Synthesis, Antitumor Cytotoxicity, and DNA-Binding of Novel *N*-5,2-Di(ω-aminoalkyl)-2,6-dihydropyrazolo[3,4,5-*kl*]acridine-5-carboxamides

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A series of DNA-binding potential antitumor agents bearing a cationic carboxamide side chain attached in position *peri* to an electron-withdrawing atom, *N*-5,2-di(ω-aminoalkyl)-2,6-dihydropyrazolo[3,4,5-*kl*]acridine-5-carboxamides, has been prepared by reaction of the appropriate 1-chloro-9-oxo-9,10-dihydro-4-acridinecarboxamides with the suitable (ω-aminoalkyl)-hydrazine. The noncovalent DNA-binding properties of these compounds have been examined using a fluorometric technique. 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. Two highly DNA-affinic and potent cytotoxic compounds, **4m**,**o**, have been identified as new leads in the antitumor strategies.

Introduction

DNA-intercalating antitumor drugs constitute an important class of derivatives in anticancer therapy. Significant examples include compounds in clinical use such as mitoxantrone[1] and doxorubicin[1] or in clinical trials such as the anthrapyrazoles,[2] the 5-nitropyrazoloacridines (**1**),[3] the acridine-4-carboxamides,[4] and the aza anthracenediones.[5] An interesting subclass of DNA-intercalating antitumor drugs is constituted by polycyclic heterocyclic carboxamides in which an important moiety is a basic carboxamide side chain attached to a terminal ring *peri* to an electron-withdrawing atom: among them the above-mentioned acridine-4-carboxamides,[4] the amsacrine-4-carboxamides,[6] the phenazine-1-carboxamides,[7] the fused tetracyclic quinoline and quinoxaline carboxamides,[8] the *N*-4-(ω-aminoalkyl)-1-[(ω-aminoalkyl)amino]acridine-4-carboxamides,[9] and the *N*-4-(ω-aminoalkyl)-1-[(ω-aminoalkyl)amino]acridone-4-carboxamides (**2**, **3**).[10] Previous results obtained by us with the acridone derivatives **2** and **3** pointed out the importance of the strong intramolecular hydrogen bond between the carbonyl in position 9 and the hydrogen of the amine in position 1 for biological activity. This bond is evidenced by the chemical shift values of the involved proton (δ range 9.80−11.21) and by the difficulty in exchanging with deuterium oxide.[10] Derivative **3**, in which the intramolecular hydrogen bond is not allowed, presents a marked drop in the cytotoxic activity and in the DNA binding strength in comparison with the related **2a** ($n = m = 2$; $R = R^1 = NMe_2$).[10] We postulate that this hydrogen bond can mimic an additional fourth ring which may be important for biological activity (Figure 1). Prompted by the above results and taking into account the noticeable anticancer properties of **2** and, especially, of **1**, we performed the synthesis of a new class of acridine derivatives: the pyrazolo[3,4,5-*kl*]acridine-5-carboxamides (**4a**−**z**), strictly related to **1** and **2**. Our aim was to verify (i) the effect on cytotoxic activity and DNA-binding of the additional pyrazole ring of compounds **4** compared to tricyclic derivatives **2** and (ii) if the substitution of the 5-nitro group in **1**, believed essential for antitumor activity, with a basic carboxamide side chain that will be in position *peri* to an electron-withdrawing atom could afford similar or enhanced biological properties.

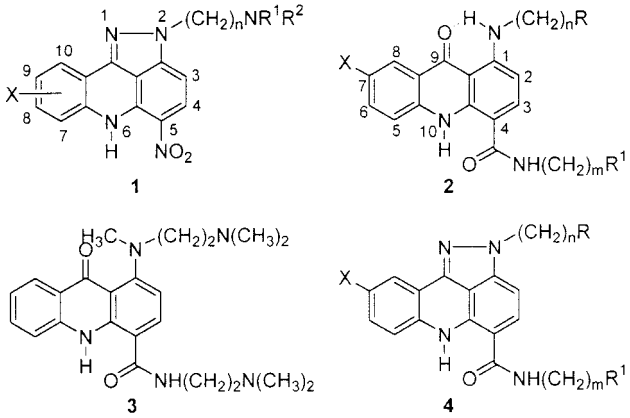

Figure 1. Structures and ring numbering for tetracyclic pyrazolo[3,4,5-*k*,*l*]acridines **1** and **4** and tricyclic acridone-4-carboxamides **2** and **3**.

In vitro cytotoxicity data against the human colon adenocarcinoma cell line HT29 and DNA-binding results, from fluorescence-based studies with calf thymus DNA and two polyoligonucleotide duplexes, are reported for these compounds.

Chemistry

Scheme 1 shows the synthetic pathway used. Condensation of *N*-4-(ω-aminoalkyl)-1-chloro-9(10*H*)-acridinone-4-carboxamides (**5a**−**g**)[10] with the appropriate (ω-aminoalkyl)hydrazine[11] in 2-ethoxyethanol at 120 °C afforded *N*-5,2-di(ω-aminoalkyl)-2,6-dihydropyrazolo-[3,4,5-*kl*]acridine-5-carboxamides **4a**−**r**; in the case of **5i**,[9] the reaction with *N*-(2-hydrazinoethyl)-*N*,*N*-dimethylamine[11] in similar conditions followed by hy-

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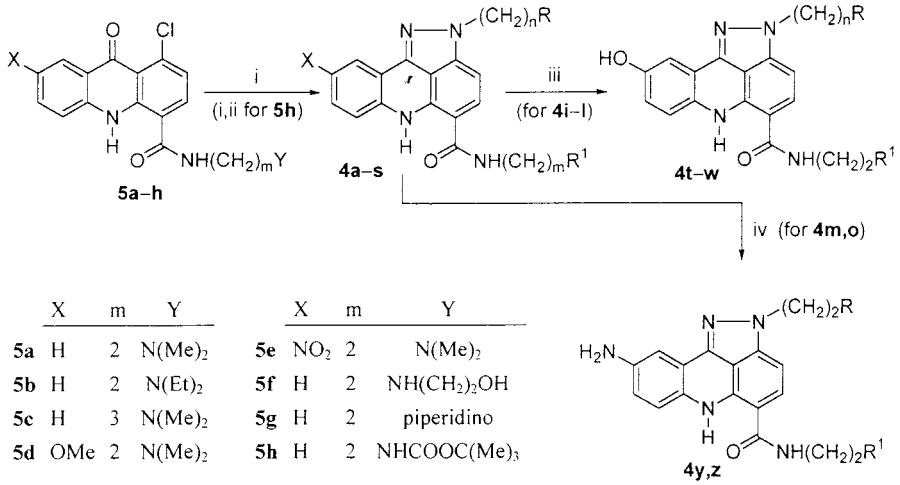
Scheme 1[a]



	X	m	Y
5a	H	2	$N(Me)_2$
5b	H	2	$N(Et)_2$
5c	H	3	$N(Me)_2$
5d	OMe	2	$N(Me)_2$

	X	m	Y
5e	NO_2	2	$N(Me)_2$
5f	H	2	$NH(CH_2)_2OH$
5g	H	2	piperidino
5h	H	2	$NHCOOC(Me)_3$

[a] Reagents: (i) $NH_2NH(CH_2)_nR$; (ii) HCl; (iii) HBr; (iv) H_2, Pd/C. Substituents of **4a**−**z** are reported in Table 1.

drolysis with aqueous HCl in dioxane at room temperature provided **4s**. Cleavage of **4i**−**l** with aqueous HBr gave the hydroxy derivatives **4t**−**w**. Reduction of nitro derivatives **4m**,**o** with hydrogen and palladium on activated carbon in acidic medium yielded **4y**,**z**.

To examine the in vitro antineoplastic activity and the DNA-binding properties of these agents, the free base forms of **4** were converted into their water-soluble hydrochlorides by the usual methods. This was not necessary for **4s**,**y**,**z** as they were directly isolated as hydrochlorides.

Results and Discussion

Cytotoxic Activity. In vitro cytotoxic potencies of target pyrazoloacridines **4a**−**z** and of reference drugs mitoxantrone (Mx) and doxorubicin (Dx) against human colon adenocarcinoma cell line (HT29) are reported in Table 1.

The results indicate that (a) **4m** emerges as the most potent among the new derivatives with an IC_{50} value of 3.9 nM; (b) all compounds **4** possess a good antiproliferative activity in the submicromolar range, with the exception of **4d**,**q**,**u** which are the least effective in the series, with values in the 1.7−2.3 μM range; (c) apart from **4m**, noticeable cytotoxic activity is also shown by **4o** with an IC_{50} value of 31 nM; (d) **4m** is more potent than Dx and than Mx itself, while **4o** is comparable with Dx. The data obtained allow us to formulate some structure−activity relationships for both side chains and substituents in position 9.

The following generalizations concerning the side chains can be deduced from the results: (i) the optimal distance between the two nitrogen atoms is two methylenes, as indicated by the decrease in potency between the pairs **4a**,**b**, **4a**,**c**, **4i**,**j**, and, especially, **4a**,**d** and **4t**,**u**; (ii) bulky substituents at the terminal nitrogen atoms (compounds **4e**−**h**) do not result in a decrease in cytotoxicity, particularly when the bulky group is only in the side chain in position 2 (**4f**,**g**); (iii) a unique substituent on the distal nitrogen of the carboxamido side chain, which parallels the side chains of mitoxantrone, (compounds **4p**−**r**) lowers the cytotoxicity, as evidenced from the values of IC_{50} of the pairs **4a**,**p**, **4g**,**r**, and, especially, **4f**,**q**; (iv) the complete absence of substituents on the terminal nitrogen atom of the carboxamido side chain (compound **4s**), which provides a side chain similar to that of the promising anticancer derivative BBR 2778,[5] preserves the cytotoxic potency; (v) in general, a dimethyl group seems to be the best substituent on the terminal nitrogen atoms of both the side chains, but in some cases a different substitution preserves (compare **4f**,**g**,**s** with **4a** or **4t** with **4w**) or increases (compare **4y** with **4z**) the level of cytotoxic activity. Generally, all these observations about the two side chains parallel what we already reported for related derivatives, with the exception of point iii.[9,10,12]

Substitution pattern in position 9 of the target compounds was found to be of relevant interest: (i) A nitro group leads to the most active compounds (**4m**,**o**) with a difference of 1 order of magnitude in activity among the components of this subclass (**4m**−**o**), the potency of **4m** being ≅10 times that of **4o** which is ≅10 times that of **4n**. This behavior was not observed with related derivatives.[10,12] (ii) A methoxy substituent leads to contrasting results with **4i** showing the highest activity in the subclass **4i**−**l** and being more active than the corresponding unsubstituted **4a**, while the other methoxy derivatives **4j**−**l** are less active than the corresponding unsubstituted **4b**,**f**,**g**, as in similar compounds.[9,10,12] (iii) A more polar hydroxy group, subclass **4t**−**w**, produces a general reduction in cytotoxicity in comparison to either parent derivatives **4i**−**l** or corresponding unsubstituted derivatives **4a**,**b**,**f**,**g**. This observation is in agreement with the similar ones regarding related tricyclic derivatives,[9,10] but in contrast to what was observed in related tetracyclic derivatives.[12] (iv) A 9-amino group, compounds **4y**,**z**, results in a drastic drop of cytotoxicity compared with the parent 9-nitro derivatives, especially in the case of **4y** (230 times less active than the parent **4m**), again in contrast with that observed in related tetracyclic derivatives.[12]

DNA-Binding Properties. Competitive displacement (C_{50}) fluorometric assays with DNA-bound ethidium can be used[13] (a) to determine '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 in

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

Compd	R	R^1	m	n	X	mp, °C[c]	yield, %	IC_{50}[d] HT29	K_{app}[e] × 10^{-7} M^{-1} CT-DNA	AT	GC	binding site preference[f]
4a	$N(Me)_2$	$N(Me)_2$	2	2	H	211-213	53	0.21	4.85	2.45	0.727	A–T (3.4)
4b	$N(Me)_2$	$N(Me)_2$	2	3	H	203-206	79	0.25	5.04	1.62	0.282	A–T (12)
4c	$N(Me)_2$	$N(Me)_2$	3	2	H	210-212	88	0.60	2.68	2.07	0.123	A–T (17)
4d	$N(Me)_2$	$N(Me)_2$	3	3	H	125-128	89	2.3	2.74	3.8	0.94	A–T (4.0)
4e	$N(Et)_2$	$N(Et)_2$	2	2	H	170-171	41	0.41	4.34	9.3	3.90	A–T (2.4)
4f		$N(Me)_2$	2	2	H	160-162	40	0.19	1.49	3.10	0.382	A–T (8.1)
4g		$N(Me)_2$	2	2	H	145-148	83	0.21	20.3	10.2	3.70	A–T (2.8)
4h			2	2	H	140-142	40	0.45	4.19	11.3	4.80	A–T (2.4)
4i	$N(Me)_2$	$N(Me)_2$	2	2	OMe	239-241	70	0.12	17.5	4.14	2.61	A–T (1.6)
4j	$N(Me)_2$	$N(Me)_2$	2	3	OMe	182-184	71	0.49	2.93	5.00	1.47	A–T (3.4)
4k		$N(Me)_2$	2	2	OMe	195-197	80	0.40	3.50	3.20	3.40	none
4l		$N(Me)_2$	2	2	OMe	205-207	68	0.26	1.54	17.3	5.70	A–T (3.0)
4m	$N(Me)_2$	$N(Me)_2$	2	2	NO_2	> 300	45	0.0039	142	6.30	1260	G–C (200)
4n		$N(Me)_2$	2	2	NO_2	249-251	32	0.36	11.0	37.1	15.7	A–T (2.4)
4o		$N(Me)_2$	2	2	NO_2	> 300	61	0.031	96.9	135	2420	G–C (18)
4p	$N(Me)_2$	$NH(CH_2)_2OH$	2	2	H	100-102	46	0.56	252	10.5	74.1	G–C (7.1)
4q		$NH(CH_2)_2OH$	2	2	H	123-125	88	1.7	3.02	6.66	6.10	none
4r		$NH(CH_2)_2OH$	2	2	H	110-112	45	0.56	15.0	9.1	14.6	G–C (1.6)
4s	$N(Me)_2$	NH_2	2	2	H	221-223	50	0.30	9.7	5.20	1.56	A–T (3.3)
4t	$N(Me)_2$	$N(Me)_2$	2	2	OH	191-193	50	0.60	3.39	9.70	1.86	A–T (5.2)
4u	$N(Me)_2$	$N(Me)_2$	2	3	OH	185-187	66	1.8	7.88	1.80	0.470	A–T (3.8)
4v		$N(Me)_2$	2	2	OH	235-238	56	0.91	12.6	7.98	0.120	A–T (66)
4w		$N(Me)_2$	2	2	OH	248-250	37	0.63	14.3	7.9	28.6	G–C (3.6)
4y	$N(Me)_2$	$N(Me)_2$	2	2	NH_2	250-252	76	0.91	28.0	5.9	17.0	G–C (2.9)
4z		$N(Me)_2$	2	2	NH_2	236-238	59	0.17	3.24	35.0	16.4	A–T (2.1)
Dx								0.026				
Mx								0.010	34.1[g]			

[a] CT-DNA, AT, and GC refer to calf thymus DNA, $[poly(dA\text{-}dT)]_2$, and $[poly(dG\text{-}dC)]_2$, respectively. [b] All the compounds were characterized and studied as salts (dihydrochlorides **4a**−**w**, trihydrochlorides **4y**,**z**). [c] All the melting points with decomposition. [d] Drug concentration (μM) required to inhibit cell growth by 50%. All assays were performed in triplicate. Human colon adenocarcinoma cell line = HT29. [e] $K_{app} = 1.26/C_{50} \times 10^7$ 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. [f] The binding site preference is considered to be significant only for [GC]/[AT] or [AT]/[GC] ratio differing by >30% from the sequence-neutral unity value (i.e., <0.7 or >1.3). The values of the [GC]/[AT] or [AT]/[GC] ratio are shown in parentheses. [g] Data from ref 10.

biological conditions. The K_{app} values of the acridine derivatives **4a**−**z**, calculated for CT-DNA, AT, and GC, are reported in Table 1. The results indicate that (a) the target compounds are excellent DNA ligands with greater affinity than ethidium and, in some cases, than Mx itself; (b) generally, the compounds **4** bind DNA more strongly than the related acridine derivatives previously studied by us;[9,10,12] (c) **4m**,**o** present exceptional binding affinity with GC; (d) almost all the derivatives show a marked, noticeable (in cases **4m**,**v**), but non-unequivocal, binding site preference. In fact, the majority of compounds **4** is AT-selective, but some substituents in position 9, such as nitro, hydroxy, or amino groups or the carboxamido side chain that mimics the Mx side chains, can reverse this selectivity.

There is not quantitative correspondence between binding with CT-DNA and in vitro potency. However, **4m**,**o**, the most cytotoxic derivatives, possess high values of K_{app}, but **4p**, with the highest value of K_{app}, is on the average of activity. It seems that the best results in cytotoxicity are obtained where high values of K_{app} with CT-DNA are combined with both very high values of K_{app} with GC and a very marked GC-selectivity (compounds **4m**,**o**).

Conclusions

The present study allows us to conclude the following: (i) The *N*-5,2-di[ω-(amino)alkyl]-2,6-dihydropyrazolo[3,4,5-*kl*]acridine-5-carboxamides (**4a**−**z**) constitute a new class of derivatives which possess potent cytotoxic

activity and relevant DNA-binding properties. (ii) In comparison with carboxamides **2**,[10] the addition of a pyrazole ring, in many cases, leads to an increase of in vitro activity and DNA-binding ability. (iii) The substitution in position 5 of the nitro group of **1**, believed essential for antitumor activity, with a basic carboxamide side chain that is in position *peri* to an electron-withdrawing atom seems to afford a similar potency in cytotoxicity, also if there are no data for a direct comparison.[3a] (iv) Finally, the pyrazolo[3,4,5-*kl*]acridine-5-carboxamides **4m**,**o** constitute new potential anticancer leads possessing cytotoxic activity in the nanomolar range against the human colon adenocarcinoma cell line HT29.

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).

***N*-5,2-Di[2-(dimethylamino)ethyl]-2,6-dihydropyrazolo-[3,4,5-*kl*]acridine-5-carboxamide (4a). Example of General Procedure for the Preparation of 4a−r.** Compound **5a**[10] (0.2 g, 0.58 mmol) and 2-(dimethylamino)ethylhydrazine[11] (0.3 g, 62.9 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 to room temperature and partitioned between $CHCl_3$ (2 × 40 mL) and an excess of 1 M aqueous Na_2CO_3 (50 mL). The organic layer was worked up to give a residue which was flash-chromatographed on silica gel column 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** (0.12 g) as a dense oil, which was directly converted into dihydrochloride by usual methods: ^{1}H NMR (DMSO-d_6) δ 2.80 (s, 12H, 4 × CH_3), 3.21−3.39 (m, 2H, CH_2), 3.52−3.80 (m, 4H, 2 × CH_2), 4.80 (t, 2H, CH_2), 6.90 (d, 1H, ar), 7.15 (t, 1H, ar), 7.40 (t, 1H, ar), 7.59 (d, 1H, ar), 7.84 (d, 1H, ar), 7.93 (d, 1H, ar), 8.80 (br t, 1H, CO-NH, ex), 10.70 (br s, 1H, ex), 10.72 (s, 1H, 6-H, ex), 10.90 (br s, 1H, ex). Anal. ($C_{22}H_{28}N_6O$·2HCl·H_2O) C, H, N.

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

***N*-5-(2-Aminoethyl)-2-[2-(dimethylamino)ethyl]-2,6-dihydropyrazolo[3,4,5-*kl*]acridine-5-carboxamide (4s).** Compound **5h**[9] (0.2 g, 0.58 mmol) and 2-(dimethylamino)ethylhydrazine[11] (0.3 g, 62.9 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 × 40 mL) and an excess of 1 M aqueous Na_2CO_3 (50 mL). The organic layer was worked up to give a residue which was flash-chromatographed on silica gel column eluted with $CHCl_3$/MeOH (9:1 v/v) to obtain a dense oil. This oil was diluted in dioxane (20 mL) and 37% HCl (2 mL) and stirred for 2 h at room temperature. The reaction mixture was evaporated to give a residue which was crystallized from EtOH to yield pure **4s**·2HCl: ^{1}H NMR (DMSO-d_6) δ 2.85 (s, 6H, 2 × CH_3), 2.94−3.15 (m, 2H, CH_2), 3.50−3.70 (m, 4H, 2 × CH_2), 4.80 (t, 2H, CH_2), 6.90 (d, 1H, ar), 7.18 (t, 1H, ar), 7.40 (t, 1H, ar), 7.58 (d, 1H, ar), 7.80−7.96 (m, 2H, ar), 8.20 (br s; 3H, NH_3^+, ex), 8.65 (br t, 1H, CO-NH, ex), 10.63 (br s, 2H, ex), 10.72 (s, 1H, 6-H, ex). Anal. ($C_{20}H_{24}N_6O$·2HCl·2H_2O) C, H, N.

***N*-5,2-Di[2-(dimethylamino)ethyl]-9-hydroxy-2,6-dihydropyrazolo[3,4,5-*kl*]acridine-5-carboxamide (4t). Example of General Procedure for the Preparation of 4t−w.** Compound **4i** (0.25 g, 0.59 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 which was flash-chromatographed on 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 afford **4t**, which was directly converted into dihydrochloride by usual methods: ^{1}H NMR (DMSO-d_6) δ 2.88 (s, 12H, 4 × CH_3), 3.15−3.34 (m, 2H, CH_2), 3.50−3.71 (m, 4H, 2 × CH_2), 4.77 (t, 2H, CH_2), 6.76 (d, 1H, ar), 6.86 (d, 1H, ar), 7.20 (s, 1H, ar), 7.40 (d, 1H, ar), 7.85 (d, 1H, ar), 8.65 (br t, 1H, CO-NH, ex), 10.48−10.67 (m, 2H, ex), 10.72 (s, 1H, 6-H, ex). Anal. ($C_{22}H_{28}N_6O_2$·2HCl·2H_2O) C, H, N.

Derivatives **4u**−**w** were prepared in a similar manner.

***N*-5,2-Di[2-(dimethylamino)ethyl]-9-amino-2,6-dihydropyrazolo[3,4,5-*kl*]acridine-5-carboxamide (4y). Example of General Procedure for the Preparation of 4y,z.** A mixture of the 9-nitro derivative **4m** (0.2 g, 0.46 mmol), Pd/C (0.2 g, 5%), and aqueous HCl (1 mL of 37% w/w) in MeOH (30 mL) was stirred under hydrogen atmosphere (30 psi) for 1 h at room temperature. The reaction mixture was filtered then evaporated to yield a residue which was treated with boiling EtOH (10 mL). The mixture was cooled to room temperature, filtered, and washed with ether to give **4y**·3HCl: ^{1}H NMR (DMSO-d_6) δ 2.86 (s, 12H, 4 × CH_3), 3.21−3.39 (m, 2H, CH_2), 3.52−3.78 (m, 4H, 2 × CH_2), 4.84 (t, 2H, CH_2), 6.94 (d, 1H, ar), 7.38 (d, 1H, ar), 7.70 (d, 1H, ar), 7.83 (s, 1H, ar), 7.94 (d, 1H, ar), 8.80 (br t, 1H, CO-NH, ex), 10.25−10.90 (m, 6H, ex). Anal. ($C_{22}H_{29}N_7O$·2HCl·2.5H_2O) C, H, N.

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

Biophysical Evaluation. 1. 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 reported as the mean from three determinations. Apparent equilibrium binding constants were calculated from the C_{50} values (in μM) using the following equation: $K_{app} = (1.26/C_{50}) \times K_{ethidium}$, and with a value of $K_{ethidium} = 10^7$ M^{-1} for ethidium bromide.[14a]

2. In Vitro Cytotoxicity. Human Colon Adenocarcinoma Experimental Protocol. Establishment details of human colon adenocarcinoma carcinoma cell line (HT29) have been previously described.[16] Drug solutions of appropriate concentration were added to a culture containing HT29 cells at 2.5 × 10^4 cells/mL of medium, and the drug exposure was protracted for 144 h. All assays were performed in triplicate, as previously described.[16]

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

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