Kinetic Studies of the Binding of Acridinecarboxamide Topoisomerase Poisons to DNA: Implications for Mode of Binding of Ligands with Uncharged Chromophores

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We have used stopped-flow spectrophotometry and the sodium dodecyl sulfate sequestration technique to study the kinetics of dissociation of DNA complexes of the mixed topoisomerase I/II poison *N*-[2-(dimethylamino)ethyl]acridine-4-carboxamide (termed DACA) and a range of related linear tricyclic carboxamides with neutral chromophores. Complexes of DACA and related acridine and phenazinecarboxamides bearing an *N*,*N*-dimethylaminoethyl side chain dissociate from calf thymus DNA by a kinetic pathway involving four discernible steps in a manner similar to complexes of *N*-[(2-dimethylamino)ethyl]-9-aminoacridine-4-carboxamide (termed 9-amino-DACA). We infer from these findings that the side chains of DACA, its phenazine homologue, and 9-amino-DACA make comparable interactions with the DNA base pairs. In the case of 9-amino-DACA, a selective topoisomerase II poison, these are known, by crystallographic analysis, to involve hydrogen-bonding interactions between the protonated dimethylammonium group of the side chain and the O6/N7 atoms of guanine and to include a bridging water molecule hydrogen bonded to the carboxamide group and a phosphate oxygen. By contrast, we find that other linear tricyclic carboxamides with neutral chromophores which lack a peri nitrogen atom and are biologically inactive dissociate from DNA by a different mechanism in which it appears their side chains fail to interact with guanine. We conclude that the ability of the carboxamide group to lie preferentially in the plane of the chromophore, so facilitating the dimethylammonium−guanine hydrogen bond and ensuring maintenance of the water-bridged carboxamide−phosphate interaction, is a critical requirement for antitumor activity among ligands of the linear tricyclic carboxamide class. However, unlike the situation for 9-amino-DACA, for ligands with uncharged chromophores containing peri nitrogen atoms such as DACA, this outcome is possible with the 4-carboxamide group rotated cis or trans with respect to the ring nitrogen. This difference may have relevance to the ability of DACA to be a dual poison of both topoisomerases I and II.

Introduction

DNA-binding agents that intercalate into DNA and poison topoisomerases I and II are important drugs in the treatment of cancer.[1,2] Denny and colleagues have established that 9-aminoacridine-4-carboxamides with *N*,*N*-dimethylaminoethyl side chains, for example, compound **1** (9-amino-DACA, Figure 1), are potent topoisomerase II-poisoning cytotoxins with activity against mouse leukaemia models in vivo[3−5] and that derivatives with electron-withdrawing substituents in the 5 position are also active against experimental solid tumors.[6,7] The origins of solid tumor activity have been attributed to the fact that the electron-withdrawing group lowers the 9-aminoacridine pK so as to produce a neutral, rather than a positively charged, chromophore at physiological pH and that this facilitates tissue distribution and promotes better penetration into tumor masses.[7] The related des-9-amino acridine-4-carboxamide, compound **3** (DACA, NSC 601316), also has a neutral chromophore and poisons both topoisomerases I and II.[5,8−10] It has a wide spectrum of activity against solid tumors in animal models, attributed in part to its ability to be a dual topoisomerase poison, and is currently in clinical trial.[8,9,11,12] A search for solid tumor activity amongst a range of compounds possessing neutral linear tricyclic chromophores, with similarly positioned *N*,*N*-dimethylaminoethyl side chains, revealed that only those agents with a nitrogen atom peri to the carboxamide are biologically active, although no rationale for this finding was apparent.[13]

There are well-defined relationships between ligand structure, cytotoxicity, and DNA binding kinetics for the 9-aminoacridine-4-carboxamide class of compounds,[3,4,6,7,14] which implies that the *N*,*N*-dimethylaminoethyl side chain makes specific interactions with the DNA that are sensed by topoisomerase II in the ternary complex. Kinetic studies show that 9-amino-DACA dissociates from calf thymus DNA by a complex mechanism involving four discernible steps, and experiments with synthetic polynucleotides containing AT, GC, and IC base

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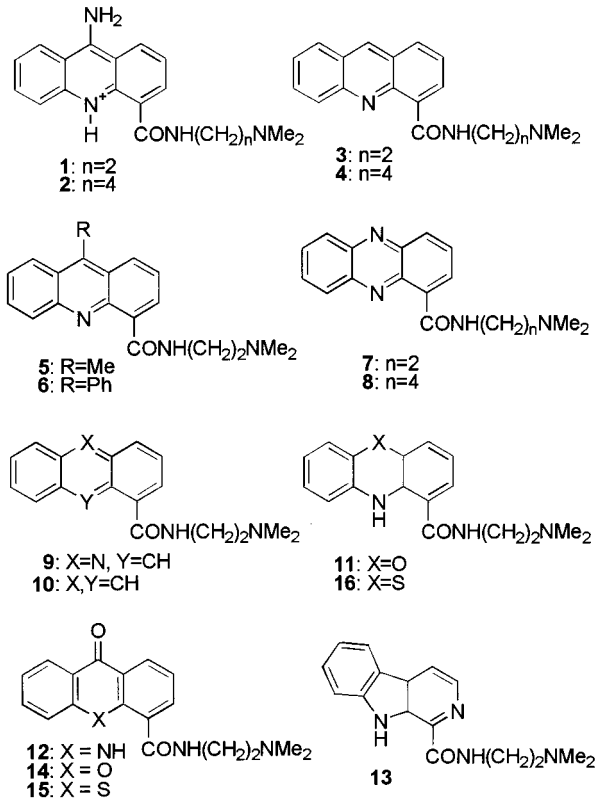

Figure 1. Structures of compounds studied.

pairs imply that these processes are sequential.[14] In addition, the same studies confirmed that the kinetic profiles seen with calf thymus DNA characterize dissociation from GC-rich nucleotide sequences, as expected given the GC selectivity of 9-amino-DACA revealed by DNase I footprinting.[15] Lengthening the 4-carboxamide side chain, so that there are three or more methylene groups between the carboxamide NH and the *N*,*N*-dimethylammonium nitrogen, results in loss of biological activity[3,6] and loss of the slowest transients in the calf thymus DNA and poly d[G−C] dissociation profiles.[14]

To help provide insight into the nature of the molecular interactions between the ligand side chain and DNA, we have presented crystal structures of 9-amino-DACA and its 5-fluoro derivative bound to the hexanucleotide d(CGTACG)$_2$[16,17] and of 6-Br-9-amino-DACA bound to the brominated hexanucleotide d(CG^{5Br}-UACG)$_2$,[18] where the ligands are intercalated at the CpG dinucleotide steps. In these complexes, the 4-carboxamide group lies in the plane of the chromophore, and the carbonyl oxygen atom forms an internal hydrogen bond with the protonated N10 nitrogen of the acridine ring. The side chain lies in the DNA major groove with its protonated *N*,*N*-dimethylamino group forming hydrogen-bonding interactions with the O6 and N7 atoms of guanine G2. In each case, a hydrogen-bonded water molecule bridges the NH of the carboxamide group to the guanine G2 phosphate at the intercalation site. These structures have provided a molecular rationale for understanding the structure−activity relationships for antitumor activity and enabled a mechanistic interpretation of the dependence of kinetics on ligand structure.[16,17] In particular, they have permitted the critical step in the dissociation kinetics profile that correlates with cytotoxicity and antitumor activity to be identified with the side chain−guanine interaction.[14,16,17] By contrast, little is known about the nature of the DNA complexes of DACA and related active compounds with neutral chromophores, save their DNA binding affinities and their helix unwinding angles.[8,13,19] Crystallographic approaches have yet to yield structures of their intercalated complexes, and currently there is no understanding of the structural requirement for a nitrogen atom peri to the carboxamide for antitumor activity, nor of the structural origins of the capacity of DACA to be a dual topoisomerase I/II poison.

Here we describe the results of studies of the kinetic stability of DNA complexes of acridine- and phenazine-4-carboxamides which suggest that their *N*,*N*-dimethylaminoethyl side chains make interactions with DNA in a manner similar to the side chain of 9-amino-DACA. By contrast, other linear tricyclic carboxamides with neutral chromophores which lack a peri nitrogen dissociate from DNA very rapidly by a different mechanism, which suggests their side chains are unable to interact favorably with guanine O6/N7 atoms. We conclude that the ability of the carboxamide group to lie preferentially in the plane of the chromophore, so facilitating the dimethylammonium−guanine hydrogen bond and ensuring maintenance of the water-bridged carboxamide−phosphate interaction, is a critical requirement for antitumor activity among ligands of the linear tricyclic carboxamide class. However, unlike the case of 9-amino-DACA,[16−18] for ligands with uncharged chromophores containing peri nitrogen atoms such as DACA, this outcome is possible with the 4-carboxamide group rotated cis or trans with respect to the ring nitrogen. This difference may have relevance to the ability of DACA to be a dual poison of both topoisomerases I and II.

Results

Spectrophotometric Measurements. In Table 1 we present the visible absorption spectra of the tricyclic carboxamides studied, free in aqueous solution, bound to calf thymus DNA and when sequestered into SDS micelles. The compounds have previously been shown to bind to DNA by intercalation as revealed by their ability to remove and reverse the supercoiling of covalently closed circular DNA (see Table 3 and references therein). The 9-aminoacridine-4-carboxamides, **1** and **2**, exhibit a bathochromic shift of 13−14 nm and a reduction in extinction coefficient of about 50% on binding,[14] whereas the acridine-4- and phenazinecarboxamides, **3**−**8**, show a wavelength shift of ≈5 nm accompanied by a hypochromicity of about 40%. Such perturbations are characteristic of the stacking interactions involved in the intercalation process. Compound **9**, the acridine-1-carboxamide, which has a −CH group peri to the side chain, is notable in having only a very small bathochromic shift on binding, which suggests a possible difference in the geometry of its intercalated complex compared to its acridine-4- and phenazinecarboxamide congeners. By contrast, the complexes of the anthracene-, phenoxazine-, and acridonecarboxamides (compounds **10**−**12**), which also have a −CH or −NH group peri to the carboxamide, have large bathochromic shifts of 9−22 nm. The xanthenone- and thioacridonecarboxa-

Table 1. Spectroscopic Properties of Tricyclic Carboxamides

compound			free		micelle-bound		DNA-bound		
no.	peri	chromophore	λ^a	$\epsilon^b \times 10^{-3}$	λ	$\epsilon \times 10^{-3}$	λ	$\epsilon \times 10^{-3}$	$\delta\epsilon^c \times 10^{-3}$
1[d]	NH^+	9-aminoacridine, $n = 2$	407	9.4	415	9.4	421	4.9	4.5
2[d]	NH^+	9-aminoacridine, $n = 4$	407	10.2	415	10.7	420	5.4	4.7
3	N	acridine, $n = 2$	357	10.2	359	10.6	361	6.1	4.3
4	N	acridine, $n = 4$	357	9.9	358	10.2	362	5.8	4.1
5	N	9-methylacridine	358	9.2	361	9.5	363	5.1	4.2
6	N	9-phenylacridine	358	10.8	362	11.1	364	6.4	4.8
7	N	phenazine, $n = 2$	368	14.9	368	15.0	373	9.0	4.7
8	N	phenazine, $n = 4$	367	14.3	367	14.3	370	8.7	4.1
9	CH	acridine-1-carboxamide	356	11.0	357	11.4	357	7.0	4.2
10	CH	anthracene	386	6.0	387	6.0	395	3.7	2.5
11	NH	phenoxazine	372	5.1	374	6.4	394	5.4	1.5
12	NH	acridone	407	8.9	412	9.6	421	4.4	4.0
13	NH	pyridoindole	366	5.6	368	5.9	370	3.5	2.5
14	O	xanthenone	346	7.7	340	8.3	346	4.8	3.9
15	S	thioacridone	362	5.2	364	5.1	363	3.1	2.1

[a] Wavelength of absorbance maximum, nm. [b] Molar extinction coefficient. [c] Difference in molar extinction coefficient between SDS-bound and DNA-bound ligand at the wavelength used in the kinetic studies. [d] Data taken from ref 14.

Table 2. Dissociation Time Constants for Tricyclic Carboxamides

compound			time constants				amplitudes					mean time constant	
no.	peri	chromophore	$\tau 1$ (ms)	$\tau 2$ (ms)	$\tau 3$ (ms)	$\tau 4$ (ms)	$A1$ (%)	$A2$ (%)	$A3$ (%)	$A4$ (%)	F^a (%)	τ^b (s)	τ^{-1c} (s^{-1})
1[d]	NH^+	9-aminoacridine, $n = 2$	6	28	86	428	14	34	34	18	90	0.12	8.6
2[d]	NH^+	9-aminoacridine, $n = 4$	4	16	52		31	29	40		85	0.027	37
3	N	acridine, $n = 2$	3	12	31	123	14	25	39	25	90	0.046	21
4	N	acridine, $n = 4$	7	17	50		27	38	35		85	0.026	38
5	N	9-methylacridine	7	30	90	530	13	21	41	25	97	0.18	5.7
6	N	9-phenylacridine	5	50	230	750	13	16	39	31	95	0.33	3.0
7	N	phenazine, $n = 2$	2	8	32	124	16	25	35	24	90	0.043	23
8	N	phenazine, $n = 4$	2	9	40		14	45	41		87	0.021	48
9	CH	acridine-1-carboxamide	7	38			70	30			32	0.006	170[e]
10	CH	anthracene	3	8	53		39	46	15		33	0.005	200[e]
11	NH	phenoxazine	4	15			28	72			22	0.003	330[e]
12	NH	acridone	3	12	45		19	35	46		60	0.016	63[e]
13	NH	pyridoindole	4	15			58	42			38	0.004	250[e]
14	O	xanthenone	3	16			46	54			38	0.005	200[e]
15	S	thioacridone	5				100				18	0.005	200[e]
16	NH	phenothiazine	too fast to measure										500[e]

[a] Fraction of the equilibrium absorbance change accounted for in the kinetic analysis. [b] Average dissociation time constant ($\tau = \Sigma_r A_r \tau_r$). [c] Mean dissociation rate ($\tau^{-1} = 1/\tau$). [d] Data from ref 14. [e] Lower limit to mean dissociation rate.

Table 3. Equilibrium and Biological Properties of Tricyclic Carboxamides Studied

compound					log K^c		Unwinding	IC_{50} L1210[e]	ILS (%)[f]	
no.	peri	chromophore	R_m[a]	pK^b	(AT)	(GC)	angle[d]	(nM)	P388	LL
1	NH^+	9-aminoacridine, $n = 2$	−1.11	8.30	7.08	7.55	17	15	98	NA
2	NH^+	9-aminoacridine, $n = 4$	−0.89	8.30[g]	7.49	7.43	17[h]	430		
3	N	acridine, $n = 2$	−0.20	3.54	6.12	6.54	21	98	91	(6)
4	N	acridine, $n = 4$	0.01	3.54[g]	6.47	6.40	21[h]	160	143	100
5	N	9-methylacridine	−0.33	4.34	6.48	5.97	16	150	NA	
6	N	9-phenylacridine	0.26	3.49	5.84	5.98	17	1370	99	
7	N	phenazine, $n = 2$	−0.29	0.84	5.74	6.04	18	1715	88	57
8	N	phenazine, $n = 4$	−0.11	0.84[g]	6.30	6.34	18[h]	1170	49	NA
9	CH	acridine-1-carboxamide	−0.57	4.24	6.04	6.43	12	17000	NA	NA
10	CH	anthracene	−0.02		5.78	5.25	8	5300	NA	NA
11	NH	phenoxazine	0.07		5.20	5.39	10	4000	NA	
12	NH	acridone	−0.28		5.04	5.40	14	1700	NA	
13	NH	pyridoindole	−0.29		5.34	5.26	19	6600	NA	
14	O	xanthenone	−0.26		5.29	5.05	16	6500	NA	
15	S	thioacridone	−0.16		5.51	5.18	12	6300	NA	
16	NH	phenothiazine	0.13		5.78	5.83	10	4100	NA	

[a] Chromatographic measure of lipophilicity; data from refs 3, 8, and 13. [b] Data from refs 3, 8, and 13. [c] Binding constant to poly[d(A−T)] or poly[d(G−C)], determined by ethidium bromide displacement; data from refs 3, 8, and 13. [d] Helix unwinding angle; data from refs 3, 8, and 13. [e] Concentration of drug that inhibits growth of L1210 cells by 50%; data from refs 3, 8, and 13. [f] Increase in life span of drug-treated mice bearing P388 leukemia or Lewis lung carcinoma; data from refs 3, 8, and 13. [g] Estimated from measured value of ethyl homologue. [h] Estimated from measured value of propyl homologue.

mides (compounds **14** and **15**), which have an oxygen or sulfur atom in the peri position, undergo minimal, if any, changes in wavelength on binding, and the pyridoindolecarboxamide, **13**, has a bathochromic effect similar to that of the acridine-4-carboxamides. When sequestered into SDS micelles, absorption maxima

generally move to slightly longer wavelengths, but there is little effect on molar extinction coefficients (Table 1). As a prerequisite to the use of the detergent sequestration technique for measuring dissociation kinetics, we verified spectrophotometrically that adding SDS to preformed complexes at equilibrium causes their complete dissociation (data not shown).

Kinetic Measurements. Kinetic measurements were made at, or close to, the wavelength of maximum absorption of the ligand in SDS, where the differential absorbance between DNA-bound versus micelle-bound ligand is greatest (see Table 1). No time-resolvable changes were seen when solutions of free ligand and detergent were mixed in the stopped-flow instrument, indicating that the changes observed in the presence of DNA can be attributed solely to dissociation of the DNA−ligand complexes. The multiple first-order dissociation curves observed were deconvoluted into their exponential components as previously described.[20] Data from 10 kinetic runs were analyzed separately and the results averaged. Table 2 gives the resolved time constants, τ, their associated amplitudes, A, and the sum of the values of the individual amplitudes expressed as a proportion of the equilibrium absorbance change, F, for all the compounds studied. Where F is ≥85% we take it to indicate that, within experimental error, all of the steps within the dissociation process are within the time range of the instrument. Lower values of F indicate that some kinetic processes are occurring faster than can be detected. The arithmetic mean time constants and mean dissociation rates are also included in Table 2 as single kinetic measures to aid in intercompound comparisons and to assist in identifying relationships between kinetics and thermodynamics, biological activity, and ligand structure. At the DNA-to-ligand input ratio and DNA concentrations used, the observed kinetic profiles likely represent dissociation from the highest affinity sites of each ligand, which in the case of DACA and 9-amino-DACA is known to be at GC-rich sequences.[8,15]

The measured dissociation curves for the acridine-4- and phenazinecarboxamides, all of which possess a nitrogen atom peri to the side chain, account fully for the equilibrium absorbance changes observed on binding (Table 2). The dissociation profile for DACA, **3**, consists of four exponentials, which reduces to three, by loss of the slowest component, on lengthening the distance between the carboxamide nitrogen and the *N*,*N*-dimethylammonium group by two methylene units (compound **4)**. This finding parallels the signal behavior within the corresponding 9-amino series, compounds **1** and **2**,[14] where the slowest component in the 9-amino-DACA dissociation spectrum has been identified with the guanine−side chain interaction.[16] Thus, we infer that the DACA side chain is able to make similar hydrogen bonding interactions with guanine O6/N7 atoms to 9-amino-DACA[16] and that these contacts are not accessible to the longer side chain homologues in the acridinecarboxamide series. The average dissociation rate for DACA is some 2.5-fold faster than that of 9-amino-DACA, which accords with DACA's lower binding affinity, resulting from it being a mono- rather than a di-cation (see Table 3 and Crenshaw et al.[19]). The thermodynamic data of Crenshaw et al.[19] enable us to calculate an average bimolecular association rate constant to calf thymus DNA under our experimental conditions of 1.6×10^6 M^{-1} s^{-1} for DACA and 3.5×10^6 M^{-1} s^{-1} for 9-amino-DACA. Thus, it appears that having a positive charge on the chromophore doubles the rate of ligand binding.

The 9-methyl and 9-phenyl acridine-4-carboxamides, compounds **5** and **6**, also dissociate by a full four-component kinetic pathway, indicating that they share the binding mechanism of DACA and 9-amino-DACA. However, the lifetimes of the complexes of both these ligands are longer than that of DACA and, notwithstanding their neutral chromophores, their complexes are even more kinetically stable than that of their 9-amino counterpart. The effect is quite pronounced, since the DNA binding constants of **5** and **6** are 35-fold lower than that of 9-amino-DACA, yet their average dissociation rates are some 1.5-to-3-fold *slower*. It seems likely that the explanation for this lies in the fact that the 9-methyl and 9-phenyl substituents have to pass through the helix during dissociation, a "threading" process that is well-known to slow dissociation in related 9-anilinoacridine-4-carboxamides.[21] The finding that the 9-phenyl substituent has a greater effect on dissociation rate than a 9-methyl group is sterically consistent with this view. The difference in kinetic behavior of the paired phenazines **7** and **8** once again matches that of 9-amino-DACA and its side chain homologue, which strongly suggests that the *N*,*N*-dimethylaminoethylguanine interaction extends to the phenazinecarboxamides as well. The kinetic and thermodynamic properties of the complexes of the phenazine- and acridine-4-carboxamides, compounds **3**, **4**, **7**, and **8**, are practically indistinguishable (Tables 2 and 3).

The kinetic profiles for dissociation of the acridine-1- and anthracenecarboxamides, compounds **9** and **10**, in which the peri group is CH, contrast sharply with those of the foregoing compounds in that only about one-third of the reaction falls in the stopped-flow time range (Table 2). Moreover, each compound has lost the slowest component in its relaxation spectrum, and its average dissociation rate is at least 8- to-10-fold faster than that of DACA. Clearly, although both ligands are good intercalating agents (see Table 3), their mechanism of dissociation is different from that of the 9-aminoacridine-4-, acridine-4-, and phenazinecarboxamides, and the kinetic data suggest that the *N*,*N*-dimethylammonium groups of their side chains are no longer able to interact favorably with the guanine O6/N7 atoms. Similar conclusions can be drawn for the mechanism of dissociation and the side chain−DNA interactions of the phenoxazine-, acridone-, pyridoindole-, and phenothiazinecarboxamides, compounds **11**−**13** and **16**, all of which bear a peri NH group. The phenothiazinecarboxamide, **16**, dissociates so fast that none of the kinetic profile can be captured by the stopped-flow spectrophotometer; we see only two fast components in the case of the phenoxazine- and pyrido-indolecarboxamides, **11** and **13**, which sum to 22% and 38% of the equilibrium absorbance change, and for the acridonecarboxamide, **12**, the slowest "signal" transient is missing in a deconvoluted spectrum that accounts for 60% of the observed reaction (Table 2). The average dissociation rate for the acridonecarboxamide is at least 3-fold faster

than that of DACA, whereas for the other compounds in the "peri NH" group, this factor rises to between at least 12- to- 25-fold. In the case of the phenothiazinecarboxamide, **16**, the extraordinarily rapid kinetics may be attributable, at least in part, to the fact that the ring is not flat but butterfly-shaped.[22] Nevertheless, this buckling of the chromophore does not inhibit intercalative binding.[13]

The xanthenone and thioacridonecarboxamides, compounds **14** and **15**, possess oxygen and sulfur atoms peri to the side chain, and their DNA complexes dissociate very fast with only 38% and 18%, respectively, of the reaction occurring in the stopped-flow time range (Table 2). Both have average dissociation rates at least an order of magnitude larger than that of DACA, their kinetic profiles having only one or two resolvable components. Thus, given their kinetic behavior, we conclude that the carboxamide side chain is unable to bond successfully with the guanine O6/N7 atoms in complexes of these heterocyclic aromatic ketones.

Discussion

Our principal concern in this study was to discover whether the *N*,*N*-dimethylaminoethyl side chain of a range of intercalated tricyclic carboxamides with neutral chromophores can form hydrogen-bonding interactions with the O6/N7 atoms of guanine in a manner similar to that of 9-amino-DACA.[16−18] If this was so, a second question was whether this property correlates with the known structural requirement for a nitrogen atom peri to the side chain that is a prerequisite for biological activity of these compounds.[8,13] To this end we used the detergent sequestration technique to investigate the kinetics of dissociation of their DNA complexes, a method that has enabled detection of the side chain−guanine interaction in solution by observation of the complexity of the dissociation spectrum.[14,16,17,21] We have found that in the phenazine and acridine-4-carboxamide series the kinetic analyses indicate that the *N*,*N*-dimethylaminoethyl side chain does interact with guanine. By contrast, when the side chain is peri to a CH group, as in the acridine-1-carboxamide and anthracene compounds, **9** and **10**, the side chain fails to make this interaction. An important difference between these two ligand classes is that, like 9-amino-DACA,[16−18] the carboxamide groups of the acridine-4- and phenazine compounds can lie in the plane of the chromophore, whereas in the case of the acridine-1- and anthracene ligands, steric interaction with the peri hydrogen atom prevents the carboxamide from being coplanar. Thus, we conclude that coplanarity of the carboxamide and chromophore is a prerequisite for effective side chain−guanine interactions among the linear tricyclic carboxamides such as DACA and its phenazine analogues.

Given this conclusion, the question arises as to the detailed nature of the side chain−DNA interactions for DACA and its analogues, and whether they are distinguishable from those in the 9-amino-DACA complexes. The answer attracts added importance since DACA is a dual topoisomerase I/II poison, unlike 9-amino-DACA, and there are suggestions that its superior activity against solid tumors may derive, in part, from its capacity to poison both topoisomerases I and II simultaneously. Indeed, much effort is currently expended on the development of dual topoisomerase I/II poisons with the view to discovering novel agents with broad solid tumor activity.[23−25] Unfortunately, we have been unable to answer the key question directly by determining the crystal structure of an intercalated DNA complex of DACA or its phenazine analogue. Attempts to crystallize these and related agents with $d(CGTACG)_2$ have yielded an unusual quadruplex which does not add to our understanding of their complexes with duplex DNA,[26−28] and other DNA sequences have failed to give diffracting crystals. Thus, in the absence of direct structural information, and in light of our present findings and the known similarities in the DNA binding characteristics of DACA and 9-amino-DACA, it is appropriate to consider potential DNA−DACA side chain interactions in terms of minimal perturbations to the known structure of the 9-amino-DACA-DNA complexes.

Since the chromophore of DACA is uncharged, and its peri nitrogen atom thus unprotonated,[16−18,29] its carboxamide group can lie in one of two orientations in the plane of the chromophore, viz, with the carbonyl oxygen pointing toward the peri nitrogen, the cis position, or away, the trans position. In these conformations the resonance stabilization between carboxamide and chromophore is maximized, and in the trans position there is the added possibility that the carboxamide NH group can form an internal hydrogen bond with the peri nitrogen. The relative energies of these two conformers is unknown, but in the case of the neutral forms of 9-amino-DACA and compound **2**, the internal hydrogen bond, i.e., the trans conformer, is observed in crystal structures of the free ligands.[29] Using molecular mechanics, Hudson et al.[29] calculated that the trans conformation is more stable than the cis by about 2.5 kcal/mol. Given that the electron density on the N10 atoms of DACA and phenazinecarboxamide **7** is less than that on the ring nitrogen in 9-amino-DACA, as revealed by their p*K* values of 3.5, 0.84, and 8.3, respectively (Table 3), it is probable that in the acridine and phenazine cases the two conformers are even more comparable in energy. Thus, it is necessary to consider both orientations in possible models of the DACA and phenazine complexes, since each might be populated depending on the fine details of the energetics of the chromophore base pair, bridging water molecule and side chain−guanine interactions.

Accordingly, in Figure 2 we present potential models for the DNA−DACA complex, with the carboxamide group in the cis and trans orientation and the side chain hydrogen bonding to guanine, which are consistent with our kinetic findings and the known DNA binding properties of DACA. In this figure, the geometry of the DNA and the positions of the acridine ring and the phosphate-bridging water molecule are unchanged from that found in the crystal structure of the 9-amino-DACA-$d(CGTACG)_2$ complex (Figure 2a).[16] In the case of the cis orientation, Figure 2b, the carboxamide lies fully in the plane of the chromophore, and the *N*,*N*-dimethylammonium group hydrogen bonds to the N7 of guanine without unacceptable van der Waals interactions. In Figure 2c, the carboxamide is in the trans orientation with an internal hydrogen bond to the acridine N10 nitrogen. This rotation flips the side chain

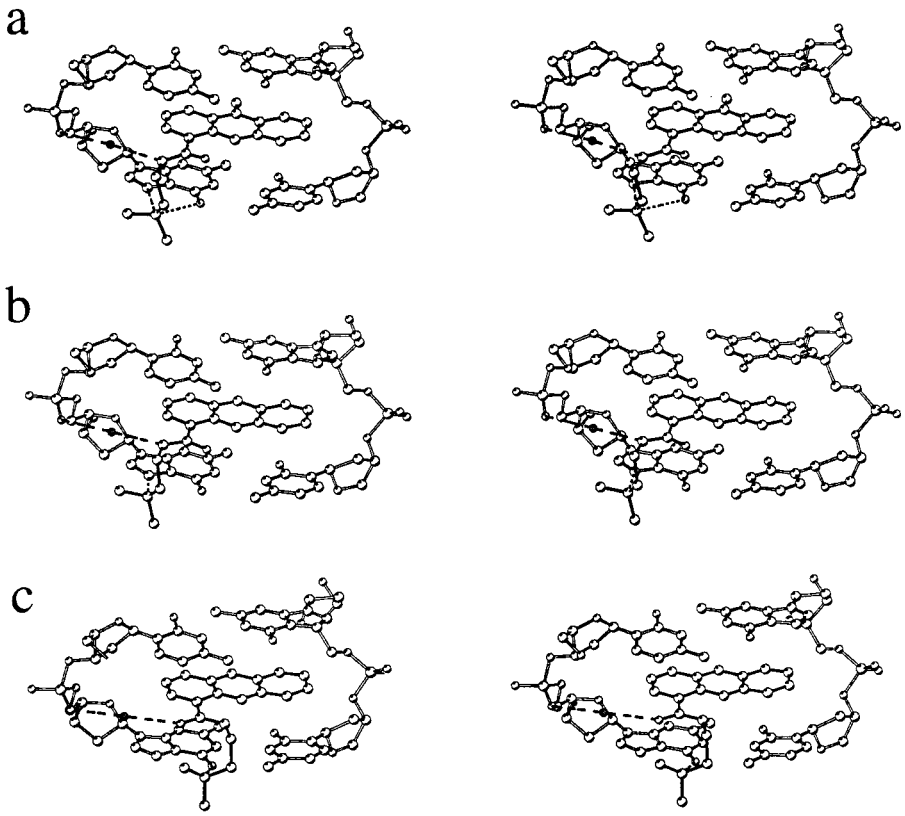

Figure 2. Stereoviews of the crystal structure and molecular models of d(CpG)$_2$/9-amino-DACA and d(CpG)$_2$/DACA complexes. (a) Crystal structure of the 9-amino-DACA complex taken from Adams et al.[16] showing side chain−guanine interactions and water-bridged carboxamide-to-phosphate interaction. (b) Proposed model of DACA complex with carboxamide group in cis orientation showing side chain−guanine and water-bridged phosphate interaction. (c) Proposed model of DACA complex with carboxamide group in trans orientation showing side chain−guanine and water-bridged phosphate interaction.

toward the center of the helix, and now the dimethylammonium group is able to hydrogen bond to the guanine O6 oxygen, again without unacceptable van der Waals contacts. In both models, the phosphate-bridging water molecule hydrogen bonds to the 4-carboxamide group: to the NH group in the cis orientation and to the carbonyl oxygen in the trans orientation. Thus, it appears likely that DACA has the capacity to form DNA complexes with the carboxamide in either the cis or trans orientation, while at the same time maintaining the phosphate-bridging water molecule and dimethylammonium−guanine interaction. The trans conformation is unavailable to 9-amino-DACA, and perhaps it is this feature that underlies the ability of DACA to poison both topoisomerases I and II.

The phenoxazine-, acridone-, pyridoindole-, and phenothiazinecarboxamides, compounds **11**−**13** and **16**, share an NH structural motif which, in principle, would not necessarily prevent the peri carboxamide from being coplanar with the chromophore since the carbonyl oxygen could form an internal hydrogen bond in the cis configuration. Indeed, the crystal structure of the free phenothiazinecarboxamide, **16**, reveals the carboxamide group to be doing just this, although in this case the tricyclic ring is buckled.[22] However, notwithstanding this hydrogen-bonding potential, the side chains of none of these compounds appear to interact with guanine. One possible explanation is that the internal hydrogen bond is intrinsically weak, since the NH group is uncharged, and made yet weaker in the DNA intercalation complex where π-orbital stacking with the base pairs and interaction with the electrostatic field derived from the phosphate charges can modify chromophore orbital energies and electron distributions.[30] Clearly, for these ligands the trans carboxamide orientation is prohibited by steric collision of the two NH groups, which will force an angular conformation between chromophore and carboxamide. The cis carboxamide configuration in the xanthenone- and thioacridonecarboxamides, **14** and **15**, which also fail to bind guanine, is not likely to lead to a stable internal hydrogen bond given the degree of electronegativity of their heteroatoms in chromophores of this type. Moreover, in the case of the thioacridone, the bulky sulfur atom would sterically hinder both coplanar carboxamide conformations. Thus, taking into consideration all of the available data, it appears that resonance stabilization alone does not provide sufficient energy to hold the carboxamide coplanar with the chromophore in the DNA complex of the tricyclic carboxamides and that some form of internal hydrogen bond is required.

The compounds studied fall into two distinct groups when comparing inter-relationships between their capacity to form hydrogen-bonding interactions with guanine, the kinetic stability of their DNA complexes, and their biological activity. In the 9-aminoacridine series, the correlations are strong between cytotoxic potency, in vivo antileukemia activity, capacity to inhibit topoisomerase II, ability of the side chain to interact with guanine, and the absolute lifetime of the complex.[4,5,14,16,17] As mentioned previously, these relationships have been taken to imply that the structures of

the 9-aminoacridine-4-carboxamide/DNA complexes revealed by crystallography represent the DNA lesion that poisons topoisomerase II.[16−18] Consistent with this set of correlations, we find that the side chains of neutral tricyclic carboxamides which lack a peri nitrogen are unable to make bonding interactions with guanine, presumably because their carboxamide groups are unable to lie stably within the plane of the chromophore and that the compounds are biologically inactive (cf. Tables 2 and 3). In the other grouping falls the phenazine and acridine-4-carboxamides which share some of these interdependencies but not others. For example, in both these series, the lead compounds, DACA and **7**, fit the paradigm in so much as their side chains interact with guanine, they inhibit topoisomerases, and they are active against both leukemic and solid tumor models in vivo.[5,8−10,13] However, when the distance between the 4-carboxamide and *N*,*N*-dimethylamino group is lengthened, the interaction with guanine is lost, but now biological activity is retained (cf. Tables 2 and 3). Thus, while the structural criteria for inhibition of topoisomerase II are met by the *N*,*N*-dimethylaminoethyl homologues of these agents, it seems that those with longer side chains are able to access a separate cytotoxic mechanism.

Finally, we note that for DNA intercalating agents a broad positive correlation has been reported between cytotoxic potency and DNA residence times (see, for example, ref 31). The linear tricyclic carboxamides studied to date fall into this broad correlation, with the 9-aminoacridine-4-carboxamides and their 5-substituents being potent cytotoxins (IC_{50}s, P388 leukemia in the range 0.5−15 nM),[14,17] the neutral chromophore analogues with side chain−guanine interactions being less cytotoxic (compounds **3**, **5**−**7** this work, IC_{50}s 100−1700 nM[13]), and compounds with even faster dissociation kinetics (compounds **9**−**16**, this work) being the least cytotoxic (IC_{50}s 1700−17000 nM[13]).

Experimental Section

Abbreviations. DACA, *N*-[2-(dimethylamino)ethyl]acridine-4-carboxamide; 9-amino-DACA, 9-amino-[*N*-(2-dimethylamino)ethyl]acridine-4-carboxamide; SDS, sodium dodecyl sulfate.

Materials. All tricyclic carboxamides were synthesized and purified in the Auckland Cancer Society Research Centre as previously described.[7,13] They were used as their hydrochloride salts, purity being assayed by thin-layer chromatography, and stock solutions prepared in ethanol and kept at −20 °C. Calf thymus DNA was purchased from the Sigma Chemical Company, St. Louis, MO.

Spectrophotometry. Molar extinction coefficients at the wavelength of maximum absorption in the visible spectrum were determined for the tricyclic carboxamides free in solution, bound to calf thymus DNA, and when sequestered into SDS micelles, using a Carey 219 UV/visible spectrophotometer. Measurements were made in 0.1 SHE buffer [2 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 10 μM EDTA, and 99.4 mM NaCl, pH 7.0] at 20 °C. Spectra were recorded at a drug concentration of 50 μM, with DNA and micelle-bound spectra determined in the presence of 1 mM calf thymus DNA (nucleotide pairs) and 10 mM SDS (monomer concentration), respectively.

Stopped-Flow Kinetics Measurements. Kinetic measurements were performed on a Dionex D110 stopped-flow spectrophotometer with a coupled DC amplifier, double-beam storage oscilloscope, and personal computer. The spectrophotometer was fitted with a 20 mm light path optical cuvette, giving the apparatus a dead time of about 2 ms, and was operated in transmittance mode. Data were collected and processed using software[20] that allows a wide choice of data sampling frequencies, up to every 0.1 ms. The spectrophotometer was operated with a time constant of 0.1 ms and an optical bandwidth of 3 nm. To investigate the kinetics of dissociation of tricyclic carboxamide−DNA complexes, we used the detergent sequestration technique originally described by Muller and Crothers,[32] and used by us previously,[14,17,21] wherein the free ligand maintaining the reversibly bound complex is rapidly sequestered into SDS micelles. Generally, solutions of DNA−ligand complexes containing 400 μM DNA (nucleotide pairs) and 20 μM ligand in 0.1 M SHE buffer were mixed with an equal volume of 20 mM SDS (monomer concentration) in the same buffer at 20 °C. On occasions where the maximum difference in molar extinction coefficient between DNA-bound and micelle-bound ligand was found to be less than 3×10^3 (compounds **10**, **11**, **13**, and **15**), the concentration of DNA and ligand in the initial complex was raised to 800 μM and 40 μM, respectively. Thus, the final DNA base pair-to-ligand input ratio was 20:1 in all cases, with the final DNA concentration being either 200 μM or 400 μM. The molecular weight of the DNA was lowered by sonicating solutions containing 2 mg/mL of DNA in 0.2 M SHE buffer at 0 °C for 5 min, followed by exhaustive dialysis into 0.1 M SHE buffer. Solutions were freed of particulates by passing them through 0.45 μm Millipore filters.

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