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Original article

Synthesis, characterisation and cytotoxic properties of the N^1,N^4-diarylidene-*S*-methyl-thiosemicarbazone chelates with Fe(III) and Ni(II)

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Abstract

Reactions of 2-hydroxy-3-methoxy, 2-hydroxy-4-methoxy-benzaldehyde with 3- and 4-methoxy-substituted salicylaldehyde *S*-methyl-thiosemicarbazones in the presence of $FeCl_3$ and $NiCl_2$ resulted in the corresponding methoxy-substituted N^1,N^4-diarylidene-*S*-methyl-thiosemicarbazone chelates. Characterisation of the compounds in the [Fe(**L**)Cl] and [Ni(**L**)] general formula was accomplished by means of elemental analysis, conductivity and magnetic measurements, ^{1}H NMR, UV–vis, IR and mass spectroscopy. Cytotoxicity and proliferation experiments using K562 chronic myeloid leukemia cell line and ECV 304 human endothelial cell line imply that the iron(III) chelates may have anti-leukemic effects with 3.5 μg/dl LD_{50} dose.

Keywords: *S*-Methyl-thiosemicarbazone; Iron chelate; Nickel chelate; Cytotoxicity; MTT

1. Introduction

For many years, thiosemicarbazones and their metal complexes have been the subject of most structural and medicinal studies because of their biological potential [1–3]. These biological effects were observed as early as 1950s [4].

It has been believed that the thiosemicarbazones are efficient on certain biological mechanisms because of their chelating ability towards trace metal ions. After the chemotherapeutic effective platin complexes of thiosemicarbazide derivatives were synthesized [5], these kinds of chemicals have raised considerable interest due to their pharmacological properties [6]. In addition to antitumor effect of transition metal complexes of thiosemicarbazones [7–9], it is well known that they are also antiviral, even anti-HIV chemicals [10–14]. Numerous thiosemicarbazones, especially their Cu(II) complexes, have been studied for their antibacterial and antifungal properties [15–18]. Further, it can be mentioned that the thiosemicarbazones have anticonvulsant [19], anti-malarial [20], antiamoebic [21,22] and antioxidant properties [23] as other biological activities.

Research on interactions of DNA and thiosemicarbazone molecules has received significant attention over the last years. These papers include the comprehensive investigation to determine the details of DNA binding for the thiosemicarbazone metal complexes [24–27]. It has been proven that some thiosemicarbazone derivatives (such as Triapin) have ribonucleotide reductase (RR) inhibitory effect. RR is a critical enzyme of RNA conversion to DNA [28]. Triapin has been tested in phase I trial for patients with advanced cancer [29].

Transition metal complexes of various thiosemicarbazone derivatives have been intensively studied in view of their structural chemistry and biological potential, however, there are limited metal complexes with *S*-alkyl-thiosemicarbazones [30]. Although there are some pharmacological studies for *S*-alkyl-thiosemicarbazone derivatives [31,32], their metal complexes have been investigated with respect to structural and analytical properties [33–35].

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We present the structural and cytotoxic properties of four new N^1,N^4-diarylidene-*S*-methyl-thiosemicarbazone chelates with Fe(III) and Ni(II). The *ONNO* chelates (Fig. 1) were synthesized and characterized by elemental analysis, molar conductivity, magnetic susceptibility, electronic, infrared, ^{1}H NMR and mass spectroscopy.

2. Chemistry

The diarylidene chelates of the Fe(III) and Ni(II) salts (**I**–**IV**) were isolated in three steps. Firstly, 2-hydroxy-3-(and 4)-methoxy-thiosemicarbazones were prepared according to the traditional methods. After that, the *S*-methyl derivatives were synthesized by reaction of the thiosemicarbazones and methyliodide in ethanol. Finally, the interaction of the *S*-methyl-thiosemicarbazones ($\mathbf{L_{I-II}}$) and the 2-hydroxyaldehydes in the presence of metal ion in 1:1:1 molar ratio yielded a stable solid complex of the general formula [Fe(**L**)Cl] and [Ni(**L**)] (Fig. 1). The formation reaction of the Fe(III) chelate **I** is given as an example in Fig. 2.

3. Pharmacology

Cytotoxic effects of the compounds were evaluated by MTT test for K562 chronic myeloid leukemia cell line and ECV 304 human umbilical vein endothelial cell line.

Cell cytotoxicity was evaluated by using a system based on the tetrazolium compound 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT, Sigma M-5655) which is reduced by living cells to yield a soluble formazan product that can be assayed colorimetrically. Colorimetric analysis was performed by ELISA multiwell spectrophotometer (Diagnostics Pasteur LP 400).

Data were calculated according to the % cytotoxicity formula and expressed as mean ± S.D. (Table 1). Statistical analysis was performed using the Statistical Package for Social Statistics (SPSS). Students *t* test was used to compare K562 cells to ECV 304 cells, $p < 0.05$ is considered as significant difference.

Lethal concentration 50 values (LC is the concentration that kills 50% of the cells) were derived by interpolation from log-linear plot of concentration–toxicity (Table 2).

Because DMSO was used as solvent for thiosemicarbazones, same concentrations of DMSO was added to control DMSO groups for evaluating possible toxic effects of DMSO ($n = 6$). Higher optical density value than control groups considered as negative cytotoxicity attributed proliferation.

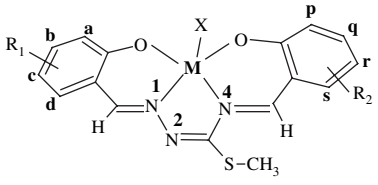

Fig. 1. M/**X**/R_1/R_2: Fe/Cl/3-CH_3O/4-CH_3O (**I**); Fe/Cl/4-CH_3O/3-CH_3O (**II**); Ni/–/3-CH_3O/4-CH_3O (**III**); Ni/–/4-CH_3O/3-CH_3O (**IV**).

4. Results and discussion

4.1. Some physical properties of the chelates (*I–IV*)

The bright coloured complexes form as a mixture of amorphous and crystal material. The purity of the compounds was controlled by Thin Layer Chromatography. When the amorphous and crystal forms were separated mechanically under a microscope, it was seen that both amorphous and crystal form gave same results on the spectroscopic and analytical techniques. We tried both forms in DMSO on leukemia cells and endothelial cells. Last of all, we saw that there was no difference between both of them.

The products are very soluble in DMSO and DMF while their solubility in alcohols and halocarbone solvents are poor.

The μ_{eff} values of iron chelates (**I** and **II**), 5.88 and 5.82, respectively, are equivalent to five unpaired electrons and so high-spin state of iron(III) indicates [Fe(**L**)Cl] composition. The low-spin complex which is in [$(FeL)_2O$] formula is isolated by using a organic base such as triethylamine in the template reaction [36,37]. Reactions of the Fe(III) chelates, **I** and **II**, with $AgNO_3$ in ethanol/water mixture yielded a white precipitate of silver chloride in the course of time. The amount of white precipitate increased after a period of 1/2 h. The chloride fragment was also recorded in the mass spectra. Moreover, M^+, MH^+ and $M^+ - H$ peaks certainly prove the [Fe(**L**)Cl] composition. The μ_{eff} values of nickel chelates (**III** and **IV**), 0.19 and 0.28, respectively, indicate that these complexes are diamagnetic and, in addition, the compounds did not react with $AgNO_3$ solution.

Molar conductivity values of **I**–**IV** are between 9.8 and 18.3 $\Omega^{-1}\,cm^2\,mol^{-1}$, therefore it can be said that the relative low molar conductances indicate non-ionic character of the chelates. The iron chelates are more conductive because of the coordinated chloride atom.

4.2. Spectral data

The absorption spectra of the free ligands in DMSO revealed bands at 248 and 312 nm for ($\mathbf{L_I}$) and at 257 and 336 nm for ($\mathbf{L_{II}}$) which can be assigned to $\pi \rightarrow \pi^*$ and $n \rightarrow \pi^*$ transitions associated with the thiosemicarbazone moiety and aromatic ring. These bands in the spectra of the chelates (**I**–**IV**) were detected in 257–264 and 315–320 nm ranges. The UV–vis spectra of the Fe(III) complexes recorded in DMSO show one band at 412 (for **I**) and at 414 (for **II**) which can be attributed to charge-transfer transition. However, as with the majority of high-spin Fe(III) complexes, the spectra of these complexes contained no bands that could be ascribed to d–d transition in the visible range. The UV–vis spectra of the Ni(II) complexes recorded in DMSO showed the charge transfer and dd bands. According to results published earlier, the low intensities of the two bands between 544 and 548 nm and between 762 and 764 nm for **III** and **IV**,

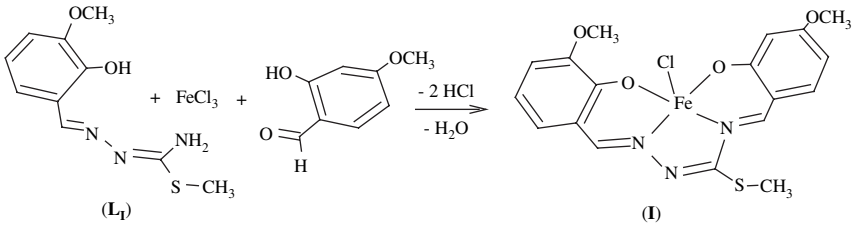

Fig. 2. Template reaction of the *S*-methyl-thiosemicarbazones.

respectively, indicate that these bands could be assigned to d–d, *Laporte* forbidden, spin-allowed transitions of the Ni(II) ion [38,39]. The medium-intensity bands at 404 (for **III**) and 406 nm (for **IV**) are due to metal–ligand charge-transfer processes.

In the infrared spectra of the 3- and 4-methoxy-*S*-methyl-thiosemicarbazones ($\mathbf{L_I}$ and $\mathbf{L_{II}}$), the ν(OH), ν_a(NH), ν_s(NH) and $\delta(NH_2)$ bands were observed at 3064 ($\mathbf{L_I}$) and 3129 ($\mathbf{L_{II}}$), 3412 ($\mathbf{L_I}$) and 3453 ($\mathbf{L_{II}}$), 3306 ($\mathbf{L_I}$) and 3303 ($\mathbf{L_{II}}$) and 1651 cm^{-1} ($\mathbf{L_I}$ and $\mathbf{L_{II}}$), respectively, and all the bands were absent by chelating.

The strong ν(C=N) bands of **I–IV** were monitored in the 1620–1612, 1607–1597 and 1597–1578 cm^{-1} regions. For the chelates, it can be observed that a new azomethine group (N^4=C) occurs by template condensation, and so the azomethine bands, ν(C=N^1) and ν(N^4=C), were seen at *ca*. 1616 and 1587 cm^{-1}, respectively. To analyse these azomethine bands is rather difficult, however, it can be proposed that the first azomethine bands (C=N^1) shift towards lower energies *ca*. 15 cm^{-1} by chelating [40]. The ν(C–O) bands of the phenolic oxygens are in medium intensity at around 1120–1150 cm^{-1}, the bands shift to lower frequencies *ca*. 25–30 cm^{-1} because of coordination of the oxygens to the metal ions.

The ^{1}H NMR spectra of 3- and 4-methoxy-salicylaldehyde *S*-methyl-thiosemicarbazones ($\mathbf{L_I}$ and $\mathbf{L_{II}}$) were recorded in a systematic pattern of isomer peaks of the azomethine, *S*-methyl and amide protons. These *cis*/*trans* and *syn*/*anti* isomerisms resulted from the double bonds such as C=N^2 and HC=N^1, respectively [41,42].

In the ^{1}H NMR spectra of the nickel chelates (**III** and **IV**), the peaks belonging to the isomerism and amide protons of $\mathbf{L_I}$ (at 6.84 ppm) and $\mathbf{L_{II}}$ (at 6.68 ppm) were not observed due to template complex formation. The spectra of the **III–IV** show also some systematic chemical shifts, for example, the **c** and **r** protons in 5-position in aromatic rings shifted to higher field according to $\mathbf{L_I}$ and $\mathbf{L_{II}}$. The chemical shifts of the other protons displayed more or less changes compared to the *S*-methyl-thiosemicarbazones upon complexation. The differences between these spectra are regular, and so it is seen clearly that the chelating reaction of $\mathbf{L_I}$ and $\mathbf{L_{II}}$ in the presence of Ni(II) resulted in a systematic constitution.

The mass spectra of the iron chelates (**I** and **II**) gave the M^+ (462), MH^+ (463), $M^+ - H$ (461) and $M^+ - Cl$ (427) peaks. M^+, MH^+ and $M^+ - H$ peaks prove the [Fe(**L**)Cl] composition.

The analytical and spectral data confirm the structure of the chelate compounds (**I–IV**) in Fig. 1.

4.3. Cytotoxicity results

Cytotoxicity assays imply that the iron chelates (**I** and **II**) are efficiently cytotoxic for leukemia cells and mildly proliferative for endothelial cells at same doses. Especially, the chelate **I** has clearly different effects on leukemic and non-cancer cell lines and these effects rose in a dose dependent manner.

The Fe(III) chelate **I** had only one of four assayed compounds, the LC_{50} dose of which was <5 μg for K562 cell lines. Same dose (3.5 μg/ml) of **I** showed minimal toxicity for non-cancer endothelial cell line ECV 304. With these cytotoxic properties, these iron chelates may have anti-leukemic drug potential. Furthermore, experimental values indicate that the chelate **I** would have some proliferative effect on ECV 304

Table 1
Cytotoxicity results for two different cell lines

Cell line	Compound	50 μg/ml	10 μg/ml	5 μg/ml	1 μg/ml	0.1 μg/ml	0.01 μg/ml
K562	**I**	78.25 ± 4.36	77.030 ± 2.88	62.13 ± 6.25[a]	21.73 ± 1.54[a]	9.95 ± 3.35[a]	9.17 ± 3.93
	II	68.06 ± 1.60[a]	21.235 ± 6.60[a]	12.91 ± 3.57[a]	−6.01 ± 15.45	−3.51 ± 4.12	−1.07 ± 1.95
	III	34.20 ± 5.83[a]	22.55 ± 2.73	10.42 ± 5.19	3.085 ± 11.86	−3.77 ± 6.74	−9.20 ± 8.39
	IV	64.02 ± 1.01[a]	−6.69 ± 6.25[a]	−1.98 ± 11.01[a]	−1.20 ± 5.82	16.31 ± 3.18	13.89 ± 4.36
	DMSO[b]	2.28 ± 4.69	−2.12 ± 2.70	−2.18 ± 4.12	−4.30 ± 5.44	3.5 ± 2.62	2.07 ± 4.92
ECV 304	**I**	76.40 ± 10.33	59.16 ± 4.98	8.45 ± 23.36	−25.83 ± 7.56	−15.27 ± 7.18	−7.23 ± 7.97
	II	27.24 ± 14.80	−13.45 ± 6.93	−13.91 ± 10.54	−6.89 ± 4.71	−0.61 ± 4.14	−3.79 ± 17.97
	III	71.85 ± 1.65	31.13 ± 9.14	9.80 ± 6.39	−5.70 ± 5.23	−1.97 ± 8.18	−2.64 ± 13.15
	IV	54.28 ± 4.00	19.15 ± 7.03	9.20 ± 3.64	−7.67 ± 10.66	−9.71 ± 5.10	−11.68 ± 12.48
	DMSO[b]	0.63 ± 5.65	−6.76 ± 4.44	−4.94 ± 4.32	−4.57 ± 7.94	−1.38 ± 6.36	−2.39 ± 7.90

[a] Mean differences are significant between K562 and ECV 304.
[b] Control DMSO is equal to DMSO concentrations which are prepared for thiosemicarbazone solution.

Table 2
Lethal concentration 50% (LC_{50}) for K562 cells and ECV 304

	LC_{50} (μg/ml)			
	I	**II**	**III**	**IV**
K562	3.5	38	>50	41
ECV 304	9.25	>50	30	42.5

cells in a dose-dependent manner between 1 and 0.01 μg/ml (Fig. 3). Considering the endothelial tissue damage contributed to pathogenesis of some diseases which have high morbidity and mortality rate, as diabetic microvasculopathies, atherosclerosis and hypertension, this proliferating effect on ECV 304 cell might be as important as anti-leukemic effect.

The second iron chelate (**II**) influencing both cell lines resemble chelate **I**, but LC_{50} dose of **II** was approximately 10 times higher than **I** (38 μg/ml and 3.5 μg/ml, respectively). On the other hand, cytotoxicity results of the nickel chelate (**IV**), denoted reverse effects on two cell lines, toxic for ECV 304, but proliferative for K562; these effects are statistically significant between 50 and 55 μg/ml. Because **I** and **III** have the methoxy substituents in same positions except metal residue, Fe(III) and Ni(II), respectively, and demonstrated different cytotoxic features, these results anticipated that the key component of anti-leukemic feature might be presence of iron. In fact, it has been known that metal chelates of thiosemicarbazones have relatively high antitumoral effects against MCF-7, T47D and C6 glioma cells [43–46].

It should be noticed that the iron chelate **I** in the N^1-3-methoxy/N^4-4-methoxy system is more active than the chelate **II** in the N^1-4-methoxy/N^4-3-methoxy system; besides the results indicate that the position of a substituent on the aromatic ring considerably specifies the biological activity.

For the determination of probable correlations between substituent and biological potential on the N^1,N^4-diarylidene chelates, detailed studies are in progress.

5. Conclusion

To explain cytotoxicity mechanisms and anti-leukemic effects, both should be investigated in more detail with experimental studies *in vivo*. If necessary an estimation of enzymatic mechanisms should be taken into consideration to explain biological effect of chemicals. The inhibition of ribonucleoside diphosphate reductase is one of these mechanisms, and some iron-thiosemicarbazone complexes have a function in the inhibition as known [47,48]. Considering quite high activities of iron chelates from the iron and nickel chelates (**I**–**IV**) in the same N^1,N^4-diarylidene ligand system (Fig. 1), ribonucleoside reductase inhibition could be suggested as a mechanism for the effect of the iron chelates on the K562 and ECV 304 cells.

6. Experimental protocols

6.1. Chemistry

All chemicals and solvents were of reagent grade. IR spectra were recorded (KBr disks) on a Mattson 1000 FT-IR spectrometer. Analytical data were obtained with a Thermo Finnigan Flash EA 1112 analyser and Unicam Solar 929 atomic absorption spectrometer. ^{1}H NMR spectra were obtained on a Varian INOVA 500 MHz spectrometer (Istanbul University). UV–vis spectra were recorded using a ATI-Unicam UV2 spectrophotometer. Molar conductivities were measured using a digital CMD 750 conductivity meter. Magnetic measurements were carried out at room temperature by the Gouy technique with an MK I model device obtained from Sherwood Scientific.

The APCI-MS analyses were carried out in positive and negative ion modes using a Thermo Finnigan LCQ Advantage MAX LC/MS/MS. The mobile phase consisted of a gradient mixture of 60% MeOH and 40% H_2O. Hypersil Betabasic-8 (5 μm, 100 mm × 4.6 mm) column was used at a flow rate

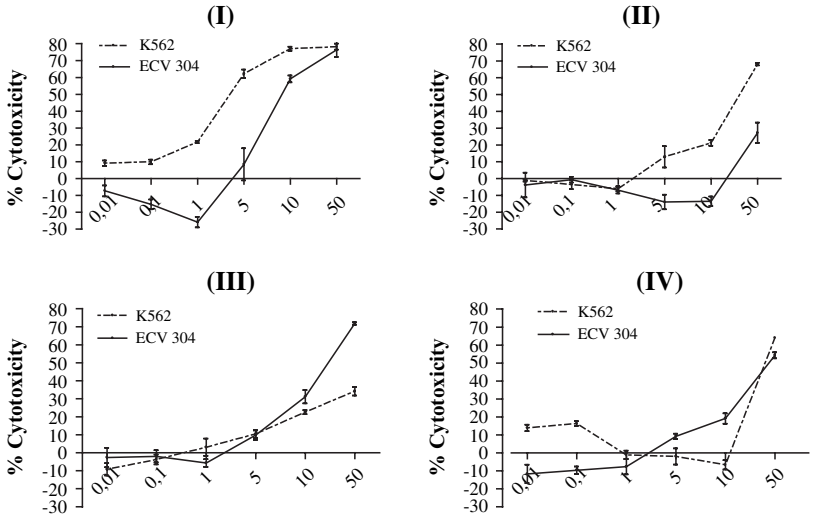

Fig. 3. Effect of iron and nickel chelates (**I**–**IV**) on K562 cells and ECV 304 cells in 0.01, 0.1, 1, 5, 10, 50 μg/ml.

of 0.2 ml/min at 25 °C. APCI-MS inlet conditions in the positive/negative-ion mode: heated capillary temperature, 200/200 °C; vaporizer temperature, 250/250 °C; sheath gas flow rate, 50/50 units; capillary voltage, 3/−8 V and tube lens offset, 45/−35 V.

6.2. Synthesis

6.2.1. S-Methylisothiosemicarbazones (L_{I-II})

The *S*-methylthiosemicarbazones of 2-hydroxy-3-methoxy- (**L_I**) and 2-hydroxy-4-methoxy-benzaldehyde (**L_{II}**) were prepared according to the literature [41]. The colours, m.p. (°C), yields (%), R_f (solvent), microanalyses % founded (calculated) and spectral data of **L_{I-II}** are given as follows.

Compound **L_I**: Cream, 164−165, 90, 0.12 ($CHCl_3$/CH_2Cl_2), $C_{10}H_{13}N_3O_2S$ (239.29 g/mol): C 50.23 (50.19), H 5.44 (5.48), N 17.53 (17.56), S 13.45 (13.40). FT-IR (KBr, cm^{-1}): ν_a(NH) 3412, ν_s(NH) 3306, ν(OH) 3129, δ(NH) 1651, ν(C=N^1), ν(N^2=C) 1628, 1601, ν(C−O) 1154. ^{1}H NMR (DMSO-d_6, 25 °C, ppm): 11.58, 10.71 (*cis*/*trans* ratio: 2:1, s, 1H, OH), 8.44, 8.30 (*syn*/*anti* ratio: 2:3, s, 1H, CH=N^1), 6.84 (s, 2H, NH_2), 7.14−6.99 (dd, $J = 7.43$ Hz, $J = 1.25$ Hz, 1H, d), 6.80 (t, 1H, $J = 8.04$ Hz, c), 6.94 (d, $J = 6.92$ Hz, 1H, b), 2.42, 2.37 (*cis*/*trans* ratio: 3:2, s, 3H, S−CH_3), 3.77 (s, 3H, OCH_3).

Compound **L_{II}**: Beige, 170−171, 91, 0.15 ($CHCl_3$/CH_2Cl_2), $C_{10}H_{13}N_3O_2S$ (239.29 g/mol): C 50.18 (50.19), H 5.46 (5.48), N 17.58 (17.56) S 13.42 (13.40). FT-IR (KBr, cm^{-1}): ν_a(NH) 3453, ν_s(NH) 3303, ν(OH) 3064, δ(NH) 1651, ν(C=N^1), ν(N^2=C) 1624, 1601, ν(C−O) 1150. ^{1}H NMR (DMSO-d_6, 25 °C, ppm): 11.78, 10.11 (*cis*/*trans* ratio: 2:1, s, 1H, OH), 8.38, 8.26 (*syn*/*anti* ratio: 1:2, s, 1H, CH=N^1), 6.68 (s, 2H, NH_2), 7.41−7.28 (dd, $J = 8.3$ Hz, 1H, d), 6.48 (split. d, 1H, $J = 8.25$ Hz, c), 6.43 (d, $J = 2.37$ Hz, 1H, a), 2.42, 2.37 (*cis*/*trans* ratio: 1:1, s, 3H, S−CH_3), 3.76 (s, 3H, OCH_3),

6.2.2. Monochloro N^1-3-methoxysalicylidene-N^4-4-methoxysalicylidene-S-methyl-thiosemicarbazidato-iron(III) (*I*)

$FeCl_3 \cdot 6H_2O$ (1.13 g, 1 mmol) was dissolved in 25 ml EtOH and *ortho*-formic ester (6.5 ml) was added to the solution. The mixture was allowed to stand for 4 h at room temperature. After this period, a solution of 3-methoxysalicylaldehyde-*S*-methylisothiosemicarbazone (1.0 g, 1 mmol) and 4-methoxysalicylaldehyde (0.64 g, 1 mmol) in EtOH (25 ml) was added with stirring to the metal salt solution. After 24 h, the resulting bright black precipitate was collected by filtration. The product was washed with a mixture of ethanol/ether (1:1, 10 ml) and dried over P_2O_5 *in vacuo* (yield 0.41 g, 21%).

6.2.3. N^1-3-methoxysalicylidene-N^4-4-methoxysalicylidene-S-methylthiosemicarbazidato-nickel(II) (*III*)

3-Methoxysalicylaldehyde-*S*-methylisothiosemicarbazone (1 g, 1 mmol) and 4-methoxysalicylaldehyde (0.64 g, 1 mmol) were dissolved in 25 ml EtOH. The solution was then added by stirring to a solution of 0.99 g $NiCl_2 \cdot 6H_2O$ (1 mmol) and *ortho*-formic ester (approx. 7 ml) in EtOH (25 ml). After 1 h at room temperature, triethylamine (*ca*. 8.0 ml) was added into the reaction mixture. After 24 h, the precipitated red crystals were filtered off, washed with EtOH/Et_2O (1:1, 10 ml) and dried over P_2O_5 *in vacuo* (yield 0.92 g, 51%).

The iron(III) and nickel(II) complexes (**II** and **IV**) were prepared in a similar manner. The colours, m.p. (°C), yields (%), μ_{eff} (BM), λ_M (Ω^{-1} cm^2 mol^{-1}), R_f (solvent), microanalyses % founded (calculated) and spectral data of **I**−**IV** are given as follows.

Compound (**I**): Bright black, >399, 21, 5.88, 18.3, 0.35 ($CHCl_3$/MeOH 20:1.5), $C_{18}H_{17}N_3O_4SFeCl$ (462.3 g/mol): C 46.56 (46.72), H 3.52 (3.68), N 8.98 (9.08), Fe 12.06 (12.08). FT-IR (KBr, cm^{-1}) ν(C=N^1) 1612, ν(N=C) 1601, ν(N^4=C) 1578, ν(C−O)$_{arom}$ 1158, 1131. *m*/*z* (−c APCI): 462 (M^+, 100.00), 463 (MH^+, 22.65), 461 ($M^+ - H$, 4.62), 460 ($M^+ - H_2$, 9.84), 356 (MH^+, $-C_6H_4-OCH_3$), 266 ($M^+ - H$, $-SH-CN-CH-C_6H_3-OCH_3-OH$, 5.62), (+c APCI): 427 (M^+, −Cl, 100.00), 428 (MH^+, −Cl, 22.77).

Compound (**II**): Bright black, 309 (dec), 28, 5.82, 16.9, 0.34 ($CHCl_3$/MeOH 20:1.5), $C_{18}H_{17}N_3O_4SFeCl$ (462.3 g/mol): C 46.68 (46.72), H 3.66 (3.68), N 9.15 (9.08), Fe 12.11 (12.08). FT-IR (KBr, cm^{-1}): ν(C=N^1) 1612, ν(N=C) 1605, ν(N^4=C) 1578, ν(C−O)$_{arom}$ 1154, 1123; (−c APCI): 462 (M^+, 100.00), 463 (MH^+, 18.48), 461 ($M^+ - H$, 6.05), (+c APCI): 427 ($M^+ - Cl$, 100.00), 428 ($MH^+ - Cl$, 26.18), 426 ($M^+ - H$, −Cl, 1.89).

Compound (**III**): Bright claret, 310 (dec), 51, 0.19, 12.3, 0.72 ($CHCl_3$/MeOH 20:1), $C_{18}H_{17}N_3SO_4Ni$ (429, 71 g/mol): C 50.18 (50.27), H 4.06 (3.96), N 9.82 (9.77), Ni 13.54 (13.66). FT-IR (KBr, cm^{-1}): ν(C=N^1) 1620, ν(N=C) 1607, ν(N^4=C) 1597, ν(C−O)$_{arom}$ 1152, 1129. ^{1}H NMR (DMSO-d_6, 25 °C, ppm): 8.44 (s, 1H, CH=N^1), 8.08 (s, 1H, CH=N^4), 7.64 (d, $J = 8.93$ Hz, 1H, d), 7.12 (d, $J = 7.30$ Hz, 1H, s), 6.88 (d, $J = 7.22$ Hz, 1H, b), 6.58 (t, $J = 7.62$ Hz, 1H, c), 6.46 (s, 1H, p), 6.42 (dd, $J = 8.97$ Hz, $J = 2.2$ Hz, 1H, r).

Compound (**IV**): Bright claret, 399, 42, 0.28, 9.8, 0.76 ($CHCl_3$/MeOH 20:1), $C_{18}H_{17}N_3SO_4Ni$ (429, 71 g/mol): C 50.34 (50.27), H 4.01 (3.96), N 9.61 (9.77), Ni 13.54 (13.66). FT-IR (KBr, cm^{-1}): ν(C=N^1) 1612, ν(N=C) 1597, ν(N^4=C) 1584, ν(C−O)$_{arom}$ 1152, 1123. ^{1}H NMR (DMSO-d_6, 25 °C, ppm): 8.09 (s, 1H, CH=N^1), 7.95 (s, 1H, CH=N^4), 7.18 (d, $J = 7.84$ Hz, 1H, d), 7.04 (d, $J = 6.95$ Hz, 1H, s), 6.69 (d, $J = 5.08$ Hz, 1H, q), 6.37 (t, $J = 6.87$ Hz, 1H, r), 6.17 (s, 1H, a), 6.08 (d, $J = 8.29$ Hz, 1H, c).

6.3. Cell cultures

For cell cultures' growth, humidified CO_2 incubator at 37 °C (Nuaire), IMDM (Sigma I-3390) for K562 and M 199 (Sigma M 0393) for ECV 304, newborn calf serum (NCS, PAA B15-001) 15 and 10%, respectively, 96-well plate, polystyrene cell culture flasks (Greiner Inc.) were used.

K562 human chronic myeloid leukemic cells were cultivated in suspension culture in Iscoves' Modified Dulbecco's Medium (IMDM) supplemented with 15% newborn calf

serum and gentamycin (625 μl/l). For experimental procedure, K562 cells were suspended at 5×10^5 cells/ml in IMDM with serum. Ninety microliters of this suspension was dispended into 96-well plates, 10 μl of corresponding stock solutions added and incubated at 37 °C in the humidified CO_2 incubator for 72 h. Ten microliter medium serum mixture was added to positive control groups in place of stock solutions and medium serum mixtures which did not contain any cells were used for negative controls.

ECV 304 human umbilical vein endothelial cells were cultivated in M 199 supplemented with 10% newborn calf serum and gentamycin (40 mg/l). For experimental procedure, cells detached by 0.5% trypsin in NM3 solution, washed two times with PBS, resuspended in M 199 at 5×10^5 cells/ml density. Incubation procedure was performed as for K562.

6.4. Cytotoxicity assay

Stock solutions of the thiosemicarbazones were prepared in DMSO (Sigma D-5879,) 5 mg/ml. The six aqueous concentrations (500, 100, 50, 10, 1, 0.1 μl/ml) were prepared from each solution. Then 10 μl of the concentrations was diluted with 90 μl cell suspension in IMDM into 96-well microplates. Thus final concentrations of compounds were 50, 10, 5, 1, 0.1, 0.01 μl/ml medium, respectively. Cytotoxicity assay was repeated six times for each concentration of all compounds.

After 72 h of incubation, 10 μl MTT (5 mg/ml) solution in phosphate buffered saline (PBS) was added to each well and the plates were incubated 4 h at 37 °C. Then the supernatants were removed from all wells and *iso*-propyl alcohol solution (pH: 5.5) was added to the wells to solubilize the MTT crystals. Then microplates were left in the dark room overnight and optical density (OD) was measured with 540 nm test wavelength and a 620 nm reference wavelength on an ELISA multiwell spectrophotometer. Hundred microliter Iscoves' Modified Dulbecco's Medium (IMDM, Sigma) that contained 15% newborn calf serum (NCS, PAA B15-001) was used as negative control for optical density (OD).

Cytotoxicity index was calculated with the formula: % Cytotoxicity = [1 − experiment (OD)/control (OD)] × 100.

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