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Original article

Synthesis, crystal structures, and biological evaluation of manganese(II) and nickel(II) complexes of 4-cyclohexyl-1-(1-(pyrazin-2-yl)ethylidene) thiosemicarbazide

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ABSTRACT

4-Cyclohexyl-1-(1-(pyrazin-2-yl)ethylidene)thiosemicarbazide (HL) and its transition metal complexes formulated as $[Mn(L)_2]$ (**1**) and $[Ni(L)_2]$ (**2**) have been prepared in 55–75% yield and characterized by elemental analysis, IR, MS, NMR and single-crystal X-ray diffraction studies. Biological activities of the synthesized compounds have been evaluated against selected Gram positive bacteria *Bacillus subtilis*, Gram negative bacteria *Pseudomonas aeruginosa* and the K562 leukemia cell line, respectively. The cytotoxicity data suggest that these compounds may be endowed with important biological properties, especially the nickel complex **2** with MIC = 31.2 μg/mL and IC_{50} = 0.53 μM, respectively. Effect of the free ligand and its two complexes on Mitochondria membrane potential (MMP) and PI-associated fluorescence intensity as well as their effect on cell apoptosis in K562 leukemia cell line was also studied. The tested compounds may exert their cytotoxicity activity via induced loss of MMP.

1. Introduction

Heterocyclic thiosemicarbazones (TSCs) and their transition metal complexes have received considerable attention due to their coordination chemistry and the beneficial pharmacological properties, notable for antiparasital, antibacterial and antitumor activities [1–6]. Currently the most promising drug candidate of this class of compounds is Triapine (3-aminopyridine-2-carboxaldehyde thiosemicarbazone, 3-AP), which entered several phase I and II clinical trials as an antitumor chemotherapeutic agent [4]. The biological activities of thiosemicarbazones often showed a high dependence on their substituents. Minor modifications in the thiosemicarbazones can lead to widely different biological activities. Earlier reports on N(4)-substituted thiosemicarbazones have concluded that the presence of a bulky group at the terminal nitrogen considerably increases biological activity [7]. Moreover, the biological properties of thiosemicarbazones are often modulated by metal ion coordination. In some cases, the highest biological activity is associated with a metal and some side effects may decrease upon complexation [8]. Their mechanism of action is still controversial in many respects, but it is known that TSCs act by inhibiting ribonucleotide reductase, a key enzyme in the biosynthesis of DNA precursors [9].

Our previous studies on a variety of substituted thiosemicarbazones and their diverse metal complexes showed that both 2-acetylpyrazine thiosemicarbazone and 2-acetylpyrazine N(4)-methylthiosemicarbazone exhibited remarkable biological activity in vitro against K562 leukemia cell lines [10]. In particular, 2-acetylpyrazine N(4)-methylthiosemicarbazone shows a lower IC_{50} value (25.9 μM) than the 2-acetylpyrazine thiosemicarbazone (47.5 μM), which indicates that the presence of bulky group at position N(4) of the thiosemicarbazone moiety enhanced the antitumor activities [10b]. On the other hand, their metal complexes also exhibited significant antitumor activity [10]. Stimulated by these encouraging and promising results, it seemed useful and desirable to us to initiate systematic investigation of 2-acetylpyrazine N(4)-substituted thiosemicarbazones and their metal complexes. Particularly, information on the mechanism of these compounds is sparse and valuable.

In the present work, with the main aim of comparison, we have tested the biological activity of 4-cyclohexyl-1-(1-(pyrazin-2-yl) ethylidene)thiosemicarbazide (HL) (Scheme 1) and its transition metal complexes formulated as $[Mn(L)_2]$ (**1**) and $[Ni(L)_2]$ (**2**) against selected Gram positive bacteria *Bacillus subtilis*, Gram negative

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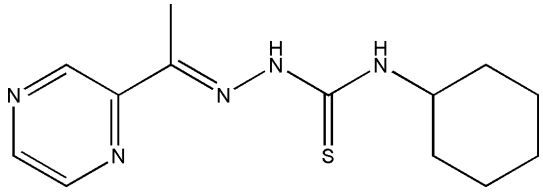

Scheme 1. 4-Cyclohexyl-1-(1-(pyrazin-2-yl)ethylidene)thiosemicarbazide, HL.

Table 1
IR spectral assignments (cm^{-1}) of HL and complexes **1** and **2**.

Compound	ν(C=N)	ν(N–N)	ν(C=S)	ρ(pz)
HL	1568	1109	866	620
1	1523	1148	827	639
2	1538	1144	832	687

bacteria *Pseudomonas aeruginosa* and the K562 leukemia cell line, respectively. It is worth noting that cytotoxic agents can induce cell death through various pathways that include necrosis and apoptosis [11a]. Apoptosis is a common process of programmed cell death and is the focus of current oncology research. To explore their biochemical mechanism of cytotoxicity initially, effect of the title three compounds on Mitochondria membrane potential (MMP) and PI-associated fluorescence intensity as well as their effect on cell apoptosis in K562 leukemia cell line are also studied. In addition, we also describe the synthesis and single-crystal X-ray crystal structures of complexes **1** and **2** here.

2. Chemistry

4-Cyclohexyl-1-(1-(pyrazin-2-yl)ethylidene)thiosemicarbazide (HL) and its transition metal complexes formulated as $[Mn(L)_2]$ (**1**) and $[Ni(L)_2]$ (**2**) have been synthesized. The complexes **1** and **2** were characterized by elemental analysis, IR, MS and single-crystal X-ray diffraction studies. Biological activities of these compounds have been investigated.

The tentative assignments of the significant IR spectral bands of HL and complexes **1** and **2** are presented in Table 1. The infrared spectral bands most useful for determining the mode of coordination of the ligand are the ν(C=N), ν(N–N) and ν(C=S) vibrations. The ν(C=N) band of thiosemicarbazone in complexes **1** and **2** undergoes a negative shift of wavenumber compared to that of the ligand, a clear sign of coordination via the imine nitrogen atom [11b]. The increase in the frequency of ν(N–N) band of the thiosemicarbazone in the spectra of complexes is due to the increase in the bond strength, again confirms the coordination via the imine nitrogen [12]. The thioamide band, which contains considerable ν(C=S) character, is less intense in the complexes and is found at a lower frequency, suggesting coordination of the metal through sulfur [13]. The breathing motion of the pyrizine ring is shifted to a higher frequency upon complexation and is consistent with pyrizine ring nitrogen coordination. These observations have also been confirmed by X-ray single-crystal structure analysis.

3. Results and discussion

3.1. X-ray crystallography

Table 2 summarizes crystal and refinement data for **1** and **2**. The molecular structures of **1** and **2** along with the atomic numbering scheme are shown in Figs. 1 and 2, respectively. Selected bond lengths and angles are listed in Table 3.

In view of the structural similarity of $[Mn(L)_2]$ (**1**) and $[Ni(L)_2]$ (**2**), only complex **1** was described in some detail. As shown in Fig. 1, the manganese(II) ion is in a slightly distorted octahedral environment, where two 4-cyclohexyl-1-(1-(pyrazin-2-yl)ethylidene) thiosemicarbazide units deprotonated act as N_2S tridentate ligands coordinated to the central manganese atom via the pyrazine nitrogen, imine nitrogen and sulfur atoms. One sulfur atom, one imine nitrogen atom and one pyrazine nitrogen atom from one ligand and one imine nitrogen atom from another ligand occupy the basal positions, the two remaining positions in the octahedral geometry are the axial ones which are occupied by one sulfur atom and one pyrazine nitrogen atom from the second ligand. The

Table 2
Summary of crystal data and refinement results for complexes **1** and **2**.

Compound	**1**	**2**
Empirical formula	C_{26} H_{36} Mn N_{10} S_2	$C_{26}H_{36}$ N_{10} Ni S_2
Formula weight	607.71	611.48
Crystal size (mm)	0.37 × 0.19 × 0.17	0.37 × 0.18 × 0.15
Crystal system	Monoclinic	Monoclinic
Space group	C2/*c*	C2/*c*
a (Å)	17.4629 (19)	29.395
b (Å)	13.0277 (14)	13.743
c (Å)	14.3027 (15)	21.170
V (Å^3)	3183.1 (6)	6155.2
β/°	101.970 (2)	133.97
D_c (g cm^{-3})	1.268	1.320
Z	4	8
μ (mm^{-1})	0.578	0.800
θ (°)	1.97–25.00	1.77–25.00
F (000)	1276	2576
hkl Range	$-20 \le h \le 14$, $-14 \le k \le 15$, $-16 \le l \le 16$	$-34 \le h \le 30$, $-16 \le k \le 16$, $-17 \le l \le 25$
T (K)	296 (2)	293 (2)
Refl. measured	2796	5261
Refl. unique	2306	3882
R_{int}	0.0243	0.0412
Parameters	177	352
R_1,wR_2 [$I \ge 2\sigma$ (I)]	0.0580, 0.1591	0.0420, 0.1217
R_1,wR_2 (all date)	0.0688, 0.1670	0.0585, 0.1283
Goodness-of-fit on F^2	1.089	0.967
$\Delta\rho_{max,\ min}$(e Å^{-3})	1.491, −1.0840	1.572, −0.276

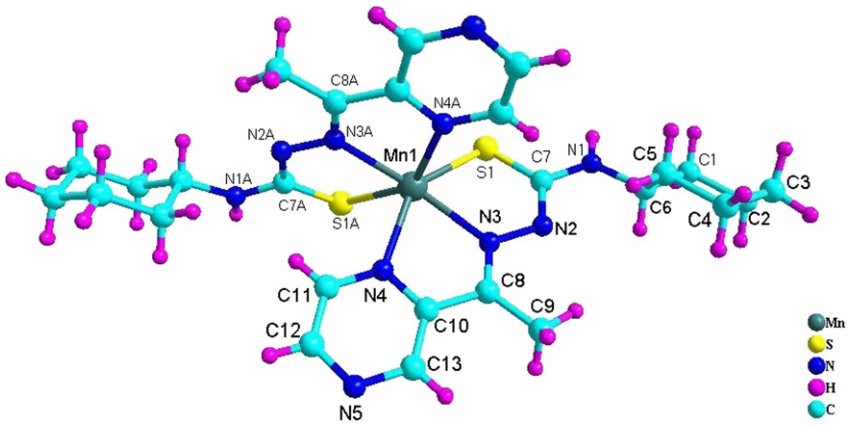

Fig. 1. Structure of complex **1** with atomic numbering scheme.

pseudo-macrocyclic coordination mode of each ligand affords two five-membered chelate rings.

The C(7)–S(1) bond length of 1.743 (4) is within the normal range of C–S single bonds, indicating that the thiosemicarbazone moieties adopt the thiol tautomeric form [14]. The C–N and N–N bond lengths in L^- are intermediate between formal single and double bonds, pointing to an extensive electron delocalization over the entire molecular skeleton. The two thiosemicarbazone ligands have slightly different Mn–N(pyrizine) bond distances and they are longer than the Mn–N(imine) distances, this may be attributed to the fact that the imine nitrogen is a stronger base compared with the pyrizine nitrogen [15].

Complex **1** is stabilized by intermolecular hydrogen bonds (see Fig. 3, Table 4). The hydrogen bond involves the terminal nitrogen atom N(1) and the uncoordinated pyrazine nitrogen atom N(5) with N(1)···N(5) 3.055 (5) Å and the angle N(1)–H(1A)···N(5) being 173.6° (symmetry code: $x-1/2, y-1/2, z$). Similarly, intermolecular hydrogen bonds of complex **2** also link the different components to stabilize the crystal structure (see Fig. 4, Table 4), differing only in the hydrogen bond donors and acceptors. These intermolecular hydrogen bonds include: terminal nitrogen atoms N(1) and N(6), hydrazine nitrogen atom N(2) and the coordinated sulfur atom S(2), respectively.

3.2. Antibacterial activity

In view of the antimicrobial activity of thiosemicarbazone [6], we have tested the inhibition ability of the obtained compounds against selected Gram positive bacteria *B. subtilis* and Gram negative bacteria *P. aeruginosa* by the disc diffusion method. Based on the minimum inhibitory concentration (Table 5), generally, the title three compounds display more inhibitory properties against Gram positive bacteria *B. subtilis* than against Gram negative bacteria *P. aeruginosa* and HL and **2** are more active than complex **1**. It should be emphasized that HL and **2** show higher activities against Gram negative bacteria *P. aeruginosa* than positive control antibiotics ampicillin (Amp), streptomycin (Str), kanamycin sulfate (Kan), respectively. In addition, although the MIC values of the parent ligand and its complexes did not reveal meaningful differences which is consistent with the previously reported that of 2-acetylpyridine N(4)-cyclohexylthiosemicarbazone and its manganese(II) and nickel(II) complexes, 2-acetylpyrazine N(4)-phenylthiosemicarbazone and its diorganotin(IV) complexes [16], in general these results are still very gratifying which are encouraging further screening in vitro and/or in vivo as well as evaluation of mechanism.

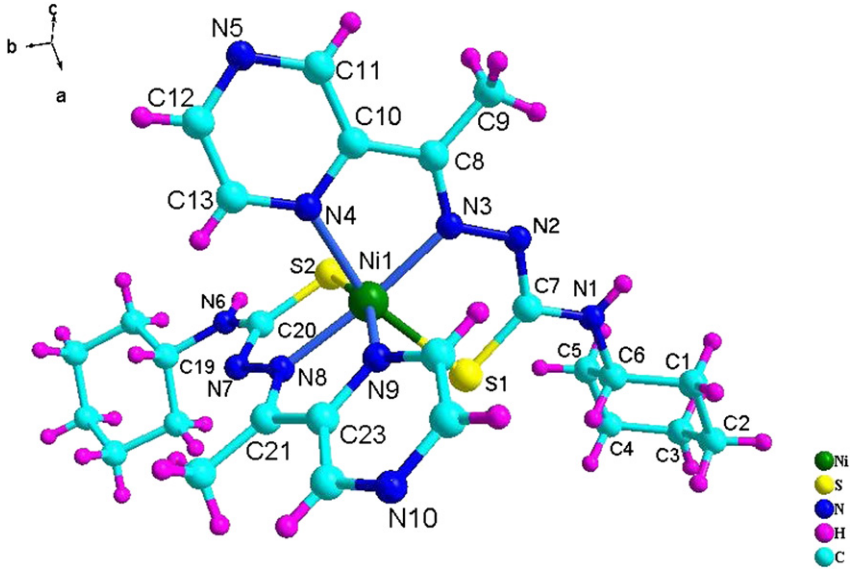

Fig. 2. Structure of complex **2** with atomic numbering scheme.

Table 3
Selected bond lengths (Å) and angles (°) of complexes **1** and **2**.

1		**2**	
Mn(1)–N(3)	2.257 (3)	Ni(1)–N(3)	2.010 (2)
Mn(1)–N(4)	2.350 (3)	Ni(1)–N(4)	2.120 (2)
Mn(1)–S(1)	2.531 (1)	Ni(1)–S(1)	2.411 (1)
S(1)–C(7)	1.743 (4)	S(1)–C(7)	1.709 (3)
N(3)–C(8)	1.303 (4)	N(3)–C(8)	1.311 (3)
N(3)–N(2)	1.362 (4)	N(3)–N(2)	1.351 (2)
N(1)–C(7)	1.343 (4)	N(1)–C(7)	1.343 (3)
N(3)–Mn(1)–N(3A)	145.51 (14)	N(3)–Ni(1)–N(8)	173.80 (7)
N(3)–Mn(1)–N(4)	70.14 (10)	N(3)–Ni(1)–N(4)	78.78 (8)
N(4)–Mn(1)–N(4A)	98.55 (16)	N(4)–Ni(1)–N(9)	92.03 (7)
N(3)–Mn(1)–S(1)	76.13 (7)	N(3)–Ni(1)–S(1)	81.96 (5)
N(4)–Mn(1)–S(1A)	90.60 (8)	N(4)–Ni(1)–S(2)	90.41 (6)

Table 4
Hydrogen bond lengths (Å) and bond angles (°) in complexes **1** and **2**.

D–H···A	*d*(H···A)	*d*(D···A)	∠(DHA)
1			
N(1)–H(1A)···N(5)[a]	2.20	3.055 (5)	173.6
2			
N(1)–H(1A)···S(2)[b]	2.64	3.484 (2)	167.1
N(6)–H(6A)···N(2)[c]	2.17	3.029 (3)	178.9

Symmetry transformation used to generate the equivalent atoms is given in the footnotes listed below.
[a] $x-1/2, y-1/2, z$.
[b] $-x+3/2, y-1/2, -z+1/2$.
[c] $-x+3/2, y+1/2, -z+1/2$.

3.3. Antitumor evaluation

In terms of the cytotoxic activity of thiosemicarbazones [17,18], we have tested the ability of HL, **1** and **2** to inhibit tumor cell growth against the K562 leukemia cell line. In our experiments, IC_{50} values (compound concentration that produces 50% of cell death) in micro molar units were calculated. The investigation has clearly shown that 4-cyclohexyl-1-(1-(pyrazin-2-yl)ethylidene)thiosemicarbazide and its metal complexes show significant antitumor activity against K562 leukemia cell line (Fig. 5), due to the NNS tridentate system [18,19]. Furthermore, HL shows much lower IC_{50} value (9.92 μM) than the previously reported cases of the 2-acetylpyrazine N(4)-methylthiosemicarbazone (25.9 μM) and the 2-acetylpyrazine thiosemicarbazone (47.5 μM) which indicate that the presence of bulky group at position N(4) of the thiosemicarbazone moiety greatly enhanced the antitumor activities [10b]. In addition, it is clearly observed that complexation with metals has a synergetic effect on the antitumor activity of these compounds and the antitumor activity depends upon the type of metal ion. As can be expected, coupling of HL to Mn(II) (2.78 μM) or Ni(II) (0.53 μM) increases the antitumor activity of their parent ligand, in a similar way to that observed other Mn(II) and Ni(II) compounds [11b,16a,20]. This confirms the conclusion that the antitumor activities of thiosemicarbazones can be enhanced by the linkage to metal ions. Importantly, in our previous unpublished studies, cisplatin only exhibited the inhibitory percentage of 9.46% at a concentration of 50 μM for 24 h against K562 leukemia cell line, suggesting that these studied compounds indicated stronger growth inhibition activities than that of cisplatin. Therefore, the title complexes may be endowed with important antitumor properties and merit further investigation for further development as anticancer agents.

Mitochondria was considered to be a major site of antitumor agents through electron leakage from the electron transport chain [21,22], the decreased MMP may open the mitochondrial permeability transition (MPT) and trigger the release of cytochrome c which activate caspase cascade, causing the cell death. In the present report, rhodamine 123 (Rh123), a mitochondrial specific stain that is dependent on the transmembrane potential, and another one, Propidine iodide (PI), were applied for the determination of MMP and apoptosis cells as well as necrosis cells, respectively. As shown in Figs. 6–8, the number of apoptosis cells and PI-associated fluorescence intensity in K562 leukemia cells were increased in a concentration-dependent manner companying decreased MMP after incubation with the tested compounds for 48 h, indicating the remarkable antitumor activity in vitro. The present results also revealed that the complexes **1** and **2** exhibited more potent effects than their parent ligand, which was consistent with their actions in MTT assay.

4. Conclusions

In summary, 4-cyclohexyl-1-(1-(pyrazin-2-yl)ethylidene)thiosemicarbazide and its manganese(II) and nickel(II) complexes were synthesized and characterized. Biological studies showed that the title three compounds exhibited significant and different biological activities. The title compounds may exert their cytotoxicity activity

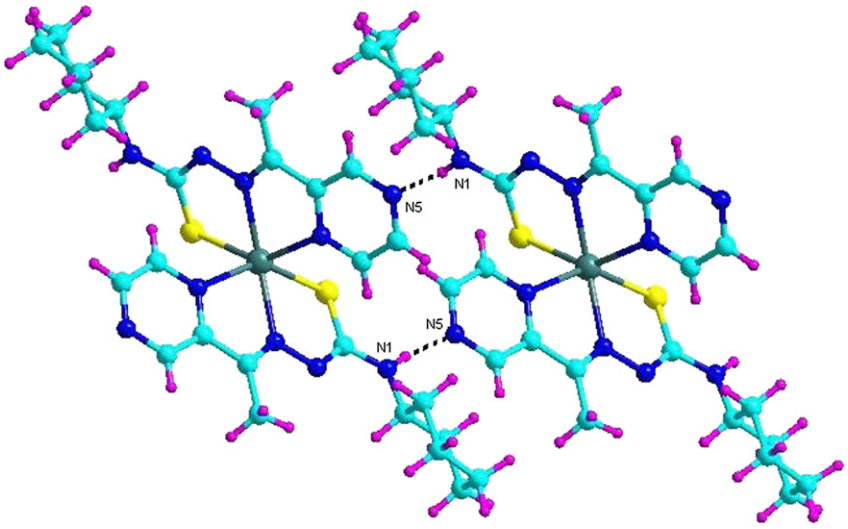

Fig. 3. Hydrogen bond indicated by dashed lines in complex **1**.

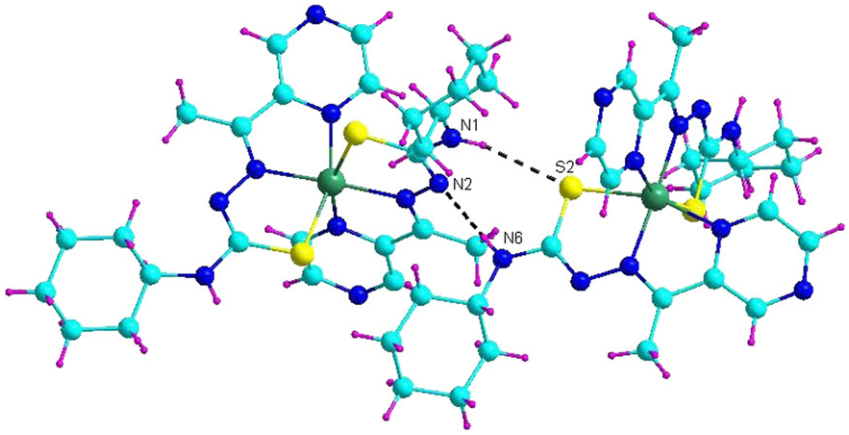

Fig. 4. Hydrogen bond indicated by dashed lines in complex **2**.

via induced loss of MMP. These promising results will be essential for antibacterial and anticancer drug discovery and medical practice.

5. Experimental

5.1. Materials and chemicals

All chemicals were of reagent grade quality obtained from commercial sources and used without further purification. Instrumentation: Elemental analysis of C, H and N was performed with a Perkin–Elmer240 analyzer. The infrared spectra were recorded from KBr discs with a Nicolet 170 FT infrared spectrophotometer. ^{1}H NMR spectra were recorded in DMSO-d_6 using a BrukerAV-400 spectrometer. The mass spectra were carried out on an Esquire 3000 LC–MS mass spectrophotometer.

5.2. Synthesis

5.2.1. Synthesis of ligand

Cyclohexyl isothiocyanate (1.41 g, 10 mmol) and hydrazine hydrate (0.50 g, 10 mmol), each dissolved in 20 mL methanol were mixed with constant stirring. The stirring was continued for 1 h and the white product, N(4)-cyclohexyl thiosemicarbazide formed was filtered, washed, dried and recrystallized from methanol. A methanolic solution of N(4)-cyclohexyl thiosemicarbazide (0.52 g, 3 mmol) was then refluxed with 2-acetylpyrazine (0.37 g, 3 mmol) in 10 mL methanol continuously for 4 h after adding a few drops of acetic acid. Yellow crystals separated on cooling were washed and recrystallorized from methanol. Yield: 75% –C_{13} H_{19} N_5 S (277): calcd. C 56.32, H 6.86, N 25.27; found C 55.74, H 6.71, N 25.58. ^{1}H NMR (DMSO-d_6, δ ppm): 10.37 (s, 1H, NH), 9.58 (d, J = 7.2Hz, 1H, $C_4N_2H_3$), 8.62–8.61(m, 2H, $C_4N_2H_3$, NH), 8.30 (d, J = 7.2Hz, 1H, $C_4N_2H_3$), 1.86–1.13 (m, 11H, C_6H_{11}), 2.35(s, 3H, CH_3). ESI-MS (m/z): 300.1 = [HL + Na^+], calc. mass = 300.1.

Table 5
Antibacterial activities of the tested compounds.

Microorganisms	MIC (μg/mL)					
	HL	**1**	**2**	Amp	Str	Kan
B. subtilis	15.6	31.2	31.2	5	20	10
P.aeruginosa	31.2	62.5	31.2	–[a]	–	40

[a] No inhibition or MIC >200 μg/mL.

5.2.2. Synthesis of complex *1*

A methanol solution containing $Mn(ClO_4)_2 \cdot 6H_2O$ (0.072 g, 0.2 mmol) was added dropwise to a solution of 4-cyclohexyl-1-(1-(pyrazin-2-yl)ethylidene)thiosemicarbazide (0.111 g, 0.4 mmol) dissolved in 20 mL methanol. The solution immediately turned deep red. After stirring for 0.5 h, the resultant solution was filtered. Deep-red crystals suitable for X-ray studies were obtained by slow evaporation of its ethanol solution. Yield: 58% –C_{26} H_{36} Mn N_{10} S_2 (607.71): calcd. C 51.34, H 5.92, N 23.04; found C 51.76, H 5.71, N 23.38. ESI-MS (m/z): 608.3 = [Mn(L)(HL)]$^+$, calc. mass = 608.2.

5.2.3. Synthesis of complex *2*

Complex **2** was prepared by a similar procedure to that of complex **1** using $Ni(ClO_4)_2 \cdot 6H_2O$ in place of $Mn(ClO_4)_2 \cdot 6H_2O$. Red crystals suitable for X-ray studies were obtained by slow evaporation of its ethanol solution. Yield: 55% –C_{26} H_{36} N_{10} Ni S_2 (611.48): calcd. C 51.02, H 5.89, N 22.90; found C 51.68, H 5.64, N 22.51. ESI-MS (m/z): 611.3 = [Ni(L)(HL)]$^+$, calc. mass = 611.2.

5.3. X-ray crystallography

Intensities of the title complexes were collected on a Siemens SMART-CCD diffractometer equipped with a graphite-monochromatic Mo-$K\alpha$ (λ = 0.71073 Å) radiation using the SMART and SAINT programs. The structure was solved by direct method and refined on F^2 by full-matrix least-squares techniques with SHELXTL *version* 5.1 [23,24]. All of the non-hydrogen atoms were refined with anisotropic thermal displacement parameters. The hydrogen

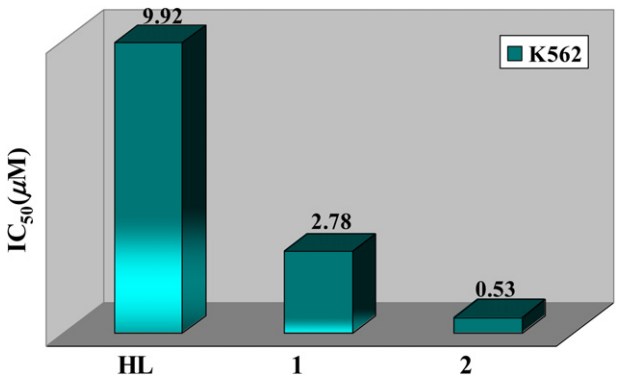

Fig. 5. The antitumor activity of HL, **1** and **2** against K562 leukemia cell line.

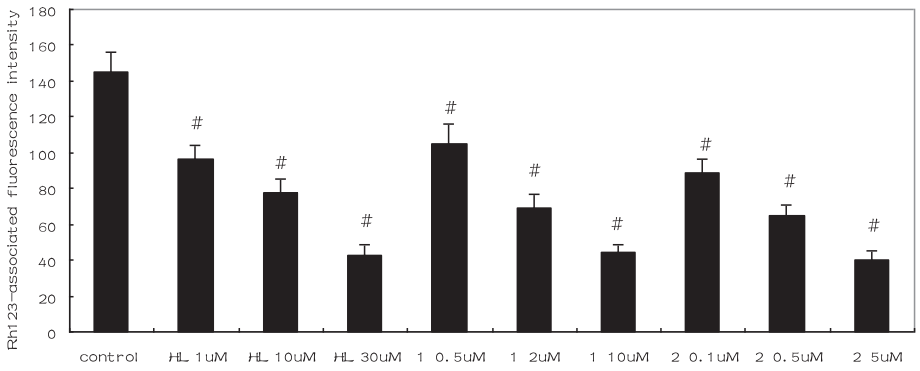

Fig. 6. Effect of HL, **1** and **2** on MMP in K562 eukaemia cell line. Each value represents the mean ± S.D. from four experiments. #: $p < 0.05$ vs control.

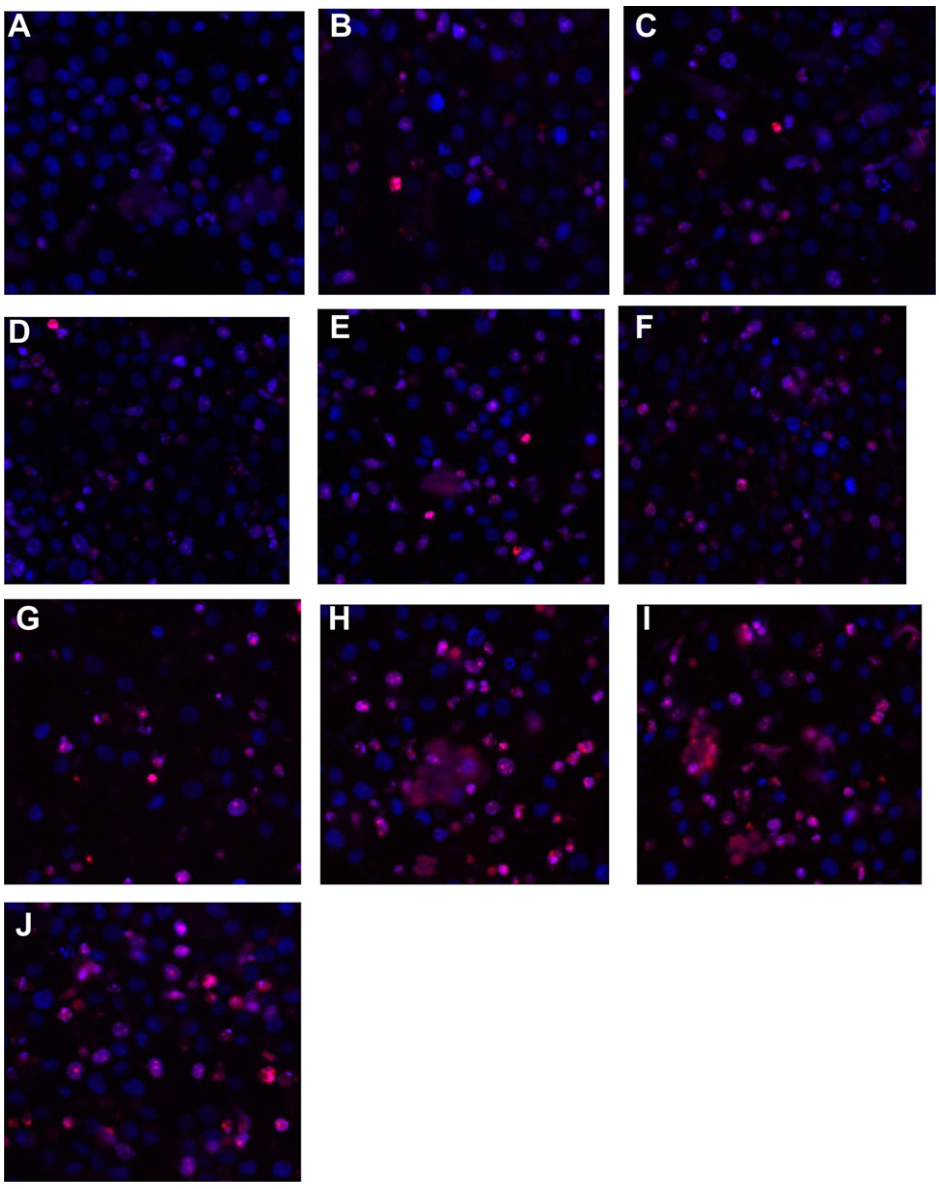

Fig. 7. Effect of HL, **1** and **2** on cell apoptosis in K562 leukemia cell line.

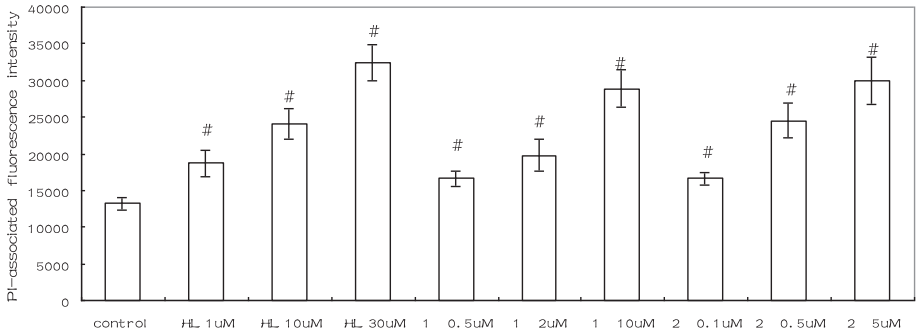

Fig. 8. Effect of HL, **1** and **2** on PI-associated fluorescence intensity in K562 leukemia line. Each value represents the mean ± S.D. from four experiments. #: $p<0.05$ vs control.

atoms were positioned according to theoretical models. Crystallographic data of **1** and **2** are summarized in Table 2.

CCDC 798836 and 798837 contain the supplementary crystallographic data for **1** and **2**, respectively. These data can be obtained free of charge from the Cambridge Crystallographic Centre via www.ccdc.cam.ac.uk/data_request/cif.

5.4. Antibacterial experiments

The in vitro antibacterial activity of the title compounds was investigated against selected Gram positive bacteria *B. subtilis* and Gram negative bacteria *P. aeruginosa*. The minimal inhibitory concentrations (MIC, μg/mL) were estimated by the disk diffusion method. The final concentration of bacteria in Mueller–Hinon agar (MHA) was adjusted to 10^6 cfu/mL and used for inoculation in the MIC test. Serial dilutions of the test compounds, previously dissolved in dimethyl sulfoxide (DMSO) were prepared at concentrations of 0–2000 μg/mL. To each plate was inoculated with 0.1 mL of the prepared bacterial cultures. Similarly, each plate carried a blank disc, with solvent DMSO only in the center to serve as negative control, as well as positive control antibiotics ampicillin (Amp), streptomycin (Str), kanamycin sulfate (Kan). The inoculated plates were then incubated at 37 °C for 18–20 h. The minimal inhibitory concentration (MIC) was detected as the lowest concentration of drug in plate for which no visible growth took place by macroscopic evaluation. All determinations were performed in triplicate and confirmed by three separate experiments.

5.5. Antitumor experiments

K562 leukemia cell line (purchased from the Institute of Biochemistry and Cell Biology, SIBS, CAS) was cultured in RPMI-1640 medium supplemented with 10% FBS, 100 U mL^{-1} of penicillin, 100 μg (200 μL per well) of streptomycin at 37 °C in humid air atmosphere of 5% CO_2. Cell cytotoxicity was assessed by the MTT assay. Briefly, cells were placed into a 96-well-plate (5×10^3 cells per well). The next day the compound diluted in culture medium at various concentrations was added (200 μL per well) to the wells. 24 h later 20 μL of MTT (0.5 mg mL^{-1} MTT in PBS) was added and cells were incubated for a further 4 h. 200 μL of DMSO were added to each culture to dissolve the MTT crystals. The MTT-formazan product dissolved in DMSO was estimated by measuring absorbance at 570 nm with a micro plate reader. Then the inhibitory percentage of each compound at various concentrations was calculated, and the IC_{50} value was determined.

5.6. Measurement of mitochondrial membrane potential (MMP)

After incubation with the tested compounds with different concentration for 48 h, the K562/DOX cells were preloaded with Rh123 (2 μM) and Hoechst 33342 (3 mg/ml) for 30 min at 37 °C, and then rinsed with freshly prepared PBS. The fluorescence intensity was measured at emission wavelength of 530 nm and excitation wavelengths of 480 nm by High-Content Screening Reader (Arrary Scan VTI 600, USA).

Statistical analysis: All the data were expressed as mean ± S.D. and analyzed using analysis of ariance (ANOVA) followed by Student's *t*-test. Differences were considered statistically significant at $p < 0.05$.

5.7. PI staining

After incubation with the tested compounds with different concentration for 48 h, the K562/DOX cells were preloaded with PI (100 μg/ml) and Hoechst 33342 (3 mg/ml) for 30 min at 37 °C, and then rinsed with freshly prepared PBS. The fluorescence intensity was measured at emission wavelength of 620 nm and excitation wavelengths of 488 nm by High-Content Screening Reader (Arrary Scan VTI 600, USA), the stained cells were observed and taken photographs.

Statistical analysis: All the data were expressed as mean ± S.D. and analyzed using analysis of ariance (ANOVA) followed by Student's *t*-test. Differences were considered statistically significant at $p < 0.05$.

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