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# Synthesis, cytotoxic activity and DNA-interaction studies of novel anthraquinone–thiosemicarbazones with tautomerizable methylene group

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ABSTRACT

A series of novel anthraquinone–thiosemicarbazone derivatives in a tautomerizable keto-imine form was synthesized and tested for their *in vitro* cytotoxic activity against human cancer cells (HeLa, MDA-MB-361, MDA-MB-453, K562, A549) and human normal MRC-5 cells. Several compounds efficiently inhibited cancer cell growth at micromolar concentrations, especially against K562 and HeLa cells. As determined by flow cytometric analysis, anthraquinone–thiosemicarbazone caused significant increase in the number of sub-G1 phase of HeLa cells and apoptosis in a concentration-dependent manner. Also, inhibition of caspase-3, -8, and -9 with specific caspase inhibitors reduced the apoptosis mediated by the tested compounds in HeLa cells. All anthraquinone–thiosemicarbazones exhibit calf thymus DNA-binding activity, but no cleavage of plasmid DNA was observed.



## 1. Introduction

Many natural and synthetic anthraquinones represent an important class of compounds with a great biological utility, especially in the role of antitumor agents [1–6]. Among them, the anthracyclines, like doxorubicin and daunorubicin have been widely applied as chemotherapeutic drugs with broad clinical indications as anticancer compounds [7], but with limited use due to their cardiotoxicity [8,9]. Anthraquinones generally act as intercalators [10,11] and inhibitors of telomerase [12–14] and DNA topoisomerase through stabilization of the cleavable complex between topoisomerase II and DNA formed in the catalytic cycle of the enzyme [15–18]. These compounds are also involved in a redox cycle system by the presence of quinone component and the generation of reactive oxygen species (ROS) which may have influence on cell proliferation in multistage carcinogenesis [19,20].

Certainly, the antitumor activity of anthraquinone derivatives is strongly affected by the introduction of the different substituents into aromatic planar system. These side groups can stabilize electrostatic interactions with phosphate backbone of the polynucleotide chain and meet requirements for effective inhibition of topoisomerase II [21]. Thiosemicarbazones belong to a group of antitumor pharmacophores [22,23] that could stabilize the cleavable complex between topoisomerase II and DNA through alkylation of thiol on the topo II–DNA complex [24]. In addition, thiosemicarbazones can inhibit iron-containing enzyme ribonucleotide reductase by metal ion chelation [25] because iron has been shown to be an essential molecular target in proliferation of cancer cells [26] and generation of reactive oxygen species [27].

Besides thiosemicarbazone substituent, the presence of an additional tautomerizable group linked to the pharmacophoric anthraquinone unit might be very useful in terms of introduction of specific oncogene recognition elements into structure of compound with an antitumor potential. For example, curcumin exhibits antitumor activity through inhibition of glyoxalase 1 (Glo 1). The possibility of existence of this polyphenol in two tautomeric forms

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suggests that keto-enol structural segment directly interacts with the active site of Glo 1 and appears to be crucial for antitumor activity [28,29].

On the basis of above mentioned facts, we supposed that combining anthraquinone and thiosemicarbazone pharmacophore with a tautomerism possibility could result in compounds with favorable biological potential. Consequently, in this study we designed and synthesized a series of new tautomerizable anthraquinone–thiosemicarbazone conjugate to evaluate their antiproliferative activity against different cancer cell lines.

## 2. Results and discussion

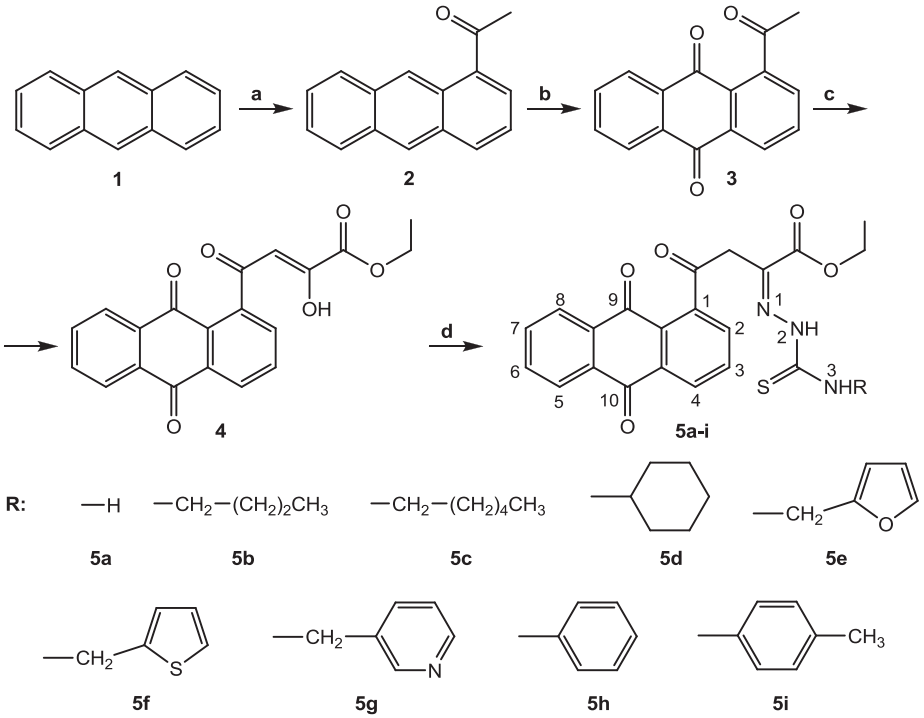
### 2.1. Chemistry

The preparation of target conjugates is presented in Scheme 1. The synthesis started with commercially available anthracene **1** which was acylated with acetyl chloride to 1-acetylanthracene **2** according to known literature procedure [30]. The compound **2** was then oxidized by chromium(VI) oxide in glacial acetic acid yielding 1-acetylanthraquinone **3** [31]. After recrystallization from ethanol, 1-acetylanthraquinone **3** was directly condensed with diethyl oxalate in the presence of sodium ethoxide affording anthraquinone ester **4** in an excellent yield. This compound was used in the next step without further purification and reacted with a series of substituted thiosemicarbazides to give the final compounds **5a**–**i** in moderate to good yields. The exact structure of all compounds was confirmed by means of $^{1}H$, $^{13}C$, NOESY, COSY, HSQC, HMBC, $^{1}H$-$^{15}N$ HSQC and $^{1}H$-$^{15}N$ HMBC spectra using compound **5a** as an illustrative example (see Supplementary material, Figs. S4–S12). The position of ketone carbonyl group was determined according to the HMBC spectrum which displays a three-bond correlation between its carbon atom and the nearest proton of the aromatic core at 7.78 ppm. This indicates that keto carbonyl group is linked directly to the anthraquinone moiety.

Eight AQ-thiosemicarbazones (AQ denotes anthraquinone moiety substituted in position **1**) have been characterized in two isomer types, *Z* and *E*, with respect to the azomethine C=N bond, whereas **5a** exists only in the form of *E* isomer. The stereochemistry of these compounds was confirmed by a NOESY experiment which indicated a correlation between hydrogens of tautomerizable methylene group and N2 hydrogens from the thiosemicarbazone moiety. The ratio of *Z* and *E* geometrical isomers is time-dependent and all values presented in Supplementary material are obtained by recording NMR spectra of freshly prepared solutions in DMSO-$d_6$.

Also, NMR spectra showed that all synthesized AQ-thiosemicarbazones exist exclusively in keto-imine tautomeric form in DMSO-$d_6$ solution (Fig. 1).

This form is confirmed by the presence of a singlet at 4.35–4.44 ppm (integral = 2) assigned to the methylene group protons. It is interesting that $^{1}H$ NMR spectrum of AQ-ester **4** (see Supplementary material, Figs. S1 and S2) unambiguously indicates keto-enol II form which can be easily confirmed by a singlet for olefinic proton at 6.54 ppm (integral = 1) and a broad singlet at 13.61 ppm attributed to proton of the hydroxyl group. An imine derivative **6** with poor antiproliferative activity ($IC_{50}$ ~ 100 μM) obtained by condensation of **4** and aniline exists only in a form of keto-enamine tautomer. However, phenylhydrazone derivative **7** existing in the keto-imine tautomeric form showed significantly better cytotoxic activity ($IC_{50}$ = 40–50 μM) against all cells in comparison with compound **6**. Although the participation of thiosemicarbazone moiety is crucial for biological potential, it seems that keto-imine form is another important prerequisite for good cytotoxicity of the tested anthraquinone compounds. The same tautomeric forms for all compounds are also observed in $CDCl_3$ indicating that the applied deutero-solvent has no influence on their tautomeric behavior.




**Scheme 1.** Reagents and conditions: a) $CH_3COCl$, $AlCl_3$, $CH_2Cl_2$, 2 h, 0 °C, 5 M HCl; b) $CrO_3$, $CH_3COOH$, 5 min reflux, $H_2O$; c) $(COOEt)_2$, NaOEt, dioxane, 1 h, stirring, r.t., 2 M HCl; d) $H_2NHNC(=S)NHR$, EtOH, 4 h, reflux.

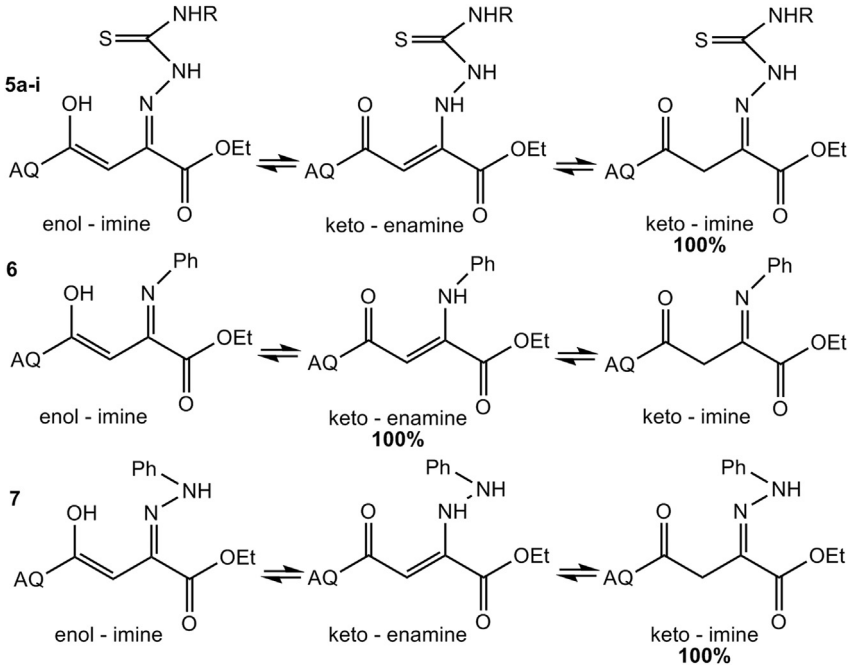



**Fig. 1.** The possible tautomeric forms of the compounds **5a**–**i**, **6** and **7**. AQ denotes anthraquinone core substituted in position **1**.

## 2.2. Biology

### 2.2.1. In vitro cytotoxicity

The *in vitro* cytotoxicity of the AQ-thiosemicarbazones **5a**–**i** against five human cancer cell lines, cervix adenocarcinoma cell line (HeLa), human breast cancer (MDA-MB-361 and MDA-MB-453), chronic myelogenous leukemia (K562), non-small cell lung carcinoma (A549) and a non-cancerous cell line, MRC-5 (human embryonic lung fibroblast) was investigated by a tetrazolium based (MTT) colorimetric assay and compared with those of cisplatin (an anticancer drug used widely in the clinic) as positive control. The results of cytotoxic activity *in vitro* are expressed as $IC_{50}$, the concentration of compound (in μM) that inhibits a survival of the cells by 50% as compared to control untreated cells (Table 1).

Generally, the compounds demonstrated good cytotoxic activity, $IC_{50}$ values range from 2.17 to 50.54 μM against all the tested cell lines. The K562 cell line is much more sensitive to the compounds than the other cell lines; in this case, the activity of compounds can even be compared with the activity of cisplatin ($IC_{50}$ values range from 2.17 to 7.99 μM). This opens the way for further investigations of these compounds as potential drug candidates in the treatment of leukemia. Compounds **5c**, **5d**, **5f** and **5h** exhibit the strongest activity against all five cancer cell lines. The $IC_{50}$ values of these compounds against all cancer cell lines are in a micromolar range similar or better to that of the antitumor drug cisplatin. Compound **5h** exhibits the highest activity against A549 cancer cell lines and **5c** against HeLa cancer cell line. Selectivity was observed for **5c** against resistant MDA-MB-361 cell lines ($IC_{50}$ value of 4.45 μM) while **5g** exhibited an excellent selectivity against K562 cell line ($IC_{50}$ value of 2.17 μM). Compounds **5c**, **5d**, **5f** and **5h** can be considered as agents with potential antitumor activity, and therefore as candidates for further stages of screening *in vitro* and/or *in vivo*. In the non-cancerous lung fibroblasts (MRC-5), most of the compounds were slightly less cytotoxic than cisplatin. The $IC_{50}$ of cisplatin in this cell line was 14.44 μM and compounds **5a**–**i** ranged from 11.79 to 42.13 μM. The cytotoxicities of **5c** and **5f** in the lung fibroblasts were marked by $IC_{50}$ values of 42.13 and 24.35 μM, respectively, indicating that they are several times less cytotoxic in the healthy cells compared to almost all the tested cancerous cells.

### 2.2.2. Cell-cycle analysis

To determine whether treatment with compounds **5a**–**i** could affect the cell cycle of HeLa cells, the cells treated for 24 h and the control cells were stained with propidium iodide (PI) and analyzed

**Table 1**
Concentrations of compounds **5a**–**i** that induced a 50% decrease in HeLa, MDA-MB-361, MDA-MB-453, K562, A549 and MRC-5 cell survival (expressed as $IC_{50}$ (μM)). All compounds were incubated with cells for 72 h.

| Compound | IC50 (μM)[a] | | | | | |
|---|---|---|---|---|---|---|
| | HeLa | MDA-MB-361 | MDA-MB-453 | K562 | A549 | MRC-5 |
| **5a** | 14.40 ± 1.82 | 30.66 ± 1.35 | 13.61 ± 4.10 | 7.99 ± 2.33 | 28.96 ± 5.26 | 36.19 ± 3.67 |
| **5b** | 10.87 ± 1.53 | 30.83 ± 1.11 | 11.51 ± 0.51 | 3.80 ± 1.04 | 19.56 ± 0.55 | 21.14 ± 0.80 |
| **5c** | 7.66 ± 2.44 | 4.45 ± 0.68 | 19.55 ± 3.92 | 5.86 ± 3.05 | 43.62 ± 2.08 | 42.13 ± 4.92 |
| **5d** | 9.15 ± 1.97 | 32.63 ± 8.31 | 4.64 ± 1.77 | 2.35 ± 0.08 | 13.87 ± 4.49 | 13.60 ± 5.90 |
| **5e** | 18.96 ± 0.63 | 47.75 ± 0.21 | 19.36 ± 1.45 | 2.63 ± 0.22 | 22.62 ± 2.74 | 38.57 ± 9.19 |
| **5f** | 8.25 ± 1.98 | 25.39 ± 1.58 | 13.47 ± 2.39 | 2.80 ± 0.26 | 12.88 ± 7.57 | 24.35 ± 0.05 |
| **5g** | 18.09 ± 1.5 | 50.54 ± 1.87 | 17.68 ± 1.03 | 2.17 ± 0.02 | 23.39 ± 7.74 | 13.60 ± 7.65 |
| **5h** | 8.19 ± 1.93 | 6.61 ± 1.51 | 6.91 ± 0.56 | 3.59 ± 1.67 | 9.89 ± 1.94 | 11.79 ± 0.36 |
| **5i** | 21.01 ± 1.01 | 32.61 ± 2.01 | 11.31 ± 0.49 | 3.97 ± 0.24 | 22.51 ± 0.09 | 30.27 ± 7.99 |
| **Cisplatin** | 2.1 ± 0.20 | 14.74 ± 0.36 | 3.75 ± 0.12 | 5.54 ± 1.03 | 11.92 ± 2.19 | 14.44 ± 1.90 |

[a] $IC_{50}$ values were expressed as the mean ± SD determined from the results of MTT assay in three independent experiments.

by flow cytometry. Fig. 2 shows a representative cell-cycle distribution of HeLa cells incubated in the absence or presence of **5c**, **5f** and **5h** for 24 h, the approximate doubling time of this cell line.

The obtained DNA histograms indicate that treatment with $IC_{50}$ or 2 × $IC_{50}$ for 24 h leads to a significant increase in the number of sub-G1 phase cells and a decrease in the number of G2/M of HeLa cells after treatment with these compounds. Treatment with **5c**, **5f** and **5h** at 2 × $IC_{50}$ resulted in the highest percentage of cells (37.32%, 36.27% and 23.74%, respectively) in sub-G1 phase of the cell cycle. Obviously, the sub-G1 phase cell population of the compound-treated cells increased in a dose-dependent manner. Therefore, it has been shown that the activity of these compounds could be related to apoptotic cell death. Similar results were also found when HeLa cells were exposed to all the other compounds **5a**–**i** (data not shown).

### 2.2.3. Apoptosis induced by AQ-thiosemicarbazones is mediated by a caspase dependent pathway

Fig. 3 shows that the apoptosis induced by compounds **5a**–**i** was mediated by stimulating caspase-3, -8, and -9 activities.

To investigate whether compounds **5a**–**i** induced apoptosis through the caspase cascades-dependent pathway, cells were individually pretreated with inhibitors of caspase-3, -8, and -9 before the treatment with the compounds concentrations corresponding to $IC_{90}$ values for 24 h. Fig. 3 shows that these specific inhibitors significantly suppressed the caspase activities and increased the cell viability in analyzed HeLa cells. As shown in Fig. 3, compound **5f** does not show the expected response to caspase inhibitors; this indicates that the induction of apoptosis in this compound involved other mechanisms. On the other hand, the inhibitors of caspase-3, and -9 exerted almost no influence on the activity of compound **5g**. We conclude that compound **5g** induces apoptotic cell death mainly via mitochondrial pathway through activation of caspase-8 in HeLa cells. Finally, our results suggest that the activation of caspases-3, -8, and -9 might be involved in apoptosis induced by compounds **5a**–**i** in HeLa cells.

### 2.2.4. Antioxidant activity

Reactive oxygen species (ROS) are able to induce cell death and DNA damage leading to increased tumor proliferation [32]. The antioxidants which scavenge intracellular ROS may inhibit carcinogenesis by affecting the molecular events in the initiation, promotion and progression stages [33]. Antioxidant activity of the synthesized AQ-thiosemicarbazones has been based on scavenging activity of stable DPPH radicals, as presented in Table 2.

If antioxidant potential cause cytotoxic behavior, bioreducible groups of anthraquinone might be responsible for anticancer activity. However, if the cytotoxicity depends only on intercalative capacity of the planar anthraquinone scaffold, it might be affected by substituents that enhance the binding affinity to DNA. Although all tested AQ-thiosemicarbazones show good to excellent antioxidant activity, it is difficult to establish a relationship between their antioxidant and cytotoxic potential as a consequence of different mechanisms of anticancer action. Nevertheless, by comparing the antioxidant activities of two compounds of similar structures (**5h** and **5i**), it can be observed that **5h** with much better scavenging potential exhibits much stronger cytotoxicity.

### 2.2.5. DNA-interaction studies

In general, non-covalent binding of small molecules with DNA occurs in three modes: intercalation binding, minor or major groove binding and electrostatic binding. Anthraquinone and its derivatives as good intercalators of DNA have been frequently used as anticancer drugs [11]. To deduce the binding mode of anthraquinone derivatives with thiosemicarbazone substituents which were used for investigation, the UV–Vis spectrometry analysis was performed. Electronic absorption spectra of all compounds recorded at different concentrations without or with fixed concentration of CT-DNA are shown in Fig. 4. It was found that the spectra of all

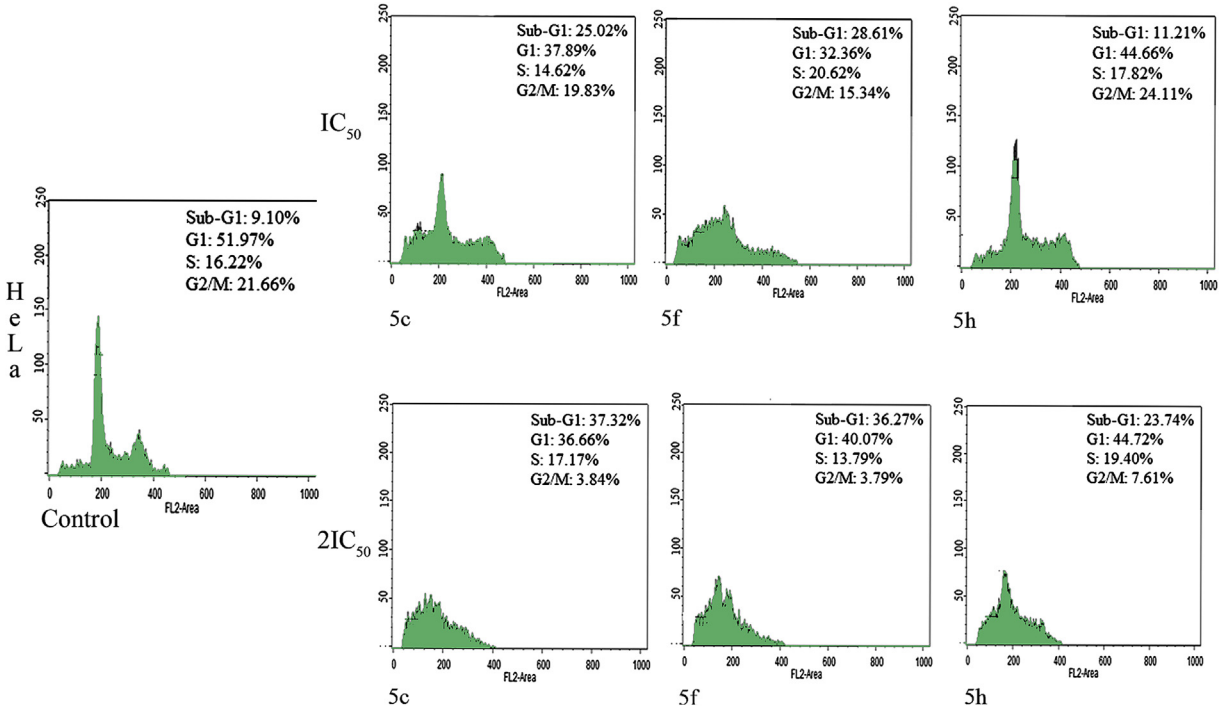



**Fig. 2.** Cell-cycle distribution after 24 h of continuous action of ($IC_{50}$ and 2 × $IC_{50}$) compounds **5c**, **5f** and **5h** in HeLa cell lines. Representative histograms of cell-cycle distribution of HeLa measured by flow cytometric analysis of DNA content after treatment with these compounds.

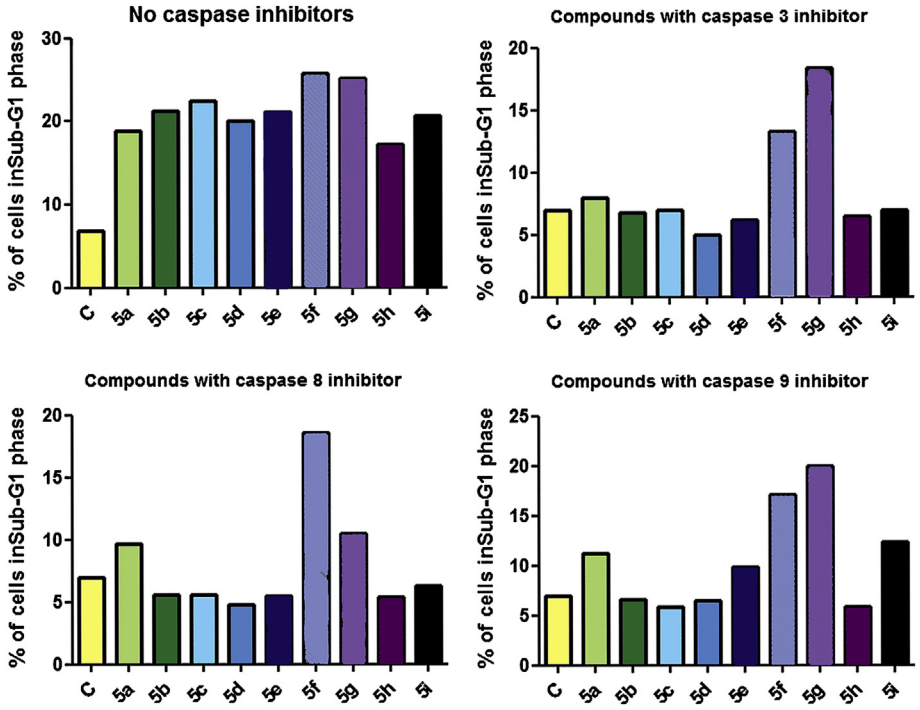



**Fig. 3.** Effects of **5a**–**i** compounds on caspase inhibitors. HeLa cells were exposed to $IC_{90}$ of compounds **5a**–**i** for 24 h in the presence of specific caspase inhibitors (final concentration – 40 μM) as described under Experimental.

anthraquinones exhibit peaks at 255 nm and 278 nm. The parent compound and the derivatives with alkyl substituents on thiosemicarbazone residue exhibit a third peak positioned at 300 nm, while aromatic substituents and cyclohexyl derivatives had a shoulder at 311–350 nm. The peaks at 278 nm disappeared after interaction of the anthraquinones with CT-DNA, followed by an increase of absorption maximum at 255 nm and 311 nm indicating the formation of an anthraquinone–CT-DNA complex. Hypochromism observed with lower concentrations of the compounds indicates intercalation as the most probable interaction. With increase of concentration hyperchromism occurs as the result of possible non-covalent or electrostatic interactions between anthraquinone moiety and double helix of DNA. These additional interactions were most prominent with the compounds with aromatic and cyclic moieties.

In order to obtain information on affinity of the anthraquinone–CT-DNA complex, spectroscopic titrations of the solution of **5a**–DNA, **5h**–DNA and **5i**–DNA with increasing concentration of CT-DNA were performed. The absorbance at 255 nm was monitored for each concentration of DNA. The binding constant $K_B$ was determined using the equation (1) [34]

$$[\mathrm{DNA}]\times(\varepsilon_A-\varepsilon_F)^{-1}=[\mathrm{DNA}]\times(\varepsilon_B-\varepsilon_F)^{-1}+K_B^{-1}\times(\varepsilon_B-\varepsilon_F)^{-1} \quad (1)$$

where $\varepsilon_A$, $\varepsilon_F$, $\varepsilon_B$ are absorbance/[compound], extinction coefficient of the free compound and the extinction coefficient of the bound compound, respectively. The intrinsic binding constant $K_B$ (Fig. 5) of **5a**–DNA, **5h**–DNA and **5i**–DNA complexes were: $0.55 \times 10^9$ $M^{-1}$, $9.3 \times 10^9$ $M^{-1}$ and $2.91 \times 10^9$ $M^{-1}$, respectively. The values are higher than the values of $K_B$ described in the literature for classical intercalators, for example, ethidium–DNA, $7 \times 10^7$ $M^{-1}$ [35]. Since the phenyl derivative **5h** was always the most active compound of these three derivatives, it is very likely that the cytotoxic activity is the consequence of DNA binding.

The ability of the anthraquinone derivatives to cleave DNA was tested using pUC18 plasmid, which might undergo transformation into relaxed circular form (FII) from supercoiled form (FI). Under applied conditions, i.e. 2 mM anthraquinone and 15.4 nM DNA, the results of agarose gel electrophoresis showed that there was no significant cleavage with any of synthesized derivatives (Fig. 6).

Since DNA damage by quinone/hydroquinone compounds is often mediated by oxygen radicals, which can be generated by reduced forms (semiquinone or hydroquinone), strand scission ability was tested under reducing conditions, i.e. in the presence of iron(II). Iron(II) per se is involved in the damage of DNA [36], and pUC18 was no exception to that (Fig. 7, lanes P + Fe(II)). When anthraquinone compounds and iron were used in mole ratio 1:1, the anthraquinones abolished the effects of iron (Fig. 7a) probably by complex formation with the thiosemicarbazone moiety. When the mole ratio quinone–iron was 1:2, DNA damage by iron could be detected (Fig. 7b). Iron(III) had no effect under any conditions. These results indicate that DNA cleavage could not be the cause of cytotoxicity.

**Table 2**
$IC_{50}$ values of tested AQ-thiosemicarbazones, and two standard compounds on DPPH free radical inhibition.

| Compound | $IC_{50}$ (μM) |
|---|---|
| **5a** | 20.5 |
| **5b** | 25.8 |
| **5c** | 26.1 |
| **5d** | 16.4 |
| **5e** | 35.8 |
| **5f** | 66.5 |
| **5g** | 46.9 |
| **5h** | 26.2 |
| **5i** | 41.0 |
| **Ascorbic acid** | 15.9 |
| **BHT** | 33.9 |

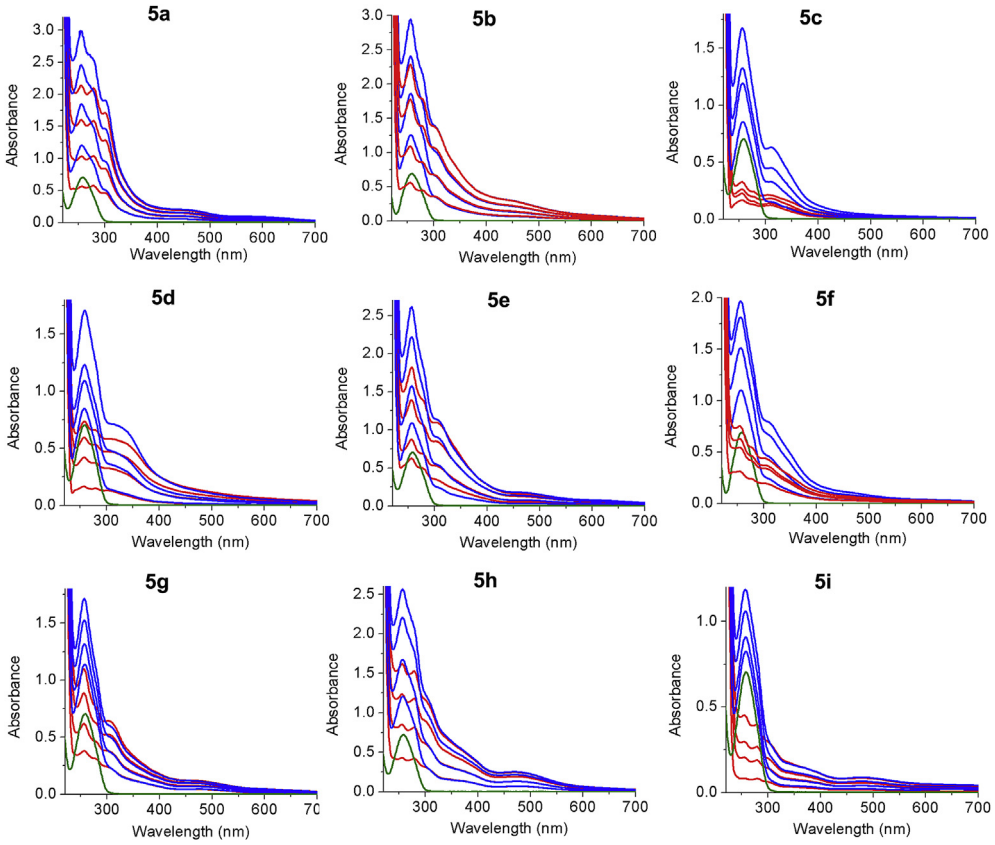



**Fig. 4.** Absorption spectra of **5a**–**i** (20 μM, 40 μM, 60 μM and 80 μM) without CT-DNA ($1.2 \times 10^{-4}$ M, red line); with CT-DNA ($1.2 \times 10^{-4}$ M, blue line); CT-DNA ($1.2 \times 10^{-4}$ M, green line).(For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

## 3. Conclusion

The novel tautomerizable anthraquinone–thiosemicarbazone derivatives have been prepared and shown to be active against several cancer cell lines, especially K562 cells with a better cytotoxic potential than cisplatin as a widely used chemotherapy drug. We demonstrated that newly synthesized compounds inhibited proliferation leading to an increase in sub-G1 phase and promoted apoptosis by a caspase dependent manner in cervix adenocarcinoma HeLa cells. The cytotoxic activity is at least in part a consequence of DNA binding.

## 4. Experimental

### 4.1. Physical measurements and methods

Melting points were determined on a Mel-Temp capillary melting points apparatus, model 1001 and are uncorrected. Elemental (C, H, N, S) analysis of the samples was carried out in the Center for Instrumental Analysis, Faculty of Chemistry, Belgrade. IR spectra were recorded on a Perkin Elmer Spectrum One FT-IR spectrometer with a KBr disc. $^{1}$H and $^{13}$C NMR spectra were recorded on a Bruker Avance III 500 MHz spectrometer. The full assignments of all reported NMR signals were made by use of 1D and 2D NMR experiments. A Rayleigh UV-1800 UV/VIS Spectrophotometer was used for determination of antioxidant activity.

Five thiosemicarbazides used for the synthesis of **5e**–**5i** were synthesized according to the reported procedure [37], while other three used for the synthesis of **5b**–**5d** were prepared according to Ref. [38].

### 4.2. General procedure for the preparation of *5a–i*

To the mixture of 1-acetylanthraquinone **3** (0.50 g, 2.00 mmol) and diethyl oxalate (1.17 g, 8.00 mmol) in dioxane (20.0 mL), sodium ethoxide 21 wt.% solution in denatured ethanol (3.1 mL, 8.20 mmol) was slowly added. The resulting dark mixture was then stirred for 1 h at room temperature. Afterward, the solution was diluted with $H_2O$ (500 mL) and 2 M HCl was added until pH 2 was reached, followed with a formation of precipitate. The mixture was left standing at the room temperature for 1 h and compound **4** was filtered off, washed with $H_2O$ and dried over $CaCl_2$. Compound **4** can be further purified by recrystallization from EtOH.

A mixture of **4** (0.20 g, 0.57 mmol) and substituted thiosemicarbazide (0.58 mmol) in EtOH was refluxed for 4 h. After cooling the flask the amount of the precipitate formed during the reflux increases. Then, the precipitate (**5a**–**i**) was filtered off, washed with a small amount of EtOH and dried over $CaCl_2$.

#### 4.2.1. Ethyl 4-(9,10-dioxo-9,10-dihydroanthracen-1-yl)-2-hydroxy-4-oxobut-2-enoate (*4*)

Light orange powder; yield: 0.62 g (89%); mp: 157–158 °C (Dec.); $^{1}$H NMR (500 MHz, DMSO-$d_6$): 1.37, (t, 3H, $J = 7.0$ Hz, $CH_3$); 4.36, (q, 2H, $J = 7.0$ Hz, $OCH_2$); 6.54, (s, 1H, olefinic); 7.65, (dd, 1H at C2, $J = 7.5$ and 1.0 Hz); 7.83, (m, 2H at C6 and C7); 7.89, (t, 1H at C3, $J = 7.5$ Hz); 8.23, (m, 1H at C5); 8.32, (m, 1H at C8); 8.46, (dd, 1H at C4, $J = 7.5$ and 1.0 Hz); $^{13}$C NMR (125 MHz, DMSO-$d_6$): 14.03, ($CH_3$); 62.52, ($OCH_2$); 103.95, (olefinic); 127.35, (Ar-H); 127.53, (Ar-H); 129.09, (Ar-H); 130.81, (Ar-H); 132.20, (Ar-H); 132.83, (Ar-H); 133.05, (Ar-H); 134.03, (Ar-H); 134.10, (Ar-H); 134.54, (Ar-H); 134.63, (Ar-H); 140.49, (Ar-H); 161.95, (COO); 162.00, (olefinic); 182.17, (C=O, AQ); 182.72,

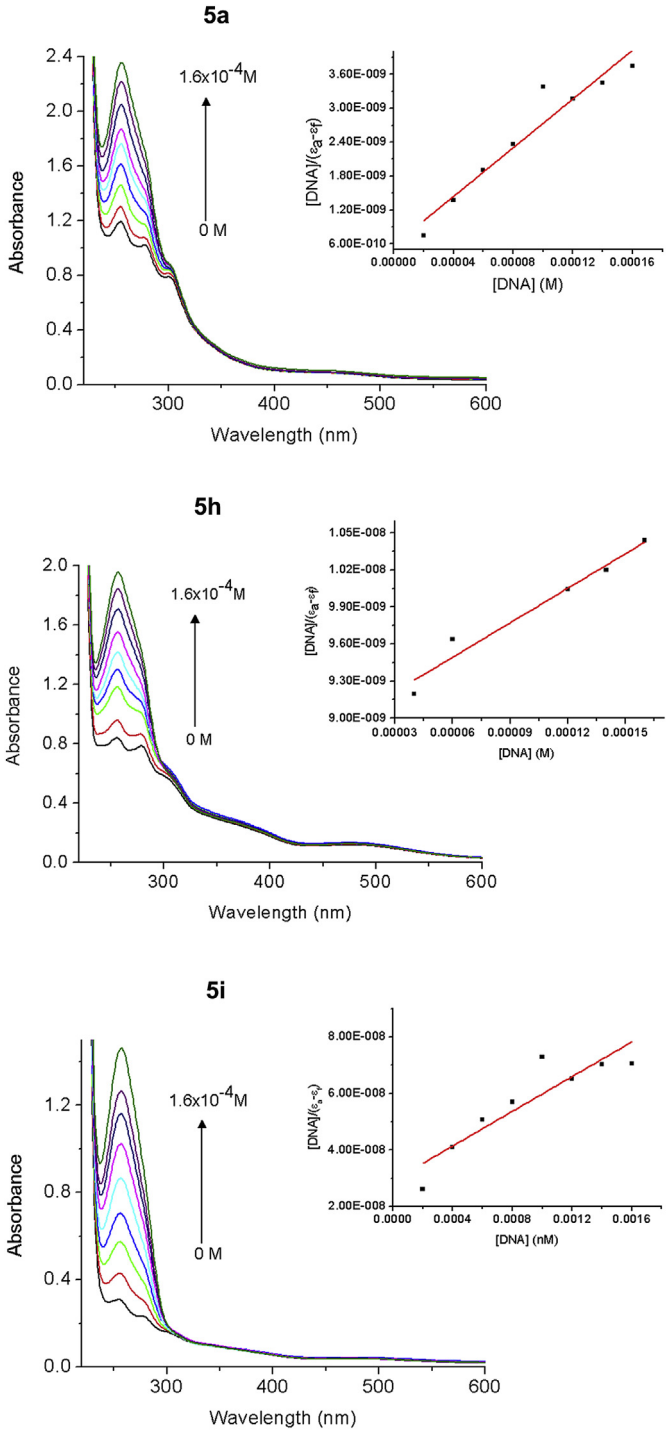



**Fig. 5.** Determination of binding constants by absorption titration of **5a** (40 μM), **5h** (40 μM) and **5i** (40 μM) with CT-DNA at different concentrations (0.2, 0.4, 0.6, 0.8, 1.0, 1.2 and 1.6) × $10^{-4}$ M. The arrows show the changes in absorbance upon increasing amounts of CT-DNA. Insets: plot of [DNA]/($\varepsilon_A - \varepsilon_F$) versus [DNA].

(C=O, AQ); 200.08, (C=O); IR (KBr, $cm^{-1}$): 3435; 1736; 1725; 1672; 1641; 1324; 1288; 1266; 710; Anal. Calcd. For $C_{20}H_{14}O_6$ (350.33 g/mol): C, 68.57; H, 4.03; Found: C, 68.04; H, 4.13.

*4.2.2. Ethyl 4-(9,10-dioxo-9,10-dihydroanthracen-1-yl)-4-oxo-2-(2-carbamothioylhydrazono)- butanoate (**5a**)*

Light yellow powder; yield: 0.55 g (52%); mp: 199–200 °C (Dec.); $^1$H NMR (500 MHz, DMSO-$d_6$): 1.28, (t, 3H, $J$ = 7.0 Hz, $CH_3$); 4.24, (q, 2H, $J$ = 7.0 Hz, $OCH_2$); 4.36, (s, 2H, $CH_2$); 7.78, (dd, 1H at C2, $J$ = 7.5 and 1.5 Hz); 7.82, (bs, 1H, $NH_2$); 7.97, (m, 2H at C6 and C7); 8.04, (t, 1H at C3, $J$ = 7.5 Hz); 8.23, (m, 1H at C5); 8.32, (m, 1H at C8); 8.32, (dd, 1H at C4, $J$ = 7.5 and 1.5 Hz); 8.84, (s, 1H, $NH_2$); 10.81, (s, 1H, NH); $^{13}$C NMR (125 MHz, DMSO-$d_6$): 13.99, ($CH_3$); 40.88, ($CH_2$); 61.34, ($OCH_2$); 126.79, (Ar-H); 127.50, (Ar-H); 127.99, (Ar-H); 130.23, (Ar-H); 131.49, (Ar-H); 132.69, (Ar-H); 132.74, (Ar-H); 133.25, (Ar-H); 134.57, (C=N); 134.68, (Ar-H); 134.81, (Ar-H); 135.08, (Ar-H); 141.95, (Ar-H); 163.91, (COO); 179.77, (C=S); 181.95, (C=O, AQ); 183.25, (C=O, AQ); 199.80, (C=O); IR (KBr, $cm^{-1}$): 3457; 3341; 3315; 1712; 1697; 1673; 1659; 1600; 1287; 706; Anal. Calcd. For $C_{21}H_{17}N_3O_5S$ (423.45 g/mol): C, 59.57; H, 4.05; N, 9.92; S, 7.57; Found: C, 59.53; H, 4.10; N, 9.93; S, 7.27.

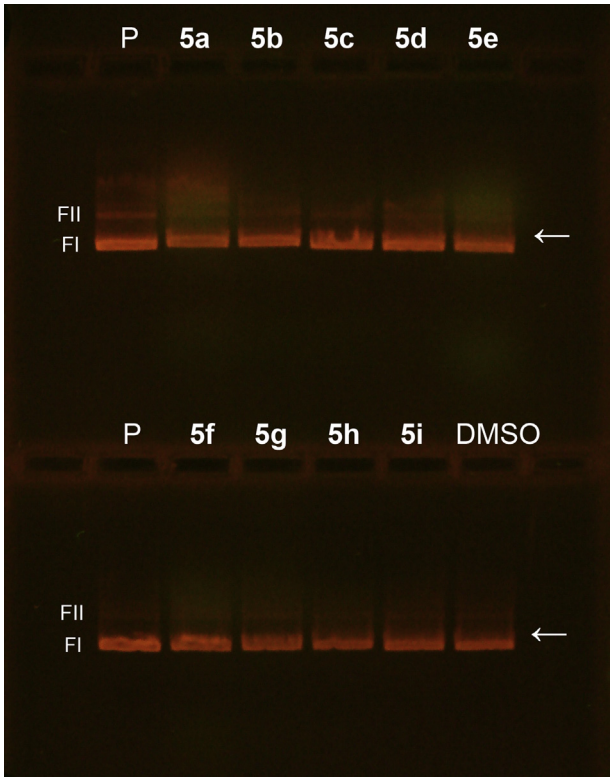



**Fig. 6.** Agarose gel electrophoresis of the supercoiled form FI and the open circular form FII of plasmid pUC18 (15.4 nM) (lanes P) after incubation with anthraquinones (2 mM): **5a**–**i**. Lane DMSO denotes the control. The arrows show the changes of the bands.

*4.2.3. Ethyl 4-(9,10-dioxo-9,10-dihydroanthracen-1-yl)-4-oxo-2-(2-(butylcarbamothioyl) hydrazono)-butanoate × $H_2O$ (**5b**, main isomer)*

Brown powder; yield: 0.20 g (70%); mp: 135–136 °C (Dec.); $^1$H NMR (500 MHz, DMSO-$d_6$): 0.92, (t, 3H, $J$ = 7.0 Hz, $CH_3$, Bu); 1.27, (t, 3H, $J$ = 7.0 Hz, $CH_3$); 1.31, (m, 2H, $CH_2$, Bu); 1.58, (m, 2H, $CH_2$, Bu); 3.60, (m, 2H, NH–$CH_2$); 4.24, (q, 2H, $J$ = 7.0 Hz, $OCH_2$); 4.35, (s, 2H, $CH_2$); 7.77, (d, 1H at C2, $J$ = 7.5 Hz); 7.96, (m, 2H at C6 and C7); 8.03, (t, 1H at C3, $J$ = 7.5 Hz); 8.21, (m, 1H at C5); 8.30, (m, 1H at C8); 8.31, (d, 1H at C4, $J$ = 7.5 Hz); 8.46, (t, 1H, $J$ = 6.0 Hz, NH–Bu); 10.81, (s, 1H, NH); $^{13}$C NMR (125 MHz, DMSO-$d_6$): 13.72, ($CH_3$, Bu); 13.99, ($CH_3$); 19.56, ($CH_2$, Bu); 30.43, ($CH_2$, Bu); 40.91, ($CH_2$); 43.61, ($CH_2$, Bu); 61.33, ($OCH_2$); 126.79, (Ar-H); 127.47, (Ar-H); 127.99, (Ar-H); 130.21, (Ar-H); 131.53, (Ar-H); 132.68, (Ar-H); 132.72, (Ar-H); 133.23, (Ar-H); 134.12, (C=N); 134.67, (Ar-H); 134.81, (Ar-H); 135.09, (Ar-H); 141.92, (Ar-H); 163.85, (COO); 178.04, (C=S); 181.93, (C=O, AQ); 183.23, (C=O, AQ); 199.85, (C=O); IR (KBr, $cm^{-1}$): 3303; 2957; 1715; 1704; 1674; 1663; 1287; 709; Anal. Calcd. For $C_{25}H_{25}N_3O_5S$ × $H_2O$

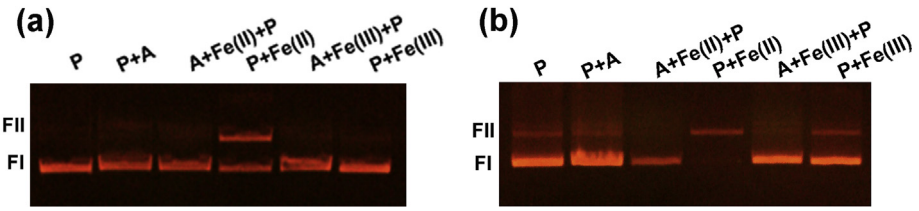



**Fig. 7.** Effect of Fe concentration on AQ-thiosemicarbazone (A) induced pUC18 DNA damage (a) **5c** quinone–iron mole ratio 1:1; (b) **5c** quinone–iron mole ratio 1:2.

(497.57 g/mol): C, 60.35; H, 5.47; N, 8.45; S, 6.44; Found: C, 60.65; H, 5.34; N, 8.67; S, 6.61.

*4.2.4. Ethyl 4-(9,10-dioxo-9,10-dihydroanthracen-1-yl)-4-oxo-2-(2-(hexylcarbamothioyl) hydrazono)- butanoate × $H_2O$ (**5c**, main isomer)*

Beige powder; yield: 0.19 g (63%); mp: 154–155 °C (Dec.); $^1$H NMR (500 MHz, DMSO-$d_6$): 0.87, (t, 3H, $J$ = 6.5 Hz, $CH_3$, Hx); 1.27, (t, 3H, $J$ = 7.0 Hz, $CH_3$); 1.29, (m, 6H, $CH_2$, Hx); 1.59, (m, 2H, $CH_2$, Hx); 3.59, (m, 2H, $CH_2$, Hx); 4.23, (q, 2H, $J$ = 7.0 Hz, $OCH_2$); 4.36, (s, 2H, $CH_2$); 7.77, (dd, 1H at C2, $J$ = 7.5 and 1.0 Hz); 7.96, (m, 2H at C6 and C7); 8.03, (t, 1H at C3, $J$ = 7.5 Hz); 8.22, (m, 1H at C5); 8.30, (m, 1H at C8); 8.32, (dd, 1H at C4, $J$ = 7.5 and 1.0 Hz); 8.46, (t, 1H, $J$ = 6.0 Hz, NH–Hx); 10.81, (s, 1H, NH); $^{13}$C NMR (125 MHz, DMSO-$d_6$): 13.90, ($CH_3$, Hx); 13.99, ($CH_3$); 22.05, ($CH_2$, Hx); 25.99, ($CH_2$, Hx); 28.23, ($CH_2$, Hx); 30.96, ($CH_2$, Hx); 40.90, ($CH_2$); 43.88, ($CH_2$, Hx); 61.32, ($OCH_2$); 126.79, (Ar-H); 127.48, (Ar-H); 127.99, (Ar-H); 130.21, (Ar-H); 131.54, (Ar-H); 132.67, (Ar-H); 132.71, (Ar-H); 133.22, (Ar-H); 134.10, (C=N); 134.67, (Ar-H); 134.81, (Ar-H); 135.09, (Ar-H); 141.92, (Ar-H); 163.85, (COO); 178.02, (C=S); 181.92, (C=O, AQ); 183.23, (C=O, AQ); 199.84, (C=O); IR (KBr, cm$^{-1}$): 3311; 3231; 2927; 1717; 1698; 1677; 1664; 1288; 1171; 709; Anal. Calcd. For $C_{27}H_{29}N_3O_5S \times H_2O$ (525.63 g/mol): C, 61.70; H, 5.94; N, 7.99; S, 6.10; Found: C, 61.84; H, 6.22; N, 8.09; S, 6.17.

*4.2.5. Ethyl 4-(9,10-dioxo-9,10-dihydroanthracen-1-yl)-4-oxo-2-(2-(cyclohexylcarbamothioyl) hydrazono)-butanoate × 0.5$H_2O$ (**5d**)*

Light yellow powder; yield: 0.16 g (54%); mp: 189–190 °C (Dec.); $^1$H NMR (500 MHz, DMSO-$d_6$): 1.26, (m, 1H, Cy); 1.26, (t, 3H, $J$ = 7.0 Hz, $CH_3$); 1.38, (m, 4H, Cy); 1.57, (m, 1H, Cy); 1.68, (m, 2H, Cy); 1.91, (m, 2H, Cy); 4.21, (m, 1H, Cy); 4.23, (q, 2H, $J$ = 7.0 Hz, $OCH_2$); 4.35, (s, 2H, $CH_2$); 7.78, (dd, 1H at C2, $J$ = 7.5 and 1.0 Hz); 7.97, (m, 2H at C6 and C7); 8.03, (t, 1H at C3, $J$ = 7.5 Hz); 8.10, (d, 1H, $J$ = 8.0 Hz, NH–Cy); 8.23, (m, 1H at C5); 8.29, (m, 1H at C8); 8.32, (dd, 1H at C4, $J$ = 7.5 and 1.0 Hz); 10.89, (s, 1H, NH); $^{13}$C NMR (125 MHz, DMSO-$d_6$): 13.94, ($CH_3$); 24.31, (Cy); 24.99, (Cy); 31.32, (Cy); 40.86, ($CH_2$); 52.40, (Cy); 61.35, ($OCH_2$); 126.80, (Ar-H); 127.44, (Ar-H); 128.00, (Ar-H); 130.22, (Ar-H); 131.57, (Ar-H); 132.69, (Ar-H); 132.72, (Ar-H); 133.23, (Ar-H); 133.97, (C=N); 134.68, (Ar-H); 134.79, (Ar-H); 135.09, (Ar-H); 141.90, (Ar-H); 163.74, (COO); 176.89, (C=S); 181.94, (C=O, AQ); 183.20, (C=O, AQ); 199.82, (C=O); IR (KBr, cm$^{-1}$): 3297; 3159; 2930; 1734; 1707; 1675; 1663; 1287; 709; Anal. Calcd. For $C_{27}H_{27}N_3O_5S \times 0.5H_2O$ (514.60 g/mol): C, 63.02; H, 5.48; N, 8.17; S, 6.23; Found: C, 63.19; H, 5.62; N, 8.23; S, 6.26.

*4.2.6. Ethyl 4-(9,10-dioxo-9,10-dihydroanthracen-1-yl)-4-oxo-2-(2-(furan-2-ylmethylcarbamo thioyl)hydrazono)-butanoate × 0.5$H_2O$ (**5e**, main isomer)*

Light yellow powder; yield: 0.16 g (55%); mp: 162–163 °C (Dec.); $^1$H NMR (500 MHz, DMSO-$d_6$): 1.26, (t, 3H, $J$ = 7.0 Hz, $CH_3$); 4.24, (q, 2H, $J$ = 7.0 Hz, $CH_2$); 4.85, (d, 2H, $J$ = 5.5 Hz, $CH_2$–Fur); 6.34, (d, 1H, $J$ = 3.0 Hz, Fur); 6.43, (dd, 1H, $J$ = 3.0 and 2.0 Hz, Fur); 7.61, (dd, 1H, $J$ = 2.0 and 1.0 Hz, Fur); 7.78, (dd, 1H at C2, $J$ = 7.5 and 1.0 Hz); 7.97, (m, 2H at C6 and C7); 8.03, (t, 1H at C3, $J$ = 7.5 Hz); 8.23, (m, 1H at C5); 8.30, (m, 1H at C8); 8.32, (dd, 1H at C4, $J$ = 7.5 and 1.0 Hz); 8.78, (t, 1H, $J$ = 5.5 Hz, NH–$CH_2$–Fur); 10.98, (s, 1H, NH); $^{13}$C NMR (125 MHz, DMSO-$d_6$): 14.01, ($CH_3$); 40.67, ($CH_2$–Fur); 40.99, ($CH_2$); 61.38, ($OCH_2$); 107.73, (Fur); 110.55, (Fur); 126.80, (Ar-H); 127.45, (Ar-H); 128.00, (Ar-H); 130.22, (Ar-H); 131.53, (Ar-H); 132.68, (Ar-H); 132.72, (Ar-H); 133.24, (Ar-H); 134.68, (C=N); 134.80, (Ar-H); 134.89, (Ar-H); 135.09, (Ar-H); 141.91, (Ar-H); 142.22, (Fur); 151.17, (Fur); 163.76, (COO); 178.57, (C=S); 181.94, (C=O, AQ); 183.22, (C=O, AQ); 199.77, (C=O); IR (KBr, cm$^{-1}$): 3307; 3148; 1716; 1702; 1674; 1662; 1288; 1165; 709; Anal. Calcd. For $C_{26}H_{21}N_3O_6S \times 0.5H_2O$ (512.54 g/mol): C, 60.93; H, 4.33; N, 8.20; S, 6.26; Found: C, 60.80; H, 4.39; N, 8.24; S, 6.30.

*4.2.7. Ethyl 4-(9,10-dioxo-9,10-dihydroanthracen-1-yl)-4-oxo-2-(2-(thiophen-2-ylmethyl carbamothioyl)hydrazono)-butanoate × $H_2O$ (**5f**, main isomer)*

Grey powder; yield: 0.19 g (62%); mp: 208–209 °C (Dec.); $^1$H NMR (500 MHz, DMSO-$d_6$): 1.26, (t, 3H, $J$ = 7.0 Hz, $CH_3$); 4.24, (q, 2H, $J$ = 7.0 Hz, $CH_2$); 4.37, (s, 2H, $CH_2$); 5.02, (d, 2H, $J$ = 6.0 Hz, $CH_2$–Thio); 6.99, (dd, 1H, $J$ = 5.0 and 3.5 Hz, Thio); 7.10, (d, 1H, $J$ = 3.5 Hz, Thio); 7.41, (dd, 1H, $J$ = 5.0 and 1.5 Hz, Thio); 7.78, (dd, 1H at C2, $J$ = 7.5 and 1.0 Hz); 7.97, (m, 2H at C6 and C7); 8.03, (t, 1H at C3, $J$ = 7.5 Hz); 8.23, (m, 1H at C5); 8.30, (m, 1H at C8); 8.31, (dd, 1H at C4, $J$ = 7.5 and 1.0 Hz); 8.98, (t, 1H, $J$ = 6.0 Hz, NH–$CH_2$–Thio); 10.94, (s, 1H, NH); $^{13}$C NMR (125 MHz, DMSO-$d_6$): 14.01, ($CH_3$); 41.02, ($CH_2$); 42.22, ($CH_2$–Thio); 61.37, ($OCH_2$); 125.46, (Thio); 126.42, (Thio); 126.54, (Thio); 126.80, (Ar-H); 127.48, (Ar-H); 128.01, (Ar-H); 130.22, (Ar-H); 131.53, (Ar-H); 132.70, (Ar-H); 132.72, (Ar-H); 133.25, (Ar-H); 134.69, (C=N); 134.81, (Ar-H); 134.96, (Ar-H); 135.10, (Ar-H); 140.90, (Thio); 141.91, (Ar-H); 163.76, (COO); 178.29, (C=S); 181.95, (C=O, AQ); 183.24, (C=O, AQ); 199.78, (C=O); IR (KBr, cm$^{-1}$): 3303; 1711; 1698; 1674; 1659; 1288; 1176; 709; Anal. Calcd. For $C_{26}H_{21}N_3O_5S_2 \times H_2O$ (537.62 g/mol): C, 58.09; H, 4.31; N, 7.82; S, 11.93; Found: C, 58.17; H, 4.40; N, 7.92; S, 12.12.

*4.2.8. Ethyl 4-(9,10-dioxo-9,10-dihydroanthracen-1-yl)-4-oxo-2-(2-(pyridin-3-ylmethylcarbamo thioyl)hydrazono)-butanoate × $H_2O$ (**5g**, main isomer)*

Light brown powder; yield: 0.18 g (59%); mp: 137–138 °C (Dec.); $^1$H NMR (500 MHz, DMSO-$d_6$): 1.26, (t, 3H, $J$ = 7.0 Hz, $CH_3$); 4.24, (q, 2H, $J$ = 7.0 Hz, $CH_2$); 4.39, (s, 2H, $CH_2$); 4.89, (d, 2H, $J$ = 6.0 Hz, $CH_2$–Py); 7.39, (dd, 1H, $J$ = 7.5 and 4.5 Hz, Py); 7.78, (d, 2H, $J$ = 7.5 Hz, C2 and Py); 7.96, (m, 2H at C6 and C7); 8.03, (t, 1H at C3, $J$ = 7.5 Hz); 8.23, (m, 1H at C5); 8.30, (m, 1H at C8); 8.32, (d, 1H at C4, $J$ = 7.5 Hz); 8.48, (d, 1H, $J$ = 4.5 Hz, Py); 8.59, (s, 1H, Py); 9.15, (t, 1H, $J$ = 6.0 Hz, NH–$CH_2$–Py); 10.95, (s, 1H, NH); $^{13}$C NMR (125 MHz, DMSO-$d_6$): 14.02, ($CH_3$); 41.08, ($CH_2$); 44.80, ($CH_2$–Py); 61.40, ($OCH_2$); 123.47, (Py); 126.81, (Ar-H); 127.46, (Ar-H); 128.01, (Ar-H); 130.22, (Ar-H); 131.54, (Ar-H); 132.69, (Ar-H); 132.71, (Ar-H); 133.24, (Ar-H); 134.32, (Py); 134.68, (C=N); 134.81, (Ar-H); 135.11, (Ar-H); 135.16, (Ar-H); 135.44, (Py); 141.90, (Ar-H); 148.06, (Py); 148.88, (Py); 163.80, (COO); 178.95, (C=S); 181.94, (C=O, AQ); 183.25, (C=O, AQ); 199.81, (C=O); IR (KBr, cm$^{-1}$): 3302; 1709; 1699; 1674; 1661; 1288; 1166; 709; Anal. Calcd.

For $C_{27}H_{22}N_4O_5S \times H_2O$ (532.58 g/mol): C, 60.89; H, 4.54; N, 10.52; S, 6.02; Found: C, 60.93; H, 4.73; N, 10.32; S, 5.92.

#### 4.2.9. Ethyl 4-(9,10-dioxo-9,10-dihydroanthracen-1-yl)-4-oxo-2-(2-(phenylcarbamothioyl) hydrazono)-butanoate × $H_2O$ (**5h**, main isomer)

Beige powder; yield: 0.39 g (66%); mp: 165–166 °C (Dec.); $^1$H NMR (500 MHz, DMSO-$d_6$): 1.30, (t, 3H, $J$ = 7.0 Hz, $CH_3$); 4.26, (q, 2H, $J$ = 7.0 Hz, $CH_2$); 4.44, (s, 2H, $CH_2$); 7.25, (m, 1H, Ph); 7.41, (t, 2H, $J$ = 7.5 Hz, Ph); 7.60, (dd, 2H, $J$ = 7.5 and 1.0 Hz, Ph); 7.82, (dd, 1H at C2, $J$ = 7.5 and 1.0 Hz); 7.96, (m, 2H at C6 and C7); 8.04, (t, 1H at C3, $J$ = 7.5 Hz); 8.23, (m, 1H at C5); 8.31, (m, 1H at C8); 8.34, (dd, 1H at C4, $J$ = 7.5 and 1.0 Hz); 10.15, (s, 1H, NH–Ph); 11.13, (s, 1H, NH); $^{13}$C NMR (125 MHz, DMSO-$d_6$): 14.01, ($CH_3$); 41.09, ($CH_2$); 61.44, ($OCH_2$); 125.14, (Ph); 125.77, (Ph); 126.81, (Ar-H); 127.46, (Ar-H); 128.03, (Ar-H); 128.38, (Ph); 130.27, (Ar-H); 131.58, (Ar-H); 132.71, (Ar-H); 132.74, (Ar-H); 133.26, (Ar-H); 134.68, (C=N); 134.81, (Ar-H); 134.92, (Ar-H); 135.11, (Ar-H); 138.53, (Ph); 141.91, (Ar-H); 163.90, (COO); 177.41, (C=S); 181.96, (C=O, AQ); 183.25, (C=O, AQ); 199.81, (C=O); IR (KBr, $cm^{-1}$): 3284; 1704; 1673; 1655; 1159; 707; Anal. Calcd. For $C_{27}H_{21}N_3O_5S \times H_2O$ (517.56 g/mol): C, 62.66; H, 4.48; N, 8.12; S, 6.20; Found: C, 62.96; H, 4.54; N, 8.26; S, 6.32.

#### 4.2.10. Ethyl 4-(9,10-dioxo-9,10-dihydroanthracen-1-yl)-4-oxo-2-(2-(p-tolylcarbamothioyl) hydrazono)-butanoate (**5i**, main isomer)

Beige powder; yield: 0.33 g (56%); mp: 200–201 °C (Dec.); $^1$H NMR (500 MHz, DMSO-$d_6$): 1.31, (t, 3H, $J$ = 7.0 Hz, $CH_3$); 2.32, (s, 3H, $CH_3$–Ph); 4.26, (q, 2H, $J$ = 7.0 Hz, $CH_2$); 4.43, (s, 2H, $CH_2$); 7.20, (d, 2H, $J$ = 8.5 Hz, Ph); 7.46, (d, 2H, $J$ = 8.5 Hz, Ph); 7.81, (dd, 1H at C2, $J$ = 7.5 and 1.0 Hz); 7.98, (m, 2H at C6 and C7); 8.05, (t, 1H at C3, $J$ = 7.5 Hz); 8.24, (m, 1H at C5); 8.31, (m, 1H at C8); 8.33, (dd, 1H at C4, $J$ = 7.5 and 1.0 Hz); 10.06, (s, 1H, NH–Ph); 11.08, (s, 1H, NH); $^{13}$C NMR (125 MHz, DMSO-$d_6$): 14.01, ($CH_3$); 20.61, ($CH_3$–Ph); 41.07, ($CH_2$); 61.44, ($OCH_2$); 122.60, (Ph); 125.09, (Ph); 126.82, (Ar-H); 127.47, (Ar-H); 128.03, (Ar-H); 128.84, (Ph); 130.27, (Ar-H); 131.58, (Ar-H); 132.72, (Ar-H); 132.75, (Ar-H); 133.27, (Ar-H); 134.69, (C=N); 134.82, (Ar-H); 134.95, (Ar-H); 135.10, (Ar-H); 135.96, (Ph); 141.92, (Ar-H); 163.91, (COO); 177.39, (C=S); 181.98, (C=O, AQ); 183.26, (C=O, AQ); 199.84, (C=O); IR (KBr, $cm^{-1}$): 3301; 1731; 1703; 1671; 1659; 1287; 1153; 710; Anal. Calcd. For $C_{28}H_{23}N_3O_5S$ (513.57 g/mol): C, 65.48; H, 4.51; N, 8.18; S, 6.24; Found: C, 65.26; H, 4.36; N, 8.23; S, 6.25.

### 4.3. Antioxidant activity

All the synthesized compounds were evaluated for antioxidant activity and compared with corresponding standards (ascorbic acid and BHT). The activity was evaluated using the DPPH method [39]. The 200 μM solution of DPPH (2 mL) in methanol was added to tested sample solutions (2 mL each) of different concentrations (8–1000 μM) in methanol. Due to the low solubility, compound **5f** was dissolved in the mixture of DMSO and MeOH (2:1). The resulting solution was allowed to react in dark at room temperature for 30 min. Afterward, the absorbance values were measured spectrophotometrically at 517 nm and converted into the percentage antioxidant activity (IC) using formula

$$\mathrm{IC}(\%) = \left[\left(A_{\mathrm{DPPH}} - A_{\mathrm{sample}}\right) \Big/ A_{\mathrm{DPPH}}\right] \times 100,$$

where $A_{DPPH}$ was the absorbance of DPPH solution and $A_{sample}$ was the absorbance of sample solution with DPPH. To correct any influence due to color of the tested compounds blank solutions (without DPPH) were prepared and used as a reference for the measuring of $A_{sample}$. The radical scavenging activities of the samples were expressed in terms of $IC_{50}$ (concentration required for a 50% decrease in absorbance of DPPH radical) by plotting the obtained IC values against concentration of the tested sample using OriginPro8 statistical software [40].

### 4.4. Biological experiments

#### 4.4.1. Compounds and solutions

Stock solutions of investigated compounds, were prepared in DMSO at concentrations of 20 mM and afterward they were diluted with complete nutrient medium (RPMI-1640 without phenol red) supplemented with 3 mM L-glutamine, 100 μg/mL streptomycin, 100 IU/mL penicillin, 10% heat inactivated fetal bovine serum (FBS), and 25 mM: 2-[4-(2-hydroxyethyl)piperazinyl]ethanesulfonic acid (HEPES) adjusted to pH 7.2 by bicarbonate solution. The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was dissolved (5 mg/mL) in phosphate buffer saline pH 7.2 and filtered (0.22 μm) before use.

RPMI-1640, FBS, HEPES, MTT and L-glutamine were products of Sigma Chemical Co., St. Louis, MO.

#### 4.4.2. Cell culture

Cervix adenocarcinoma cell line (HeLa), human breast cancer (MDA-MB-361) and (MDA-MB-453) cell lines, human chronic myelogenous leukemia (K562) cells, non-small cell lung carcinoma (A549) and a non-cancerous cell line, MRC-5 (human embryonic lung fibroblast) were grown in RPMI-1640 medium (Sigma). Media were supplemented with 10% fetal bovine serum, L-glutamine, and penicillin-streptomycin (Sigma). CLL lymphocytes were separated from the heparinized blood samples of patients with chronic lymphocytic leukemia and resuspended in RPMI-1640 supplemented with 10% fetal bovine serum (FBS).

#### 4.4.3. Treatment of cell lines

Target cells HeLa (2000 cells per well), MDA-MB-361 (7000 cells per well), MDA-MB-453 (3000 cells per well), K562 (5000 cells per well), A549 (5000 cells per well) and non-cancerous MRC-5 (5000 cells per well) were seeded into wells of a 96-well flat-bottomed microtitre plate. Twenty-four hours later, after the cell adherence, different concentrations of investigated compounds were added to the wells, except for the control cells to which a nutrient medium only was added. Final concentrations achieved in treated wells were 5 μM, 10 μM, 25 μM, 50 μM, and 100 μM. The final concentration of DMSO solvent never exceeded 0.5%, which was non-toxic to the cells. Especially, compounds were applied to the suspension of K562 cells 2 h after the cell seeding. All concentrations were set up in triplicate. Nutrient medium with corresponding concentrations of investigated compounds, but without cells, was used as a blank, also in triplicate. The cultures were incubated for 72 h.

#### 4.4.4. Determination of cell survival

The effect of the prepared compounds on cancer cell survival was determined by the microculture tetrazolium test (MTT) according to Mosmann [41] with modification by Ohno and Abe [42] 72 h after addition of the compounds, as described earlier. Briefly, 20 μL of MTT solution (5 mg/mL phosphate-buffered saline) was added to each well. Samples were incubated for a further 4 h at 37 °C in a humidified atmosphere of 95% air/5% $CO_2$ (v/v). Then 100 μL of 100 g/L sodium dodecyl sulfate was added to extract the insoluble product formazan resulting from conversion of the MTT dye by viable cells. The number of viable cells in each well was proportional to the intensity of the absorbance of light, which was read in an enzyme-linked immunosorbent assay (ELISA) plate reader at 570 nm. The absorbance ($A$) at 570 nm was measured 24 h

later. To determine cell survival (%), the *A* of a sample with cells grown in the presence of various concentrations of the investigated compounds was divided by the control optical density (the *A* of control cells grown only in nutrient medium) and multiplied by 100. It was implied that the *A* of the blank was always subtracted from the *A* of the corresponding sample with target cells. $IC_{50}$ was defined as the concentration of an agent inhibiting cell survival by 50% compared with a vehicle-treated control. As positive controls, *cis*-diaminedichloroplatinum (*cis*-DDP) and doxorubicin were used. All experiments were done in triplicate.

*4.4.5. Flow cytometry analysis*

Cellular DNA content and cell distribution were quantified by flow cytometry using propidium iodide (PI). Cells ($3 \times 10^5$ cells/well) were seeded in 6-well plates and incubated with or without $IC_{50}$ and $2 \times IC_{50}$ concentrations of investigated compounds for 24 h. After treatment, the cells were collected by trypsinization, and fixed in ice-cold 70% ethanol at −20 °C overnight. After fixation, the cells were washed in PBS and pellets obtained by centrifugation were treated with RNase (100 μg/mL) at 37 °C temperature for 30 min and then incubated with propidium iodide (PI) (40 μg/mL) for at least 30 min. DNA content and cell-cycle distribution were analyzed using a Becton Dickinson FACSCalibur flow cytometer. Flow cytometry analysis was performed using a CellQuestR (Becton Dickinson, San Jose, CA, USA), software on a minimum of 10,000 cells per sample [43].

*4.4.6. Determination of target caspases*

In order to examine the role of caspases involved in the apoptotic cell death induced by the investigated extracts, the percentages of HeLa cells pretreated with caspase inhibitors in sub-G1 phase were determined. HeLa cells were preincubated for 2 h with specific caspase inhibitors (final concentration − 40 μM): Z-DEVD-FMK (caspase-3 inhibitor), Z-IETD-FMK (caspase-8 inhibitor) and Z-LEHD-FMK (caspase-9 inhibitor). Caspase inhibitors were purchased from R&D Systems (Minneapolis, USA). Five tested extracts were applied to target HeLa cells at concentrations which corresponded to $IC_{90}$ values obtained for 72 h. For each extract, one sample of HeLa cells was not treated with inhibitor and served as a referent sample. After 24 h of incubation, cells were harvested, fixed in 70% ethanol on ice. Samples were stored at −20 °C for one week before PI staining. Changes in the percentages of cells in sub-G1 phase were determined using a FACSCalibur Flow Cytometer and analyzed using CellQuest Software.

*4.5. DNA-interaction studies*

*4.5.1. DNA-binding experiments*

Calf thymus DNA (lyophilized, highly polymerized, obtained from Serva, Heidelberg) (CT-DNA) was dissolved in Tris buffer (10 mM Tris–HCL pH 7.9) overnight at 4 °C. This stock solution was stored at 4 °C and was stable for several days. A solution of CT-DNA in water gave a ratio of UV absorbance at 260 and 280 nm, $A_{260}/A_{280}$ of 1.89–2.01, indicating that DNA was sufficiently free of protein. The concentration of DNA (2.6 mg/mL) was determined from the UV absorbance at 260 nm using the extinction coefficient $\varepsilon_{260} = 6600\ M^{-1}cm^{-1}$ [44].

All AQ-thiosemicarbazone compounds were dissolved in dimethyl sulfoxide in concentrations of 10 mM and these solutions were used as stock solutions.

Reaction mixtures (1 mL in 40 mM bicarbonate buffer, pH 8.4) consisting of different concentrations of compounds (20 μM, 40 μM, 60 μM and 80 μM) and 0.12 mM of CT-DNA were incubated at 37 °C for 1.5 h with occasional vortexing. UV–Vis spectra were recorded on a UV-1800 Shimadzu UV/Visible spectrometer operating from 200 to 800 nm in 1.0 cm quartz cells. Primary spectra of all spectrometric measurements were imported into OriginPro 8.0.

The absorbance titrations were performed at a fixed concentration of anthraquinone compound and varying the concentration of double stranded CT-DNA. For an individual experiment, each DNA solution (0, 2.5, 5, 7.5, 10, 12.5, 15, 17.5 and 20 μL of CT-DNA stock solution) was pipetted into a separate vial, to which 4 μL of a stock solution of compound was added and the volume adjusted to 1 mL with the bicarbonate buffer and incubated at 37 °C for 1.5 h with occasional vortexing. UV–Vis spectra were recorded as described above.

*4.5.2. DNA cleavage experiments*

*4.5.2.1. Preparation and purification of plasmid DNA.* The plasmid pUC18 (pUC18, 2686 bp, purchased from Sigma–Aldrich, USA) was prepared by its transformation in chemically competent cells *Escherichia coli* strain XL1 blue. Amplification of the clone was done according to the protocol for growing *E. coli* culture overnight in LB medium at 37 °C [45] and purification was performed using Qiagen Plasmid plus Maxi kit. Finally, DNA was eluted in 10 mM Tris–HCl buffer and stored at −20 °C. The concentration of plasmid DNA (131 ng/μL) was determined by measuring the absorbance of the DNA containing solution at 260 nm. One optical unit corresponds to 50 μg/mL of double stranded DNA.

The cleavage reaction of supercoiled pUC18 DNA by AQ-thiosemicarbazones (2 mM) was investigated by incubation of 262 ng of pUC18 in a 20 μL reaction mixture in 40 mM bicarbonate buffer pH 8.4 at 37 °C, for 90 min. The reaction mixtures were vortexed from time to time. The reaction was terminated by short centrifugation at 10,000 rpm and addition of 5 μL of loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF and 30% glycerol in TAE buffer, pH 8.24 (40 mM Tris–acetate, 1 mM EDTA)). The samples were subjected to electrophoresis on 1% agarose gel (Amersham Pharmacia-Biotech, Inc) prepared in TAE buffer pH 8.24. The electrophoresis was performed at a constant voltage (80 V) until bromophenol blue had passed through 75% of the gel. A Submarine Mini-gel Electrophoresis Unit (Hoeffer HE 33) with an EPS 300 power supply was used. After electrophoresis, the gel was stained for 30 min by soaking it in an aqueous ethidium bromide solution (0.5 μg/mL). The stained gel was illuminated under a UV transilluminator Vilber-Lourmat (France) at 312 nm and photographed with a Panasonic DMC-LZ5 Lumix Digital Camera through filter DEEP YELLOW 15 (TIFFEN, USA).

Interactions of the tested compounds with pUC18 in the presence of Fe(II) and Fe(III) were done as follows. Solutions of 2 μL $FeSO_4$ (40 mM or 20 mM, freshly prepared in a sterile water) or 2 μL $FeCl_3$ (40 mM or 20 mM, freshly prepared in a sterile water) with 4 μL of 10 mM solution of AQ-thiosemicarbazone in 14 μL of bicarbonate buffer were incubated at 37 °C, for 60 min, then 2 μL of pUC18 were added to reaction mixtures and incubated further for 90 min. The reaction mixtures were vortexed from time to time and terminated by short centrifugation at 10,000 rpm and addition of 5 μL of loading buffer and analyzed as described previously.

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## Appendix A. Supplementary information

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2013.03.071.

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