Design and Synthesis of Modified Quinolones as Antitumoral Acridones[1]

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The bacterial topoisomerase II (DNA gyrase) and the mammalian topoisomerase II represent the cellular targets for quinolone antibacterials and a wide variety of anticancer drugs, respectively. In view of the mechanistic similarities and sequence homologies exhibited by the two enzymes, tentative efforts to selectively shift from an antibacterial to an antitumoral activity was made by synthesizing a series of modified tricyclic quinolones, in which the essential 3-carboxylic function is surrogated by phenolic OH and the classic C-6 fluorine atom is replaced by a NH_2 group. The resulting 7-amino-9-acridone derivatives were assayed for their antibacterial as well as cytotoxic activities. No antibacterial activity was found. On the other hand, many derivatives showed significant cytotoxic activity against both HL-60 and P388 leukemias and a wide panel of human and rodent solid tumor cells, derivatives **25** and **26** displaying the best overall antiproliferative activity. Against the LoVo cell line, derivative **25** exhibited higher cytotoxic effects than etoposide.

Introduction

DNA topoisomerases are a group of ubiquitous enzymes that are essential for cell survival and proliferation in both prokaryotic and eukaryotic organisms.[2] These enzymes catalyze the interconversion of different topological forms of DNA through concerted sequential DNA breaking−passing−resealing processes.[3] The indispensable nature of the enzyme makes it the target of choice for broad-spectrum antibacterial drugs and potent antitumor agents.[4] Quinolone antibacterials are potent broad-spectrum drugs that target the bacterial type II DNA topoisomerases (DNA gyrase and topoisomerase IV),[5] while several different classes of antitumor agents such as acridines, anthracyclines, ellipticines, and epipodophyllotoxins target the mammalian type II enzyme.[6] The principal action mechanism of all these agents is quite similar. These compounds actually act as cellular poisons, trapping the topoisomerase on DNA in a frozen enzyme−DNA cleavage complex that leads to the generation of cytotoxic events.[7]

In view of mechanistic similarities and sequence homologies in prokaryotes and eukaryotes, the possibility exists that some agents interact with both enzymes. It is generally recognized that quinolones act quite selectively since DNA gyrase is at least 2 orders of magnitude more sensitive than eukaryotic topoisomerase.[8] However, a number of quinolones, characterized by a 7-pyridinyl[9] or 7-(4-hydroxyphenyl)[10] group at the C-7 position, besides exhibiting an excellent antibacterial spectrum, are also reported to exhibit inhibitory mammalian topoisomerase II activity. Hence, they can be considered as potential antitumor agents. Other modified quinolones, such as tricyclic isothiazoloquinolones[11] and tetracyclic pyridophenoxazines,[12] are cytotoxic to mammalian cells and also inhibit DNA gyrase.

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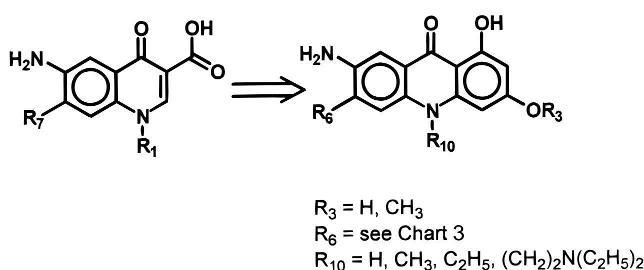
Chart 1



On the basis of these considerations and in connection with our research on quinolone structural manipulation,[13] we synthesized a series of acridones as modified quinolones in an attempt to shift the activity from antibacterial to antineoplastic. In the resulting 9-acridone derivatives (Chart 1), the 3-carboxylic function of the classical quinolone is surrogated by a phenolic OH and the C-6 fluorine atom, the basic structural feature of all new quinolones, is replaced by an NH_2 group which, as we recently discovered,[14] is a good fluorine replacer. In addition, typical substituents known to confer topoisomerase inhibitory activity were linked to the C-6 and N-10 positions of the acridone structure.

This drug design was strengthened by the observation that both tricyclic antibacterial isothiazoloquinolones[11] and natural acridine derivatives, acronycin[15] and glyfoline,[16] have a γ-ketooxidrylic system just as in our target compounds and are indicated as potential anticancer drugs. The same system can also be found in isoflavones such as ginestein and orobol,[17] which are known to inhibit mammalian DNA topoisomerase II (Chart 2).

In this paper, we furnish the experimental details of the synthesis of 7-amino-9-acridone derivatives, their antibacterial activity, their inhibitory activity of mam-

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Chart 2

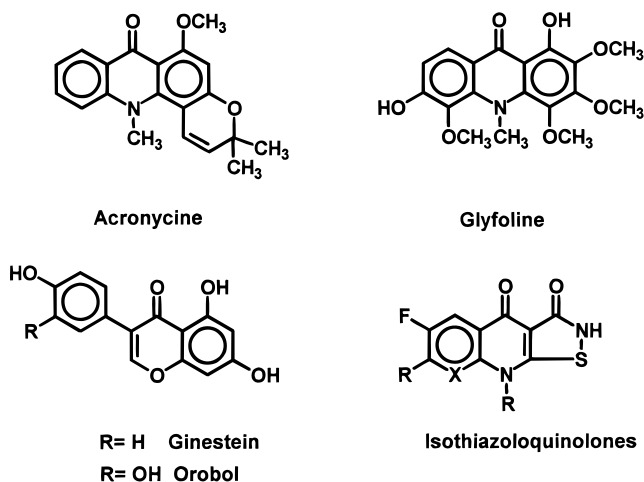
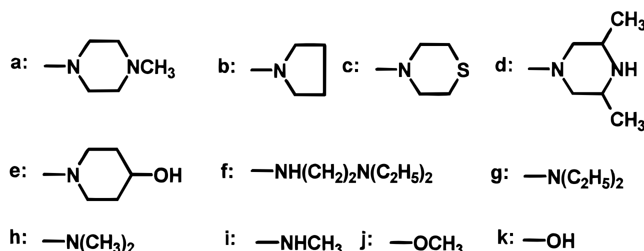


Chart 3. Side Chains Employed as the R_6 Substituent



malian topoisomerase II, a SAR study for antiproliferative in vitro and in vivo activity, and their cyclospecificity.

Chemistry

The 7-amino-9-acridone derivatives were prepared according to Scheme 1 and are listed in Table 1. Ullman reaction of 2,4-dichloro-5-nitrobenzoic acid (**1**) with 3,5-dimethoxyaniline gave diphenylamine carboxylic acid **2** which was cyclized to give the 7-nitro-9-acridone precursor **3**. The 7-amino-9-acridone target derivatives were obtained following four sequential steps: N-alkylation, nucleophilic substitution at C-6 with selected nucleophiles (see Chart 3), reduction of nitro group, and finally de-*O*-methylation. In particular, the alkylation of **3** with 2-(diethylamino)ethyl chloride was assisted by crown ether; the de-*O*-methylation with 48% HBr gave 1,3-dihydroxyacridones **15a, 17a, 19a**−**g**, and **20a**−**c**,**e**,**i**−**k** and 1-hydroxy-3-methoxyacridones **16a** and **18a**.

Direct reduction of 6-chloro derivatives **5** and **6** followed by complete or selective de-*O*-methylation afforded the 1,3-dihydroxy-6-chloro derivatives **24** and **26**, as well as the 1-hydroxy-3-methoxy-6-chloro derivatives **23** and **25**. Starting with 2,4-dichlorobenzoic acid and using the same procedure as that used to prepare compound **24**, it was possible to obtain compound **27,** as an example of a 7-desamino-9-acridone derivative (see Experimental Section).

Results and Discussion

A preliminary screening on a set of representative derivatives (**19a**−**c**, **20a**, **18a**, **24)** to evaluate antibacterial activity on five Gram-positive and eight Gram-negative bacteria ruled out any antibacterial activity (data not shown). These results are in agreement with those previously observed for other acridone analogues.[18] On the other hand, all tested compounds, when assayed for their ability to inhibit the growth of murine lymphocytic leukemia P388 and human promyelocytic leukemia HL-60 (Table 2), showed significant cytotoxic activity, except for **19b**,**c** and **27**. These results indicate that the structural manipulation made on the quinolone moiety shifted the activity from an antibacterial to an antitumor one. Moreover, some compounds demonstrated a remarkable cytotoxic activity, comparable to that of etoposide (a topoisomerase II inhibitor used as a clinically useful antitumor agent).

On the basis of the results reported in Table 2, some important structural features in relation to the cytotoxic activity have been observed. The presence of a substituent at N-10 position of the acridone moiety is crucial for cytotoxic activity. Indeed the N-unsubstituted **15a** is much less active when compared with the corresponding N-substituted analogues **17a**, **19a**, and **20a**. The presence of an amino group at the C-7 position seems to be crucial for the antitumor activity; in fact, the 7-desamino derivative **27** lacks any cytotoxic activity.

The head-to-head comparison between 1,3-dihydroxy-*N*-ethyl derivatives **19a**−**c**,**e** and **24** and the corresponding 1,3-dihydroxy-*N*-diethylaminoethyl derivatives **20a**−**c**,**e** and **26** suggests that the presence of a diethylaminoethyl side chain generally increases the cytotoxic activity of the compounds; for example, the ethyl derivative **19e** is 3−7 times less active than **20e**.

A comparison between the cytotoxic activity of 1,3-dimethoxy derivatives and their 1,3-dihydroxy analogues (**21** vs **24**, **22** vs **26**, **13a** vs **19a**, **14a** vs **20a**) shows that 1,3-dihydroxy-9-acridones are more active; the only exception was 1,3-dimethoxy derivative **14a** which was more cytotoxic than **20a**.

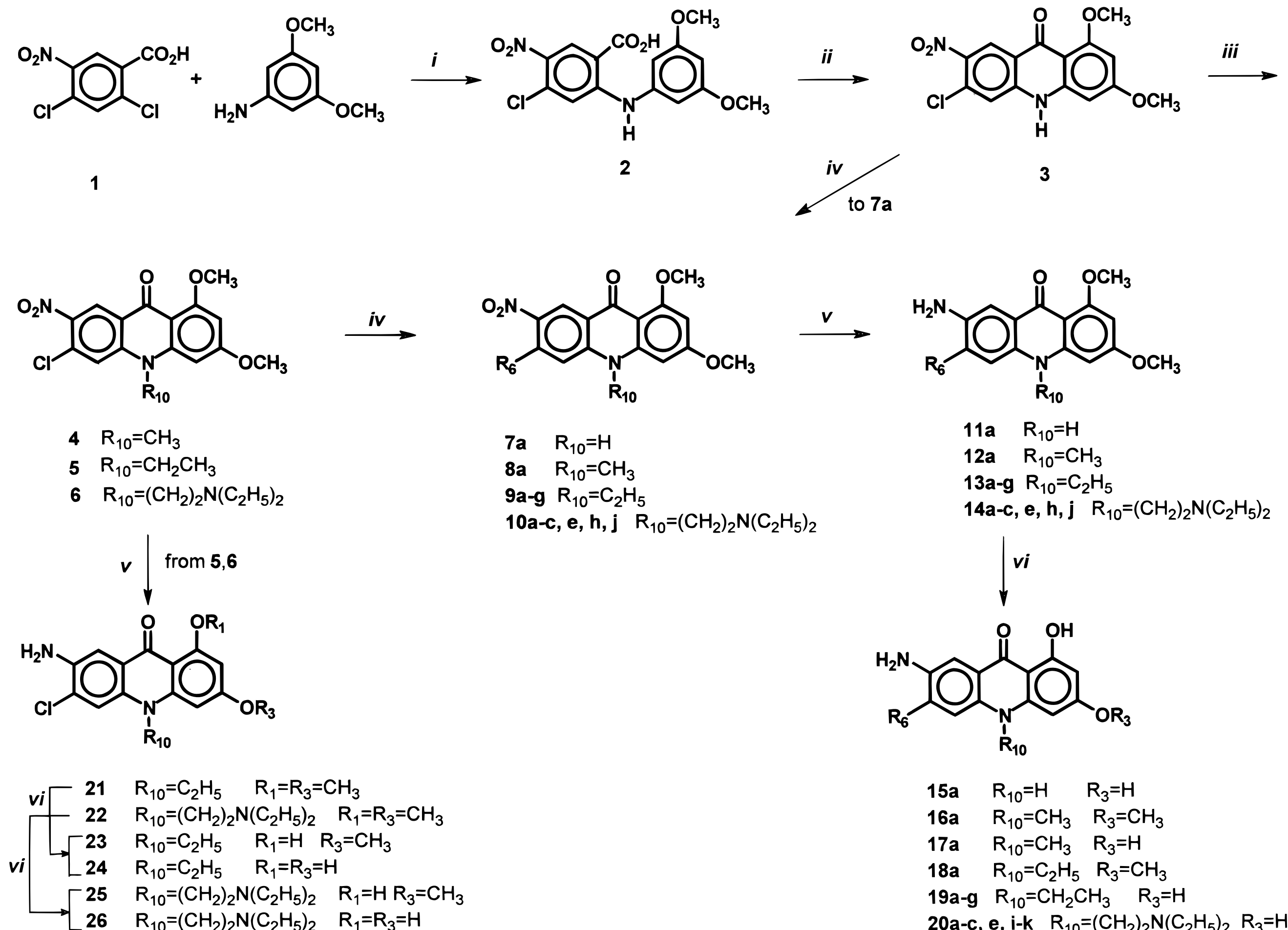
As in the 1,3-dihydroxy derivatives, high cytotoxic activity was also maintained in the 1-hydroxy-3-methoxy derivatives (**16a**, **18a**, **25**). These results show that the presence of an intramolecular hydrogen bond between the hydroxyl group at C-1 and the peri carbonyl function could play a role in cytotoxicity in these compounds as demostrated for glyfoline congeners.[16] In contrast, the substituent at the C-3 position does not greatly influence the activity.

Looking at the C-6 substituent in the series 1,3-dihydroxy-*N*-diethylaminoethyl, the cytotoxic potency follows this order: hydroxypiperidine > chlorine > OCH_3 > $NHCH_3$ > methylpiperazine > thiomorpholine > pyrrolidine> OH. The same trend was also displayed by the corresponding *N*-ethyl derivatives.

An overall evaluation of the in vitro screening on leukemic cell lines showed that the 6-(4-hydroxypiperidine)-*N*-diethylaminoethyl derivative **20e** was the most active, displaying IC_{90} values against P388 and HL-60 cell lines greater than or similar to those observed for etoposide. Good antitumoral activity was also displayed by its *N*-ethyl analogue **19e**, some 6-chloro derivatives (**24**−**26**), the C-6 aminomethyl derivative **20i**, and the C-6 methoxy derivative **20j**.

The compounds more active on leukemia cells were selected and assayed against a panel of human (LoVo)

Scheme 1[a]



[a] Reagents: (i) KOAc, $Cu(OAc)\cdot H_2O$, Et_3N, 2-propanol, reflux; (ii) PPA, 110 °C; (iii) RX, K_2CO_3, dry DMF, 60−120 °C; (iv) RH, dry DMF, 120 °C; (v) $SnCl_2\cdot 2H_2O$, 8 N HCl, reflux; (vi) 48% HBr, reflux.

and rodent (B16, LLC, CHO) solid tumor cells (Table 3). It should be noted that the 6-chloro derivatives **25** and **26** were the most potent against solid tumors. The IC_{50} and IC_{90} values for derivative **25** against LoVo were 1.5 and 6.3 μM, respectively, better than those exhibited by etoposide (2.3 and 44 μM). It should be noted that the results with derivative **20e**, which was the most active against the leukemic lines, failed to reach the IC_{90} against some solid tumors; this result suggests a selectivity in the action spectrum.

The cytotoxic activity of some selected acridone derivatives was also tested against normal human bone marrow cells (BM) from six different healthy donors with a second-stage screening model.[19] Comparing the results with those obtained with HL-60 cells (see ratio of the means, Table 4) leads to the identification of compounds which are more toxic against normal cells than tumor cells (group N) and focuses attention on compounds characterized by higher activity against tumor cells than against normal cells (group A). In a third group (group X), compounds with indeterminable antitumor activity are ranked. In this model, compound **25** was ranked in group A, like the reference compounds mitoxantrone and etoposide. Morever, **18a** was ranked in group X showing indeterminable antitumor activity, while the other compounds were ranked in the N group.

Compound **19a**, as a series prototype, and compounds **25** and **26**, showing good overall antiproliferative activity, were tested for the in vivo antineoplastic activity in mice bearing P388 leukemia (Table 5). Only the **19a** derivative with 131.6 T/C% at 60 mg/kg could be considered to be active since it surpassed the 125 T/C% limit. The in vivo inactivity of compounds **25** and **26** could be due to poor ip absorption. In fact, at the autopsy, the product was present at the injection site and the mice died without developing intraperitoneal leukemia. All attempts to improve the solubility by preparing hydrochloride or methanesulfonic salts failed.

To clarify the possible mode of action, compounds **19a**, **25**, and **26** were tested for their effect on cell cycle via a flow cytometric analysis. P388 cells treated with the test compounds at a dose of 32 μM accumulated in the S-phase with a maximum after 8 h of contact. When the cells were treated with 128 μM of the compounds for 1 h and the cell cycle was evaluated during the recovery period, the treated cells accumulated in the S-phase after 4 h and in the G2/M-phases after 8 h. These results are typical of, but not restricted to, topoisomerase-targeting compounds.

Thus, the same compounds were tested for DNA-topoisomerase II inhibitory activity by both cleavage and decatenation assays. When tested for their ability to enhance topoisomerase II-mediated DNA cleavage, the compounds **19a**, **25**, and **26** induced detectable levels of DNA damage at concentrations above 100, 40, and 50 μM, respectively (etoposide being active at 1 μM). It

Table 1. Physical Properties for 7-Amino-9-acridones Tested in This Study

compd	R_6	R_{10}	R_1	R_3	purification method[a]	% yield[b]	mp, °C	formula[c]
21	Cl	C_2H_5	CH_3	CH_3	A	75[d]	>300	$C_{17}H_{17}ClN_2O_3$
23	Cl	C_2H_5	H	CH_3	B	10	>300	$C_{16}H_{15}ClN_2O_3$
24	Cl	C_2H_5	H	H	D	60	>300	$C_{15}H_{13}ClN_2O_3$
22	Cl	$(CH_2)_2N(C_2H_5)_2$	CH_3	CH_3	A	85[d]	>300	$C_{21}H_{26}ClN_3O_3$
25	Cl	$(CH_2)_2N(C_2H_5)_2$	H	CH_3	B	20	186-189	$C_{20}H_{24}ClN_3O_3$
26	Cl	$(CH_2)_2N(C_2H_5)_2$	H	H	C	40	225-227	$C_{19}H_{22}ClN_3O_3$
15a	–N NCH$_3$	H	H	H	C	40	> 300 dec	$C_{18}H_{20}N_4O_3$
16a	"	CH_3	H	CH_3	E	15	263-268	$C_{20}H_{24}N_4O_3$
17a	"	CH_3	H	H	E	40	>300	$C_{19}H_{22}N_4O_3$
13a	"	C_2H_5	CH_3	CH_3	A	90[d]	260-262	$C_{22}H_{28}N_4O_3$
18a	"	C_2H_5	H	CH_3	B	20	230-232	$C_{21}H_{26}N_4O_3$
19a	"	C_2H_5	H	H	B	45	285-289	$C_{20}H_{24}N_4O_3$
14a	"	$(CH_2)_2N(C_2H_5)_2$	CH_3	CH_3	A	80[d]	>300	$C_{26}H_{37}N_5O_3$
20a	"	$(CH_2)_2N(C_2H_5)_2$	H	H	B	40	229-231	$C_{24}H_{33}N_5O_3$
19b	–N	C_2H_5	H	H	B	25	>300	$C_{19}H_{21}N_3O_3$
20b	"	$(CH_2)_2N(C_2H_5)_2$	H	H	C	60	210-212	$C_{23}H_{30}N_4O_3$
19c	–N S	C_2H_5	H	H	B	30	291-293	$C_{19}H_{21}N_3SO_3$
20c	"	$(CH_2)_2N(C_2H_5)_2$	H	H	B	20	238-240	$C_{23}H_{30}N_4SO_3$
19e	–N –OH	C_2H_5	H	H	B	20	>300	$C_{20}H_{23}N_3O_4$
20e	"	$(CH_2)_2N(C_2H_5)_2$	H	H	B	10	>300	$C_{24}H_{32}N_4O_4$
19d	CH$_3$ –N NH CH$_3$	C_2H_5	H	H	B	50	>300	$C_{21}H_{26}N_4O_3$
19f	$HN(CH_2)_2N(C_2H_5)_2$	C_2H_5	H	H	E	55	186-188	$C_{21}H_{28}N_4O_3$
19g	$N(C_2H_5)_2$	C_2H_5	H	H	B	43	201-203	$C_{19}H_{23}N_3O_3$
20i	$NHCH_3$	$(CH_2)_2N(C_2H_5)_2$	H	H	B	40	191-193	$C_{20}H_{26}N_4O_3$
20j	OCH_3	$(CH_2)_2N(C_2H_5)_2$	H	H	B	20	210-213	$C_{20}H_{25}N_3O_4$
20k	OH	$(CH_2)_2N(C_2H_5)_2$	H	H	B	30	218-220	$C_{19}H_{23}N_3O_4$

[a] (A) Crystallized by EtOH; (B) the bromohydrate was filtered, dissolved in a little water, reprecipitated with Na_2CO_3 at pH 8, washed again with water, dried, and purified by column chromatography eluting with CH_2Cl_2/EtOH/CH_3OH, 93:2:5; (C) after purification as reported in method A, the compound was crystallized by EtOH; (D) the bromohydrate was filtered, dissolved in a little water, reprecipitated with Na_2CO_3 at pH 8, and washed again with water; (E) the acid reaction mixture was made basic to pH 8 with Na_2CO_3 solution, and the monohydroxy derivative was obtained from the first extraction with $CHCl_3$, while the dihydroxy derivative was obtained as a precipitate by concentration of successive extractions. [b] Yield refers to the final step (48% HBr de-*O*-methylation). [c] All compounds had elemental analyses within ±0.4% of theoretical value. [d] Yield is that obtained from the $SnCl_2$ reduction step as the final step.

is worth noting that these concentrations are well above the IC_{50} values rerported in Tables 2 and 3 for the same compounds. In addition, in the decatenation assay, the test drugs targeted topoisomerase II only at concentrations above 250 μM. The poor ability of the compounds to poison topoisomerase II in in vitro experiments could be explained by the need for intracellular activation.

More likely, the results presented here suggest that topoisomerase II targeting is just one, and probably not the most important, of the mechanisms of action responsible for the cytotoxic activity of these compounds. Mechanistic studies based on assays other than topoisomerase II have yet to be performed for this new series.

Table 2. Cytotoxic Activity (IC_{50} and IC_{90})[a] of 9-Acridones against Murine Lymphocytic Leukemia P388 and Human Promyelocytic Leukemia HL-60

	P388		HL-60	
compd	IC_{50}	IC_{90}	IC_{50}	IC_{90}
19a	4.6	13	23	34
18a	2.9	8.9	17	24
19b	>100	>100	NT[b]	NT[b]
19c	>100	>100	NT[b]	NT[b]
24	1.6	13	13	74
15a	57	>100	>100	>100
13a	16	26	61	99
19e	3.4	9.4	5.4	9.2
19d	6.2	18	21	32
19f	4.3	11	19	46
26	1.4	3.5	3.9	10
20a	7.2	13	11	19
19g	24	50	24	58
23	16	>100	NT[b]	NT[b]
25	3.8	8.0	4.2	8.4
17a	7.2	11	24	39
16a	3.4	6.8	23	33
20c	8.8	19	NT[b]	NT[b]
20e	0.62	1.6	2.2	3.6
20i	3.9	7.0	9.7	27
20j	2.8	5.9	8.2	27
20k	26	56	>100	>100
20b	9.7	21	96	>100
22	4.3	14	8.6	29
14a	3.4	5.8	8.3	17
21	14	33	16	62
27	>100	>100	>100	>100
ETP[c]	0.24	0.63	1.2	3.1

[a] Concentration (μM) of agent required to reduce cell viability by 50% (IC_{50}) or 90% (IC_{90}). [b] NT, not tested. [c] ETP, etoposide.

Table 3. Cytotoxic Activity (IC_{50} and IC_{90})[a] of Selected 9-Acridones against Some Cell Lines Deriving from Solid Tumors[b]

	B16		CHO		LoVo		LLC	
compd	IC_{50}	IC_{90}	IC_{50}	IC_{90}	IC_{50}	IC_{90}	IC_{50}	IC_{90}
19a	9.9	19	5.8	19	17	47	11	17
18a	8.6	12	6.0	11	10	15	9.3	11
24	2.8	28	5.8	71	15	>100	16	>100
19e	26	>50	9.9	38	NT[c]	NT[c]	NT[c]	NT[c]
19d	22	32	12	38	21	37	29	43
19f	10	38	8.1	17	19	30	25	46
26	1.0	5.9	1.5	6.3	5.4	14	3.6	11
20a	16	23	7.1	16	17	21	24	35
25	1.6	4.0	1.1	5.9	1.5	6.3	5.0	11
20e	1.7	NO[d]	23	NO[d]	>32	>32	8.7	NO[d]
20i	3.8	24	8.2	36	26	44	23	64
20j	3.4	11	5.6	20	11	36	10	32
ETP[e]	0.32	1.9	0.35	7.7	2.3	44	1.1	4.5

[a] Concentration (μM) of agent required to reduce cell viability by 50% (IC_{50}) or 90% (IC_{90}). [b] B16, murine melanoma; CHO, Chinese hamster ovaric carcinoma; LoVo, human colon adenocarcinoma; LLC, Lewis lung carcinoma. [c] NT, not tested. [d] NO, not observed. [e] ETP, etoposide.

Experimental Section

Thin-layer chromatography (TLC) was performed on precoated sheets of silica gel $60F_{254}$ (Merck) and visualized by using UV. Column chromatography separations were carried out on Merck silica gel 40 (mesh 70−230). Melting points were determined in capillary tubes (Büchi melting point apparatus) and are uncorrected. Elemental analyses were performed on a Carlo Erba elemental analyzer, model 1106, and the data for C, H, and N are within ±0.4% of the theoretical values. ^{1}H NMR spectra were recorded at 200 MHz (Bruker AC-200) with Me_4Si as internal standard, and chemical shifts are given in ppm (δ). The spectral data are consistent with the assigned structures. Reagents and solvents were purchased from common commercial suppliers and were used as received. Organic solutions were dried over anhydrous Na_2SO_4 and concentrated with a Büchi rotary evaporator at low pressure. Yields were of purified product and were not optimized. All starting materials were commercially available unless otherwise indicated. The physical properties of target acridone derivatives are summarized in Table 1.

3-Nitro-4-chloro-*N*-(3,5-dimethoxyphenyl)anthranilic Acid (2). A mixture of 2,4-dichloro-5-nitrobenzoic acid (**1**)[20] (3.37 g, 14.3 mmol), 3,5-dimethoxyaniline (2 g, 13.1 mmol), KOAc (3.83 g, 39.1 mmol), $Cu(OAc)_2 \cdot H_2O$ (0.078 g, 0.39 mmol), and Et_3N (1.99 mL, 14.3 mmol) in 2-propanol (50 mL) was refluxed for 50 h. The solvent was then removed on a rotary evaporator, and the residue was partitioned between 50 mL of CH_2Cl_2 and 50 mL of 2 N HCl solution. The organic phase was washed with water, dried, and concentrated to one-half volume. The product that crystallized from the solution was collected, dried, and recrystallized by MeOH to give **2** (2.8 g, 56%): mp 288−290 °C; ^{1}H NMR (DMSO-d_6) δ 3.80 (6 H, s, OCH_3), 6.40−6.60 (3 H, m, H-2′, H-4′, H-6′), 7.15 (1 H, s, H-5), 8.65 (1 H, s, H-2), 10.20 (1 H, bs, NH). Anal. ($C_{15}H_{13}ClN_2O_6$) C, H, N.

6-Chloro-1,3-dimethoxy-7-nitroacridin-9-one (3). Compound **2** (2.5 g, 7.1 mmol) was treated with PPA (12.5 g) in an oil bath (110 °C) for 2 h with stirring. After cooling, the mixture was triturated with ice−water, and the precipitate was collected by filtration, washed with 10% Na_2CO_3 solution and water, and then recrystallized from MeOH/DMF to give **3** (1.9 g, 80%): mp > 300 °C; ^{1}H NMR (TFA) δ 4.15 and 4.40 (each 3 H, s, OCH_3), 6.85 and 7.05 (each 1 H, bs, H-2 and H-4), 8.15 (1 H, s, H-5), 9.10 (1 H, s, H-8). Anal. ($C_{15}H_{11}ClN_2O_5$) C, H, N.

6-Chloro-1,3-dimethoxy-10-ethyl-7-nitroacridin-9-one (5). A mixture of **3** (4.5 g, 13.5 mmol), EtI (5,4 mL, 68 mmol), and K_2CO_3 (4.65 g, 33.7 mmol) in dry DMF (35 mL) was heated at 85 °C for 12 h. After cooling, the precipitate was filtered, washed with water, and recrystallized by DMF to give **5** (3.5 g, 72%) as a yellow crystalline solid: mp > 300 °C; ^{1}H NMR (TFA) δ 1.75 (3 H, t, J = 6 Hz, CH_2CH_3), 4.20 and 4.40 (each 3 H, s, OCH_3), 4.90 (2 H, q, J = 6 Hz, CH_2), 6.90 and 7.05 (each 1 H, bs, H-2 and H-4), 8.20 (1 H, s, H-5), 9.20 (1 H, s, H-8). Anal. ($C_{17}H_{15}ClN_2O_5$) C, H, N.

6-Chloro-1,3-dimethoxy-10-methyl-7-nitroacridin-9-one (4). Starting from **3** and using MeI, compound **4** was obtained by the above procedure, except that the temperature and time of the reaction were 60 °C for 8 h. It was obtained in 70% yield: mp > 310 °C; ^{1}H NMR (DMSO-d_6/TFA) δ 4.20 (3 H, s, NCH_3), 4.35 and 4.40 (each 3 H, s, OCH_3), 6.95 and 7.10 (each 1 H, bs, H-2 and H-4), 8.30 (1 H, s, H-5), 9.25 (1 H, s, H-8). Anal. ($C_{16}H_{13}ClN_2O_5$) C, H, N.

6-Chloro-10-[2-(diethylamino)ethyl]-1,3-dimethoxy-7-nitroacridin-9-one (6). KOH (0.74 g, 13.2 mmol), K_2CO_3 (0.99 g, 7.2 mmol), and 18-crown-6 (0.32 g, 1.2 mmol) were added to a suspension of **3** (4 g, 12 mmol) in dry DMF (25 mL), and the mixture was stirred at room temperature for 2 h. After this time, a solution of 2-(diethylamino)ethyl chloride (1.8 g, 13.3 mmol) in dry DMF (5 mL) was added dropwise, and the mixture was heated at 120 °C for 10 h. After cooling, the precipitate was filtered, washed with DMF and EtOH, and dried to give **6** (1.5 g, 30%). An additional amount of **6** (2.2 g, 44%) was obtained from the filtrate by adding water, filtering the precipitate, and purifying by column chromatography eluting with EtOAc/CH_2Cl_2 (2:98): mp >330 °C; ^{1}H NMR (DMSO-d_6) δ 0.90 (6 H, t, J = 7 Hz, CH_2CH_3), 2.30−2.60 (4 H, m, CH_2CH_3), 2.80 (2 H, t, J = 6 Hz, CH_2), 3.90 and 4.00 (each 3 H, s, OCH_3), 4.50 (2 H, t, J = 6 Hz, CH_2), 6.50 and 6.70 (each 1 H, bs, H-2 and H-4), 7.95 (1 H, s, H-5), 8.70 (1 H, s, H-8). Anal. ($C_{21}H_{24}ClN_3O_5$) C, H, N.

1,3-Dimethoxy-10-ethyl-6-(4-methyl-1-piperazinyl)-7-nitroacridin-9-one (9a). A mixture of 6-chloro derivative **5** (2 g, 5.5 mmol) and *N*-methylpiperazine (2.75 g, 27.5 mmol) in dry DMF (5 mL) was heated at 120 °C for 30 min. After cooling, the precipitate was filtered off and washed with DMF to give **9a** (1.9 g, 81%): mp 282−285 °C; ^{1}H NMR (TFA) δ 1.70 (3 H, t, J = 6 Hz, CH_2CH_3), 3.20 (3 H, d, NCH_3), 3.45−4.10 (8 H, m, piperazine H), 4.20 and 4.40 (each 3 H, s, OCH_3),

Table 4. Relative Toxicity of Selected 9-Acridones as Evaluated by Using an in Vitro Human Model[19]

	bone marrow samples		HL-60		ratio of the means[b] and indices			
compd	range IC_{50}[a] (μM)	range IC_{90}[a] (μM)	range IC_{50}[a] (μM)	range IC_{90}[a] (μM)	IC_{50} BM/ IC_{50} HL	IC_{90} BM/ IC_{90} HL	IC_{50}BM/ IC_{90} HL	group[c]
24	3.55−7.08	22.4−74.1	5.36−17.0	81.5−97.5	0.6 (≈)	0.5 (−)	0.1 (−)	N
25	1.30−3.26	8.13−14.8	1.70−1.86	2.52−2.93	1.2 (≈)	4.1 (+)	0.8 (≈)	A
26	0.85−1.52	2.14−6.46	3.15−3.61	4.92−5.74	0.3 (−)	0.7 (≈)	0.2 (−)	N
18a	5.66−10.7	38.9−89.1	17.4−19.1	20.9−22.9	0.4 (−)	2.7 (+)	0.4 (−)	X
19a	2.79−3.27	13.6−20.3	20.8−27.7	29.5−42.6	0.1 (−)	0.5 (−)	0.1 (−)	N
19e	1.54−1.80	9.99−15.9	3.15−6.64	5.76−11.68	0.4 (−)	1.5 (≈)	0.2 (−)	N
20e	0.096−0.168	0.419−0.672	0.212−0.510	0.387−0.657	0.4 (−)	1.1 (≈)	0.3 (−)	N
ETP[d]	0.41−0.99	2.25−7.02	0.34−0.367	0.70−0.73	1.8 (≈)	5.6 (+)	0.9 (≈)	A
DHAD[e]	8.36−27.56	38.7−179.4	3.02−3.35	6.40−8.03	4.8 (+)	11.6 (+)	2.1 (+)	A

[a] The lower value is equal to antilog (mean log IC_{50}−2SD), and the higher value is equal to antilog (mean log IC_{50}+2SD). [b] Ratio of antilog (mean log IC_{50} or IC_{90}) in BM and antilog (mean log IC_{50} or IC_{90}) in HL-60 (HL). The IC_{50} or IC_{90} values in BM samples were compared with the range IC_{50} [antilog (mean log IC±DS)] or IC_{90} in HL. If more than 70% of the sample ICs were higher than the higher range value, then the index was considered positive (+); if more than 70% of the sample ICs were lower than the lower range value, then the index was considered negative (−); if the above conditions were not satisfied, then the index was considered indifferent (≈). [c] In function of the indices, three groups were considered: compounds with no negative index and one or more positive indices were classified in group A (compounds with potential antitumor activity); compounds with no positive indices and one or more negative indices were classified in group N (compounds with no antitumor activity); compounds with positive and negative indices or only indifferent indices were classified in group X (compounds with indeterminable antitumor activity). [d] ETP, etoposide. [e] DHAD, mitoxantrone.

Table 5. In Vivo Antitumor Activity of Selected 7-Amino-9-acridones against Murine P388 Leukemia

compd	dose (mg/kg)	T/C (%)[a]
19a	6.67	115
	20	116
	60	132
25	6.67	83
	20	97
	60	100
26	25	105
	50	109
	75	110
	100	111

[a] (Median survival time of treated mice)/(median survival time of controls) × 100. T/C (%) values > 125 are considered indicative of significant activity.

4.80 (2 H, q, *J* = 6 Hz, CH_2CH$_3$), 6.90 and 7.05 (each 1 H, bs, H-2 and H-4), 7.40 (1 H, s, H-5), 9.25 (1 H, s, H-8). Anal. ($C_{22}H_{26}N_4O_5$) C, H, N.

In analogous procedures, compounds **9b**−**g** were prepared from **5**, compound **7a** from **3**, and compound **8a** from **4** by reaction with the appropriate nucleophile.

10-[2-(Diethylamino)ethyl]-1,3-dimethoxy-6-(4-methyl-1-piperazinyl)-7-nitroacridin-9-one (10a). A mixture of **6** (0.43 g, 1 mmol) and *N*-methylpiperazine (0.2 g, 2 mmol) in DMF dry (4 mL) was heated at 120 °C for 1 h. The solution was evaporated to dryness, and EtOH was added to the residue. The product was filtered and washed with EtOH to give pure **10a** (0.32 g, 64%): mp 235−238 °C; ^{1}H NMR (TFA) δ 1.20 (6 H, t, *J* = 6 Hz, CH$_2$CH_3), 3.10−3,60 (6 H, m, CH_2-CH$_3$ and CH$_2$), 3.80 and 4.00 (each 3 H, s, OCH$_3$), 4.90−5.10 (2 H, m, CH$_2$), 6.60 and 6.65 (each 1 H, bs, H-2 and H-4), 7.80 (1 H, s, H-5), 8.85 (1 H, s, H-8). Anal. ($C_{26}H_{35}N_5O_5$) C, H, N.

In an analogous procedure, compounds **10b**,**c**,**e**,**h**,**j** were prepared from **6** by reaction with the appropriate nucleophile.

General Procedure for the Reduction of the 7-Nitro Group. Preparation of 7-Amino-1,3-dimethoxy-10-ethyl-6-(4-methyl-1-piperazinyl)acridin-9-one (13a). A solution of $SnCl_2 \cdot 2H_2O$ (3 g, 13.3 mmol) in 8 N HCl (10 mL) was added at room temperature with stirring to a solution of nitro derivative **9a** (1.9 g, 4.5 mmol) in 8 N HCl (22 mL). The mixture was heated under reflux for 30 min. After cooling, the precipitate was filtered, dissolved in water (50 mL), and made basic with saturated Na_2CO_3 solution. The product was collected, washed with water, and crystallized from EtOH to give **13a** (1.7 g, 95%): mp 260−262 °C; ^{1}H NMR (DMSO-d_6) δ 1.45 (3 H, t, *J* = 6 Hz, CH$_2$CH_3), 2.35 (3 H, s, NCH$_3$), 2.45−2.70 and 2.95−3.20 (each 4 H, m, piperazine CH$_2$), 3.85 and 3.95 (each 3 H, s, OCH$_3$), 4.40 (2 H, q, *J* = 6 Hz, CH_2CH$_3$), 4.75 (2 H, br s, NH$_2$), 6.30 and 6.50 (each 1 H, bs, H-2 and H-4), 6.95 (1 H, s, H-8), 7.50 (1 H, s, H-5). Anal. ($C_{22}H_{28}N_4O_3$) C, H, N.

In an analogous procedure, compounds **11a**, **12a**, **13b**−**g**, and **14a**−**c**,**e**,**h**,**j** were prepared starting from the corresponding nitroacridones **7a**, **8a**, **9b**−**g**, and **10a**−**c**,**e**,**h**,**j**, while compounds **21** and **22** were prepared from the corresponding 6-chloro-7-nitroacridones **5** and **6**.

7-Amino-10-ethyl-1-hydroxy-3-methoxy-6-(4-methyl-1-piperazinyl)acridin-9-one (18a) and 7-Amino-1,3-dihydroxy-10-ethyl-6-(4-methyl-1-piperazinyl)acridin-9-one (19a). A mixture of **13a** (1.9 g, 4.8 mmol) in 48% HBr (1.2 mL) was heated at reflux for 6 h. TLC (CHCl$_3$/MeOH, 8:2) showed that two products were formed. The reaction mixture was worked up and purified following method A (see Table 1). Thus, after cooling, the bromohydrate precipitate was collected by filtration, solubilized in water, and made basic with Na_2CO_3 solution. The precipitate so obtained was filtered, dried, and chromatographed. Elution with CH$_2$Cl$_2$/EtOH/MeOH (93:2:5) (method A) gave compound **18a** (0.187 g, 10.4%) followed by compound **19a** (1.6 g, 88.2%). Compound **18a**: mp 228−230 °C; ^{1}H NMR (DMSO-d_6) δ 1.40 (3 H, t, *J* = 6 Hz, CH$_2$CH_3), 2.25 (3 H, s, NCH$_3$), 2.40−2.65 and 2.95−3.20 (each 4 H, m, piperazine CH$_2$), 3.90 (3 H, s, OCH$_3$), 4.45 (2 H, q, *J* = 6 Hz, CH_2CH$_3$), 4.90 (2 H, bs, NH$_2$), 6.15 and 6.40 (each 1 H, bs, H-2 and H-4), 7.05 (1 H, s, H-8), 7.55 (1 H, s, H-5), 15.45 (1 H, s, OH-1). Anal. ($C_{21}H_{26}N_4O_3$) C, H, N. Compound **19a**: mp 293−295 °C; ^{1}H NMR (DMSO-d_6) δ 1.35 (3 H, t, *J* = 6 Hz, CH$_2$CH_3), 2.25 (3 H, s, NCH$_3$), 2.40−2.65 and 2.95−3.25 (each 4 H, m, piperazine CH$_2$), 4.35 (2 H, q, *J* = 6 Hz, CH_2CH$_3$), 4.85 (2 H, bs, NH$_2$), 6.00 and 6.30 (each 1 H, bs, H-2 and H-4), 7.00 (1 H, s, H-8), 7.50 (1 H, s, H-5), 15.35 (1 H, s, OH-1). Anal. ($C_{20}H_{24}N_4O_3$) C, H, N.

In an analogous demethylation procedure from **11a**, **13b**−**g**, and **14a**−**c**,**e**, only the corresponding dihydroxy derivatives **15a**, **19b**−**g**, and **20a**−**c**,**e** were obtained. While from the demethylation of **12a**, **21**, and **22**, both monohydroxy and dihydroxy derivatives **16a** and **17a**, **23** and **24**, **25** and **26** were obtained, respectively. The dihydroxy-6-methylamino derivative **20i** was obtained as the only product by the demethylation of the 6-dimethylamino derivative **14h**, while the demethylation of the 6-methoxy derivative **14j** gave the corresponding dihydroxy derivative **20j** together with the trihydroxy derivative **20k**. The workup and purification details of the above products are given in Table 1.

6-Chloro-1,3-dihydroxy-10-ethylacridin-9-one (27). It was obtained using the same procedure employed to obtain **24** using 2,6-dichlorobenzoic acid instead of 2,6-dichloro-5-nitrobenzoic acid in the first step: mp 323−325 °C; ^{1}H NMR (DMSO-d_6) δ 1.40 (3 H, t, *J* = 7.20 Hz, CH$_2$CH_3), 4.40 (2 H, q, *J* = 7.20 Hz, CH_2CH$_3$), 6.20 and 6.50 (each 1 H, d, *J* = 1.5 Hz,

H-2 and H-4), 7.40 (1 H, dd, $J = 1.5$ and 9 Hz, H-7), 7.90 (1 H, bs, H-5), 8.30 (1 H, d, $J = 9$ Hz, H-8), 14.75 (1 H, s, OH-1). Anal. ($C_{15}H_{12}ClNO_3$) C, H, N.

In Vitro Antibacterial Activity. The minimum inhibitory concentrations (MIC, μg/mL) were determined by the microdilution technique using nutrient broth, according to NCCLS.[21]

In Vitro Antitumor Activity. Compound solubilization: Compounds were solubilized in DMSO plus water. The use of DMSO + PEG-8000 or DMSO + γ-cyclodextrin did not facilitate solubilization. The highest DMSO concentration used (0.2%) had no cytotoxic effect on our testing systems.

Cell culture: The following cell lines were used: P388 murine lymphocytic leukemia, HL-60 human promyelocytic leukemia, CHO Chinese hamster ovaric carcinoma, LLC Lewis lung carcinoma, B16 murine melanoma, and LoVo human colon adenocarcinoma.

With the exception of LoVo and CHO cells, cell lines were grown in RPMI-1640 medium supplemented with 10 mM HEPES buffer and 15% (P388) or 5% (LLC) heat-inactivated new-born calf serum or 10% (B16) or 15% (HL-60) heat-inactivated fetal calf serum. LoVo and CHO cells were maintained in HAM's F12 medium supplemented with 10% heat-inactivated fetal calf serum. Antibiotics (penicillin 100 U/mL, streptomycin 100 μg/mL, gentamicin 50 μg/mL) and 3 mM glutamine were added to all cell culture media. 2-Mercaptoethanol (10 nM) was also added to P388 and HL-60, while 1% vitamins and nonessential amino acids were added to LoVo, CHO, and B16. The cells were grown in a humidified atmosphere of 5% CO_2 at 37 °C and maintained in logarithmic cell growth.

BM cells were obtained from BM of healthy donors and were the unused quota following homologous BM transplantation procedures.[19] Briefly, the mononuclear cell fraction was mixed with soybean agglutinin (2 mg/mL in PBS). The unagglutinated fraction (SBA^-) was washed twice and resuspended in the same medium used for HL-60 culture (10^7 cells/mL). Before testing, cells were kept for 3−4 days in a humidified atmosphere of 5% CO_2 at 37 °C.

Antiproliferative assay: To determine cytotoxic activity, a partially modified tetrazolium-based colorimetric assay was used.[22] Various concentrations of each drug were placed with tumor cell suspensions (CHO, 2.5×10^3 cells/well; B16, 5×10^3 cells/well; P388 and LLC, 10^4 cells/well; HL-60 and LoVo, 5×10^4 cells/well). Cell growth was determined 68 h later by adding 50 μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2.5 mg/mL. This was reduced by mitochondrial dehydrogenase of viable cells in an insoluble blue formazan product during the 4-h contact period at 37 °C. After the supernatant was removed, the formazan crystals were solubilized by adding DMSO (100 μL). The plates were read at 550 nm with a microELISA reader. At each dose level of the compounds tested, cell growth inhibition was expressed as a fractional decrease of 550-nm absorbance in the treated cultures with respect to control cultures.

Second-stage screening model: The assay used to evaluate cytotoxic activity was developed for predictive evaluation of tumor chemosensitivity and had been previously used in in vitro studies of new compounds with potential antitumor activity.[19] Four wells of various concentrations of each drug (prestored at −20 °C) were placed with tumor cell suspensions (HL-60, 5×10^4 cells/well; BM cells, 5×10^5 cells/well) and kept at 37 °C. After 48 h of incubation, DNA synthesis was evaluated by adding 5-[^{125}I]iodo-2′-deoxyuridine (0.1 μCi/well) together with 2′-deoxy-5-fluorouridine (0.01 μg/well) to the cultured cells for an additional 18 h. Harvesting was done by a multiple suction filtration apparatus (Mash II) on a fiberglass filter (Whittaker Co.). Paper disks containing the aspirated cells were read in a gamma-scintillation counter. For each concentration of compounds tested, cell growth inhibition was expressed as the fractional decrease of radioisotope incorporation in the treated cultures compared to untreated controls. In control samples, the cpm ranged from 1230 to 5560 (mean 3219) in BM from different donors, and valid data were obtained from every sample used.

Data analysis: The median-effect concentration required for 50% (IC_{50}) or 90% (IC_{90}) inhibition was calculated using Chou's median-effect equation:

$$F_i/(1 - F_i) = (C/IC_{50})^h$$

where C represents the concentration of drug which produces a determined fractional inhibition on the system, F_i is the fractional inhibition value, and h is the Hill-type coefficient. This coefficient indicates the degree of sigmoid shape in the dose−effect curve. If the correlation coefficient for the regression line is greater than 0.9, the equation fits the dose−effect relationship. In our analyses the correlation coefficient value was always greater than 0.95.

To evaluate the range of concentration inhibiting cell growth by 50% or 90%, using different assays, the logs of concentration values were obtained for each assay and the mean (m) and standard deviation (SD) calculated. The lower range value was equal to the m−2SD antilog and the higher to the m+2SD antilog. To calculate the ratio of the mean between BM and HL-60 inhibiting concentrations, the m antilog was used. The human "in vitro therapeutic indices" were ranked as reported.[19]

In Vivo Antitumor Activity. The test animals used in this experiment were female BD2F1 mice (8 weeks old, 20−22 g), purchased from Charles River, Italy. Each experimental group contained eight mice which were injected ip with 10^5 P388 cells on day 0; 24 h later, a micronized suspension of drugs in 0.5% carboxymethylcellulose (methocel) was injected ip once a day for 9 days. Mice were observed daily for signs of toxicity and survival. The date of death was recorded for each animal during the 33-day study. The median survival time (MST) for each treatment group was calculated, and the percent T/C was determined by using the following formula: % T/C = [(MST treated)/(MST control)] × 100.

Topoisomerase II-Mediated DNA Decatenation. Assays were carried out as previously reported.[23] The effects of drugs were examined over a concentration range 2−270 μM.

Topoisomerase II-Mediated DNA Cleavage. Assays were carried out as previously reported.[24] The effects of drugs were examined over a concentration range 0.1−100 μM.

Flow Cytometric Analysis. Cell cycle was analyzed by performing a flow cytometric analysis. Briefly, tumor cells incubated for the specified time with the test substance were washed in PBS and resuspended in 1.5 mL of hypotonic solution of propidium iodide (PI; 50 mg/mL in 0.1% Na-citrate containing 0.1% Triton X-100), and the tube was placed at 4 °C in the dark for 1−16 h before the flow cytometric analysis. The PI fluorescence of isolated nuclei was measured with a FACSCAN (Becton Dickinson, Mountain Wiew, CA) flow cytometer and the percentage of nuclei in the different cell cycle phases calculated using Lysis II research software.

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Supporting Information Available: ^{1}H NMR data of acridone target derivatives not reported in the test. This material is available free of charge via the Internet at http://pubs.acs.org.

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