Novel Cyano- and *N*-Isopropylamidino-Substituted Derivatives of Benzo[*b*]thiophene-2-carboxanilides and Benzo[*b*]thieno[2,3-*c*]quinolones: Synthesis, Photochemical Synthesis, Crystal Structure Determination, and Antitumor Evaluation. 2

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Received June 11, 2004

Derivatives of 3-chlorobenzo[*b*]thiophene-2-carboxanilides and their "cyclic" analogues benzo[*b*]thieno[2,3-*c*]quinolones were synthesized. Spectroscopic study of the interactions of some representatives of "cyclic" derivatives and their "acyclic" precursors with ds-DNA/RNA supported strong intercalative binding of the former and weak nonintercalative binding of the latter group of compounds. All tested compounds showed a certain antiproliferative effect on a series of human tumor cells and on a normal cell line. Among the compounds, those with one amidino-substituent have shown the best effect. The most active benzo[*b*]thieno[2,3-*c*]quinolones induced apparent S and G2/M arrests of the cell cycle, which resulted in apoptosis. These results strongly suggest that the compounds may act as topoisimerase "poisons", which is in good agreement with their intercalative mode of binding to ds-DNA/RNA, in contrast to the studied "acyclic" group of derivatives. **6a** and **6d** showed the best selectivity by inhibiting the growth of tumor cells but not of normal fibroblasts.

Introduction

There are not many recent literature data about the synthesis and antitumor evaluation of heterocyclic condensed quinolones. Novel quinoline−quinone, e.g. streptonigrin (SN), is an antitumor antibiotic that has activity against a broad range of tumors.[1−3] SN was studied clinically as an antitumor agent, but its use was limited by reports of delayed myelotoxicity.[4,5] Nevertheless, positive results were reported for SN both as a single agent[6,7] and in combination chemotherapy.[8,9] SN is an excellent substrate for oxidoreductase (NQ01).[10]

Pyranoquinoline-2-ones were synthesized and evaluated for their in vitro cytotoxicity against a panel of human tumor cell lines,[11] while 2-arylquinazolinones displayed significant growth inhibitory action against tumor cell lines, some of them being potent inhibitors of tubulin polymerization. Some displayed selective activity against P-gp-expressing epidermoid carcinoma of the nasopharynx.[12] Besides, trifluoromethyl-substituted pyranoquinolinone was prepared and tested for the ability to modulate the transcriptional activity of the human androgen receptor (HAR).[13]

Synthesis and structure−activity relationships of novel 7-substituted 1,4-dihydro-4-oxo-1-(2-thiazolyl)-1,8-naphthyridine-3-carboxylic acids as antitumor agents have been described recently.[14]

The authors investigated the structure−activity relationships in this series of compounds by changing N-1 and C-7 positions and the core ring structure itself and evaluated the synthesized compounds against several murine and human tumor cell lines. These modifications led to the following findings. The 2-thiazolyl group at the N-1 position of the naphthyridine structure is the best substituent for antitumor activity. Regarding the core ring structure, the naphthyridine derivative is the most active, followed by the pyridopyrimidine analogue. At the C-7 position, aminopyrrolidine derivatives are more effective than other amines or thioether derivatives. Finally, the *trans*-3-amino-4-methoxypyrrolidinyl derivative and the 3-amino-3-methylpyrrolidinyl derivative as well as 3-aminopyrrolidinyl derivative were found to be effective in in vitro and in vivo antitumor assays, and their activity was comparable to that of etoposide.

Indolo-, pyrrolo-, and benzofuroquinolones and anilinoindoloquinolone derivatives were synthesized and evaluated in vitro against a three-cell line panel consisting of MCF7, NCI-H460, and SF268. The results have shown that cytotoxicity decreases in the order: anilinoindoloquinolones > indoloquinolones > pyrroloquinolones > benzofuroquinolones.[15] New synthesized hydroxymethyl- and methoxymethylfuro[2,3-*h*]quinolin-2(1*H*)-ones inhibited topoisomerase II, leading to a moderate antiproliferative activity in mammalian cells. The antiproliferative activity was also tested upon UVA

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10.1021/jm049541f CCC: $30.25
Published on Web 02/17/2005

irradiation in mammalian cells: all compounds showed higher activity than 8-MOP, without mutagenicity and skin phototoxicity.[16]

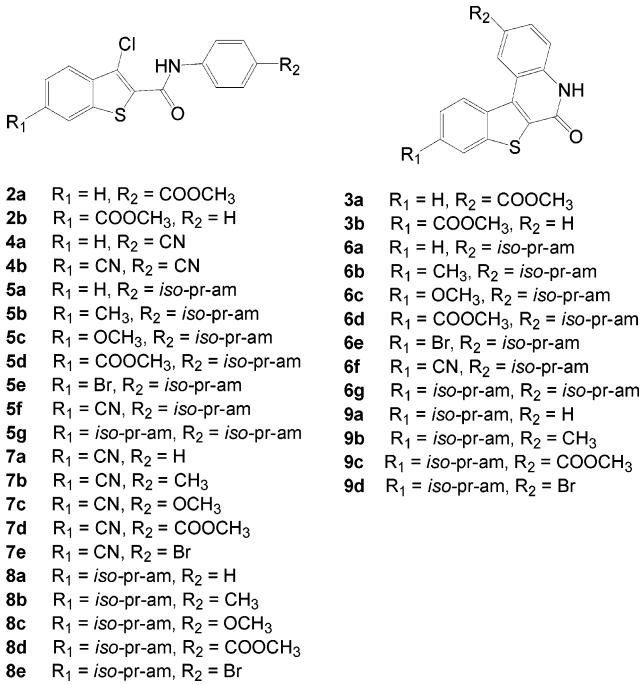
Recent data describe the quinolone−metal complexes as antraquinone intercalators investigated on leukaemia L1210 cells**.** A new series of quinolone−platinum(II) conjugates, [Pt(Q′-NH_2)(dmso)X_2] and cis-[Pt(Q″-en)X_2], where Q′ and Q″ are quinolones (flumequine, nalidixic acid, or oxolinic acid) linked to monodentate and bidentate amine ligands, respectively, and X_2 is Cl_2 or 1,1-cyclobutanedicarboxylate, have been synthesized with the aim of examining the synergetic antitumor activity of quinolone intercalation and platinum(II) chelation.[17]

Searching for compounds related to heterocyclic quinolones as antitumor active compounds, we have prepared new derivatives of thieno[3′,2′:4,5]thieno[2,3-*c*]quinolones and examined their antitumor activity.[18] Later on, a class of substituted benzo[*b*]thieno[2,3-*c*]-quinolones containing a 3-dimethylaminopropyl substituent on the quinolone nitrogen or a 3-dimethylaminopropyl substituent in the amide part of the molecule was prepared. All prepared compounds were tested on several human tumor cell lines and exhibited strong inhibitory activities in vitro against all the cell lines tested. The compounds that bear a dimethylaminopropyl substituent on the quinolone nitrogen showed higher antitumor activity than compounds bearing the same substituent on the amidic nitrogen.[19]

In this work, we have prepared a new group of substituted derivatives of benzo[*b*]thieno-2-carboxanilides (**4a**, **4b**, **5a**−**g**, **7a**−**e**, and **8a**−**e**) and benzo[*b*]-thieno[2,3-*c*]quinolones bearing a cyano or a substituted amidino group in different positions (**6a**−**g** and **9a**−**d**) of the condensed ring and evaluated their antitumor activity.

Chemistry

All compounds (**2a**−**9d**) shown in Figure 1 were prepared according to Scheme 1, starting from 3-chlorobenzo[*b*]thiophene-2-carbonyl chloride **1a** or 6-substituted 3-chlorobenzo[*b*]thiophene-2-carbonyl chlorides **1b**−**f**, which were already known from the literature.[20] 2- and 9-methoxycarbonyl-substituted benzo[*b*]thieno-[2,3-*c*]quinolones **3a** and **3b** were prepared from the corresponding carboxanilides **2a** and **2b**. Carboxanilides **2a** and **2b** served as reference compounds in the biological examination, while quinolones **3a** and **3b** could not be examined for their antitumor activity because of insolubility. Novel derivatives of cyano-substituted 3-chlorobenzo[*b*]thiophene-2-carboxanilides **4a**, **4b**, and **7a**−**e** were prepared in the reaction of the corresponding carbonyl chlorides with p-cyanoaniline or with 6-cyano-substituted 3-chlorobenzo[*b*]thiophene-2-carbonyl chloride **1f** with the corresponding *p*-substituted anilines. Novel *N*-isopropylamidino-substituted 3-chlorobenzo[*b*]thiophene-2-carboxanilides **5a**, **5f**, and **8a**−**e** were prepared in the Pinner[21] reaction from cyano derivatives (**4a**, **4b**, and **7a**−**e**) while carboxanilides **5b**−**e** were prepared by direct condensation of 3-chlorobenzo[*b*]thiophene-2-carbonyl chlorides **1b**−**e** with 4-(*N*-isopropylamidino)aniline, as well as diamidino-substituted carboxanilide **5g**. All prepared 6-substituted 3-chlorobenzo[*b*]thiophene-2-carboxanilides were obtained in 15−81% yields. All substituted benzo[*b*]thieno-[2,3-*c*]quinolones **3a**, **3b**, **6a**−**g**, and **9a**−**d** were prepared by the photochemical dehydrohalogenation reaction in very good yields.



2a R_1 = H, R_2 = $COOCH_3$
2b R_1 = $COOCH_3$, R_2 = H
4a R_1 = H, R_2 = CN
4b R_1 = CN, R_2 = CN
5a R_1 = H, R_2 = *iso*-pr-am
5b R_1 = CH_3, R_2 = *iso*-pr-am
5c R_1 = OCH_3, R_2 = *iso*-pr-am
5d R_1 = $COOCH_3$, R_2 = *iso*-pr-am
5e R_1 = Br, R_2 = *iso*-pr-am
5f R_1 = CN, R_2 = *iso*-pr-am
5g R_1 = *iso*-pr-am, R_2 = *iso*-pr-am
7a R_1 = CN, R_2 = H
7b R_1 = CN, R_2 = CH_3
7c R_1 = CN, R_2 = OCH_3
7d R_1 = CN, R_2 = $COOCH_3$
7e R_1 = CN, R_2 = Br
8a R_1 = *iso*-pr-am, R_2 = H
8b R_1 = *iso*-pr-am, R_2 = CH_3
8c R_1 = *iso*-pr-am, R_2 = OCH_3
8d R_1 = *iso*-pr-am, R_2 = $COOCH_3$
8e R_1 = *iso*-pr-am, R_2 = Br

3a R_1 = H, R_2 = $COOCH_3$
3b R_1 = $COOCH_3$, R_2 = H
6a R_1 = H, R_2 = *iso*-pr-am
6b R_1 = CH_3, R_2 = *iso*-pr-am
6c R_1 = OCH_3, R_2 = *iso*-pr-am
6d R_1 = $COOCH_3$, R_2 = *iso*-pr-am
6e R_1 = Br, R_2 = *iso*-pr-am
6f R_1 = CN, R_2 = *iso*-pr-am
6g R_1 = *iso*-pr-am, R_2 = *iso*-pr-am
9a R_1 = *iso*-pr-am, R_2 = H
9b R_1 = *iso*-pr-am, R_2 = CH_3
9c R_1 = *iso*-pr-am, R_2 = $COOCH_3$
9d R_1 = *iso*-pr-am, R_2 = Br

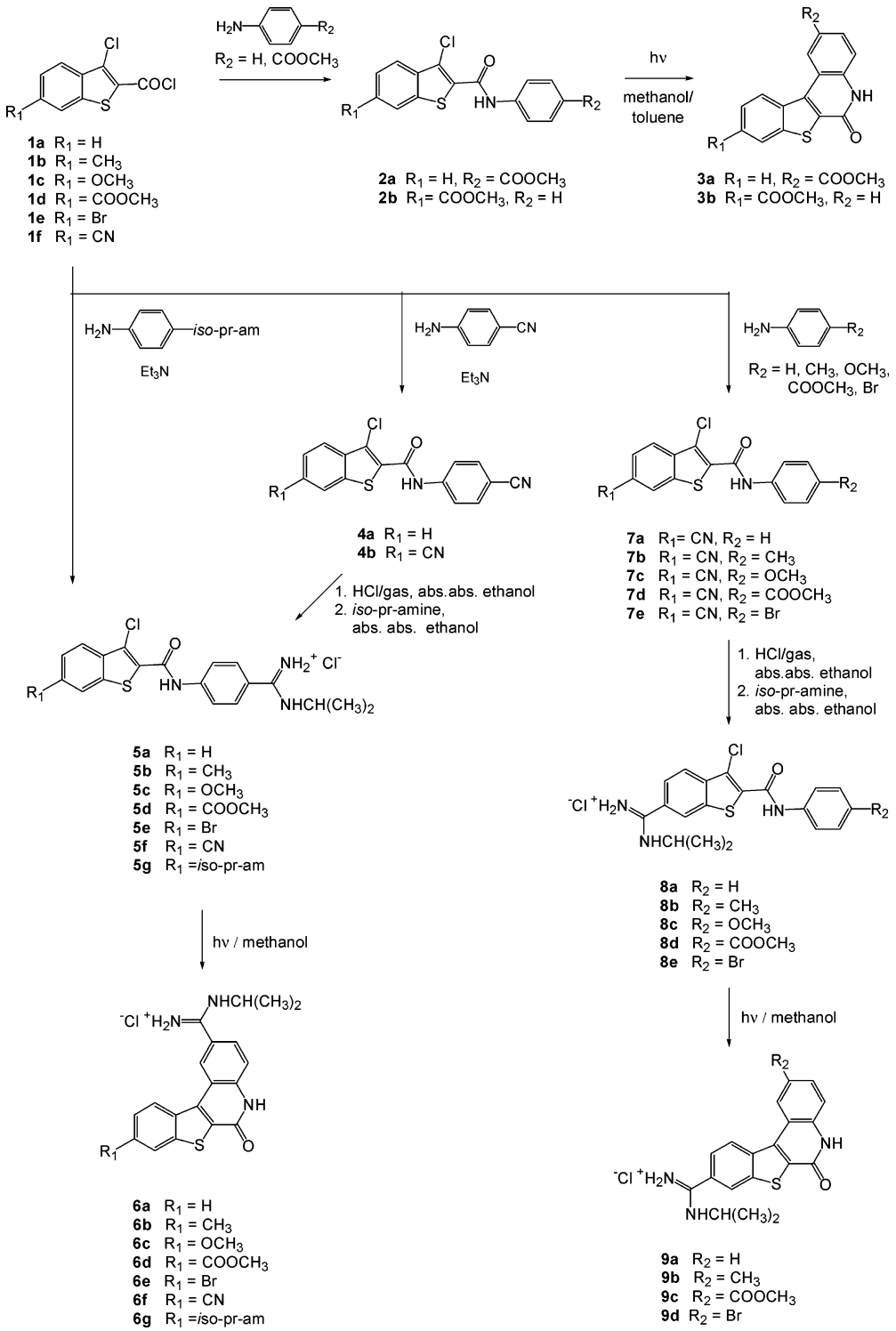
Figure 1. Substituted 3-chlorobenzo[*b*]thiophene-2-carboxamides and benzo[*b*]thieno[2,3-*c*]qinolones (**2a**, **2b**, **4a**, **4b**, **5a**−**g**, **7a**−**e**, **8a**−**e**, and **3a**, **3b**, **6a**−**g**, **9a**−**d**).

Crystal Structure of 9a. The molecular and crystal structures of compound **9a** were determined by single-crystal X-ray diffractometry. The ORTEP view (Figure 2; Cl^- ions have been omitted for clarity) of the molecular structure clearly shows that the asymmetric unit contains two crystallographically independent molecules, which differ only in the conformation of the isopropyl fragment. The π-electron delocalization through the molecules includes planar benzo[*b*]thieno[2,3-*c*]-quinolone moieties, while the *N*-isopropylamidino fragment does not lie in the plane of the remaining molecules. Protonation of the amidine group is confirmed by the equal bond distance values of C11−N13 and C11−N12 bonds (of the first molecule) and C21−N23 and C21−N22 bonds (of the second molecule).

All hydrogen atoms, belonging either to the amidino nitrogen atoms or to the nitrogen atoms of the quinolonic part, participate in hydrogen-bond formations of the N−H···Cl^- or N−H···O type. The geometry of the N−H···Cl^- hydrogen bonds is characterized by the N···Cl^- distances being in the range 3.183(7)−3.264(7) Å and the angles in the range 154(7)°−172(5)°. Nitrogen atoms N12 and N22 form intermolecular hydrogen bonds with O1 and O2 atoms (N22−H7···O2 and N12−H8···O1 distances are 2.814(8) and 2.852(8) Å, respectively).

Molecular Structure of 9a and Its Correlation with Binding to DNA/RNA. Most aromatic compounds built of three or more condensed aromatic units base their biological activity on intercalation.[22] The determined crystal structure of compound **9a** shows that the benzo[*b*]thieno[2,3-*c*]quinolone moiety is planar and aromatic, while the *N*-isopropylamidino substituent is in extended conformation and lies only slightly out of

Scheme 1



the plane (Figure 3a). Although the aromatic surface of **9a** is more than large enough for intercalation,[22] it should be stressed that the longer axis of **9a** (13.6 Å) significantly surpasses longer axes of some classical intercalators (ethidium bromide 9.8 Å, proflavine 9.5 Å). The question therefore arises whether insertion of **9a** and its analogues between DNA/RNA basepairs is possible.

To study if the sterical properties of **9a** allow intercalation into the double stranded helix, we have chosen the intercalative ds-5′-d(CpGpCpG)-3′/Flexi-Di complex, whose crystal structure was previously determined.[23] Flexi-Di (an analogue of ditercalinium)[24] consists of two linked 7*H*-pyridocarbazole units (Figure 3b), which are actually close structural analogues of the here studied benzo[*b*]thieno[2,3-*c*]quinolones (e.g. **9a**, Figure 3a). Flexi-Di was converted into two separate molecules of **9a** by deleting the aliphatic linker and modifying the structure of 7*H*-pyridocarbazole into **9a** by introducing parameters of atoms taken from the crystal structure of the latter compound.

Structures represented in Figure 4 indicate that the curved shape of **9a** allowed its intercalation into ds-DNA without steric clashes with the polynucleotide backbone.

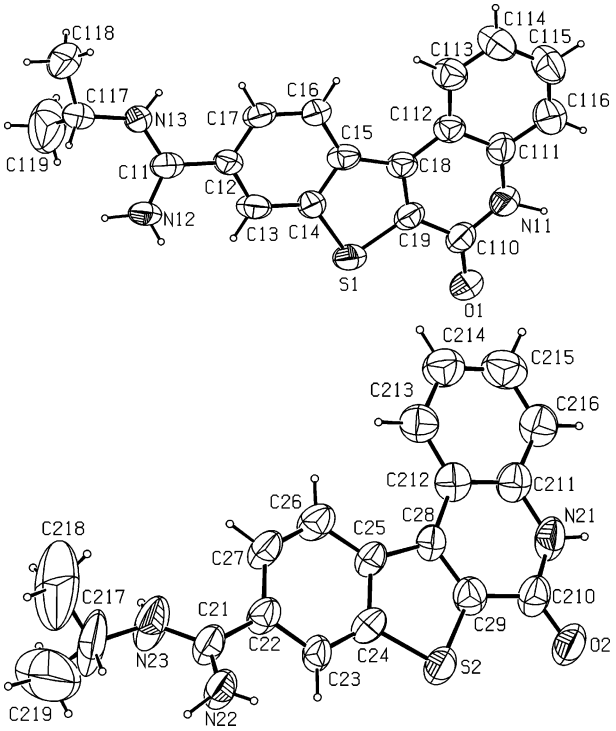
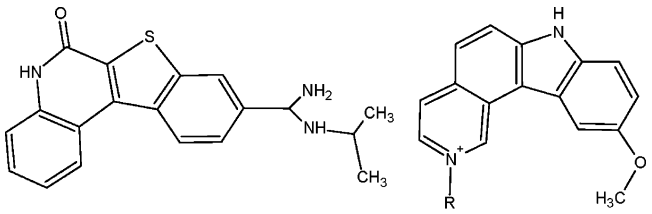


Figure 2. (a and b) Molecular structure of **9a** showing two crystallographically independent molecular ions. Chloride ions have been omitted.



A **B**

Figure 3. Single molecule of **9a** isolated from its crystal structure (A). 7*H*-Pyridocarbazole unit taken from the crystal structure of 5′-d(CpGpCpG)-3′/Flexi-Di complex[23] (B).

Both shown positions of the amidinium substituent allow formation of additional hydrogen bonds (Figure 4a).

Interactions of Acyclic Derivatives (5d, 8d) and Their Cyclic Analogues (6d, 9c) with Double-Stranded DNA and RNA. Compounds studied in this paper can be divided into two major groups, "acyclic" derivatives (Figure 1; compounds **2**, **4**, **5**, **7**, **8**) and their "cyclic" analogues (Figure 1; compounds **3**, **6**, **9**). Cyclic derivatives consist of a large planar moiety (see X-ray data, Figure 3a), which should form stable intercalative complexes with double-stranded (ds) DNA and RNA (as shown in Figure 4). On the other hand, acyclic derivatives are much more flexible and possess no condensed aromatic moiety large enough for stable intercalation into ds-polynucleotides.[22] Owing to the mentioned structural differences, acyclic and cyclic derivatives can differ in the modes of their biological action.

To shed more light on the structure−biological activity relationship, we have studied interactions of the close structural analogues **5d**, **8d**, **6d**, and **9c** (Figure 1) with ds-polynucleotides, namely natural calf thymus (ct) DNA and synthetic RNA homo-polynucleotides. Since application of spectrophotometric methods was necessary for these studies, we have characterized aqueous solutions of the compounds by means of electronic absorption (UV/vis) and fluorescence emission spectra.

Absorbencies of aqueous solutions of **5d**, **8d**, **6d**, and **9c** are proportional to their concentrations up to 1×10^{-4} mol dm^{-3}, indicating that there is no significant intermolecular stacking that could give rise to hypochromicity effects. The pH dependence of the absorption spectra of **6d** and **9c** under neutral and basic conditions can be attributed to the protonation of amidine substituents, commonly having pK = 8−9.[25] To have predominantly protonated amidines under the experimental conditions used (more than 99%), we performed our studies at pH = 6.2.

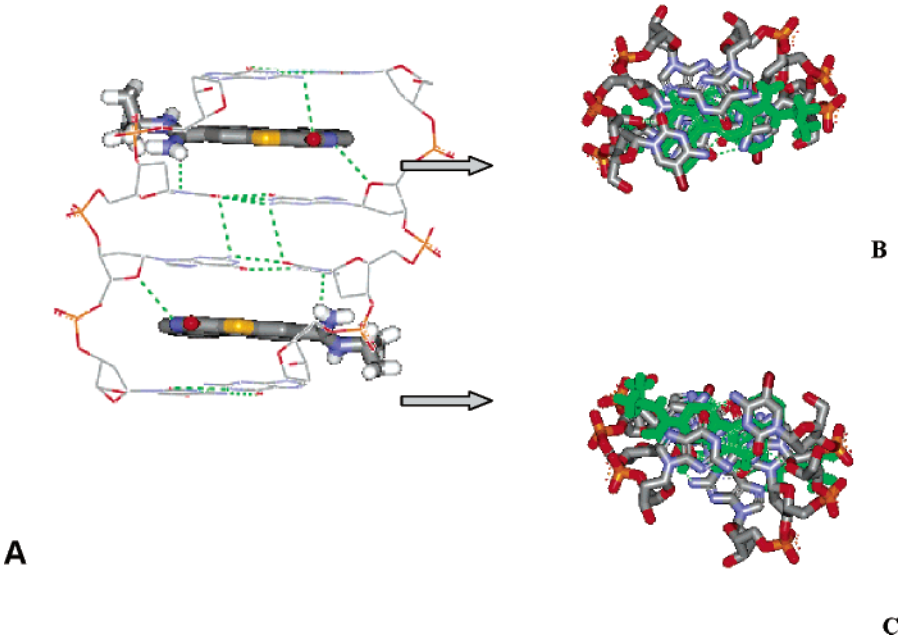


Figure 4. Single molecule of **9a** taken from its crystal structure and inserted into ds-5′-d(CpGpCpG)-3′ on positions of the bis-intercalator Flexi-Di.[23] Green dotted lines on structure A mark possible hydrogen bonds. Views **B** and **C** show the overlapping of basepairs with the aromatic system of **9a** (green).

Table 1. Electronic Absorption Data of **5d**, **8d**, **6d** and **9c**[a]

	λ_{max}/ nm	$\epsilon \times 10^3$/ dm^3 mol^{-1} cm^{-1}		λ_{max}/ nm	$\epsilon \times 10^3$/ dm^3 mol^{-1} cm^{-1}
5d	250	30.2	**6d**	264	43.5
	304	35.8		354	9.0
8d	250	24.0		370	8.2
	304	25.0	**9c**	260	36.9
				353	6.6
				368	6.1

[a] Sodium cacodilate/HCl buffer, I = 0.05 mol dm^{-3}, pH = 6.2.

Cyclization of **5d** and **8d** into **6d** and **9c**, respectively, resulted in pronounced bathochromic shifts of the absorption maxima of acyclic compounds (Table 1). Comparison of ϵ values revealed that all ϵ(**5d**) > ϵ(**8d**) and also ϵ(**6d**) > ϵ(**9c**), strongly suggesting that the positioning of amidine and carbmethoxy substituents has a systematic impact on the electron absorption properties of studied compounds and the likely electron distribution in the studied systems. Aqueous solutions of all studied compounds were shown to be stable over longer periods and also at increased temperature during shorter periods (a few hours at up to 100 °C).

All studied compounds exhibit characteristic fluorescence emission with maxima at about 400 nm, their excitation spectra being in good agreement with the absorption spectra. Fluorescence intensities were found to be linearly concentration dependent up to 4×10^{-6} mol dm^{-3}. Under the same conditions, cyclic compounds **6d** and **9c** exhibit 14 and 80 times stronger emission than acyclic analogues **5d** and **8d**, respectively.

Addition of ct-DNA to cyclic compounds ($c_{\mathbf{6d,9c}} = 6 \times 10^{-5}$ mol dm^{-3}) resulted in strong hypochromic effects (27% for **6d** and 43% for **9c**) and pronounced broadening of UV/vis spectra, which resulted in the merging of two maxima (354 and 370 nm) into one at 361 nm. The observed changes in the UV/vis spectra of **6d** and **9c** strongly suggest intercalation as the dominant binding mode.[26] For both compounds, titration with ct-DNA yielded measurable changes only in excess of the studied compounds; consequently, titration ended at an equimolar ratio of c(**6d**, **9c**)/c(ct-DNA phosphates). This observation suggested high values of the binding constants, $K_s > 10^5$ M^{-1}. Since aqueous solutions of **6d** and **9c** exhibit strong fluorescence, it was possible to perform fluorimetric titrations at up to 100 times lower concentrations than used in UV/vis experiments. Addition of any ds-polynucleotide strongly quenched emission of **6d** and **9c**. Processing the titration data by means of the Scatchard equation[27] gave the binding constants and ratios n ([bound compound]/[polynucleotide phosphate]) presented in Table 2.

Unlike **6d** and **9c**, titration with ct-DNA induced only minor changes in the UV/vis spectra of their acyclic analogues **5d** and **8d** (19% hypochromic effect and negligible bathochromic shift of the maximum at 304 nm). Titration with ds-RNA polynucleotides in most cases yielded even smaller changes, some of them close to the error of the instrument. Due to the low fluorescence of the aqueous solutions of **5d** and **8d**, fluorimetric titrations were not possible. Therefore, UV/vis titration data (when more than 10 data points could be collected) were processed by means of the Scatchard equation[27] to give the binding constants and ratios n (Table 2).

Table 2. Stability Constants (log K_s) and Ratios n ([bound compound]/[polynucleotide]) Calculated According to UV/Vis Titrations of **5d** and **8d** and Fluorimetric Titrations of **6d** and **9c** with Polynucleotides[a,b]

	ct-DNA		polyA−polyU		polyG−polyC	
	log K_s	n	log K_s	n	log K_s	n
5d	5.07	0.60	5.07	0.86	*c*	*c*
8d	5.05	0.37	*c*	*c*	*c*	*c*
6d	5.96	0.25	5.74	0.10	5.25	0.08
9c	6.21	0.25	5.79	0.20	5.91	0.03

[a] UV/vis titrations were performed at pH = 6.2 (sodium cacodilate/HCl buffer, I = 0.05 mol dm^{-3}). [b] Accuracy of n ± 30%, consequently varying values log K_s ± 0.5. [c] To small changes (total less than ΔAbs = 0.05) for evaluation of binding.

Table 3. ΔT_m Values[a] (°C) of Different ds-Polynucleotides with **5d**, **8d**, **6d**, and **9c** at pH = 6.2 (sodium cacodilate/HCl buffer, I = 0.05 mol dm^{-3})

	r[b]	**5d**	**8d**	**6d**	**9c**
ct-DNA	0.1	0.8	>0.5	2.6	2.6
	0.2	1.9	>0.5	5.2	3.6
	0.3	2.3	>0.5	5.9	4.2
	0.5	3.0	>0.5	7.7	6.4
polyA−polyU	0.1	>0.5	>0.5	1.2	1.9
	0.2	>0.5	>0.5	1.9	3.1
	0.3	>0.5	>0.5	2.3	3.5/11.8[c]
	0.5	>0.5	>0.5	3.1/23.8[c]	3.9/16.9[c]

[a] Error in ΔT_m, ±0.5 °C. [b] r = [compound]/[polynucleotide]. [c] Biphasic curves.

Results of the titrations reveal significantly higher affinity of cyclic **6d** and **9c** toward ds-DNA and RNA than found for their acyclic analogues **5d** and **8d**. This finding can be correlated with the size of the aromatic surface, since cyclic analogues are characterized by a planar system more than large enough for intercalation into ds-DNA/RNA.[22] Also, the obtained ratios n vary considerably; only those found for cyclic **6d** and **9c** agree well with the concentration of the intercalative binding sites.[28]

Interactions of **5d**, **8d**, **6d**, and **9c** with ds-polynucleotides were studied also by thermal melting experiments (Table 3). Addition of cyclic **6d** and **9c** significantly stabilized the double helices of ct-DNA and polyA−polyU, revealing pronounced DNA selectivity; both observations are characteristic of the intercalative binding mode.[22,28] Biphasic curves observed for polyA−polyU at higher ratios r can be explained by two coexistent modes of binding. The first transition (ΔT_m value almost identical to ΔT_m values at lower ratios r) can be attributed to intercalation of the studied compound into the double helix, while the second transition is very likely a consequence of additional nonintercalative binding (electrostatic binding of a positively charged compound to the negatively charged phosphate backbone) of the compound in excess over the intercalation binding sites (r > 0.25).[29] Acyclic derivatives **5d** and **8d** do not stabilize double helices in most cases, with the exception of the weak stabilization of *ct*-DNA observed upon addition of **5d** (Table 3).

As a general conclusion, studies of interactions of ds-polynucleotides with acyclic derivatives **5d** and **8d** and their cyclic analogues **6d** and **9c** strongly support weak, nonintercalative binding of the former and strong, intercalative binding of the latter compounds.

Table 4. In Vitro Inhibition of the Compounds on the Growth of Tumor Cells and Normal Human Fibroblasts (WI 38)

	IC_{50} (μM)[a]					
compd	HeLa	MCF-7	MiaPaCa-2	Hep-2	SW 620	WI 38
2a	>100	>100	>100	>100	>100	44.34
2b	>100	>100	>100	>100	95.46	53.62
4a	0.86	g100	>100	6.50	>100	8.12
5a	12.9	1.967	6.99	15.3	27.8	36.4
5b	3.0	3.49	4.44	3.94	3.53	3.3
5c	2.7	3.7	4.67	2.55	3.86	4.01
5d	2.24	1.23	3.49	5.37	3.67	2.85
5e	2.15	2.54	3.51	2.37	3.93	2.99
5f	22.2	23.9	8.72	21	8.02	25.1
5g	25.6	15.6	75.9	67.3	>100	86.9
6a	25.3	1.36	7.3	16.6	32.2	≥100
6b	4.3	5.4	5.98	8.59	7.57	18.66
6c	2.34	38	6.73	34.7	20.6	40.6
6d	4.79	65.1	5.64	45.9	48.8	>100
6e	1.7	5.78	3.71	6.95	6.84	19
6f	6.14	>100	42.1	≥100	>100	≥100
6g	31.6	78.5	86.9	>100	≥100	≥100
7a	0.35	84.93	>100	0.69	≥100	≥100
7b	>100	>100	>100	>100	>100	0.75
7c	40.35	17.56	90.87	48.38	>100	23.43
7d	24.10	>100	>100	59.06	4.62	2.71
7e	>100	>100	>100	>100	>100	5.99
8a	4.26	1.27	7.23	23.8	4.57	4.7
8b	2.35	7.26	5.11	2.96	3.54	2.03
8c	5.83	17.37	22.49	9.70	8.24	29.81
8d	5.65	2.36	7.10	8.16	3.60	6.90
8e	3.05	2.62	2.54	3.14	4.18	3.64
9a	49.2	21.3	18	20.9	3.76	37.4
9b	6.43	5.58	6.67	8.51	22.67	19.07
9c	15.76	5.56	6.93	97.02	8.11	5.33
9d	8.92	6.28	7.99	9.79	13.16	3.88

[a] IC_{50}, the concentration that causes a 50% reduction of the cell growth.

Biological Results and Discussion

Compounds **2a**−**9d** were tested for their potential antiproliferative effect on a panel of six human cell lines, five of which derived from five cancer types: HeLa (cervical carcinoma), MCF-7 (breast carcinoma), SW 620 (colon carcinoma), MiaPaCa-2 (pancreatic carcinoma), Hep-2 (laryngeal carcinoma), and WI 38 (diploid fibroblasts).

All tested compounds showed a certain antiproliferative effect (Table 4). **2a** and **2b** produced little or no growth inhibition except at the highest concentration tested, but when the cyano group was introduced into the molecule as a R_1 or R_2 substituent, slightly more pronounced activity was obtained (**4a**, **7a**, **7c**, **7d**; Figure 5E). In contrast, carboxanilides that bear an isopropylamidino substituent on the quinolone part of the acyclic molecule (R_2) were much more active, in general showing mostly "nondifferential" cytotoxicity at the highest concentrations tested (**5b**, **5c**, **5d**, **5e**; Figure 5A), except for analogues bearing strongly polarizable groups **5f** (R1 = CN) and **5g** [R1 = isopropylamidino (iso-pr-am)]. A comparable, but less pronounced effect was obtained with the compounds that bear isopropylamidino as R_1 substituent (**8a**−**e**; Figure 5B).

To check whether, due to structural differences, the "cyclic" analogues (compounds **6** and **9**) will have significantly different activity compared to the above-discussed "acyclic" ones (compounds **2**, **5**, **7**, **8**), the

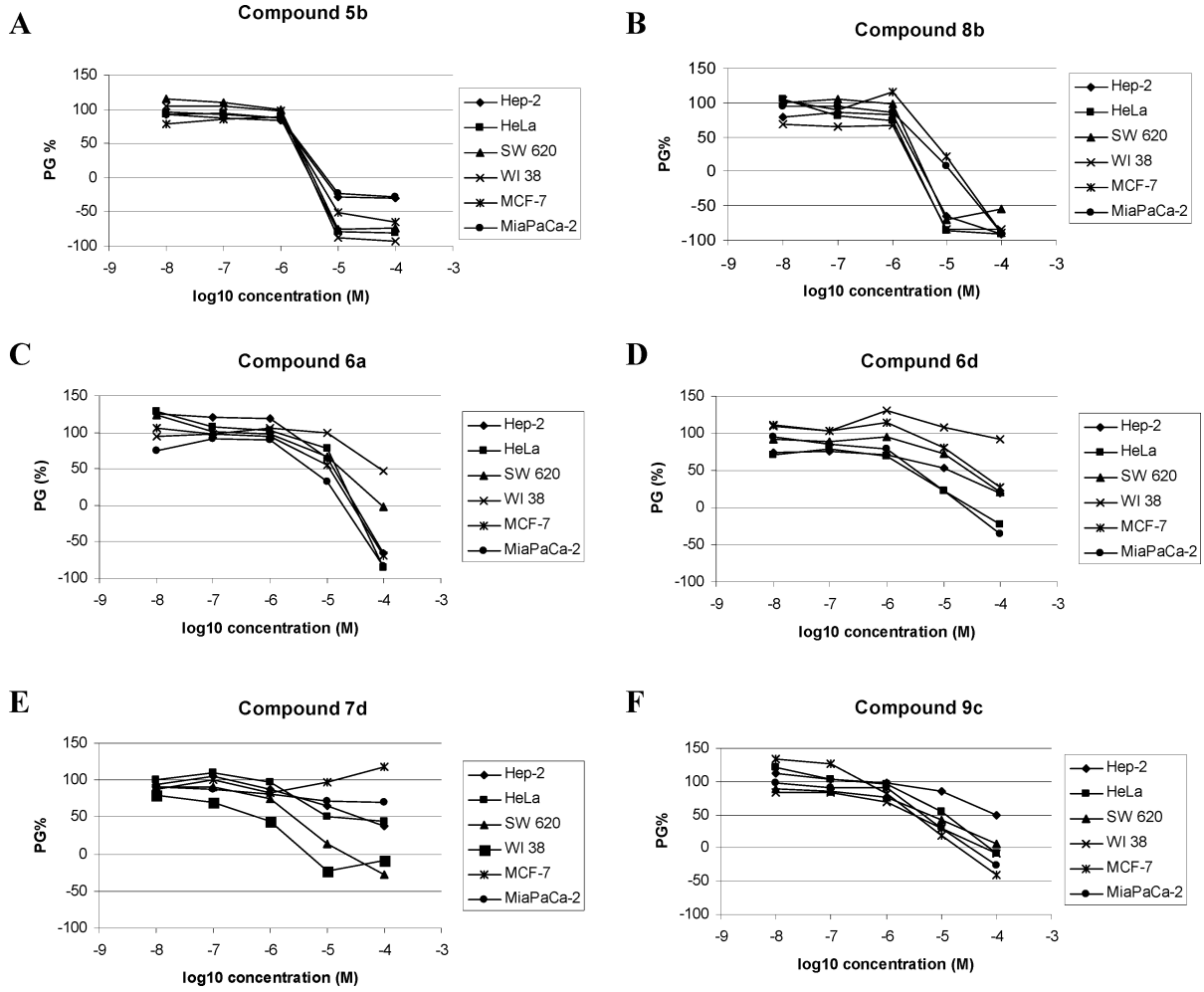


Figure 5. Dose−response profiles for compounds **5b** (A), **8b** (B), **6a** (C), **6d** (D), **7d** (E), and **9c** (F). PG = percentage of growth.

antiproliferative effect on the same cell lines was also studied. It was definitely shown that condensed quinolones with an isopropylamidino substituent at the R_2 position have distinct antiproliferative and rather differential effects (Figure 5C,D), again except for those with strongly polarizable cyano (**6f**) and isopropylamidino groups (**6g**) as R_1 substituents. Similar, but somewhat less obvious, effects were achieved with condensed quinolones that bear the isopropylamidino group as R_1 substituent (**9a**−**d**; Figure 5F). Interestingly, comparison of the IC_{50} values of all compounds indicates that compounds **6a** and **6d** showed the best selective effect; they selectively inhibited the growth of tumor cells but not of normal fibroblasts (WI38) (Figure 5C,D).

Compounds that manifest "differential" growth inhibition and/or cytotoxicity (especially without an inhibitory effect on normal fibroblasts) are mostly of particular interest as the basis for additional research applications, as well as for the selection and prioritization of compounds for in vivo evaluation. We therefore selected several compounds that manifested the most interesting differential effect and subjected them to further testing to obtain a more precise insight into the possible mechanism of their antiproliferative effect. First of all, to establish whether the cell growth inhibition was caused by specific perturbation of cell cycle-related events, flow cytometric analyses were performed for compounds **6a**, **6b**, **6d**, **7d**, **9c**, and **9d**, at concentrations of 5×10^{-6} and 10^{-5} mol/L after 24, 48, and 72 h. DNA contents of MiaPaCa-2, HeLa, SW 620, and WI 38 cells were measured. The results revealed that all tested compounds noticeably influenced the distribution of cell cycle phases; however, the results vary among the cell lines. For instance, no significant changes in the cell cycle population were observed in WI 38 after treatment with compounds **6a**, **6b**, **6d**, and **9c** for as long as 72 h, except for a slight reduction in the percentage of the cells in the S and G2/M phases and a slight increase in the G0/G1 phase (Figures 6J−L and 7D−F), which is in agreement with the cell proliferation assay. On the other hand, the most distinct changes of DNA histograms were observed after the treatment of MiaPaCa-2 and HeLa cells with compounds **6d** and **9c** (Figures 6D−I and 7A−C). This caused a decrease in the G0/G1 fraction and accumulation of cells in the S and G2/M phases after 24 h. This initial S and G2/M arrest persisted for the following 48 h, when an increase in the subG1 fraction (apoptotic cells) was detected, but only in cells treated with the higher concentration (10 μM). Parallel with these results, morphological changes of the treated cells were noticed, such as the presence of giant cells, characteristic of G2/M-arrested cells. Similar results (G2/M arrest) were obtained with the MiaPaCa-2 cells treated with compounds **6a**, **6b**, and **9d** (data not shown). Interestingly, after treatment of the SW 620 cells with compound **6d**, the percentage of arrested cells in the G2/M phase was much lower than in MiaPaCa-2 and HeLa cells, which correlates with the most pronounced growth inhibition of these two cell lines: IC_{50} (SW 620) = 48.8 ± 17.5 μM, while IC_{50} (MiaPaCa-2) = 5.6 ± 1.7 μM and IC_{50} (HeLa) = 4.8 ± 1.5 μM (Figure 5D).

On the other hand, compound **7d** induced completely different effects on the tested cell lines. While it did not induce any substantial changes in the cell cycle phases distribution in the MiaPaCa-2 cells, it induced massive apoptosis in the SW 620 and HeLa cells (Figure 8), but only at the higher tested concentration (10 μM), correlating again with the growth inhibition results IC_{50} (SW 620) = 4.6 ± 2.9 μM, while IC_{50} (MiaPaCa-2) > 100 μM and IC_{50} (HeLa) = 24.1 ± 44.7 μM.

Induction of apoptosis was further confirmed by the Annexin-V assay, an immunofluorescence technique for detection and quantification of apoptotic cells at the single cell level (Table 5).

Both the mechanism of cell death and the overall cytotoxic effect seem to depend on at least three different parameters, including: (1) pharmacological factors (e.g. dose, exposure time), (2) cell type, and (3) expression of certain oncogenes and oncogene suppressors.[30] For a given cell type, different mechanisms of cell death may be triggered off as a function of dose, which was obviously the case after cell treatment with **6a**, **6b**, **6d**, **9c**, and **9d**. Lower doses resulted in a mixture of cytostatic and delayed cytotoxic effects, and the cells ultimately died of delayed apoptosis. However, different cell types responded differently to the same compound, so they have diverse tolerance toward drug-induced lesions. One of the most important factors that influence the response to chemotherapeutic drug treatment is the endogenous status of the p53 suppressor gene. In most cases, a functional p53 is required for drug-induced apoptosis. However, as HeLa, MiaPaCa-2, and SW 620 have all either mutated or inactivated p53, another mechanism of partial resistance of colon carcinoma cells SW 620 to even higher doses of compound **6d** is relevant. One of the possible explanations is that colon carcinoma cells have a high level of natural resistance, which could be due to the expression of different resistance mechanisms.[30,33]

Conclusions

Among the studied compounds, those with one amidino group substituent have shown the best antiproliferative effect on the cell lines tested. This could be explained by the permanent positive charge placed on the amidino group, which may start a number of additional interactions. Unexpectedly, however, the introduction of two amidino groups diminished the biological activity, possibly due to the steric effects.

The presented cell-cycle distribution related results strongly suggest that compounds **6a**, **6b**, **6d**, **9c**, and **9d** may act as topoisimerase "poisons". In addition, studies on interactions of compounds **6d** and **9c** with ds-DNA and RNA have shown that these compounds efficiently bind to double-stranded polynucleotides by intercalation. Since it is well-known that intercalators act as topoisimerase inhibitors and/or "poisons", it is reasonable to propose intercalation of **6d** and **9c** but also of other cyclic analogues (groups **6** and **9**) as the main mode of action in the treated cell lines. For final confirmation, detailed mechanistic studies based on topoisomerase assays should be additionally performed.

Representatives of the "acyclic" group of derivatives (**5d** and **8d**) either do not bind to ds-DNA and RNA (Tables 2, 3) or exhibit weak nonintercalative interactions. Therefore, the mode of their action in the cell lines is not likely to be targeted at nucleic acids.

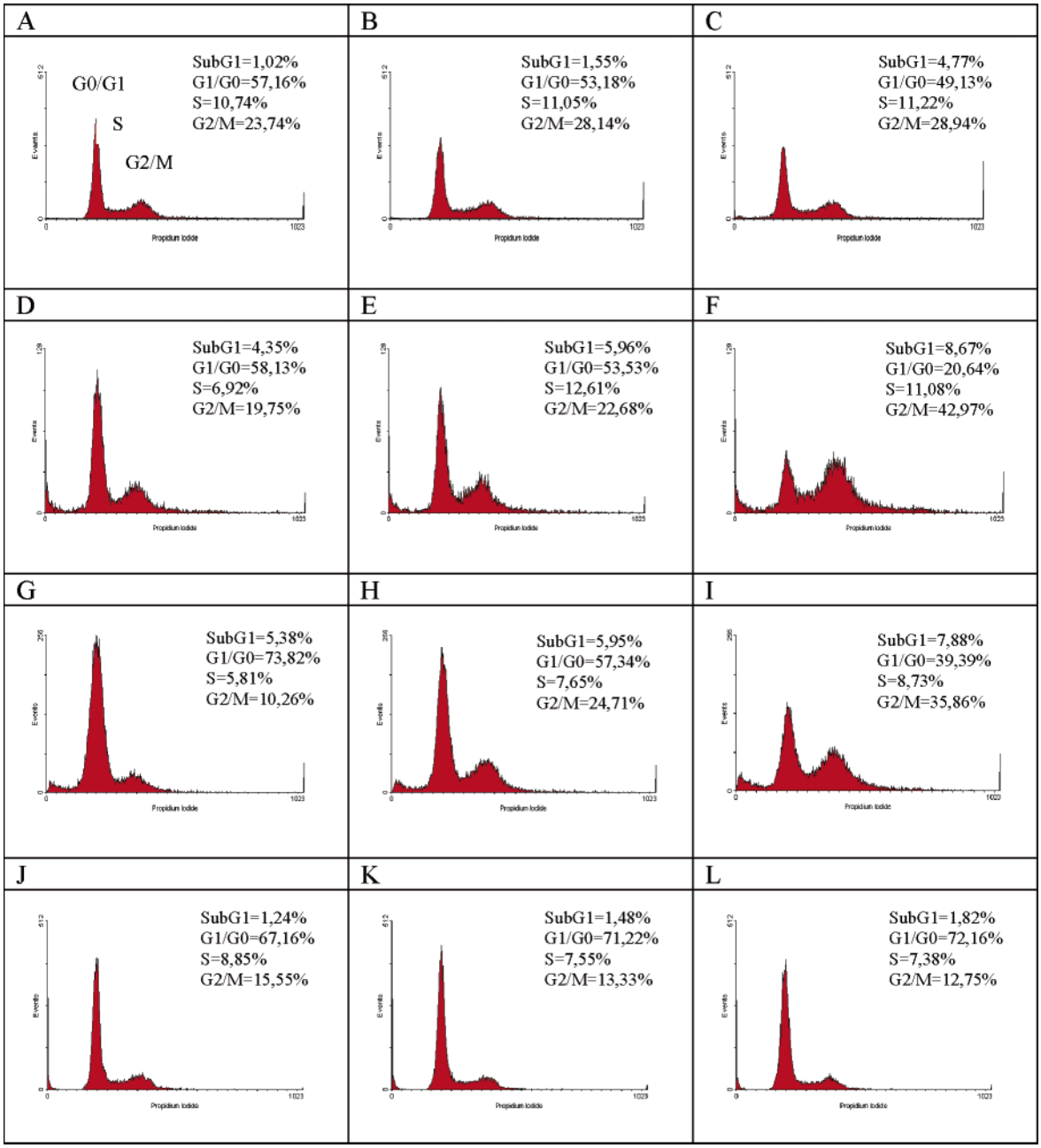


Figure 6. Effect of the compound **6d** on the cell cycle of SW 620 (A−C), HeLa (D−F), MiaPaCa-2 (G−I), and WI 38 (J−L) cells. The cells were untreated (A, D, G, and J) or treated with 5 μM (B, E, H, and K) or 10 μM (C, F, I, and L) concentration of compound **6d** for 72 h, fixed, and stained with propidium iodide to determine the DNA content. Percentage of the cells in each phase of the cell cycle was obtained by flow cytometric analysis.

Comparison of the IC_{50} values of all compounds indicates that compounds **6a** (R_1 = H, R_2 = isopropyamidino) and **6d** (R_1 = $COOCH_3$, R_2 = isopropyamidino) showed the best selective effect; they selectively inhibited the growth of tumor cells but not of normal fibroblasts (WI38).

On the basis of the presented results (the superior properties of derivatives bearing a positively charged substituent, amidine), future prospects in the here presented area should be focused on the benzo[*b*]thieno-[2,3-*c*]quinolone system substituted by groups bearing more permanent positive charges (e.g. spermine derivatives) or stronger positive charges (e.g. guanidinium).

Experimental Section

Chemistry. Melting points were determined on a Kofler hot stage microscope and are uncorrected. IR spectra were recorded on a Nicolet Magna 760 spectrophotometer with KBr disks. ^{1}H and ^{13}C NMR spectra were recorded on either a Varian Gemini 300 or a Bruker Avance DPX 300 spectrometer using TMS as an internal standard in DMSO-d_6. Elemental analysis for carbon, hydrogen, and nitrogen were performed on a Perkin-Elmer 2400 elemental analyzer. Where analyses are indicated only as symbols of elements, analytical results obtained are within 0.4% of the theoretical value. Irradiation was performed at room temperature with a water-cooled immersion well with an "Original Hanau" 400-W high-pressure mercury arc lamp using Pyrex filter. All compounds were routinely checked by TLC with Merck silica gel 60F-254 glass plates.

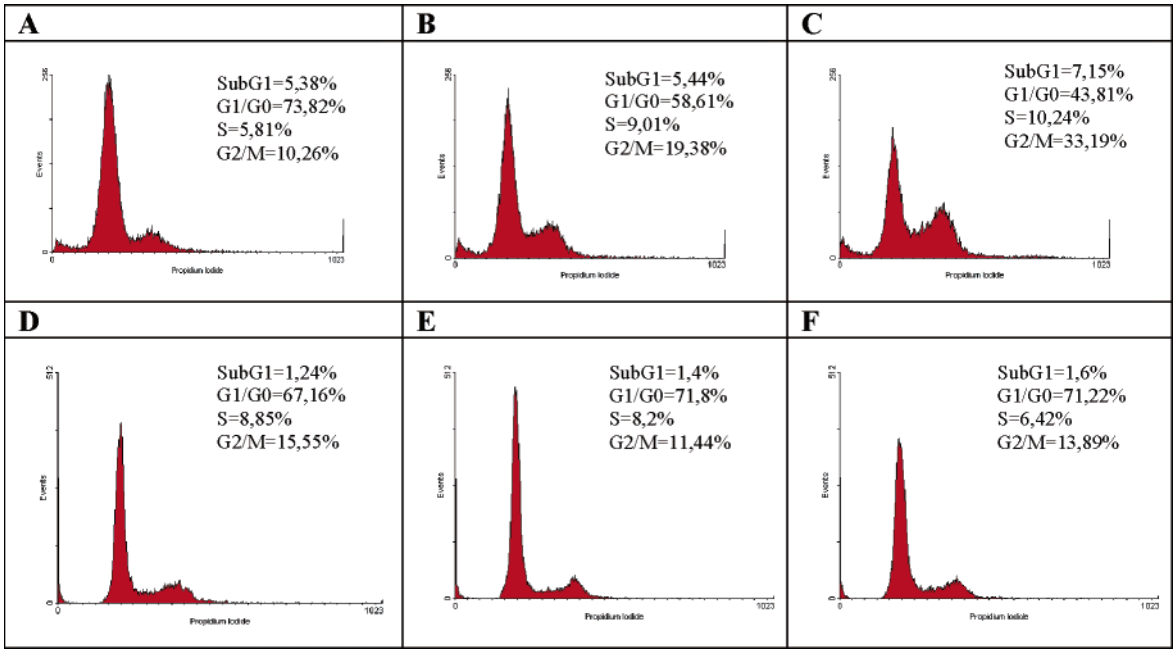


Figure 7. Effect of the compound **9c** on the cell cycle of MiaPaCa-2 (A−C) and WI 38 (D−F) cells. The cells were untreated (A and D) or treated with 5 μM (B and E) or 10 μM (C and F) concentration of compound **9c** for 72 h, fixed, and stained with propidium iodide to determine the DNA content. Percentage of the cells in each phase of the cell cycle was obtained by flow cytometric analysis.

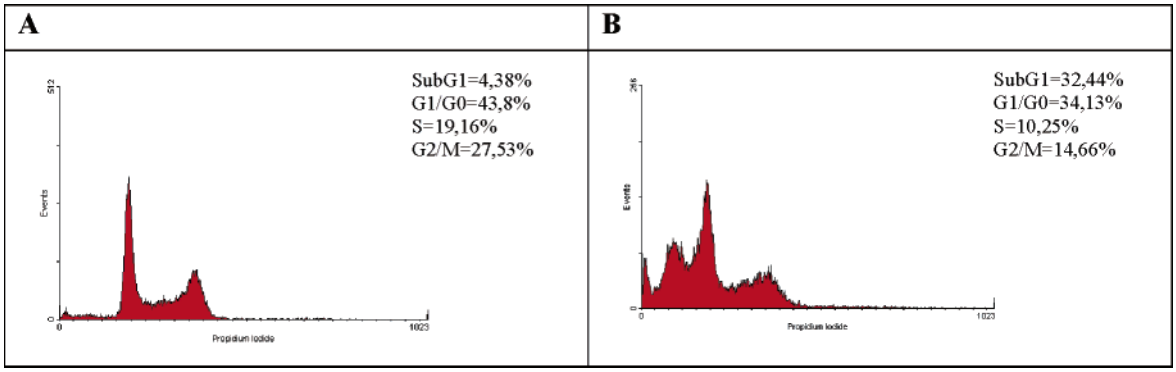


Figure 8. Effect of the compound **7d** on the cell cycle of HeLa cells. The cells were untreated (A) and treated with 10 μM (B) concentration of compound **7d** for 72 h, fixed, and stained with propidium iodide to determine the DNA content. Percentage of the cells in each phase of the cell cycle was obtained by flow cytometric analysis.

Table 5. Percentage of Apoptosis Induced by Compounds **6a**, **6b**, **6d**, **7d**, **9c**, and **9d** in MiaPaCa-2, HeLa, and SW 620 Cells after 72 h

	apoptotic cells (%)												
	control	**6a**		**6b**		**6d**		**7d**		**9c**		**9d**	
cell line	0 μM	5 μM	10 μM	5 μM	10 μM	5 μM	10 μM	5 μM	10 μM	5 μM	10 μM	5 μM	10 μM
MiaPaCa-2	2	4	11	3	17	4	13	3	9	6	14	3	11
HeLa	2	9	11	6	8	12	17	6	52	13	15	10	15
SW 620	4	9	13	15	16	7	9	6	71	6	7	9	13

4′-Carbmethoxy-*N*-phenyl-3-chlorobenzo[*b*]thiophene-2-carboxamide (2a). To a solution of **1a** (1.00 g, 3.05 mmol) in dry toluene (45 mL) was added a solution of methyl 4-aminobenzoate (1.02 g, 10.9 mmol) in dry toluene (5 mL) dropwise, followed by the addition of Et_3N (0.6 mL, 4.3 mmol). The mixture was refluxed for 2.5 h. After cooling, precipitated crystals were filtered off and washed with diluted HCl and water. After recrystallization from acetone, 1.23 g (81.8%) of white crystals was obtained: mp 189−190 °C; IR (KBr) (ν_{max}/cm^{-1}) 3420, 1710, 1640, 1590; ^{1}H NMR (DMSO-d_6) (δ ppm) 10.91 (s, 1H, NH_{amide}), 8.17 (dd, 1H, J_1 = 6.25 Hz, J_2 = 2.89 Hz, $H_{arom.}$), 8.99 (d, 2H, J = 8.72 Hz, $H_{anilide}$), 7.95 (dd, 1H, J_1 = 5.89 Hz, J_2 = 3.39 Hz, $H_{arom.}$), 7.87 (d, 2H, J = 8.73 Hz, $H_{anilide}$), 7.635 (d, 1H, J = 6.09 Hz, $H_{arom.}$), 7.63 (d, 1H, J = 6.09 Hz, $H_{arom.}$), 3.84 (s, 3H, $COOCH_3$); ^{13}C NMR (DMSO-d_6) (δ ppm) 166.2, 159.9, 143.0, 137.3, 136.1, 132.1, 130.8 (2C), 128.2, 126.7, 125.6, 124.0, 123.1, 120.6, 120.1 (2C), 52.4. Anal. ($C_{17}H_{12}ClNO_3S$) C, H, N.

Methyl 2-(*N*-Phenylcarbamoyl)-3-chlorobenzo[2,3-*c*]-thiophene-6-carboxylate (2b). Compound **2b** was prepared using the method described for the preparation of **2a**, from **1d** (1.00 g, 4.30 mmol) in dry THF (23 mL) and aniline (1.31 g, 8.70 mmol) in dry THF (5 mL) after refluxing for 3 h; 0.34 g (33%) of ivory crystals was obtained: mp 192−194 °C (lit.[19] mp 197−198 °C).

Methyl 6-Oxo-5,6-dehydro[1]benzothieno[2,3-*c*]quinolin-2-carboxylate (3a). A solution of **2a** (0.1 g, 0.29 mmol) in 10% methanol/toluene solution (18.7 mL) was irradieted at

room temperature with 400-W high-pressure mercury lamp using a Pyrex filter for 1.5 h. The air was bubbled through the solution. The solution was concentrated and the thus obtained solid was filtered off and washed with acetone. After recrystallization from DMF, 0.083 g (93.7%) of white crystals was obtained: mp >300 °C; IR (KBr) (ν_{max}/cm^{-1}) 3000, 2960, 1720, 1655, 1610; ^{1}H NMR (DMSO-d_6) (δ ppm) 12.46 (s, 1H, $NH_{quinolone}$), 9.13 (s, 1H, $H_{arom.}$), 8.65 (d, 1H, J = 7.81 Hz, $H_{arom.}$), 8.29 (d, 1H, J = 7.55 Hz, $H_{arom.}$), 8.09 (d, 1H, J = 8.21 Hz, $H_{arom.}$), 7.77−7.67 (m, 2H, $H_{arom.}$), 7.59 (d, 1H, J = 8.65 Hz, $H_{arom.}$), 3.94 (s, 3H, $COOCH_3$); ^{13}C NMR (DMSO-d_6) (δ ppm) 165.9, 158.1, 141.4, 140.9, 135.3, 135.1, 133.2, 129.1, 127.7, 126.3, 124.9, 124.7, 124.4, 123.6, 117.0, 116.9, 52.3. Anal. ($C_{17}H_{11}NO_3S$) C, H, N.

Methyl 6-Oxo-5,6-dehydro[1]benzothieno[2,3-*c*]quinolin-9-carboxylate (3b). Compound **3b** was prepared using the method described for the preparation of **3a**, from **2b** (0.095 g, 0.27 mmol) in 10% methanol/toluene solution (16.5 mL) and 2 h of irradiation. After recrystallization from DMF, 0.076 g (89.6%) of white crystals was obtained: mp >300 °C (lit.[19] mp >300 °C).

***N*-(4′-Cyanophenyl)-3-chlorobenzo[*b*]thiophene-2-carboxamide (4a).** To a solution of **1a** (2.08 g, 9.0 mmol) in dry toluene (40 mL) was added dropwise a solution of 4-aminobenzonitrile (1.063 g, 9.0 mmol) in dry toluene (90 mL), followed by the addition of Et_3N (2 mL, 14 mmol). The mixture was refluxed for 4 h. After cooling, precipitated crystals were filtered off and washed with diluted HCl and water to yield 2.34 g (83%) of white crystals: mp 211−213 °C; IR (KBr) (ν_{max}/cm^{-1}) 3880, 2220, 1660, 1590; ^{1}H NMR (DMSO-d_6) (δ ppm) 11.00 (s, 1H, NH_{amide}), 8.19 (m, 1H, $H_{arom.}$), 7.98 (m, 1H, $H_{arom.}$), 7.92 (d, 2H, J = 9.07 Hz, $H_{anilide}$), 7.87 (d, 1H, J = 8.98 Hz, $H_{anilide}$), 7.65 (dd, 2H, J_1 = 5.88 Hz, J_2 = 6.26 Hz, $H_{arom.}$); ^{13}C NMR (DMSO-d_6) (δ ppm) 159.6, 142.3, 136.8, 135.3, 133.3 (2C), 131.3, 127.7, 126.2, 123.5, 122.6, 120.3, 120.17 (2C), 118.8, 106.2. Anal. ($C_{16}H_9ClN_2OS$) C, H, N.

6-Cyano-*N*-(4′-cyanophenyl)-3-chlorobenzo[*b*]thiophene-2-carboxamide (4b). To a solution of 4-aminobenzonitrile (1.18 g, 10.0 mmol) in dry toluene (90 mL) was added dropwise a suspension of **1f** (1.78 g, 8.7 mmol) in dry toluene (50 mL), followed by the addition of Et_3N (3.5 mL, 25 mmol). The mixture was refluxed for 14 h. After cooling, precipitated crystals were filtered off and washed with diluted HCl, water, and acetone to yield 0.81 g (38.6%) of white crystals: mp 290−293 °C; IR (KBr) (ν_{max}/cm^{-1}) 3400, 2220, 1680, 1600; ^{1}H NMR (DMSO-d_6) (δ ppm) 11.17 (s, 1H, NH_{amide}), 8.84 (dd, 1H, J_1 = 1.35 Hz, J_2 = 0.63 Hz, $H_{arom.}$), 8.13 (dd, 1H, J_1 = 8.44 Hz, J_2 = 0.61 Hz, $H_{arom.}$), 8.01 (dd, 1H, J_1 = 8.39 Hz, J_2 = 1.42 Hz, $H_{arom.}$), 7.92 (d, 2H, J = 9.22 Hz, $H_{anilide}$), 7.88 (d, 2H, J = 9.19 Hz, $H_{anilide}$); ^{13}C NMR (DMSO-d_6) (δ ppm) 159.0, 142.1, 138.3, 136.7, 135.9, 133.4 (2C), 128.9, 128.6, 123.6, 120.2 (2C), 118.7, 118.4, 109.7, 106.4. Anal. ($C_{17}H_8ClN_3OS$) C, H, N.

***N*-[4′-(*N*′-Isopropylamidino)phenyl]-3-chlorobenzo[*b*]thiophene-2-carboxamide hydrochloride (5a).** Dry HCl was bubbled for 4 h into the suspension of **4a** (2 g, 0.106 mol) in absolute ethanol (100 mL). The suspension was stirred at room temperature until the −CN band was undetectable (IR). After anhydrous diethyl ether was added, the corresponding iminoether was collected by filtration. The product was suspended in absolute ethanol (10 mL), and isopropylamine (11 mL, 0.13 mol) was added. The mixture was refluxed for 17 h. The volume was reduced by evaporation and the product was filtered off and washed with hot acetone. After recrystallization from ethanol, 0.8 g (83%) of white crystals was obtained: mp >300 °C; IR (KBr) (ν_{max}/cm^{-1}) 3360, 3200, 3000, 1650, 1605; ^{1}H NMR (DMSO-d_6) (δ ppm) 10.91 (bs, 1H, NH_{amide}), 9.44 (bs, 1H, $NH_{amidine}$), 9.29 (bs, 1H, $NH_{amidine}$), 8.93 (bs, 1H, $NH_{amidine}$), 8.12 (dd, 1H, J_1 = 5.2 Hz, J_2 = 3.9 Hz, $H_{arom.}$), 7.90 (dd, 1H, J_1 = 6.38 Hz, J_2 = 2.89 Hz, $H_{arom.}$), 7.707 (d, 2H, J = 8.60 Hz, $H_{anilide}$), 7.705 (d, 2H, J = 8.61 Hz, $H_{anilide}$), 7.58 (m, 2H, $H_{arom.}$), 3.98−3.95 (m, 1H, J = 6.32 Hz, CH_{i-pr}), 1.22 (d, 6H, J = 6.38 Hz, $2CH_{3\,i-pr}$); ^{13}C NMR (DMSO-d_6) (δ ppm) 161.1, 159.6, 142.5, 136.8, 135.6, 131.5, 129.4 (2C), 127.7, 126.6, 124.4, 123.5, 122.6, 120.3, 119.7 (2C), 44.9, 21.2 (2C). Anal. ($C_{19}H_{19}Cl_2N_3OS$) C, H, N.

6-Methyl-*N*-[4′-(*N*′-isopropylamidino)phenyl]-3-chlorobenzo[*b*]thiophene-2-carboxamide hydrochloride (5b). To a solution of 6-methyl-3-chlorobenzo[*b*]thiophene-2-carbonyl chloride (1.15 g, 4.7 mmol) in dry toluene (40 mL) was added dropwise a solution of *p*-(*N*-isopropyl)amidinoaniline (1 g, 4.7 mmol) in dry DMF (70 mL), followed by the addition of Et_3N (1 mL, 7 mmol). The mixture was refluxed for 6 h. After cooling, precipitated crystals were filtered off, washed with hot chloroform, and recrystallized from DMF to produce 0.54 g (27%) of white crystals: mp >300 °C; IR (KBr) (ν_{max}/cm^{-1}) 3360, 3200, 3000, 1660, 1600; ^{1}H NMR (DMSO-d_6) (δ ppm) 10.88 (s, 1H, NH_{amide}), 9.51 (1H, J = 8.18 Hz, $NH_{amidine}$), 9.47 (s, 1H, $NH_{amidine}$), 9.01 (s, 1H, $NH_{amidine}$), 7.995 (d, 2H, J = 6.41 Hz, $H_{anilide}$), 7.91 (s, 1H, $H_{arom.}$), 7.85 (d, 1H, J = 8.23 Hz, $H_{arom.}$), 7.78 (d, 2H, J = 8.77 Hz, $H_{anilide}$), 7,47 (dd, 1H, J_1 = 8.35 Hz, J_2 = 0.96 Hz, $H_{arom.}$), 4.097−4.007 (m, 1H, J = 6.76 Hz, CH_{i-pr}), 3.3 (s, 3H, CH_3), 1.29 (d, 6H, J = 6.39 Hz, $2CH_{3\,i-pr}$); ^{13}C NMR (DMSO-d_6) (δ ppm) 161.2, 159.6, 142.5, 138.0, 137.0, 133.6, 132.0, 131.0, 129.3, 127.9, 124.3, 122.9, 122.3, 119.6 (2C), 44.9, 21.2 (2C). Anal. ($C_{20}H_{21}Cl_2N_3OS$) C, H, N.

General Method for the Synthesis of 6-Substituted-*N*-[4′-(*N*′-isopropylamidino)phenyl]-3-chlorobenzo[*b*]thiophene-2-carboxamide hydrochloride (5c−f). To a solution of *p*-(*N*-isopropyl)amidinoaniline in dry DMF was added dropwise a solution of 6-substituted-3-chlorobenzo[*b*]-thiophene-2-carbonyl chloride (**1c−f**) in dry THF (10 mL), followed by the addition of Et_3N. The mixture was refluxed for 4−24 h. After cooling, precipitated crystals were filtered off, washed with hot chloroform, and recrystallized.

6-Methoxy-*N*-[4′-(*N*′-isopropylamidino)phenyl]-3-chlorobenzo[*b*]thiophene-2-carboxamide hydrochloride (5c): from *p*-(*N*-isopropyl)amidinoaniline (0.41 g, 1.92 mmol) in dry DMF (30 mL), 6-methoxy 3-chlorobenzo[*b*]thiophene-2-carbonyl chloride (0.5 g, 1.91 mmol) in dry THF (10 mL), and Et_3N (2.27 mL, 1.94 mmol) after refluxing for 24 h: 0.49 g (58%) of white crystals; mp 292−295 °C; IR (KBr) (ν_{max}/cm^{-1}) 3450, 3200, 2900, 2660, 1660, 1600; ^{1}H NMR (DMSO-d_6) (δ ppm) 10.92 (s, 1H, H_{amide}), 9.52 (d, 1H, J = 7.86 Hz, $H_{amidine}$), 9.39 (s, 1H, $H_{amidine}$), 9.02 (s, 1H, $H_{amidine}$), 7.91 (d, 2H, J = 8.64 Hz, $H_{anilide}$), 7.83 (d, 1H, J = 8.87 Hz, $H_{arom.}$), 7.76 (d, 2H, J = 8.95 Hz, $H_{anilide}$), 7.758 (s, 1H, $H_{arom.}$), 7.23 (dd, 1H, J_1 = 8.87 Hz, J_2 = 2.19 Hz, $H_{arom.}$), 4.07−4.03 (m, 1H, J = 6.76 Hz, CH_{i-pr}), 3.88 (s, 3H, OCH_3), 1.27 (d, 6H, J = 6.47 Hz, $CH_{3\,i-pr}$); ^{13}C NMR (DMSO-d_6) (δ ppm) 161.2, 159.5, 142.6, 138.8, 129.5, 129.3 (2C), 128.5, 124.2, 123.6, 120.3, 119.6 (2C), 116.6, 105.6, 55.8, 44.9, 21.2 (2C). Anal. ($C_{20}H_{21}Cl_2N_3O_2S$) C, H, N.

Methyl 2-[*N*-4′-(*N*′-isopropylamidino)phenylcarbamoyl]-3-chlorobenzo[*b*]thiophene-6-carboxylate hydrochloride (5d): from **1d** (0.8 g, 2.8 mmol) in dry THF (15 mL), *p*-(*N*-isopropyl)amidinoaniline (0.534 g, 2.5 mmol) in dry DMF (30 mL), and Et_3N (0.56 mL, 4 mmol), after refluxing for 21 h and recrystallization from the mixture of methanol and DMF (1:1): 0.19 g (16.3%) of white powder; mp 286−289 °C; IR (KBr) (ν_{max}/cm^{-1}) 3360, 3200, 3000, 1720, 1670, 1610; ^{1}H NMR (DMSO-d_6) (δ ppm) 11.1 (s, 1H, NH_{amide}), 9.52 (d, 1H, J = 7.51 Hz, $NH_{amidine}$), 9.37 (s, 1H, $NH_{amidine}$), 8.99 (s, 1H, $NH_{amidine}$), 8.89 (s, 1H, $H_{arom.}$), 8.15 (d, 1H, J = 8.57 Hz, $H_{arom.}$), 8.075 (d, 1H, J = 8.54 Hz, $H_{arom.}$), 7.93 (d, 2H, J = 8.53 Hz, $H_{anilide}$), 7.78 (d, 2H, J = 8.29 Hz, $H_{anilide}$), 4.04−4.01 (m, 1H, J = 6.45 Hz, CH_{i-pr}), 3.93 (s, 3H, $COOCH_3$), 1.28 (d, 6H, J = 6.11 Hz, $2CH_{3\,i-pr}$); ^{13}C NMR (DMSO-d_6) (δ ppm) 161.3, 159.2, 142.3, 138.7, 136.7, 129.3 (2C), 128.5, 126.3, 125.5, 124.6, 122.9, 119.7 (3C). Anal. ($C_{21}H_{21}Cl_2N_3O_3S$) C, H, N.

6-Bromo-*N*-[4′-(*N*′-isopropylamidino)phenyl]-3-chlorobenzo[*b*]thiophene-2-carboxamide hydrochloride (5e): from **1e** (0.62 g, 2.0 mmol) in dry THF (10 mL), *p*-(*N*-isopropyl)-amidinoaniline (0.43 g, 2.0 mmol) in dry DMF (30 mL), and Et_3N (0.42 mL, 3 mmol), after refluxing for 20 h and recrystallization from DMF: 0.19 g (15.2%) of white powder; mp >300 °C; IR (KBr) (ν_{max}/cm^{-1}) 3320, 3000, 1670, 1650, 1610; ^{1}H NMR (DMSO-d_6) (δ ppm) 10.99 (s, 1H, NH_{amide}), 9.51 (d, 1H, J = 7.65 Hz, $NH_{amidine}$), 9.37 (s, 1H, $NH_{amidine}$), 9.02 (s,

1H, $NH_{amidine}$), 8.51 (s, 1H, $H_{arom.}$), 7.90 (d, 2H, J = 8.64 Hz, $H_{anilide}$), 7.87 (d, 1H, J = 9.31 Hz, $H_{arom.}$), 7.76 (d, 3H, J = 8.65 Hz, $2H_{anilide}$, $1H_{arom.}$), 4.077−4.007 (m, 1H, J = 6.64 Hz, CH_{i-pr}), 1.26 (d, 6H, J = 6.32 Hz, $2CH_{3\,i-pr}$); ^{13}C NMR (DMSO-d_6) (δ ppm) 161.3, 159.3, 142.5, 138.4, 134.8, 132.3, 129.6, 129.4 (2C), 126.2, 124.5, 124.4, 121.0, 120.7, 120.2, 119.7 (2C), 45.0, 21.3 (2C). Anal. ($C_{19}H_{18}BrCl_2N_3OS$) C, H, N.

6-Cyano-*N*-[4′-(*N*′-isopropylamidino)phenyl]-3-chlorobenzo[*b*]thiophene-2-carboxamide hydrochloride (5f): from **1f** (0.7 g, 2.7 mmol) in dry toluene (40 mL), *p*-(*N*-isopropyl)amidinoaniline (0.49 g, 2.3 mmol) in dry DMF (30 mL), and Et_3N (0.56 mL, 4 mmol), after refluxing for 4 h and recrystallization from a mixture of DMF and methanol (1:1): 0.39 g (39.2%) of white powder; mp 292−295 °C; IR (KBr) (ν_{max}/cm^{-1}) 3340, 3200, 3000, 2200, 1670, 1600; ^{1}H NMR (DMSO-d_6) (δ ppm) 11.0 (s, 1H, NH_{amide}), 9.51 (d, 1H, J = 7.81 Hz, $NH_{amidine}$), 9.37 (bs, 1H, $NH_{amidine}$), 9.00 (bs, 1H, $NH_{amidine}$), 8.84 (s, 1H, $H_{arom.}$), 8.13 (d, 1H, J = 8.48 Hz, $H_{arom.}$), 8.01 (dd, 1H, J_1 = 8.46 Hz, J_2 = 1.32 Hz, $H_{arom.}$), 7.93 (d, 2H, J = 8.82 Hz, $H_{anilide}$), 7.79 (d, 2H, J = 8.81 Hz, $H_{anilide}$), 4.09−4.02 (m, 1H, J = 6.58 Hz, CH_{i-pr}), 1.29 (d, 6H, J = 6.0 Hz, $2CH_{3\,i-pr}$); ^{13}C NMR (DMSO-d_6) (δ ppm) 161.1, 159.0, 142.3, 128.4, 136.7, 136.1, 129.4 (2C), 128.9, 128.6, 124.6, 123.7, 120.0, 199.7 (2C), 118.5, 109.7, 44.9, 21.3 (2C). Anal. ($C_{20}H_{18}Cl_2N_4OS$) C, H, N.

6-*N*″-Isopropylamidino-*N*-[4′-(*N*′-isopropylamidino)-phenyl]-3-chlorobenzo[*b*]thiophene-2-carboxamide dihydrochloride (5g). Compound **5g** was prepared using the method described for the preparation of **5a**; from **4b** (0.75 g, 2.5 mmol) in dry methoxyethanol (80 mL). The obtained imino ether was collected by filtration after 7 days and suspended in absolute ethanol (40 mL). Isopropylamine (1.8 mL, 21 mmol) was added and the mixture was stirred at room temperature for 3 days. Crude product was recrystallized from methanol and then from water to yield 0.25 g (24.4%) of white crystals: mp 306−308 °C; IR (KBr) (ν_{max}/cm^{-1}) 3320, 3180, 3000, 1670, 1610; ^{1}H NMR (DMSO-d_6) (δ ppm) 9.75−9.49 (bs, 6H, $NH_{amidine}$), 8.64 (s, 1H, $H_{arom.}$), 8.15 (d, 1H, J = 8.44 Hz, $H_{arom.}$), 7.95 (d, 2H, J = 8.83 Hz, H_{anilid}), 7.92 (d, 2H, J = 8.76 Hz, $H_{arom.}$), 7.80 (d, 2H, J = 8.61 Hz, $H_{anilide}$), 4.06−4.02 (m, 2H, CH_{i-pr}), 1.35 (d, 6H, J = 6.62 Hz, $2CH_{3\,i-pr}$), 1.29 (d, 6H, J = 6.50 Hz, $2CH_{3\,i-pr}$); ^{13}C NMR (DMSO-d_6) (δ ppm) 161.5, 161.3, 159.2, 142.4, 138.6, 136.5, 135.3, 129.5 (2C), 128.4, 125.9, 124.7, 122.9, 120.0, 119.9 (2C), 45.4, 45.1, 21.4 (2C), 21.3 (2C). Anal. ($C_{23}H_{28}Cl_3N_5OS$) C, H, N.

General Method for the Synthesis of 6-Oxo-5,6-dihydro-[1]benzothieno[2,3-*c*]quinoline (6a−g). A solution of 6-substituted *N*-[4′-(*N*′-isopropylamidino)phenyl]-3-chlorobenzo[2,3-*c*]thiophene-2-carboxamide (**5a**−**g**) (0.24 mmol) in methanol (10 mL) was irradiated at room temperature with 400-W high-pressure mercury lamp using a Pyrex filter for 1.5 h. The air was bubbled through the solution. The solution was concentrated and the obtained solid was filtered off and washed with acetone. After recrystallization from methanol, white crystals were obtained.

2-(*N*′-Isopropylamidino)-6-oxo-5,6-dihydro[1]-benzothieno[2,3-*c*]quinoline hydrochloride (6a). Compound **6a** was prepared using the general method described for the preparation of **6a**−**g**; a solution of **5a** (0.106 g, 0.25 mmol) in methanol (10 mL) was irradiated for 1 h. The formed precipitate was filtered off and recrystallized from ethanol to give 0.061 g (64%) of white crystals: mp >300 °C; IR (KBr) (ν_{max}/cm^{-1}) 3060, 2940, 1650, 1620; ^{1}H NMR (DMSO-d_6) (δ ppm) 12.62 (bs, 1H, $NH_{quinolone}$), 9.72 (bs, 1H, $NH_{amidine}$), 9.62 (bs, 1H, $NH_{amidine}$), 9.18 (bs, 1H, $NH_{amidine}$), 8.92 (d, 1H, J = 8.32 Hz, $H_{arom.}$), 8.90 (d, 1H, J = 1.69 Hz, $H_{arom.}$), 8.23 (dd, 1H, J_1 = 7.64 Hz, J_2 = 1.21 Hz, $H_{arom.}$), 7.83 (dd, 1H, J_1 = 8.60 Hz, J_2 = 1.74 Hz, $H_{arom.}$), 7.69 (dd, 1H, J_1 = 7.02 Hz, J_2 = 1.06 Hz, $H_{arom.}$), 7.67−6.55 (m, 2H, $H_{arom.}$), 4.12−4.07 (m, 1H, CH_{i-pr}), 1.28 (d, 6H, J = 6.41 Hz, $2CH_{3\,i-pr}$); ^{13}C NMR (DMSO-d_6) (δ ppm) 161.1, 158.0, 141.4, 140.7, 135.3, 135.1, 133.4, 128.5, 127.9, 126.2, 126.0, 124.28, 124.24, 123.2, 116.8 (2C), 45.2, 21.4 (2C). Anal. ($C_{19}H_{18}ClN_3OS$) C, H, N.

2-(*N*′-Isopropylamidino)-9-methyl-6-oxo-5,6-dihydro[1]-benzothieno[2,3-*c*]quinoline hydrochloride (6b): yield 0.074 g (81%); mp >300 °C; IR (KBr) (ν_{max}/cm^{-1}) 3220, 3000, 1650, 1610; ^{1}H NMR (DMSO-d_6) (δ ppm) 12.58 (s, 1H, $NH_{quinolone}$), 9.86 (bs, 1H, $NH_{amidine}$), 9.65 (bs, 1H, $NH_{amidine}$), 9.11 (bs, 1H, $NH_{amidine}$), 8.95 (s, 1H, $H_{arom.}$), 8.85 (d, 1H, J = 8.44 Hz, $H_{arom.}$), 8.11 (s, 1H, $H_{arom.}$), 7.87 (d, 1H, J = 8.60 Hz, $H_{arom.}$), 7.69 (d, 1H, J = 8.61 Hz, $H_{arom.}$), 7.59 (d, 1H, J = 8.65 Hz, $H_{arom.}$), 4.11−4.07 (m, 1H, J = 6.39 Hz, CH_{i-pr}), 3.39 (s, 3H, CH_3), 1.35 (d, 6H, J = 6.35 Hz, $2CH_{3\,i-pr}$); ^{13}C NMR (DMSO-d_6) (δ ppm) 161.6, 157.9, 141.7, 140.7, 138.0, 125.3, 132.7, 132.4, 128.3, 127.6, 125.7, 124.2, 123.8, 123.0, 116.75, 116.73, 45.1, 21.3 (2C), 21.08. Anal. ($C_{20}H_{20}ClN_3OS$) C, H, N.

2-(*N*′-Isopropylamidino)-9-methoxy-6-oxo-5,6-dihydro-[1]benzothieno[2,3-*c*]quinoline hydrochloride (6c): yield 0.074 g (81%); mp >300 °C; IR(KBr) (ν_{max}/cm^{-1}) 3400, 3100, 2920, 2840, 1670, 1640, 1600; ^{1}H NMR (DMSO-d_6) (δ ppm) 12.56 (s, 1H, $NH_{quinolone}$), 9.71 (bs, 2H, $NH_{amidine}$), 9.18 (bs, 1H, $NH_{amidine}$), 8.91 (s, 1H, $H_{arom.}$), 8.85 (d, 1H, J = 9.19 Hz, $H_{arom.}$), 7.89 (s, 1H, $H_{arom.}$), 7.875 (d, 1H, J = 7.86 Hz, $H_{arom.}$), 7.69 (d, 1H, J = 8.47 Hz, $H_{arom.}$), 7.325 (d, 1H, J = 8.70 Hz, $H_{arom.}$), 4.13−4.11 (m, 1H, J = 5.04 Hz, CH_{i-pr}), 4.08 (s, 3H, OCH_3), 1.35 (d, 6H, J = 5.98 Hz, $2CH_{3\,i-pr}$); ^{13}C NMR (DMSO-d_6) (δ ppm) 162.0, 159.7, 144.3, 141.2, 135.8, 131.5, 130.0, 128.9, 127.4, 124.6, 123.5, 117.2, 117.1, 116.5, 106.8, 56.2, 45.6, 21.8 (2C). Anal. ($C_{20}H_{20}ClN_3O_2S$) C, H, N.

Methyl 2-(*N*′-isopropylamidino)-6-oxo-5,6-dihydro[1]-benzothieno[2,3-*c*]quinolin-9-carboxylate hydrochloride (6d): yield 0.083 g (83.5%); mp 280−283 °C; IR (KBr) (ν_{max}/cm^{-1}) 3200, 3020, 1710, 1670, 1650; ^{1}H NMR (DMSO-d_6) (δ ppm) 12.70 (bs, 1H, $NH_{quinolone}$), 9.72 (bs, 2H, $NH_{amidine}$), 9.16 (bs, 1H, $NH_{amidine}$), 9.11 (d, 1H, J = 8.61 Hz, $H_{arom.}$), 8.98 (s, 1H, $H_{arom.}$), 8.97 (s, 1H, $H_{arom.}$), 8.23 (dd, 1H, J_1 = 8.72 Hz, J_2 = 1.61 Hz, $H_{arom.}$), 7.90 (d, 1H, J = 8.46 Hz, $H_{arom.}$), 7.72 (d, 1H, J = 8.64 Hz, $H_{arom.}$), 4.12−4.08 (m, 1H, J = 6.38 Hz, CH_{i-pr}), 3.96 (s, 3H, $COOCH_3$), 1.36 (d, 6H, J = 6.33 Hz, $2CH_{3\,i-pr}$); ^{13}C NMR (DMSO-d_6) (δ ppm) 165.7, 161.5, 157.7, 141.3, 140.7, 138.3, 136.6, 134.6, 128.7, 128.4, 126.3, 125.8, 125.7, 124.2, 123.3, 116.9, 116.6, 52.6, 45.2, 21.3 (2C). Anal. ($C_{21}H_{20}ClN_3O_3S$) C, H, N.

2-(*N*′-Isopropylamidino)-9-bromo-6-oxo-5,6-dihydro[1]-benzothieno[2,3-*c*]quinoline hydrochloride (6e): yield 0.094 g (90%); mp >300 °C; IR (KBr) (ν_{max}/cm^{-1}) 3200, 3000, 2820, 2780, 1650, 1610; ^{1}H NMR (DMSO-d_6) (δ ppm) 12.70 (s, 1H, $NH_{quinolone}$), 9.73 (s, 1H, $H_{amidine}$), 9.64 (s, 1H, $NH_{amidine}$), 9.12 (s, 1H, $NH_{amidine}$), 9.82 (d, 2H, J = 8.56 Hz, $H_{arom.}$), 8.66 (d, 1H, J = 1.49 Hz, $H_{arom.}$), 7.91 (d, 1H, J = 6.99 Hz, $H_{arom.}$), 7.89 (d, 1H, J = 5.42 Hz, $H_{arom.}$), 7.70 (d, 1H, J = 8.63 Hz, $H_{arom.}$), 4.12−4.09 (m, 1H, J = 7.59 Hz, CH_{i-pr}), 1.35 (d, 6H, J = 6.29 Hz, $CH_{3\,i-pr}$); ^{13}C NMR (DMSO-d_6) (δ ppm) 161.9, 158.3, 143.6, 141.2, 134.8, 134.2, 134.0, 129.5, 129.1, 128.1, 127.3, 124.6, 123.7, 121.7, 117.3, 116.9, 45.6, 21.8 (2C). Anal. ($C_{19}H_{17}BrClN_3OS$) C, H, N.

2-(*N*′-Isopropylamidino)-9-cyano-6-oxo-5,6-dihydro[1]-benzothieno[2,3-*c*]quinoline hydrochloride (6f): yield 0.059 g (65%); mp >300 °C; IR (KBr) (ν_{max}/cm^{-1}) 3400, 3000, 2220, 1650, 1610; ^{1}H NMR (DMSO-d_6) (δ ppm) 12.47 (bs, 1H, $NH_{quinolone}$), 9.68 (bs, 3H, $NH_{amidine}$), 9.13 (d, 1H, J = 8.65 Hz, $H_{arom.}$), 8.91 (s, 2H, $H_{arom.}$), 8.09 (d, 1H, J = 8.64 Hz, $H_{arom.}$), 7.89 (d, 1H, J = 8.64, $H_{arom.}$), 7.70 (d, 1H, J = 8.64, $H_{arom.}$), 4.15−4.11 (m, 1H, J = 6.43 Hz, CH_{i-pr}), 1.34 (d, 6H, J = 6.32 Hz, $CH_{3\,i-pr}$); ^{13}C NMR (δ ppm) (DMSO-d_6) 161.7, 140.9, 138.2, 135.5, 131.8, 129.4, 129.1, 128.5, 127.3, 124.4, 123.6, 119.0, 117.2, 117.0, 110.1, 45.4 (2C), 21.4. Anal. ($C_{20}H_{17}ClN_4OS$) C, H, N.

2-(*N*′-Isopropylamidino)-9-(*N*″-isopropylamidino)-6-oxo-5,6-dihydro[1]benzothieno[2,3-*c*]quinoline dihydrochloride (6g). Compound **6g** was prepared using the general method described for the preparation of **6a**−**g**; a solution of **5g** (0.10 g, 0.19 mmol) in methanol (12 mL) was irradiated for 1.5 h. The formed precipitate was filtered off and recrystallized from methanol to yield 0.06 g (64%) of white crystals: mp >300 °C; IR (KBr) (ν_{max}/cm^{-1}) 3040, 1650, 1640, 1610; ^{1}H NMR (DMSO-d_6) (δ ppm) 12.82 (s, 1H, $NH_{quinolone}$), 9.93 (d, 1H, J = 9.35 Hz, $NH_{amidine}$), 9.20 (d, 1H, J = 9.46 Hz, $NH_{amidine}$), 9.80 (s, 1H, $NH_{amidine}$), 9.78 (s, 1H, $NH_{amidine}$), 9.39

(s, 1H, $NH_{amidine}$), 9.26 (s, 1H, $NH_{amidine}$), 9.18 (d, 1H, $J = 8.81$ Hz, $H_{arom.}$), 8.97 (s, 1H, $H_{arom.}$), 8.75 (d, 1H, $J = 1.63$ Hz, $H_{arom.}$), 8.01 (dd, 1H, $J_1 = 8.67$ Hz, $J_2 = 1.70$ Hz, $H_{arom.}$), 7.93 (dd, 1H, $J_1 = 8.64$ Hz, $J_2 = 1.74$ Hz, $H_{arom.}$), 7.74 (d, 1H, $J = 8.66$ Hz, $H_{arom.}$), 4.19−4.14 (m, 2H, $J = 6.55$ Hz, CH_{i-pr}), 1.35 (d, 6H, $J = 6.41$ Hz, $2CH_{3\,i-pr}$), 1.33 (d, 6H, $J = 6.41$ Hz, $2CH_{3\,i-pr}$); ^{13}C NMR (DMSO-d_6) (δ ppm) 161.4, 161.3, 157.7, 140.9, 140.8, 138.0, 136.0, 134.8, 128.9, 128.1, 126.4, 125.3, 124.8, 124.4, 123.3, 116.9, 116.5, 45.3, 45.2, 21.3 (2C), 21.2 (2C). Anal. ($C_{23}H_{27}Cl_2N_5OS$) C, H, N.

General Method for the Synthesis of 4′-Substituted-6-cyano-*N*-phenyl-3-chlorobenzo[*b*]thiophene-2-carboxamide (7a−e). To a solution of 6-cyano-3-chlorobenzo[2,3-*c*]thiophene-2-carbonyl chloride (**1f**) was added dropwise a solution of 4-substituted aniline. The mixture was refluxed for 1−2.5 h. After cooling, precipitated crystals were filterd off, washed with diluted HCl and water, and recrystallized from acetone.

6-Cyano-*N*-phenyl-3-chlorobenzo[*b*]thiophene-2-carboxamide (7a): from **1f** (2.3 g, 9.0 mmol) in dry toluene (70 mL) and aniline (1.68 g, 18.0 mmol) after refluxing for 1 h: 2.16 g (76%) of light yellow crystals; mp 216−218 °C; IR (KBr) (ν_{max}/cm^{-1}) 3320, 3040, 2220, 1640, 1600; ^1H NMR (DMSO-d_6) (δ ppm) 10.73 (s, 1H, NH_{amide}), 8.30 (s, 1H, $H_{arom.}$), 8.11 (d, 1H, $J = 8.43$ Hz, $H_{arom.}$), 7.91 (dd, 1H, $J_1 = 8.43$ Hz, $J_2 = 1.38$ Hz, $H_{arom.}$), 7.72 (d, 2H, $J = 7.68$ Hz, $H_{2',6'}$), 7.40 (t, 2H, $J = 7.89$ Hz, $H_{3',5'}$), 7.18 (t, 1H, $J = 7.37$ Hz, $H_{4'}$); ^{13}C NMR (DMSO-d_6) (δ ppm) 158.3, 157.5, 138.4, 137.9, 136.8, 136.6, 128.8 (2C), 128.5, 124.6, 123.5, 120.2 (2C), 119.4, 118.5, 109.5. Anal. ($C_{16}H_9ClN_2OS$) C, H, N.

6-Cyano-*N*-(4′-methylphenyl)-3-chlorobenzo[*b*]thiophene-2-carboxamide (7b): from **1f** (1.0 g, 3.9 mmol) in dry toluene (40 mL) and *p*-methylaniline (1.25 g, 11.7 mmol) in dry toluene (40 mL) after refluxing for 2.5 h: 0.92 g (72.4%) of light brown crystals; mp 238−240 °C; IR (KBr) (ν_{max}/cm^{-1}) 3400, 2220, 1650, 1600; ^1H NMR (DMSO-d_6) (δ ppm) 10.63 (s, 1H, NH_{amide}), 8.81 (d, 1H, $J = 0.625$ Hz, $H_{arom.}$), 8.10 (d, 1H, $J = 8.42$ Hz, $H_{arom.}$), 7.98 (dd, 1H, $J_1 = 8.45$ Hz, $J_2 = 1.33$ Hz, $H_{arom.}$), 7.60 (d, 2H, $J = 8.30$ Hz, $H_{anilide}$), 7.20 (d, 2H, $J = 8.35$ Hz, $H_{anilide}$), 2.29 (s, 3H, CH_3); ^{13}C NMR (DMSO-d_6) (δ ppm) 158.3, 138.6, 137.0, 135.7, 133.9, 129.4 (2C), 128.9, 128.7, 123.6, 120.3 (2C), 119.5, 118.7, 109.6, 20.6. Anal. ($C_{17}H_{11}ClN_2OS$) C, H, N.

6-Cyano *N*-(4′-methoxyphenyl)-3-chlorobenzo[*b*]thiophene-2-carboxamide (7c): from **1f** (1.2 g, 4.7 mmol) in dry toluene (50 mL) and *p*-methoxyaniline (1.46 g, 11.9 mmol) in dry toluene (20 mL) after refluxing for 1.5 h: 0.95 g (58.7%) of yellow crystals; mp 211 °C; IR (KBr) (ν_{max}/cm^{-1}) 3400, 3040, 1650, 1600; ^1H NMR (DMSO-d_6) (δ ppm) 10.58 (s, 1H, NH_{amide}), 8.81 (d, 1H, $J = 1.24$ Hz, $H_{arom.}$), 8.10 (d, 1H, $J = 8.43$ Hz, $H_{arom.}$), 8.09 (dd, 1H, $J_1 = 8.46$ Hz, $J_2 = 1.34$ Hz, $H_{arom.}$), 7.46 (d, 2H, $J = 8.99$ Hz, $H_{anilide}$), 6.97 (d, 2H, $J = 9.02$ Hz, $H_{anilide}$), 3.76 (s, 3H, OCH_3); ^{13}C NMR (DMSO-d_6) (δ ppm) 158.4, 156.7, 139.0, 137.5, 137.0, 131.6, 129.3, 129.0, 124.0, 122.9 (2C), 119.8, 119.0, 114.5 (2C), 109.9, 55.7. Anal. ($C_{17}H_{11}ClN_2O_2S$) C, H, N.

6-Cyano-*N*-(4′-carbmethoxyphenyl)-3-chlorobenzo[*b*]thiophene-2-carboxamide (7d): from **1f** (0.86 g, 3.36 mmol) in dry THF (10 mL), methyl 4-aminobenzoate (1.12 g, 6.7 mmol) in dry THF (30 mL), and Et_3N (0.47 mL, 3.4 mmol) after refluxing for 2 h: 0.81 g (64.6%) of white crystals; mp 193−196 °C; IR (KBr) (ν_{max}/cm^{-1}) 3387, 2987, 2229, 1714, 1669, 1597; ^1H NMR (DMSO-d_6) (δ ppm) 11.05 (s, 1H, NH_{amide}), 8.84 (d, 1H, $J = 0.62$ Hz, $H_{arom.}$), 8.12 (d, 1H, $J = 8.46$ Hz, $H_{arom.}$), 8.01 (d, 1H, $J = 7.65$ Hz, $H_{arom.}$), 7.99 (d, 2H, $J = 8.46$ Hz, $H_{anilide}$), 7.87 (d, 2H, $J = 8.82$ Hz, $H_{anilide}$), 3.85 (s, 2H, $COOCH_3$); ^{13}C NMR (DMSO-d_6) (δ ppm) 166.2, 159.4, 142.8, 138.9, 137.2, 136.7, 130.8 (2C), 129.4, 129.1, 125.8, 124.1, 120.5, 120.1 (2C), 119.0, 110.4, 52.5. Anal. ($C_{18}H_{11}ClN_2O_3S$) C, H, N.

6-Cyano-*N*-(4′-bromophenyl)-3-chlorobenzo[*b*]thiophene-2-carboxamide (7e): from **1f** (1.0 g, 3.9 mmol) in dry toluene (50 mL) and *p*-bromoaniline (2.01 g, 11.7 mmol) in dry toluene (40 mL) after refluxing for 1.5 h: 1.05 g (68.8%) of light yellow crystals; mp 252−255 °C; IR (KBr) (ν_{max}/cm^{-1}) 3480, 2220, 1710, 1680, 1620; ^1H NMR (DMSO-d_6) (δ ppm) 10.85 (s, 1H, NH_{amide}), 8.82 (s, 1H, $H_{arom.}$), 8.10 (d, 1H, $J = 8.43$ Hz, $H_{arom.}$), 7.99 (d, 1H, $J = 8.44$ Hz, $H_{arom.}$), 7.695 (d, 1H, $J = 8.84$ Hz, $H_{anilide}$), 7.585 (d, 2H, $J = 8.42$ Hz, $H_{anilide}$); ^{13}C NMR (DMSO-d_6) (δ ppm) 158.9, 138.9, 137.9, 137.1, 136.9, 132.3 (2C), 129.4, 129.1, 124.1, 122.6 (2C), 119.0, 116.9, 110.1. Anal. ($C_{16}H_8BrClN_2OS$) C, H, N.

General Method for the Synthesis of 4′-Substituted-6-*N*′′-isopropylamidino-*N*-phenyl-3-chlorobenzo[*b*]thiophene-2-carboxamide hydrochloride (8a−e). Dry HCl was bubbled for 4 h into a suspension of 4′-substituted-6-*N*′′-cyano *N*-phenyl-3-chlorobenzo[*b*]thiophene-2-carboxamide **(7a−e)** in absolute ethanol. The suspension was stirred at room temperature until the −CN band was undetectable (IR). After anhydrous diethyl ether was added, the corresponding imino ether was collected by filtration. The product was suspended in absolute ethanol (20 mL), isopropylamine was added, and the mixture was refluxed for 17−27 h. The volume was reduced by evaporation, and the product was filtered off and washed with hot acetone.

6-*N*′′-Isopropylamidino-*N*-phenyl-3-chlorobenzo[*b*]thiophene-2-carboxamide hydrochloride (8a): from **7a** (1.00 g, 3.20 mmol) in absolute EtOH (65 mL), which gave the corresponding imino ether after 14 days. After addition of isopropylamine (1.3 mL, 15.30 mmol) the mixture was refluxed for 20 h. The crude product was recrystallized from ethanol to yield 0.61 g (49.6%) of white crystals: mp 260−264 °C; IR (KBr) (ν_{max}/cm^{-1}) 3420, 3200, 3020, 1660, 1590; UV (methanol) (λ_{max}/nm) 262, 350, 366; ^1H NMR (DMSO-d_6) (δ ppm) 10.74 (s, 1H, NH_{amide}), 9.50 (bs, 3H, $NH_{amidine}$), 8.62 (s, 1H, $H_{arom.}$), 8.14 (d, 1H, $J = 8.45$ Hz, $H_{arom.}$), 7.89 (d, 1H, $J = 8.47$ Hz, $H_{arom.}$), 7.73 (d, 2H, $J = 7.90$ Hz, $H_{2',6'}$), 7.41 (t, 2H, $J = 7.53$ Hz, $H_{3',5'}$), 7.18 (t, 1H, $J = 7.28$ Hz, $H_{4'}$), 4.11−4.08 (m, 1H, $J = 5.99$ Hz, CH_{i-pr}), 1.31 (d, 6H, $J = 6.09$ Hz, $2CH_{3\,i-pr}$); ^{13}C NMR (DMSO-d_6) (δ ppm) 165.2, 157.6, 141.3, 138.7, 138.1, 137.6, 135.3, 129.1, 128.8, 128.3, 125.9, 125.4, 123.5, 122.8, 120.2, 117.1, 116.8, 45.7, 21.6 (2C). Anal. ($C_{19}H_{19}Cl_2N_3OS$) C, H, N.

6-*N*′′-Isopropylamidino-*N*-(4′-methylphenyl)-3-chlorobenzo[*b*]thiophene-2-carboxamide hydrochloride (8b): from **7b** (0.35 g, 1.10 mmol) in absolute EtOH (70 mL), which gave the corresponding imino ether after 12 days. After addition of isopropylamine (4.6 mL, 53.60 mmol) the mixture was refluxed for 17 h. The crude product was recrystallized from methanol to yield 0.38 g (81.9%) of yellow crystals: mp 286−290 °C; IR (KBr) (ν_{max}/cm^{-1}) 3400, 3160, 3000, 1660, 1600; ^1H NMR (DMSO-d_6) (δ ppm) 10.55 (s, 1H, NH_{amide}), 9.55 (bs, 3H, $NH_{amidine}$), 8.60 (s, 1H, $H_{arom.}$), 8.12 (d, 1H, $J = 8.50$ Hz, $H_{arom.}$), 7.90 (d, 1H, $J = 5.56$ Hz, $H_{arom.}$), 7.615 (d, 2H, $J = 8.21$ Hz, $H_{anilide}$), 7.21 (d, 2H, $J = 8.37$ Hz, $H_{anilide}$), 4.16−4.11 (m, 1H, $J = 6.36$ Hz, CH_{i-pr}), 2.31 (s, 3H, CH_3), 1.32 (d, 6H, $J = 6.34$ Hz, $2CH_{3\,i-pr}$); ^{13}C NMR (DMSO-d_6) (δ ppm) 161.4, 158.4, 138.6, 136.2, 136.1, 135.7, 133.7, 129.3 (2C), 128.0, 125.7, 124.6, 122.7, 120.3 (2C), 119.3, 45.3, 21.3 (2C), 20.6. Anal. ($C_{20}H_{21}Cl_2N_3OS$) C, H, N.

6-*N*′′-Isopropylamidino *N*-(4′-methoxyphenyl)-3-chlorobenzo[*b*]thiophene-2-carboxamide hydrochloride (8c): from **7c** (0.45 g, 1.30 mmol) in absolute EtOH (80 mL), which gave the corresponding imino ether after 10 days. After addition of isopropylamine (4.5 mL, 52.50 mmol) the mixture was refluxed for 20 h. The crude product was recrystallized from methanol to yield 0.39 g (67.5%) of yellow crystals: mp 264−267 °C; IR (KBr) (ν_{max}/cm^{-1}) 3400, 3200, 3000, 1650, 1600; ^1H NMR (DMSO-d_6) (δ ppm) 10.57 (s, 1H, NH_{amide}), 9.73−9.40 (bs, 3H, $NH_{amidine}$), 8.61 (s, 1H, $H_{arom.}$), 8.135 (d, 1H, $J = 8.50$ Hz, $H_{arom.}$), 7.89 (d, 1H, $J = 8.51$ Hz, $H_{arom.}$), 7.65 (d, 2H, $J = 8.66$ Hz, $H_{anilide}$), 6.98 (d, 2H, $J = 8.71$ Hz, $H_{anilide}$), 4.12−4.03 (m, 1H, $J = 6.22$ Hz, CH_{i-pr}), 3.76 (s, 3H, OCH_3), 1.31 (d, 6H, $J = 6.26$ Hz, $2CH_{3\,i-pr}$); ^{13}C NMR (DMSO-d_6) (δ ppm) 161.4, 158.1, 138.6, 136.2 (2C), 131.1, 128.1, 125.7, 124.5, 122.7, 121.9 (2C), 119.1, 114.0 (2C), 55.2, 45.3, 21.3 (2C). Anal. ($C_{20}H_{21}Cl_2N_3O_2S$) C, H, N.

6-*N*′′-Isopropylamidino-*N*-(4′-carbmethoxyphenyl)-3-chlorobenzo[*b*]thiophene-2-carboxamide hydrochloride

(8d): from **7d** (0.49 g, 1.3 mmol) in absolute ethanol (70 mL), which gave the corresponding imino ether after 30 days. After addition of isopropylamine (4 mL, 0.047 mol), the mixture was refluxed for 3 h. The crude product was recrystallized from methanol to yield 0.23 g (38%) of white crystals: mp 193−196 °C; IR (KBr) (ν_{max}/cm^{-1}) 3460, 3000, 1710, 1660, 1590; ^{1}H NMR (DMSO-d_6) (δ ppm) 11.06 (s, 1H, NH_{amide}), 9.78 (d, 1H, J = 7.76 Hz, $NH_{amidine}$), 9.58 (s, 1H, $NH_{amidine}$), 9.21 (s, 1H, $NH_{amidine}$), 8.56 (s, 1H, J = 0.91 Hz, $H_{arom.}$), 8.08 (d, 1H, J = 8.45 Hz, $H_{arom.}$), 7.94 (dd, 2H, J_1 = 8.95 Hz, J_2 = 2.29 Hz, $H_{anilide}$), 7.83 (dd, 1H, J_1 = 8.33 Hz, J_2 = 1.58 Hz, $H_{arom.}$), 7.82 (d, 2H, J = 8.68 Hz, $2H_{anilide}$), 4.04−4.02 (m, 1H, J = 6.27 Hz, CH_{i-pr}), 3.78 (s, 3H, $COOCH_3$), 1.28 (d, 6H, J = 6.41 Hz, $2CH_{3\ i-pr}$); ^{13}C NMR (DMSO-d_6) (δ ppm) 165.6, 161.4, 158.9, 142.3, 138.4, 136.3, 135.4, 130.2 (2C), 128.1, 125.6, 125.2, 124.5, 122.7, 119.9, 119.6 (2C), 51.9, 45.2, 21.2 (2C). Anal. ($C_{21}H_{21}Cl_2N_3O_3S$) C, H, N.

6-*N*′′-Isopropylamidino-*N*-(4′-bromophenyl)-3-chlorobenzo[*b*]thiophene-2-carboxamide hydrochloride (8e): from **7e** (0.43 g, 1.10 mmol) in absolute EtOH (50 mL), which gave the corresponding imino ether after 17 days. After addition of isopropylamine (4.6 mL, 53.60 mmol), the mixture was refluxed for 27 h. The crude product was recrystallized from methanol to yield 0.33 g (61%) of yellow crystals: mp 275−278 °C; IR (KBr) (ν_{max}/cm^{-1}) 3380, 3040, 1670, 1630, 1590; ^{1}H NMR (DMSO-d_6) (δ ppm) 10.88 (s, 1H, NH_{amide}), 9.57 (bs, 3H, $NH_{amidine}$), 8.62 (s, 1H, $H_{arom.}$), 8.14 (d, 1H, J = 8.52 Hz, $H_{arom.}$), 7.90 (dd, 1H, J_1 = 7.97 Hz, J_2 = 1.30 Hz, $H_{arom.}$), 7.20 (d, 2H, J = 8.83 Hz, $H_{anilide}$), 7.60 (d, 2H, J = 8.88 Hz, $H_{anilide}$), 4.12−4.06 (m, 1H, J = 6.17 Hz, CH_{i-pr}), 1.31 (d, 6H, J = 6.36 Hz, $2CH_{3\ i-pr}$); ^{13}C NMR (DMSO-d_6) (δ ppm) 161.9, 159.1, 138.9, 137.9, 136.8, 136.1, 132.2 (2C), 128.7, 126.2, 125.0, 123.3, 122.7 (2C), 120.2, 116.9, 45.7, 21.7 (2C). Anal. ($C_{19}H_{18}BrCl_2N_3OS$) C, H, N.

General Method for the Synthesis of 2-Substituted-9-(*N*′-isopropylamidino)-6-oxo-5,6-dihydro[1]benzothieno-[2,3-*c*]quinoline hydrochloride (9a−d). A solution of 4′-substituted-6-*N*′′-isopropylamidino-*N*-phenyl-3-chlorobenzo[*b*]thiophene-2-carboxamide hydrochloride (**8a,b,d,e**) in methanol or water was irradiated at room temperature with a 400-W high-pressure mercury lamp using a Pyrex filter for 1−14 h. The air was bubbled through the solution. The solution was concentrated and the obtained solid was filtered off and washed with acetone.

9-(*N*′-Isopropylamidino)-6-oxo-5,6-dihydro[1]benzothieno[2,3-*c*]quinoline hydrochloride (9a). Method a. A solution of **8a** (0.12 g, 0.29 mmol) in methanol (10 mL) was irradiated for 1 h. The formed precipitate was filtered off and recrystallized from ethanol to yield 0.082 g (74.5%) of white crystals.

Method b. A solution of **8a** (0.10 g, 0.25 mmol) in distilled water (13 mL) was irradiated for 2 h. The formed precipitate was filtered off and recrystallized from ethanol to yield 0.085 g (93%) of white crystals: mp >300 °C; IR (KBr) (ν_{max}/cm^{-1}) 3040, 1630; ^{1}H NMR (DMSO-d_6) (δ ppm) 12.46 (s, 1H, $NH_{quinolone}$), 9.87 (d, 1H, J = 6.06 Hz, $NH_{amidine}$), 9.67 (s, 1H, $NH_{amidine}$), 9.32 (s, 1H, $NH_{amidine}$), 9.14 (d, 1H, J = 8.53 Hz, $H_{arom.}$), 8.82 (d, 1H, J = 8.08 Hz, $H_{arom.}$), 8.72 (d, 1H, J = 1.51 Hz, $H_{arom.}$), 7.95 (dd, 1H, J_1 = 8.60 Hz, J_2 = 1.52 Hz, $H_{arom.}$), 7.47−7.43 (m, 1H, $H_{arom.}$), 6.64−6.62 (m, 2H, $H_{arom.}$), 4.15−4.12 (m, 1H, J = 6.98 Hz, CH_{i-pr}), 1.33 (d, 6H, J = 6.22 Hz, $2CH_{3\ i-pr}$); ^{13}C NMR (DMSO-d_6) (δ ppm) 161.5, 157.6, 140.9, 138.5, 137.7, 135.4, 134.9, 129.3, 127.9, 126.0, 125.3, 124.8, 123.7, 122.9, 117.1, 116.9, 45.3, 21.2 (2C). Anal. ($C_{19}H_{18}ClN_3OS$) C, H, N.

2-Methyl-9-(*N*′-isopropylamidino)-6-oxo-5,6-dihydro[1]benzothieno[2,3-*c*]quinoline hydrochloride (9b). A solution of **8b** (0.10 g, 0.24 mmol) in methanol (11 mL) was irradiated for 14 h. The formed precipitate was filtered off and recrystallized from methanol to yield 0.035 g (38%) of yellow crystals: mp >300 °C; IR (KBr) (ν_{max}/cm^{-1}) 3000, 1620; ^{1}H NMR (DMSO-d_6) (δ ppm) 12.37 (bs, 1H, $NH_{quinolone}$), 9.62 (bs, 3H, $NH_{amidine}$), 9.18 (d, 1H, J = 8.67 Hz, $H_{arom.}$), 8.97 (s, 1H, $H_{arom.}$), 8.56 (s, 1H, $H_{arom.}$), 7.945 (d, 1H, J = 8.51 Hz, $H_{arom.}$), 7.51 (d, 1H, J = 8.36 Hz, $H_{arom.}$), 7.45 (d, 1H, J = 8.42 Hz, $H_{arom.}$), 4.16−4.12 (m, 1H, J = 6.09 Hz, CH_{i-pr}), 2.55 (s, 3H, CH_3), 1.34 (d, 6H, J = 6.21 Hz, $2CH_{3\ i-pr}$); ^{13}C NMR (DMSO-d_6) (δ ppm) 161.6, 157.5, 140.9, 138.6, 135.8, 135.3, 135.1, 132.4, 130.6, 127.9, 126.3, 125.4, 124.8, 123.1, 117.1, 116.9, 45.4, 21.3 (2C), 20.9. Anal. ($C_{20}H_{20}ClN_3OS$) C, H, N.

2-Carbmethoxy-9-(*N*′-isopropylamidino)-6-oxo-5,6-dihydro[1]benzothieno[2,3-*c*]quinoline hydrochloride (9c). A solution of **8d** (0.10 g, 0.22 mmol) in methanol (14 mL) was irradiated for 2 h and 10 min. The formed precipitate was filtered off and recrystallized from methanol to yield 0.059 g (63%) of white crystals: mp 280−283 °C; IR (KBr) (ν_{max}/cm^{-1}) 3260, 3040, 3960, 1710, 1620; ^{1}H NMR (DMSO-d_6) (δ ppm) 12.78 (s, 1H, $NH_{quinolone}$), 9.68 (bs, 3H, $NH_{amidine}$), 9.16 (s, 1H, $H_{arom.}$), 8.83 (d, 1H, J = 8.77 Hz, $H_{arom.}$), 8.75 (s, 1H, $H_{arom.}$), 8.14 (dd, 1H, J_1 = 8.57 Hz, J_2 = 0.96 Hz, $H_{arom.}$), 8.06 (d, 1H, J = 8.47 Hz, $H_{arom.}$), 7.67 (d, 1H, J = 8.64 Hz, $H_{arom.}$), 4.16−4.12 (m, 1H, J = 6.30 Hz, CH_{i-pr}), 3.95 (s, 3H, $COOCH_3$), 1.34 (d, 6H, J = 6.28 Hz, $2CH_{3\ i-pr}$); ^{13}C NMR (DMSO-d_6) (δ ppm) 167.0, 162.6, 159.1, 142.3, 142.2, 139.3, 137.0, 136.0, 130.7, 129.4, 127.0, 126.4, 126.3, 125.9, 125.0, 118.5, 117.9, 53.7, 46.6, 22.5 (2C). Anal. ($C_{21}H_{20}ClN_3O_3S$) C, H, N.

2-Bromo-9-(*N*′-isopropylamidino)-6-oxo-5,6-dihydro[1]-benzothieno[2,3-*c*]quinoline hydrochloride (9d). A solution of **8e** (0.10 g, 0.21 mmol) in methanol (13 mL) was irradiated for 1.5 h. The formed precipitate was filtered off and recrystallized from ethanol to yield 0.059 g (62.5%) of white crystals: mp >300 °C; IR (KBr) (ν_{max}/cm^{-1}) 3000, 1630; ^{1}H NMR (DMSO-d_6) (δ ppm) 12.57 (bs, 1H, $NH_{quinolone}$), 9.71 (bs, 3H, $NH_{amidine}$), 9.06 (d, 1H, J = 8.71 Hz, $H_{arom.}$), 8.79 (d, 1H, J = 1.50 Hz, $H_{arom.}$), 8.71 (s, 1H, $H_{arom.}$) 7.95 (d, 1H, J = 8.60 Hz, $H_{arom.}$), 7.80 (dd, 1H, J_1 = 8.83 Hz, J_2 = 1.31 Hz, $H_{arom.}$), 7.56 (d, 1H, J = 8.81 Hz, $H_{arom.}$), 4.15−4.11 (m, 1H, J = 6.36 Hz, CH_{i-pr}), 1.36 (d, 6H, J = 6.31 Hz, $2CH_{3\ i-pr}$). ^{13}C NMR (DMSO-d_6) (δ ppm) 161.3, 157.3, 140.7, 137.9, 136.8, 136.0, 133.9, 131.9, 127.9, 125.8, 125.4, 125.2, 124.7, 118.9, 118.5, 114.9, 45.3, 21.2 (2C). Anal. ($C_{19}H_{17}BrClN_3OS$) C, H, N.

Interactions with DNA and RNA. The electronic absorption spectra were obtained on Varian Cary 100 Bio spectrometer, and fluorescence emission spectra were recorded on Varian Eclipse fluorimeter, in both cases using quartz cuvettes (1 cm).

Polynucleotides were purchased from Sigma and Aldrich and used without further purification. Polynucleotides were dissolved in the sodium cacodylate buffer, 0.05 mol dm^{-3}, pH = 7, and their concentration was determined spectroscopically as the concentration of phosphates. The measurements were performed in aqueous buffer solution (pH = 6.2; sodium cacodilate/HCl buffer, I = 0.05 mol dm^{-3}). Under the experimental conditions used, the absorbance and fluorescence intensities of studied compounds were proportional to their concentrations. In fluorimetric titrations, excitation wavelengths of 310 nm (**5d**, **8d**) and 370 nm (**6d**, **9c**) were used to avoid inner filter effects caused by absorption of excitation light of added polynucleotides, and changes of emission were monitored at 400 nm. The stability constants (K_s) and [bound compound]/[polynucleotide phosphate] ratio (n) were calculated according to the Scatchard equation[28] by nonlinear least-squares fitting.[29] Values for K_s and n given in Table 2 all have satisfying correlation coefficients (>0.999). Thermal melting curves for DNA, RNA, and their complexes were determined as previously described[34] by following the absorption change at 260 nm as a function of temperature. The absorbance of the ligands was subtracted from every curve, and the absorbance scale was normalized. T_m values are the midpoints of the transition curves, determined from the maximum of the first derivative or graphically by a tangent method.[34] ΔT_m values were calculated by subtracting T_m of the free nucleic acid from T_m of the complex. Every ΔT_m value here reported was the average of at least two measurements, the error in ΔT_m is ± 0.5 °C.

Antitumor Activity Assays. The HeLa (cervical carcinoma), MCF-7 (breast carcinoma), SW 620 (colon carcinoma),

MiaPaCa-2 (pancreatic carcinoma), Hep-2 (laryngeal carcinoma), and WI 38 (diploid fibroblasts) cells were cultured as monolayers and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin in a humidified atmosphere with 5% CO_2 at 37 °C.

The growth inhibition activity was assessed according to the slightly modified procedure performed at the National Cancer Institute, Developmental Therapeutics Program.[31] The cells were inoculated onto standard 96-well microtiter plates on day 0. The cell concentrations were adjusted according to the cell population doubling time (PDT): 1×10^4/mL for HeLa, Hep-2, MiaPaCa-2, and SW 620 cell lines (PDT = 20−24 h); 2×10^4/mL for MCF-7 cell lines (PDT = 33 h); and 3×10^4/ml for WI 38 (PDT = 47 h). Test agents were then added in five 10-fold dilutions (10^{-8} to 10^{-4} mol/L) and incubated for a further 72 h. Working dilutions were freshly prepared on the day of testing. The solvent was also tested for eventual inhibitory activity by adjusting its concentration to be the same as in working concentrations. After 72 h of incubation, the cell growth rate was evaluated by performing the MTT assay,[32] which detects dehydrogenase activity in viable cells. The absorbency (OD, optical density) was measured on a microplate reader at 570 nm. The percentage of growth (PG) of the cell lines was calculated according to one or the other of the following two expressions:

$$\text{If } (\text{mean OD}_{\text{test}} - \text{mean OD}_{\text{tzero}}) = 0, \text{ then}$$

$$\text{PG} = 100(\text{mean OD}_{\text{test}} - \text{mean OD}_{\text{tzero}})/(\text{mean OD}_{\text{ctrl}} - \text{meanOD}_{\text{tzero}})$$

$$\text{If } (\text{mean OD}_{\text{test}} - \text{mean OD}_{\text{tzero}}) < 0, \text{ then}$$

$$\text{PG} = 100(\text{mean OD}_{\text{test}} - \text{mean OD}_{\text{tzero}})/\text{OD}_{\text{tzero}}$$

where mean OD_{tzero} = the average of optical density measurements before exposure of cells to the test compound, mean OD_{test} = the average of optical density measurements after the desired period of time, and mean OD_{ctrl} = the average of optical density measurements after the desired period of time with no exposure of cells to the test compound.

Each test point was performed in quadruplicate in three individual experiments. The results are expressed as IC_{50}, which is the concentration necessary for 50% inhibition. The IC_{50} values for each compound are calculated from dose−response curves using linear regression analysis by fitting the test concentrations that give PG values above and below the reference value (i.e. 50%). If, however, for a given cell line all of the tested concentrations produce PGs exceeding the respective reference level of effect (e.g. PG value of 50), then the highest tested concentration is assigned as the default value, which is preceded by a ">" sign. Each result is a mean value from three separate experiments.

Cell Cycle Analysis. A total of 1×10^6 cells were seeded per 100 mm plate. After 24 h the tested compounds were added at concentrations of 5×10^{-6} and 10^{-5} mol/L. After the desired length of time, the attached cells were trypsinized, combined with floating cells, washed with phosphate buffer saline (PBS), and fixed with 70% ethanol. Immediately before the analysis, the cells were washed with PBS and stained with 1 μg/mL of propidium iodide (PI) with the addition of 0.2 μg/μL of RNAse A. The stained cells were then analyzed with a Becton Dickinson FACScalibur flow cytometer (20 000 counts were measured). The percentage of the cells in each cell cycle phase was determined using the WinMDI software based on the DNA histograms. Statistical analysis was performed in SigmaStat 2.0 by using the one-way ANOVA test.

Annexin-V Assay. Detection and quantification of apoptotic cells at the single-cell level was performed using an Annexin-V−FLUOS staining kit (Roche), according to the manufacturer's recommendations. After the desired length of time, both floating and attached cells were collected. The cells were then washed with PBS, pelleted, and resuspended in staining-solution (Annexin-V−fluorescein labeling reagent and propidium iodide (PI) in Hepes buffer). The cells were then analyzed under a fluorescence microscope. Annexin-V (green fluorescent) cells were determined to be apoptotic and Annexin-V and PI cells were determined to be necrotic. The percentage of apoptotic cells was expressed as the number of fluorescent cells in relation to the total cell number (fluorescent and nonfluorescent cells), which was expressed as 100%.

Acknowledgment. Support for this study by the Ministry of Science, Education and Sport of Croatia is gratefully acknowledged (Projects 0125005, 00981104, 0119633, 0098053).

Supporting Information Available: Elemental analysis of novel compounds, X-ray crystal structure analysis, and selected crystallographic data. This material is available free of charge via the Internet at http://pubs.acs.org.

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JM049541F