2,6-Di(ω-aminoalkyl)-2,5,6,7-tetrahydropyrazolo[3,4,5-*mn*]pyrimido[5,6,1-*de*]-acridine-5,7-diones: Novel, Potent, Cytotoxic, and DNA-Binding Agents

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Received July 30, 2001

DNA-binding agents with potential antitumor activities bearing two cationic side chains, the 2,6-di(ω-aminoalkyl)-2,5,6,7-tetrahydropyrazolo[3,4,5-*mn*]pyrimido[5,6,1-*de*]acridine-5,7-diones (**4a**−**r**), have been prepared either by reaction of the appropriate 2-(ω-aminoalkyl)-6-chloro-2,3-dihydro-1*H*,7*H*-pyrimido[5,6,1-*de*]acridine-1,3,7-trione with the appropriate (ω-aminoalkyl)hydrazine or by cyclization of the requisite *N*-6,2-di(ω-aminoalkyl)-2,6-dihydropyrazolo[3,4,5-*kl*]acridine-6-carboxamide with phosgene. In vitro cytotoxic properties of these derivatives against three human colon adenocarcinoma cell lines (HT29, LoVo, and LoVo/Dx) and against some cell lines of the NCI panel are described and compared to that of reference drugs. Some of the new compounds showed outstanding potency while lacking cross-resistance with anthracyclines. Structure−activity relationships are discussed, and a mechanistic analysis is performed using the COMPARE procedure. The mechanism and efficiency of noncovalent DNA binding of these compounds are examined using gel electrophoresis and fluorometric techniques. The 2,6-di(ω-aminoalkyl)-2,5,6,7-tetrahydropyrazolo[3,4,5-*mn*]pyrimido[5,6,1-*de*]-acridine-5,7-diones (**4**) constitute a new class of potent, cytotoxic DNA-binding agents not cross-resistant with doxorubicin.

Introduction

Acridine derivatives play an important role in the class of DNA-intercalating anticancer drugs, which are structurally characterized by the presence of a planar or semiplanar chromophore portion possibly capable of intercalation into DNA. The linear tricyclic ring system of acridine is a versatile moiety in the synthesis of this family of antitumor agents and may constitute either the whole or part of the chromophore. Some noticeable examples clinically used or in clinical trials are constituted by amsacrine,[1] its derivative CI-921,[2] the imidazo-[4,5,1-*de*]acridinones,[3] the 5-nitropyrazolo[3,4,5-*kl*]acridine,[4] and the acridine-4-carboxamide DACA.[5]

In the past, we have studied a number of acridine derivatives with interesting antitumor properties, including the acridone-4-carboxamides (**1**),[6] the pyrazolo-[3,4,5-*kl*]acridine-5-carboxamides (**2**),[7] the pyrimido-[5,6,1-*de*]acridines (**3**),[8,9] the pyrimido[4,5,6-*kl*]acridines,[10,11] and the bis-functionalized acridine-4-carboxamides.[12] Since there are a few examples of acridine derivatives in which the acridine is a part of a pentacyclic chromophore, we planned the synthesis of the pyrazolo[3,4,5-*mn*]pyrimido[5,6,1-*de*]acridine-5,7-diones (**4**). The rational choice of this particular structure was prompted by its strict correlation with derivatives

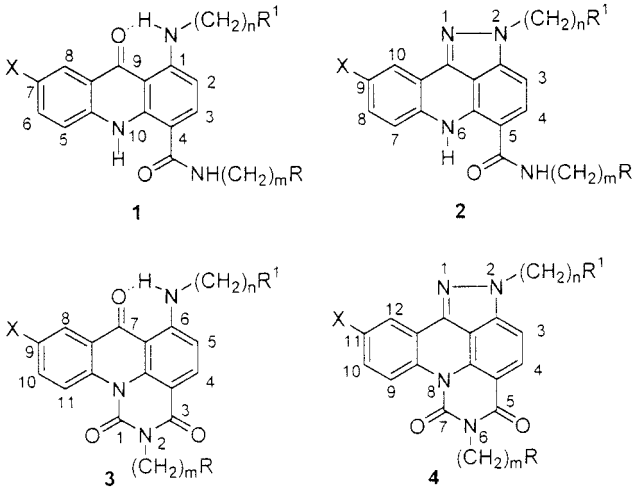

Figure 1. Structures and ring numbering of parent compounds **1**−**3** and of target derivatives **4**.

1−**3**. Indeed, compounds **4** can be formally considered as cyclization products of the above series. Moreover, we previously noted the importance, to ensure biological activity, of the strong intramolecular hydrogen bond in the acridonecarboxamides **1** and in the pyrimidoacridines **3**, as evidenced in Figure 1.[6,9] We postulated that this hydrogen bond mimics an additional ring that may play an important role in cytotoxic activity and in DNA binding.[9] Prompted by the above rationale, we synthesized compounds **4a**−**r** and investigated biologically relevant properties such as in vitro cytotoxicity, mode and affinity of DNA-binding results, and possible mechanistic similarities with known anticancer agents.

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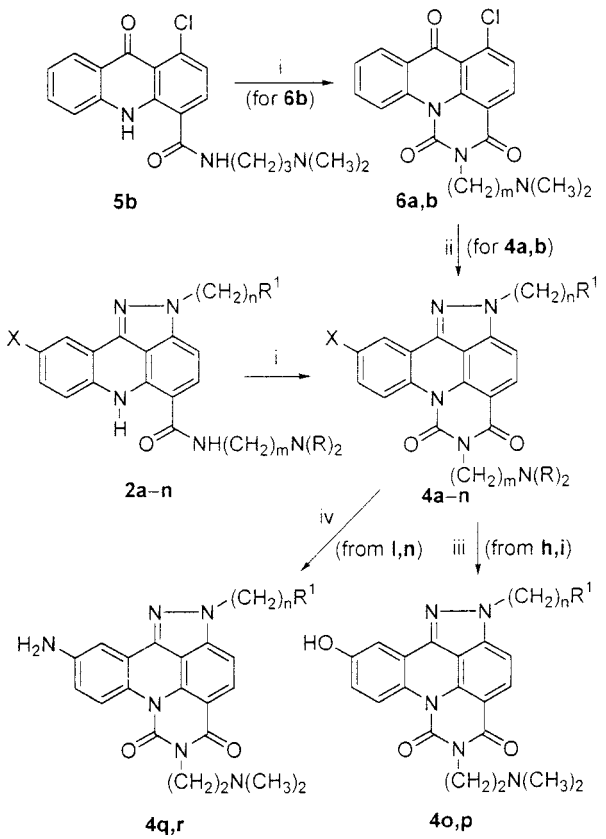
10.1021/jm011004x CCC: $22.00
Published on Web 12/21/2001

Scheme 1[a]



[a] Reagents: (i) $COCl_2$, NEt_3; (ii) $H_2N-NHCH_2CH_2N(CH_3)_2$; (iii) HBr 48%; (iv) H_2, Pd/C. For X, *m*, *n*, R, and R^1, see Table 1.

Chemistry

Scheme 1 shows two synthetic pathways leading to derivatives **4**. According to one, condensation of compound **6a**[9] or of the new derivative **6b** with the *N*-(2-hydrazinoethyl)-*N*,*N*-dimethylamine[13] in 2-ethoxyethanol at 120 °C afforded the pyrazolo[3,4,5-*mn*]pyrimido-[5,6,1-*de*]acridines **4a** or **4b**, respectively. The 1-chloropyrimido[5,6,1-*de*]acridine (**6b**) was prepared by cyclization with phosgene in chloroform at room temperature of the *N*-4-[3-(dimethylamino)propyl]-1-chloro-9-oxo-9,-10-dihydro-4-acridinecarboxamide (**5b**).[6]

Alternatively, the target compounds **4a**−**n** can also be obtained by reaction of the corresponding parent pyrazolo[3,4,5-*kl*]acridine-5-carboxamides (**2a**−**n**)[7] with phosgene in chloroform at room temperature. The 11-hydroxy derivatives **4o**,**p** were obtained by refluxing the corresponding 11-methoxy derivatives **4h**,**i** in aqueous HBr, while the reduction of the nitro derivatives **4l**,**n** with hydrogen and palladium on activated carbon in acidic medium yielded **4q**,**r** as trihydrochlorides.

To examine the DNA-binding properties and the in vitro antineoplastic activity of these agents, the free base forms of **4a**−**p** were converted into their water-soluble hydrochloride salts by the usual methods.

Results and Discussion

Cytotoxic Activity. In vitro cytotoxic potencies of target pyrazolopyrimidoacridines **4a**−**r** and of reference drugs mitoxantrone (Mx) and doxorubicin (Dx) against human colon adenocarcinoma cell line (HT29) are tabulated in Table 1. The results indicate that (a) all compounds **4** possess an excellent antiproliferative activity with IC_{50} values in the low nanomolar range, many of them being remarkably more potent than Dx and Mx, (b) **4k** emerges as the most active among the new derivatives (IC_{50} = 0.04 nM), (c) apart from **4k**, potent cytotoxicity is shown also by **4j**,**n**,**r** with IC_{50} values less than 1 nM and by **4g**,**l**,**m**,**q** with IC_{50} values in the range 1−4 nM. The data obtained allow us either to formulate some structure−activity relationships for both side chains and substituents in position 11 and to compare the target derivatives **4** with the related compounds **1**−**3**.

Concerning the side chains, the following generalizations can be deduced: (i) for both the side chains, the optimal distance between the two nitrogen atoms corresponds to two methylene groups, as indicated by the results obtained with the pairs **4a**,**c**, **4a**,**d**, **4h**,**i**, and **4o**,**p**; (ii) a distance of three methylene units is well tolerated for the side chain in position 6 only (compound **4b**); (iii) substituents at the terminal nitrogen atoms bulkier than methyl increase cytotoxic activity, in contrast to previous observations with parent derivatives **1**−**3**;[6−8] (iv) the best results are obtained for R^1 = piperidino (compounds **4g**,**k**,**n**,**r**).

The substitution pattern in position 11 of the target compounds was also found to be relevant for activity. (i) A methoxy substituent leads to a noticeable improvement in cytotoxic activity, as can be seen from the IC_{50} values of the corresponding unsubstituted/substituted pairs **4a**,**h**, **4c**,**i**, **4f**,**j**, and **4g**,**k**, with a potency increment of 2−100 times. This result again contrasts with the data obtained for the parent compounds **1**−**3**, where the methoxy substitution in corresponding positions leads to a diminished cytotoxicity with respect to unsubstituted derivatives.[6−9] (ii) Also, a nitro group leads to a general increase in cytotoxic activity, as indicated by the IC_{50} values of the corresponding unsubstituted/substituted pairs **4a**,**l**, **4f**,**m**, and **4g**,**n**, with a potency increment of 7−40 times. It is interesting to note that the nitro group performs better than the methoxy in the pair **4h**,**l**, but not in the pairs **4j**,**m** and **4k**,**n**. This behavior is in agreement with our previous observations with parent compounds **2**[7] but not with related compounds **1** and **3**.[6,9] (iii) A hydroxy group, as in derivatives **4o**,**p**, does not vary the activity in comparison to the corresponding unsubstituted derivatives **4a**,**c**. A similar trend was observed with derivatives **1** and **2**,[6,7] not with **3**, where the hydroxy derivative is the most potent in the series.[9] (iv) An amino group, as in compounds **4q**,**r**, results in an increase in cytotoxic potency when compared to the corresponding unsubstituted **4a**,**g** and in a moderate variation in activity in comparison with the parent 11-nitro derivatives **4l**,**n**. Interestingly, in the **2** series the 9-amino derivatives exhibited an activity comparable to that of unsubstituted congeners but are much less potent than 9-nitro compounds,[7] whereas in the **3** series the 9-amino derivative is almost as active as the unsubstituted one but is much more potent than the 9-nitro analogue.[9]

The 2,6-di[2-(dimethylamino)ethyl]-2,5,6,7-tetrahydropyrazolo[3,4,5-*mn*]pyrimido[5,6,1-*de*]acridine-5,7-dione (**4a**) and its 11-substituted derivatives (**4h**,**l**,**o**,**q**) were selected to evaluate their activity on a multidrug-resistant (MDR) cell line. Their structures were chosen to allow a comparison with related compounds **3** having

Table 1. Substituents, Melting Points, Yields, Cytotoxicity, and CT-DNA Binding of Target Compounds **4a**−**r** (Reference Drugs: Doxorubicin (Dx) and Mitoxantrone (Mx))

Compd	R	R^1	m	n	X	mp, °C [a]	yield, % [b]	IC_{50} (SD) [c] HT29	C_{50} [d] (μM) CT-DNA
4a	Me	$N(Me)_2$	2	2	H	181-182 (282-284)	45 (80)	37 (19)	0.0018
4b	Me	$N(Me)_2$	3	2	H	154-155 (149-151)	37 (42)	37 (19)	0.12
4c	Me	$N(Me)_2$	2	3	H	161-162 (283-285)	59	68 (3.7)	0.12
4d	Me	$N(Me)_2$	3	3	H	185-187 (178-180)	23	135 (109)	0.56
4e	Et	$N(Et)_2$	2	2	H	(265-267) [e]	38	29 (17)	0.051
4f	Me		2	2	H	155-156 (287-290)	10	9.2 (5.3)	0.049
4g	Me		2	2	H	157-158 (265-267)	41	4.1 (2.1)	0.050
4h	Me	$N(Me)_2$	2	2	OMe	186-187 (278-281)	30	11 (3.6)	0.052
4i	Me	$N(Me)_2$	2	3	OMe	200-201 (253-255)	24	37 (12)	0.037
4j	Me		2	2	OMe	190-192 (273-274)	20	0.17 (0.05)	0.065
4k	Me		2	2	OMe	202-204 (290-292)	39	0.040 (0.01)	0.0099
4l	Me	$N(Me)_2$	2	2	NO_2	216-217 (261-263)	58	0.98 (0.32)	0.070
4m	Me		2	2	NO_2	210-212 (294-297)	26	1.4 (0.46)	0.048
4n	Me		2	2	NO_2	210-212 (281-285)	85	0.21 (0.05)	0.010
4o	Me	$N(Me)_2$	2	2	OH	289-290 (266-268)	59	39 (23)	0.056
4p	Me	$N(Me)_2$	2	3	OH	241-242 (295-297	90	61 (16)	0.0066
4q	Me	$N(Me)_2$	2	2	NH_2	(283-285) [e]	66	3.7 (0.58)	0.0009
4r	Me		2	2	NH_2	(280-283) [f]	61	0.17 (0.05)	0.006
Dx								26	
Mx								10	0.037 [f]

[a] In parentheses are the hydrochloride melting points, all with decomposition. [b] In parentheses is the percent yield according to reaction ii. [c] Drug concentration (nM) required to inhibit cell growth by 50%. SD = standard deviation. All assays were performed in triplicate. [d] Drug concentration required to produce a 50% drop in fluorescence of DNA-bound ethidium. [e] Isolated as hydrochloride. [f] Data from ref 6.

Table 2. Cytotoxic Activity on LoVo and LoVo/Dx Cell Lines of Selected Compounds in Comparison with Doxorubicin after 1 and 144 h of Drug Exposure[a]

	IC_{50} (μM)[b] 1 h exposure			IC_{50} (μM)[b] 144 h exposure		
compd	LoVo	LoVo/Dx	RI[c]	LoVo	LoVo/Dx	RI[c]
4a	0.29	0.079	0.27	0.012	0.0039	0.33
3a				0.022	0.029	1.3
4h	0.29	0.14	0.48	0.013	0.0018	0.14
3h				0.011	0.42	38
4l	0.52	0.19	0.37	0.016	0.013	0.81
3l				0.5	5.8	12
4o	0.22	0.080	0.36	0.011	0.17	15
3o				0.0017	1.0	590
4q	0.13	0.55	4.2	0.00052	0.016	31
3q				0.049	2.8	57
Dx	0.86	9.7	11	0.024	2.4	100

[a] The data at 144 h of exposure are also compared with the data of the corresponding derivatives **3**. Data are from ref 9. *n*, *m*, R, R^1, and X (Figure 1) are the same as those of the corresponding **4** derivatives. [b] Drug concentration required to inhibit cell growth by 50%. [c] RI (resistance index) is the IC_{50} ratio of LoVo/Dx to LoVo.

the same *m*, *n*, R, R^1, and X substitutions (see Figure 1). In Table 2, we report the cytotoxic activity on human colon adenocarcinoma cell lines (LoVo sensitive and LoVo/Dx doxorubicin-resistant) and the resistance index (RI = IC_{50} ratio of LoVo/Dx to LoVo) of selected compounds **4** in comparison with the corresponding derivatives **3** and with Dx. Already after 1 h of exposure, the new pentacyclic compounds **4** show an excellent cytotoxicity against both LoVo cell lines with no cross-resistance with Dx (except **4q**). At 144 h of exposure, all the new derivatives show cytotoxic activity on the LoVo-sensitive cell line in the nanomolar range, especially **4q** with an IC_{50} of 0.52 nM. However, and even more important, the unsubstituted derivative **4a**, the 11-methoxy-substituted **4h**, and the 11-nitro-substituted **4l** are not cross-resistant with Dx at all. Besides, **4a** and **4h** seem to possess a selective cytotoxicity against the LoVo/Dx MDR cell line. If we compare derivatives **4** with related compounds **3**, we can observe that the new pentacyclic chromophore confers greater cytotoxic potency and much lower RI values with respect to the tetracyclic chromophore. Also, if the compounds substituted with hydrophilic groups such as hydroxy and amino (**4o** and **4q**, respectively) show some cross-resistance with Dx, certainly they are less cross-resistant than the corresponding **3o**,**q**. Moreover, the 11-methoxy-substituted and the 11-nitro-substituted **4h**,**l** are not cross-resistant, unlike the corresponding 9-methoxy- and 9-nitro-substituted **3h**,**l**. These results suggest that the novel compounds are hardly recognized by the protein machinery governing multidrug resistance.

Compounds **4a**,**h**,**l**,**o**,**q** and **4k**, the most potent in the series, were selected for the National Cancer Institute (NCI) screening on 60 human tumor lines. This screen is designed to discover the spectrum of activity and, eventually, selectivity of drugs. The data from this assay can be presented in several different formats. Since it is not practical to report all experimental data available, in Table 3 we choose to describe the antiproliferative

Table 3. Percent Growth[a] of Some NCI Cell Lines Exposed for 48 h at Three Increasing Concentrations (10^{-8}, 10^{-6}, and 10^{-4} M) of Selected Compounds

	4a			**4h**			**4l**			**4o**			**4q**			**4k**		
cell line	10^{-8}	10^{-6}	10^{-4}	10^{-8}	10^{-6}	10^{-4}	10^{-8}	10^{-6}	10^{-4}	10^{-8}	10^{-6}	10^{-4}	10^{-8}	10^{-6}	10^{-4}	10^{-8}	10^{-6}	10^{-4}
leucemia: K-562	61	−27	−67	47	−14	−81	82	6	−38	84	7	−66	45	7	−15	39	12	−12
lung-nsc: NCI-h460	30	−44	−79	9	−60	−95	47	−19	−94	71	−23	−74	16	−28	−64	22	−36	−57
colon: HCT-15	48	−29	−85	36	−31	−80	68	−4	−89	85	21	−68	54	−25	−75	24	−34	−61
cns: SNB-19	66	−3	−41	62	−15	−62	75	2	−93	65	−1	−72	43	−14	−69	55	8	−54
melanoma: LOX IMVI	65	−32	−93	62	−19	−80	79	5	−89	63	−48	−79	47	−28	−50	*b*	*b*	*b*
ovarian: OVCAR-4	51	−36	−56	40	−73	−82	57	−55	−96	70	−19	−60	55	−47	−51	15	−84	−89
renal: 786-0	27	−42	−90	28	−26	−96	36	−25	−99	43	−11	−82	23	−44	−95	20	−41	−67
prostate: DU-145	60	−16	−37	54	−11	−77	82	−16	−94	71	0	−50	59	−11	−67	39	−51	−91
breast: MCF7	63	−41	−95	49	−27	−89	81	−12	−93	60	−17	−64	62	−6	−65	42	4	−12
mean of the cell lines	52	−30	−71	43	−31	−82	67	−13	−87	68	−9	−68	45	−21	−61	32	−28	−55

[a] The negative values indicate the percent of cell killed. [b] Not tested.

activity of selected compounds against one cell line of each NCI subpanel and the mean of the activity on these nine cell lines in one of the possible formats. For each compound, the percent growth at three different increasing concentrations (10^{-8}, 10^{-6}, and 10^{-4} M) is reported to give an idea of the cytostatic (positive values) and cell-killing (negative values) capacity of the selected derivatives. Generally, the data at 10^{-8} M are parallel to what is observed with the HT29 cell line, with a noticeable cytotstatic activity in the order **4k** > **4h** ≅ **4q** > **4a** > **4l** ≅ **4o**. In contrast to what is observed with HT29, in these cell lines the nitro group in the 11 position of **4l** leads to a lesser cytostatic activity in comparison to that of the unsubstituted **4a**. At 10^{-6} M drug concentration, there is a general cell-killing capacity in the order **4h** ≅ **4a** ≅ **4k** > **4q** > **4l** ≅ **4o**. At 10^{-4} M there is a very marked cell-killing capacity for all the compounds tested in the order **4l** > **4h** > **4a** > **4o** > **4q** > **4k**. Besides, it is worth noting the selectivity of all the compounds tested against the NCI-H460 (lung-NSC cancer) and 786-0 (renal cancer) cell lines.

Cytotoxic Mechanism. A COMPARE analysis[14] was performed with compounds **4a**, **4h**, **4l**, **4o**, and **4q** to check whether they resemble previously identified anticancer drugs. The results obtained for **4h**, **4l**, and **4o** were very similar, indicating a closely related mechanism of action. However, no satisfactory resemblance was found with any other of the compounds in the NIH database, the highest correlation coefficient (for etoposide) being about 0.5. The results for the other two compounds, characterized by an H (**4a**) or a NH_2 (**4q**) at position 11, are distinct from the previous set and are different from each other. The closest match for **4q** is nitroimidazole (PCC 0.65), which possibly indicates a mechanism related to redox reactions. These results correlate with the lack of cross-resistance discussed above and further suggest that the test acridones represent a new family of cytotoxic agents.

Since these compounds represent the evolution of an older series, we compared the cytotoxicity exhibited by **4a**, **4h**, **4o**, **4l**, and **4q** with the activity of the correlated analogues belonging to the class of pyrimido[5,6,1-*de*]-acridines (**3**), shown in Figure 1. The average GI_{50} values of the drugs tested in the NCI panel of cell lines were plotted and are reported in the bar graph of Figure 2. It is immediately evident how all derivatives of series **4** are more active than those of series **3**, although the improvement in potency is modulated by the nature of the substituent at corresponding positions in the aromatic ring system and is maximal for the **l** and **a** derivatives, bearing respectively a nitro and a hydrogen substituent. A further correlation was done comparing the growth inhibition parameters for each couple of analogues (i.e., **3a** and **4a**, **3h** and **4h**, etc.) for every specific cell line of the multiple panel, showing also that compounds **4** are consistently more active than compounds of the older series. Hence, the novel tetrahydropyrazoloacridone class represents a successful evolution of the previous families of compounds.

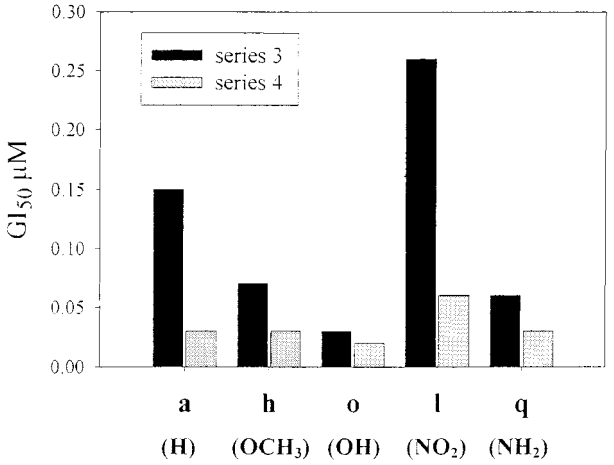


Figure 2. Average GI_{50} values (μM) of the compounds **a**, **h**, **o**, **l**, and **q**, belonging to the series **3** or **4** of derivatives tested in the NCI 60-cell lines panel. In brackets the chemical structure of the substituent at position 9 (series **3**) or 11 (series **4**) is reported.

DNA-Binding Properties. Given the structural characteristics of the test derivatives, we investigated the process of DNA binding in terms of mechanism and affinity.

To assess whether the novel acridones are able to intercalate into the double helix, we performed DNA-unwinding studies using ethidium bromide as a control. The example reported in Figure 3 shows that derivative **4k** alters dramatically the migration of the supercoiled form of the plasmid through the agarose gel. In fact, at drug concentrations up to 10 μM the gel mobility decreases, while further addition of compound causes an actual increase in the plasmid mobility. This behavior is consistent with unwinding of the DNA duplex so that the number of negative supercoils is progressively reduced to obtain a form migrating at the relaxed position, and further untwisting by the drug binding is compensated by the formation of positive supercoils,

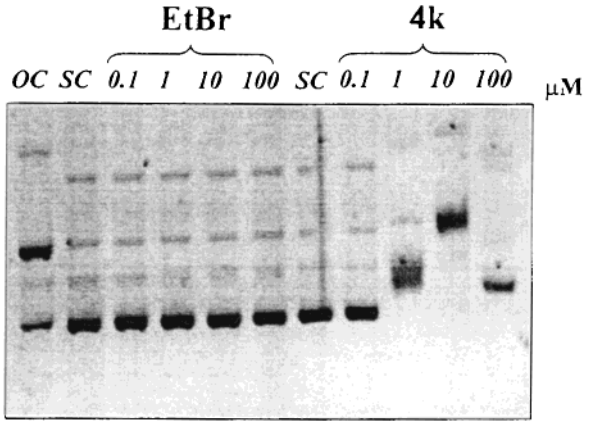


Figure 3. Acridone **4k** unwinds DNA. Increasing concentrations of ethidium bromide (EtBr) or **4k** were incubated with negatively supercoiled pBR322 (0.25 μg) for 1 h at 25 °C in TE buffer, pH 8.0, and then run for 4 h at 5 V/cm on a 1% agarose gel (Tris-borate 50 mM, EDTA 1mM). OC refers to the open circular form of the plasmid and SC to the negatively supercoiled form of pBR322.

characterized by increased rate migration. Hence, we can conclude that the mechanism of DNA binding of **4k**, as well as of the other DNA-binding derivatives belonging to this family (not shown), is intercalation. It is noteworthy that the drug−DNA complex formed upon brief incubation is stable even upon dilution in the gel wells and during the gel run (the drug was not present in the agarose gel), whereas ethidium bromide under the same conditions dissociates from its DNA target in the electric field and no variation of plasmid mobility is observed at any concentration tested (Figure 3). In fact, the outcome of this assay is largely affected by the drug's in and out kinetics, diffusion, and charge rather than by the absolute value of the compound's affinity for DNA.

The binding of the test drugs to DNA was evaluated using a competitive-displacement fluorometric assay with DNA-bound ethidium.[15] This allows determination of the drug concentration required to reduce ethidium concentration by 50% (C_{50}), which is inversely related to binding affinity.[16] In the present study, fluorescence displacement assays were performed at pH 7.0 to mimic biological conditions. The C_{50} values for the acridine derivatives **4a**−**r** are reported in Table 1. Our results indicate that (a) the target compounds are excellent DNA binders, comparable, in some cases much superior, to Mx, (b) generally, compounds **4** bind DNA more strongly than the related acridine derivatives previously studied by us,[6,7,9] and (c) **4k**,**m**,**p**,**r** and especially **4a**,**q** apparently exhibit very high binding affinity. No quantitative correlation is found when comparing C_{50} and IC_{50} values (Table 1). In any event, prominent DNA binding is generally accompanied by very remarkable cytotoxicity, with IC_{50} values in the nanomolar range. Different pharmacokinetic properties may well account for the lack of strict binding/potency correlation. In addition, the COMPARE results reported above suggest novel mechanisms of cytotoxicity and raise the issue of the existence of previously unexploited cellular targets for the test acridones, not necessarily related to DNA.

Conclusions

From the present study we can conclude that our hypothesis that the strong intramolecular hydrogen bond of derivatives **3**, evidenced in Figure 1, can mimic an additional fifth ring important for biological activity[9] is supported by the results obtained with the 2,6-di(ω-aminoalkyl)-2,5,6,7-tetrahydropyrazolo[3,4,5-*mn*]pyrimido[5,6,1-*de*]acridine-5,7-diones (**4a**−**r**). In fact, the target derivatives **4** exhibit enhanced cytotoxic activity, superior efficacy against the MDR LoVo/Dx cell line, and higher DNA affinity than the tetracyclic parent pyrimido[5,6,1-*de*]acridines **3**, already endowed with remarkable activity. Also, with respect to the other related acridine derivatives **1** and **2** in Figure 1, compounds **4** possess higher cytotoxicity and better DNA-binding capacities.

An important novelty for this kind of compound is the efficacy for cytotoxic activity of the side chain in the 2 position when R^1 is a piperidino group. The 6-[2-(dimethylamino)ethyl]-11-methoxy-2-(2-piperidinoethyl)-2,5,6,7-tetrahydropyrazolo[3,4,5-*mn*]pyrimido[5,6,1-*de*]-acridine-5,7-dione (**4k**), with an IC_{50} of 0.04 nM on HT29 cell line, emerges as the most potent of the new acridine derivatives.

Finally, the 2,6-di(ω-aminoalkyl)-2,5,6,7-tetrahydropyrazolo[3,4,5-*mn*]pyrimido[5,6,1-*de*]acridine-5,7-diones (**4a**−**r**) represent a new class of potential antitumor drugs endowed with potent and broad spectrum cytotoxicity, no cross-resistance with Dx on LoVo/Dx cell line, and a relevant affinity with DNA.

Experimental Section

Synthetic Chemistry. Melting points were determined on a Büchi 510 apparatus and are uncorrected. Thin-layer chromatography (TLC) was accomplished using plates precoated with silica gel 60 F-254 (Merck). All ^{1}H NMR spectra were recorded on a Varian VXR 300 instrument**.** Chemical shifts are reported as δ values (ppm) downfield from internal Me_4Si in the solvent shown. The following NMR abbreviations are used: br (broad), s (singlet), d (doublet), t (triplet), m (multiplet), ar (aromatic proton), ex (exchangeable with D_2O). Elemental analyses were performed on a model 1106 elemental analyzer (Carlo Erba Strumentazione).

6-Chloro-2-[3-(dimethylamino)propyl]-2,3-dihydro-1*H*,7*H*-pyrimido[5,6,1-*de*]acridine-1,3,7-trione (6b). To a mixture of **5b**[6] (0.33 g, 0.92 mmol) and triethylamine (1 mL) in $CHCl_3$ (20 mL), a solution of $COCl_2$ (20% in toluene, 1.87 mL, 3.74 mmol) in $CHCl_3$ (10 mL) was added dropwise at 0 °C. After being stirred at room temperature for 15 min, the mixture was partitioned between $CHCl_3$ and aqueous 1 M Na_2CO_3. The organic layer was worked up to give a residue, which was chromatographed on a silica gel column and eluted with $CHCl_3$/MeOH (14:1 v/v). The residual dense oil obtained solidified on washing with Et_2O (0.28 g, 79%) and was used for the next step.

2,6-Di[2-(dimethylamino)ethyl]-2,5,6,7-tetrahydropyrazolo[3,4,5-*mn*]pyrimido[5,6,1-*de*]acridine-5,7-dione (4a). Example of General Procedure for the Preparation of 4a−n According to Reaction i. To a mixture of the dihydrochloride of *N*-5,2-di[2-(dimethylamino)ethyl]-2,6-dihydropyrazolo[3,4,5-*kl*]acridine-5-carboxamide[7] (**2a**, 0.05 g, 0.11 mmol) and triethylamine (0.2 mL) in $CHCl_3$ (10 mL), $COCl_2$ (20% in toluene, 0.35 mL, 0.7 mmol) in $CHCl_3$ (10 mL) was added dropwise under stirring at 0 °C. The stirring was protracted for 20 min at room temperature. The mixture was partitioned between $CHCl_3$ (2 × 20 mL) and an excess of 1 M aqueous Na_2CO_3 (30 mL). The organic layer was worked up to give a residue that was flash-chromatographed on a silica gel column and was eluted first with $CHCl_3$/MeOH (1:1 v/v), then

with $CHCl_3$/MeOH (1:1 v/v) and 32% aqueous NH_3 (10 mL for 1 L of eluent) to give **4a**: ^{1}H NMR ($CDCl_3$) δ 2.31 (s, 6H, 2 × CH_3), 2.38 (s, 6H, 2 × CH_3), 2.68 (t, 2H, CH_2), 2.89 (t, 2H, CH_2), 4.37 (t, 2H, CH_2), 4.51 (t, 2H, CH_2), 7.10 (d, 1H, ar), 7.44 (t, 1H, ar), 7.51 (t, 1H, ar), 8.00 (d, 1H, ar), 8.19 (d, 1H, ar), 9.45 (d, 1H, ar). Anal. ($C_{23}H_{26}N_6O_2$) C, H, N.

Derivatives **4b**−**n** were prepared in a similar manner.

2,6-Di[2-(dimethylamino)ethyl]-2,5,6,7-tetrahydropyrazolo[3,4,5-*mn*]pyrimido[5,6,1-*de*]acridine-5,7-dione (4a). Example of General Procedure for the Preparation of 4a,b According to Reaction ii. The pyrimido[3,4,5-*kl*]acridine **5h**[7] (0.2 g, 0.54 mmol) and 2-(dimethylamino)ethylhydrazine[11] (0.26 g, 2.5 mmol) in 2-ethoxyethanol (10 mL) were stirred at 120 °C until the TLC showed the disappearance of the starting material. The mixture was cooled at room temperature and partitioned between $CHCl_3$ (2 × 30 mL) and an excess of 1 M aqueous Na_2CO_3 (40 mL). The organic layer was worked up to give a residue that was flash-chromatographed on a silica gel column and was eluted first with $CHCl_3$/MeOH (1:1 v/v), then with $CHCl_3$/MeOH (1:1 v/v) and 32% aqueous NH_3 (10 mL for 1 L of eluent) to give **4a**.

Derivative **4b** was prepared in a similar manner.

2,6-Di[2-(dimethylamino)ethyl]-11-hydroxy-2,5,6,7-tetrahydropyrazolo[3,4,5-*mn*]pyrimido[5,6,1-*de*]acridine-5,7-dione (4o). Example of General Procedure for the Preparation of 4o,p. 4h (0.14 g, 0.31 mmol) was suspended in aqueous HBr 48% (2 mL) and refluxed until the TLC showed the disappearance of the starting material. The reaction mixture, diluted with water (20 mL), was partitioned between $CHCl_3$ (3 × 50 mL) and an excess of 1 M aqueous Na_2CO_3 (100 mL). The organic layer was worked up to give a residue that was washed with Et_2O to afford pure **4t**: ^{1}H NMR (DMSO-d_6) δ 2.20 (s, 6H, 2 × CH_3), 2.25 (s, 6H, 2 × CH_3), 2.53 (t, 2H, CH_2), 2.78 (t, 2H, CH_2), 4.16 (t, 2H, CH_2), 4.56 (t, 2H, CH_2), 6.96 (d, 1H, ar), 7.37−7.45 (m, 2H, ar), 7.80 (d, 1H, ar), 9.21 (d, 1H, ar). Anal. ($C_{23}H_{26}N_6O_3$) C, H, N.

Derivative **4p** was prepared in a similar manner.

11-Amino-2,6-di[2-(dimethylamino)ethyl]-2,5,6,7-tetrahydropyrazolo[3,4,5-*mn*]pyrimido[5,6,1-*de*]acridine-5,7-dione Trihydrochloride (4q·3HCl). Example of General Procedure for the Preparation of 4q,r. A mixture of the 9-nitro derivative **4l** (0.13 g, 0.28 mmol), Pd/C (0.2 g, 5%), and aqueous HCl (1 mL of 37% w/w) in MeOH (20 mL) was stirred under a hydrogen atmosphere (30 psi) for 1 h at room temperature. The reaction mixture was filtered and then evaporated to yield a residue that was treated with boiling EtOH (10 mL). The mixture was cooled to room temperature, filtered, and washed with Et_2O to give **4q**·3HCl: ^{1}H NMR (DMSO-d_6) δ 2.90 (s, 12H, 4 × CH_3), 3.45 (m, 2H, CH_2), 3.70 (m, 2H, CH_2), 4.20 (br s, 7H, ex), 4.45 (m, 2H, CH_2), 5.01 (m, 2H, CH_2), 7.10 (d, 1H, ar), 7.51−7.67 (m, 2H, ar), 7.97 (d, 1H, ar), 9.22 (d, 1H, ar), 10.16 (br s, 1H, ex), 10.96 (br s, 1H, ex). Anal. ($C_{23}H_{27}N_7O_2$·3HCl·2H_2O) C, H, N.

Derivative **4r** was prepared in a similar manner.

Biophysical Evaluation. 1. In Vitro Cytotoxicity. Human Colon Adenocarcinoma Experimental Protocol. Establishment details of human colon adenocarcinoma carcinoma cell lines (HT29, LoVo sensitive, and LoVo/Dx resistant) have been previously described.[10,17,18] Drug solutions of appropriate concentration were added to a culture containing HT29 cells at 2.5 × 10^4 cells/mL of medium[10] or to a culture containing LoVo or LoVo/Dx cells at 2.5 × 10^5 cells/mL of medium.[17] All assays were performed in triplicate.

2. Fluorescence Binding Studies. The fluorometric assays have been described previously.[19] The C_{50} values for ethidium displacement from CT-DNA were determined using aqueous buffer (10 mM Na_2HPO_4, 10 mM NaH_2PO_4, 1 mM EDTA, pH 7.0) containing 1.26 μM ethidium bromide and 1 μM CT-DNA.[19,20]

All measurements were made in 10 mm quartz cuvettes at 20 °C using a Perkin-Elmer LS5 instrument (excitation at 546 nm; emission at 595 nm) following serial addition of aliquots of a stock drug solution (~5 mM in DMSO). The C_{50} values are defined as the drug concentrations that reduce the fluorescence of the DNA-bound ethidium by 50% and are calculated as the mean from three determinations.

3. DNA Unwinding. To test the drug's ability to unwind DNA, we performed a direct assay employing negative supercoiled pBR322, ethidium bromide as a control, and the derivative **4k**. A total of 0.25 μg of plasmid was incubated in TE buffer, pH 8.0, with 0.1, 1, 10, and 100 μM of either compound for 1 h at room temperature. After the incubation period, the complex was directly loaded onto a 1% agarose gel in 5 mM Tris-borate and 1 M EDTA and was run at 5 V/cm for 4 h. The gel was then stained with ethidium bromide to show the change in plasmid mobility upon complex formation.

Supporting Information Available: Detailed information on target compounds (^{1}H NMR, purification procedure). This material is available free of charge via the Internet at http://pubs.acs.org.

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JM011004X