4-Hydroxymethyl-3-aminoacridine Derivatives as a New Family of Anticancer Agents

Franck Charmantray,[‡] Martine Demeunynck,*[,‡] Danièle Carrez,[§] Alain Croisy,[§] Amélie Lansiaux,[#] Christian Bailly,[#] and Pierre Colson[||]

LEDSS, UMR CNRS 5616, Université Joseph Fourier, BP 53, 38041 Grenoble Cedex 9, France, INSERM UR-350, Institut Curie Recherche, Laboratoire Raymond Latarjet, Centre Universitaire, 91405 Orsay Cedex, France, INSERM UR-524 and Laboratoire de Pharmacologie Antitumorale du Centre Oscar Lambret, IRCL, Place de Verdun, 59045 Lille, France, and Biospectroscopy Laboratory, Department of Chemistry, University of Liege, Belgium

Received September 10, 2002

3-Amino- and 3-alkylamino-4-hydroxymethylacridines bearing various substituents on the C ring have been prepared by regioselective electrophilic aromatic substitution of the corresponding 3-aminoacridines and ring opening of the dihydrooxazinoacridine key intermediates. Most of the new compounds show potent cytotoxic activities against murine L1210 (leukemia), human A549 (lung), and HT29 (colon) cancer cell lines. The most cytotoxic molecules, **1** and **13**, are active at nanomolar concentrations. As predicted for acridine derivatives, the new compounds intercalate in DNA, but interestingly they do not interfere with topoisomerase I and II activities. The mode of action remains uncertain because intracellular distribution indicated very different behaviors for **1** and **13**. Compound **13** is uniformly distributed in the cell both in the cytoplasm and in the nucleus, whereas compound **1** is essentially localized in cytoplasmic granules.

Introduction

DNA remains one of the major targets for cytotoxic anticancer drugs. The planar structure of acridines confers to the molecules the ability to strongly bind DNA by intercalation and therefore to interfere with critical metabolic processes. A large number of natural alkaloids and synthetic acridine derivatives have been designed and tested as anticancer agents.[1−3] Among them, amsacrine, a 9-anilinoacridine, and ledakrin, a nitroacridine, are used in the clinic, and several derivatives such as the 4-carboxamidoacridine DACA (XR5000), imidazoacridinones,[4] and pyrazolo[3,4,5-*kl*]nitroacridine (PZA or NSC366140)[5] are currently undergoing clinical trials. The observed cytotoxicity may also be related to potent enzyme inhibition because it has been shown that topoisomerase[6] and telomerase[7] activities may be strongly affected by acridines. The affinity of acridines for DNA has been used to prepare intercalating alkylating agents in which a DNA alkylating group is tethered to the acridine nucleus. Denny and his group have extensively developed this approach by introducing various alkylating moieties to intercalating heterocycles.[8−11] All these synthetic intercalating alkylating agents interact by positioning the alkylating group in one groove, allowing formation of drug−DNA adducts.[12] We have prepared in the past a family of heterocyclic molecules derived from 3,6-diaminoacridine. To modulate the intercalation properties of the drugs, various substituents and/or new rings (10-aminobenzo[*b*][1,7]phenanthroline derivatives) were introduced on the acridine ring.[13−16] With the benzo[*b*][1,7]phenanthroline derivatives, pharmacological data indicated that the highest cytotoxicity was found with molecules substituted at position 11, in particular with hydroxymethyl group or dihydrooxazine analogues. Similar trends were obtained with proflavine (3,6-diaminoacridine) and 3-aminoacridine analogues. Furthermore, it turned out that some of these molecules, tested in vivo, displayed positive activities on tumor regression and increased the survival time ratio, the molecules containing the hydroxymethyl group in a position ortho to the amino group showing the highest activities (general structures shown in Figure 1).[13] We anticipated that such molecules would intercalate in DNA and generate an electrophilic quinone−imine−methide intermediate directly inside the intercalation complex, thus favoring the reaction with nucleophilic sites of nucleic acid bases.

To test this hypothesis, we examined more closely the chemical reactivity of the two molecules 3-methylamino- and 3-(dimethylamino)-4-hydroxymethylacridines **2** and **3**, possessing a benzylic-type hydroxyl group in a position ortho to the amino substituent. Compounds **2** and **3** were chosen as the simplest models for the molecules of the family.[17] The electrophilic properties of **2** and **3** were evidenced by studying their reactivity in methanol and 2-propanol. The corresponding 4-methoxy and 4-isopropyloxy derivatives were obtained quantitatively. Comparison of the solvolytic behavior of the free bases and of their *p*-toluenesulfonate salts clearly demonstrated the importance of protonation of the acridine ring on the reaction rate.

From these results, we propose that an intramolecular acid−base catalysis involving the acridinium **2** and **3** (Figure 2), with the protonated heterocyclic nitrogen in the peri position relative to the reactive hydroxymethyl group, allows the efficient catalysis of hydroxyl elimination and generation of the electrophilic quinone−imine−methide under mild conditions at room temperature.

* To whom correspondence should be addressed. Phone: +33 (0)476 514429. Fax: +33 (0)476 514946. E-mail: martine.demeunynck@ujf-grenoble.fr.
[‡] Université Joseph Fourier.
[§] Centre Universitaire.
[#] IRCL.
[||] University of Liege.

10.1021/jm020389w CCC: $25.00
Published on Web 02/15/2003

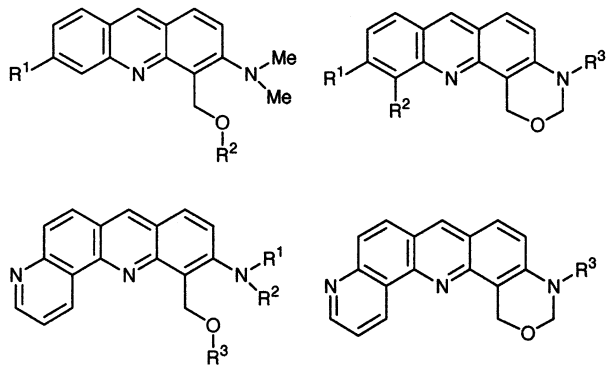


Figure 1.

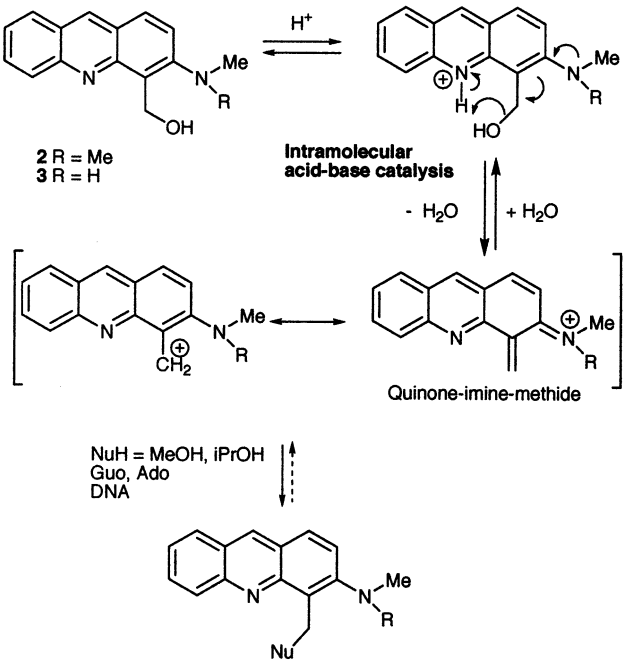


Figure 2.

Covalent binding of the two drugs with calf-thymus DNA was evidenced.[17] For molecules **2** and **3**, a high level of binding was found corresponding to one adduct for 16 or 14 base pairs, indicating that almost 50% of the drug was covalently bound. These results were in favor of the intercalation−alkylation hypothesis as a potential mode of action for these series of compounds at the molecular level. We proposed that the two molecules may form a noncovalent complex with DNA and then slowly react as electrophiles to covalently bind to DNA. These compounds would therefore constitute a new type of intercalating−alkylating agents because the electrophilic species would be generated at the intercalation site, i.e., in a "sandwich" position between base pairs and not in a groove as observed for other intercalating−alkylating drugs.

In this paper, we extend the series to novel 3-amino- and 3-alkylamino-4-hydroxymethylacridines. We report the synthesis, in vitro biological activity, structure−activity relationships for substituted derivatives of **2** and **3**, and their cyclic dihydrooxazino precursors.

Results and Discussion

Synthesis. As shown in Figure 3, the molecules described in this paper may be grouped into three

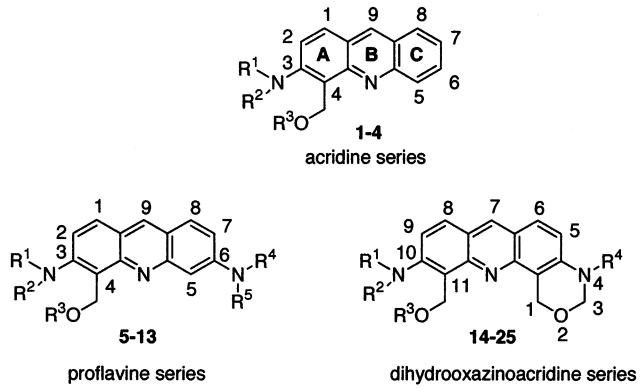
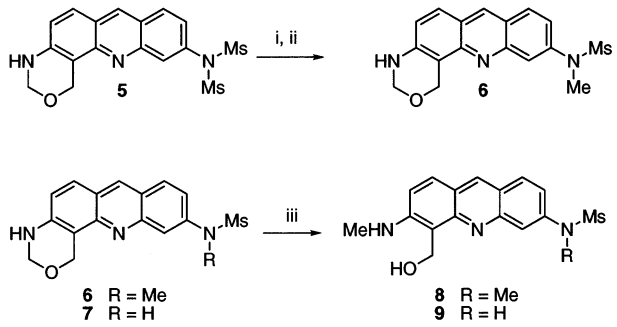


Figure 3.

Scheme 1[a]

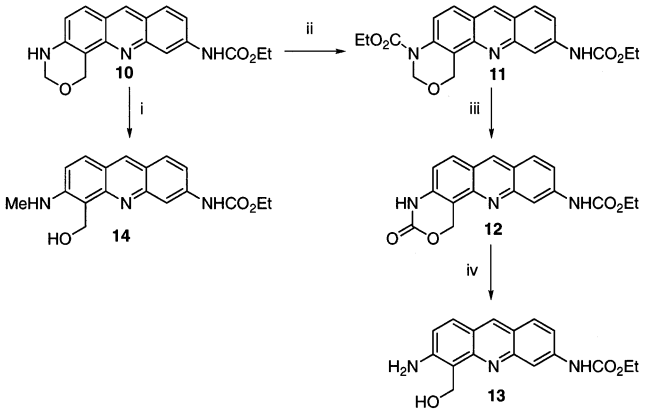


[a] (i) K_2CO_3/DMF/H_2O, 80 °C, 1 h and then (ii) MeI in excess; (iii) $LiAlH_4$, 10 equiv, 0 °C to room temp.

families depending on the presence and nature of substituents on the C ring of the acridine nucleus. The synthesis of the three series of molecules is based on regioselective aromatic electrophilic substitution of 3-aminoacridine derivatives to form dihydrooxazinoacridines. As previously reported,[18,19] dihydrooxazinoacridines may be prepared in good yields by reacting N-protected 3-amino- or 3,6-diaminoacridines with formaldehyde. Depending on the nature of the protecting group (acetyl, methanesulfonyl, or ethoxycarbonyl) and of the acid (methanesulfonic acid or hydrochloric acid) used as solvent and catalyst, N-unsubstituted, *N*-methyl-, or *N*-acyldihydrooxazines may be selectively obtained. These dihydrooxazines are key intermediates in the synthesis of the *o*-hydroxymethylalkylamines because ring opening of N-unsubstituted dihydrooxazine by LAH yields the corresponding *o*-hydroxymethylmethylamines in good yield.[17]

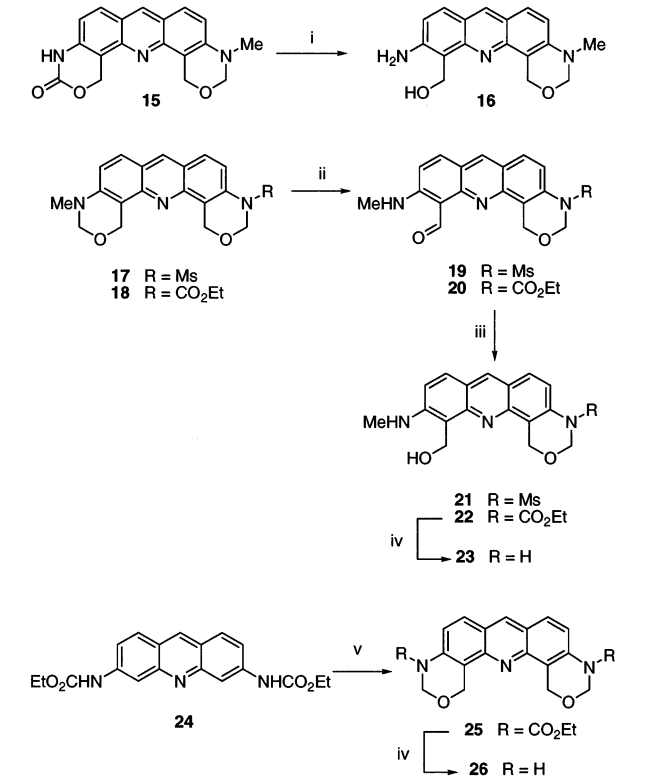
Under these conditions *N*-methyl- or *N*-acyl derivatives remain unchanged; however, *N*-methyldihydrooxazines react with DDQ to give the corresponding *o*-methylamino aldehydes,[18] which may be reduced to the corresponding alcohols with sodium borohydride. The synthesis of the molecules **1**−**4** belonging to the acridine series has been already published.[17,20] As outlined in Scheme 1, methanesulfonylproflavine alcohols **8** and **9**, which differ by the presence of a methyl substituent on the sulfonamido group, were prepared in 68% yield by LAH reduction of the corresponding dihydrooxazine **6** (itself obtained from **5** in a one-pot reaction) and **7**, respectively. The *N*-ethoxycarbonylproflavines **13** and **14**, which differ by the degree of substitution of the 3-amino group, were prepared from dihydrooxazine **10** (Scheme 2). LAH-catalyzed ring

Scheme 2[a]



[a] (i) LiAlH4, THF, 0 °C; (ii) $ClCO_2Et$ in excess/pyr, 0 °C to room temp; (iii) MsOH, 65 °C; (iv) NaOH, 0.3 to 1 N in THF (2:3, v/v).

Scheme 3[a]



[a] (i)NaOH, 0.3 N in THF (2:1, v/v); (ii) DDQ (2 equiv) in dioxane followed by 5 N HCl, 2 h, room temp; (iii) $NaBH_4$ in excess, room temp; (iv) (NaOH, 30%)/EtOH/THF/H_2O, 65 °C, 5 h; (v) $(HCHO)_n$, MsOH, room temp, 24 h.

opening afforded 3-*N*-methylamino derivative **14** in 70% yield. 3-Amino analogue **13** was obtained in 70% yield by alkaline hydrolysis of the corresponding dihydrooxazinone **12**, itself prepared in two steps from **10** by the method previously used to prepare **4**.[20] In a similar way, in the dihydrooxazinoacridine series depicted in Scheme 3, compound **16** was obtained by ring opening in an alkaline medium of the corresponding oxazinone **15**.[19] Synthesis of methylamino derivatives **21**−**23** exploited the reactivity of *N*-methyldihydrooxazine with DDQ reported previously.[18] Treatment of the bis-dihydrooxazines **17** and **18** with DDQ selectively cleaves the *N*-methyl-substituted dihydrooxazine ring, affording the corresponding aldehydes **19** and **20** in 71% and 80% yields, respectively. Reduction of the aldehydes to the corresponding alcohols was achieved by treatment with sodium borohydride. **21** and **22** were isolated in 55% and 84% yields, respectively. Compound **22** was converted to **23** by careful hydrolysis of the ethoxycarbonyl substituent under basic conditions. Reaction of *N*,*N*′-bis-(ethoxycarbonyl)proflavine with paraformaldehyde in methanesulfonic acid afforded the symmetrical bis-ethoxycarbonyldihydrooxazine **25** (71% yield), which was converted to the unsubstituted analogue **26** in 76% yield by hydrolysis.

In Vitro Cytotoxic Activities against Murine and Human Cell Lines. The biological activity of the new series of acridine derivatives has been measured using murine L1210 (leukemia) and human A549 (lung) and HT29 (colon) cancer cell lines. The results are collected in Table 1. Except for one molecule (**11**), all compounds are cytotoxic at micromolar concentrations or below. The nature of the substituents located on the A ring of the acridine appears to play a major role in cytotoxicity as observed previously.[13−16] Cytotoxicity decreases when the degree of substitution of the amino group ortho to the hydroxymethyl substituent increases (compare compounds **1**−**4** or **12**−**14**). Irrespective of the presence or nature of substituents on ring C, compounds carrying a primary amine ortho to the hydroxymethyl group (**1**, **13**, **16**) are among the most active molecules. Cyclic analogues such as dihydrooxazine or dihydrooxazinone appear less cytotoxic than the acyclic amino alcohols, and the presence of the electron-withdrawing function (dihydrooxazinone or *N*-ethoxycarbonyldihydrooxazine) results in loss of cytotoxicity (compare **10**, **12**, and **13**).

The nature of the substituent introduced on the C ring of the acridine does not influence the biological activity to a major extent, and no general structure−activity relationship may be established. Yet, an intriguing point emerges from this study: the *N*-methyl-substituted methanesulfonamido derivatives **6**/**8** are 10 times more cytotoxic than their unsubstituted analogues **7**/**9**. This difference in biological activity is associated with a difference in log *P*, **7/9** being much less lipophilic than **7/9** as reflected by lower log *P* values. These results are probably related to the existence of a tautomeric equilibrium for **7/9** with a charged limit form due to the presence of the very acidic NHMs proton (Scheme 4). Major differences in the UV−vis spectra of NMeMs vs NHMs compounds reinforce this hypothesis (data not shown).

DNA Binding. To evaluate the relative affinities of the acridines for DNA, melting temperature (T_m) measurements were performed with calf thymus DNA (42% GC bp) and the alternating polynucleotide poly(dAT)$_2$. The difference in T_m values between the drug−DNA complexes and free DNA or polynucleotide in solution provides a useful means to assess the strength of the interaction of the molecules with double-stranded DNA. The ΔT_m values ($\Delta T_m = T_m^{complex} - T_m^{DNA}$) measured with each compound in BPE buffer (16 mM Na ions) at a drug/DNA-phosphate (D/P) ratio of 0.5 are collected in Table 1. Apart from two compounds (**12** and **22**) that gave complex bi- or triphasic melting curves, simple melting curves were obtained for all the other compounds and the T_m values could be precisely measured from the first-derivative plots of the melting profiles.

Table 1. Biological and Biophysical Properties

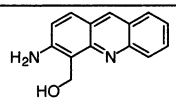
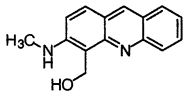
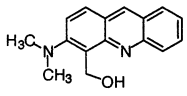
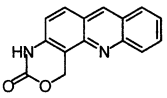
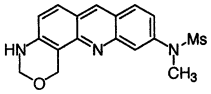
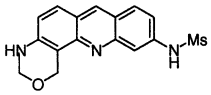
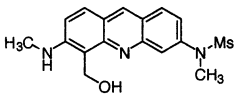
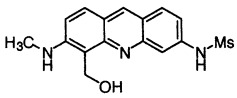
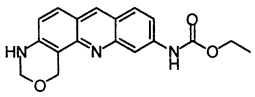
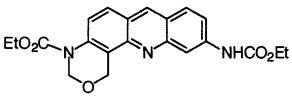
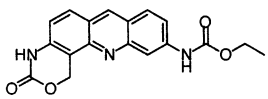
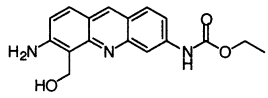
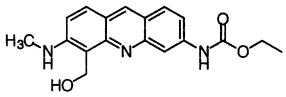
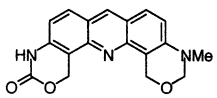
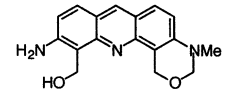
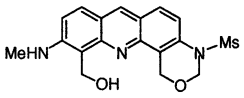
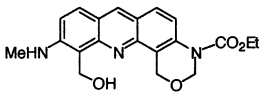
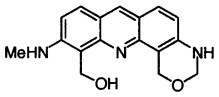
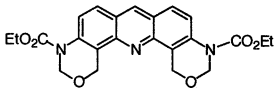
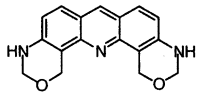
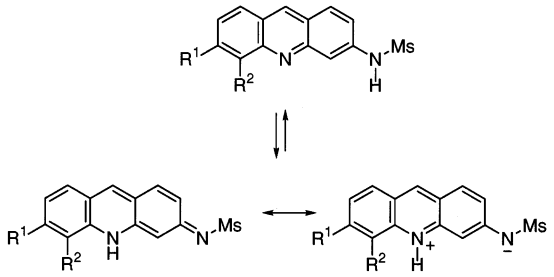
COMPOUNDS	N°	L1210 IC_{50} (μM)	A549 IC_{50} (μM)	HT29 IC_{50} (μM)	ΔTm^a CT DNA	ΔTm^a poly(dAT)	LOG P
	**1**	0.02	0.005	0.025	1.9	5.3	1.77
	**2·TsOH**	1.1	1.4	2.5	2.9	8	2.10
	**3·TsOH**	6	13	14	2.9	2.6	2.20
	**4**	2.8	7	17	0	1.3	1.64
	**6**	0.6	1.3	2.3	-	-	1.54
	**7**	6.2	6	12	0	1.3	0.70
	**8**	0.2	0.3	1.2	0	0	1.56
	**9**	2.8	4.4	29	0	0	0.55
	**10**	0.08	> 0.05	1.3	2.9	10.7	2.07
	**11**	84	19	>100	-	-	3.12
	**12**	2.7	2.2	11	0	2.5	2.00
	**13**	0.01	0.01	0.04	2.7	9.1	1.55
	**14**	0.09	0.03	0.35	2.7	10.4	1.97
	**15**	1	0.45	9	0	1.2	1.70

Table 1 (Continued)

COMPOUNDS	N°	L1210 IC_{50} (μM)	A549 IC_{50} (μM)	HT29 IC_{50} (μM)	ΔTm[a] CT DNA	ΔTm[a] poly(dAT)	LOG P
	**16**	0.65	0.05	0.3	2.9	10.9	1.64
	**21**	1.6	0.1	3.7	0	0	1.2
	**22**	1.5	0.35	4.2	0	1.2	1.75
	**23**	0.5	0.1	2	4.1	13	1.65
	**25**	>200	3.5	1	1.3	3	2.50
	**26**	0.35	0.02	1.5	0	0	2.17

[a] Variation in melting temperature ($\Delta T_m = T_m^{complex} - T_m^{DNA}$). T_m measurements were performed in BPE buffer, pH 7.1 (6 mM Na_2HPO_4, 2 mM NaH_2PO_4, 1 mM EDTA), using 10 μM drug and 20 μM calf thymus DNA (CT) or poly(dAdT)$_2$ (dAT) at 260 nm with a heating rate of 1 °C/min.

Scheme 4



The higher ΔT_m values measured with the poly(dAT)$_2$ compared to CT DNA reflect a greater susceptibility of the synthetic polymer to heat denaturation than the natural DNA. For this reason, a comparison can be made between the compounds in terms of relative DNA binding affinities and not sequence selectivity. In some cases, the compounds that were found to bind strongly to DNA correspond to the most cytotoxic drugs. For example, compounds **10**, **13**, and **14**, which are highly cytotoxic, gave ΔT_m values of ≥9 °C with poly(dAT)$_2$, whereas weakly cytotoxic compounds such as **7**, **15**, and **25** gave $\Delta T_m \leq 3$ °C. However, there is no direct relationship between DNA binding and cytotoxicity. A strong stabilization of the DNA duplex structure, as is the case with **23**, does not necessarily lead to a particularly high cytotoxic effect, and conversely, a high cytotoxicity can be obtained with a compound such as **1**, which exhibits a modest affinity for DNA. As is generally the case with such aromatic molecules, DNA is a potential, but not a unique target.

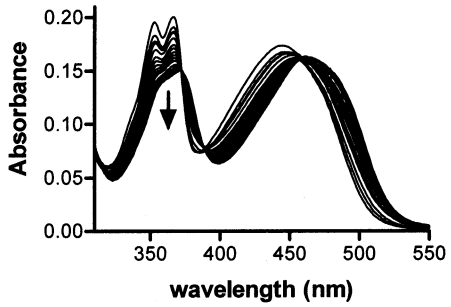


Figure 4. DNA titrations of **1**. To 3 mL of drug solution at 20 μM were added aliquots of a concentrated calf thymus DNA solution in 1 mM sodium cacodylate buffer, pH 7.0. The DNA-phosphate/drug ratio increased from 0 to 20 (top to bottom curves, at 350 nm).

In general, the binding was homogeneous and clear isosbestic behaviors were observed on titration of the compounds with DNA. Typical UV−visible titration spectra of **1** with calf thymus DNA (Figure 4) show both strong hypochromic and bathochromic effects. The drug absorption band centered at 360 nm is strongly decreased upon addition of DNA, and an 18 nm red shift (444 to 462 nm) occurs in the band characteristic of the acridine ring. This reflects the intercalation of the drug between DNA base pairs. The intercalative mode of binding was fully characterized by means of electric linear dichroism (ELD) experiments, which provide direct information on the orientation of the compounds with respect to the DNA helix. Typical ELD sets of data obtained with compounds **2** and **3** are shown in Figure 5. Whatever the sequence of the DNA substrate, the

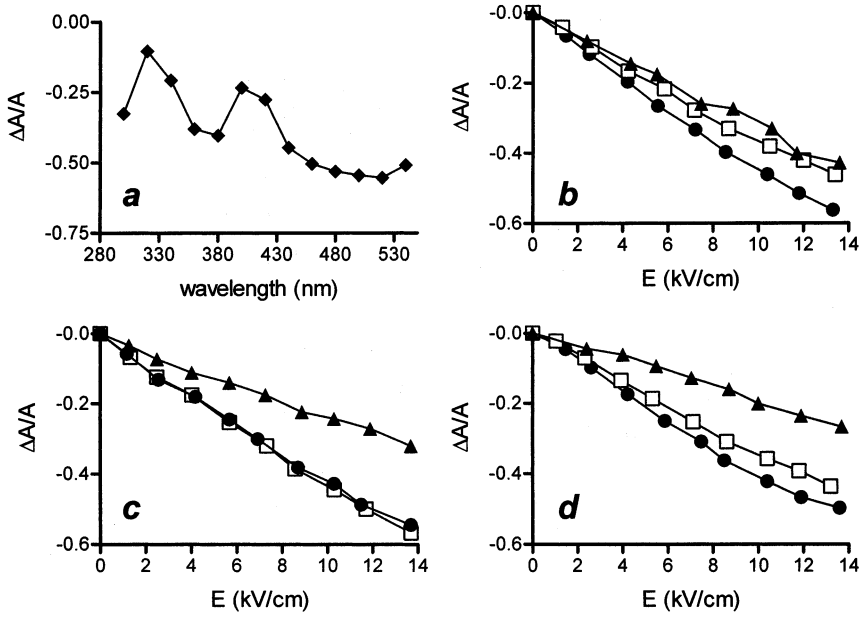


Figure 5. Electric linear dichroism data: (a) ELD spectrum of **2** bound to calf thymus DNA; (b−d) dependence of the reduced dichroism ($\Delta A/A$) on electric field strength for (b) calf thymus DNA, (c) poly(dAT)$_2$, and (d) poly(dGC)$_2$ alone (triangles) or complexed with **2** (circles) and **3** (squares). Measurements were performed at 260 nm for the DNA and polynucleotides alone and at 500 nm for the drug−DNA complexes, at a DNA-phosphate/drug (P/D) ratio of 20, in 1 mM sodium cacodylate buffer, pH 7.0.

reduced dichroism $\Delta A/A$ is negative in the drug absorption band, as expected for an orientation of the chromophore roughly perpendicular to the helix axis (or electric field direction). The study of the dependence of the reduced dichroism $\Delta A/A$ as a function of the electric field strength shows slight differences between the mono- and dimethylamino compounds, suggesting that their relative orientation along the DNA may be slightly distinct, but there is no doubt that they both intercalate into DNA. Footprinting experiments (not shown) indicated that the binding is not sequence-selective, since it is generally the case with proflavine-type acridine intercalators.

The compounds were also tested for their potential inhibitory activity against topoisomerases. Plasmid DNA relaxation assays[21] were employed with human topoisomerases I and II, but in both cases, none of the compounds were able to promote DNA cleavage by the enzyme. The acridine derivatives do not stabilize topoisomerase−DNA covalent complexes (data not shown).

Intracellular Distribution. HT29 colon carcinoma cells and B16 melanoma cells were used to compare the capacities of the drugs to stain the cells and their nuclei (Figure 6). The intrinsic fluorescence properties of the acridine nucleus were exploited to assess the cellular uptake and intracellular distribution of the drugs by confocal microscopy. The cells were treated with each compound at 20 μM for 24 h at 37 °C, washed, fixed with 2% paraformaldehyde, and then counterstained with the far-red fluorescent dye TOTO-3, which is routinely used to stain cell nuclei and to label nucleic acids.[22] The dye is detected under far-red fluorescence (647 nm) excitation.

Two types of intracellular fluorescence profiles were observed. For compounds such as **1**−**3**, the fluorescence was essentially localized in cytoplasmic granules with little staining of the cell nuclei. In other cases, such as with compound **13**, the fluorescence is more uniformly distributed in the cells and both the cytoplasm and the nucleus were strongly fluorescent. The marked yellow fluorescence seen in the HT29 cells doubly stained with **13** and TOTO-3 suggests that a significant proportion of the drug molecule is bound to DNA. For this particular compound, it is possible that the cytotoxic action is linked to nuclear DNA interaction, but this is not generally the case. For example, compound **1** shows little nuclear accumulation despite its high cytotoxic property.

Conclusion

Twenty aminoacridine derivatives have been prepared. The new compounds bear a methylene-type substituent ortho to the 3-amino group and differ by the degree of alkylation of the amino group and the presence of substituents on the C ring. Most of them display interesting cytotoxicities against murine and human cancer cell lines ($IC_{50} < 1$ μM). To understand their mode of action, we investigated their interaction with DNA. As expected for acridine derivatives, they interact with DNA by intercalation, and some of them may covalently bind to the macromolecules as evidenced previously.[17] Unlike other acridine antitumor agents such as DACA or amsacrine, the compounds in this novel acridine series do not interfere with topoisomerase activities. The results of intracellular distribution of the most cytotoxic molecules **1** and **13** raise new questions about the mode of action and provide a novel challenge for future studies. For compound **1**, being clearly located in the cytoplasm, it seems obvious that nuclear DNA is not its cellular target, and the mechanisms of action of these compounds are still to be found.

The picture is different with compound **13**, which appears to be located in the cytoplasm and the nucleus. Identifying the target(s) and understanding the mechanisms of cellular entry and localization are important issues for reengineering related acridines with superior antitumor activities. The potential target for these compounds, DNA, is found not only in the nucleus but

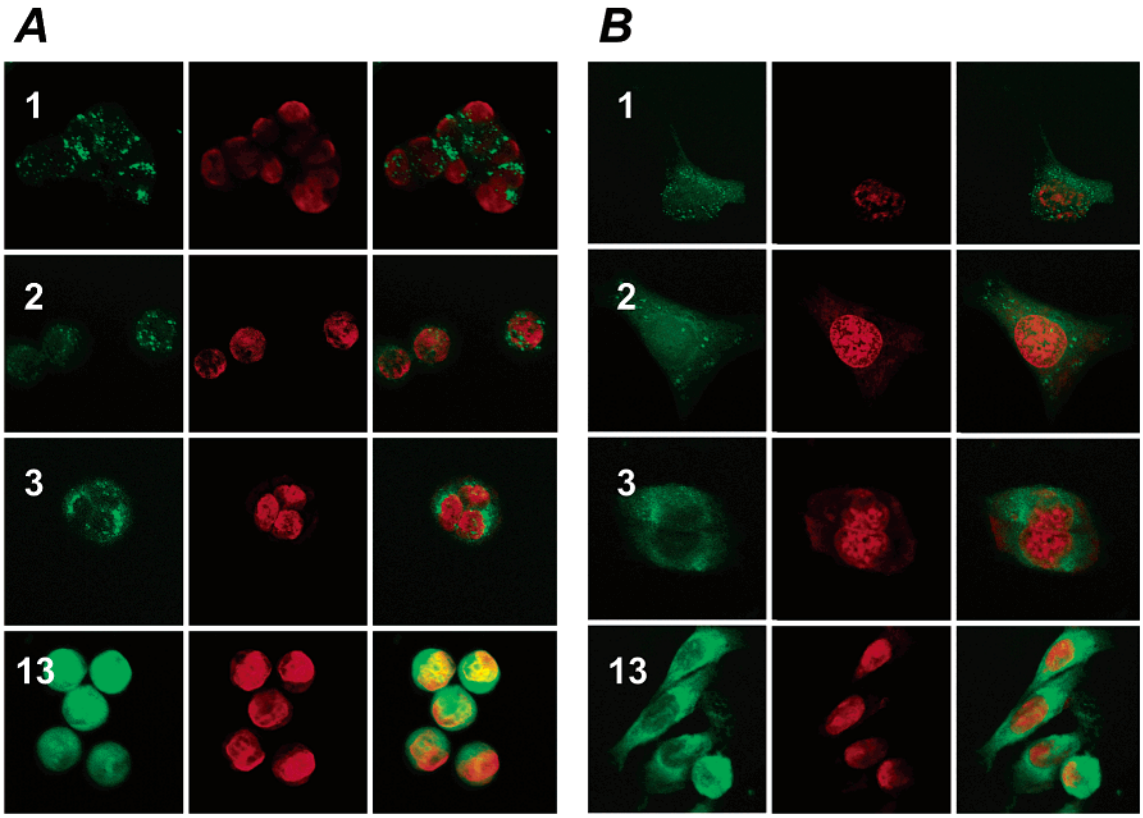


Figure 6. Confocal laser scanning microscopy of (A) HT29 colon carcinoma cells and (B) B16 melanoma cells treated with the indicated compound at 20 μM for 24 h. The green and red images correspond to the subsequent incubation of the same cells with the acridine drugs and the dye TOTO-3 (0.5 μM), and the images on the right side correspond to the superimposed green and red fluorescence.

also in mitochondria. The possibility exists that mitochondrial DNA and its associated proteins, such as topoisomerases,[23,24] represent bioreceptors for these acridines. This study raises new opportunities to distribute DNA-targeted drugs in specific intracellular organelles.

Experimental Section

Melting points were determined using a Reicher Thermovar apparatus and are uncorrected. NMR spectra were recorded on Bruker AC 200 and AM 300 spectrometers using solvent as the internal reference (DMSO-d_6 at 2.49 ppm, $CDCl_3$ at 7.24 ppm). The chemical shifts are reported in ppm, in δ units. The high-resolution mass spectra of pure products were recorded on Varian Mat 311 and AET MS 30 instruments and were obtained from "Centre Regional de Mesures Physiques de l'Ouest", Université de Rennes. Other mass spectral data were obtained from CERMAV/CNRS-Grenoble. They were recorded on a Nermag R10-10 quadrupolar mass spectrometer. Absorption spectra were obtained on a Perkin-Elmer Lambda UV−vis spectrometer. Microanalyses were performed by the "Service Central de Microanalyses du CNRS", Lyon. Reversed-phase HPLC was performed with a μ-bondapak C18 analytical column (Waters Associates). A Waters chromatographic system was used, with two M-510 pumps and a Waters 996 photodiode array detector using Millenium 32 software. A linear gradient from 0% to 100% methanol in H_2O, pH 2.5 (phosphoric acid), 2 mL/min flow rate, was used.

Syntheses. The synthesis of compounds **1**−**4** has been previously reported.[17,20]

3,4-Dihydro-10-(*N*-methanesulfonyl-*N*-methylamino)-1*H*-[1,3]oxazino[4,5-*c*]acridine (6). Compound **5**[19] (125 mg, 0.30 mmol) was dissolved in a 6:1 DMF/water mixture (10 mL) in the presence of potassium carbonate (210 mg, 1.53 mmol). The reaction was heated at 80 °C for 1 h. After the mixture was cooled, iodomethane (0.66 mL, 10.3 mmol) was added to the solution. The mixture was then allowed to react for 6 h at room temperature. The solvents were removed in vacuo, and the resultant solid was dissolved in water and extracted with ether. The organic phases were collected and coevaporated with silica gel (0.5 g) pretreated with ammonium hydroxide. The crude product was subjected to column chromatography, eluting with a AcOEt/CH_2Cl_2 (1:1, v/v) mixture. Compound **6** was obtained as a yellow powder (50 mg, 48%). Mp 300 °C. ^{1}H NMR (200 MHz, $CDCl_3$): δ 8.92 (1H, s, H-7), 7.93 (1H, d, J = 1.9 Hz, H-11), 7.88 (1H, d, J = 8.9 Hz, H-8), 7.71 (1H, d, J = 8.9 Hz, H-6), 7.58 (1H, dd, J = 1.9, 8.9 Hz, H-9), 7.01 (1H, d, J = 8.9 Hz, H-5), 5.46 (2H, s, Ar−CH_2−O), 4.89 (2H, s, O−CH_2−N), 3.48 (3H, s, N(Ms)CH_3), 2.89 (3H, s, SO_2−CH_3). ^{13}C NMR (75 MHz, DMSO-d_6): δ 149.2 (C quat), 148.2 (C quat), 144.0 (C quat), 143.3 (C quat), 136.3 (CH), 129.6 (CH), 128.4 (CH), 125.1 (CH), 124.2 (C quat), 123.0 (C quat), 122.7 (CH), 121.5 (CH), 113.9 (C quat), 75.4 (O−CH_2−N), 66.3 (Ar−CH_2−O), 38.8 (SO_2−CH_3), 35.8 (N(Ms)CH_3). MS (FAB (+) glycerol) *m/e*: 344 $(M + H)^+$. HRMS (LSIMS with Cs^+, positive mode, mNBA): $(M + H)^+_{found}$ 344.1069; $(M + H)^+_{calcd}$ for $C_{17}H_{18}N_3O_3S$ 344.1069. UV (ethanol 95%) λ_{Max} (ϵ): 428 (5550), 377 (7770), 270 (34 710), 250 (28 020) nm.

4-Hydroxymethyl-3-methylamino-6-(*N*-methanesulfonyl, *N*-methylamino)acridine (8). $LiAlH_4$ (30 mg, 0.8 mmol) was added to a solution of **6** (25 mg, 0.073 mmol) in dried THF (5 mL), and the mixture was cooled to 0 °C. The solution was stirred for 1 h under nitrogen atmosphere in the dark. The reaction was quenched by pouring the mixture onto ice. The aqueous solution was made alkaline (pH 8−9) with 0.1 N NaOH solution. Ethyl acetate was added, and the organic layer was separated, washed with water and with brine, and dried over sodium sulfate. The solvent was removed in vacuo to give **8** (17 mg, 68%) as an orange powder. Mp 150 °C. ^{1}H NMR (200 MHz, DMSO-d_6): δ 8.78 (1H, s, H-9), 7.99 (2H, m, Ar−H), 7.90 (1H, d, J = 1.9 Hz, H-5), 7.45 (1H, dd, J = 1.9, 8.9 Hz, H-7),

7.35 (1H, d, J = 8.9 Hz, Ar−H), 6.28 (1H, m, NHMe), 5.28 (2H, s, Ar−CH_2−OH), 3.41 (3H, s, N(Ms)−CH_3), 3.00 (3H, d, J = 4.8 Hz, (NH)CH_3), 2.89 (3H, s, SO_2−CH_3). ^{13}C NMR (75 MHz, DMSO-d_6): δ 149.6 (C quat), 148.3 (C quat), 148.2 (C quat), 143.1 (C quat), 135.7 (CH), 129.3 (CH), 128.9 (CH), 122.5 (CH), 122.3 (CH), 121.9 (C quat), 120.4 (C quat), 116.0 (CH), 112.4 (C quat), 54.7 (Ar−CH_2−O), 37.7 (SO_2−CH_3), 35.3 (N(Ms)−CH_3)), 29.9 (NH−CH_3). MS (FAB (+) glycerol) *m/e*: 346 (M + H)$^+$. HRMS (LSIMS with Cs^+, positive mode, mNBA): $(M + H)^+_{found}$ 346.1218; $(M + H)^+_{calcd}$ for $C_{17}H_{20}N_3O_3S$ 346.1225; $(M - OH)^+_{found}$ 328.1118; $(M - OH)^+_{calcd}$ 328.1120. UV (ethanol 95%) λ_{Max} (ϵ): 455 (6930), 364 (6350), 284 (43 480), 245 (27 330) nm.

4-Hydroxymethyl-3-methylamino-6-methanesulfonylaminoacridine (9). 9 was prepared as above from dihydrooxazine **7**[19] and was isolated as an orange solid (46% yield). Mp >350 °C. ^{1}H NMR (200 MHz, DMSO-d_6): δ 10.19 (1H, s, NH−Ms), 8.68 (1H, s, H-9), 7.94 (1H, d, J = 8.9 Hz, H-1 or H-8), 7.89 (1H, d, J = 8.9 Hz, H-8 or H-1), 7.67 (1H, d, J = 1.9 Hz, H-5), 7.28 (1H, dd, J = 1.9, 8.9 Hz, H-7), 7.23 (1H, d, J = 8.9 Hz, H-2), 6.2 (1H, s, NH−Me), 5.22 (2H, s, Ar−CH_2−OH), 5.00 (1H, s, OH), 3.12 (3H, s, SO_2−CH_3), 2.97 (3H, d, J = 4.8 Hz, NCH_3). MS (FAB (+) mNBA) *m/e*: 332 (M + H)$^+$. HRMS (LSIMS with Cs^+, positive mode, mNBA): $(M + H)^+_{found}$ 332.1068; $(M + H)^+_{calcd}$ for $C_{16}H_{18}N_3O_3S$ 332.1069. UV (ethanol 95%) λ_{Max} (ϵ): 481 (21 670), 380 (8550), 272 (43 520), 251 (33 460) nm.

4-Ethoxycarbonyl-10-ethoxycarbonylamino-3,4-dihydro-1*H*-[1,3]oxazino[4,5-*c*]acridine (11). Ethyl chloroformate (0.120 mL, 1 mmol) was added to a solution of **10**[19] (100 mg, 0.31 mmol) in pyridine (4 mL) cooled at 0 °C. The mixture was stirred for 2 h at room temperature. Pyridine was removed in vacuo. The resinous residue was dissolved in water (100 mL) and then extracted twice with ethyl acetate. The combined organic layers were successively washed with 0.1 N HCl and 0.1 N NaOH solutions. After drying over sodium sulfate, the organic phase was concentrated. The residue was purified on silica. eluting with 1% EtOH in CH_2Cl_2. Compound **11** was thus obtained as a yellow solid (90 mg, 74%). Mp 174−175 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.53 (1H, s, H-7), 8.10 (1H, d, J = 2.1 Hz, H-11), 7.87 (2H, m, H-5 or H-6, and H-8), 7.76 (1H, d, J = 9.6 Hz, H-6 or H-5), 7.61 (1H, dd, J = 2.1, 9.1 Hz, H-9), 5.57 (2H, s, Ar−CH_2−O), 5.31 (2H, s, N−CH_2−O), 4.33 (2H, q, J = 7.2 Hz, CO_2−CH_2), 4.29 (2H, q, J = 7.2 Hz, CO_2−CH_2), 1.37 (3H, t, J = 7.2 Hz, CH_3), 1.35 (3H, t, J = 7.2 Hz, CH_3). ^{13}C NMR (75 MHz, $CDCl_3$): δ 154.2 (CO), 153.5 (CO), 149.4 (C quat), 146.7 (C quat), 139.8 (C quat), 138.3 (C quat), 135.4 (CH), 129.4 (CH), 126.5 (CH), 123.4 (C quat), 122.9 (C quat), 122.8 (CH), 121.4 (C quat), 119.9 (CH), 114.7 (CH), 75.3 (CH_2), 66.7 (CH_2), 62.8 (CH_2), 61.8 (CH_2), 14.7 (CH_3), 14.6 (CH_3). IR (KBr): 3340, 2983, 1734 (CO), 1684 (CO) cm^{-1}. MS (FAB (+) glycerol) *m/e*: 395 (M)$^{+\bullet}$. HRMS (LSIMS with Cs^+, positive mode, mNBA): $(M + H)^+_{found}$ 396.1562; $(M + H)^+_{calcd}$ for $C_{21}H_{22}N_3O_5$ 396.1559. UV (ethanol 95%) λ_{Max} (ϵ): 376 (10 000), 271 (10 630) nm.

10-Ethoxycarbonylamino-3(4*H*)-one-1*H*-[1,3]oxazino-[4,5-*c*]acridine (12). A solution of compound **11** (100 mg, 0.25 mmol) in 99% methanesulfonic acid (15 mL) was stirred for 40 min at 75 °C. After cooling, the solution was added dropwise into water (100 mL) cooled in an ice bath, and the solution was extracted twice with ethyl acetate. The aqueous layer was made alkaline (pH 12) by addition of ammonium hydroxide and was extracted three times with ethyl acetate. The organic layer was washed with water and brine, dried over sodium sulfate, and evaporated to dryness. Raw compound **12** was obtained in 80% yield (>85% purity as estimated by HPLC). The pure sample of compound **12** was obtained by crystallization from CH_2Cl_2/hexane. Mp 235−236 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 10.47 (1H, s, NH), 10.12 (1H, s, NH), 8.85 (1H, s, H-7), 8.25 (1H, d, J = 1.8 Hz, H-11), 8.02 (1H, d, J = 9.0 Hz, H-5 or H-6), 8.0 (1H, d, J = 9.1 Hz, H-8), 7.58 (1H, dd, J = 1.8, 9.1 Hz, H-9), 7.17 (1H, d, J = 9.0 Hz, H-6 or H-5), 5.93 (2H, s, Ar−CH_2−O), 4.19 (2H, q, J = 7.2 Hz, CH_2), 1.29 (3H, t, J = 7.2 Hz, CH_3). ^{13}C NMR (75 MHz, DMSO-d_6): δ 153.5 (CO), 151.1 (CO), 149.5 (C quat), 144.9 (C quat), 141.4 (C quat), 137.5 (C quat), 136.4 (CH), 130.0 (CH), 129.4 (CH), 121.9 (C quat), 121.7 (C quat), 119.6 (CH), 115.5 (CH), 112.2 (CH), 108.6 (C quat), 66.2 (CH_2), 60.6 (CH_2), 14.4 (CH_3). IR (KBr): 1710 (CO), 1648 (CO) cm^{-1}. MS (FAB (+) glycerol) *m/e*: 338 (M + H)$^+$. HRMS (LSIMS with Cs^+, positive mode, mNBA): $(M + H)^+_{found}$ 338.1138; $(M + H)^+_{calcd}$ for $C_{18}H_{16}N_3O_4$ 338.1141. UV (ethanol 95%) λ_{Max} (ϵ): 384 (15 350), 272 (51 250) nm.

6-Ethoxycarbonylamino-4-hydroxymethyl-3-aminoacridine (13). A solution of dihydrooxazinone **12** (155 mg, 0.46 mmol) in a mixture of 0.4 N NaOH and THF (2:3, v/v, 60 mL) was heated at 65 °C for 5 h. After cooling, the mixture was poured into water (150 mL). The aqueous solution was extracted three times with ethyl acetate (150 mL). The organic layers were washed with 0.1 N HCl solution, saturated sodium hydrogenocarbonate solution, and water. The solvent was evaporated in vacuo. The residue was triturated in a 1:1 ether/pentane mixture, and **13** was obtained as a yellow solid (100 mg, 70%). Mp 135 °C. ^{1}H NMR (200 MHz, DMSO-d_6): δ 9.98 (1H, s, NH), 8.56 (1H, s, H-9), 8.13 (1H, d, J = 2.1 Hz, H-5), 7.87 (1H, d, J = 8.9 Hz, H-8), 7.73 (1H, d, J = 9.0 Hz, H-1 or H-2), 7.45 (1H, dd, J = 2.1, 8.9 Hz, H-7), 7.07 (1H, d, J = 9.0 Hz, H-2 or H-1), 6.00 (2H, s, Ar−NH_2), 5.16 (2H, s, Ar−CH_2), 4.20 (2H, q, J = 7.2 Hz, CH_2), 1.33 (3H, t, J = 7.2 Hz, CH_3). ^{13}C NMR (75 MHz, DMSO-d_6): δ 153.9 (CO), 149.7 (C quat), 149.1 (C quat), 149.0 (C quat), 140.8 (C quat), 135.6 (CH), 129.2 (CH), 128.8 (CH), 120.7 (CH), 120.5 (C quat), 120.4 (C quat), 117.9 (CH), 112.9 (CH), 111.9 (C quat), 60.8 (CH_2), 55.6 (CH_2), 14.9 (CH_3). IR (KBr): 1717 (CO) cm^{-1}. MS (FAB (+) glycerol) *m/e*: 312 (M + H)$^+$. HRMS (LSIMS with Cs^+, positive mode, mNBA): $(M + H)^+_{found}$ 312.1357; $(M + H)^+_{calcd}$ for $C_{17}H_{18}N_3O_3$ 312.1348; $(M - OH)^+_{found}$ 294.1265; $(M - OH)^+_{calcd}$ 294.1243. UV (ethanol 95%) λ_{Max} (ϵ): 440 (6230), 384 (6990), 268 (26 770) nm.

6-Ethoxycarbonylamino-4-hydroxymethyl-3-methylaminoacridine (14). Compound **10**[19] (100 mg, 0.31 mmol) dissolved in dried THF (10 mL) was stirred at 0 °C for 10 min. LAH (23 mg, 1.3 mmol) was added portionwise to the solution. The reaction mixture was stirred at 0 °C until the starting material completely disappeared (TLC elution with 8:2 AcOEt/MeOH mixture). The solution was poured onto ice and diluted with water (150 mL) and ethyl acetate. The organic layer was separated, dried over sodium sulfate, and evaporated to dryness. The residue was dissolved in methylene chloride, and the insoluble part was removed by filtration. The filtrate was triturated in pentane to give compound **14** (70 mg, 70%) as a yellow solid. Mp 145 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 9.96 (1H, s, NH), 8.62 (1H, s, H-9), 8.13 (1H, d, J = 2.0 Hz, H-5), 7.89 (1H, d, J = 9.3 Hz, H-1 or H-2), 7.87 (1H, d, J = 9.0 Hz, H-8), 7.44 (1H, dd, J = 2.0, 9.0 Hz, H-7), 7.24 (1H, d, J = 9.2 Hz, H-2 or H-1), 6.16 (1H, m, NHMe), 5.23 (2H, s, CH_2), 4.19 (2H, q, J = 7.1 Hz, CH_2), 2.97 (3H, d, J = 5.0 Hz, NCH_3), 1.29 (3H, t, J = 7.1 Hz, CH_3). ^{13}C NMR (75 MHz, DMSO-d_6): δ 153.5 (CO), 149.3 (C quat), 149.1 (C quat), 148.3 (C quat), 140.5 (C quat), 135.6 (CH), 129.2 (CH), 129.0 (CH), 120.1 (C quat), 119.5 (C quat), 117.6 (CH), 114.8 (CH), 112.3 (CH), 60.4 (CH_2), 54.9 (CH_2), 29.9 (CH_3), 14.5 (CH_3). IR (KBr): 3426, 3983, 1713 (CO) cm^{-1}. MS (FAB (+) glycerol) *m/e*: 326 (M + H)$^+$. HRMS (LSIMS with Cs^+, positive mode, mNBA): $(M + H)^+_{found}$ 326.1502; $(M + H)^+_{calcd}$ for $C_{18}H_{20}N_3O_3$ 326.1505; $(M - OH)^+_{found}$ 308.1379; $(M - OH)^+_{calcd}$ 308.1399. UV (ethanol 95%) λ_{Max} (ϵ): 467 (11 410), 386 (12 570), 295 (33 660), 272 (39 630), 255 (35 680) nm.

10-Amino-11-hydroxymethyl-3,4-dihydro-4-methyl-1*H*-[1,3]oxazino[4,5-*c*]acridine (16). 16 was prepared by alkaline hydrolysis of the corresponding oxazinone **15**,[19] following the procedure described above for **13**. Mp 235−240 °C. ^{1}H NMR (200 MHz, DMSO-d_6): δ 8.50 (1H, s, H-7), 7.76 (1H, d, J = 9.0 Hz, Ar−H), 7.68 (1H, d, J = 9.0 Hz, Ar−H), 7.18 (1H, d, J = 9.0 Hz, Ar−H), 7.01 (1H, d, J = 9.0 Hz, Ar−H), 5.96 (2H, s, NH_2), 5.29 (2H, s, Ar−CH_2−O), 5.14 (2H, s, Ar−CH_2−OH), 4.72 (2H, s, N−CH_2−O), 3.03 (1H, s, CH_3), OH not detected. MS (FAB (+) glycerol) *m/e*: 296 (M + H)$^+$. Anal. ($C_{17}H_{17}N_3O_2$·

0.5$CHCl_3$) C, H, N. UV (ethanol 95%) λ_{Max} (ϵ): 408 (11 300), 272 (38 000) nm.

11-Formyl-4-methanesulfonyl-10-methylamino-3,4-dihydro-1*H*-[1,3]oxazino[4,5-*c*]acridine (19). A mixture of **17**[19] (50 mg, 0.13 mmol) and DDQ (66 mg, 0.29 mmol) in MeOH (5 mL) was stirred overnight at room temperature. The solution was added dropwise to a solution of 5 N HCl (40 mL) with stirring for 90 min. The orange solid was filtered and added to diluted ammonium hydroxide (20 mL). The suspension was stirred for 30 min. The solid was filtered off and was washed with ether and cold methanol. Compound **19** was isolated as a yellow powder (0.034 g, 71%). Mp 122 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 11.31 (1H, s, CHO), 10.50 (1H, m, NH), 8.39 (1H, s, H-7), 7.86 (1H, d, J = 9.5 Hz, H-8), 7.72 (1H, d, J = 9.4 Hz, H-5 or H-6), 7.68 (1H, d, J = 9.4 Hz, H-5 or H-6), 7.10 (1H, d, J = 9.5 Hz, H-9), 5.57 (2H, s, Ar−CH_2−O), 5.28 (2H, s, N−CH_2−O), 3.14 (3H, d, J = 5.3 Hz, NCH_3), 2.97 (3H, s, SO_2CH_3). ^{13}C NMR (75 MHz, $CDCl_3$): δ 193.1 (CHO), 155.8 (C quat), 150.8 (C quat), 145.8 (C quat), 137.4 (C quat), 137.1 (CH), 136.6 (CH), 127.4 (CH), 124.0 (C quat), 123.0 (CH), 122.8 (C quat), 119.9 (C quat), 115.6 (CH), 107.1 (C quat), 77.8 (CH_2), 66.4 (CH_2), 39.8 (CH_3−SO_2), 29.6 (NCH_3). IR (KBr): 3237, 2935, 2845, 1635 (CHO), 1343 (SO_2), 1158(SO_2) cm^{-1}. MS (FAB (+) glycerol) *m/e*: 372 (M + H)$^+$. HRMS (LSIMS with Cs^+, positive mode, mNBA): (M + H)$^+_{found}$ 372.1013; (M + H)$^+_{calcd}$ for $C_{18}H_{18}N_3O_4S$ 372.1018. UV (ethanol 95%) λ_{Max} (ϵ): 452 (9400), 428 (11 340), 343 (16 400), 269 (69 600) nm.

4-Ethoxycarbonyl-11-formyl-10-methylamino-3,4-dihydro-1*H*-[1,3]oxazino[4,5-*c*]acridine (20). 20 was prepared as above from **18** to give **20** as a yellow solid (89% yield). Mp 240−241 °C. ^{1}H NMR (200 MHz, $CDCl_3$): δ 11.28 (1H, s, CHO), 10.41 (1H, m, N−H), 8.27 (1H, s, H-7), 7.85−7.63 (3H, m, H-5, H-6, and H-8), 7.04 (1H, d, J = 9.6 Hz, H-9), 5.48 (2H, s, Ar−CH_2−O), 5.29 (2H, s, N−CH_2−O), 4.32 (2H, q, J = 7.1 Hz, CH_2), 3.10 (3H, d, J = 5.5 Hz, NCH_3), 1.36 (3H, t, J = 7.1 Hz, CH_3). ^{13}C NMR (75 MHz, $CDCl_3$): δ 193.4 (CHO), 155.6 (C quat), 154.1 (C quat), 150.5 (C quat), 146.0 (C quat), 138.8 (C quat), 137.2 (CH), 136.2 (CH), 126.4 (CH), 121.7 (CH), 121.6 (C quat), 121.3 (C quat), 119.3 (C quat), 114.8 (CH), 107.2 (C quat), 75.3 (O−CH_2−N), 66.7 (Ar−CH_2−O), 62.8 (CH_2), 29.6 (NCH_3), 14.6 (CH_3). IR (KBr): 2990, 2845, 1709 (CO), 1638 (CHO) cm^{-1}. MS (FAB(+) glycerol) *m/e*: 366 (M + H)$^+$. HRMS (LSIMS with Cs^+, positive mode, mNBA): (M + H)$^+_{found}$ 366.1460; (M + H)$^+_{calcd}$ for $C_{20}H_{20}N_3O_4$ 366.1454. UV (ethanol 95%) λ_{Max} (ϵ): 450 (9640), 426 (1240), 374 (13 400), 348 (17 000), 266 (71 140) nm.

11-Hydroxymethyl-4-methanesulfonyl-10-methylamino-3,4-dihydro-1*H*-[1,3]oxazino[4,5-*c*]acridine (21). $NaBH_4$ (0.03 g, 0.8 mmol) was added in portions to a suspension of **18** (29 mg, 0.08 mmol) in methanol (4 mL). The reaction was monitored by TLC (elution with ether/pentane, 3:1, v/v). After having been stirred for 90 min, the solution was diluted in a large volume of water and the organic layer was removed by evaporation. The aqueous layer was extracted with ethyl acetate. The organic layer was washed twice with water, dried over sodium sulfate, and concentrated. Addition of pentane induced precipitation of an orange product that was filtered and washed with ether to give compound **21** (16 mg, 55%). Mp 205 °C. ^{1}H NMR (200 MHz, $CDCl_3$): δ 8.52 (1H, s, H-7), 7.86 (1H, d, J = 9.6 Hz, H-8), 7.75 (2H, m, H-5, H-6), 7.26 (1H, d, J = 9.6 Hz, H-9), 5.59 (2H, s, Ar−CH_2−O), 5.34 (2H, s, Ar−CH_2−OH), 5.29 (2H, s, N−CH_2−O), 3.09 (3H, s, NCH_3), 2.97 (3H, s, SO_2−CH_3), OH and NH not detected. MS (FAB (+) glycerol) *m/e*: 374 (M + H)$^+$. HRMS (LSIMS with Cs^+, positive mode, mNBA): (M + H)$^+_{found}$ 374.1176; (M + H)$^+_{calcd}$ for $C_{18}H_{20}N_3O_4S$ 374.1175. UV (ethanol 95%) λ_{Max} (ϵ): 445 (8780), 366 (7600), 282 (61 420), 248 (39 360) nm.

4-Ethoxycarbonyl-11-hydroxymethyl-10-methylamino-3,4-dihydro-1*H*-[1,3]oxazino[4,5-*c*]acridine (22). 22 was prepared as above from **20** to give **22** as an orange powder (84% yield). Mp 145−147 °C. ^{1}H NMR (200 MHz, $CDCl_3$): δ 8.46 (1H, s, H-7), 7.85−7.33 (3H, m, H-5, H-6, and H-8), 7.21 (1H, d, J = 9.6 Hz, H-9), 5.53 (2H, s, N−CH_2−O), 5.30 (4H, m, Ar−CH_2−O and Ar−CH_2−OH), 4.95 (1H, s, NH), 4.32 (2H, q, J = 7.0 Hz, CH_2), 3.07 (3H, s, NCH_3), 1.36 (3H, t, J = 7.0 Hz, CH_3), OH not detected. IR (KBr): 3415, 1713 (CO) cm^{-1}. MS (FAB (+) glycerol) *m/e*: 368 (M + H)$^+$. HRMS (LSIMS with Cs^+, positive mode, mNBA): (M + H)$^+_{found}$ 368.1610; (M + H)$^+_{calcd}$ for $C_{20}H_{22}N_3O_4$ 368.1610; (M − OH)$^+_{found}$ 350.1509; (M − OH)$^+_{calcd}$ 350.1505. UV (ethanol 95%) λ_{Max} (ϵ): 438 (7050), 372 (8340), 276 (46 000), 253 (28 600) nm.

11-Hydroxymethyl-10-methylamino-3,4-dihydro-1*H*-[1,3]oxazino[4,5-*c*]acridine (23). A suspension of **22** (50 mg, 0.14 mmol) in a mixture of THF, 95% EtOH, water, and 30% NaOH (20 mL, 20:10:1:6, v/v/v/v) was stirred at 65 °C for 5 h. After cooling, the mixture was poured into water (150 mL). The organic solvents were evaporated, and the aqueous phase was extracted with ethyl acetate. The organic layer was washed with water, dried over sodium sulfate, and evaporated in vacuo. The viscous residue was dissolved in a minimum of CH_2Cl_2. Adding pentane to the solution afforded **23** as an orange powder (29 mg, 72%). Mp 104−106 °C. ^{1}H NMR (200 MHz, $CDCl_3$): δ 8.36 (1H, s, H-7), 7.75 (1H, d, J = 8.9 Hz, Ar−H), 7.61 (1H, d, J = 8.9 Hz, Ar−H), 7.12 (1H, d, J = 8.9 Hz, Ar−H), 6.83 (1H, d, J = 8.9 Hz, Ar−H), 5.53 (2H, s, Ar−CH_2−O), 5.27 (2H, s, N−CH_2−O), 4.86 (2H, d, J = 5.1 Hz, Ar−CH_2−OH), 4.52 (1H, s, NH), 3.05 (1H, s, NCH_3), NH and OH not detected. ^{13}C NMR (75 MHz, $CDCl_3$): δ 148.6 (C quat), 148.2 (C quat), 146.5 (C quat), 142.7 (C quat), 136.6 (CH), 129.4 (CH), 127.8 (CH), 120.0 (C quat), 119.8 (C quat), 118.7 (CH), 113.9 (CH), 113.8 (C quat), 112.7 (C quat), 75.0 (CH_2), 65.8 (CH_2), 58.4 (CH_2), 30.9 (CH_3). MS (FAB (+) glycerol) *m/e*: 296 (M + H)$^+$. HRMS (LSIMS with Cs^+, positive mode, mNBA): (M + H)$^+_{found}$ 296.1396; (M + H)$^+_{calcd}$ for $C_{17}H_{18}N_3O_2$ 296.1399; (M − OH)$^+_{found}$ 278.1285; (M − OH)$^+_{calcd}$ 278.1293. UV (ethanol 95%) λ_{Max} (ϵ): 475 (15 670), 422 (11 000), 273 (40 500) nm.

3,6-Diethoxycarbonylaminoacridine (24). A solution of 3,6-diaminoacridine (1.0 g, 4.78 mmol) in pyridine (15 mL) was cooled to 0 °C. Ethyl chloroformate (1.83 mL, 19.1 mmol) was carefully added to the mixture, and the reaction mixture was stirred at room temperature until an orange precipitate appeared. The pyridine was removed in vacuo. The syrup was diluted with a minimum of methanol and was added dropwise to cold diluted aqueous ammonium hydroxide. The yellow solid was filtered off and was washed with water and ether. Compound **24** was obtained as a beige solid (1.63 g, 95%). An analytical sample was purified on silica, eluting with 5% ethyl acetate in CH_2Cl_2. Mp 235−236 °C. ^{1}H NMR (200 MHz, DMSO-d_6): δ 8.79 (1H, s, H-9), 8.22 (2H, s, H-4 and H-5), 7.99 (2H, d, J = 9.3 Hz, H-1 and H-8), 7.57 (2H, dd, J = 1.7, 9.3 Hz, H-2 and H-7), 4.20 (4H, q, J = 7.2 Hz, CH_2), 1.28 (6H, t, J = 7.2 Hz, CH_3). ^{13}C NMR (75 MHz, $CDCl_3$): δ 153.5 (CO), 149.8 (C quat), 140.9 (C quat), 135.2 (CH), 129.1 (CH), 121.9 (C quat), 119.4 (CH), 112.4 (CH), 60.5 (CH_2), 14.5 (CH_3). IR (KBr): 3433, 3346, 1724 (CO) cm^{-1}. MS (FAB (+) glycerol) *m/e*: 354 (M + H)$^+$. UV (ethanol 95%) λ_{Max} (ϵ): 384 (21 530), 271 (80 810) nm. Anal. ($C_{19}H_{19}N_3O_4$) C, H, N.

4,10-Diethoxycarbonyl-3,4,10,11-tetrahydro-1*H*,13*H*-bis[1,3]oxazino[4,5-*c*:5′,4′-*h*]acridine (25). Paraformaldehyde (6.8 g, 227 mmol) was added to a solution of **24** (400 mg, 1.14 mmol) in 99% methanesulfonic acid (105 mL). The suspension was sonicated at room temperature until complete dissolution. The mixture was stirred overnight. The reaction was quenched with water (400 mL), and the resulting solution was stored at 4 °C for 3 h. The solid was filtered and poured into diluted aqueous ammonium hydroxide. The yellow solid was filtered off and was washed with water and ether to give **25** (350 mg, 71%). Mp 195−196 °C. ^{1}H NMR (200 MHz, $CDCl_3$): δ 8.59 (1H, s, H-7), 7.93 (2H, d, J = 9.6 Hz, H-5/9 or H-6/8), 7.80 (2H, d, J = 9.6 Hz, H-6/8 or H-5/9), 5.52 (4H, s, Ar−CH_2−O), 5.32 (4H, s, N−CH_2−O), 4.33 (4H, q, J = 7.0 Hz, CH_2), 1.37 (6H, t, J = 7.0 Hz, CH_3). ^{13}C NMR (75 MHz, $CDCl_3$): δ 154.1 (CO), 145.4 (C quat), 138.5 (C quat), 135.6 (CH), 126.4 (CH), 123.2 (C quat), 123.1 (CH), 121.5 (C quat), 75.4 (CH_2), 66.5 (CH_2), 62.9 (CH_2), 14.6 (CH_3). IR (KBr): 1718 (CO) cm^{-1}. MS (FAB (+) glycerol) *m/e*: 438 (M + H)$^+$. HRMS (LSIMS with Cs^+, positive mode, mNBA) *m/e*: $M^{+\bullet}_{found}$ 437.1577;

M^+_{calcd} for $C_{23}H_{24}N_3O_6$ 437.1587. UV (ethanol 95%) λ_{Max} (ϵ): 373 (17 780), 271 (111 140) nm.

3,4,10,11-Tetrahydro-1*H*,13*H*-bis[1,3]oxazino[4,5-*c*:5′,4′-*h*]acridine (26). Compound **25** (300 mg, 0.69 mmol) was dissolved in a mixture of THF, 95% EtOH, water, and 30% aqueous sodium hydroxide (20:10:1:6, v/v/v/v). The solution was stirred for 8 h at 60−65 °C. The mixture was slowly poured into water (400 mL). The organic solvents were evaporated in vacuo, and the resulting aqueous phase was extracted with ethyl acetate. After evaporation to dryness, the residue was dissolved in a minimum of ether. Pouring the solution into a large volume of pentane afforded **26** as a yellow solid that was crystallized from chloroform (153 mg, 76%). Mp 235−240 °C. ^{1}H NMR (200 MHz, $CDCl_3$): δ 8.37 (1H, s, H-7), 7.64 (2H, d, J = 8.9 Hz, H-5/9 or H-6/8), 6.89 (2H, d, J = 8.9 Hz, H-6/8 or H-5/9), 5.40 (4H, s, Ar−CH_2−O), 4.87 (4H, d, J = 6.2 Hz, N−CH_2−O), 4.51 (2H, s, NH). ^{13}C NMR (75 MHz, DMSO-d_6): δ 146.0 (C quat), 143.4 (C quat), 135.6 (CH), 127.5 (CH), 118.9 (C quat), 117.9 (CH), 110.1 (C quat), 73.7 (CH_2), 64.8 (CH_2). MS (FAB (+) glycerol) *m/e*: 294 $(M + H)^+$. HRMS (LSIMS with Cs^+, positive mode, mNBA): $(M + H)^+_{found}$ 294.1238; $(M + H)^+_{calcd}$ for $C_{17}H_{16}N_3O_2$ 294.1238. UV (ethanol 95%) λ_{Max} (ϵ): 402 (9840), 294 (22 260), 271(42 150) nm.

log *P* Determination. The log *P* (octanol/water) was determined according to the procedure by Kessel et al.[25]

In Vitro Screening. L1210 cells (mouse leukæmia, ATCC CCL 219) were cultivated in Dulbecco's MEM supplemented with 10% fetal calf serum (FCS). Exponentially growing cells were seeded (10^5 cells/mL) in microwell plates (24 × 1 mL) and incubated for 24 h. After that time, the cell density was approximatively 3 × 10^5 cells/mL and increasing concentrations of the tested compounds were added, in duplicate, as DMSO solutions to a maximum volume of 5 μL/well. Control wells received 5 μL of DMSO alone. After a further 24 h of incubation, cells were enumerated using a Coulter counter ZM (Coultronics inc.). Growth inhibition was measured in relation to DMSO-treated controls.

HT29 cells (human colon adenocarcinoma, ATCC HTB 38) were cultivated in Dulbecco's MEM supplemented with 10% FCS. Cells from log-phase culture were seeded in 24-microwell plates (1 mL, 5 × 10^4 cells/well) and incubated for 2 days. Test compounds in DMSO solution were added to a maximum volume of 5 μL. Controls cells received 5 μL of DMSO alone. Plates were incubated for 24 h, and then the medium was removed and cells were washed twice with phosphate-buffered saline (PBS) before addition of fresh medium free of drug. Plates were reincubated for 3 days before evaluation of the cell survival using the MTT assay[26] using 30 min of incubation with 100 μg/well of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT, Sigma). After removal of the medium, formazan crystals were taken up with 100 μL of DMSO and absorbance at 540 nm was measured with a microplate reader (model 450 Bio-Rad). Survival was expressed as a percentage of DMSO-treated controls.

A549 cells (human pulmonary adenocarcinoma, ATCC CCL 185) were grown in Kaighn modified ham F12 medium (F12-K) supplemented with 10% FCS. Cells were plated in 3 cm diameter multiwell plates (200 cells/well). After 24 h, test compounds were added at various concentrations and cells were incubated for another 24 h. Cells were washed with PBS and then reincubated for 14 days with fresh medium free of drug. Colonies were numbered after washing with PBS and staining with Giemsa. Survival was expressed as a percentage of untreated controls.

All incubations were carried out at 37 °C in a water-jacketed CO_2 incubator (5% CO_2, 100% relative humidity). Cytostatic/cytotoxic activities were expressed as IC_{50}, the concentration that reduced by 50% the number of treated cells relative to controls. IC_{50} values were extracted from regression curves obtained with experimental points.

Absorption Spectrophotometry and Melting Temperature Studies. Absorption spectra and melting curves were measured using a Uvikon 943 spectrophotometer coupled to a Neslab RTE111 cryostat. Titrations of the drug with DNA, covering a large range of DNA-phosphate/drug ratios (P/D), were performed by adding aliquots of a concentrated DNA solution to a drug solution at constant ligand concentration (20 μM). For each series of T_m measurements, 12 samples were placed in a thermostatically controlled cell holder and the quartz cuvettes (10 mm path length) were heated by circulating water. The measurements were performed in BPE buffer, pH 7.1 (6 mM Na_2HPO_4, 2 mM NaH_2PO_4, 1 mM EDTA). The temperature inside the cuvette was measured with a platinum probe; it was increased over the range 20−100 °C with a heating rate of 1 °C/min. The "melting" temperature T_m was taken as the midpoint of the hyperchromic transition.

Electric Linear Dichroism. ELD measurements were performed with a computerized optical measurement system using the procedures previously outlined.[27] All experiments were conducted with a 10 mm path length Kerr cell having 1.5 mm electrode separation. The samples were oriented under an electric field strength varying from 1 to 14 kV/cm. The drug under test was present at 10 μM with the DNA at 200 μM unless otherwise stated.

Topoisomerase I and II Mediated DNA Cleavage Assays. The assays were performed as previously described.[28,29] Supercoiled pKMp27 DNA (0.5 μg) was incubated with 4 units of human topoisomerase I or II (TopoGen Inc.) at 37 °C for 30 min in relaxation buffer (50 mM Tris, pH 7.8, 50 mM KCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM EDTA and ATP) in the presence of varying concentrations of the drug under study. Reactions were terminated by adding SDS to 0.25% and proteinase K to 250 μg/mL. DNA samples were then added to the electrophoresis dye mixture (3 mL) and electrophoresed in a 1% agarose gel containing ethidium bromide (1 mg/mL) at room temperature for 2 h at 120 V. Gels were washed and photographed under UV light.

Confocal Microscopy. The cells (20 000 cells/cm^2) were incubated at 37 °C with the test compound at 20 μM for 24 h. The medium was removed, and cells were rinsed with ice-cold PBS (10 min) prior to the fixation with a 2% paraformaldehyde solution for 20 min at +4 °C. A drop of antifade solution containing the nuclear specific dye TOTO-3 (0.5 μM) was added, and the treated portion of the slide was covered with a glass coverslip. The fluorescence of the drug was detected by confocal microscopy using a Leica DMIRBE microscope controlled by a Leica TCS-NT workstation (Leica Microsystems, Bensheim, Germany) with a 63 × 1.32 NA oil objective, equipped with a 75 mW argon−krypton and Coherent Innova-90 UV laser lines. The emission signal was observed through a dichroic mirror (DD488/568) followed by a filter set (RSP 580, BF 530/30, BP 600/30. and BP460/30 for UV excited probes). The optical sections were obtained in the *Z*-axis and stored on the computer with a scanning mode. In all cases, the operating conditions were such that detectable images could not be obtained for cell samples not treated with drugs.

Acknowledgment. The authors thank the Service Commun d'Imagerie Cellulaire de l'IMPRT (IFR114) for access to the fluorescence microscope and Dr. Zohar Mishal (CNRS, Villejuif, France) for his contributions to the confocal measurements.

Supporting Information Available: Biphasic melting curves obtained with compounds **12** and **22** interacting with the polymer poly(dAT)$_2$. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Denny, W. A. Acridine-based antitumour agents. *Chemistry of antitumour agents*; Blackie and Son Ltd: Glasgow and London, 1990; pp 1−29.
(2) Skaltsounis, A.-L.; Mikatu, S.; Tillequin, F. Acridone alkaloids. *The alkaloids: chemistry and biology*; Academic Press: New York, 2000; pp 259−377.
(3) Demeunynck, M.; Charmantray, F.; Martelli, A. Interest of acridine derivatives in the anticancer chemotherapy. *Curr. Pharm. Des.* **2001**, *7*, 1703−1724.

(4) Cholody, W. M.; Hernandez, L.; Hassner, L.; Scudiero, D. A.; Djurickovic, D. B.; Michejda, C. J. Bisimidazoacridones and related compounds: new antineoplastic agents with high selectivity against colon tumors. *J. Med. Chem.* **1995**, *38*, 3043−3052.
(5) Adjei, A. A. Current status of pyrazoloacridine as an anticancer agent. *Invest. New Drugs* **1999**, *17*, 43−48.
(6) Denny, W. A. Dual topoisomerase I/II poisons as anticancer drugs. *Expert Opin. Invest. Drugs* **1997**, *6*, 1845−1851.
(7) Read, M.; Harrison, R. J.; Romagnoli, B.; Tanious, F. A.; Gowan, S. H.; Reszka, A. P.; Wilson, W. D.; Kelland, L. R.; Neidle, S. Structure-based design of selective and potent G quadruplex-mediated telomerase inhibitors. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 4844−4849.
(8) Fan, J.-Y.; Tercel, M.; Denny, W. A. Synthesis, DNA binding and cytotoxicity of 1-[[*ω*-(9-acridinyl)amino]alkyl]carbonyl-3-(chloro-methyl)-6-hydroxyindolines, a new class of DNA-targeted alkylating agents. *Anti-Cancer Drug Des.* **1997**, *12*, 277−293.
(9) Gaffney, B. L.; Marky, L. A.; Jones, R. A. The influence of the purine 2-amino group on DNA conformation and stability-II. Synthesis and physical characterization of d[CGT(2-NH2)ACG], d[CGU(2-NH2)ACG], and d[CGT(2-NH2)AT(2-NH2)ACG]. *Tetrahedron* **1984**, *40*, 3−13.
(10) Gourdie, T. A.; Prakash, A. S.; Wakelin, L. P. G.; Woodgate, P. D.; Denny, W. A. Synthesis and evaluation of DNA-targeted spatially separated bis(aniline mustards) as potential alkylating agents with enhanced DNA cross-linking capability. *J. Med. Chem.* **1991**, *34*, 240−248.
(11) Lee, H. H.; Palmer, B. D.; Baguley, B. C.; Chin, M.; McFadyen, W. D.; Wickham, G.; Thorsbourne-Palmer, D.; Wakelin, L. P. G.; Denny, W. A. DNA-directed alkylating agents. 5. Acridinecarboxamide derivatives of (1,2-diaminoethane)dichloroplatinum(II). *J. Med. Chem.* **1992**, *35*, 2983−2987.
(12) Denny, W. A. DNA minor groove alkylating agents. *Expert Opin. Ther. Patents* **2000**, *10*, 459−474.
(13) Wardani, A.; Lhomme, J.; Bigg, D. C. H.; Salez, H. Novel benzo-[*b*]phenanthroline-1,7 derivatives and their preparation and therapeutic applications. Patent WO 91 07,403, 1991; *Chem. Abstr.* **1991**, *115*, 207973h.
(14) Duflos, A.; Bigg, D. C. H.; Lhomme, J.; Fixler, N.; Etievant, C.; Demeunynck, M. Oxazino-acridine substituée: leur préparation et les applications en thérapeutique (Substituted oxazinoacridines: their preparation and applications in therapeutics). Patent FR 2 712,290, 1993; *Chem. Abstr.* **1995**, *123*, 313985p.
(15) Lhomme, J.; Bigg, D. C. H.; Duflos, A.; Bonnaud, B.; Demeunynck, M.; Etievant, C.; Kiss, R. Novel 3-dimethylaminoacridine derivatives. Their preparation and their application in therapeutic. *Chem. Abstr.* **1995**, **122**, 239555j; Patent WO 94 25,439, 1994.
(16) Duflos, A.; Bigg, D. C. H.; Lhomme, J.; Bonnaud, B.; Etievant, C. Nouveaux dérivés de 4-amidométhyl 3-diméthylamino acridines substituées, leur préparation et leur application en thérapeutique (Novel derivatives of substituted 4-amidomethyl-3-dimethylaminoacridines, their preparation and their application in therapeutics). Patent FR 2 716,454, 1995; *Chem. Abstr.* **1996**, *124*, 117108m.
(17) Charmantray, F.; Duflos, A.; Lhomme, J.; Demeunynck, M. Synthesis and study of 4-hydroxymethyl-3-alkylaminoacridines as models of a new class of DNA intercalating−alkylating agents. *J. Chem. Soc, Perkin Trans.1* **2001**, 2962−2968.
(18) Charmantray, F.; Wardani, A.; Demeunynck, M.; Lhomme, J. *N*-Methyl-3,4-dihydro-[3,1]oxazino-acridine, a useful intermediate in the synthesis of polysubstituted acridines. *Synth. Commun.* **2001**, *31*, 2001−2009.
(19) Fixler, N.; Demeunynck, M.; Duflos, A.; Lhomme, J. Reaction of proflavine derivatives with formaldehyde: access to mono- and bis(3,4-dihydro[1,3]oxazino)acridines of pharmaceutical interest. *Heterocycles* **1998**, *48*, 755−767.
(20) Charmantray, F.; Demeunynck, M.; Lhomme, J.; Duflos, A. New route to extended angular polyaza-heterocycles. *J. Org. Chem.* **2001**, *66*, 8222−8226.
(21) Bailly, C. DNA relaxation and cleavage assays to study topoisomerase I inhibitors. *Methods Enzymol.* **2001**, *340*, 610−623.
(22) Suzuki, T.; Fujikura, K.; Higashiyama, T.; Takata, K. DNA staining for fluorescence and laser confocal microscopy. *J. Histochem. Cytochem.* **1997**, *45*, 49−53.
(23) Wang, Y.; Lyu, Y. L.; Wang, J. C. Dual localization of human DNA topoisomerase IIIalpha to mitochondria and nucleus. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 12114−12119.
(24) Zhang, H.; Barcelo, J. M.; Lee, B.; Kohlhagen, G.; Zimonjic, D. B.; Popescu, N. C.; Pommier, Y. Human mitochondrial topoisomerase I. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 10608−10613.
(25) Kessel, D. Effects of Photoactivated Porphyrins at the Cell Surface of Leukemia L1210 Cells. *Biochemistry* **1977**, *16*, 3443−3449.
(26) Mosmann, T. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *J. Immunol. Methods* **1983**, *65*, 55−63.
(27) Colson, P.; Bailly, C.; Houssier, C. Electric linear dichroism as a new tool to study sequence preference in drug binding to DNA. *Biophys. Chem.* **1996**, *58*, 125−140.
(28) Dassonneville, L.; Bonjean, K.; De Pauw-Gillet, M. C.; Colson, P.; Houssier, C.; Quetin-Leclercq, J.; Angenot, L.; Bailly, C. Stimulation of topoisomerase II-mediated DNA cleavage by three DNA-intercalating plant alkaloids. Cryptolepine, matadine and serpentine. *Biochemistry* **1999**, *38*, 7719−7726.
(29) Dassonneville, L.; Wattez, N.; Baldeyrou, B.; Mahieu, C.; Lansiaux, A.; Banaigs, B.; Bonnard, I.; Bailly, C. Inhibition of topoisomerase II by the marine alkaloid ascididemin and induction of apoptosis in leukemia cells. *Biochem. Pharmacol.* **2000**, *60*, 527−537.

JM020389W