Design, Synthesis, and Biological Properties of New Bis(acridine-4-carboxamides) as Anticancer Agents

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Received February 21, 2003

To enhance the outstanding biological response shown by the corresponding monomers **4** and **5**, two classes of bis-acridine-4-carboxamides, **9**, with a linker between the 4,4′ positions, and **13**, with a linker between the 1,1′ positions, have been prepared as DNA-binding and potential antitumor agents. The noncovalent DNA-binding properties of these compounds have been examined using gel-electrophoresis and fluorometric techniques. The results indicate that (i) target compounds intercalate DNA; (ii) the bis derivatives with the optimal linker are considerably more DNA-affinic than corresponding monomers; (iii) overall affinity is sensitive to the nature of the linker, of the chromophores, and of the substituents at 7,7′; (iv) often, the bis derivatives show a marked AT-preferential binding. In vitro cytotoxic potency of these derivatives toward the human colon adenocarcinoma cell line (HT29) is described and compared to that of reference drugs. Structure−activity relationships are discussed. Some highly DNA-affinic and potent cytotoxic compounds, **9b**,**f** and **13b**,**c**, have been selected for the National Cancer Institute (NCI) screening on 60 human tumor cell lines and identified as new leads in the antitumor strategies.

Introduction

Connecting two planar intercalating moieties to obtain a bis derivative generally increases the DNA binding affinity and the drug's residence times in the DNA-bound form. This rational has led to the development of an interesting bis-intercalator family possessing noticeable antitumor properties. Some successful examples are the bis(naphthalimide) analogue, DMP 840 (**1**), reported to be a topoisomerase II poison,[1] presently undergoing clinical trials,[2] and the related LU 79553 (**2**), highly effective against tumor xenografts in vivo.[3] Moreover, a series of bisimidazoacridones (e.g., WMC-26, **3**) exhibited highly selective cytotoxicity toward human colon carcinoma cells both in culture and in xenografts.[4] However, for the latter derivatives, bis-intercalation appears an unlikely process, and a groove binding mode has been proposed, although the precise nature of this interaction is still to be clarified.[4] Recently, bis(acridine-4-carboxamides) and dicationic bis-(9-methylphenazine-1-carboxamides) have been synthesized and studied.[5,6] Interestingly, the cytotoxic profile and in vitro activity of these derivatives are consistent with topoisomerase I rather than topoisomerase II poisoning effects. Hence, different modes of DNA-binding and cytotoxic action can be observed, which are still compatible with prominent antitumor efficacy. Altogether, the above results confirm that bis-intercalators can be considered as a promising class of anticancer compounds. Nevertheless, according to the information thus far available, general structure−activity relationships are still far from being firmly established, also given the fact that different cellular targets are possibly recognized by different drugs.

Our aim is to exploit the potential of new acridine-based bis-intercalators as anticancer drugs. The novel compounds are derived from two interesting series of acridine-4-carboxamides we have previously examined.[7,8] In these derivatives the chromophore moiety is either 9-acridone (e.g., **4**)[7] or acridine (e.g., **5**).[8] Given the outstanding biological response shown by the above monomers, we synthesized and investigated the biochemical and pharmacological properties of a series of bis analogues derived from these monomers.

Chemistry

Schemes 1 and 2 show the synthetic pathways leading to target derivatives **9** and **13**. According to the first scheme, the reaction of either 1-chloro-9-oxo-9,10-dihydroacridine-4-carboxylic acid (**6**)[9] or 1-chloro-7-methoxy-9-oxo-9,10-dihydroacridine-4-carboxylic acid (**7**)[7] with the suitable diamine, by the “mixed anhydride” method,[10] afforded the intermediate bis(acridine-4-carboxamides) **8a**−**f**. All the diamines were commercially available, except the N^1,N^2-bis(2-aminoethyl)-N^1,N^2-dimethyl-1,2-ethanediamine, needed for **8d**, prepared according to the literature.[11] Nucleophilic substitution of **8a**−**f** with N^1,N^1-dimethyl-1,2-ethanediamine yielded the target bis(acridine-4-carboxamides) **9a**−**f**. Cleavage with aqueous HBr of the methoxy derivatives **9e**,**f** gave the hydroxy derivatives **9g**,**h**, respectively. Finally, the bis-

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10.1021/jm030820x CCC: $25.00
Published on Web 06/07/2003

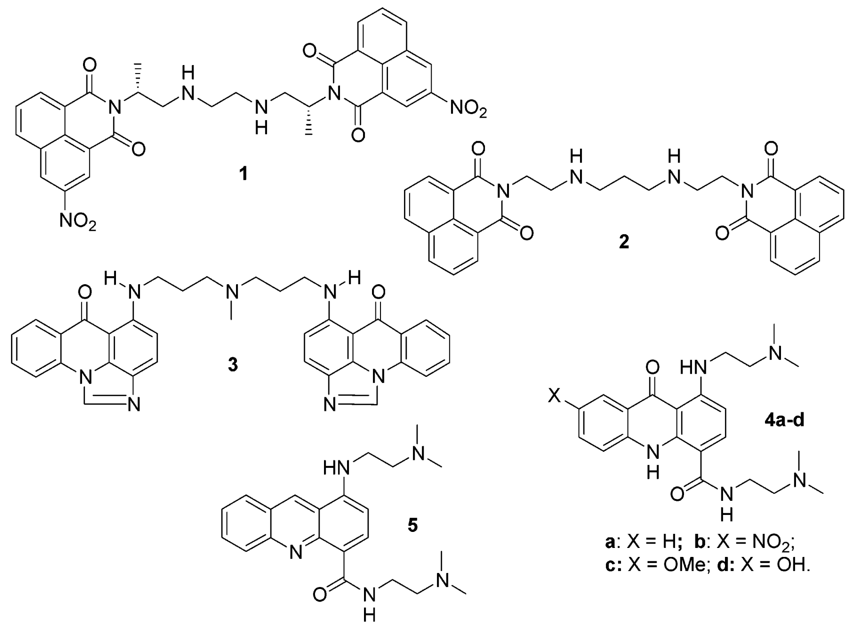
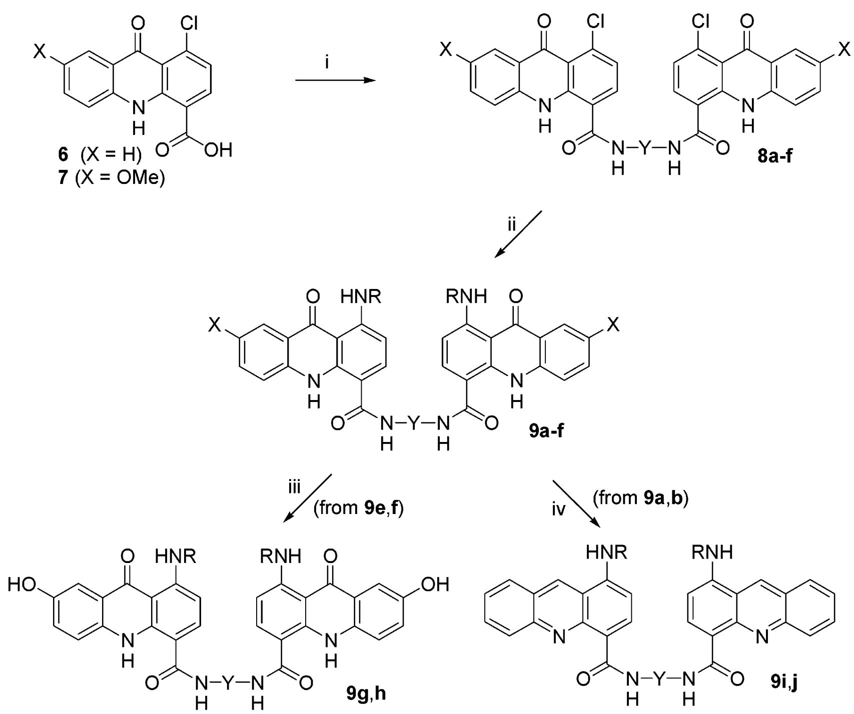


Figure 1. Structures of DMP 840 (**1**), LU 79553 (**2**), WMC-26 (**3**), bis-functionalized 9-acridones (**4**), and bis-functionalized acridine (**5**).

Scheme 1[a]



[a] Reagents: (i) ClCOOEt, $N(Et)_3$, $H_2N-Y-NH_2$; (ii) H_2NR; (iii) HBr 48%; (iv) Al/Hg. Structures: R = $(CH_2)_2N(CH_3)_2$ for **9a**−**j**; X = H for **a**−**d**; X = OMe for **e**, **f**; Y = $(CH_2)_2N(Me)(CH_2)_2$ for **a**, **e**, **g**, and **i**; Y = $(CH_2)_3N(Me)(CH_2)_3$ for **b**, **f**, **h**, and **j**; Y = $(CH_2)_3$ for **c**; Y = $(CH_2)_2N(Me)(CH_2)_2N(Me)(CH_2)_2$ for **d**.

acridines **9i**,**j** were obtained by reduction with aluminum amalgam of bis-9-acridones **9a**,**b**, respectively.

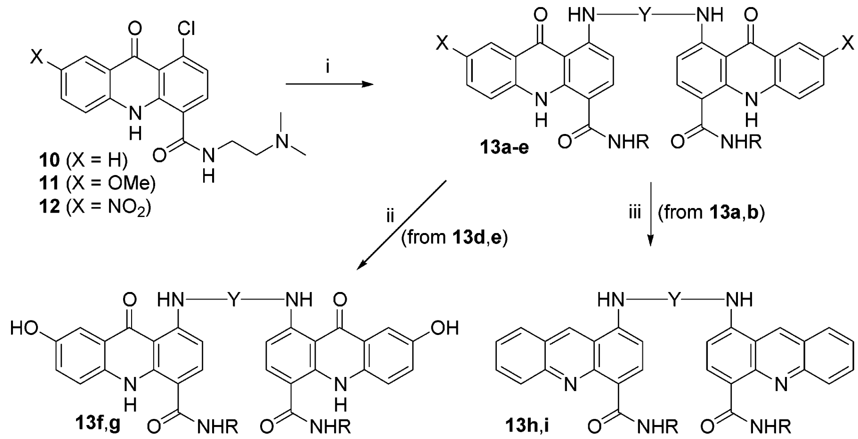
As shown in Scheme 2, the target compounds **13a**−**e** were prepared by reaction of the appropriate 9-acridone-4-carboxamide (**10**−**12**)[7] with either *N*[1]-(2-aminoethyl)-*N*[1]-methyl-1,2-ethanediamine or *N*[1]-(3-aminopropyl)-*N*[1]-methyl-1,3-propanediamine in 2-ethoxyethanol at 120 °C. The hydroxy derivatives **13f**,**g** were obtained by refluxing the corresponding methoxy derivatives **13d**,**e** in aqueous HBr, while the reduction with aluminum amalgam of the 9-acridone derivatives **13a**,**b** afforded the acridine derivatives **13h**,**i**.

All the target compounds **9** and **13** were examined, as water-soluble hydrochloride salts obtained by usual methods, for their DNA-binding properties and their antineoplastic activity.

Results and Discussion

DNA-Binding Properties. To assess whether the new compounds are able to intercalate into the double helix, we performed DNA unwinding studies using 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 (PPAC),[12] an acridone derivative that

Scheme 2[a]



[a] Reagents: (i) $H_2N-Y-NH_2$; (ii) HBr 48%; (iii) Al/Hg. Structures: R = $(CH_2)_2N(CH_3)_2$ for **a**−**i**; X = H for **a**,**b**; X = NO_2 for **c**; X = OMe for **d**,**e**; Y = $(CH_2)_2N(Me)(CH_2)_2$ for **a**, **d**, **f**, and **h**; Y = $(CH_2)_3N(Me)(CH_2)_3$ for **b**, **c**, **e**, **g**, and **i**.

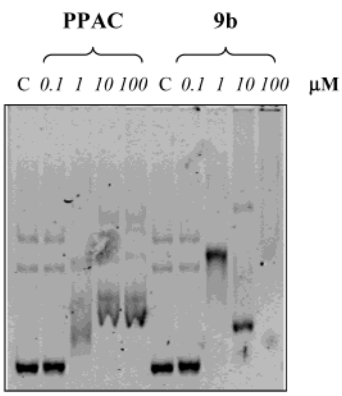


Figure 2. The bis-acridine derivative **9b** unwinds plasmid DNA. Increasing concentrations of PPAC or **9b** were incubated with negatively supercoiled pBR322 (0.25 μg) for 1 h at 25 °C in TE buffer pH 8.0, and then run for 4 h at 5 V/cm on a 1% agarose gel (Tris-borate 50 mM, EDTA 1 mM). C refers to the negatively supercoiled form of pBR322.

showed the ability to bind to DNA in an intercalative mode, as control. The example reported in Figure 2 shows that derivative **9b** alters the migration of the supercoiled form of the plasmid through the agarose gel more dramatically than our positive control. In fact, at drug concentrations up to 1 μM, the gel mobility decreases consistently, while further addition of compound causes an increase in the plasmid mobility. At 100 μM, precipitation of the DNA in the well occurs. This electrophoretic behavior is consistent with unwinding of the DNA duplex so that the number of negative supercoils is progressively reduced, and further untwisting by the drug binding is compensated for by the formation of positive supercoils, characterized by increased rate of migration. Hence, we can conclude that the mechanism of DNA binding of **9b**, as well as of the other DNA binding derivatives belonging to this family (not shown), is intercalation, and the degree of unwinding of the bis derivatives is higher than that of the mono-acridone derivative used as control. It is noteworthy that the drug−DNA complex formed upon brief incubation is stable even upon dilution in the gel wells and during the overnight gel run (the drug was not present in the agarose gel).

Competitive displacement (C_{50}) fluorometric assays with DNA-bound ethidium can be used[13] (a) to determine, as shown in Table 1, 'apparent' equilibrium constants (K_{app}) for drug binding, as the C_{50} value is approximately inversely proportional to the binding constant,[14] and (b) to establish possible base- or sequence-preferential binding.[15] In the present study, fluorescence displacement assays were performed at pH 7 to enable comparison under biological conditions.

The K_{app} values of the new derivatives with CT-DNA, AT, and GC are reported in Table 1. The results indicate that the target compounds are excellent DNA ligands, generally possessing greater affinity than ethidium, but lower than mitoxantrone (Mx); two exceptions are **9c** with a K_{app} of 3.3×10^5 and **9j** with a K_{app} of 1.4×10^{10}; the low affinity of **9c** is probably due to a linker that is too short and devoid of a basic nitrogen (Y = -$(CH_2)_3$-) to allow an efficient interaction with DNA. The exceptional affinity of **9j** is difficult to explain, without considering the K_{app} value (1.5×10^9) of related monomer **5**.[8] **9d** also shows a remarkable K_{app} value, indicating that even when Y = -$(CH_2)_2N(CH_3)(CH_2)_2N(CH_3)$-$(CH_2)_2$-, an efficient binding can be achieved.

For the derivatives with Y = -$(CH_2)_nN(CH_3)(CH_2)_n$-, the following should be noted: (a) The respective K_{app} values of all the related pairs with $n = 2$ or 3 (**8a**,**b**, **9a**,**b**, **9e**,**f**, **9g**,**h**, **9i**,**j**, **13a**,**b**, **13d**,**e**, **13f**,**g**, and **13h**,**i**) always indicate a binding more (2.3−100 times) efficient for compounds with $n = 3$, clearly showing that the best results in terms of DNA affinity are achieved with the longest linker either in the 4,4′ or in the 1,1′ positions. (b) The K_{app} values of the intermediates **8a**,**b**, compared to that of the related target compounds **9a**,**b**, indicate that **9a**,**b** are more DNA affinic (4−17 times) than **8a**,**b**, pointing out the importance of the basic side chains in positions 1,1′ for binding. (c) The substitution at positions 7,7′ leads to different results in the subseries **9** and **13**: for derivatives **9** the affinity rank order is **9e**,**f** (X = OMe) > **9a**,**b** (X = H) > **9g**,**h** (X = OH); for **13** the order is **13f**,**g** (X = OH) > **13a**,**b** (X = H) > **13d**,**e** (X = OMe). (d) Generally, the related monomers **4** and **5** possess DNA affinity inferior to that of target derivatives with $n = 3$, but superior to that of target derivatives with $n = 2$. A noticeable exception is constituted by the case of **5** compared with the pair **13h**,**i**: the monomer is much more DNA affinic than both components of the pair. (e) Effect of the chromophore: com-

Table 1. Melting Points,[a] Yields, Formula,[b] DNA Binding,[c] and Cytotoxic Activity against Human Colon Adenocarcinoma (HT29) of Target Compounds **9a**–**j** and **13a**–**i**, of Intermediate Derivatives **8a**,**b**, and of Related Monomers **4a**–**d** and **5**. Reference Drug: Mitoxantrone (Mx).

compd	mp, °C	yield, %	formula	K_{app}[d] × 10^{-7} M^{-1} AT	CT-DNA	GC	binding site preference[e]	IC_{50} (μM)[f] HT29
9a	116–117(238–240)	79	$C_{41}H_{49}N_9O_4$	2.9	2.6	1.5	A–T (1.9)	0.48
9b	119–120 (226–228)	85	$C_{43}H_{53}N_9O_4$	8.2	6.3	0.79	A–T (10)	0.057
8a	(>300)[g]	38	$C_{33}H_{28}Cl_3N_5O_4$	0.09	0.15	0.0034	A–T (26)	5.5
8b	(245–247 dec) [g]	46	$C_{35}H_{32}Cl_3N_5O_4$	1.5	1.6	1.6	none	0.34
4a[h]	-	-	-	-	3.7	-	none	0.094
9c	220–221 (198–200)	47	$C_{39}H_{44}N_8O_4$	0.041	0.033	0.020	A–T (2.0)	>10
9d	(268–270)[g]	46	$C_{44}H_{60}Cl_4N_{10}O_4$	32	15	40	none	0.29
9e	217–218 (270–273 dec)	74	$C_{43}H_{53}N_9O_6$	5.8	4.4	4.3	A–T (1.3)	0.89
9f	143–144 (250–252 dec)	66	$C_{45}H_{57}N_9O_6$	13	20	5.2	A–T (2.5)	0.0020
4c[h]	-	-	-	-	1.8	-	none	0.50
9g	162–163 (262–264 dec)	64	$C_{41}H_{49}N_9O_6$	1.9	1.9	1.9	none	8.9
9h	221–222 (250–251)	45	$C_{43}H_{53}N_9O_6$	13	4.4	8.3	A–T (1.6)	0.80
4d[h]	-	-	-	-	3.4	-	none	2.0
9i	196–197 (248–249 dec)	25	$C_{41}H_{49}N_9O_2$	12	13	6.3	A–T (1.9)	2.0
9j	166–167 (240–242 dec)	50	$C_{43}H_{53}N_9O_2$	1260	1400	43	A–T (29)	0.23
5[i]	-	-	-	2.8	150	23	G–C (8.2)	0.007
13a	135–136 (258–260 dec)	39	$C_{41}H_{49}N_9O_4$	8.4	1.5	3.8	A–T (2.2)	0.41
13b	112–113 (235–236 dec)	48	$C_{43}H_{53}N_9O_4$	9.3	10	7.5	none	0.00043
13c	152–153 (255–257 dec)	67	$C_{43}H_{51}N_{11}O_8$	19	14	23	none	<0.0001
4b[h]	-	-	-	-	7.4	-	A–T (1.3)	0.19
13d	160–162 (250–252 dec)	35	$C_{43}H_{53}N_9O_6$	1.3	1.8	0.88	A–T (1.5)	3.3
13e	160–161 (230–233 dec)	40	$C_{45}H_{57}N_9O_6$	26	6.2	14	A–T (1.9)	0.039
13f	190–192 (254–255)	78	$C_{41}H_{49}N_9O_6$	1.3	2.0	1.4	none	>10
13g	186–188 (270–271)	49	$C_{43}H_{53}N_9O_6$	13	12	7.4	A–T (1.8)	6.9
13h	138–139 (255–257 dec)	57	$C_{41}H_{49}N_9O_2$	2.1	5.3	0.72	A–T (2.9)	0.47
13i	130–131 (250–252 dec)	25	$C_{43}H_{53}N_9O_2$	66	12	9.4	A–T (7.0)	0.047
Mx					34			0.010

[a] In parentheses hydrochlorides melting points, dec = decomposition. [b] Analyses for C, H, and N. [c] CT-DNA, AT, and GC refer to calf thymus DNA, [poly(dA-dT)]$_2$, and [poly(dG-dC)]$_2$, respectively. [d] $K_{app} = 1.26/C_{50} \times 10^7$ in which 1.26 is the concentration (μM) of ethidium in ethidium–DNA complex, C_{50} is drug concentration (μM) to effect 50% drop in fluorescence of bound ethidium, and 10^7 is the value of K_{app} assumed for ethidium in the complex. [e] The binding site preference is considered to be significant only for [AT]/[GC] ratio differing by > 30% from the sequence-neutral unity value (i.e., <0.7 or >1.3). In parentheses the values of [AT]/[GC] ratio ([GC]/[AT] for **5**). [f] Drug concentration required to inhibit cell growth by 50%, all assays were performed in triplicate. [g] Isolated as hydrochloride salts. [h] Data from ref 7. [i] Data from ref 8.

parison of the related pairs **9a**,**i**, **9b**,**j**, **13a**,**h**, and **13b**,**i** indicate that the "acridinic" chromophore generally yields higher affinity than the "acridonic" one. The most relevant difference is shown by the pair **9b**,**j**, **9j** being 220 times more affinic than **9b**. (f) Effect of the linker position: contrasting results can be observed by comparing compounds **9** (linker in positions 4,4′) with related **13** (linker in positions 1,1′). The rank affinity order for unsubstituted compounds is **9a** > **13a**, **9b** < **13b**, **9i** > **13h**, and **9j** ≫ **13i**; for methoxy derivatives is **9e**,**f** > **13d**,**e**, while for hydroxy is **9g**,**h** < **13f**,**g**.

The binding behavior of target compounds with synthetic polynucleotides reflects what observed for CT-DNA. The observed binding site preference indicates a frequent, remarkable in some cases, preference for binding to AT-rich duplexes. Previously, in contrast to other acridine/acridone derivatives, we have noted a moderate AT preference for monomers **4** related to "acridonic" dimers.[7] Compounds with "acridinic" chromophore, **9i**,**j** and **13h**,**i**, show AT ≫ GC preference, in contrast with related monomer **5** endowed with GC ≫ AT preference, suggesting a different binding mechanism between "acridinic" dimers and monomer **5**.

Cytotoxic Activity. In vitro cytotoxic potencies of target compounds **9a**–**j** and **13a**–**i**, of intermediates **8a**,**b**, of related monomers **4a**–**d** and **5**, and of reference drug Mx against human colon adenocarcinoma cell line (HT29) are tabulated in Table 1. The results indicate that compounds **9f** and **13b**,**c** possess excellent antiproliferative activity, with IC_{50} values in the low/sub nM range, being remarkably more potent than Mx itself.

The following remarks can be made:

(i) Regarding the linker: (a) In the unsubstituted (X = H) subseries **9a**–**d** with four different linkers, **9b**, with Y = -$(CH_2)_3N(CH_3)(CH_2)_3$-, appears to be the most active (IC_{50} in the high nM range); on the other hand, **9c**, does not inhibit the 50% cell growth even at highest concentration (10 μM) tested; **9a**,**d**, with IC_{50} in sub μM range, are in the middle. (b) Comparison of the homologous pairs (**8a**,**b**, **9a**,**b**, **9e**,**f**, **9g**,**h**, **9i**,**j**, **13a**,**b**, **13d**,**e**, **13f**,**g**, and **13h**,**i**) clearly indicates that the best results are always obtained with Y = -$(CH_2)_3N(CH_3)(CH_2)_3$- either at the 4,4′ or 1,1′ positions, according to what was observed for similar derivatives.[5] (c) Analysis of the linker position leads to contrasting results: in the pairs with X = H (**9a**,**b**, **9i**,**j**, **13a**,**b**, and **13h**,**i**, respectively), compounds **13** (linker at 1,1′) are more cytotoxic than **9** (linker at 4,4′). For the pairs with X = OMe or OH (**9e**,**f**, **9g**,**h**, **13d**,**e**, and **13f**,**g**, respectively), the opposite is observed (**9** more cytotoxic than **13**).

(ii) Regarding the side chains of subseries **9**, the IC_{50} comparison of related pairs **8a**,**b** and **9a**,**b** underlines the importance of the basic side chains in 1,1′ for the cytotoxic activity. The intermediate **8a**,**b**, without these side chains, are 6–11 times less active than corresponding target **9a**,**b**, bearing the side chains at 1,1′.

(iii) In respect to substituent groups at the 7,7′ positions, it can be noted that the nature of the substituents remarkably influences the activity, but with a different trend in subseries **9** and **13**. In subseries **9**, the cytotoxicity rank order of derivatives with the same linker is **9f** (X = OMe) ≫ **9b** (X = H) ≫ **9h** (X = OH)

Table 2. Percent Growth of Some NCI Cell Lines Exposed 48 h at Three Increasing Concentrations (10^{-8}, 10^{-6}, and 10^{-4} or 10^{-5} M) of Selected Compounds. The Negative Values Indicate the Percent of Cell Killed

	9b			**9f**			**13b**			**13c**		
cell line	10^{-8}	10^{-6}	10^{-4}	10^{-8}	10^{-6}	10^{-4}	10^{-8}	10^{-6}	10^{-5}	10^{-8}	10^{-6}	10^{-5}
leucemia: RPMI-8226	32	−23	−46	4	−36	−44	16	−37	−49	12	−44	−58
lung-NSC: NCI−H460	42	11	−86	9	−10	−90	12	−52	−90	28	−31	−95
colon: HCT-116	20	−14	−90	17	−23	−97	20	−35	−96	31	−5	−94
cns: SNB-19	44	4	−88	38	3	−85	38	−20	−55	42	−7	−96
melanoma: LOX IMVI	26	−23	−82	31	−2	−73	30	−65	−93	28	−40	−93
ovarian: OVCAR-4	19	−22	−97	38	−21	−96	46	−47	−74	67	16	−72
renal: 786−0	47	13	−88	28	1	−98	30	−26	−98	41	6	−95
prostate: DU-145	52	5	−100	25	−25	−77	11	−31	−72	55	−2	−93
breast: MCF7	20	−8	−78	18	0	−85	22	−6	−86	26	0	−86
mean of the cell lines	34	−6	−84	23	−13	−83	25	−35	−79	32	−28	−87

and **9a** (X = H) > **9e** (X = OMe) ≫ **9g** (X = OH). In subseries **13**, the cytotoxicity rank order of derivatives with the same linker is **13c** (X = NO_2) > **13b** (X = H) ≫ **13d** (X = OMe) ≫ **13g** (X = OH) and **13a** (X = H) ≫ **13d** (X = OMe) > **13g** (X = OH). In general, can be remarked that hydroxy derivatives are always the least cytotoxic target drugs, while **13c**, with X = NO_2, the linker at 1,1′ and Y = -$(CH_2)_3N(CH_3)(CH_2)_3$-, is the most active compound. Unfortunately, it was not possible synthesize the corresponding derivative for **9** group.

(iv) Regarding the chromophore: comparing the IC_{50} of **9a**,**b** and **13a**,**b** (9-acridone chromophore) with that of the corresponding **9i**,**j** and **13h**,**i** (acridine chromophore), the activity rank order of related pairs is **9a** > **9i**, **9b** > **9j**, **13a** ≅ **13h**, and **13b** ≫ **13i**. It is clear that in both the subseries **9** and **13** the best results are obtained with the "acridonic" chromophore, in contrast to that observed with related monomers **4a** and **5**. However, it should be noted that monomers **4a**−**d** generally possess antiproliferative activity less than or much less than that of related bis compounds with Y = -$(CH_2)N(CH_3)(CH_2)_3$-, but greater than that of related bis compounds with Y = -$(CH_2)_2N(CH_3)(CH_2)_2$-; instead, monomer **5** is more active than all the related bis **9i**,**j** and **13h**,**i**. A possible explanation can derive from the DNA binding behavior: the "acridonic" bis derivatives, more active than the corresponding monomers **4**, are also more DNA affinic; in the "acridinic" subseries, **9i**,**j** and **13h**,**i**, only **9j** is more DNA affinic than corresponding monomer **5**, but with a very remarkable AT preference, while **5** has a noticeable GC preference. So, the opposite binding site preference may influence the potency of cytotoxic activity.

Generally, there is no quantitative correlation between IC_{50} and K_{app} values. However, some generalizations can be made: (i) in the homologous pairs, the shortest linker, Y = -$(CH_2)_2N(CH_3)(CH_2)_2$-, always corresponds to an inferior cytotoxicity and DNA affinity in respect to the longest linker, Y = -$(CH_2)_3N(CH_3)$-$(CH_2)_3$-; (ii) compound **9c**, the weakest DNA ligand, is not cytotoxic; (iii) the most active derivatives, **9f** and **13b**,**c**, also possess very high K_{app} values (range $1-2 \times 10^8$); (iv) the hydrophilic hydroxy groups at 7,7′ lead to compounds, **9g**,**h** and **13f**,**g**, endowed with a good DNA affinity, but with a poor cytotoxicity, indicating that other factors, e.g., cellular uptake, may influence the activity in these cases.

Compounds **9b**,**f** and **13b**,**c** were selected for the National Cancer Institute (NCI) screening on 60 human tumor cell 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, we choose to describe in Table 2, in one of the possible formats, the antiproliferative activity of selected compounds against one cell line of each NCI subpanel and the mean of the activity on these nine cell lines. So, for each compound, we report the percent growth of some cell lines exposed 48 h at three different increasing concentrations (10^{-8}, 10^{-6}, and 10^{-4} or 10^{-5} M, respectively) of drug. Positive values represent the percent growth of each cell line in respect to the untreated control (100% growth) and give an indication of the cytostatic action. Negative values represent the percent of death cells in respect to the initial number and give an indication of the derivative's cell killing capacity. The data show that all the compounds explicate a strong cytostatic action (70−80% average of cell growth inhibition) at 10 nM concentration; the differences are not so marked as observed with the HT29 cell line at 144 h of drug exposure, the antiproliferative activity average being in the order: **9f** ≅ **13b** > **13c** ≅ **9b**. At 10^{-6} M concentration there is not only a general stop of cellular growth, but often it begins a significant reduction of the initial cell number (cell killing): the activity rank order is **13b** > **13c** > **9f** > **9b**, reflecting the rank observed with HT29 cell line. A very marked cell killing capacity (80−90%) is shown by **9b**,**f** at 10^{-4} M and by **13b**,**c** at 10^{-5} M. It is worth noting the potency and the broad spectrum of activity of all four compounds. Indeed, a COMPARE analysis[16] was performed with these compounds to check whether they resemble previously identified anticancer drugs. The results are similar, with **13b** and **13c** being more closely related to each other than the other two sets of compounds. However, the highest correlation (correlation coefficient 0.77) was found for **9f** with rapamycin, an antibiotic with immunosuppressant activity. Other very toxic immunosuppressant or antiblastic agents with various mechanisms of action were found with this analysis, consistent with the high cytotoxicity exhibited by this class of bis-acridine derivatives.

Conclusions

From the present study we can conclude the following: (i) Our expectation, that the outstanding biological response shown by the monomers **4** and **5** could be increased with bis derivatives **9** and **13**, was fulfilled for target compounds with "acridonic" chromophores. In fact, **9b**,**f**,**h** and **13b**,**c**,**e** exhibit enhanced cytotoxic activity and higher DNA affinity than corresponding monomers. (ii) The most potent compounds, **9b**,**f** and **13b**,**c**, represent new leads in the field of anticancer acridine derivatives, being endowed with excellent DNA affinity, a broad spectrum of activity, and remarkable cytotoxic potency. (iii) However, further studies, to better understand the mechanism of action of these bis derivatives, are needed.

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).

1,7-Bis(1-chloro-9-oxo-9,10-dihydroacridine-4-carbonyl)-4-methyl-1,4,7-triazaheptane (8a). Example of General Procedure for the Preparation of 8a−f. To a mixture of **6**[9] (0.5 g, 1.83 mmol) and triethylamine (0.26 mL, 1.83 mmol) in $CHCl_3$ (20 mL)was added a solution of ClCOOEt (0.26 mL, 1.83 mmol) in $CHCl_3$ (10 mL) dropwise at 0 °C. After stirring at room temperature for 1 h, the mixture was again cooled at 0 °C and N^2-methyldiethylentriamine (0.12 mL, 0.915 mmol) was added. The resulting mixture was stirred for 4 h at room temperature. The precipitate solid was filtered and washed with boiling MeOH (2 × 10 mL) to yield pure **8a** as hydrochloride salt: ^{1}H NMR (DMSO-d_6) δ 2.80−3.11 (m, 7H, CH_3 + 2 × CH_2), 3.53−3.75 (m, 4H, 2 × CH_2), 7.09 (d, 2H, ar), 7.15 (t, 2H, ar), 7.35 (d, 2H, ar), 7.52 (t, 2H, ar), 8.00−8.11 (m, 4H, ar), 9.20 (br t, 2H, 2 × C−NH, ex), 9.68 (br s, 1H, ex), 12.50 (s, 2H, 10-H + 10′-H, ex).

Derivatives **8b**−**f** were prepared in a similar manner from the suitable 1-chloroacridine-4-carboxylic acid and the appropriate amine.

1,7-Bis{1-[(2-(dimethylamino)ethyl)amino]-9-oxo-9,10-dihydroacridine-4-carbonyl}-4-methyl-1,4,7-triazaheptane (9a). Example of General Procedure for the Preparation of 9a−f. The hydrochloride salt of **8a** (0.15 g, 0.23 mmol) was refluxed in *N*,*N*-dimethylethylendiamine (2 mL) for 1 h. After cooling, 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 which was flash-chromatographed on silica gel column eluted with $CHCl_3$/MeOH/NH_3 (1:1:0.01 v/v) to give pure **9a**: ^{1}H NMR ($CDCl_3$) δ 2.31 (s, 12H, 4 × CH_3), 2.50 (s, 3H, CH_3), 2.60 (t, 4H, 2 × CH_2), 2.77−2.87 (m, 4H, 2 × CH_2), 3.54−3.65 (m, 4H, 2 × CH_2), 5.80 (d, 2H, ar), 6.78 (br t, 2H, 2 × CONH, ex), 7.10−7.21 (m, 4H, ar), 7.41−7.52 (m, 4H, ar), 8.28 (d, 2H, ar), 10.85 (br t, 2H, 2 × 1-NH, ex), 13.18 (s, 2H, 10-H + 10′-H, ex).

Derivatives **9b**−**f** were prepared in a similar manner from the corresponding intermediates **8b**−**f**.

1,7-Bis{1-[(2-(dimethylamino)ethyl)amino]-7-hydroxy-9-oxo-9,10-dihydroacridine-4-carbonyl}-4-methyl-1,4,7-triazaheptane (9g). Example of General Procedure for the Preparation of 9g,h. 9e (0.13 g, 0.16 mmol) was suspended in aqueous HBr 48% (3 mL) and refluxed for 1 h. The mixture was cooled at room temperature and partitioned between $CHCl_3$ (2 × 20 mL) and an excess of 1 M aqueous Na_2CO_3 (20 mL). The organic layer was worked up to give a residue which was flash-chromatographed on silica gel column eluted with $CHCl_3$/MeOH/NH_3 (1:1:0.01 v/v) to give pure **9g**: ^{1}H NMR (DMSO-d_6) δ 2.23 (s, 12H, 4 × CH_3), 2.34 (s, 3H, CH_3), 2.51−2.74 (m, 8H, 4 × CH_2), 3.17−3.22 (m, 4H, 2 × CH_2), 3.42−3.46 (m, 4H, 2 × CH_2), 6.05 (d, 2H, ar), 7.16 (d, 2H, ar), 7.41−7.50 (m, 4H, ar), 7.89 (d, 2H, ar), 8.22 (br t, 2H, 2 × CONH, ex), 9.65 (br s, 2H, 2 × OH, ex), 10.83 (br t, 2H, 2 × 1-NH, ex), 13.62 (s, 2H, 10-H + 10′-H, ex).

Derivative **9h** was prepared in a similar manner from **9f**.

1,7-Bis{1-[(2-(dimethylamino)ethyl)amino]acridine-4-carbonyl}-4-methyl-1,4,7-triazaheptane (9i). Example of General Procedure for the Preparation of 9i,j. The hydrochloride salt of **9a** (0.35 g, 0.42 mmol) was refluxed in EtOH/H_2O (3:1, 30 mL). Portions of aluminum foil (0.35 g) were amalgamated in a solution of $HgCl_2$ (1.3 g) in EtOH (25 mL) and added to the above boiling solution over 30 min. The mixture was refluxed for other 30 min and then filtered and the solid washed with hot EtOH (20 mL). The filtrate was diluted with H_2O (50 mL), and $FeCl_3$ (1.2 g) was added. The resulting mixture was partitioned between $CHCl_3$ (3 × 30 mL) and an excess of 1 M aqueous Na_2CO_3 (30 mL). The organic layer was worked up to give a residue which was flash-chromatographed on silica gel column eluted with $CHCl_3$/MeOH/NH_3 (1:1:0.01 v/v) to give pure **9i**: ^{1}H NMR ($CDCl_3$) δ 2.46 (s, 12H, 4 × CH_3), 2.62 (s, 3H, CH_3), 2.75−2.90 (m, 4H, 2 × CH_2), 2.90−3.06 (m, 4H, 2 × CH_2), 3.30−3.44 (m, 4H, 2 × CH_2), 3.81−3.97 (m, 4H, 2 × CH_2), 5.74 (br t, 2H, 2 × CONH, ex), 6.27 (d, 2H, ar), 7.07 (t, 2H, ar), 7.32 (t, 2H, ar), 7.51 (d, 2H, ar), 7.71 (d, 2H, ar), 8.32 (s, 2H, ar), 8.50 (d, 2H, ar), 11.60 (s, 2H, 2 × 1-NH, ex).

Derivative **9j** was prepared in a similar manner from **9b**.

1,7-Bis{4-[*N*-(2-(dimethylamino)ethyl)carbamoyl]-9-oxo-9,10-dihydroacridin-1-yl}-4-methyl-1,4,7-triazaheptane (13a). Example of General Procedure for the Preparation of 13a−e. *N*[4]-[2-(Dimethylamino)ethyl]-1-chloro-9-oxo-9,10-dihydro-4-acridinecarboxamide[7] (**10**, 0.5 g, 1.45 mmol), N^2-methyldiethylentriamine (0.1 mL, 0.72 mmol), and triethylamine (0.5 mL) were stirred in 2-ethoxyethanol (10 mL) at 120 °C for 5 h. The resulting mixture was partitioned between $CHCl_3$ (2 × 30 mL) and an excess of 1 M aqueous Na_2CO_3 (30 mL). The organic layer was worked up to give a residue which was flash-chromatographed on a silica gel column eluted with $CHCl_3$/MeOH/NH_3 (1:1:0.01 v/v) to give pure **13a**: ^{1}H NMR ($CDCl_3$) δ 2.32 (s, 12H, 4 × CH_3), 2.48 (s, 3H, CH_3), 2.59 (t, 4H, 2 × CH_2), 2.83 (t, 4H, 2 × CH_2), 3.34−3.44 (m, 4H, 2 × CH_2), 3.45−3.55 (m, 4H, 2 × CH_2), 6.16 (d, 2H, ar), 6.88 (br t, 2H, 2 × CONH, ex), 7.10 (7, 2H, ar), 7.25 (d, 2H, ar), 7.49−7.60 (m, 4H, ar), 8.20 (d, 2H, ar), 10.97 (br t, 2H, 2 × 1-NH, ex), 13.20 (s, 2H, 10-H + 10′-H, ex).

Derivative **13b**−**e** were prepared in a similar manner from the appropriate acridine-4-carboxamide and the suitable triamine.

1,7-Bis{4-[*N*-(2-(dimethylamino)ethyl)carbamoyl]-7-hydroxy-9-oxo-9,10-dihydroacridin-1-yl}-4-methyl-1,4,7-triazaheptane (13f). Example of General Procedure for the Preparation of 13f,g. 13d (0.28 g, 0.35 mmol) was suspended in aqueous HBr 48% (3 mL) and refluxed for 1 h. The mixture was cooled at room temperature and partitioned between $CHCl_3$ (2 × 20 mL) and an excess of 1 M aqueous Na_2CO_3 (20 mL). The organic layer was worked up to give a residue which was flash-chromatographed on silica gel column eluted with $CHCl_3$/MeOH/NH_3 (1:1:0.01 v/v) to give pure **13f**: ^{1}H NMR (DMSO-d_6) δ 2.25 (s, 12H, 4 × CH_3), 2.38−2.50 (m, 7H, CH_3 + 2 × CH_2), 2.75−2.93 (m, 4H, 2 × CH_2), 3.32−3.77 (m, 8H, 4 × CH_2), 6.31 (d, 2H, ar), 7.10 (d, 2H, ar), 7.41−7.54 (m, 4H, ar), 7.98 (d, 2H, ar), 8.30 (br t, 2H, 2 × CONH, ex), 9.64 (br s, 2H, 2 × OH, ex), 10.96 (br t, 2H, 2 × 1-NH, ex), 13.69 (s, 2H, 10-H + 10′-H, ex).

Derivative **13g** was prepared in a similar manner from **13e**.

1,7-Bis{4-[*N*-(2-(dimethylamino)ethyl)carbamoyl]acridin-1-yl}-4-methyl-1,4,7-triazaheptane (13h). Example of General Procedure for the Preparation of 13h,i. The hydrochloride salt of **13a** (0.19 g, 0.26 mmol) was refluxed in EtOH/H_2O (3:1, 15 mL). Portions of aluminum foil (0.19 g) were amalgamated in a solution of $HgCl_2$ (0.76 g) in EtOH (18 mL) and added to the above boiling solution over 30 min. The mixture was refluxed for other 30 min and then filtered and the solid washed with hot EtOH (20 mL). The filtrate was diluted with H_2O (40 mL), and $FeCl_3$ (0.6 g) was added. The resulting mixture was partitioned between $CHCl_3$ (3 × 30 mL) and an excess of 1 M aqueous Na_2CO_3 (30 mL). The organic layer was worked up to give a residue which was flash-chromatographed on silica gel column eluted with $CHCl_3$/$MeOH/NH_3$ (1:1:0.01 v/v) to give pure **13h**: ^1H NMR ($CDCl_3$) δ 2.45 (s, 12H, 4 × CH_3), 2.57 (s, 3H, CH_3), 2.70 (t, 4H, 2 × CH_2), 2.90−3.02 (m, 4H, 2 × CH_2), 3.40−3.53 (m, 4H, 2 × CH_2), 3.68−3.82 (m, 4H, 2 × CH_2), 5.80 (br t, 2H, 2 × CONH, ex), 6.48 (d, 2H, ar), 6.97−7.17 (m, 4H, ar), 7.58 (t, 2H, ar), 7.78 (d, 2H, ar), 8.28 (s, 2H, ar), 8.73 (d, 2H, ar), 11.65 (br t, 2H, 2 × 1-NH, ex).

Derivative **13i** was prepared in a similar manner from **13b**.

Biophysical Evaluation. 1. DNA Unwinding. To test the drug's ability to unwind DNA, we performed a direct assay employing negative supercoiled pBR322 and PPAC as control. Plasmid (0.25 μg) 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 on a 1% agarose gel in Tris-borate 50 mM, EDTA 1 mM, and run at 5 V/cm for 4 h. The gel was then stained with ethidium bromide to visualize the change in plasmid mobility upon complex formation.

2. Fluorescence Binding Studies. The fluorometric assays have been described previously.[13] The C_{50} values for ethidium displacement from CT-DNA and from synthetic [poly-(dA-dT)]$_2$ (AT) and [poly(dG-dC)]$_2$ (GC) oligonucleotides 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, AT, and GC, respectively.[13,14]

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 which reduce the fluorescence of the DNA-bound ethidium by 50% and are calculated as the mean from three determinations.

3. 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.[17−19] Drug solutions of appropriate concentration were added to a culture containing HT29 cells at 2.5 × 10^4 cells/mL of medium[17] or to a culture containing LoVo or LoVo/Dx cells at 2.5 × 10^5 cells/mL of medium.[18] All assays were performed in triplicate.

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

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JM030820X