2,3-Dihydro-1*H*,7*H*-pyrimido[5,6,1-*de*]acridine-1,3,7-trione Derivatives, a Class of Cytotoxic Agents Active on Multidrug-Resistant Cell Lines: Synthesis, Biological Evaluation, and Structure–Activity Relationships

Ippolito Antonini,*,[†] Paolo Polucci,[†] Lloyd R. Kelland,[‡] Ernesto Menta,[§] Nicoletta Pescalli,[§] and Sante Martelli[†]

Department of Chemical Sciences, University of Camerino, Via S. Agostino 1, 62032 Camerino, Italy, Cancer Research Campaign Cancer Therapeutics Centre, The Institute of Cancer Research, Cotswold Road, Sutton, Surrey SM2 5NG, U.K., and Boehringer Mannheim Italia, Research Center, 20052 Monza, Italy

A series of DNA-intercalating potential antitumor agents, (amino)alkyl-substituted 2,3-dihydro-1*H*,7*H*-pyrimido[5,6,1-*de*]acridine-1,3,7-triones, has been prepared by aminolysis of the corresponding 6-chloro derivative with a suitable ω-aminoalkylamine. The noncovalent DNA-binding properties of these compounds have been examined using a fluorometric technique. In vitro cytotoxic potencies of these derivatives toward eight tumor cell lines, including human colon adenocarcinoma (HT29, LoVo sensitive and LoVo/Dx (doxorubicin-resistant)) and human ovarian carcinoma (A2780 sensitive, A2780cisR (cisplatin-resistant), CH1, CH1cisR (cisplatin-resistant), and SKOV-3) cells, are described and compared to that of reference drugs. The cytotoxic activity often parallels the observed DNA affinities, for almost all the target compounds. Interesting structure–activity relationships have been found. The octanol/water partition coefficients have also been calculated, but there was no correlation either with cytotoxicity values or with resistance index. Three highly DNA-affinic analogues, **9** and **15f,15h**, have been identified with a useful broad spectrum of cytotoxic activity.

Introduction

Up to now, the only hope to cure systemic cancers, such as leukemia and lymphoma, and unifocal tumors that have spread by metastasis resides in systemic treatments such as chemotherapies and immunotherapies, with the former being of major clinical importance. Unfortunately, the prolonged use of chemotherapeutic agents often leads to the appearance of cell populations resistant to multidrug-based chemotherapy. This phenomenon, known as "multidrug resistance" (MDR), constitutes currently the major problem to achieve cures in patients. Although the problem of MDR of tumor cells has been known for almost 30 years, it has not yet been successfully circumvented in the clinic.

In 1995 we published a paper describing the synthesis and biological activities of bis-functionalized pyrimido-[5,6,1-*de*]acridines, a novel group of anticancer agents active on a MDR cell line, that exhibit cytostatic and antitumor activity.[1] The obtained compounds (**1a**–**e**, Figure 1) are characterized by the presence of two basic side chains in positions 2 and 6 and by a chromophore in which is present a 9-acridone moiety fused with a uracil to form a tetracyclic system.

In vitro and in vivo activity of some of the derivatives of **1** is very high, but their most important property is the very good activity on the MDR cell line LoVo/Dx. For some compounds of this novel group the resistance factor (RI) is close to 1.[1] The activity on MDR tumor cells makes pyrimido[5,6,1-*de*]acridines "lead compounds" in the class of acridine derivatives.
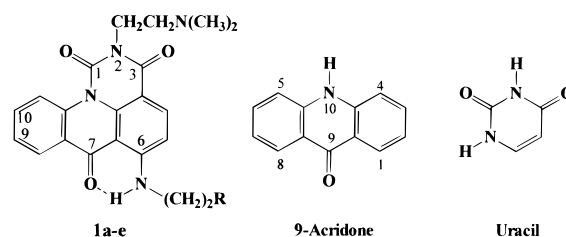

Figure 1. Structure and ring numbering of parent pyrimido-[5,6,1-*de*]acridines **1** [**a**, R = N(Me)₂; **b**, R = N(Et)₂; **c**, R = CH₂N(Me)₂; **d**, R = NH(CH₂)₂OH; **e**, R = 1-piperazinyl].[1] The chromophore results from the formal fusion of 9-acridone and uracil moieties.

To better understand the structure–activity relationships and the biological properties of these compounds, we decided to synthesize new derivatives, both modifying the side chains in positions 2 and 6 and introducing substituents in positions 9 and 10. In vitro cytotoxicity data of target pyrimidoacridines and reference compounds against eight tumor cell lines, including human colon adenocarcinoma (HT29, LoVo sensitive, and LoVo/Dx (doxorubicin-resistant)) and human ovarian carcinoma (A2780 sensitive, A2780cisR (cisplatin-resistant), CH1, CH1cisR (cisplatin-resistant), and SKOV-3) cells, are described. DNA-binding results from fluorescence-based studies are reported for these compounds with calf thymus DNA and two polyoligonucleotide duplexes. Attempts to correlate DNA-binding and cytotoxic activity were also made. The octanol/water partition coefficients have been also calculated and related to cytotoxicity values or with resistance index.

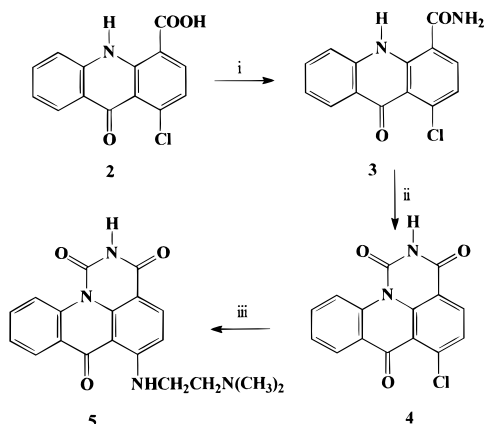## Rational Approach and Chemistry

(i) Since the second basic side chain in position 6 seems to be essential for the activity,[1] we decided to

* Address correspondence to this author.
† University of Camerino.
‡ The Institute of Cancer Research.
§ Boehringer Mannheim Italia.

Scheme 1[a]

[a] Reagents: (i) 1,1′-carbonyldiimidazole/NH$_3$; (ii) ClCOOC$_2$H$_5$; (iii) H$_2$N(CH$_2$)$_2$N(CH$_3$)$_2$.

synthesize the derivative **5**, with only one side chain in position 6, to prove if both the chains in positions 2 and 6 are important or only that in position 6. Thus, according to Scheme 1, the carboxylic acid **2**[2] was reacted with 1,1′-carbonyldiimidazole and ammonia in chloroform to afford the carboxamide **3**, which by refluxing in ethyl chloroformate cyclized to pyrimido-acridine **4**. Nucleophilic substitution of **4** with *N,N*-dimethylethylenediamine gave the desired compound **5**.

(ii) There is a strong intramolecular hydrogen bond between the carbonyl in position 7 and the hydrogen of the amine in position 6, as can be seen from chemical shift values of the involved proton (δ range 9.90−11.18) and from the difficulty with which this proton exchanged with deuterium oxide.[1] It may be postulated that this hydrogen bond can mimic an additional fifth ring which may be important for biological activity (Figure 1). To verify this hypothesis, we synthesized compound **7**, in which this hydrogen bond is not possible, by nucleophilic substitution of **6**[1] with *N,N,N*-trimethylethylenediamine (Scheme 2).

Scheme 2[a]
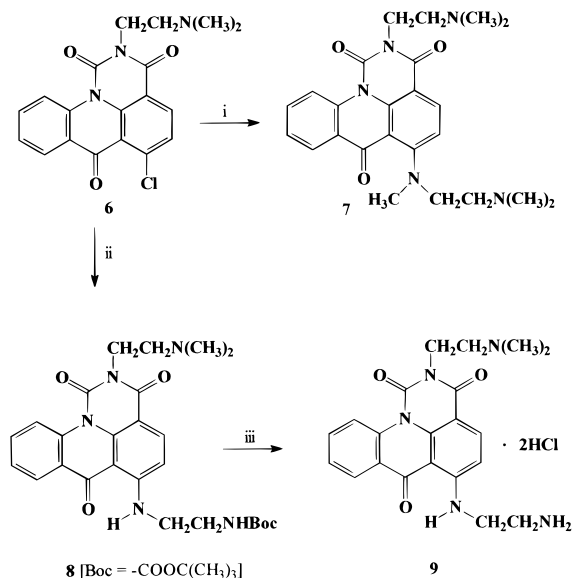
[a] Reagents: (i) CH$_3$NH(CH$_2$)$_2$N(CH$_3$)$_2$; (ii) H$_2$N(CH$_2$)$_2$NHBoc; (iii) H$^+$.

(iii) Because an aminoethylamino basic chain seems to be important in some intercalating antitumor agents,[3] compound **9**, with this kind of side chain in position 6, was obtained by nucleophilic substitution of **6**[1] with *N*-Boc-ethylenediamine and subsequent deprotection of the amino group in acidic medium, which gave directly the dihydrochloride **9** (Scheme 2).

(iv) Compounds **15a**−**h** with substituents in positions 9 and 10, often able to increase the activity of similar intercalating antitumor compounds,[4] were synthesized according to Schemes 3 and 4. Thus, the intermediate carboxamide **12a** was obtained by condensation of 2-bromo-4,5-dimethoxybenzoic acid with methyl 2-amino-4-chlorobenzoate[2] followed by cyclization with PPE to yield the ester **10**, which was hydrolyzed to carboxylic acid **11**. Treatment of **11** with 1,1′-carbonyldiimidazole

Scheme 3[a]
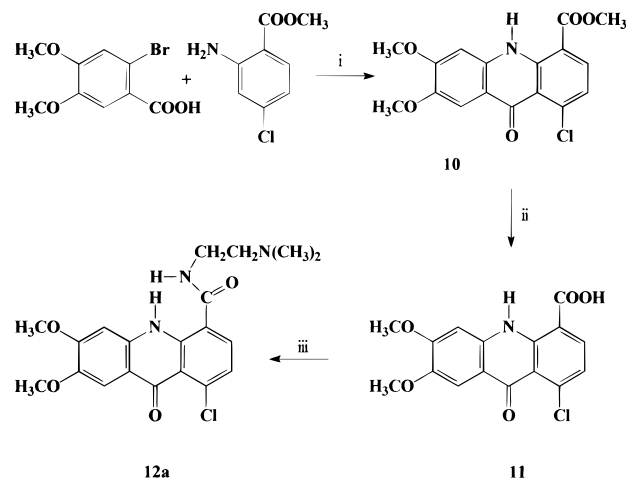
[a] Reagents: (i) (1) Cu(OAc)$_2$/1-methyl-2-pyrrolidinone/*N,N*-di-isopropylethylamine, (2) PPE (polyphoshoric acid ethyl ester); (ii) NaOH/EtOH; (iii) 1,1′-carbonyldiimidazole/H$_2$N(CH$_2$)$_2$N(Me)$_2$.

Scheme 4[a]
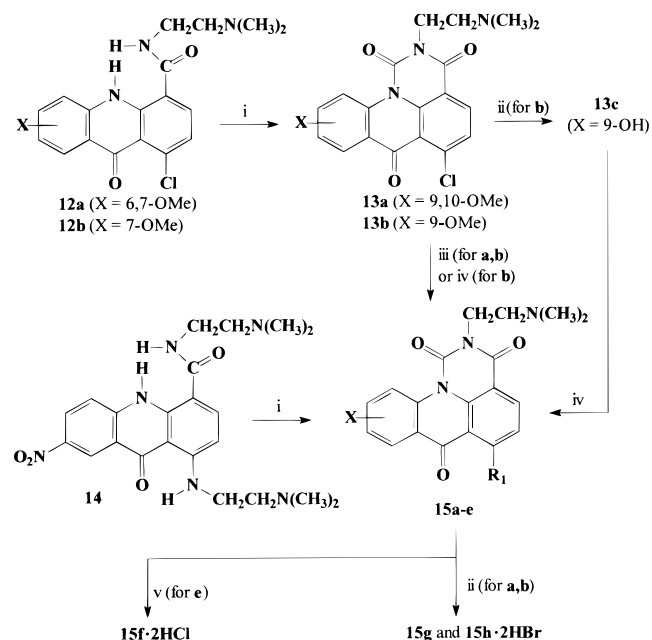
[a] Reagents: (i) COCl$_2$; (ii) HBr; (iii) H$_2$N(CH$_2$)$_2$N(CH$_3$)$_2$; (iv) H$_2$N(CH$_2$)$_2$NH(CH$_2$)$_2$OH; (v) H$_2$, Pd/C. Structures: for compounds **15a**−**h**, see Table 1.

Table 1. Substituents, Melting Points, Yields, and Formulas of Target Compounds **5**, **7**, **9**, and **15a−h**
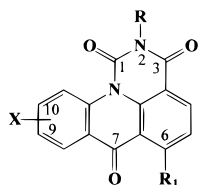
no.	R	R_1	X	mp (°C)^a	% yield	formula^b
5	H	NHA^c		253−254 (282−284)	12	$C_{19}H_{18}N_4O_3$
7	A^c	N(Me)A^c		114−115 (264−265 dec)	61	$C_{24}H_{29}N_5O_3$
9·2HCl	A^c	NHB^d		194−196	94	$C_{21}H_{25}Cl_2N_5O_3$
15a	A^c	NHA^c	9,10-OMe	186−187 (235−237 dec)	85	$C_{25}H_{31}N_5O_5$
15b	A^c	NHA^c	9-OMe	270−272 dec (>300)	77	$C_{24}H_{29}N_5O_4$
15c	A^c	NHC^e	9-OMe	229−231 dec (>300)	55	$C_{24}H_{29}N_5O_5$
15d	A^c	NHC^e	9-OH	120−122 dec (260−262 dec)	30	$C_{23}H_{27}N_5O_5$
15e	A^c	NHA^c	9-NO$_2$	175−176 (256−258 dec)	64	$C_{23}H_{26}N_6O_5$
15f·3HCl	A^c	NHA^c	9-NH$_2$	278−280 dec	56	$C_{23}H_{31}Cl_3N_6O_3$
15g	A^c	NHA^c	9,10-OH	170−171 (268−270 dec)	83	$C_{23}H_{27}N_5O_5$
15h·2HBr	A^c	NHA^c	9-OH	>300	74	$C_{23}H_{29}Br_2N_5O_4$

^{a} In parentheses is the melting point of the dihydrochloride (hydrochloride for **5**); dec, decomposition. ^{b} Analyses for C, H, and N. ^{c} A, $(CH_2)_2N(Me)_2$. ^{d} B, $(CH_2)_2NH_2$. ^{e} C, $(CH_2)_2NH(CH_2)_2OH$.

and dimethylethylenediamine in chloroform afforded **12a** (Scheme 3). The carboxamides **12a**,**b**[5] were cyclized with phosgene in chloroform to pyrimidoacridines **13a**,**b**. The 9-hydroxy derivative **13c** was obtained by hydrolysis of **13b** with 48% HBr. Nucleophilic substitution of **13a**,**b** with *N*,*N*-dimethylethylenediamine and of **13b**,**c** with *N*-(2-hydroxyethyl)ethylenediamine gave the target compounds **15a**,**b** and **15c**,**d**, respectively. The cyclization with phosgene of bis-functionalized acridone **14**[5] gave the 9-nitro derivative **15e**, which was reduced to the corresponding 9-aminopyrimidoacridine **15f** (isolated as trihydrochloride) with hydrogen and palladium on activated carbon in acidic medium. Hydrolysis with 48% HBr afforded the 9,10-dihydroxy and 9-hydroxy derivatives **15g**,**h** (isolated as dihydrobromide) from **15a**,**b**, respectively (Scheme 4).

To examine the DNA-binding properties and the in vitro antineoplastic activity of these agents, the free bases **5**, **7**, and **15a−e**,**g** were converted into their water-soluble dihydrochlorides (hydrochloride for **5**) by usual methods. In Table 1 are reported structure, melting point, yield, and formula of target derivatives **5**, **7**, **9**, and **15a−h**.

Results and Discussion

DNA-Binding Properties. Competitive displacement (C_{50}) fluorometric assays with DNA-bound ethidium can be used[6] to (a) determine 'apparent' equilibrium constants (K_{app}) for drug binding, as the C_{50} value is approximately inversely proportional to the binding constant,[7] and (b) establish possible base- or sequence-preferential binding.[8]

In the present study, fluorescence displacement assays were performed at pH 7 to enable comparison in biological conditions. The K_{app} values of pyrimidoacridines in Table 2 indicate that: (a) the bis(amine-functionalized) pyrimidoacridines generally are DNA-binding ligands stronger than ethidium with the exception of **1e**, **7**, and **15d**; (b) the N-methylation of proximal nitrogen on the side chain in the 6 position, compound **7**, is detrimental for binding as well as the contemporary presence of a hydroxyl group on the same side chain and in position 9, compound **15d**, or as the presence of

a bulky substituent on the distal nitrogen of the same side chain, compound **1e**; (c) the side chain in position 6 seems to be more important for binding than that in position 2 (see **5** and **6** K_{app} values), but only the contemporary presence of both side chains strongly stabilizes the binding.

The K_{app} values (Table 2), determined for ethidium displacement from synthetic [poly(dA-dT)]$_2$ (AT) and [poly(dG-dC)]$_2$ (GC) by pyrimidoacridines **1a−e**, **5−7**, **9**, and **15a−h**, indicate the same trend observed with CT-DNA, but particularly relevant is the binding site preference showed by compounds **9** and **15a−h**: the binding on GC sequence is 4−20-fold more efficient than binding on AT sequence.

Cytotoxic Activity. In vitro cytotoxic potencies of parent compounds (**1a−e** and **6**), of target pyrimidoacridines (**5**, **7**, **9**, and **15a−h**), and of reference compounds, mitoxantrone (Mx) and doxorubicin (Dx), against three human colon adenocarcinoma cell lines (HT29, LoVo sensitive, and LoVo/Dx (doxorubicin-resistant) are reported in Table 2.

Regarding the HT29 and LoVo sensitive cell lines, the following can be observed. (i) Cytotoxic activity values of compound **5**, with only one side chain in position 6, compared to that of compound **6**, with only one side chain in position 2, indicate that the side chain in position 6 is more important for cytotoxic potency than the side chain in position 2, but the comparison with many of the derivatives bearing two side chains in positions 2 and 6 proves that the contemporary presence of both side chains is needed for optimal activity. This observation parallels with that on DNA binding. (ii) The values for the *N*-methyl derivative **7** are at least 1 order of magnitude lower than that of parent **1a**, clearly indicating the importance of an intramolecular hydrogen bond between the carbonyl in position 7 and the hydrogen of the amine in position 6. Also in this case there is correspondence between cytotoxic activity and DNA binding. (iii) The introduction of an aminoethylamino side chain in position 6, compound **9**, produces cytotoxicity comparable to the best parent compound **1a** and to Dx. (iv) The substitution in position 9 leads to different results. The 9-hydroxy derivative of **1a**, com-

Table 2. In Vitro Cytotoxicity against Three Human Colon Adenocarcinoma (HT29, LoVo Sensitive, and LoVo/Dx Resistant) Cell Lines, DNA Binding,[a] and Log P[b] of Target Compounds **5**, **7**, **9**, and **15a−h**, of Parent Compounds **1a−e** and **6**,[1] and of Reference Compounds Mitoxantrone (Mx) and Doxorubicin (Dx)

compd	IC$_{50}$ (μM)[c]			RI[d]	K_{app}[e] $\times$ 10^{-7}			binding site preference[h]	log P
	HT29	LoVo	LoVo/Dx		CT-DNA[f]	AT	GC[g]		
1a	0.022	0.022[i]	0.029[i]	1.3[i]	1.7 (0.58)	0.68	1.2(1.8)	GC	1.94 ± 0.73
1b	0.21	0.052[i]	0.10[i]	1.9[i]	1.7 (0.57)	0.75	1.9(2.5)	GC	3.00 ± 0.73
1c	0.22	0.19[i]	0.16[i]	0.84[i]	1.4 (0.70)	0.77	1.7(2.2)	GC	2.27 ± 0.73
1d	0.071	0.03[i]	0.23[i]	7.7[i]	1.1 (0.93)	0.35	1.0(2.9)	GC	0.91 ± 0.75
1e	0.30	0.85[i]	1.0[i]	1.2[i]	0.52 (1.9)	0.37	0.33(0.89)	none	1.70 ± 0.79
5	0.90	0.36	1.0	2.8	0.28 (4.5)				2.02 ± 0.69
6	6.7	1.2[i]	1.4[i]	1.2[i]	0.043 (29)	0.029	0.065(2.2)	GC	1.56 ± 0.69
7	0.90	0.57	8.2	14	0.37 (3.4)	0.24	0.73(3.0)	GC	1.12 ± 0.71
9	0.069	0.033	0.61	18	2.4 (0.53)	0.66	7.9(12)	GC	1.21 ± 0.72
15a	0.37	0.36	5.6	16	1.5 (0.82)	0.40	3.2(8.0)	GC	1.39 ± 0.77
15b	0.067	0.011	0.42	38	1.3 (0.99)	0.73	3.3(4.5)	GC	1.28 ± 0.76
15c	0.42	0.015	9.7	650	3.6 (0.35)	0.76	3.4(4.5)	GC	0.25 ± 0.77
15d	3.38	4.50	>10		0.31 (4.1)	0.014	0.28(20)	GC	0.47 ± 0.76
15e	0.31	0.5	5.8	12	0.79 (1.6)	0.31	2.6(8.4)	GC	2.32 ± 0.75
15	0.055	0.049	2.8	57	3.9 (0.32)	1.1	5.7(5.2)	GC	0.67 ± 0.74
15g	4.71	6.59	>10		1.6 (0.77)	0.56	2.9(5.2)	GC	1.29 ± 0.75
15h	0.022	0.0017	1.0	590	3.5 (0.36)	0.79	3.2(4.1)	GC	1.50 ± 0.74
Mx	0.010	0.046	1.29	28	19 (0.053)	24	32(1.3)	GC?	
Dx	0.026	1.0	91	91					

[a] CT-DNA, AT, and GC refer to calf thymus DNA, [poly(dA-dT)]$_2$, and [poly(dG-dC)]$_2$, respectively. [b] Log P is the calculated value (log$_{10}$) of the octanol/water partition coefficient. [c] Drug concentration required to inhibit cell growth by 50%. [d] RI, resistance index is the IC$_{50}$ ratio of LoVo/Dx on LoVo. [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] In parentheses are the C_{50} values. [g] In parentheses are the values of [GC]/[AT] ratio. [h] The binding site preference is considered to be significant only for [GC]/[AT] ratio differing by >30% from the sequence-neutral unity value (i.e., <0.7 or >1.3). [i] Data from ref 1.

pound **15h**, is the most potent cytotoxic agent in the pyrimidoacridine series, comparable to (HT29) or more potent than (LoVo) **1a**, Dx, and Mx itself. Also 9-methoxy derivatives **15b,c** and the 9-amino derivative **15f** are endowed of relevant activity, comparable to that of **1a**, Dx, and Mx. It is noticeable that the 9-nitro derivative **15e** is scarcely cytotoxic, while its reduction product **15f** possesses cytotoxicity 1 order of magnitude higher. The contemporary substitution of 9 and 10 positions is detrimental for cytotoxic activity leading to scarcely (9,10-dimethoxy derivative **15a**) or not (9,10-dihydroxy derivative **15g**) active compounds. Also the presence of two hydroxyl groups, compounds **15d,g**, which make these derivatives hydrophilic and water soluble as free bases, implicates lack of activity.

Regarding the LoVo/Dx (doxorubicin-resistant) cell line, it can be seen that none of the target compounds approach the potency of the best parent compound **1a**, and the resistance index (RI) values clearly indicate that substituent groups in 9 or 9,10 positions are detrimental in overcoming the cross resistance induced by doxorubicin. It is interesting to note that the RI value goes from 1.3 (parent compound **1a**), to 12 (**15e**, 9-nitro derivative of **1a**), to 38 (**15b**, 9-methoxy derivative of **1a**), to 57 (**15f**, 9-amino derivative of **1a**), to 590 (**15h**, 9-hydroxy derivative of **1a**), showing that also the hydrophilicity of the substituent group in position 9 is important. Also the lack of the strong intramolecular hydrogen bond between the carbonyl in position 7 and the hydrogen of the amine in position 6 influences negatively the RI which increases from 1.3 (**1a**) to 14 (**7**, *N*-methyl derivative of **1a**). Moreover, the demethylation of the distal nitrogen of the side chain in 6 position (compare the structure of **1a** and **9**) implicates an increase of RI from 1.3 to 18, and the 6-aminoalkylamino derivative **9** becomes cross-resistant with Dx on this cell line.

The log values of octanol/water partition coefficients (log P) for pyrimidoacridines of Table 2 have also been calculated using ACD/Labs software (Advanced Chemistry Development, Inc., Toronto, Ontario M5H 2L3, Canada). It can be noted that bis-functionalized pyrimidoacridines with log P values < 1 are characterized by low cytotoxicity against LoVo/Dx cell line. However, attempts to quantitatively correlate log P to cytotoxicity values or to resistance index failed. It can be noted also a qualitative correspondence between binding site preference ([GC]/[AT] ratio values) and RI. Pyrimidoacridines with [GC]/[AT] values > 2.9 are cross-resistant with Dx on the LoVo/Dx cell line.

The attempt to find a quantitative correlation between binding (log C_{50} values) with CT-DNA and in vitro activity (log IC$_{50}$ values) on the HT29 cell line of compounds **1a−e**, **5−7**, **9**, and **15a−f,h** is described in Figure 2. There is some grade of correlation ($r = 0.828$) for all pyrimidoacridines of Table 2, except derivative **15g**, which is never included in this kind of correlation. In fact, **15g** is a good DNA binder but devoid of cytotoxic activity against all cell lines tested. It may be hypothesized that the 9,10-hydroxy groups do not interfere with DNA in the binding process (the K_{app} value is very similar to that of 9,10-unsubstituted **1a**) but make the derivative hydrophilic; in fact the compound is water soluble also as free base, and perhaps for this reason it cannot be taken up into cells or is rapidly metabolized. Similar behavior is observed in regard to LoVo sensitive cell line with a regression line, according to the equation log C_{50} = 0.430 log IC$_{50}$ + 0.493 (r = 0.745). No correlation has been found regarding the LoVo/Dx cell line.

In vitro cytotoxic potencies of new pyrimidoacridines, **5**, **7**, **9**, and **15a−h**, and of reference compounds, cisplatin (Cs) and Dx, against five human ovarian carcinoma (A2780 sensitive, A2780cisR (cisplatin-
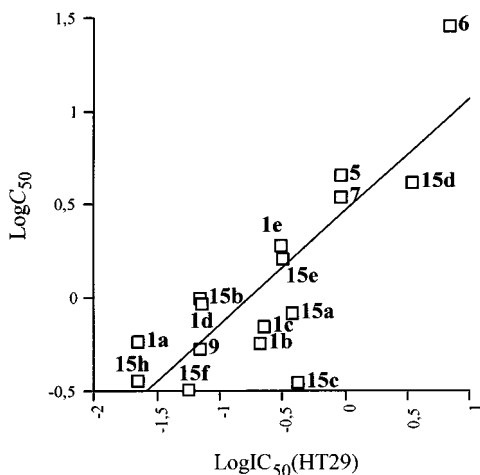
Figure 2. Correlation between log C_{50} (CT-DNA) and log IC$_{50}$ (HT29), according to the equation: log $C_{50} = 0.607$ log IC$_{50}$ + 0.460 ($r = 0.828$), for all pyrimidoacridines of Table 2, except derivative **15g**.

Figure 3. Correlation between log C_{50} (CT-DNA) and log IC$_{50}$ (SKOV-3), according to the equation: log $C_{50} = 0.565$ log IC$_{50}$ + 0.272 ($r = 0.919$), for target pyrimidoacridines of Table 3, except derivative **15g**.

Table 3. In Vitro Cytotoxicity (IC$_{50}$)^a against Five Human Ovarian Carcinoma (A2780, A2780cisR, CH1, CH1cisR, and SKOV3) Cell Lines of Target Compounds **5**, **7**, **9**, and **15a−h** and of Reference Compounds Cisplatin (Cs) and Doxorubicin (Dx)

compd	A2780	A2780cisR	RI^b	CH1	CH1cisR	RI^b	SKOV-3
5	2.1	2.0	0.95	1.4	2.45	1.7	3.85
7	0.12	0.16	1.3	0.35	0.41	1.2	1.35
9	0.021	0.022	1.1	0.020	0.022	1.1	0.046
15	0.14	0.32	2.3	0.34	0.35	1.0	0.56
15b	0.023	0.031	1.3	0.056	0.47	8.4	0.16
15c	0.046	0.049	1.1	0.058	0.076	1.3	0.14
15d	2.1	1.3	0.62	0.96	2.2	2.3	4.7
15e	0.11	0.11	1.0	0.24	0.27	1.1	0.58
15f	0.022	0.026	1.2	0.026	0.027	1.0	0.12
15g	1.6	1.4	0.87	1.1	1.3	1.2	3.4
15h	0.0041	0.0055	1.3	0.0076	0.015	2.0	0.042
Cs	0.89	3.4	3.8	0.15	2.4	16	3.4
Dx	0.0096	0.017	1.8	0.0063	0.45	71	0.078

^{a} Drug concentration (μM) required to inhibit cell growth by 50%. ^{b} See Table 2.

resistant), CH1, CH1cisR (cisplatin-resistant), and SKOV-3) cell lines are reported in Table 3. Also regarding the A2780, CH1, and SKOV-3 cell lines, observations similar to that previously reported for HT29 and LoVo cells can be made: (i) it is confirmed that only one side chain in position 6, compound **5**, is not sufficient for optimal activity; (ii) the cytotoxicity of the *N*-methyl derivative **7** is not relevant confirming the importance of an intramolecular hydrogen bond between the carbonyl in position 7 and the hydrogen of the amine in position 6; (iii) compound **9**, with the aminoethylamino side chain in position 6, shows very interesting values of cytotoxicity; (iv) again the best cytotoxic agent in the series is compound **15h**, with IC$_{50}$ values in the nanomolar range lower than Dx itself, and derivatives **15b,c,f** possess relevant activity; also in these cases the reduction of NO$_2$ to NH$_2$ group (**15e** to **15f**) increases the cytotoxicity by almost 1 order of magnitude, while the contemporary presence of two hydroxyl groups (**15d,g**) produces lack of activity.

However, the most relevant feature on ovarian carcinoma cells seems to be the activity of the target derivatives in regard to the resistant cell lines A2780cisR and CH1cisR. The RI values are often close to 1 clearly
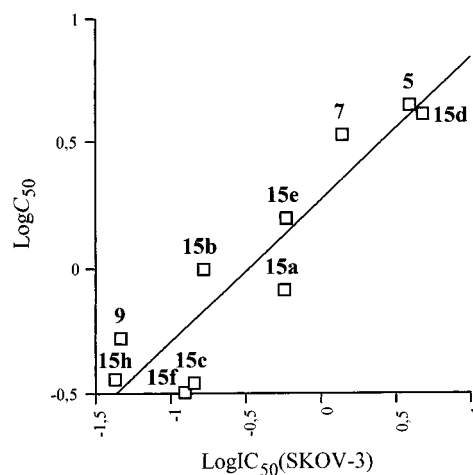
indicating that these compounds are not affected by cross resistance induced by Cs on these cell lines.

In Figure 3 a good correlation ($r = 0.919$) between binding (log C_{50} values) with CT-DNA and in vitro activity (log IC$_{50}$ values) on the SKOV-3 cell line of target compounds, except **15g**, is described. Very similar behavior is shown with A2780, A2780cisR, CH1, and CH1cisR cell lines, with r values 0.850, 0.838, 0.888, and 0.895, respectively.

Conclusions

The present study allows us to conclude the following: (i) Both basic side chains are needed for cytotoxic potency and efficient DNA binding. (ii) The fifth ring mimed by an intramolecular hydrogen bond plays an important role either in cytotoxic activity and in DNA binding. This hypothesis has been confirmed by the synthesis of new derivatives in which there is a formal fifth ring in the chromophore moiety, and these compounds are extremely potent cytotoxic agents, with IC$_{50}$ values in the sub-nanomolar range.[9] (iii) The 6-aminoethylamino side chain seems to be of relevant interest also for the pyrimidoacridine class. (iv) The substitution at 9 and 9,10 positions does not give univocal results. The 9,10-disubstituted compounds **15a,g** show DNA binding similar to unsubstituted **1a**, but much lower cytotoxicity. The 9-substituted derivatives of **1a**, **15b,e,f,h**, present interesting values of cytotoxicity, sometimes better than the parent compound, with the exception of 9-nitro derivative **15e**, which is scarcely cytotoxic: in the 9-substituted series the polarity of the group plays a role in DNA binding (K_{app} values of 0.79 $\times 10^7$, 1.3×10^7, 3.5×10^7, and 3.9×10^7 with growing π values of corresponding 9-nitro, 9-methoxy, 9-hydroxy, and 9-amino groups). The 9-substituted derivatives of **1d**, **15c,d**, present very different values of cytotoxicity and DNA binding: the 9-methoxy **15c** shows relevant capacity of binding DNA ($K_{app} = 3.6 \times 10^7$, >3 times the **1d** value) and relevant cytotoxic activity on many of the tested cell lines; instead, the 9-hydroxy derivative **15d** shows a $K_{app} = 0.31 \times 10^7$, <3 times the **1d** value, and is scarcely or not cytotoxic against all the cell lines tested suggesting that the contemporary presence of two

hydroxy groups, one on the chromophore moiety and the other on the side chain in position 6, is detrimental for both DNA binding and cytotoxic activity, differently from **15g** where the contemporary presence of two hydroxy groups in the chromophore moiety is detrimental only for cytotoxic activity. (v) In regard to the cytotoxic activity on resistant cell lines, the chromophore substitution in 9 or 9,10 positions, compounds **15a−h**, as well as the introduction of an aminoethylamino side chain in position 6, compound **9**, or the N-methylation of the proximal nitrogen of the side chain in position 6, compound **7**, lead to different results in the three cell lines studied. While all the new bis-functionalized compounds show inferior capacity compared to parent compounds in overcoming cross resistance induced by Dx in the LoVo/Dx cell line with RI values in the range of 12−650, they overcome the cross resistance induced by Cs in A2780cisR and CH1cisR cell lines with many RI values close to 1. This fact is not surprising, since the mechanism of resistance induced by doxorubicin on LoVo/Dx cells and by cisplatin on A2780cisR and CH1cisR cells is different. A faster drug efflux on LoVo/Dx cells than on the sensitive line was observed,[10] perhaps due to P-glycoprotein overexpression in the resistant cells. Notably, the two acquired cisplatin-resistant cell lines have been shown to differ in their primary mechanisms of resistance: CH1cisR being resistant due to enhanced DNA repair or tolerance to platinum−DNA adducts and A2780cisR being resistant due to a combination of decreased drug transport, increased intracellular glutathione levels, and enhanced DNA repair.[11] Anyway a very important characteristic of the pyrimido[5,6,1-*de*]acridine derivatives is that, in the complex, they constitute a class of cytotoxic agents capable to overcome cross resistance induced by different drugs in different cell lines and acting by different mechanism of resistance. (vi) Among the new derivatives, three compounds, **9** and **15f**,**h**, emerge as leads for their cytotoxic potency and large spectrum of action.

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$_4$Si in the solvent shown. The following NMR abbreviations are used: br (broad), s (singlet), d (doublet), dd (double doublet), t (triplet), q (quartet), m (multiplet), ar (aromatic proton), ex (exchangeable with D$_2$O), d ex (exchangeable with D$_2$O, but with difficulty). Elemental analyses were performed on a model 1106 elemental analyzer (Carlo Erba Strumentazione); all analytical values for C, H, and N were within ±0.4% of the theoretical values.

1-Chloro-9-oxo-9,10-dihydro-4-acridinecarboxamide (3). The carboxylic acid **2**[2] (1 g, 3.65 mmol) and 1,1′-carbonyldiimidazole (1.1 g, 6.8 mmol) in DMF (10 mL) were stirred until it became homogeneous. The mixture was cooled to 10 °C, and a saturated solution of NH$_3$ in CHCl$_3$ (5 mL) was added. After 30 min at room temperature, the precipitate was filtered and washed with Et$_2$O to give crude **3** (0.8 g, 81%), which was used for the next step.

6-Chloro-2,3-dihydro-1*H*,7*H*-pyrimido[5,6,1-*de*]acridine-1,3,7-trione (4). Compound **3** (0.8 g, 2.93 mmol) was refluxed with ClCOOEt (20 mL) for 4 days. The solvent was evaporated to give a residue which was washed with hot MeOH (3 × 5 mL) to yield the crude pyrimidoacridine **4** (0.44 g, 51%), which was used for the next step.

6-[[2-(Dimethylamino)ethyl]amino]-2,3-dihydro-1*H*,7*H*-pyrimido[5,6,1-*de*]acridine-1,3,7-trione (5). Example of General Procedure for Preparation of 5, 7, 8, and 15a−d. A mixture of the 6-chloro derivative **4** (0.25 g, 0.84 mmol) and *N,N*-dimethylethylenediamine (0.25 g, 2.85 mmol) in CHCl$_3$ (10 mL) was stirred for 15 days at room temperature. The reaction mixture was partitioned between CHCl$_3$ (70 mL) and an excess of 1 M aqueous Na$_2$CO$_3$ (50 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 (49:1 v/v) to afford pure **5**: ^{1}H NMR (DMSO-d_6) δ 2.31 (s, 6H, 2 × CH$_3$), 2.67 (t, 2H, CH$_2$), 3.40 (q, 2H, CH$_2$), 6.41 (d, 1H, ar), 7.30 (t, 1H, ar), 7.67−7.80 (m, 2H, ar), 7.95 (d, 1H, ar), 8.20 (d, 1H, ar), 10.98 (br t, 1H, 6-NH, d ex), 11.11 (br s, 1H, 2-H, ex).

The pyrimidoacridines **7**, **8**, and **15a−d** were prepared in a similar manner by analogous treatment of the appropriate 6-chloro derivative with the suitable amine.

6-[(2-Aminoethyl)amino]-2-[2-(dimethylamino)ethyl]-2,3-dihydro-1*H*,7*H*-pyrimido[5,6,1-*de*]acridine-1,3,7-trione (9). The derivative **8** (0.09 g, 0.18 mmol) and aqueous HCl (1 mL of 37% w/w) in dioxane (10 mL) were stirred for 2 h at room temperature. The residue of evaporation was crystallized from absolute EtOH to yield **9** as the dihydrochloride: ^{1}H NMR (DMSO-d_6) δ 2.91 (s, 6H, 2 × CH$_3$), 3.02−3.17 (m, 2H, CH$_2$), 3.42−3.56 (m, 2H, CH$_2$), 3.70−3.87 (m, 2H, CH$_2$), 4.40 (t, 2H, CH$_2$), 7.10 (d, 1H, ar), 7.60 (t, 1H, ar), 7.85 (t, 1H, ar), 8.04−8.25 (m, 4H, 1 ar + NH$_3^+$ ex), 8.31 (d, 1H, ar), 8.75 (d, 1H, ar), 10.10 (br s, 1H, N$^+$−H, ex), 11.74 (br t, 1H, NH, l ex).

Methyl 1-Chloro-6,7-dimethoxy-9-oxo-9,10-dihydro-4-acridinecarboxylate (10). A suspension of methyl 2-amino-4-chlorobenzoate[2] (0.8 g, 4.3 mmol), commercially available 2-bromo-4,5-dimethoxybenzoic acid (1.1 g, 4.3 mmol), and Cu-(OAc)$_2$·H$_2$O (0.86 g, 4.3 mmol) in 1-methyl-2-pyrrolidinone (5 mL) and *N,N*-diisopropylethylamine (10 mL) was stirred for 6 h at 160 °C. After cooling, dilution with water, and acidification to pH 2, the dried collected precipitate was stirred with Et$_2$O and then filtered to give a residue which was refluxed with PPE (40 g) in CHCl$_3$ (50 mL) until all solid was dissolved. The reflux condenser was removed, and the CHCl$_3$ was allowed to evaporate to give an oil which was heated for 1 h at 100 °C. The mixture was diluted cautiously with MeOH (5 mL) and water (10 mL) and then extracted with CHCl$_3$ (3 × 20 mL). The concentrated chloroform extracts were chromatographed on silica gel using CHCl$_3$/C$_6$H$_6$ (9:1 v/v) to yield the ester **10** (0.71 g, 47%): mp 277−278 °C; ^{1}H NMR (CDCl$_3$) δ 3.95 (s, 3H, CH$_3$), 4.00−4.01 (m, 6H, 2 × CH$_3$), 6.70 (s, 1H, ar), 7.18 (d, 1H, 2-H, ar), 7.75 (s, 1H, ar), 8.10 (d, 1H, 3-H, ar), 12.05 (br s, 1H, NH, ex).

1-Chloro-6,7-dimethoxy-9-oxo-9,10-dihydro-4-acridinecarboxylic Acid (10). The ester **10** (1 g, 2.88 mmol) was suspended in EtOH (100 mL) and 2 M NaOH (100 mL) and heated under reflux for 30 min. The resulting mixture was acidified with 4 M HCl and stirred at room temperature for 20 min; the precipitate was collected and washed with water, MeOH, and Et$_2$O to give crude **11** (0.72 g, 75%) which was used for next step.

***N*4-[2-(Dimethylamino)ethyl]-1-chloro-6,7-dimethoxy-9-oxo-9,10-dihydro-4-acridinecarboxamide (12a).** The carboxylic acid **11** (1 g, 3.0 mmol) and 1,1′-carbonyldiimidazole (1.1 g, 6.8 mmol) in DMF (10 mL) were stirred until became homogeneous. The mixture was cooled to 10 °C, and *N,N*-dimethylethylenediamine (0.88 g, 10 mmol) was added. After 15 min at room temperature, the mixture was partitioned between CHCl$_3$ and aqueous 1 M Na$_2$CO$_3$. The organic layer was worked up to give a residue, which solidified on washing with Et$_2$O to yield pure carboxamide **12a** (0.91 g, 75%): mp 221−222 °C; ^{1}H NMR (CDCl$_3$) δ 2.35 (s, 6H, 2 × CH$_3$), 2.60 (t, 2H, CH$_2$), 3.50−3.60 (m, 2H, CH$_2$), 4.00−4.01 (m, 6H, 2 × O−CH$_3$), 6.71 (s, 1H, ar), 7.18 (d, 1H, 2-H, ar), 7.30 (br s, 1H, CO−NH ex), 7.68 (d, 1H, 3-H, ar), 7.80 (s, 1H, ar), 12.72 (br s, 1H, NH, ex).

6-Chloro-2-[2-(dimethylamino)ethyl]-9-methoxy-2,3-dihydro-1*H*,7*H*-pyrimido[5,6,1-*de*]acridine-1,3,7-trione (13b).

Example of General Procedure for Preparation of 13a and 15e. To a mixture of **12b** (0.7 g, 1.87 mmol) and anhydrous Et_3N (0.76 g, 7.48 mmol) in $CHCl_3$ (20 mL) was added a solution of $COCl_2$ (20% in toluene, 1.87 mL, 3.74 mmol) in $CHCl_3$ (10 mL) dropwise at 0 °C. After stirring at room temperature for 15 min, the mixture was partitioned between $CHCl_3$ and aqueous 1 M Na_2CO_3. The organic layer was worked up to give a residue, which solidified on washing with Et_2O to yield pure **13b** (0.62 g, 76%): mp >300 °C; ^1H NMR (DMSO-d_6) δ 2.90 (s, 6H, 2 × CH_3), 3.42−3.56 (m, 2H, CH_2), 3.95 (s, 3H, O−CH_3), 4.42 (t, 2H, CH_2), 7.51 (dd, 1H, ar), 7.65 (s, 1H, ar), 7.77 (d, 1H, ar), 8.43 (d, 1H, ar), 8.53 (d, 1H, ar), 10.15 (br s, 1H, N^+−H, ex).

The pyrimidoacridines **13a** and **15e** were prepared in a similar manner by analogous treatment of **12a** and **14**, respectively. Compound **15e** was purified by chromatography on silica gel using $CHCl_3$/MeOH (9:1 v/v).

2-[2-(Dimethylamino)ethyl]-6-[[2-(dimethylamino)ethyl]amino]-9,10-dihydroxy-2,3-dihydro-1H,7H-pyrimido-[5,6,1-de]acridine-1,3,7-trione (15g). Example of General Procedure for Preparation of 13c and 15h. Compound **15a** (0.13 g, 0.27 mmol) in 48% HBr (50 mL) was refluxed for 1 h. The reaction mixture was partitioned between $CHCl_3$ and 0.5 M aqueous Na_2CO_3. Workup of the organic phase and flash chromatography on silica gel with $CHCl_3$/MeOH (1:1 v/v) and 32% aqueous NH_3 (10 mL for 1 L of eluent) afforded **15g**: ^1H NMR (DMSO-d_6) δ 2.18 (s, 6H, 2 × CH_3), 2.20 (s, 6H, 2 × CH_3), 2.47−2.59 (m, 4H, 2 × CH_2), 3.30−3.41 (m, 2H, CH_2), 4.07 (t, 2H, CH_2), 4.60−4.62 (m, 2H, 2 × OH), 6.73 (d, 1H, ar), 7.48 (s, 1H, ar), 7.99 (d, 1H, ar), 8.10 (s, 1H, ar), 10.90 (br t, 1H, NH, ex).

By analogous treatment of **13b** and **15b** and by cooling the reaction mixture, the pyrimidoacridines **13c** and **15h** were obtained directly as the hydrobromide and dihydrobromide, respectively. These salts were purified by washing sequentially with MeOH, EtOH, and then Et_2O.

9-Amino-2-[2-(dimethylamino)ethyl]-6-[[2-(dimethylamino)ethyl]amino]-2,3-dihydro-1H,7H-pyrimido[5,6,1-de]acridine-1,3,7-trione (15f). A mixture of 9-nitro derivative **15e** (0.08 g, 0.17 mmol), Pd/C (0.04 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 diluted with MeOH (30 mL) and filtered, and the filtrate was evaporated to yield a residue which was crystallized (absolute EtOH) to give **15f·**$3HCl$: (DMSO-d_6) δ 2.85 (d, 6H, 2 × CH_3), 2.90 (d, 6H, 2 × CH_3), 3.20−3.52 (m, 4H, 2 × CH_2), 3.55−5.00 (m, 7H, 2 × CH_2 + NH_3^+ ex), 7.10 (d, 1H, ar), 7.40 (dd, 1H, ar), 7.82 (s, 1H, ar), 8.19 (d, 1H, ar), 8.65 (d, 1H, ar), 10.31 (br s, 1H, N^+−H, ex), 10.70−10.96 (m, 2H, NH + N^+−H, ex).

Biophysical Evaluation. 1. Fluorescence Binding Studies. The fluorometric assays have been described previously.[6] 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.[6,7]

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: $K_{app} = (1.26/C_{50}) \times K_{ethidium}$, with a value of $K_{ethidium} = 10^7$ M^{-1} for ethidium bromide.[7a]

2. In Vitro Cytotoxicity. A. Human Colon Adenocarcinoma Experimental Protocol. Established details of human colon adenocarcinoma cell lines (HT29, LoVo sensitive, and LoVo/Dx Resistant) have been previously described.[3,10,12]

Drug solutions of appropriate concentrations were added to a culture containing HT29 cells at 2.5×10^4 cells/mL of medium[12] or to a culture containing LoVo or LoVo/Dx cells at 2.5×10^5 cells/mL of medium.[3] All assays were performed in duplicate.

2. B. Human Ovarian Carcinoma Experimental Protocol. Established details and biological properties of human ovarian carcinoma cell lines (A2780, A2780cisR, CH1, CH1cisR, and SKOV-3) have been described previously.[11] The sulforhodamine B (SRB) experimental protocol used has been described previously.[6,11] Cells were plated (100−5000 cells) in 96-well microtiter plates and left overnight to adhere prior to drug treatment. Aqueous drug solutions at pH 7.0 were then added to the cells at various concentrations following dilution of a stock DMSO solution. After 96 h of continuous drug exposure at 37 °C, growth inhibition was assessed using SRB protein staining. IC$_{50}$ values (drug dose required for 50% growth inhibition compared to drug-free controls) were determined by comparing treated and untreated cells.

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