Synthesis and Cytotoxic Evaluation of Novel Spirohydantoin Derivatives of the Dihydrothieno[2,3-*b*]naphtho-4,9-dione System

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The synthesis and cytotoxic evaluation of 3-(alkyl)(alkyl-substituted)spiro[(dihydroimidazo-2,4-dione)-5,3′-(2′,3′-dihydrothieno[2,3-*b*]naphtho-4′,9′-dione)]derivatives are described. Evaluation of these analogues against the MCF-7 human breast carcinoma and SW 620 human colon carcinoma cell lines uncovered for most of the compounds a cytotoxic potency comparable to or greater than that of doxorubicin. Compound **15** exhibited remarkable cytotoxic activity against several other human solid tumor cell lines. Interestingly, only a partial cross-resistance to compound **15** in selected tumor cell sublines known to be resistant to doxorubicin (MCF-7/Dx and A2780/Dx) was observed, whereas a total absence of cross-resistance in a tumor cell subline selected for resistance to cisplatin was found (A2780/DDP).

Introduction

The quinone group forms the basis of a number of clinical and experimental anticancer drugs, notably the natural antibiotic doxorubicin and the synthetic compound mitoxantrone. Although these two drugs in particular occupy a prominent position in the chemotherapeutic control of a number of human cancers, they have markedly reduced efficacy in resistant disease and against slowly growing tumors.[1] The clinical importance of this class of antitumor agents has stimulated the development of new agents that, retaining the "core" quinonic moiety, could exhibit different spectra of potency with an improved tolerance profile.[2−6]

In general, the structural characteristics of the quinone derivatives are characterized by two common features which seem to determine their antineoplastic activity: a planar, polycyclic nucleus capable of binding to the DNA by intercalation and one or two side chains containing different substituents in a defined orientation to the chromophore. It has been also suggested that the side chain has the function of additionally stabilizing a drug−DNA complex, binding electrostatically to the phosphate backbone of the polynucleotide.[7,8]

In the course of a medicinal chemistry program aimed at discovering new quinone derivatives endowed with antitumor activity, we have recently developed a series of 3-amino-3-ethoxycarbonyl-2,3-dihydrothieno[2,3-*b*]-naphtho-4,9-dione derivatives substituted at the position 3 with different amino acids.[9] The rationale for using such amino acids has been to extend the overall conjugation of the planar chromophore by amide groups, as a side arm, thereby leading to more effective target-binding activity.[10,11] The most active of these derivatives, the 3-(glycyl)amino-3-ethoxycarbonyl-2,3-dihydrothieno[2,3-*b*]naphtho-4,9-dione, showed remarkable cytotoxic activity at submicromolar concentration not only against several human leukaemia and solid tumor cell lines but also toward resistant human cell lines. The results obtained on dihydrothieno[2,3-*b*]naphtho-4,9-dione derivatives showed that the stereochemistry at C-3 of these compounds is not an essential feature for cytotoxic activity.[9] These results have also highlighted the potential value of the DTNQ as template in the development of more effective antitumoral agents (DTNQ = 3-amino-3-ethoxycarbonyl-2,3-dihydrothieno[2,3-*b*]-naphtho-4,9-dione).

Herein, we designed a set of spirohydantoin derivatives in which the planar chromophore (DTNQ) was combined, through the hydantoin ring, to an alkyl side chain. To determine how the steric and electronic properties of the side chain may influence the cytotoxic activity, we examined several derivatives containing different alkyl (methyl, ethyl, propyl, butyl, *sec*-butyl, and *tert*-butyl) and alkyl-substituted chains (hydroxyethyl, hydroxypropyl, aminoethyl, aminopropyl, aminobutyl, (*N*,*N*-dimethyl)aminoethyl, (*N*,*N*-dimethyl)-aminopropyl, (*N*,*N*-diethyl)aminoethyl, and (*N*,*N*-diethyl)-aminopropyl) in position 3 of the hydantoin ring. The cytotoxic activity was evaluated toward the human breast carcinoma (MCF-7) and human colon carcinoma (SW 620) cell lines. Moreover one of the most active compounds was tested against several other human tumor derived cell lines as well as against the catalytic activity of human topoisomerase II.

Chemistry

The new 3-(alkyl)(alkyl-substitued)spiro[(dihydroimidazo-2,4-dione)-5,3′-(2′,3′-dihydrothieno[2,3-*b*]naphtho-4′,9′-dione)] derivatives (**3**−**17**) were prepared applying the synthetic route shown in Scheme 1.

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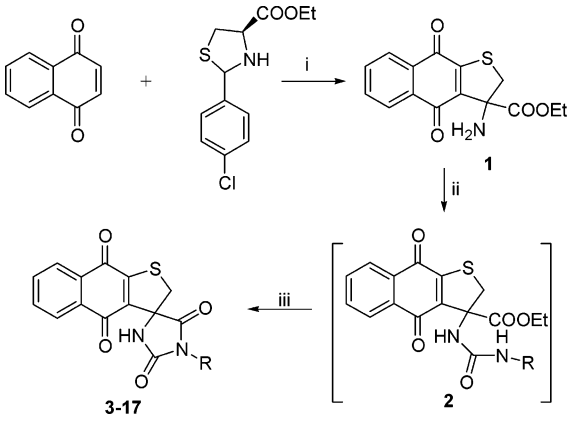
Scheme 1. General Synthetic Pathway[a]



[a] Reagents and conditions: (i) Na_2CO_3, DBU, acetonitrile, rt, 12 h, then 1 N HCl, 1 h, and $H_2O/CHCl_3$; (ii) triphosgen, TEA, THF, rt, 10 min, then $R-NH_2$; (iii) EtOH, TEA, reflux, and 1−3 h.

The starting 3-amino-3-ethoxycarbonyl-2,3-dihydrothieno[2,3-*b*]naphtho-4,9-dione (**1**, DTNQ), was obtained by a cycloaddition reaction between the 2-(4′-chlorophenyl)-thiazolidine and naphthoquinone as we previously described.[12] The *N*-carbamoyl derivatives of general formula **2** were obtained by reaction of the 3-ethoxycarbonyl-3-isocyanato-2,3-dihydrothieno[2,3-*b*]naphtho-4,9-dione, generated in situ from **1** with bis(trichloromethyl)carbonate[13] in the presence of triethylamine, with the corresponding amines. In these conditions, the HPLC analysis of the crude reaction showed a partial cyclization of the linear compounds (ratio of linear/cyclic compound 1:1−2). All attempts to isolate linear compounds, by semipreparative HPLC or flash chromatography, failed as a result of a further cyclization during the purification process. The complete cyclization of derivatives **2** was performed in EtOH in the presence of TEA, and the corresponding spirohydantoin derivatives (**3**−**17**) were obtained in 45−55% overall yields.

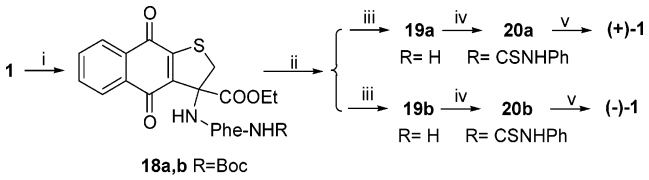
The final compounds **11**−**13** were obtained as TFA salts after removing the BOC protecting group. In their turn, the compounds **14**−**17**, as HCl salts, were obtained after treatment of the corresponding free bases with a solution of ethylic ether saturated with HCl.

Since the synthetic pathway used for the preparation of the starting DTNQ generates an asymmetric carbon (C-3) in this system,[12] the final compounds were obtained as racemic mixtures. To verify and evaluate the importance of the stereochemistry of this chiral center on the activity in this series of compounds, the synthesis of the enantiomerically pure compounds **11** and **15** was performed starting from pure enantiomers of DTNQ. The racemic mixture of starting DTNQ (**1**) was resolved in the two corresponding enantiomers following the Evans method[12,14] as reported in Scheme 2 (see experimental section). Subsequently, reaction of enantiomers (+)-**1** and (−)-**1** with *N*-BOC-ethylenediamine or *N*,*N*-dimethylaminopropylamine, following the general procedure described in Scheme 1, gave the corresponding (+)-**11**, (+)-**15**, (−)-**11**, and (−)-**15** derivatives.

Results and Discussion

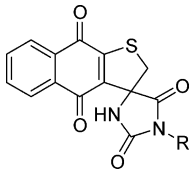
In Vitro Cytotoxicity. Preliminarily, the cytotoxicity of all compounds was evaluated against the MCF-7 human breast carcinoma and SW 620 human colon carcinoma cell lines in comparison with the reference drug doxorubicin.

Scheme 2. Enantiomeric Resolution of Compound **1**[a]



[a] Reagents and conditions: (i) BOC-Phe, HBTU, HOBt, DIEA, THF/DMF, rt, and 48 h; (ii) TFA, CH_2Cl_2, rt, 2 h, then TEA; (iii) chromatographic separation; (iv) phenylisothiocyanate, CH_2Cl_2, reflux, and 1 h; (v) TFA in CH_2Cl_2, rt, and 1 h.

Table 1. Cytotoxic Activity of Compounds Synthesized



compds	R	IC_{50} (μM ± SD)[a] MCF-7[b]	SW 620[c]
3	$-CH_3$	0.023 ± 0.003	0.108 ± 0.014
4	$-CH_2CH_3$	0.116 ± 0.020	0.203 ± 0.018
5	$-(CH_2)_2CH_3$	0.104 ± 0.010	0.379 ± 0.016
6	$-(CH_2)_3CH_3$	0.178 ± 0.020	0.740 ± 0.015
7	$-CH(CH_3)CH_2CH_3$	0.178 ± 0.040	0.562 ± 0.017
8	$-C(CH_3)_3$	0.331 ± 0.020	0.900 ± 0.141
9	$-(CH_2)_2OH$	0.069 ± 0.020	0.198 ± 0.027
10	$-(CH_2)_3OH$	0.040 ± 0.010	0.119 ± 0.002
11	$-(CH_2)_2NH_2$[d]	0.022 ± 0.008	0.035 ± 0.005
(+)-**11**	$-(CH_2)_2NH_2$[d]	0.020 ± 0.008	0.034 ± 0.005
(−)-**11**	$-(CH_2)_2NH_2$[d]	0.031 ± 0.008	0.047 ± 0.005
12	$-(CH_2)_3NH_2$[d]	0.025 ± 0.004	0.052 ± 0.007
13	$-(CH_2)_4NH_2$[d]	0.035 ± 0.005	0.068 ± 0.006
14	$-(CH_2)_2N(CH_3)_2$[e]	0.032 ± 0.001	0.057 ± 0.005
15	$-(CH_2)_3N(CH_3)_2$[e]	0.019 ± 0.002	0.058 ± 0.001
(+)-**15**	$-(CH_2)_3N(CH_3)_2$[e]	0.019 ± 0.002	0.060 ± 0.001
(−)-**15**	$-(CH_2)_3N(CH_3)_2$[e]	0.027 ± 0.002	0.065 ± 0.001
16	$-(CH_2)_2N(CH_2CH_3)_2$[e]	0.021 ± 0.001	0.050 ± 0.001
17	$-(CH_2)_3N(CH_2CH_3)_2$[e]	0.026 ± 0.001	0.054 ± 0.005
doxorubicin		0.022 ± 0.008	0.178 ± 0.003

[a] Data represent mean values (±SD) for three independent determinations. [b] Human breast carcinoma cell line. [c] Human colon carcinoma cell line. [d] Evaluated as TFA salts. [e] Evaluated as HCl salts.

As shown in Table 1, the first tested, compound **3**, showed a potent cytotoxic activity on MCF-7 and SW 620 cell lines, similar to that of doxorubicin, with IC_{50} values of 23 and 108 nM, respectively. A reduced activity of derivatives **4**−**8** was observed, presumably due to the increasing lipophilic nature and/or the steric hindrance of the side chain.

The incorporation of the primary or tertiary final amine to the ethyl, propyl, or butyl side chain afforded the most potent spirohydantoin derivatives (**11**−**17**), which retained cytotoxic levels similar to those of compound **3** and doxorubicin on the MCF-7 cell line; also, these compounds were more active on the SW 620 cell line compared to doxorubicin. These results indicate that the side chain length, determined by the number of methylene groups separating the chromophore and the remote protonatable amine groups in the pendant arm, appears not to have an important effect upon cytotoxicity. Congeners with a hydroxyl group (com-

Table 2. Antiproliferative Activity of Compound **15** and Doxorubicin (Dx) on a Panel of Different Human Tumor Cell Lines

human tumor	cell line	IC_{50} (μM ± SD)[a]		RI[b]	
		15	Dx	**15**	Dx
lung	NCI-H460	0.260 ± 0.002	0.010 ±0.008		
	A549	0.110 ± 0.001	0.064 ± 0.005		
colon	LoVo	0.230 ± 0.001	0.033 ± 0.004		
melanoma	MeVo	0.250 ± 0.002	0.820 ± 0.100		
epidermal	KB	0.100 ± 0.001	0.021 ± 0.002		
	Hep-2	0.170 ± 0.001	0.059 ± 0.004		
ovarian	A2780	0.068 ± 0.009	0.007 ± 0.001		
	A2780/Dx[c]	0.130 ± 0.007	0.290 ± 0.002	1.9[e]	41
	A2780/DDP[d]	0.110 ± 0.010	0.006 ± 0.001	1.6[f]	
breast	MCF-7	0.019 ± 0.002	0.022 ± 0.008		
	MCF-7/Dx[c]	0.173 ± 0.012	5.300 ± 0.400	9.1[e]	240

[a] Data represent mean values (±SD) for three independent determinations. [b] Resistance index: ratio between the IC_{50} of **15** or Dx on resistant tumor cells over the IC_{50} of **15** or Dx on sensitive tumor cell lines. [c] Subline resistant to doxorubicin. [d] Subline resistant to cisplatin. [e] RI for cisplatin on A2780/DDP was 28.

pounds **9** and **10**) were less potent with respect to their primary and tertiary amine analogues.

The biological results obtained imply that the relative activity of these compounds is not determined only by the physicochemical properties of the different substituents in side chains. We might only hypothesize that while compound **3** is well located in the molecular target, the higher homologues **4**−**10** are not well tolerated. On the other hand, the enhancement of cytoxicity obtained with the aminoalkyl derivatives (**11**−**17**) might be due to an additional electrostatic interaction of the positively charged ammonium cation with a negatively charged site of the molecular target.[7] Another possibility is that these aminoalkyl derivatives could be actively transported into cells similarly to the polyamines as recently hypothesized by other authors for anthracenes and indenoisoquinoline derivatives.[15,16]

Additionally, the enantiomers (+)-**11**, (−)-**11**, (+)-**15**, and (−)-**15** showed an activity similar to the corresponding racemic mixture. These results suggest that the stereochemistry of C-3 does not have any influence on the cytotoxic activity of these compounds, in agreement with our previous results obtained on 3-acylamino-3-ethoxycarbonyl-2,3-dihydrothieno[2,3-*b*]naphtho-4,9-dione series.[9]

The antiproliferative activity of one of the most potent compounds, **15**, was analyzed against a panel of human tumor cell lines, including selected cellular subclones of the ovarian carcinoma resistant to doxorubicin (A2780/Dx) and to cisplatin (A2780/DDP) and the MCF-7/Dx mammary carcinoma resistant to doxorubicin (Table 2). In all tested cells systems, compound **15** showed marked cytotoxic potency with IC_{50} in the range 0.019−0.26 μM. Although the observed IC_{50} values were higher than that of the reference compound doxorubicin in 5 out of 11 cell lines, it is remarkable that the doxorubicin-resistant MCF-7/Dx and A2780/Dx cell lines exhibited a low level of cross-resistance to compound **15** (resistance indexes were 9.1 and 1.9, respectively), whereas very low cross-resistance was observed in the subline cisplatin-resistant A2780/DDP (resistance index was 1.6).

Topoisomerase II Inhibition. As far as the mechanism of action is concerned, we analyzed the possibility that this class of compounds could inhibit the activity of topoisomerase II (topo II), a well-known target of several quinone containing anticancer drugs.[17] However compounds **11** and **15** did not inhibit topo II catalytic activity in the concentration range 1−10 μM (Figure 1). These results are in agreement with the low level of cross-resistance observed on cell lines resistant to doxorubicin and suggest that this series of compounds has a distinct mechanism of action.

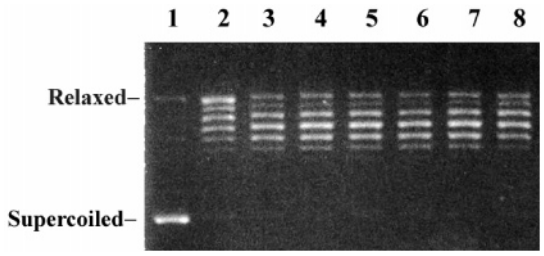


Figure 1. Effects of compounds **15** and **11** on the DNA-relaxing activity of human topo II. Supercoiled plasmid pBR322 was incubated with purified human topo II in the presence or absence of the tested agents: (lane 1) no enzyme control; (lane 2) enzyme control; (lanes 3−5) 1, 5, and 10 μM of compound **15**; (lanes 6−8) 1, 5, and 10 μM of compound **11**.

Conclusions

We reported here the synthesis and biological evaluation of new spirohydantoin derivatives of the dihydrothieno[2,3-*b*]naphtho-4,9-dione system as potential cytotoxic agents. Several of these compounds, carrying a distal lipophilic amine moiety, showed a similar or greater cytotoxic potency than doxorubicin against the human breast (MCF-7) and colon (SW 620) tumor cell lines. In particular, compound **15** showed a high efficacy in cell lines resistant to doxorubicin (MCF-7/Dx and A2780/Dx) and to cisplatin (A2780/DDP). The preliminary data indicate that these spirohydantoin derivatives represent a potential starting point in discovering new and potent antitumoral agents with cytotoxic activity on ovarian and breast cancer. Studies that include an examination of impact of the differently functionalized side chain on cytotoxic activity of these and other spiro derivatives of dihydrothieno[2,3-*b*]naphtho-4,9-dione system are ongoing.

However, since the molecular basis of the cytotoxic activity shown by the derivatives examined in this study remains to be determined, further experiments aimed at defining the target and the mechanisms of the growth-inhibitory effect shown by these molecules are currently underway.

Experimental Procedures

General. Reagents, starting material, and solvents were purchased from commercial suppliers and used as received. Analytical TLC was performed on a 0.25 mm layer of silica gel 60 F_{254} from Merck, and preparative TLC was performed on 20 cm × 20 cm glass plates coated with a 2 mm layer of silica gel PF_{254} 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, operating at 500 MHz. Chemical shifts are reported in δ values (ppm) relative to internal Me_4Si, and J values are reported in hertz (Hz). Mass spectra were obtained using a FABMS spectrometer. Analytical RPHPLC was performed on a Vydac C-18 (25 cm × 0.46 cm) columm, using a

tunable UV detector set at 215 nm. Elution was performed with a linear gradient from 10 to 60% of acetonitrile in 0.1% aqueous TFA over 55 min at a flow rate of 1 mL/min. The 3-amino-3-ethoxycarbonyl-2,3-dihydrothieno[2,3-*b*]naphtho-4,9-dione system was synthesized according to the procedure previously described,[9] using ethyl 2-(4-chloro)phenyl-1,3-thiazolidine-4-carboxylate and naphthoquinone as starting material.

General Procedure for the Synthesis of the 3-(Alkyl)-(alkyl-substituted)spiro[(dihydroimidazo-2,4-dione)-5,3′-(2′,3′-dihydrothieno[2,3-*b*]naphtho-4′,9′-dione)] Derivatives (3−17). Bis(trichloromethyl)carbonate (118 mg, 0.4 mmol) was added to a solution of DTNQ (**1**, 300 mg, 1 mmol) in dry THF (25 mL) at room temperature. Then TEA (0.3 mL, 2 mmol) was added dropwise throughout 5 min, continuing the stirring for an additional 10 min. Afterward, a solution of corresponding amines (1.1 equiv), methylamine, ethylamine, propylamine, butylamine, *sec*-butylamine, *tert*-butylamine, hydroxyethylamine, hydroxypropylamine, *N*-BOC-ethylenediamine, *N*-BOC-propylenediamine, *N*-BOC-butylenediamine, *N*,*N*-dimethylaminoethylamine, *N*,*N*-dimethylaminopropylamine, *N*,*N*-diethylaminoethylamine, and *N*,*N*-diethylaminopropylamine, in dry THF (5 mL) and TEA (0.3 mL, 2 mmol) was added, and the mixture was stirred for 1 h. Then, the reaction mixture was diluted with chloroform, washed with water, dried over Na_2SO_4, and evaporated to dryness. The residue was dissolved into EtOH (20 mL) and TEA (0.15 mL, 1 mmol) and was added to this solution. The reaction mixture was stirred for 1−3 h at reflux temperature, then neutralized with 1 N HCl and diluted with $CHCl_3$. The organic extract was washed with 10% $NaHCO_3$ and water, dried over Na_2SO_4, and evaporated to dryness. Flash chromatography of the residues using $CHCl_3$ or a gradient of 0−20% MeOH in $CHCl_3$ as eluents yielded, in each case, the correspondent spirohydantoin derivatives.

The final compounds **11**−**13** were obtained, as trifluoroacatate salts, after BOC removal using a 50% TFA/CH_2Cl_2 solution. On the other hand, the compounds **14**−**17** were obtained as hydrochloride salts by treatment of the corresponding free bases with a HCl (g)/diethyl ether solution.

3-Methylspiro[(dihydroimidazo-2,4-dione)-5,3′-(2′,3′-dihydrothieno[2,3-*b*]naphtho-4′,9′-dione)] (3). Yellow solid (54%), mp 190−191 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ, 3.21 (s, 3H, CH_3), 3.38−3.41 (d, 1H, 2′-H, $J_{2',2''}$ = 12.9 Hz), 4.08−4.11 (d, 1H, 2″-H), 5.89 (s, 1H, NH), 7.72−7.76 (m, 2H, 6′-H and 7′-H), 8.01−8.03 (d, 1H, 8′-H), 8.10−8.12 (d, 1H, 5′-H). HPLC t_R 21.2 min. MS [M$^+$] calcd for $C_{15}H_{10}N_2O_4S$: 314.04; found, 314.31. Anal. ($C_{15}H_{10}N_2O_4S$) C, H, N, S.

3-Ethylspiro[(dihydroimidazo-2,4-dione)-5,3′-(2′,3′-dihydrothieno[2,3-*b*]naphtho-4′,9′-dione)] (4). Yellow solid (50%), mp 199−200 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ, 1.33−1.36 (m, 3H, CH_3), 3.37−3.40 (1H, 2′-H, $J_{2',2''}$ = 12.5 Hz), 3.66−3.68 (m, 2H, CH_2), 4.05−4.08 (d, 1H, 2″-H), 6.58 (s, 1H, NH), 7.63−7.69 (m, 2H, 6′-H and 7′-H), 8.04−8.06 (d, 1H, 8′-H), 8.09−8.11 (d, 1H, 5′-H). HPLC t_R 22.0 min. MS [M$^+$] calcd for $C_{16}H_{12}N_2O_4S$: 328.04; found, 328.41. Anal. ($C_{16}H_{12}N_2O_4S$) C, H, N, S.

3-Propylspiro[(dihydroimidazo-2,4-dione)-5,3′-(2′,3′-dihydrothieno[2,3-*b*]naphtho-4′,9′-dione)] (5). Yellow solid (55%), mp 237−239 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ, 0.93−0.95 (m, 3H, CH_3) 1.70−1.74 (m, 2H, CH_2), 3.30−3.33 (d, 1H, 2′-H, $J_{2',2''}$ = 12.4 Hz), 3.49−3.51 (m, 2H, CH_2), 3.97−4.00 (d, 1H, 2″-H), 6.31 (s, 1H, NH), 7.63−7.69 (m, 2H, 6′-H and 7′-H), 7.93−7.95(d, 1H, 8′-H), 8.07−8.09 (d, 1H, 5′-H). HPLC t_R 22.9 min. MS [M$^+$] calcd for $C_{17}H_{14}N_2O_4S$: 342.07; found, 342.01. Anal. ($C_{17}H_{14}N_2O_4S$) C, H, N, S.

3-Butylspiro[(dihydroimidazo-2,4-dione)-5,3′-(2′,3′-dihydrothieno[2,3-*b*]naphtho-4′,9′-dione)] (6). Yellow solid (52%), mp 219 °C (dec). ^{1}H NMR (500 MHz, $CDCl_3$) δ, 0.89−0.91 (m, 3H, CH_3), 1.42−1.47 (m, 2H, CH_2), 1.73−1.79 (m, 2H, CH_2), 3.37−3.40 (d, 1H, 2′-H, $J_{2',2''}$ = 12.4 Hz), 3.62−3.65 (m, 2H, CH_2), 4.06−4.09 (d, 1H, 2″-H), 5.92 (s, 1H, NH), 7.70−7.76 (m, 2H, 6′-H and 7′-H), 8.02−8.04 (d, 1H, 8′-H), 8.09−8.11 (d, 1H, 5′-H). HPLC t_R 23.2 min. MS [M$^+$] calcd for $C_{18}H_{16}N_2O_4S$: 356.08; found, 356.01. Anal. ($C_{18}H_{16}N_2O_4S$) C, H, N, S.

3-*sec*-Butylspiro[(dihydroimidazo-2,4-dione)-5,3′-(2′,3′-dihydrothieno[2,3-*b*]naphtho-4′,9′-dione)] (7). Yellow solid (47%), mp 259−260 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ, 1.53−1.56 (m, 6H, CH_3), 1.80−1.84 and 2.06−2.10 (m, 2H, CH_2), 3.34−3.37 (d, 1H, 2′-H, $J_{2',2''}$ = 12.5 Hz), 4.05−4.08 (d, 1H, 2″-H), 4.15−4.17 (m, 1H, CH), 5.76 (s, 1H, NH), 7.72−7.77 (m, 2H, 6′-H and 7′-H), 8.04−8.06 (d, 1H, 8′-H), 8.09−8.11 (d, 1H, 5′-H). HPLC t_R 23.9 min. MS [M$^+$] calcd for $C_{18}H_{16}N_2O_4S$: 356.08; found, 356.12. Anal. ($C_{18}H_{16}N_2O_4S$) C, H, N, S.

3-*tert*-Butylspiro[(dihydroimidazo-2,4-dione)-5,3′-(2′,3′-dihydrothieno[2,3-*b*]naphtho-4′,9′-dione)] (8). Yellow solid (45%), mp 264−266 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ, 1.72 (s, 9H, CH_3), 3.32−3.35 (d, 1H, 2′-H, $J_{2',2''}$ = 12.3 Hz), 4.03−4.06 (d, 1H, 2″-H), 5.65 (s, 1H, NH), 7.71−7.77 (m, 2H, 6′-H and 7′-H), 8.04−8.06 (d, 1H, 8′-H), 8.09−8.11 (d, 1H, 5′-H). HPLC t_R 24.52 min. MS [M$^+$] calcd for $C_{18}H_{16}N_2O_4S$: 356.08; found, 356.00. Anal. ($C_{18}H_{16}N_2O_4S$) C, H, N, S.

3-(2-Hydroxyethyl)spiro[(dihydroimidazo-2,4-dione)-5,3′-(2′,3′-dihydrothieno[2,3-*b*]naphtho-4′,9′-dione)] (9). Yellow solid (48%), mp 193 °C (dec). ^{1}H NMR (500 MHz, $CDCl_3$) δ, 3.41−3.44 (d, 1H, 2′-H, $J_{2',2''}$ = 12.4 Hz), 3.85−3.87 (m, 2H, CH_2), 3.97−3.99 (m, 2H, CH_2), 4.10−4.13 (d, 1H, 2″-H), 6.16 (s, 1H, NH), 7.74−7.77 (m, 2H, 6′-H and 7′-H), 8.03−8.05 (d, 1H, 8′-H), 8.10−8.13 (d, 1H, 5′-H). HPLC t_R 18.6 min. MS [M$^+$] calcd for $C_{16}H_{12}N_2O_5S$: 344.05; found, 344.35. Anal. ($C_{16}H_{12}N_2O_5S$) C, H, N, S.

3-(3-Hydroxypropyl)spiro[(dihydroimidazo-2,4-dione)-5,3′-(2′,3′-dihydrothieno[2,3-*b*]naphtho-4′,9′-dione)] (10). Yellow solid (51%), mp 212 °C (dec). ^{1}H NMR (500 MHz, $CDCl_3$) δ, 1.21−1.24 (m, 2H, CH_2), 1.90−1.93 (m, 2H, CH_2), 3.39−3.42 (d, 1H, 2′-H, $J_{2',2''}$ = 12.5 Hz), 3.72−3.76 (m, 2H, CH_2), 3.99−4.02 (d, 1H, 2″-H), 6.97 (s, 1H, NH), 7.69−7.74 (m, 2H, 6′-H and 7′-H), 7.96−7.98 (d, 1H, 8′-H), 8.04−8.06 (d, 1H, 5′-H). HPLC t_R 19.4 min. MS [M$^+$] calcd for $C_{17}H_{14}N_2O_5S$: 358.07; found, 358.33. Anal. ($C_{17}H_{14}N_2O_5S$) C, H, N, S.

3-(2-Aminoethyl)spiro[(dihydroimidazo-2,4-dione)-5,3′-(2′,3′-dihydrothieno[2,3-*b*]naphtho-4′,9′-dione)] Trifluoroacetate (11). Yellow solid (48%), mp 174−176 °C. ^{1}H NMR (500 MHz, CD_3OD) δ, 3.06−3.08 (m, 2H, CH_2), 3.20−3.22 (m, 2H, CH_2), 3.42−3.45 (d, 1H, 2′-H, $J_{2',2''}$ = 12.5 Hz), 3.90−3.93 (d, 1H, 2″-H), 6.70 (s, 1H, NH), 7.66−7.70 (m, 2H, 6′-H and 7′-H), 7.88−7.90 (d, 1H, 8′-H), 7.97−7.99 (d, 1H, 5′-H). HPLC t_R 19.8 min. MS [M$^+$] calcd for $C_{16}H_{14}N_3O_4S{\cdot}CF_3COO$: 457.06; found, 457.10. Anal. ($C_{16}H_{13}N_3O_4S$) C, H, N, S.

3-(3-Aminopropyl)spiro[(dihydroimidazo-2,4-dione)-5,3′-(2′,3′-dihydrothieno[2,3-*b*]naphtho-4′,9′-dione)] Trifluoroacetate (12). Yellow solid (50%), mp 223−224 °C. ^{1}H NMR (500 MHz, CD_3OD) δ, 1.95−1.97 (m, 2H, CH_2), 3.03−3.05 (m, 2H, CH_2), 3.37−3.40 (d, 1H, 2′-H, $J_{2',2''}$ = 12.5 Hz), 3.55−3.58 (m, 2H, CH_2), 3.83−3.86 (d, 1H, 2″-H), 6.70 (s, 1H, NH), 7.64−7.67 (m, 2H, 6′-H and 7′-H), 7.84−7.86 (d, 1H, 8′-H), 7.93−7.95 (d, 1H, 5′-H). HPLC t_R 20.6 min. MS [M$^+$] calcd for $C_{17}H_{16}N_3O_4S{\cdot}CF_3COO$: 471.06; found, 471.43. Anal. ($C_{17}H_{15}N_3O_4S$) C, H, N, S.

3-(4-Aminobutyl)spiro[(dihydroimidazo-2,4-dione)-5,3′-(2′,3′-dihydrothieno[2,3-*b*]naphtho-4′,9′-dione)] Trifluoroacetate (13). Yellow solid (46%), mp 231−233 °C. ^{1}H NMR (500 MHz, CD_3OD) δ, 1.89−1.91 (m, 2H, CH_2), 2.40−2.43 (m, 2H, CH_2), 2.73−2.79 (m, 2H, CH_2), 3.37−3.40 (d, 1H, 2′-H, $J_{2',2''}$ = 12.4 Hz), 3.49−3.53 (m, 2H, CH_2), 4.06−4.09 (d, 1H, 2″-H), 5.49 (s, 1H, NH), 7.82−7.84 (m, 2H, 6′-H and 7′-H), 8.02−8.04 (d, 1H, 8′-H), 8.12−8.10 (d, 1H, 5′-H). HPLC t_R 21.3 min. MS [M$^+$] calcd for $C_{18}H_{18}N_3O_4S{\cdot}CF_3COO$: 483.07; found, 483.01. Anal. ($C_{18}H_{17}N_3O_4S$) C, H, N, S.

3-[2-(*N*,*N*-Dimethyl)aminoethyl]spiro[(dihydroimidazo-2,4-dione)-5,3′-(2′,3′-dihydrothieno[2,3-*b*]naphtho-4′,9′-dione)] Hydrochloride (14). Yellow solid (51%), mp 219−222 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ, 2.83−2.85 (m, 2H, CH_2), 2.97 (s, 6H, N−CH_3), 3.43−3.46 (d, 1H, 2′-H, $J_{2',2''}$ = 12.5 Hz), 3.79−3.83 (m, 2H, CH_2), 4.06−4.09 (d, 1H, 2″-H), 6.40 (s, 1H, NH), 7.72−7.75 (m, 2H, 6′-H and 7′-H), 7.98−8.00 (d, 1H, 8′-H),

8.10−8.12 (d, 1H, 5′-H). HPLC t_R 16.0 min. MS [M$^+$] calcd for $C_{18}H_{17}N_3O_4S$·HCl: 407.59; found, 407.91. Anal. ($C_{18}H_{17}N_3O_4S$) C, H, N, S.

3-[3-(*N*,*N*-Dimethyl)aminopropyl]spiro[(dihydroimidazo-2,4-dione)-5,3′-(2′,3′-dihydrothieno[2,3-*b*]naphtho-4′,9′-dione) Hydrochloride (15). Yellow solid (50%), mp 238−240 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ, 2.37−2.39 (m, 2H, CH_2), 2.89 and 2.90 (2s, 6H, N−CH_3), 3.31−3.34 (m, 2H, CH_2), 3.46−3.49 (d, 1H, 2′-H, $J_{2',2''}$= 12.5 Hz), 3.76−3.78 (m, 2H, CH_2), 4.03−4.06 (d, 1H, 2″-H), 6.67(s, 1H, NH), 7.71−7.76 (m, 2H, 6′-H and 7′-H), 8.03−8.05 (d, 1H, 8′-H), 8.09−8.11 (d, 1H, 5′-H). HPLC t_R 16.7 min. MS [M$^+$] calcd for $C_{19}H_{19}N_3O_4S$·HCl: 421.61; found, 421.42. Anal. ($C_{19}H_{19}N_3O_4S$) C, H, N, S.

3-[2-(*N*,*N*-Diethyl)aminoethyl]spiro[(dihydroimidazo-2,4-dione)-5,3′-(2′,3′-dihydrothieno[2,3-*b*]naphtho-4′,9′-dione)] Hydrochloride (16). Yellow solid (45%), mp 241−243 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ, 1.11−1.19 (m, 6H, CH_3), 2.46−2.51 (m, 4H, N−CH_2), 2.83−2.85 (m, 2H, CH_2), 3.43−3.46 (d, 1H, 2′-H, $J_{2',2''}$ = 12.5 Hz), 3.86−3.89 (d, 1H, 2″-H), 4.00−4.03 (m, 2H, CH_2), 6.45 (s, 1H, NH), 7.72−7.75 (m, 2H, 6′-H and 7′-H), 7.99−8.01 (d, 1H, 8′-H), 8.09−8.11 (d, 1H, 5′-H). HPLC t_R 17.0 min. MS [M$^+$] calcd for $C_{20}H_{21}N_3O_4S$·HCl: 435.40; found, 435.71. Anal. ($C_{20}H_{21}N_3O_4S$) C, H, N, S.

3-[3-(*N*,*N*-Diethyl)aminopropyl]spiro[(dihydroimidazo-2,4-dione)-5,3′-(2′,3′-dihydrothieno[2,3-*b*]naphtho-4′,9′-dione)] Hydrochloride (17). Yellow solid (47%), mp 251−253 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ, 1.05−1.12 (m, 6H, CH_3), 1.94−1.98 (m, 2H, CH_2), 2.50−2.58 (m, 4H, N−CH_2), 2.61−2.64 and 2.66−2.68 (m, 2H, CH_2), 3.41−3.44 (d, 1H, 2′-H, $J_{2',2''}$ = 12.5 Hz), 3.66−3.69 (m, 2H, CH_2), 4.04−4.07 (d, 1H, 2″-H), 6.80 (s, 1H, NH), 7.71−7.75 (m, 2H, 6′-H and 7′-H), 7.98−8.00 (d, 1H, 8′-H), 8.08−8.10 (d, 1H, 5′-H). HPLC t_R 17.9 min. MS [M$^+$] calcd for $C_{21}H_{23}N_3O_4S$·HCl: 449.64; found, 449.66. Anal. ($C_{21}H_{23}N_3O_4S$) C, H, N, S.

Resolution of the (+)-1 and (−)-1 Enantiomers. The DTNQ (1 mmol) was dissolved in (3/1) THF/DMF (20 mL), and BOC-Phe (1 equiv), HBTU (1.2 equiv), HOBt (1.2 equiv), and DIEA (2 equiv) were added successively to the solution that was stirred continuously at room temperature for 48 h. Afterward, the solvents were evaporated; the residue was dissolved in $CHCl_3$, washed successively with 10% citric acid solution (2 × 25 mL), 10% $NaHCO_3$ (2 × 25 mL), and H_2O (25 mL), dried over Na_2SO_4, and evaporated. Flash chromatography of the residue with 20−50% gradient of EtOAc in *n*-hexane yielded the diastereoisomeric mixture of 3-(*tert*-butoxycarbonyl-L-phenylalanyl)amino-3-etoxycarbonyl-(2,3)-dihydrothieno-[2,3-*b*]naphtho-4,9-dione (**18a**,**b**). After treatment of this mixture with TFA in CH_2Cl_2 at room temperature for 2 h, the solution was neutralized with triethylamine and the solvent evaporated to dryness. Flash chromatography of the residue with AcOEt yielded, in decreasing order of R_f, the 3-(L-phenylalanyl)amino-3-etoxycarbonyl-(2,3)-dihydrothieno[2,3-*b*]naphtho-4,9-dione **19a** and **19b**. The analytical and spectroscopy data are in agreement to those previously reported.[9]

To a solution of corresponding **19a** or **19b** (170 mg, 0.35 mmol) in CH_2Cl_2, phenylisothiocyanate (60 mg, 0.44 mmol) and triethylamine (88 mg, 0.8 mmol) were added. After 1 h of stirring at reflux temperature, the solvent was evaporated to dryness. Flash cromatography of the resulting residues with $CHCl_3$ yielded, in each case, the 3-[(*N*-phenylthiocarbamoyl)-(L-phenylalanyl)]amino-3-etoxycarbonyl-(2,3)-dihydrothieno [2,3-*b*]naphtho-4,9-dione **20a** as a yellow solid (160 mg, 80%); mp 239−240 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ, 1.20−1.24 (m, 3H, CH_3), 3.10−3.11 (s, 2H, β-CH_2), 3.64−3.70 (dd, 2H, 2 and 2′-H, $J_{2,2'}$ = 12.5 Hz), 4.21−4.29 (m, 2H, CH_2), 5.23−5.27 (m, 1H, α-H), 6.36 (s, 1H, NH), 7.09−7.20 (m, 5H, aryl), 7.31−7.36 (m, 5H, aryl), 7.73 (s, 1H, NH), 7.70−7.76 (m, 2H, 6-H and 7-H), 8.02−8.03 (d, 1H, 8-H), 8.10−8.11 (d, 1H, 5-H). MS [M$^+$] calcd for $C_{31}H_{27}N_3O_5S_2$: 435.40; found, 435.71.

20b. Yield 174 mg, 87%; mp 245−247 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ, 1.21−1.24 (m, 3H, CH_3), 3.00−3.05 (m, 1H, β-CH_2), 3.11−3.01 (m, 1H, β-CH_2), 3.69−3.71 (d, 1H, 2-H, $J_{2,2'}$ = 12.5 Hz), 3.73−3.75 (d, 1H, 2′-H), 4.25−4.28 (m, 2H, CH_2), 5.24−5.28 (m, 1H, α-H), 6.53 (s, 1H, NH), 7.08−7.22 (m, 5H, aryl), 7.26−7.32 (m, 5H, aryl), 7.68−7.75 (m, 2H, 6-H and 7-H), 7.97 (s, 1H, NH), 8.00−8.01 (d, 1H, 8-H), 8.07−8.08 (d, 1H, 5-H). MS [M$^+$] calcd for $C_{31}H_{27}N_3O_5S_2$: 435.40; found, 435.71.

TFA (2 mL) was added to a solution of the carbamoyl derivatives **20a** or **20b**. After 1 h at room temperature, the solution was neutralized with triethylamine, and the solvents were evaporated to dryness. Flash cromatography of the residues with ethyl ether yielded, in each case, the corresponding enantiomer (+)-**1** as an orange oil, $[\alpha]^D_{20}$ = +31.0° (*c*, 1.2, MeOH), and (−)-**1** as an orange oil $[\alpha]^{20}_D$ −30.1° (*c* 1.0, MeOH).

Successively, according to the general procedure described above, these enantiomers were used as starting material for the synthesis of corresponding 3-(2-aminoethyl)spiro[(dihydroimidazo-2,4-dione)-5,3′-(2′,3′-dihydrothieno[2,3-*b*]naphtho-4′,9′-dione)] (+)-**11** ($[\alpha]^{20}_D$ +30.5° (*c* 1.2, MeOH), mp 175−176 °C) and (−)-**11**($[\alpha]^{20}_D$ −28.8° (*c* 1.0, MeOH), mp 174−175) and for the synthesis of 3-[3-(*N*,*N*-dimethyl)aminopropyl]spiro-[(dihydroimidazo -2,4-dione)-5,3′-(2′,3′-dihydrothieno[2,3-*b*]-naphtho-4′,9′-dione) (+)-**15** ($[\alpha]^{20}_D$ +28.0° (*c* 1.1, MeOH), mp 240−241 °C) and (−)-**15** ($[\alpha]^{20}_D$ −26.9° (*c* 1.0, MeOH), mp 239−241 °C).

Cell Lines. Human tumor cell lines were used in this study: NCI-H460 and A459 lung carcinomas, MCF-7 breast carcinoma, SW 620 and LoVo colon carcinoma, KB and Hep-2 epidermal carcinoma, and A2780 ovarian carcinoma, as well as two sublines selected for resistance to doxorubicin (A2780/Dx and MCF-7/Dx) and a subline selected for resistance to cisplatin (A2780/DDP). Sensitive tumor cells were obtained from American Type Culture Collection, whereas resistant tumor cells were from Istituto Tumori of Milan. All cell lines were grown as monolayers in RPMI-1640 (Life Technologies, Inc.) containing 10% fetal bovine serum (Life Technologies, Inc., NY).

Drug Treatment. Cells were grown in a volume of 100 μL at approximately 10% confluency in 96-well multititer plates and were allowed to attach and recover for another 24 h. Varying concentrations of drugs alone were then added to each well, and the plates were incubated in an atmosphere of 5% CO_2 and 95% air at 37 °C for an additional 24 h; then the plates were washed to remove the drug and incubated for 48 h. Control cultures included equivalent amounts of vehicle used to solubilize each molecule. Experimental agents **3**−**10** were solubilized in DMSO, while **11**−**17** and doxorubicin were dissolved in water.

Sulforhodamine B Assay. At the end of the treatment, cell viability was assessed by the sulforhodamine B (SRB) assay.[18] Data were expressed as %(T/C) = (OD of treated cells/OD of control cells) × 100, and the concentration of the test compound causing a 50% inhibition of cell growth (IC_{50}) was calculated from the dose/effect curve for each tested compound. Every assay was performed in triplicate, and the drug IC_{50} of each cell line was the average of at least three independent experiments.

Human Topoisomerase IIα Relaxation Assay. Purified topo II was purchased from TopoGEN (Columbus, OH). For the assay, 0.2 μg of supercoiled pBR322 DNA (Takara Shuzo Co., Ltd., Otsu, Japan) was relaxed by one unit of topo II in a total volume of 20 μL of assay buffer in the presence of various concentrations of the drugs for 1 h at 30 °C. At the end of the incubation period, the DNA samples were subjected to electrophoresis in a 1% agarose gel with TBE running buffer at a voltage of 1 V/cm for approximately 18 h. After the run, the gel was stained with 1 μg/mL ethidium bromide for 30 min followed by destaining in distilled water for 30 min. The DNA topoisomers were photographed under UV light.

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Supporting Information Available: Table of experimental analysis data for all compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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