Synthesis and Biological Evaluation of New Asymmetrical Bisintercalators as Potential Antitumor Drugs

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The good results obtained in the past decade with various types of potential bisintercalating agents, e.g., LU 79553, DMP 840, BisBFI, MCI3335, WMC-26, BisAC, BisPA, and the asymmetrical derivative WMC-79 (Chart 1), prompted us to investigate a new series of asymmetrical bisintercalators, compounds **1a**−**t** (Chart 2), which can combine the potentiality of bisintercalation with a possible different mechanism of action due to two diverse chromophores. The DNA-binding properties of these compounds have been examined using fluorometric techniques: target compounds are excellent DNA ligands, with a clear preference for binding to AT-rich duplexes. In vitro cytotoxicity of these derivatives toward human hormone-refractory prostate adenocarcinoma cell line (PC-3) is described. Apoptosis assays of four selected compounds are also reported. Very potent cytotoxic compounds, some of them capable of inducing early apoptosis, have been identified.

Introduction

Some new potential anticancer drugs are composed of two planar intercalating monomers connected by an appropriate linker to give bis derivatives, which generally present higher DNA affinity and prolonged drug residence time in DNA with respect to the monomer. Thus, we can expect to enhance the biological properties of monointercalators. In the past decade, on the basis of this rationale, many positive results have been achieved with various types of potential bisintercalating agents. In Chart 1 are reported some successful examples: bis(benzoisoquinolines) LU 79553[1] and DMP 840,[2] bis(benzofuroisoquinolines) BisBFI[3] and MCI3335,[4] bis(imidazoacridine) WMC-26,[5] bis(acridine-4-carboxamide) BisAC,[6] bis(pyrimidoacridine) BisPA,[7] and the asymmetrical bis derivative WMC-79.[8] In particular, LU 79553 has shown potent antitumor activity and has been positively tested in the clinic for the treatment of solid tumors;[1a,b] it does form bisintercalation complexes,[1c] and it binds selectively to alternating purine−pyrimidine motifs, particularly those containing GpT (ApC) and TpG (CpA) steps,[1d] but only weakly inhibits topoisomerase II.[1a] Also DMP 840 has demonstrated high-level antitumor activity and has reached clinical trials.[2a] It is a potent DNA binder, but appears to be only a monointercalator;[2b] the capacity to inhibit topoisomerase II seems responsible for its cytotoxicity.[2c] BisBFI showed promising in vitro and in vivo antiproliferative activity, was more potent than parent compound LU 79553, and seems to behave as a bisintercalator.[3] MCI3335 has been shown to display a very high cytotoxicity toward CEM human leukemia cells. The high cytotoxic potential of MCI3335 is attributed to its enhanced capacity to bind to DNA with a marked preference for GC sites, but it does not promote DNA cleavage by topoisomerase I or II.[4] WMC-26 showed high and selective biological activity against colon cancer in the NCI in vitro screening, further confirmed in vivo in nude mice bearing colon adenocarcinoma xenografts; preliminary experimental and modeling results suggested that bifunctional WMC-26 does not bind to DNA by bisintercalation.[5a] Further studies showed that WMC-26 binds to DNA in an unsymmetrical fashion, wherein one aromatic residue intercalates into DNA, whereas the other binds to a groove; furthermore, WMC-26 is endowed with some sequence selectivity, preferring to bind to GC-rich DNA.[5b] BisAC and BisPA exhibited intriguing biological properties: high DNA affinity, a remarkable preference for binding to AT-rich duplexes, very broad and potent spectrum of cytotoxic activity.[6,7]

The hypothesis previously reported of an unsymmetrical binding to DNA for WMC-26 focused our interest on asymmetrical dimeric compounds constituted by two different chromphores properly linked. So far, very few examples of asymmetrical bis derivatives have been reported in the literature.[8−10] However, this appears an intriguing approach which may combine the potentiality of bisintercalation with a possible different mechanism of action of two diverse chromophores. The asymmetrical bis derivative WMC-79 (Chart 1) is a DNA-binding agent endowed with potent and selective antitumor activity. This compound induces apoptosis in sensitive cells at low nanomolar concentrations and positive in vivo activity in a human colon cancer xenograft in nude mice.[8]

Thus (Chart 2), we designed and synthesized a series of asymmetrical bis derivatives, compounds **1a**−**t** with two different chromophores, usually connecting the reagents shown by the polyamine, found to be the most appropriate among our bisintercalators.[6,7] In a more detailed manner, we can say that compounds **1a**−**c** represent a mix of BisAC and BisPA (AC−PA subseries), compounds **1d**−**k** are a mix of BisAC and LU 79553 (AC−MIT subseries), and compounds **1l**−**r** are a mix of BisPA and LU 79553 (PA−MIT subseries); compound **1s** is structurally related to WMC-79, while **1t** possesses a linker different from those of the other target compounds, but similar to that of LU 79553.

Chemistry

Schemes 1 and 2 show the synthetic pathways leading to the target derivatives **1a**−**t**. According to Scheme 1, the suitable 1-chloroacridone-4-carboxamides **2a**−**e**[11−13] were allowed to react with the bis(3-aminopropyl)methylamine in 2-ethoxyethanol and triethylamine at 80 °C to afford the desired intermediates

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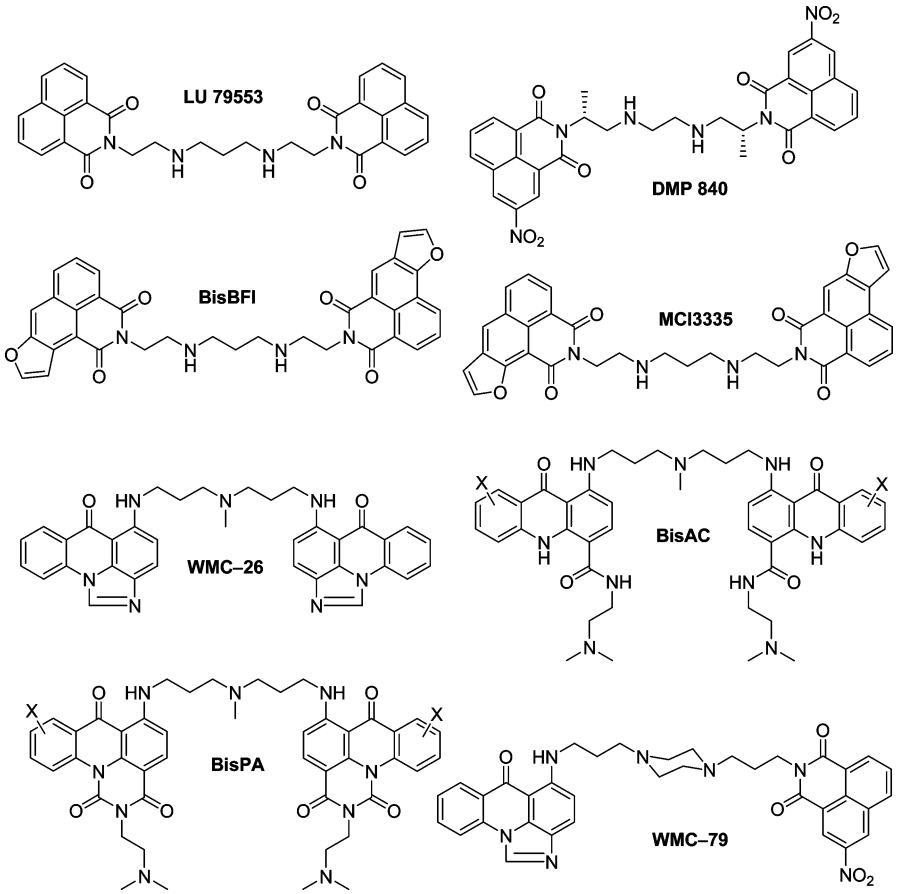
Chart 1. Structures of LU 79553, DMP 840, BisBFI, MCI3335, WMC-26, BisAC, BisPA, and Asymmetrical Bis Derivative WMC-79



6a−**e**; in a similar way, **6f** was obtained from **2a** and 1,4,7,-10-tetraazadecane. Target compounds **1a,b** were obtained by condensation of **3a**[12] with **6a**,**b**, respectively, in the above experimental conditions, while target compounds **1d**−**k** and **1t** were prepared by treatment of the suitable intermediates **6a**−**f** either with **5a** or with **5b** in 2-ethoxyethanol at 80 °C.

As shown in section A of Scheme 2, the reaction of the appropriate 6-chloro-2,3-dihydro-1*H*,7*H*-pyrimido[5,6,1-*de*]acridine-1,3,7-triones **3a**−**d**[12−14] with bis(3-aminopropyl)methylamine, in ethoxyethanol and triethylamine at 80 °C, gave the compounds **7a**−**d**. In the same experimental conditions, treatment of **7d** with **2e**[11] yielded **1c**, showing that the AC−PA subseries may be prepared starting from either compound **2** or compound **3**. Target compounds **1l**−**r** were obtained by reaction of the suitable intermediates **7a**−**d** either with **5a** or with **5b** in 2-ethoxyethanol at 80 °C. Finally, section B of Scheme 2 describes the condensation of (i) 5-chloro-8-methyl-6*H*-[1,2,3]-triazolo[4,5,1-*de*]acridin-6-one (**4**)[15] with bis(3-aminopropyl)-methylamine to yield **8** and (ii) **8** with **5a** to give target compound **1s**, always in 2-ethoxyethanol at 80 °C.

All target compounds **1a**−**t** were examined as water-soluble hydrochloride salts, prepared by the usual methods, to estimate their DNA-binding properties and their antineoplastic activity.

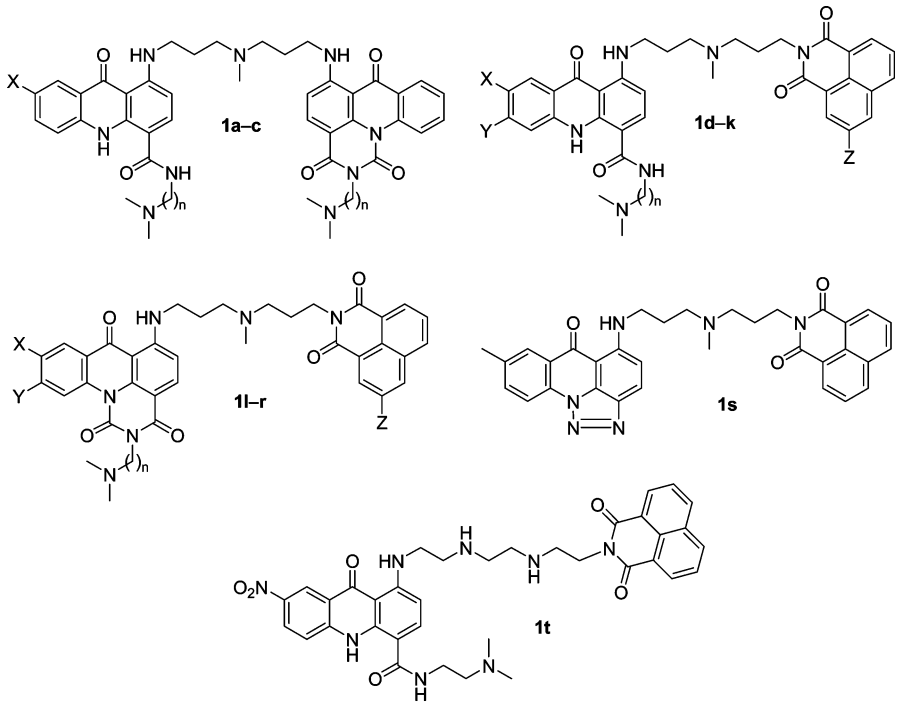
Results and Discussion

DNA-Binding Properties. As shown in Table 1, competitive displacement (C_{50}) fluorometric assays[16] with DNA-bound ethidium was used (a) to determine "apparent" equilibrium constants (K_{app}) for drug binding, as the C_{50} value is approximately inversely proportional to the binding constant,[17] and (b) to establish possible base- or sequence-preferential binding.[18] In the present study, fluorescence displacement assays were performed at pH 7 to enable comparison in biological conditions.

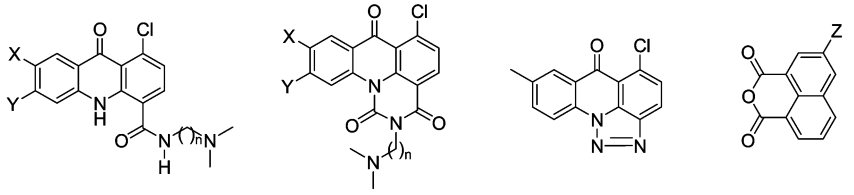
In Table 1 are reported the K_{app} values, related to CT-DNA, AT, and GC, of the new derivatives **1a**−**t**. Mitonafide,[1d] a monomeric naphthalimide the chromophore of which is the base of many bis derivatives reported here, was chosen as a reference drug. Mitonafide binds tightly to DNA with a marked GC specificity,[19] and its K_{app} value related to CT-DNA was determined. The results indicate that target compounds possess excellent DNA affinity, generally greater or much greater than that of ethidium and mitonafide. Some considerations can be made about the CT-DNA K_{app} values. (i) In the AC−PA subseries, compounds **1a**−**c**, it may be evinced first that **1a,b** are the strongest DNA binders among all target derivatives and second that the nature of substituent X does not affect the DNA affinity, whereas the concurrent lengthening of the carboxylic and imidic side chains is detrimental for DNA binding. (ii) In the AC−MIT subseries, compounds **1d**−**k** and **1t**, we can say that a nitro group as the Z substituent and a methoxy group as the substituent X significantly increase the DNA affinity, whereas two methoxy groups as substituents X and Y significantly decrease the affinity. Comparing the pair **1f** ↔ **1t**, endowed with equal chromophores and different linkers, it can be outlined that the linker of **1t** seems much more efficient in terms of DNA binding. (iii) In the PA−MIT subseries, compounds **1l**−**r**, what was previously observed concerning substituent groups can be confirmed. (iv) Finally, compound **1s** is the weakest DNA ligand of all target derivatives.

Generally, the binding behavior of target compounds with synthetic polynucleotides reflects what we observed for CT-DNA. A clear, very remarkable in some cases, preference for binding to AT-rich duplexes is to be outlined, with the only

Chart 2. Structures of Target Compounds **1a**−**t** and Reagents **2**−**5**[a]



Reagents



2a (X=Y=H; n=2)
2b (X=NO_2; Y=H; n=2)
2c (X=OMe; Y=H; n=2)
2d (X=Y=OMe; n=2)
2e (X=Y=H; n=3)

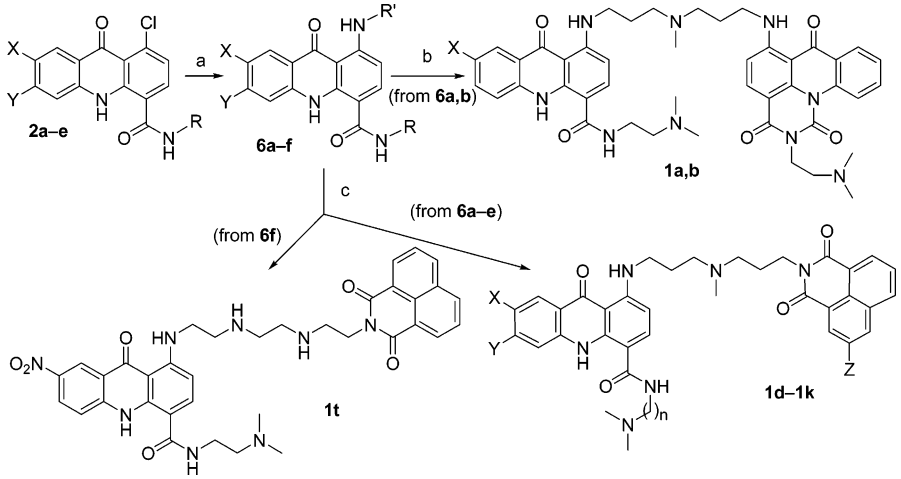
3a (X=Y=H; n=2)
3b (X=OMe; Y=H; n=2)
3c (X=Y=OMe; n=2)
3d (X=Y=H; n=3)

4

5a (Z=H)
5b (Z=NO_2)

[a] For substituents of **1a**−**r** see Table 1.

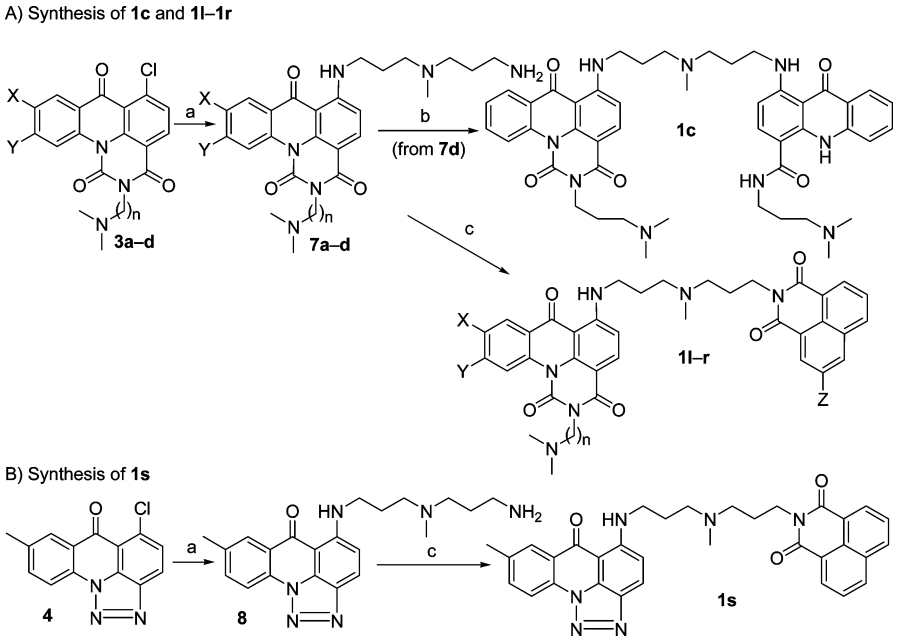
Scheme 1[a]



[a] Reagents and conditions: (a) bis(3-aminopropyl)methylamine for **6a**−**e** or 1,4,7,10-tetraazadecane for **6f**, in 2-ethoxyethanol/Et_3N, 80 °C; (b) **3a** in 2-ethoxyethanol/Et_3N, 80 °C; (c) **5a** for **1d**,**f**,**h**,**i**,**j**,**t** or **5b** for **1e**,**g**,**k**, in 2-ethoxyethanol, 80 °C. Substituents: X = Y = H for **6a**,**f**, X = NO_2 and Y = H for **6b**, X = OMe and Y = H for **6c**, X = Y = OMe for **6e**, R = $(CH_2)_2N(CH_3)_2$ for **6a**−**d** and **6f**, R = $(CH_2)_3N(CH_3)_2$ for **6e**, R′ = $(CH_2)_3N(CH_3)(CH_2)_3NH_2$ for **6a**−**e**, R′ = $(CH_2)_2NH(CH_2)_2NH(CH_2)_2NH_2$ for **6f**. For compounds **1** see Table 1.

exception compound **1t**, devoid of any preference. However, this is not surprising for the subseries **1a**−**c**, a mix of BisAC and BisPA which present the same binding site preference,[6,7] but also compounds **1d**−**t** with a chromophore moiety similar

Scheme 2[a]



[a] Reagents and conditions: (a) bis(3-aminopropyl)methylamine in 2-ethoxyethanol/Et_3N, 80 °C; (b) **2e** in 2-ethoxyethanol/Et_3N, 80 °C; (c) **5a** for **1l**,**n**,**q**,**s** or **5b** for **1m**,**o**,**p**,**r**, in 2-ethoxyethanol, 80 °C. Substituents: X = Y = H for **7a**,**d**, X = OMe and Y = H for **7b**, X = Y = OMe for **7c**, $n = 2$ for **7a**−**c**, $n = 3$ for **7d**. For compounds **1** see Table 1.

Table 1. Substituents,[a] DNA Binding,[b] and Cytotoxic Activity[c] against Human Hormone-Refractory Prostate Adenocarcinoma (PC-3) of Target Derivatives **1a**−**t**[d]

					binding ($K_{app} \times 10^{-7}$ M^{-1})			cytotoxic activity		
compd	X	Y	Z	*n*	AT[e]	CT-DNA	GC	GI_{50}	TGI	LC_{50}
1a	H				26.8 (1.3)	20.7	21.3	0.10	1.0	8.3
1b	NO_2				22.5 (1.9)	20.0	12.1	0.007	0.16	1.6
1c					5.04 (14)	3.39	0.359	0.25	0.60	2.5
1d	H	H	H	2	1.84 (4.1)	1.31	0.444	0.52	2.0	5.4
1e	H	H	NO_2	2	7.12 (7.1)	9.69	1.00	0.60	1.0	4.4
1f	NO_2	H	H	2	8.75 (1.4)	3.30	6.24	0.089	0.63	3.0
1g	NO_2	H	NO_2	2	14.8 (2.1)	9.10	7.08	0.008	1.3	4.1
1h	OMe	H	H	2	5.06 (30)	6.27	0.169	0.00016	0.014	3.6
1i	OMe	OMe	H	2	0.391 (1.3)	0.846	0.303	0.15	3.8	>50
1j	H	H	H	3	3.74 (6.9)	2.32	0.541	0.60	0.99	4.1
1k	H	H	NO_2	3	4.86 (6.1)	4.55	0.792	0.050	0.090	3.6
1l	H	H	H	2	2.57 (2.4)	1.97	1.08	0.023	2.2	8.4
1m	H	H	NO_2	2	4.52 (4.3)	4.01	1.06	0.023	0.010	2.6
1n	OMe	H	H	2	4.81 (4.5)	5.19	1.06	0.051	0.85	4.0
1o	OMe	H	NO_2	2	4.34 (4.8)	5.86	0.913	0.083	0.50	9.3
1p	OMe	OMe	NO_2	2	1.24 (1.6)	0.86	0.792	0.017	0.34	4.0
1q	H	H	H	3	3.13 (3.4)	2.27	0.906	0.42	0.83	3.8
1r	H	H	NO_2	3	11.3 (20)	6.81	0.548	1.70	3.2	6.6
1s					0.452 (1.7)	0.269	0.264	0.017	1.6	5.1
1t					13.1 (none)	14.5	12.5	0.062	0.39	2.5
MIT						0.340		0.15	3.8	56

[a] See the structures in Schemes 1 and 2. [b] CT-DNA, AT, and GC refer to calf thymus DNA, $[poly(dA-dT)]_2$, and $[poly(dG-dC)]_2$, respectively. $K_{app} = 1.26/C_{50} \times 10^7$, in which 1.26 is the concentration (μM) of ethidium in the ethidium−DNA complex, C_{50} is the drug concentration (μM) that effects a 50% drop in the fluorescence of bound ethidium, and 10^7 is the value of K_{app} assumed for ethidium in the complex. [c] Drug concentration (μM) required to inhibit cell growth by 50% (GI_{50}) and by 100% (TGI) or to kill 50% of the initial cell number (LC_{50}) after 48 h of drug exposure. All assays were performed in triplicate. [d] Reference compound mitonafide (MIT). [e] In parentheses is the binding site preference, considered to be significant only for the [A−T]/[G−C] ratio differing by >30% from the sequence-neutral unity value (i.e., <0.7 or >1.3).

to that of mitonafide, which has a marked GC specificity, are AT preferential. A different binding fashion was outlined for LU 79553 and MCI3335, which possess chromophores related to that of mitonafide. Anyway, in the case of BisPA we found an opposite binding preference among monomers and dimers (GC vs AT, respectively),[7] and a different binding mechanism between a monomer and a bis derivative may be assumed, because the linker too can play an important role.

Cytotoxic Activity. In Table 1 in vitro cytotoxic activities of target asymmetrical bis derivatives **1a**−**t** and of reference compound mitonafide against the human hormone-refractory prostate adenocarcinoma cell line (PC-3) are reported. The results are expressed in terms of growth inhibition 50, GI_{50}, and total growth inhibition, TGI, which represent the drug concentration required to inhibit cell growth by 50% and by 100%, respectively, giving an idea of the cytostatic action of the drugs, and lethal concentration 50, LC_{50}, which represents the drug concentration required to kill 50% of the initial cell number, giving an idea of the cytocide action of the drugs. Each quoted value is the mean of triplicate experiments. Almost all

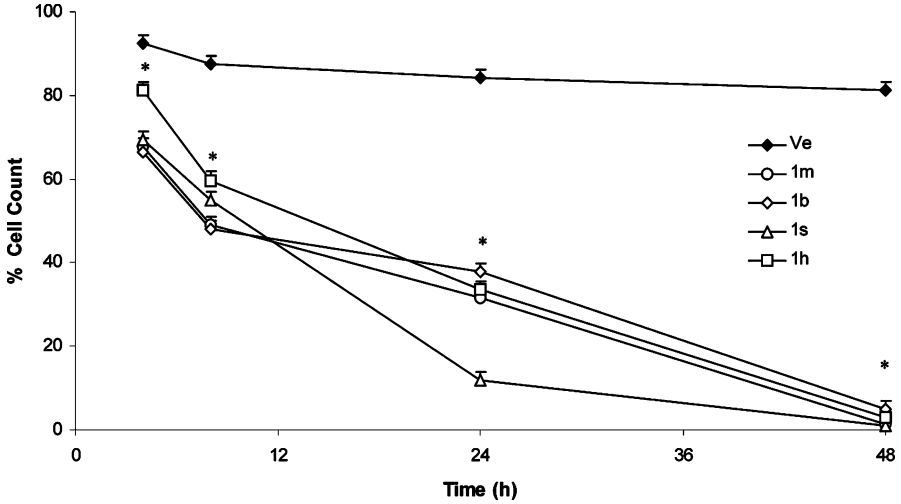


Figure 1. Selected compounds affect the cell viability of PC-3 prostate cancer cells. The viability of PC-3 cells was evaluated by FDA staining. PC-3 cells were treated with vehicle or the LC_{50} of the target compounds at 37 °C, were incubated for 5 min at 37 °C with 0.125 μg/μL FDA, and were analyzed by flow cytometric analysis (Ve = vehicle). Data expressed as the percentage of FDA positive cells are the mean + SD of three separate experiments. Statistical analysis was performed by Student's *t* test. The asterisk indicates $p < 0.01$.

derivatives possess GI_{50} values in the low nanomolar and the submicromolar range; the most active appears to be **1h** (GI_{50} in the subnanomolar range). The TGI values are often located in the submicromolar range and in two cases in the low nanomolar range (**1h** and **1m**). All the LC_{50} values are in the low micromolar range. Overall, these data indicate that the target derivatives are potent cytotoxic agents against PC-3 cells, much more than the reference compound mitonafide. This fact is not surprising as with a bis derivative we expect to enhance the biological properties of monointercalators. However, LU 79553 gave an IC_{50}, after 78 h of drug exposure, of 0.32 μM;[3] even if the data are relative to another experiment and cannot be directly compared with the GI_{50} values of Table 1, we can say that many target derivatives are much more active in vitro than LU 79553 vs the PC-3 cell line. Other bis derivatives related to **1a**−**c** possess similar potent cytotoxic activity against the same cell line: (i) BisAC 1 and BisAC 2 (Chart 1, X = H and X = 7,7′-NO_2, respectively), IC_{50} = 19 and 33 nM, correspondingly, after 144 h of drug exposure (data not published); (ii) BisPA 1 and BisPA 2 (Chart 1, X = H and X = 9,9′-OMe, respectively) IC_{50} = 1.6 and 1.2 nM, correspondingly, after 144 h of drug exposure.[7]

The following remarks can be made.

(i) In the AC−PA subseries (compounds **1a**−**c**), considering **1a** as the parent compound, the introduction of a NO_2 group as the X substituent (**1b**) leads to the best results, while the lengthening of the side chains (**1c**) affords small contrasting effects on the activity profile.

(ii) In the AC−MIT subseries (compounds **1d**−**k** and **1t**), considering **1d** as the parent compound, the introduction of a NO_2 group as the Z substituent (**1e**) does not significantly change the in vitro activity, whereas the introduction of a NO_2 group as the X substituent (**1f**) enhances the cytostatic and cytotoxic activities, as was mentioned above in (i). The concurrent introduction of two NO_2 groups as X and Z substituents (**1g**) leads to a GI_{50} value of 8 nmol, although the TGI and LC_{50} values are similar to those of the parent **1d**. A methoxy group as the X substituent (**1h**) leads to the best derivative in the subseries, while two methoxy groups as X and Y substituents (**1i**) contribute to a decrease in the cytostatic and cytotoxic activities with respect to those of the monosubstituted **1h**. The homologation of the side chain (**1j,k** compared to **1d,e**) as well as the use of a different linker (**1t** compared to **1f**) generally leads to a moderate increment of the in vitro activity.

(iii) In the PA−MIT subseries (compounds **1l**−**r**), considering **1l** as the parent compound, the introduction of a NO_2 group as the Z substituent (**1m**) yields the same GI_{50} value, but much lower TGI and LC_{50} values compared to those of the parent compound. The introduction of one or two methoxy groups, either in the presence or in the absence of a nitro Z substituent (compounds **1n**−**p**), does not cause any dramatic change in terms of the activity profiles. In this subseries the homologation of the side chain generally produces a decrease in the activity (**1q** and, especially, **1r** compared to **1l** and **1m**), in contrast with what was previously reported in (ii).

(iv) Finally, compound **1s**, the only asymmetrical bis derivative made of a triazoloacridone chromophore, linked with a benzoisoquinolinedione, shows a good in vitro activity profile.

As previously noted with BisAC and BisPA,[6,7] there is not a good correlation between cytotoxic activity and DNA-binding properties; nevertheless, some considerations can be made. Thus, we found a very high correlation for **1h** vs **1i** (compounds **1d**−**k**): the addition of two vs one methoxy group, which causes a 7-fold decrease in the DNA binding affinity (K_{app}(CT-DNA, **1h**) = 6.27 vs K_{app}(CT-DNA, **1i**) = 0.846), induces an almost complete loss of cytotoxic activity for **1i**. Compounds **1b**, **1h**, and **1m**, three of the most potent cytotoxic derivatives, are also very attracted to DNA (4−20 times more than ethidium). However, **1s**, the weakest DNA ligand, shows high cytotoxic activity.

On the basis of their activity profiles and structure diversities, four compounds (**1b,h,m,s**) were selected for further studies. We analyzed whether the treatment of PC-3 cells with these antitumor agents would be able to induce apoptotic and/or necrotic cell death.

To this aim, we evaluated the changes in the PC-3 cell viability by flow cytometry. In any case, the percentage of dead PC-3 cells increases according to the treatment time, indicating that the different compounds affect the PC-3 cell viability in a time-dependent manner. At 24−48 h a massive reduction of cell viability was observed (Figure 1).

Moreover, biparametic flow cytometric analysis allows us to establish whether the different compounds induce a distinct pathway of cell death, e.g., apoptosis or necrosis, by annexin

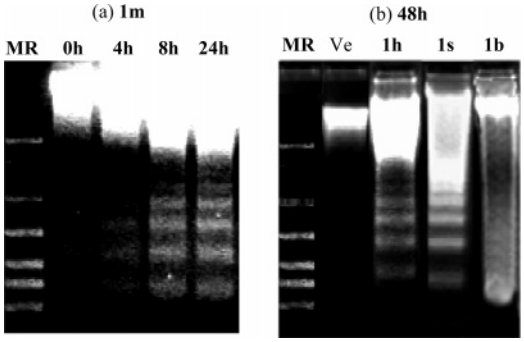


Figure 2. Selected compounds induce in a time-dependent manner distinct pathways of PC-3 prostate cancer cell death. (a) Time-course analysis of DNA fragmentation from PC-3 cells untreated and treated with **1m** target compound for different times (4, 8, and 24 h) was performed by agarose gel (1.7%) electrophoresis. (b) Analysis of DNA fragmentation from cells treated with vehicle (Ve) and **1h**, **1s**, and **1b** target compounds (LC_{50}) for 48 h was performed as described above. Data shown are representative of three separate experiments.

Table 2. Selected Compounds Induce PS Exposure in PC-3 Prostate Cancer Cells in a Time-Dependent Manner[a]

	$PI^-/AnnV^-$	$PI^+/AnnV^-$	$PI^-/AnnV^+$	$PI^+/AnnV^+$
		Time 4 h		
vehicle	92.3 ± 1.9	6.3 ± 0.9	0.2 ± 0.1	1.2 ± 0.1
1m	67.7 ± 2.3	16.3 ± 1.8	12.5 ± 1.0	3.5 ± 0.4
1b	66.5 ± 2.4	28.6 ± 2.6	1.9 ± 0.2	3.0 ± 0.5
1s	69.4 ± 2.0	28.6 ± 2.0	0.9 ± 0.1	1.1 ± 0.2
1h	81.1 ± 1.9	16.4 ± 1.6	1.3 ± 0.2	1.2 ± 0.1
		Time 8 h		
vehicle	83.5 ± 2.7	14.3 ± 0.9	0.2 ± 0.1	2.0 ± 0.5
1m	49.0 ± 1.9	26.9 ± 1.4	16.3 ± 1.2	7.8 ± 0.9
1b	53.0 ± 2.6	37.9 ± 2.3	3.6 ± 0.6	5.5 ± 1.2
1s	54.8 ± 1.9	42.0 ± 2.0	1.3 ± 0.2	1.9 ± 0.1
1h	62.7 ± 2.0	32.9 ± 1.2	1.5 ± 0.1	2.9 ± 0.6
		Time 24 h		
vehicle	81.2 ± 2.9	15.6 ± 1.2	0.4 ± 0.1	2.8 ± 0.6
1m	31.5 ± 1.3	28.6 ± 2.4	33.6 ± 1.9	6.3 ± 1.0
1b	37.5 ± 2.0	44.0 ± 2.6	2.9 ± 0.6	15.6 ± 1.2
1s	11.9 ± 1.2	33.0 ± 1.6	39.0 ± 1.4	16.1 ± 2.1
1h	35.5 ± 1.4	22.3 ± 1.2	25.9 ± 2.1	16.3 ± 1.9
		Time 48 h		
vehicle	79.1 ± 2.6	17.0 ± 1.2	0.6 ± 0.1	3.4 ± 1.2
1m	1.2 ± 0.4	87.3 ± 2.9	4.0 ± 0.6	7.5 ± 2.1
1b	5.0 ± 0.9	55.7 ± 2.4	4.1 ± 0.9	35.2 ± 2.6
1s	1.0 ± 0.2	27.3 ± 2.1	57.0 ± 1.9	15.7 ± 1.9
1h	2.3 ± 0.1	24.4 ± 1.9	59.0 ± 2.4	14.3 ± 1.8

[a] The apoptosis of PC-3 cells treated at different times (4, 8, 24, and 48 h) with the LC_{50} of the selected compounds was evaluated by biparametric cytofluorimetric analysis using PI and FITC-conjugated annexin V. Data expressed as the percentage of positive cells are the mean ± SD of three separate experiments.

V (AnnV) and propidium iodide (PI) treatment. In fact, a characteristic feature of necrotic cell death is the loss of plasma membrane integrity. This damage can be highlighted by treatment with nonvital PI dye, which is allowed to penetrate into the cell, intercalating DNA and turning it fluorescent. The phosphatidylserine (PS) exposure represents an early and widespread hallmark of apoptotic cells. During the early phases of apoptosis, when the cell membrane remains intact, PS translocates from the inner to the outer layer of the plasma membrane. Thus, apoptotic cells may be evidenced by annexin V, which binds to negatively charged PS. Therefore, $PI^-/AnnV^-$ cells are living cells, $PI^+/AnnV^-$ cells are necrotic, $PI^-/AnnV^+$ cells are early apoptotic cells, and $PI^+/AnnV^+$ cells are late apoptotic or necrotic cells.

The results of biparametric flow cytometric analysis for the selected compounds are reported in Table 2. Treatment with target compound **1m** induces a significant reduction (from 92.3% to 1.2%) of $PI^-/AnnV^-$ intact cells and early (4 h) translocation of PS in about 12.5% of the PC-3 cells ($PI^-/AnnV^+$). PS exposure of **1m**-treated PC-3 cells increased in a time-dependent manner, and about 33.6% of the PC-3 cells displayed PS 24 h after the treatment. Target compound **1b** induces necrosis as proved by the increased percentage (from 6.3% to 55.7%) of $PI^+/AnnV^-$ PC-3 cells and the corresponding decrease (from 92.3% to 5.0%) of $PI^-/AnnV^-$ intact cells. Late (24 h) PS exposure ($PI^-/AnnV^+$) was observed with target compounds **1s** and **1h**.

Figure 2 shows that, in accordance with cytofluorimetric analysis, agarose gel electrophoresis evidences necrosis or apoptosis of PC-3 cells depending on the different compounds used. MR is a molecular ruler indicating 200, 400, 600, etc. base pairs. Indeed, time-course analysis of target compound **1m** shows very early apoptosis induction as demonstrated by DNA ladder formation already 4 h after treatment. DNA ladder formation increases at later times, such as 8−24 h. PC-3 cell treatment with target compound **1s** induces apoptosis as proved by the characteristic ladder pattern of DNA, whereas the target compound **1b** displays a diffuse smearing of degraded DNA, indicating cell necrosis. PC-3 cells treated with the target compound **1h** show both necrotic and apoptotic cell death. Neither apoptosis nor necrosis is observed in PC-3 cells treated with vehicle.

It seems that the presence of two "acridinic"-type chromophores (compound **1b**) results in cellular death by necrosis, while the presence of a benzoisoquinolinedione chromophore (compounds **1h,m,s**) may confer the capacity of inducing apoptosis.

As previously noted, DMP 840 has been shown to interfere with topoisomerase II activity.[2c] Thus, as a hypothesis, we can say that the proapoptotic activity of the **1h**, **1m**, and **1s** compounds is probably related to their ability to stabilize topoisomerase−DNA cleavable complexes convertible into double-strand-DNA breaks (DSBs), which trigger apoptosis of PC-3 prostate cancer cells. The difference in the time course of **1m** vs **1h** and **1s** (4−8 h vs 24 h) may be related to the different capabilities of these compounds to interact with and stabilize the DNA−topoisomerase II complexes or to other differences in the mechanisms responsible for DSB generation. However, the ability of **1b** to induce PC-3 cell death, by a necrotic instead of an apoptotic mechanism, suggests the presence of a different mechanism of action. Compound **1b** seems to activate a topoisomerase-independent cell death, as evidenced by the absence of the double-strand-DNA breaks characteristic of apoptosis, but induces massive DNA degradation, the hallmark of necrosis. Thus, the cytotoxicity of compound **1b**, with a very high DNA-binding capability, also regarding $[poly(dG-dC)]_2$, may be due to its DNA-binding properties as recently proposed by Bailly for similar compounds.[4]

Conclusions

From the present successful study we can state the following: (i) The asymmetrical bis derivatives **1a**−**t** represent a new class of interesting antitumor agents endowed with noticeable DNA-binding properties and antiproliferative activity. (ii) The selected compound **1b,h,m,s** may act as new leads in the field of anticancer derivatives. (iii) In particular, **1m**, showing high DNA affinity, very potent cytostatic and cytocide action, and an intriguing capacity of early apoptosis induction, may be a good candidate for in vivo preclinical studies.

Experimental Section

Synthetic Chemistry. Melting points were determined on a Büchi 540 apparatus and are uncorrected. Thin-layer chromatography (TLC) was accomplished using plates precoated with silica gel 60 F-254 (Merck). All ^{1}H NMR spectra were recorded on a Varian VXR 300 instrument. Chemical shifts are reported as δ values (ppm) downfield from internal Me_4Si in the solvent shown. The following NMR abbreviations are used: br (broad), s (singlet), d (doublet), t (triplet), m (multiplet), ar (aromatic proton), ex (exchangeable with D_2O). Elemental analyses were performed on an EA1108CHAZ-O elemental analyzer (Fisons Instruments).

***N*4-[2-(Dimethylamino)ethyl]-1-({3-[(3-aminopropyl)-methylamino]propyl}amino)-9-oxo-9,10-dihydro-4-acridinecarboxamide (6a). Example of the General Procedure for the Preparation of 6a−f, 7a−d, and 8.** *N*4-[2-(Dimethylamino)ethyl]-1-chloro-9-oxo-9,10-dihydro-4-acridinecarboxamide (**2a**; 0.3 g, 0.97 mmol), bis(3-aminopropyl)methylamine (0.32 mL, 2.0 mmol), and triethylamine (0.5 mL) were stirred in 2-ethoxyethanol (10 mL) at 80 °C for 2 h. The resulting mixture was partitioned between $CHCl_3$ (2 × 30 mL) and an excess of 1 M aqueous Na_2CO_3 (30 mL). The organic layer was worked up to give a residue which was purified by flash chromatography on a silica gel column eluted first with $CHCl_3$/MeOH (7:3, v/v) and then with $CHCl_3$/MeOH (7:3, v/v) containing 32% aqueous NH_3 (15 mL for 1 L of eluent) to obtain **6a** (0.22 g, 50% yield) as a dense oil, pure enough to be employed in the next step: ^{1}H NMR ($CDCl_3$) δ 1.55 (m, 2H, CH_2), 1.82 (m, 2H, CH_2), 2.15 (s, 3H, CH_3), 2.22 (s, 6H, 2 × CH_3), 2.27−2.53 (m, 6H, 3 × CH_2), 2.64 (t, 2H, CH_2), 3.25 (m, 2H, CH_2), 3.40 (m, 2H, CH_2), 6.10 (d, 2H, ar), 7.02 (t, 2H, NH_2, ex), 7.17 (t, 1H, ar), 7.25 (d, 2H, ar), 7.49 (t, 1H, ar), 7.59 (d, 1H, ar), 8.23 (d, 1H, ar), 10.85 (t, 1H, NH, ex), 13.40 (br s, 1H, 10−H, ex).

In a similar manner, the following compounds were prepared: (i) **6b**−**f** from the appropriate 1-chloro-9-oxo-9,10-dihydro-4-acridinecarboxamide **2b**−**e** and the suitable amine; (ii) **7a**−**d** from the appropriate 6-chloro-2,3-dihydro-1*H*,7*H*-pyrimido[5,6,1-*de*]-acridine-1,3,7-trione **3a**−**d** and the suitable amine; (iii) **8** from **4** and bis(3-aminopropyl)methylamine. All the intermediate derivatives **6a**−**f**, **7a**−**d**, and **8** were not characterized, but used in the next step after purification by flash chromatography.

***N*4-[2-(Dimethylamino)ethyl]-1-({3-[[3-({2-[2-(dimethylamino)ethyl]-1,3,7-trioxo-2,3-dihydro-1*H*,7*H*-pyrimido[5,6,1-*de*]acridin-6-yl}amino)propyl]methylamino]propyl}amino)-9-oxo-9,10-dihydro-4-acridinecarboxamide (1a). Example of the General Procedure for the Preparation of 1a−t.** Compound **6a** (0.21 g, 0.46 mmol), 6-chloro-2-[2-(dimethylamino)ethyl]-2,3-dihydro-1*H*,7*H*-pyrimido[5,6,1-de]acridine-1,3,7-trione (**3a**; 0.37 g, 1 mmol), and triethylamine (0.5 mL) were stirred in 2-ethoxyethanol (10 mL) at 80 °C for 2 h. The mixture was cooled at room temperature and partitioned between $CHCl_3$ (4 × 20 mL) and an excess of 1 M aqueous Na_2CO_3 (20 mL). The organic layer was worked up to give a residue which was flash-chromatographed on a silica gel column eluted first with $CHCl_3$/MeOH (1:1, v/v) and then with $CHCl_3$/MeOH (1:1, v/v) and 32% aqueous NH_3 (5 mL for 1 L of eluent) to give pure **1a** (0.3 g, 83%): mp 160−162 °C; hydrochloride mp 233−235 °C (EtOH); ^{1}H NMR ($CDCl_3$) δ 1.86−2.04 (m, 4H, 2 × CH_2), 2.23−2.40 (m, 15H, 5 × CH_3), 2.55−2.73 (m, 8H, 4 × CH_2), 3.20−3.40 (m, 4H, 2 × CH_2), 3.43−3.56 (m, 2H, CH_2), 4.31 (t, 2H, CH_2), 6.00 (d, 1H, ar), 6.45 (d, 1H, ar), 6.79 (t, 1H, NH, ex), 7.05−7.15 (m, 2H, ar), 7.30−7.55 (m, 4H, ar), 7.98 (d, 1H, ar), 8.20 (t, 2H, ar), 8.62 (d, 1H, ar), 10.73 (t, 1H, NH, ex), 10.82 (t, 1H, NH, ex), 13.21 (s, 1H, NH, ex). Anal. ($C_{44}H_{60}Cl_3N_9O_8$) C, H, N.

In the same experimental conditions, derivatives **1b**,**c** from **6b** and **7d** were prepared, respectively. Derivatives **1d**−**t** were also prepared in a similar manner from the suitable intermediates **6a**−**f**, **7a**−**d**, or **8**, but without using triethylamine. All the target compounds **1a**−**t** were converted to water-soluble hydrochloride salts by the usual methods.

Data for *N*4-[2-(dimethylamino)ethyl]-1-({3-[[3-({2-[2-(dimethylamino)ethyl]-1,3,7-trioxo-2,3-dihydro-1*H*,7*H*-pyrimido-[5,6,1-*de*]acridin-6-yl}amino)propyl]methylamino]propyl}amino)-7-nitro-9-oxo-9,10-dihydro-4-acridinecarboxamide (1b): yield 71%; mp 152−154 °C; hydrochloride mp 201−202 °C (EtOH); ^{1}H NMR ($CDCl_3$) δ 1.86−2.04 (m, 4H, 2 × CH_2), 2.23−2.50 (m, 15H, 5 × CH_3), 2.50−2.78 (m, 8H, 4 × CH_2), 3.18−3.38 (m, 4H, 2 × CH_2), 3.43−3.59 (m, 2H, CH_2), 4.30 (t, 2H, CH_2), 6.08 (d, 1H, ar), 6.30 (d, 1H, ar), 7.01 (d, 2H, ar), 7.30 (t, 1H, NH, ex), 7.39−7.57 (m, 2H, ar), 7.86 (d, 1H, ar), 8.00 (d, 1H, ar), 8.12 (d, 1H, ar), 8.60 (d, 1H, ar), 8.96 (s, 1H, ar), 10.55 (t, 1H, NH, ex), 10.70 (t, 1H, NH, ex), 13.63 (s, 1H, NH, ex). Anal. ($C_{44}H_{57}Cl_3N_{10}O_9$) C, H, N.

Data for *N*4-[3-(dimethylamino)propyl]-1-({3-[[3-({2-[3-(dimethylamino)propyl]-1,3,7-trioxo-2,3-dihydro-1*H*,7*H*-pyrimido-[5,6,1-*de*]acridin-6-yl}amino)propyl]methylamino]propyl}amino)-9-oxo-9,10-dihydro-4-acridinecarboxamide (1c): yield 71%; mp 119−121 °C; hydrochloride mp 88−90 °C (EtOH); ^{1}H NMR ($CDCl_3$) δ 1.78−2.09 (m, 8H, 4 × CH_2), 2.23−2.40 (m, 15H, 5 × CH_3), 2.40−2.70 (m, 8H, 4 × CH_2), 3.20−3.42 (m, 4H, 2 × CH_2), 3.50 (t, 2H, CH_2), 4.24 (t, 2H, CH_2), 6.00 (d, 1H, ar), 6.52 (d, 1H, ar), 7.04−7.18 (m, 2H, ar), 7.30−7.58 (m, 4H, ar), 8.03 (d, 1H, ar), 8.21 (t, 2H, ar), 8.52 (m, 1H, NH, ex), 8.64 (d, 1H, ar), 10.80 (m, 2H, 2 × NH, ex), 13.45 (s, 1H, NH, ex). Anal. ($C_{46}H_{62}Cl_3N_9O_7$) C, H, N.

Data for *N*4-[2-(dimethylamino)ethyl]-1-({3-[[3-(1,3-dioxo-2,3-dihydro-1*H*-benzo[*de*]isoquinolin-2-yl)propyl]methylamino]-propyl}amino)-9-oxo-9,10-dihydro-4-acridinecarboxamide (1d): yield 82%; mp 138−140 °C; hydrochloride mp 196−198 °C (EtOH); ^{1}H NMR ($CDCl_3$) δ 1.82−2.04 (m, 4H, 2 × CH_2), 2.28 (s, 3H, CH_3), 2.38 (s, 6H, 2 × CH_3), 2.55 (t, 4H, 2 × CH_2), 2.63 (t, 2H, CH_2), 3.30−3.43 (m, 2H, CH_2), 3.48−3.62 (m, 2H, CH_2), 4.23 (t, 2H, CH_2), 6.25 (d, 1H, ar), 7.02−7.38 (m, 3H, 2ar + NH ex), 7.58 (t, 1H, ar), 7.68 (t, 3H, ar), 8.20 (d, 2H, ar), 8.30 (d, 1H, ar), 8.58 (d, 2H, ar), 10.95 (t, 1H, NH, ex), 13.55 (s, 1H, NH, ex). Anal. ($C_{37}H_{46}Cl_2N_6O_6$) C, H, N.

Data for *N*4-[2-(dimethylamino)ethyl]-1-({3-[[3-(5-nitro-1,3-dioxo-2,3-dihydro-1*H*-benzo[*de*]isoquinolin-2-yl)propyl]-methylamino]propyl}amino)-9-oxo-9,10-dihydro-4-acridinecarboxamide (1e): yield 71%; mp 157−159 °C; hydrochloride mp 128−130 °C (EtOH); ^{1}H NMR ($CDCl_3$) δ 1.80−2.06 (m, 4H, 2 × CH_2), 2.29 (s, 3H, CH_3), 2.36 (s, 6H, 2 × CH_3), 2.48−2.72 (m, 6H, 3 × CH_2), 3.29−3.49 (m, 2H, CH_2), 3.50−3.62 (m, 2H, CH_2), 4.22 (t, 2H, CH_2), 6.25 (d, 1H, ar), 6.98 (t, 1H, NH, ex), 7.18 (t, 1H, ar), 7.22−7.31 (m, 1H, ar), 7.55 (t, 1H, ar), 7.68 (d, 1H, ar), 7.82 (t, 1H, ar), 8.18−8.38 (m, 2H, ar), 8.66 (d, 1H, ar), 9.04 (s, 1H, ar), 9.22 (s, 1H, ar), 10.91 (t, 1H, NH, ex), 13.38 (s, 1H, NH, ex). Anal. ($C_{37}H_{45}Cl_2N_7O_8$) C, H, N.

Data for *N*4-[2-(dimethylamino)ethyl]-1-({3-[[3-(1,3-dioxo-2,3-dihydro-1*H*-benzo[*de*]isoquinolin-2-yl)propyl]methylamino]-propyl}amino)-7-nitro-9-oxo-9,10-dihydro-4-acridinecarboxamide (1f): yield 63%; hydrochloride mp 213−215 °C; ^{1}H NMR ($CDCl_3$) δ 1.83−2.03 (m, 4H, 2 × CH_2), 2.23−2.38 (m, 9H, 3 × CH_3), 2.46−2.62 (m, 6H, 3 × CH_2), 3.33−3.58 (m, 4H, 2 × CH_2), 4.22 (t, 2H, CH_2), 6.42 (d, 1H, ar), 7.07 (t, 1H, NH, ex), 7.28 (d, 1H, ar), 7.65−7.78 (m, 3H, ar), 8.18 (d, 2H, ar), 8.32 (d, 1H, ar), 8.55 (d, 2H, ar), 9.10 (s, 1H, ar), 10.72 (t, 1H, NH, ex), 13.90 (s, 1H, NH, ex). Anal. ($C_{37}H_{45}Cl_2N_7O_8$) C, H, N.

Data for *N*4-[2-(dimethylamino)ethyl]-1-({3-[[3-(5-nitro-1,3-dioxo-2,3-dihydro-1*H*-benzo[*de*]isoquinolin-2-yl)propyl](methyl)-amino]propyl}amino)-7-nitro-9-oxo-9,10-dihydro-4-acridinecarboxamide (1g): yield 63%; hydrochloride mp 230−232 °C (EtOH); ^{1}H NMR ($CDCl_3$) δ 1.80−2.03 (m, 4H, 2 × CH_2), 2.21−2.42 (m, 9H, 3 × CH_3), 2.43−2.70 (m, 6H, 3 × CH_2), 3.30−3.60 (m, 4H, 2 × CH_2), 4.22 (t, 2H, CH_2), 6.35 (d, 1H, ar), 7.04 (t, 1H, NH, ex), 7.19−7.28 (m, 1H, ar), 7.65 (d, 1H, ar), 7.83 (t, 1H, ar), 8.22−8.40 (m, 2H, ar), 8.60−8.78 (m, 1H, ar), 8.99 (d, 2H, ar), 9.17 (s, 1H, ar), 10.65 (t, 1H, NH, ex), 13.83 (s, 1H, NH, ex). Anal. ($C_{37}H_{44}Cl_2N_8O_{10}$) C, H, N.

Data for *N*4-[2-(dimethylamino)ethyl]-1-({3-[[3-(1,3-dioxo-2,3-dihydro-1*H*-benzo[*de*]isoquinolin-2-yl)propyl]methylamino]-propyl}amino)-7-methoxy-9-oxo-9,10-dihydro-4-acridinecarboxamide (1h): yield 82%; mp 169−171 °C; hydrochloride mp

79−81 °C (EtOH); ^{1}H NMR ($CDCl_3$) δ 1.83−2.03 (m, 4H, 2 × CH_2), 2.29 (s, 3H, CH_3), 2.40 (s, 6H, 2 × CH_3), 2.50−2.61 (m, 4H, 2 × CH_2), 2.68 (t, 2H, CH_2), 3.30−3.46 (m, 2H, CH_2), 3.52−3.63 (m, 2H, CH_2), 3.88 (s, 3H, OCH_3), 4.22 (t, 2H, CH_2), 6.25 (d, 1H, ar), 7.16−731 (m, 3H, 2 ar + NH ex), 7.62−7.84 (m, 4H, ar), 8.18 (d, 2H, ar), 8.55 (d, 2H, ar), 11.03 (t, 1H, NH, ex), 13.44 (s, 1H, NH, ex). Anal. ($C_{38}H_{48}Cl_2N_6O_7$) C, H, N.

Data for *N*4-[2-(dimethylamino)ethyl]-1-({3-[[3-(1,3-dioxo-2,3-dihydro-1*H*-benzo[*de*]isoquinolin-2-yl)propyl]methylamino]-propyl}amino)-6,7-dimethoxy-9-oxo-9,10-dihydro-4-acridinecarboxamide (1i): yield 63%; mp 158−160 °C; hydrochloride mp 204−207 °C (EtOH); ^{1}H NMR ($CDCl_3$) δ 1.83−2.00 (m, 4H, 2 × CH_2), 2.21−2.37 (m, 9H, 3 × CH_3), 2.45−2.60 (m, 6H, 3 × CH_2), 3.30−3.43 (m, 2H, CH_2), 3.47−3.58 (m, 2H, CH_2), 3.98 (s, 3H, 2 × OCH_3), 4.22 (t, 2H, CH_2), 6.25 (d, 1H, ar), 6.72 (s, 1H, ar), 7.06 (m, 1H, NH, ex), 7.63 (s, 1H, ar), 7.67−7.77 (m, 3H, ar), 8.18 (d, 2H, ar), 8.58 (d, 2H, ar), 11.00 (t, 1H, NH, ex), 13.47 (s, 1H, NH, ex). Anal. ($C_{39}H_{50}Cl_2N_6O_8$) C, H, N.

Data for *N*4-[3-(dimethylamino)propyl]-1-({3-[[3-(1,3-dioxo-2,3-dihydro-1*H*-benzo[*de*]isoquinolin-2-yl)propyl]methylamino]-propyl}amino)-9-oxo-9,10-dihydro-4-acridinecarboxamide (1j): yield 62%; mp 123−125 °C; hydrochloride mp 75−77 °C (EtOH); ^{1}H NMR ($CDCl_3$) δ 1.70−2.04 (m, 6H, 3 × CH_2), 2.25 (s, 3H, CH_3), 2.35 (s, 6H, 2 × CH_3), 2.56 (t, 6H, 3 × CH_2), 3.30−3.43 (m, 2H, CH_2), 3.50−3.62 (m, 2H, CH_2), 4.23 (t, 2H, CH_2), 6.29 (d, 1H, ar), 7.17−7.38 (m, 2H, ar), 7.51−7.60 (m, 2H, ar), 7.78 (t, 2H, ar), 8.20 (d, 2H, ar), 8.30 (d, 1H, ar), 8.58 (d, 2H, ar), 8.70 (t, 1H, NH, ex), 10.97 (t, 1H, NH, ex), 13.70 (s, 1H, NH, ex). Anal. ($C_{38}H_{48}Cl_2N_6O_6$) C, H, N.

Data for *N*4-[3-(dimethylamino)propyl]-1-({3-[[3-(5-nitro-1,3-dioxo-2,3-dihydro-1*H*-benzo[*de*]isoquinolin-2-yl)propyl]-methylamino]propyl}amino)-9-oxo-9,10-dihydro-4-acridinecarboxamide (1k): yield 76%; mp 108−110 °C; hydrochloride mp 118−120 °C (EtOH); ^{1}H NMR ($CDCl_3$) δ 1.70−2.10 (m, 6H, 3 × CH_2), 2.24 (s, 3H, CH_3), 2.40 (s, 6H, 2 × CH_3), 2.45−2.63 (m, 6H, 3 × CH_2), 3.25−3.42 (m, 2H, CH_2), 3.50−3.62 (m, 2H, CH_2), 4.23 (t, 2H, CH_2), 6.22 (d, 1H, ar), 7.18 (t, 1H, ar), 7.21−7.30 (m, 1H, ar), 7.50−7.60 (m, 2H, ar), 7.82 (t, 1H, ar), 8.09−8.38 (m, 3H, ar), 8.60−8.71 (m, 2H, ar + NH ex), 9.04 (s, 1H, ar), 9.21 (s, 1H, ar), 10.89 (t, 1H, NH, ex), 13.36 (s, 1H, NH, ex). Anal. ($C_{38}H_{47}Cl_2N_7O_8$) C, H, N.

Data for 2-[2-(dimethylamino)ethyl]-6-({3-[[3-(1,3-dioxo-2,3-dihydro-1*H*-benzo[*de*]isoquinolin-2-yl)propyl]methylamino]-propyl}amino)-2,3-dihydro-1*H*,7*H*-pyrimido[5,6,1-*de*]acridine-1,3,7-trione (1l): yield 45%; mp 156−158 °C; hydrochloride mp 124−126 °C (EtOH); ^{1}H NMR ($CDCl_3$) δ 1.83−2.03 (m, 4H, 2 × CH_2), 2.29 (s, 3H, CH_3), 2.54 (s, 6H, 2 × CH_3), 2.59 (t, 4H, 2 × CH_2), 2.70 (t, 2H, CH_2), 3.40−3.58 (m, 2H, CH_2), 4.20−4.38 (m, 4H, 2 × CH_2), 6.78 (d, 1H, ar), 7.43 (t, 1H, ar), 7.60−7.79 (m, 3H, ar), 8.18−8.28 (m, 3H, ar), 8.38 (d, 1H, ar), 8.58 (d, 2H, ar), 8.77 (d, 1H, ar), 10.92 (t, 1H, NH, ex). Anal. ($C_{38}H_{44}Cl_2N_6O_7$) C, H, N.

Data for 2-[2-(dimethylamino)ethyl]-6-({3-[[3-(5-nitro-1,3-dioxo-2,3-dihydro-1*H*-benzo[*de*]isoquinolin-2-yl)propyl]-methylamino]propyl}amino)-2,3-dihydro-1*H*,7*H*-pyrimido[5,6,1-*de*]acridine-1,3,7-trione (1m): yield 41%; mp 119−121 °C; hydrochloride mp 133−135 °C (EtOH); ^{1}H NMR ($CDCl_3$) δ 1.85−2.03 (m, 4H, 2 × CH_2), 2.32 (s, 3H, CH_3), 2.39 (s, 6H, 2 × CH_3), 2.59 (t, 4H, 2 × CH_2), 2.70 (t, 2H, CH_2), 3.40−3.58 (m, 2H, CH_2), 4.20−4.38 (m, 4H, 2 × CH_2), 6.78 (d, 1H, ar), 7.40 (t, 1H, ar), 7.65 (t, 1H, ar), 7.86 (t, 1H, ar), 8.19−8.40 (m, 3H, ar), 8.60−8.75 (m, 2H, ar), 9.08 (s, 1H, ar), 9.25 (s, 1H, ar), 10.92 (t, 1H, NH, ex). Anal. ($C_{38}H_{43}Cl_2N_7O_9$) C, H, N.

Data for 2-[2-(dimethylamino)ethyl]-6-({3-[[3-(1,3-dioxo-2,3-dihydro-1*H*-benzo[*de*]isoquinolin-2-yl)propyl]methylamino]-propyl}amino)-9-methoxy-2,3-dihydro-1*H*,7*H*-pyrimido[5,6,1-*de*]acridine-1,3,7-trione (1n): yield 57%; mp 117−119 °C; hydrochloride mp 263−265 °C (EtOH); ^{1}H NMR ($CDCl_3$) δ 1.83−2.03 (m, 4H, 2 × CH_2), 2.28 (s, 3H, CH_3), 2.36 (s, 6H, 2 × CH_3), 2.56 (t, 4H, 2 × CH_2), 2.65 (t, 2H, CH_2), 3.42−3.57 (m, 2H, CH_2), 3.91 (s, 3H, OCH_3), 4.18−4.36 (m, 4H, 2 × CH_2), 6.78 (d, 1H, ar), 7.22 (d, 1H, ar), 7.60−7.79 (m, 3H, ar), 8.14−8.26 (m, 3H, ar), 8.55 (d, 2H, ar), 8.71 (d, 1H, ar), 10.95 (t, 1H, NH, ex). Anal. ($C_{39}H_{46}Cl_2N_6O_8$) C, H, N.

Data for 2-[2-(dimethylamino)ethyl]-6-({3-[[3-(5-nitro-1,3-dioxo-2,3-dihydro-1*H*-benzo[*de*]isoquinolin-2-yl)propyl]-methylamino]propyl}amino)-9-methoxy-2,3-dihydro-1*H*,7*H*-pyrimido[5,6,1-*de*]acridine-1,3,7-trione (1o): yield 87%; mp 148−150 °C; hydrochloride mp 257−259 °C (EtOH); ^{1}H NMR ($CDCl_3$) δ 1.83−2.03 (m, 4H, 2 × CH_2), 2.29 (s, 3H, CH_3), 2.37 (s, 6H, 2 × CH_3), 2.55 (t, 4H, 2 × CH_2), 2.65 (t, 2H, CH_2), 3.42−3.57 (m, 2H, CH_2), 3.90 (s, 3H, OCH_3), 4.20−4.37 (m, 4H, 2 × CH_2), 6.77 (d, 1H, ar), 7.21 (d, 1H, ar), 7.71 (s, 1H, ar), 7.88 (t, 1H, ar), 8.22 (d, 1H, ar), 8.39 (d, 1H, ar), 8.62−8.70 (m, 2H, ar), 9.07 (s, 1H, ar), 9.22 (s, 1H, ar), 10.93 (t, 1H, NH, ex). Anal. ($C_{39}H_{45}Cl_2N_7O_{10}$) C, H, N.

Data for 2-[2-(dimethylamino)ethyl]-6-({3-[[3-(5-nitro-1,3-dioxo-2,3-dihydro-1*H*-benzo[*de*]isoquinolin-2-yl)propyl]-methylamino]propyl}amino)-9,10-dimethoxy-2,3-dihydro-1*H*,7*H*-pyrimido[5,6,1-*de*]acridine-1,3,7-trione (1p): yield 87%; mp 188−190 °C; hydrochloride mp 122−124 °C (EtOH); ^{1}H NMR ($CDCl_3$) δ 1.83−2.03 (m, 4H, 2 × CH_2), 2.30 (s, 3H, CH_3), 2.38 (s, 6H, 2 × CH_3), 2.57 (t, 4H, 2 × CH_2), 2.69 (t, 2H, CH_2), 3.41−3.55 (m, 2H, CH_2), 3.93 (s, 3H, OCH_3), 4.02 (s, 3H, OCH_3), 4.20−4.37 (m, 4H, 2 × CH_2), 6.75 (d, 1H, ar), 7.67 (s, 1H, ar), 7.86 (t, 1H, ar), 8.22 (d, 1H, ar), 8.37 (d, 1H, ar), 8.41 (s, 1H, ar), 8.70 (d, 1H, ar), 9.05 (s, 1H, ar), 9.19 (s, 1H, ar), 10.97 (t, 1H, NH, ex). Anal. ($C_{40}H_{47}Cl_2N_7O_{11}$) C, H, N.

Data for 2-[3-(dimethylamino)propyl]-6-({3-[[3-(1,3-dioxo-2,3-dihydro-1*H*-benzo[*de*]isoquinolin-2-yl)propyl]methylamino]-propyl}amino)-2,3-dihydro-1*H*,7*H*-pyrimido[5,6,1-*de*]acridine-1,3,7-trione (1q): yield 85%; hydrochloride mp 175−177 °C (EtOH); ^{1}H NMR ($CDCl_3$) δ 1.83−2.03 (m, 6H, 3 × CH_2), 2.22−2.34 (m, 9H, 3 × CH_3), 2.42−2.60 (m, 6H, 3 × CH_2), 3.40−3.58 (m, 2H, CH_2), 4.14−4.28 (m, 4H, 2 × CH_2), 6.78 (d, 1H, ar), 7.43 (t, 1H, ar), 7.62−7.79 (m, 3H, ar), 8.16−8.23 (m, 3H, ar), 8.38 (d, 1H, ar), 8.58 (d, 2H, ar), 8.70 (d, 1H, ar), 10.95 (t, 1H, NH, ex). Anal. ($C_{39}H_{46}Cl_2N_6O_7$) C, H, N.

Data for 2-[3-(dimethylamino)propyl]-6-({3-[[3-(5-nitro-1,3-dioxo-2,3-dihydro-1*H*-benzo[*de*]isoquinolin-2-yl)propyl]-methylamino]propyl}amino)-2,3-dihydro-1*H*,7*H*-pyrimido[5,6,1-*de*]acridine-1,3,7-trione (1r): yield 88%; mp 106−108 °C; hydrochloride mp 123−125 °C (EtOH); ^{1}H NMR ($CDCl_3$) δ 1.85−2.03 (m, 6H, 3 × CH_2), 2.29 (s, 9H, 3 × CH_3), 2.42−2.60 (m, 6H, 3 × CH_2), 3.42−3.58 (m, 2H, CH_2), 4.17−4.32 (m, 4H, 2 × CH_2), 6.78 (d, 1H, ar), 7.39 (t, 1H, ar), 7.64 (t, 1H, ar), 7.86 (t, 1H, ar), 8.19−8.42 (m, 3H, ar), 8.62−8.78 (m, 2H, ar), 9.08 (s, 1H, ar), 9.23 (s, 1H, ar), 10.90 (t, 1H, NH, ex). Anal. ($C_{39}H_{45}Cl_2N_7O_9$) C, H, N.

Data for 2-[3-(methyl{3-[(8-methyl-6-oxo-6*H*-^{1,2,3}triazolo-[4,5,1-*de*]acridin-5-yl)amino]propyl}amino)propyl]-2,3-dihydro-1*H*-benzo[*de*]isoquinoline-1,3-dione (1s): yield 80%; mp 187−189 °C; hydrochloride mp 229−231 °C (EtOH); ^{1}H NMR ($CDCl_3$) δ 1.88−2.07 (m, 4H, 2 × CH_2), 2.35 (s, 3H, CH_3), 2.53 (s, 3H, CH_3), 2.61 (t, 4H, 2 × CH_2), 3.58−3.69 (m, 2H, CH_2), 4.22−4.30 (m, 2H, CH_2), 7.01 (d, 1H, ar), 7.62−7.77 (m, 3H, ar), 8.12−8.20 (m, 3H, ar), 8.25 (s, 1H, ar), 8.40 (d, 1H, ar), 8.57 (d, 2H, ar), 9.42 (t, 1H, NH, ex). Anal. ($C_{33}H_{33}ClN_6O_4$) C, H, N.

Data for *N*4-[2-(dimethylamino)ethyl]-1-({2-[(2-{[2-(1,3-dioxo-2,3-dihydro-1*H*-benzo[*de*]isoquinolin-2-yl)ethyl]amino}-ethyl)amino]ethyl}amino)-7-nitro-9-oxo-9,10-dihydro-4-acridinecarboxamide (1t): yield 28%; hydrochloride mp 270−272 °C (EtOH)h ^{1}H NMR ($CDCl_3$) δ 2.20 (br s, 2H, 2 × NH, ex), 2.35 (s, 6H, 2 × CH_3), 2.60 (t, 2H, CH_2), 2.83−3.10 (m, 6H, 3 × CH_2), 3.17−3.34 (m, 4H, 2 × CH_2), 3.60−3.70 (m, 2H, CH_2), 4.38 (t, 2H, CH_2), 6.15 (d, 1H, ar), 7.02 (br s, 1H, NH, ex), 7.28 (d, 1H, ar), 7.40 (t, 2H, ar), 7.59 (d, 1H, ar), 7.85 (d, 2H, ar), 8.10−8.27 (m, 3H, ar), 8.90 (s, 1H, ar), 10.62 (br s, 1H, NH, ex), 13.65 (br s, 1H, NH, ex). Anal. ($C_{36}H_{47}Cl_3N_8O_9$) C, H, N.

Biophysical Evaluation. 1. Fluorescence Binding Studies. The fluorometric assays have been described previously.[16] 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.[16,17]

All measurements were made in 10 mm quartz cuvettes at 20 °C using a Perkin-Elmer LS5 instrument (excitation at 546 nm, emission at 595 nm) following serial addition of aliquots of a stock drug solution (~5 mM in DMSO). The C_{50} values are defined as the drug concentrations which reduce the fluorescence of the DNA-bound ethidium by 50% and are calculated as the mean from three determinations.

2. In Vitro Cytotoxicity. The human androgen-independent prostate adenocarcinoma cell line (PC-3) was used for cytotoxicity testing in vitro using the FDA and SRB (sulforhodamine B) assay.[20] Cells were maintained as stocks in DMEM (Gibco) supplemented with 10% fetal bovine serum (Gibco) and 2 mM L-glutamine (Gibco). Cell cultures were passaged twice weekly using trypsin−EDTA to detach the cells from their culture flasks. The rapidly growing cells were harvested, counted, and incubated under the appropriate concentrations (7×10^5 cells/well) in 96-well microtiter plates. After incubation for 24 h, target and reference compounds dissolved in culture medium were applied to the culture wells in quadruplicate and incubated for 48 h at 37 °C in a 5% CO_2 atmosphere and 95% relative humidity. At the same time a plate was tested to value the cell population before the drug addition (T_z). Culture fixed with cold trichloroacetic acid (TCA) (J.T. Baker B.V., Deventer, Holland) was stained by 0.4% sulforhodamine B (SRB) (Sigma-Aldrich, Milan, Italy) dissolved in 1% acetic acid. Bound stain was subsequently solubilized with 10 mM Trizma (Sigma-Aldrich, Milan, Italy) and the absorbance read on the microplate reader Dynatech model MR 700 at a wavelength of 520 nm. The cytotoxic activity was evaluated by measuring the drug concentration resulting in a 50% reduction in the net protein increase (as measured by SRB staining) in control cells during the drug incubation (GI_{50}), the drug concentration resulting in total growth inhibition (TGI), and the drug concentration resulting in a 50% reduction in the measured protein at the end of the drug treatment as compared to that at the beginning (LC_{50}). The percentage of growth inhibition was calculated as $[(T_i - T_z)/(C - T_z)] \times 100$ for concentrations for which $T_i \geq T_z$ and as $[(T_i - T_z)/T_z] \times 100$ for concentrations for which $T_i < T_z$, where T_z = absorbance at time zero, C = absorbance in the presence of vehicle, and T_i = absorbance in the presence of drug at different concentrations. GI_{50}, TGI, and LC_{50} were obtained by interpolation on a graph of the percentage of growth versus log(M). Each quoted value represents the mean of triplicate experiments.

3. Viability and Apoptotic Assays. a. Cytofluorimetric Analyses. The viability of PC-3 cells was evaluated by FDA staining.[21,22] PC-3 cells treated with vehicle or with about the LC_{50} of target compounds at 37 °C, 5% CO_2, were incubated for 5 min at 37 °C with 0.125 μg/μL FDA, an agent that is metabolized to fluorescein in living cells, in 0.2 mL of binding buffer. Apoptosis of PC-3 cells treated with about the LC_{50} of target compounds was evaluated by annexin V binding[19] and biparametric PI/annexin V cytofluorimetric analysis.[22] To detect early stages of apoptosis, the expression of annexin V, a Ca^{2+}-dependent phospholipid-binding protein with high affinity for phosphatidylserine was employed. Moreover, simultaneous staining of cells with FITC−Annexin V and with PI allows the discrimination of intact cells (annexin V^-/PI^-), early apoptotic cells (annexin V^+/PI^-), and late apoptotic or necrotic cells (annexin V^+/PI^+). Apoptotic cells become annexin V^+ after nuclear condensation has started, but before the cells become permeable to PI. Briefly, 2×10^6 PC-3 cells treated with the LC_{50} of selected compounds for 6 h were resuspended in 0.2 mL of binding buffer (10 mM Hepes/NaOH, pH 7.4, 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$) in the presence of 5 μL of FITC−annexin V (Bender MedSystem, Vienna, Austria) and were incubated for 10 min at room temperature in the dark. The cells were washed, resuspended in 0.2 mL of binding buffer containing 10 μL of PI (20 μg/mL in PBS) (Molecular Probes, Eugene, OR), and then analyzed as mentioned above. The percentage of positive cells determined over 10000 events was analyzed on an FACScan cytofluorimeter (Becton Dickinson, San Jose, CA) using the CellQuest software. Fluorescence intensity is expressed in arbitrary units on a logarithmic scale.

b. DNA Fragmentation Assay. The time course of selected compound (**1b,h,m,s**) induced nucleosomal DNA fragmentation was performed by agarose gel electrophoresis.[23] Briefly, 5×10^5 cells/mL were cultured at 37 °C, 5% CO_2, and treated with the LC_{50} of selected compounds for different times (2, 4, 6, and 8 h). After treatment, the cells were washed, and DNA was extracted using the Genomix Cells and Tissues Mini Preparations kit (Talent, Trieste, Italy). The DNA samples were electrophoresed on a 1.7% agarose gel and stained with ethidium bromide, and the assays were acquired by a ChemiDoc (BioRad, Milano, Italy).

Supporting Information Available: Elemental analysis results for target compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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