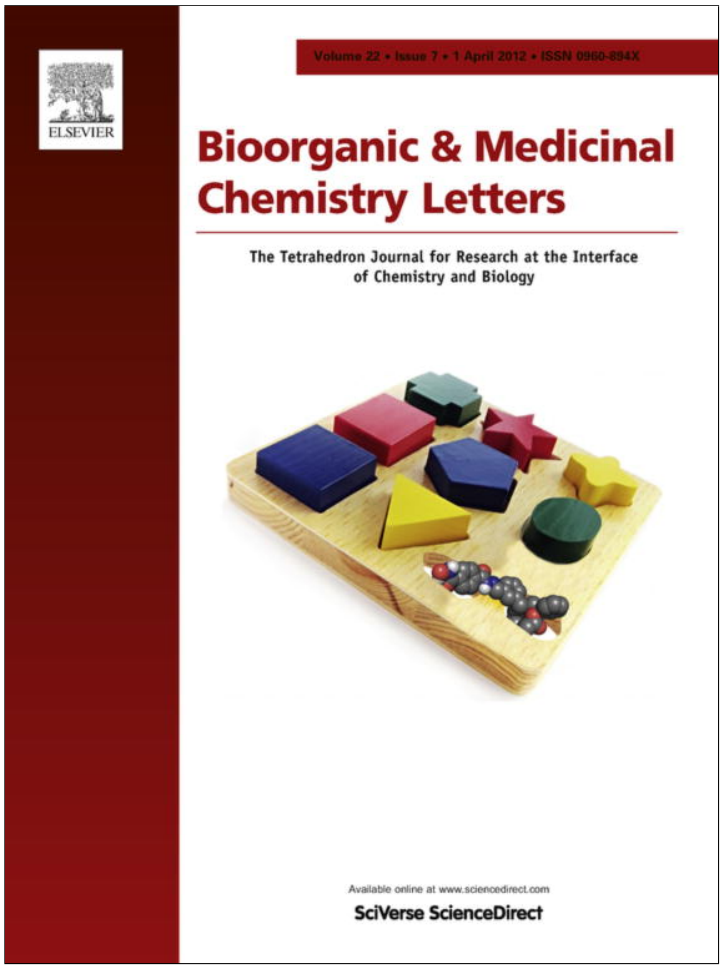

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Synthesis, crystal structures, in vitro biological evaluation of zinc(II) and bismuth(III) complexes of 2-acetylpyrazine *N*(4)-phenylthiosemicarbazone

Ming-Xue Li *, Li-Zhi Zhang, Min Yang, Jing-Yang Niu *, Jing Zhou

Institute of Molecular and Crystal Engineering, College of Chemistry and Chemical Engineering, Henan University, Kaifeng 475004, PR China

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ABSTRACT

Two metal complexes formulated as $[Zn(L)_2]_2{\cdot}H_2O$ (**1**) and $[Bi(L)(NO_3)_2(CH_3OH)]$ (**2**), where HL = 2-acetylpyrazine *N*(4)-phenylthiosemicarbazone, have been synthesized and characterized by elemental analysis, IR, MS, NMR and single-crystal X-ray diffraction studies. Biological studies, carried out in vitro against selected bacteria and the K562 leukemia cell lines, respectively, have shown that the free ligand and its two complexes may be endowed with important biological properties, especially HL with MIC = 3.90 μg/mL against *Pseudomonas aeruginosa*, the zinc(II) complex **1** with IC_{50} = 1.0 μM against K562 leukemia cell lines, respectively. The compounds HL and **1** may exert their cytotoxicity activity via induced loss of mitochondria membrane potential (MMP).

Heterocyclic thiosemicarbazones and their metal complexes have received considerable attention in chemistry and biology, primarily because of their marked and various biological properties.[1] In general, thiosemicarbazones are well-known chelators of metal ions and are obtained by condensation of the corresponding thiosemicarbazide with aldehydes or ketones. The mechanism of action of thiosemicarbazones is due to their ability to inhibit the biosynthesis of DNA, possibly by blocking ribonucleotide diphosphate reductase.[2–4] Moreover, the biological properties of thiosemicarbazones are often related and modulated by metal ion coordination.[5] For example, lipophilicity, which controls the rate of entry into the cell, is modified by coordination and some side effects may decrease upon complexation.[6–8]

In recent years we have been working on the structural and biological properties of heterocyclic thiosemicarbazones and their metal complexes.[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.[9d] 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 anticancer activities. Furthermore, their metal complexes also exhibited significant cytotoxicity.[9e–h]

The widespread use of bismuth compounds in the medicinal realm for centuries is coming from their high effectiveness and low toxicity in the therapy of diverse microbial infections, involving syphilis, diarrhea, gastritis and colitis.[10] In addition to antimicrobial activity, bismuth compounds revealed anticancer activities, Radioisotope ^{212}Bi and ^{213}Bi compounds have been applied as targeted radio-therapeutic agents for cancer therapy,[11–13] and furthermore they have the ability to reduce the side-effects of cisplatin (*cis*-DDP) in carcinoma treatment. Bismuth is also considered nowadays as the least toxic heavy metal and the bismuth(III) atom with a larger ionic radius has one inert electron pair ($6s^2$) and forms the complexes of the non-transition metal center with higher coordination numbers. Importantly, comparatively few structural studies of bismuth(III) complexes containing thiosemicarbazone are published,[14] which makes their structural characterization interesting and meaningful. On the other hand, the zinc(II) complexes are important. Intracellular distribution of several zinc(II) complexes have been tracked in different cancer cell lines.[15] In particular, zinc(II) complexes are well-known for their significant pharmaceutical properties[16] and in most cases were found to be generally more active than their free ligands.[17]

Stimulated by above-mentioned encouraging and promising results, it seemed useful and desirable to us to initiate systematic investigation of 2-acetylpyrazine N(4)-substituted thiosemicarbazones and their bismuth(III) and zinc(II) complexes. Particularly, information on the mechanism of these compounds is important and valuable. In the present work, with the main aim of

* Corresponding authors. Tel./fax: +86 378 2853650.
E-mail addresses: limingxue@henu.edu.cn (M.-X. Li), jyniu@henu.edu.cn (J.-Y. Niu).

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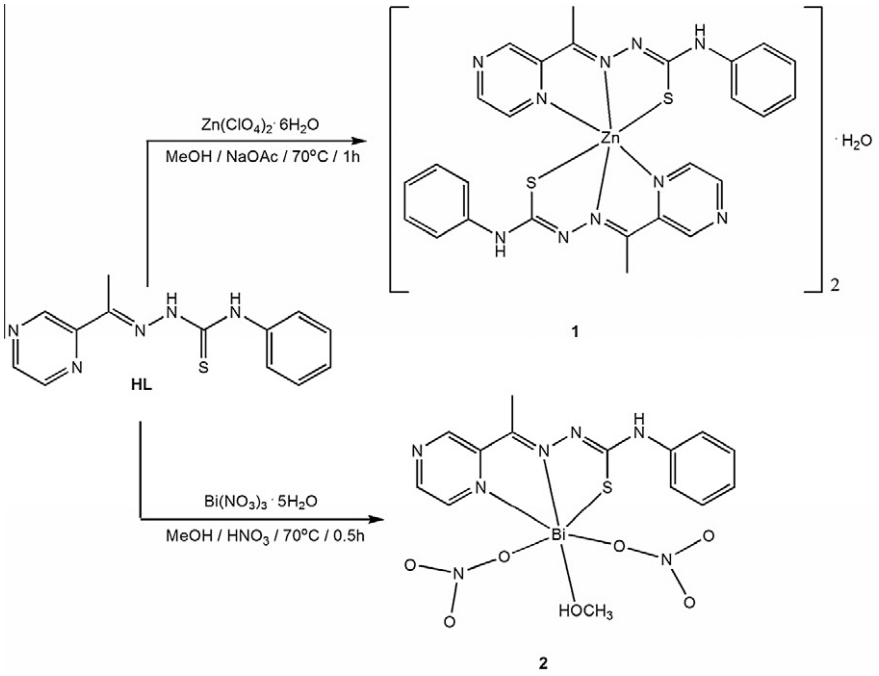

Scheme 1. The reaction scheme for the synthesis of **1** and **2**.

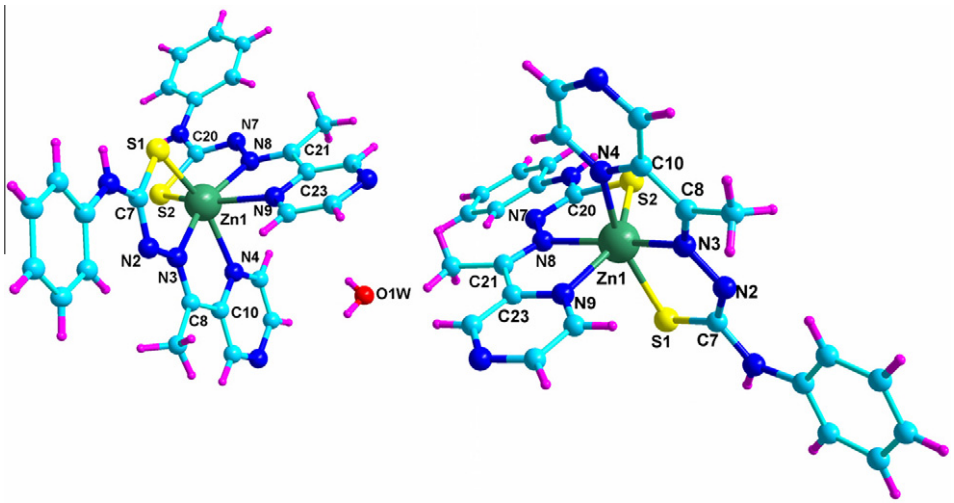

Figure 1. The molecular structure of complex **1** with atomic numbering scheme.

comparison, we have tested the biological activity of 2-acetylpyrazine *N*(4)-phenylthiosemicarbazone and its metal complexes formulated as $[Zn(L)_2]_2{\cdot}H_2O$ (**1**) and $[Bi(L)(NO_3)_2(CH_3OH)]$ (**2**) (Scheme 1). It is worth noting that cytotoxic agents can induce cell death through various pathways that include necrosis and apoptosis.[18] 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 free ligand and its zinc(II) complex **1** with excellent activity on mitochondria membrane potential (MMP) and PI-associated fluorescence intensity in K562 leukemia cell lines are performed. In addition, we also describe X-ray single crystal structures of **1** and **2** herein.

2-Acetylpyrazine *N*(4)-phenylthiosemicarbazone was prepared as described previously,[9e] whereas complexes **1** and **2** were synthesized by reacting 2-acetylpyrazine *N*(4)-phenylthiosemicarbazone and $Zn(ClO_4)_2{\cdot}6H_2O$ (2:1 ligand–metal molar ratio) and $Bi(NO_3)_3{\cdot}5H_2O$ (1:1 ligand–metal molar ratio) in methanol, respectively.[19]

X-Ray analysis shows that the zinc(II) ion in complex $[Zn(L)_2]_2{\cdot}H_2O$ (**1**) is in a slightly distorted octahedral environment with the monoanionic ligand bound through the pyrazine nitrogen, azomethine nitrogen and sulfur atoms (Fig. 1).[20] 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, whereas the axial positions are occupied by one sulfur atom and one pyrazine nitrogen atom from the other ligand. The pseudo-macrocyclic coordination mode of each ligand affords two five-membered chelate rings which is the typical coordination mode for α-N-heterocyclic N_2S tridentate thiosemicarbazones. The C(7)–S(1) bond length of 1.722(3) Å is within the normal range of C–S single bonds, indicating that the thiosemicarbazone moieties adopt the thiol tautomeric form.[9e]

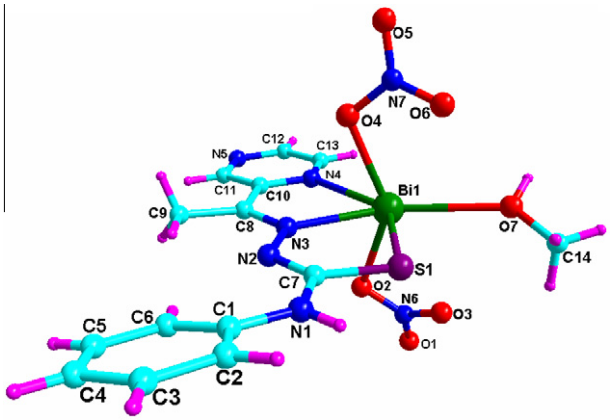

Figure 2. The molecular structure of complex **2** with atomic numbering scheme.

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.

As shown in Figure 2,[21] the molecular structure of **2** is depicted as a monomeric, 7-coordinate distorted pentagonal bipyramidal geometry with the pentagonal plane defined by the tridentate N_2S thiosemicarbazone, one electron pair ($6s^2$) of the bismuth(III) atom and the monodentate methanol, whereas the axial positions are occupied by two monodentate NO_3^- ions. The tridentate deprotonated thiosemicarbazone ligand coordinates to the bismuth atom with its imine nitrogen, thiol sulfur and pyrazine nitrogen atoms, forming two five membered chelate rings, which are near planar, the dihedral angle between the chelate rings is 0.5°. The measured C(7)–S(1) bond distances of 1.774(7) Å is within the normal range of a C–S single bond, indicating that the ligand adopted a *thiol* tautomeric form and acted as a mononegative ligand.[22] The C(7)–N(2) and N(2)–N(3) bond distances of 1.277(8) Å and 1.372(3) Å, respectively, were intermediate between formal single and double bonds, pointing to the extensive electron delocalization over the entire molecular skeleton. The O(4) atom was coordinated to the bismuth(III) atom from one side of the plane formed by S(1), N(3), N(4), O(7) and an electron pair which occupied one corner of the pentagonal (basal) plane (the sum of angles N(4)–Bi(1)–N(3), N(3)–Bi(1)–S(1), S(1)–Bi(1)–O(7) and N(4)–Bi(1)–O(7) is 359.9°), while the O(2) atom was coordinated from the opposite side. The bond distances around the bismuth(III) atom of **2** (Bi(1)–S(1) 2.564(8), Bi(1)–N(4) 2.624(6), Bi(1)–N(3) 2.428(6), Bi(1)–O(2) 2.504(6), Bi(1)–O(4) 2.389(5), Bi(1)–O(7) 2.752(5)) are compared with other data in related bismuth(III) complexes.[14b] The longer Bi–N bonds and Bi–S bond are resulting from the larger ionic radius of Bi^{3+} compared to that of Mn^{2+}, Zn^{2+} and Ni^{2+}, respectively.[9b]

Complex **2** is stabilized by intermolecular hydrogen bonds (see Fig. 3). The hydrogen bonds involving the terminal nitrogen N(1) atom, the uncoordinated nitrogen N(5) atom of the pyrazine ring and the oxygen atoms O(1), O(4) and O(5) of the nitrate ions and the oxygen atom O(7) of the methanol. The terminal N(1) hydrogen atom participates in forming two hydrogen bonds. One is with oxygen atom O(4) of nitrate ion interaction (N(1)···O(4) 3.020(8) Å) (symmetry code: x, $y-1$, z) and the other with the oxygen atom O(5) of nitrate ion interaction (N(1)···O(5) 3.297(9) Å) (symmetry code: x, $y-1$, z). The O(7) atom on the methanol also participates in forming two hydrogen bonds. One is with oxygen atom O(1) of nitrate ion interaction (O(7)···O(1)2.992(8) Å) (symmetry code: x, $-y+3/2$, $-z+1/2$) and the other with the uncoordinated nitrogen N(5) atom of the pyrazine ring (O(7)···N(5) 2.876(8) Å) (symmetry code: $-x+1$, $y+1/2$, $-z+1/2$) interaction.

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) bands of the ligand and its complexes **1** and **2** are found at 1587, 1534 and 1551 cm^{-1}, respectively. The decrease in frequency of this band in the spectra of

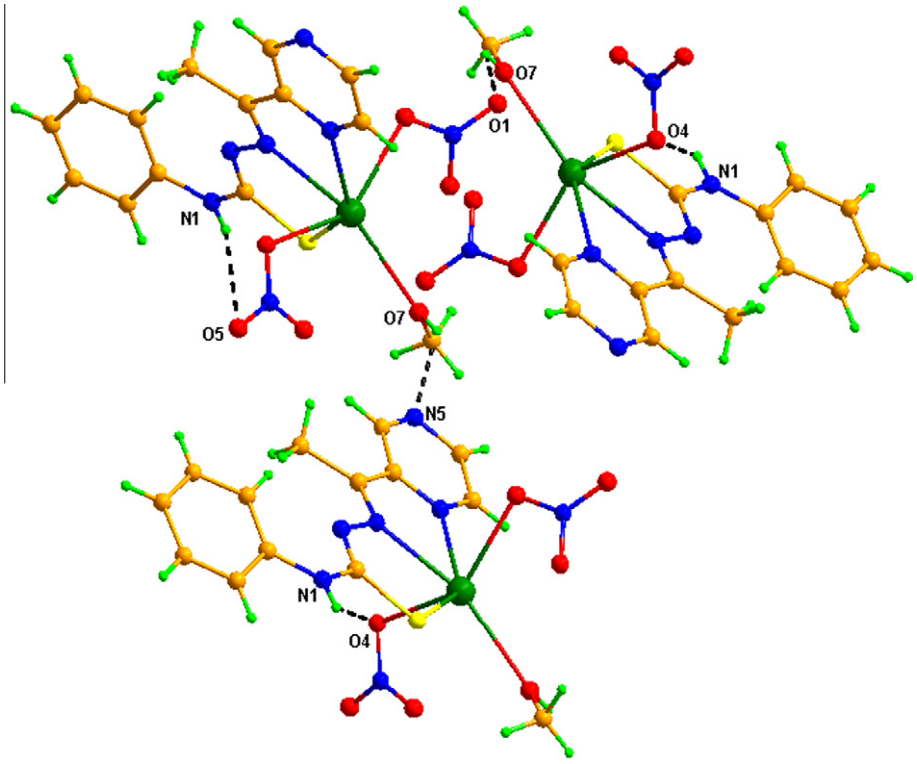

Figure 3. Hydrogen bond indicated by dashed lines in complex **2**.

Table 1
Minimal inhibitory concentration (μg/mL) of the tested compounds

Microorganism	MIC(μg/mL)							
	HL	**1**	**2**	$Zn(ClO_4)_2{\cdot}6H_2O$	$Bi(NO_3)_3{\cdot}5H_2O$	**A**mp	Cm	Kan
B. subtilis	15.62	7.81	31.25	125	1000	0.24	15.62	15.62
S. aureus	31.25	500	31.25	–[a]	1000	0.48	62.5	7.81
B. cereus	125	–	15.62	–	500	–	31.25	62.5
S. lutea	7.81	500	31.25	–	500	0.48	15.62	125
P. aeruginosa	3.90	62.5	15.62	–	250	1.95	31.25	3.90
S. typhimurium	15.62	–	15.62	–	500	125	31.25	31.25
E. coli	–	–	–	–	500	250	31.25	31.25
A. tumefaciens	62.50	–	62.5	500	500	0.48	15.62	3.90

[a] No inhibition or MIC > 1000 μg/mL.

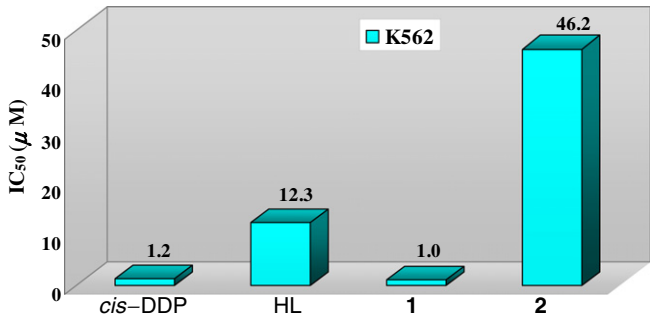

Figure 4. The anticancer activities of *cis*-DDP, HL, **1** and **2** against K562 leukemia cell lines.

the complexes is an evidence for the coordination via the azomethine nitrogen atom.[23] The increase in the frequency of *ν*(N–N) band of the thiosemicarbazone in the spectra of its metal complexes is due to the increase in the bond strength, again confirms the coordination via the azomethine nitrogen. In the free ligand, a band at 801 cm^{-1} is assigned to *ν*(C=S), whereas in the complexes **1** and **2**, this band is found to be shifted to lower frequency (786 cm^{-1} for **1**, 749 cm^{-1} for **2**), indicating the coordination of sulfur to the metal ions center. These observations have also been confirmed by X-ray single crystal structure analysis.

In view of the antimicrobial activity of thiosemicarbazones,[24,25] we have tested the inhibition ability of the obtained compounds as well as the starting compounds $Zn(ClO_4)_2{\cdot}6H_2O$ and $Bi(NO_3)_3{\cdot}5H_2O$ against the selected four Gram positive bacteria and four Gram negative bacteria by the disc diffusion method. Based on the minimum inhibitory concentrations (Table 1), generally, both the free ligand and **2** exhibit broad and effective activities against the tested microorganisms. However, both $Bi(NO_3)_3{\cdot}5H_2O$ alone and $Zn(ClO_4)_2{\cdot}6H_2O$ alone are inactive and complex **1** only showed excellent activity against *Bacillus subtilis* (MIC = 7.81 μg/mL) under same experimental conditions. Importantly, the remarkable antibacterial activity is observed for the free ligand against Gram positive bacteria *Sarcina lutea* (MIC = 7.81 μg/mL) and Gram negative bacteria *Pseudomonas aeruginosa* (MIC = 3.9 μg/mL), respectively. It should be emphasized that the bismuth(III) complex **2** show higher activities against Gram positive bacteria *Bacillus cereus* and Gram negative bacteria *Salmonella typhimurium* than positive control antibiotics ampicillin (Amp), chloramphenicol (Cm), kanamycin sulfate (Kan), respectively. We concluded that the structure factors which govern antimicrobial activities are strongly dependent on the central metal ion. These gratifying results are encouraging further screening in vitro and/or in vivo and evaluation of mechanism.

In terms of the cytotoxicity of thiosemicarbazones,[26] we have tested the ability of the obtained compounds to inhibit tumor cell growth against the K562 human leukemia cell lines.[27] 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 2-acetylpyrazine *N*(4)-phenylthiosemicarbazone and its metal complexes **1** and **2** exhibit IC_{50} values in the low micromolar range and show significant anticancer activity against K562 leukemia cell lines (Fig. 4), due to the NNS tridentate system.[28] It is worth mentioning that the zinc(II) complex **1** with the lower IC_{50} value (1.0 μM) indicated slightly stronger growth inhibition activity than cisplatin and also shows enhanced in vitro anticancer activity in comparison with its free ligand (12.3 μM). Therefore, complex **1** merits further investigation. On the other hand, the bismuth(III) complex with IC_{50} value (46.2 μM) revealed a decrease in activity, which indicate that the cytotoxicity of thiosemicarbazones can be enhanced/decreased by coordinating the ligand to metal cation, in a similar way to that observed other compounds.[29,30]

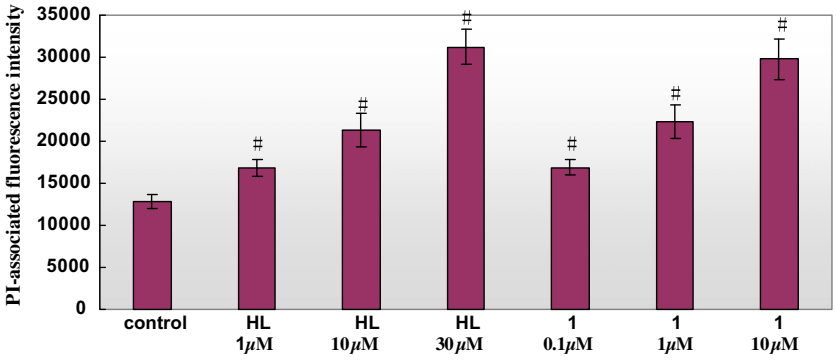

Figure 5. Effect of HL and **1** on PI-associated fluorescence intensity in K562 leukemia lines. Each value represents the mean ± S.D. from four experiments. $^{\#}p$ <0.05 versus control.

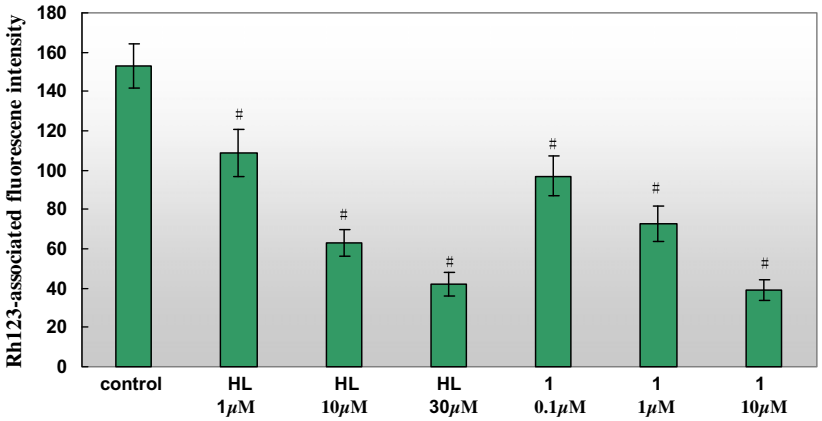

Figure 6. Effect of HL and **1** on MMP in K562 leukemia cell lines. Each value represents the mean ± S.D. from four experiments. $^{\#}p$ <0.05 versus control.

Mitochondria was considered to be a major site of antitumor agents through electron leakage from the electron transport chain,[31,32] the decreased mitochondria membrane potential (MMP) may open the mitochondrial permeability transition (MPT) and trigger the release of cytochrome c which activate caspase cascade, causing the cell death. Due to the excellent biological activities of 2-acetylpyrazine *N*(4)-phenylthiosemicarbazone and its zinc(II) complex **1**, in the present report, rhodamine 123 (Rh123), a mitochondrial specific stain that is dependent on the transmembrane potential, and another one, Propidine iodide (PI)[33] were applied for the determination of MMP[34] and apoptosis cells as well as necrosis cells, respectively. As shown in Figures 5 and 6, the PI-associated fluorescence intensity in K562 leukemia cells was 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 complex **1** exhibited more potent effects than its parent ligand, which was consistent with their actions in MTT assay.

In summary, the zinc(II) and bismuth(III) metal complexes derived from 2-acetylpyrazine *N*(4)-phenylthiosemicarbazone were synthesized and structurally characterized. Biological studies, carried out in vitro against selected bacteria and K562 leukemia cell lines, respectively, have shown that the free ligand and its complexes may be endowed with important biological properties, especially HL with MIC = 3.90 μg/mL against *Pseudomonas aeruginosa*, the zinc(II) complex **1** with IC_{50} = 1.0 μM against K562 leukemia cell lines, respectively. The compounds HL and **1** may exert their cytotoxicity via induced loss of MMP. These promising results will be essential for antibacterial and anticancer drug discovery and medical practice.

Supplementary data

CCDC 806018 and 827307 contain the supplementary crystallographic data for complexes **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.

Acknowledgment

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19. *Synthesis of* $[Zn(L)_2]_2{\cdot}H_2O$ (**1**): A methanol solution (10 mL) containing $Zn(ClO_4)_2{\cdot}6H_2O$ (0.074 g, 0.2 mmol) was added dropwise to a methanol solution (20 mL) of 2-acetylpyrazine *N*(4)-phenyl thiosemicarbazone (0.108 g, 0.4 mmol) and NaOAc (0.032 g, 0.4 mmol). The solution immediately turned yellow. After stirring for 1 h, the resultant solution was filtered. Products separated were recrystallised from hot methanol and dried over P_4O_{10} in vacuo. Yield: 60% –C_{52} H_{50} N_{20} O S_4 Zn_2: Calcd C 50.73, H 4.06, N 22.76; Found C 50.58, H 4.12, N 22.53. ESI-MS (*m*/*z*): 940.1 $[Zn_2(L)_3]^+$. ^{1}H NMR(400 MHz, $CDCl_3$,

δppm): 8.99 (s, 1H, NH), 8.54 (s, 1H, Pz), 7.96 (s, 1H, Pz), 7.68 (s, 1H, Pz), 7.44–7.34 (m, 2H, Ph), 7.28 (s, 2H, Ph), 7.10–7.06 (m, 1H, Ph), 2.81(s, 3H, CH_3). Yellow crystals suitable for X-ray studies were obtained by slow evaporation of its methanol solution.

Synthesis of [Bi(L)(NO$_3$)$_2$(CH$_3$OH)] (**2**): $Bi(NO_3)_3{\cdot}5H_2O$ (0.097 g, 0.2 mmol) solution dissolved in methanol with the help of a few drops of nitrate acid was added dropwise to a methanol solution (20 mL) of 2-acetylpyrazine *N*(4)-phenyl thiosemicarbazone (0.054 g, 0.2 mmol). After refluxing with stirring for 0.5 h, the resultant solution was filtered. This crude product obtained was further recrystallized from methanol and dried over P_4O_{10} in vacuo. Yield: 78% –C_{14} H_{16} BiN_7 SO_7: Calcd C 26.46, H 2.54, N 15.43; Found C 26.25, H 2.46, N 15.52; ESI-MS (*m*/*z*): 512.1 $[Bi(L)(CH_3O)]^+$. ^{1}H NMR(400 MHz, DMSO-d_6, δppm): 10.21 (s, 1H, NH), 9.80 (s, 1H, Pz), 9.51 (s, 1H, Pz), 9.49 (s, 1H, Pz), 7.73–7.62 (m, 2H, Ph), 7.40–7.30 (m, 2H, Ph), 7.09–7.00 (m, 1H, Ph), 2.76(s, 3H, CH_3). Brown crystals suitable for X-ray studies were obtained by slow evaporation of its methanol solution.

20. *Crystal data for [Zn(L)$_2$]$_2$·H$_2$O* (**1**): M = 1230.10, monoclinic, space group P2(1)/c, a = 13.3333(9) Å, b = 13.7659(10) Å, c = 16.3200(12) Å, α = 90.00(1)°, β = 103.6120(10), γ = 90.00(1)°, V = 2911.3(4) Å^3, Z = 2, D_c = 1.403 Mg°m^{-3}, μ = 1.024 mm^{-1}, F(000) = 1268, T = 296(2) K. A crystal with approximate dimensions of 0.26 × 0.23 × 0.20 mm^3 was mounted on a glass fiber in a random orientation. Crystallographic data were collected with a Siemens Smart-CCD diffractometer with graphite-monochromated MoK_α radiation (λ = 0.71073 Å). A total of 14597 reflections was measured by ω scan technique at 296(2) K within $1.96 \leqslant \theta \leqslant 25.00°$, of which 3535 were independent with R_{int} = 0.0406, and 5110 were observed with $I \geqslant 2\sigma$ (I). The structure was solved by Direct Methods and refined by full-matrix least-squares on F^2 with anisotropic displacement parameters for all non-hydrogen atoms using Shelxtl-97 program package. The hydrogen atoms were added in idealized geometrical positions. Final R indices [$I \geqslant 2\sigma$ (I)]: R_1 = 0.0888, wR_2 = 0.2037.
21. *Crystal data for [Bi(L)(NO$_3$)$_2$(CH$_3$OH)]* (**2**): M = 635.38, monoclinic, space group P2(1)/c, a = 16.533(2) Å, b = 7.4178(10) Å, c = 17.363(2) Å, α = 90.00(1)°, β = 111.462(2), γ = 90.00(1)°, V = 1981.7(5) Å^3, Z = 4, D_c = 2.130 Mg°m^{-3}, μ = 9.056 mm^{-1}, F(000) = 1216, T = 296(2) K. A crystal with approximate dimensions of 0.23 × 0.18 × 0.13 mm^3 was mounted on a glass fiber in a random orientation. Crystallographic data were collected with a Siemens Smart-CCD diffractometer with graphite-monochromated MoK_α radiation (λ = 0.71073 Å). A total of 9785 reflections was measured by ω scan technique at 296(2) K within $2.38 \leqslant \theta \leqslant 25.00°$, of which 2495 were independent with R_{int} = 0.0757, and 3501 were observed with $I \geqslant 2\sigma$ (I). The structure was solved by Direct Methods and refined by full-matrix least-squares on F^2 with anisotropic displacement parameters for all non-hydrogen atoms using Shelxtl-97 program package. The hydrogen atoms were added in idealized geometrical positions. Final R indices [$I \geqslant 2\sigma$ (I)]: R_1 = 0.0332, wR_2 = 0.0618.
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27. *3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was carried out to evaluate cytotoxicity in K562 leukemia cell lines*: Cells were placed into 96-well plates at a density of 1×10^4 cells per well and allowed to grow in a CO_2 incubator. After 24 h, the medium was removed and replaced by fresh medium containing the tested compounds which were dissolved in DMSO at 0.01 M and diluted to various concentrations with phosphate-buffered saline (PBS) before the experiment. The final concentration of DMSO is lower than 1%. After 24 h incubation, cultures were incubated in 100 μL of medium with 10 μL of 5 mg/mL MTT solution for 4 h at 37 °C. The medium with MTT was removed, and 100 μL of DMSO was added to each well to dissolve the formazan. The absorbance at 570 nm was measured with a microplate reader (Bio-Tek ELX800, USA). The inhibitory percentage of each compound at various concentrations was calculated, and the IC_{50} value was determined.
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33. 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).
Statistical analysis: All the data were expressed as mean ± S.D. and analyzed using analysis of variance (ANOVA) followed by Student's *t*-test. Differences were considered statistically significant at p <0.05.
34. 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 variance (ANOVA) followed by Student's *t*-test. Differences were considered statistically significant at p <0.05.