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Synthesis and cytotoxic properties of 4,11-bis[(aminoethyl)amino]anthra-[2,3-*b*]thiophene-5,10-diones, novel analogues of antitumor anthracene-9,10-diones

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ABSTRACT

We developed the synthesis of a series of thiophene-fused tetracyclic analogues of the antitumor drug ametantrone. The reactions included nucleophilic substitution of methoxy groups in 4,11-dimethoxyanthra[2,3-*b*]thiophene-5,10-diones with ethylenediamines, producing the derivatives of 4,11-diaminoanthra[2,3-*b*]thiophene-5,10-dione in good yields. Several compounds showed marked antiproliferative potency against doxorubicin-selected, P-glycoprotein-expressing tumor cells and $p53^{-/-}$ cells. The cytotoxicity of some novel compounds for P-glycoprotein-positive cells is highly dependent on N-substituent at the terminal amino group of ethylenediamine moiety. The cytotoxic potency of selected compounds correlated with their ability to attenuate the functions of topoisomerase I and telomerase, strongly suggesting that these enzymes are the major targets of antitumor activity of anthra[2,3-*b*]thiophene-5,10-dione derivatives.

1. Introduction

Throughout the decades the anthracycline antibiotics and their analogues remained a key class of compounds for cancer chemotherapy.[1] However, the efficacy of treatment is frequently limited by organ toxicity (mostly blood, bone marrow and heart) and the emergence of pleiotropic tumor cell resistance (multidrug resistance). Efforts aimed at the design of synthetic analogues of anthracyclines with lower organ toxicity identified the derivatives of anthracene-9,10-dione, for example, mitoxantrone and ametantrone, as the promising class of chemotherapeutic drugs. Indeed, ametantrone (**3**) does not generate superoxide radicals but rather exhibits an antioxidant activity.[2] Individual heterocyclic analogues of aminoalkylaminoanthraquinones such as pixantrone[3] (the aza analogue of ametantrone), pyrazolantrones[4] and pyrazolacridines,[5] demonstrated high antitumor potency and low, if any, cardiotoxicity.[6]

However, drug resistance remains a serious reason for therapeutic failures in patients treated with anthracyclines.[7] This clinically unfavorable phenomenon is directly related to the chemical structure of anthracyclines. First, these compounds can be effluxed out of the cells by molecular transporters of the ATP binding cassette superfamily (in particular, by 140–170 kDa transmembrane P-glycoprotein, Pgp).[8] Second, planar rings of anthracyclines are suited for intercalation into the DNA, thereby triggering DNA damage cell responses in which p53 is a major regulatory molecule.[9] It is therefore not surprising that Pgp overexpression and loss of proapoptotic p53 functions are the main determinants of tumor cell resistance to anthracyclines. Thus, optimization of chemical structure presumes that new generations of synthetic anthracyclines should be poorer substrates of Pgp-mediated efflux; in the same time, novel compounds should trigger p53-independent cytocidal pathways. The latter mechanisms can be employed due to the ability of anthracyclines to interfere with DNA-dependent enzymes such as topoisomerases and telomerase.[10]

In our earlier work we have reported the synthesis and antitumor properties of derivatives of naphtho[2,3-*f*]indole-5,10-dione

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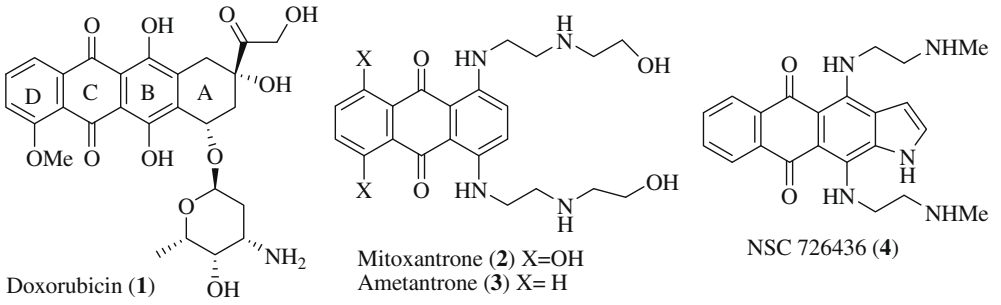


(such as NSC 726436, **4**).[11] These compounds demonstrated remarkable cytotoxicity against human cancer cell lines and higher potency than doxorubicin or mitoxantrone for drug-resistant tumor cells. Supposedly, an additional heterocyclic moiety might substantially increase the binding of ametantrone analogues to their intracellular targets. Indeed, in doxorubicin the A ring is important for the formation of a tertiary complex that includes the drug, the duplex DNA and topoisomerase II.[12] Krapcho and colleagues have shown high potency of thioanalogues of ametantrone against cultured L1210 leukemia cells and in in vivo models.[13] In further search for potential chemotherapeutic agents, particularly for evaluating the role of the heterocyclic moiety in cytotoxicity, we synthesized and tested a series of thiophene-fused tetracyclic analogues of ametantrone, the derivatives of anthra[2,3-*b*]thiophene-5,10-dione. We report herein high cytotoxic activity of novel compounds for a variety of tumor cell lines including the isogenic drug-resistant counterparts. Importantly, the cytotoxicity of selected compounds was associated with in vitro attenuation of topoisomerase I (topo I) and telomerase activities.

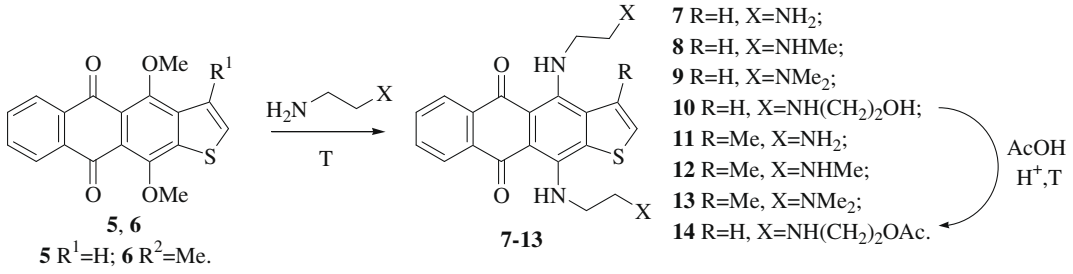
2. Results and discussion

2.1. Chemistry

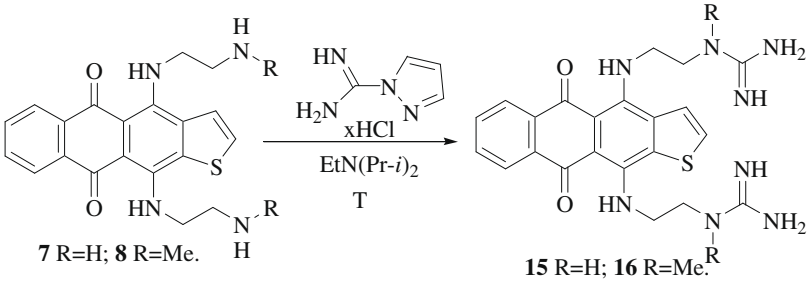
To synthesize naphthoindoledione analogues of ametantrone, for example, compound **4,** we have used the reaction of nucleophilic substitution of 4,11-dimethoxy groups with ethylenediamine and its derivatives.[11] We applied a similar approach to obtain thiophene containing tetracyclic analogues of ametantrone. Previously we have developed the method for the synthesis of 4,11-dialkoxyanthra[2,3-*b*]thiophene-5,10-diones.[14] Using this synthetic approach, we started from quinizarine and obtained 4,11-dimethoxyanthra[2,3-*b*]thiophene-5,10-dione (**5**) and its 3-methyl derivative (**6**) in 4 stages. The thiophene analogues of ametantrone **7**–**13** with different distal amino groups (Scheme 1) were synthesized by substituting the methoxy groups in anthrathiophenediones **5** and **6** by heating with ethylenediamines.

Some derivatives were synthesized by modifying functional groups in the side chains of thiophene-fused ametantrone analogues. For instance, the hydroxyl groups in **10** were turned into ester groups with acetic acid, yielding diacetoxy derivative **14.**

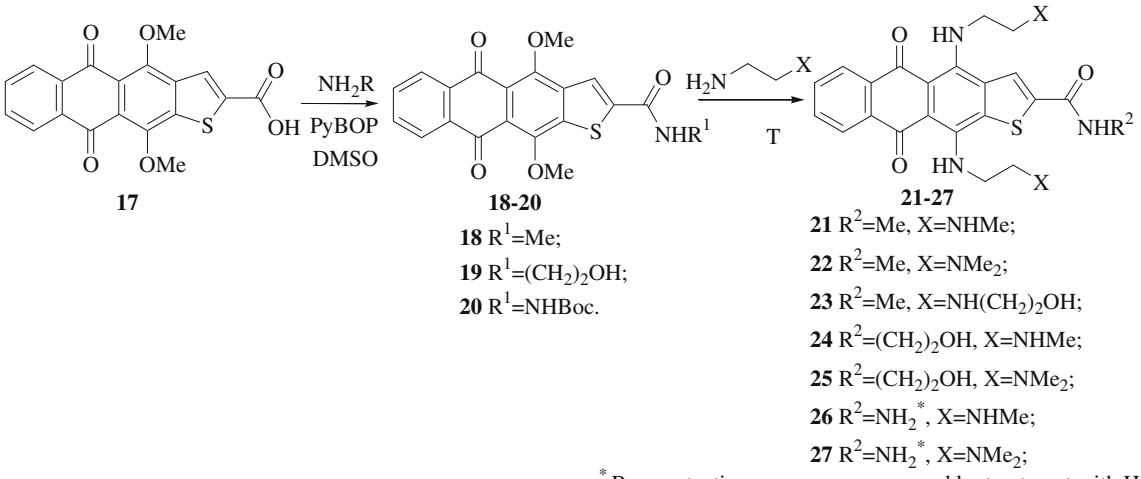
Interaction with DNA, a major intracellular target of anthracyclines, is a critical prerequisite for induction of cell death. Along with the intercalation of anthracyclines and their analogues into the duplex and inhibition of topoisomerases I and II,[15,16] these agents can interfere with telomeric DNA. SAR studies of telomeric G-quadruplex ligands revealed that, due to big square of the quadruplexes, the distance between the terminal amino groups and the chromophore should be longer than the respective distance in the duplex ligands.[17] To obtain the compounds with potentially dual inhibitory activity against topoisomerases and telomerase, the derivatives with additional distal basic groups were synthesized. The terminal amino groups in **7** and **8** were transformed into



Scheme 1.



Scheme 2.



Scheme 3.

guanidine groups (compounds **15, 16;** Scheme 2). The specificity of delocalization of charges in guanidine groups suggests that these compounds can intercalate into the DNA duplex as well as interact with the telomeric G-quadruplex.

Another series of ametantrone analogues was synthesized based on the previously reported anthrathiophene-2-carbonic acid **17**.[18] To prepare the derivatives of acid **17**, it is worth noting that the efficacy of transformation of **17** is limited by its poor solubility in major organic solvents. To synthesize the amides of the acid **17**, we used its chloroanhydride formed by heating in benzene with an excess of thionyl chloride. Subsequent treatment of chloroanhydride of **17** with methylamine resulted in *N*-methylamide **18**; however, its yield was <50%. More efficient transformation of **17** into amides was achieved using condensation with Castro reagents (BOP or PyBOP) in dimethylsulfoxide (DMSO). These reactions produced *N*-methylamide **18,** *N*-ethanolamide **19** and *N*′-Boc-hydrazide **20**, all in practically quantitative yields (Scheme 3). Treatment of 4,11-dimethoxyanthrathiophenediones **18**–**20** with ethylenediamines led to thiophene analogues of ametantrone **21**–**27** (Scheme 3). In case of derivatives of *N*′-Boc-hydrazide **20** the protective group was removed by treatment with HCl in methanol, yielding hydrazides **26**, **27** in reasonable yields.

2.2. Biological testing

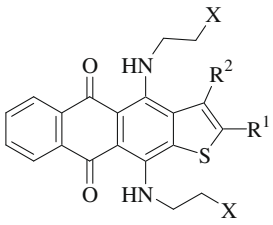
We used a panel of tumor cell lines to test the cytotoxicity of the new compounds. Importantly, this panel included the cell lines and their isogenic sublines with the determinants of drug resistance: murine leukemia L1210, T-lymphocyte cell lines Molt4/C8 and CEM, human leukemia K562 and its MDR subline K562/4 that overexpressed P-glycoprotein, and the colon carcinoma HCT116 and its subline HCT116p53KO with deleted *p53*. The above determinants alter the response of cells to many anticancer drugs including doxorubicin. Data on cytotoxic (antiproliferative) activity are presented in Tables 1 and 2 in which IC_{50} values represent the concentrations that inhibit cell proliferation by 50%.

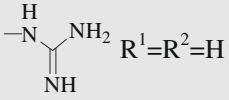
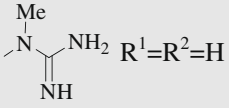
Almost all final derivatives of anthra[2,3-*b*]thiophene-5,10-dione **7**–**14, 21**–**27** (excluding the amide **23**) were cytotoxic for L1210, Molt4/C8 and CEM cell lines (Table 1). The most potent compounds were **8**, **11**, **21** and especially **9** with the dimethylamino group in the side chains. Potency of these compounds was close to that of doxorubicin. Introduction of methyl group in the position 3 of the chromophore (compounds **11**–**13**) did not significantly change the cytotoxicity, except for the ethylenediamine derivative **11** that was more potent than its 3-unsubstituted analogue **7**. Comparison of the thiophene analogue of ametantrone **8** and structurally similar derivatives of naphtho[2,3-*f*]indole-5,10-dione **4** (which are less active) illustrated the key role of the heteroatom in cytotoxicity (Table 1).

The presence and structure of distal amino groups is especially important for activity against drug resistant cells. Compounds **8**, **9**, **12** and **13** with methylamino- and dimethylamino groups in the side chains were capable of circumventing drug resistance (Table 1). In contrast, the derivatives with amino- and ethanolamino groups **7** and **10** were inactive against doxorubicin-selected Pgp-positive cells. Introduction of hydrophilic substituents (such as ethanolamide or carbohydrazide groups) in the position 2 of the chromophore led to loss of cytotoxiciy for K562 cells (compounds **23**, **24**, **27**), whereas the derivatives with methyl or dimethylamino groups in the side chains were less potent against the Pgp-positive K562/4 subline. Importantly, the derivative **22** with the methylamide group was more potent than doxorubicin for both K562 and K562/4 cells. The congeners with guanidine residue in the side chain (compounds **15** and **16**) were less active against L1210, Molt4/C8 and CEM cell lines. However, guanidination of the amino group in the side chain of **7** (compound **15**) led to a substantial increase of activity against the K562/4 subline. For **8** similar modification of the side chain (compound **16**) completely abrogated the activity against both wild type K562 cells and their resistant variant (Table 1).

Next, we were interested whether p53 plays a role in cytotoxicity of derivatives of anthra[2,3-*b*]thiophene-5,10-diones. It is well documented that p53-inactivating mutations are the most frequent events in cancer, and the loss of p53 can confer resistance to DNA damaging agents including doxorubicin.[19] The derivatives **8** and **9** containing an unsubstituted heterocyclic moiety were as toxic for HCT116 and HCT116p53KO cells as naphthoindoledione **4** (Table 2). These derivatives inhibited cell proliferation independently of the p53 status (Table 2). Introduction of a methylcarboxamide group in the position 2 of the chromophore (compound **22**) increased the activity against HCT116 cells, suggesting that the cytotoxic potency of this compound is mediated via a p53-dependent mechanism(s). Intriguing data were obtained with guanidine derivatives **15** and **16**. Introduction of the guanidine groups into the side chains resulted in an increased potency of **15** and **16** for HCT116 cells compared to **7** and **8**, respectively. However, **15** and **16** exerted virtually no toxicity for the $p53^{-/-}$ subline (Table 2). Together, the cytotoxicity assays showed that individual novel compounds were potent for human tumor cell lines at submicromolar concentrations, similarly to the potency of the reference drug

Table 1
Anti-proliferative activity (IC_{50}) of anthra[2,3-*b*]thiophene-5,10-dione derivatives



#	X, R^1, R^2	L1210	Molt4/C8	CEM	K562	K562/4	RI[a]
7	X = NH_2, R^1 = R^2 = H	1.9 ± 0.5	1.1 ± 0.4	1.2 ± 0.5	>50	>50	—
8	X = NHMe, R^1 = R^2 = H	0.95 ± 0.2	0.5 ± 0.1	0.4 ± 0.1	0.5 ± 0.1	0.7 ± 0.2	1.4
9	X = NMe_2, R^1 = R^2 = H	0.4 ± 0.1	0.3 ± 0.04	0.4 ± 0.2	0.3 ± 0.1	0.7 ± 1.5	2.3
10	X = $NH(CH_2)_2OH$, R^1 = R^2 = H	2.2 ± 0.6	8.5 ± 1.2	5.6 ± 1.6	4.0 ± 0.5	>50	>12
11	X = NH_2, R^1 = H, R^2 = Me	0.8 ± 0.3	0.3 ± 0.1	0.4 ± 0.04	0.1 ± 0.06	0.5 ± 0.1	5
12	X = NHMe, R^1 = H, R^2 = Me	1.3 ± 0.5	1.7 ± 0.5	2.0 ± 0.2	0.1 ± 0.02	0.2 ± 0.1	2
13	X = NMe_2, R^1 = H, R^2 = Me	1.5 ± 0.4	1.8 ± 0.4	1.7 ± 0.5	0.5 ± 0.15	0.9 ± 0.2	1.8
14	X = $NH(CH_2)_2OAc$, R^1 = R^2 = H	0.95 ± 0.4	0.9 ± 0.5	0.7 ± 0.3	2.8 ± 0.2	>50	>18
15	X=  R^1=R^2=H	20.1 ± 6.2	10.8 ± 3.2	11.3 ± 2.0	1.1 ± 0.1	3.7 ± 0.4	3.4
16	X=  R^1=R^2=H	51.2 ± 5	12.1 ± 4	25.3 ± 4	>50	>50	—
21	X = NHMe, R^1 = CONHMe, R^2 = H	0.6 ± 0.3	1.0 ± 0.4	0.8 ± 0.5	4.5 ± 0.7	>50	>11
22	X = NMe_2, R^1 = CONHMe, R^2 = H	1.3 ± 0.1	1.1 ± 0.4	2.1 ± 0.2	0.8 ± 0.1	0.9 ± 0.2	1.1
23	X = $NH(CH_2)_2OH$, R^1 = CONHMe, R^2 = H	>500	210 ± 11	207 ± 48	>50	>50	—
24	X = NHMe, R^1 = $CONH(CH_2)_2OH$, R^2 = H	7.8 ± 1.5	5.5 ± 2.6	6.0 ± 2.5	>50	>50	—
25	X = NMe_2, R^1 = $CONH(CH_2)_2OH$, R^2 = H	3.5 ± 0.6	1.4 ± 0.3	1.7 ± 0.2	8.6 ± 0.2	>50	>6
26	X = NHMe, R^1 = $CONHNH_2$, R^2 = H	38.2 ± 6	13.1 ± 4	10.2 ± 4	>50	>50	—
27	X = NMe_2, R^1 = $CONHNH_2$, R^2 = H	2.8 ± 2.1	1.6 ± 0.5	1.6 ± 0.2	0.6 ± 0.1	>50	>90
4		1.2 ± 0.1	0.87 ± 0.2	1.1 ± 0.1	0.8 ± 0.1	2.5 ± 0.2	3.1
Dox		0.37 ± 0.07	0.20 ± 0.02	0.06 ± 0.01	0.14 ± 0.03	7.2 ± 0.9	51

[a] **RI,** resistance index = IC_{50}(K562/4)/IC_{50}(K562). Mean ± S.D. of three experiments. (—) RI was not calculated for non-toxic agents. Naphtho[2,3-*f*]indole-5,10-dione **4** and doxorubicin (**Dox**) were used as reference compounds.

Table 2
Anti-proliferative activity (IC_{50}) of anthra[2,3-*b*]thiophene-5,10-diones against colon carcinoma HCT116 cell line and HCT116p53KO ($p53^{-/-}$) variant

#	HCT116	HCT116p53KO	RI[a]
8	8.3 ± 0.2	5.3 ± 0.3	0.6
9	18.4 ± 0.7	10.6 ± 0.8	0.6
11	0.6 ± 0.1	>50	>83
12	1.4 ± 0.2	3.8 ± 0.4	2.7
13	2.8 ± 0.2	23.5 ± 0.2	8.4
15	2.0 ± 0.2	>50	>25
16	2.1 ± 0.1	>50	>24
22	2.3 ± 0.1	9.7 ± 0.8	4.2
27	2.0 ± 0.1	>50	>25
4	11.1 ± 0.4	11.3 ± 0.4	1.0
Dox	1.4 ± 0.1	4.4 ± 0.4	3.1

[a] **RI,** resistance index = IC_{50}(HCT116p53KO)/IC_{50}(HCT116).

doxorubicin. Importantly, the selected novel compounds were more active than doxorubicin for cells with the determinants of altered drug response, that is, Pgp overexpression or non-functional p53.

We next studied intracellular targets critical for antitumor properties of anthra[2,3-*b*]thiophene-5,10-diones. One can suppose that the derivatives of anthra[2,3-*b*]thiophene-5,10-diones bind DNA and interfere with DNA-dependent enzymes. Therefore, we tested the thiophene analogues of ametantrone **7** and **8**, as well as their guanidine derivatives **15** and **16** for the ability to modulate topo I-mediated relaxation of supercoiled DNA. Figure 1 shows the topoisomers of pHot plasmid produced in the presence of **7**, **8**, **15** and **16**. The highest inhibitory effect on DNA relaxation was evoked by **8**. At 1 μM of **8** DNA relaxation was almost completely blocked, whereas other tested compounds at equimolar concentrations were less potent. The data on topo I inhibition correlated well with cytotoxic potency of the compounds. Guanidination of distal amino groups in **7** increased topo I inhibitory activity of **15** as well as its toxicity against K562 cells and the K562/4 Pgp-positive subline. An opposite trend was found for compounds **8** and **16**: guanidination of methylamino groups decreased both cytotoxicity and topo I poisoning. Thus, the cytotoxicity of thiophene analogues of ametantrone correlated with topo I inhibitory activity. It remains to be elucidated whether the cytotoxic anthra[2,3-*b*]thiophene-5,10-diones inhibit topo I function via DNA binding and interference with topo I-mediated duplex unwinding or by a camptothecin-like mechanism, that is, the drug-enzyme-DNA tertiary complex formation.

Finally, we tested whether our thiophene analogues of ametantrone are capable of inhibiting telomerase function. At 1 μM each anthra[2,3-*b*]thiophene-5,10-diones **8, 15** and **16** inhibited telomerase activity (Fig. 2A). The inhibitory effects of these compounds were enzyme-specific because the internal controls were

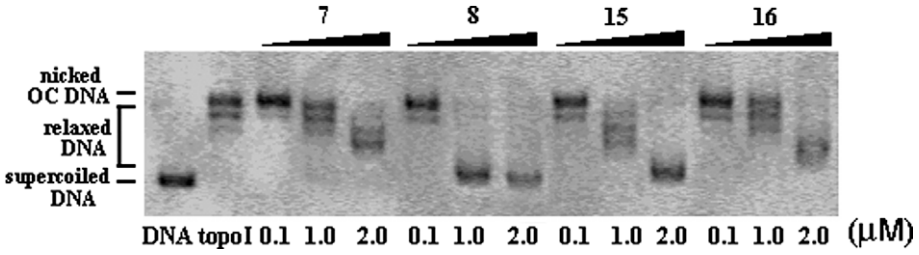


Figure 1. Electrophoretic mobility of p-Hot plasmid after topo I-mediated relaxation in the presence of anthra[2,3-*b*]thiophene-5,10-diones **7, 8, 15** and **16**. Positions of topoisomers are shown to the left.

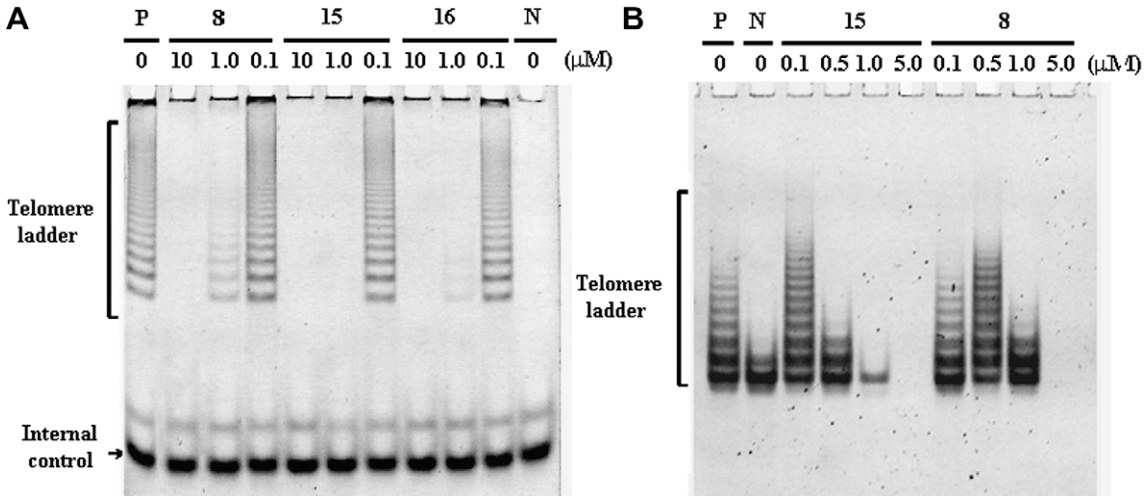


Figure 2. Inhibition of telomerase activity by anthra[2,3-*b*]thiophene-5,10-diones **8**, **15** and **16.** (A) Tested concentration of anthra[2,3-*b*]thiophene-5,10-diones **8, 15** and **16** were 0.1, 1.0 and 10.0 μM. (B) Tested concentration of anthra[2,3-*b*]thiophene-5,10-diones **8** and **15** were 0.1, 0.5, 1.0 and 5.0 μM. P—positive control (no inhibitor); N—negative control (RNase A-treated cell extract, no inhibitor).

unaffected (Fig. 2A). The most potent agent **15** completely blocked telomerase activity at 1 μM. Comparison of the potency of **8** and **16** revealed that guanidination of distal amino groups led to a somewhat higher inhibitory activity. Furthermore, although both **8** and **15** inhibited telomerase activity at submicromolar concentrations, the guanidine derivative **15** was more potent than the derivative **8** with methylamino groups in the side chains (Fig. 2B). These data provide an important insight into the SAR of thiophene analogues of ametantrone as the inhibitors of telomerase.

2.3. Conclusions

We performed chemical synthesis of series of novel anthra[2,3-*b*]thiophene-5,10-diones. The selected novel anthrathiophenediones demonstrated excellent antiproliferative activity for a variety of tumor cell lines, both wild type and those that express major determinants of altered anticancer drug response such as the efflux pump P-gp and non-functional p53. The cytotoxic properties of selected new compounds correlated with topo I inhibition, strongly suggesting that topo I is a major candidate target of antitumor potency of derivatives of this chemical class. Finally, we identified novel derivatives of anthra[2,3-*b*]thiophene-5,10-diones as potent inhibitors of telomerase activity.

3. Experimental

3.1. Chemistry

NMR spectra were registered on a Varian VXR-400 instrument operated at 400 MHz (^{1}H NMR). Chemical shifts were measured in DMSO-d_6, $CDCl_3$ or D_2O using tetramethylsilane as internal standard. Analytical TLC was performed on Silica Gel F_{254} plates (Merck) and column chromatography on Silica Gel Merck 60. Melting points were determined on a Buchi SMP-20 apparatus and are uncorrected. Mass spectra were obtained on SSQ 710 Finnigan and MALDI TOF Bruker BIFLEX III instruments. UV spectra were recorded on Hitachi-U2000 spectrophotometer. HPLC was performed using Shimadzu Class-VP V6.12SP1 system. All solutions were dried over Na_2SO_4 and evaporated at reduced pressure on a Buchi-R200 rotary evaporator at the temperature below 45°C. All products were vacuum dried at room temperature.

3.1.1. 4,11-Bis[(2-aminoethyl)amino]anthra[2,3-*b*]thiophene-5,10-dione (7)

A mixture of 4,11-dimethoxyanthra[2,3-*b*]thiophene-5,10-dione (**5**[14]; 0.10 g, 0.3 mmol) and ethylenediamine (3.0 mL) was heated at 50 °C for 1.5–2 h. During this time, the yellow color of the reaction mixture changed to dark blue, and after complete conversion of **5** (as determined by TLC) the solution was cooled and quenched with water. Aqueous solution of HCl (1.0%) was added to reach pH 8.0, the solution was saturated with NaCl, and the product was extracted with warm *n*-butanol (3 × 30 mL). The extract was washed twice with brine, dried and evaporated. The residue was purified by column chromatography with chloroform–methanol–concd NH_4OH (10:2:0 → 10:4:1) as eluting solvent. The solid residue obtained after evaporation was crystallized from ethanol–1,4-dioxane (1:1) to afford **7** (76 mg, 65%) as dark blue crystals, mp 143–144 °C; dihydrochloride mp >250 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.54 (t, 1H, J = 5.3 Hz, NH), 12.43 (t, 1H,

J = 7.5 Hz, NH), 8.27 (m, 2H, 6-H, 9-H), 8.16 (d, 1H, *J* = 5.6 Hz, 2-H), 8.04 (d, 1H, *J* = 5.6 Hz, 3-H), 7.75 (m, 2H, 7-H, 8-H), 3.91 (dd, 2H, ^1J = 6.0 Hz, ^2J = 5.3 Hz, HNCH_2), 3.79 (dd, 2H, ^1J = 6.0 Hz, ^2J = 5.0 Hz, HNCH_2), 2.97 (t, 2H, *J* = 5.0 Hz, CH_2NH$_2$), 2.92 (t, 2H, *J* = 6.0 Hz, CH_2NH$_2$); MS *m/z* 380 (M$^+$, 8), 295 (43), 205 (65), 42 (100). Anal. Calcd for $C_{20}H_{20}N_4O_2S$: C, 63.14; H, 5.30; N, 14.73; S, 8.43. Found: C, 62.95; H, 5.18; N, 14.56; S, 8.75.

3.1.2. 4,11-Bis{[2-(methylamino)ethyl]amino}anthra[2,3-*b*]thiophene-5,10-dione (8)

This was prepared similarly from anthrathiophenedione **5**[14] and *N*-methylethylenediamine (50 °C, 3–3.5 h). Dark blue needles, yield 68%, mp 129–130 °C (benzene); dihydrochloride mp >250 °C; ^{1}H NMR (400 MHz, CDCl$_3$) δ 12.21 (t, 1H, *J* = 5.0 Hz, NH), 11.90 (t, 1H, *J* = 4.7 Hz, NH), 8.37 (m, 2H, 6-H, 9-H), 7.90 (d, 1H, *J* = 5.6 Hz, 2-H), 7.66 (m, 2H, 7-H, 8-H), 7.62 (d, 1H, *J* = 5.6 Hz, 3-H), 4.07 (dd, 2H, ^1J = 5.8 Hz, ^2J = 5.6 Hz, HNCH_2), 3.90 (dd, 2H, ^1J = 5.7 Hz, ^2J = 5.5 Hz, HNCH_2), 3.07 (t, 2H, *J* = 6.1 Hz, CH_2NHMe), 3.03 (t, 2H, *J* = 5.9 Hz, CH_2NHMe), 2.57 (s, 3H, Me), 2.55 (s, 3H, Me); MS *m/z* 408 (M$^+$, 16), 365 (34), 333 (43), 321 (65), 307 (100), 44 (54). Anal. Calcd for $C_{22}H_{24}N_4O_2S$: C, 64.68; H, 5.92; N, 13.71; S, 7.85. Found: C, 65.01; H, 5.86; N, 13.96; S, 8.03. Hydrochloride mp 223–225 °C; ^{1}H NMR (400 MHz, D$_2$O) δ 7.77 (d, 1H, *J* = 5.3 Hz, 2-H), 7.37 (m, 3H, 3-H, 6-H, 9-H), 7.24 (m, 2H, 7-H, 8-H), 3.81 (t, 2H, *J* = 6.8 Hz, HNCH_2), 3.68 (t, 2H, *J* = 6.3 Hz, HNCH_2), 3.25 (m, 4H, CH_2NHMe), 2.87 (3H, s, Me), 2.86 (3H, s, Me).

3.1.3. 4,11-Bis{[2-(dimethylamino)ethyl]amino}anthra[2,3-*b*]thiophene-5,10-dione (9)

A mixture of anthrathiophenedione **5**[14] (0.10 g, 0.31 mmol) and *N*,*N*-dimethylethylenediamine (3.0 mL) was heated at 50 °C for 4–5 h. After complete conversion of **5** (as determined by TLC) the reaction mixture was cooled, quenched with water, and pH was adjusted to 8.0 by aqueous solution of HCl (1.0%). The product was extracted with ethyl acetate (4 × 30 mL). The residue obtained after the evaporation of the extract was purified by column chromatography with chloroform–methanol (10:1 → 2:1) as eluent. The solid product was crystallized from benzene–*n*-heptane mixture (1:4) and dried to afford anthrathiophenedione **9** (0.10 g, 76%) as dark blue needles, mp 132–134 °C; dihydrochloride mp >250 °C; ^{1}H NMR (400 MHz, CDCl$_3$) δ 12.36 (t, 1H, *J* = 4.9 Hz, NH), 12.21 (t, 1H, *J* = 5.1 Hz, NH), 8.40 (m, 2H, 6-H, 9-H), 7.94 (d, 1H, *J* = 5.5 Hz, 2-H), 7.57 (m, 3H, 3-H, 7-H, 8-H), 4.06 (dd, 2H, ^1J = 5.2 Hz, ^2J = 6.4 Hz, HNCH_2), 3.76 (dd, 2H, ^1J = 4.0 Hz, ^2J = 5.4 Hz, HNCH_2), 2.76 (m, 4H, 2CH_2NMe$_2$), 2.40 (s, 6H, NMe$_2$), 2.38 (s, 6H, NMe$_2$); MS *m/z* 436 (M$^+$, 34), 333 (44), 58 (100). UV (ethanol) λ_{max} (log ε) 235 sh (4.2), 265 (4.6), 285 sh (4.0), 325 sh (3.7), 394 (3.2), 532 sh (3.8), 568 (4.1), 613 (4.2) nm; Anal. Calcd for $C_{24}H_{28}N_4O_2S$: C, 66.03; H, 6.46; N, 12.83; S, 7.33. Found: C, 66.05; H, 6.52; N, 12.65; S, 7.58.

3.1.4. 4,11-Bis[{2-[(2-hydroxyethyl)amino]ethyl}amino]anthra[2,3-*b*]thiophene-5,10-dione (10)

This was prepared from anthrathiophenedione **5**[14] and 2-[(2-hydroxyethyl)amino]ethylamine as described for compound **7** (50 °C, 2–3 h). Yield 56%, mp 148–150 °C (ethanol); dihydrochloride mp 225–227 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.38 (br s, 1H, NH), 12.30 (br s, 1H, NH), 8.22 (m, 2H, 6-H, 9-H), 8.05 (d, 1H, *J* = 5.9 Hz, 2-H), 7.99 (d, 1H, *J* = 5.9 Hz, 3-H), 7.72 (m, 2H, 7-H, 8-H), 3.95 (m, 2H, HNCH_2), 3.75 (m, 2H, HNCH_2), 3.53 (m, 4H, CH_2OH), 2.94 (m, 4H, CH_2NH), 2.70 (m, 4H, NHCH_2); MS *m/z* 468 (M$^+$, 8), 395 (56), 333 (45), 308 (100), 293 (16). Anal. Calcd for $C_{24}H_{28}N_4O_4S.2HCl.2H_2O$: C, 49.91; H, 5.93; N 9.70; S, 5.55. Found: C, 50.32; H, 5.96; N, 9.61; S, 5.71.

3.1.5. 4,11-Bis[(2-aminoethyl)amino]-3-methylanthra[2,3-*b*]thiophene-5,10-dione dihydrochloride (11)

This was prepared from anthrathiophenedione **6**[14] and ethylenediamine as described for compound **7** (50 °C, 2–3 h). The residue obtained after purification by chromatography was dissolved in warm 1 N HCl and re-precipitated with acetone. The precipitated crystals were filtered, washed with acetone and dried to yield 59% dihydrochloride **11**, mp >250 °C; ^{1}H NMR (400 MHz, D$_2$O) δ 7.55 (m, 2H, 6-H, 9-H), 7.42 (s, 1H, 2-H), 7.28 (m, 2H, 7-H, 8-H), 3.88 (t, 2H, *J* = 5.3 Hz, HNCH_2), 3.27 (m, 4H, CH$_2$), 2.97 (t, 2H, *J* = 5.5 Hz, CH_2NH$_2$), 2.45 (s, 3H, Me). Anal. Calcd for $C_{21}H_{22}N_4O_2S{\cdot}2HCl{\cdot}2H_2O$: C, 50.10 H, 5.61; N, 11.13; S, 6.37. Found: C, 50.19; H, 5.78; N, 11.24; S, 6.56.

3.1.6. 4,11-Bis{[2-(methylamino)ethyl]amino}-3-methylanthra[2,3-*b*]thiophene-5,10-dione dihydrochloride (12)

This was prepared from anthrathiophenedione **6**[14] and *N*-methylethylenediamine as described for compound **11** (50 °C, 2–3 h). Yield 67%, mp >250 °C; ^{1}H NMR (400 MHz, D$_2$O) δ 7.64 (m, 2H, 6-H, 9-H), 7.46 (s, 1H, 2-H), 7.37 (m, 2H, 7-H, 8-H), 3.95 (t, 2H, *J* = 5.3 Hz, HNCH_2), 3.32 (m, 4H, CH$_2$), 3.03 (t, 2H, *J* = 5.5 Hz, CH_2NH), 2.80 (s, 3H, NMe), 2.62 (s, 3H, NMe), 2.54 (s, 3H, Me); MS *m/z* 422 (M$^+$, 24), 379 (34), 347 (28), 330 (64), 321 (100). Anal. Calcd for $C_{23}H_{26}N_4O_2S{\cdot}2HCl{\cdot}H_2O$: C, 53.80; H, 5.89; N, 10.91; S, 6.24. Found: C, 53.53; H, 6.15; N, 10.82; S, 6.35.

3.1.7. 4,11-Bis{[2-(dimethylamino)ethyl]amino}-3-methylanthra[2,3-*b*]thiophene-5,10-dione (13)

This was prepared from anthrathiophenedione **6**[14] and *N*,*N*-dimethylethylenediamine (50 °C, 3–4 h) as dark blue needles. Yield 62%, mp 111–112 °C (benzene); dihydrochloride mp 252–254 °C; ^{1}H NMR (400 MHz, CDCl$_3$) δ 12.06 (t, 1H, *J* = 5.2 Hz, NH), 9.77 (t, 1H, *J* = 5.4 Hz, NH), 8.33 (m, 2H, 6-H, 9-H), 7.66 (m, 2H, 7-H, 8-H), 7.31 (s, 1H, 2-H), 4.06 (dd, 2H, ^1J = 5.2 Hz, ^2J = 6.5 Hz, HNCH_2), 3.76 (dd, 2H, ^1J = 5.4 Hz, $^2J^2J$ = 6.7 Hz, HNCH_2), 2.78 (t, 2H, *J* = 6.5 Hz, CH_2NMe$_2$), 2.71 (s, 3H, Me), 2.45 (t, 2H, *J* = 6.7 Hz, CH_2NMe$_2$), 2.40 (s, 6H, NMe$_2$), 2.22 (s, 6H, NMe$_2$); MS *m/z* 450 (M$^+$, 100), 347 (47), 58 (67); UV (ethanol) λ_{max} (log ε) 225 (4.1), 269 (4.6), 315 sh (3.7), 396 (3.3), 517 sh (3.8), 557 (4.0), 595 sh (3.9) nm; Anal. Calcd for $C_{25}H_{30}N_4O_2S$: C, 66.64; H, 6.71; N, 12.43; S, 7.12. Found: C 66.23, H 6.82, N 12.53; S, 7.34.

3.1.8. 4,11-Bis[{2-[(2-acetoxyethyl)amino]ethyl}amino]anthra[2,3-*b*]thiophene-5,10-dione dihydrochloride (14)

To the stirring solution of hydrochloride of anthrathiophendione **10** (54 mg, 0.1 mmol) in hot acetic acid (30.0 mL) thionyl chloride (0.1 mL, 1.2 mmol) was added, the mixture was refluxed for 2 h and evaporated. The resulting dark-blue solid was re-precipitated from water with acetone–Et$_2$O mixture (1:1). The precipitate was filtered and washed with Et$_2$O, yielding 53 mg (82%) of dihydrochloride **14** after drying; mp 233–236 °C (dec); ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.92 (br s, 1H, NH), 11.74 (br s, 1H, NH), 9.56 (br s, 4H, 2NH$_2$), 8.29 (d, 1H, *J* = 5.5 Hz, 2-H), 8.21 (m, 2H, 6-H, 9-H), 8.01 (d, 1H, *J* = 5.5 Hz, 3-H), 7.77 (m, 2H, 7-H, 8-H), 4.31 (t, 4H, *J* = 4.8 Hz, 2CH_2), 4.24 (br m, 2H, CH_2), 4.09 (br m, 2H, CH_2), 3.35 (m, 8H, 4CH_2), 2.07 (s, 3H, Ac), 2.06 (s, 3H, Ac); MS *m/z* 294 (M$^+$-2C$_6$H$_{11}$NO$_2$, 100). Anal. Calcd for $C_{28}H_{32}N_4O_6S.2HCl.2H_2O$: C, 50.83; H, 5.79; N, 8.47; S, 4.85. Found: C, 50.58; H, 6.06; N 8.11; S, 5.14.

3.1.9. 4,11-Bis[(2-guanidinoethyl)amino]anthra[2,3-b]-thiophene-5,10-dione dihydrochloride (15)

To the stirring solution of free base of anthrathiophenedione **7** (95 mg, 0.25 mmol) in DMSO (10.0 mL) ethyldiisopropylamine (1.0 mL, 6.0 mmol) and 0.5 g (3.4 mmol) hydrochloride of pyrazole-1-carboxamidine were added. The mixture was stirred for

5 h at 60 °C and then cooled. The product was precipitated by treatment with acetone and collected by filtration. The blue solid was twice re-precipitated from hot water with acetone, washed with acetone and, after drying, yielded dihydrochloride **15** (82 mg, 74%); mp 190–192 °C (dec); HPLC Kromasil-100-C-18 column (6 × 250 mm, LW = 260 nm), eluent: A—H_3PO_4 (0.01M), B—MeCN; gradient B 10 → 40% (30 min.), elution time 16.1 min, purity 96%. ^{1}H NMR (400 MHz, D_2O) δ 7.73 (br s, 1H, 2-H), 7.25 (br m, 3H, 3-H, 6-H, 9-H), 7.04 (m, 2H, 7-H, 8-H), 3.42 (br m, 4H, HNCH_2), 3.20 (m, 4H, CH_2NH); MS *m/z* 294 (M^+-2$C_3H_7N_3$, 100); MALDI *m/z* 465 (M^++H); UV (ethanol) λ_{max} (log ε) 230 sh (4.2), 266 (4.6), 285 sh (4.0), 333 sh (3.7), 400 (31), 532 sh (3.8), 565 (4.1), 604 (4.1) nm; Anal. Calcd for $C_{22}H_{24}N_8O_2S.2HCl.2H_2O$: C, 46.07; H, 5.27; N, 19.54; S, 5.59. Found: C, 45.68; H, 5.14; N, 19.14; S, 6.01.

3.1.10. 4,11-Bis{[2-(1-methylguanidino)ethyl]amino}anthra[2,3-*b*]thiophene-5,10-dione dihydrochloride (16)

This was prepared from anthrathiophenedione **8** as described for compound **15** (60 °C, 8–10 h), mp 203–204 °C (dec); HPLC Kromasil-100-C-18 column (6 × 250 mm, LW = 260 nm), eluent: A—H_3PO_4 (0.01M), B—MeCN; gradient B 10 → 40% (30 min), elution time 18.3 min, purity 94%. ^{1}H NMR (400 MHz, D_2O) δ 7.73 (d, 1H, *J* = 5.5 Hz, 2-H), 7.70 (m, 2H, 6-H, 9-H), 7.51 (m, 2H, 7-H, 8-H), 7.41 (d, 1H, *J* = 5.5 Hz, 3-H), 3.76 (t, 2H, *J* = 5.2 Hz, HNCH_2), 3.76 (t, 2H, *J* = 5.5 Hz, HNCH_2), 3.54 (t, 2H, *J* = 5.2 Hz, CH_2NMe), 3.50 (t, 2H, *J* = 5.5 Hz, CH_2NMe), 3.08 (s, 3H, NMe), 2.98 (s, 3H, NMe); MS *m/z* 294 (M^+-2$C_4H_9N_3$, 100); MALDI *m/z* 493 (M^++H). Anal. Calcd for $C_{24}H_{28}N_8O_2S{\cdot}2HCl{\cdot}2H_2O$: C, 47.92; H, 5.70; N, 18.63; S, 5.33. Found: C, 47.54; H, 5.89; N, 18.22; S, 5.78.

3.1.11. *N*-Methylamide 4,11-dimethoxyanthra[2,3-*b*]thiophene-5,10-dione-2-carboxylic acid (18)

Anthrathiophendione-2-carboxylic acid **17**[18] (184 mg, 0.5 mmol) was dissolved by stirring and heating in DMSO (50 mL), and solution was cooled to room temperature. To this solution 0.1 g (1.5 mmol) hydrochloride of methylamine, 0.15 mL (1.1 mmol) ethyldiisopropylamine and 0.3 g (0.6 mmol) PyBOP® were added. The mixture was stirred for 20 min and quenched with water (150 mL). The precipitated yellow solid was collected by filtration, washed with water and dried. Yield 90%, mp >250 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.03 (q, 1H, *J* = 4.6 Hz, NH), 8.36 (s, 1H, 3-H), 8.12 (m, 2H, 6-H, 9-H), 7.87 (m, 2H, 7-H, 8-H), 4.03 (s, 3H, OMe), 4.01 (s, 3H, OMe), 3.97 (d, 3H, *J* = 4.6 Hz, NMe); MS *m/z* 381 (M+, 100), 352 (29), 338 (12). Anal. Calcd for $C_{20}H_{15}NO_5S$: C, 62.98; H, 3.96; N, 3.67; S, 8.41. Found: C, 63.14; H, 4.05; N, 3.82; S, 8.76.

3.1.12. *N*-(2-Hydroxyethyl)amide 4,11-dimethoxyanthra[2,3-*b*]thiophene-5,10-dione-2-carboxylic acid (19)

This was prepared from acid **17**[18] and ethanolamine as described for amide **18.** Yield 96%, yellow powder, mp 233–235 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.09 (t, 1H, *J* = 5.7 Hz, NH), 8.45 (s, 1H, 3-H), 8.11 (m, 2H, 6-H, 9-H), 7.86 (m, 2H, 7-H, 8-H), 4.86 (t, 1H, *J* = 5.7 Hz, OH), 4.03 (s, 3H, OMe), 4.00 (s, 3H, OMe), 3.54 (dd, 2H, *J* = 5.7 Hz, NCH_2), 3.35 (dd, 2H, *J* = 5.7 Hz, NCH_2); MS *m/z* 411 (M^+, 28), 395 (11), 370 (23), 351 (15), 322 (100). Anal. Calcd for $C_{21}H_{17}NO_6S$: C, 61.30; H, 4.16; N, 3.40; S, 7.79. Found: C, 61.53; H, 4.25; N, 3.53; S, 7.96.

3.1.13. *N*′–(*tert*-Butoxycarbonyl)hydrazide 4,11-dimethoxyanthra[2,3-*b*]thiophene-5,10-dione-2-carboxylic acid (20)

This was prepared from acid **17**[18] and *tert*-butyl carbazate as described for amide **18.** Yield 93%, yellow powder, mp 194–196 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.78 (s, 1H, NH), 9.20 (s, 1H, NH), 8.47 (s, 1H, 3-H), 8.10 (m, 2H, 6-H, 9-H), 7.86 (m, 2H, 7-H, 8-H), 4.03 (s, 3H, OMe), 4.01 (s, 3H, OMe), 1.44 (s, 9H, *t*-Bu); MS *m/z* 482 (M^+, 15), 382 (100); UV (ethanol) λ_{max} (log ε) 227 (4.0), 268 (4.5), 275 (4.5), 314 sh (4.0), 403 (3.9) nm. Anal. Calcd for $C_{24}H_{22}N_2O_7S$: C, 59.74; H, 4.60; N, 5.81; S, 6.65. Found: C 59.65; H, 4.44; N, 5.74; S, 6.94.

3.1.14. *N*-Methylamide 4,11-bis{[2-(methylamino)ethyl]amino}anthra[2,3-*b*]thiophene-5,10-dione-2-carboxylic acid (21)

This was prepared from amide **18** and *N*-methylethylenediamine as described for anthrathiophenedione **7** (60 °C, 2–3 h). Yield 68%, dark blue needles, mp 123–125 °C (benzene); dihydrochloride mp 241–244 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 12.12 (t, 1H, *J* = 5.2 Hz, NH), 12.10 (t, 1H, *J* = 5.6 Hz, NH), 8.25 (m, 2H, 6-H, 9-H), 8.11 (s, 1H, 3-H), 7.63 (m, 2H, 7-H, 8-H), 7.35 (q, 1H, *J* = 4.5 Hz, NH), 3.96 (dd, 2H, ^1J = 6.0 Hz, ^2J = 5.2 Hz, HNCH_2), 3.72 (dd, 2H, ^1J = 5.9 Hz, ^2J = 5.6 Hz, HNCH_2), 3.04 (d, 3H, *J* = 4.5 Hz, NMe), 3.00 (t, 2H, *J* = 6.0 Hz, CH_2 N), 2.95 (t, 2H, *J* = 5.9 Hz, CH_2 N), 2.57 (s, 3H, NHMe), 2.55 (s, 3H, NHMe); MS *m/z* 465 (M^+, 11), 422 (21), 374 (42), 365 (34), 350 (100), 336 (72). Anal. Calcd for $C_{24}H_{27}N_5O_3S$: C, 61.92; H, 5.85; N, 15.04; S, 6.89. Found: C, 61.61; H, 5.58; N, 15.12; S, 7.12.

3.1.15. *N*-Methylamide 4,11-bis{[2-(dimethylamino)ethyl]amino}anthra[2,3-*b*]thiophene-5,10-dione-2-carboxylic acid (22)

This was prepared similarly from amide **18** and *N,N*-dimethylethylenediamine as described for anthrathiophenedione **9** (60 °C, 3–4 h). Yield 74%, dark blue crystals, mp 126–127 °C (benzene); dihydrochloride mp >250 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 12.14 (br s, 1H, NH), 12.10 (br s, 1H, NH), 8.32 (m, 2H, 6-H, 9-H), 8.14 (s, 1H, 3-H), 7.65 (m, 2H, 7-H, 8-H), 7.21 (br s, 1H, NH), 3.96 (dd, 2H, ^1J = 5.3 Hz, ^2J = 5.5 Hz, HNCH_2), 3.70 (br m, 2H, HNCH_2), 3.04 (d, 3H, *J* = 3.1 Hz, NMe), 2.71 (t, 2H, *J* = 5.7 Hz, C$H_2$$NMe_2$), 2.65 (t, 2H, *J* = 5.8 Hz, C$H_2$$NMe_2$), 2.38 (s, 6H, NMe_2), 2.35 (s, 6H, NMe_2); MS *m/z* 493 (M^+, 100), 448 (64), 432 (18), 390 (76), 377 (54), 362 (87). Anal. Calcd for $C_{26}H_{31}N_5O_3S$: C, 63.26; H, 6.33; N, 14.19; S, 6.50. Found: C, 63.03; H, 6.58; N, 14.34; S, 6.83.

3.1.16. *N*-Methylamide 4,11-bis[{2-[(2-hydroxyethyl)amino]ethyl}amino]anthra[2,3-*b*]thiophene-5,10-dione-2-carboxylic acid dihydrochloride (23)

This was prepared from amide **18** and 2-[(2-hydroxyethyl)amino]ethylamine as described for anthrathiophenedione **7** (50 °C, 2–3 h). The residue obtained after purification by chromatography was dissolved in warm 1 N HCl and re-precipitated with acetone. The precipitated crystals were filtered, washed with Et_2O and dried to yield 63% of dihydrochloride **23** as dark-blue powder, mp 245–246 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.71 (t, 1H, *J* = 5.4 Hz, NH), 11.66 (t, 1H, *J* = 5.3 Hz, NH), 9.54 (q, 1H, *J* = 4.7 Hz, NH), 9.24 (br s, 2H, NH_2), 9.15 (br s, 2H, NH_2), 8.60 (s, 1H, H^3), 8.18 (m, 2H, 6-H, 9-H), 7.74 (m, 2H, 7-H, 8-H), 4.15 (m, 4H, 2NCH_2), 3.70 (m, 4H, 2NCH_2), 3.35 (m, 4H, 2NCH_2), 3.12 (m, 4H, 2NCH_2), 2.83 (d, 3H, *J* = 4.7 Hz, NMe); MS *m/z* 351 (M^+–2C_4H_9NO, 100). Anal. Calcd for $C_{26}H_{31}N_5O_5S{\cdot}2HCl{\cdot}2H_2O$: C, 49.21; H, 5.88; N, 11.04; S, 5.05. Found: C, 48.91; H, 5.24; N, 10.80; S, 5.32

3.1.17. *N*-(2-Hydroxyethyl)amide 4,11-bis{[2-(methylamino)ethyl]amino}anthra[2,3-*b*]thiophene-5,10-dione-2-carboxylic acid (24)

This was prepared from amide **19** and *N*-methylethylenediamine as described for anthrathiophenedione **7** (60 °C, 3–4 h). Yield 77%, dark blue needles, mp 145–148 °C (benzene); ^{1}H NMR (400 MHz, $CDCl_3$) δ 11.94 (t, 1H, *J* = 4.8 Hz, NH), 11.48 (t, 1H, *J* = 5.3 Hz, NH), 8.40 (t, 1H, *J* = 5.2 Hz, NH), 8.07 (m, 2H, 6-H, 9-H), 7.93 (s, 1H, 3-H), 7.55 (m, 2H, 7-H, 8-H), 3.93 (dd, 2H, *J* = 4.7 Hz,

CH_2), 3.72 (dd, 2H, ^1J = 4.8 Hz, ^2J = 5.3 Hz, HNCH_2), 3.61 (m, 4H, 2CH_2), 2.58 (t, 2H, J = 5.3 Hz, CH_2NMe_2), 2.58 (t, 2H, J = 5.8 Hz, CH_2NMe_2), 2.60 (s, 3H, NHMe), 2.49 (s, 3H, NHMe); MS *m/z* 495 (M^+, 5), 417 (19), 404 (25), 395 (31), 381 (100), 366 (24). Anal. Calcd for $C_{25}H_{29}N_5O_4S$: C, 60.59; H, 5.90; N, 14.13; S, 6.47. Found: C, 60.72; H, 5.74; N, 14.44; S, 6.81.

3.1.18. *N*-(2-Hydroxyethyl)amide 4,11-bis{[2-(dimethylamino)-ethyl]amino}anthra[2,3-*b*]thiophene-5,10-dione-2-carboxylic acid (25)

This was prepared from amide **19** and *N,N*-dimethylethylenediamine (60 °C, 3–4 h) as described for anthrathiophenedione **9**. Yield 79%, dark blue crystals, mp 163–165 °C (benzene); ^{1}H NMR (400 MHz, $CDCl_3$) δ 12.17 (t, 1H, J = 5.3 Hz, NH), 12.00 (t, 1H, J = 4.8 Hz, NH), 8.24 (m, 2H, 6-H, 9-H), 7.99 (s, 1H, 3-H), 7.97 (t, 1H, J = 4.9 Hz, NH), 7.61 (m, 2H, H[7,8]), 3.96 (dd, 2H, J = 5.1 Hz, CH_2), 3.78 (dd, 2H, ^1J = 5.3 Hz, ^2J = 6.4 Hz, HNCH_2), 3.63 (m, 4H, 2CH_2), 2.58 (t, 4H, J = 6.4 Hz, 2CH_2NMe_2), 2.33 (s, 6H, NMe_2), 2.32 (s, 6H, NMe_2); MS *m/z* 523 (M^+, 100), 505 (24), 478 (54). Anal. Calcd for $C_{27}H_{33}N_5O_4S$: C, 61.93; H, 6.35; N, 13.37; S, 6.12. Found: C, 61.61; H, 6.58; N, 13.49; S, 6.45.

3.1.19. Hydrazide of 4,11-bis{[2-(methylamino)ethyl]-amino}anthra[2,3-*b*]thiophene-5,10-dione-2-carboxylic acid trihydrochloride (26)

This was prepared from hydrazide **20** and *N*-methylethylenediamine as described for anthrathiophenedione **7** (60 °C, 2–3 h). The residue obtained after purification by chromatography was dissolved in hot methanol followed by the addition of a solution of HCl in methanol. The mixture was stirred for 4 h, the precipitated crystals were filtered, the collected solid was re-precipitated from hot water with acetone, washed with acetone and dried to yield 72% of trihydrochloride **27** as dark blue powder, mp 235–237 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.65 (t, 1H, J = 5.4 Hz, NH), 11.49 (t, 1H, J = 5.7 Hz, NH), 9.23 (br s, 3H, 3NH), 8.71 (s, 1H, 3-H), 8.18 (m, 2H, 6-H, 9-H), 7.77 (m, 2H, 7-H, 8-H), 4.13 (m, 4H, 2CH_2), 3.32 (br m, 4H, 2CH_2), 2.65 (s, 3H, NHMe), 2.64 (s, 3H, NHMe); MS *m/z* 466 (M^+, 5), 464 (24), 451 (14), 406 (76), 351 (100). Anal. Calcd for $C_{23}H_{26}N_6O_3S{\cdot}3HCl{\cdot}2H_2O$: C, 45.14; H, 5.44; N, 13.73; S, 5.24. Found: C, 45.51; H, 5.72; N, 13.77; S, 5.66.

3.1.20. Hydrazide of 4,11-bis{[2-(dimethylamino)ethyl]amino}anthra[2,3-*b*]thiophene-5,10-dione-2-carboxylic acid trihydrochloride (27)

This was prepared from hydrazide **20** and *N,N*-dimethylethylenediamine as described for anthrathiophenedione **9** (60 °C, 3–4 h). The residue obtained after purification by chromatography was dissolved in hot methanol, and the solution of HCl in methanol was added. The mixture was stirred for 4 h, the precipitated crystals were filtered, the collected solid was re-precipitated from hot water with acetone, washed with acetone and dried to yield 65% of dark-blue powder of trihydrochloride **27**, mp 244–247 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.78 (t, 1H, J = 4.3 Hz, NH), 11.66 (t, 1H, J = 5.2 Hz, NH), 10.96 (br s, 1H, NH), 8.79 (s, 1H, 3-H), 8.14 (m, 2H, 6-H, 9-H), 7.74 (m, 2H, 7-H, 8-H), 4.21 (br m, 4H, 2CH_2), 3.51 (m, 4H, 2CH_2), 2.93 (s, 6H, NMe_2), 2.87 (s, 6H, NMe_2); UV (ethanol) λ_{max} (log ε) 240 (4.2), 275 (4.6), 335 sh (3.8), 412 (3.2), 547 sh (3.8), 584 (4.1), 626 (4.1) nm. Anal. Calcd for $C_{25}H_{30}N_6O_3S{\cdot}3HCl{\cdot}2H_2O$: C, 46.92; H, 5.83; N, 13.13; S, 5.01. Found: C, 49.60; H, 6.12; N, 13.01; S, 5.14.

3.2. Cell lines, drug treatment and viability assay

The K562 human leukemia cell line (American Type Culture Collection; ATCC, Manassas, VA) and its Pgp-positive subline K562/4 selected for survival in the continuous presence of doxorubicin (gift of A. Saprin, Moscow), the HCT116 colon carcinoma cell line (ATCC) with wild type p53 ($p53^{+/+}$) and the HCT116p53KO subline (both p53 alleles deleted[20]) ($p53^{-/-}$) and human lung carcinoma H1299 cell line (ATCC) were cultured in RPMI-1640 supplemented with 5% fetal calf serum (HyClone, Logan, UT), 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 °C, 5% CO_2 in humidified atmosphere. The murine leukemia L1210, human lymphocyte Molt4/C8 and CEM cells were cultured in RPMI-1640 supplemented with 10% fetal calf serum, 0.075% $NaHCO_3$ and 2 mM L-glutamine. Cells in logarithmic phase of growth were used in all experiments. All reagents were from Sigma Chemical Co., St. Louis, MO unless specified otherwise. Novel compounds were dissolved in 10% aqueous DMSO as 10 mM stock solutions followed by serial dilutions in water immediately before experiments. The cytotoxicity was determined in a formazan conversion assay (MTT-test). Briefly, cells (5×10^3 in 190 μL of culture medium) were plated into a 96-well plate (Becton Dickinson, Franklin Lakes, NJ) and treated with 0.1% DMSO (vehicle control) or with increasing concentrations of tested compounds (each dose in duplicate) for 48 h (L1210 cells) or 72 h (all other cell lines). After the completion of drug exposure, 50 μg of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide were added into each well for an additional 2 h. Formazan was dissolved in DMSO, and the absorbance at λ = 540 nm was measured. Cell viability at a given drug concentration was calculated as the percentage of absorbance in wells with drug-treated cells to that of vehicle control cells (100%). The IC_{50} (50% inhibitory concentration) was defined as the concentration of the compound that inhibited cell viability by 50%. In some experiments viable (Trypan blue negative) cells were counted in a Coulter counter.

3.3. Topo I assay

The ability of anthrathiophenediones **7**, **8**, **15**, **16** to modulate the activity of topo I in vitro was determined in a DNA relaxation assay.[21] Briefly, 4 units of purified topo I (Amersham Biosci., UK) were incubated with 0.25 μg of supercoiled pHot plasmid DNA in the buffer (10 mM Tris–HCl, pH 7.9, 1 mM ethylenediamine tetraacetic acid disodium salt, 0.15 M NaCl, 0.1% bovine serum albumin, 0.1 mM spermidine, 5% glycerol) in the presence of 0.1% DMSO (vehicle control) or compounds **7**, **8**, **15**, **16** (at concentrations indicated in Fig. 1) at 37 °C for 30 min. The reaction was terminated by the addition of 1% sarcosyl. DNA topoisomers were resolved by electrophoresis in 1% agarose gel (3 h, 70 V) in the buffer containing 40 mM Tris–acetate, pH 7.6, 1 mM ethylenediamine tetraacetic acid disodium salt. After electrophoresis gels were stained with 0.5 μg/mL ethidium bromide.

3.4. Telomere repeat amplification protocol (TRAP assay)

Telomerase activity in the presence of anthrathiophenediones **8**, **15** and **16** was detected by a modified TRAP assay[22,23] Telomerase products were resolved by electrophoresis in 10% polyacrylamide gel and visualized after staining with SYBR Green. As a source of telomerase, the total cell lysates of H1299 cell line were used. Total protein concentration was assayed using Bradford reagent (BioRad, Hercules, CA).

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