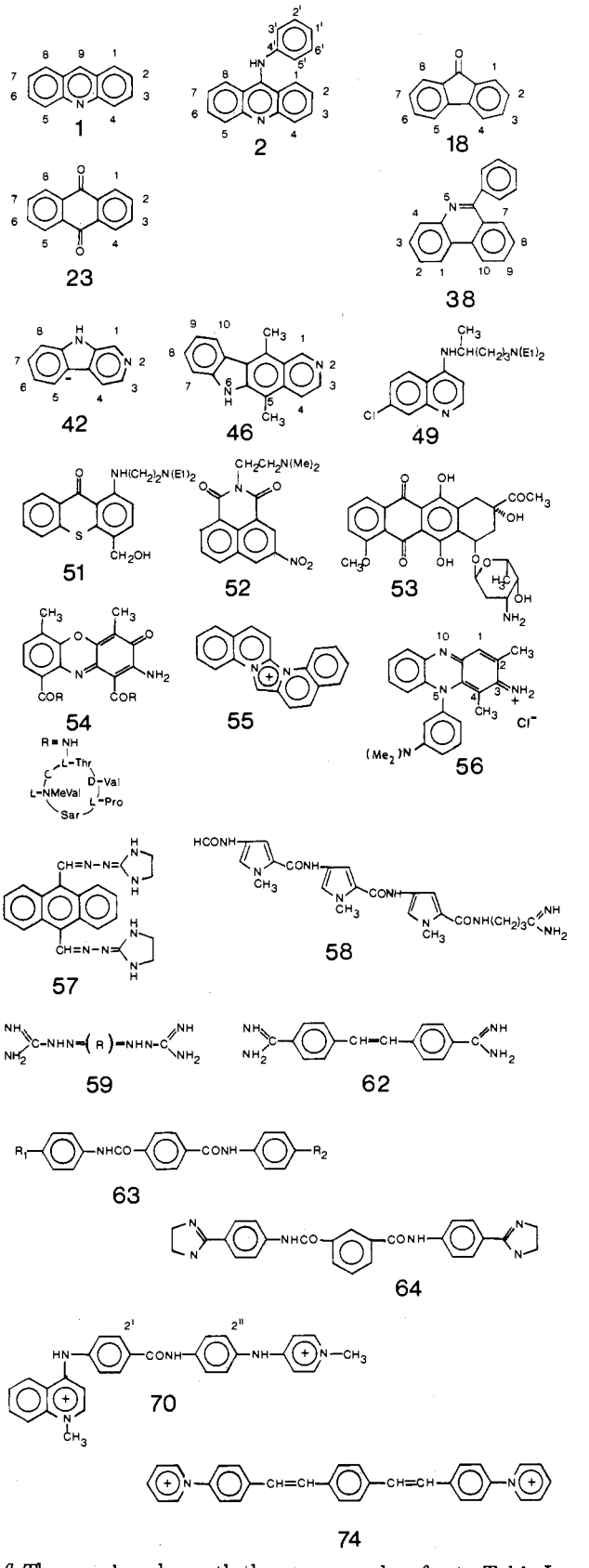
also been shown to have a profound effect on the ability of drugs to inhibit the B to Z transition in poly(dG-dC).[27]

A variety of methods have been used to investigate ligand–DNA complex formation in solution, including measurement of changes in sedimentation,[8,9] fluorescence,[28–30] and electric and circular dichroism.[31,32] Mode of binding has generally been determined by the unwinding assays or change in sedimentation velocity, which usually allows classification of the drugs as either intercalators or outside binders. Specificity for A·T or G·C base pairs has been studied by equilibrium dialysis experiments with A·T- and G·C-rich DNAs.[33–35] Drug–DNA binding kinetics have been investigated by temperature-jump and stopped-flow UV spectroscopic techniques, particularly for the antitumor agents actinomycin and derivatives,[36,37] as well as ethidium[38–40] and acridine analogues.[41,42] Very complex structure-dependent kinetic behavior has been observed for the binding of these compounds to DNA, and, at this point, relatively few compounds have been investigated.

High-resolution NMR is a powerful tool for studying ligand–nucleic acid complexes in solution, since signals from both the ligand and the DNA substrate can be monitored. Most NMR work to date has been on the complexes of ligands with short synthetic oligo(deoxyribonucleotides),[43–47] although there have been some studies with repeating-sequence deoxyribonucleic acid duplexes, especially poly(dA-dT),[48–50] at temperatures near the duplex to strand transition.

The number of ligands studied has been quite limited, and the most extensively studied compounds (e.g., ethidium bromide, proflavine, and 9-aminoacridine) are of little current clinical interest, and among the currently used antitumor agents, actinomycin D[47–49] and daunomycin[51] are among the few that have been examined in detail by NMR. From the standpoint of biomedical relevance, it

Chart I. Structures of the Parent Compounds for the Drugs Studied[a]



[a] The numbers beneath the compounds refer to Table I, where the complete set of drugs investigated is listed. The numbering system for the various compounds is given where necessary in order to identify positions of side chains on derivatives; in some cases, variations in substituents are indicated by R.

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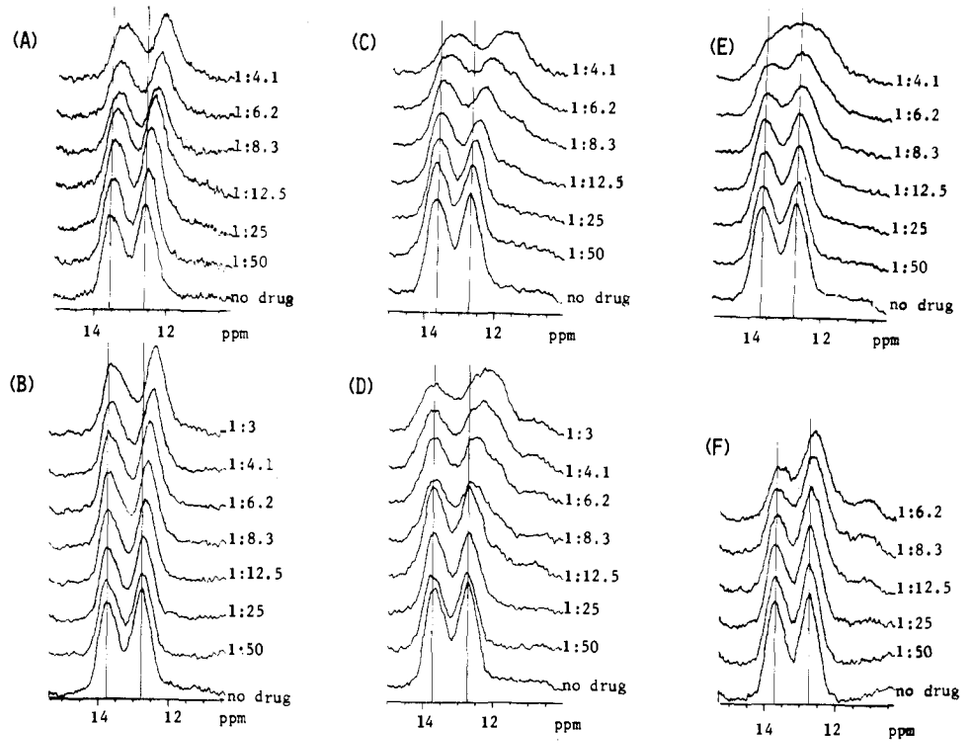

Figure 1. ^{1}H NMR (300 MHz) spectra of the low-field resonances of DNA as a function of intercalating drug level at 35 °C. The drug/DNA base-pair ratios are given by the spectra. (A) Methylharman (**44**) is near fast exchange with the DNA and is G·C specific. (B) Chloroquin (**49**) is in fast exchange and is G·C specific. (C) Mitonafide (**52**) is in slow exchange and is G·C specific. (D) Actinomycin D (**54**) is in slow exchange and is very G·C specific. (E) Anthracenedione derivative RMI10024 (**26**) is in intermediate exchange and is A·T specific. The compound produces only small upfield shifts on the DNA resonances. (F) Bisantrene (**57**) is in slow exchange and is tentatively considered to be A·T specific. Spectra were obtained in the correlation mode and required 23 min of signal averaging; 4000 transients were collected with a sweep time of 0.3 s and a predelay of 50 ms. Spectra are line broadened (exponential multiplication) by 2–5 Hz.

would obviously be desirable to study the interaction of drugs with native DNA of natural origin. In the past, NMR studies of this type have been limited by a lack of samples in the right size range, but with the development of techniques for obtaining large quantities of short, random-sequence DNA,[52,53] it was possible to study the binding of a large number of different compounds to DNA. Thus, a survey of the DNA-binding characteristics of more than 70 clinical and experimental antitumor drugs of current interest was carried out (Chart I). This survey included representatives of all of the major structural classes of drug that are thought to act by virtue of their DNA-binding ability.[54-59]

In this work the focus is entirely on the low-field resonances of DNA arising from the imino protons of G and T in Watson–Crick base pairs. Thus, the effects of drug binding on the DNA are probed directly, with little or no interference from the drug resonances themselves. The results obtained extend the usefulness of NMR in determining general drug-binding characteristics, including mode of binding, base-pair specificity, and kinetics.

Results

The hydrogen-bonded imino protons of the Watson–Crick base pairs in DNA give rise to two groups of resonances centered at 13.72 and 12.75 ppm for the A·T and G·C base pairs, respectively. The half-widths of these two envelopes are about 190 Hz, and each envelope is comprised of many overlapping lines with varying chemical shifts due primarily to ring-current shifts on the imino protons by neighboring base pairs. A variety of different effects on the low-field spectra of the DNA were observed upon addition of drugs. These changes, which were monitored as a function of increasing drug level at 35 °C, fell into the following three major classes.

(1) Intercalators (I). For the majority of drugs, *upfield shifts* of both the A·T and the G·C resonances were observed, often accompanied by some broadening of the resonance envelopes. These spectral effects are indicative of intercalative binding (vide infra). Many of these drugs had been previously classified as intercalators by other methods,[60] but the mode of binding of several of the drugs (**28–31**, **42–45**, **49**, and **55–57**) was untested or unclear. Low-field spectra of the DNA with several different intercalators are given in Figure 1. Although all of these drugs cause upfield shifts of the A·T and G·C imino proton resonances, the shape of the resulting spectral envelopes varied considerably due to differences in kinetics and base-pair specificity (vide infra).[61]

(2) Groove Binders (G). Very different spectral changes were observed upon addition of certain drugs, as

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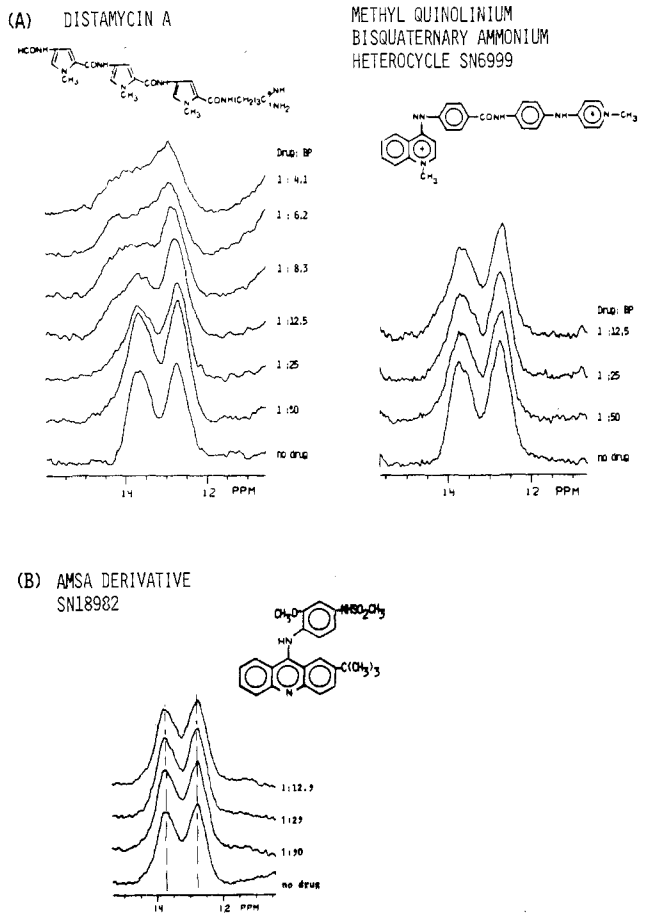


Figure 2. Effect of outside binders on the low-field ^1H NMR spectra of DNA at 35 °C. Spectra show the effects of increasing drug levels on the imino proton spectra of DNA. The drug/DNA base-pair ratios are given to the right of each spectrum. (A) Distamycin A (**58**) and SN6999 (methylquinolinium bisquaternary ammonium heterocycle) (**70**) are groove binders. (B) AMSA derivative SN18982 (**9**) is a nonspecific outside binder. Instrumental conditions are given in Figure 1.

shown in Figure 2A. For this class of drugs, *downfield shifts* of the A·T, and to a smaller extent the G·C, resonances were observed.[46] Most of these drugs had been previously shown not to bind by intercalation and to have a high A·T specificity, and a binding site along the minor groove of the DNA had been proposed for some of them. The spectral changes observed are consistent with binding of the drugs in a groove of the DNA.[47]

(3) Nonspecific Outside Binders (NSO). Figure 2B shows that with some drugs very little or no spectral changes were observed in the low-field spectral region, although by other criteria (e.g., UV spectrophotometry, equilibrium dialysis, etc.) the drugs had been shown to bind to DNA. For this group of drugs, binding to the outside of the DNA in an unspecified manner (possibly by electrostatic interactions with the phosphate backbone) is proposed.

Framework for Analysis of the Effects of Drug Binding on DNA Spectra. To provide a framework for interpreting the experimental results obtained on drug binding to DNA, we first discuss the different types of spectra for several different general types of binding. At least three properties of the drugs are expected to be important in terms of how the drugs affect the DNA spectrum.

(1) Mode of Binding. The three major modes of binding are (1) intercalation, (2) outside binding in groove, and (3) nonspecific outside binding. With certain compounds, more than one mode of binding might be possible, leading to spectra that are difficult to interpret. Since the DNA solutions contained 10 mM $MgCl_2$ and 0.1 M NaCl, intercalative binding is favored over outside binding for drugs that could bind either way.

(2) Kinetics of Binding. The lifetime of the drug at any specific binding site has a very profound effect on the way in which the drug affects the DNA spectrum,[61] and it is relatively easy to predict the type of spectra that will be observed in limiting cases.

(3) Sequence Specificity. Some of the drugs studied here have a preference for binding adjacent to either A·T or G·C base pairs, and in many cases it is possible to predict the sort of spectral changes expected. We now consider each of the major types of drug binding and discuss the way in which kinetics and sequence specificities affect spectra.

Intercalators. For purposes of discussion, consider a simple-sequence DNA [e.g., poly(dA-dT)·poly(dA-dT), poly(dA)·poly(dT), or poly(dG)·poly(dC)] that exhibits only a single low-field resonance. When a drug intercalates into the DNA, the ring-current shifts exerted on each base pair at the intercalation site by the other base pair is replaced by the generally larger ring-current shift from the drug. Thus, for each drug bound, the resonances from the two adjacent base pairs will be affected. If the lifetime of the drug in the binding site is very long, a new upfield-shifted resonance(s) will appear, and the intensity of the original peak will be correspondingly decreased. Conversely, if the binding-site lifetime is very short, there will be an averaging effect due to chemical exchange among DNA binding sites, and the *entire* lowfield resonance will gradually be shifted upfield as more drug is added. The situation is more complicated with the random-sequence DNA. Each base pair gives rise to a resonance whose chemical shift is determined mainly by its intrinsic chemical shift (~14.6 ppm for A·T and ~13.6 for G·C)[44,48] and by the ring-current shifts exerted on it by both its nearest-neighbor and, to a smaller extent, its next-nearest-neighbor base pairs. Thus, at least 256 different resonance positions for the base pairs in random-sequence DNA are expected. These resonances overlap in such a way that only two broad envelopes of resonances are seen, and these can be clearly differentiated as arising from A·T base pairs (centered at 13.72 ppm) and G·C base pairs (centered at 12.75 ppm). The nature of the spectral changes induced by the addition of drug will depend upon both the kinetics of the binding and the sequence specificities.

(a) Slow Exchange Limit. If exchange of the drug among different DNA binding sites is slow, two upfield-shifted resonances will appear for each drug bound, and the remaining drug-free DNA base-pair resonances will remain unshifted (neglecting second-neighbor ring-current shifts). Since there are many possible binding sites, there will also be a range of resonance positions for the upfield-shifted resonances at the intercalation site, and these too will appear as two envelopes of overlapping resonances (one set from the A·T and one set from the G·C base pairs) just as in the free DNA. If $\nu_A - \nu_B$ is the average upfield shift (in hertz) of resonances induced by the drug, then the criterion for observation of a resolved shifted resonance is

$$t > [\pi(\nu_A - \nu_B)]^{-1} \quad (1)$$

where t is the lifetime of the drug in a binding site. For many intercalators, $\nu_A - \nu_B$ is typically ~300 Hz at our field; hence, a lifetime significantly longer than 1 ms is required to observe the shifted resonance. (When the observed line width of the shifted resonances is less than

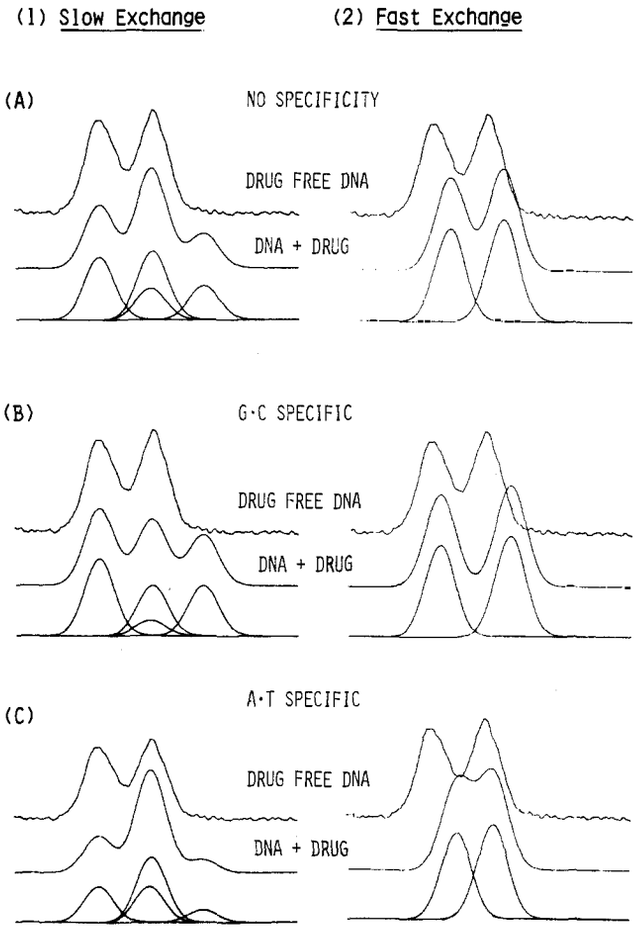


Figure 3. Simulations of the effects of drug binding at a level of 1 drug/6 base pairs on the imino proton spectra of DNA, in the limits of (1) slow and (2) fast exchange of the drug with the DNA. An average upfield shift of 0.9 ppm for both the A·T and the G·C resonances at the intercalation site was used in making the simulations: (A) no base-pair specificity; (B) 2:1 specificity for G·C base pairs; (C) 2:1 specificity for A·T base pairs. The top spectrum in each case is a real drug-free DNA spectrum. The simulated spectrum is directly below, with the component resonance envelopes shown at bottom. In making the simulations, we assumed that only resonances from base pairs at the intercalation site would be upfield ring current shifted by the drug, so that at 1 drug/6 base pairs, 1 in 3 resonances are affected. For each simulation, the free DNA spectrum was fit with two 100% Gaussian lines, one each for the A·T and G·C resonance envelopes. For the slow exchange cases, the appropriate amount of intensity was subtracted from each of the resonance envelopes and placed 0.9-ppm upfield, keeping the same line widths. For the fast exchange cases, the resonance envelopes were upfield shifted by the weighted average of bound and free resonance positions.

100 Hz, a lifetime of greater than about 5 ms can be inferred.[61])

(b) Fast Exchange Limit. If the lifetime, t, of the drug in any specific binding site is short, i.e.,

$$t < [\pi(\nu_A - \nu_B)]^{-1} \quad (2)$$

then all resonances in the spectrum will shift upfield by an amount that depends on the fraction of drug bound per base pair.

In Figure 3A are shown simulations of spectra that might be observed for the two limiting cases (fast and slow exchange) for an intercalating drug that exhibits no preference for binding to A·T or G·C base pairs and causes an average upfield shift of 0.9 ppm. The case of intermediate exchange is more difficult to treat because it leads to spectra that are more complex and, therefore, more difficult to analyze. The actual spectral results observed on drug binding to DNA deviate somewhat from those shown in Figure 3, due partially to the fact that the drug kinetics are intermediate between slow and fast exchange with the DNA. However, even for the limiting cases, differences between the real and calculated spectra are observed, and this is attributed to the assumptions made in the simulations. These include (a) the same average upfield shift of 0.9 ppm for both A·T and G·C resonances, (2) no second-neighbor effects, (3) the same relative chemical-shift distribution for bound and free sites, and (4) no other effects, except those due to ring currents. In reality, a range of upfield shifts are observed depending on the chromophore. In considering the experimental data, it should be noted that the 1-m time constant used to classify compounds as exhibiting either "slow" or "fast" exchange kinetics is an arbitrary one imposed by our spectrometer frequency and the chemical shifts induced by the drug. Note further that drugs exhibiting very different binding lifetimes might both be classified the same.

(c) Effects of Specificity. In the discussion on kinetics, we tacitly assumed all binding sites have the same occupation probability, i.e., that the drugs showed no site specificity on binding. We now extend the analysis to cases where the drugs do show a specificity for binding next to A·T or G·C base pairs. If a drug is somewhat G·C specific, and in slow exchange, a greater fraction of G·C resonances will be affected than A·T resonances. Simulated results are shown in Figure 3B for a drug with a 2:1 specificity for G·C vs. A·T base pairs. The situation would be reversed for A·T-specific intercalators (Figure 3C). Many intercalators cause an average upfield shift of about 1 ppm, and in those cases, in slow exchange the upfield-*shifted* A·T resonances will appear at about the same resonance positions as the *unshifted* G·C resonances. This results in an apparent increase in intensity of the unshifted G·C resonances relative to the A·T resonances.

If the binding is A·T specific, then the loss at the A·T peak would be even more pronounced. However, if the G·C specificity is sufficiently high (2:1), then a loss of the G·C peak relative to the A·T peak will be observed. By comparing the intensity of the upfield-shifted G·C resonances relative to the total integrated intensity in the spectrum, the preference for G·C base pairs can be deduced. The intensity of the unshifted A·T peak relative to the total intensity provides another index of specificity. Finally, the relative intensity of the unshifted A·T and G·C peaks can be used as a qualitative index of sequence preference.

In the fast exchange limit, the shapes of the envelopes of the A·T and the G·C resonances would be predicted to remain more or less unaffected, but their peak positions would shift upfield as illustrated in Figure 3. By comparing the relative magnitudes of the shifts of the A·T and the G·C peaks, the preference of the drugs for binding either adjacent to A·T or G·C base pairs can be determined *provided* it is assumed that binding of the drug produces comparable upfield shifts of both A·T and G·C resonances. Experimental results obtained with drugs of known specificity support this assumption;[61] therefore, when the G·C peak receives a larger upfield shift than the A·T peak due to binding of a drug, we will infer that the compound has a preference for binding adjacent to G·C base pairs. Some plots of the A·T and G·C peak chemical shifts as a function of drug/base pair ratio for drugs at, or near, fast exchange are shown in Figure 4. Specificity for A·T or G·C base pairs can be determined from the relative slopes of the lines for the A·T and G·C chemical shifts vs. drug level for

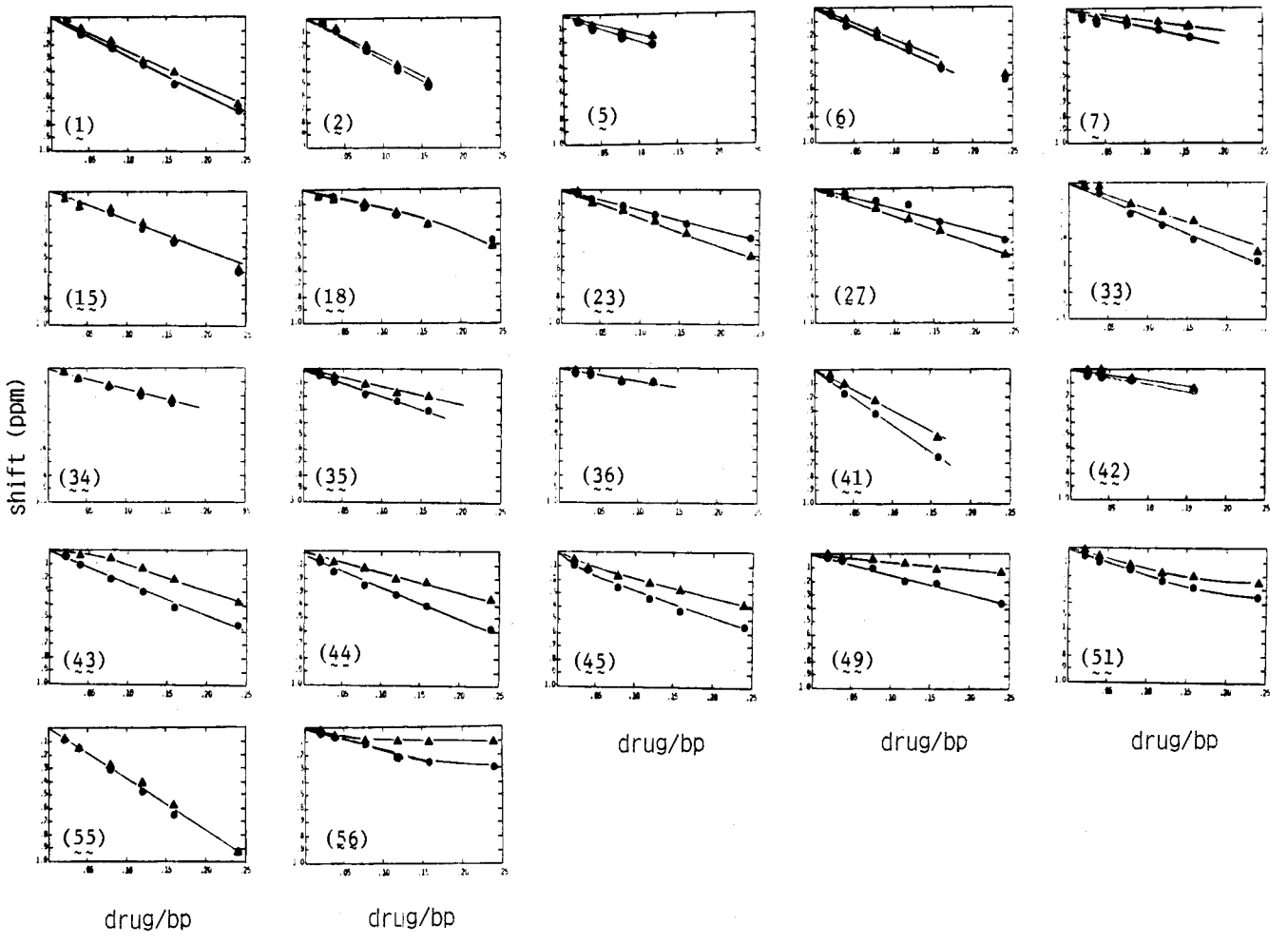


Figure 4. Plots of average chemical shift of A·T (▲) and G·C (●) resonance envelopes vs. drug level for intercalators at or near fast exchange with the DNA. Specificity for A·T and G·C base pairs can be determined by the relative slopes of the lines, if it is assumed that the resonances of both the A·T and the G·C base pairs receive the same upfield shift from bound drug. The shift index (SI) that is derived from these plots is given for the appropriate drugs in Table I and represents the slopes of these plots for the G·C and A·T imino proton resonance shifts vs. drug level.

a given drug. The relative A·T/G·C slopes are given as the shift index (SI) in Table I.

The examples shown in Figure 3 indicate that information on the kinetics and the specificity of the drug binding can be deduced from the spectra, provided the kinetics approach either the slow or fast exchange limit and the ring-current shifts from the drugs are large.

With intermediate exchange it is difficult to make judgments about A·T/G·C specificity, but the lifetime of the drug in the bound state is more accurately fixed for the following reason. The broadening, $\Delta\nu_{1/2}$, of the shifted resonances due to the finite lifetime of the drug in a binding site is

$$\Delta\nu_{1/2} = (\pi t)^{-1} \quad (3)$$

where $\Delta\nu_{1/2}$ is the width of the individual resonance at half height. Judging from an analysis of the effects of broadening on the lowfield spectra of short DNA,[53] we conclude that the shifted resonances would begin to be lost as separate, resolved resonances when $\Delta\nu_{1/2}$ is on the order of 100 Hz, corresponding to a lifetime of 3 ms.

For drugs that intrinsically give small "bound" site shifts, these binding kinetics will be very difficult to obtain, since the spectral changes will look similar for fast and slow exchange.

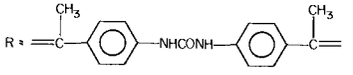
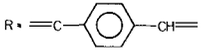
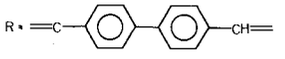
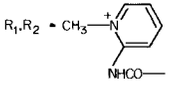
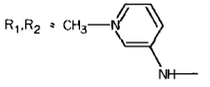
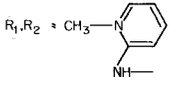
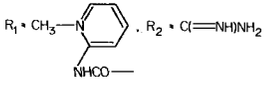
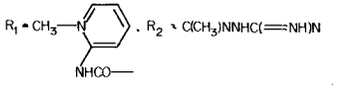
Groove Binders. Previous studies have shown that minor groove binders, such as distamycin A, have a preference for binding near A·T base pairs[62] and cause small downfield shifts of the imino protons.[47] In principle, the aromatic residues of a drug could bind in the major groove with a geometry that would produce upfield shifts of the imino proton resonances, but no such results were observed in this study. Therefore, drugs that induced downfield shifts of the imino proton resonance have been classified in this study as groove binders. It is not possible by this method to distinguish between binding in the major vs. the minor groove. Since the observed shifts are smaller for groove binders than for intercalators, the criteria for fast and slow exchange are accordingly altered. Differences are also to be expected because some groove binders contain several aromatic residues, in which case a single drug might affect resonances from several base pairs. Because of these factors and the fact that fewer groove binders were studied, analysis of the kinetics of their binding can only be considered tentative and in most cases was not attempted.

Nonspecific Outside Binders. Drugs that bind to the outside of DNA in a nonspecific manner through electrostatic and/or hydrogen-bonding interactions to the phosphate groups have little effect on the *low-field* resonances of the DNA, since the aromatic chromophore would be too far away from the bases to exert much of an effect. All of the drugs that produce no spectral changes in the low-field spectra are classified as nonspecific outside

(62) Krey, A. K. *Prog. Mol. Subcell. Biol.* **1980**, *8*, 43–87.

Table I. A Summary of NMR and Other Physical Measurements on the DNA-Binding Properties of Selected Drugs

				NMR results					literature measurements				
					kinetics[c]								
no.	formula no.	name	side chain in formula[a]	mode[b]	F	I	S	specificity of GC or AT;[d] SI = GC/AT[e]	unwinding angle, deg	site size, bp[f]	log K_D	ionic strength, M	ref
1	1	9-aminoacridine	9-NH_2	I	*	*		GC; 3.0/2.6	16		5.7	0.01	*j*
2	2	AMSA	1'-$NHSO_2CH_3$	I	*	*		(GC); 3.2/3.0	21		5.63	0.01	*k, l*
3	2	SN 8552[g]	2'-$NHSO_2CH_3$	NSO/(I)							5.80	0.01	*k*
4	2	SN 18493	3'-$NHSO_2CH_3$	NSO/(I)							5.72	0.01	*k*
5	2	amsacrine	1'-$NHSO_2CH_3$, 3'-OCH_3	I	*	*		GC; 2.0/1.5	21		5.14	0.01	*j*
6	2	SN 15247	1'-$NHSO_2CH_3$, 3'-CH_3	I	*	*		2.5/2.4					
7	2	SN 19643	1'-$NHSO_2CH_3$, 3'-O-*i*-Pr	I	*	*		GC; 1.4/0.8					
8	2	SN 19799	1'-$NHSO_2CH_3$, 3',5'-$(OCH_3)_2$	NSO									
9	2	SN 18982	1'-$NHSO_2CH_3$, 3'-OCH_3, 2-$C(CH_3)_3$	NSO									
10	2	SN 14477	1'-$NHSO_2CH_3$, 3'-OCH_3, 4-$CONH_2$	I	*								
11	2	SN 14771	1'-$NHSO_2CH_3$, 3'-OCH_3, 4-$CONHCH_2CONH_2$	I			*	GC					
12	2	SN 20779	1'-$NHSO_2CH_3$, 3'-OCH_3, 4-$CONHCH_2CONH(CH_2)_2NH(CH_2)_2OH$	I			*	GC					
13	2	SN 16713	1'-$NHSO_2CH_3$, 3'-OCH_3, 4-$CONH(CH_2)_2N(CH_3)_2$	I			*	GC					
14	2	SN 16712	1'-$NHSO_2CH_3$, 3'-OCH_3, 4-$CONH(CH_2)_3N(CH_3)_2$	I			*	GC					
15	2	SN 9796	1'-$NHSO_2(CH_2)_4NH_2$	I	*	*		2.0/2.0					
16	2	SN 13657	1'-$NHSO_2(CH_2)_4NHC(=NH)NH_2$, 3-$NO_2$	I		*							
17	2	SN 13774	1'-$NHSO_2(CH_2)_4NHC(=NH)NH_2$	I		*							
18	18	tilorone	2,7-$[O(CH_2)_2N(Et)_2]_2$	I	*	*		1.3/1.3	13	3.6	5.64		*m, o*
19	18	RMI 2481[h]	2,7-$[CCO(CH_2)_2N(Et)_2]_2$	I		*	*						
20	18	RMI 2515	2,7-$[COO(CH_2)_3N(Et)_2]_2$	I		*	*						
21	18	RMI 9597	2,7-$[CCO(CH_2)_4N(Et)_2]_2$	I		*		(AT); 1.1/1.4					
22	18	RMI 10117	2,7-$[COO(CH_2)_6N(Et)_2]_2$	I		*		(AT)					
23	23	RMI 10761	1,5-$[O(CH_2)_2N(Et)_2]_2$	I	*	*		AT; 1.5/2.0					
24	23	RMI 10662	1,8-$[O(CH_2)_2N(Et)_2]_2$	I		*							
25	23	RMI 10781	2,7-$[O(CH_2)_2N(Et)_2]_2$	I		*	*						
26	23	RMI 10024	2,6-$[O(CH_2)_2N(Et)_2]_2$	I		*	*	AT					
27	23	RMI 10471	1,4-$[O(CH_2)_2N(Et)_2]_2$	I	*	*		AT; 1.5/2.0					
28	23	ametantrone	1,4-$[NH(CH_2)_2NH(CH_2)_2OH]_2$	I		*			20				*q*
29	23	NSC 278467[i]	1,4-$[NH(CH_2)_3NH(CH_2)_2OH]_2$	I		*	*						
30	23	nitroxantrone	1,4-$[NH(CH_2)_2NH(CH_2)_2OH]_2$, 5,8-$(OH)_2$	I		*	*		22				*q*
31	23		1,5-$[NH(CH_2)_2N(Et)_2]_2$	I		*	*	(AT)					
32	32			I			*	AT					
33	1	proflavine	3,6-$(NH_2)_2$	I	*	*		GC; 2.6/2.0	17				*p, q*
34	1	dimethylproflavine	2,7-$(CH_3)_2$, 3,6-$(NH_2)_2$	I	*	*		1.5/1.5	18				*q*
35	1	diethylproflavine	2,7-$(Et)_2$, 3,6-$(NH_2)_2$	I	*	*		GC, W; 2.5/1.3	16				*q*
36	1	diisopropylproflavine	2,7-(*i*-Pr)$_2$, 3,6-$(NH_2)_2$	I/C	*	*		0.8	0				*q*
37	1	di-*tert*-butylproflavine	2,7-(*t*-Bu)$_2$, 3,6-$(NH_2)_2$	G				(AT)	0	3			*p, q*
38	38	ethidium	3,8-$(NH_2)_2$, 5-Et	I			*		26	2	5.7	0.1	*z, aa*
39	38	dimidium	3,8-$(NH_2)_2$, 5-CH_3	I			*		26	2	5.7	0.1	*z*
40	38	8-deaminoethidium	3-NH_2, 5-Et	I			*						
41	38	3-deaminoethidium	8-NH_2, 5-Et	I	*			GC; 4.8/3.0		2.4	4.9	0.1	*bb*
42	42	norharman		I	*				17	3.9	5.34	0.002	*w*

43	42	methylnorharman	2,9-$(CH_3)_2$	I	*			GC; 2.3/1.0				
44	42	methylharman	1,2,9-$(CH_3)_3$	I	*			GC; 2.8/1.5	9			q
45	42	methylharmine	1,2,9-$(CH_3)_3$, 7-OCH_3	I	*	*		GC; 2.8/1.8				
46	46	ellipticine		I		*	*				5.18	0.1	n, y
47	46	9-hydroxyellipticine	9-OH	I		*	*	(GC)			.30	0.1	n
48	46	9-hydroxy-2-methylellipticine	2-CH_3, 9-OH	I		*	*	(GC)			6.11	0.1	n
49	49	chloroquine		I	*			GC; 1.5/0.7	17	5.0	4.25	0.02	s, t
50	1	mepacrine	9-$NHC(CH_3)(CH_2)_3N(Et)_2$, 2-OCH_3, 6-Cl	I	*	*			17		4.25	0.2	v
51	51	mycanthone		I	*	*		(GC); 2.0/1.5	15				o
52	52	mitonafide		I			*	GC	11	1.7	5.16	0.01	u
53	53	daunomycin		I			*	GC	11	2.6	6.49	0.02	o
54	54	actinomycin D		I			*	GC	26				o
55	55	imidazodiquinolinium cation		I		*		3.8	14	3	4.70	0.11	q
56	56	4-methyl-5-phenyl-Neutral Red		I	*			GC; 1.5/1.0			3.64	0.19	x
57	57	disantrene		I			*	AT	14				q
58	58	distamycin A		G			*	AT	0	4.6	6.06	0.05	r
59	59	DDUG		G					0				j
60	59	SN 20440		G				AT					
61	59	SN 20452		G									
62	62	stilbamidine		NSO									
63	63	NSC 53312	R_1, R_2 = $C(=NH)NH_2$	G				AT					
64	64	NSC 53212		G									
65	63	SN 5752		G					0				
66	63	SN 6053		G			*	AT					
67	63	SN 6058		G			*	AT					
68	63	SN 6136		G									
69	63	SN 6134		(G)									
70	70	SN 6999		G			*	AT	0	5	6.90	0.01	j
71	70	SN 7006	6-NH_2	G					0				j
72	70	SN 7122	2'-NH_2	G									
73	70	SN 7164	2''-NH_2	G				(AT)					

[a] Formulas are given in Chart I, with the numbers given below each apparent compound. [b] Mode of binding as determined by NMR: I, intercalation; NSO, nonspecific outside binding; G, groove binding. [c] Kinetics of binding (lifetime in the bound state) as determined by NMR, i.e., fast, intermediate, or slow on the NMR time scale. Fast to intermediate is indicated by an asterisk in both columns and likewise for intermediate to slow. [d] A general specificity for AT or GC base pairs is indicated, but the extent of specificity is not determined. Marginal specificity is indicated by parentheses. [e] Shift index (SI) is the slope of the curves for chemical-shift change vs. drug level for intercalators in or near fast exchange with the DNA. Values are given for GC/AT resonances. See Figure 4 for further details. [f] Excluded site size in base pairs. [g] SN = screen number, Cancer Research Laboratory, Auckland, New Zealand. [h] RMI = Richardson-Merrill, Inc. [i] NSC = National Service Center, National Cancer Institute. [j] Braithwaite, A. W.; Baguley, B. C. *Biochemistry* **1980**, *19*, 1101. [k] Baguley, B. C.; Denny, W. A.; Atwell, G. J.; Cain, B. F. *J. Med. Chem.* **1981**, *24*, 170. [l] Waring, M. J. *Eur. J. Cancer* **1976**, *12*, 995. [m] Chandra, P.; Woltersdorf, M.; Wright, G. J. *Antibiotics (N.Y.)* **1979**, *5* 385–413. [n] Paoletti, C.; LePecq, J. B.; Dat-Xuong, N.; Jurey, P.; Garnier, H.; Amtel, J.-L.; Rouesse, J. *Recent Results Cancer Res.* **1980**, *74*, 107. [o] Waring, M. J. *J. Mol. Biol.* **1970**, *54*, 247. [p] Müller, W.; Crothers, D. M.; Waring, M. J. *Eur. J. Biochem.* **1973**, *39*, 223. [q] Unpublished data, Cancer Research Laboratory. [r] Luck, G.; Truehel, H.; Waring, M. J.; Zimmer, C. *Nucleic Acids Res.* **1974**, *1*, 503. [s] Jones, R. L.; Lanier, A. C.; Keel, R. A.; Wilson, W. D. *Nucleic Acids Res.* **1980**, *8*, 1613. [t] Yielding, K. L.; Blodgett, L. W.; Sternglanz, H.; Gaudin, D. *Mol. Subcell. Biol.* **1971**, *2*, 69. [u] Waring, M. J.; Gonsalez, A.; Jimenez, A.; Vazquez, D. *Nucleic Acids. Res.* **1979**, *7*, 217. [v] Jones, R. L.; Wilson, W. D. *J. Am. Chem. Soc.* **1980**, *102*, 7776. [w] Hayashi, K.; Nagao, M.; Sugimura, T. *Nucleic Acids Res.* **1977**, *4*, 3679. [x] Müller, M.; Bunemann, H.; Dattagupta, N. *Eur. J. Biochem.* **1975**, *54*, 279. [y] Kohn, K. W.; Waring, M. J.; Glaubiger, D.; Friedman, G. A. *Cancer Res.* **1975**, *35*, 71. [z] Jacquemin-Sablon, H.; Le Bret, M.; Jacquemin-Sablon, A.; Paoletti, C. *Biochemistry* **1979**, *18*, 128. [aa] Bontemps, J.; Fredericq, E. *Biophys. Chem.* **1974**, *2*, 1. [bb] Pauluhn, J.; Zimmermann, H. W. *Ber. Bunsenges. Phys. Chem.* **1979**, *83*, 76.

binders; i.e., they do not intercalate, but the geometry of the binding is unknown. Thus, it is possible that some groove binders may be in this category.

In Table I are summarized the NMR results for over 70 different drugs, along with additional literature data, when available, on their DNA-binding properties. In each case the drug has been classified according to mode of binding, kinetics and base-pair specificity when possible.

Discussion

Having presented an overview of the various types of spectral behavior to be expected, we now discuss the experimental data for specific drugs and comment on the various drug-binding properties that may be deduced from the NMR measurements carried out at 35 °C and 0.11 M ionic strength. Unless otherwise noted, all kinetics refer to results at 35 ± 1 °C.

AMSA Derivatives. The largest number of derivatives of a single drug type studied are those (**2–17**) of 9-acridinylmethanesulfonanilide (AMSA, **2**).[63] This compound is derived from 9-aminoacridine (**1**) by addition of an anilino side chain at the 9 position. This group of compounds was of interest because a very large number of them have been synthesized, and the effect of substituents on their antileukemic activity has been accurately determined.[55,64] One member of this group of compounds (**5**) is currently in widespread clinical use, especially for the treatment of leukemias and lymphomas.[65]

Low-field spectra of the DNA titrated with several of these drugs are shown in Figure 5 and illustrate the range of spectral effects observed within this one group. On the basis of the NMR results, all but two of the drugs are classified as intercalators, consistent with results from unwinding assays,[66] but the kinetics vary from slow to fast. Specificity of binding, where determined (see Table I), is for G·C base pairs. For drugs near fast exchange (**2**, **5**, and **6**), large upfield shifts are observed, while others (**3**, **4**, and **7**) produce only small upfield shifts of the low-field resonances at the same ratios of drug/DNA. Because the chromophore is the same for all the drugs in this series, relative magnitudes of the chemical shifts can be justifiably compared.

It is of interest to compare the results of the biological studies with the NMR results obtained here. The parent AMSA (**2**) is near fast exchange with the DNA and causes large (0.4–0.5 ppm at 1 drug/8.3 base pairs) upfield shifts of both the A·T and G·C resonances. Similar results are observed for compounds **5** and **6**, which have a OCH_3 and a CH_3 substituent, respectively, on the 3′ position of the anilino ring, although the shifts for **5** are somewhat smaller. In contrast, compound **7**, which has a more bulky 3′-isopropyloxy substituent, causes relatively small upfield shifts (~0.2 ppm at a drug/base pair ratio of 1:8.3). The antileukemic activity of these compounds is sensitive to group size, falling off virtually to zero for the 3′-O-*i*-Pr compound.[55]

Compounds **3** and **4**, in which the 1′-$NHSO_2CH_3$ group is moved to positions 2′ and 3′, respectively, shift the low-field resonances even less than **7**, with maximum shifts of 0.13 and 0.04 ppm, respectively, being observed. (Due to the insolubility of these compounds, maximum drug levels were probably only 1:12.5.) Movement of the 1′-

(63) Cain, B. F.; Atwell, G. J.; Denny, W. A. *J. Med. Chem.* **1975**, *18*, 1110.
(64) Baguley, B. C.; Denny, W. A.; Atwell, G. J.; Cain, B. F. *J. Med. Chem.* **1981**, *24*, 520.
(65) Arlin, Z. A.; Sklaroff, R. B.; Gee, T. S. *Cancer Res.* **1980**, *40*, 3304.
(66) Waring, M. J. *Eur. J. Cancer* **1976**, *12*, 995.

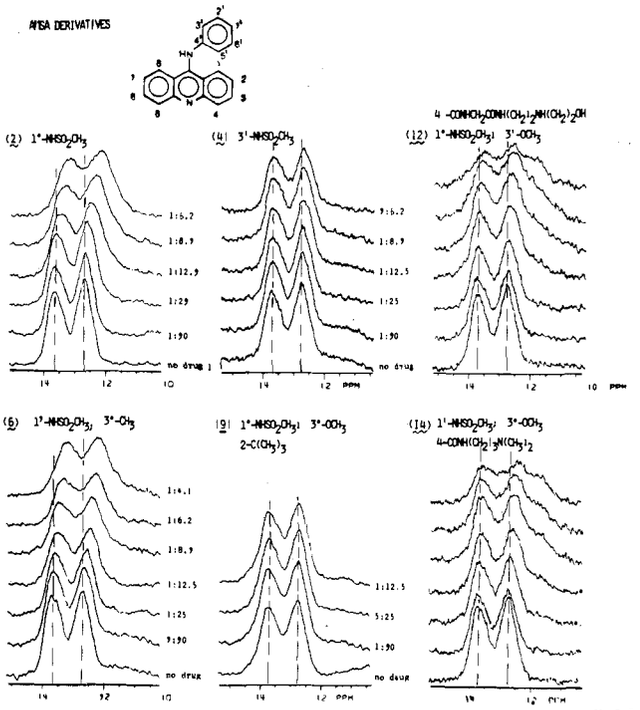


Figure 5. Effect of various AMSA derivatives on the low-field spectra of DNA at 35 °C. The drug/DNA base-pair ratios are given with the spectra. The parent AMSA (**2**), and derivative (**6**) bind by intercalation and are in intermediate to fast exchange. Derivative **4** intercalates, but only to a very small extent, and derivative **9** binds on the outside only. Derivatives **12** and **14** bind by intercalation, are in slow exchange with the DNA, and show G·C specificity. For instrumental conditions, see Figure 1.

$NHSO_2CH_3$ group to other positions on the anilino ring also dramatically decreases biological activity, with the 2′-$NHSO_2CH_3$ compound (**3**) being much less active than **2**, and the 3′-$NHSO_2CH_3$ compound (**4**) being inactive.[55]

The disubstituted 3′,5′-$(OCH_3)_2$ derivative (**8**) appears not to intercalate at all, since no effect on the low-field resonances was observed, up to the highest ratio obtained (1:12.5). This compound is biologically inactive. Thus, of compounds with varying substituents on the anilino ring (**2–8**), only the three (**2**, **5**, and **6**) that give large upfield shifts show much biological activity. The substituents on compounds **3**, **4**, **7**, and **8** apparently introduce steric restrictions that inhibit intercalative binding.

The 2-*t*-Bu-AMSA (**9**) derivative also does not appear to intercalate and is devoid of antileukemic activity, consistent with other derivatives where steric bulk is placed at the 2-position of the acridine ring.[55]

Early quantitative structure–activity work demonstrated that the biological activity of AMSA derivatives was closely related to their DNA association constants, with the more tightly binding drugs being the most active.[54,67] However, such equations did not account very well for the dramatic loss of activity that accompanied attachment of bulky groups in the 2-position, since the binding constants of these derivatives remain substantial. The present work supports the view that the loss of activity shown by compounds such as **8** and **9** is due not to a lack of binding to DNA but to a change in the mode of binding from intercalation to binding of the drug in some undefined way on the outside of the helix.

Compounds **10–14** are derivatives of **5** (amsacrine) with various carboxamido side chains at the 4-position of the acridine ring. The 4-$CONH_2$ derivative (**10**) shows kinetics similar to AMSA at 36 °C, but all the other derivatives (**11–14**) are in slow exchange with the DNA. The charged side chains of compounds **12–14** enable these dicationic molecules to bind very tightly to the DNA, but it is noteworthy that a second charge in the side chain is not necessary for the molecule to exhibit slow exchange, as the glycinamide derivative (**11**) also appears to be slow (although insolubility limited the drug/base pair ratio to 1:12). The remaining three derivatives in this series (**15–17**) bear a charged group in place of the 1′-$NHSOCH_3$. Compound **16** also has a 3-NO_2. All bind by intercalation, with large upfield shifts, but the kinetics are more difficult to classify. The charged substituent seems to have a small effect on the residence times, slowing the kinetics somewhat.

Tilorone and Anthracenedione Derivatives. Compounds **19–27** in Table I are analogues of tilorone (**18**), a dibasic compound that has been widely studied as an experimental antitumor and antiviral agent and as a small-molecule inducer of interferon.[68,69] Tilorone itself binds tightly to DNA by intercalation, with an unwinding angle of 13°[13] (Table I), but it is unusual among intercalating drugs in that it shows a strong preference for A·T-rich regions[68] and that no charge residues on the chromophore. The NMR results on a related anthracenedione derivative, RMI 10024 (**26**), are shown in Figure 1.

Anthracenediones **28–31** are similar in structure to the tilorone analogues but bear alkylamino side chains at the 1- and 4-positions. This class of compounds was initially studied as simpler analogues of the antitumor anthracycline antibiotics[70] and as a class are strongly active in animal tumor systems. Two derivatives, ametantrone (**28**) and mitoxantrone (**30**), are in clinical trial.[71] The presence of the side chains at positions 1 and 4 vs. 2 and 7 on tilorone would presumably result in both side chains being in one groove of the DNA, as has been suggested for daunomycin.

The NMR results indicate that all of the tilorone and anthracenedione derivatives bind by intercalation, in spite of the long side chains at various positions around the ring. This was of interest, since unwinding angles for many of these derivatives have not yet been determined. It was also of considerable interest to see if the previously determined A·T specificity of tilorone (and its derivatives) would be confirmed. Unfortunately, none of the derivatives are clearly in the fast or slow exchange limit; moreover, the relatively small chemical shifts observed make classification of kinetics tentative. Higher temperature spectra of several of these analogues (**18**, **21**, **25**, **26**, **28**, and **30**) bound to DNA unexpectedly showed a decrease in the A·T peak relative to the G·C peak, although the G·C peak shifted only slightly further upfield (unpublished results). This suggests a more complicated interaction than presented here. A low-temperature (9 °C) spectrum of **23** at a drug/base pair ratio of 1:6.2 clearly shows a shoulder of upfield-shifted resonances at ~12.1 ppm (unpublished results). This is consistent both with the small shifts observed for this series (presumably due to the interruption of the conjugated ring system in the center of the molecule) and with a marked A·T specificity of the drug.

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It is also consistent with the rather odd spectral behavior observed for some of these derivatives (**19**, **26**, and **31**) at high drug/DNA levels, where the A·T and G·C peaks merge into one broad envelope. Relative chemical shifts of the A·T and G·C resonance envelopes for the derivatives in intermediate to fast exchange also are consistent with A·T specificity, with the exception of tilorone.

Proflavines. The proflavine analogues (**33–37**) constitute an interesting series of compounds, where the mode of binding to DNA changes across the series with increasing size of the substituent groups at the 2- and 7-positions. Proflavine itself (**33**) has a low but definite in vivo antitumor activity.[54] The 2,7-dimethyl analogue, acridine yellow (**34**), also shows some antitumor activity, but the other molecules in the series are inactive in vivo. The interaction of proflavine with DNA has been widely studied and appears to be complicated kinetically;[38,42,72,73] at moderate drug/DNA ratios under most salt conditions, the strong binding is by intercalation, and there is a small G·C specificity. Di-*tert*-butylproflavine (**37**), with two bulky *tert*-butyl groups on the chromophore, binds not by intercalation but on the outside of the DNA and is A·T specific.[74]

The NMR results indicate that proflavine and the dimethyl, diethyl, and the diisopropyl derivatives all bind to some extent by intercalation. The kinetics appear to be intermediate to fast. Fairly large upfield shifts are observed for proflavine itself, although they are somewhat smaller than those for **1**, from which it is derived by addition of NH_2 at the 3- and 6-positions. Upfield shifts decrease along the series, with the diethyl and dimethyl shifts being about the same and only very small shifts observed for the diisopropyl derivative. Di-*tert*-butylproflavine has no effect on the G·C peak up to the highest level (1:12.5) tested, but some intensity from the A·T peak is shifted to *lower* field. This is consistent with its classification as an outside binder by other methods and gives evidence that it binds in a groove of the DNA. The diisopropyl derivative actually exhibits behavior that indicates it may bind both by intercalation and on the outside, since the low-field edge of the A·T peak also moves downfield. Thus, it appears that these molecules can bind either by intercalation or on the outside of the DNA, presumably in a groove, with intercalation being the more predominant mode for derivatives with small substituents and virtually impossible for the bulky *tert*-butyl derivative.

Subsequent to the NMR experiments, all of the derivatives were tested for the ability to unwind closed-circular DNA. Compounds **33–35** showed similar unwinding angles indicative of intercalation, while **36** and **37** did not unwind the DNA at all.[54]

Ethidium Derivatives. The ethidium derivatives (**38–41**) all bind by intercalation, as determined by previous studies and by the upfield shifts they produce on the DNA resonances.[61] The three ethidium derivatives (**38–40**) that possess a 3-NH_2 group are in slow exchange with the DNA at 35 °C, whereas the derivative (**41**) lacking the 3-NH_2 group is in fast exchange. This observation is in agreement with other studies that suggest that formation of a hydrogen bond between the 3-NH_2 group and the DNA makes an important contribution to the stability of the ligand–DNA complex[38] and provides a possible explanation for the fact that 3-NH_2-substituted ethidium derivatives are much more potent inhibitors of DNA polymerase in vitro than analogues lacking the 3-NH_2 group.

Pyridoindoles. The pyrido[3,4-*b*]indole derivatives (**42–45**) were studied as simpler analogues of the important ellipticine series of antitumor alkaloids. Norharman (**42**) occurs naturally in some foods and possesses a wide spectrum of biological activities, including antifungal, antihistaminic, and hypotensive behavior. It is also a potent inhibitor of DNA repair enzymes, monoamine oxidase, acetylcholinesterase, and mixed function oxidases.[75] Although there have been conflicting reports[76,77] about the binding of norharman to DNA, the NMR results clearly indicate that the protonated form binds by intercalation and is in fast exchange with the DNA at 35 °C (see Table I). The NMR results on the other three pyrido[3,4-*b*]indole derivatives (**43–45**) all show fairly large upfield shifts of the low-field resonances, indicative of the intercalative binding in the fast-exchange limit. The NMR results from binding of methylharmane (**44**) to DNA are illustrated in Figure 1. The relative G·C/A·T shifts indicate marked G·C specificity for all of these drugs.

Ellipticines. Compounds **46–48** represent the important class of ellipticine alkaloids, which have been extensively studied as both experimental and clinical antitumor agents by LePecq and co-workers.[78] Ellipticine itself (**46**) shows high activity in several in vivo animal tumor systems, and 9-hydroxyellipticine (**47**) was identified early as a primary metabolite with greater toxicity than the parent. Both 9-hydroxy- and 9-methoxyellipticine have a wider spectrum of action than the parent, and the latter drug has been used successfully against human leukemia.[78] Ellipticine and analogues bind to DNA by intercalation, and studies have shown a positive correlation between the DNA binding constant and cytotoxicity for a series of derivatives. Quaternization of the 2-nitrogen of 9-hydroxyellipticine gives **48**, which has a wider spectrum of cytotoxicity, as well as greatly decreased bacterial mutagenic activity.[79] These desirable properties have resulted in a great deal of interest in **48** as an antitumor agent, and a number of clinical trials are now in progress.

All three ellipticine derivatives give NMR results that indicate that they bind by intercalation. Relatively small overall shifts are observed, but a temperature study of ellipticine with the DNA clearly indicates that these molecules are in intermediate to slow exchange, in contrast to the pyrido[3,4-*b*]indoles. Unlike the other drugs found so far to have slow exchange kinetics, the ellipticines do not have a bulky side chain off the chromophore that might help to stabilize the intercalated complex.

Chloroquines. The antimalarial drugs chloroquine (**49**) and mepacrine (quinacrine, **50**) were included in this study, although they have no antitumor activity, to compare the NMR results with the many other studies of the binding of these drugs to DNA. Chloroquine has a rather low affinity for DNA (log K = 4.52 at 0.02 M salt), even at neutral pH where the drug exists as the diprotonated

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species.[80] There has been some doubt over whether the quinoline ring of chloroquine is large enough to favor binding by intercalation, and other modes of binding in the major groove have been proposed. Waring found that the results in the closed-circular DNA assay were consistent with intercalative binding but noted that the low binding affinity made an unequivocal assignment difficult.[9] The NMR results for chloroquine are illustrated in Figure 1 and show clearly that chloroquine binds by intercalation and is in fast exchange with the DNA. Although upfield shifts are fairly small, especially for A·T resonances, there is no broadening of the resonance lines as in other more ambiguous cases, and the relative A·T/G·C shifts indicate a marked G·C specificity. Since an appreciable fraction of the drug may not be bound even at the high concentrations employed, the size of the upfield shift clearly confirms that the major binding mode for chloroquine is by intercalation, with the drug undergoing fast exchange. The antimalarial mepacrine (**50**) is similar in structure to chloroquine, but with the addition of another aromatic ring to the chromophore. The resulting acridine chromophore increases DNA binding by favoring intercalation; mepacrine unwinds closed-circular supercoiled DNA with an unwinding angle of 17°.[81] In the NMR it shows essentially the same behavior as chloroquine.

Hycanthone and Mitonafide. Hycanthone (**51**) has a considerably different structure from many of the compounds examined, with an uncharged thioxanthenone chromophore bearing a side chain off one of the terminal rings. It is currently the drug of choice for the treatment of schistosomiasis, in spite of documented hepatotoxicity.[82] It is also an active antitumor agent in animal systems and a radiation potentiator. In spite of the fact that the thioxanthenone chromophore carries no positive charge, hycanthone binds fairly tightly to DNA by intercalation, with an unwinding angle of 15°.[9] It shows intercalative behavior in the NMR, similar to that of mepacrine; thus, the presence of a charge on the chromophore is not mandatory for efficient intercalative binding. This is well demonstrated also by the experimental antitumor drug mitonafide (**52**), which is effective against several animal tumor lines both in vivo and in vitro and is a potent selective inhibitor of nucleic acid synthesis.[83] Mitonafide binds tightly to DNA (log K = 5.16 at 0.01 M salt) by intercalation, with an unwinding angle of 11° and an apparent site size of 1.7 base pairs. The intercalative mode of binding is confirmed by the NMR results shown in Figure 1. The drug is in slow exchange with the DNA, with a large upfield-shifted G·C peak appearing at ~11.7 ppm. The results indicate marked G·C specificity. The slow kinetics are a surprising result in view of the small uncharged chromophore and may help to explain the potent inhibition of nucleic acid synthesis this molecule demonstrates; the unexpectedly high DNA-binding affinity and slow-binding kinetics of mitonafide make this drug type worthy of further developmental studies.

Daunomycin and Actinomycin D. The antitumor antibiotics daunomycin (**53**) and actinomycin D (**54**) are among the most useful of the clinical drugs[84] and the most well-characterized as far as their interaction with DNA is concerned.[85,86] Both bind tightly by intercalation.[9] The binding of actinomycin D involves intercalation of the phenoxazone chromophore, followed by slow rearrangement of the pentapeptide side chains.[26,87] Both compounds show strong G·C specificity,[88] with specific interactions proposed between the drugs and the 2-NH_2 group of the guanine.[1] The NMR spectra of actinomycin D bound to DNA, shown in Figure 1, confirm the slow kinetics and very marked G·C specificity described above. In addition, a small downfield shift of the low-field edge of the A·T peak was observed, perhaps due to binding of the side chains in a groove. Daunomycin (**53**) is also an intercalator in slow exchange, but the spectrum is complicated by the appearance of low-field drug resonances.

Imidazodiquinolinium Cation. The imidazolodiquinolinium compound (**55**) is an antiparasitic and anthelmintic agent in both animals and humans (French patent M3438; British patent 1 011 311) that shows an exceptionally high fluorescence quantum yield, which is dramatically reduced in the presence of DNA (W. Leupin, unpublished results). The binding constant is 5×10^4 M^{-1} at 0.11 M NaCl. An unwinding angle of 14° was determined by the reduced viscosity technique. This compound caused the largest upfield shifts of the low-field resonances of all the drugs studied, presumably due to the large ring-current shielding capability of the extended chromophore. It shows no base-pair specificity and is at or near fast exchange.

Neutral Red Derivative. 4-Methyl-5-phenyl-Neutral Red (compound **56**) was of interest because it shows the highest G·C specificity of a large number of 5-phenylphenazinium analogues examined by Müller et al.[34] They recorded a binding constant (log K) of 3.64 for the binding of **56** to *Bacillus subtilis* DNA at 0.19 M salt. The phenylphenazinium compounds were assumed to form intercalated complexes, although no corroborating evidence was offered.

The NMR results confirm the assumption that the molecule intercalates into the DNA, but relatively small spectral effects are observed, and they level off at a drug/base-pair ratio of 1:12.5. The kinetics appear to be intermediate to fast, and there is a marked preferential effect on the G·C envelope, consistent with the G·C specificity found by Müller et al.[34]

Bisantrene. The anthracene 9,10-dihydrazone (**57**, bisantrene) is a member of a relatively new class of antitumor agents; the example shown possesses antitumor activity against leukemia and melanoma in mouse systems and is currently in clinical trial.[89,90] The molecule gives NMR results consistent with binding by intercalation in slow exchange (see Figure 1). There is a very marked decrease in the A·T peak relative to the G·C peak, and a small peak of upfield-shifted intensity appears at ~11.2 ppm. This molecule may thus be preferentially binding near A·T base pairs. However, because of the nature of the side chains and the fact that they might be interacting with the DNA in a way that would affect the chemical

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shifts or intensity of the low-field resonances, a temperature study of the spectra at a 1 drug to 6.2 base-pair ratio was done. The relative differences in the A·T and G·C peaks persist up to 53 °C; most likely, the molecule is still in slow exchange with the DNA at this temperature. Confirmation of the A·T specificity will have to await further study; but an unwinding assay using the reduced viscosity method gave an unwinding angle of 14°, in agreement with the NMR determination of an intercalative binding mode.

Distamycin. The polypyrrole antibiotic distamycin A (**58**) has significant antiviral and antibacterial activity[91] and is an effective inhibitor of DNA polymerase. It binds tightly to DNA, and much interest has centered on the geometry of this binding. A number of different studies have concluded that distamycin A and the related antibiotic netropsin bind on the outside of the helix, specifically by lodgement in the minor groove.[15,45,92] The effect of distamycin on the low-field spectra of DNA is illustrated in Figure 2. At low drug/DNA ratios, the A·T envelope is mainly affected, consistent with the high selectivity shown by distamycin A for A·T-rich sites.[15,93] At higher drug/DNA ratios, the available favored binding sites (at least three contiguous A·T base pairs) should be saturated, and the G·C envelope begins to be affected. Both resonance envelopes shift downfield with a particular growth of resonance intensity ~0.55-ppm downfield of the A·T envelope. Thus, the NMR results are consistent with a proposed mode of binding in the minor groove and high A·T specificity. Binding of the related antibiotic netropsin to the self-complementary DNAs d(GGTTAACC) and d(CGCGAATTCGCG) has been studied by Patel et al.;[45,46] they observed a downfield shift of 0.5 ppm for one of the A·T imino proton resonances and concluded that the drug was in slow exchange with the DNA at 5 °C under his experimental conditions. Although the drugs cannot be directly compared, since distamycin A lacks the second cationic charge of netropsin, it appears that the two drugs are binding with similar kinetics and geometry.

The dramatic effects seen on the low-field spectra of DNA when distamycin is bound clearly indicate that the molecule is in slow exchange with the DNA. However, for most of the outside binders studied, we were unable to determine binding kinetics because the observed shifts are small.

Aromatic Bis(guanylhydrazone)s. Distamycin A is one of a large number of compounds of generally similar topology: linear, almost coplanar molecules with cationic charges at one or both ends, possessing a wise spectrum of biological activities. Further examples of this type of compound are the aromatic bis(guanylhydrazone)s (**59–61**). DDUG [**59**, 4,4′-diacetyldiphenylurea bis(guanylhydrazone)]. DDUG shows high potency against the L1210 leukemia in vivo[94] and is a potent inhibitor of DNA-dependent DNA polymerase in vitro,[16,17] a property that has been related to its DNA-binding properties.[95] DDUG binds preferentially to A·T sites and does not unwind closed-circular supercoiled DNA; thus, it has been classed as a minor groove binder. The insolubility of DDUG limited the drug/DNA base-pair level that could be studied to >1:25. Small but significant downfield shifts of both the A·T and G·C envelopes were observed, consistent with its classification as a groove binder, although there appeared to be no preference for A·T sites. Somewhat higher drug levels were obtained with the related SN20440 (**60**), and in this case the A·T peak was preferentially affected. The G·C peak did not shift, but a slight narrowing of the overall line width was noted. SN20452 (**61**) also had a solubility limitation of 1:12.5 drugs per base pair. The A·T peak was preferentially affected with a slight downfield shift, and there was little or no effect on the G·C peak. Because of the insolubility of these compounds, some of the DNA may have precipitated out with the drug at higher levels, so that the effects seen may not actually be representative of the total effect. Nevertheless, the results at least allow all three of these drugs to be classified as groove binders.

Stilbamidine. Stilbamidine (**62**) and the closely related berenil are widely used antiprotozoal and antitrypanosomal drugs.[96] The latter compound was shown by Waring not to unwind closed-circular supercoiled DNA,[9] although it binds tightly to DNA. Stilbamidine gave spectral results similar to those seen for SN20440 (**60**), with a slight narrowing of the overall G·C peak line width, but the effect on the A·T envelope was too small to classify it definitely as a groove binder. Thus, we are not able to decide if this class of rather short linear molecules are minor groove binders or attach themselves less specifically by Coulomb interactions only.

Phthalanilides. Compounds **63** and **64** are representatives of the large class of phthalanilides, which were made and tested during the 1960's after the observation by Hirt and Berchtold that the compounds had a broad spectrum of activity against murine tumor models.[97] The compounds bind tightly to DNA without unwinding closed-circular supercoiled DNA and have been classified as minor groove binders.[14] Three members of the class have been evaluated for antitumor activity in man, and some therapeutic responses were seen, but clinical toxicity was severe.[98] Insolubility limited the drug/DNA level that could be studied, and the spectral changes observed were small but consistent with binding of the molecules in a groove of the DNA.

Bisquaternary Ammonium Heterocycles. The bisquaternary ammonium heterocycles (**65–73**) are representatives of the large group of these compounds made and tested by Cain and co-workers as potential antitumor agents.[2] Like the phthalanilides that they resemble in structural type, these compounds show a broad spectrum of activity against animal tumor models and are selective inhibitors of bacteriophage production. They bind tightly to DNA without intercalation and have been proposed to bind in the minor groove.[14] Like all such compounds, the bisquaternary ammonium heterocycles bind preferentially to A·T sites, but the degree of this preference varies widely. A quantitative structure–activity relationship study of the antitumor activity of 176 active derivatives of this class showed that activity was related not simply to the level of DNA binding of a compound but to the degree of specificity showed for A·T over G·C sites [defined as the ratios of the binding to poly(dG-dC) and poly(dA-dT)].[2] Compounds **65–67** are examples of early members of this class of drugs. The *p*- and *m*-pyridinium analogues show high

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activity against leukemia in vivo but show delayed toxicity.[99] In contrast, the o-pyridinium analogues (e.g., **67**) are not tumor active, although they have similar physical properties. In the NMR, all three derivatives had similar effects, giving results typical of groove binders.

Derivatives **68** and **69**, bearing different charged groups at each end, were made as conceptual bridges between the bisquaternary ammonium heterocycles and, respectively, the phthalanilides and bis(guanylhydrazone)s. They are highly active against the L1210 leukemia, and the level of this activity is well-fitted by the derived equation.[2] No evaluation of their toxicity has been made. These drugs were very insoluble. Only very small changes were observed for compound **68**, but they were consistent with binding in a groove. Even smaller changes were observed for compound **69**, and it is tentatively assigned as a groove binder, although the distinction between groove and nonspecific outside binding for this drug is not unambiguous.

Methylquinolinium Compounds. Compounds **70–73** are representative of the large series of bisquaternary ammonium heterocycles made and tested by Cain and coworkers[2,99] as potential antitumor agents. They bind tightly to DNA without intercalation, and have been proposed to bind in the minor groove.[14] Like all such compounds, the bisquaternary ammonium heterocycles bind preferentially to A·T sites, but the degree of this preference varied widely. A quantitative structure–activity relationship study of the antitumor activity of 176 active derivatives of this class showed that activity was related not simply to the level of DNA binding of a compound but to the degree of specificity showed for A·T over G·C sites [defined as the ratios of the binding to poly(dG-C) and poly(A-dT)].[2] Compound **71** (NSC176319, quinolinium dibromide) has completed extensive preclinical pharmacology and toxicology testing at the National Cancer Institute. In the NMR, compounds **70–73** all showed behavior characteristic of minor groove binders. The NMR results obtained with SN6999 (**70**) are shown in Figure 2.

Although the spectral changes observed varied somewhat among these derivatives, with compounds **70** and **71** showing the most downfield-shifted intensity and compounds **72** and **73** showing the least, the changes were not large enough to draw any significant conclusions. These compounds (**70–73**) were of some interest in this study, since from the structure it was not obvious if the drugs should intercalate or bind on the outside.

Conclusions

The study of the general class of drugs known as the DNA adductors or DNA binding agents is important in delineating the forces involved in the binding of these drugs to DNA, the resulting geometry and kinetics of the interaction, and the relation of these properties to the therapeutic effectiveness of the drugs as cytotoxic agents.

Mode of Binding. In the work described in this paper, the use of readily available chicken erythrocyte DNA allowed a large number of different compounds to be studied, representative of all the major classes of antitumor drugs. This DNA represents a more biologically relevant system than defined-sequence synthetic DNAs for broadly surveying biologically active compounds. Most compounds studied fit clearly into one of the three spectral categories illustrated in Figures 3 and 4, and it is pleasing that in all cases where independent tests have been performed, the NMR results which indicated binding by intercalation were consistent with results found in the literature. The fact that the NMR method can distinguish between molecules that bind by intercalation and those that bind on the outside of the DNA is clearly illustrated by such molecules as the di-*tert*-butylproflavine derivative (**37**) and 2-*tert*-butylamsacrine (**9**). Although these chromophores normally bind by intercalation, the bulky *tert*-butyl group apparently places steric restrictions on intercalative binding. These molecules have been independently found not to unwind closed-circular supercoiled DNA, although their binding constants to DNA are substantial and were, thus, classed as outside binders.[55,74] The NMR results indicate that both drugs bind on the outside of the DNA, with the di-*tert*-butylproflavine giving results typical of groove binding.

The NMR results did provide some information about compounds whose binding mode was previously unknown or in doubt. Thus, all the anthracenedione derivatives (**28–32**) show behavior indicative of intercalative binding, as does norharman (**42**) and the other pyridoindoles (**43–45**). The antihelmintic imidazolodiquinolinium derivative (**55**), the phenylphenazinium derivative (**56**), and the antitumor drug bisantrene (**57**) are shown here to bind by intercalation, as would be expected from their chemical structure.

From the compounds now examined by the NMR technique and by the unwinding assays, it is clear that the possession of three or more fused benzenoid rings virtually assures that the compound will bind primarily by intercalation of that chromophore, in the absence of bulky substituents that prevent intercalactive binding. For compounds with only two fused rings, this is less certain; thus, chloroquine (**49**) and the bisquaternary ammonium heterocycle derivatives (**70–73**) both possess the 4-aminoquinoline moiety, but the former intercalates while the latter bind in a groove of the DNA.

The existence of a relationship between the mode of binding and antitumor activity for a series of drugs is clearly illustrated by the AMSA derivatives. Those derivatives that caused little or no upfield shift of the low-field resonances also showed little or no antitumor activity.[55] The substituents in compounds **3**, **4**, **7**, and **8** apparently placed steric restrictions on intercalative binding; the resulting loss of antitumor activity implies that for this series, intercalative binding is required for activity.

Kinetics. The average residence time of a ligand at a particular site on the DNA is a property that may have some importance in determining the cytotoxicity of DNA-binding drugs, and for intercalating drugs, the NMR method makes it possible to place limits on the lifetime of a drug in the intercalation site. If distinct new resonances are seen for the imino protons at the drug-binding site, the molecule is said to be in slow exchange between available sites on the NMR time scale; at 300 MHz and with a shift of about 1 ppm, this implies an average residence time at a site of appreciably more than a millisecond. If only an averaged resonance position is seen on drug binding (fast exchange), the average drug residence time at a particular site is appreciably less than a millisecond. It is interesting that, under our conditions (0.11 M NaCl, 35 °C), differences in kinetic behavior for different drugs are seen (Table I). While the structural features required for a ligand to bind to DNA by intercalation seem fairly clear, the results in Table I make it obvious that the features determining the kinetics of the binding of such ligands to DNA are less well defined. It is reasonable that the attachment of cationic side chains to an intercalating chromophore might increase residence time just by providing additional Coulombic interactions,

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but our results indicate that the effect depends on the position, length, and formula of the side chain. For the amsacrine derivatives, attachment of charged side chains off the 4-position, which places the chromophore between the anilino ring and the charged side chain, results in drugs with long residence times (11–14) for all but the derivative with the shortest side chain (10). In contrast, charged side chains at the 1′-position of the anilino ring have a much smaller effect on the kinetics. The disubstituted tilorones and anthracenediones show a range of kinetics that vary with the position and formula of the side chain. Some singly charged compounds can have slow kinetics, as shown by the ethidium derivatives (38–41). Removal of the 3-NH_2 group to give 41 abolishes a presumptive H bond in the DNA complex and results in faster kinetics for this molecule compared to the other three. Most other singly charged molecules are in or near fast exchange, such as the proflavine derivatives (33–36), the imidazolodiquinolinium cation (55), the phenylphenazinium compound (56), hycanthone (51), and the pyridoindoles (42–45). However, addition of another ring to the latter to give the related ellipticine alkaloids results in compounds (46–48) with slower kinetics, without additional charges or H-bonding functionality. Mitonafide (52) represents a surprising example of a small chromophore with only a singly charged side chain that, nevertheless, is in slow exchange under the experimental conditions, whereas the doubly charged chloroquine (49) and mepacrine (50) are in fast exchange. Finally, actinomycin D (54), which is uncharged but possesses two complex pentapeptide side chains, is the classical example of a ligand with slow exchange kinetics.[87]

Although little can be said at this time about relationships between the structure of drugs, their observed kinetics, and their biological activity, the NMR work presented here represents a step toward remedying this deficiency.

If the cytotoxicity of DNA-intercalating drugs is a result of their ability to block polymerase enzymes during replication and transcription[26] and to induce strand breaks,[100] then, as noted in the introduction, drugs with long residence times at a DNA site might show enhanced cytotoxicity and antitumor activity. While this is certainly a very simplistic viewpoint, it is interesting that for the wide variety of DNA-intercalating drugs looked at in this study, the results support it. The clinically useful drugs looked at in this study, the results support it. The clinically useful drugs daunomycin (53), actinomycin D (54), and the ellipticines (46–48) all show slow exchange kinetics at 35 °C, as do the promising new drugs bisantrene (57), mitonafide (52), and the anthracenediones (28–31), although for the latter the kinetics are less clear but appear to be intermediate to slow. Compounds known to have little or no antitumor activity, e.g., 9-aminoacridine (1), the tilorones (18–27), the pyridoindoles (42–45), the proflavines (33–37), and the antimalarials chloroquine (49) and mepacrine (50), are in fast exchange. It is particularly striking that in closely related compounds, such as the pyridoindoles (42–45) vs. the ellipticines (46–48), and the 1,4-disubstituted tilorone analogue (27) vs. the anthracenediones (28–31), structural changes that result in a slowing of the exchange kinetics generate antitumor activity.

The correspondence is not complete among the drugs studied. The ethidium analogues with the 3-NH_2 group are in slow exchange but do not show marked antitumor activity, although ethidium itself was sufficiently active in animal tests to warrant a phase I clinical trial at one point. (There is, however, a correlation between antitrypanosomal activity of ethidium derivatives and slow kinetics.[61]) Conversely hycanthone, although in fast exchange, does possess significant antitumor activity in animals, although this has not been substantiated in humans. Amsacrine (5) also has substantial clinical activity but is in intermediate exchange under our experimental conditions. The general conclusion from this study is that drug/DNA binding kinetics are an important determinant of biological activity in this type of molecule and need to be considered along with other parameters, such as hydrophobicity, metabolic stability, and pharmacology, in the design of new agents.

Base-Pair Specificity. For the intercalators, in the limit of fast or slow exchange, a specificity for binding to G·C vs. A·T sites can be determined. The reliability of this method is limited by the fact that for fast exchange the assumption of equal shifts for the A·T and G·C resonances next to a bound drug has to be made, and for the drugs in slow exchange, the overlapping of the upfield-shifted G·C resonances with the unshifted resonance makes integration of the peaks difficult. Nevertheless, the conclusions about relative base-pair specificity derived from the NMR data were consistent with the literature, except in the case of tilorone. No simple correlation between base-pair specificity and antitumor activity was noted. Positive correlations between degree of A·T specificity and antitumor activity have been made for many of the groove binders.[2,95] These drugs also show a specific effect on the A·T resonances in the NMR study, but in general the effects were too small to determine if one drug was much more A·T specific than the other.

Summary

The random-sequence DNA–drug binding studies described in this paper give important qualitative information on drug binding. In order to obtain details of the geometry of binding of drugs to specific sites on the DNA, it will be necessary to use short DNA duplexes of defined sequence. However, the random-sequence DNA system has some distinct advantages: (1) it provides an inexpensive and quick screen of mode of binding, kinetics, and base-pair specificity; (2) an averaged effect of binding to all possible sites rather than to one or more specific sites is observed, and this may be more biologically relevant; and (3) a wide range of temperatures can be studied. Finally, this system provides an excellent method for selecting specific drugs for study with synthetic DNA duplexes.

The kinetic information determined by this method show that, at least for the intercalating compounds, cytotoxicity and useful antitumor activity are associated with long drug/DNA residence times. This provides a reason for the previously puzzling observation that while many of the most useful antitumor drugs are DNA intercalators, there exist many ligands that bind tightly to DNA by intercalation, yet do not possess cytotoxic activity.

Experimental Section

Preparation of DNA. Gram quantities of nucleosome-length (~150 base pairs) DNA were prepared from chicken erythrocytes as described elsewhere.[52] This DNA was then further digested to fragments of 15–100 base pairs in length with S_1 and DNase II nucleases, by modification of the method described by Early and Kearns.[53] In a typical preparation, ~1.5 g of nucleosome-length DNA was dissolved in ~20 mL of digestion buffer (0.2 M sodium acetate, pH 5.0, with acetic acid, 2 mM $ZnCl_2$). The solution was placed in a dialysis bag in a vacuum dialysis setup, and the DNA was dialyzed vs. the digestion buffer prior to and

(100) Ross, W. F.; Glaubiger, D.; Kohn, K. W. *Biochim. Biophys. Acta* **1979**, *562*, 41.

during the digestion. The reaction was initiated with the addition of 50 μL (40 units/μL) of nuclease S_1, followed by 300 μL of DNase II (10 mg/mL, 22 units/μL); additional aliquots of DNase II were added as needed during the course of the digestion (200 μL at t = 1 and 3 h). The progress of the reaction was monitored by ^{1}H NMR. As the digestion proceeds, the intensity of the low-field spectrum initially increases and then begins to decrease while the resolution (peak-to-valley ratio) increases. The reaction was quenched with phenol after ~8 h when the resolution of the spectra indicated that DNA of appropriately short length had been obtained.[53] The DNA solution was removed from the dialysis bag, and 0.5 vol of buffer-saturated phenol plus 0.5 vol of chloroform/isoamyl alcohol, 24:1, were added. This mixture was stirred for several minutes and then centrifuged to separate the phenol and water layers. The water layer was extracted once with the phenol and chloroform mixture and then with the 24:1 chloroform/isoamyl alcohol. The DNA in the water layer was ethanol precipitated and placed in the −5 °C freezer overnight. The ethanol precipitate was centrifuged, and the pellet was lyophilized and then redissolved and dialyzed exhaustively vs. NMR buffer (0.01 M sodium cacodylate, pH 7.0, 0.1 M NaCl, 0.01 M $MgCl_2$). This buffer was chosen in order to aid in removal of Zn^{2+} ions, to stabilize the lower molecular weight DNA against denaturation, and to favor intercalation over outside binding in the drug studies. After dialysis, the DNA was again ethanol precipitated, lyophilized, and stored frozen until use.

Average size and distribution of the DNA was determined by polyacrylamide gel electrophoresis and by ^{31}P NMR (ratio of terminal-to-interior phosphates). For a typical preparation at least 90% of the DNA was between 100 to 15 base-pairs long, with a median molecular length (weight average) of ~35 base pairs. The number average molecular length (from ratio of internal-to-terminal phosphates determined by ^{31}P NMR) was ~25 base pairs. Almost all material less than 15 base-pairs long is fractionated out by ethanol precipitation and dialysis.

Drugs. Materials purchased from commercial sources were used without purification after checking sample composition on TLC. Compounds not obtained from commercial sources are noted below. The structures of the parent compounds for the drugs studied are shown in Chart I. The numbers given below the compounds refer to Table I, where the complete set of drugs investigated is listed. Daunomycin (**53**), actinomycin D (**54**), hycanthone (**51**), mitoxantrone (**30**), and the anthracene derivative (**57**) were gifts from the Warner-Lambert Co., together with their proprietary compounds (**28**, **31**, and **55**). Tilorone and analogues (**18–27**) were a gift from Richardson-Merrell Inc. through Dr. William L. Albrecht. DDUG and analgoues (**59–61**) were a gift from Dr. E. Mihich, Roswell Park. Ellipticine and derivatives (**46–48**) were a gift from Professor J.-B. LePecq. NSC278467 (**29**) was obtained from the NCI through the courtesy of Dr. C. C. Cheng, and the phthalanilide analogues (**63** and **64**) were from the same source by courtesy of Professor Corwin Hansch. 8-Deaminoethidium (**49**) was a gift from Professor H. W. Zimmermann. The alkyl proflavines (**33–37**),[54] the 9-anilinoacridine derivatives (**2–17**), and the bisquaternary ammonium heterocycles (**65–73**) were prepared in the Cancer Chemotherapy Research Laboratory, University of Auckland School of Medicine.[55] Mitonafide (**52**), 3-deaminoethidium (**41**), 4-methyl-5-phenyl-Neutral Red (**56**), and the quaternary pyridoindoles (**43–45**) were prepared in the University of California laboratory by known procedures[56–59] and had physical constants in agreement with the literature cited.

Unwinding angles for several of the drugs were measured in the Cancer Chemotherapy Research Laboratory following the procedure of Revet et al.[60] The viscometer had a bore size of 0.4 mm and a flow time for distilled water of 92.6 s. It was filled with 1.3 mL of a dust-free solution of PM2 DNA (150–250 μM in phosphate) in 0.01 M buffer (9.4 mM NaCl, 2 mM, Na^+–Hepes, pH 7.0, and 20 μM EDTA). Reagents were added from a microsyringe as concentrated (200–860 μM) solutions in water. Flow times were measured 2–4 times for each addition, and the reduced viscosities were calculated by standard methods. The data reported are triplicate determinations. Experience with other derivatives indicates a standard deviation on determinations of ±4%.

NMR Spectra. NMR spectra were obtained with a Varian HR 300 ^{1}H NMR spectrometer modified to operate as a correlation spectrometer. A 41 mM (in base pairs) solution of DNA (120 μL) in 0.1 M NaCl, 0.01 M Mg, and 0.01 M sodium cacodylate buffer at pH 7.0 was placed in a Wilmad microcell. Many of the drugs were relatively insoluble in water (solubility was often markedly increased in the presence of DNA, especially for the intercalating drugs) making it difficult to prepare sufficiently concentrated drug solutions to add to the DNA samples without serious dilution factors. Thus, bulk solutions of a drug of known concentration were made up in aqueous ethanol, and the appropriate amounts were pipetted out into 0.5-mL Eppendorf tubes. The solvents were evaporated under a stream of dry nitrogen. A series of samples was prepared for each drug, to correspond to a cumulative ratio of 1:50, 1:25, 1:12.5, 1:8.3, 1:6.2, and 1:4.1 drug molecules per base pair when dissolved in the 120-μL DNA sample. After the spectrum of the free DNA sample was recorded, the sample was removed from the microcell, placed in the Eppendorf tube, and vortexed or, if necessary, mixed with a small glass rod until all the drug was dissolved. The sample was then returned to the NMR microcell, and the spectrum was remeasured. Transfer of the sample to the successive tubes containing drug allowed determination of the spectrum for a series of drug/DNA ratios without serious loss or dilution of the DNA, and thus without the need to apply correction factors. Originally all NMR spectra were collected at a temperature of 34–36 °C. This relatively high temperature was chosen in order to keep the resonance envelopes as sharp as possible and also to work close to the physiological temperature at which the drugs express their biological activity. As an additional check on binding kinetics, the effect of temperature on at least one drug from each major class was studied at a drug/DNA base pairs ratio of 1:6.2.

Binding constants for many of the drugs studied have been determined (in other laboratories) by a number of different methods, and under the low salt conditions used in the current experiments (0.11 M ionic strength) these binding constants lie in the range $10–10^7$ M^{-1} (see Table I). Thus, at the DNA concentrations used (41 mM), essentially all of the drug will be bound, and, it is, therefore, permissible to compare results obtained at a fixed ratio of added drug to DNA.

Acknowledgment. This work was supported by the American Cancer Society (Grant CH32 to D.R.K.). W.L. was supported by a fellowship from the Swiss National Science Foundation. W.A.D. acknowledges support from the Auckland Division of the Cancer Society of New Zealand and the Medical Research Council of New Zealand. The authors thank Dr. Simon Twigden for determining unwinding angles for several compounds.

Registry No. **1**, 90-45-9; **2**, 53478-38-9; **3**, 64894-99-1; **4**, 80260-78-2; **5**, 51264-14-3; **6**, 57164-89-3; **7**, 88412-52-6; **8**, 86955-71-7; **9**, 88476-66-8; **10**, 76708-54-8; **11**, 76708-63-9; **12**, 88476-67-9; **13**, 88476-68-0; **14**, 88476-69-1; **15**, 80259-83-2; **16**, 80261-23-0; **17**, 80259-91-2; **18**, 27591-97-5; **19**, 88476-70-4; **20**, 88476-71-5; **21**, 88476-72-6; **22**, 57993-51-8; **23**, 88476-73-7; **24**, 88476-74-8; **25**, 88476-75-9; **26**, 35697-33-7; **27**, 88476-76-0; **28**, 64862-96-0; **29**, 65271-79-6; **30**, 65271-80-9; **31**, 1614-59-1; **33**, 92-62-6; **34**, 92-26-2; **35**, 83951-93-3; **36**, 83951-94-4; **37**, 33244-11-0; **38**, 3546-21-2; **39**, 20566-69-2; **40**, 74444-52-3; **41**, 74444-53-4; **42**, 244-63-3; **43**, 37689-96-6; **44**, 37689-97-7; **45**, 88476-77-1; **46**, 519-23-3; **47**, 51131-85-2; **48**, 58337-34-1; **49**, 54-05-7; **50**, 83-89-6; **51**, 3105-97-3; **52**, 54824-17-8; **53**, 20830-81-3; **54**, 50-76-0; **55**, 75769-83-4; **56**, 88476-78-2; **57**, 78186-34-2; **58**, 636-47-5; **59**, 13284-07-6; **60**, 1945-65-9; **61**, 23140-11-6; **62**, 122-06-5; **63**, 13202-07-8; **64**, 5697-54-1; **65**, 88476-79-3; **66**, 88476-80-6; **67**, 20719-27-1; **68**, 88495-90-3; **69**, 88495-91-4; **70**, 68772-09-8; **71**, 88495-92-5; **72**, 88495-93-6; **73**, 88495-94-7.