# Influence of Substituent Modifications on DNA Binding Energetics of Acridine-Based Anticancer Agents†

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ABSTRACT: The DNA binding energetics of a series of analogues derived from the anticancer agent *N*-[2-(dimethylamino)ethyl]-9-aminoacridine-4-carboxamide (AAC) are investigated. The effects of substituent modification at the C5 position of the acridine chromophore on the interaction of AAC with DNA are determined using spectrophotometry and isothermal titration calorimetry (ITC). The binding affinity and binding free energy associated with the interaction of AAC with DNA are significantly enhanced upon substitution at the C5 position. Energetic profiles describing ligand−DNA complex formation obtained from ITC indicate that C5 substitution significantly enhances binding enthalpy relative to the parent AAC. In many cases, the enhanced binding enthalpies of the C5-substituted analogues correlate with anticancer activity. Because of the cationic character of AAC and its analogues, the DNA binding properties of these compounds are dependent on ionic strength. To quantitate the ionic contributions to complex formation, the observed binding free energy of each compound is parsed into its polyelectrolyte and nonelectrostatic components. Enhanced nonelectrostatic contributions to the overall binding free energies observed with C5-substituted analogues relative to the parent AAC suggest that C5 substituents play a critical role in directing both thermodynamic mechanisms associated with complex formation and molecular interactions between the ligand and its DNA binding site. These studies have demonstrated that substitution of AAC at the C5 position results in enhanced DNA binding affinity and energetics.

The DNA intercalating agent *N*-[2-(dimethylamino)ethyl]-9-aminoacridine-4-carboxamide (AAC)[1] is a potent cellular cytotoxin that exhibits excellent in vivo activity against disseminated cancer cell lines such as leukemia but relatively low activity against remotely implanted solid tumors such as Lewis lung carcinoma (*1*). To elicit its biological activity, AAC modulates a critical step in the catalytic cycle of the cellular enzyme topoisomerase II such that normally transient double-stranded DNA breaks are allowed to accumulate to cytotoxic levels (*2*−*4*). Studies of equilibrium binding to synthetic DNAs (*5*, *6*) and DNase footprinting (*7*) have shown that AAC binds preferentially to GC-rich sequences; the 4-carboxamide side chain has been identified as the primary determinant of this sequence specificity. The GC selectivity of AAC and a number of analogues has been correlated with in vivo anticancer activity (*7*). Furthermore, kinetics studies of AAC have revealed strong correlations between longer ligand residence time on the target DNA and in vivo antitumor activity (*8*, *9*).

The parent AAC is dicationic at neutral pH, with a charged N10 acridine ring nitrogen (p$K_a$ 8.3) and 4-carboxamide side chain distal nitrogen (p$K_a$ 10) (*5*). Structure−activity relationships of AAC analogues substituted on the acridine chromophore with groups of varying electronic character, hydrophobicity, and steric bulk revealed that substitution at the C5 position (Figure 1) was an important determinant of biological activity (*5*). Enhanced in vitro antileukemic activity of AAC was observed upon C5 substitution, with several analogues exhibiting a 10-fold or greater enhancement in cytoxicity relative to the unsubstituted parent compound. Although possessing potent in vitro efficacy, the range of in vivo activity of the hydrophilic, dicationic 9-aminoacridine-4-carboxamides appeared to be limited by their poor cellular distribution that prevented them from reaching remote targets. To broaden the spectrum of activity, analogues were developed in which electron withdrawing groups, such as trifluoromethyl and methylsulfonyl, were added to the C5 position of the acridine chromophore of AAC. These groups strengthened the acidity of the acridine ring by up to three units, yielding predominantly monocationic analogues at neutral pH. The uncharged chromophores improved biodistribution, as evidenced by the excellent activity of the monocationic analogues against the remotely implanted solid cell Lewis lung carcinoma, an experimental tumor in mice (*5*).

Previous studies with related anilinoacridines suggested a correlation between thermodynamic properties associated with ligand−DNA complex formation and anticancer activity

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[1] Abbreviations: AAC, *N*-[2-(dimethylamino)ethyl]-9-aminoacridine-4-carboxamide; DACA, *N*-[2-(dimethylamino)ethyl]acridine-4-carboxamide; ITC, isothermal titration calorimetry.

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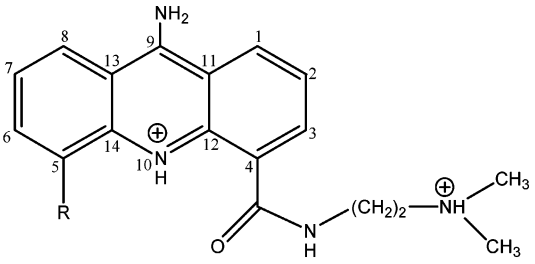

| R | Compound |
|---|---|
| H | AAC (parent) |
| $CH_3$ | 5-$CH_3$ AAC |
| $OCH_3$ | 5-$OCH_3$ AAC |
| $NH_2$ | 5-$NH_2$ AAC |
| $CF_3$ | 5-$CF_3$ AAC |
| $SO_2CH_3$ | 5-$SO_2CH_3$ AAC |

FIGURE 1: Structures of *N*-[2-(dimethylamino)ethyl]-9-aminoacridine-4-carboxamide (AAC) and analogues. R represents the C5 substituent on the acridine chromophore.

(*10*). In the present work, changes in the DNA binding energetics of AAC resulting from C5 substitution are investigated to further elucidate the relationship between energetics and anticancer activity. Since protonation of the acridine ring varies considerably with substituent modification, effects of ionic strength on DNA binding affinities and overall binding free energies are investigated. The nonelectrostatic contributions to overall binding free energies are determined to help identify the intrinsic characteristics of each ligand exclusive of its charge, such as molecular interactions between the ligand and its DNA binding site, that stabilize the ligand−DNA complex.

## EXPERIMENTAL PROCEDURES

*Buffer Preparation.* Buffers were prepared in Milli-Q Ultrapure water and filtered through 0.45 $\mu$m filters (Millipore). Buffer designations are as follows: BPES, 10 mM total phosphate (6.13 mM $Na_2HPO_4$ and 3.87 mM $NaH_2PO_4$), 1 mM disodium ethylenediaminetetraacetic acid ($Na_2$-EDTA), and 100 mM NaCl, pH 7.0.

*Drug Preparation.* All compounds in this study were synthesized as the chloride salts as previously described (*5*, *6*, *11*). Stock drug solutions were prepared in BPES buffer immediately prior to use. Optical data were collected on a Cary 400 UV−vis spectrophotometer (Varian) equipped with a Lauda R61 circulating water bath. Molar absorptivities of the free and bound species of AAC and selected analogues were determined experimentally at the wavelength of maximal absorption as previously described (*12*) and are given in the Supporting Information.

*DNA Preparation*. Calf thymus DNA (Sigma Co.) was prepared for DNA binding studies using the method described by Chaires et al. (*13*). DNA concentrations were determined by UV absorbance at 260 nm using a molar absorptivity of 13 200 $M(bp)^{-1}$ $cm^{-1}$ (*14*).

*DNA Binding Studies.* Absorbance titrations were carried out in 10 cm cylindrical quartz cells at a constant temperature of 25 °C. DNA binding isotherms were obtained by the addition of aliquots of ligand to fixed concentrations of DNA. To minimize aggregation, the level of free ligand was maintained below 5 $\mu$M throughout all binding studies.

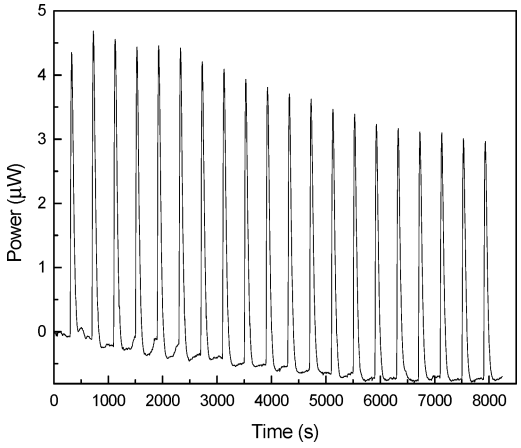


FIGURE 2: Representative ITC ligand−DNA titration.

Concentrations of bound ligand were determined by monitoring changes in the absorbance of ligand. Theoretical fits of the binding data were calculated using a nonlinear least-squares analysis (Origin, Microcal Inc.) of the noncooperative neighbor exclusion model of McGhee and von Hippel (*15*)

$$\frac{r}{C_f} = K_{obs}(1 - nr)\left[\frac{1 - nr}{1 - (n-1)r}\right]^{(n-1)}$$

where $r$ is the molar ratio of bound ligand per base pair, $C_f$ is the free ligand concentration, $K_{obs}$ is the intrinsic binding constant for the interaction of the ligand with an isolated DNA site, and $n$ is the neighbor exclusion parameter, or the number of base pairs per ligand binding site.

Using the spectrophotometrically determined $K_{obs}$ values, binding free energies were determined using the equation

$$\Delta G_{obs}^{\circ} = -RT \ln K_{obs}$$

*DNA Binding Energetics.* Isothermal titration calorimetry (ITC) was performed at 25 °C for each compound using a Model 4200 Calorimetry Sciences Corporation isothermal titration calorimeter. All solutions were thoroughly degassed prior to use. To obtain enthalpies of binding ($\Delta H_{obs}^{\circ}$), the calorimeter cell was filled to capacity (1.328 mL) with calf thymus DNA (1 mM (bp)), and ligand (1 mM) was injected in 20 aliquots of 10 $\mu$L each from a 250 $\mu$L microsyringe at 300 s intervals. Because the ligand was titrated into a solution with a large excess of DNA binding sites, all added ligand was considered bound. Raw data (Figure 2) were obtained as power ($\mu$W) versus time (s); following baseline adjustment, the heat of each injection was obtained by integrating each titration peak using BindWorks (Calorimetry Sciences Corporation). Because the heats of injection were relatively constant, the heat of reaction could be obtained directly without the use of model-dependent curve fitting (*16*). To calculate enthalpies of reaction, the average heat of injection was divided by the number of moles of ligand added per injection. The heat of dilution was determined by titrating the drug into BPES; the enthalpy of dilution was then subtracted from the enthalpy of reaction to give the enthalpy of binding. Entropies of binding were determined from the equation

$$\Delta S_{obs}^{\circ} = \frac{\Delta H_{obs}^{\circ} - \Delta G_{obs}^{\circ}}{T} \qquad (3)$$

Table 1: DNA Binding Profiles of AAC and C5-Substituted Analogues Including p$K_a$ Values, Equilibrium Binding Constants, and Binding Site Sizes[a]

| compound | p$K_a$[b] | $K_{obs}/10^5$ ($M^{-1}$)[c] | $n$ (base pairs)[c] |
|---|---|---|---|
| AAC (parent) | 8.30 | 4.1 ± 0.1 | 2.7 |
| 5-$CH_3$ AAC | 8.01 | 22 ± 0.8 | 2.6 |
| 5-$OCH_3$ AAC | 7.80 | 15 ± 0.5 | 2.8 |
| 5-$NH_2$ AAC | 7.41 | 8.9 ± 0.3 | 2.7 |
| 5-$CF_3$ AAC | 5.89 | 4.6 ± 0.2 | 2.0 |
| 5-$SO_2CH_3$ AAC | 5.15 | 1.2 ± 0.1 | 2.3 |

[a] For binding to calf thymus DNA in 100 mM NaCl BPES at 25 °C and pH 7.0. The top portion is compounds that are predominantly dicationic at neutral pH, and the bottom is compounds that are monocationic. [b] Determined spectrophotometrically; data from ref *5*. [c] Obtained by fitting the McGhee and von Hippel neighbor exclusion model (eq 1) to absorbance titration data.

*Determination of Electrostatic Binding Parameters.* The influence of ionic strength on binding affinity was investigated using the fixed ratio method (*17*). The ionic strength of the binding solution was varied by titration of a concentrated sodium chloride solution (5 M) into a 10 cm path length cylindrical quartz cell containing a ligand−DNA complex in a ratio of one ligand bound per 10 base pairs. Absorbances were recorded at the $\lambda_{max}$ of the free ligand and were corrected for dilution effects. Binding constants were calculated at each salt concentration using the neighbor exclusion model (eq 1). Data were plotted as ln $K_{obs}$ versus ln[$Na^+$]; the slope $S$ of each plot was determined by linear least-squares analysis. These slopes were used to calculate the charge of each ligand and the polyelectrolyte contribution to the overall binding free energy.

## RESULTS

*Equilibrium Binding Studies.* DNA binding isotherms constructed for each compound from UV−vis titration data were subjected to nonlinear least-squares analysis of the neighbor exclusion model of McGhee and von Hippel to determine the intrinsic binding constant, $K_{obs}$, and the neighbor exclusion parameter, $n$ (Table 1). As expected, the dicationic AAC analogues were found to bind DNA more strongly than the monocationic analogues due to enhanced polyelectrolyte contributions of the dicationic species (*18*, *19*). Resonance-stabilizing electron-donating groups (i.e., methyl, methoxy, or amino) substituted at the C5 position were shown to enhance DNA binding affinity up to 5-fold relative to that of the parent AAC. The methyl group induced the greatest enhancement in binding affinity, followed by the methoxy group and the amino group, respectively. In contrast, substituting electron-withdrawing groups (i.e., trifluoromethyl or methylsulfonyl) at the C5 position produced variable effects on DNA binding affinity. When the trifluoromethyl group was substituted at C5, the DNA binding affinity of AAC was relatively unchanged. Conversely, addition of the methylsulfonyl group resulted in a 4-fold decrease in binding affinity relative to the parent compound. The neighbor exclusion parameter, $n$, calculated from binding isotherm data indicated binding site sizes of approximately three and two base pairs for the dicationic and monocationic analogues, respectively.

*Energetics of DNA Binding.* Energetic profiles, including overall binding free energies and enthalpy and entropy components, describing the interactions of AAC and each of the C5-substituted analogues with DNA are provided in Table 2. Overall binding free energies ($\Delta G_{obs}°$) calculated using experimentally determined intrinsic binding constants revealed that substitution at the C5 position produced favorable $\Delta G_{obs}°$ enhancements of up to 1 kcal/mol. The increased binding affinities of the dicationic analogues were translated into significant $\Delta G_{obs}°$ enhancements of 0.4−1.0 kcal/mol relative to that of the parent AAC, while the more weakly binding monocationic analogues exhibited binding free energies either equivalent to (5-$CF_3$ AAC) or slightly less favorable than (5-$SO_2CH_3$ AAC) the parent compound.

Table 2: Thermodynamic DNA Binding Profiles of AAC and Analogs[a]

| compound | $\Delta G_{obs}°$ (kcal $mol^{-1}$)[b] | $\Delta H_{obs}°$ (kcal $mol^{-1}$)[c] | $\Delta S_{obs}°$ (cal $deg^{-1}$ $mol^{-1}$)[d] |
|---|---|---|---|
| AAC | −7.7 ± 0.02 | −5.4 ± 0.01 | +7.7 ± 0.03 |
| 5-$CH_3$ AAC | −8.7 ± 0.04 | −6.5 ± 0.14 | +7.4 ± 0.14 |
| 5-$OCH_3$ AAC | −8.4 ± 0.03 | −6.5 ± 0.02 | +6.4 ± 0.04 |
| 5-$NH_2$ AAC | −8.1 ± 0.03 | −7.6 ± 0.18 | +1.7 ± 0.18 |
| 5-$CF_3$ AAC | −7.7 ± 0.04 | −5.7 ± 0.17 | +6.7 ± 0.18 |
| 5-$SO_2CH_3$ AAC | −6.9 ± 0.08 | −8.1 ± 0.05 | −4.0 ± 0.10 |

[a] For binding to calf thymus DNA in 100 mM NaCl BPES at 25 °C and pH 7.0. [b] $\Delta G_{obs}° = -RT \ln K_{obs}$. [c] Enthalpy values obtained from isothermal titration calorimetry. [d] Entropy values calculated from $\Delta S_{obs}° = (\Delta H_{obs}° - \Delta G_{obs}°)/T$.

The distinct effects of C5 substitution on the DNA binding energetics of AAC were demonstrated by parsing the observed binding free energy into enthalpy and entropy components. Binding enthalpies were found to vary over a range of 3 kcal/mol, from −5.4 kcal/mol for the interaction of the parent AAC with DNA to −8.1 kcal/mol for the 5-$SO_2CH_3$ analogue. Substitution at the C5 position of AAC was highly influential in determining binding enthalpy ($\Delta H_{obs}°$) and resulted in favorable increases of 0.3−2.7 kcal/mol in $\Delta H_{obs}°$ relative to the parent compound. While C5 substitution enhanced the binding enthalpy of AAC, it produced an unfavorable effect on binding entropy—the binding entropy ($\Delta S_{obs}°$) of AAC (+7.7 eu) was reduced up to 11.7 eu upon C5 substitution. Despite the reduction in $\Delta S_{obs}°$ resulting from C5 substitution, the DNA binding events of all but one analogue, 5-$SO_2CH_3$ AAC, remained entropically favorable, with $\Delta S_{obs}°$ values ranging from +1.7 to +7.1 eu.

*Polyelectrolyte Effects.* In accordance with polyelectrolyte theory, studies of the ionic strength dependency of ligand−DNA binding affinities provide data that may be used to parse the overall binding free energy ($\Delta G_{obs}°$) into its polyelectrolyte ($\Delta G_{pe}°$) and nonelectrostatic ($\Delta G_t°$) components. The intercalation of ligands, especially those bearing a positive charge, into DNA is accompanied by the displacement of monovalent cations that are territorially associated with DNA. This counterion displacement results in a favorable entropy of dilution that can substantially contribute to the overall binding free energy (*20*−*22*). The number of counterions released upon the binding of one ligand is equivalent to the effective charge of the ligand. Various relationships have been developed relating effective charge to the ionic strength dependence of binding affinities. Record and co-workers (*20*) were among the first to describe the relationship between binding affinity and ionic strength with

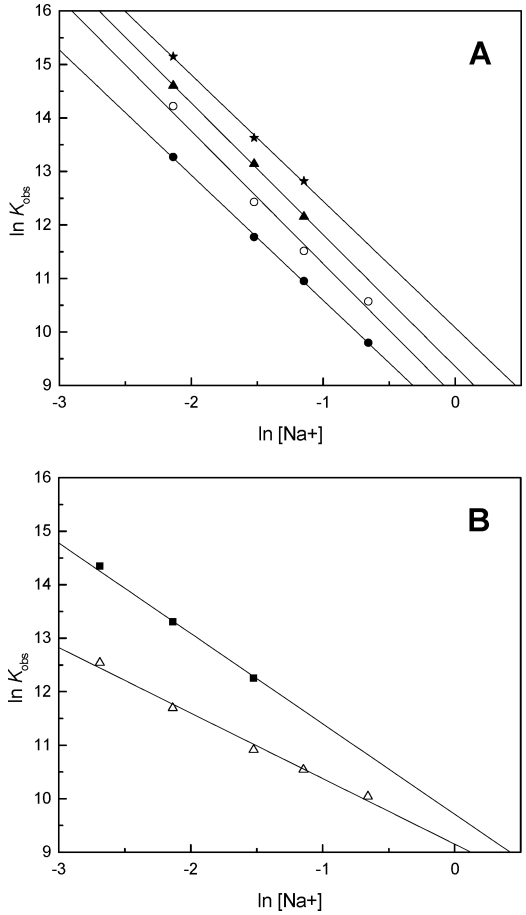


FIGURE 3: Ionic strength dependence of intrinsic binding constants for dicationic (A) and monocationic (B) aminoacridinecarboxamides at pH 7.0 and 25 °C. Symbols represent the following AAC compounds: (A) stars, 5-$CH_3$; closed triangles, 5-$OCH_3$; open circles, 5-$NH_2$; closed circles, 5-H (parent AAC); (B) squares, 5-$CF_3$ and open triangles, 5-$SO_2CH_3$. Solid lines represent the linear least-squares fit of each data set.

the equation

$$S = \frac{\delta \ln K_{obs}}{\delta \ln[Na^+]} = -Z\Psi \qquad (4)$$

where $Z$ is the number of counterions released per ligand binding, and $\Psi$ is a constant equal to the fraction of counterions associated per phosphate (0.88 for B-DNA). From the slope $S$ of a linear least-squares analysis of ln $K_{obs}$ versus ln[$Na^+$] (Figure 3), the number of counterions released from DNA upon ligand binding, and thus the charge on the ligand, can be determined. Record's equation predicts $S$ values of −0.88 and −1.76 for monocationic and dicationic ligands, respectively. However, this equation neglects the additional release of counterions that accompanies DNA perturbation upon formation of the intercalative complex. During the intercalation process, adjacent base pairs must move apart to accommodate an intercalating ligand. The distance between adjacent phosphates is increased; thus, the charge density of DNA is lowered, necessitating the release of counterions. Insertion of the intercalated ligand follows this conformational change. If the intercalator is positively charged, a release of additional counterions accompanies the ligand binding step due to ion pair formation between the ligand and the DNA. Wilson and Lopp (*21*) modified Record's polyelectrolyte theory to account for the additional counterion release brought about by the unwinding of the DNA helix during intercalative ligand binding

$$S = \frac{\delta \ln K_{obs}}{\delta \ln[Na^+]} = -2n(\Psi - \Psi^*) - Z^*\Psi^* \qquad (5)$$

where $n$ is the number of base pairs involved in the intercalation conformational change per ligand, equal to the McGhee−von Hippel neighbor exclusion parameter; $\Psi^*$ is a constant equal to 0.82 counterions associated per phosphate of I-DNA (DNA in its intercalation conformation); and $Z^*$ is the fraction of counterions released per phosphate from I-DNA upon ligand binding, which is equivalent to the effective charge of the ligand. For the monocationic and dicationic ligands of this study, eq 5 predicts $S$ values of −1.06 and −2.00, respectively.

Table 3: Effective Charge Determinations of AAC and Analogues, Calculated from Ionic Dependence of DNA Binding Affinities

| compound | $S^a$ | $Z^b$ | $Z^{*c}$ |
|---|---|---|---|
| AAC | −2.34 ± 0.03 | 2.7 ± 0.03 | 2.5 ± 0.03 |
| 5-$CH_3$ AAC | −2.36 ± 0.09 | 2.7 ± 0.10 | 2.5 ± 0.10 |
| 5-$OCH_3$ AAC | −2.46 ± 0.06 | 2.8 ± 0.07 | 2.6 ± 0.06 |
| 5-$NH_2$ AAC | −2.48 ± 0.18 | 2.8 ± 0.20 | 2.6 ± 0.19 |
| 5-$CF_3$ AAC | −1.69 ± 0.05 | 1.9 ± 0.06 | 1.8 ± 0.05 |
| 5-$SO_2CH_3$ AAC | −1.22 ± 0.06 | 1.4 ± 0.07 | 1.1 ± 0.05 |

[a] $S = \delta \ln K_{obs}/\delta \ln[Na^+]$; slopes of the linear fits of Figure 3. [b] $Z$ = effective charge calculated from the Record and Manning equation (eq 4). [c] $Z^*$ = effective charge calculated from the Wilson and Lopp equation (eq 5), which takes intercalative effects into account.

The calculated experimental values of $S$, $Z$, and $Z^*$ for each analogue are provided in Table 3. From the slopes of the ln $K_{obs}$ versus ln[$Na^+$] plots describing ionic strength dependency of binding affinities (Figure 3), the mean effective charge ($Z^*$) according to eq 5 of the 5-$CH_3$, 5-$OCH_3$, and 5-$NH_2$ AAC analogues and the parent AAC was found to be 2.5, indicative of dicationic species, whereas 5-$SO_2CH_3$ and 5-$CF_3$ analogues were confirmed to be monocationic, with a mean $Z^*$ of 1.4.

The slope $S$ of the ln $K_{obs}$ versus ln[$Na^+$] plot can also be used to calculate the polyelectrolyte contribution ($\Delta G_{pe}°$) to the overall DNA binding free energy. Record and Spolar (*22*) offer an expression relating the polyelectrolyte binding free energy to $S$ and the concentration of monovalent cation

$$\Delta G_{pe}° = -SRT \ln[Na^+] \qquad (6)$$

The polyelectrolyte ($\Delta G_{pe}°$) and nonelectrostatic ($\Delta G_t°$) contributions can be dissected from the overall binding free energy ($\Delta G_{obs}°$) according to the expression

$$\Delta G_{obs}° = \Delta G_{pe}° + \Delta G_t° \qquad (7)$$

The values of $\Delta G_{pe}°$ for the binding of each compound to DNA calculated using eq 6 indicated that the variation in overall binding free energy among the AAC and its analogues is a manifestation of both polyelectrolyte and nonelectrostatic effects (Table 4). Polyelectrolyte effects contributed an average of −3.3 and −2.0 kcal/mol to the overall binding free energies of the dicationic and monocationic analogues, respectively. The nonelectrostatic contributions to overall binding free energies calculated from eq 7 showed that

Table 4: Polyelectrolyte and Nonelectrostatic Components of Overall DNA Binding Free Energies for AAC and Analogues[a]

| compound | $\Delta G_{obs}^{\circ}$ [b] (kcal mol$^{-1}$) | $\Delta G_{pe}^{\circ}$ [c] (kcal mol$^{-1}$) | $\Delta G_{t}^{\circ}$ [d] (kcal mol$^{-1}$) | $\Delta\Delta G_{t}^{\circ}$ [e] (kcal mol$^{-1}$) |
|---|---|---|---|---|
| AAC | −7.7 | −3.2 | −4.5 | 0 |
| 5-$CH_3$ AAC | −8.7 | −3.2 | −5.5 | −1.0 |
| 5-$OCH_3$ AAC | −8.4 | −3.4 | −5.0 | −0.5 |
| 5-$NH_2$ AAC | −8.1 | −3.4 | −4.7 | −0.2 |
| 5-$CF_3$ AAC | −7.7 | −2.3 | −5.4 | −0.9 |
| 5-$SO_2CH_3$ AAC | −6.9 | −1.7 | −5.2 | −0.7 |

[a] For binding to calf thymus DNA in 100 mM NaCl BPES at 25 °C and pH 7.0. [b] $\Delta G_{obs}^{\circ}$ = overall binding free energy, calculated from $\Delta G_{obs}^{\circ} = -RT \ln K_{obs}$. [c] $\Delta G_{pe}^{\circ}$ = polyelectrolyte contribution to overall binding free energy at 100 mM NaCl, calculated from $\Delta G_{pe}^{\circ} = -SRT \ln[Na^+]$. [d] $\Delta G_{t}^{\circ}$ = nonelectrostatic component of the overall binding free energy, calculated from $\Delta G_{t}^{\circ} = \Delta G_{obs}^{\circ} - \Delta G_{pe}^{\circ}$. [e] Differences in nonelectrostatic binding free energy relative to the parent AAC.

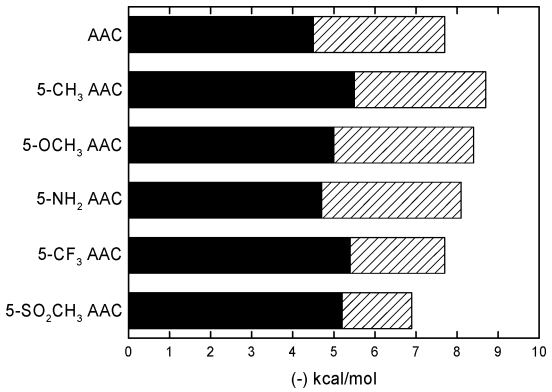


FIGURE 4: Polyelectrolyte ($\Delta G_{pe}^{\circ}$) and nonelectrostatic ($\Delta G_{t}^{\circ}$) contributions to the overall DNA binding free energies of AAC and analogues. The contributions of $\Delta G_{t}^{\circ}$ (dark area) and $\Delta G_{pe}^{\circ}$ (hatched area) to the total binding free energy are shown.

$\Delta G_{t}^{\circ}$ of the parent AAC was enhanced up to 1.0 kcal/mol as a result of C5 substitution (Figure 4).

## DISCUSSION

The primary aim of this study was to discern the effects of substituent modification on the energetics associated with the interaction of AAC with DNA. The C5 substituent of AAC exerts significant influences on both the anticancer effectiveness and the thermodynamic properties associated with ligand−DNA complex formation. Determination of the biophysical properties associated with the DNA binding of AAC and five C5-substituted analogues allowed the elucidation of potential relationships between the energetics of complex formation and anticancer activity.

*Equilibrium Binding Studies.* The compounds examined in this study bind DNA through an intercalative mechanism with binding affinities on the order of $10^5-10^6$ M$^{-1}$. Addition of electron-donating substituents to the C5 position of AAC results in substantial enhancements (up to 5-fold) in DNA binding affinity. A direct correlation between the intrinsic binding constant ($K_{obs}$) and acridine basicity is evident, wherein the analogues with higher p$K_a$ values at the N10 position exhibit enhanced DNA binding affinities (*23*). In addition to increasing the affinity for binding to calf thymus DNA, addition of substituents to the C5 position has been shown to enhance the specificity of AAC for GC-rich sites (*5*). Calculations of binding free energies from experimentally derived equilibrium binding constants reveal an enhancement in overall binding free energy upon AAC substitution, with one exception. Addition of the methylsulfonyl group results in a markedly weaker binding affinity and a less favorable binding free energy than the parent AAC. The atypical effects observed with the 5-$SO_2CH_3$ substituent could be a consequence of interference with intercalation arising from the substantial steric bulk of the methylsulfonyl group.

*DNA Binding Energetics.* Relative to the parent AAC, the interaction of each C5-substituted analogue with DNA involves an enthalpic stabilization and an entropic destabilization of the ligand−DNA complex. The significant enhancement of binding enthalpy resulting from C5 substitution can be attributed to a number of factors that may stabilize the intercalation complex. The recently reported crystal structures of AAC and its 5-F analogue complexed to a DNA hexamer solved at 1.55 Å resolution revealed increased stacking interactions of the substituted analogue with cytosine in the CpG binding site (*9*). If this finding is applied to the series of C5-substituted compounds as a whole, then a substantial contribution toward the enhanced binding enthalpies of the C5-substituted analogues could arise from the improved stacking between the substituted analogues and the DNA bases in the binding site. Enhancement of base stacking interactions between the ligand chromophore and the $\pi$-system of the DNA bases results from substituent-induced changes in electron density distribution in the acridine ring. The hydrogen bond acceptor sites of the methoxy and methylsulfonyl groups and donor sites of the amino group may provide an additional source of ligand−DNA binding enthalpy enhancement. It should be noted that because ligand p$K_a$ values have been shown to increase upon intercalation (*19*), the enthalpy values reported here may include a small contribution due to the enthalpy of ionization in addition to the enthalpy of ligand binding.

In contrast to the enhancement in binding enthalpy that results from C5 substitution, the binding entropy of AAC is rendered less favorable upon C5 substitution. Although the binding entropies observed for most analogues are positive and thus favorable toward complex formation, the interaction of each C5-substituted analogue with DNA is less entropically favorable than that observed with the parent AAC. During the intercalation process, a major contributor to favorable binding entropy is the entropic driving force of the hydrophobic effect that accompanies the release of water from the ordered shell surrounding the ligand as the ligand intercalates into the hydrophobic interior of DNA. Potential steric hindrance at the intercalation site by the C5 substituent could explain the overall decrease in $\Delta S_{obs}^{\circ}$ of AAC upon C5 substitution. This effect is exemplified by the 5-$SO_2CH_3$ analog—addition of the bulky methylsulfonyl group to the C5 position resulted in the only compound in this study whose entropy of binding opposed ligand−DNA complex formation. In addition to steric hindrance at the binding site, sources of the reduction in $\Delta S_{obs}^{\circ}$ of AAC upon C5 substitution may include a reduction in rotational entropy brought about by interactions between the C5 substituent and the DNA binding site. For example, the methylsulfonyl group possesses two sites for potential hydrogen bonding within the DNA binding site; the restrictions in rotational freedom brought about by these two bonds could contribute to the significant decrease in binding entropy upon addition of the 5-$SO_2CH_3$ group to AAC.

Table 5: Antitumor Activities of AAC and Analogues in Relation to DNA Binding Affinity, Enthalpy, and the Nonelectrostatic Contribution to Overall Binding Free Energy[a]

| compound (AAC) | $K_{obs}/10^5$ ($M^{-1}$) | $\Delta H_{obs}°$ (kcal/mol) | $\Delta G_t°$ (kcal/mol) | in vitro activity | | in vivo activity | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | P388 | | Lewis lung | |
| | | | | L1210 $IC_{50}$[b] | HCT-8 $IC_{50}$ | OD[c] | $ILS_{max}$[d] | OD | $ILS_{max}$ |
| AAC | 4.1 ± 0.1 | −5.4 ± 0.01 | −4.5 ± 0.1 | 15 | 66 | 4.5 | 98 | 4.5 | NA |
| 5-$CH_3$ | 22 ± 0.8 | −6.5 ± 0.14 | −5.5 ± 0.3 | 0.5 | 11 | 2.6 | 107 | 1.8 | NA[e] |
| 5-$OCH_3$ | 15 ± 0.5 | −6.5 ± 0.02 | −5.0 ± 0.2 | 4.3 | 89 | 3.9 | 81 | 3.9 | NA |
| 5-$NH_2$ | 8.9 ± 0.3 | −7.6 ± 0.18 | −4.7 ± 0.3 | 18 | 120 | 5.9 | 32 | 13.3 | NA |
| 5-$CF_3$ | 4.6 ± 0.2 | −5.7 ± 0.17 | −5.4 ± 0.2 | 5.7 | —[f] | 13.3 | 115 | 13.3 | 40 |
| 5-$SO_2CH_3$ | 1.2 ± 0.1 | −8.1 ± 0.05 | −5.2 ± 0.1 | 2.8 | 451 | 30 | 138 | 65 | 106 |

[a] Ref *5*. [b] $IC_{50}$ = concentration of drug in nM required to inhibit growth of murine leukemia (L1210) or human colon tumor (HCT-8) cells in culture by 50%, following a 40 h exposure. [c] OD = optimal dose of drug in mg $kg^{-1}$ $day^{-1}$, administered interperitoneally as a solution in 0.1 mL of 30% v/v ethanol/water on days 1, 5, and 9 after intraperitoneal inoculation of $10^6$ P388 leukemia cells or on days 5, 9, and 13 after intravenous inoculation of $10^6$ Lewis lung carcinoma cells. [d] $ILS_{max}$ = the percentage increase in lifespan of drug-treated tumor-bearing animals as compared to that of untreated tumor-bearing controls when treated at the optimal dose; values above 20% for P388 and above 40% for Lewis lung are considered statistically significant. [e] NA = compound is inactive at all dose levels. [f] No data available. In the case of the in vitro and in vivo studies, the values presented are averages of at least three independent determinations with coefficients of variation ranging from 12 to 18%.

*Polyelectrolyte Effects.* Examination of the ionic strength dependency of DNA binding affinities substantiated the influence of the polyelectrolyte effect on the interaction of AAC and its analogues with DNA. While the average slopes $S$ of the ln $K_{obs}$ versus ln[$Na^+$] plots for the dicationic ($S = -2.4$) and monocationic compounds ($S = -1.4$) of this study are higher than the values predicted by Record et al. (*20*), they lie in close agreement with the values predicted by Wilson and Lopp (*21*), which account for conformational changes in the DNA helix during intercalation. As a single dicationic or monocationic AAC compound interacts with DNA, approximately 2.5 or 1.5 $Na^+$ counterions, respectively, are released. For AAC and its analogues, ligand charge correlates with the polyelectrolyte contribution to the overall binding free energy ($\Delta G_{pe}°$), with the dicationic species contributing an average of −3.3 kcal/mol and the monocationic analogues contributing −2.0 kcal/mol (Table 4). Thus, consistent with previous studies comparing AAC and its monocationic des-amino analogue DACA (*12*), the polyelectrolyte binding free energies of the dicationic AAC compounds are approximately 1 kcal/mol more favorable than that of the monocationic analogues.

Both salt-dependent and -independent factors contribute to the enhanced $\Delta G_{pe}°$ of the dicationic versus monocationic AAC compounds. According to the ion release theory, the entropy of dilution as counterions are released into the bulk solution contributes favorably to the overall binding free energy (*20*). In addition to the salt-dependent contribution to $\Delta G_{pe}°$, salt-independent phenomena, including the Coulombic attraction and desolvation that accompany ligand−DNA interactions, also influence complex formation. The model by Honig and Sharp (*19*, *24*, *25*) based on the nonlinear Poisson−Boltzmann (NLPB) equation affirms that salt effects on ligand binding have additional electrostatic contributions other than the entropy of ion release. The NLPB model describes ligand−DNA binding as a balance between the opposing forces of the favorable Coulombic attraction between a cationic ligand and polyanionic DNA, and the unfavorable desolvation of both the ligand and the DNA as polar and charged groups are buried at the binding interface. The cationic character of AAC and its analogues ensures a strong Coulombic attraction between the ligand and the DNA that will overcome any opposition to complex formation due to desolvation. Increasing the charge of the ligand strengthens the Coulombic attraction, as demonstrated by the enhanced $\Delta G_{pe}°$ of dicationic versus monocationic AAC compounds.

As shown in Figure 4, the addition of substituents to the C5 position of the parent AAC results in significant enhancements of up to 1.0 kcal/mol in the nonelectrostatic contribution ($\Delta G_t°$) to the overall binding free energy. The $\Delta G_t°$ component is derived primarily from molecular interactions, including hydrogen bonding, van der Waals interactions, and hydrophobic effects accompanying the intercalation of the ligand chromophore between DNA base pairs (*22*). The increase in favorable $\Delta G_t°$ upon substitution reflects the enhanced intermolecular interactions between the ligand and the DNA binding site that are facilitated by the C5 substituent during intercalation. Substituents at C5 may enhance intercalative interactions between the ligand chromophore and the DNA base pairs through changes in the electron density of the acridine chromophore that result in a more favorable $\pi-\pi$ overlap between the aromatic systems of the ligand and DNA bases. DNA duplex unwinding angle data support this assertion—C5-substituted AAC analogues produced greater unwinding angles upon intercalation than the parent compound, indicative of more efficient drug intercalation of the substituted compounds (*2*). However, $\Delta G_t°$ does not exclusively reflect molecular interactions within the ligand binding site. Other microscopic free energies constitute $\Delta G_t°$, including the free energy of transfer of the ligand from solution to its binding site and the free energy describing conformational changes (*16*). Although additional components of $\Delta G_t°$ must be considered, the parsing of polyelectrolyte effects from the overall binding free energy has allowed the enhancement of ligand−DNA interactions upon C5 substitution of AAC to be considered an intrinsic result of the modification itself rather than merely a result of differences in charge.

*Effects of Substitution on Biological Activity.* The anticancer activity of various acridine-based compounds has been linked to numerous aspects associated with ligand−DNA complex formation including binding affinity, kinetics, binding site specificity, and thermodynamics of binding (*8*, *9*). When the relationship between AAC substituent modification and antitumor activity was investigated, notable differences among the analogues were apparent (Table 5). Substitution at C5 of AAC resulted in increases of up to 30-fold in in vitro antileukemic potency (*5*). With the

dicationic analogues, a correlation between enhanced in vitro cytotoxicity against leukemia cell lines and increased DNA binding affinity is evident. The placement of electron-withdrawing groups (trifluoromethyl and methylsulfonyl) at the C5 position of AAC yielded monocationic analogues with moderate but significant in vivo activity against Lewis lung solid tumor. The enhanced activities of both monocationic and dicationic AAC analogues may be related to the significant favorable increases in binding enthalpies incurred upon modification of the parent compound. The finding that AAC analogues with enhanced binding enthalpies exhibited higher levels of antitumor activity is similar to that previously reported for related amsacrine analogues (*10*). Additionally, a positive correlation may exist between favorable nonelectrostatic binding free energy ($\Delta G_t°$) and drug dose potency. It is difficult to extract any such relationship from these data since the interaction of many of the analogues with DNA yields very similar $\Delta G_t°$ values; however, close examination of Table 5 reveals that those compounds with the most favorable $\Delta G_t°$ values do follow this trend. The one exception to each of the aforementioned relationships between ligand−DNA interactions and biological activity is 5-$NH_2$ AAC, whose activity is consistently lower than that of the parent AAC despite its improved binding affinity, enthalpy, and nonelectrostatic binding free energy.

In general, when considering DNA binding data of a comprehensive collection of structurally diverse antitumor agents, DNA binding characteristics are not the only predictor of cytotoxicity. The biological activity of AAC and its analogues depends on a variety of factors, including not only interactions with DNA but also the abilities of the compounds to inhibit the enzyme topoisomerase II, thus generating cytotoxic double-stranded DNA breaks (*3*, *26*). However, for the structurally related 9-aminoacridine-4-carboxamides of this study, correlations between thermodynamic binding parameters and anticancer activity have been extracted, yielding information that could potentially contribute to the rational design of new related anticancer agents. By revealing relationships between anticancer activity and enthalpic and nonelectrostatic contributions to the overall binding free energy, this study demonstrates the important link between the energetics of ligand−DNA binding and biological activity.

## SUPPORTING INFORMATION AVAILABLE

Spectral properties of AAC and C5-substituted analogues. This material is available free of charge via the Internet at http://pubs.acs.org.

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