Bisimidazoacridones and Related Compounds: New Antineoplastic Agents with High Selectivity against Colon Tumors

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A new class of potent and highly selective antitumor agents has been synthesized. Bisimidazoacridones, where the tetracyclic ring systems are held together by either a N^2-methyldiethylenetriamine or 3,3'-diamino-*N*-methyldipropylamine linker, and related asymmetrical compounds, where one of the imidazoacridone ring system was replaced by a triazoloacridone ring system, were found to be cytostatic and cytotoxic *in vitro*. Some of these compounds, such as 5,5'-[(methylimino)bis(3,1-propanediylimino)]bis[6*H*-imidazo[4,5,1-*de*]acridin-6-one] (**4b**) showed remarkably high activity and selectivity for colon cancer in the National Cancer Institute screen. This antitumor effect was also apparent in colony survival assays utilizing the colon cancer line, HCT-116, and in *in vivo* assays involving xenografts of tumor derived from HCT-116 in nude mice. The tested compounds exhibited relatively low acute toxicity and were well-tolerated by the treated animals. The bisimidazoacridones interact with nucleic acids *in vitro* but preliminary experimental and modeling data indicate that in spite of their structure, they may not be bis-intercalators. While the precise mode of action of these compounds is not yet understood, they appear to be excellent candidates for clinical development.

Introduction

The effectiveness of intercalating compounds in the treatment of cancer has been known for some time. This class of drugs includes doxorubicin which continues to be the agent with the broadest spectrum of useful activity of all the currently available drugs.[1] A great number of modified natural and synthetic agents that supposedly interact with DNA by intercalation has been synthesized and tested for antitumor activity, including ellipticines,[2] anthracenediones,[3] benzothiopyranoindazoles,[4] anthrapyrazoles,[5] pyrazoloacridines,[6] imidazoacridones,[7] triazoloacridones,[8] and naphthalimides.[9] The presence of planar polycyclic aromatic residues in these molecules, which are able to stack between base pairs at the intercalation site, is the common basic structural feature for these agents.

The mechanism of anticancer action of intercalating drugs is unclear. It is known that intercalators induce conformational changes in the DNA helix that affect activity of different nuclear proteins and enzymes which operate on DNA. It is generally thought that antitumor activity of many intercalating agents is related to the inhibition of topoisomerase II.[10] It was suggested that intercalators trap the enzyme at the cleavable complex stage.[11] The molecular details of the way in which drugs interfere with topoisomerase II remain unknown, but it is most likely that the drug-induced site-specific structural distortions of DNA are a determinant. Very recently, inhibition of helicase activity by intercalating agents was suggested to be responsible for their antitumor activity.[12]

It has been shown that dimerization of various intercalating moieties can lead to a very large increase in the DNA binding affinity.[13,14] Such compounds are able to bind to DNA by bis-intercalation, which produces binding sites that are at least twice as large as mono-intercalation sites and which cause much more pronounced structural changes of DNA. Echinomycin[15] and ditercalinium[16] are examples of antitumor agents that bind to DNA by bis-intercalation. It has been shown that in the echinomycin—DNA complex base-pairing arrangement was changed from Watson—Crick to Hoogsteen pairing,[17,18] but the real importance of these structural changes is unknown. On the other hand, ditercalinium causes significant bending of DNA.[16,19] It has been suggested that the DNA structural alterations effected by this agent can be recognized by DNA repair enzymes and that the DNA repair system induces cell death by its inability to carry out the repair successfully.[20]

The complete structural requirements for bifunctional intercalators to show antitumor activity are unknown. Both the physicochemical characteristics of ring systems and the type and characteristics of the linker by which they are joined appear to be important, and it is impossible at this stage to predict a new structure that will possess antitumor activity.

It is generally accepted that intercalation is not a sufficient condition for antitumor activity. There is a great number of compounds that bis-intercalate into DNA but are devoid of significant cytotoxic activity. These include simple bisacridines,[21] ethidium homo- and heterodimers,[22] ellipticine dimers,[23] and substituted 7*H*-pyridocarbazole dimers.[24] However, as bis-intercalation constitutes one of the most powerful ways in which relatively small molecules can interact with DNA and modify its structure, we believe that there is an opportunity to find new antitumor agents among compounds that potentially can bis-intercalate into DNA.
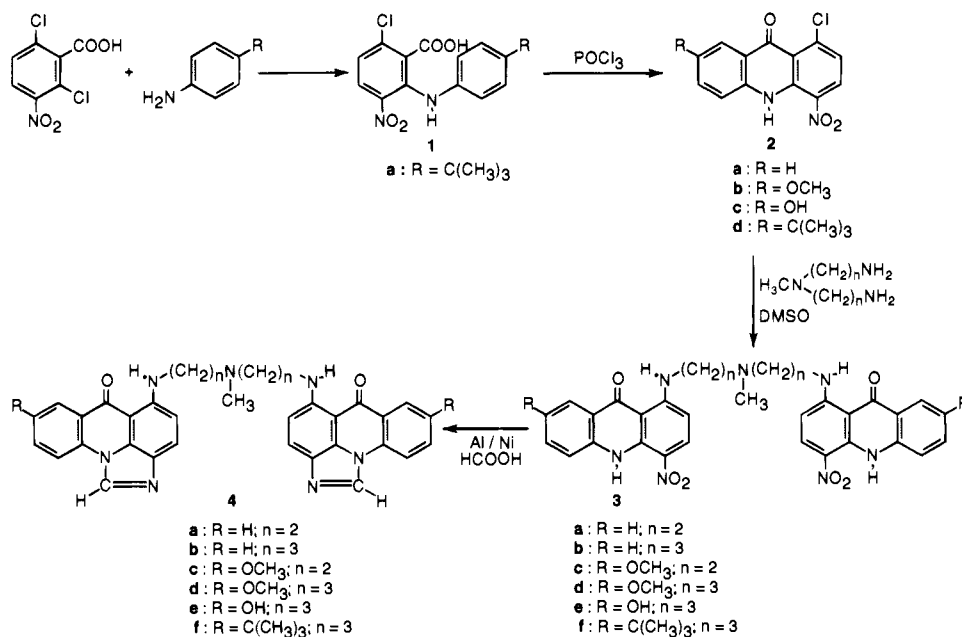
The new drugs described in this paper were designed

Scheme 1

Chemistry

The synthesis of symmetrical bisimidazoacridones (4) was carried out according to Scheme 1. The starting acridones 2a–c were obtained from known 2-(phenylamino)-6-chloro-3-nitrobenzoic acids according to previously reported methods.[6,26] The new acridone 2d and its precursor 1a were synthesized in an analogous manner under slightly modified conditions. The acridones were condensed with N^2-methyldiethylenetriamine or 3,3'-diamino-N-methyldipropylamine in dimethyl sulfoxide in the presence of triethylamine to give bisnitroacridones 3 which were transformed into bisimidazoacridones 4 by refluxing in formic acid in presence of aluminum–nickel alloy. Due to the extreme insolubility of the bisnitroacridones 3 in most of the commonly used solvents, only small samples were crystallized from N,N-dimethylformamide–water for elemental analysis. The major part of product was heated to boiling in N,N-dimethylformamide to wash out impurities, and the residue was used in the next step without crystallization. The final derivatives 4 were purified by crystallization or, if necessary, by column chromatography on silica gel.

The synthetic pathway leading to the asymmetrical compounds 8 is given in Scheme 2. The mono-substituted compound 5 obtained in reaction of 1-chloro-4-nitroacridone 2a with an excess of 3,3'-diamino-N-

to take advantage of the generally higher DNA binding efficiency of bis-intercalators with the already established antitumor activity of imidazoacridones[7] and triazoloacridones.[8] We expected to observe useful activity for these new agents at lower concentration than their monointercalating counterparts, which would have an effect of enhancing the therapeutic index. The data presented in this paper show clearly that some of the new compounds are excellent drug candidates, but the original hypothesis was shown to be faulty. We demonstrated that the compounds do interact with DNA, but the nature of the interaction does not appear to involve bis-intercalation or minor groove binding.
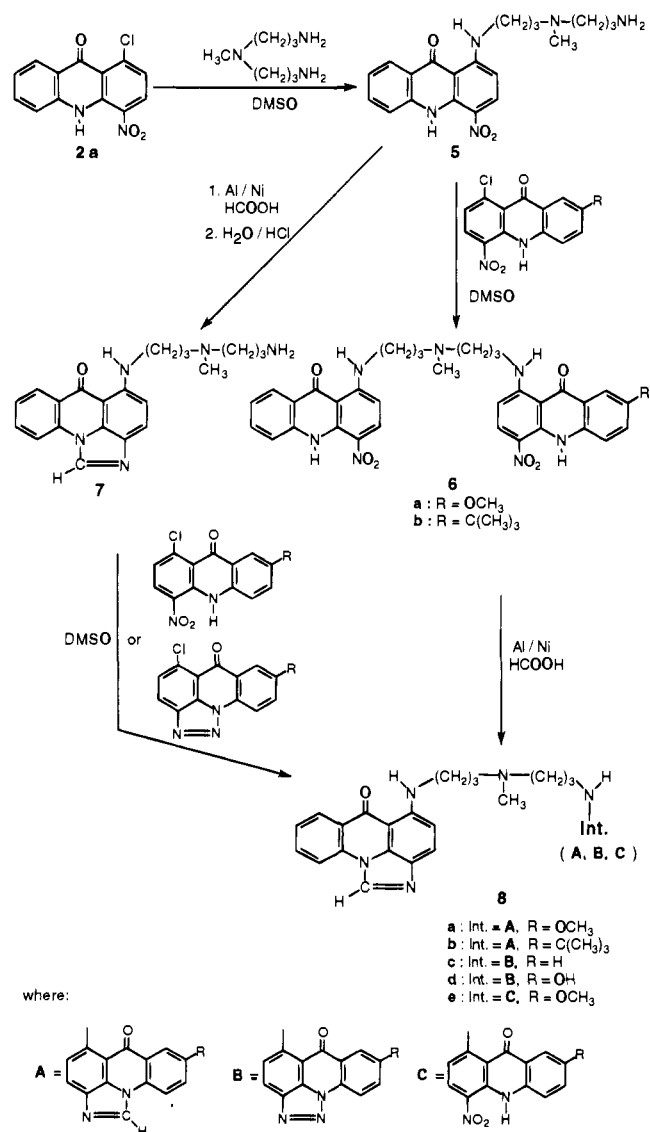
methyldipropylamine was condensed with a suitable acridone to give asymmetrical bisnitroacridones 6 or was transformed into mono-imidazoacridone 7 by refluxing in formic acid in presence of aluminum–nickel alloy. Under the reaction conditions, the distal amino group was formylated, so the product was hydrolyzed to liberate the amino group. Compound 7 was then condensed with the corresponding acridone or 5-chlorotriazoloacridones. The latter compounds were synthesized according to the previously reported methods.[8] Compounds 6 were transformed into the final structures 8a and 8b in reaction with formic acid and aluminum–nickel alloy. NMR spectroscopy, along with elemental analyses, was used to establish the structural identity of all of the reported compounds.

Results and Discussion

On the basis of structural analogy to such well-known bis-intercalators as acridine dimers,[21] ethidium dimers,[13] ditercalinium, and its flexible analog Flexi-Di,[19] we assumed that our compounds should bind into DNA by bis-intercalation and that their ability for bis-intercalation would be reflected in their biological activity. With this in mind, we synthesized a set of structures in which the ability to bis-intercalate should be significantly altered by suitable modifications. From the studies on acridine dimers, it was known that bis-intercalation requires a linker with at least eight atoms.[21] Because of this we introduced 3,3'-diamino-N-methyldipropylamine linker (nine atoms) into our structures, and for comparison, a shorter N^2-methyldiethylenetriamine linker (seven atoms). As it is known that a bulky *tert*-butyl group attached to a suitable position of a chromophore can totally prevent intercalation,[27] we synthesized the mono- and di-*tert*-butyl derivatives (4e and 8b). Additionally, because of the reported importance of general drug symmetry (not to be confused with the optical symmetry) for biological activity in ditercalinium analogs,[28] we synthesized the asymmetrical compounds 8.

a. **Evaluation of Cytotoxicity.** All the final compounds shown in general structures 4 and 8, and for

Scheme 2

but low activity on the TGI and LC_{50} levels (the latter data not shown). Very similar patterns were found for compounds **4b**, **8a**, and **8e**. The remaining compounds did not show such significant selectivity. These data suggest that compounds **4b**, **8a**, **8c**, and **8e** are cytostatic but not cytotoxic. However, as this assay involves only a 48 h development period, it was possible that there was not enough time to fully reveal the killing potency of these compounds. This may be especially true, if the compounds exert their activity by "delayed cytotoxicity", previously observed for ditercalinium.[32]

The GI_{50} data in Figure 1 also show how drastically this activity was modified by subtle structural changes. Comparison of **4b** and **7** indicates that the presence of the second ring system is necessary for selective cytostatic activity. On the other hand, compound **4a** revealed the importance of the linker length and compound **8b** showed that presence of bulky *tert*-butyl group even in only one of the two chromophores is deleterious to selectivity.

b. Colony Survival Assay. The data from the NCI primary screening clearly suggested that some of the bifunctional compounds discussed here possess selective cytostatic activity toward colon tumor cells. To additionally confirm this activity, we performed a colony survival test on the HCT-116 colon adenocarcinoma cell line which was one of the more sensitive lines in the NCI assay. Figure 2 shows the results of HCT-116 cell colony survival after exposure to compounds **4a**, **4b**, **4d**, **7**, **8b**, **8c**, and ditercalinium which was used as a control. This assay measured the ability of colon tumor cells to survive the effect of a short (2 h) exposure to the compounds and to recover over longer period of time. The results clearly show that the previously observed cytostatic effect of some compounds is the determining factor in their final cytotoxicity. Compounds **4b** and **8c** were toxic at close to nanomolar concentrations, closely reflecting their GI_{50} concentrations. Compounds **4a**, **4d**, and **8b**, in turn, showed much lower toxicities, further highlighting the importance of a longer linker and the absence of *tert*-butyl group(s) for maximum toxicity. The monofunctional compound **7** and ditercalinium showed comparable cytotoxicities in the micromolar range.

c. *In Vivo* Antitumor Activity. On the basis of the in vitro results, two drugs, **4b** and **4d**, were selected for *in vivo* antitumor activity assays in nude mice xenografted with HCT-116 tumors. Initially, acute toxicity assays in mice were performed for each individual compound. Surprisingly, despite their relatively high cytotoxic activity *in vitro*, the tested compounds were not acutely toxic in mice. For example, a dose of 200 mg/kg given for three consecutive days (total dose of 600 mg/kg) was well-tolerated for compounds **4a**, **4b**, **4f**, **8a**, **8c**, and **8d**. *tert*-Butyl derivatives **4e** and **8b** as well as methoxy derivatives **4c** and **4d**, however, were found to be much more toxic.

Figure 3 presents the relative growth of tumors in animals treated with compounds **4b**, **4d**, and doxorubicin as a positive control. In all cases there was a dramatic reduction in the growth rate of treated tumors versus controls. This effect was greater than that of doxorubicin treatment at 3 × 4 mg/kg (which was the maximally tolerated dose in our assay) and was dose-related in the case of **4b**, but not for **4d**. Curiously, in the case of that compound, a dose of 3 × 25 mg/kg gave

comparison compound **7**, were tested for cytotoxicity in the National Cancer Institute screening system on 60 human tumor lines.[29,30] This primary antitumor screen is designed to discover selective, disease-specific drugs. The data from this assay can be presented in several different formats. The mean graph histogram[31] is particularly instructive and is shown in a highly abbreviated form in Figure 1. The dose—response data for a particular drug are plotted on a logarithmic scale as horizontal bars to the right (more active) or to the left (less active) than the mean value for the activity of the drug against all 60 cell lines after 48 h of exposure. Normally, there are three histograms for each compound which represent the concentration of the drug required for 50% cell growth inhibition (GI_{50}), total cell growth inhibition (TGI), and 50% cell kill (LC_{50}). This method of presentation allows one to quickly ascertain the activity of a given drug against specific tumor cell lines. Figure 1 shows only the GI_{50} values for five selected drugs and three tumor types.

Although the compounds presented in this paper have closely related structures, the results obtained from the NCI screen showed dramatic differentiation of activity on the GI_{50} level. Compound **8c** showed high selective activity toward all colon cancer lines on the GI_{50} level

Compound	4a		4b		7		8b		8c	
Panel/Cell Line	Log GI50	GI50	Log GI50	GI50	Log GI50	GI50	Log GI50	GI50	Log GI50	GI50
Leukemia										
CCRF-CEM	-4.02		< -8.00		-6.46		-5.16		-5.30	
HL-60(TB)	> -4.00		-7.35		-6.55		-5.32		-4.99	
K-562	-4.55		< -8.00		-6.09		-6.14		-4.75	
MOLT-4	-4.23		< -8.00		-6.45		-5.51		> -4.00	
RPMI-8226	-4.55		-6.84		-5.94		-5.54			
Colon Cancer										
COLO 205	-4.65		-6.30		-5.94		-5.76		-6.43	
HCC-2998	-4.60		-7.03		-5.87		-5.77			
HCT-116	-5.65		< -8.00		-6.18		-5.91		< -8.00	
HCT-15	-4.56		-6.50		-5.46		-5.75		-7.32	
HT29	-5.36		-7.78		-6.20		-5.80		< -8.00	
KM12	-5.57		-6.53		-5.97		-5.81		-7.51	
SW-620	-4.95		< -8.00		-6.20		-5.89		< -8.00	
Melanoma										
MALME-3M	-4.88		-6.11		-5.79		-5.76		-4.92	
MI4	-5.39		> -4.00		-5.75		-5.69		> -4.00	
SK-MEL-2	-4.56		-4.86		-5.84		-5.80		-4.29	
SK-MEL-28	-5.55		> -4.00		-5.81		-5.74		-4.35	
SK-MEL-5	-5.23		-4.89		-5.81		-5.77		-4.77	
UACC-257	-5.09		> -4.00		-5.75		-5.84		> -4.00	
UACC-62	-4.66		-5.09		-5.79		-5.72		-4.42	
Median Value for 60 Lines (log 10)	-4.88		-5.94		-5.94		-5.71		-5.52	

Figure 1. Selected growth inhibition data for three tumor types (leukemia, colon and melanoma) from the National Cancer Institute *in vitro* screen. These results are an abridged version of the complete data set which include results for 60 human tumor cell lines and also provide total growth inhibition (TGI) and half-lethal concentration (LC50) end points.
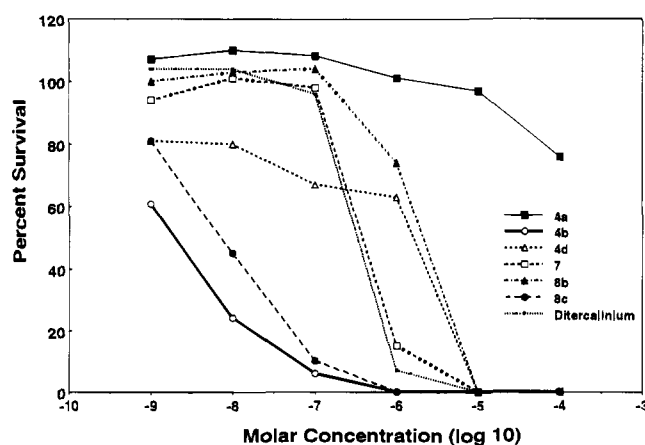

Figure 2. Effect of selected compounds on HCT-116 cells colony survival. Cells were exposed for 2 h to the compounds at different concentrations, washed, and allowed to grow in fresh medium for 1–2 weeks. Control cells received vehicle alone. Colonies of greater than 30 cells were scored as survivors. The data shown here are a mean of three independent experiments.

better results than twice that amount, using this particular schedule. It is worth noting that **4d** was more acutely toxic to the animals than **4b**.

d. In Vitro Interactions with DNA. All the compounds discussed in this paper showed strong fluorescence when excited at 430 nm with maximum of emission at about 525 nm. The intensity of this fluorescence was modified after addition of double-stranded DNAs, depending on the molecule structure. The unsubstituted compound **4b** showed the strongest enhancement of fluorescence (40–100-fold) from the series, which depended on the conditions used, mainly on the ratio [drug]:[base pairs], and the kind of DNA (the complete data will be published in a separate paper). The enhancement of fluorescence for **4b** was stronger with poly(dA)·poly(dT) than with poly(dG)·poly-(dC). In the case of the unsubstituted derivative with a shorter linker (**4a**) or with two *tert*-butyl substituents (**4f**), the change of fluorescence intensity after addition of DNA was very weak.

The initial fluorescence studies, gel mobility assays
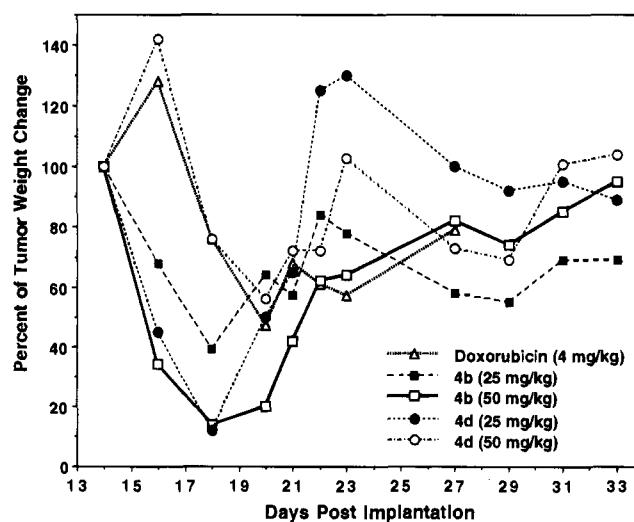
Figure 3. Effect of compounds **4b**, **4d**, and doxorubicin on HCT-116 colon tumor xenografts in nude mice. There were six mice in each treatment and control group. The drugs were administered by intraperitoneal injections of drug solution in 5% glucose, three times, once every fourth day, commencing with day 8 postimplantation of the xenograft. Control animals received 5% glucose solution (negative control) or doxorubicin at a dose of 4 mg/kg (positive control). Median tumor weight changes were determined as a percentage of median control tumor weight commencing at day 14 when the tumors had a minimum of 63 mg/tumor. In this representation the control group would be represented by a horizontal line at 100%. The maximum scatter in the individual tumor weights was about ±50%, with the greatest variation occuring toward the end of the experiment, when the tumors were large. All animals treated with doxorubicin were sacrificed after day 27 due to a pronounced loss of body weight.

and DNase I footprinting experiments[33] suggested that some of the bisimidazoacridones strongly interacted with double-stranded DNAs and that bis-intercalation may be the most likely nature of this interaction. To get additional evidence for this mode of binding, we studied thermal denaturation of different DNAs after addition of **4b**, the compound which revealed the strongest enhancement of fluorescence. Figure 4 presents selected data from these studies (the complete results will be published in a separate paper). **4b** was
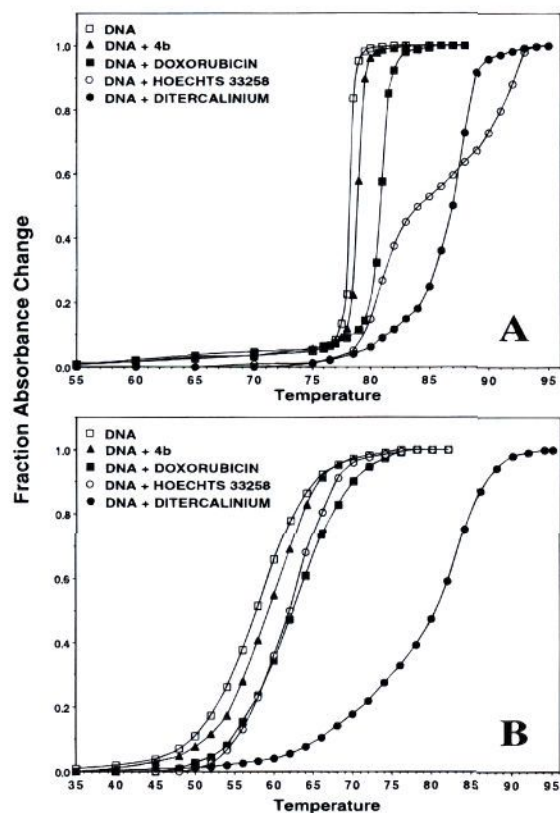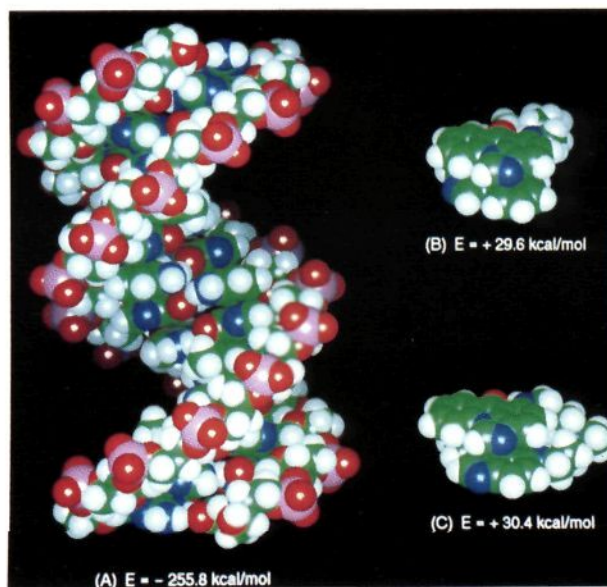
Figure 4. Thermal denaturation profiles for poly(dA)·poly-(dT) (A), d(CATATGCGCATATG) (B), and their complexes with **4b**, doxorubicin, Hoechts 33258, and ditercalinium. Base pairs concentrations were 30 μM in CNE buffer, and the ratio [base pairs]/[ligand] = 4.

Figure 5. Minimized structures of d(GATATGCGCATATC) (**A**) and compound **4b** (**B** and **C**) created by INSIGHT II package. There are two conformers of **4b** with comparable energies.

compared with doxorubicin, a classical mono-intercalator, ditercalinium, an authentic bis-intercalator, and Hoechts 33258, a minor groove binder. Surprisingly, these studies showed that, although **4b** interacted with double-stranded DNAs, it had almost no ability to stabilize their double helical structure, independent of the type of DNA. The effect of **4b** on the melting temperatures of the various DNAs was negligible when compared to the authentic bis-intercalator ditercalinium and also to doxorubicin and Hoechts 33258. Binding of Hoechts 33258 with poly(dA)·poly(dT) gave a somewhat unusual curve (Figure 4, part A), suggesting that under the conditions used in this experiment, the agent probably formed two different types of complexes, one weak (unspecific) and second one which was specific and much stronger. This observation is in a good agreement with the results of extensive studies on interaction of this agent with various DNAs reported in literature.[34]

Taken together, our initial studies on binding of **4b** with DNA revealed that the nature of this interaction does not appear to involve intercalation (mono or bis) or minor groove binding, and other modes of binding have to be considered.

e. Molecular Modeling. Molecular modeling is very useful in assessing drug–DNA interactions despite various theoretical and practical limitations.[35–37]

The results of thermal denaturation studies prompted us to investigate other possible modes of binding of these compounds to DNA by means of molecular modeling. We used self-complementary tetradecamer sequence 5′-GATATGCGCATATC-3′ in the form of double-helical

B-DNA and compound **4b** protonated on the central nitrogen of the linker. The sequence of DNA was changed a little from that used in thermal denaturation studies as we found that the original sequence 5′-CATATGCGCATATG-3′ gave unnaturally distorted structure after minimization due to end effects. Initially, both of the interacting molecules (in this study the DNA double helix was treated as one molecule) were subjected to energy refinement by molecular mechanics at infinite separation from each other, as described in the Experimental Section. Figure 5 presents minimized structures for DNA fragment (**A**) and the drug (**B** and **C**) shown in the CPK representation. It is worth noting that the energy refinement of the drug revealed two minimized structures with very similar energies which differ in the orientation of the aromatic ring systems. In the next step, the bis-intercalation site was built in the central part of the DNA fragment into which the drug molecule was inserted and the energy of the complex was minimized. Figure 6 presents structures of the minimized classical bis-intercalated complex **D** in which nearest neighbor exclusion rule is obeyed[38] as well as two other lower energy nonintercalative complexes **E** and **F** which can be created on the basis of the drug structure.

Table 1 presents the calculated energy analysis of the structures presented in Figure 6. The energy designated as E_{COM} represents the total calculated energy of each of the complexes. It should be remembered that the molecules are identical in all three structures; only the conformations are different. On this basis, structure **F** is the most stable ($E = -286.9$ kcal/mol). This involves a π-stacked conformation of the drug resident in the major groove, with the charged linker oriented in such a way that it makes a good electrostatic contact with the phosphate backbone. The second most stable structure is **E** ($E = -273.6$ kcal/mol) where one imidazoacridone ring system sits in in the major groove, while the other is in the minor groove, and the linker maintains electrostatic interaction with the backbone.
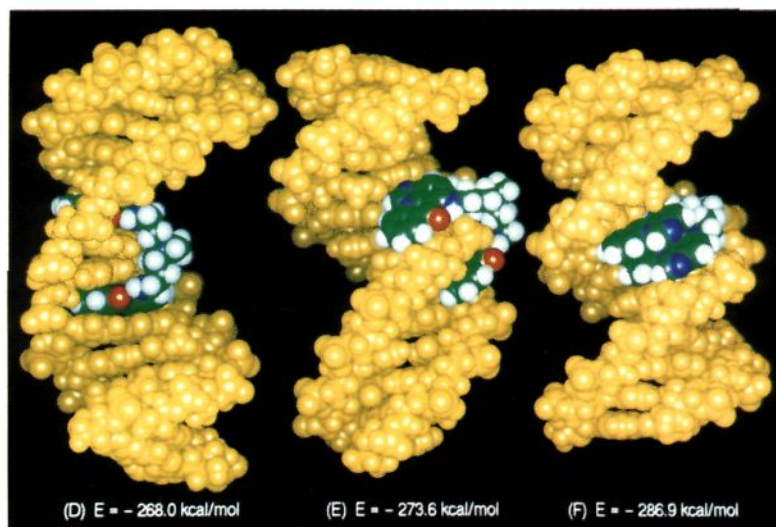
Figure 6. Computer image of three minimized structures for possible complexes formed by **4b** with d(GATATGCGCATATC): (**D**) classical bis-intercalating complex, (**E**) minor−major groove binding complex, and (**F**) major groove binding complex. Bis-intercalating complex is enegetically the least favorable.

It is significant to note that while structure **F** involves very little distortion of the DNA, there is considerable bending of the double helix in structure **E**. Structure **D** is the classical bis-intercalated conformation and is the least stable one. The other quantities in Table 1 are also worth mentioning. The column labeled ΔE gives an estimate of the crude drug−DNA binding energy, and the order is the same as the total energy. The columns labeled E_{DNA} and E_{DRUG} are the internal energies of the two components of the individual complexes. These quantities were used to derive the last two columns ΔE_{DNA} and ΔE_{DRUG}. These latter quantities give an approximation of the energy cost to bring the DNA and the drug to their conformations in the individual complexes relative to their fully relaxed forms. These data are very instructive, although it must be realized that the absolute numbers from which they were obtained are very approximate. It can be seen that the energy required for DNA and drug deformation to achieve structure **D** is very high relative to the calculated binding energy. This is not the case for structures **E** and especially **F**, where the binding energies (ΔE) are higher than the sum of the destabilization energies. Thus, the model predicts that DNA bis-intercalation is unlikely and that these drugs are probably bound in the grooves, although the precise nature of this binding remains to be determined.

Conclusions

The bisimidazoacridones such as **4b** and the closely related asymmetrical congeners such as **8c** described in this paper constitute a new class of highly selective and potent agents against colon cancer.

The high, selective biological activity against colon cancer initially found in the NCI *in vitro* screening was further confirmed in the colony survival assays *in vitro* and *in vivo* in nude mice bearing colon adenocarcinoma xenografts. The *in vitro* experiments revealed that the presence of two aromatic ring systems in the molecule of drug was essential for high selectivity toward specific tumor types (**4b** vs **7**). However, even small structural changes in the the bifunctional agents can dramatically modify their activity and selectivity (**4b** vs **8b** and **8c**).

Table 1. Energy Analysis for the DNA−Drug Complexes from Figure 6[a]

complex	E_{COM}[b]	ΔE[c]	E_{DNA}[d]	E_{DRUG}[e]	ΔE_{DNA}[f]	ΔE_{DRUG}[g]
D	−268.0	−41.8	−209.3	52.1	46.5	22.6
E	−273.6	−47.4	−245.8	50.4	10.0	20.9
F	−286.9	−60.7	−246.3	31.6	9.4	2.0

[a] All energies are given in kcal/mole. [b] The total of intermolecular and internal energies in the drug−DNA complex. [c] The binding energy defined as the energy of the drug−DNA complex (E_{COM}) minus the total energies of the drug and the B-DNA helix. [d] The internal energy of the DNA helix in the drug−DNA complex. [e] The internal energy of the drug in the drug−DNA complex. [f] Helix destabilization energy. [g] Drug destabilization energy.

For example, the exchange of one carbon atom by a nitrogen atom in one of the heteroaromatic moieties (compare **8c** with **4b**) totally removed the high activity against leukemias. This feature seems to be very promising as far as further structural modifications in this class are concerned. Compounds **4b** and **4d** offer another interesting contrast. Compound **4d** was less cytostatic in the National Cancer Institute screen than **4b**; however, in contrast to **4b**, it exhibited about the same level of activity at the LC$_{50}$ level. This suggests that **4d** exerts its cytotoxic effect more rapidly than **4b**. **4d** was also more acutely toxic to the mice than **4b** but had good activity against HCT-116 xenograft *in vivo*. It is also possible that **4b** and **4d** exert their antitumor effects by somewhat different mechanisms.

The compounds described here were initially designed to act as bis-intercalators. While it has not been proved conclusively that they are not, preliminary experimental and modeling results suggest that these compounds have a different mode of binding to nucleic acids, which does not involve intercalation. It is worth noting that the authentic bis-intercalator, ditercalinium, is much less cytotoxic to HCT-116 colon cancer cells *in vitro* than compounds **4b** and **8c** (Figure 2). This suggests that the ability to bis-intercalate does not, in itself, affect the ability of these compounds to act as cytotoxic agents. Ongoing studies will probe the mechanism of action of this new class of drugs, but the data presented in this paper already suggest that several members of the series are potential candidates for clinical development.

Experimental Section

All solvents were reagent grade. All reagents were obtained either from Aldrich Chemicals or from Fluka and were used as received. Melting points were taken on an Electrothermal capillary melting point apparatus and are uncorrected. ¹H NMR spectra were recorded on a Varian VXR-S spectrometer operating at 500 MHz. Chemical shifts are reported as ∂ units in ppm downfield from internal tetramethylsilane. NMR abbreviations used are as follows: br (broad), s (singlet), d (doublet), t (triplet), m (multiplet). Coupling constants are given in hertz. Elemental analyses were performed by Atlantic Microlab, Inc., Norcross, GA, and were within ±0.4% of theoretical values for C, H, and N.

2-[(4-*tert*-Butylphenyl)amino]-6-chloro-3-nitrobenzoic Acid (1a). A mixture of 2,6-dichloro-3-nitrobenzoic acid (4.72 g, 0.02 mol), 4-*tert*-butylaniline (4.47 g, 0.03 mol), lithium carbonate (1.48 g, 0.02 mol), and 1-propanol (10 mL) was refluxed with stirring for 24 h. The solvent was evaporated, and to the residue were added water (30 mL) and benzene (50 mL). The mixture was made strongly basic with sodium hydroxide and stirred for 1 h. The orange precipitate was collected by filtration and washed with benzene and ether. The dry precipitate was dissolved in water and acidified with diluted hydrochloric acid. The resulting precipitate was collected and crystallized twice from acetone–water to give 4.55 g (65%) of the desired product, mp 208–211 °C. Anal. ($C_{17}H_{17}N_2O_4Cl$) C, H, N.

7-*tert*-Butyl-1-chloro-4-nitro-9(10*H*)-acridinone (2d). To a solution of **1a** (3.485 g, 0.01 mol) in 1,2-dichloroethane (40 mL) was added POCl₃ (15 mL, 0.16 mol), and the mixture was refluxed for 4.5 h. Solvents were removed under reduced pressure. To the residue was added 40 mL of a 1,4-dioxane–water mixture (8:1), and the mixture was acidified with concentrated hydrochloric acid and refluxed with stirring for 2 h. Water (100 mL) was added, and the precipitate was collected by filtration and crystallized from *N,N*-dimethylformamide–water to give 3.106 g (94%) of the desired product, mp above 300 °C. Anal. ($C_{17}H_{15}N_2O_3Cl$) C, H, N.

General Procedure for the Preparation of Bisnitroacridinones 3: 1,1′-[(Methylimino)bis(2,1-ethanediylimino)]bis[7-methoxy-4-nitro-9(10*H*)-acridinone] (3c). To a suspension of 1-chloro-7-methoxy-4-nitro-9(10*H*)-acridone (1.522 g, 0.005 mol) in dimethyl sulfoxide (20 mL) were added triethylamine (1mL) and *N*²-methyldiethylenetriamine (0.293 g, 0.0025 mol), and the solution was stirred at 80 °C for 3 h. Water (200 mL) was added to the mixture, which then was made basic with aqueous sodium hydroxide and stirred for 20 min. The precipitate was collected and washed with water and methanol. The crude product was heated to boiling in *N,N*-dimethylformamide (50 mL), filtered, and washed with water to afford 1.437 g (88%) of orange **3c**, mp 268–270 °C. Anal. ($C_{33}H_{31}N_7O_8$) C, H, N.

The following compounds were obtained in an analogous manner:

1,1′-[(Methylimino)bis(2,1-ethanediylimino)]bis[4-nitro-9(10*H*)-acridinone] (3a): yield 86%; mp 265–267 °C. Anal. ($C_{31}H_{27}N_7O_6$) C, H, N.

1,1′-[(Methylimino)bis(3,1-propanediylimino)]bis[4-nitro-9(10*H*)-acridinone] (3b): yield 82%; mp 255–257 °C. Anal. ($C_{33}H_{31}N_7O_6 \cdot 0.5H_2O$) C, H, N.

1,1′-[(Methylimino)bis(3,1-propanediylimino)]bis[7-methoxy-4-nitro-9(10*H*)-acridinone] (3d): yield 93%; mp 256–259 °C. Anal. ($C_{35}H_{35}N_7O_8$) C, H, N.

1,1′-[(Methylimino)bis(3,1-propanediylimino)]bis[7-hydroxy-4-nitro-9(10*H*)-acridinone] (3e): yield 79%; mp 256–258 °C. Anal. ($C_{33}H_{31}N_7O_8$) C, H, N.

1,1′-[(Methylimino)bis(3,1-propanediylimino)]bis[7-*tert*-butyl-4-nitro-9(10*H*)-acridinone] (3f): yield 74%; mp 219–221 °C. Anal. ($C_{41}H_{47}N_7O_6$) C, H, N.

General Procedure for the Preparation of Bisimidazoacridinones (4). A mixture of the bisnitroacridinone derivative (**3a–f**) (0.002 mol), 3.0 g of Raney alloy, and 96% formic acid (30 mL) was refluxed with energetic stirring for 36 h. Methanol (100 mL) was added to the reaction mixture, and the catalyst and inorganic salts were removed by filtration

and washed with MeOH. The filtrate was evaporated under reduced pressure. The residue dissolved in MeOH containing 1% methanesulfonic acid was decolorized by heating with charcoal, and the charcoal was removed by filtration. The filtrate was condensed, and product was precipitated by addition of acetone or ether. The precipitate was collected by filtration and recrystallized from MeOH–ether. The dry methanesufonate salt was dissolved in water, and the solution was made alkaline with sodium hydroxide (in the case of **4e** with NH₄OH) to give a precipitate of the free base, which was collected by filtration. The product was purified by crystallization from suitable solvent mixtures or, in the case of **4f**, by column chromatography on silica gel using a chloroform–methanol (15:1) mixture as eluent.

5,5′-[(Methylimino)bis(2,1-ethanediylimino)]bis[6*H*-imidazo[4,5,1-*de*]acridin-6-one] (4a): yield 58%; mp >300 °C (crystallized from DMF–H₂O); ¹H NMR (Me₂SO-*d₆*) 8.89 (s, 2H, C1-H), 8.85 (t, 2H, *J* = 5.2, Ar-NHCH₂), 8.15 (m, 2H, C10-H), 8.01 (m, 2H, C7-H), 7.76 (m, 2H, C9-H), 7.75 (d, 2H, *J* = 8.8, C3-H), 7.32 (m, 2H, C8-H), 6.69 (d, 2H, *J* = 8.8, C4-H), 3.48 (m, 4H, NHCH₂CH₂), 2.83 (m, 4H, CH₂CH₂NCH₃), 2.50 (s, 3H, NCH₃). Anal. ($C_{33}H_{27}N_7O_2 \cdot 0.5H_2O$) C, H, N.

5,5′-[(Methylimino)bis(3,1-propanediylimino)]bis[6*H*-imidazo[4,5,1-*de*]acridin-6-one] (4b): yield 70%; mp 253–254 °C (crystallized from DMF–H₂O); ¹H NMR (Me₂SO-*d₆*) 8.98 (s, 2H, C1-H), 8.74 (t, 2H, *J* = 4.9, ArNHCH₂), 8.17 (m, 2H, C7-H), 8.16 (m, 2H, C10-H), 7.71 (m, 2H, C9-H), 7.62 (d, 2H, *J* = 8.9, C3-H), 7.36 (m, 2H, C8-H), 6.50 (d, 2H, *J* = 8.9, C4-H), 3.36 (m, 4H, NHCH₂CH₂), 2.55 (m, 4H, CH₂CH₂NCH₃), 2.28 (s, 3H, NCH₃), 1.90 (m, 4H, CH₂CH₂CH₂). Anal. ($C_{35}H_{31}N_7O_2 \cdot 0.5H_2O$) C, H, N.

5,5′-[(Methylimino)bis(2,1-ethanediylimino)]bis[8-methoxy-6*H*-imidazo[4,5,1-*de*]acridin-6-one] (4c): yield 67%; mp 275–277 °C (crystallized from DMF–H₂O); ¹H NMR (Me₂SO-*d₆*) 8.81 (t, 2H, *J* = 5.1, Ar-NHCH₂), 8.75 (s, 2H, C1-H), 8.00 (m, 2H, C10-H), 7.71 (d, 2H, *J* = 8.8, C3-H), 7.30 (m, 4H, C7-H and C9-H), 6.62 (d, 2H, *J* = 8.8, C4-H), 3.75 (s, 6H, CH₃-OAr), 3.44 (m, 4H, NHCH₂CH₂), 2.81 (m, 4H, CH₂CH₂NCH₃), 2.50 (s, 3H, NCH₃). Anal. ($C_{35}H_{31}N_7O_4$) C, H, N.

5,5′-[(Methylimino)bis(3,1-propanediylimino)]bis[8-methoxy-6*H*-imidazo[4,5,1-*de*]acridin-6-one] (4d): yield 71%; mp 252–254 °C (crystallized from DMF–H₂O); ¹H NMR (Me₂SO-*d₆*) 8.85 (s, 2H, C1-H), 8.69 (t, 2H, *J* = 4.8, ArNHCH₂), 8.00 (m, 2H, C10-H), 7.53 (m, 2H, C7-H), 7.53 (d, 2H, *J* = 8.7, C3-H), 7.20 (m, 2H, C9-H), 6.36 (d, 2H, *J* = 8.7, C4-H), 3.79 (s, 6H, CH₃OAr), 3.38 (m, 4H, NHCH₂CH₂), 2.56 (m, 4H, CH₂CH₂NCH₃), 2.28 (s, 3H, NCH₃), 1.91 (m, 4H, CH₂CH₂CH₂). Anal. ($C_{37}H_{35}N_7O_4$) C, H, N.

5,5′-[(Methylimino)bis(3,1-propanediylimino)]bis[8-hydroxy-6*H*-imidazo[4,5,1-*de*]-acridin-6-one] (4e): yield 59%; mp >300 °C (crystallized from DMF–H₂O); ¹H NMR (Me₂SO-*d₆*) 9.90 (br s, 2H, C8-OH), 8.92 (s, 2H, C1-H), 8.79 (t, 2H, *J* = 5.0, ArNHCH₂), 8.07 (m, 2H, C10-H), 7.66 (d, 2H, *J* = 8.7, C3-H), 7.60 (m, 2H, C7-H), 7.19 (m, 2H, C9-H), 6.53 (d, 2H, *J* = 8.7, C4-H), 3.39 (m, 4H, NHCH₂CH₂), 2.54 (m, 4H, CH₂CH₂NCH₃), 2.27 (s, 3H, NCH₃), 1.89 (m, 4H, CH₂CH₂CH₂). Anal. ($C_{35}H_{31}N_7O_4 \cdot H_2O$) C, H, N.

5,5′-[(Methylimino)bis(3,1-propanediylimino)]bis[8-*tert*-butyl-6*H*-imidazo[4,5,1-*de*]acridin-6-one] (4f): yield 61%; mp 150–152 °C (crystallized from chloroform–hexane); ¹H NMR (Me₂SO-*d₆*) 8.99 (s, 2H, C1-H), 8.76 (t, 2H, *J* = 5.1, ArNHCH₂), 8.20 (m, 2H, C7-H), 8.12 (m, 2H, C10-H), 7.82 (m, 2H, C9-H), 7.62 (d, 2H, *J* = 8.7, C3-H), 6.50 (d, 2H, *J* = 8.7, C4-H), 3.36 (m, 4H, NHCH₂CH₂), 2.54 (m, 4H, CH₂CH₂NCH₃), 2.27 (s, 3H, NCH₃), 1.89 (m, 4H, CH₂CH₂CH₂), 1.29 (s, 18H, (CH₃)₃CAr). Anal. ($C_{43}H_{47}N_7O_2$) C, H, N.

1-[3-[*N*-(3-Aminopropyl)-*N*-methylamino]propyl]amino]-4-nitro-9(10*H*)-acridinone (5). To a suspension of 1-chloro-4-nitro-9(10*H*)-acridone (2.747 g, 0.01 mol) in dimethyl sulfoxide (50 mL) was added 3,3′-diamino-*N*-methyldipropylamine (5.81 g, 0.04 mol), and the mixture was stirred at room temperature for 3 h. Water (200 mL) was added, and the mixture was stirred for 10 min. The precipitate was collected by filtration and washed with water. It was then transferred into water (100 mL), acidified with hydrochloric acid, and stirred for 15 min. Undissolved material was separated by

filtration. The solution was made basic with sodium hydroxide and the product was extracted with chloroform. The extract was dried, solvent was evaporated, and the crude product was crystallized from benzene—hexane to give 3.11 g (81%) of yellow **5**: mp 114—116 °C; ^{1}H NMR (Me$_2$SO-d_6) 11.86 (br s, 1H, N10-**H**), 8.36 (d, 1H, J = 9.8, C3-**H**), 8.20 (m, 1H, C8-**H**), 7.94 (m, 1H, C5-**H**), 7.77 (m, 1H, C6-**H**), 7.40 (m, 1H, C7-**H**), 6.58 (d, 1H, J = 9.8, C2-**H**), 3.48 (m, 2H, NHCH$_2$CH$_2$), 2.34 (m, 2H, CH$_2$CH$_2$NH$_2$), 2.42 (m, 2H, CH$_2$CH$_2$NCH$_3$), 2.34 (m, 2H, NCH$_3$CH$_2$CH$_2$), 2.16 (s, 3H, NCH$_3$), 1.82 (m, 2H, CH$_2$CH$_2$-CH$_2$), 1.50 (m, 2H, CH$_2$CH$_2$CH$_2$). Anal. (C$_{20}$H$_{25}$N$_5$O$_3$·0.5H$_2$O) C, H, N.

5-[[3-[N-(3-Aminopropyl)-N-methylamino]propyl]-amino]-6H-imidazo[4,5,1-de]acridin-6-one (7). A mixture of **5** (1.534 g, 0.004 mol), 3.0 g of Raney alloy, and 96% formic acid (40 mL) was refluxed with energetic stirring for 30 h. Methanol (100 mL) was added to the reaction mixture, and the catalyst and inorganic salts were removed by filtration and washed with MeOH. The filtrate was evaporated under reduced pressure. To the residue were added 100 mL of a MeOH—water (1:1) mixture and 3 mL of concentrated hydrochloric acid, and the solution was refluxed for 8 h. Solvents were evaporated under reduced pressure. The residue was dissolved in MeOH and acidified with hydrogen chloride. The product was precipitated by addition of acetone, collected by filtration, and crystallized from MeOH—ether. The dry hydrochloride was dissolved in water, and the solution was made alkaline with sodium hydroxide to give an oily precipitate of free the base which was extracted with chloroform. The extract was dried, the solvent was evaporated, and product was crystallized from benzene—hexane to give 0.989 g (68%) of yellow **7**: mp 101—102 °C; ^{1}H NMR (Me$_2$SO-d_6) 9.21 (s, 1H, C1-**H**), 8.91 (t, 1H, J = 5.6, NHCH$_2$), 8.42 (m, 1H, C7-**H**), 8.39 (m, 1H, C10-**H**), 8.00 (d, 1H, J = 8.9, C3-**H**), 7.93 (m, 1H, C9-**H**), 7.59 (m, 1H, C8-**H**), 6.85 (d, 1H, J = 8.9, C4-**H**), 3.45 (m, 2H, NHCH$_2$CH$_2$), 3.31 (m, 2H, CH$_2$CH$_2$NH$_2$), 2.43 (m, 2H, CH$_2$CH$_2$NCH$_3$), 2.34 (m, 2H, CH$_2$CH$_2$NCH$_3$), 2.17 (s, 3H, NCH$_3$), 1.83 (m, 2H, CH$_2$CH$_2$CH$_2$), 1.52 (m, 2H, CH$_2$CH$_2$CH$_2$). Anal. (C$_{21}$H$_{25}$N$_5$O·H$_2$O) C, H, N.

7-Methoxy-4-nitro-1-[[3-[methyl[3-[(4-nitro-9-oxo-9,10H-acridin-1-yl)amino]propyl]amino]propyl]amino]-9(10H)-acridinone (6a). A mixture of **5** (0.768 g, 0.002 mol), 1-chloro-7-methoxy-4-nitro-9(10H)-acridinone (0.610 g, 0.002 mol), dimethyl sulfoxide (20 mL), and triethylamine (1 mL) was stirred at 70 °C for 6 h. To the reaction mixture was added water (200 mL), and the solution was made alkaline with potassium hydroxide and stirred for an additional 10 min. The precipitate was collected by filtration and washed with water. The crude product was crystallized from DMF—water to give 1.20 g (86%) of yellow **6a**: mp 193—195 °C; ^{1}H NMR (Me$_2$SO-d_6) 12.15 (s, 1H), 12.08 (s, 1H), 11.73 (s, 1H), 11.61 (s, 1H), 8.11 (m, 1H), 8.01 (m, 1H), 7.93 (m, 1H), 7.55 (m, 3H), 7.24 (m, 1H), 7.19 (m, 1H), 7.09 (m, 1H), 6.27 (m, 1H), 6.19 (m, 1H), 3.72 (s, 3H), 3.37 (m, 4H), 2.36 (m, 2H), 2.34 (m, 2H), 2.25 (s, 3H), 1.88 (m, 4H). Anal. (C$_{34}$H$_{33}$N$_7$O$_7$·H$_2$O) C, H, N.

7-tert-Butyl-4-nitro-1-[[3-[methyl[3-[(4-nitro-9-oxo-9,10H-acridin-1-yl)amino]propyl]amino]propyl]amino]-9(10H)-acridinone (6b). This compound was prepared in a manner analogous to the preparation of **6a**: yield 82%; mp 206—208 °C (crystallized from DMF-H$_2$O); ^{1}H NMR (Me$_2$SO-d_6) 12.14 (s, 1H), 12.12 (s, 1H), 11.64 (br m, 2H), 8.13 (m, 1H), 8.05 (m, 1H), 7.91 (br m, 2H), 7.70 (m, 1H), 7.58 (m, 2H), 7.42 (m, 1H), 7.11 (m, 1H), 6.33 (m, 1H), 6.21 (m, 1H), 3.39 (m, 2H), 3.34 (m, 2H), 2.54 (m, 4H), 2.25 (s, 3H), 1.88 (m, 4H), 1.33 (s, 9H). Anal. (C$_{37}$H$_{39}$N$_7$O$_6$) C, H, N.

8-Methoxy-5-[[3-[methyl[3-[(6-oxo-6H-imidazo[4,5,1-de]-acridin-5-yl]amino]propyl]amino]propyl]amino]-6H-imidazo[4,5,1-de]acridin-6-one (8a). This compound was prepared from **6a** according to the general procedure for the preparation of bisimidazoacridinones: yield 60%; mp 216—219 °C (crystallized from DMF—H$_2$O); ^{1}H NMR (Me$_2$SO-d_6) unsubstituted chromophore 8.96 (s, 1H, C1-**H**), 8.75 (t, 1H, J = 4.8, NHCH$_2$), 8.17 (m, 1H, C7-**H**), 8.16 (m, 1H, C10-**H**), 7.72 (m, 1H, C9-**H**), 7.67 (d, 1H, J = 8.8, C3-**H**), 7.38 (m, 1H, C8-**H**), 6.51 (d, 1H, J = 8.8, C4-**H**); 8-methoxy chromophore 8.87 (s, 1H, C1-**H**), 8.66 (t, 1H, J = 4.9, NHCH$_2$), 8.00 (m, 1H, C10-

H), 7.51 (m, 1H, C7-**H**), 7.46 (d, 1H, J = 8.9, C3-**H**), 7.17 (m, 1H, C9-**H**), 6.32 (d, 1H, J = 8.9, C4-**H**), 3.75 (s, 3H, OCH$_3$); aliphatic protons 3.35 (m, 2H, NHCH$_2$CH$_2$), 3.28 (m, 2H, NHCH$_2$CH$_2$), 2.55 (m, 4H, CH$_2$CH$_2$NCH$_3$), 2.28 (s, 3H, NCH$_3$), 1.90 (m, 4H, CH$_2$CH$_2$CH$_2$). Anal. (C$_{36}$H$_{33}$N$_7$O$_3$) C, H, N.

8-tert-Butyl-5-[[3-[methyl[3-[(6-oxo-6H-imidazo[4,5,1-de]acridin-5-yl]amino]propyl]amino]propyl]amino]-6H-imidazo[4,5,1-de]acridin-6-one (8b). This compound was prepared from **6b** according to the general procedure for the preparation of bisimidazoacridinones: yield 51%; mp 153—156 °C (crystallized from methylene chloride—hexane); ^{1}H NMR (Me$_2$SO-d_6) 8.99 (s, 1H), 8.95 (s, 1H), 8.76 (m, 1H), 8.73 (m, 1H), 8.21 (m, 1H), 8.16 (m, 1H), 8.14 (m, 1H), 8.13 (m, 1H), 7.86 (m, 1H), 7.69 (m, 1H), 7.65 (m, 1H), 7.60 (m, 1H), 7.32 (m, 1H), 6.57 (m, 1H), 6.46 (m, 1H), 3.37 (m, 2H), 3.33 (m, 2H), 2.54 (m, 4H), 2.28 (s, 3H), 1.90 (m, 4H), 1.34 (s, 9H). Anal. (C$_{39}$H$_{39}$N$_7$O$_2$·0.5H$_2$O) C, H, N.

5-[[3-[methyl[3-[(6-oxo-6H-imidazo[4,5,1-de]acridin-5-yl)amino]propyl]amino]propyl]amino]-6H-v-triazolo[4,5,1-de]acridin-6-one (8c). This compound was prepared in an analogous manner as **8d**: yield 59%; mp 205—208 °C (crystallized from benzene—hexane); ^{1}H NMR (CHCl$_3$-d) 9.18 (m, 1H), 8.91 (m, 1H), 8.37 (m, 2H), 8.31 (s, 1H), 8.29 (m, 1H), 7.77 (m, 1H), 7.75 (m, 1H), 7.66 (m, 1H), 7.58 (m, 1H), 7.50 (m, 1H), 7.47 (m, 1H), 7.20 (m, 1H), 6.59 (m, 1H), 6.49 (m, 1H), 3.45 (m, 4H), 2.63 (m, 4H), 2.35 (s, 3H), 2.00 (m, 4H). Anal. (C$_{34}$H$_{30}$N$_8$O$_2$·0.5H$_2$O) C, H, N.

8-Hydroxy-5-[[3-[methyl[3-[(6-oxo-6H-imidazo[4,5,1-de]-acridin-5-yl)amino]propyl]amino]propyl]amino]-6H-v-triazolo[4,5,1-de]acridin-6-one (8d). A mixture of **7** (0.364 g, 0.001 mol), 5-chloro-8-hydroxy-6H-v-triazolo[4,5,1-de]acridin-6-one[8] (0.272 g, 0.001 mol), dimethyl sulfoxide (8 mL), and triethylamine (1 mL) was stirred at 70 °C for 12 h. To the reaction mixture was added water (100 mL), and the precipitate was collected by filtration and washed with water. The crude product was crystallized from DMF—water to give yellow **8d**: mp 240—242 °C; ^{1}H NMR (Me$_2$SO-d_6) triazoloacridone chromophore 10.17 (br s, 1H, 8-OH), 9.18 (t, 1H, J = 5.2, NHCH$_2$), 8.07 (m, 1H, C10-**H**), 7.82 (d, 1H, J = 9.2, C3-**H**), 7.55 (m, 1H, C7-**H**), 7.23 (m, 1H, C9-**H**), 6.71 (d, 1H, J = 9.2, C4-**H**); imidazoacridone chromophore 8.93 (s, 1H, C1-**H**), 8.73 (t, 1H, J = 5.0, NHCH$_2$), 8.13 (m, 1H, C7-**H**), 8.09 (m, 1H, C10-**H**), 7.66 (m, 1H, C9-**H**), 7.65 (d, 1H, J = 8.8, C3-**H**), 7.31 (m, 1H, C8-**H**), 6.53 (d, 1H, J = 8.8, C4-**H**); aliphatic protons 3.45 (m, 2H, NHCH$_2$CH$_2$), 3.36 (m, 2H, NHCH$_2$CH$_2$), 2.55 (m, 4H, CH$_2$CH$_2$NCH$_3$), 2.29 (s, 3H, NCH$_3$), 1.90 (m, 4H, CH$_2$CH$_2$-CH$_2$). Anal. (C$_{34}$H$_{30}$N$_8$O$_3$·0.5H$_2$O) C, H, N.

5-[[3-[Methyl[3-[(7-methoxy-4-nitro-9-oxo-9,10H-acridin-1-yl)amino]propyl]amino]propyl]amino]-6H-imidazo[4,5,1-de]acridin-6-one (8e). This compound was obtained in an analogous manner as **6a** but **7** was used instead of **5**: yield 73%; mp 237—239 °C (crystallized from DMF—H$_2$O); ^{1}H NMR (Me$_2$SO-d_6) 12.09 (s, 1H), 11.68 (br s, 1H), 8.94 (s, 1H), 8.71 (br s, 1H), 8.10 (m, 2H), 7.91 (m, 1H), 7.68 (m, 1H), 7.61 (m, 2H), 7.29 (m, 2H), 7.17 (m, 1H), 6.52 (m, 1H), 6.06 (m, 1H), 3.77 (s, 3H), 3.34 (m, 4H), 2.55 (m, 4H), 2.27 (s, 3H), 1.89 (m, 4H). Anal. (C$_{35}$H$_{33}$N$_5$O$_5$·H$_2$O) C, H, N.

***In Vitro* Cytotoxicity Assay.** The cellular response to drugs was evaluated utilizing the sulforhodamine B assay as previously described.[29,39] Briefly, the human tumor cell lines making up the NCI cancer screening panel were routinely grown in RPMI 1640 medium containing 5% fetal bovine serum and 2 mM L-glutamine. Cells were inoculated into 96-well microtiter plates in 100 μL of complete medium at densities ranging from 5000 to 40 000 cells/well. The microtiter plates containing cells were incubated for 24 h prior to the addition of experimental drugs. Following the addition of drugs, the plates were incubated for an additional 48 h, and cells were fixed with TCA, washed, and stained with sulforhodamine B (Sigma Chemical Co., St. Louis, MO) at 0.4% (w/v) in 1% acetic acid. After washing with 1% acetic acid, the stain was solubilized with 10 mM unbuffered Tris base and the absorbance was measured on a Bio-Tek microplate reader. Dose—response parameters were calculated as previously reported.[29]

Colony Survival Assay. HCT-116 cells were obtained from American Type Culture Collection (Rockville, MD) and grown in Dulbecco's Minimum Essential Medium containing 10% fetal bovine serum and standard antibiotics (Sigma). Ditercalinium was a gift from Drug Synthesis & Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD. Cells were maintained at 37 °C in complete humidity and a 5% CO$_2$ atmosphere during subculturing and all experiments.

Assay consisted of seeding dilute cultures of approximately 40 cells per well in 6-well plates and allowing them to attach for 24 h. Compounds were added in microliter volumes directly to wells from concentrated stocks in dimethyl sulfoxide or in water (ditercalinium) to a final range of concentrations from milimolar to nanomolar. Control wells received vehicle alone. After 2 h exposure, wells were washed and fresh medium added to cells. Cultures were observed daily for 1–2 weeks, and cells were fixed in methanol and stained with a 1% Crystal Violet solution in methanol according to standard procedures.[40] Colonies of greater than 30 cells were scored as survivors.

***In Vivo* Antitumor Activity.** Athymic (nude) mice were provided by the Laboratory of Animal Sciences Program of the Frederick Cancer Research and Development Center. Weanling male mice weighing approximately 20 g received (on day 0) fresh subcutaneous implants of tumor fragments (30 mg) established previously from HCT-116 cells through *in vivo* passage in similar mice. There were six mice in each treatment and control group. Treatment with compounds was initiated on day 8, when tumors were reliably measurable. Treatment consisted of three intraperitoneal injections, every fourth day, of varying concentrations of compounds, used in the form of their water-soluble methanesulfonate salts, from fresh stocks prepared in a 5% glucose aqueous solution. Control animals received 5% glucose solution (negative control) or doxorubicin at a dose of 4 mg/kg (positive control). Doxorubicin was a gift from Drug Synthesis & Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD. Toxic doses were determined by prior studies by giving control mice a series of three consecutive daily injections of different doses of compound and recording deaths, survivors, and white blood cell counts at the end of 1 week after the initial dose.

Animal weights and tumor sizes were recorded daily according to the NCI *in vivo* cancer model procedures.[41] Briefly, caliper measurements of tumor dimensions were recorded in millimeters and tumor net weights obtained using the formula for a prolate ellipsoid. Median tumor weight changes were calculated as a percentage of median control tumor weights after a minimum weight of 63 mg per tumor was achieved (day 14). All animals treated with doxorubicin were sacrificed after day 27 due to a pronounced loss of body weight.

Thermal Melting Studies. Thermal melting curves for poly(dA)·poly(dT), d(CATATGCGCATATG), and their complexes with drugs were determined as previously described[42] by following the absorption change at 260 nm as a function of temperature. Poly(dA)·poly(dT) was purchased from Pharmacia, d(CATATGCGCATATG) was synthesized by Oligonucleotide Synthesis Laboratory, ABL-BRP, NCI-FCRDC, Frederick, MD. Solutions of DNAs (60 μM calculated on per base pair basis) were prepared in CNE buffer (10 mM sodium cacodylate (pH = 6), 300 mM NaCl, and 0.1 mM EDTA); 0.1 mM solutions of drugs in water were diluted with CNE buffer to 15 μM concentration and mixed with equivalent volume of the DNA solution to give experimental solutions with the ratio [base pairs]/[drug] = 4. The solutions were equilibrated for 1 h at 35 °C before measurement. A reference cuvette contained solution of drug at concentation 7.5 μM.

Computational Details. The modeling studies were conducted with Insight II[43] software from BIOSYM running on a Silicon Graphics IRIS Indigo-XZ workstation equipped with a R4000 processor. The B-DNA fragment was built using the Biopolymer module, and the drug molecule using the Builder module of Insight II. Partial atomic charges were calculated for the drug using the semiempirical quantum mechanical method MNDO which is contained in the MOPAC package.

The DNA fragment and the drug molecule were subjected to molecular mechanics energy refinement using the Discover program (version 2.95) utilizing the AMBER forcefield parameters. The 1–4 nonbond interactions were scaled by a factor of 0.5; the others parameters were scaled by 1. A distance-dependent dielectric constant $c = 3.5$ was used in the Coulombic electrostatic term to simulate the shielding influence of solvent. To diminish the probability that false local energy wells were minimized, a combination of molecular mechanics (MM) and molecular dynamics (MD) was used. After rough minimization by MM (RMS $\leq$ 0.1), the model was subjected to 1000 steps of MD at 500 K and 1 fs for each step of simulation, and the results of every 100 steps were recorded and next minimized using MM until RMS was less than 0.01. These calculations were carried out on a CRAY-YMP computer at the Biomedical Supercomputing Center at FCRDC.

The bis-intercalation binding site was built according to the method described by Rao[36] by inserting two molecules of 9-aminoacridine into the desired sequence. After initial energy refinement the molecules of 9-aminoacridine were removed from the binding site, and the molecule of DNA fragment was fully constrained. A properly constructed molecule of the drug was inserted into the preformed binding site and the complex was subjected to energy refinement, first with fully constrained DNA, and next without constraints. New partial atomic charges were calculated for the minimized conformation of drug built into the binding site using MOPAC. In the last step of refinement, the complex was minimized using a combination of MD and MM. Complex **E** was built by suitable adjustment of the molecule of the drug to a minimized DNA fragment, and the complex **F** by simple closing of the two minimized molecules.

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