Kinetic and Equilibrium Binding Studies of Amsacrine-4-carboxamides: A Class of Asymmetrical DNA-Intercalating Agents Which Bind by Threading through the DNA Helix

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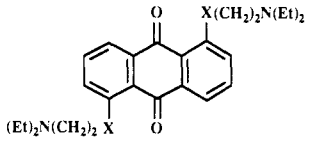
Detailed equilibrium and kinetic studies of the DNA interaction of the amsacrine-4-carboxamide class of compounds suggest that they bind by intercalating the acridine chromophore at near-maximal overlap with the base pairs, locating their two dissimilar side chains in specific grooves of the double helix. The first step is a fast bimolecular association to form an outside-bound complex (probably in the major groove). Insertion of the less bulky carboxamide side chain then occurs in a process governed largely by the rate of transient opening of the double helix by natural "breathing" motions and is followed by further monomolecular rearrangements to allow the carboxamide side chain to find its highest affinity binding sites in the minor groove. Dissociation of the complexes are much more ligand structure dependent, but also involve opening of the double helix to allow disengagement. Compounds of this type, which locate their two distinguishable side chains one in each DNA groove, form a unique class of DNA-binding ligand, with considerable potential for regiospecific delivery of reactive functionality to DNA. Although natural products which also have such specific binding modes are known (e.g. nogalamycin), the amsacrine-4-carboxamides discussed here are the first class of readily modified synthetic compounds with this property.

Ligands which bind to DNA by intercalation are of interest, both as probes for studies of ligand/DNA binding[1,2] and because of their antibacterial, cytotoxic, and antitumor activity.[3] The most active antitumor agents of this class contain additional side chains on the intercalating ligand, and it has been suggested[1,2,4] that the important effect of such side chains is to reduce the rate of dissociation of the ligand-DNA complex, thereby increasing the residence time of the ligand at particular binding sites.

There has been much discussion concerning the location of these ligand side chains in the DNA complex, but in the majority of cases these appear to lie in the minor rather than the major groove. Molecules bearing two side chains off a single intercalating chromophore have similar options, with both side chains able to lie in the same DNA groove (major or minor). However, a third and more interesting possible binding mode exists for these ligands, where one side chain lies in each groove with the molecule thus "threaded" through the DNA helix. Because of the activation energy necessary to thread one of the chains through the helix, this orientation is likely to be kinetically unfavored and only important when it allows considerably greater chromophore overlap with the DNA intercalation site than any alternative binding mode.

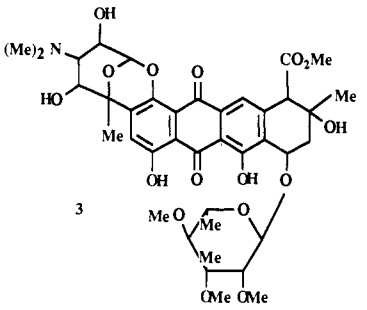
Several symmetrical DNA-binding ligands with the required topology are known, for example 1,5-disubstituted anthraquinone diether **1**. This compound has been shown



1: X = O

2: X = NH

by high-field NMR to bind by intercalation,[2] and to do so it must place one chain in each groove. Molecular modeling studies of the closely related anthraquinone **2** showed that only this binding mode was energetically favorable.[5] A series of naphthalenediimides was also shown to bind to DNA by intercalation,[6] and these must reside similarly with one side chain in each groove. A recent NMR study[7] of the unsymmetrical antitumor antibiotic nogalamycin (**3**) bound to the oligonucleotide d(GCATGC)$_2$ has shown



that very specific binding can occur. Nogalamycin binds by threading through the helix, with the nogalose sugar side chain located in the minor groove and the other fused bicyclic side chain in the major groove.

This "threaded" binding mode is of interest for two reasons. Firstly, dissociation of such ligand-DNA complexes necessitates passing one of the side chains through the base-pair stack, which will result in slower rates of dissociation. Since there is increasing evidence that this property may contribute to the antitumor activity of DNA-intercalating agents,[4,8] this may be a useful drug-design strategy. Secondly, compounds with dissimilar side chains which locate in specific DNA grooves (such as nogalamycin) have potential as "carriers" for the regiospecific delivery of reactive functionality to DNA.[3] However, the complexity of this natural antibiotic makes structural

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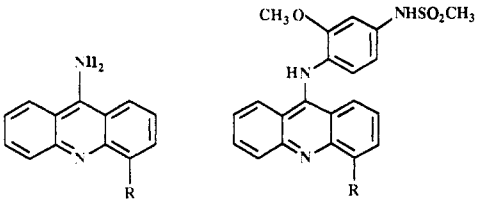
Table I. Structures and Spectral Characteristics



4,5 6-14

compd	R	free drug[a] max	free drug[a] $10^{-3}\epsilon$	DNA-bound drug[b] max	DNA-bound drug[b] $10^{-3}\epsilon$	micelle-bound drug[c] max	micelle-bound drug[c] $10^{-3}\epsilon$
4	H	399	10.1	406	5.0	402	10.2
5	$CONH(CH_2)_2N(CH_3)_2$	407	9.4	421	4.9	412	9.4
6	H	434	12.1	439	8.8	437	12.2
7	$CONHCH_3$	438	11.6	457	2.4	449	12.6
8	$CONH(CH_2)_2OH$	441	10.4	457	7.9	450	12.2
9	$CONH(CH_2)_3OH$	439	10.9	456	8.3	450	12.8
10	$CONHCH_2CH(OH)CH_2OH$	425	10.4	455	7.4	448	11.4
11	$CONH(OH_2)_2N(CH_3)_2$	442	12.9	462	8.8	449	13.9
12	$CONH(CH_2)_2NH(CH_2)_2OH$	441	11.6	460	8.2	450	12.8
13	$CONH(CH_2)_3N(CH_3)_2$	441	11.6	458	8.5	449	13.3
14	$CONHCH_2CONH(CH_2)_2NH(CH_2)_2OH$	440	12.3	460	8.8	450	14.3

[a] 50 μM drug in 0.1 SHE buffer (see the text). [b] 50 μM drug and 1 mM DNA (base pairs) in 0.1 SHE. [c] 50 μM drug, 1 mM DNA (base pairs) and 10 mM SDS in 0.1 M SHE.

modifications to the ligand very difficult, while virtually all of the simpler synthetic "DNA-threading" agents studied so far are symmetric compounds with identical side chains which cannot possess the desired specificity.

We report here on a class of synthetic compounds (amsacrine-4-carboxamides **7–14**) which are potential asymmetric DNA-threading agents suitable (by virtue of their synthetic availability) as regiospecific "carriers". These agents are composites of two previously well-studied groups of DNA-intercalating ligands, represented by the parent compounds amsacrine (**6**) and 9-aminoacridine-4-carboxamide **5**. We present here detailed equilibrium and kinetic studies of their interaction with DNA, aimed at elucidating their mode of binding.

Results

Equilibrium Binding Studies. The spectroscopic data recorded in Table I for the equilibrium binding of the ligands to sonicated calf thymus DNA show that in all cases the expected bathochromic and hypochromic shifts of the free drug spectrum observed on addition of DNA could be completely reversed by the addition of 10 mM of SDS, indicating complete sequestration of the ligand by the detergent micelles. While the ϵ values for the free ligand and ligand in the presence of SDS were identical for amsacrine (**6**), all the compounds containing a 4-carboxamide group (**5**, **7–14**) showed significantly (average 15%) higher ϵ values in the presence of SDS. Such increases are usually attributed to partial self-association of the free drug in solution. 9-Aminoacridine itself (**4**) binds strongly to DNA, showing no selectivity for different sites (Table II). The bulky side chain of amsacrine lowers DNA binding but does not alter selectivity. However, the compounds (**7–10**) containing a neutral 4-carboxamide side chain not only show increased levels of binding but also considerable selectivity for GC sites, in the case of diol **10** by a factor of nearly 14-fold. Dicationic 9-aminoacridine-4-carboxamide **5** showed greatly increased DNA binding as expected, but it also had significant GC selectivity. The highest level of binding was shown by compound **11**, which is the closest composite of the two parent compounds (**5** and **6**), although this compound did not show the levels of GC selectivity of amsacrinediol **10**.

Table II. Equilibrium Binding Parameters and Antitumor Activity

compd	binding constant[a] ($k(o) \times 10^{-5}$ M^{-1}) dA-dT	binding constant[a] ($k(o) \times 10^{-5}$ M^{-1}) dG-dC	angle, deg	NMR shift (GC)[c]
4	12	14	17	1.4
5	224	500	16	–
6	2.9	2.6	20.5 ± 0.4	1.06
7	3.5	15.1	17.5	1.50
8	8.9	24.0		–
9	9.5	81.2		–
10	25.1	371	15	–
10	245	660	25	ca. 1.0
12	219	490		–
13	23.4	39.8	21	ca. 1.0
14	282	661	21	ca. 1.05

[a] Association constants for binding either to poly(dA-dT) or poly(dG-dC) in 0.01 SHE buffer, measured by the ethidium displacement method (ref 20). [b] DNA-unwinding angle (degrees), measured using *E. coli* pN2 116 DNA (see the text). [c] Maximum shift, in parts per million, of the center of mass of the GC imino proton envelopes (data from ref 2).

Unwinding angles were determined for representative compounds, and the results (Table II) suggest that all compounds bind by intercalation as expected. The unwinding angle for amsacrine (**6**) has been previously reported[9] as 20.5°, and that[10] for 9-aminoacridine-4-carboxamide **5** was 16°. The composite compounds (**11** and **14**) have unwinding angles closer to that of amsacrine, indicating some participation by the bulky side chain in the unwinding process.

For a series of compounds such as this, with an identical intercalating chromophore, a comparative measure of the chromophore/DNA base pair stacking interactions (and thus the degree of overlap and chromophore orientation) can be obtained from the drug-induced perturbations of the DNA base pair imino protons by high-field NMR. Such studies have been reported[2] for several of the compounds of Table I (**7**, **11**, **13**, and **14**), and all these com-

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(10) Atwell, G. J.; Cain, B. F.; Baguley, B. C.; Finlay, G. J.; Denny, W. A. *J. Med. Chem.* **1984**, *27*, 1481.

Table III. Association Kinetic Parameters for the Compounds of Table I

compd	time constants,[a] ms			relative % amplitudes[b]			F^c
	1	2	3	A1	A2	A3	
4	too fast to measure						
5	4	19	–	52	48	–	7
6	too fast to measure						
7	2	7	–	52	48	–	54
8	4	31	100	14	64	22	90
9	4	32	95	9	66	25	85
10	8	40	110	20	40	40	77
11	5	24	110	41	43	16	62
12	3	18	66	30	40	30	69
13	3	15	66	31	39	30	79
14	5	25	93	31	33	36	66

[a] Time constants describing the association profile of calf thymus DNA–drug complexes in 0.1 SHE buffer at 20 °C. Final [DNA] = 200 μM (base pairs) and final [drug] = 10 μM. Measurements were made at the wavelengths shown in Table I. [b] Amplitudes of the processes characterized by the corresponding time constants, expressed as a percentage of the sum of the amplitudes evaluated in the kinetic experiment. [c] The fraction of the observed equilibrium absorbance change (on addition of DNA to the free drug) accounted for in the kinetic analysis.

Table IV. Dissociation Kinetic Parameters for the Compounds of Table I

compd	time constants,[a] ms				relative % amplitudes[b]				F^c
	T1	T2	T3	T4	A1	A2	A3	A4	
4	3	15			15	12			50
5	6	28	86	428	15	33	34	18	89
6	2	6	–	–	75	25	–	–	48
7	11	29	–	–	45	55	–	–	79
8	81	208	–	–	45	55	–	–	80
9	58	157	–	–	35	65	–	–	72
10	14	103	328	1830	4	35	49	12	88
11	65	490	2100	6100	7	15	37	41	97
12	79	278	1000	2900	4	14	37	45	100
13	26	230	570	–	7	55	38	–	92
14	28	190	960	2900	5	10	45	40	100

[a] Time constants describing the dissociation profile of calf thymus DNA–drug complexes in 0.1 SHE buffer at 2.0 °C. Final [DNA] = 200 μM (base pairs), final [drug] = 10 μM, and final [SDS monomer] = 10 mM. Measurements were made at the wavelengths shown in Table I. [b] As for footnote b, Table III. [c] The fraction of the observed equilibrium absorbance change (on addition of SDS to the drug–DNA complex) accounted for in the kinetic analysis. The total equilibrium absorbance changes were in the range 0.07–0.12. The errors in the values of the time constants and amplitudes are estimated to be ±20%.

pounds induced upfield shifts of the imino protons of similar magnitude (ca. 1 ppm) to the maximum induced shift calculated[2,11] for both 9-aminoacridine (**4**) and amsacrine (**6**).

Association Kinetics. Kinetics of association to calf thymus DNA were measured for all the compounds at a ligand/nucleotide pair ratio of 1:20 and a DNA concentration of 200 μM (Table III). Deconvolution of the absorbance trace was carried out by curve stripping,[12] with data from at least six independent kinetic runs being analyzed separately and the results averaged. To check whether all the association process was observed, the total absorbance change in the kinetic experiments was compared to that found under equilibrium conditions. The rates of association of compounds **4–6** were too fast to measure, and only about half of the equilibrium absorbance change was seen (resolvable into two exponentials with time constants of 2 and 7 s) for 4-methylcarboxamide **7**. However, for the remainder of the compounds (**8–14**), essentially all of the association processes were in the stopped-flow time range (Table III). All the compounds showed very similar kinetic profiles, with three exponentials of time constants about 10, 30, and 90 s. The association reaction was repeated for each compound at a variety of DNA concentrations, keeping the ligand/DNA ratio constant at 0.05, but the kinetic parameters were in each case found to be essentially independent of DNA concentration over at least a 3-fold and (for compound **13**) over a 6-fold range (data not shown).

For the three compounds (**11**, **12**, and **14**) displaying the slowest kinetics, association rates were also studied for a range of ligand/DNA ratios (Table V). In each case there was no change in the number of resolvable exponentials or their relative amplitudes, but the magnitudes of the time constants increased essentially linearly with the ligand/DNA ratio.

Finally, association kinetics were determined for the binding of compound **11** to a variety of DNAs of different base composition (Table VI). The process with poly(dG-dC) was broadly similar to that with the random-sequence calf thymus DNA, showing three time constants of similar

Table V. Variation of On and Off Rates with D/P Ratio[a]

On Rates

compd	D/P	time constants,[b] ms			relative % amplitudes[c]			F^d
		T1	T2	T3	A1	A2	A3	
11	0.05	5	24	110	41	43	16	62
	0.10	8	35	130	41	46	13	72
	0.20	12	53	300	39	48	13	60
12	0.05	3	18	66	30	40	30	69
	0.10	7	36	130	40	49	11	68
	0.20	16	60	300	44	48	8	60
14	0.05	5	25	93	31	33	36	66
	0.10	8	37	100	33	30	37	74
	0.20	10	71	220	39	48	13	65

Off Rates

compd	D/P	time constants,[b] ms				relative % amplitudes[c]				F^d
		T1	T2	T3	T4	A1	A2	A3	A4	
11	0.05	65	490	2100	6100	7	15	37	41	97
	0.2	63	434	2160	6170	6	20	43	31	95

[a] Drug/DNA base pair ratio in final solution. [b–d] See footnotes a–c of Tables III and IV.

Table VI. Kinetic Behavior of Complexes of Compound 11 with Synthetic DNAs

Association

DNA	time constants,[a] ms			relative % amplitudes[b]			F^c
	T1	T2	T3	A1	A2	A3	
CT	5	24	107	41	43	16	62
(dG-dC)	6	16	58	25	30	45	76
(dA-dT)	3	16		64	36		25
(dI-dC)	5	20		69	31		28

Dissociation

DNA	time constants,[a] ms				relative % amplitudes[b]				F^c
	T1	T2	T3	T4	A1	A2	A3	A4	
CT	65	490	2100	6100	7	15	37	41	97
(dG-dC)	50	1640	3500		2	13	87		99
(dA-dT)	7	38			19	81			70
(dI-dC)	12	73	193		5	17	78		85

[a–c] See footnotes a–c of Tables III and IV.

(11) Denny, W. A.; Atwell, G. J.; Baguley, B. C. *J. Med. Chem.* **1983**, *26*, 1625.
(12) Roos, I. A. G.; Wakelin, L. P. G.; Hakkennes, Coles, J. *Anal. Biochem.* **1985**, *146*, 287.

magnitude (although somewhat different relative amplitudes). However, association to both poly(dA-dT) and poly(dI-dC) was much more rapid, with complete loss of the slowest exponential.

Dissociation Kinetics. Kinetic measurements were made at or near the wavelength of maximum drug absorption of the drug in SDS solution, the exact position corresponding to the maximum difference between the DNA-bound and SDS-micelle-bound drug. Checks were carried out to show that dissociation rates for typical compounds were independent of SDS concentration, and there were no time-resolvable changes seen when free-drug solutions were mixed with SDS. Thus we infer that the transient absorbance changes measured with drug–DNA complexes are due solely to dissociation of the complex. As reported previously,[8] the rate of dissociation of amsacrine (**6**) was so fast that only about 50% of the process occurred in the stopped-flow time range, with much of the absorbance change occurring in less than 1 ms. However, for all the other compounds studied the amplitudes for the resolved exponential decays summed to at least 85% of the equilibrium absorbance change, indicating that virtually all the dissociation processes were observed.

Table IV shows the rates of SDS-induced dissociation of the compounds from random-sequence DNA (calf thymus), when initially bound at a ratio of one ligand molecule to 20 base pairs. 4-Methylcarboxamide **7** had slightly slower dissociation kinetics than amsacrine, the process being characterized by two time constants of 11 and 29 ms. The more complex neutral side chains of 8 and **9** resulted in additional slowing of the dissociation kinetics of these molecules, but again only two processes with approximately equal amplitudes were observed In contrast, carboxamide diol **10** showed a much slower and more complex dissociation behavior, with four resolvable exponential processes (or families of processes) of relative amplitudes (A1–A4) of approximately 1:9:12:3, with the longest time constant being 1.83 s. This behavior is similar to that of 9-aminoacridine-4-carboxamide **5**, which also showed[4] four processes of relative amplitudes 1:3:3:1.

However, the slowest dissociation kinetics were shown by compound **11**, which has both the bulky side chain of amsacrine and the cationic side chain of 9-aminoacridine-4-carboxamide. This compound showed much slower overall dissociation kinetics than either parent compound (the reciprocal harmonic mean time constant $1/t^*$ for **11** is 0.3 s^{-1}, compared to 330 s^{-1} for **6** and 9 s^{-1} for **5**). In addition, the slowest process, with a time constant of 6.1 s, had the largest amplitude (41% of the total). Extension of the cationic side chain by one methylene unit to give **13** resulted in faster overall kinetics but, more interestingly, in complete loss of the slowest transient T4. The dissociation of **11** from calf thymus DNA was also studied at a ligand/DNA ratio of 0.2 (Table V), but the results were essentially identical, indicating that the dissociation is independent of binding ratio.

The dissociation of compound **11** from DNAs of varying base composition was also studied (Table VI) and (like the association process) showed significant variation with base composition. Dissociation from pol(dG-dC) occurred in a similar time range to that from calf thymus DNA, although the longest lived transient T4 was lost. In contrast, dissociation from poly(dA-dT) was a very rapid, two-component process, and that from poly(dI-dC) was also much faster than from random-sequence DNA.

Discussion

The amsacrine-4-carboxamides have the correct topology to be "DNA-threading" agents, in that maximal overlap between the acridine chromophore and the DNA base pairs (the major driving force for intercalative binding) can only take place if the ligand is oriented with one side chain in each DNA groove. It is accepted that 9-aminoacridine itself, which has few other constraints on its binding mode, intercalates with maximal overlap.[13] NMR studies show that, on binding, 9-aminoacridine causes a 1.0–1.4 ppm upfield shift of the imino proton resonances belonging to the base pairs on either side of the intercalation site, with the magnitude of this "ring current shift" being a characteristic of both the chemistry and the binding orientation of the chromophore.[2] Amsacrine itself (**6**) elicits a similar shift of ca. 1 ppm. The fact that representative amsacrine-4-carboxamides (**7**, **11**, **13**, and **14**) have both unwinding angles and imino proton shifts of similar magnitude to those of amsacrine (Table II) suggests that the acridine chromophore of these compounds intercalates with similar geometry, with its long axis parallel to the base pair long axis. In this case, the compounds must bind by "threading" through the double helix, with one side chain lying in each groove.

To understand the mechanism by which the ligands attain this binding orientation, the kinetics of their association with DNA were studied as a function of DNA concentration, with a constant ligand/base pair ratio of 1:20 (Table III). The association of the two parent compounds (**5** and **6**) bearing single side chains was very fast, with essentially all the absorbance changes occurring within the dead time of the instrument, since for each compound intercalation can occur by direct insertion of the chromophore. For the amsacrine-4-carboxamides (**8**–**14**) with bulky 4-side chains, the association was much slower, with a considerable proportion (70–90%) of the equilibrium absorbance changes observable in the stopped-flow time range. The observed time constants for these processes were independent of DNA concentration, indicating that they are due to monomolecular rearrangements of the ligand on the DNA. The remainder of the equilibrium absorbance change not accounted for in the kinetic experiments is assumed to be due to bimolecular association processes, which are known[8,14] to be very fast ($>10^6$ M^{-1} s^{-1}) for acridine derivatives.

The first step in the monomeric rearrangement of the amsacrine-4-carboxamide ligands on the DNA after the fast bimolecular association must involve insertion of one of the side chains through the helix, in order to allow the acridine to attain maximum overlap. For the methylcarboxamide with the smallest side chain (**7**), the rearrangement was still a very rapid, two-exponential process, suggesting that the compound intercalates via insertion of the carboxamide. The remainder of the compounds (**8**–**14**) bear much larger carboxamide side chains of quite variable structure and associate more slowly via a three-exponential process. The kinetic parameters of the association are essentially the same for all compounds, showing little dependence on side-chain structure. Although it is impossible to decide from this data which side chain is inserted, it seems likely to be the carboxamide side chain rather than the bulky and geometrically constrained anilino ring.

The effect of the ligand/DNA binding ratio on association kinetics was investigated for compounds **11**, **12**, and **14**, which bear carboxamide side chains of quite different structures. The relative amplitudes of the three resolvable processes varied little with binding ratio (Table V), sug-

(13) Seeman, N. C.; Day, R. O.; Rich, A. *Nature* **1975**, *253*, 324.
(14) Capelle, N.; Barbet, J.; Dessen, P.; Blanquet, S.; Roques, P. B.; Le Pecq, J-B. *Biochemistry* **1979**, *18*, 3354.

gesting that they were probably not the result of parallel processes, where ligand binding occurs independently to DNA sites of varying affinity. If this were the case, one would expect variations in the amplitudes (A1–A3), since at low binding ratios binding to the high-affinity sizes would dominate.[15] In contrast, the magnitudes of the time constants varied significantly with binding ratio. While the ratios T1:T2:T3 did not alter, being approximately 1:5:20 for all three compounds at all D/P ratios, the magnitudes of the time constants increased linearly with increasing D/P ratio. This suggests that the rate-determining step in the association process is determined by the ability of the DNA double helix to open sufficiently for the ligand-insertion process to occur. The slower kinetics at higher ligand/DNA ratios could then be due to a slowing down to these effects by externally bound drug "stiffening" the DNA or simply occluding potential insertion sites. The influence of the DNA structure is further seen in the data of Table VI, which shows that the kinetics of association of compound 11 to synthetic DNAs varies significantly with base composition. Association to poly(dG-dC) is not significantly different than to random-sequence DNA, but is much faster to both poly(dA-dT) and poly(dI-dC). These two polymers have much more rapid "breathing" motions than poly(dG-dC) because they have only two Watson–Crick H bonds per base pair and consequently provide more open sites for drug intercalation.

The dissociation kinetics of the amsacrine-4-carboxamides from DNA are also complex. Amsacrine itself (6) has been shown to exhibit very rapid dissociation kinetics, with only about half of the process being in the stopped-flow time range.[8] Two processes with time constants of 1.7 and 5.5 ms could be observed (Table IV). This is faster than the dissociation of the parent chromophore 9-aminoacridine (time constants 2.4 and 15 ms,[8] which runs counter to the usual observation that attachment of a bulky side chain slows the dissociation kinetics of a chromophore[1,16] and led to the suggestion[8] that amsacrine might bind with the methanesulfonanilide side chain in the major groove.

A detailed kinetic study[4] of the dissociation kinetics of a series of 9-aminoacridine-4-carboxamides including compound 5 showed the importance of the nature of the carboxamide side chain. Selectivity for GC binding sites and the presence of the fourth T4 transient in the kinetic profile (Table IV) were shown only by those compounds (e.g. 5) bearing a (dialkylamino)ethyl side chain. The additional binding stability conferred by this side chain was considered[4] to come primarily from the formation in the minor groove of a complex stabilized by two hydrogen bonds from the secondary amide and protonated dialkylamino hydrogens to the O2 group of a cytosine in one of the base pairs flanking the DNA intercalation site, although this view has recently been questioned.[17]

In the present study, amsacrine-4-carboxamides 7–9 showed dissociation kinetic profiles similar to that of amsacrine (6), with only two resolvable exponentials, albeit with considerably longer time constants. However, diol 10 showed a more complex profile, closer to that of 5, with four clearly resolvable processes. Although the longest of these accounted for only 12% of the total amplitude, its time constant of 1.83 s makes it readily detectable. The most slowly dissociating compound was (dimethylamino)ethyl derivative 11, which, as mentioned above, is the closest composite of the two parent compounds (5 and 6). For this compound the longest time constant, accounting for over 40% of the total absorbance change, 6.1 s. Hydroxyethyl derivative 12 shows an essentially similar profile, although with slightly smaller time constants, but extension of the side chain of 11 by one methylene unit to give 13 results in considerable destabilization of the complex, with the most striking effect being complete loss of the longest lived T4 transient. This is mirrored by an order-of-magnitude reduction in the equilibrium binding constant for this compound (Table II). These changes among compounds 11, 12, and 13 exactly parallel those seen among compound 5 and its corresponding side chain homologues.[4] This is compelling evidence that the amsacrine-4-carboxamides orient their carboxamide side chains in the DNA minor groove, in the same way as the 9-aminoacridine-4-carboxamides are thought to do.[4]

Studies carried out with compound 11 show that the dissociation is independent of ligand/DNA ratio (Table Vb), which is additional evidence (see above) that the process probably involves sequential rather than parallel steps, since parallel dissociation from binding sites of differing affinity would result in variation of the amplitudes with D/P ratio.[15]

Although the dissociation of the amsacrine-4-carboxamides is highly ligand-dependent (unlike their association), a study of the dissociation of compound 11 from DNAs of varying base composition (Table VI) shows that it is also dependent on DNA structure. As for the association kinetics (see above), the process is much faster for poly(dA-dT) and poly(dI-dC) than for random-sequence DNA and poly(dG-dC), suggesting that opening of the double helix to allow disengagement of one of the side chains is still a limiting step (although by no means the only one) in the dissociation process.

It is interesting to compare these kinetic data with those for the asymmetric DNA-threading ligand nogalamycin (3). The dissociation of this compound from poly(dG-dC) (under slightly different conditions: 5 μM nogalamycin and 150 μM DNA at 25 °C with an ionic strength of 0.1) can be described by a single exponential of time constant of 2.26 s and that from poly(dA-dT) by a two-exponential process with time constants 52 and 279 ms.[18] Even its dissociation from the more complex calf thymus DNA could be fitted by only three exponentials, with time constants 20, 228, and 1540 ms.[18] Thus, although it is a much more complex molecule, nogalamycin dissociates more rapidly from DNA than some of the amsacrine-carboxamides and shows somewhat similar dissociation profiles.

Conclusions

The data discussed above suggest that, at equilibrium, the amsacrine-4-carboxamides bind to DNA in a definite orientation, with the acridine in a position of maximum overlap with the DNA base pairs, the carboxamide side chain in the minor groove, and the bulky anilino chain in the major groove. The initial rapid bimolecular step (not observed in the stopped-flow) is most likely external binding of the drug in the major groove of the helix. The first slow step is then insertion of the carboxamide side chain through a transient opening in the double helix caused by the natural "breathing" motion of the DNA, with the acridine chromophore subsequently attaining near-maximal overlap and the carboxamide side chain binding in the minor groove. If there are favored DNA sequences

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for this minor groove binding for a particular side chain, it seems likely that a final (slower) process might then be "shuffling" of the drug by partial dissociation followed by reinsertion until the most favored minor groove binding sequences for the particular carboxamide side chain are found. Such a process is supported by the dissociation kinetic data (Table IV), which show clear relationships between the stability of the complexes and the nature of the carboxamide side chain (see above).

Although such kinetic data is only indirect evidence for the proposed binding mode, it is difficult to obtain more direct information. NMR studies of drug–oligonucleotide complexes depend on a level of kinetic stability of the complex not usually attained by simple (and therefore synthetically accessible) chromophores.

Compounds which bind to DNA by intercalation of a chromophore between the base pairs and location of two distinguishable side chains one in each DNA groove form a unique class of DNA binding ligand, with considerable potential as "carriers" for regiospecific delivery of reactive functionality to DNA. The amsacrine-4-carboxamides discussed here are a class of readily modified synthetic compounds which appear to have this property.

Experimental Section

General Procedures. The syntheses of amsacrine-4-carboxamides 7–14 have been reported.[19,20] The buffer used (designated 0.01 SHE) has been reported.[4] All ligands were dissolved in 0.01 SHE buffer at a concentration of 1 mM and stored at −20 °C. The molecular weight of calf thymus DNA was reduced to approximately 10^6 by sonicating solutions containing 0.2 mg/mL of DNA in 0.2 SHE buffer at 0 °C for 5 min on a Branson 150 sonicator. The sonicated DNA was exhaustively dialyzed into 0.01 SHE buffer, and solutions were filtered through Millipore 0.45-μm filters.

Equilibrium Measurements. Association constants for binding to poly(dA-dT) and poly(dG-dC) were determined by the ethidium displacement method and are corrected for quenching.[21] Unwinding angles were determined[22] with closed circular DNA from the *Escherichia coli* plasmid pNZ 116. Measurements were carried out at 25.0 ± 0.1 °C, and the helix unwinding angle was calculated as $26° \times r(e)/r(d)$, where $r(e)$ and $r(d)$ are respectively the bound drug/DNA phosphate molar ratios at the equivalence point for ethidium bromide and the compounds of Table I. Molar extinction coefficients at the wavelengths of maximum absorption in the visible spectrum were determined for the compounds free in solution, bound to DNA, and when sequestered into detergent micelles (sodium dodecyl sulfate, SDS), with a Cary 219 UV/visible spectrophotometer. All spectra were measured at a drug concentration of 50 μM in 0.1 SHE buffer, DNA- and micelle-bound spectra being determined in the presence of 1 mM calf thymus DNA and 10 mM SDS (monomer concentration), respectively.

Kinetic Measurements. These were performed with a Dionex D110 stopped-flow spectrophotometer coupled to a 64K Apple II microcomputer as described previously.[8,12] For dissociation kinetic studies, solutions of drug–DNA complex containing 400 μM calf thymus or synthetic DNA (in nucleotide pairs) and 20 μm of drug in 0.1 SHE buffer were mixed with an equal volume of SDS (20 mM monomer concentration) in the same buffer at 20 °C. For association kinetics studies, the mixing solutions were drug (20 mM) and DNA (400 μM in nucleotide pairs). The spectrometer was operated with a time constant of 1 ms and the optical bandwidth set to 3 nm.

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