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Novel thiosemicarbazone derivatives as potential antitumor agents: Synthesis, physicochemical and structural properties, DNA interactions and antiproliferative activity

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Abstract—The paper describes synthesis of several novel thiosemicarbazone derivatives. Furthermore, crystal and molecular structure of 4-diethylamino-salicylaldehyde 4-phenylthiosemicarbazone revealed planarity of conjugated aromatic system, which suggested the possibility of DNA binding by intercalation, especially for here studied naphthalene derivatives. However, here presented DNA binding studies excluded this mode of action. Physicochemical and structural properties of novel derivatives were compared with previously studied analogues, taken as reference compounds, revealing distinctive differences. In addition, novel thiosemicarbazone derivatives (**1**, **2** and **5**–**8**) clearly display stronger antiproliferative activity on five tumor cell lines than the reference compounds **3** and **4**, which supports their further investigation as potential antitumor agents.

1. Introduction

Thiosemicarbazones exhibit various biological activities and have therefore attracted considerable pharmaceutical interest.[1] They have been evaluated over the last 50 years as antiviral, antibacterial and anticancer therapeutics. Thiosemicarbazones belong to a large group of thiourea derivatives, whose biological activities are a function of parent aldehyde or ketone moiety.[2,3] Conjugated N–N–S tridentate ligand system of thiosemicarbazide (NH_2–CS–NH–NH_2) seems essential for anticancer activity, possibly due to the observation that structural alterations that hinder a thiosemicarbazone's ability to function as a chelating agent tend to destroy or reduce its medicinal activity.[4] Furthermore, the most active thiosemicarbazones are those which possess the *trans* isomer (i.e., hydrogen bonding involving O···HN).

Several mechanisms of antitumor action of thiosemicarbazones were proposed. For example, they could stabilize cleavable complexes formed by topoisomerase II (topoII) and DNA leading to apoptosis. The stabilizing effect is mainly due to the alkylation of thiol residues on the topo II–DNA complex.[5] Besides, thiosemicarbazones could inhibit ribonucleotide reductase (RR) activity. RR catalyzes the synthesis of deoxyribonucleotides required for DNA synthesis. Since deoxyribonucleotides are present in extremely low levels in mammalian cells, it is a crucial and rate-controlling step in the pathway leading to the biosynthesis of DNA. Mammalian ribonucleotide reductase (RR) is composed of two dissimilar proteins, (R1), which contains polythiols and (R2), which contains non-heme iron and a free tyrosyl radical. Both the R1 and R2 subunits contribute to the active site of the enzyme.[6] Since thiosemicarbazones are known iron chelators and as such can destabilize or damage the non-heme iron-stabilized tyrosyl free radical and thus inhibit the catalytical function of RR.

Very few studies were done on the non-covalent interactions of thiosemicarbazones with double stranded (ds-) DNA, although DNA damage was often proposed as possible mechanism of action. For example, biological

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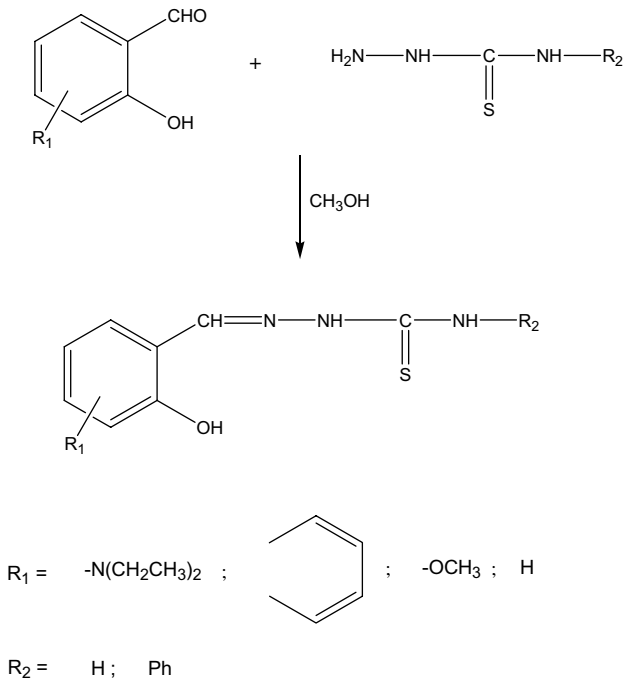


Scheme 1. The general synthetic procedure and the structures of all studied compounds.

activity of some thiosemicarbazones substituted by condensed aromatic moieties was based on prior formation of non-covalent complexes with DNA.[7] Furthermore, 5-nitroimidazole-thiosemicarbazone damaged both pyrimidine and purine bases[8] but it was not clear by which mode of interaction the active compound approached DNA. However, a huge number of thiosemicarbazone derivatives with the potential of direct DNA binding, like, for example, many derivatives containing condensed aromatic moieties,[5,9] were never tested for interactions with ds-DNA.

Therefore, condensation of various naphthyl- and salicyl- or 3-methoxy-salicylaldehyde analogues with thiosemicarbazide to form thiosemicarbazone derivatives (Scheme 1) may result in highly biologically active compounds because of all afore-mentioned reasons.

Table 1. General and crystal data, summary of intensity data collection and structure refinement for the compound **(5)**

	(5)
Empirical formula	$C_{18}H_{22}N_4OS$
Formula weight	342.47
Habit and color	Prismatic, colorless
Crystal dimension (mm^3)	$0.62 \times 0.21 \times 0.20$
Crystal system, space group	Monoclinic, $P2_1/c$
Unit cell dimensions (Å, °)	
a	8.8570(3)
b	18.1129(6)
c	11.4739(3)
β	94.974(3)
Volume ($Å^3$)	1833.78(10)
Z	4
D_{calc} ($g\ cm^{-3}$)	1.240
μ (mm^{-1})	0.188
F(000)	728
2θ range for data collection (°)	3.74–27.99
h, *k*, *l* range	−11 to 11, −23 to 23, −15 to 15
Reflections observed/ independent (R_{int})	30,150/4410 (0.0324)
Observed reflections [$I > 2\sigma(I)$]	2576
Data/restraints/parameters	4410/0/305
Goodness-of-fit on F^2	0.951
R/*wR* [$I > 2\sigma(I)$][a]	0.0415/0.1014
R/*wR* (all data)[b]	0.0749/0.1211
Largest diff. peak and hole (e^- Å^3)	0.375 and −0.219

[a] $R = \sum \|F_o\| - \|F_c\| / \sum F_o$, $w = 1/[\sigma^2(F_o^2) + (g_1P)^2 + g_2P]$ where $P = (F_o^2 + 2F_c^2)/3$, $S = \Sigma[w(F_o^2 - F_c^2)^2/(N_{obs} - N_{param})]^{1/2}$.
[b] $wR = [\Sigma(F_o^2 - F_c^2)^2/\Sigma(F_o^2)^2]^{1/2}$.

2. Results and discussion

2.1. Chemistry

All thiosemicarbazones (**1**–**8**) were prepared according to the modified procedure described in the literature[10] starting from corresponding aldehyde and thiosemicarbazide or 4-phenylthiosemicarbazide (Scheme 1). Compounds are white or yellow powders easily soluble in warm DMF, DMSO, acetone, dichloromethane or

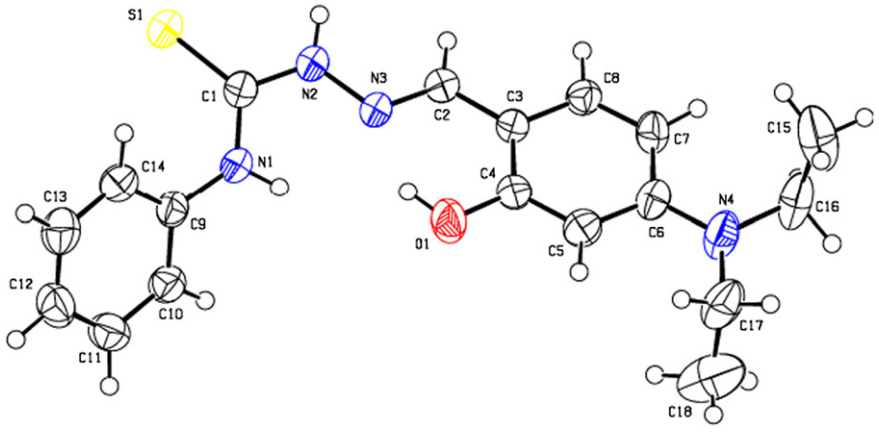


Figure 1. ORTEP plot of the (**5**) compound, showing atom-labeling scheme and displacement ellipsoids are drawn at 50% probability level. Hydrogen atoms are shown as spheres of arbitrary small radii.

Table 2. Selected bond lengths (Å)

C1–S1	1.6792(15)	C1–N1	1.346(2)
C1–N2	1.342(2)	C2–N3	1.286(2)
C6–N4	1.3734(19)	C9–N1	1.412(2)
C16–N4	1.456(3)	C17–N4	1.457(2)
C4–O1	1.3507(19)	N2–N3	1.3866(17)

chloroform. The IR spectra of powders are in accordance to the literature data for the same type of compounds,[11] namely the characteristic intense bands in the range of 1652–1610 cm^{-1} are associated with the ν(C=N) and δ(N–H) frequencies. The ν(OH) band of phenolic oxygen occurs almost at the same frequency for all thiosemicarbazones at 3337 or 3339 cm^{-1}. The in-

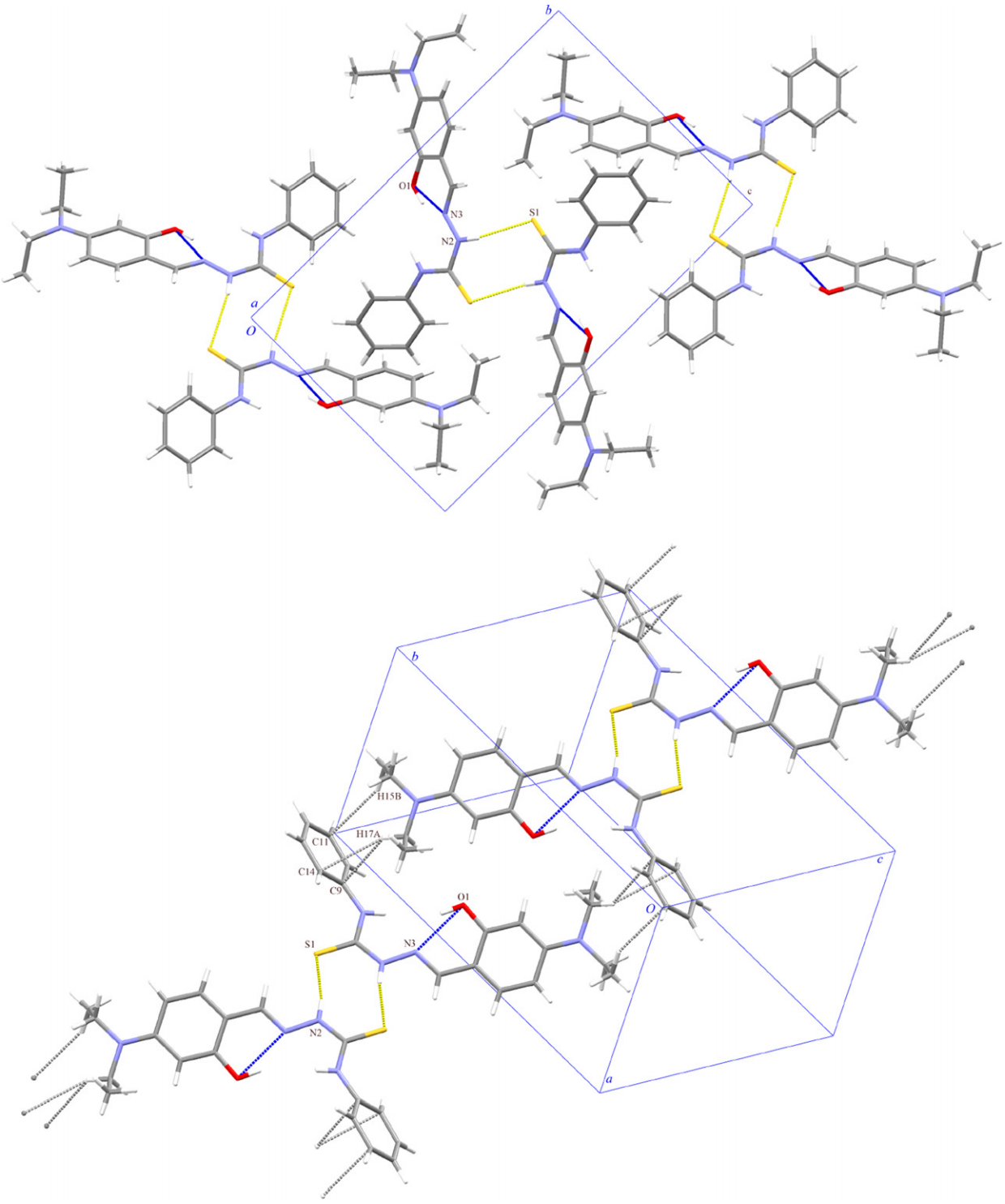


Figure 2. (Top) Centrosymmetric dimers of (**5**) are held together by N–H···S hydrogen bonds ($R_2^2(8)$). Intramolecular O–H···N hydrogen bonds are also shown ($S_1^1(6)$). View is projected down the crystallographic *a* axis. (Bottom) T-shaped C–H···π interactions are added to the above-mentioned interactions.

tense ν(C–S) band is observed in the range of 760–775 cm^{-1}.

The yellow prismatic crystals of 4-diethylamino-salicylaldehyde 4-phenylthiosemicarbazone (**5**) suitable for X-ray crystallography were obtained from a dichloromethane solution by slow evaporation.

General and crystal data for the compound (**5**) are given in Table 1.

Like in many thiosemicarbazones, especially ones derived from salicylaldehyde, the thione form (Fig. 1) dominates in the solid state (C–S bond distance of 1.6792(15) is comparable to the one found in thiourea[12]). The *trans* configuration of the S1 atom with respect to N3 is present (and with N1 in the *cis* configuration with respect to N3). It has been seen that the above-mentioned configuration of similar thiosemicarbazones changes upon complexation.[13]

The molecule (Fig. 1) can be separated in two planar fragments: salicylthiosemicarbazone (consisting of S1, C1–C8, N2, N3, N4 and O1) and *N*-phenyl part (consisting of N1, C9–C14) which are inclined by 58.21(4)°. Planarity of the salicylthiosemicarbazone part of molecule enhances delocalization of π electrons, which also can be seen easily from the bond length distribution (Table 2). It is interesting to see how π-system spreads on the diethylamino moiety by achieving additional planarity which is accompanied by shortening of C6–N4 distance. Because of steric hindrance, the methyl groups (C15 and C18 with attached hydrogen atoms) of the diethylamino moiety are found on the opposite sides of the plane defined by C16, N4 and C17 atoms. The endocyclic bond distances and angles found in the phenyl rings are similar to those in normal sp^2 hybridized moieties.

As in many non-protonated thiosemicarbazones derived from salicylaldehide derivatives,[14] the salicylaldimine part of molecule is characterized by an intramolecular six-membered heteronuclear resonance-assisted hydrogen bond (Fig. 2). The thiosemicarbazone part is characterized by an intramolecular five-membered (non-planar) weak hydrogen.

Molecules are arranged in sheets parallel to the crystallographic (0 2 1) plane and connected by the T-shaped π interactions and relatively close H···H contacts (Table 3). Mentioned sheets are interconnected by N–H···S hydrogen bonds ($R_2^2(8)$) forming centrosymmetric dimers (Fig. 2).

Table 3. Intermolecular contacts (Å)

Type		Distance
C–H···π	C9···H17A^{iii}	2.892(23)
	C11···H15B^{iii}	2.861(36)
	C14···H17A^{iii}	2.885(23)
H···H	H10···H7^{iii}	2.393(23)

iii = $-x$, $-y+1$, $-z$.

2.2. Study of compounds 1–8 in aqueous solutions

2.2.1. Electronic absorption spectra of 1–8. All studied compounds (**1**–**8**) were scarcely soluble in water, forming colloids after agitation in the ultrasonic bath. Only upon heating some compounds dissolved in water at about 10^{-5} mol dm^{-3} concentrations, which were not suitable for the preparation of stock solutions. Therefore, for easier manipulation in further experiments, stock solutions of **1**–**8** were prepared in DMSO at $c = 5\times10^{-3}$–1×10^{-2} mol dm^{-3}. For all experiments small aliquots of DMSO stock solutions were added into the aqueous medium to give homogeneous solutions with DMSO content of less than 5%. The UV/vis spectra of **1**–**8** are linearly dependent on the concentration of compounds up to 8×10^{-6} mol dm^{-3}.

Comparison of the UV/vis spectra (Fig. 3) of **1**–**4** with those of **5**–**8** revealed that introduction of phenyl-substituent on the amino group of thiosemicarbazone influenced only the intensity of the electronic absorption properties of studied compounds, in most cases increasing the molar absorptivity. However, electronic properties of the substituent attached to the salicylic part of studied thiosemicarbazones had much more pronounced influence on the UV/vis spectra of **1**–**8** (Fig. 3). If we consider compound **4** with unsubstituted salicylic moiety as a reference, introduction of electron-donating substituents such as diethylamino- (**1**) and methoxy- (**2**) caused merging of two absorption maxima into one. However, only diethylamino- (**1**) substituent-induced additionally strong batochromic shift of maximum (ΔAbs_{max}(**1**–**2**) = 51 nm). The shape of the UV/vis spectrum of the naphthalene analogue (**3**) is quite similar to the spectrum of **4** but it is shifted toward longer wavelengths by about 25 nm. Such a shift is quite common between most benzene and naphthalene analogues. The same substituent-induced effects are observed for **5**–**7** in comparison to **8**.

2.2.2. Thermal dependence of the UV/vis spectra of 1–8. The aqueous solutions of **1**–**8** were successively heated up to 85 °C and the changes were monitored by UV/vis spectrophotometry. Upon heating from 25 to 85 °C the UV/vis spectra of **1, 2, 4** changed less than 5% and upon cooling back to 25 °C the starting spectra are fully recovered. Heating of aqueous solutions of naphthalene derivatives **3** and **7** caused partial precipitation. In contrast to **1**, **2**, **4**, the UV/vis spectra of their phenyl-substituted analogues **5**, **6**, **8** changed considerably upon heating (Fig. 4). Upon cooling back to 25 °C only starting spectrum of **6** was fully recovered, while those of **5** and **8** remained significantly changed, most likely due to the chemical changes related to the phenyl-substituent.

2.2.3. Fluorimetric properties of 1–8. Solutions of **1**–**8** exhibited strong fluorescence (Fig. 5), emission intensity being linearly dependent on their concentration up to 5×10^{-6} mol dm^{-3}. Similar to that observed in the UV/vis spectra, introduction of phenyl-substituent on the amino group of thiosemicarbazone (**5**–**8**) influenced only the emission intensity but not the shape of the fluorescence spectra of **1**–**4**. The only exception is significant difference between fluorimetric spectra of **3** and **7**, where introduction of phenyl- (**7**) induced

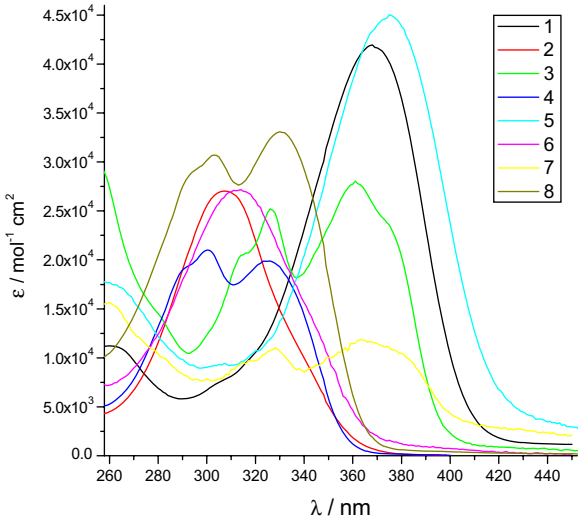
extraordinary strong batochromic shift of emission maxima (ΔAbs_{max}(**7**–**3**) = 71 nm). The linear dependence of the UV/vis and fluorimetric spectrum of **7** on the concentration does not support intermolecular aggregates. Therefore, such an effect could be attributed to the strong intramolecular interaction between naphthalene- and phenyl-moiety of **7**.

If we consider compound **4** with the unsubstituted salicylic moiety as a reference, the introduction of diethylamino-substituent (**1**) results in hypsocromic shift of emission maximum, while methoxy-substituent (**2**) yields batochromic shift. Obviously, the electron-donating nature of both substituents is not controlling fluorescence properties of **1** and **2**. It is interesting to note that replacement of benzene (**4**) with naphthalene (**3**) caused batochromic shift of emission maximum.

2.3. Study of interactions of 1–8 with *calf thymus* (ct-) DNA in aqueous solutions

The spectroscopic properties of all studied compounds in buffered aqueous solutions (Na cacodylate, pH 7, I = 0.05 M) are comparable to those in pure water (Figs. 4 and 5). The low solubility of naphthalene derivatives **3** and **7** hampered UV/vis titrations and thermal denaturation experiments with *calf thymus* (ct-) DNA. Thermal instability of **5** and **8** did not allow accurate thermal denaturation experiments with ct-DNA. Thus, as the representatives of salicylic derivatives (excluding naphthalene analogues **3** and **7**), compounds **2** and **4** were tested for binding to the double stranded ct-DNA.

Addition of ct-DNA did not yield any measurable change in the UV/vis spectra of **2** and **4**, while fluorimetric titrations revealed only very weak quenching of **2** and **4** fluorescence (<10%) upon titration with ct-DNA. Processing of the fluorimetric titration data by Scatchard equation[15] gave us the only possible result values of ratio $n_{[\text{bound compd}]/[\text{ct-DNA}]} \gg 1$ and $K_s \approx 10^5$ M. In thermal denaturation experiments addition of **2** or **4** at ratios $r_{[\text{compd}]/[\text{ct-DNA}]} = 0.3$ did not alter thermal denaturation properties of DNA, therefore intercalation of **2** and **4** within DNA double helix can be excluded. Furthermore, compounds **2** and **4** do not possess any positive charge, which would allow significant interaction with the DNA phosphates. On the other hand, neutral, aromatic molecules of **2** and **4** are strongly hydrophobic and therefore should tend to accumulate within the lyp-



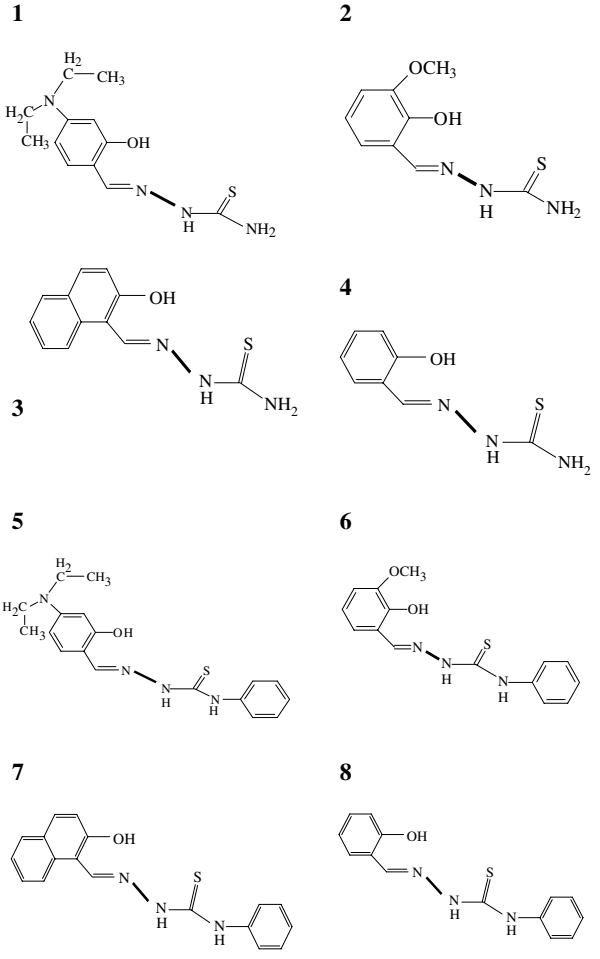


Figure 3. The UV/vis spectra of **1**–**8** in water.

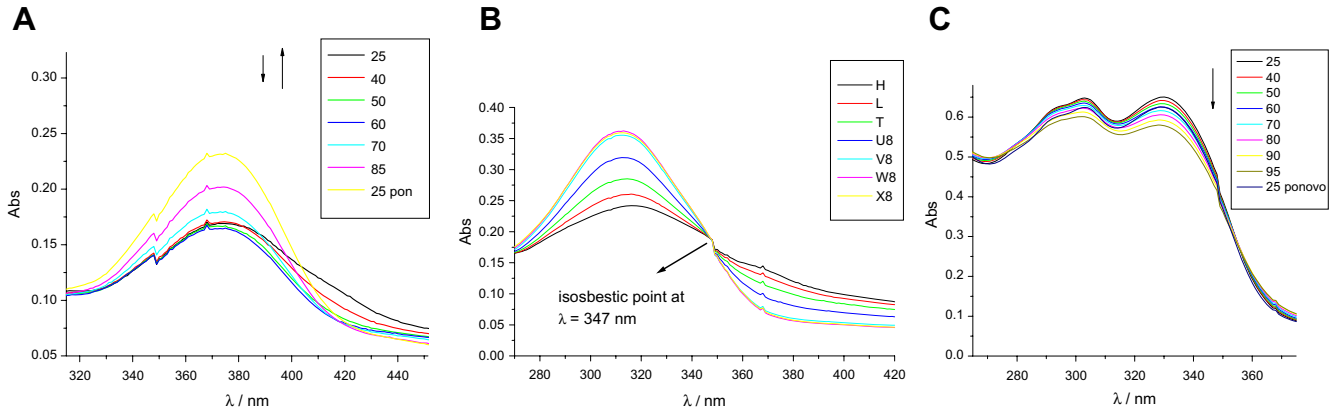


Figure 4. The dependence of the UV/vis spectra of aqueous solutions of **5** (A), **6** (B) and **8** (C) on the temperature.

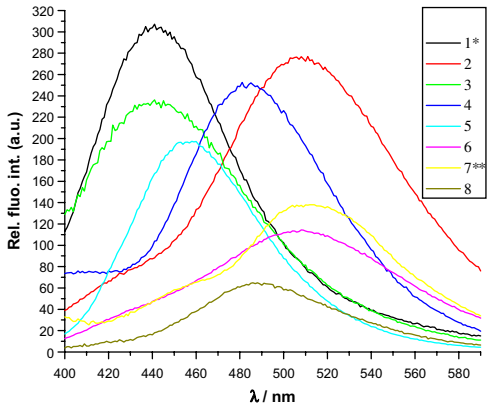


Figure 5. Fluorescence emission spectra of **1**–**8** in water, 25 °C, c(**1**–**8**) = 1.4×10^{-6} mol dm^{-3}; excitation wavelength see longer λ_{max} in Supplementary Material. *__1__ and **__7__: due to the very low emission intensities wider slits had to be used, thus intensity of the shown spectra is not comparable with the spectra of **2**, **3**, **4**, **5**, **6**, and **8**.

ophilic DNA grooves. Therefore, changes in fluorescence spectra of **2** and **4** upon addition of ct-DNA most likely originate from aromatic interactions between molecules of **2** (or **4**) within the DNA grooves and do not represent interaction of **2** (or **4**) with the ct-DNA. Such agglomerates of **2** (or **4**) would allow presence of more small molecules per one DNA base pair than it is theoretically possible for intercalation or minor groove binding and therefore calculated Scatchard ratio $n_{[\text{bound compd}]/[\text{ct-DNA}]}$ is significantly higher than 1. Consequently, calculated binding constants (K_s) are mainly related to the interactions between molecules of **2** or **4**, respectively.

In conclusion, compounds **2** and **4** as representatives of other salicylic derivatives (**1**, **5**, **6**, and **8**), do not show noteworthy interactions with double-stranded DNA. The naphthalene derivatives **3** and **7** could not be tested due to their low solubility.

2.4. The effect of thiosemicarbazones on the proliferation of tumor and normal cells

Compounds **1**–**8** were tested for their potential antiproliferative effects using MTT test (as described in Section 4) on a panel of six human cell lines, five of which were derived from different cancer types including HeLa (cervical carcinoma), MCF-7 (breast carcinoma), SW620 (colon carcinoma), MiaPaCa-2 (pancreatic carcinoma), Hep-2 (laryngeal carcinoma) and one from normal diploid fibroblasts, WI 38 (Table 4). All tested compounds showed noticeable antiproliferative effect having IC_{50} values in the low micromolar, or submicromolar range (Table 4). Regarding the structure–activity relationship, the lowest activity exhibited previously published compounds **4** and **3**,[3] which can be considered as reference

Table 4. In vitro inhibition of thiosemicarbazones **1**–**8** on the growth of tumor cells and normal human fibroblasts (WI 38)

Compound	IC_{50} (μM)[a]					
	Hep-2	HeLa	MiaPaCa-2	SW620	MCF-7	WI 38
1	0.9 ± 0.1	2 ± 2	>5	3 ± 1	0.4 ± 0.2	0.2 ± 0.1
2	1.4 ± 0.3	⩾5	0.4 ± 0.1	0.5 ± 0.5	0.3 ± 0.1	0.3 ± 0.2
3	0.2 ± 0.2	0.2 ± 0.06	>5	4.7 ± 0.3	>5	⩾5
4	⩾5	⩾5	>5	>5	⩾5	⩾5
5	0.3 ± 0.3	0.7 ± 0.08	0.2 ± 0.3	0.6 ± 0.5	1.2 ± 1	0.3 ± 0.1
6	1.6 ± 0.9	1.7 ± 0.2	2.5 ± 0.7	1.6 ± 0.8	3 ± 2	1.4 ± 1.2
7	0.7 ± 0.5	2 ± 0.7	0.6 ± 0.1	0.3 ± 0.2	3.3 ± 1.3	0.6 ± 0.08
8	2.4 ± 0.4	1.5 ± 0.3	2.4 ± 0.8	2 ± 0.08	4 ± 1	1.3 ± 1.1

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

compounds due to unsubstituted salicylic and amino-parts. The introduction of phenyl-substituent on the amino group of **3** and **4** increased the activity (compounds **7** and **8**, respectively). The exception is a very strong activity of **3** toward HeLa and Hep-2 cells. Furthermore, the introduction of polar, electron-donating substituents as diethylamino- (**1**) and methoxy- (**2**) to the salicylic moiety of **4** also increased the activity. Moreover, the introduction of phenyl-substituents (**5**) and (**6**) abolished the selectivity among different cell lines observed for unsubstituted analogues **1** and **2**.

The antiproliferative activity of compounds **3** and **4** has already been described by Lovejoy and Richardson,[3] whereby compound **4** had lowest activity, which is in perfect accordance with our results. Moreover, in our experiments, as well as in the above-mentioned study, MCF-7 cells were least affected by the treatment with **3** among tumor cells tested. However, although **3** is in our experiments less active on normal human fibroblast (WI 38) than on HeLa and Hep-2 tumor cells, it is equally active as on other tumor cells. Therefore, significantly lower rate of proliferation of WI 38 cells did not cause the resistance of these cells to **3**, pointing to cytotoxic mode of action. Therefore, the hypothesis that thiosemicarbazone **3** shows selectivity in regard to normal cells, as discussed by Lovejoy and Richardson,[3] could not be generalized. Nevertheless, here described novel thiosemicarbazone derivatives (**1**, **2** and **5**–**8**) clearly display stronger antiproliferative activity than the parent compounds **3** and **4**, which supports their further investigation as potential antitumor agents.

Another perspective of these novel compounds is their potential as promising chelators for a number of biologically relevant cations. Namely, metal chelation is a very important process, useful to afford new chemical features to metal complexes in order to make them suitable for practical purposes (e.g., pharmacological applications). For example, copper(II) complexes, containing aromatic thiosemicarbazones were determined to induce apoptosis in human leukemia cell lines.[16] Therefore, further studies of these novel thiosemicarbazones with various metal cations are underway.

3. Conclusions

The crystal and molecular structure of one of the novel thiosemicarbazone derivatives (**5**) revealed the planarity of the conjugated aromatic system, which suggested the possibility of DNA binding by intercalation. However, the presented DNA binding studies excluded this mode of action. Physicochemical and structural properties of novel derivatives (**1**, **2** and **5**–**8**) were compared with previously studied analogues (**3** and **4**), taken as reference compounds, revealing distinctive differences. Most likely as a consequence of substituent effects, the novel thiosemicarbazone derivatives displayed stronger antiproliferative activity on five tumor cell lines than the reference compounds **3** and **4**, which supports their further investigation as potential antitumor agents.

4. Experimental

4.1. Materials

All aldehydes, 4-phenylthiosemicarbazide and thiosemicarbazide, were of reagent grade and used as purchased. C, H, N analyses were provided by the Analytical Services Laboratory of Ruđer Bošković Institute, Zagreb. ^{1}H and ^{13}C NMR spectra were recorded on either a Varian Gemini 300 or a Bruker Avance DPX 300 spectrometer using TMS as an internal standard in DMSO-d_6.

IR spectra were recorded as KBr pellets using Perkin-Elmer Fourier-Transform Spectrum RX1 Spectrophotometer in the region 4500–450 cm^{-1}.

Diffracted intensities were collected on the Oxford Diffraction Xcalibur 3 diffractometer using graphite monochromated Mo Kα radiation at 293 K. The programs CrysAlis CCD[17] and CrysAlis RED[17] were used for data collection, cell refinement and data reduction. The structure was solved by direct methods (SHELXS[18]). Refinement procedure (SHELXL-97[19]) by full-matrix least squares methods based on F^2 values against all reflections included anisotropic displacement parameters for all non-H atoms. The two last-mentioned programs operated under the WinGX[20] program package. The hydrogen atom positions were obtained from a difference Fourier map and they were included in the refinement process with isotropic displacement parameters. A summary of crystal data is presented in Table 1. Programs PLATON,[21] PARST,[22] ORTEP[23] and Mercury[24] were used for analysis of the structure and drawings preparation.

4.2. Synthesis

4.2.1. Synthesis of 4-diethylamino-salicylaldehyde thiosemicarbazone (1). Thiosemicarbazide (0.92 g, 10 mmol) was dissolved in 40 mL of dry methanol with stirring and warming over a period of 30 min. To the warm thiosemicarbazide solution, 4-diethylamino-salicylaldehyde (1.94 g, 10 mmol) in 20 mL of dry methanol was added and the mixture was stirred and slowly refluxed for 2 h. The mixture was then cooled down to room temperature when the yellow crystalline compound precipitated. The compound was collected by filtration, washed well with cold methanol and dried in vacuum.

The product is soluble in warm acetone, methanol, ethanol, dichloromethane, chloroform, DMF and DMSO.

Yield: 1.15 g (43%). Anal. Calcd for $C_{12}H_{18}N_4OS$: C, 54.11; H, 6.81; N, 21.03; S, 12.04. Found: C, 54.00; H, 6.90; N, 21.11; S, 11.98%. IR (cm^{-1}) in KBr: 3337 (m), 3150 (s), 2996 (m), 1622 (m), 1610 (m),

1596 (s), 760 (m); ^{1}H NMR (δ, DMSO-d_6, 25 °C, ppm): 11.07 (s, 1H, NHCS), 9.61 (s, 1H, OH), 8.19 (s, 1H, CH=N), 7.66 and 7.87 (2br s, 1H each, NH_2), 3.33 (q, 4H, CH_2N), 1.10 (t, 6H, CH_3), 6.09–7.52 (m, 3H, aromatic); ^{13}C NMR (δ, DMSO-d_6, 25 °C, ppm): 176.75 (C=S), 142.45 (CH=N), 43.94 (CH_2), 12.96 (CH_3), 158.26, 150.20, 129.12, 107.51, 104.08, 97.40 (C-aromatic).

4.2.2. Synthesis of 3-methoxy-salicylaldehyde thiosemicarbazone (2). Thiosemicarbazide (0.92 g, 10 mmol) was dissolved in 40 mL of dry methanol with stirring and warming over a period of 30 min. To the warm thiosemicarbazide solution, 3-methoxy-salicylaldehyde (1.53 g, 10 mmol) in 10 mL of dry methanol was added and the mixture was stirred and slowly refluxed for 2 h. The mixture was then cooled down to room temperature when the white crystalline compound precipitated. The compound was collected by filtration, washed well with cold methanol and dried in vacuum.

The product is soluble in warm acetone, methanol, ethanol, dichloromethane, chloroform, DMF and DMSO.

Yield: 0.85 g (38%). Anal. Calcd for $C_9H_{11}N_3O_2S$: C, 47.98; H, 4.92; N, 18.65; S, 14.23. Found: C, 47.76; H, 5.00; N, 18.67; S, 14.20%. IR (cm^{-1}) in KBr: 3337 (m), 3130 (s), 2986 (m), 1632 (s), 1613 (s), 1576 (s), 765 (m); ^{1}H NMR (δ, DMSO-d_6, 25 °C, ppm): 11.39 (s, 1H, NHCS), 9.17 (s, 1H, OH), 8.40 (s, 1H, CH=N), 7.88 and 8.10 (2br s, 1H each, NH_2), 3.81(s, 3H, OCH_3), 6.77–7.53 (m, 3H, aromatic); ^{13}C NMR (δ, DMSO-d_6, 25 °C, ppm): 178.05 (C=S), 139.83 (CH=N), 56.25 (CH_3O), 148.26, 146.34, 121.14, 119.33, 118.52, 113.19 (C-aromatic).

4.2.3. Synthesis of 2-hydroxy-naphthaldehyde thiosemicarbazone (3). Thiosemicarbazide (0.92 g, 10 mmol) was dissolved in 40 mL of dry methanol with stirring and warming over a period of 30 min. To the warm thiosemicarbazide solution, 2-hydroxy-naphthaldehyde (1.73 g, 10 mmol) in 20 mL of dry methanol was added and the mixture was stirred at room temperature for 2 h. The yellow crystalline compound obtained after standing at room temperature over the night was filtered, washed well with cold methanol and dried in vacuum.

The product is soluble in warm acetone, methanol, ethanol, dichloromethane, chloroform, DMF and DMSO.

Yield: 1.27 g (52%). Anal. Calcd for $C_{12}H_{11}N_3OS$: C, 58.76; H, 4.52; N, 17.13; S, 13.07. Found: C, 58.68; H, 4.50; N, 17.07; S, 13.11%. IR (cm^{-1}) in KBr: 3337 (m), 3118 (s), 2986 (m), 1615 (s), 1623 (m), 1586 (s), 764 (m); ^{1}H NMR (δ, DMSO-d_6, 25 °C, ppm): 11.38 (s, 1H, NHCS), 10.41 (s, 1H, OH), 9.05 (s, 1H, CH=N), 7.81 and 8.20 (2br s, 1H each, NH_2), 7.19–8.51 (m, 6H, aromatic); ^{13}C NMR (δ, DMSO-d_6, 25 °C, ppm): 177.41 (C=S), 142.92 (CH=N), 156.49, 132.35, 131.40, 128.56, 127.95, 127.75, 123.33, 122.70, 118.23, 109.61 (C-aromatic).

4.2.4. Synthesis of salicylaldehyde thiosemicarbazone (4). Thiosemicarbazide (0.92 g, 10 mmol) was dissolved in 40 mL of dry methanol with stirring and warming over a period of 30 min. To the warm thiosemicarbazide solution, salicylaldehyde (1.21 g, 10 mmol) in 10 mL of dry methanol was added and the mixture was stirred and slowly refluxed for 2 h. The mixture was then cooled down to room temperature when the white crystalline compound precipitated. The compound was collected by filtration, washed well with cold methanol and dried in vacuum.

The product is soluble in warm acetone, methanol, ethanol, dichloromethane, chloroform, DMF and DMSO.

Yield: 0.97 g (50%). Anal. Calcd for $C_8H_9N_3OS$: C, 49.23; H, 4.61; N, 21.54; S, 16.42. Found: C, 49.09; H, 4.50; N, 21.41; S, 16.39%. IR (cm^{-1}) in KBr: 3337 (m), 3133 (s), 2886 (m), 1622 (s), 1610 (s), 1576 (s), 775 (m); ^{1}H NMR (δ, DMSO-d_6, 25 °C, ppm): 11.39 (s, 1H, NHCS), 9.88 (s, 1H, OH), 8.39 (s, 1H, CH=N), 8.12 and 7.94 (2br s, 1H each, NH_2), 6.82–7.91 (m, 4H, aromatic); ^{13}C NMR (δ, DMSO-d_6, 25 °C, ppm): 177.56 (C=S), 139.55 (CH=N), 156.28, 130.97, 126.65, 120.22, 119.15, 115.92 (C-aromatic).

4.2.5. Synthesis of 4-diethylamino-salicylaldehyde 4-phenylthiosemicarbazone (5).

4.2.5.1. Method A. 4-Phenylthiosemicarbazide (1.67 g, 10 mmol) was dissolved in 40 mL of dry methanol with stirring and warming over a period of 30 min. To the cold thiosemicarbazide solution, 4-diethylamino-salicylaldehyde (1.94 g, 10 mmol) in 20 mL of dry methanol was added. A yellow product began to immediately separate from the red solution. The mixture was stirred additionally at room temperature for 3 h. The solution was evaporated and product was filtered and dried in vacuum without washing.

The product is soluble in warm acetone, methanol, ethanol, dichloromethane, chloroform, DMF and DMSO.

Yield: 0.97 g (28%). Anal. Calcd for $C_{18}H_{22}N_4OS$: C, 63.13; H, 6.48; N, 16.36; S, 9.36. Found: C, 63.09; H, 6.32; N, 16.30; S, 9.34. IR (cm^{-1}) in KBr: 3339 (m), 3131 (s), 2980 (m), 1652 (s), 1623 (s), 1577 (s), 765 (s); ^{1}H NMR (δ, DMSO-d_6, 25 °C, ppm): 11.49 (s, 1H, NHCS), 9.85 (s, 1H, NHPh), 9.58 (s, 1H, OH), 8.33 (s, 1H, CH=N), 3.33 (q, 4H, CH_2N), 1.10 (t, 6H, CH_3), 7.16–7.61 (m, 3H, aromatic), 6.12–7.70 (m, 5H, C_6H_5); ^{13}C NMR (δ, DMSO-d_6, 25 °C, ppm): 174.52 (C=S), 142.53 (CH=N), 43.95 (CH_2), 12.65 (CH_3), 139.43, 128.06, 125.24, 124.86 (C_6H_5), 158.43, 150.36, 129.20, 107.49, 104.07, 97.32 (C-aromatic).

4.2.5.2. Method B. 4-Diethylamino-salicylaldehyde (0.24 g, 1.24 mmol) was dissolved in 5 mL of dichloromethane and the solution of 4-phenylthiosemicarbazide (0.21 g, 1.25 mmol) in 20 mL of dichloromethane was added. The reaction mixture was then stirred at room temperature for 3 h. The resulting yellow prismatic crystals were collected by filtration and dried over KOH.

The product is soluble in warm acetone, methanol, ethanol, dichloromethane, chloroform, DMF and DMSO.

Yield: 0.42 g (100%). Anal. Calcd for $C_{18}H_{22}N_4OS$: C, 63.13; H, 6.48; N, 16.36; S, 9.36. Found: C, 63.19; H, 6.42; N, 16.35; S, 9.30. IR (cm^{-1}) in KBr: 3339 (m), 3131 (s), 2980 (m), 1652 (s), 1623 (s), 1577 (s), 765 (s); ^1H NMR (δ, DMSO-d_6, 25 °C, ppm): 11.49 (s, 1H, NHCS), 9.85 (s, 1H, NHPh), 9.58 (s, 1H, OH), 8.33 (s, 1H, CH=N), 3.33 (q, 4H, CH_2N), 1.10 (t, 6H, CH_3), 7.16–7.61 (m, 3H, aromatic), 6.12–7.70 (m, 5H, C_6H_5); ^{13}C NMR (δ, DMSO-d_6, 25 °C, ppm): 174.52 (C=S), 142.53 (CH=N), 43.95 (CH_2), 12.65 (CH_3), 139.43, 128.06, 125.24, 124.86 (C_6H_5), 158.43, 150.36, 129.20, 107.49, 104.07, 97.32 (C-aromatic).

4.2.6. Synthesis of 3-methoxy-salicylaldehyde 4-phenylthiosemicarbazone (6). 4-Phenylthiosemicarbazide (1.67 g, 10 mmol) was dissolved in 40 mL of dry methanol with stirring and warming over a period of 30 min. To the cold thiosemicarbazide solution, 3-methoxy-salicylaldehyde (1.53 g, 10 mmol) in 10 mL of dry methanol was added and mixture was stirred at room temperature for a 4 h. Within 10 min a white product began to separate out. After standing at room temperature over the night, the product was filtered off, washed with small volume of cold methanol and dried in vacuum.

The product is soluble in warm acetone, methanol, ethanol, dichloromethane, chloroform, DMF and DMSO.

Yield: 1.65 g (54%). Anal. Calcd for $C_{15}H_{15}N_3O_2S$: C, 59.81: H, 15.12; N, 13.95; S, 10.64. Found: C, 59.65; H, 14.99; N, 13.78; S, 10.43%. IR (cm^{-1}) in KBr: 3339 (m), 3159 (s), 2976 (m), 1642 (s), 1633 (s), 1576 (s), 768 (m). ^1H NMR (δ, DMSO-d_6, 25 °C, ppm): 11.74 (s, 1H, NHCS), 10.03 (s, 1H, NHPh), 9.26 (s, 1H, OH), 8.53 (s, 1H, CH=N), 3.82 (s, 3H, OCH_3), 6.80–7.71 (m, 3H, aromatic), 7.19–7.58 (m, 5H, C_6H_5); ^{13}C NMR (δ, DMSO-d_6, 25 °C, ppm): 175.82 (C=S), 140.04 (CH=N), 55.99 (CH_3O), 139.23, 128.10, 125.76, 125.25 (C_6H_5), 146.23, 147.99, 120.76, 119.02, 118.58, 113.11 (C-aromatic).

4.2.7. Synthesis of 2-hydroxy-naphthaldehyde 4-phenylthiosemicarbazone (7). 4-Phenylthiosemicarbazide (1.67 g, 10 mmol) was dissolved in 40 mL of dry methanol with stirring and warming over a period of 30 min. To the cold thiosemicarbazide solution, 2-hydroxynaphtaldehyde (1.73 g, 10 mmol) in 20 mL of dry methanol was added and the mixture was stirred at room temperature for 4 h. After 30 min the yellow crystalline product began to separate out. The product was filtered, washed with small volume of cold methanol and dried in vacuum.

The product is soluble in warm acetone, methanol, ethanol, dichloromethane, chloroform, DMF and DMSO.

Yield: 0.88 g (27%). Anal. Calcd for $C_{18}H_{15}N_3OS$: C, 67.27; H, 4.70; N, 13.07; S, 9.98. Found: C, 67.11; H, 4.58; N, 16.97; S, 9.78%. IR (cm^{-1}) in KBr: 3349 (m), 3157 (s), 2876 (m), 1641 (s), 1635 (s), 1577 (s), 766 (m); ^1H NMR (δ, DMSO-d_6, 25 °C, ppm): 11.79 (s, 1H, NHCS), 10.68 (s, 1H, OH), 10.11 (s, 1H, NHPh), 9.20 (s, 1H, CH=N), 7.21–8.51 (m, 6H, aromatic), 7.21–7.62 (m, 5H, C_6H_5); ^{13}C NMR (δ, DMSO-d_6, 25 °C, ppm): 175.85 (C=S), 143.65 (CH=N), 139.32, 128.33, 125.23, 125.22 (C_6H_5), 156.78, 132.73, 131.76, 128.19, 128.89, 127.95, 123.63, 122.62, 118.63, 109.83 (C-aromatic).

4.2.8. Synthesis of salicylaldehyde 4-phenylthiosemicarbazone (8). 4-Phenylthiosemicarbazide (1.67 g, 10 mmol) was dissolved in 40 mL of dry methanol with stirring and warming over a period of 30 min. To the cold thiosemicarbazide solution, salicylaldehyde (1.23 g, 10 mmol) in 10 mL of dry methanol was added and mixture was stirred at room temperature for a 4 h. Within 10 min a white product began to separate out. After standing at room temperature over the night, the product was filtered off, washed with small volume of cold methanol and dried in vacuum.

The product is soluble in warm acetone, methanol, ethanol, dichloromethane, chloroform, DMF and DMSO.

Yield: 1.65 g (60%). Anal. Calcd for $C_{14}H_{13}N_3OS$: C, 61.99; H, 4.79; N, 15.49; S, 11.82. Found: C, 61.72; H, 4.68; N, 15.40; S, 11.78%. IR (cm^{-1}) in KBr: 3359 (m), 3259 (s), 2986 (m), 1641 (s), 1630 (s), 1566 (s), 758 (m); ^1H NMR (δ, DMSO-d_6, 25 °C, ppm): 11.79 (s, 1H, NHCS), 10.06 (s, 1H, NHPh), 9.99 (s, 1H, OH), 8.53 (s, 1H, CH=N), 6.85–8.10 (m, 4H, aromatic), 7.20–7.60 (m, 5H, C_6H_5); ^{13}C NMR (δ, DMSO-d_6, 25 °C, ppm): 176.49 (C=S), 140.80 (CH=N), 139.91, 128.79, 126.41, 125.92 (C_6H_5), 157.36, 132.09, 127.86, 121.00, 119.99, 116.81 (C-aromatic).

4.3. Spectroscopic measurements

The electronic absorption spectra were recorded on Varian Cary 100 Bio spectrometer, and fluorescence emission spectra were recorded on Varian Eclipse fluorimeter, in all cases using quartz cuvettes (1 cm). The measurements were performed in the aqueous buffer solution (pH 7.0; sodium cacodylate buffer, $I = 0.05$ mol dm^{-3}). Under the experimental the conditions used absorbance and fluorescence intensities of studied compounds were proportional to their concentrations.

4.3.1. Interactions with DNA. The *calf thymus* DNA (ct-DNA) was purchased from Aldrich, dissolved in the sodium cacodylate buffer, $I = 0.05$ mol dm^{-3}, pH 7.0, additionally sonicated and filtered through a 0.45 μm filter and the concentration of corresponding solution determined spectroscopically as the concentration of phosphates. In fluorimetric titrations, excitation wavelength of $\lambda > 300$ nm was used to avoid inner filter effects caused by absorption of excitation light by added polynucleotide. Spectroscopic titrations were performed by adding portions of polynucleotide solution into the solution of the studied compound. The stability constant (K_s) and [bound compound]/[polynucleotide phosphate] ratio (n) were calculated according to the

Scatchard equation by non-linear least-square fitting,[15] giving excellent correlation coefficients (>0.999) for obtained values for K_s and n.

Thermal melting curves for ct-DNA and its complexes with studied compounds were determined as previously described by following the absorption change at 260 nm as a function of temperature.[25] The absorbance of the ligand was subtracted from every curve, and the absorbance scale was normalized. Obtained T_m values are the midpoints of the transition curves, determined from the maximum of the first derivative or graphically by a tangent method. Given ΔT_m values were calculated subtracting T_m of the free nucleic acid from T_m of complex. Every ΔT_m value here reported was the average of at least two measurements, the error in ΔT_m is ±0.5 °C.

4.3.2. Antiproliferative activity assay. The HeLa (cervical carcinoma), Hep-2 (laryngeal carcinoma), MCF-7 (breast carcinoma), SW620 (colon carcinoma), MiaPaCa-2 (pancreatic carcinoma) and Hep-2 (laringeal carcinoma) and WI 38 (normal diploid fibroblasts) cells (obtained from American Type Culture Collection (ATCC, Rockville, MD, USA)) were cultured as monolayers and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin in a humidified atmosphere with 5% CO_2 at 37 °C. The growth inhibition activity was assessed as described previously, according to the slightly modified procedure of the National Cancer Institute, Developmental Therapeutics Program.[25] The cells were inoculated onto standard 96-well microtiter plates on day 0. The cell concentrations were adjusted according to the cell population doubling time (PDT): 1×10^4/mL for HeLa, Hep-2, MiaPaCa-2 and SW620 cell lines (PDT = 20–24 h), 2×10^4/mL for MCF-7 cell line (PDT = 33 h) and 3×10^4/mL for WI 38 (PDT = 47 h). Test agents were then added in five dilutions (10^{-8} to 5×10^{-6} mol/l) and incubated for a further 72 h. Working dilutions were freshly prepared on the day of testing. After 72 h of incubation, the cell growth rate was evaluated by performing the MTT assay, which detects dehydrogenase activity in viable cells. Each test was performed in quadruplicate in three individual experiments. The results are expressed as IC_{50}, which is the concentration necessary for 50% of inhibition. The IC_{50} values for each compound are calculated from concentration–response curves using linear regression analysis by fitting the test concentrations that give PG values above and below the reference value (i.e., 50%). If however, for a given cell line all of the tested concentrations produce PGs exceeding the respective reference level of effect (e.g., PG value of 50), then the highest tested concentration is assigned as the default value, which is preceded by a '>' sign. Each result is a mean value from three separate experiments.

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Supplementary data

Additional crystallographic and spectroscopic data. Crystallographic data have been deposited with the CCDC, 12 Union Road, Cambridge, CB2 1EZ, UK (fax: +44 1223 366033; e-mail: deposit@ccdc.cam.ac.uk or www://www.ccdc.cam.ac.uk) and are available on request, quoting the deposition number 674277 for the compound **5**. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2008.03.006.

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