Relationships between DNA-Binding Kinetics and Biological Activity for the 9-Aminoacridine-4-carboxamide Class of Antitumor Agents

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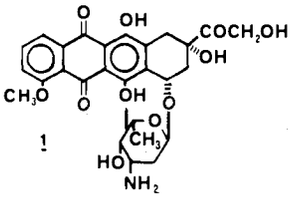
The kinetics of dissociation of calf thymus DNA complexes of the new intercalating antitumor drug *N*-[2-(dimethylamino)ethyl]-9-aminoacridine-4-carboxamide (**5**) and selected derivatives have been investigated by using the surfactant-sequestration method. The derivatives studied include those where the position (**14** and **15**) and nature of attachment (**20** and **21**) of the cationic side chain is modified, those where the distance (**16–19**) and composition (**22–24**) of the cationic group are varied, and those in which the chromophore is further substituted (**25–31**). While all of the compounds dissociate by a mechanism that involves at least three intermediate bound forms, derivatives bearing a 4-$CONH(CH_2)_2NR_1R_2$ side chain (where R_1 and R_2 are groups that permit the nitrogen to be protonated at neutral pH) have access to an additional binding mode of greater kinetic stability. A positive correlation is found between in vivo antitumor activity, selectivity of binding to GC-rich DNAs, and the presence of this fourth, long-lived transient species. We have interpreted our kinetic findings in terms of a molecular model for acridinecarboxamide–DNA complexes that accounts for the appearance of the fourth component. The acridine chromophore is postulated to intercalate from the narrow groove, its major axis lying at an angle to the major axis of the base pairs so that the CH atoms of positions 5 and 6 protrude into the groove. An important feature of the model is a bifurcated hydrogen bond between the O2 oxygen atom of a cytosine base adjacent to the binding site and the NH atoms of the carboxamide and protonated terminal amino functions of the drug molecule. Since the structural features required to form this bonding interaction are necessary, although not sufficient, conditions for in vivo antitumor activity, it is suggested that the model may describe the essential characteristics of the biologically active form of the bound drug. These findings further attest to the value of investigating the kinetics of DNA–drug interaction in studies of the mode of action of antitumor intercalating agents.

The class of drugs usually described as the "antitumor antibiotics", which includes adriamycin (**1**), actinomycin D (**2**), ellipticine (**3**), and their close analogues, has long been important in cancer chemotherapy. The origin of these compounds as natural products has perhaps obscured the fact that they also belong to the wider general category of DNA-intercalating agents. However, the recent development of a number of synthetic intercalating antitumor drugs has led to renewed interest in the mode of action of intercalators and their structural and physicochemical requirements for biological activity.



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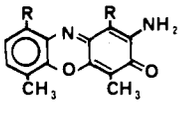

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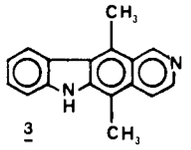

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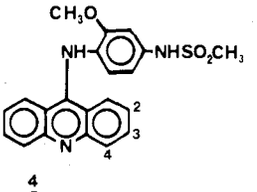

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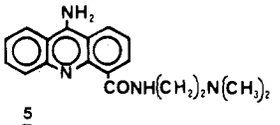

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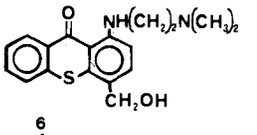

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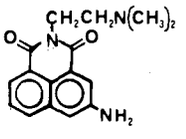

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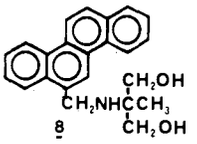

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Although intercalating agents have been well-characterized as inhibitors of the template functions of DNA,[1,2] it appears that their primary mode of cytotoxicity is related to the induction of DNA breaks.[3,4] It is not clear which of a number of different types of strand breaks are the more important, but a concensus favors the introduction of double-strand breaks as being the lethal event. The exact mechanisms by which intercalating agents induce such lesions is not yet understood. In whole-cell systems, the clinically used drugs adriamycin, amsacrine, and ellipticine cause a high proportion of protein-associated breaks, and it has been suggested that the mechanism involves trapping of a DNA–enzyme intermediate at a stage where the enzyme has already effected a strand break. Recent work[5,6] showing that amsacrine is a very potent inhibitor of the DNA nicking-closing enzyme topoisomerase II makes this an attractive putative target for intercalating agents. For amsacrine, the formation of a ternary DNA–drug–enzyme complex is postulated,[5] but the fact that this and the other two drugs mentioned readily undergo redox reactions and have been found[7,8] to break DNA efficiently in cell-free systems in the presence of oxygen, copper, and a reducing agent has also to be considered.

In terms of structure–activity relationships, a necessary requirement for in vivo antitumor activity among compounds of this class is that they bind to DNA by intercalation of a fused tricyclic aromatic moiety. Compounds that do not possess this necessary minimum surface area[9]

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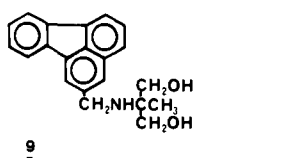
for effective intercalation are not biologically active. The importance of the intercalative mode of binding is emphasized in the amsacrine series of compounds (4) where introduction of a *tert*-butyl group at positions 2, 3, or 4 on the acridine nucleus is sufficient to prevent intercalation, and although these compounds still have high affinity for DNA and possess the appropriate lipophilicity, they are not active.[10] However, efficient intercalative binding in itself appears to be a necessary but not sufficient condition for in vivo antitumor activity, as the vast majority of DNA-intercalating chromophores (e.g., 9-aminoacridine and 1,4-diaminoanthraquinone) are inactive. A study of the recent literature shows that the DNA-intercalating agents currently under development as antitumor drugs share the common structural features of an aromatic nucleus to which is appended either one or two flexible cationic side chains. Of the former type, the 9-aminoacridine-4-carboxamides (5) and the hycanthone analogues (6)[11] are in laboratory development, and the benz[*de*]isoquinolinedione (7)[12] and the amino diol derivatives (8 and 9)[13] are in clincal trial, while in the latter group, bisantrene (10),[14] ametantrone (11),[15] mitoxantrone (12)[16] and anthrapyrazole (13)[17] are all in human trials.



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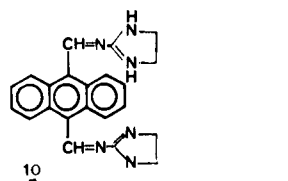

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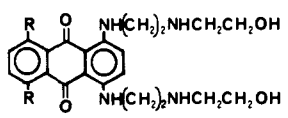
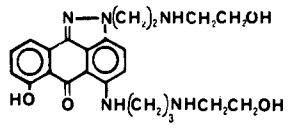


11, R = H
12, R = OH



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While the appended cationic functions in these compounds undoubtedly increase DNA-binding affinity, an equally important role may well be their effects on the kinetics of the drug–DNA interaction. Several studies[18-20] have highlighted the importance of the kinetic as well as the geometric aspects of drug–DNA complexes as a determinant of biological activity and have shown that, for intercalating agents, slow dissociation rates from DNA and long residence times at particular binding sites correlate with greater cytotoxic potency and in vivo antitumor activity.

We have recently reported preliminary structure–activity relationships for the 9-aminoacridine-4-carboxamide class of compounds[21,22] exemplified by the parent *N*-[2-(dimethylamino)ethyl]-9-aminoacridine-4-carboxamide (5). These reveal the critical nature of the position of the cationic side chain with respect to the intercalating chromophore for biological activity: shifting the link away from the 4-position or even lengthening the joining chain by one methylene unit is sufficient to abolish in vivo activity.[21] Concomitant with these changes in biological activity, the inactive compounds also show a greatly decreased ability to cause DNA breaks in cultured cells.[23] Further study of a limited series of monosubstituted acridine derivatives of 5 showed[22] that the in vivo activity varies more with the position of the substituent group on the acridine chromophore rather than with the nature (e.g., lipophilic, electronic) of the groups themselves, suggesting a predominantly steric effect. A number of 1-, 2-, 5-, and 6-substituted compounds are active, but only the 2- and 5-substituted derivatives have activity comparable to that of the parent compound 5. Apart from relatively small and predictable effects on overall lipophilicity, the substituents do not significantly alter the key physicochemical properties usually considered important for the biological activity of DNA-intercalating agents. Although some modification to the basicity of the acridine chromophore is to be expected, the pK_a of the parent compound 5 is sufficiently high (8.30) that the derivatives would exist predominantly as dications at physiological pH. All the compounds intercalate very tightly to DNA (equilibrium constants above 10^7 M^{-1}), with helix unwinding angles of 13–17°.

In the light of this apparent inability to account for the structure–activity relationships of the 9-aminoacridine-4-carboxamides in terms of variations in their mode and strength of DNA binding, and considering previous work suggesting the importance of drug–DNA kinetic parameters for biological activity, we have studied the kinetic stability of complexes of selected 9-aminoacridine-4-carboxamides with calf thymus DNA, using stopped-flow spectrophotometry and the surfactant-sequestration technique.[24] We report here the results of those studies and their relevance to understanding the observed structure–activity relationships for this new class of antitumor drugs.

Chemistry

Most of the compounds listed in Table I have been reported previously.[21,22] Compounds 20 were prepared by coupling 9-chloroacridine-4-carbonyl chloride[21] with *N*,*N*,*N*′-trimethylethylenediamine by using published methods.[21] The 9-chloroacridine-4-sulfonyl chloride required for 21 was prepared from 2-phenyliodonium-2-carboxylate[25] and 2-aminobenzenesulfonic acid, followed by sequential treatment with $POCl_3$ and $SOCl_2$.

Results

As a preliminary to the use of the surfactant-sequestration method, it is necessary to demonstrate that the DNA–drug complexes are completely dissociated by ad-

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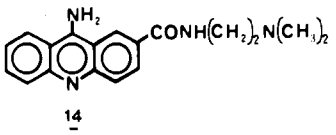
Table I. Spectroscopic and Affinity Data for the Interaction of 9-Aminoacridinecarboxamides with DNA



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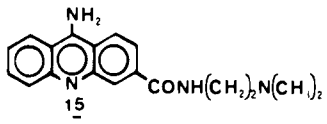

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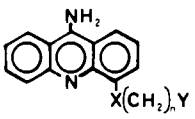

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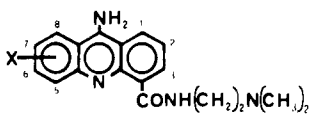

25 - 31

				free drug[a]		DNA-bound drug[b]		micelle-bound drug[c]				log K^e		
no.	x	n	Y	λ_{max}	$10^{-3}E$	λ_{max}	$10^{-3}E$	λ_{max}	$10^{-3}E$	λ	$10^{-3} \times \Delta E^d$	AT	GC	Δ(GC-AT)
9-aa (9-aminoacridine)				399	10.1	406	5.0	402	10.2	402	5.9	6.08	6.11	0.07
14				401	7.3	408	3.8	403	7.4	403	3.6	7.08	7.55	0.47
15				409	8.3	417	4.2	415	8.3	415	3.3	7.18	7.66	0.48
5	CONH	2	$N(CH_3)_2$	407	9.4	421	4.9	415	9.4	415	4.5	7.35	7.70	0.35
16	CONH	3	$N(CH_3)_2$	407	10.1	420	5.3	415	10.7	415	4.5	7.51	7.60	0.09
17	CONH	4	$N(CH_3)_2$	407	10.2	420	5.4	415	10.7	415	4.7	7.49	7.43	−0.06
18	CONH	5	$N(CH_3)_2$	407	9.8	420	5.5	415	10.5	415	4.6	7.45	7.45	0.00
19	CONH	6	$N(CH_3)_2$	406	10.2	420	5.5	415	11.0	415	4.5	7.41	7.40	−0.01
20	$CON(CH_3)$	2	$N(CH_3)_2$	403	10.9	410	5.8	405	10.9	405	5.1	6.37	6.37	0.00
21	SO_2NH	2	$N(CH_3)_2$	403	7.8	410	4.5	405	8.9	405	4.3			
22	CONH	2	$N(CH_2CH_3)_2$	407	10.4	420	5.4	412	10.9	411	6.4	7.26	7.59	0.33
23	CONH	2	$N(CH_2CH_2)_2O^f$	406	9.4	422	4.9	412	10.0	415	6.4	7.29	7.58	0.29
24	CONH	2	$NH(CH_2)_2OH$	408	10.5	421	5.5	413	10.9	413	6.2	7.34	7.73	0.39
25	1-OCH_3			428	9.2	441	5.4	433	10.0	433	5.2	7.31	7.52	0.21
26	2-OCH_3			423	9.3	439	5.5	427	10.1	427	5.9	7.68	8.44	0.76
27	3-OCH_3			398	8.6	408	5.3	402	9.6	402	4.6	7.21	7.12	−0.09
28	5-OCH_3			421	8.6	437	4.6	424	8.9	424	4.8	7.62	7.83	0.21
29	6-OCH_3			395	8.6	406	4.7	396	8.7	396	4.4	7.33	7.71	0.38
30	7-OCH_3			421	8.2	435	4.3	426	8.5	426	4.6	7.64	7.66	0.02
31	8-OCH_3			426	9.3	435	5.1	426	9.5	426	4.8	7.68	7.55	−0.18

[a] 50 μM drug in 0.1 SHE buffer (see 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 SHE. [d] Difference in molar extinction coefficient between SDS micelle-bound and DNA-bound drug at wavelength used in the kinetic measurements. [e] log K is the logarithm of the association constant for binding either to poly·[d(A-T)] or poly·[d(G-C)], measured by the ethidium displacement method of ref 34. Most values are taken from ref 21 and 22. [f] Morpholino group.

dition of detergent and to verify that optical changes observed in the stopped-flow spectrophotometer are not related to processes associated with absorption of the drug into the surfactant micelles. Accordingly, spectroscopic measurements were used to show that sodium dodecyl sulfate (SDS) fully dissociates the DNA complexes of all the ligands studied, and the relevant spectral characteristics of free, DNA-bound, and SDS-micelle-bound drug are recorded in Table I. All of the unsubstituted compounds with the side chain at the 4-position have an absorption maximum around 407 nm with molar extinction coefficients of about 10^4. Substitution of the acridine ring with methoxy groups considerably perturbs the wavelength of this maximum; for the 3- and 6-methoxy derivatives, the peak moves down to 395–398 nm while substitution at all other positions results in a shift to 421–428 nm. On addition of excess DNA all the compounds exhibit the expected bathochromic and hypochromic changes associated with intercalative binding. In SDS solution the absorption spectra are reminiscent of those of the free drug in buffer with modifications generally limited to moderate bathochromic and/or slight hyperchromic changes.

Kinetic measurements were made at, or near, the wavelength of maximum absorption of the drug in SDS solution since this wavelength corresponds to the maximum in the DNA-bound vs. micelle-bound difference spectrum. Since no time-resolvable changes are detectable in the stopped-flow apparatus when DNA-free drug solutions are mixed with SDS solutions, we infer that the transient absorbance changes measured in the presence of DNA are attributable solely to dissociation of the drug–DNA complex. Control experiments were performed to show that dissociation rates of the complexes are independent of SDS concentration in the range 5–50 mM at constant ionic strength. All of the complexes studied have dissociation curves that can be deconvoluted into two or more exponential components. Numerical analysis was performed by curve stripping, with data from at least six kinetic runs being analyzed separately and the results averaged.[26] The resolved time constants T and their associated amplitudes A are given in Table II. It is evident that the sum of the amplitudes for 9-aminoacridine and compounds **20** and **21** fall far short of the equilibrium absorbance change characterizing complete dissociation. Thus, for these compounds, there are clearly additional processes in the dissociation pathway of their DNA complexes that occur more rapidly than the time resolution of the stopped-flow instrument. Bearing in mind the errors associated with the analysis of multiple exponential transients, it is difficult to know whether similar "missed" processes occur for the other compounds studied. Practically speaking, where the sum of the amplitudes reaches 90% or more of the equilibrium absorbance change, we believe the kinetic spectrum to be fully accounted for, whereas sums in the vicinity of 80% we take to imply the presence of additional transients that are too fast to measure.

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Table II. DNA Dissociation Kinetics and Biological Activity for the Compounds of Table I

	time constants,[b] ms				ratios[c]			amplitudes,[d] %					L1210	P388	
no.[a]	T_1	T_2	T_3	T_4	T_2/T_1	T_3/T_2	T_4/T_3	A_1	A_2	A_3	A_4	F[e]	IC_{50}[f]	OD[g]	ILS[h]
9-aa	2.5	15			6.3			88	12			50	2600	20	*i*
14	10	27	90		2.7	3.37		16	57	27		91	>2000	12.5	*i*
15	6	27	98		4.5	3.6		14	59	27		80	>2000	250	*i*
5	6	28	86	428	4.7	3.1	5.0	14	34	34	18	89	15	4.5	98
16	4	15	44		3.8	2.9		22	48	30		80	157	50	*i*
17	4	16	52		4.0	3.3		31	29	40		76	430	*j*	
18	4	16	58		4.0	3.6		39	21	40		81	1370	*j*	
19	4	19	80		4.8	4.2		41	21	38		83	1540	*j*	
20	2	11			5.5			68	32			33	5300	30	*i*
21	5	21			4.2			57	43			40	13000	65	*i*
22	3	13	51	280	4.3	3.9	5.5	15	26	46	13	86	5.5	5.9	70
23	4	40	135	590	10.0	3.3	4.4	13	32	40	15	84	1025	30	*i*
24	5	17	49	214	3.4	2.9	4.4	8	27	49	16	77	77	20	73
25	22	102	355	2270	4.6	3.5	6.4	12	37	36	15	90	0.6	8.9	27
26	20	79	311	2050	4.0	3.9	6.6	20	39	28	13	90	2.9	5.9	72
27	7	20	87		2.9	4.4		40	28	22		85	300	20	*i*
28	17	69	299	1080	4.1	4.3	3.6	4	20	53	23	96	4.3	3.9	81
29	26	83	271	1000	3.2	3.3	3.7	8	24	42	26	95	15	8.9	*i*
30	9	25	64	330	2.8	2.6	5.2	9	42	47	7	95	670	13.3	*i*
31	6	30	168		5.0	5.6		22	61	17		87	220	45	*i*

[a] Number of the compound in Table I. [b] Time constants describing the dissociation profile of calf thymus DNA–drug complexes in 0.1 SHE buffer at 20 °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. [c] Ratios of the noted time constants. [d] Amplitudes of the processes characterized by the appropriate time constants, expressed as a percentage of the sum of the amplitudes evaluated in the kinetic experiment. [e] F is 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%. [f] IC_{50} is the nanomolar concentration of drug that when added to cultures of L1210 cells for a period of 70 h reduces cell numbers of 50% of control cultures. Most of the data are taken from ref 21 and 22. [g] OD is the optimal drug dose (in mg/kg per day), administered intraperitoneally as a solution in 0.1 mL of 30% v/v EtOH–water on days 1, 5, and 9 after inoculation of 10^6 P388 leukemia cells. For active compounds, OD is the dose at which the highest ILS was obtained and for inactive compounds is the highest nontoxic dose. Most of the data are taken from ref 21 and 22. [h] ILS is the percentage increase in life span of treated animals over those of controls injected with tumor alone. Values of ILS greater than 20% are considered statistically significant. Most of the data are taken from ref 21 and 22. [i] No activity (ILS < 20%) at all dose levels. [j] Not tested in vivo.

Addition of the cationic side chain to 9-aminoacridine at positions 2 or 3 (**14** and **15**) significantly stabilizes the drug–DNA complex, both kinetically (Table II) and thermodynamically (Table I), an effect that no doubt is due in large part to enhanced electrostatic interactions. By contrast to 9-aminoacridine where only two optical transients are resolvable, the kinetic profiles for compounds **14** and **15** can be deconvoluted into three components whose respective time constants and amplitudes are similar. Moving the side chain to the 4-position (to give the biologically active compound **5**) leaves these three transients essentially unperturbed but causes the appearance of a fourth, slower process. Although the latter accounts for only 18% of the absorbance change, it is readily detectable, being the longest lived transient, and is clearly absent from the kinetic spectrum of compounds **14** and **15**. The next four compounds in Table II retain the side chain at the 4-position, but the distance between their cationic center and chromophore is lengthened by sequential addition of methylene groups. Extension by only one methylene unit to give **16** results in an approximately 2-fold reduction in the value of the first three time constants (T_1, T_2, and T_3), but the most striking finding is the complete loss of the fourth transient (T_4). Incrementing the methylene chain as far as hexyl (compound **19**) has little further effect, the kinetic behavior of compounds **16–19** being virtually identical with respect to the number of resolvable transients, their amplitudes, and time constants.

Altering the nature of the link group at position 4, but keeping the (dimethylamino)ethylamine function of **5**, to give compounds **20** and **21**, has profound effects on kinetic stability. Methylating the carboxamide (**21**) or replacing it by sulfonamide (**20**) destabilizes the DNA complex to the extent that, as mentioned above, only about one-third of the dissociation reaction occurs in the stopped-flow time range, resolvable as two closely coupled transients. By contrast, modifications to the structure of the terminal amino function of the side chain of **5** (**22–24**) have only minor effects, with all four kinetic processes associated with the parent compound remaining clearly detectable. Specifically, the time constants for the diethyl (**22**) and hydroxyethyl (**24**) derivatives are somewhat reduced compared to those of **5**, whereas those for the morpholino compound (**23**) are slightly enhanced.

Previous work with the set of seven methoxy positional isomers (**25–31**) revealed that although they bind tightly to DNA by intercalation, their in vivo biological activity varies widely.[22] These compounds are of special interest since they provide an opportunity for probing the orientation of the acridine chromophore of **5** when bound in its intercalation site. In Table II we see that, with the exceptions of compounds **27** and **31**, the 3- and 8-methoxy derivatives, respectively, the dissociation profiles of their calf thymus DNA complexes resolve into four components. The data for the 7-methoxy derivative **30** are very similar to those for the parent compound **5**, except that the amplitude of the longest exponential, T_4, is noticeably smaller and accounts for only 7% of the total absorbance change. The time constants for the other four compounds (**25, 26, 28,** and **29**) are substantially greater than those of the parent compound, indicating that methoxy substitution in the 1-, 2-, 5-, and 6-positions significantly stabilizes the DNA complexes of these analogues. The overall similarity of the shape of the kinetic profiles of compounds **25, 26,** and **28–30** with that of **5** suggests that the substitutions have not significantly altered the nature of the DNA–ligand complex. However, the 3- and 8-methoxy derivatives have lost the fourth kinetic transient and have more labile complexes, and it appears that substitution in the 3- or

Table III. Kinetic Parameters for the Dissociation of Compounds 5 and 17 from Synthetic Polynucleotides[a]

DNA and compd	time constants, ms			ratios		amplitudes			
	T_1	T_2	T_3	T_2/T_1	T_3/T_2	A_1	A_2	A_3	F
poly(dG-dC), 5	3	76	230	25	30	6	17	77	106
poly(dA-dT), 5	4	8		2.0		33	67		70
poly(dI-dC), 5	5	15		3.0		46	54		66
poly(dG-dC), 17	4	26	86	6.5	3.3	26	15	59	99

[a] Symbols and conditions as described in Tables I and II and the text for interaction with calf thymus DNA.

8-position severely perturbs the configuration of the intercalated complex.

To help understand the multiple steps in the dissociation pathways of the calf thymus DNA complexes of **5** and **17**, and to investigate the dependence of the kinetic spectrum on nucleotide sequence, measurements were made with synthetic polynucleotides (Table III). Dissociation of **5** from poly(dG-dC)·poly(dG-dC) is characterized by three resolvable transients, the slowest of which stands out as the dominant feature with its amplitude contributing three-fourths of the total absorbance change. It is interesting to note that although the number and values of the time constants for dissociation from the copolymer and calf thymus DNA are different, both reactions take place within a similar time domain. In sharp contrast, dissociation of the poly(dA-dT)·poly(dA-dT)–**5** complex is very rapid, the two resolvable transients representing only two-thirds of the complete reaction (Table III). Measurements with an inosine-containing polymer reveal that removing the 2-amino group of guanine has major consequences for the kinetic stability of DNA complexes of **5**. The poly(dI-dC)·poly(dI-dC) complex dissociates quickly, its kinetic parameters being similar to those found for poly(dA-dT)·poly(dA-dT) and quite unlike those seen with poly(dG-dC)·poly(dG-dC) and calf thymus DNA (Tables II and III). Compound **17**, whose calf thymus DNA complex lacks the slow T_4 component, dissociates from poly(dG-dC)·poly(dG-dC) more rapidly than **5** (Table III), the time constants of the three components of its GC–copolymer complex being comparable to those of its calf thymus complex (Tables II and III).

Discussion

The 9-aminoacridinecarboxamides represent a new class of DNA-binding antitumor drug with well-defined structure–activity relationships that cannot be satisfactorily explained by alterations in either their strength or general mode of binding to DNA, since these properties are essentially similar across the series. However, the results presented here do show a clear relationship between in vivo biological activity and the kinetics of dissociation of the drug–DNA complexes. Of the three positional isomers **5**, **14**, and **15**, only the 4-substituted compound **5** is biologically active, and only this derivative displays a fourth, long-lived transient (T_4) in its dissociation from calf thymus DNA. The tight structure–activity relationships for this class of compound are exemplified by the fact that even extension of the side chain at position 4 by one methylene unit abolishes in vivo activity and simultaneously causes loss of the fourth kinetic transient (Table II) and a significant loss of selective binding to GC base pairs (Table I). Alteration of the nature of the link group, which has such a profound effect on the kinetic stability of the complexes of **20** and **21**, also leads to loss of in vivo activity and GC selectivity. The only modifications to the side chain that do not result in loss of biological activity (varying the nature of the cationic nitrogen) are also those that do not change the kinetic spectrum of the resulting DNA complexes. Given this, the inactivity of the morpholino derivative **23** is puzzling in view of the observed kinetics, but lack of activity may be related to the low pK_a (6.7) of this side chain[21] and/or to some form of metabolic deactivation. A broadly similar picture is seen for the methoxy derivatives **25–31**. The active compounds are the 1-, 2-, and 5-substituted derivatives **25**, **26**, and **28**, which display both selective binding to GC nucleotide pairs, enhanced stability of their DNA complexes, and four components in their dissociation kinetics. In contrast, the inactive 3- and 8-methoxy derivatives **27** and **31** have lost both GC selectivity (Table I) and the fourth kinetic transient. Although dissociation of the inactive 7-methoxy derivative **30** also shows four components, T_4 has but a very small amplitude, implying that there is little of the bound form characterized by T_4 present at equilibrium. Only the 6-methoxy derivative **29**, which is inactive in vivo in spite of GC-selective binding and a dissociation process that includes a significant T_4 transient, convincingly fails to fit the pattern (although it should be noted that this compound retains potent in vitro cytotoxicity).

The compounds studied here constitute a set of carefully selected variants of the biologically active lead compound **5**, where the position (compounds **14** and **15**) and nature (compounds **20** and **21**) of attachment of the cationic side chain has been explored, together with the distance (compounds **16–19**) and structure (compounds **22–24**) of the cationic group and finally the substitution of chromophore (compounds **25–31**). For the great majority of these compounds, it is evident that there is a positive correlation between in vivo biological activity, GC-selective binding to DNA, and the presence of the fourth transient in the dissociation kinetics of their complexes with calf thymus DNA. It is not possible from the present data to determine if the kinetic spectra observed represent parallel dissociations from a number of classes of binding site of different affinity or if the dissociation is a sequential process, where all of the drug molecules have to proceed through a series of different bound states before final release from the DNA. However, bearing in mind the flexible nature of the drug side chains, the finding of complex kinetics with the synthetic DNAs of limited base composition, and the fact that the measurements with calf thymus DNA were made at low binding levels (one drug molecule per two turns of the helix) to promote population of only the highest affinity sites, we suspect that the observed mechanisms are sequential. At any event, and irrespective of the exact binding mechanism, it is clear that compounds bearing a 4-$CONH(CH_2)_2NR_1R_2$ side chain (where R_1 and R_2 are groups allowing a basic nitrogen, protonated near physiological pH) have access to a binding mode characterized by T_4 that is not available to the other derivatives. The question then arises as to the nature of this unique complex.

Notwithstanding the usual caveats associated with extrapolating properties of synthetic copolymers to small regions of similar sequence in natural DNA, it appears, both from equilibrium and kinetic measurements, that the highest affinity intercalation sites for compound **5** in calf thymus DNA are GC rich. Given this, plus the finding that

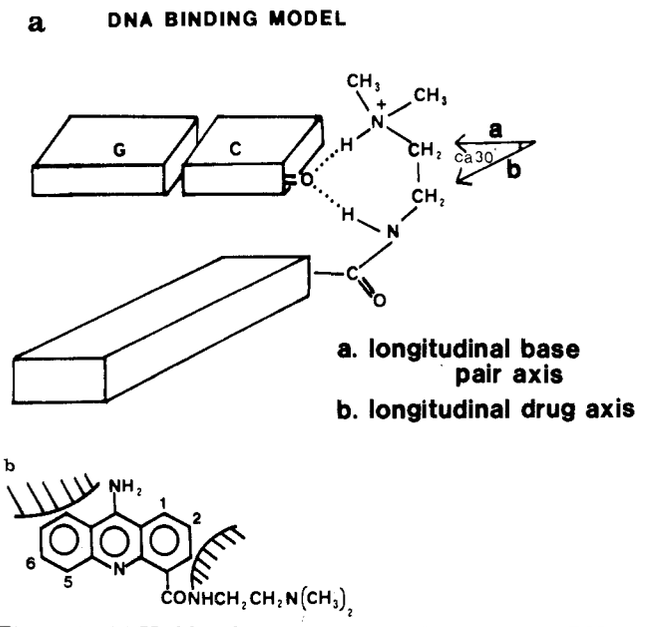

Figure 1. (a) Highly schematic diagram of the proposed DNA–drug complex of highest stability, resulting in the observed T_4 transient. (b) Diagram indicating approximate areas of bulk intolerance due to close proximity of the DNA ribophosphate chains in the complex proposed in (a).

the electrostatic potential is greater in the minor compared to the major groove in B-type DNA,[27] as well as the examples of echinomycin–,[28] actinomycin–,[29] ethidium–,[30] and daunomycin–DNA[31] complexes where the bulk of the ligand attached to the intercalating chromophore resides in the minor groove, we suggest that the dicationic **5** intercalates from the narrow groove adjacent to a GC pair. Previous measurements[21] show that intercalation of **5** unwinds the DNA helix by 16°. With the long axes of the drug chromophore and the base pairs twisted away from the alignment of maximum overlap, so that the acridine 5- and 6-positions protrude into the groove, and the 4-carboxamide group in the trans configuration held at right angles to the acridine plane, the carboxamide NH atoms are well placed to make a hydrogen-bonding contact with the O2 oxygen of cytosine. Although the minimum-energy conformation of the dication of **5** has the amide coplanar with the acridine ring,[32] the energy barrier to full rotation about the amide carbonyl bond is very low.[32] Such an interaction is, of course, available to all 4-carboxamide derivatives. The additional binding mode inferred from the fourth, long-lived transient shown by all compounds possessing a 4-$COHN(CH_2)_2NR_1R_2$ group (the exceptions of compounds **27** and **31** are noted below) is postulated to be one where a second H-bond is formed to the cytosine O2 from the hydrogen of the protonated nitrogen at the end of the chain (Figure 1a). Such "double" H-bonds are well-documented to occur when the geometry is favorable.[33]

In the present case, CPK models show that only when the side chain contains two methylene groups is such an interaction possible. If the carboxamide hydrogen is lost (as in compounds **20** and **21**), the stability of the resulting complexes is similar to that of 9-aminoacridine, pointing to the importance of this hydrogen in binding. Although we describe the cytosine oxygen–NH contacts as hydrogen bonds, we note that the interaction may be primarily electrostatic given the large negative electric field in the vicinity of base carbonyls in DNA[27] and the positive residual charge on the hydrogen atoms of amides and protonated amines. The observed GC selectivity of these compounds indicates that similar bonding does not take place with thymidine O2 atoms, which lie in broadly equivalent positions in B-DNA, but it is not possible to determine the reason for this from the present data.

In order to optimize the bonding interactions postulated, the major axis of the acridine chromophore has to lie at an angle of about 30° from the plane of maximum overlap (with the GC base pair). Such an orientation of the acridine ring forces the 3-, 7-, and 8-positions close to the deoxyribophosphate chains (Figure 1b). The closeness of these contacts accounts for the loss of GC selectivity by the corresponding 3-, 7-, and 8-methoxy derivatives (**27**, **30**, and **31**) and the loss (**27** and **31**) or severe diminution in amplitude (**30**) of the fourth kinetic transient T_4 since with these substitution patterns the postulated complex is geometrically unobtainable or very unfavorable even though the compounds possess the necessary 4-$CONH(CH_2)_2NR_1R_2$ side chain. It seems unlikely that the hydrogen-bonding scheme we describe could be formed with the base carbonyls in the DNA wide groove, as these oxygen atoms lie close to the helix axis and the asymmetry of the ligand causes its side chain to occupy space toward the edge of a groove.

The proposed model accounts for the observed pattern of GC-selective binding as well as the dissociation kinetics of the family of 9-aminoacridine-4-carboxamides studied. The fact that only compounds that have access to the binding mode postulated for compound **5** are biologically active in vivo is more difficult to explain. The slower dissociation of the active compounds could favorably alter pharmacokinetics, allowing longer drug half-lives. However, a marked correlation of the DNA-breakage ability of a limited number of these compounds with their DNA binding kinetics has been shown.[23] The active parent compound **5** is a more potent DNA-breaking agent than the *N*-methyl carboxamide analogue **20**, the $(CH_2)_4$ analogue **17**, and 9-aminoacridine by factors of >600, >50, and >100, respectively.[23] Thus it seems likely that the observed correlation between in vivo antitumor activity and DNA-binding kinetics for these compounds is due more to effects at the cellular level than to altered pharmacokinetics. This is supported by the fact that the correlation between in vitro cytotoxicity and kinetics is even more complete (e.g., compound **29**). If the cytotoxic lesion is eventually identified with the clastogenic activity of these compounds as a result of poisoning topoisomerase II, it may be that the DNA complex postulated for **5** defines the correct stereochemical features for efficient antagonism of this enzyme by 9-aminoacridine-4-carboxamides.

While it is not possible at present to causally relate slow drug–DNA dissociation rates, DNA breakage, and biological activity at the molecular level, the above hypothesis

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provides a framework on which to base further development of this series. This work is being undertaken, together with molecular-level studies of the complexes of selected derivatives with DNA, to substantiate the above hypotheses.

Experimental Section

Chemistry. 2-[(2-Carboxyphenyl)amino]benzenesulfonic Acid. A mixture of diphenyliodonium-2-carboxylate[25] (25 g, 0.077 mol), 2-aminobenzenesulfonic acid (25 g, 0.145 mol), potassium carbonate (10.6 g, 0.077 mol), and cupric acetate (0.5 g) in DMF (100 mL) was heated at 100 °C for 72 h, and the solvent was removed under vacuum. The residue was dissolved in 0.1 N NH_4OH, and the solution was filtered and extracted with EtOAc. Acidification with HCl gave a crystalline precipitate of 2-[(2-carboxyphenyl)amino]benzenesulfonic acid, mp 260 °C dec (10.55 g, 47%). Anal. ($C_{13}H_{11}NO_5S·2H_2O$) C, H, N, S.

***N*-[(2-Dimethylamino)ethyl]-9-aminoacridine-4-sulfonamide Dihydrochloride (Compound 9 of Table I).** A suspension of 2-[(2-carboxyphenyl)amino]benzenesulfonic acid (5 g, 0.017 mol) in $POCl_3$ (150 mL) was heated at reflux until homogeneous (ca. 1 h) and for a further 30 min. The $POCl_3$ was removed under vacuum and the residue dissolved in $SOCl_2$ (50 mL, containing 2 drops of DMF). After 30 min at reflux temperature, the $SOCl_2$ was removed under vacuum and the residue dissolved in an ice-cold solution of *N,N*-dimethylethylenediamine (5 mL) and triethylamine (10 mL) in ethanol-free $CHCl_3$ (200 mL). Subsequent to stirring for 5 min the solution was washed with water (3×), dried, and evaporated. The residue was dissolved in phenol (50 g) and treated with a stream of ammonia gas at 120 °C for 30 min. The cooled solution was diluted with 2 N HCl and the phenol removed by washing with EtOAc. The aqueous layer was basified with NH_4OH and the oily product extracted into EtOAc. Removal of the solvent and chromatography on neutral alumina (hexane/EtOAc) gave a bright yellow product, which was dissolved in MeOH and treated with HCl gas until the solution was acidic. Addition of EtOAc gave a precipitate, which was recrystallized from MeOH/EtOAc, yielding N-[2-(dimethylamino)ethyl]-9-aminoacridine-4-sulfonamide dihydrochloride, mp 278–280 °C (2.1 g, 30% based on the sulfonic acid). Anal. ($C_{17}H_{20}N_4O_2S·2HCl$) C, H, N, S, Cl.

***N*-Methyl-*N*-[2-(dimethylamino)ethyl]-9-aminoacridine-4-carboxamide Dihydrochloride (Compound 8 of Table I).** This compound was prepared from 9-chloroacridine-4-carbonyl chloride and *N,N,N'*-trimethylethylenediamine essentially by the method of ref 14 to give the dihydrochloride as yellow crystals from MeOH/EtOAc, mp 313–314 °C. Anal. ($C_{19}H_{22}N_4O·2HCl$) C, H, N, Cl.

Spectroscopic Measurements. Molar extinction coefficients at the wavelength of maximum absorption in the visible spectrum were determined for the compounds free in solution, bound to DNA, and when sequestered into SDS micelles by using a Carey 219 UV/visible spectrophotometer. Measurements were made in 0.1 SHE buffer, a solvent containing 2 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid, 10 μM EDTA, and 99.4 mM NaCl, pH 7.0. Spectra were recorded at a drug concentration of 50 μM, DNA- and micelle-bound spectra being determined in the presence of 1 mM calf thymus DNA (nucleotide pairs) and 10 mM SDS (monomer concentration), respectively. To investigate the ability of SDS to dissociate DNA–drug complexes, spectra of drug–DNA mixtures (50 μM drug, 1 mM DNA) were measured before and after the addition of surfactant to a final monomer concentration of 10 mM.

Kinetic Measurements. Kinetic measurements were performed with a Dionex D110 stopped-flow spectrophotometer coupled via a dc amplifier and double-beam storage oscilloscope to a 64-kilobyte Apple II microcomputer equipped with two floppy-disc drives, an 8-bit analogue-to-digital converter, and a high-precision clock. Software has been developed to permit collection, storage, editing, and analysis of up to 500 points per dataset, with a wide choice of sampling frequency.[26] The fastest rate of data collection was 0.1 ms per point. The spectrophotometer was fitted with a 20-mm-light-path optical cuvette, giving the apparatus a deadtime of about 2 ms, and was operated in transmittance mode. Solutions of drug–DNA complexes containing 400 μM calf thymus DNA (nucleotide pairs) and 20 μM drug in 0.1 SHE buffer were mixed with an equal volume of 20 mM SDS (monomer concentration) in the same buffer at 20 °C. The spectrophotometer was operated with a time constant of 0.1 ms and the optical bandwidth set to 3 nm. The molecular weight of the DNA used in both the spectroscopic and kinetic measurements was reduced by sonicating solutions containing 2 mg/mL of DNA in 0.2 SHE buffer at 0 °C for 5 min on a Branson 150-W sonicator. The sonicated DNA was exhaustively dialyzed into 0.1 SHE buffer, and solutions were freed of particulate material by passing through 0.45 μM Millipore filters.

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Registry No. **5**, 89459-43-8; **5**·poly(dG-dC), 107009-86-9; **5**·poly(dA-dT), 107009-85-8; **5**·poly(dI-dC), 107009-84-7; **8**·2HCl, 106988-42-5; **9**·2HCl, 107009-87-0; **9**-aa, 90-45-9; **14**, 91549-70-1; **15**, 91549-71-2; **16**, 89459-44-9; **17**, 91549-79-0; **17**·poly(dG-dC), 107009-83-6; **18**, 91549-80-3; **19**, 91549-81-4; **20**, 103554-57-0; **21**, 106988-41-4; **22**, 89459-45-0; **23**, 91549-84-7; **24**, 89459-30-3; **25**, 100113-16-4; **26**, 100113-19-7; **27**, 100113-21-1; **28**, 89459-52-9; **29**, 89459-57-4; **30**, 89459-61-0; **31**, 100113-24-4; *o*-$HO_2CC_6H_4NHC_6H_4SO_3H$-*o*, 39189-36-1; *o*-$NH_2C_6H_4SO_3H$, 88-21-1; $Me_2N(CH_2)_2NH_2$, 108-00-9; $Me_2N(CH_2)_2NHMe$, 142-25-6; diphenyliodonium-2-carboxylate, 1488-42-2; 9-chloroacridine-4-carbonyl chloride, 89459-22-3.