A New Series of Ellipticine Derivatives (1-(Alkylamino)-9-methoxyellipticine). Synthesis, DNA Binding, and Biological Properties

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A new series of ellipticine derivatives, 1-(alkylamino)-5,11-dimethyl-9-methoxy-6*H*-pyrido[4,3-*b*]carbazoles, were synthesized as potential DNA intercalating antitumor drugs. The structure of these compounds were confirmed by ^{1}H NMR spectroscopy and mass spectrometry. These compounds are able to bind to DNA with an affinity of about 10^6 M^{-1}, and their intercalating characteristics (lengthening and unwinding of DNA) depend upon the length of the chain in position 1. The cytotoxicities of these compounds on L1210 and NIH-3T3 cells are quite similar, and fluorescence techniques showed that the compounds are localized mainly in the cytoplasmic granules of the cells. One of these compounds appears to show a very high antitumor activity (equivalent to the more active known ellipticine analogues: 10-[[γ-(diethylamino)propyl]amino]-6-methyl-5*H*-pyrido[3′,4′:4,5]pyrollo[2,3-*g*]isoquinoleine (BD40), 1-[[γ-(diethylamino)propyl]amino]-9-methoxyellipticine (BD84) and 2-[β-(diethylamino)ethyl]-9-hydroxyellipticinium chloride (DEAE).

Ellipticine is a molecule belonging to the 6*H*-pyridocarbazole family. Many derivatives of ellipticine have been synthesized in our and other groups[1,2] in an attempt to improve the antitumor properties of this plant alkaloid.

Among these drugs, which exhibit antitumor properties, 2-methyl 9-hydroxyellipticinium (NMHE) has been introduced in the treatment of breast cancer[3,4] while 9-methoxyellipticine (MeOE) has been described as an antitumor agent.[5] From data relating the structures of these compounds to their pharmacological properties, some trends have emerged. Among them, the importance of an alkyl or alkylamino substitution is noteworthy. For instance, it has been established that the 11-demethylellipticine is far less toxic and is less active on the L1210 leukemia, following a single injection, than its parent compound.[6] Substitution by a [(diethylamino)alkyl]amino group in position 10 of the azaellipticine (BD40), which corresponds to position 1 in the ellipticine series,[7,8] in position 1 of 9-methoxyellipticine (BD84),[8,9] in position 2 of 9-hydroxyellipticine (DEAE),[10] or in position 2 of ellipticine[11] improves the antitumor efficiency.

Ellipticine and many of its analogues intercalate into the DNA double helix.[12–14] The antitumor activity of these compounds has been reported as being related to their high DNA binding affinity,[12] but no conclusive data permit one to correlate the intercalation of these compounds to their pharmacological action. Therefore the mode of interaction between DNA and these compounds is still of the utmost importance.

Among the steric constraints that control this interaction, the presence of alkyl and alkylamino substituents on the molecule is certainly important as far as drug DNA binding is concerned, as illustrated and discussed for a series of methylated phenanthroline derivatives[15] and a series of quinoline derivatives.[16]

Here, we report the synthesis of a new series of compounds obtained by replacing the hydrogen at position C1 of 9-methoxyellipticine (MeOE), by alkylamino chains. Moreover, a physicochemical and pharmacological study of these new compounds was undertaken. These fluorescent compounds bind to DNA and their intercalation potency depends upon the length of the alkylamino side chain. These derivatives are still cytotoxic and retain some antitumor properties. It appears that a direct correlation does not exist between their cytotoxicity and their ability to intercalate between the DNA base pairs. Furthermore, these derivatives when incubated with cells are mainly located into the cytoplasm, whereas no fluorescence can be detected in the nuclei.

Results

Chemistry. From 1-chloro-9-methoxyellipticine (**1a**), which was already described,[17] compounds **2** and **3** have been obtained by substitution in liquid ammonia (120 °C,

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Table I. New 1-(Alkylamino)pyrido[4,3-*b*]carbazole Derivatives

no.	mp, °C	method[a] (time in days)	temp, °C	yield, %	formula[b]	anal.	purifn proced[c]
2	260	A(7)	120	51[d]	$C_{18}H_{17}N_3O\cdot C_4H_4O_4$	C, H, N, O	I
3	252	A(3)	150	18[d]	$C_{20}H_{21}N_3O\cdot C_4H_4O_4$	C, H, N, O[e]	II
4	240	B(14)	50	23[d]	$C_{21}H_{23}N_3O\cdot C_4H_4O_4$	C, H, N, O	II
5	195	B(12)	70	54	$C_{22}H_{25}N_3O$	C, H, N, O	I
6	245	B(2)	90	47[d]	$C_{23}H_{27}N_3O\cdot C_4H_4O_4$	C, H, N, O	II
7	154	B(1)	150	33[d]	$C_{28}H_{37}N_3O\cdot C_4H_4O_4$	C, H, N, O	II
8		B(2)	90	49	$C_{29}H_{31}N_3O$		I
9	266	C		92[d]	$C_{22}H_{25}N_3O\cdot C_4H_4O_4$	C, H, N, O	II

[a] Methods: A = heating the chloroellipticine with the approriate amine in autoclave for the indicated time; B = substitution performed at normal pressure; C = this compound was obtained by catalytic hydrogenation of 8 in ethanol with use of a Pd/C catalyst, 24 h at 60 °C in a hydrogen atmosphere. [b] Unless otherwise stated, microanalyses are within 0.4% of the theoretical values for C, H, N, and O corresponding to the mentioned empirical formulas. [c] Recrystallization solvents: I = ethanol; II = acetone. [d] The reported yields are calculated on the maleate salts obtained by treatment of the alkylamino ellipticine with maleic acid, in acetone. [e] Anal. Calcd for $C_{20}H_{21}N_3O\cdot C_4H_4O_4$: C, 66.19. Found: C, 65.61.

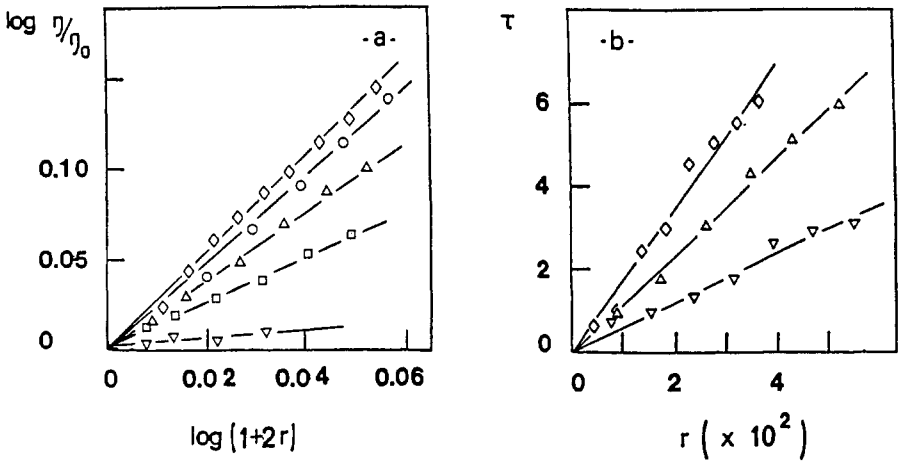
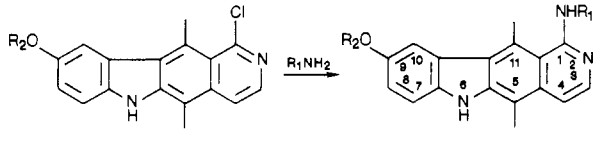


Figure 1. Determination of length increase and unwinding angle of DNA by the 1-(alkylamino)-9-methoxyellipticines. (a) Viscosimetric measurement of the length increase of sonicated calf thymus DNA. The slope of log η/η_0 versus log $(1 + 2r)$ was determined for each drug. η and η_0 are the intrinsic viscosities of sonicated DNA in the presence and absence of ligand and r represents the bound drug per nucleotide. (b) Unwinding of DNA induced by three derivatives. The mean number of superhelical turns τ in plasmid DNA samples relaxed by topoisomerase I in the presence of various drug concentrations is plotted versus r. The unwinding angle is proportional to the slope of the line: (◇) compound **2**, (○) compound **3**, (△) compound **4**, (□) compound **5**, (▽) compound **6**.

under pressure in a steel vessel) and in aqueous ethylamine with use of similar conditions (150 °C).



1a: $R_2=CH_3$
b: $R_2=CH_2C_6H_5$

2: $R_1=H$; $R_2=CH_3$
3: $R_1=C_2H_5$; $R_2=CH_3$
4: $R_1=n\text{-}C_3H_7$; $R_2=CH_3$
5: $R_1=CH_2CH(CH_3)_2$; $R_2=CH_3$
6: $R_1=CH_2CH_2CH(CH_3)_2$; $R_2=CH_3$
7: $R_1=(CH_2)_5CH_3$; $R_2=CH_3$
8: $R_1=CH_2CH_2CH(CH_3)_2$; $R_2=CH_2C_6H_5$
9: $R=CH_2CH_2CH(CH_3)_2$; $R_2=H$

Other 1-(alkylamino)-substituted ellipticines were prepared by refluxing the required chloro derivative **1a** or **1b**[17] in the corresponding pure free amine for compounds **4–6** and **8** and by heating at 160 °C for derivative **7**.[17-19] 1-[[(3-Methyl)butyl]amino]-5,11-dimethyl-9-hydroxy-6*H*-pyrido[4,3-*b*]carbazole (**9**) was obtained by catalytic hydrogenation of the benzylated derivative **8** (Table I).

Biological Results. Interaction with DNA. DNA binding constants were determined by a fluorescence technique.[37] Fluorescence is enhanced when a fluorescent drug is bound to DNA and a classical shift of the maximum

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Table II. DNA Binding Parameters

	K_{app},[a] 10^{-6} M^{-1}	r_{app}	$\phi°$[b]	slope[c]
MeOE	0.9	0.20	10 (14)	2.9
2	$\simeq 1$[d]	$\simeq 0.20$[d]	24	2.8
3	2.15	0.20	16 (14)	2.6
4	2.00	0.20	15	2.1
5	2.35	0.17	12	1.2
6	3.75	0.20	7	0.2

[a] K_{app} value is determined by direct fluorimetric titration, at pH 5. The value obtained for MeOE was determined by competition with ethidium bromide. [b] $\phi°$ is referred to as DNA unwinding angle caused by the drug binding. The values in parentheses were determined by viscosimetric method. [c] The slope of log η/η_0 versus log $(1 + 2r)$ was determined by viscosimetry where η and η_0 are the intrinsic viscosities of sonicated DNA in the presence and absence of ligand and r represents the bound drug per nucleotide. [d] At high concentration of drug, a precipitation of DNA takes place; we then estimated these two values with the first points of the binding isotherm.

of excitation (about 10 nm) toward higher wavelengths occurs. At optimal excitation and emission wavelengths, the bound and the free drugs exhibit a fluorescence ratio equal to 20. For all the tested compounds, the affinity for DNA (K_{app}) and the maximum number of sites (r_{app}) are quite similar: 2×10^6 M^{-1} and 0.2, respectively (Table II). The length of the alkylamino chain does not seem to influence the affinity and the number of sites of these drugs for the DNA. Under the same conditions, MeOE and NMHE display DNA binding affinities of 0.9×10^6 M^{-1} and 1.1×10^6 M^{-1}, respectively.[6,21]

The enhancement of fluorescence observed when these drugs are bound to DNA is not by itself a proof of intercalation. In order to determine whether these compounds are intercalated between the DNA base pairs, we have studied the consequences of their binding on the hydrodynamic properties of the DNA: unwinding of covalently closed circular DNA and DNA length increase of sonicated calf thymus DNA, by using viscosimetric techniques[21] or gel electrophoresis.[22]

Using viscosimetry, we measured an unwinding angle of $14 \pm 1°$ for MeOE and compound **3**. For the other derivatives, we used the gel electrophoresis technique.[14,22] In Figure 1b, we have represented the variation of τ as a function of r for these derivatives. From the slope of the straight line, we determined the unwinding angle corresponding to the different compounds (Table II). The unwinding angle depends upon the length of the alkylamino chain: the longer the alkylamino chain, the smaller the unwinding angle.

The theoretical treatment of the DNA length increase[23,24] indicates that if log η/η_0 is plotted as a function of log $(1 + 2r)$, where η and η_0 are the intrinsic viscosities of sonicated calf thymus DNA in the presence and absence of dye, respectively, and r is the number of bound drug per nucleotide, the slope is expected to be between 2 and 3 for a monointercalating agent.[24,26] For the alkylamino compounds, we observe (Figure 1a and Table II) that the slope varies as the inverse of the length of the alkylamino chain.

From fluorescence data, it appears that the 1-alkylamino derivatives of MeOE are bound to the DNA with the same affinities. If they are intercalated between the DNA base pairs, they should induce a detorsion ($\theta \geq 12°$) and a lengthening (slope ≥ 2) of the DNA. Actually, only compounds **2–4** behave like intercalators (Table II). Furthermore, we observed a regular decrease of the slope and of the unwinding angle of these compounds as a function of the length of the alkylamino chain.

In Vitro Cytotoxicity. The cytotoxicities of compounds **2–6**, measured in L1210 cells and NIH-3T3 cells by growth inhibition, are shown in Table III. The dose–effect relationship was determined from the regression lines drawn from the percentage of cell growth inhibition plotted as a function of the logarithm of the dose. The ID_{50} is defined as the concentration of drug that induces a 50% cell growth inhibition. All compounds, except compound **6**, show an ID_{50} at 48 h on L1210 of about 10^{-7} M. The ID_{50} for compound **6** is equal to about 8×10^{-7} M. Under the same conditions, the ID_{50} corresponding to 2-methylellipticinium (NME) is 7×10^{-7} M at 48 h. It thus appears that for an alkylamino chain greater than four carbon atoms, the cytotoxicity decreases. This phenomenon is also observed, but less marked, in the case of the NIH-3T3 system.

Uptake of the Drugs into NIH-3T3 Cells. The NIH-3T3 cells were used to study the cytofluorescence localization of the derivatives. NIH-3T3 cells in the exponential growth phase were incubated for 3 h in the presence of two different concentrations of drug: 5×10^{-7} and 10^{-6} M. The ID_{50} at 48 h in this cell line is about equal to 5×10^{-7} M, and we can then consider that the cells are alive under conditions of the experiments. Four fluorescent compounds, **3–6**, were examined. It appears that the fluorescence is located in the cytoplasm, and that no fluorescence can be detected in the nucleus (Figure 2). The fluorescence appears as a granular pattern for the tested compounds. For low concentrations, the drug is located mainly into well-defined regions of the cytoplasm, except for compound **6** whose fluorescence appears more diffuse. When compound **7** was tested in the same conditions, we did not observe any fluorescence in the cells. The hydrophobic chain probably inhibits the uptake of the drug.

Contrary to what was previously observed for other drugs in the ellipticine series, NME[27] and NMHE-amino acids adducts (OPC),[28] a 30-min irradiation of the cells in presence of drug does not induce any nuclear fluorescence (excitation wavelength in the range of 270–380 nm, and emission in the range of 410–580 nm). The intensity and the distribution of this fluorescence remain constant.

In Vivo Antitumor Effects. The highest nonlethal dose (LD_0) and lethal dose (LD_{100}) of each drug were determined after a single injection to mice by the intraper-

Table III. In Vitro and in Vivo Toxicity and Antitumor Properties of 1-(Alkylamino)-9-methoxyellipticines

	ID_{50},[a] μM				ILS:[c] L1210		
	L1210	NIH-3T3	LD_0,[b] mg/kg	LD_{100}, mg/kg	1	2	3
MeOE	0.1	0.3	70	nd	nd	nd	20
2	0.1	nd	60	70	21	39 (1)	93 (2)
3	0.05	0.3	70	90	2	13	42
4	0.1	0.3	100	200	0	2	nd
5	0.09	0.3	>200	?	nd	nd	nd
6	0.8	0.5	300	350	10	5	nd

[a] Dose that reduces by 50%, after 48 h, the cells growth in vitro as compared to controls. [b] Highest nonlethal dose (ip treatment). [c] Increase in life of span over controls, 10^5 L1210 cells per mouse of 20 g, by ip route, single injection, 24 h after cell grafting. The drug concentrations were equal to: $LD_0/10$ (1), $LD_0/5$ (2), $LD_0/2$ (3). Number of animals cured is indicated in parentheses.

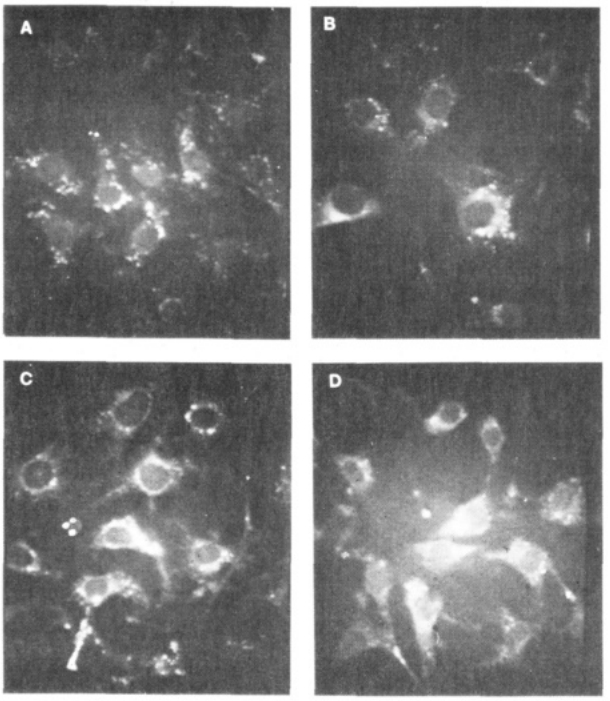

Figure 2. Fluorescence microscopy of NIH-3T3 cells in presence of the different compounds. Cells were prepared as described in the Experimental Section, incubated 3 h in growth medium containing drugs, and analyzed by fluorescence microscopy: (A) compound **3**, (B) compound **4**, (C) compound **5**, (D) compound **6**.

itoneal route. The results are shown on Table III. The shorter the 1-alkylamino chain, the higher the toxicity. We observe a good correlation between the LD_0 and the number of carbon atoms on the 1-alkylamino chain ($R^2 = 0.96$). We have not seen any delayed toxicity (5 months after inoculation) for all these compounds. Compound **9** is an analogue of compound **6**, substituted by an hydroxyl group instead of a methoxy group. It is 5 times more toxic than compound **6**. As already observed,[11] the presence of the hydroxyl group on the 9-position of the molecule increase the toxicity.

The antitumor activity of compound **3** was determined on L1210 and P388 tumors. We have determined the ILS at different doses and found an ILS of 42 on L1210 leukemia and 40 on P388 leukemia at $LD_0/2$. This drug shows a moderate antitumor property. Compound **2** was tested on L1210 leukemia, and we observed an ILS of 93 with two mice surviving after 3 months. From this observation, compound **2** is as active as the most active ellipticine derivatives, 1-[[γ-(diethylamino)propyl]-amino]-9-methoxyellipticine (BD84), 2-[β-(diethylamino)ethyl]-9-hydroxyellipticinium chloride (DEAE), and ellipticine analogue (10-[(γ-(diethylamino)propyl]-amino]-6-methyl-5*H*-pyrido[3′,4′:4,5]pyrollo[2,3-g]isoquinoleine (BD40). Compounds **4** and **6** were also tested, and no antitumor effect was observed (Table III).

Conclusion

The new series of ellipticine derivatives that we are presently describing appears to be fluorescent and cytotoxic and is able to bind to DNA.

The intercalation ability of these compounds depends upon the number of carbons of the side chain. From the hydrodynamic properties of the DNA–drug complexes, it appears that, together with the lengthening of the side chain, a diminution in the parameters classically associated with the intercalation is observed. The average unwinding angle induced by the drug decreases together with the slope of the straight line representative of log η/η_0 as a function of log $(1 + 2r)$. Such a situation has been already described for other compounds[8,18,29] and could be accounted for by assuming the existence of two types of interaction with DNA: intercalation and outside groove binding. In our series, the *N*-isobutyl-substituted compound **5** has lost its potential for being an intercalator while retaining its affinity for DNA together with an enhancement of the fluorescence associated to its DNA binding. This fluorescence change indicates that this molecule, once bound to DNA, is in an hydrophobic site. Such binding could therefore correspond to an outside groove binding. The progressive decrease of the parameters corresponding to the intercalation is very well correlated ($r^2 = 0.988$) with the number of carbons of the substituting chain, leading to the assumption that the length of the side chain could be responsible for the passage of one type of binding to the other, the longer chain favoring outside groove binding.

We do not observe any good correlation between the cytotoxicity and the intercalation, suggesting that the important parameter for the cytotoxicity is not the intercalation per se but the affinity for the DNA. Nevertheless, the affinity is not the only parameter to be taken into account to explain the cytotoxicity of the ellipticine derivatives. In our series, compound **6** is about 10 times less toxic than the other compounds with the same affinity for DNA. In this case, the hydrophobicity of the drug could induce a loss of accessibility for a target responsible of the cytotoxicity, through a strong binding to the cellular membrane, leading to a modification of the intracellular distribution. In accordance with this point, the data concerning the distribution of these compounds into the cell suggest that the cellular distribution of compound **6** is different from the distribution of compounds **3** or **4**. As shown in Figure 2, the distribution of compound **6** is more uniform and diffuse than the observed granulation corresponding to compound **3** or **4**. The granulate shape fluorescence observed for compounds **3** and **4** is characteristic of the ellipticine derivatives, and, referring to previous studies dealing with adriamycin,[30] BD40,[31,32] OPC derivatives,[28] and 2-*N*-methylellipticinium,[27] we can assume that this granular cytoplasmic distribution of fluorescence corresponds to a capture of the compounds by lysosomes and that an overconcentration in these regions would reflect the transmembrane potential of these organelles.[33,34]

Regarding antitumor activity, two compounds (**2** and **3**) out of the three intercalating agents manifest antitumor effects in vivo. However, a direct correlation between intercalation and antitumor activity cannot be drawn as long as in vivo metabolism of these compounds can lead to O-demethylated metabolites.[41]

In conclusion, we describe in this work a new series of ellipticine derivatives with physicochemical properties challenging enough, once correlated with their pharmacological properties, to be used in trying to understand the mechanism of action of this family of molecules.

Experimental Section

Chemistry. All melting points, uncorrected, were determined with a Kofler apparatus. Purification procedures described in Table I were checked by thin-layer chromatography on silica gel

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(Kieselgel, 60 F 254, Merck) or alumina (neutral, type E, 60 F 254, Merck). The eluent was a mixture of dichloromethane–ethanol at variable concentration.

General Procedure for the Preparation of 1-(Alkylamino)-Substituted Ellipticines 2–8. The chloro derivatives **1a** and **1b** (200 mg) and the requisite amine (10 mL) were heated in the conditions noted in Table I. After elimination of excess amine under reduced pressure, the residue was treated with 50 mL of 0.5 N sodium hydroxide solution, and the compounds were extracted with dichloromethane (10 × 30 mL), dried over sodium sulfate, and evaporated. The crude material was taken up in the solvents listed in Table I or transformed into the corresponding maleate salts.

^{1}H NMR spectra of 1-(alkylamino)-substituted ellipticines were recorded in $(CD_3)_2SO$ with a Varian XL-100 NMR spectrometer. Chemical shifts of compound **3** are given as typical example. **3** ($R_2 = CH_3$; $R_1 = CH_3CH_2$) as maleate: δ 8.23 (s broad, 1,6-NH), 7.84 (d, 1, J_{8-10} = 2.4 Hz, 10-H), 7.60 (d, 1 J_{7-8} = 8.9 Hz, 7-H), 7.54 (d, 1, J_{3-4} = 7.3 Hz, 3-H), 7.34 (d, 1,4-H), 7.27 (dd, 1,8-H), 6.07 (s, 4, CH maleic acid), 3.94 (s, 3, OCH_3), 3.65 (q, 2, CH_2CH_3), 3.37 (s, 3, 11-CH_3), 2.75 (s, 3, 5-CH_3), 1.43 (t, 3, $J_{CH_3-CH_2}$ = 7 Hz, CH_3CH_2).

Each drug (as a maleate salt, except for the compound **5**) was dissolved in 50% DMSO and 50% water at a concentration of 5×10^{-3} M. This stock solution was stored at −20 °C and diluted before use in the appropriate buffer (as indicate in the text). No loss of activity was observed after 8 days at 37 °C.

The purity assessed by thin-layer chromatography, high-pressure liquid chromatography (gradient), and mass spectrophotometry was more than 97%. Furthermore these compounds are stable in cell culture medium (48 h at 37 °C).

The presence of an aliphatic chain in these compounds increases their hydrophobicity as a function of the chain length. The HPLC retention index of the drugs on a reverse-phase column (C_{18}) can be considered to reflect their lipophilicity and is closely related to the octanol/water partition coefficient.[13,35] We have determined the capacity factor ($K_D = (T_R - T_0)/T_0$) of these drugs; where T_R represents the retention time of the derivatives and T_0 represents the retention time of DMSO. The values of K_D are correlated to the number of carbons of the substituting chains (R^2 = 0.98). (Figure 3, supplementary material).

These derivatives show fluorescent properties ($\lambda_{exc} \simeq 300$ nm and $\lambda_{em} \simeq 450$ nm).

The compounds **2–6** are able to self-associate, and by assuming a simple dimerisation model,[36] we can derive the association constant of the dimerization. These constants were found equal to about 10^4 M^{-1} for the different drugs, except for the compound **2** ($K = 10^5$ M^{-1}).

Biology. Interaction with DNA. The calf thymus DNA (Boehringer) binding constants were determined at pH 5.2 in acetate buffer (10 mM) and NaCl (100 mM) at 20 °C. The drug-binding parameters were determined by fluorescence titration[37] with a SFM25 spectrofluorimeter (Kontron). Fluorescence excitation was 310 nm, and fluorescence emission was followed at 540 nm. Binding curves were plotted as previously described by Scatchard.[38] K_{app} is the apparent association constant and r_{app} is the apparent number of binding site per nucleotide.

The length increase of short calf thymus DNA segments was determined by measuring the increase in the intrinsic viscosity of the DNA in the presence of increasing concentrations of drugs.[24] The calf thymus DNA was sonicated and digested by Endonuclease of *Neurospora crassa* (Boehringer). Viscosimetric measurements were performed in a semimicrodilution capillary viscosimeter, Cannon Ubbehlode semimicro 9722-H50, CUSMC (Cannon Instrument Co., State College, PA) at pH 7.4 in Tris buffer (10 mM) and NaCl (100 mM).

The unwinding of the DNA helix was measured by two different techniques: (i) viscosimetry was used in the case of MeOE and of compound **3**, with bacteriophage PM2 DNA (Boehringer) in presence of increasing concentrations of drugs[23] and (ii) relaxation of a supercoiled DNA was used for all the molecules by the Keller's method[22] with some modifications. Aliquots of 1 μg of pUC13 DNA (2730 pb) were incubated, at 37 °C during 2 h, with increasing concentrations of drugs (from 4×10^{-7} to 8×10^{-6} M) in presence of rat liver topoisomerase 1.[41] The total volume of reaction is equal to 30 μL, and the buffer is sodium acetate (20 mM), NaCl (200 mM), EDTA (1 mM), and BSA (20 μg/mL), pH 5. The reaction is stopped, at 37 °C, with 170 μL of 1% SDS in TE buffer (Tris (10 mM); EDTA (1 mM); pH 7.4). Fifty microliters of NaCl (5 M) and 250 μL of chloroform/isoamylic alcohol (v/v, 24:1) are successively added. The solution is vortexed for 30 s and centrifuged for 5 min at 4 °C. To the supernatant is added 4 μL of tRNA (5 mg/mL), and the nucleic acids are precipitated with 700 μL of cold ethanol (2 h, −70 °C). Neutral gel electrophoresis are then performed in 0.7% agarose containing different concentrations of chloroquine (from 0.3 to 1.2 μg/mL), at 1 V/cm overnight. The gels are then stained with ethidium bromide (0.25 μg/mL) and photographed through an UV transilluminator. The negatives are scanned on a microdensitometer (Joyce-Loebl). The distribution of topoisomers is fitted to a Gaussian curve on a MINC computer. From the curves thus obtained, we measure the number of superhelical turns on circular DNA (τ) as a function of drug concentration. The unwinding angle is then $\sigma = [\Delta\tau/\Delta r]360/2N$ where r is the number of drug molecules bound per nucleotide, computed from the known binding parameters, and N is the number of base pairs in the plasmid DNA molecule.

Cell Culture, in Vitro Cytotoxicity Determination, and Fluorescence Distribution. Cell Culture. The L1210 leukemia cells adapted to stationary suspension cultures were provided by Dr. J. Belehradek (Institut Gustave Roussy, Villejuif, France). L1210 cells were grown in suspension in RPMI (GIBCO, medium 1640, batch number 61F0147) supplemented with 8% fetal calf serum in presence of penicillin (100 units/mL) and streptomycin (100 μg/mL) in a 6% CO_2-humidified incubator at 37 °C. The doubling time of the cells, in these conditions, is 9 ± 1 h. The NIH-3T3 mouse fibroblasts were provided by J. F. Nicolas (Institut Pasteur, Paris, France). The cells were grown in Dulbecco's modified Eagle's Medium (DMEM) containing 5% fetal calf serum, penicillin (100 units/mL) and streptomycin (100 μg/mL), in a 6% CO_2-humidified incubator at 37 °C. The doubling time of the cells, in these conditions, was 16 ± 1 h.

Cytotoxicity Determination. L1210: At day 1, 10^4 cells/mL in RPMI supplemented with 16% fetal calf serum in exponential phase were suspended in a volume of 6 mL in a NUNC 25 bottle. Then, increasing amounts of drugs were added to the culture in duplicate vials, in a volume of 6 mL of RPMI without serum. Final concentrations were in the range of 10^{-5}–10^{-8} M. The controls without drug were done in the same conditions. Forty-eight hours later, cell number was determined by using a 6300A cytograf (Bio/physics systems, Inc.), and viability was estimated by the trypan blue exclusion test. The ID_{50} was then computed.

NIH-3T3: at day 1, 7.5×10^3 cells/cm^2 were seeded in 25-cm^3 NUNC flasks in a volume of 9 mL. Drug was added 24 h after seeding, and growth followed for three days. Trypsinized cells are counted with a 6300 A cytograf.

Fluorescence Distribution (NIH-3T3). The cells were seeded on specially treated coverslips at a density of 1.5×10^5 cells per slip (21 cm^2). After 48 h, the slips were mounted in perfusion chambers build to fit under the microscope. New medium containing the drug was then added, and the cells were incubated under normal growth conditions. The cells were kept at 37 °C for 3 h in presence of low concentrations of drugs (5×10^{-7} and 10^{-6} M). Under these conditions, corresponding to the ID_{50} at 48 h, no apparent toxicity was detected after 3 h of incubation. The treated cells were then observed under a Leitz Orthoplan microscope fitted with the Ploemopak 2.1 fluorescence system. Filter 12/3 (Leitz Ploemopak) was used. To process the images thus obtained, a SIT video camera (Bosch) was interfaced with a Magiscan 2 automated image analysis system. Images were digitised into a 512 × 512 matrix at 64 levels with or without multiple addition. Linearity of response to fluorescence was previously established[40] with Rhodamin B.

Determination of in Vivo Toxic Doses and Antitumor Test. Drugs were diluted in water without DMSO; the lethal dose (LD_{100}) and the highest nonlethal dose (LD_0) were determined for each compound after a single injection into random DBA/2 female mice of 20 g by intraperitoneal route (two to five mice for each drug concentration). Mice died in 5 days, and the survivors were kept 5 months (no delayed death was observed).

Random DBA/2 female mice of 20 g each were inoculated intraperitoneally with 10^5 L1210 leukemia cells (provided from

Dr. Atassi, EORTC) or 10^6 P388 leukemia cells. The drugs were administered by the same route as a single dose 24 h after the inoculation of the leuekmia cells (10 animals per dose, 200 μL, and 20 animals for the control). In the case of compounds **4** and **6**, which are less soluble than others compounds, we could not administer the highest dose ($LD_0/2$) in this volume. Dead mice were counted each day at the same hour. The comparison of the mean of the median survival time of the controls (C) (9 ± 0.3 days for L1210 and 9.5 ± 0.6 days for P388) and of the treated (T) animals allows us to estimate the antitumor activity of the tested compounds. Antitumor efficiency is expressed in term of ILS (increase in life span) over controls: (T − C) × 100/C. Survivors are not included in the ILS determination.

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Registry No. **1a**, 72237-98-0; **1b**, 72237-99-1; **2**, 115464-61-4; **3**, 115464-63-6; **4**, 115464-65-8; **5**, 115464-66-9; **6**, 115464-68-1; **7**, 115464-70-5; **8**, 115464-71-6; **9**, 115464-73-8; H_2NEt, 75-04-7; H_2NPr, 107-10-8; H_2NBu-*i*, 78-81-9; $H_2N(CH_2)_2CH(CH_3)_2$, 107-85-7; $H_2N(CH_2)_9CH_3$, 2016-57-1.

Supplementary Material Available: Figure 3 containing hydrophobic properties of compounds **3–6** (2 pages). Ordering information is given on any current masthead page.

Synthesis and Antineoplastic Evaluation of 1,4-Bis(aminoalkanamido)-9,10-anthracenediones

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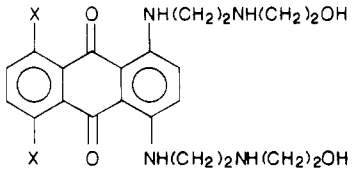
The effect of the replacement of amino groups, attached to the anthraquionone ring in [(aminoalkyl)amino]-anthraquinones, by an amido function on DNA binding, cytotoxicity, and antileukemic activity has been studied. The corresponding 1,4-bis(aminoalkanamido)-9,10-anthracenediones have been synthesized and examined. It has been concluded that such modification does not exclude the DNA binding and cytotoxicity of mentioned compounds but decreases or abolishes the in vivo antileukemic activity.

Among the anthraquinone derivatives with antitumor activity the 1,4-dihydroxy-5,8-bis[[2-[(2-hydroxyethyl)-amino]ethyl]amino]-9,10-anthracenedione (mitoxantrone, **1**) and its dideoxy analogue (ametantrone **2**) are the most promising as potential clinical drugs.[1-3] Mitoxantrone is



1: X = OH
2: X = H

active against a broad range of experimental tumors[3,4] and possibly less cardiotoxic than adriamycin.[3,5-9] At present, intercalative binding of mitoxantrone to DNA[1,9,10] is considered to be responsible for cytotoxic activity, but also metabolically generated free radical species[11] can contribute to the damage of DNA. However, the significantly lower rate of metabolic activation in comparison with anthracyclines may be correlated with the lower cardiotoxicity of this drug.[12] The encouraging properties of this compound have promoted many studies on structural modifications in order to obtain more active compounds and for a better understanding of molecular nature of their properties. Some of these studies have been directed toward developing new molecules with modified anthracenedione nuclei[13] and were based on the concept, previously applied to anthracyclines, that chromophore modifications might diminish cardiotoxicity by reducing the ability to mediate the electron transfer and thus decreasing the formation of reactive oxygen species.[17]

In our studies on the elucidation of the role of structural factors of natural and synthetic anthracenedione derivatives in their biological properties, attention has been drawn to the role of heteroatoms in 1,4-bis-substituted 9,10-anthracenediones side chains. In this paper we examine the influence of modification of properties of nitrogen atoms attached directly to the anthraquinone nucleus at positions 1 and 4. These heteroatoms exert significant effect on the anthraquinone moiety, influencing

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