Journal of Medicinal Chemistry

ARTICLE

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Design, Synthesis, and Cytotoxic Evaluation of Acyl Derivatives of 3-Aminonaphtho[2,3-*b*]thiophene-4,9-dione, a Quinone-Based System

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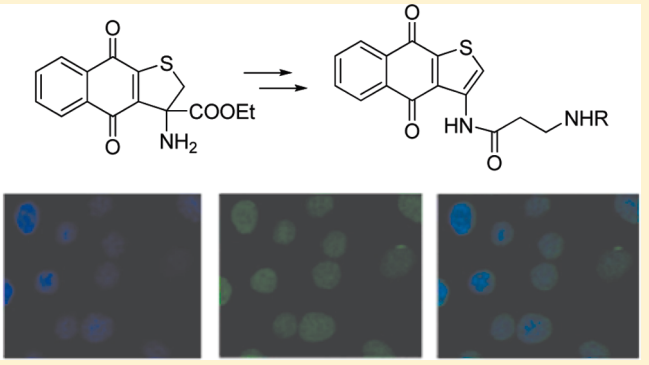
Supporting Information

ABSTRACT: A series of 3-acyl derivatives of the dihydronaphtho[2,3-*b*]thiophen-4,9-dione system were studied with respect to cytotoxicity and topoisomerase II inhibitory activity. These analogues were designed as electron-deficient anthraquinone analogues with potential intercalation ability. Derivatives 3-(diethylamino)-*N*-(4,9-dioxo-4,9-dihydronaphtho-[2,3-*b*]thiophen-3-yl)propanamide (**11m**) and 3-(2-(dimethyl-amino)ethylamino)-*N*-(4,9-dioxo-4,9-dihydronaphtho[2,3-*b*]-thiophen-3-yl)propanamide (**11p**) showed a high efficacy in cell lines that were highly resistant to treatment with doxorubicin, such as MDA-MB435 (melanoma), IGROV (ovarian), and SF-295 (glioblastoma) human cell lines. Both compounds inhibit topoisomerase II mediated relaxation of DNA, while only **11p** incites arrest at the S phase in Caco-2 cells, inducing a delay of cell cycle progression and an increase of cell differentiation. The ability of these derivatives to modulate small heat shock proteins and cardiotoxicy effects was also explored. In addition, the DNA-binding properties of these compounds were investigated and discussed.



INTRODUCTION

Anthracyclines are among the most effective and useful anticancer agents developed, and they are used to treat more types of cancer than any other chemotherapy agent.[1,2] Their clinical importance has stimulated wide research[3−6] directed to the development of new structurally related compounds with the goal of bypassing significant problems that limit their utility, such as their failure in resistant tumors expressing the ABCB1 (MDR1) gene[7−9] and the emergence of severe short- and long-term side effects associated with bone marrow and myocardial cell toxicity.[10,11] With this aim, our research group has developed different series of quinone-based compounds containing the 3-amino-3-(ethoxycarbonyl)-2,3-dihydrothieno[2,3-*b*]-naphtho-4,9-dione system (**4**, DTNQ) as chromophore (Figure 1).[12] The effected modifications on this template and the analysis of the structure−activity relationship (SAR) on the different synthesized series showed that the incorporation of a distal protonated alkylamine linked to the chromophore DTNQ system through a five- or six-membered heterocycle or the presence of a cycloalkyl as the fifth ring was an effective approach to identify new compounds endowed with potent cytotoxic activity and able to overcome multidrug resistance of tumor cells. Thus, the 3-glycylamino-3-(ethoxycarbonyl)-2,3-dihydrothieno[2,3-*b*]naphtho-4,9-dione (**5**),[13] the spirohydantoin derivatives 3-[2-(*N*,*N*-dimethylamino)ethyl or propyl]-spiro[(dihydroimidazo-2,4-dione)-5,3′-(2′,3′-dihydrothieno[2,3-*b*]-naphtho-4′,9′-dione)] (**6a**,**b**),[14] and the spirodiketopiperazine derivatives 4-[(2-*N*,*N*-dimethyl)amino]ethylspiro[(dihydropirazin-2,5-dione)-6,3′-(2′,3′-dihydrothieno[2,3-*b*]naphtho-4′,9′-dione) (**7**)[15] and spiro[(hexahydropyrrolo[1,2-*a*]pyrazine-1,4-dione)-6,3′-(2′,3′-dihydrothieno[2,3-*b*]naphtho-4′,9′-dione)] (**8**)[16] showed remarkable cytotoxic activity against several solid tumors and doxorubicin- and *cis*-platinum-resistant human cell lines.

Received: January 27, 2011
Published: May 09, 2011

In addition, STD NMR spectroscopy investigation performed on compounds **7** and **8** demonstrated that these derivatives interact with DNA with a dual binding mode: intercalative for the dihydrothieno[2,3-*b*]naphtho-4,9-dionetricyclic core and external considering the side chain moiety.[15,16] However, even though these derivatives had many of the structural characteristics of classical quinone-based DNA intercalating agents, they were not able to inhibit topoisomerase II (topo II) at equicytotoxic concentrations, indicating that other factors such as differences in cellular uptake, distribution within the cell, and additional targets within the cell might also affect the cytotoxicity of these derivatives.[17]

Now we have considered the possibility of using a new DTNQ derivative, the 3-aminonaphtho[2,3-*b*]thiophene-4,9-dione (**9**, TNQ) recently synthesized in our laboratories,[18] as a more planar chromophore. This quinone-based amine system showed interesting cytotoxic activity toward the MCF-7 human breast carcinoma (IC_{50} = 3.2 μM) and SW 620 human colon carcinoma cell lines (IC_{50} = 4.0 μM), indicating its potential as a template in the development of efficient cytotoxic agents. The new system presents a more "planar core" compared to initial DTNQ structure and an amine group able to be functionalized with appropriate side chain in a defined orientation with respect to the chromophore, thus guaranteeing two of the main structural requisites for the antineoplastic activity of intercalating agents. According to literature data, among heterocyclic quinones endowed with cytotoxic activity, those containing a thiophene nucleus fused to a quinone system have received little attention, despite the antitumoral activity of thiophene analogues of daunomycin and mitoxantrone described by the work groups of Kita[19] and Krapcho,[20] respectively.

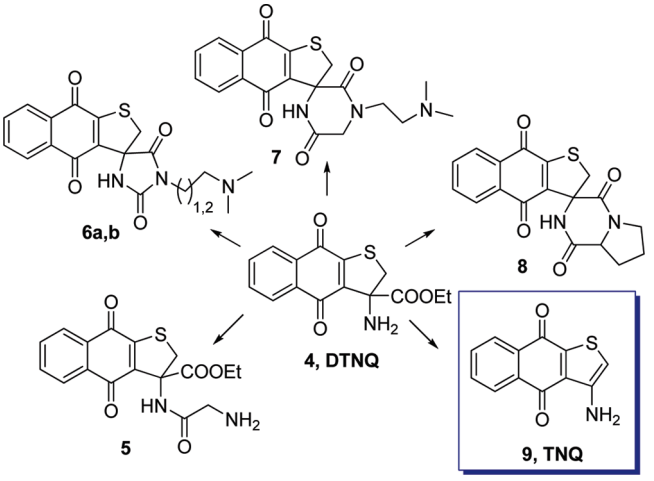
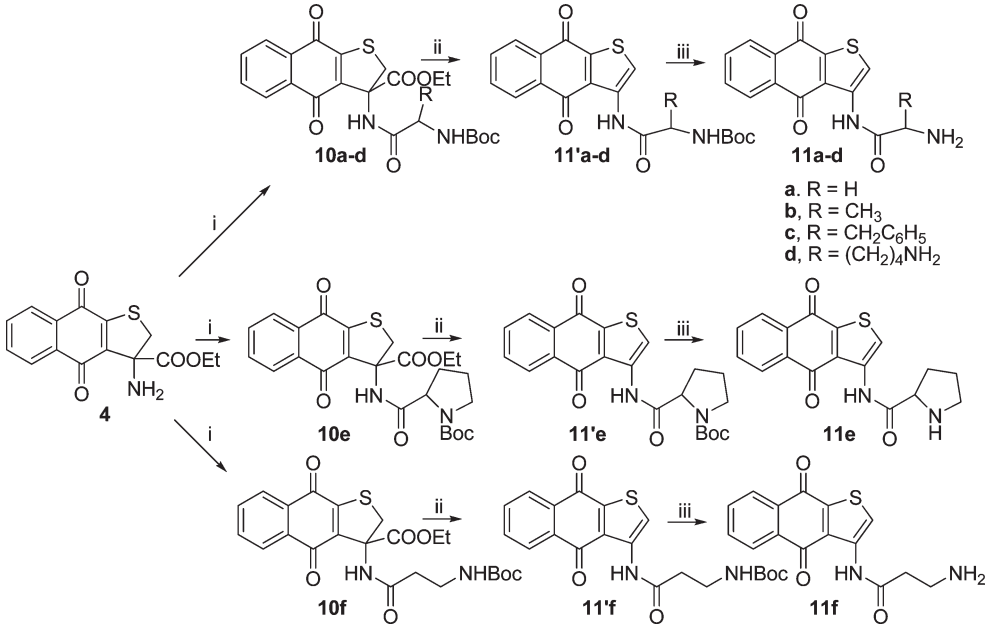


Figure 1. Structures of some DTNQ derivatives and the new TNQ system.

Thus, we developed a series of 3-substituted aminonaphtho-[2,3-*b*]thiophene-4,9-dione derivatives in which the amine group of the planar chromophore (TNQ) was linked to several amino acids (Gly, Ala, Phe, Lys, Pro, β-Ala), substituted alkylcarbonyl chains (hydroxyacetyl, hydroxypropionyl, (*N*,*N*-diethyl)aminoacetyl, (*N*,*N*-diethyl)aminopropionyl, 2-morpholinacetyl, 3-morpholinpropionyl, (*N*′,*N*′-methyl)(*N*-aminoethyl)aminopropionyl, thioacetyl, thiopropionyl), and carbamoyl chains (propyl, aminoethyl), which represent the side chain functionalities of the more active compounds of the precedent series. The objectives of this investigation are (a) validation of the TNQ system as template in the development of new quinone-based antitumoral agents, thus exploring new chemical spaces; (b) identification of the structural parameters that are important for the cytotoxic activity, through a comparative study of the structure−activity relationships (SARs) of TNQ derivatives; and (c) exploration of the basic biochemical events correlated to cytotoxic activity of new derivatives. The present paper deals with the preliminary studies concerning the synthesis of novel TNQ derivatives, their

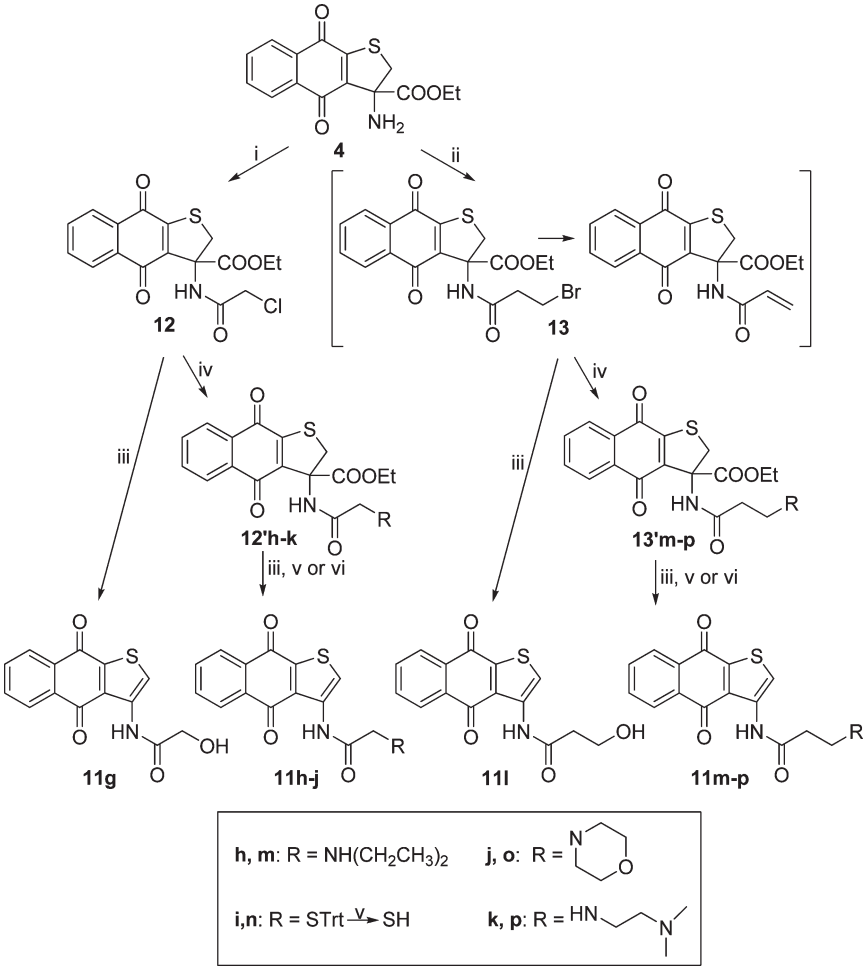
Scheme 1[a]



[a] Reagents and conditions: (i) Boc-Aaa-OH, HBTU, HOBt, DIPEA in DMF, room temperature; (ii) DBU in MeOH/H_2O, room temperature; (iii) TFA/DCM, TES.

dx.doi.org/10.1021/jm200094h |*J. Med. Chem.* 2011, 54, 4077–4091

Scheme 2[a]



[a] Reagents and conditions: (i) chloroacetyl chloride, TEA in THF; (ii) bromopropionyl chloride, TEA in THF; (iii) DBU in MeOH/H_2O, room temperature; (iv) nucleophilic reagents in THF, TEA, reflux temperature; (v) for **11i** and **11n** 20% TFA in dichloromethane; (vi) for **11j**, **11k**, **11m**, **11o**, and **11p** HCl (g)/diethyl ether solution.

cytotoxic activity, interaction with topo II and DNA, influence on cell cycle progression, modulation of small heat shock proteins, and cardiotoxicity effects.

RESULT AND DISCUSSION

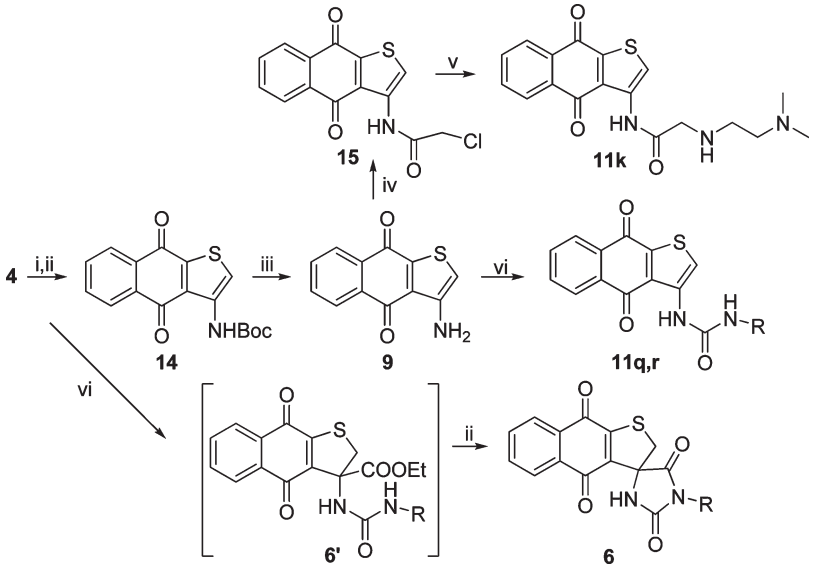
Chemistry. The synthetic approach to new 3-substituted aminonaphtho[2,3-*b*]thiophene-4,9-dione derivatives was based on the capacity of the DTNQ system and its 3-*N*-acyl derivatives to undergo oxidative decarboxylation during the hydrolysis of 3-ethyl ester group under basic conditions, as we recently described.[18] Condensation of 3-amino-3-ethoxycarbonyl-2,3-dihydrothieno[2,3-*b*]naphtho-4,9-dione (**4**, DTNQ) with different Boc-amino acids (**a** = Gly, **b**= Ala, **c** = Phe, **d** = Lys, **e** = Pro, **f** = β-Ala,) using HBTU, HOBt, and DIPEA in DMF afforded, with high yields (50−65%), the appropriate pseudodipeptide intermediates **10′a**−**f**, as shown in Scheme 1. Treatment with DBU in MeOH/H_2O medium directly gave the corresponding decarboxylated intermediates **11′a**−**f** in 76−82% yields. Finally, after removal of the Boc protecting group using 20% TFA in dichloromethane and triethylsilane as scavenger, the final compounds **11a**−**f** were obtained as trifluoroacetate salts in 40−48% overall yields.

Two homologue series of compounds containing a linear substituted alkyl chain were synthesized from 3-(2′-chloro)-acetamide-3-ethoxycarbonyl-2,3-dihydrothieno[2,3-*b*]naphtho-4,9-dione (**12**) and 3-(acrylamido)-3-ethoxycarbonyl-2,3-dihydrothieno[2,3-*b*]naphtho-4,9-dione (**13**), respectively, following a similar methodology (Scheme 2).

Condensation of **4** with chloroacethyl chloride in THF, using triethylamine as base, afforded the (2′-chloro)acetamide derivative **12** with 92% yield. Under these conditions, the reaction of **4** with bromopropionyl chloride gave the 3-bromopropionamide intermediate (90% yield), which partially evolved to β-elimination product 3-(acrylamido)-3-ethoxycarbonyl-2,3-dihydrothieno-[2,3-*b*]naphtho-4,9-dione (**13**) during workup of reaction. Decarboxylation performed on **12** and **13** intermediates gave directly the 2-hydroxyacetamide (**11g**) and 3-hydroxypropyonamide (**11l**) as final compounds, respectively. Nucleophilic displacement of the chlorine atom (**12**) or Michael-type addiction to acrylamido moiety (**13**) using diethylamine, triphenylmethanethiol, morpholine, or *N*,*N*-diethylethylendiamine, in THF and triethylamine at reflux, readily provided the corresponding

dx.doi.org/10.1021/jm200094h |*J. Med. Chem.* 2011, 54, 4077–4091

Scheme 3[a]



[a] Reagents and conditions: (i) $(Boc)_2O$; (ii) DBU in MeOH/H_2O, room temperature; (iii) 50% TFA/dichloromethane; (iv) chloroacetyl chloride, TEA in THF; (v) (*N*,*N*-dimethyl)ethylenediamine in THF, TEA, reflux temperature; (vi) triphosgene, TEA, THF, room temperature, 10 min, then R-NH_2.

acetamide (**12′h**−**k**) or propionamide (**13′m**−**p**) analogues. Basic hydrolysis of these derivatives afforded the corresponding decarboxylated compounds (**11h**−**j** and **11m**−**p**) except in the case of **12′k** (R = $HNCH_2CH_2N(CH_3)_2$). In fact, under the cited conditions, this intermediate gave the cyclic derivative 4-[(2-*N*,*N*-dimethyl)amino]ethylspiro[(dihydropirazin-2,5-dione)-6,3′-(2′,3′-dihydrothieno[2,3-*b*]naphtho-4′,9′-dione)] (**7**) previously described.[15] Then final compounds presenting an amine functionality **11j**, **11k**, **11m**, **11o**, and **11p** were treated with a solution of gaseous hydrochloric acid in diethyl ether to provide corresponding hydrochloride salts. This was found to both aid purification and provide an improved solubility profile for the biological assays. The final thioacetamide **11i** and thiopropyonamide **11n** derivatives were obtained after S-Trt deprotection using 20% TFA in dichloromethane in quantitative yields.

For the synthesis of compound **11k** and the urea-based derivatives **11q** and **11r** we chose an alternative route that implied the use of 3-aminonaptho[2,3-*b*]thiophene-4.9-dione (TNQ, **9**) as starting material (Scheme 3). The condensation of **9**, obtained after deprotection of the corresponding *N*-Boc TNQ (**14**) using 50% TFA in dichloromethane,[18] with chloroacetyl chloride afforded the (2′-chloro)acetamide intermediate **15** (88% yield). Reaction of **15** with diethylamine in THF and triethylamine at reflux afforded the final derivative **11k**. Compounds **11q** and **11r** were obtained by treatment of **9** with triphosgene and TEA in THF followed by addiction of propylamine or *N*,*N*-dimethylethylendiamine. Also in this case, the use of **9** as starting material was necessary because the corresponding *N*-carbamoyl derivatives of DTNQ (compounds **6′**) evolved rapidly to spirohydantoin derivatives **6** under hydrolytic conditions.[14]

In Vitro Cytotoxicity. TNQ derivatives were first examined for antiproliferative activity against the MDA231 human breast carcinoma, SW 620 human colon carcinoma, and U937 human leukemic monocyte lymphoma cell lines, and the obtained IC_{50} values are summarized in Table 1. For comparative purposes, the template **9** and doxorubicin were also included in the assay.

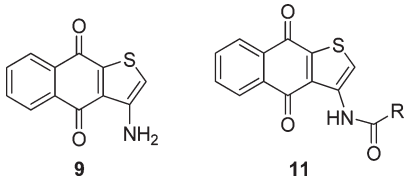
Results in Table 1 confirmed compound **9** as a potential scaffold for new antitumoral agents with a cytotoxic activity in the micromolar range on the three cell lines used in the assay. The improved antitumor activity and spectra of some of the newly synthesized compounds, compared to **9**, demonstrated that chemical modification at C-3 was an effective approach to optimize the activity profiles of TNQ moiety. The wide activity range observed for compounds **11a**−**r** (IC_{50} from 0.6 to >40 μM) indicated that the nature of substituents on the amine group at the C-3 position markedly affects the activity profile of these compounds. Incorporation through the 3-amino group of different amino acids was well tolerated in the case of linear amino acids such as glycine (**11a**). The presence of amino acids containing alkyl (Ala, **11b**) or benzyl (Phe, **11c**) side chain, relatively more rigid and more electron rich when compared to unsubstituted side chain, led to significant loss of activity, especially in the MDA231 cell line. This negative effect was more noteworthy with the introduction of an alkylamino side chain (Lys, **11d**). The incorporation of Pro gave the derivative **11e**, which turned out to be the most active in the leukemic cell line (IC_{50} = 0.9 μM).

Other interesting results were obtained with the incorporation of a primary or tertiary amine to the end of the ethyl side chains. Compounds **11f**, **11m**, and **11p** retained cytotoxic levels similar to those of doxorubicin on the SW 620 cell line, with IC_{50} of 0.8, 1.5, and 0.6 μM, respectively, and maintained the activity on the MDA231 and U937 cell lines within the micromolar range (2.0−3.7 and 1.1−1.7 μM, respectively). These derivatives were 2- to 5-fold more potent than their methylene homologues (**11a**, **11h**, and **11k**, respectively) on all the cell lines. Congeners with a hydroxyl (compounds **11g** and **11l**), thiol (compounds **11i** and **11n**), or morpholin (compounds **11j** and **11o**) groups were remarkably less potent compared to their primary and tertiary amine analogues.

Finally, the incorporation of an alkyl or alkylamino side chain through a ureide group led to a decrease of the activity in the resultant analogues **11q** and **11r**, respectively. These results

dx.doi.org/10.1021/jm200094h |*J. Med. Chem.* 2011, 54, 4077–4091

Table 1. Cytotoxic Activities of 3-(Amino)naphtho[2,3-*b*]thiophene-4,9-dione (9) and 3-[(Acyl)amino]naphtho[2,3-*b*]-thiophene-4,9-dione Derivatives (11a−f)



		IC$_{50}$ ± SD [a] (μM)			topo II activity[e]	
compd	R	MDA231[b]	SW620[c]	U937[d]	5 μM	10 μM
9		11.3 ± 0.4	4.0 ± 0.3	10.1 ± 0.4		
11a	CH_2NH_2[f]	6.2 ± 4.6	2.3 ± 0.4	7.0 ± 0.07		
11b	$CH(CH_3)NH_2$[f]	>40	12.4 ± 1.5	9.1 ± 0.2		
11c	$CH[CH_2(C_6H_5)]NH_2$[f]	>40	30.50 ± 6.4	>40	+	+
11d	$CH[(CH_2)_4NH_2]NH_2$[f]	>40	>40	20 ± 0.01		
11e	2-pyrrolidinyl[f]	6.7 ± 2.5	5.4 ± 0.1	0.9 ± 0.06		
11f	$CH_2CH_2NH_2$[f]	3.7 ± 0.9	0.8 ± 0.27	1.7 ± 0.01		
11g	CH_2OH	10.1 ± 0.2	18.5 ± 0.7	15.1 ± 0.06		
11h	$CH_2N(CH_2CH_3)_2$[g]	8.5 ± 0.12	4.0 ± 0.14	5.1 ± 0.07		
11i	CH_2SH	13.6 ± 0.15	20.9 ± 0.16	30.1 ± 0.04		
11j	CH_2-morpholine[g]	7.1 ± 0.2	10.8 ± 0.12	4.3 ± 0.02		
11k	$CH_2NH(CH_2)_2N(CH_3)_2$[g]	4.9 ± 0.4	2.1 ± 0.3	4.0 ± 0.03		
11l	$(CH_2)_2OH$	9.2 ± 0.6	20.3 ± 0.8	15 ± 0.06		
11m	$(CH_2)_2N(CH_2CH_3)_2$[g]	2.5 ± 0.1	1.5 ± 0.2	1.1 ± 0.01	+	+++
11n	$(CH_2)_2SH$	15.2 ± 0.1	20.7 ± 0.8	23.9 ± 0.34		
11o	$(CH_2)_2$-morpholine[g]	10.1 ± 1.3	20.1 ± 0.3	7.2 ± 0.07		
11p	$(CH_2)_2NH(CH_2)_2N(CH_3)_2$[g]	2.0 ± 0.1	0.6 ± 0.08	1.3 ± 0.03	++	+++
11q	$NH(CH_2)_2CH_3$[g]	9.5 ± 0.52	6.5 ± 1.20	10.1 ± 0.50		
11r	$NH(CH_2)_2N(CH_3)_2$[g]	8.7 ± 0.30	5.9 ± 0.20	9.8 ± 0.20		
doxorubicin		1.13 ± 0.01	0.12 ± 0.01	0.93 ± 0.01	0	0

[a] Data represent mean values (±SD) for three independent determinations. [b] Human melanoma cell line. [c] Human colon carcinoma cell line. [d] Human leukemic monocyte lymphoma cell line. [e] The semiquantitative evaluation of topo II mediated DNA relaxation activity was as follows: +++, high; ++, intermediate; +, low; 0, absent. The rest of the compounds were not tested. [f] Evaluated as TFA salts. [g] Evaluated as HCl salts.

imply a minor tolerance to structural modifications in this series compared to precedent series.

To further determine the antitumor spectra, the most potent compounds **11f**, **11m**, and **11p** were selected and screened against a panel of human tumor cell lines, including MDA-MB435 and SK-MEL 28 (melanoma), IGROV (ovarian), SF-295 and SNB-19 (glioblastoma), and Colo205, HT-29, and undifferentiated Caco-2 (colon). Differentiated Caco-2, a well accepted model of normal cell line because of its ability to acquire the phenotype of mature small-intestinal cell,[21] was utilized to characterize a safety profile of the compounds at least in terms of "cell selectivity".[22]

As observed in Table 2, selected compounds were more potent than doxorubicin on the melanoma, colon, and CNS human tumor cell lines, with IC$_{50}$ in the range 0.1−1.0 μM. Compounds **11m** and **11p** turned out to be the most active derivatives against SK-MEL 28 human melanoma cell line (IC$_{50}$ = 0.6 and 0.3 μM, respectively) and were equipotent to doxorubicin (IC$_{50}$ = 0.4 μM). Analogous to that observed in the previously described series,[14−16] these compounds showed a remarkable activity against tumor cell lines generally highly resistant to treatment with doxorubicin. Compounds **11m** and **11p** presented a cytotoxic activity in the micromolar range against undifferentiated Caco-2 tumor colon cell lines (IC$_{50}$ = 0.8−1.0 μM) while being 4-fold less active (IC$_{50}$ = 3.8−4.1 μM) on differentiated Caco-2 cell line. These data indicated a good profile of cell selectivity for our derivatives (selectivity index SI ≈ 0.22) especially if they are compared with the high toxicity data obtained with doxorubicin (SI = 11.1).

Subcellular Distribution of TNQ Derivatives in MCF-7 Cell Line. Distribution of the labeled forms of our derivatives within the cell was investigated by confocal microscopy in MCF-7 cell line, using 50 nM **11m** (IC$_{50}$= 0.5 μM) and **11p** (IC$_{50}$ = 0.6 μM). As shown in Figure 2, these TNQ derivatives are clearly localized in the nuclei, indicating a site of cytotoxic action similar to those of classic quinone-based intercalators.[23]

Topoisomerase Inhibition. A number of quinone antitumor drugs are thought to be cytotoxic by virtue of their ability to stabilize a covalent topo II−DNA intermediate, the cleavable complex.[24] Topo II is an essential enzyme that plays an important role in DNA replication, repair, transcription, and chromosome segregation.[25] Topo II alters the topological state of nucleic acids by passing an intact DNA helix through a transient break which generates a separate DNA helix.[26] We analyzed the possibility

Table 2. Inhibition of Multiple Human Tumor Cell Lines by Selected Compound

		$IC_{50} \pm SD$ [a] (μM)			
origin of tumor	cell line	**11f**	**11m**	**11p**	doxorubicin
melanoma	MDA-MB435	0.4 ± 0.10	0.5 ± 0.08	0.5 ± 0.09	1.3 ± 0.21
	SK-MEL 28	1.5 ± 0.08	0.6 ± 0.08	0.3 ± 0.07	0.6 ± 0.09
ovarian	IGROV	1.2 ± 0.30	2.5 ± 0.10	2.0 ± 0.20	1.3 ± 0.30
glioblastoma	SF-295	2.8 ± 0.20	0.6 ± 0.06	0.6 ± 0.09	4.4 ± 0.50
	SNB-19	1.6 ± 0.60	0.7 ± 0.04	0.9 ± 0.10	0.8 ± 0.05
colon	Colo205	0.4 ± 0.04	0.9 ± 0.05	1.1 ± 0.05	1.5 ± 0.30
	HT-29	0.6 ± 0.08	0.8 ± 0.05	0.5 ± 0.10	1.1 ± 0.20
	Caco-2[b]	2.6 ± 0.2	1.0 ± 0.6	0.8 ± 0.03	6.7 ± 0.80
	Caco-2[c]	6.1 ± 0.32	4.1 ± 0.10	3.8 ± 0.09	0.6 ± 0.05

SI [d]			
11f	**11m**	**11p**	doxorubicin
0.43	0.25	0.21	11.1

[a] Data represent mean values (SD) for three independent determinations. [b] Preconfluent Caco-2 cell line. [c] Postconfluent Caco-2 cell line. [d] SI = selectivity index = (IC_{50} on undifferentiated Caco-2 cell line)/(IC_{50} on differentiated Caco-2 cell line).

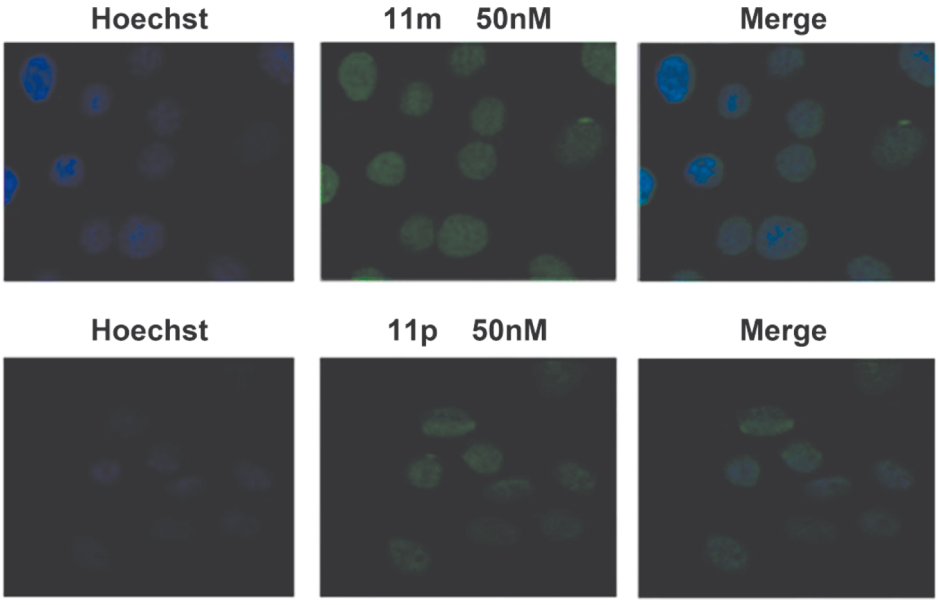


Figure 2. Distribution of labeled **11m** and **11p** in MCF-7 cells by confocal microscopy.

that compounds **11m** and **11p** could inhibit the activity of topo II. The effect of cytotoxic compounds **11m** and **11p** and of the inactive compound **11c** on the strand passage activity of topo II was determined by the enzyme-mediated negatively supercoiled pBR322 relaxation.[27]

As indicated in Figure 3, compounds **11m** and **11p** displayed significant inhibition of topo II mediated relaxation in a concentration-dependent mode, while **11c** does not inhibit this activity at the concentrations tested. These results, shown also in Table 1 as semiquantitative form, parallel the cytotoxicity data enumerated in the same table, thus suggesting a behavior similar to that of classical intercalators. Moreover, at the assay concentrations, the doxorubicin showed a lack of activity (see Supporting Information) which agrees with the results described in different studies.[28] These studies show that doxorubicin inhibits topo II only at 0.04−0.92 μM, while at higher concentration the inhibition is either diminished or totally abolished.

DNA Binding Properties by NMR. Representative compounds **11c**, **11m**, and **11p** were tested to see if they interact with DNA, using both saturation transfer difference (STD)[29] and water−ligand observed via gradient spectroscopy (WaterLOGSY) NMR techniques.[30] STD NMR and WaterLOGSY are techniques that can be used to characterize and identify binding. These techniques have become increasingly important as tools in the investigation of biomolecular recognition phenomena.[31] In the STD NMR, resonances of the macromolecule are selectively saturated, and in a binding ligand, enhancements are observed in the difference (STD NMR) spectrum resulting from subtraction of this spectrum from a reference spectrum in which the macromolecule is not saturated. All the proton resonances of

11m and **11p** were observed in the STD spectra acquired in the presence of poly(dG-dC)·poly(dG-dC) copolymer as the DNA target (Figure 4), demonstrating that **11m**/DNA and **11p**/DNA interactions did occur. In contrast, the absence of the proton resonances of **11c** in its STD spectra (Figure 4) demonstrates that **11c** does not interact with DNA. The same results were obtained using the WaterLOGSY experiment. In this experiment, the large bulk water magnetization is partially transferred via the macromolecule−ligand complex to the free ligand. Because of the very different tumbling times of the free ligand and of the macromolecule−ligand complex, LOGSY signals are typically negative for free ligands in solution and relatively less negative or positive for binders in the presence of the macromolecule. Figure 5 shows the WaterLOGSY spectra of **11c**, **11m**, and **11p** with and without the poly(dG-dC)·poly(dG-dC) copolymer. As observed, **11m** and **11p** signals became positive in the presence of DNA while **11c** signals remain negative demonstrating that **11m** and **11p** but not **11c** interact with the DNA polymer.

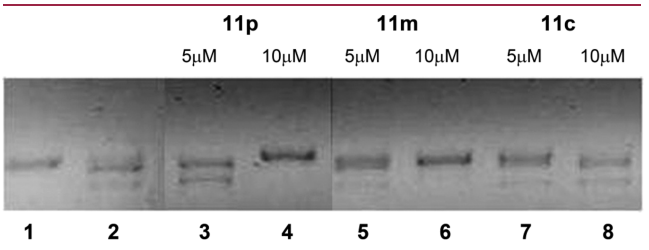


Figure 3. Effects of compounds **11p**, **11m**, and **11c** on the topo II mediated DNA cleavage. Supercoiled plasmid pBR 322 (0.5 pmol) was incubated with 1 unit of purified human topo II in the presence or absence of the tested agents: (lane 1), supercoiled DNA; (lane 2), relaxed DNA enzyme control; (lanes 3 and 4) 5 and 10 μM **11p**; (lanes 5 and 6) 5 and 10 μM **11m**; (lanes 7 and 8) 5 and 10 μM **11c**.

Furthermore, we applied the so-called DF-STD (differential frequency STD) spectroscopy,[32] to study the binding modes of **11m** and **11p** with the DNA. The method allows the discrimination of base-pair intercalators and minor-groove and external binders. The approach is based on the comparison of two parallel sets of STD experiments performed under the same experimental

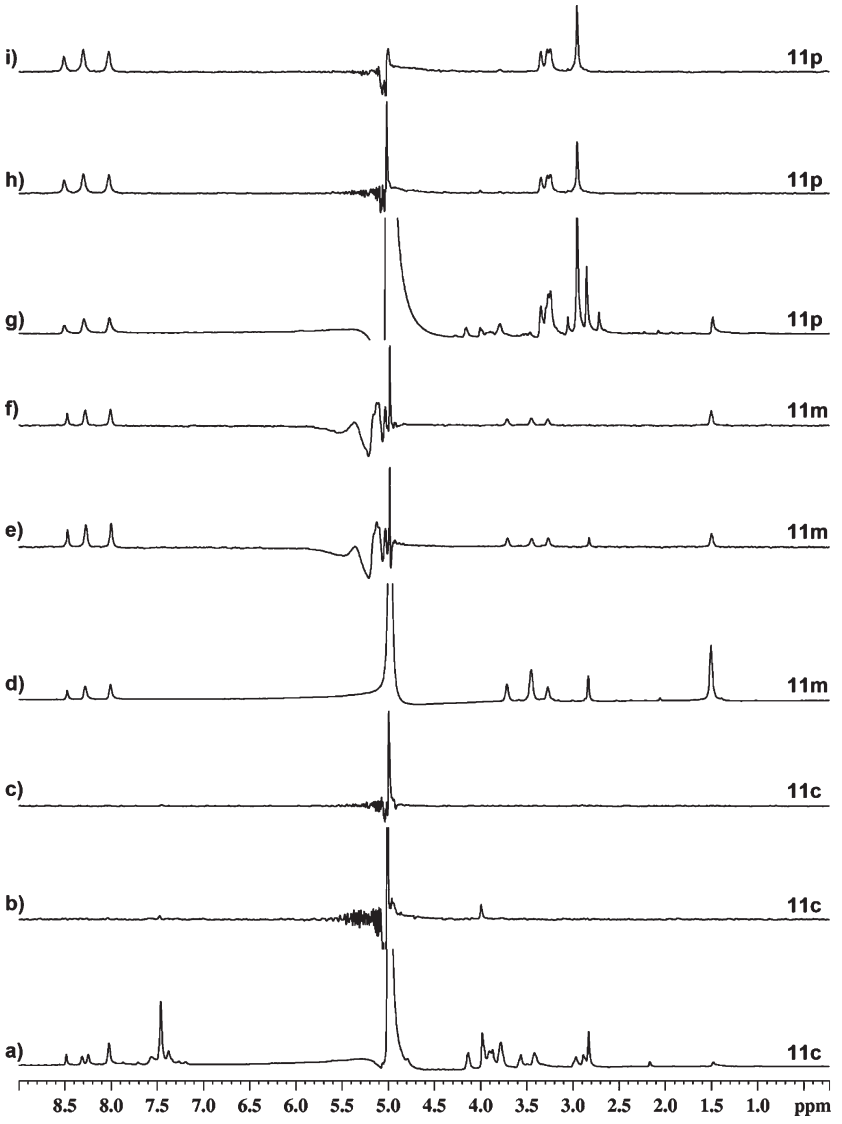


Figure 4. 1D proton spectra (a, d, g) and the corresponding STD NMR spectra recorded upon saturation at 10 ppm (b, e, h) and −1 ppm (c, f, i) of **11c**/DNA, **11m**/DNA, and **11p**/DNA complexes, respectively. The STD NMR spectra were plotted with the same noise level.

dx.doi.org/10.1021/jm200094h |*J. Med. Chem.* 2011, 54, 4077–4091

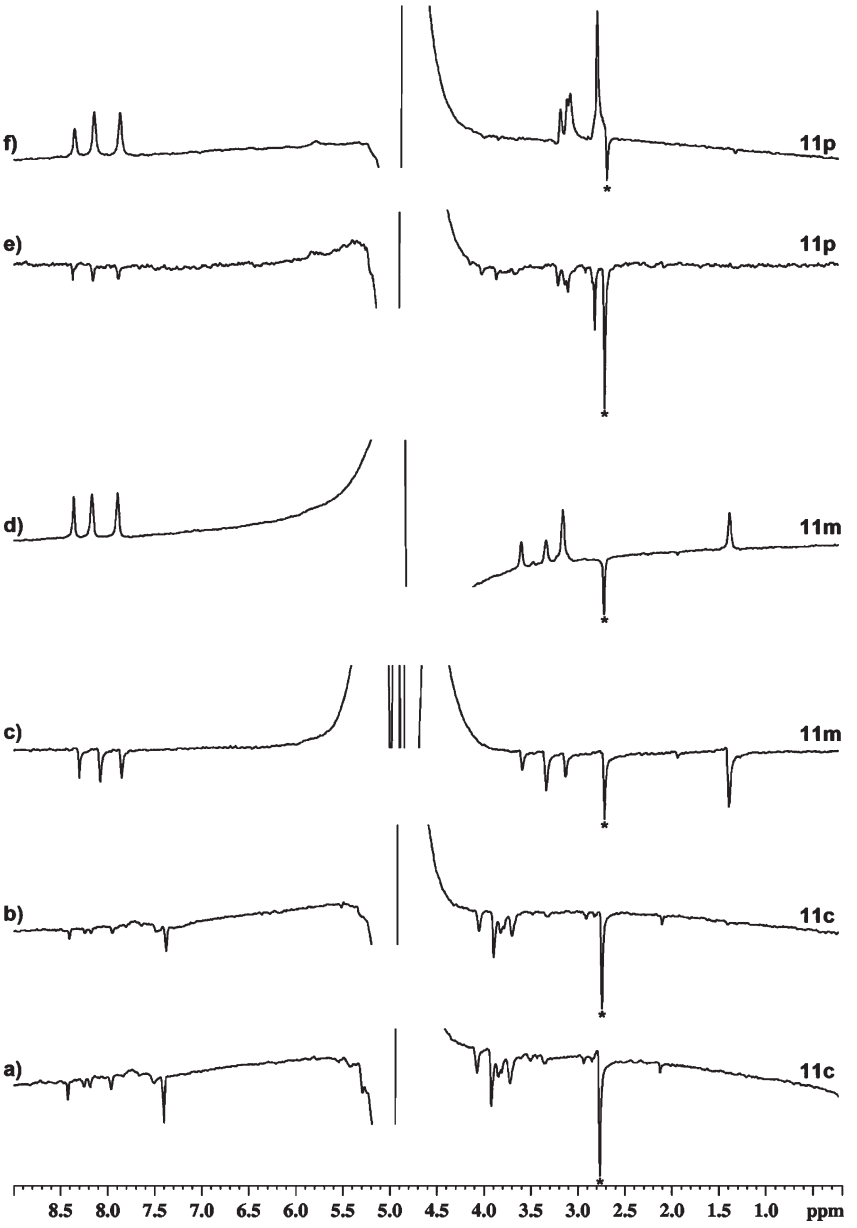


Figure 5. Water LOGSY spectra of **11c**, **11m**, and **11p** in the absence (b, d, e) or in the presence (a, c, e) of poly(dG-dC)·poly(dG-dC) copolymer. The asterisk (∗) indicates the DMSO residual signal.

conditions, in which saturation is centered either in the aromatic or in the low-field aliphatic spectral regions.

A ligand making proximate contacts with aromatic base protons, such as an intercalator sandwiched by consecutive base pairs, would receive more saturation upon irradiation of DNA aromatic protons rather than irradiation of deoxyribose protons. The converse would be true for an external ligand. The "binding mode index" (BMI), a numerical parameter that expresses the relative sensitivity of ligand protons to the perturbation arising from base versus sugar/backbone saturation was used.[32] Three BMI ranges were defined in the original contribution:[32] 0 < BMI < 0.50 for external (nonspecific) electrostatic backbone binding; 0.90 < BMI < 1.10 for minor-groove binding; 1.20 (0.90) < BMI < 1.50 for base-pair intercalation. DF-STD analysis of compound **11m** gave different BMI values: BMI = 0.86, for the aliphatic

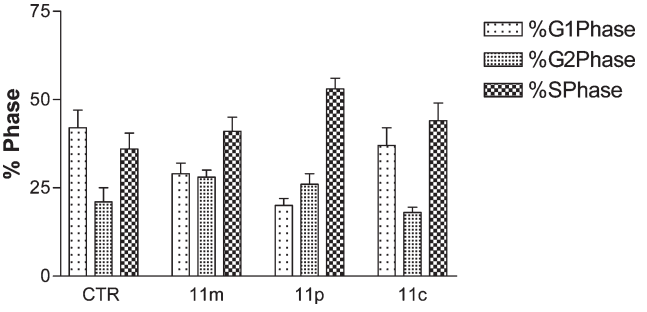


Figure 6. Effects of **11m**, **11p**, and **11c** on the distribution of Caco-2 cell populations data represent the percentage of cells in each phase of cell cycle. For **11m**: G1, 29%; G2/M, 28%; S, 41%. For **11p**: G1, 20%; G2/M, 26%, S, 53%. For **11c**: G1, 37%; G2/M, 18%, S, 44%.

signals and BMI = 1.35 for the aromatic signals. This result can be explained assuming two different DNA binding modes for **11m**. An intercalative mode of binding is sustained by its tricyclic planar core, and an external backbone binding can be attributed to its side chain. This is similar to that observed for doxorubicin[32] and for compounds **7** and **8** in our previous work.[15,16] Considering **11p**, BMI = 1.01 was measured for the aromatic protons and BMI = 0.90 for the aliphatics. These BMI values are compatible with both intercalative and minor-groove binders.

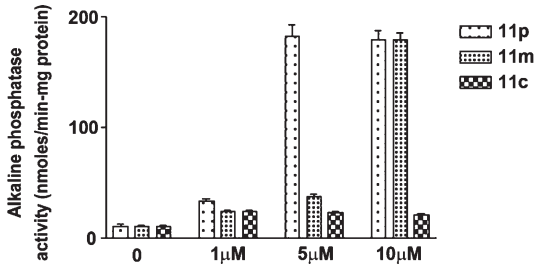


Figure 7. Differentiation of Caco-2 cells assessed by measurement of alkaline phosphatase activity after 48 h of culture in the presence of 0, 1, 5, and 10 μM **11p**, **11m**, and **11c**.

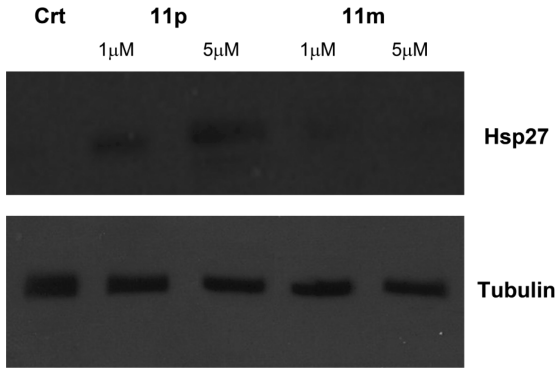


Figure 8. Effects of Caco-2 cells treatment with 1 and 5 μM **11p** and **11m** on Hsp27 expression.

Cell Cycle Effects. To investigate the cytotoxic effects of these derivatives in more detail, we examined the effects on cell cycle progression in CaCo-2 cell line. The percentage of these cells in G1, S, and G2/M phases was analyzed after 48 h of treatment with 1 μM **11m**, **11p**, and **11c** (Figure 6). Under these conditions, the control cells were in phase G1 (42%), G2/M (21%), and S (36%). The treatment with **11p** resulted in a significant accumulation of cells in the S phase, while concomitantly the G1 populations decreased. About 53% of the CaCo-2 cells treated with this compound were arrested at the S phase. Under the same conditions, the treatment with **11m** induces a weak increase of cell in both G2 and S phases while the treatment with **11c** does not modify significantly the distribution of Caco-2 cell populations

Accordingly, treatment of Caco-2 cells with 1 μM of our derivatives for 48 h induced an increase of cyclin A expression[33] only in the case of **11p** (43%; see Supporting Information), indicating that the cell cycle progression of cells in the S phase was prompted. The expression of cyclin A was not upset in treated Caco-2 cells with **11m** and **11c**.

Since cell division arrest is one of the prerequisites for cell differentiation,[34] we determined the effect of our molecules on Caco-2 differentiation. In Figure 7 we report alkaline phosphatase (ALP) activity, a marker of enterocytic differentiation correlated to the postconfluent phase.[35]

Treatment of preconfluent Caco-2 with 1 μM **11p** increased ALP activity of 35% ($p < 0.005$). A more significant increase of the differentiation, with ALP augment of >180%, was only obtained by treatment of Caco-2 cells with 5 μM **11p** or 10 μM **11m** for 48 h. All these preliminary results suggested that for this series the cell growth inhibition was not related to cell cycle perturbation.

Modulation of Heat Shock Protein (Hsp) Expression. Small heat shock proteins are involved in a variety of cellular processes including cell growth and differentiation.[36] We previously reported the ability of a DTNQ analogue, compound **8**, to modulate the heat shock protein expression on Caco-2 cells.[17] In order to evaluate the behavior of the new synthesized derivatives, we carried out a preliminary study on the effect of **11m** and **11p** at 1 and 5 μM in Hsp27 expression in Caco-2 cells for 48 h. Hsp27 is weakly expressed in Caco-2 (Figure 8), and

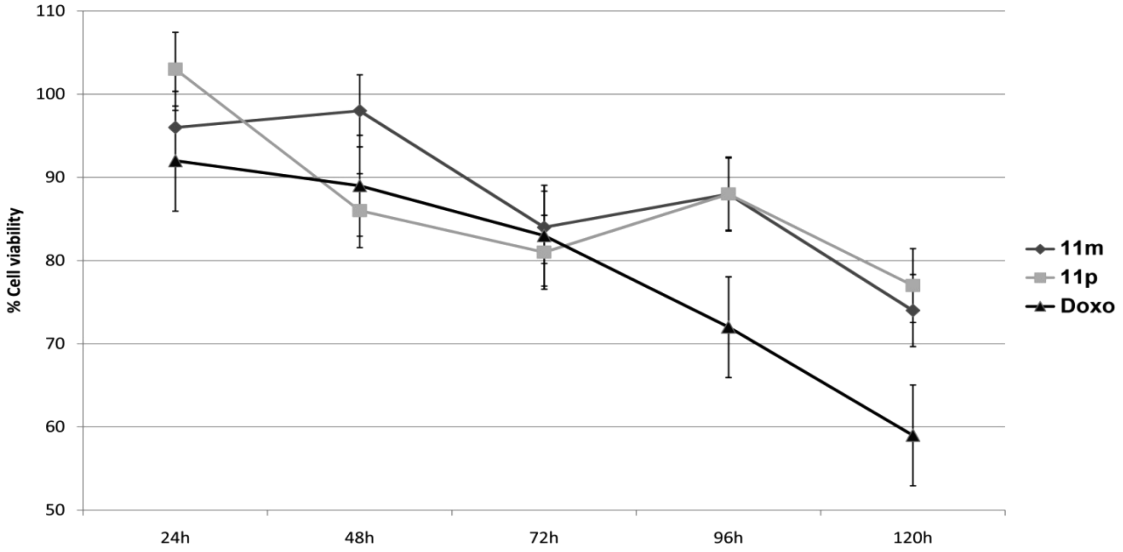


Figure 9. Results of cell viability assay of **11m**, **11p**, and doxorubicin on H9C2 cells at 1 μM.

dx.doi.org/10.1021/jm200094h |J. Med. Chem. 2011, 54, 4077–4091

treatment of this cell line with **11p** led to a significant dose-dependent increase of its expression. **11m** produced a weak enhancement of Hsp27 expression at 1 μM, which was not observed at 5 μM.

Cardiomyocyte Cell Viability. It is well-known that the clinical use of anthracyclines, specially doxorubicin, in the treatment of many neoplastic diseases is limited by cumulative cardiotoxicty.[37] One cause of this effect has been attributed to the redox process involving the quinone system which results in the formation of reactive oxygen species and ultimately in myocyte death. In order to evaluate the potential toxicity of our quinone ring, we examined the cell viability in cardiac derived H9C2 myocytes exposed to 1 μM **11m**, **11p**, and doxorubicin for 24, 48, 72, 96, and 120 h. Previous studies reported in the literature used this cell line as a model system to evaluate the cardiotoxicity caused by doxorubicin.[38]

As shown in Figure 9, treatment with doxorubicin induced cardiotoxicity in a time-dependent manner[37] while compounds **11m** and **11p** maintained good cell viability after 120 h (74% and 76%, respectively). The possible correlation between the reduced cardiotoxicity and the Hsp27 modulation[39] will be the subject of more in-depth studies.

CONCLUSIONS

We report the synthesis and biological evaluation of a series of quinone-based derivatives, designed as conjugated structures linking a planar naphtho[2,3-*b*]thiophenedione core with different acyl-substituted groups. Among the designed molecules, compounds containing a 3-(diethylamino)propanamide (**11m**) or 3-(2-(dimethylamino)ethylamino)propanamide (**11p**) protonatable side chain showed a greater cytotoxic potency than doxorubicin against cell lines that were highly resistant to treatment with this drug, such as the melanoma (MDA-MB435), glioblastoma (SF-295), and colon (SW 620, Col205, and HT-29) human tumor cell lines,

Preliminary results about the mechanism of action indicate that these derivatives had a significant effect on topoisomerase II activity targeting the nuclear DNA, which is generally considered as an attractive target for anticancer therapy. The NMR results suggested that DNA interactions do occur for highly active compounds **11m** and **11p** but not for inactive compound **11c**. Experimental data indicate that **11m** and **11p** intercalate the DNA through their aromatic portion. Furthermore, a nonintercalative mode of binding to DNA can also hold for **11p**. These data revealed significant similarities in the cytotoxic behavior and the site of action of these compounds compared to classical intercalators. However, the compounds tested showed a minor influence on the regulation of the cell cycle and only derivative **11p** prolonged the S phase of the Caco-2 cell cycle inducing both delay of cell cycle progression in responsive cells and moderate cellular differentiation. This latter compound also showed a high ability to increase Hsp27 expression. Finally, the compounds under study affect the viability of H9C2 cells after chronic treatment to a lesser extent than does doxorubicin. Further development and more in-depth studies on the mechanism of action of this series are in progress.

EXPERIMENTAL SECTION

General. Reagents, starting materials, and solvents were purchased from commercial suppliers and used as received. Analytical TLC was performed on plates coated with a 0.25 mm layer of Merck silica gel 60 F254, and preparative TLC was performed on 20 cm × 20 cm glass plates coated with a 0.5 mm layer of Merck silica gel PF254. Flash and gravity chromatographic purification was performed using 230−400 mesh silica gel unless otherwise noted. Melting points were taken on a Kofler apparatus and are uncorrected. ^{1}H NMR and ^{13}C NMR spectra were recorded with a Varian 400 spectrometer, operating at 400 and 100 MHz, respectively. Chemical shifts are reported in δ values (ppm) relative to internal Me_4Si, and J values are reported in hertz (Hz). ESI-MS experiments were performed on an Applied Biosystem API 2000 triple-quadrupole spectrometer. Combustion microanalyses were performed on a Carlo Erba CNH 1106 analyzer, and all reported values are within 0.4% of calculated values. These elemental analysis results confirmed ≥95% purity.

General Procedure for the Synthesis of 3-[(Acyl)amino]-naphtho[2,3-*b*]thiophene-4,9-dione Trifluoroacetate Salts (11a−f). The 3-amino-3-ethoxycarbonyl-2,3-dihydrothieno[2,3-*b*]-naphtho-4,9-dione system (DTNQ) (1), the 3-(*N*-*tert*-butyloxyamino-acyl)amino-3-ethoxycarbonyl-2,3-dihydrothieno[2,3-*b*]naphtho-4,9-dione (**10′a−f**), and the 3-(*N*-*tert*-butyloxyaminoacyl)aminonaphtho-[2,3-*b*]thiophene-4,9-dione (**11′a−f**) derivatives were synthesized according to the refs 12, 13, and 17, respectively. Then TFA was added to a solution of decarboxylated Boc-protected derivatives (**11′a−f**) (0.1 mmol) in DCM (10 mL), using triethylsilane as scavenger. Stirring was continued for 3−4 h at room temperature. The reaction mixture was concentrated to half volume, and ether was added. The title compounds as the trifluoroacetate salt were collected by filtration as yellow solids.

3-[(Glycyl)amino]naphtho[2,3-*b*]thiophene-4,9-dione Trifluoroacetate (11a). Yield: 45%. Mp 207−208 °C. ^{1}H NMR (400 MHz, CD_3OD) δ: 4.10 (s, 2H, CH_2); 7.85−7.87 (m, 2H, H-6 and H-7); 8.20−8.23 (m, 2H, H-5 and H-8); 8.50 (s, 1H, H-2). ^{13}C NMR (100 MHz, CD_3OD) δ: 45.7(CH_2), 119.2 (C-2), 127.5 (C-6 and C-7), 129.4 (C-3), 132.9 (C-8a), 134.2 (C-4a), 134.7 (C-5 and C-8), 135.9 (C-3a), 147.1 (C-9a), 171.8, 180.7, and 182.7 (C═O). ESI-MS m/z calcd for $C_{16}H_{11}F_3N_2O_5S$, 400.03; found, 400.11.

3-[(L-Alanyl)amino]naphtho[2,3-*b*]thiophene-4,9-dione Trifluoroacetate (11b). Yield: 45%. Mp 206 °C. ^{1}H NMR (400 MHz, CD_3OD) δ: 1.69 (d, 3H, J = 7.2 Hz, CH_3); 4.38−4.41 (q, 1H, CH); 7.87−7.89 (m, 2H, H-6 and H-7); 8.22−8.28 (m, 2H, H-5 and H-8); 8.49 (s, 1H, H-2). ^{13}C NMR (100 MHz, CD_3OD) δ: 21.7 (CH_3), 50.1 (αCH), 118.8 (C-2), 126.6 (C-6 and C-7), 129.8 (C-3), 133.1 (C-8a), 133.9 (C-4a), 134.6 (C-5 and C-8), 135.1 (C-3a), 147.2 (C-9a), 172.1, 180.9, and 182.5 (C═O). ESI-MS m/z calcd for $C_{17}H_{13}F_3N_2O_5S$, 414.05; found, 414.12.

3-[(L-Phenylyl)amino]naphtho[2,3-*b*]thiophene-4,9-dione Trifluoroacetate (11c). Yield: 41%. Mp 195−196 °C. ^{1}H NMR (400 MHz, CD_3OD) δ 2.96−3.07 (2 H, m, βCH_2); 4.44−4.47 (1 H, m, αCH); 7.12−7.22 (5 H, m, aryl); 7.87−7.89 (2 H, m, H-6 and H 7); 8.22−8.25 (2 H, m, H-5 and H 8); 8.47 (1H, s, H-2). ^{13}C NMR (100 MHz, CD_3OD) δ: 37.9 (βCH_2), 50.6 (αCH), 118.8 (C-2), 127.9 (C-6 and C-7), 125.9, 127.6, 128.3, 128.9, and 137.9 (aryl), 131.4 (C-3), 133.6 (C-8a), 134.2 (C-4a), 134.9 (C-5 and C-8), 139.0 (C-3a), 142.5 (C-9a), 172.7, 179.8, and 181.9 (C═O). ESI-MS m/z calcd for $C_{23}H_{17}F_3N_2O_5S$, 490.08; found, 490.01.

3-[(L-Lysyl)amino]naphtho[2,3-*b*]thiophene-4,9-dione Bistrifluoroacetate (11d). Yield: 42%. Mp 241−242 °C. ^{1}H NMR (400 MHz, D_2O) δ: 1.13−1.19 (m, 2H, γCH_2); 1.37−1.40 (m, 2H, δCH_2); 1.59−1.61 (m, 2H, βCH_2); 2.83−2.88 (m, 2H, εCH_2); 3.56−3.58 (m, 1H, αCH); 7.52−7.55 (m, 2H, H-6 and H-7); 7.60−7.70 (m, 2H, H-5 and H-8); 7.94 (s, 1H, H-2). ^{13}C NMR (100 MHz, CD_3OD) δ: 22.7 (γCH_2), 31.5 (δCH_2), 36.4 (βCH_2), 45.7 (εCH_2), 53.1 (αCH), 119.8 (C-2), 126.9 and 127.3 (C-6 and C-7), 128.9 (C-3), 132.2 (C-8a), 133.5 (C-4a), 134.5 (C-5 and C-8), 135.6 (C-3a), 146.2 (C-9a), 172.6, 181.7, and 182.2 (C═O). ESI-MS m/z calcd for $C_{22}H_{21}F_6N_3O_7S$, 585.10; found, 585.21.

3-[(L-Prolyl)amino]naphtho[2,3-*b*]thiophene-4,9-dione Trifluoroacetate (11e). Yield: 40%. Mp 197−198 °C. ^{1}H NMR (400 MHz, CD_3OD) δ: 2.17−2.19 (m, 2H, γCH_2); 2.25−2.29 (m, 1H, βCH_2); 2.63−2.66 (m, 1H, βCH_2); 3.45−3.50 (m, 2H, δCH_2); 4.73−4.75 (m, 1H, αCH_2); 7.87−7.88 (m, 2H, H-6 and H-7); 8.21−8.24 (m, 2H, H-5 and H-8); 8.46 (s, 1H, H-2). ^{13}C NMR (100 MHz, CD_3OD) δ: 23.9 (γCH_2), 29.5 (βCH_2), 46.2 (δCH_2), 60.7 (αCH), 120.4 (C-2), 126.8 and 127.0 (C-6 and C-7), 129.5 (C-3), 132.9 (C-8a), 133.4 (C-4a), 134.2 and 134.4 (C-5 and C-8), 135.8 (C-3a), 147.6 (C-9a), 171.9, 181.2, and 182.8 (C═O). ESI-MS *m*/*z* calcd for $C_{19}H_{15}F_3N_2O_5S$, 440.07; found, 440.13.

3-[(*β*-Alanyl)amino]naphtho[2,3-*b*]thiophene-4,9-dione Trifluoroacetate (11f). Yield: 48%. Mp 205−207 °C. ^{1}H NMR (400 MHz, CD_3OD) δ: 2.77−2.80 (t, 2H, αCH_2); 3.06−3.11 (t, 2H, $\beta CH2$); 7.88−7.91 (m, 2H, H-6 and H-7); 8.15−8.19 (m, 2H, H-5 and H-8); 8.42 (s, 1H, H-2). ^{13}C NMR (100 MHz, CD_3OD) δ: 37.4 (αCH_2), 40.1 (CH_2NH2), 119.9 (C-2), 126.9 (C-6 and C-7), 128.9 (C-3), 131.9 (C-8a), 134.2 (C-4a), 134.5(C-5 and C-8), 139.0 (C-3a), 144.1 (C-9a), 170.9, 178.7, and 181.2 (C═O). ESI-MS *m*/*z* calcd for $C_{17}H_{13}F_3N_2O_5S$, 414.05; found, 414.06.

***N*-(4,9-Dioxo-4,9-dihydronaphtho[2,3-*b*]thiophen-3-yl)-2-hydroxyacetamide (11g).** DBU (0.6 mmol) was added dropwise to a solution of 3-(2′-chloro)acetamide-3-ethoxycarbonyl-2,3-dihydrothieno[2,3-*b*]naphtho-4,9-dione (**12**)[15] (0.2 mmol) in methanol−water (9:1, 10 mL). After 30 min at room temperature the solvents were evaporated and the reaction residues were dissolved in chloroform and washed with water and dried with Na_2SO_4. The title compound was purified by flash chromatography (FC) using ethyl acetate as eluent. Yellow solid (43%). Mp 181−183 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 4.12 (s, 2H, CH_2); 7.79−7.81 (m, 2H, H-6 and H-7); 8.24−8.27 (m, 2H, H-5 and H-8); 8.50 (s, 1H, H-2); 11.23 (s, 1H, N*H*). ^{13}C NMR (100 MHz, CD_3OD) δ: 65.2(CH_2), 119.9 (C-2), 126.7 (C-6 and C-7), 129.6 (C-3), 132.6 (C-8a), 134.0 (C-4a), 134.3 (C-5 and C-8), 136.8 (C-3a), 146.2 (C-9a), 171.9, 181.5, and 183.0 (C═O). ESI-MS *m*/*z* calcd for $C_{14}H_9NO_4S$, 287.03; found, 287.19.

General Procedure for the Synthesis of *N*-(4,9-Dioxo-4,9-dihydronaphtho[2,3-*b*]thiophen-3-yl)-2-(substituted)acetamide (11h−j). To a solution of 12 (0.3−0.4 mmol) in THF (20 mL) were added the corresponding nucleophilic agents: *N*,*N*-diethylamine or triphenylmethanethiol or morpholine (3.0 equiv) and DIPEA (2 equiv). After the mixture was stirred at reflux temperature for 1−3 h, the solvent was evaporated. Then the residues (**12′h**−**j**) were dissolved in methanol−water (9:1, 20 mL) and DBU (0.9−1.2 equiv) was added dropwise to these solutions. The reaction mixtures were stirred for 0.5−1.5 h. Then the solvents were evaporated and the reaction residues were dissolved in chloroform and washed with water and dried with Na_2SO_4. The corresponding free bases of compounds **11h** and **11i** were first purified by FC using DCM/methanol 9/1 as the eluent system. Then the treatment with a HCl (g)/diethyl ether solution gave the final compounds as hydrochloride salts. Compound **11j** was obtained after Trt removal using a 50% TFA/DCM solution. All compounds were obtained as yellow solids.

2-(*N*′,*N*′-Diethylamino)-*N*-(4,9-dioxo-4,9-dihydronaphtho-[2,3-*b*]thiophen-3-yl)acetamide Hydrochloride (11h). Yield: 43%. Mp 209−210 °C. ^{1}H NMR (400 MHz, CD_3OD) δ: 1.37−1.40 (t, 6H, CH_3); 3.31−3.34 (q, 4H, CH_2); 4.38 (s, 2H, CH_2,); 7.85−7.87 (m, 2H, H-6 and H-7); 8.21−8.26 (m, 2H, H-5 and H-8); 8.52 (s, 1H, H-2). ^{13}C NMR (100 MHz, CD_3OD) δ 15.8 (CH_3), 47.9 (CH_2), 51.5 (αCH_2), 119.2 (C-2), 126.4 (C-6 and C-7), 129.5 (C-3), 132.9 (C-8a), 133.9 (C-4a), 135.1 (C-5 and C-8), 137.9 (C-3a), 144.9 (C-9a), 172.8, 179.9, and 181.9 (C═O). ESI-MS *m*/*z* calcd for $C_{18}H_{19}ClN_2O_3S$, 378.08; found, 377.98.

***N*-(4,9-Dioxo-4,9-dihydronaphtho[2,3-*b*]thiophen-3-yl)-2-(*N*′-morpholine)acetamide Hydrochloride (11i).** Yield: 43%. Mp 213-214 °C. ^{1}H NMR (400 MHz, CD_3OD) δ: 3.29−3.32 (t, 4H, CH_2N); 4.15−4.18 (t, 4H, CH_2O); 4.39 (s, 2H, CH_2,); 7.86−7.89 (m, 2H, H-6 and H-7); 8.22−8.26 (m, 2H, H-5 and H-8); 8.49 (s, 1H, H-2). ^{13}C NMR (100 MHz, CD_3OD) δ: 48.5 (CH_2N), 50.9 (αCH_2), 63.2 (CH_2O), 118.7 (C-2), 127.1 (C-6 and C-7), 129.7 (C-3), 132.9 (C-8a), 133.7 (C-4a), 135.5 (C-5 and C-8), 138.1 (C-3a), 143.6 (C-9a), 172.6, 178.7, and 181.6 (C═O). ESI-MS *m*/*z* calcd for $C_{18}H_{17}ClN_2O_4S$, 392.06; found, 392.15.

***N*-(4,9-Dioxo-4,9-dihydronaphtho[2,3-*b*]thiophen-3-yl)-2-mercaptoacetamide (11j).** FC: *n*-hexane/ethyl acetate 1/1. Yield: 43%. Mp 183−184 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 3.50 (s, 2H, CH_2); 7.79−7.81(m, 2H, H-6 and H-7); 8.25−8.27 (m, 2H, H-5 and H-8); 8.50 (s, 1H, H-2); 11.21 (s, 1H, N*H*). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 29.2 (CH_2), 119.3 (C-2), 127.5 (C-6 and C-7), 129.7 (C-3), 133.7 (C-8a), 134.1 (C-4a), 134.3 and 134.3 (C-5 and C-8), 137.9 (C-3a), 145.3 (C-9a), 170.5, 180.1, and 182.3 (C═O). ESI-MS *m*/*z* calcd for $C_{14}H_9NO_3S_2$, 303.00; found, 303.26.

2-(2-(Dimethylamino)ethylamino)-*N*-(4,9-dioxo-4,9-dihydronaphtho[2,3-*b*]thiophen-3-yl)acetamide Dihydrochloride Salt (11k). The 3-aminonaphtho[2,3-*b*]thiophene-4,9-dione (TNQ, 9,1 g, 4.4 mmol)[17] was dissolved in THF (30 mL). Chloroacetyl chloride (0.6 g, 5.3 mmol) and TEA (2.2 equiv) were added to the solution. The mixture was stirred at room temperature for 1 h, after which chloroform and water were added. The organic layer was washed with water and dried. Concentration of the chloroform phase gave the crude product 2-chloro-*N*-(4,9-dioxo-4,9-dihydronaphtho[2,3-*b*]thiophen-3-yl)acetamide as an oil (14, 1.2 g, 89%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.82 (s, 2H, CH_2,); 7.81−7.851 (m, 2H, H-6 and H-7); 8.26−8.29 (m, 2H, H-5 and H-8); 8.41 (s, 1H, H-2); 11.23 (s, 1H, N*H*). This was used directly in the next step without further purification. **14** (150 mg, 0.5 mmol) was dissolved in THF (10 mL), and the mixture was added to a solution of *N*,*N*-dimethylethylendiamine (mL, mmol) in THF (10 mL). After the mixture was stirred at reflux temperature for 6 h, the solvent was evaporated and the residue was purified by flash chromatography with DCM/MeOH 9/1. Then treatment with a HCl (g)/diethyl ether solution gave the title compound as a yellow solid (140 mg, 65%). Mp 212−213 °C. ^{1}H NMR (400 MHz, CD_3OD) δ: 2.76 (s, 6H, CH_3); 3.02−3.05 (m, 2H, CH_2); 3.19−3.21 (m, 2H, CH_2); 3.41 (sbr, 2H, CH_2), 7.84−7.86 (m, 2H, H-6 and H-7); 8.18−8.23 (m, 2H, H-5 and H-8); 8.38 (s, 1H, H-2). ^{13}C NMR (100 MHz, CD_3OD) δ: 42.0 (CH_2), 42.8 (CH_3), 45.7 (CH_2), 53.1 (CH_2), 118.8 (C-2), 126.9 (C-6 and C-7), 128.9 (C-3), 133.0 (C-8a), 133.9 (C-4a), 134.2 (C-5 and C-8), 137.5 (C-3a), 144.0 (C-9a), 171.9, 179.1, and 182.3 (C═O). ESI-MS *m*/*z* calcd for $C_{18}H_{21}C_{l2}N_3O_3S$, 429.07; found, 429.18.

3-(3′-Bromo)propanamide-3-ethoxycarbonyl-2,3-dihydrothieno[2,3-*b*]naphtho-4,9-dione and 3-Acrylamido-3-ethoxycarbonyl-2,3-dihydrothieno[2,3-*b*]naphtho-4,9-dione (13). DTNQ (3.3 mmol) was dissolved in THF. Bromopropanoyl chloride (4 mmol) and TEA (8.2 equiv) were added to the solution. The mixture was stirred at room temperature for 2 h, after which chloroform and water were added. The organic layer was washed with water and dried. Concentration of the chloroform phase gave the crude product, which was purified by flash chromatography with DCM/MeOH 9.5/0.5. The title compounds in 1:1 ratio were obtained as a yellow oil (89%). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 1.23−1.25 (m, 6H, CH_3); 2.71 (d, 1H, *J*= 6.5 Hz, CH_2); 2.82−2.85 (m, 1H, CH_2); 3.69−3.63 (m, 6H, CH_2Br and H-2); 4.21−4.30 (m, 4H, CH_2); 5.91 (d, 1H, *J* = 16.0 Hz, CH═CH_2); 5.68 (d, 1H, *J* = 9.9 Hz, CH═CH_2); 6.23 (dd, 1H, *J* = 16.2 and 10.0 Hz); 7.40 (s, 2H, N*H*); 7.67−7.71 (m, 4H, H-6 and H-7); 7.98 (d, 2H, *J* = 6.8 Hz, H-5); 8.07 (d, 2H, *J* = 7.6 Hz, H-8).

***N*-(4,9-Dioxo-4,9-dihydronaphtho[2,3-*b*]thiophen-3-yl)-3-hydroxypropionamide (11l).** DBU (0.6 mmol) was added dropwise to a solution of 13(0.1 mmol) in methanol−water (9:1, 10 mL). After 30 min at room temperature the solvents were evaporated

and the reaction residues were dissolved in chloroform and washed with water and dried with Na_2SO_4. The title compound was purified by flash chromatography (FC) using ethyl acetate as eluent. Yellow solid (56%). Mp 152−153 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 2.74−2.77 (m, 2H, CH_2); 3.77−3.79 (m, 2H, CH_2); 7.76−7.79 (m, 2H, H-6 and H-7); 8.23−8.24 (m, 2H, H-5 and H-8); 8.52 (s, 1H, H-2); 10.75 (s, 1H, NH). ^{13}C NMR (100 MHz, CD_3OD) δ: 38.1 (α-CH_2), 68.3 (CH_2OH), 119.4 (C-2), 127.4 (C-6 and C-7), 128.9 (C-3), 132.7 (C-8a), 134.1 (C-4a), 134.2 and 134.3 (C-5 and C-8), 138.5 (C-3a), 143.2 (C-9a), 171.2, 179.1, and 181.3 (C═O). ESI-MS m/z calcd for $C_{15}H_{11}NO_4S$, 301.04; found, 301.22.

General Procedure for the Synthesis of *N*-(4,9-Dioxo-4,9-dihydronaphtho[2,3-*b*]thiophen-3-yl)-3-(substituted) propanamide (11m−p). To a solution of **13** (0.1−0.3 mmol) in THF (20 mL) were added *N*,*N*-diethylamine or triphenylmethanethiol or morpholine or *N*,*N*-dimethylethylendiamine (1.1 equiv) and DIPEA (2 equiv). After the mixture was stirred at reflux temperature for 12−24 h, the solvent was evaporated. Then the residues (**13′m−p**) were dissolved in methanol−water (9:1, 20 mL) and DBU (5 equiv) was added dropwise to these solutions. The reaction mixtures were stirred for 0.5−1 h. Then the solvents were evaporated and the reaction residues were dissolved in chloroform and washed with water and dried with Na_2SO_4. The corresponding free bases of compounds **11m**, **11n**, and **11p** were first purified by FC using DCM/methanol 9/1 as the eluent system. Then treatment with a HCl (g)/diethyl ether solution gave the final compounds as hydrochloride salts and yellow solids. Compound protected **11o** was purified by FC using *n*-hexane/ethyl acetate 3/2 as eluent. Then the final compound was obtained after Trt removal with a 50% TFA/DCM solution.

3-(Diethylamino)-*N*-(4,9-dioxo-4,9-dihydronaphtho[2,3-*b*]thiophen-3-yl)propanamide Hydrochloride (11m). Yield: 43%. Mp 201−202 °C. ^{1}H NMR (400 MHz, CD_3OD) δ: 1.36−1.40 (t, 6H, CH_3); 3.10−3.13 (m, 2H, CH_2); 3.30−3.34 (q, 4H, CH_2); 3.56−3.59 (m, 2H, CH_2); 7.85−7.87 (m, 2H, H-6 and H-7); 8.20−8.24 (m, 2H, H-5 and H-8); 8.48 (s, 1H, H-2). ^{13}C NMR (100 MHz, CD_3OD) δ: 15.8 (CH_3), 35.8 (αCH_2), 47.9 (CH_2CH_3), 51.5 (βCH_2), 118.6 (C-2), 126.9 (C-6 and C-7), 129.2 (C-3), 132.7 (C-8a), 133.1 (C-4a), 134.8 (C-5 and C-8), 138.9 (C-3a), 145.0 (C-9a), 172.5, 178.9, and 182.6 (C═O). ESI-MS m/z calcd for $C_{19}H_{21}ClN_2O_3S$, 392.10; found, 390.17.

***N*-(4,9-Dioxo-4,9-dihydronaphtho[2,3-*b*]thiophen-3-yl)-3-morpholinopropanamide Hydrochloride (11n).** Yield: 48%. Mp 207−208 °C. ^{1}H NMR (400 MHz, CD_3OD) δ: 3.11−3.14 (t, 2H, αCH_2); 3.32−3.37 (m, 4H, CH_2N); 3.48−3.50 (m, 2H, βCH_2); 3.98−4.01 (m, 4H, CH_2O); 7.86−7.88 (m, 2H, H-6 and H-7); 8.19−8.24 (m, 2H, H-5 and H-8); 8.50 (s, 1H, H-2). ^{13}C NMR (100 MHz, CD_3OD) δ: 37.3 (α-CH_2), 47.5 (CH_2N), 50.9 (βCH_2), 67.2 (CH_2O), 119.1 (C-2), 126.8 (C-6 and C-7), 130.1 (C-3), 132.6 (C-8a), 133.9 (C-4a), 135.1 (C-5 and C-8), 138.7 (C-3a), 145.5 (C-9a), 172.3, 178.5, and 181.9 (C═O). ESI-MS m/z calcd for $C_{19}H_{19}ClN_2O_4S$, 406.08; found 406.17.

***N*-(4,9-Dioxo-4,9-dihydronaphtho[2,3-*b*]thiophen-3-yl)-3-mercaptopropanamide (11o).** Yield: 42%. Mp 181−182 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 2.82−2.85 (m, 2H, CH_2); 2.91−2.95 (m, 2H, CH_2); 7.79−7.81 (m, 2H, H-6 and H-7); 8.21−8.26 (m, 2H, H-5 and H-8); 8.48 (s, 1H, H-2); 10.33 (s, 1H, NH). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 23.9 (CH_2SH), 39.5 (α-CH_2), 119.9 (C-2), 127.6 (C-6 and C-7), 128.0 (C-3), 132.9 (C-8a), 134.0 (C-4a), 134.4 (C-5 and C-8), 138.9 (C-3a), 144.8 (C-9a), 172.1, 179.8, and 181.8 (C═O). ESI-MS m/z calcd for $C_{15}H_{11}NO_3S_2$, 317.02; found, 317.18.

3-(2-(Dimethylamino)ethylamino)-*N*-(4,9-dioxo-4,9-dihydronaphtho[2,3-*b*]thiophen-3-yl)propanamide Dihydrochloride (11p). Yield: 45%. Mp 227−228 °C. ^{1}H NMR (400 MHz, CD_3OD) δ: 2.98 (s, 6H, CH_3); 3.09−3.12 (t, 2H, αCH_2); 3.29−3.31 (m, 2H, $CH_2N(Me)_2$); 3.47−3.50(m, 2H, βCH_2); 3.50−3.53 (m, 2H, $NHCH_2$); 7.85−7.87 (m, 2H, H-6 and H-7); 8.20−8.25 (m, 2H, H-5 and H-8); 8.49 (s, 1H, H-2). ^{13}C NMR (100 MHz, CD_3OD) δ: 32.1 (αCH_2), 42.4 ($NHCH_2$), 42.8 (CH_3), 43.9 (βCH_2), 53.1 ($CH_2N(Me)_2$), 119.5 (C-2), 126.8 and 127.0 (C-6 and C-7), 129.0 (C-3), 132.9 (C-8a), 133.5 (C-4a), 134.2 and 134.3 (C-5 and C-8), 137.5 (C-3a), 144.0 (C-9a), 171.9, 178.9, and 182.5 (C═O). ESI-MS m/z calcd for $C_{19}H_{23}Cl_2N_3O_3S$, 443.08; found, 443.18.

General Procedure for the Synthesis of 1-(4,9-Dioxo-4,9-dihydronaphtho[2,3-*b*]thiophen-3-yl)-3-(substituted)urea (11q,r). Bis(trichloromethyl)carbonate (118 mg, 0.4 mmol) was added to a solution of TNQ (9, 230 mg, 1 mmol) in dry THF (25 mL) at room temperature. Then TEA (0.3 mL, 2 mmol) was added dropwise for 5 min, with stirring continuing for an additional 10 min. Afterward, a solution of corresponding amines, propylamine, or *N*,*N*-dimethylaminoethylamine (1.1 equiv) 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. Flash chromatography of the residues using *n*-hexane/ethyl acetate 4/1 or DCM/MeOH 9/1 as eluents yielded, in each case, the correspondent urea derivatives as yellow solids.

The final compound **11r** was obtained as the hydrochloride salt by treatment of the corresponding free bases with a HCl (g)/diethyl ether solution.

1-(4,9-Dioxo-4,9-dihydronaphtho[2,3-*b*]thiophen-3-yl)-3-propylurea (11q). Yield: 75%. Mp 198−199 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 0.98−1.02 (m, 3H, CH_3); 1.60−1.64 (m, 2H, CH_2); 3.27−3.32 (m, 2H, CH_2); 4.83 (s, 1H, NH); 7.76−7.78 (m, 2H, H-6 and H-7); 8.19−8.21 (m, 2H, H-5 and H-8); 8.23 (s, 1H, H-2); 9.51 (s, 1H, NH). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 11.5 (CH_3), 23.4 (CH_2), 42.7 ($NHCH_2$), 116.2 (C-2), 127.2 and 127.4 (C-6 and C-7), 124.9 (C-3), 131.6 (C-8a), 133.6 (C-4a), 134.0 and 134.3 (C-5 and C-8), 136.4 (C-3a), 142.9 (C-9a), 154.9, 177.6, and 182.1 (C═O). ESI-MS m/z calcd for $C_{16}H_{14}N_2O_3S$, 314.07; found, 314.22.

1-(2-(Dimethylamino)ethyl)-3-(4,9-dioxo-4,9-dihydronaphtho[2,3-*b*]thiophen-3-yl)urea Hydrochloride (11r). Yield: 69%. Mp 216−217 °C. ^{1}H NMR (400 MHz, CD_3OD) δ: 3.23−3.25 (m, 2H, CH_2); 3.30 (s, 6H, CH_3); 3.59−3.61 (m, 2H, CH_2); 7.85−7.87 (m, 2H, H-6 and H-7); 8.21 (s, 1H, H-2); 8.22−8.24 (m, 2H, H-5 and H-8). ^{13}C NMR (100 MHz, CD_3OD) δ: 35.4 ($NHCH_2$), 42.9 [$N(CH_3)_2$], 58.3 [$CH_2N(CH_3)_2$], 116.0 (C-2), 126.7 and 127.9 (C-6 and C-7), 125.3 (C-3), 132.6 (C-8a), 134.7 (C-4a), 134.1 (C-5 and C-8), 136.1 (C-3a), 143.5 (C-9a), 154.6, 178.2, and 181.1 (C═O). ESI-MS m/z calcd for $C_{17}H_{18}ClN_3O_3S$, 379.08; found, 378.97.

Biology. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), trypsin−EDTA solution (1×), penicillin and streptomycin, and phosphate-buffered saline (PBS) were from Cambrex Biosciences. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), propidium iodide (PI), Triton X-100, sodium citrate, formamide, and mouse monoclonal anti-tubulin were purchased from Sigma (Milan, Italy). Rabbit polyclonal anti-cyclin A primary antibody were from Cell Signaling Technology (Celbio; Milan, Italy). ECL reagent was obtained from Amersham Pharmacia Biotech, U.K.

Cell Culture. Human breast MDA231, human colon carcinoma SW620, Colo205, HT-29, and Caco-2, human monocytic leukemia U937, human melanoma MDA-MB435 and SK-MEL28, human ovarian cancer IGROV, and human glioblastoma SF-295 and SNB-19 cell lines were grown at 37 °C in Dulbecco's modified Eagle's medium containing 10 mM glucose (DMEM-HG) supplemented with 10% fetal calf serum and 100 units/mL each of penicillin and streptomycin and 2 mmol/L glutamine. In each experiment, cells were placed in fresh medium, cultured in the presence of synthesized compounds (from 0.1 to 25 mM) and followed for further analyses.

Cell Viability Assay. Cell viability for all cell lines was determined using the 3-[4,5-demethylthiazol-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) colorimetric assay. The test is based on the ability of mitochondrial dehydrogenase to convert, in viable cells, the yellow MTT reagent (Sigma Chemical Co., St. Louis, MO) into a soluble blue formazan dye. Cells were seeded into 96-well plates to a density of 10^5 cells/100 μL well. After 24 h of growth to allow attachment to the wells, compounds were added at various concentrations (from 0.1 to 25 mM). After 24 or 48 h of growth and after removal of the culture medium, 100 μL/well medium containing 1 mg/mL MTT was added. Cell cultures were further incubated at 37 °C for 2 h in the dark. The solution was then gently aspirated from each well, and the formazan crystals within the cells were dissolved with 100 μL of DMSO. Optical densities were read at 550 nm using a Multiskan Spectrum Thermo Electron Corporation reader. Results were expressed as percentage relative to vehicle-treated control (0.5% DMSO was added to untreated cells). IC_{50} (concentration eliciting 50% inhibition) values were determined by linear and polynomial regression. Experiments were performed in triplicate.

Topo II Mediated Supercoiled pBR322 Relaxation. DNA relaxation assays were based on the procedure of Osheroffet al.[26b] Reaction buffer contained 10 mM Tris-HCl (pH 7.9), 50 mM KCl, 50 mM NaCl, 5 mM $MgCl_2$, 0.1 mM EDTA, and 15 μg/mL bovine serum albumin (BSA), 0.15 μg of supercoiled pBR322, 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 (EB) solution and photographed through a gel document system GDS8000 (UVP). The amount of DNA bands was quantified by Gel 1D intermediate software.

Confocal Microscopy. For immunocytochemistry, cells were fixed in 0.04 g/L paraformaldehyde for 30 min at 4 °C and permeabilized with 0.01 g/L Triton X-100 for 30 min at 4 C. Cells were then washed and stained with Hoechst 33342 (Vector, Burlingame, CA). Images were acquired with a LSM510 inverted confocal microscope (Zeiss, Oberkochen, Germany) using a 63× oil objective and processed using LSM software (Zeiss).

Flow Cytometry: Analysis of Cell Cycle. CaCo-2 cells were seeded in six multiwell plates at a density of 25 × 10^5 cells/plate. After 48 h of incubation with **11m** and **11p** derivatives and doxorubicin in DMEM without serum at 37 °C, cells were washed in PBS, pelleted, centrifuged, and directly stained in a propidium iodide (PI) solution (50 mg of PI in 0.1% sodium citrate, 0.1% NP40, pH 7.4) for 30 min at 4 °C in the dark. Flow cytometric analysis was performed using a FACScan flow cytometer (Becton Dickinson, San Jose, CA). To evaluate the cell cycle, PI fluorescence was collected as FL2 (linear scale) by the ModFIT software (Becton Dickinson). For the evaluation of intracellular DNA content, at least 20 000 events for each point were analyzed in at least three different experiments giving a SD of less than 5%.

Western Blot Assay. The effects of **11m**, **11p**, and **11c** on expression of cyclin A and of **11m** and **11p** on Hsp27, were determined by Western blots. Compound-stimulated and unstimulated (control) cell lysates were prepared using an ice cold lysis buffer (50 mM Tris, 150 mM NaCl, 10 mM EDTA, 1% Triton) supplemented with a mixture of protease inhibitors containing antipain, bestatin, chymostatin, leupeptin, pepstatin, phosphoramidon, Pefabloc, EDTA, and aprotinin (Boehringer, Mannheim, Germany). Equivalent protein samples were resolved on 8−12% sodium dodecyl sulfate (SDS)−polyacrylamide gels and transferred to nitrocellulose membranes (Bio-Rad, Germany). For immunodetection, membranes were incubated overnight with specific antibody at the concentrations indicated in the manufacter's protocol (Santa Cruz Biotechnology). The two antibodies were diluted in Tris-buffered saline/Tween 20/1% milk powder. This step was followed by incubation with the corresponding horseradish peroxidase conjugated antibody (anti-rabbit-IgG 1:6000; Biosource, Germany). Bands were read by enhanced chemiluminescence (ECL kit, Amersham, Germany).

Alkaline Phosphatase Activity. Alkaline phosphatase (ALP) activity was used as marker of the degree of cell differentiation. Attached and floating cells were washed and lysed with 0.25% sodium deoxycholate, essentially as described by Herz et al.[40] ALP activity was determined using Sigma Diagnostics ALP reagent (no. 245). Total cellular protein content of the samples was determined in a microassay procedure as described by Bradford[41] using the coomassie protein assay reagent kit (Pierce). ALP activity was calculated as units of activity per milligram of protein.

H9C2 Cell Viability. Cardiomyoblasts H9C2 were cultured in Dulbecco's minimal essential medium (DMEM, GIBCO) supplemented with 0.1 g/L fetal bovine serum (FBS, GIBCO), 200 mg/mL L-glutamine, 100 units/mL penicillin, and 10 mg/mL streptomycin (Sigma-Aldrich) at 37 °C in 0.95 g/L air−0.05 g/L CO_2. The H9C2 samples were studied between passages 4 and 10. The MTT colorimetric assay (Invitrogen, San Diego, CA) was used to evaluate cell proliferation in the presence or absence of inhibitors. Briefly, H9C2 cells were plated into 96 multiwells at a density of 2000 cells/well in quadruplicate. Inhibitors (**11m**, **11p**, and doxorubicin) were added to each well at 1 μM for the indicated time points. Then an amount of 10 μL of MTT reagent was added to each well. The plate was returned to the cell culture incubator for 2 h. The absorbance in each well, including the blanks, was measured at 570 nm in a microplate plate reader.

Statistical Analysis. Data were expressed as the mean ± standard deviation (SD). Statistical significance was assessed by the Student *t* test. *P* adjustment for multiple comparisons was done by the Holm method (sequential Bonferonni correction method). $P < 0.05$ was considered statistically significant.

STD NMR and WaterLOGSY Spectroscopy. STD NMR[29] and WaterLOGSY[30] experiments were performed on a Varian Inova 700 MHz spectrometer at 25 °C. NMR samples were prepared by dissolving the ligand and the poly(dGdC)·poly(dG-dC) copolymer (Pharmacia Biochemicals) in H_2O/D_2O 9:1 (final volume of 600 μL; D_2O 99.996%, CIL Laboratories) containing phosphate buffered saline (100 mM) at pH 7.1. A high ligand−receptor molar excess (20:1) was used. In particular, the concentrations of **11c**, **11m**, and **11p** were 1.0 mM, whereas that of the DNA was 50 μM, expressed as molarity of phosphate groups. Water suppression was achieved by using the double-pulsed field gradient spin−echo (DPFGSE) scheme.[42] The STD effects of the individual protons were calculated for each compound relative to a reference spectrum with off-resonance saturation at $\delta = -16$ ppm. Typically, 512 scans were recorded for each DF-STD spectrum (saturation time of 2 s). The relative STD effect was calculated for each signal as the difference between the intensity (expressed as S/N ratio) of one signal in the on-resonance STD spectrum and that of the same signal in the off-resonance NMR spectrum divided by the intensity of the same signal in the off-resonance spectrum. BMI values were obtained as the ratio of the relative STD effects upon irradiation at 10.0 and −1.0 ppm.[31] The absence of STD effects in samples in which the DNA was not added ensured a selective macromolecule saturation. WaterLOGSY NMR experiments employed a 20 ms selective Gaussian 180° pulse at the water signal frequency and an NOE mixing time of 1.5 s.

■ ASSOCIATED CONTENT

Supporting Information. Microanalysis data for all compounds; data on the expression of cyclin A in control and induced by **11p**, **11m**, and **11c**; and data of effects of doxorubicin on the topo II mediated DNA cleavage. This material is available free of charge via the Internet at http://pubs.acs.org.

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ACKNOWLEDGMENT

The ESI-MS and NMR spectra data were provided by Centro di Servizio Interdipartimentale di Analisi Strumentale (CSIAS), Università degli Studi di Napoli "Federico II", Italy. The assistance of the staff is gratefully appreciated.

ABBREVIATIONS USED

Boc, *tert*-butyloxycarbonyl; HBTU, 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HOBt, *N*-hydroxybenzotriazole; DIPEA, *N*,*N*-diisopropylethylamine; DMF, *N*,*N*dimethylformamide; THF, tetrahydrofuran; DCM, dichloromethane; TES, triethylsilane; DBU, 1,8-diazabicyclo(5.4.9)-undec-7-ene; TLC, thin-layer chromatography; ES-MS, electrospray mass spectrometry; SD, standard deviation; STD, saturation transfer difference; DF, differential frequency; NOE, nuclear Overhauser effect; WaterLOGSY, water−ligand observed via gradient spectroscopy

ADDITIONAL NOTE

Abbreviations used for amino acids follow the rules of the IUPAC-IUB Commission of Biochemical Nomenclature in *J. Biol. Chem.* **1972**, *247*, 977−983. Amino acid symbols denote L-configuration.

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