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Novel cyano- and amidino-substituted derivatives of thieno[2,3-*b*]- and thieno[3,2-*b*]thiophene-2-carboxanilides and thieno[3′,2′:4,5]thieno- and thieno[2′,3′:4,5]thieno [2,3-*c*]quinolones: Synthesis, photochemical synthesis, DNA binding, and antitumor evaluation

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Abstract—A series of cyano- and amidino-substituted derivatives of thieno[2,3-*b*]- and thieno[3,2-*b*]thiophene-2-carboxanilides and their 'cyclic' derivatives (quinolones) were synthesized. 'Cyclic' compounds displayed a rather strong and differential antiproliferative effect on various cell lines, while the 'acyclic' amidino-substituted compounds were much more active, but showing mostly non-differential cytotoxicity, whereas cyano-substituted compounds (**2a**,**b**) produced a strikingly strong effect selectively on HeLa and Hep-2 cell lines. Antiproliferative activity of 'cyclic' derivatives is very likely caused by intercalation into DNA, while their 'acyclic' analogues use other target(s) and/or mechanisms of action.

1. Introduction

Quinolone antibacterials are a class of potent broad-spectrum drugs that target the bacterial type II DNA topoisomerases; they have been developed most fully for clinical use in human medicine.[1] Besides, it was shown that they could also inhibit mammalian topoisomerase activity and tubulin polymerization and thus act as potent antitumor drugs as well. For instance, pyranoquinoline-2-ones were synthesized and evaluated for their in vitro cytotoxicity against a panel of human tumor cell lines,[2] while 2-arylquinazolinones displayed significant growth inhibitory action against tumor cell lines. Some of them were potent inhibitors of tubulin polymerization and displayed selective activity against P-gp-expressing epidermoid carcinoma of the nasopharynx.[3] Indolo-, pyrrolo-, and benzofuro-quinolones, and anilinoindoloquinolone derivatives were synthesized and evaluated in vitro against a 3-cell line panel consisting of MCF7, NCI-H460, and SF268. The results have shown that cytotoxicity decreases in the order of anilinoindoloquinolones > indoloquinolones > pyrroloquinolones > benzofuroquinolones.[4] Moreover, 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 irradiation in mammalian cells; all compounds showed higher activity than 8-MOP, without mutagenicity and skin phototoxicity.[5,6]

As part of our continuing search for potential anticancer drug candidates related to heterocyclic quinolones, we have prepared new 3-dimethylaminopropyl-substituted derivatives of thieno[3′,2′:4,5]thieno[2,3-*c*]quinolones[7]

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and benzo[*b*]thieno-quinolones,[8] and observed considerable antitumor activity on several human cell lines. Later on, a class of substituted benzo[*b*]thieno[2,3-*c*]quinolones containing an isopropylamidino substituent, either on the quinolone or benzo[*b*]thiophene part of the condensed benzo[*b*]thieno-quinolone molecule, was prepared.[9] No significant difference in the antitumor activity regarding the position of the amidine substituent was noted for benzo[*b*]thieno[2,3-*c*] analogues. At the same time, benzo[*b*]thieno[2,3-*c*]quinolones showed strong, but more selective-antitumor activity compared to their 'acyclic' amidino-substituted precursors.[9] A 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 non-intercalative binding of the latter group of compounds.[9]

Proceeding in a systematic study of the structure–activity relationship (SAR), we have decided to prepare analogues of previously reported thieno[3′,2′:4,5]thieno[2,3-*c*]quinolones[7] with amidino-substituent positioned on the quinolone side (Fig. 1). Studies of the DNA interactions and in vitro antitumor activity of such compounds and of their precursors, when compared with previously observed results,[7] could clarify the impact of the position of the amidine substituent on monitored SAR parameters. In addition, such studies could elucidate the impact of variation of benzene vs. thiophene moiety if novel compounds are correlated with their previously reported benzo[*b*]thieno[2,3-*c*] analogues.[8,9]

In this work, we have prepared a new group of substituted thieno[2,3-*b*]thiophene-2-carboxanilides **2a**, **3a**, and **3b** and thieno[3,2-*b*]thiophene-2-carboxanilides **2b**, **3c**, and **3d** and their 'cyclic' derivatives, thieno[3′,2′:4,5]thieno[2,3-*c*]quinolones **4a**, **4b** and thieno[2′,3′:4,5]thieno[2,3-*c*]quinolones **4c**, **4d**, bearing a cyano, amidino or isopropylamidino substituent on the anilide or quinolone ring (Fig. 1). DNA binding as well as their antitumor activity were evaluated.

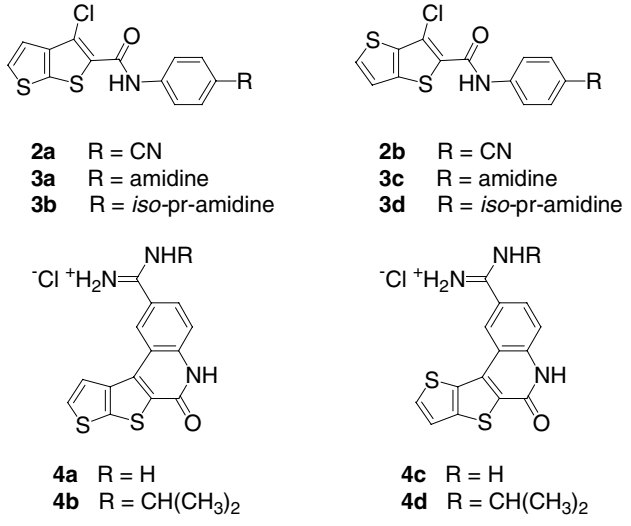


Figure 1. Substituted 3-chlorothieno[2,3-*b*]thiophene-2-carboxamides (**2a**, **3a**, and **3b**), 3-chlorothieno[3,2-*b*]thiophene-2-carboxamide (**2b**, **3c**, and **3d**), 6-oxo-5,6-dihydrothieno[3′,2′:4,5]thieno[2,3-*c*]quinolones **4a**, **4b**, and 6-oxo-5,6-dihydrothieno[2′,3′:4,5]thieno[2,3-*c*]quinolones **4c**, **4d**.

2. Results and discussion

2.1. Chemistry

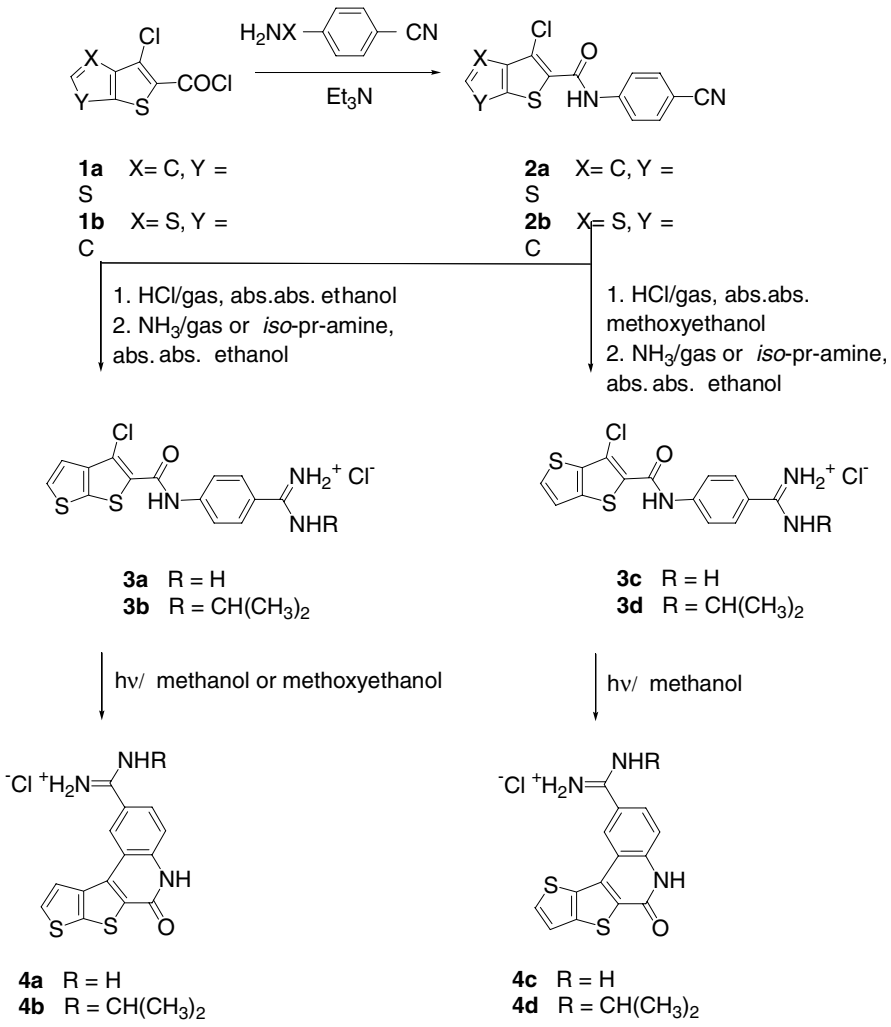
All compounds (**2a**–**4d**) shown in Figure 1 were prepared according to Scheme 1, starting from 3-chlorothieno[2,3-*b*]thiophene-2-carbonyl chloride **1a** and 3-chlorothieno[3,2-*b*]thiophene-2-carbonyl chloride **1b**, which were synthesized according to the literature procedure, in 46% and 11.3% overall yields from thiophene-2- and thiophene-3-aldehyde, respectively.[10] Novel derivatives of cyano-substituted 3-chlorothieno[2,3-*b*]thiophene-2-carboxamide **2a** and 3-chlorothieno[3,2-*b*]thiophene-2-carboxamide **2b** were prepared in the reaction of the corresponding carbonyl chlorides **1a** and **1b** with *p*-aminobenzonitrile in 70% and 60% yields, respectively. Cyano-substituted carboxanilides **2a** and **2b** were subsequently converted into amidino-substituted derivatives by a Pinner reaction.[11] Treatment of **2a** and **2b** with dry HCl gas in anhydrous ethanol or methoxy-ethanol afforded the corresponding imidate esters, which were refluxed either in methanolic ammonia or *iso*-propylamine in anhydrous ethanol, to yield *N*-4′-amidinophenyl carboxamide hydrochloride **3a**,**c** (87% and 85.2%, respectively) or *N*-4′-(*N*′-isopropylamidino)phenyl carboxamide hydrochloride **3b**,**d** (59% and 50%, respectively). 2-amidino-6-oxo-5,6-dihydrothieno[3′,2′:4,5]thieno[2,3-*c*]quinolines **4a**, **4b** and 2-amidino-6-oxo-5,6-dihydrothieno[2′,3′:4,5]thieno[2,3-*c*]quinolines **4c**, **4d** were prepared by the photochemical dehydrohalogenation reaction from the corresponding carboxamides **3a**–**d**. **4a** and **4b** were obtained after 1.5 h (**4a**; 48%) and 2 h 45 min (**4b**; 82%) irradiation with a high-pressure mercury lamp (400 W) using a Pyrex filter. Under the same conditions, **4c** and **4d** were obtained after 13 h (**4c**; 55%) and 19 h (**4d**; 70%) of irradiation.

2.2. Spectroscopic properties of compounds 2a–4d

Since application of spectrophotometric methods in the DNA binding studies was necessary, we characterized aqueous solutions of compounds by means of electronic absorption (UV/vis) and fluorescence emission spectra (compounds **4a**–**d**).

Absorbancies of aqueous solutions of compounds are proportional to their concentrations up to 2×10^{-5} mol dm^{-3} (**2a**), 3×10^{-5} mol dm^{-3} (**3a**, **3c**), 2.3×10^{-5} mol dm^{-3} (**3b**), 1.8×10^{-5} mol dm^{-3} (**2b**, **4a**), 1.2×10^{-5} mol dm^{-3} (**3d**), 4×10^{-5} mol dm^{-3} (**4b**), 3.3×10^{-5} mol dm^{-3} (**4c**), and 2.6×10^{-5} mol dm^{-3} (**4d**), indicating that there is no significant intermolecular stacking that would give rise to hypochromicity effects.

Conversion of cyano-substituted 3-chlorothieno[2,3-*b*]thiophene-2-carboxamide **2a** into amidino-substituted **3a** and **3b** resulted in bathochromic shifts and



Scheme 1.

increased ε values, while amidino-derivatives (**3c** and **3d**) of 3-chlorothieno[3,2-*b*]thiophene-2-carboxamide **2b** had increased ε values and lower absorption maxima than the corresponding cyano-derivative (Table 1). Cyclization of **3a**–**d** into 2-amidino-6-oxo-5,6-dihydrothieno[3′,2′:4,5]thieno[2,3-*c*]quinolines **4a**, **4b** and 2-amidino-6-oxo-5,6-dihydrothieno[2′,3′:4,5]thieno[2,3-*c*]quinolines **4c**, **4d** resulted in pronounced bathochromic shifts of the absorption maxima of 'acyclic' compounds **3a**–**d** (Table 2). Comparison of ε values and λ_{max} of absorption maxima strongly suggests that positioning of the sulfur atom within **4a**–**4d** and their 'acyclic' precursors, as well as the nature of the

Table 1. Electronic absorption data of **2a**,**b** and **3a**–**d**[a]

Compound	λ_{max} (nm)	$\varepsilon \times 10^3$ (dm^3 mol^{-1} cm^{-1})
2a	295	9.9
2b	321	10.4
3a	308	24.6
3b	308	25.7
3c	316	21.6
3d	315	25.3

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

Table 2. Electronic absorption data of **4a**–**d**[a]

Compound	λ_{max} (nm)	$\varepsilon \times 10^3$ (dm^{3}mol^{-1} cm^{-1})
4a	346	12.8
	331	12.5
	327	10.1
	266	37.4
	250	42.2
4b	345	9.1
	330	8.5
	316	6.3
	264	25.5
	249	31.1
4c	348	12.6
	333	12.9
	318	11.2
	272	24.0
	254	25.6
4d	348	18.0
	333	18.9
	317	16.6
	271	35.2
	253	38.8

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

substituent, have a systematic impact on electron absorption properties of studied compounds and probably electron distribution in the systems studied.

Compounds **4a–d** exhibit characteristic fluorescence emission with maxima at about 391 nm (**4a–b**), and 372 and 391 nm (**4c–d**); their excitation spectra are in good agreement with absorption spectra. Fluorescence intensities are proportional to their concentrations up to 5×10^{-6} mol dm^{-3} (**4b**, **4d**) and 2×10^{-6} mol dm^{-3} (**4a**, **4c**).

To have predominantly protonated amidines under the experimental conditions used (more than 99%), we performed our studies at pH 6.2. Aqueous solutions of all studied compounds have shown to be stable over longer periods of time.

2.3. Interactions of 'acyclic' derivatives 2a,b, 3a–d and their 'cyclic' analogues 4a–d with double-stranded ctDNA

Two series of structurally related amidino-substituted 'acyclic' 3-chlorothienothiophene-2-carboxamides **3a–d** and 'cyclic' 6-oxo-5,6-dihydrothienothienyl[2,3-*c*]quinolines **4a–d** were examined for their potential antiproliferative effects. All of them possess a certain, but non-selective, antiproliferative effect with enhanced selectivity and activity upon introduction of a cyano substituent (**2a**, **2b**).

To shed more light on the structure–biological activity relationship, we have studied interactions of 'acyclic' cyano **2a–b**, and amidino and isopropylamidino derivatives **3a–d** and their 'cyclic' analogues **4a–d** with ctDNA. Since 'cyclic' derivatives contain a large aromatic moiety, they should form an intercalative complex with the double-stranded helix of ctDNA. 'Acyclic' derivatives possessing a distinctively smaller aromatic surface and a more flexible scaffold might not be able to intercalate the double-stranded helix of DNA. The mentioned structural differences could therefore cause different modes in their biological action.

Interactions of compounds **2a–b**, **3a–d** and **4a–d** with ctDNA were evaluated by thermal melting experiments and UV/vis and fluorimetric titrations.

DNA binding properties of compounds **2a–b**, **3a–d**, and **4a–d** were evaluated by thermal melting experiments at different ratios r([compound]/[polynucleotide phosphate]). Melting temperatures (T_m) did not change in the presence of 'acyclic' derivatives **2a**, **2b**, and **3a–d** at the ratio $r = 0.3$ (compounds in excess over the intercalation binding sites). On the other hand, addition of 'cyclic' derivatives **4a–d** stabilized the double helix of ctDNA (increased T_m) at all examined ratios r ($r = 0.1$–0.3), as shown in Table 3. Melting transitions were monophasic, pointing to only one dominant binding mode (Fig. 2). All studied compounds yielded a similar stabilization of ctDNA. Increase of ΔT_m values was not proportional to the increase of ratio r, suggesting saturation of binding sites within the values of $r = 0.2$–0.3.

Binding of 'cyclic' compounds **4a–d** that stabilized ctDNA was also studied by UV/vis and fluorescence spectroscopic titrations. Addition of ctDNA to **4a** ($c = 5 \times 10^{-5}$ mol dm^{-3}), **4b** ($c = 7.1 \times 10^{-5}$ mol dm^{-3}), **4c** ($c = 3.2 \times 10^{-5}$ mol dm^{-3}), and **4d** ($c = 3.5 \times 10^{-5}$ mol dm^{-3}) resulted in strong hypochromic (41% for **4a**; 46.6% for **4b**; 46.8% for **4c** and 42.1% for **4d**) and bathochromic effects (ca. 7 nm shift of the maximum for all compounds). The observed changes in UV/vis spectra strongly suggest intercalation as the dominant binding mode and high values of binding

Table 3. ΔT_m values[a] (°C) of ctDNA with **4a–d** at pH 6.2 (sodium cacodylate/HCl buffer, $I = 0.05$ mol dm^{-3})

Compound	r[b]	4a	4b	4c	4d
ctDNA	0.1	1.9	1.9	1.4	1.9
	0.2	2.4	2.2	1.7	2.5
	0.3	2.7	2.7	2.6	3.9

[a] Error in ΔT_m: ±0.5 °C.
[b] r = [compound]/[polynucleotide].

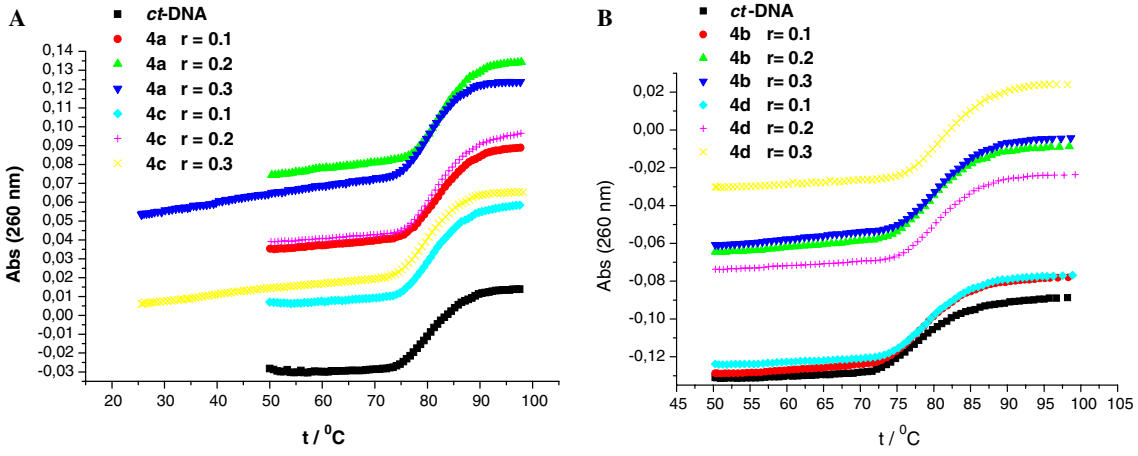


Figure 2. The normalized changes of absorbance at 260 nm ctDNA on heating in the presence of (A) **4a**, **c** and (B) **4b**, **d** at ratios r([compound]/[ctDNA]) = 0.1–0.3; sodium cacodylate/HCl buffer, $I = 0.05$ mol dm^{-3}, pH 6.2.

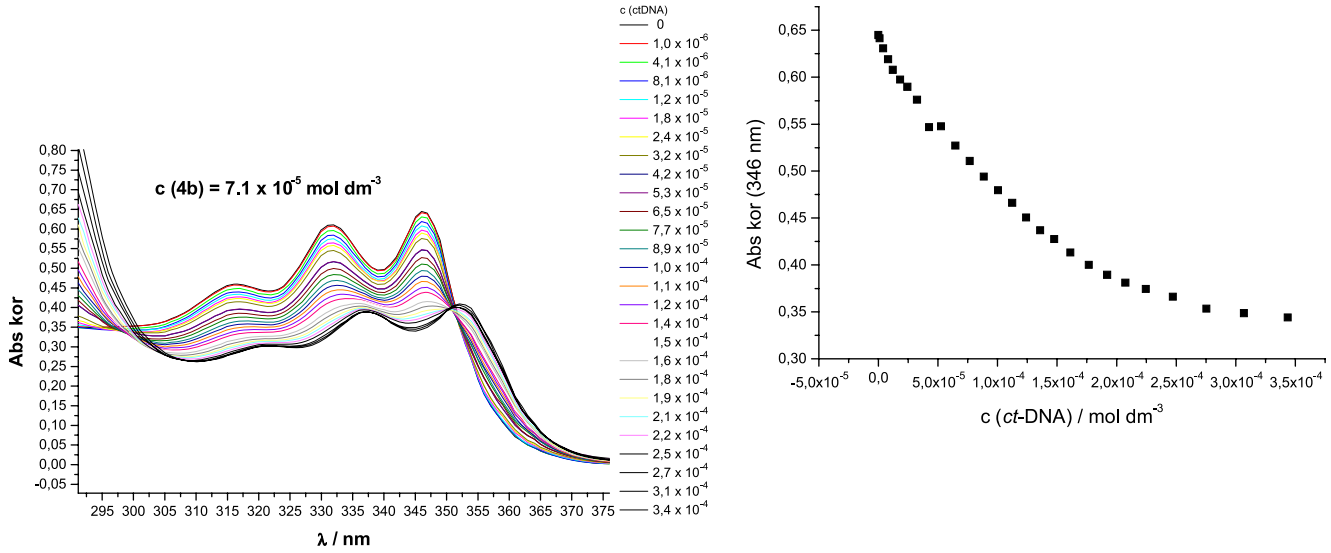


Figure 3. UV/vis spectral changes of **4b** ($c = 7.1 \times 10^{-5}$ mol dm^{-3}) upon the addition of ctDNA ($c = 6.3 \times 10^{-3}$ mol dm^{-3}); sodium cacodylate/HCl buffer, $I = 0.05$ mol dm^{-3}, pH 6.2.

constants, $K_s > 10^5$ M^{-1} (Fig. 3). However, a systematic deviation from the isosbestic point was observed for all compounds, pointing to the presence of more than one type of dominant complex. Since titration with ctDNA yielded measurable changes in absorbance only at excess of studied compounds, except for the possible intercalative mode of binding, excess of positively charged molecules could be bound on the outer polynucleotide surface. A similar effect of weaker, non-intercalative binding at a high intercalator/DNA ratio was observed for most of the classical intercalators.[12]

To make it possible to perform titrations under conditions preferable for only one type of binding, we have used strong fluorescence of aqueous solutions of **4a–d**, enabling titrations at up to 100 times lower concentrations than used in UV/vis experiments and at a high excess of the polynucleotide. Addition of *ct*-DNA strongly quenched emission of **4b–d** and caused less pronounced quenching of the emission of **4a** (Fig. 4). Increasing the amount of ctDNA, F/F_0 of **4a** reached ca. 0.69. Similar quenching was observed for **4b** and **4c** (F/F_0 ca. 0.24 and 0.17, respectively), while the complex of **4d** with ctDNA is emission inactive.

Processing of titration data by means of the Scatchard equation gave the binding constants ($\log K_s$) and ratios n([bound compound]/[polynucleotide phosphate]) presented in Table 4.

The calculated binding constants ($\log K_s$) point to somewhat higher affinity of unsubstituted amidine hydrochlorides **4a** and **4c** toward ctDNA compared to their isopropyl-substituted amidino analogues **4b** and **4d**.

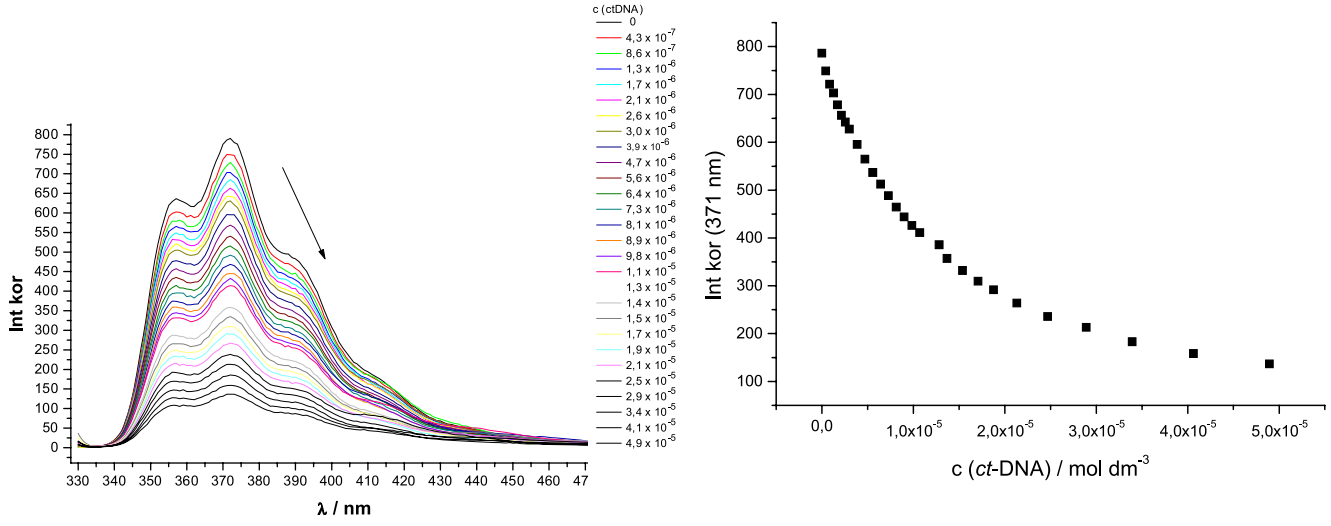


Figure 4. Fluorescence emission of **4d** ($c = 5 \times 10^{-7}$ mol dm^{-3}) upon the addition of ctDNA ($c = 2.58 \times 10^{-3}$ mol dm^{-3}); sodium cacodylate/HCl buffer, $I = 0.05$ mol dm^{-3}, pH 6.2.

Table 4. Binding constants ($\log K_s$) and ratios n([bound compound]/[polynucleotide]) calculated according to fluorimetric titrations of **4a–d** ($c = 5 \times 10^{-7}$ mol dm^{-3}) with ctDNA[a,b]

Compound	$\log K_s$	n	F/F_0[c]
4a	6.04	0.12	0.69
4b	5.53	0.3	0.24
4c	6.51	0.1	0.17
4d	5.73	0.18	—

[a] UV/vis titrations were performed at pH 6.2 (sodium cacodylate/HCl buffer, $I = 0.05$ mol dm^{-3}).
[b] Accuracy of $n \pm 30\%$, consequently varying values $\log K_s \pm 0.5$.
[c] F = fluorescence of compound–DNA complex; F_0 = fluorescence of compound without ctDNA addition.

Possible shielding of the positive charge of amidine in the latter compounds at least partially hampered additional stabilizing interactions with DNA phosphates. Position of the sulfur atom in the 6-oxo-5,6-dihydrothienothieno[2,3-*c*]quinoline moiety showed no significant impact on the affinity toward ctDNA. The calculated n values are in agreement with the concentration of intercalative binding sites and also in good agreement with the non-linear ΔT_m/ratio r dependency.

2.4. Biological results and discussion

Compounds **2a**–**4d** were tested for their potential antiproliferative effect on a panel of 6 human cell lines, 5 of which derived from 5 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). For comparison and as a control of correct functionality of the cell lines, three reference compounds (classical antitumor drugs) were used: cisplatin (alkylating agent), doxorubicin (intercalator), and etoposide (topoisomerase II inhibitor).

All tested compounds showed a certain antiproliferative effect (Table 5). Interestingly, although the compounds bearing a cyano substituent, **2a** and **2b**, did not generally show discernible antiproliferative effects, they produced strikingly strong effects selectively on HeLa and Hep-2 cell lines (Fig. 5A). A very similar effect was reported by Jarak et al.[9] using carboxanilides bearing a cyano substituent either on the anilide or benzothiophene part of the molecule. HeLa and Hep-2 cell lines have similar genetic backgrounds, both having the human papilloma virus (HPV) type 18 DNA integrated into their genomes. It has even been shown that the Hep-2 cells are HeLa cell contaminants/derivatives.[13] It is not clear, however, why these molecules are especially active in these cell lines, and further studies are needed to provide the basis for a new strategy in the antitumor therapy of HPV-related cancers.

Table 5. In vitro inhibition of the growth of tumor cells and normal human fibroblasts (WI 38)

Compound	IC_{50}[a] (μM)					
	Hep-2	HeLa	MiaPaCa-2	SW 620	MCF-7	WI 38
2a	0.7 ± 0.1	0.1 ± 0.01	36 ± 45	15.6 ± 7.8	>100	18.6 ± 30
2b	0.7 ± 0.01	0.2 ± 0.001	>100	76 ± 20	>100	⩾100
3a	3.8 ± 0.8	2.2 ± 0.08	1.6 ± 0.2	1.6 ± 0.1	3 ± 0.1	3.4 ± 1.5
3b	6.3 ± 1.3	14.5 ± 1	3.8 ± 0.03	5.5 ± 1.2	9.5 ± 3.6	1.8 ± 1.5
3c	2.7 ± 1.3	1.8 ± 0.001	1.9 ± 0.2	1.8 ± 0.1	1.9 ± 0.2	1.8 ± 0.5
3d	4.8 ± 0.6	3.8 ± 3.5	4.4 ± 2	2.7 ± 1.1	6.2 ± 9.7	1.8 ± 0.3
4a	20 ± 3.5	40 ± 14	34 ± 1.9	>100	11 ± 1.5	3.6 ± 2
4b	21 ± 0.3	28 ± 4	27.7 ± 4	36 ± 10	8.7 ± 2.8	10 ± 6.5
4c	21 ± 1.4	37 ± 9	30.5 ± 0.4	⩾100	21 ± 19	4.7 ± 0.8
4d	11 ± 5	14 ± 5	12 ± 2	18 ± 0.8	9.7 ± 6.5	6.4 ± 5
Cis	2.4 ± 0.3	2.9 ± 0.6	5.4 ± 1.6	4 ± 1.8	12 ± 6	19 ± 20
Dox	0.04 ± 0.01	0.04 ± 0.01	0.02 ± 0.01	0.02 ± 0.02	0.04 ± 0.01	0.1 ± 0.01
Eto	N.T.	2.9 ± 1	15.4 ± 14	20 ± 3.4	50 ± 30	N.T.

[a] IC_{50}, the concentration that causes a 50% reduction of the cell growth.
N.T., not tested; Cis, cisplatin; Dox, doxorubicin; Eto, etoposide.

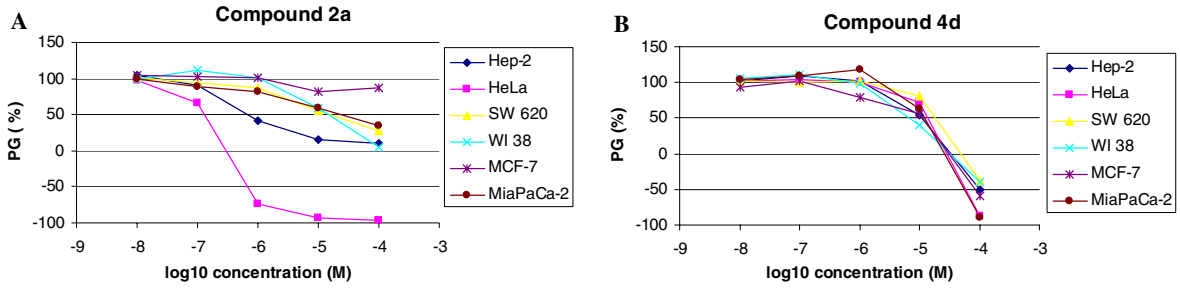


Figure 5. Dose–response profile for compounds **2a** (A) and **4d** (B) tested on various human cell lines in vitro. The cells were treated with the compounds **2a** and **4d** at different concentrations, and percentage of growth (PG) was calculated. Each point represents a mean value of four parallel samples in three individual experiments.

Similar intriguing results were also reported by Beneteau et al.[14] These authors described the synthesis of novel benzothiazoles and dioxinobenzothiazoles, among which the 2-cyano derivatives exhibit interesting in vitro antitumor activity, though on a different cell model (mouse leukemia cells, L1210). Therefore, the antiproliferative effect of cyano-substituted compounds **2a** and **2b** could be correlated with the similar effect of a related heteroaromatic system (benzothiazole-2-carbonitrile derivatives).[14] It is important to stress that the authors of the aforementioned study found that the conversion of nitrile in a number of other neutral or negatively charged substituents clearly inhibited antiproliferative activity. Some of the pathways they used are also possible under in vivo conditions if catalyzed by enzymes. This suggests that both herein studied **2a** and **2b**, and their benzothiazole analogues base their biological activity on interactions of the nitrile moiety with the molecular target(s), but not with the possible nitrile metabolic products.

In contrast, *N*-4′-amidinophenyl carboxamide hydrochlorides **3a**, **3c** and *N*-4′-(*N*′-isopropylamidino)phenyl carboxamide hydrochlorides **3b**, **3d** were much more active, but in general showing mostly 'non-differential' cytotoxicity at the highest concentrations tested. Thus, the introduction of a strong positively charged moiety resulted in a strong, but not selective (differential), antiproliferative activity, implying a different molecular target and/or mechanism of action.

Compounds **4a**, **4b**, **4c**, and **4d** display strong (**4d**) (Fig. 5B) and differential (**4a**–**c**) effects, however with a rather strong inhibitory effect on normal fibroblasts. As a result, in contrast to the results presented in our previous work,[9] where 'cyclic' analogues—benzo[*b*]thieno[2,3-*c*]qinolones displayed prominent and selective activity, the corresponding selectivity of their thieno[3′,2′:4,5]- and thieno[2′,3′:4,5]- analogues is not pronounced. Nevertheless, it has been demonstrated again that the introduction of the amidino substituent into the herein presented series of novel compounds led to the best antiproliferative effect on the cell lines tested.

3. Conclusion

Strong bathochromic and hypochromic effects in the UV/vis spectra of cyclic derivatives **4a**–**d** upon addition of ctDNA, thermal stabilization of ctDNA, the calculated characteristic values ratio *n* and high binding constants strongly support intercalation as a dominant binding mode.[15] Interactions of substituted amidine derivatives (**4b** and **4d**) with DNA are somewhat weaker compared to analogues bearing unsubstituted amidine (**4a** and **4c**), very probably due to steric hindering of the voluminous isopropyl moiety. 'Acyclic' precursors **2a**–**3d** do not interact significantly with the double-stranded helix of ctDNA.

Antiproliferative activity of 'cyclic' derivatives **4a**–**d** is very likely caused by intercalation into DNA, while their 'acyclic' analogues **3a**–**d** use some other pathway of action.

Intriguing antiproliferative effects of cyano-substituted compounds **2a** and **2b** on HeLa and Hep-2 cell lines could, similarly to the related heteroaromatic benzothiazole-2-carbonitrile derivatives,[14] base their biological activity on interactions of the nitrile moiety with the molecular target(s). Moreover, HeLa and Hep-2 cell lines have the human papilloma virus (HPV) type 18 DNA integrated into their genomes, and HPVs types 16 and 18 have oncogenic potential, attributed mainly to two small proteins called E6 and E7. Repression of E6 and E7 expression results in acquisition of the senescent phenotype and in the rescue of functional p53 and $p105^{Rb}$ pathways; therefore, therapies directed against either viral protein may be beneficial.[16,17] Compounds **2a** and **2b** could potentially inhibit the E6 and E7 expression, but further studies are needed to prove this assumption.

4. Experimental

4.1. Materials

All the solvents were of analytical grade. Melting points were determined on a Kofler hot stage microscope and are uncorrected. IR spectra were recorded on a Nicolet Magna 760 spectrophotometer in 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 analyses 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.

4.2. Synthesis

4.2.1. *N*-(4′-Cyanophenyl)-3-chlorothieno[2,3-*b*]thiophene-2-carboxamide (2a). To a solution of **1a** (2 g, 8.4 mmol) in dry toluene (90 ml) was added dropwise a solution of 4-aminobenzonitrile (1 g, 8.4 mmol) in dry toluene (60 ml), followed by the addition of Et_3N (1.4 ml, 10 mmol). The mixture was refluxed for 2.5 h. After cooling, precipitated crystals were filtered off and washed with diluted HCl, water, and acetone to yield 1.89 g (70%) of yellow crystals: mp 197–201 °C; IR (KBr) (ν_{max}/cm^{-1}): 3380, 3100, 2200, 1650, 1590; ^{1}H NMR (DMSO-d_6) (δ ppm): 10.61 (s, 1H, H_{amide}), 7.82 (d, 2H, J = 8.72 Hz, $H_{arom.}$), 7.78 (d, 2H, J = 8.75 Hz, $H_{arom.}$), 7.76 (d, 1H, J = 5.37 Hz, $H_{thiophene}$), 7.30 (d, 1H, J = 5.33 Hz, $H_{thiophene}$); ^{13}C NMR (DMSO-d_6) (δ ppm): 159.8, 144.4, 143.0, 138.8, 133.9, 133.7 (2C), 132.9, 120.7 (2C), 119.4, 119.2, 118.1, 106.4. Anal. Calcd

for ($C_{14}H_7ClN_2OS_2$): C, 52.74; H, 2.21; N, 8.79. Found: C, 52.69; H, 2.26; N, 8.72.

4.2.2. *N*-(4′-Cyanophenyl)-3-chlorothieno[3,2-*b*]thiophene-2-carboxamide (2b). To a solution of **1b** (1.48 g, 6.2 mmol) in dry toluene (50 ml) was added dropwise a solution of 4-aminobenzonitrile (0.74 g, 6.2 mmol) in dry toluene (50 ml), followed by the addition of Et_3N (1.3 ml, 9.3 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 DMF, 1.2 g (60%) of ivory crystals was obtained: mp 147–151 °C; IR (KBr) (ν_{max}/cm^{-1}): 3480, 2200, 1650, 1600; ^{1}H NMR (DMSO-d_6) (δ ppm): 10.66 (s, 1H, H_{amide}), 8.99 (d, 1H, J = 5.22 Hz, $H_{thiophene}$), 7.90 (d, 2H, J = 8.81 Hz, $H_{arom.}$), 7.83 (d, 2H, J = 8.75 Hz, $H_{arom.}$), 7.61 (d, 1H, J = 5.21 Hz, $H_{thiophene}$); ^{13}C NMR (DMSO-d_6) (δ ppm): 159.8, 143.0, 138.9, 138.6, 133.7 (2C), 132.9, 132.4, 122.1, 121.1 (2C), 120.7, 119.3, 106.5. Anal. Calcd for ($C_{14}H_7ClN_2OS_2$): C, 52.74; H, 2.21; N, 8.79. Found: C, 52.70; H, 2.11; N, 8.91.

4.2.3. *N*-(4′-Amidinophenyl)-3-chlorothieno[2,3-*b*]thiophene-2-carboxamide hydrochloride (3a). Into the suspension of **2a** (0.5 g, 1.55 mmol) in absolute ethanol (50 ml) dry HCl was bubbled for 4 h. The suspension was stirred at room temperature until the –CN band was undetectable (IR). After anhydrous diethyl ether was added, the corresponding imido-ester was collected by filtration. The product was suspended in absolute ethanol (35 ml) and dry NH_3 was bubbled into the suspension. The mixture was stirred at room temperature for 3 days. The crude product was filtered off and washed with hot acetone. After recrystallization from methanol, 0.48 g (87%) of white crystals was obtained: mp 247–251 °C; IR (KBr) (ν_{max}/cm^{-1}): 3345, 3094, 1681, 1644, 1601; ^{1}H NMR (DMSO-d_6) (δ ppm): 9.04 (br s, 4H, $H_{amidine}$), 7.87 (d, 2H, J = 8.81, $H_{arom.}$), 7.83 (d, 2H, J = 8.82, $H_{arom.}$), 7.82 (d, 1H, J = 5.3, $H_{thiophene}$), 7.34 (d, 1H, J = 5.3, $H_{thiophene}$); ^{13}C NMR (DMSO-d_6) (δ ppm): 164.4, 159.5, 140.0, 143.5, 138.1, 134.0, 132.3, 128.8 (2C), 123.6, 119.8, 118.7 (2C), 117.3. Anal. Calcd for ($C_{14}H_{11}Cl_2N_3OS_2$): C, 45.17; H, 2.98; N, 11.29. Found: C, 45.01; H, 2.86; N, 11.43.

4.2.4. *N*-[4′-(*N*′-Isopropylamidino)phenyl]-3-chlorothieno[2,3-*b*]thiophene-2-carboxamide hydrochloride (3b). Into the suspension of **2a** (0.5 g, 1.55 mmol) in absolute ethanol (50 ml), dry HCl was bubbled for 4 h. The suspension was stirred at room temperature until the –CN band was undetectable (IR). After anhydrous diethyl ether was added, the corresponding imido-ester was collected by filtration. The product was suspended in absolute ethanol (20 ml), and isopropylamine (13.8 ml, 162 mmol) was added. The mixture was refluxed for 24 h. The volume was reduced by evaporation and the product was filtered off and washed with hot acetone. After recrystallization from methanol, 0.36 g (59%) of white crystals was obtained: mp 305–307 °C; IR (KBr) (ν_{max}/cm^{-1}): 3360, 3037, 1672, 1658, 1614; ^{1}H NMR (DMSO-d_6) (δ ppm): 10.83 (s, 1H, H_{amide}), 9.47 (br s, 3H, $H_{amidine}$), 7.98 (d, 2H, J = 8.15 Hz, $H_{arom.}$), 7.83 (d, 1H, J = 5.095 Hz, $H_{thiophene}$), 7.77 (d, 2H, J = 8.24 Hz, $H_{arom.}$), 7.375 (d, 1H, J = 4.99 Hz, $H_{thiophene}$), 4.06 (m, 1H, J = 5.92 Hz, $CH_{i\text{-}Pr}$), 1.28 (d, 6H, J = 5.84 Hz, $2CH_{3i\text{-}Pr}$); ^{13}C NMR (DMSO-d_6) (δ ppm): 161.1, 159.4, 143.9, 142.7, 138.3, 133.6, 132.5, 129.3 (2C), 124.3, 119.7 (2C), 118.8, 117.5, 44.9, 21.3 (2C). Anal. Calcd for ($C_{17}H_{17}Cl_2N_3OS_2$): C, 49.27; H, 4.13; N, 10.14. Found: C, 48.95; H, 3.88; N, 10.04.

4.2.5. *N*-(4′-Amidinophenyl)-3-chlorothieno[3,2-*b*]thiophene-2-carboxamide hydrochloride (3c). Into the suspension of **2b** (0.5 g, 1.55 mmol) in absolute methoxy-ethanol (50 ml), dry HCl was bubbled for 4 h. The suspension was stirred at room temperature until the –CN band was undetectable (IR). After anhydrous diethyl ether was added, the corresponding imido-ester was collected by filtration. The product was suspended in absolute ethanol (25 ml) and dry NH_3 was bubbled into the suspension. The mixture was stirred at room temperature for 7 days. The volume was reduced by evaporation and anhydrous diethyl ether was added. The crude product was filtered off and washed with hot acetone. After recrystallization from methanol, 0.265 g (43%) of white crystals was obtained: mp +300 °C; IR (KBr) (ν_{max}/cm^{-1}): 3354, 3089, 1683, 1634; ^{1}H NMR (DMSO-d_6) (δ ppm): 10.76 (s, 1H, H_{amide}), 9.30 (s, 2H, $H_{amidine}$), 8.99 (s, 2H, $H_{amidine}$), 8.02 (d, 1H, J = 5.20 Hz, $H_{thiophene}$), 7.92 (d, 2H, J = 9.14 Hz, $H_{arom.}$), 7.87 (d, 2H, J = 9.08 Hz, $H_{arom.}$), 7.635 (d, 1H, J = 5.23 Hz, $H_{thiophene}$); ^{13}C NMR (DMSO-d_6) (δ ppm): 165.3, 159.8, 143.7, 138.9, 133.0, 132.4, 129.7 (2C), 123.3, 122.1, 120.2 (2C), 118.7. Anal. Calcd for ($C_{14}H_{11}Cl_2N_3OS_2$): C, 45.17; H, 2.98; N, 11.29. Found: C, 45.35; H, 2.72; N, 11.16.

4.2.6. *N*-[4′-(*N*′-Isopropylamidino)phenyl]-3-chlorothieno[3,2-*b*]thiophene-2-carboxamide hydrochloride (3d). Into the suspension of **2b** (0.5 g, 1.55 mmol) in absolute methoxy-ethanol (50 ml) dry HCl was bubbled for 4 h. The suspension was stirred at room temperature until the –CN band was undetectable (IR). After anhydrous diethyl ether was added, the corresponding imido-ester was collected by filtration. The product was suspended in absolute ethanol (25 ml) and isopropylamine (5 ml, 0.06 mol) was added. The mixture was refluxed for 18 h. After cooling, anhydrous diethyl ether was added. The crude product was filtered off and washed with hot acetone. After recrystallization from ethanol, 0.16 g (26%) of white crystals was obtained: mp +300 °C; IR (KBr) (ν_{max}/cm^{-1}): 3363, 3037, 1660, 1614; ^{1}H NMR (DMSO-d_6) (δ ppm): 10.14 (s, 1H, H_{amide}), 8.94 (d, 1H, J = 9.00 Hz, $H_{amidine}$), 8.79 (s, 1H, $H_{amidine}$), 8.41 (s, 1H, $H_{amidine}$), 7.47 (d, 1H, J = 5.32 Hz, $H_{thiophene}$), 7.37 (d, 2H, J = 8.67 Hz, $H_{arom.}$), 7.215 (d, 2H, J = 8.78 Hz, $H_{arom.}$), 7.09 (d, 1H, J = 5.18 Hz, $H_{thiophene}$), 3.47 (m, 1H, J = 6.43 Hz, $CH_{i\text{-}Pr}$), 1.28 (d, 6H, J = 6.28 Hz, $2CH_{3i\text{-}Pr}$); ^{13}C NMR (DMSO-d_6) (δ ppm): 116.1, 159.3, 142.6, 138.4, 138.1, 132.4, 131.9, 129.3 (2C), 124.2, 121.6, 119.7 (2C), 118.1, 44.9, 21.3 (2C). Anal. Calcd for ($C_{17}H_{17}Cl_2N_3OS_2$): C, 49.27; H, 4.13; N, 10.14. Found: C, 49.17; H, 4.27; N, 9.76.

4.2.7. General method for the synthesis of 2-amidino-6-oxo-5,6-dihydrothienothienyl[2,3-*c*]quinoline hydrochloride (4a–d). A solution of 4′-amidino-substituted *N*-phenyl-3-chlorothieno[2,3-*b*]thiophene-2-carboxamide hydrochloride (**3a**, **b**) and *N*-phenyl-3-chlorothieno[3,2-*b*]thiophene-2-carboxamide hydrochloride (**3c**, **d**) in methanol or methoxy-ethanol was irradiated at room temperature with a 400-W high-pressure mercury lamp using Pyrex filter for 1.5–19 h. The air was bubbled through the solution. The solution was concentrated and the obtained solid was filtered off, washed with hot acetone and recrystallized.

4.2.8. 2-Amidino-6-oxo-5,6-dihydrothieno[3′,2′:4,5]thienyl[2,3-*c*]quinilone hydrochloride (4a). A solution of **3a** (0.05 g, 0.13 mmol) in methoxy-ethanol (10 ml) was irradiated for 1.5 h. The formed precipitate was filtered off and recrystallized from methanol to yield 0.027 g (48%): mp +300 °C; IR (KBr) (ν_{max}/cm^{-1}): 3322, 3119, 1649, 1589; ^{1}H NMR (DMSO-d_6) (δ ppm): 12.47 (s, 1H, $H_{quinolone}$), 9.50 (s, 2H, $H_{amidine}$), 9.08 (s, 2H, $H_{amidine}$), 8.74 (d, 1H, J = 1.81 Hz, H_{arom}), 8.30 (d,1H, J = 5.54 Hz, $H_{thienyl}$), 8.02 (d, 1H, J = 5.42, $H_{thienyl}$), 7.97 (dd, 1H, J_1 = 8.73 Hz, J_2 = 1.92 Hz, H_{arom}), 7.67 (d, 1H, J = 8.7 Hz, H_{arom}); ^{13}C NMR (DMSO-d_6) (δ ppm):165.3, 158.3, 144.5, 141.7, 141.4, 135.3, 134.4, 132.4, 128.8, 124.9, 122.1, 122.0, 117.2, 116.5. Anal. Calcd for ($C_{14}H_{10}ClN_3OS_2$): C, 50.07; H, 3.00; N, 12.51. Found: C, 50.19; H, 3.12; N, 12.75.

4.2.9. 2-(*N*′-Isopropyl)amidino-6-oxo-5,6-dihydrothieno[3′,2′:4,5]thienyl[2,3-*c*]quinoline hydrochloride (4b). A solution of **3b** (0.1 g, 0.24 mmol) in methanol (14 ml) was irradiated for 2 h and 45 min. The formed precipitate was filtered off and recrystallized from ethanol to yield 0.075 g (82%): mp +300 °C; IR (KBr) (ν_{max}/cm^{-1}): 3394, 3109, 1652, 1622, 1584; ^{1}H NMR (DMSO-d_6) (δ ppm): 12.44 (s, 1H, $H_{quinolone}$), 9.63 (s, 3H, $H_{amidine}$), 8.69 (s, 1H, H_{arom}), 8.23 (d, 1H, J = 5.47 Hz, $H_{thienyl}$), 8.02 (d, 1H, J = 5.38 Hz, $H_{thienyl}$), 7.89 (d, 1H, J = 8.56 Hz, H_{arom}), 7.67 (d, 1H, J = 8.65 Hz, H_{arom}), 4.17–4.09 (1H, m, J = 6.29 Hz, $CH_{i\text{-}Pr}$), 1.34 (d, 6H, J = 6.30 Hz, $2CH_{3i\text{-}Pr}$); ^{13}C NMR (DMSO-d_6) (δ ppm): 161.8, 158.3, 144.5, 141.7, 140.9, 135.2, 134.3, 132.5, 129.1, 125.0, 123.3, 122.1, 116.9, 116.3, 45.6, 21.8 (2C). Anal. Calcd for ($C_{17}H_{16}ClN_3OS_2$): C, 54.03; H, 4.27; N, 11.11. Found: C, 54.24; H, 4.02; N, 11.21.

4.2.10. 2-Amidino-6-oxo-5,6-dihydrothieno[2′,3′:4,5]thienyl[2,3-*c*]quinoline hydrochloride (4c). A solution of **3c** (0.099 g, 0.27 mmol) in methanol (14 ml) was irradiated for 13 h. The formed precipitate was filtered off and recrystallized from methanol to yield 0.049 g (55%): mp +300 °C; IR (KBr) (ν_{max}/cm^{-1}): 3311, 3100, 1672, 1633, 1592; ^{1}H NMR (DMSO-d_6) (δ ppm): 12.53 (s, 1H, $H_{quinolone}$), 9.61 (s, 2H, $H_{amidine}$), 9.26 (S, 2H, $H_{amidine}$), 8.30 (d, 1H, J = 1.86 Hz, H_{arom}), 8.24 (d, 1H, J = 5.27 Hz, $H_{thienyl}$), 7.96 (dd, 1H, J_1 = 8.69 Hz, J_2 = 1.99 Hz, H_{arom}), 7.79 (d, 1H, J = 5.27 Hz, $H_{thienyl}$), 7.70 (d, 1H, J = 8.68 Hz, H_{arom}); ^{13}C NMR (DMSO-d_6) (δ ppm): 165.9, 158.6, 143.9, 141.5, 134.7, 133.6, 133.56, 122.5, 115.3. Anal. Calcd for ($C_{14}H_{10}ClN_3OS_2$): C, 50.07; H, 3.00; N, 12.51. Found: C, 50.27; H, 2.67; N, 12.29.

4.2.11. 2-(*N*′-Isopropyl)amidino-6-oxo-5,6-dihydrothieno[2′,3′:4,5]thienyl[2,3-*c*]quinoline hydrochloride (4d). A solution of **3d** (0.101 g, 0.24 mmol) in methanol (12 ml) was irradiated for 19 h. The formed precipitate was filtered off and recrystallized from methanol to yield 0.06 g (70%): mp +300 °C; IR (KBr) (ν_{max}/cm^{-1}): 3361, 3211, 3060, 1645; ^{1}H NMR (DMSO-d_6) (δ ppm): 12.51 (s, 1H, $H_{quinolone}$), 9.79 (s, 1H, $H_{amidine}$), 9.63 (s, 1H, $H_{amidine}$), 9.15 (s, 1H, $H_{amidine}$), 8.28 (s, 1H, $H_{arom.}$), 8.245 (d, 1H, J = 6.26 Hz, $H_{thienyl}$), 7.89 (d, 1H, J = 8.79 Hz, $H_{arom.}$), 7.81 (d, 1H, J = 5.08 Hz, $H_{thienyl}$), 7.69 (d, 1H, J = 8.64 Hz, $H_{arom.}$), 4.10 (s, 1H, $CH_{i\text{-}Pr}$), 1.34 (d, 6H, J = 6.23 Hz, $2CH_{3i\text{-}Pr}$); ^{13}C NMR (DMSO-d_6) (δ ppm): 161.5, 158.0, 143.4, 140.6, 134.2, 134.0, 133.1, 128.9, 124.3, 123.0, 120.9, 116.6, 114.7, 45.2, 21.3 (2C). Anal. Calcd for ($C_{17}H_{16}ClN_3OS_2$): C, 54.03; H, 4.27; N, 11.11. Found: C, 53.84; H, 4.44; N, 11.34.

4.3. Interactions with DNA

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

DNA was purchased from Fluka and used without further purification. DNA was dissolved in the sodium cacodylate buffer, 0.05 mol dm^{-3}, pH 7, and its concentration was determined spectroscopically as the concentration of phosphates. The measurements were performed in aqueous buffer solution (pH 6.2; sodium cacodylate/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 317 nm (**4a**, **b**) and 319 nm (**4c**, **d**) were used to avoid inner filter effects caused by absorption of excitation light of added polynucleotide, and changes of emission were monitored at 391 nm (**4a**, **b**) and 370 nm (**4c**, **d**). The binding constants (K_s) and [bound compound]/[polynucleotide phosphate] ratio n were calculated according to the Scatchard equation by non-linear least-squares fitting. Values for K_s given in Table 4 all have satisfying correlation coefficients (>0.999). The thermal melting curves for DNA and its complexes were determined as previously described by following the absorption change at 260 nm as a function of temperature. 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. 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.

4.4. Antitumor activity assays

The HeLa (cervical carcinoma), MCF-7 (breast carcinoma), SW 620 (colon carcinoma), MiaPaCa-2 (pancre-

atic 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.[9,18] 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-, ten-fold dilutions (10^{-8}–10^{-4} mol/L) and incubated for further 72 h. Working dilutions were freshly prepared on the day of testing. The solvent (DMSO) 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, which detects dehydrogenase activity in viable cells.[19] 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:

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

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

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

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

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.
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% of 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.

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