Rational Design, Synthesis, and Biological Evaluation of Bis(pyrimido[5,6,1-*de*]acridines) and Bis(pyrazolo[3,4,5-*kl*]acridine-5-carboxamides) as New Anticancer Agents

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Received April 20, 2004

The good results obtained with pyrimido[5,6,1-*de*]acridines **7** and with pyrazolo[3,4,5-*kl*]-acridinecarboxamides **8** prompted us to the synthesis of two new series of bis acridine derivatives: the bis(pyrimidoacridines) **5** and the bis(pyrazoloacridinecarboxamides) **6**. Compounds **5** can be regarded also as cyclized derivatives of bis(acridine-4-carboxamides) **3** and compounds **6** as cyclized derivatives of bis(acridine-4-carboxamides) **4**. The noncovalent DNA-binding properties of these compounds have been examined using fluorometric techniques. The results indicate that (i) the target compounds are excellent DNA ligands; (ii) the bis derivatives **5** and **6** are more DNA-affinic than corresponding monomers **7** and **8**; (iii) the new bis **5** and **6** result always less efficient in binding than related bis(acridine-4-carboxamides) **3** and **4**; and (iv) in both series **5** and **6** a clear, remarkable in some cases, preference for binding to AT rich duplexes can be noted. 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. We could identify six very potent cytotoxic compounds for further in vitro studies: a cytotoxic screening against six human cancer cell lines and the National Cancer Institute (NCI) screening on 60 human tumor cell lines. Finally, compound **6a** was selected for evaluation in a NCI in vivo hollow fiber assay.

Introduction

In past years, interest in symmetric bifunctional intercalators has been growing and a number of derivatives, synthesized employing different chromophores, have been studied.[1−10] The noticeable results in the field may be exemplified by LU 79553 (**1**, Figure 1) and WMC-26 (**2**, Figure 1), both showing high effectiveness against tumor xenografts in vivo.[6,7] We have also described the synthesis and the biological properties of two novel interesting classes of antitumor agents belonging to this type of derivative: the bis(acridine-4-carboxamides) **3** and **4** (Figure 2).[11] From them, **3a** [X = H, Y = $(CH_2)_3N(Me)(CH_2)_3$], **3d** [X = NO_2, Y = $(CH_2)_3N(Me)(CH_2)_3$], **4a** [X = H, Y = $(CH_2)_3N(Me)$-$(CH_2)_3$], and **4b** [X = OMe, Y = $(CH_2)_3N(Me)(CH_2)_3$] emerged as lead derivatives.[11] Compounds **3a**,**d** and **4a**,**b** have fulfilled our purpose to enhance the outstanding biological response shown by the corresponding bisfunctionalized acridone-4-carboxamide monomers.[12] Prompted by the above results, we designed the synthesis of two new classes of potential bis intercalators: the bis(pyrimidoacridines) **5** and the bis(pyrazoloacridinecarboxamides) **6**, which can be regarded as cyclized derivatives of **3** and **4**, respectively (Figure 2). On the other hand, the chromophore moiety of **5** and **6** being constituted by the pyrimido[5,6,1-*de*]acridine-1,3,7-trione and by the pyrazolo[3,4,5-*kl*]acridine, respectively (Figure 2), we expected also an increase of the notable antitumor properties of the corresponding monomers **7**[13] and **8**.[14] Finally, we have better investigated the relevance of the linker Y for biological activity of these compounds.

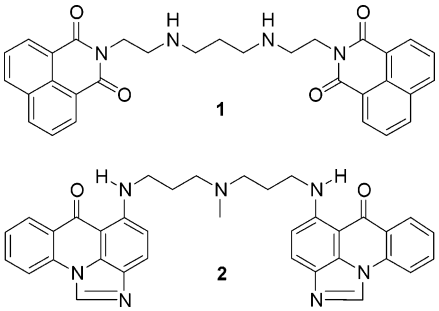

Figure 1. Structures of LU 79553 (**1**) and WMC-26 (**2**).

Chemistry

Schemes 1 and 2 show the synthetic pathways leading to target derivatives **5** and **6**. According to Scheme 1, the reaction of the suitable 6-chloro-2-[2-(dimethylamino)ethyl]pyrimido[5,6,1-*de*]acridine-1,3,7-trione (**7**)[13a,b] with the appropriate α,ω−diamine in ethoxyethanol, in the presence of triethylamine at 80 °C, afforded the target bis(pyrimidoacridines) **5a**−**j**. 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 **5g**, that was prepared according to the literature.[5] Cleavage with aqueous HBr of the methoxy derivatives **5h**,**i** gave the hydroxy derivatives **5k**,**l**, respectively. Target compound **5m** could not be prepared in the same way, due to the difficulty in obtaining the 9-nitro

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10.1021/jm049706k CCC: $27.50
Published on Web 09/11/2004

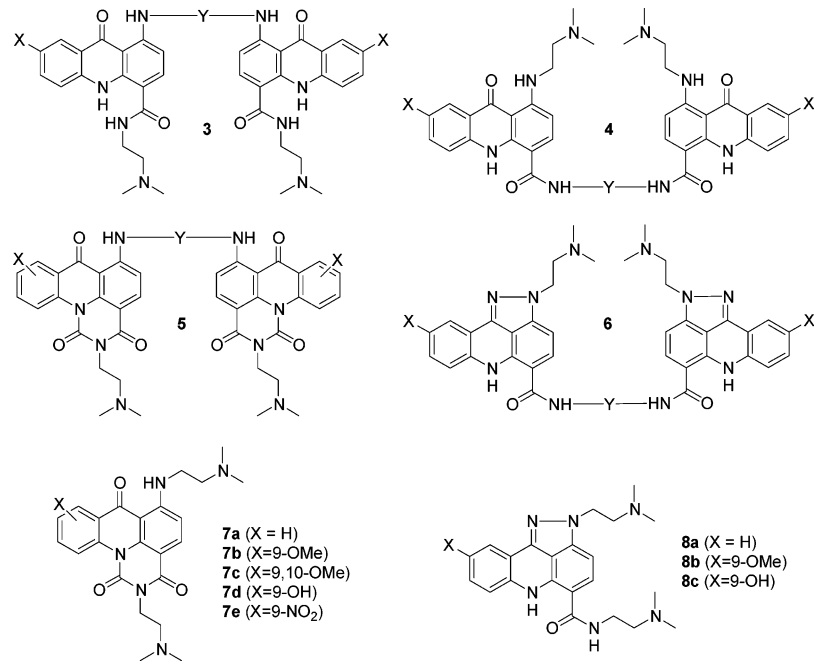
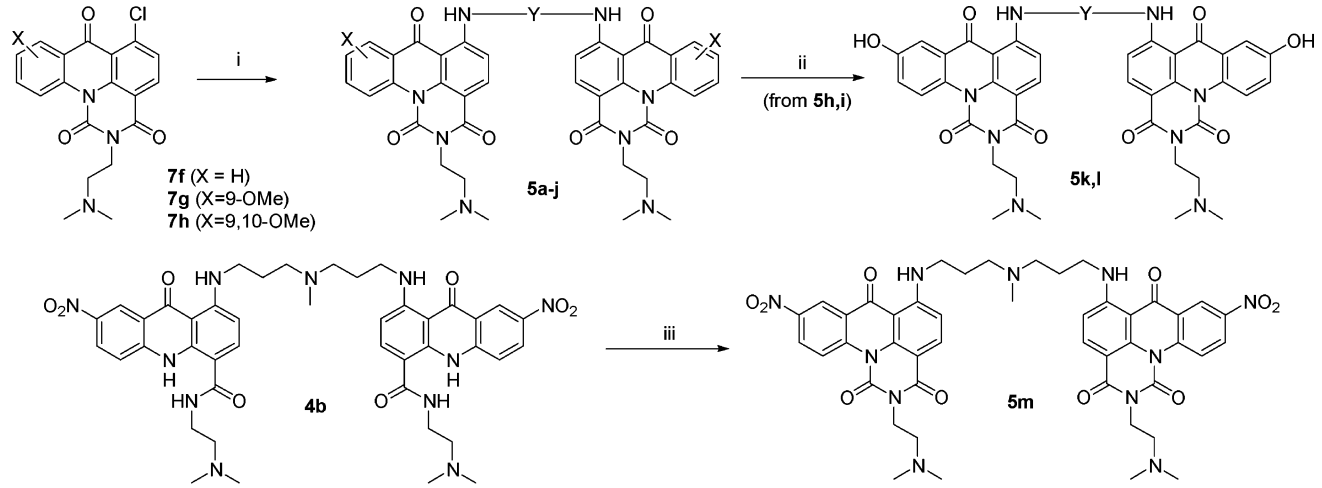


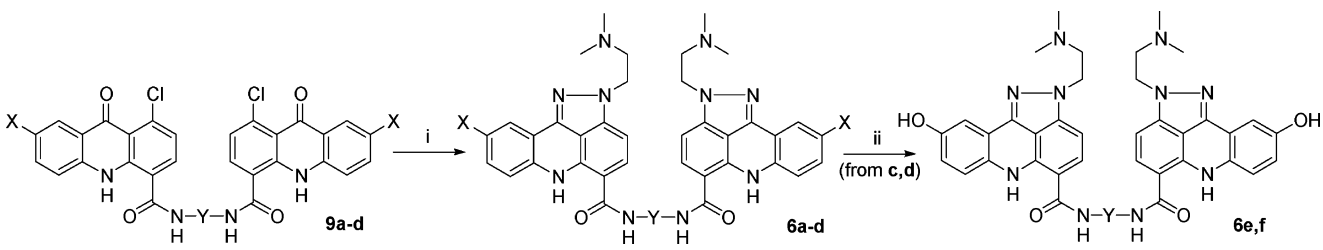
Figure 2. Rational design of **5** and **6** which can be regarded either as cyclized derivatives of the bis(acridine-4-carboxamides) **3** and **4** or as bis derivatives of the corresponding monomers **7** and **8**.

Scheme 1[a]



[a] Reagents: (i) $H_2N-Y-NH_2$, $N(Et)_3$; (ii) HBr 48%; (iii) $COCl_2$, $N(Et)_3$. Linker Y: $(CH_2)_3N(Me)(CH_2)_3$ for **5a**,**h**,**j**,**k**; $(CH_2)_2N(Me)(CH_2)_2$ for **5b**,**i**,**l**; $(CH_2)_3$ for **5c**; $(CH_2)_6$ for **5d**; $(CH_2)_8$ for **5e**; $(CH_2)_{12}$ for **5f**; $(CH_2)_2N(Me)(CH_2)_2N(Me)(CH_2)_2$ for **5g**. Substituents X: H for **5a**−**g**; 9,9′-OCH_3 for **5h**,**i**; 9,9′,10,10′-OCH_3 for **5j**.

Scheme 2[a]



[a] Reagents: (i) $H_2N-NH-(CH_2)_2N(CH_3)_2$; (ii) HBr 48%. Linker Y: $(CH_2)_3N(Me)(CH_2)_3$ for **6a**,**c**,**e**; $(CH_2)_2N(Me)(CH_2)_2$ for **6b**,**d**,**f**. Substituents X: H for **6a**,**b**; 9,9′-OMe for **6c**,**d**.

derivative of **7**.[13b] However, direct cyclization of **4b**, performed in $CHCl_3$ with $COCl_2$ and triethylamine at room temperature, afforded **5m**.

As shown in Scheme 2, the bis(pyrazolo[3,4,5-*kl*]-acridinecarboxamides) **6a**−**d** were prepared by reaction of the appropriate bis(acridine-4-carboxamides) **9a**−**d**[11] with [(2-hydrazino)ethyl]dimethylamine in 2-ethoxyethanol at 120 °C. The hydroxy derivatives **6e**,**f** were obtained by refluxing the corresponding methoxy derivatives **6c**,**d** in aqueous HBr.

Table 1. Melting Points,[a] Yields, Formula,[b] DNA Binding,[c] and Cytotoxic Activity against Human Colon Adenocarcinoma (HT29) of Target Compounds **5a**−**m** and **6a**−**f**, of Related Bis(acridine-4-carboxamides) **3** and **4**, of Corresponding Monomers **7** and **8**, and of Mitoxantrone (Mx)

compd	mp, °C	yield, %	formula	K_{app}[d] × 10^{-7} M^{-1}			binding site preference[e]	IC_{50} (nM)[f]
				AT	CT-DNA	GC		HT29
5a	143−144 (259−260 d)	59	$C_{45}H_{49}N_9O_6$	126	7.1	5.0	A−T (25)	<0.1
3a[h]				9.3	10	7.5	none	0.43
7a[i]				0.68	1.7	1.2	G−C (0.57)	22
5b	183−184 (275−277 d)	60	$C_{43}H_{45}N_9O_6$	4.0	2.1	0.94	A−T (4.3)	110
5c	222−224 (273−274)	50	$C_{41}H_{40}N_8O_6$	2.5	2.8	0.28	A−T (8.9)	390
5d	275−277 (239−241)	66	$C_{44}H_{46}N_8O_6$	8.2	3.3	0.27	A−T (30)	43
5e	242−244 (185−187)	64	$C_{46}H_{50}N_8O_6$	6.9	3.6	0.17	A−T (41)	360
5f	187−189 (254−256)	81	$C_{50}H_{58}N_8O_6$	0.49	0.36	0.041	A−T (12)	3500
5g	182−184 (>350)	29	$C_{46}H_{52}N_{10}O_6$	31	15	0.37	A−T (84)	390
5h	183−185 (258−259 d)	44	$C_{47}H_{53}N_9O_8$	7.1	2.1	3.4	A−T (2.1)	<0.1
3b[h]				26	6.2	14	A−T (1.9)	39
7b[i]				0.73	1.3	3.3	G−C (0.22)	67
5i	200−202 (253−254 d)	48	$C_{45}H_{49}N_9O_8$	0.90	0.34	0.88	none	720
5j	190−192 (240−242 d)	30	$C_{49}H_{57}N_9O_{10}$	11	6.2	2.4	A−T (4.6)	250
7c[i]				0.40	1.5	3.2	G−C (0.12)	370
5k	263−264 (265−267 d)	95	$C_{45}H_{49}N_9O_8$	13	8.5	7.8	A−T (1.7)	<0.1
3c[h]				13	12	7.4	A−T (1.8)	6900
7d[i]				0.79	3.5	3.2	G−C (0.25)	22
5l	279−280 (270−272 d)	97	$C_{43}H_{45}N_9O_8$	3.9	3.7	0.43	A−T (9.1)	390
5m	148−149 (262−263)	59	$C_{45}H_{47}N_{11}O_{10}$	26	14	11	A−T (2.4)	<0.1
3d[h]				19	14	23	none	<0.1
7e[i]				0.31	0.79	2.6	G−C (0.12)	310
6a	(>350)[g]	74	$C_{43}H_{51}N_{11}O_2{\cdot}3HCl{\cdot}3H_2O$	17	10	8.2	A−T (2.1)	3.0
4a[h]				8.2	6.3	0.79	A−T (10)	57
8a[j]				2.5	4.9	0.73	A−T (3.4)	210
6b	(230−231)[g]	34	$C_{41}H_{47}N_{11}O_2{\cdot}3HCl{\cdot}2H_2O$	20	23	5.2	A−T (4.3)	360
6c	(204−205)[g]	67	$C_{45}H_{55}N_{11}O_4{\cdot}3HCl{\cdot}3H_2O$	9.9	6.3	1.8	A−T (5.5)	3.0
4b[h]				13	20	5.2	A−T (2.5)	2.0
8b[j]				4.1	17.5	2.6	A−T (1.6)	120
6d	(245−247)[g]	60	$C_{43}H_{51}N_{11}O_4{\cdot}3HCl{\cdot}2H_2O$	5.0	1.7	1.8	A−T (2.8)	150
6e	239−240 (260−261)	40	$C_{43}H_{51}N_{11}O_4{\cdot}3HCl{\cdot}3H_2O$	25	4.3	19	A−T (1.3)	780
4c[h]				13	4.4	8.3	A−T (1.6)	800
8c[j]				9.7	3.4	1.9	A−T (5.1)	600
6f	282−283 (255−257)	39	$C_{41}H_{47}N_{11}O_4{\cdot}3HCl{\cdot}2H_2O$	8.5	4.9	3.0	A−T (2.8)	>10000
Mx					34			10

[a] In parentheses, hydrochlorides' melting points, d = decomposition. [b] Analyses for C, H, and N. [c] CT-DNA, AT, and GC refer to calf thymus DNA, $[poly(dA\text{-}dT)]_2$, and $[poly(dG\text{-}dC)]_2$, 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 the [AT]/[GC] ratio. [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 11. [i] Data from ref 13b. [j] Data from ref 14a.

All the target compounds **5** and **6** were examined as water-soluble hydrochloride salts, yielded by the usual methods, for their DNA-binding properties and their antineoplastic activity.

Results and Discussion

DNA-Binding Properties. As shown in Table 1, competitive displacement (C_{50}) fluorometric assays[15] with DNA-bound ethidium was used (a) to determine "apparent" equilibrium constants (K_{app}) for drug binding, as the C_{50} value is approximately inversely proportional to the binding constant,[16] and (b) to establish possible base- or sequence-preferential binding.[17] In the present study, fluorescence displacement assays were performed at pH 7 to enable comparison in biological conditions.

The K_{app} values of the new derivatives **5a**−**m** and **6a**−**f**, of related bis **3** and **4**, and of corresponding monomers **7** and **8** with CT-DNA, AT, and GC are reported in Table 1. The results indicate the target compounds to possess excellent DNA affinity, generally greater than ethidium, but lower than mitoxantrone (Mx). Some observations can be made about the CT-DNA K_{app} values. (a) Regarding the linker Y: in the unsubstituted sub series **5a**−**j**, we can observe the effect of the linker on binding; many K_{app} values are in the range $2-3 \times 10^7$, indicating that the nature of the linker is not decisive for binding; anyway, the weakest ligand is **5f** (Y = $(CH_2)_{12}$), with a K_{app} value of 0.36×10^7, indicating that the length and flexibility of the linker are detrimental for DNA binding; the best results are obtained with **5a**,**g** (Y = $(CH_2)_3N(CH_3)(CH_2)_3$ and Y = $(CH_2)_2N(CH_3)(CH_2)_2$, respectively), with corresponding K_{app} values of 7.1×10^7 and 15×10^7, suggesting that basic nitrogen atoms, protonated at pH 7, may increment the binding potency; considering the homologue pairs (Y = $(CH_2)_3N(CH_3)(CH_2)_3$ and Y = $(CH_2)_2N(CH_3)(CH_2)_2$, respectively), **5a** is 3.4 times more efficient than **5b**, and the same trend is confirmed for **5h**,**i**, **5k**,**l**, and **6c**,**d**; for the pairs **6a**,**b** and **6e**,**f**, the behavior is opposite. (b) Regarding the substituents X: in series **5** the rank in binding potency, for derivatives with Y = $(CH_2)_3N(CH_3)(CH_2)_3$, is 9,9′-NO_2 > 9,9′-OH > 9,9′-H >9,9′,10,10′-OMe > 9,9′-OMe, and, similarly, for derivatives with Y = $(CH_2)_2N(CH_3)(CH_2)_2$, it is 9,9′-OH > 9,9′-H > 9,9′-OMe; in series **6** the behavior is different; for derivatives with Y = $(CH_2)_3N(CH_3)(CH_2)_3$, the rank is 9,9′-H > 9,9′-OMe > 9,9′-OH, whereas, for derivatives

with Y = $(CH_2)_2N(CH_3)(CH_2)_2$, the rank is 9,9′-H > 9,9′-OH > 9,9′-OMe. (c) Regarding the chromophore: comparing the analogue pairs **5a**,**6a**, **5b**,**6b**, **5i**,**6d**, and **5l**,**6f**, the pyrazoloacridine chromophore seems more efficient than the pyrimidoacridone chromophore; only in the analogue pair **5k**,**6e** we have an opposite trend. (d) Regarding the comparison with monomers: the bis derivatives **5** and **6**, with Y = $(CH_2)_3N(CH_3)(CH_2)_3$, result always more efficient than corresponding monomers **7** and **8**, with the only exception of **6c** and **8b**. (e) Regarding the comparison with related bis(acridine-4-carboxamides): the new bis derivatives **5** and **6** result always less efficient than related bis(acridine-4-carboxamides) **3** and **4**, with the only exception of **6a** and **4a**.

Generally, the binding behavior of target compounds with synthetic polynucleotides reflects what we observed for CT-DNA. However, a clear, remarkable in some cases, preference for binding to AT rich duplexes is to be noted in both series **5** and **6**. In contrast, compounds related to bis derivatives **5** have previously shown a decided GC preference (monomers **7**) or a borderline AT preference (dimers **3**), whereas compounds **6** show a binding site preference very similar to that of related bis derivatives **4** and monomers **8**.

Cytotoxic Activity. The target compounds **5a**−**m** and **6a**−**f**, the related bis **3** and **4**, the corresponding monomers **7** and **8**, and the reference drug mitoxantrone (Mx) were examined for antiproliferative activity against the human colon adenocarcinoma cell line (HT29). The results shown in Table 1 indicate that compounds **5a**,**h**,**k**,**m** and **6a**,**c** possess very potent 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) sub series **5a**−**g**, with seven different linkers, **5a** (Y = $(CH_2)_3N(CH_3)(CH_2)_3$) appears to be the most potent derivative, with IC_{50} value < 0.1 nM (at least 3 orders of magnitude lowest in the sub series), but also **5d** (Y = $(CH_2)_6$) is very active (IC_{50} 43 nM); on the opposite site is **5f** (Y = $(CH_2)_{12}$) with the highest IC_{50} (3.5 μM); the other derivatives possess similar activity (IC_{50} range 0.11−0.39 μM). It seems that compounds **5a** and **5d** (Y = $(CH_2)_3N(CH_3)(CH_2)_3$ and Y = $(CH_2)_{12}$, respectively) ensure an optimal linker length, but also the presence of a basic nitrogen atom, compound **5a**, is important for cytotoxicity. (b) Comparison of the homologous pairs (**5a**,**b**, **5h**,**i**, **5k**,**l**, **6a**,**b**, **6c**,**d**, and **6e**,**f**) clearly indicates that the best results are always obtained with Y = $(CH_2)_3N(CH_3)(CH_2)_3$ for both series **5** and **6**, according to what is observed for related bis derivatives **3** and **4**.[11] The difference in potency is 3 orders of magnitude, at least, in series **5** and 2 orders of magnitude in series **6** for derivatives with Y = $(CH_2)_3N(CH_3)(CH_2)_3$.

(ii) Regarding the substituents X: (a) In the series **6** the cytotoxicity rank order of derivatives with the same linker is **6c**,**d** (X = 9,9′-OMe) ≅ **6a**,**b** (X = H) ≫ **6e**,**f** (X = 9,9′-OH). (b) The nature of substituents in 9,9′ positions in the series **5** does not influence the activitiy too much. In the sub series with Y = $(CH_2)_3N(CH_3)(CH_2)_3$, all the derivatives **5a**,**h**,**k**,**m**, possess very potent cytotoxicity (IC_{50} < 0.1 nM); in the sub series with Y = $(CH_2)_2N(CH_3)(CH_2)_2$, the derivatives **5b**,**i**,**l** possess IC_{50} in the range 0.11−0.72 μM, with a weak influence giving the rank order **5b** (X = H) > **5l** (X = OH) > **5i** (X = OMe). (c) The only derivative with substituents in 9,9′,-10,10′ positions, **5j** (Y = $(CH_2)_3N(CH_3)(CH_2)_3$, X = 9,9′,-10,10′-OMe), possesses IC_{50} = 0.25 μM, indicating that this kind of substitution is not detrimental for binding, but greatly diminishes cytotoxic potency.

(iii) Regarding the chromophore: Comparing IC_{50} values of the pairs with Y = $(CH_2)_3N(CH_3)(CH_2)_3$ and the same X, **5a**,**6a**, **5h**,**6c**, and **5k**,**6e**, it can be underlined that series **5** (pyrimido[5,6,1-*de*]acridine chromophore) is much more cytotoxic than series **6** (pyrazolo-[3,4,5-*kl*]acridine chromophore), especially considering the pair **5k**,**6e**. The results are in agreement with what is observed with the corresponding monomers **7** and **8**.

(iv) Finally, it should be noted that the target derivatives with Y = $(CH_2)_3N(CH_3)(CH_2)_3$, **5a**, **5h**, **5j**, **5k**, **5m**, **6a**, **6c**, and **6e**, are all more potent cytotoxic agents than the corresponding monomers **7** and **8**; they are more potent than or equally potent to related bis derivatives **3** and **4**.

Generally, there is not a great correlation between IC_{50} and K_{app} values. However, some considerations can be made: (a) In the homologous pairs of series **5**, the linker Y = $(CH_2)_2N(CH_3)(CH_2)_2$ corresponds always to an inferior cytotoxicity and DNA affinity with respect to the linker Y = $(CH_2)_3N(CH_3)(CH_2)_3$. (b) Also in the homologous pairs of series **6**, the shortest linker, Y = $(CH_2)_2N(CH_3)(CH_2)_2$, corresponds always to an inferior cytotoxicity with respect to the longest linker, Y = $(CH_2)_3N(CH_3)(CH_2)_3$, but for the DNA affinity the trend is different. (c) Compounds **5f**,**i**, the weakest DNA ligands, are scarcely cytotoxic, but also compounds **5g**,**6b**, the most potent DNA ligands, are scarcely cytotoxic; it can be deduced that DNA binding is not the only determinant for cytotoxic activity; other factors, e.g. cellular uptake, may influence the cytotoxicity.

Compounds **5a**,**h**,**k**,**m**, and **6a**,**c**, the most potent in the series, were selected for a cytotoxic screening against six human cancer cell lines (large cell lung carcinoma H460M, gastric cancer MKN45, prostatic carcinoma PC3, colon adenocarcinoma HCT116, LoVo, sensitive, and LoVo/Dx, doxorubicin-resistant). The IC_{50} (nM) values after 1 and 144 h drug exposure are reported in Table 2. Reference drugs are Mx and doxorubicin (Dx). The results indicate the following: (i) The target derivatives are extremely potent cytoxic agents, especially compounds **5**, which often present IC_{50} values inferior to the minimum drug concentration tested (10^{-3} nM). (ii) As previously noted with the HT29 cell line, compounds **5** are more potent than compounds **6**, but here we can also discriminate among the potency of selected derivatives **5**, in relation to the nature of substituents in 9,9′ positions. The rank order in potency seems to be **5k** (X = OH) > **5a** (X = H) > **5h** (X = OMe) > **5m** (X = NO_2). (iii) Finally, it can be remarked that both derivatives **5** and **6** are cross resistant with Dx, but the grade of cross resistance of compounds **5** appear to be inferior to that of derivatives **6**.

Compounds **5a**,**h**,**k**,**m**, and **6a**,**c**, were also selected by the National Cancer Institute (NCI) for a screening on a panel of 60 human tumor cell lines. This screen is designed to discover spectrum of activity and, eventually, selectivity of drugs. The data from this assay can

Table 2. Cytotoxic Screening against Six Human Cancer Cell Lines of Selected Compounds **5** and **6** after 1 h and 144 h of Drug Exposure[a]

	H460M		MKN45		PC3		HCT116		LoVo		LoVo/Dx[b]	
	1 h	144 h	1 h	144 h	1 h	144 h	1 h	144 h	1 h	144 h	1 h	144 h
5a	$<10^{-3}$	$<10^{-3}$	0.011	$<10^{-3}$	4.1	1.6	0.72	0.10	5.2	0.31	11 (2.1)	3.1 (10)
5h	0.066	$<10^{-3}$	0.057	0.0028	4.7	1.2	0.85	0.66	18	0.95	76 (4.2)	7.6 (8.0)
5k	$<10^{-3}$	$<10^{-3}$	$<10^{-3}$	$<10^{-3}$	2.0	0.011	0.060	$<10^{-3}$	98	3.9	810 (8.3)	59 (15)
5m	0.93	0.19	1.3	0.29	11	4.5	2.9	1.1	42	7.6	380 (9.0)	67 (8.8)
6a	7.2	4.4	6.8	0.60	12	6.0	12	19	150	1.1	3600 (24)	400 (360)
6c	26	4.3	51	13	>100	39	120	43	53	5.1	3100 (58)	290 (57)
Mx	0.85	0.75	6.8	12	75	7.0	110	5.8	12	3.3		
Dx									810	22	9100 (11)	2300 (100)

[a] Activity expressed as IC_{50} (nM). [b] In parentheses, RI = resistance index is the IC_{50} ratio of LoVo/Dx on LoVo.

Table 3. Percent Growth of Some NCI Cell Lines Exposed for 48 h at Three Increasing Concentrations (10^{-8}, 10^{-6}, and 10^{-5} M) of Selected Compounds[a]

	5a			**5h**			**5k**			**5m**			**6a**			**6c**		
cell line	10^{-8}	10^{-6}	10^{-5}	10^{-8}	10^{-6}	10^{-5}	10^{-8}	10^{-6}	10^{-5}	10^{-8}	10^{-6}	10^{-5}	10^{-8}	10^{-6}	10^{-5}	10^{-8}	10^{-6}	10^{-5}
leukemia: MOLT-4	9	−26	−71	6	−33	−62	−4	−37	−60	−2	−31	−50	28	7	−27	19	−28	−28
lung-NSC: NCI−H460	13	−22	−95	14	−20	−87	11	−23	−60	10	−36	−60	12	−6	−53	23	3	−58
colon: SW-620	26	−25	−89	30	−2	−49	36	10	−8	42	5	−16	37	14	−45	44	3	−39
CNS: SNB-19	32	7	−53	38	14	−48	34	−4	−17	37	−25	−66	40	−7	−31	47	0	−37
melanoma: LOX IMVI	25	−43	−82	26	−34	−91	20	−50	−77	16	−56	−69	22	5	−57	46	3	−80
ovarian: OVCAR-4	15	−53	−77	0	−37	−72	42	−37	−36	31	−54	−65	41	−17	−40	71	−5	−54
renal: 786−0	34	4	−100	37	10	−79	36	1	−52	33	−4	−63	30	5	−48	53	11	−33
prostate: DU-145	37	−13	−98	40	3	−66	32	−6	−42	35	−13	−83	25	−23	−40	70	−8	−21
breast: MCF7	31	0	−96	39	−3	−63	16	−1	−32	21	−2	−55	18	2	−35	66	7	−18
mean of the cell lines	25	−19	−85	26	−11	−69	25	−16	−43	25	−24	−59	28	−2	−42	49	−2	−41

[a] The negative values indicate the percent of cells killed.

be presented in several different formats.[18] Since it is not practical to report all experimental data available, we choose to describe in Table 3, in one of the possible formats, the antiproliferative activity of selected compounds against one cell line of each NCI sub panel and the mean of the activity on these nine cell lines. So, for each compound, the percent growth of the cell lines exposed for 48 h at three different increasing drug concentrations (10^{-8}, 10^{-6}, and 10^{-5} M, respectively) has been reported. Positive values represent the percent growth of each cell line with respect to the nontreated control (100% growth) and give an idea of the cytostatic action. Negative values represent the percent of cell deaths with respect to the initial number and give an idea of the derivative's cell killing capacity. The data at 10 nM show that all the compounds possess a strong cytostatic action, about 75% average of cell growth inhibition, except **6c**, which however has an average of 49%; compounds **5** demonstrate very similar cytostatic potency and are shown to be more cytostatic than compounds **6**. At 10^{-6} M not only is 100% of cellular growth inhibition achieved but also a significant reduction of the initial cell number often occurs (cell killing): the activity rank order is **5m** > **5a** > **5k** > **5h** > **6a** = **6c**. At 10^{-5} M a very marked cell killing capacity can be noted: the activity rank order is **5a** ≫ **5h** > **5m** ≫ **5k** ≅ **6a** ≅ **6c**. It is worth noting the potency and the broad spectrum of activity of all selected compounds.

Hollow Fiber Assay for Preliminary in Vivo Testing.[18] On the basis of these results, derivative **6a** was selected for preliminary in vivo testing. In this assay human tumor cells are cultivated in hollow fibers, which are implanted in mice. Some mice are treated with tested compound at various concentrations, whereas the control mice receive only the compound diluent. The fiber cultures are collected on the day following the last day of treatment, and the effect of antitumor activity of the tested compounds is calculated on the basis of the net increase in the tumor cell mass. Thus, the cytostatic and cytocidal capacities of the test compound can be assessed. The compound was tested against 12 human cancer cell lines. This represents a total of 4 experiments since each experiment contains three cell lines. The data are reported as % *T*/*C* for each of the two compound doses against each of the cell lines with separate values calculated for the intraperitoneal and subcutaneous samples.

A compound is selected for further in vivo testing in standard subcutaneous xenograft models on the basis of several hollow fiber assay criteria. These include (a) a % *T*/*C* of 50 or less in 10 of the 48 possible test combinations (12 cell lines × 2 sites × 2 compound doses); (b) activity at a distance (intraperitoneal drug/subcutaneous culture) in a minimum of 4 of the 24 possible combinations; and/or (c) a net cell kill of one or more cell lines in either implant site. To simplify evaluation, a points system has been adopted which allows rapid viewing of the activity of a given compound. For this, a value of 2 is assigned for each compound dose which results in a 50% or greater reduction in viable cell mass. The intraperitoneal and subcutaneous samples are scored separately so that criteria (a) and (b) can be evaluated. A compound with a combined ip + sc score ≥ 20, a sc score ≥ 8 or a net cell kill of one or more cell lines is referred for xenograft testing.

In Table 4 are presented ip score, sc score, ip + sc score, and net cell kill for compound **6a**. It can be seen that **6a** largely exceed the limits of the combined ip + sc score ≥ 20 and of the sc score ≥ 8, indicating an interesting preliminary in vivo activity worthy of in vivo testing in standard subcutaneous xenograft models.

Table 4. Results of the in Vivo Hollow Fiber Assay for **6a**[a]

ip score	40
sc score	10
total score	50
cell kill	N

[a] Ip = intraperitoneal implants, sc = subcutaneous implants, total score = ip + sc score, N = no cell kill.

Conclusions

From the present study we can conclude the following: (i) Our expectation to enhance the remarkable antitumor properties of monomers **7** and **8** was fulfilled with corresponding bis derivatives **5** and **6**. In fact, **5a**,**h**,**k**,**m**, and **6a**,**c** exhibit enhanced cytotoxic activity and higher DNA affinity than corresponding monomers. (ii) The bis derivatives **5** and **6** are also more potent cytotoxic agents than related bis **3** and **4**. (iii) It was established that the best linker for this kind of bis derivatives is for Y = $(CH_2)_3N(CH_3)(CH_2)_3$. (iv) The compounds **5a**,**k** and **6a**, being endowed of a remarkable DNA affinity, a broad spectrum of activity, an excellent cytotoxic potency, and a interesting preliminary in vivo activity (**6a**), represent good candidates for preclinical studies.

Experimental Section

Synthetic Chemistry. Melting points were determined on a Büchi 540 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 EA1108CHAZ-O elemental analyzer (Fisons Instruments).

1,9-Bis{2-[2-(dimethylamino)ethyl]-1,3,7-trioxo-2,3-dihydro-1*H*,7*H*-pyrimido[5,6,1-*de*]acridin-6-yl}-5-methyl-1,5,9-triazanonane (5a). Example of General Procedure for the Preparation of 5a−j. The 6-chloro-2-[2-(dimethylamino)ethyl]-2,3-dihydro-1*H*,7*H*-pyrimido[5,6,1-*de*]acridine-1,3,7-trione[13a] (**7f**, 0.3 g, 0.81 mmol), 5-methyl-1,5,9-triazanonane (0.07 mL, 0.405 mmol), and triethylamine (0.5 mL) were stirred in 2-ethoxyethanol (10 mL) at 80 °C for 2 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 chromatographed on a silica gel column eluted with $CHCl_3$/MeOH (4:1 v/v) to give pure **5a**: ^{1}H NMR ($CDCl_3$) δ 1.90−2.07 (m, 4H, 2 × CH_2), 2.32 (s, 3H, CH_3), 2.40 (s, 12H, 4 × CH_3), 2.60 (t, 4H, 2 × CH_2), 2.72 (t, 4H, 2 × CH_2), 3.25−3.39 (m, 4H, 2 × CH_2), 4.28 (t, 4H, 2 × CH_2), 6.43 (d, 2H, ar), 7.28 (t, 2H, ar), 7.53 (t, 2H, ar), 7.97 (d, 2H, ar), 8.21 (d, 2H, ar), 8.63 (d, 2H, ar), 10.75 (t, 2H, 2 × NH, ex).

Derivative **5b−j** were prepared in a similar manner from the appropriate 6-chloro-2-[2-(dimethylamino)ethyl]-2,3-dihydro-1*H*,7*H*-pyrimido[5,6,1-*de*]acridine-1,3,7-trione[13a,b] and the suitable $H_2N-Y-NH_2$.

1,9-Bis{2-[2-(dimethylamino)ethyl]-9-hydroxy-1,3,7-trioxo-2,3-dihydro-1*H*,7*H*-pyrimido[5,6,1-*de*]acridin-6-yl}-5-methyl-1,5,9-triazanonane (5k). Example of General Procedure for the Preparation of 5k,l and 6e,f. Compound **5h** (0.24 g, 0.28 mmol) was suspended in aqueous HBr 48% (3 mL) and refluxed for 1 h. The mixture was cooled at room temperature and partitioned between $CHCl_3$ (4 × 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 a silica gel column eluted first with $CHCl_3$/MeOH (1:1 v/v) and then with $CHCl_3$/MeOH (1:1 v/v) and 32% aqueous NH_3 (15 mL for 1 L of eluent) to give pure **5h**: ^{1}H NMR (DMSO-d_6) δ 1.79−1.98 (m, 4H, 2 × CH_2), 2.32 (s, 3H, CH_3), 2.36 (s, 12H, 4 × CH_3), 2.56−2.73 (m, 8H, 4 × CH_2), 3.22−3.52 (m, 4H, 2 × CH_2), 4.05−4.18 (m, 4H, 2 × CH_2), 6.50 (d, 2H, ar), 7.06 (d, 2H, ar), 7.43 (s, 2H, ar), 7.78 (d, 2H, ar), 8.48 (d, 2H, ar), 10.01 (b t, 2H, 2 × OH, ex), 10.67 (t, 2H, 2 × NH, ex).

Derivatives **5l** and **6e**,**f** were prepared in a similar manner from **5i** and **6c**,**d**, respectively.

1,9-Bis{2-[2-(dimethylamino)ethyl]-9-nitro-1,3,7-trioxo-2,3-dihydro-1*H*,7*H*-pyrimido[5,6,1-*de*]acridin-6-yl}-5-methyl-1,5,9-triazanonane (5m). To a mixture of **4b**[11] (0.23 g, 0.81 mmol) and triethylamine (0.75 mL) in $CHCl_3$ (10 mL) was added $COCl_2$ (20% in toluene, 0.7 mL, 1.4 mmol) in $CHCl_3$ (10 mL) dropwise with 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, which was chromatographed on a silica gel column eluted with $CHCl_3$/MeOH (1:1 v/v) to give pure **5m**: ^{1}H NMR ($CDCl_3$) δ 1.89−2.10 (m, 4H, 2 × CH_2), 2.36 (s, 15H, 5 × CH_3), 2.59−2.73 (m, 8H, 4 × CH_2), 3.32−3.48 (m, 4H, 2 × CH_2), 4.26 (t, 4H, 2 × CH_2), 6.50 (d, 2H, ar), 7.99 (d, 2H, ar), 8.28 (d, 2H, ar), 8.79 (d, 2H, ar), 8.96 (s, 2H, ar), 10.66 (t, 2H, 2 × NH, ex).

1,9-Bis{2-[2-(dimethylamino)ethyl]-2,6-dihydropyrazolo-[3,4,5-*kl*]acridine-5-carbonyl}-5-methyl-1,5,9-triazanonane·3HCl (6a). Example of General Procedure for the Preparation of 6a−d. The hydrochloride salt of **9a**[11] (0.25 g, 0.36 mmol) and [(2-hydrazino)ethyl]dimethylamine (0.37 g, 3.6 mmol) were stirred in 2-ethoxyethanol (10 mL) at 120 °C for 1 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 (1:1 v/v) and 32% aqueous NH_3 (10 mL for 1 L of eluent) to give pure **6a**, which was directly transformed to a hydrochloride salt and, as such, characterized: ^{1}H NMR (DMSO-d_6) δ 1.90−2.04 (m, 4H, 2 × CH_2), 2.73 (s, 3H, CH_3), 2.81 (s, 12H, 4 × CH_3), 2.97−3.27 (m, 4H, 2 × CH_2), 3.29−3.41 (m, 4H, 2 × CH_2), 3.50−3.63 (m, 4H, 2 × CH_2), 4.72 (t, 4H, 2 × CH_2), 6.82 (d, 2H, ar), 7.10 (t, 2H, ar), 7.32 (t, 2H, ar), 7.50 (d, 2H, ar), 7.72−7.87 (m, 4H, ar) 8.50−8.60 (m, 2H, 2 × CONH, ex), 10.55−10.83 (m, 5H, 2 × 6-H + 3 × N^+−H, ex).

Derivatives **6b−d** were prepared in a similar manner from the appropriate bis(acridine-4-carboxamide) **9**[11] and [(2-hydrazino)ethyl]dimethylamine.

Biophysical Evaluation. 1. Fluorescence Binding Studies. The fluorometric assays have been described previously.[15] The C_{50} values for ethidium displacement from CT-DNA and from synthetic $[poly(dA\text{-}dT)]_2$ (AT) and $[poly(dG\text{-}dC)]_2$ (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.[15,16]

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.

2. In Vitro Cytotoxicity. Cell Culturing. Human colon adenocarcinoma HT29 and HCT116 cell lines: The cells were cultured in D-MEM medium supplemented with 10% FBS, 2 mM glutamine, 100 units/mL penicillin, and 100 mg/mL streptomycin in a humidified atmosphere with 5% CO_2 at 37 °C. Human large cell lung carcinoma H460M, gastric cancer MKN45, and prostate carcinoma PC-3 cell lines: The cells were seeded into 75 cm^2 culture flasks in RPMI 1640 medium with supplemented with L-glutamine, 10% fetal calf serum, and penicillin-streptomycin (100 units/mg). Culturing details of human colon adenocarcinoma carcinoma LoVo sensitive and LoVo/Dx resistant cell lines have been previously described.[19]

Cell Growth Inhibition Assays. For the cytotoxicity evaluation of the compounds the 3-(4,5-dimethylthiazol-2-yl)-

2,5-diphenyl tetrazolium bromide (MTT) colorimetric assay was used.[20] Briefly, cells at appropriate densities, depending on the growth characteristics of particular cell lines, were seeded in 96 microwell plates and preincubated for 24 h. After this time, the cells were exposed to drugs and reincubated for 144 h (drug exposure time) at 37 °C in an atmosphere of 5% CO_2. The IC_{50} values were determined as previously described.[21] All assays were performed in duplicate.

NCI Assays.[18] The NCI uses the sulforhodamine B assay for assessing the cytotoxicity of test agents in their panel of 60 cell lines.[22] Briefly, cell lines were inoculated into a series of 96-well microtiter plates, with varied seeding densities depending on the growth characteristics of particular cell lines. Following a 24-h drug-free incubation, test agents were added routinely at five 10-fold dilutions with a maximum concentration of 10^{-4} M. After 2 days of drug exposure, the change in protein stain optical density allowed the inhibition of cell growth to be analyzed.

3. In Vivo Hollow Fiber Assay.[18] Human tumor cells were cultivated in polyvinylidene fluoride (PVDF) hollow fibers, and samples of the cells were implanted into two physiologic compartments of each mouse. The mice were treated by the intraperitoneal route with the analogues over time, the fibers were harvested the day following the last treatment, and the degree of cell kill compared to controls was measured by optical density. Each compound was tested at two different doses against a minimum of 12 human cancer cell lines.

Supporting Information Available: ^{1}H NMR data of target compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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JM049706K