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Article

Antitumor Agents 6. Synthesis, Structure#Activity Relationships, and Biological Evaluation of Spiro[imidazolidine-4,3#-thieno[2,3-*g*]quinoline]-tetraones and Spiro[thieno[2,3-*g*]quinoline-3,5#-[1,2,4]triazinane]-tetraones with Potent Antiproliferative Activity

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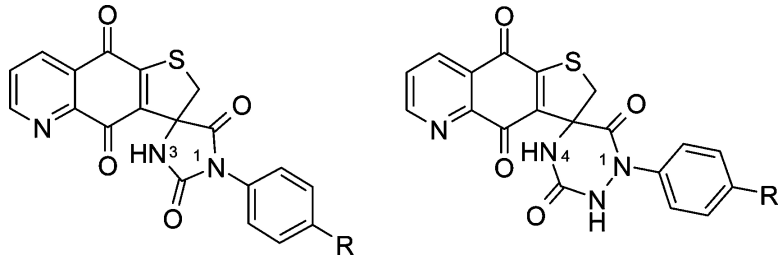

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Antitumor Agents 6. Synthesis, Structure−Activity Relationships, and Biological Evaluation of Spiro[imidazolidine-4,3′-thieno[2,3-*g*]quinoline]-tetraones and Spiro[thieno[2,3-*g*]quinoline-3,5′-[1,2,4]triazinane]-tetraones with Potent Antiproliferative Activity†

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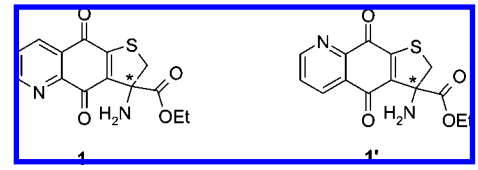
Two series of quinolinquinone derivatives, 2′*H*-spiro[imidazolidine-4,3′-thieno[2,3-*g*]quinoline]-2,4′,5,9′-tetraones (**2a**−**n**) and 2*H*-spiro[thieno[2,3-*g*]quinoline-3,5′-[1,2,4]triazinane]-3′,4,6′,9-tetraones (**3a**−**e**), were designed and synthesized using the previously described ethyl 3-amino-4,9-dioxo-2,3,4,9-tetrahydrothieno[2,3-*g*]quinoline-3-carboxylate (**1**) as a starting material. All compounds were evaluated for their antiproliferative activity against a panel of representative liquid and solid human tumor cell lines and exhibit IC_{50} values in the micromolar/submicromolar range. Series **2** displayed higher cytotoxicity than did series **3**. The nature of the substituents on both imidazoline and triazinane N1 nitrogen markedly affected the activity profile of these series. Spectrophotometric and fluorescence measurements as well as unwinding assays performed on the most cytotoxic compounds, **2c**, **2g**, and **2k**, showed that they are nonintercalative DNA agents and inhibit the catalytic activity of Topo II in a concentration-dependent mode. **2g** was the most active Topo II inhibitor with activity levels comparable to those of VP-16.

Introduction

Current chemotherapy commonly uses quinone-containing drugs to interfere with DNA replication and induce apoptosis in replicating cells.[1−4] Numerous quinone derivatives are powerful anticancer agents characterized by a DNA intercalating mechanism of action because of the planar structure of the α,β-unsaturated system. Furthermore, they can induce the formation of semiquinone radicals, which can transfer an electron to oxygen and produce a superoxide anion originating the ROS cascade.[5−8] This process is catalyzed by flavoenzymes such as NADPH-cytochrome P-450 reductase. Both superoxide and semiquinone radical anions can generate the hydroxyl radical, which is known to cause DNA strand breaks and enzyme modification, devastating effects leading to determine cell death. These multimodal activities of quinone drugs, using different weapons to attack the neoplastic cells contemporaneously, give high therapeutic relevance to this class of antitumor drugs. Very significant examples of these compounds are the well-known actinomicyn D and doxo[a].[3]

Chart 1. Chemical Structures of Quinolindiones **1** and **1′**



In a previous paper, we reported the synthesis of ethyl 3-amino-4,9-dioxo-2,3,4,9-tetrahydrothieno[2,3-*g*]quinoline-3-carboxylate (**1**, Chart 1 and Scheme 1) and of ethyl 3-amino-4,9-dioxo-2,3,4,9-tetrahydrothieno[3,2-*g*]quinoline-3-carboxylate (**1′**), two quinolindione derivatives arising from a 1,4-Michael reaction of quinolin-5,8-dione with a thiolate species formed by the opening of 2-aryl-1,3-thiazolidine ethyl esters in basic medium.[9] Both **1** and **1′,** which can be regarded as cyclic cysteine derivatives, were suspected of interacting with topoisomerases.[9−12]

Moreover, the antiproliferative activity of **1** and **1′**, evaluated against representative leukemia and solid human neoplastic cell lines, exhibited a significantly high in vitro cytotoxicity, with **1** ($IC_{50} \approx 0.01$ μM) being 10 times more active than **1′**.[9] Because of our ongoing interest in the search for new antitumor compounds containing the quinolin-5,8-dione motif, we explored structural modifications of the dihydrothiophene ring of the most active quinone **1**, keeping the quinolin-5,8-dione moiety unchanged. Because some imidazoline-diones,[13] pyrimidine-diones (ER-37328),[14] and piperazine-diones (ICRF-159)[15] (Chart 2) are potent antitumor drugs whose mechanisms of action include metal chelation[16] and inhibition of the enzymatic activity of Topo II,[17] four new compounds, two 2′*H*-spiro[imidazolidine-4,3′-thieno[2,3-*g*]quinoline]-2,4′,5,9′-tetraones, (+)-**2** and (−)-**2**, and two 2*H*-spiro[thieno[2,3-*g*]quinoline-3,5′-[1,2,4]triazinane]-3′,4,6′,9-tetraones, (+)-**3** and (−)-**3** (Chart 3), were designed and synthesized to be scaffolds for the development of new potential antitumor drugs useful in chemotherapy.

† Dedicated to Professor Rodolfo A. Nicolaus, a pioneer of the melanin chemistry.

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[a] Abbreviations: VP-16, etoposide; doxo, doxorubicin; CCRF-CEM, human acute T-lymphoblastic leukemia; CCRF-SB, human acute B-lymphoblastic leukemia; MT-4, human $CD4^+$ T cells expressing the TAT gene of HTLV-1; HT-29, human colon adenocarcinoma; MCF-7, human breast adenocarcinoma; HeLa, cervix carcinoma; ACHN, human renal adenocarcinoma; MRC-5, normal human lung fibroblasts; SAR, structure−activity relationships; LNR, linear DNA; CC, circularized DNA; SC, supercoiled DNA; RLX, relaxed DNA; ATCC, American Type Culture Collection; FCS, fetal calf serum; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; BSA, bovine serum albumin; Topo, topoisomerase; HBTU, O-Benzotriazole-*N*,*N*,*N*′,*N*′-tetramethyl-uronium-hexafluoro-phosphate; HOBt, *N*-hydroxybenzotriazole; HBT, 1-hydroxybenotriazole; DIEA, diisopropylethylamine.

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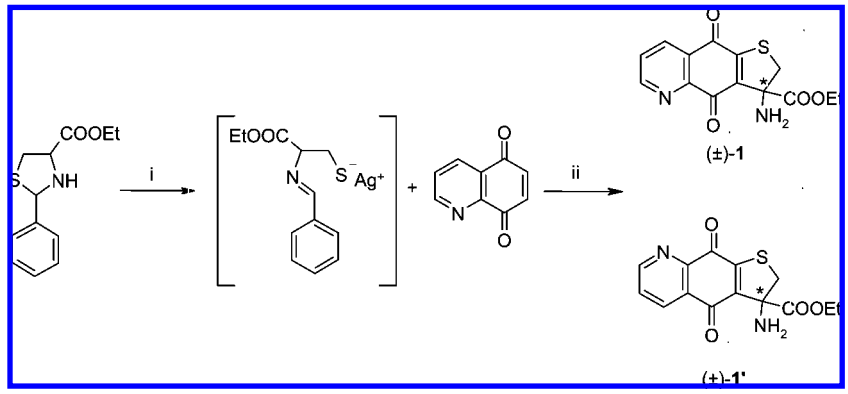
Scheme 1. Synthesis of Compounds (±)-**1** and (±)-**1′**[a]



[a] Reagent and conditions: (i) Ag_2CO_3, DBU, acetonitrile, rt, 12 h; (ii) 1N HCl, 1 h, $H_2O/CHCl_3$.

Chart 2. Chemical Structures of Some Potent Antitumor Dione Compounds

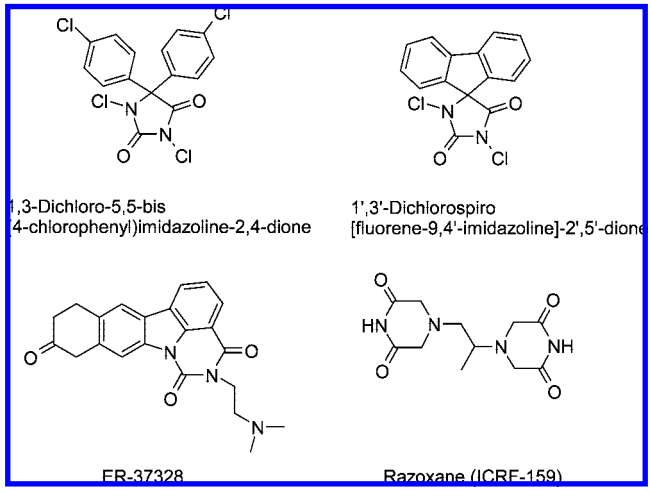
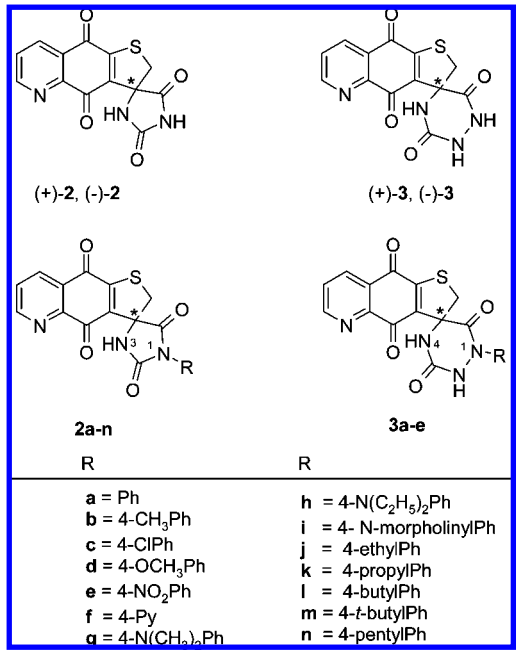


Chart 3. Chemical Structures of the Newly Designed and Synthesized Quinolindione Derivatives



The enantiomeric couples of **2** and **3** (Chart 3), which were preliminarily tested against MT-4 and HT-29 cell lines (Table 1), showed a promising cytotoxicity against both leukemia/lymphoma and carcinoma cells, independently of their stereochemical difference. With the aim of increasing the compound lypophilicity, a substituted pendant phenyl group was introduced to scaffolds **2** and **3**, and the effect of a set of electron-donating and -withdrawing substituents at the four-position of the phenyl ring was investigated to better delineate SAR (compounds **2a**−**e** and **3a**−**e**). Positively chargeable polar substituents (compounds **1f**−**i**) and highly lypophilic neutral groups (compounds **1j**−**n**) were also placed on the pendant phenyl of the more active series **1**. The synthesis and assessment of the in vitro antiproliferative activity of quinolinediones **1** and **2** (Chart 3) are described in the present article.

Table 1. Cytotoxic Activity of **2** and **3** Enantiomers

	IC_{50} (μM)[a]	
compd	MT-4 [b]	HT-29
(+)-**2**	0.13 ± 0.02	0.19 ± 0.02
(−)-**2**	0.12 ± 0.004	0.18 ± 0.04
(+)-**3**	0.55 ± 0.03	1.58 ± 0.05
(−)-**3**	0.50 ± 0.09	1.60 ± 0.03
doxo[b]	0.003 ± 0.001	0.002 ± 0.001

[a] Data represent mean values (±SD) for three independent determinations. [b] doxo was used as control.

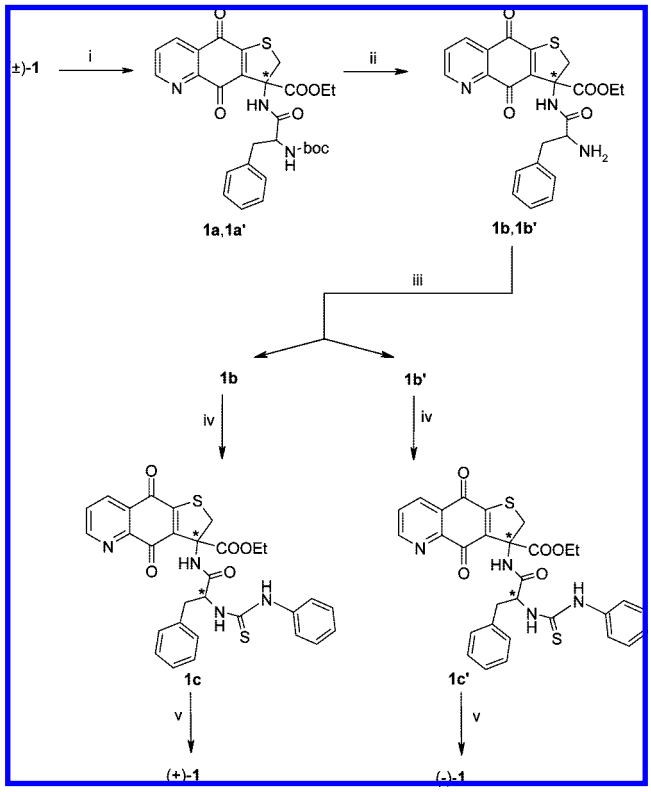
Chemistry

The racemic mixture of ethyl 3-amino-4,9-dioxo-2,3,4,9-tetrahydrothieno[2,3-*g*]quinoline-3-carboxylate ((±)-**1**) was obtained from the in situ 1,4-Michael addition of a thiolate species arising from the ethyl 2-phenylthiazolidine-4-carboxylate demolition in basic medium, followed by hydrolysis, as reported in a previous article.[9] A small amount of isomer (±)-**1′** was also formed in the reaction mixture (Scheme 1).

(±)-**1** was resolved according to the Evans method (Scheme 2),[18] a useful alternative procedure to the salt crystallization technique. (±)-**1** was coupled to boc-L-Phe in a THF/DMF (3:1 v/v) anhydrous solution (using the coupling reagents HBTU, HOBT, and DIEA) to give the mixture of protected diastereomer phenylalanylamides **1a** and **1a′** (1:1). Removal of the boc group, followed by neutralization, gave the diastereomer amides **1b** and **1b′**, which were separated by flash chromatography. The diasteroisomers **1b** and **1b′** were warmed, respectively, with phenylisothiocyanate to form the corresponding thiocarbamoyl derivatives **1c** and **1c′,** which in turn yielded (+)-**1** and (−)-**1** by TFA treatment.

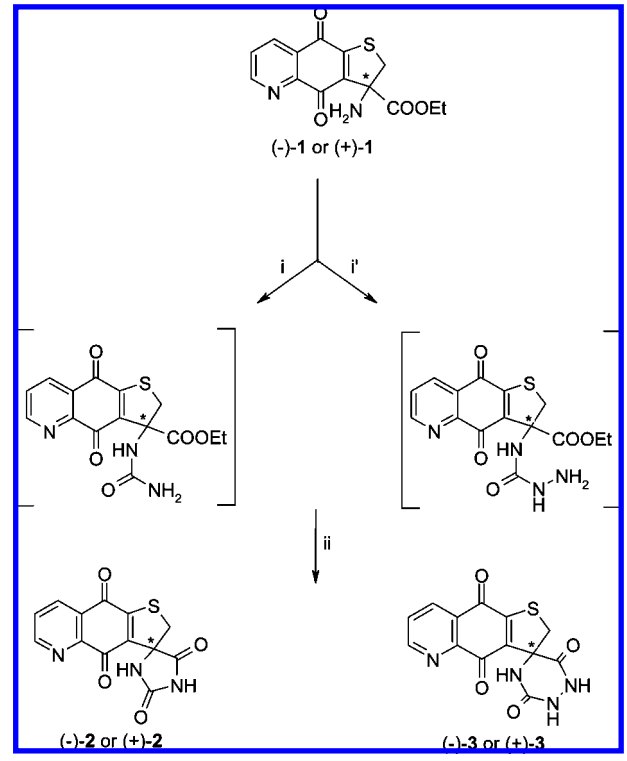
The pure enantiomers (+)-**1** and (−)-**1** were used to synthesize the couples (+)-**2** and (−)-**2** and (+)-**3** and (−)-**3**, respectively, as reported in Scheme 3.

Scheme 2. Resolution of (+)-**1** and (−)-**1** Enantiomers[a]



[a] Reagents and conditions: (i) Boc-L-Phe, HBTU, HOBt, DIEA, THF/DMF, rt, 48 h; (ii) TFA, CH_2CH_2, rt, 2 h, then TEA; (iii) flash chromatography separation; (iv) CSNHPh, CH_2Cl_2, reflux 1 h; (v) TFA, CH_2Cl_2, rt, 1 h.

Scheme 3. Synthesis of Enantiomers (+)-**2**, (−)-**2**, (+)-**3**, and (−)-**3**[a]



[a] Reagents and conditions: (i) $(CHCl_3)_2CO$, TEA, THF, rt, 30 min, then NH_4Cl, TEA; (i′) $(CHCl_3)_2CO$, TEA, THF, rt, 30 min, then NH_2-NH_2; (ii) EtOH, TEA, reflux (1 or 2 h).

(+)-**1** and (−)-**1** were treated, respectively, with bis(trichloromethyl)carbonate, a very handy phosgene analog, in presence of TEA and ammonium chloride or hydrazine in THF. The parenthesized urea intermediates were not isolated and afforded (+)-**2** and (−)-**2** and (+)-**3** and (−)-**3**, respectively, by the subsequent addition of EtOH and TEA at reflux temperature. This synthetic pathway consists of a sequence of three imidation reactions. The first two steps involve the highly favored nucleophilic attack of an amine group on the electron-poor carbonyl carbon of the bis(trichloromethyl)carbonate. The third step, an ester−urea cyclization, needed further heating. Series **2a**−**n** and **3a**−**e** were prepared according to this general procedure by the use of appropriate anilines and aminopyridine or hydrazines (Scheme 4), and their structures were determined on the basis of the physical−chemical and spectroscopic properties and elemental analysis.

Results and Discussion

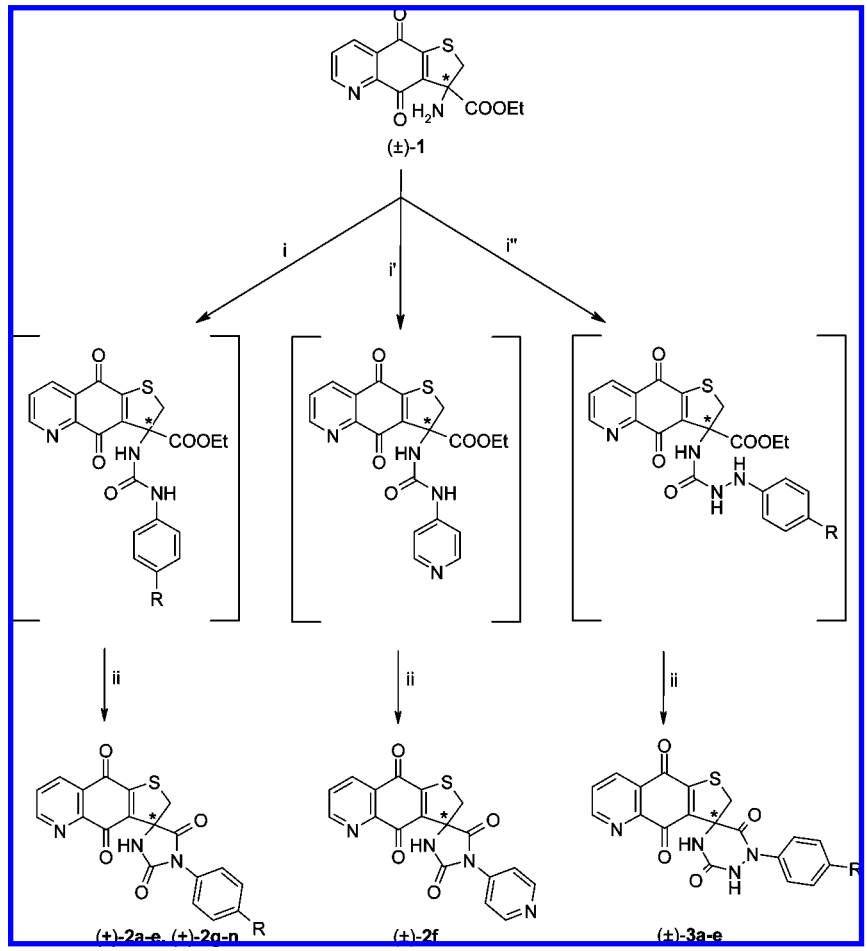
In Vitro Cytotoxicity. Table 1 shows the cytotoxic activity of the separated enantiomers of lead compounds **2** and **3** tested against the lymphoid MT-4 and the carcinoma HT-29 cell lines compared with doxo. The reported data suggest that both enantiomers of **2** and **3** are active at micromolar concentration and that the activity is independent of the compound chirality.

Because no substantial difference was observed between the activities of the enantiomeric couples of **2** and **3**, the further synthesized derivatives were tested as racemic mixtures. Table 2 shows the in vitro cytotoxic activity of compounds **2**, **2a**−**e**, **3**, and **3a**−**e** tested on a panel of nine human tumor cell lines representative of leukemia and solid human tumors. The most active compounds were also tested on MRC-5, a normal human lung fibroblast cell line. Data for doxo and VP-16 were included as control.

The cytotoxicities of compounds reported in Tables 1 and 2 revealed that lead **3** was 10 times less active than **2** and that the substituted phenyl derivatives **2a**−**e** and **3a**−**e** were more cytotoxic than **2** and **3**, respectively. In general, series **2a**−**e** was more active than series **3a**−**e**. On the basis of the median of the IC_{50} values, the cytotoxic activity seems to decrease according to the nature of the substituent in the order 4-NO_2Ph < 4-OCH_3Ph < Ph ≤ 4-CH_3Ph < 4-ClPh in both series **2a**−**e** and **3a**−**e**. We note that the human CNS SF-268 and XF 498 cells were 10−1000 times less sensitive than the other tumor lines to the antiproliferative effect of compounds **2a**−**e** and **3a**-**e**. In series **2a**−**e**, the phenyl derivative **2a** (median $IC_{50} = 0.02$ μM), the 4-methylphenyl derivative **2b** (median $IC_{50} = 0.02$ μM), and the 4-chlorophenyl derivative **2c** (median $IC_{50} = 0.01$ μM) were the most cytotoxic compounds, exhibiting an activity comparable or superior to that of doxo (median $IC_{50} = 0.03$ μM) and superior to that of VP-16 (median $IC_{50} = 2.15$ μM). Compounds **2a**−**d** displayed better specificity as antiproliferative agents against carcinoma than against leukemia cell lines. A selective inhibitory effect was observed for the proliferation of HT-29 cells with IC_{50} values ranging from 0.001 to 0.002 μM. Surprisingly, the 4-nitrophenyl derivative **2e**, which is weakly cytotoxic against carcinoma cells, showed a selective activity against lymphoma/leukemia cells (range of 0.001 to 0.008 μM).

As illustrated in Table 1, the normal human MRC-5 lung fibroblasts were about 100 times less sensitive to the inhibitory activity of the most cytotoxic compounds, **2c**, **2g**, and **2k**. This finding points to the highly differential (selective) effect between tumor and normal cells of the aforementioned compounds. It is

Scheme 4. Synthesis of Compounds (±)-**2a**−**n** and (±)-**3a**−**e**[a]



[a] Reagents and conditions: (i) $(CHCl_3)_2CO$, TEA, THF, rt, 30 min, then substituted anilines; (i′) $(CHCl_3)_2CO$, TEA, THF, rt, 30 min, then aminopyridine; (i″) $(CHCl_3)_2CO$, TEA, THF, rt, 30 min, then substituted hydrazines; (ii) EtOH, TEA, reflux (1 or 2 h).

noteworthy that **2c**, **2g**, and **2k** showed significantly stronger activity than VP-16 against normal cells but showed lower activity in comparison with doxo.

In regards to the less-active series **3a**−**e**, Table 2 shows that the compounds were more active against lymphoma/leukemia than carcinoma cells. Among the lymphoma cell lines, the CCRF-CEM and the CCRF-SB cells were most prone to the antiproliferative effect of **3a**−**e**. The 4-nitrophenyl derivative **3e** revealed a distinct specificity against CCRF-CEM, CCRF-SB, and MT-4 cells. The activity on lymphoma/leukemia cells of both **2e** and **3e** was 10 times higher than that of doxo (range of 0.002 to 0.005 μM). These compounds, both containing a reductable nitro group, could give rise to cell-damaging reactive amine species generated by some bioreductive processes.[19] However, to sustain this hypothesis, a more detailed investigation should be carried out.

Because series **2a**−**e** was found to be more active than series **3a**−**e**, the scaffold of **2** was selected to develop the derivatives **2f**−**n** further (Chart 3). Compounds **2f**−**i**, which have cationic groups, were designed on the basis of the fact that many quinones or polycarbonylic compounds bearing a cationic side chain such as intoplicine,[20] TAS-103,[21] and BBR-2778[22] (Chart 4) are potent inhibitors of Topo I and II, important targets of numerous antiproliferative drugs.

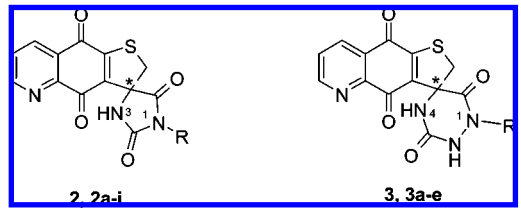
Moreover, given that the lipophilicity is a physical parameter that can contribute to in vitro cytotoxicity,[23] the 4-substituted phenyl derivatives **2j**−**n**, which contain highly apolar substituents, were also synthesized and evaluated for their antiproliferative activity. Table 2 reports the cytotoxic activity of the new derivatives **2f**−**n**.

Compounds **2f**, **2g**, and **2h**, which hold, respectively, a pyridine-, 4-*N*-dimethylphenyl-, and 4-*N*-diethylphenyl substituent, showed a nanomolar cytotoxic activity against lymphoma/leukemia cells and a lower antiproliferative effect on carcinoma cells compared with compounds **2a**−**e**. Only compound **2f** displayed high cytotoxicity against HT-29 carcinoma cells (IC_{50} = 0.002 μM). The 4-morfolinophenyl derivative **2i** was the least active compound of the series, suggesting that an electronegative oxygen far from the phenyl ring and the further bulky six-membered ring decreases the activity of the molecule.

For the highly lipophilic derivatives **2j**−**n**, the ethyl and propyl derivatives **2j** and **2k** showed the highest potency against almost all tested cell lines, including the CNS cells, SF-268 and XF-498. Extending the chain length from methyl to ethyl and propyl chain (compounds **2b**, **2j**, and **2k**), an increase in potency was recorded, whereas longer side chains resulted in a dramatic loss of potency (compounds **2l**, **2m**, and **2n**). This result could mean that too many lipophilic compounds, trapped in the outer lipid bilayer, could not reach their target to exert biological activity.

DNA Binding Properties. To test the propensity of the most active series **2** to interact with DNA, we carried out spectrophotometric and fluorescence measurements. Moreover, unwinding assays were undertaken to observe whether these compounds could intercalate DNA. The most representative compounds of series **2**, **2c**, **2g**, and **2k**, were examined.

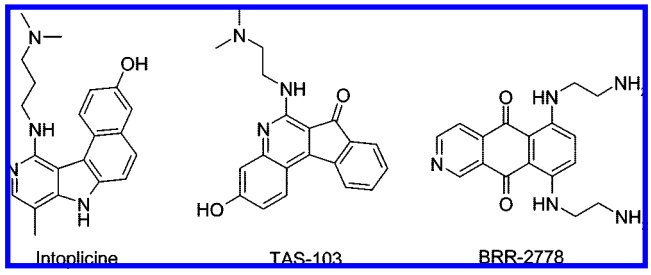
Table 2. Antiproliferative Activity of Compounds (±)-**2**, (±)-**2a**−**e**, (±)-**3**, and (±)-**3a**−**e**



		IC_{50} (μM)[a]									
compd	R	CCRF-CEM	CCRF-SB	MT-4	HT-29	MCF-7	HeLa	ACHN	SF-268	XF-498	MRC-5 normal cells
2	H	0.12	0.15	0.13	0.19	0.12	0.15	0.08	3.10	3.12	
2a	Ph	0.01	0.03	0.025	0.002	0.001	0.003	0.021	1.70	1.51	
2b	4-CH_3Ph	0.02	0.02	0.027	0.001	0.002	0.002	0.012	0.66	1.35	
2c	4-ClPh	0.01	0.01	0.01	0.002	0.004	0.002	0.004	0.58	0.59	0.80
2d	4-OCH_3Ph	0.05	0.01	0.07	0.002	0.05	0.001	0.05	0.65	0.65	
2e	4-NO_2Ph	0.001	0.002	0.008	0.25	0.35	0.10	0.10	1.52	1.03	
2f	4-Py	0.002	0.001	0.003	0.002	0.01	0.01	0.08	1.10	1.12	
2g	4-$N(CH_3)_2$Ph	0.001	0.001	0.015	0.02	0.01	0.01	0.09	0.95	0.80	1.00
2h	4-$N(C_2H_5)_2$Ph	0.020	0.009	0.008	0.05	0.03	0.05	0.02	0.96	0.73	
2i	4-N-morpholinylPh	0.60	0.65	0.80	0.75	0.75	0.20	0.14	1.98	1.70	
2j	4-ethylPh	0.01	0.01	0.001	0.001	0.05	0.005	0.005	0.45	1.20	
2k	4-propylPh	0.001	0.002	0.001	0.001	0.002	0.005	0.001	0.30	0.45	0.85
2l	4-butylPh	0.15	0.19	0.20	0.05	0.07	0.09	0.05	0.40	0.95	
2m	4-*t*-butylPh	0.20	0.20	0.20	0.15	0.13	0.10	0.15	0.50	0.85	
2n	4-pentylPh	0.25	0.15	0.19	0.25	0.25	0.20	0.25	0.52	0.83	
3	H	0.45	0.048	0.55	1.60	1.25	0.98	1.05	4.82	5.00	
3a	Ph	0.09	0.039	0.15	0.55	0.15	0.21	0.94	2.20	4.55	
3b	4-CH_3Ph	0.07	0.037	0.10	0.20	0.41	0.21	0.50	2.10	3.80	
3c	4-ClPh	0.05	0.051	0.15	0.22	0.11	0.15	0.36	2.00	2.50	
3d	4-OCH_3Ph	0.035	0.035	0.08	0.65	0.23	0.15	0.43	2.85	3.80	
3e	4-NO_2Ph	0.002	0.002	0.005	1.01	0.55	0.61	0.65	3.55	4.80	
doxo[b]		0.02	0.03	0.01	0.01	0.05	0.07	0.04	0.20	0.16	0.30
VP-16		1.20	0.10	1.25	2.50	1.00	3.00	2.15	3.25	3.45	3.90

[a] Compound concentration required to reduce cell proliferation by 50%, as determined the MTT method, under conditions allowing untreated controls to undergo at least three consecutive rounds of multiplication. Data represent mean values for three independent determinations. [b] Control.

Chart 4. Chemical Structures of Potent Inhibitors of Topo I and II



The spectroscopic properties of the selected compounds, in the presence and absence of calf thymus DNA, were studied by conventional optical spectroscopy. Characterization of this mixture was made by comparing the UV−vis absorption spectrum of the complex with the spectra of **2c**, **2g**, and **2k**, respectively.

The absorption spectrum of compound **2c** (data not shown) was totally unaffected by the addition of DNA. The absorption maximum of the free drug at 425 nm remains unchanged, and the hypochromism is extremely weak (3%), suggesting that **2c** has no significant interaction with the DNA double helix. Moreover, we used fluorescence spectroscopy to examine the abilities of the test drugs to compete with a DNA-binding ligand for available binding sites. In these experiments, the fluorescent ligand used was the intercalating drug ethidium bromide, which is highly fluorescent in the presence of DNA. Almost no quenching was observed with compounds **2c**, **2g**, and **2k.** Figure 1 shows the fluorescence quenching of **2c**. The results of the fluorescence quenching experiments are totally consistent with

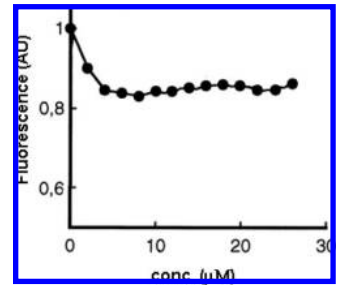


Figure 1. Quenching of fluorescence intensity of ethidium bromide−DNA complex by compound **2c**. Experimental conditions: 20 μM calf thymus DNA, 2 μM ethidium bromide (λ_{exc} = 546 nm, λ_{em} = 595 nm), in 0.01 M ionic-strength buffer (9.3 mM NaCl, 2 mM Na−acetate, and 0.1 mM EDTA).

the absorption measurements and demonstrate that compound **2c** has minimal, if any, interaction with DNA. Similar spectrophotometric and fluorescence results were recorded for compounds **2g** and **2k**.

To further investigate whether compounds belonging to series **2** intercalate in DNA, we performed an unwinding assay using linearized pBR322 and T_4 DNA ligase. In this assay, DNA intercalators first unwind the linearized DNA, which results in a change in the twist of the duplex helix. Circulation of drug-bound DNA by T_4 DNA ligase freezes the linking number of the unwound DNA. Upon drug removal, the twist changes back to normal, whereas the linking number remains constant, which causes the introduction of negative superhelicity to the DNA. Figure 2 shows the separation of the products on an agarose gel. In lanes 3−5, the inhibition of the activity of the T_4 ligase by doxo was observed, which was due to the strong intercalation

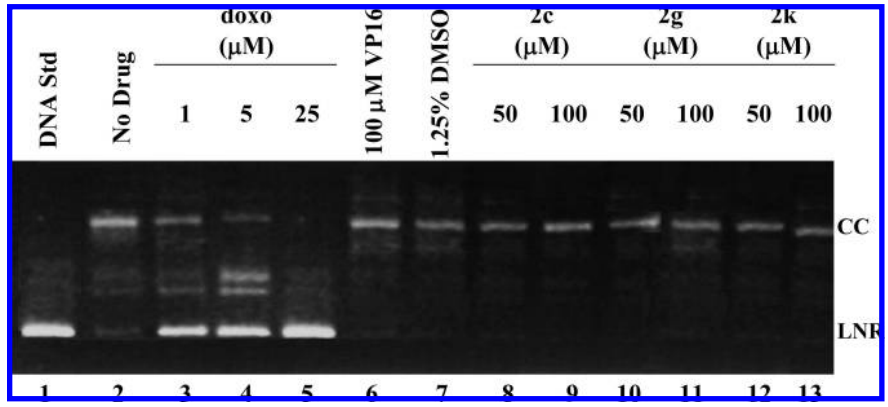


Figure 2. Effect of compounds **2c**, **2g**, and **2k** on the DNA unwinding assay with T_4 DNA ligase. In this assay, linearized plasmid DNA was incubated with T_4 DNA ligase in the presence of testing compounds. Linear DNA is shown for reference. The positions of LNR and CC DNA are indicated.

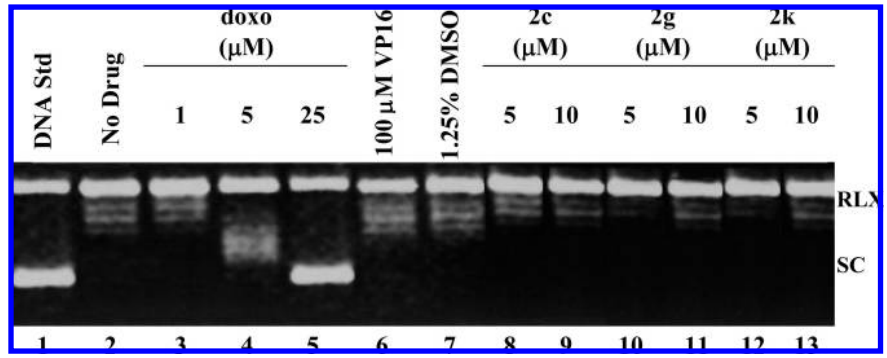


Figure 3. Effect of **2c**, **2g**, and **2k** on the DNA unwinding assay with DNA Topo I. In this assay, supercoiled plasmid DNA was incubated with Topo I. Negatively supercoiled pBR322 (DNA Std) is shown for reference. The positions of SC and RLX DNA are indicated.

ability of doxo.[24] However, the negative control VP16 and the compounds **2c**, **2g**, and **2k**, which were chosen to be representative of series **2**, had no significant influence on the DNA topoisomers, even at concentrations of 100 μM.

To verify the results, another unwinding assay was performed. In this assay, supercoiled DNA is relaxed by Topo I in the presence of the testing compounds. The intercalation of the compounds into a closed circular DNA reduces the twisting number of the DNA. Upon Topo I addition, the linking number is adjusted to the twist of the intercalated DNA. Following relaxation, samples are phenol-extracted to remove the intercalator, and the twisting number is restored, thus leaving the DNA in a new supercoiled state. As shown in Figure 3, doxo converts the topoisomers to a less-relaxed form at a low concentration (5 μM) and to a complete supercoiled form at a higher concentration (25 μM). On the contrary, VP16, **2c**, **2g**, and **2k** have no effect. From these two experiments, we concluded that compound **2c**, **2g**, and **2k** and the whole series **2** are nonintercalative agents.

Inhibition of the Activity of Topo II by 2c, 2g, and 2k. Topo II is an essential enzyme that plays an important role in DNA replication, repair, transcription, and chromosome segregation. Topo II alters DNA topology by catalyzing the passing of an intact DNA double helix through a transient double-stranded break made in a second helix.[25,26] A number of antitumor drugs, including quinonic structures, are thought to be cytotoxic by virtue of their ability to stabilize a covalent Topo II−DNA intermediate, the cleavable complex.[27]

The effect of compounds **2c**, **2g**, and **2k** on the strand passage activity of Topo II was determined by the enzyme-mediated negatively supercoiled pBR322 relaxation. As shown in Figure 4, **2c**, **2g**, and **2k** displayed significant inhibition of this reaction in a concentration-dependent mode. It appears that compounds **2c**, **2g**, and **2k** are potent Topo II inhibitors, with **2g** being the most potent. This result suggests that the cationic group promotes more favorable interactions with the enzyme.

Conclusions

This study reports the synthesis and the SARs of two series of quinolindione derivatives, the spiro[(dihydroimidazo-2,4-dione)-5,3′-(2′,3′-dihydrothieno[2,3-*b*]quinoline-4′,9′-diones)] (**2a**−**n**) and the spiro[(1,2,4-triazinane-3,6-dione)-6,3′-(2′,3′-dihydrothieno[2,3-*g*]quinoline-4′,9′-dione)] (**3a**−**e**), analogues of the 3-amino-3-(ethoxycarbonyl)-2,3-dihydrothieno[2,3-*b*]quinoline-2,5-dione (**1**), which exhibits a broad spectrum of cytotoxicity against leukemia and solid tumor cell lines at micromolar and submicromolar concentrations. Series **2** displays higher cytotoxicity compared with the corresponding series **3**. Among the compounds **2a**−**e**, the 4-nitrophenyl derivative **2e** showed potent activity against lymphoma/leukemia cells (range of 0.001 to 0.008 μM). The positively charged compounds **2f**, **2g**, and **2h** displayed nanomolar cytotoxic activity against lymphoma/leukemia cells and, when compared with compounds **2a**−**e**, a lower antiproliferative effect on carcinoma cells. Among the lipophilic compounds **2j**−**n**, the propyl derivative **2k** showed the highest potency against almost all tested cell lines, including the CNS cells, SF-268 and XF-498.

Spectrophotometric and fluorescence measurements as well as unwinding assays performed on the most active compounds **2c**, **2g**, and **2k**, showed that they are nonintercalative DNA agents. The inhibition of these compounds on the catalytic activity of Topo II was also examined, and **2g** was the most

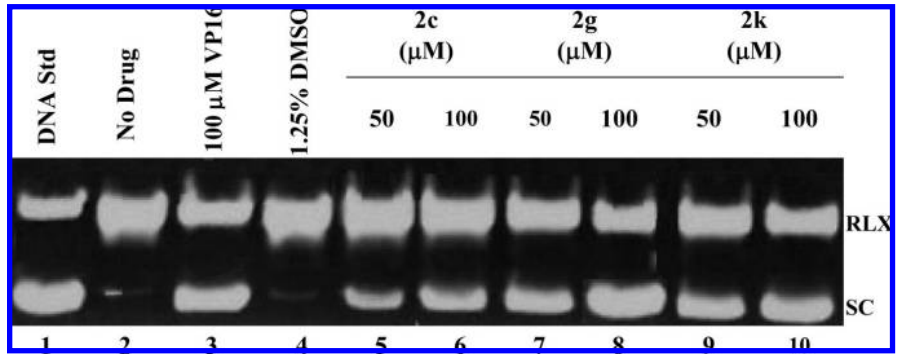


Figure 4. Effect of compounds **2c**, **2g**, and **2k** on Topo-II-mediated supercoiled pBR322 relaxation. Negatively supercoiled pBR322 (DNA Std) and relaxed pBR322 (no drug) are shown for reference. The positions of SC and RLX are reported.

active compound, showing an inhibitory effect comparable to that of VP-16. An attempt to understand the mechanism of action of this series of new nonintercalative Topo II inhibitors further is being undertaken in our laboratory.

Experimental Section

General. Reagents, starting material, and solvents were purchased from commercial suppliers and were used as received. Analytical TLC was performed on a 0.25 mm layer of silica gel 60 F254 from Merck, and preparative TLC was performed on 20 × 20 cm^2 glass plates coated with a 2 mm layer of silica gel PF254 from Merck. Silica gel 60 (300−400 mesh), Merck, was used for flash chromatography. Melting points were taken on a Kofler apparatus and are uncorrected. Optical rotations were determined with a Perkin-Elmer-241 MC polarimeter. ^{1}H NMR spectra were recorded with a Bruker-500 spectrometer. Chemical shifts are reported in δ relative to internal Me_4Si, and J values are reported in Hz. Mass spectra were obtained using an EI at 70 eV on a ZAB 2F spectrometer and a FABMS spectrometer.

Resolution of the (+)-1 and (−)-1 Enantiomers. (±)-**1** (1 mmol, 304 mg) was dissolved in a solution of THF/DMF (20 mL; 2:1 v/v), and L-Boc-Phe (1 equiv), HBTU (1.2 equiv), HOBt (1.2 equiv), and DIEA (2 equiv) were added to the stirring solution at room temperature for 48 h. The reaction was evaporated in vacuo, and the solid residue, dissolved in $CHCl_3$, was washed with a 10% citric acid (2 × 25 mL), 10% $NaHCO_3$ (2 × 25 mL), and H_2O (25 mL) solutions. The organic layer was dried over Na_2SO_4 and evaporated. Flash chromatography (20−50% gradient of EtOAc in *n*-hexane) of the solid residue gave the diastereoisomeric mixture of the ethyl 3-(2-(*tert*-butoxycarbonylamino)-3-phenylpropanamido)-4,9-dioxo-2,3,4,9-tetrahydrothieno[2,3-*g*]quinoline-3-carboxylates (**1a**, **1a′**). The mixture, which was treated with TFA in CH_2Cl_2 at room temperature for 2 h, was neutralized by TEA, and the solvent was evaporated to dryness. Flash chromatography of the residue by the use of AcOEt as eluent yielded the ethyl 3-(2-amino-3-phenylpropanamido)-4,9-dioxo-2,3,4,9-tetrahydrothieno[2,3-*g*]quinoline-3-carboxylates (**1b**, R_f less) and (**1b′**, R_f greater). Phenylisothiocyanate (60 mg, 0.44 mmol) and TEA (88 mg, 0.8 mmol) were added to the respective stirring solutions of **1b** and **1b′** (170 mg, 0.35 mmol) in CH_2Cl_2. After 1 h at reflux temperature, the solvent was evaporated to dryness, and the residues were analyzed by flash chromatography using $CHCl_3$ as eluent. The yellow solids ethyl 4,9-dioxo-3-(3-phenyl-2-(3-phenylthioureido)propanamido)-2,3,4,9-tetrahydrothieno[2,3-*g*]quinoline-3-carboxylates **1c** and **1c′** (∼106 mg) were treated with TFA (2 mL). After 1 h at room temperature, the reaction mixtures were neutralized with TEA and evaporated to dryness. Flash chromatography, using ethyl ether as eluent, yielded the crude residues of the enantiomers (+)-**1** (orange oil, $[\alpha]_{20}^{D} = +31.0°$ (*c* 1.2, MeOH)) and (−)-**1** (orange oil, $[\alpha]_{20}^{D} = -30.1°$ (*c* 1.0, MeOH)).

Ethyl 4,9-Dioxo-3-(3-phenyl-2-(3-phenylthioureido)propanamido)-2,3,4,9-tetrahydrothieno[2,3-*g*]quinoline-3-carboxylates (1c). Yellow solid (70%). mp: dec > 220. MS *m/z*: 586 (M+). ^{1}H NMR (δ, $CDCl_3$): 8.90 (1H, dd, J = 4.3, 2.2 Hz), 8.36 (1H, dd, 7.3, 2.2 Hz), 7.70 (1H, bs, NH), 7.57 (1H, dd, J = 7.3, 4.3 Hz), 7.35 (5H, m), 7.10 (6H, m, 5H + NH), 6.32 (1H, bs, NH), 5.25 (1H, m, αCH), 4.25 (2H, q, CH_2), 3.81 (1H, d, J = 12.5 Hz), 3.70 (1H, d, J = 12.5 Hz), 3.10 (2H, m, βCH_2), 1.22 (3H, t, CH_3). Anal. ($C_{30}H_{26}N_4O_5S_2$) C, H, N, S.

Ethyl 4,9-Dioxo-3-(3-phenyl-2-(3-phenylthioureido)propanamido)-2,3,4,9-tetrahydrothieno[2,3-*g*]quinoline-3-carboxylates (1c′). Yellow solid (70%). mp: dec > 220. MS *m/z*: 586 (M+). ^{1}H NMR (δ, $CDCl_3$): 8.90 (1H, dd, J = 4.3, 2.2 Hz), 8.36 (1H, dd, 7.3, 2.2 Hz), 7.71 (1H, bs, NH), 7.57 (1H, dd, J = 7.3, 4.3 Hz), 7.35 (5H, m), 7.10 (6H, m, 5H + NH), 6.32 (1H, bs, NH), 5.27 (1H, m, αCH), 4.25 (2H, q, CH_2), 3.81 (1H, d, J = 12.5 Hz), 3.70 (1H, d, J = 12.5 Hz), 3.12 (2H, m, βCH_2), 1.22 (3H, t, CH_3). Anal. ($C_{30}H_{26}N_4O_5S_2$) C, H, N, S.

***2′H*-Spiro[imidazolidine-4,3′-thieno[2,3-*g*]quinoline]-2,4′,5,9′-tetraones (2, 2a−n) and 2*H*-Spiro[thieno[2,3-*g*]quinoline-3,5′-[1,2,4]triazinane]-3′,4,6′,9-tetraones (3, 3a−e). General Procedure.** Bis(trichloromethyl)carbonate (118 mg, 0.4 mmol) was added to a solution of the quinoline-dione 1, (300 mg, 1 mmol) in dry THF (25 mL) at room temperature. TEA (0.3 mL, 2 mmol) was added dropwise under stirring. After 10 min, a solution in dry THF (5 mL) of TEA (0.3 mL, 2 mmol) and ammonium chloride, aniline, substituted anilines (4-methyl-, 4-chloro-, 4-methoxy-, and 4-nitro), or 4-aminopyridine (2 equiv) was added under stirring. After 1 h, the reaction mixtures were diluted with chloroform, washed with water, dried over Na_2SO_4, and evaporated to dryness and afforded the crude compounds **2** and **2a**−**n**, respectively. All compounds were purified by flash chromatography (80% $CHCl_3$, 20 MeOH). To prepare compounds (**3**, **3a**−**e**), according to the above-reported procedure, hydrazine, phenylhydrazine, and the corresponding substituted phenylhydrazines (4-methyl, 4-chloro, 4-methoxy, and 4-nitro) were used instead of the titled anilines.

***2′H*-Spiro[imidazolidine-4,3′-thieno[2,3-*g*]quinoline]-2,4′,5,9′-tetraone (2).** Pale yellow solid (43%). mp: 188−189 °C. ^{1}H NMR (500 MHz, $CDCl_3$, δ): 10.5 (1H, s, NH-1), 9.02 (1H, d, J = 4.2 Hz, 6′-H), 8.42 (1H, d, J = 7.7 Hz, 8′-H), 7.65 (1H, dd, J = 7.7, 4.2 Hz, 7′-H), 5.90 (1H, s, NH-3), 3.40 (1H, d, J = 12.9 Hz, 2′a-H), 4.12 (1H, d, J = 12.9 Hz, 2′b-H). MS [M^+] Calcd for $C_{13}H_7N_3O_4S$: 301; Found. Anal. ($C_{13}H_7N_3O_4S$) C, H, N, S. (+)-**2** ($[\alpha]_{20}^{D} = +29.9°$ (*c* 1.2, MeOH)), (−)-**2** ($[\alpha]_{20}^{D} = -30.1°$ (*c* 1.1, MeOH)).

1-Phenyl-2′*H*-spiro[imidazolidine-4,3′-thieno[2,3-*g*]quinoline]-2,4′,5,9′-tetraone (2a). Pale yellow solid (45%). mp: 193−194 °C. ^{1}H NMR (500 MHz, $CDCl_3$, δ): 9.02 (1H, d, J = 4.2 Hz, 6′-H), 8.42 (1H, d, J = 7.7 Hz, 8′-H), 7.65 (1H, dd, J = 7.7, 4.2 Hz, 7′-H), 7.64 (2H, d, J = 7.2 Hz), 7.57 (2H, t, J = 6.9 Hz), 7.30 (1H, t, J = 6.5 Hz), 5.90 (1H, s, NH-3), 3.40 (1H, d, J = 12.9 Hz, 2′a-H), 4.12 (1H, d, J = 12.9 Hz, 2′b-H). MS [M^+] Calcd for $C_{19}H_{11}N_3O_4S$: 377; Found. Anal. ($C_{19}H_{11}N_3O_4S$) C, H, N, S.

1-*p*-Tolyl-2′*H*-spiro[imidazolidine-4,3′-thieno[2,3-*g*]quinoline]-2,4′,5,9′-tetraone (2b). Pale yellow solid (44%). mp: 196−197 °C. ^{1}H NMR (500 MHz, $CDCl_3$, δ): 9.02 (1H, d, J = 4.2 Hz, 6′-H),

8.42 (1H, d, J = 7.7 Hz, 8′-H), 7.65 (1H, dd, J = 7.7, 4.2 Hz, 7′-H), 7.58 (2H, d, J = 7.2 Hz), 7.35 (2H, d, J = 7.2 Hz), 5.90 (1H, s, NH-3), 4.12 (1H, d, J = 12.9 Hz, 2′b-H), 3.40 (1H, d, J = 12.9 Hz, 2′a-H), 2.32 (3H, s). MS [M^+] Calcd for $C_{20}H_{13}N_3O_4S$: 391; Found. Anal. ($C_{20}H_{13}N_3O_4S$) C, H, N, S.

1-(4-Chlorophenyl)-2′*H*-spiro[imidazolidine-4,3′-thieno[2,3-*g*]quinoline]-2,4′,5,9′-tetraone (2c). Pale yellow solid (43%). mp: 197−198 °C. ^{1}H NMR (500 MHz, $CDCl_3$, δ): 9.02 (1H, d, J = 4.2 Hz, 6′-H), 8.42 (1H, d, J = 7.7 Hz, 8′-H), 7.65 (1H, dd, J = 7.7, 4.2 Hz, 7′-H), 7.50 (2H, d, J = 7.2 Hz), 7.30 (2H, d, J = 7.2 Hz), 5.90 (1H, s, NH-3), 4.12 (1H, d, J = 12.9 Hz, 2′b-H), 3.40 (1H, d, J = 12.9 Hz, 2′a-H). MS [M^+] Calcd for $C_{19}H_{10}ClN_3O_4S$: 411, 413 (M + 2); Found. Anal. ($C_{19}H_{10}ClN_3O_4$) C, H, N, S.

1-(4-Methoxyphenyl)-2′*H*-spiro[imidazolidine-4,3′-thieno[2,3-*g*]quinoline]-2,4′,5,9′-tetraone (2d). Pale yellow solid (46%). mp: >200 °C. ^{1}H NMR (500 MHz, $CDCl_3$, δ): 9.02 (1H, d, J = 4.2 Hz, 6′-H), 8.42 (1H, d, J = 7.7 Hz, 8′-H), 7.65 (1H, dd, J = 7.7, 4.2 Hz, 7′-H), 7.40 (2H, d, J = 7.2 Hz), 7.15 (2H, d, J = 7.2 Hz), 5.90 (1H, s, NH-3), 4.12 (1H, d, J = 12.9 Hz, 2′b-H), 3.75 (3H, s), 3.40 (1H, d, J = 12.9 Hz, 2′a-H). MS [M^+] Calcd for $C_{20}H_{13}N_3O_5S$: 407; Found. Anal. ($C_{20}H_{13}N_3O_5S$) C, H, N, S.

1-(4-Nitrophenyl)-2′*H*-spiro[imidazolidine-4,3′-thieno[2,3-*g*]quinoline]-2,4′,5,9′-tetraone (2e). Orange solid (23%). mp: >200 °C. ^{1}H NMR (500 MHz, $CDCl_3$, δ): 9.02 (1H, d, J = 4.2 Hz, 6′-H), 8.42 (1H, d, J = 7.7 Hz, 8′-H), 8.05 (2H, d, J = 7.2 Hz), 7.70 (2H, d, J = 7.2 Hz), 7.65 (1H, dd, J = 7.7, 4.2 Hz, 7′-H), 5,90 (1H, s, NH-3), 4.12 (1H, d, J = 12.9 Hz, 2′b-H). 3.40 (1H, d, J = 12.9 Hz, 2′a-H), MS [M^+] Calcd for $C_{19}H_{10}N_4O_6S$: 422; Found. Anal. ($C_{19}H_{10}N_4O_6S$) C, H, N, S.

1-(Pyridin-4-yl)-2′*H*-spiro[imidazolidine-4,3′-thieno[2,3-*g*]quinoline]-2,4′,5,9′-tetraone (2f). Pale yellow solid (45%). mp: >200 °C. ^{1}H NMR (500 MHz, $CDCl_3$, δ): 9.02 (1H, d, J = 4.2 Hz, 6′-H), 8.70 (2H, d, J = 5.8 Hz), 8.42 (1H, d, J = 7.7 Hz, 8′-H), 7.65 (1H, dd, J = 7.7, 4.2 Hz, 7′-H), 7.61 (2H, d, J = 5.8 Hz), 5.90 (1H, s, NH-3), 3.40 (1H, d, J = 12.9 Hz, 2′a-H), 4.12 (1H, d, J = 12.9 Hz, 2′b-H). MS [M^+] Calcd for $C_{18}H_{10}N_4O_4S$: 378; Found. Anal. ($C_{18}H_{10}N_4O_4S$) C, H, N, S.

1-(4-(Dimethylamino)phenyl)-2′*H*-spiro[imidazolidine-4,3′-thieno[2,3-*g*]quinoline]-2,4′,5,9′-tetraone (2g). Pale yellow solid (40%). mp: >200 °C. ^{1}H NMR (500 MHz, CD_3OD, δ): 9.03 (1H, d, J = 4.2 Hz, 6′-H), 8.42 (1H, d, J = 7.7 Hz, 8′-H), 7.67 (1H, dd, J = 7.7, 4.2 Hz, 7′-H), 7.50 (2H, d, J = 7.2 Hz), 7.15 (2H, d, J = 7.2 Hz), 5.90 (1H, s, NH-3), 4.12 (1H, d, J = 12.9 Hz, 2′b-H), 3.40 (1H, d, J = 12.9 Hz, 2′a-H), 2.32 (6H, s). MS [M^+] Calcd for $C_{21}H_{16}N_4O_4S$: 420; Found. Anal. ($C_{21}H_{16}N_4O_4S$) C, H, N, S.

1-(4-(Diethylamino)phenyl)-2′*H*-spiro[imidazolidine-4,3′-thieno[2,3-*g*]quinoline]-2,4′,5,9′-tetraone (2h). Pale yellow solid (39%). mp: >200 °C. ^{1}H NMR (500 MHz, CD_3OD, δ): 9.03 (1H, d, J = 4.2 Hz, 6′-H), 8.42 (1H, d, J = 7.7 Hz, 8′-H), 7.67 (1H, dd, J = 7.7, 4.2 Hz, 7′-H), 7.50 (2H, d, J = 7.2 Hz), 7.15 (2H, d, J = 7.2 Hz), 5.90 (1H, s, NH-3), 4.12 (1H, d, J = 12.9 Hz, 2′b-H), 3.40 (5H, m), 1.15 (6H, t, J = 7.4 Hz). MS [M^+] Calcd for $C_{23}H_{20}N_4O_4S$: 448; Found. Anal. ($C_{23}H_{20}N_4O_4S$) C, H, N, S.

1-(4-Morpholinophenyl)-2′*H*-spiro[imidazolidine-4,3′-thieno[2,3-*g*]quinoline]-2,4′,5,9′-tetraone (2i). Pale yellow solid (39%). mp: >200 °C. ^{1}H NMR (500 MHz, CD_3OD, δ): 9.03 (1H, d, J = 4.2 Hz, 6′-H), 8.42 (1H, d, J = 7.7 Hz, 8′-H), 7.67 (1H, dd, J = 7.7, 4.2 Hz, 7′-H), 7.50 (2H, d, J = 7.2 Hz), 7.15 (2H, d, J = 7.2 Hz), 5.90 (1H, s, NH-3),4.12 (1H, d, J = 12.9 Hz, 2′b-H), 3.65 (4H, m), 3.40 (1H, d, J = 12.9 Hz, 2′a-H), 3.32 (4H, m). MS [M^+] Calcd for $C_{23}H_{18}N_4O_5S$: 462; Found. Anal. ($C_{23}H_{18}N_4O_5S$) C, H, N, S.

1-(4-Ethylphenyl)-2′*H*-spiro[imidazolidine-4,3′-thieno[2,3-*g*]quinoline]-2,4′,5,9′-tetraone (2j). Pale yellow solid (44%). mp: 198−199 °C. ^{1}H NMR (500 MHz, $CDCl_3$, δ): 9.02 (1H, d, J = 4.2 Hz, 6′-H), 8.42 (1H, d, J = 7.7 Hz, 8′-H), 7.65 (1H, dd, J = 7.7, 4.2 Hz, 7′-H), 7.58 (2H, d, J = 7.2 Hz), 7.35 (2H, d, J = 7.2 Hz), 5.90 (1H, s, NH-3), 4.12 (1H, d, J = 12.9 Hz, 2′b-H), 3.40 (1H, d, J = 12.9 Hz, 2′a-H), 2.58 (2H, q, J = 6.7 Hz), 1.20 (3H, t, J = 6.7 Hz). MS [M^+] Calcd for $C_{21}H_{15}N_3O_4S$: 405; Found. Anal. ($C_{21}H_{15}N_3O_4S$) C, H, N, S.

1-(4-Propylphenyl)-2′*H*-spiro[imidazolidine-4,3′-thieno[2,3-*g*]quinoline]-2,4′,5,9′-tetraone (2k). Pale yellow solid (44%). mp: 196−197 °C. ^{1}H NMR (500 MHz, $CDCl_3$, δ): 9.02 (1H, d, J = 4.2 Hz, 6′-H), 8.42 (1H, d, J = 7.7 Hz, 8′-H), 7.65 (1H, dd, J = 7.7, 4.2 Hz, 7′-H), 7.58 (2H, d, J = 7.2 Hz), 7.35 (2H, d, J = 7.2 Hz), 5.90 (1H, s, NH-3), 4.12 (1H, d, J = 12.9 Hz, 2′b-H), 3.40 (1H, d, J = 12.9 Hz, 2′a-H), 2.54 (2H, t, J = 6.7 Hz), 1.60 (2H, m), 0.98 (3H, t, J = 6.7 Hz). MS [M^+] Calcd for $C_{22}H_{17}N_3O_4S$: 419; Found. Anal. ($C_{22}H_{17}N_3O_4S$) C, H, N, S.

1-(4-Butylphenyl)-2′*H*-spiro[imidazolidine-4,3′-thieno[2,3-*g*]quinoline]-2,4′,5,9′-tetraone (2l). Pale yellow solid (44%). mp: 198−199 °C. ^{1}H NMR (500 MHz, $CDCl_3$, δ): 9.02 (1H, d, J = 4.2 Hz, 6′-H), 8.42 (1H, d, J = 7.7 Hz, 8′-H), 7.65 (1H, dd, J = 7.7, 4.2 Hz, 7′-H), 7.58 (2H, d, J = 7.2 Hz), 7.35 (2H, d, J = 7.2 Hz), 5.90 (1H, s, NH-3), 4.12 (1H, d, J = 12.9 Hz, 2′b-H), 3.40 (1H, d, J = 12.9 Hz, 2′a-H), 2.60 (2H, t, J = 6.7 Hz), 1.58 (2H, m), 1.36 (2H, m), 0.90 (3H, t, J = 6.7 Hz). MS [M^+] Calcd for $C_{23}H_{19}N_3O_4S$: 433; Found. Anal. ($C_{23}H_{19}N_3O_4S$) C, H, N, S.

1-(4-*tert*-Butylphenyl)-2′*H*-spiro[imidazolidine-4,3′-thieno[2,3-*g*]quinoline]-2,4′,5,9′-tetraone (2m). Pale yellow solid (44%). mp: 198−199 °C. ^{1}H NMR (500 MHz, $CDCl_3$, δ): 9.02 (1H, d, J = 4.2 Hz, 6′-H), 8.42 (1H, d, J = 7.7 Hz, 8′-H), 7.65 (1H, dd, J = 7.7, 4.2 Hz, 7′-H), 7.48 (2H, d, J = 7.2 Hz), 7.30 (2H, d, J = 7.2 Hz), 5.90 (1H, s, NH-3), 4.12 (1H, d, J = 12.9 Hz, 2′b-H), 3.40 (1H, d, J = 12.9 Hz, 2′a-H), 1.35 (9H, s). MS [M^+] Calcd for $C_{23}H_{19}N_3O_4S$: 433; Found. Anal. ($C_{23}H_{19}N_3O_4S$) C, H, N, S.

1-(4-Pentylphenyl)-2′*H*-spiro[imidazolidine-4,3′-thieno[2,3-*g*]quinoline]-2,4′,5,9′-tetraone (2n). Pale yellow solid (44%). mp: 198−199 °C. ^{1}H NMR (500 MHz, $CDCl_3$, δ): 9.02 (1H, d, J = 4.2 Hz, 6′-H), 8.42 (1H, d, J = 7.7 Hz, 8′-H), 7.65 (1H, dd, J = 7.7, 4.2 Hz, 7′-H), 7.58 (2H, d, J = 7.2 Hz), 7.35 (2H, d, J = 7.2 Hz), 5.90 (1H, s, NH-3), 4.12 (1H, d, J = 12.9 Hz, 2′b-H), 3.40 (1H, d, J = 12.9 Hz, 2′a-H), 2.62 (2H, t, J = 6.7 Hz), 1.60 (2H, m), 1.34 (4H, m), 0.95 (3H, t, J = 6.7 Hz). MS [M^+] Calcd for $C_{24}H_{21}N_3O_4S$: 447; Found. Anal. ($C_{24}H_{21}N_3O_4S$) C, H, N, S.

2*H*-Spiro[thieno[2,3-*g*]quinoline-3,5′-[1,2,4]triazinane]-3′,4,6′,9-tetraone (3). Pale yellow solid (40%). mp: 198−199 °C. ^{1}H NMR (500 MHz, CD_3OD, δ): 9.01 (1H, d, J = 4.2 Hz, 6′-H), 8.40 (1H, d, J = 7.7 Hz, 8′-H), 7.65 (1H, dd, J = 7.7, 4.2 Hz, 7′-H), 3.52 (1H, d, J = 12.9 Hz, 2′a-H), 4.20 (1H, d, J = 12.9 Hz, 2′b-H). MS [M^+] Calcd for $C_{13}H_8N_4O_4S$: 316; Found. Anal. ($C_{13}H_8N_4O_4S$) C, H, N, S. (+)-**3** ($[\alpha]_{20}^{D}$ = +27.5° (*c* 1.2, MeOH)), (−)-**3** ($[\alpha]_{20}^{D}$ = −28.1° (*c* 1.2, MeOH)).

1′-Phenyl-2*H*-spiro[thieno[2,3-*g*]quinoline-3,5′-[1,2,4]triazinane]-3′,4,6′,9-tetraone (3a). Yellow solid (32%). mp: >200 °C. ^{1}H NMR (500 MHz, CD_3OD, δ): 9.01 (1H, d, J = 4.2 Hz, 6′-H), 8.40 (1H, d, J = 7.7 Hz, 8′-H), 7.65 (3H, m), 7.57 (2H, t, J = 6.9 Hz), 7.30 (1H, t, J = 6.5 Hz), 3.52 (1H, d, J = 12.9 Hz, 2′a-H), 4.20 (1H, d, J = 12.9 Hz, 2′b-H). MS [M^+] Calcd for $C_{19}H_{12}N_4O_4S$: 392; Found. Anal. ($C_{19}H_{12}N_4O_4S$) C, H, N, S.

1′-*p*-tolyl-2*H*-spiro[thieno[2,3-*g*]quinoline-3,5′-[1,2,4]triazinane]-3′,4,6′,9-tetraone (3b). Yellow solid (32%). mp: >200 °C. ^{1}H NMR (500 MHz, CD_3OD, δ): 9.01 (1H, d, J = 4.2 Hz, 6′-H), 8.40 (1H, d, J = 7.7 Hz, 8′-H), 7.65 (1H, dd, J = 7.7, 4.2 Hz, 7′-H), 7.38 (2H, d, J = 7.2 Hz), 7.20 (2H, d, J = 7.2 Hz), 4.20 (1H, d, J = 12.9 Hz, 2′b-H), 3.52 (1H, d, J = 12.9 Hz, 2′a-H), 2.30 (3H, s). MS [M^+] Calcd for $C_{20}H_{14}N_4O_4S$: 406; Found. Anal. ($C_{20}H_{14}N_4O_4S$) C, H, N, S.

1′-(4-Chlorophenyl)-2*H*-spiro[thieno[2,3-*g*]quinoline-3,5′-[1,2,4]triazinane]-3′,4,6′,9-tetraone (3c). Yellow solid (32%). mp: >200 °C. ^{1}H NMR (500 MHz, CD_3OD, δ): 9.01 (1H, d, J = 4.2 Hz, 6′-H), 8.40 (1H, d, J = 7.7 Hz, 8′-H), 7.65 (1H, dd, J = 7.7, 4.2 Hz, 7′-H), 7.43 (2H, d, J = 7.2 Hz), 7.20 (2H, d, J = 7.2 Hz), 4.20 (1H, d, J = 12.9 Hz, 2′b-H), 3.52 (1H, d, J = 12.9 Hz, 2′a-H). MS [M^+] Calcd for $C_{19}H_{11}ClN_4O_4S$: 426, 428 (M + 2); Found. Anal. ($C_{19}H_{11}ClN_4O_4S$) C, H, N, S.

1′-(4-Methoxyphenyl)-2*H*-spiro[thieno[2,3-*g*]quinoline-3,5′-[1,2,4]triazinane]-3′,4,6′,9-tetraone (3d). Yellow solid (32%). mp: >200 °C. ^{1}H NMR (500 MHz, CD_3OD, δ): 9.01 (1H, d, J = 4.2 Hz, 6′-H), 8.40 (1H, d, J = 7.7 Hz, 8′-H), 7.65 (1H, dd, J = 7.7, 4.2 Hz, 7′-H), 7.43 (2H, d, J = 7.2 Hz), 7.20 (2H, d, J = 7.2 Hz),

4.20 (1H, d, J = 12.9 Hz, 2′b-H), 3.98 (3H,s), 3.52 (1H, d, J = 12.9 Hz, 2′a-H). MS [M^+] Calcd for $C_{20}H_{14}N_4O_5S$: 422; Found. Anal. ($C_{20}H_{14}N_4O_5S$) C, H, N, S.

1′-(4-Nitrophenyl)-2*H*-spiro[thieno[2,3-*g*]quinoline-3,5′-[1,2,4]triazinane]-3′,4,6′,9-tetraone (3e). Orange solid (32%). mp: >200 °C. ^{1}H NMR (500 MHz, CD_3OD, δ): 9.01 (1H, d, J = 4.2 Hz, 6′-H), 8.40 (1H, d, J = 7.7 Hz, 8′-H), 8.20 (2H, t, J = 7.2 Hz),7.65 (1H, dd, J = 7.7, 4.2 Hz, 7′-H), 7.53 (2H, d, J = 7.2 Hz), 4.20 (1H, d, J = 12.9 Hz, 2′b-H), 3.52 (1H, d, J = 12.9 Hz, 2′a-H). MS [M^+] Calcd for $C_{19}H_{11}N_5O_6S$: 437; Found. Anal. ($C_{19}H_{11}N_5O_6S$) C, H, N, S.

Biology. Compounds. Test compounds were dissolved in DMSO at an initial concentration of 200 μM and were then serially diluted in culture medium.

Cells. Cell lines were from American type Culture Collection (ATCC). MRC-5 normal human lung fibroblasts and leukemia- and lymphoma-derived cells were grown in RPMI 1640 containing 10% fetal calf serum (FCS), 100 U/mL penicillin G, and 100 μg/mL streptomycin. Solid-tumor-derived cells were grown in their specific media supplemented with 10% FCS and antibiotics. Cell cultures were incubated at 37 °C in a humidified 5% CO_2 atmosphere. Cell cultures were periodically checked for the absence of mycoplasma contamination by the Hoechst staining method.

Antiproliferative Assays. Exponentially growing normal, leukemia, and lymphoma cells were resuspended at a density of 1×10^5 cells/mL in RPMI containing serial dilutions of the test drugs. Cell viability was determined after 96 h at 37 °C by the MTT method. The activity against solid-tumor-derived cells was evaluated in exponentially growing cultures seeded at 5×10^4 cells/mL and allowed to adhere for 16 h to culture plates before the addition of drugs. Cell viability was determined by the MTT method 4 days later.

Linear Regression Analysis. Tumor cell growth at each drug concentration was expressed as the percentage of untreated controls, and the concentration resulting in 50% (IC_{50}) growth inhibition was determined by linear regression analysis.

Spectrophotometry. Molar extinction coefficients at the wavelength of maximum absorption in the visible spectrum were determined for **2c** free in solution, bound to calf thymus DNA (Sigma-Aldrich, Milano, Italy) using a Cary 5000 UV−vis-NIR spectrophotometer. Measurements were made in 0.1 SHE buffer (2 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 10 mM EDTA, and 99.4 mM NaCl, pH 7.0) at 25 °C. Titrations of **2c**, **2g**, and **2k** with DNA, covering a large range of phosphate-DNA/drug ratios, were performed by the addition of aliquots of a concentrated DNA solution to a drug solution at constant ligand concentration (20 μM). DNA blanks at the same nucleotide concentrations were concomitantly prepared and used as a reference in the recording of absorption spectra.

Fluorescence Measurements. Fluorescence measurements were carried out on a Perkin-Elmer LS50B spectrofluorimeter. All measurements were made using a 10 mm lightpath cuvette in a 0.01 M ionic-strength buffer (9.3 mM NaCl, 2 mM Na acetate, and 0.1 mM EDTA) using 20 μM DNA and 2 μM ethidium bromide. The DNA−ethidium complex was excited at 546 nm, and the fluorescence was measured at 595 nm.[28]

Materials. Supercoiled pBR322 DNA, *Eco*RI restriction endonuclease, T_4 DNA ligase, and SDS were purchased from the New England Biolab (U.K.). DNA Topo I and II were purchased from TopoGen (Columbus, OH). VP16 and doxo were obtained from Sigma-Aldrich (Milano, Italy). **2c**, **2g**, **2k**, and control drugs were solubilized at 10^4 μM in DMSO as stock solution. All stock solutions were stored at −20 °C and diluted with double-distilled water just before the test. Testing compound (or DMSO for control reactions to control for the solvent's effect) (2 μL) was added to the reaction mixture.

Unwinding Assay. DNA unwinding effects of the compounds **2c**, **2g**, and **2k** were assayed according to the method described by Yamashita et al.[29] Plasmid DNA was linearized by *Eco*RI restriction endonuclease and recovered by phenol extraction and ethanol precipitation. Reaction mixtures (20 μL) containing 30 mM Tris-HCl (pH 7.8), 10 mM $MgCl_2$, 10 mM DTT, 0.5 mM ATP, 0.1 μg of linearized DNA, and testing compounds were equilibrated at 20 °C for 30 min and were then incubated with excess amounts of T_4 DNA ligase at 20 °C for 30 min. The reactions were stopped by shifting the temperature to 55 °C. Afterward, the compounds were removed from the reaction mixture by extraction with phenol. DNA bands were analyzed by 1% agarose gel electrophoresis.

Another unwinding assay was employed according to the Topo-I-mediated supercoiled pBR322 relaxation except that the DNA−compound complexes were dissociated by extraction of the reaction mixture with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1 v/v/v). Then, the samples were subjected to electrophoresis and were photographed under UV light.

Topo-II-Mediated Supercoiled pBR322 Relaxation. DNA relaxation assays were based on the procedure of Osheroff et al.[30] Reaction buffer contained 10 mM Tris-HCl (pH 7.9), 50 mM KCl, 50 mM NaCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 15 μg/mL BSA, 1 mM ATP, 0.15 μg supercoiled pBR322, and 4 units of Topo II in a total of 20 μL. Relaxation was employed at 37 °C for 6 min and stopped by the addition of 3 μL of stop solution (100 mM EDTA, 0.5% SDS, 50% glycerol, 0.05% bromophenol blue). Electrophoresis was carried out in a 1% agarose gel in 0.5× TBE (89 mM Tris base, 89 mM boric acid, and 2 mM EDTA) at 4 V/cm for 1 h. DNA bands were stained with 0.5 μg/mL ethidium bromide solution and were photographed under UV light.

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Supporting Information Available: Elemental analysis data for compounds **1c**, **1c′**,**2**, **2a**−**n**, **3**, and **3a**−**e**. This material is available free of charge via the Internet at http://pubs.acs.org.

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