Pergamon

Bioorganic & Medicinal Chemistry 10 (2002) 887–895

BIOORGANIC & MEDICINAL CHEMISTRY

Synthesis, Characterization and Antitumor Studies of Mn(II), Fe(III), Co(II), Ni(II), Cu(II) and Zn(II) Complexes of *N*-Salicyloyl-*N*′-*o*-hydroxythiobenzhydrazide

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Received 18 June 2001; accepted 17 September 2001

Abstract—A new ligand *N*-salicyloyl-*N*′-*o*-hydroxythiobenzhydrazide (H_2Sotbh) forms complexes [Mn(HSotbh)$_2$], [Fe(Sotbh-H)(H_2O)$_2$], [M(Sotbh)] [M = Co(II), Cu(II) and Zn(II)] and [Ni(Sotbh)(H_2O)$_2$], which were characterized by various physico-chemical techniques. Mössbauer spectrum of [Fe(Sotbh-H)(H_2O)$_2$] reveals the quantum admixture of 5/2 and 3/2 spin-states. Mn(II), Cu(II) and Ni(II) complexes were observed to inhibit the growth of tumor in vitro, whereas, Fe(III), Co(II), Zn(II) complexes did not. In vivo administration of Mn(II), Cu(II) and Ni(II) resulted in prolongation of survival of tumor bearing mice. Tumor bearing mice administered with Mn(II), Cu(II) and Ni(II) complexes showed reversal of tumor growth associated induction of apoptosis in lymphocytes. The paper discusses the possible mechanisms and therapeutic implication of the H_2Sotbh and its metal complexes in tumor regression and tumor growth associated immunosuppression.

Introduction

One of the major applications of the transition metal complexes has been the testing of their antibacterial and antititumor activities with an aim to find an effective and safe therapeutic regimen for the treatment of cancer and infections. The coordination chemistry of nitrogen–sulfur donor ligands such as substituted thiosemicarbazide,[1] thiosemicarbazones[2–5] and dithiocarbazates[6] is well documented in literature. A few papers have appeared on the synthetic and structural aspects of the 3d-metal complexes of thiohydrazides.[7–11] In addition to the antifungal[12] and antibacterial[13] properties, some of the transition metal complexes of nitrogen–sulfur donor ligands also exhibit antitumor activity against a variety of tumor cells.[14,15] Thiosemicarbazones such as 3-ethoxy-2-oxobutyraldehyde bis(thiosemicarbazonato) copper(II) complexes (CuKTS) have been found to exhibit antitumor activity[16,17] due to their binding with DNA.[18] Thiosemicarbazones of 1-formylisoquinoline and 2-formyl pyridine and their derivatives were also demonstrated to be effective against animal tumor[19,20] by inhibiting ribonucleoside diphosphate reductase activity and synthesis of DNA.[21,22] We have reported that Mn(II), Fe(III), Ni(II), Cu(II) and Zn(II) complexes of *N*-aroyl-*N*′-thiohydrazide have antitumor activity against different tumor cells.[23–25] Many chemotherapeutic agents have also been reported to possess immunomodulatory properties.[26–28] However, the immunomodulatory potential of the metal complexes with antitumor activity has not been explored adequately. Although, thiohydrazides are structurally quite similar to thiosemicarbazides, scarcity of work on the antineoplastic and immunomodulatory activity of transition metal complexes of thiohydrazides prompted us to synthesize, characterize and study the antitumor activity of transition metal complexes of newly synthesized ligand *N*-salicyloyl-*N*′-o-hydroxythiobenzhydrazide. Further, we also investigated the immunomodulatory action of these complexes in a tumor bearing host.

Results and Discussion

The analytical data correspond to the 1:1 metal to ligand stoichiometry, except for Mn(II) where it is 1:2, and the complexes are colored. These complexes were formed by loss of two or three protons from the ligand. The complexes are soluble in polar coordinating solvent, DMSO, and melt above 300 °C, except [Cu(Sotbh)] which melts at 223 °C. The composition of

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0968-0896/02/$ - see front matter
PII: S0968-0896(01)00373-X

Table 1. Analytical and Physical Properties of H_2Sotbh and its Complexes

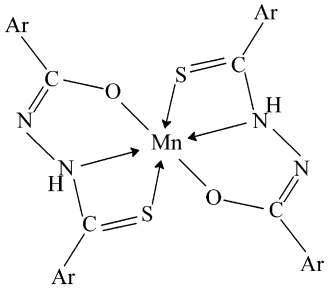
Compound	Colour	m.p. (°C)	Yield (%)	Found (calcd) %					μ_{eff} B.M.
				M	S	C	H	N	
H_2Sotbh	White	220	61	–	11.4 (11.1)	58.7 (58.3)	3.9 (4.1)	9.5 (9.7)	–
[Mn(Sotbh)$_2$]	Greenish Black	>300	54	8.9 (8.7)	10.5 (10.1)	53.6 (53.4)	3.1 (3.4)	9.1 (8.9)	5.9
[Fe(Sotbh-H)(H_2O)$_2$]	Black	>300	66	14.4 (14.8)	8.9 (8.5)	44.2 (44.6)	3.5 (3.4)	7.5 (7.4)	4.7
[Co(Sotbh)]	Greenish Brown	>300	62	16.8 (17.1)	8.9 (9.2)	48.2 (48.7)	2.8 (2.9)	7.7 (8.1)	2.43
[Ni(Sotbh)(H_2O)$_2$]	Light Green	210[d]	64	15.1 (15.4)	8.9 (8.4)	44.3 (44.1)	3.2 (3.6)	7.6 (7.4)	2.83
[Cu(Sotbh)]	Blackish Brown	223	70	17.9 (18.2)	9.4 (9.1)	48.2 (48.1)	2.7 (2.9)	7.7 (8.0)	1.9
[Zn(Sotbh)]	Light Yellow	>300	71	19.1 (18.6)	8.8 (9.1)	47.7 (47.8)	2.4 (2.8)	8.1 (8.0)	Dia

[d] = decompose

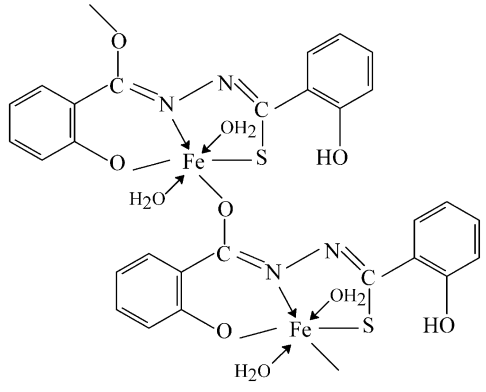
the complexes along with color, mp magnetic susceptibility and elemental analyses are given in Table 1. The proposed structure for the complexes is given in Figure 1.

Magnetic properties and electronic spectra

The magnetic moments of Mn(II) and Ni(II) complexes suggest their octahedral geometry which is further supported by the electronic spectral data. [Mn(HSotbh)$_2$] exhibits a μ_{eff} of 5.9 B.M. and shows two electronic bands at 15,430 and 22,320 cm^{-1} assigned to the $^6A_{1g} \rightarrow {}^4T_{1g}$ and $^6A_{1g} \rightarrow {}^4T_{2g}$ transitions, respectively, for high-spin, octahedral Mn(II). A magnetic moment of 2.83 B.M. for [Ni(Sotbh)(H_2O)$_2$] suggests an octahedral geometry around Ni(II). It displays bands at 16,780 and 25,510 cm^{-1} for the $^3A_2g \rightarrow {}^3T_{1g}$, $^3T_{1g}$(P) transitions, respectively, characteristic of an octahedral system. In addition two spin forbidden bands at 15,575 and 24,040 cm^{-1}, one due to the transition to ^1E_g (near $^3T_{1g}$, for common spin allowed transition systems with Dq/B near unity), and the second, primarily due to the transition $^1T_{2g}$ [between the $^3T_{1g}$ and $^3T_{1g}$(P) spin allowed transitions][29] were also observed. The bands at 26,880 and 28,250 cm^{-1} are assigned to the charge transfer and intraligand transitions, respectively.[30] A magnetic moment of 4.7 B.M. for [Fe(Sotbh-H)(H_2O)$_2$] suggests an intermediate spin state for iron(III) centre with a distorted octahedral geometry and the presence of a band at 19,840 cm^{-1} assigned to the $^6A_{1g} \rightarrow {}^4T_{2g}$ transition further supports the above geometry around Fe(III). The magnetic moments of 2.43 and 1.9 B.M. for [Co(Sotbh)] and [Cu(Sotbh)], respectively, suggest a square-planar geometry around Co(II) and Cu(II). [Cu(Sotbh)] displays a broad band at 17,480 cm^{-1} assigned to the envelope of $^2B_{1g} \rightarrow {}^2A_{1g}$, $^2B_{2g}$, ^2E_g transitions further supporting a square-planar geometry around Cu(II).[31] Very limited information is available on the electronic spectra of square-planar complexes of Co(II). Nishida and Kida[32] have reported that the electronic spectra of square-planar Co(II) complexes exhibit three bands at 4000, 7000 and 18000 cm^{-1} mainly due to the transitions $^2A_{1g} \rightarrow {}^2B_{2g}$, ^2E_g and $^2E'_{2g}$, respectively. [Co(Sotbh)] exhibits a band at 18,115 cm^{-1} assigned to the $^2A_{1g} \rightarrow {}^2E'_{2g}$ transition supporting square-planar geometry around Co(II). Other bands at 23,585 and 25,640 cm^{-1} have been attributed to the charge-transfer transitions.



[Mn(HSotbh)$_2$]



[Fe(Sotbh-H)(H_2O)]

Figure 1. Proposed structure of the complexes of H_2Sotbh.

IR spectra

The IR spectrum of H_2Sotbh shows bands at 3452 and 3171 cm^{-1} assigned to ν(OH) and ν(NH), respectively. The bands occurring at 1630, 1439, 1346 and 958 cm^{-1} are assigned to ν(C=O), thioamide I [β(NH) + ν(CN)], thioamide II [ν(CN) + β(NH)] and ν(C=S), respectively. A broad band at 3427 cm^{-1} observed in the spectrum of

$[Mn(HSotbh)_2]$ may be assigned to ν(OH) of the ligand. A new band appears at 1608 cm^{-1}, due to the ν(C=N) of NCO in place of ν(C=O), suggesting that one hydrazinic proton is lost via enolization and bonding of the resulting enolic oxygen takes place with the metal ion. The thioamide I and II bands at 1439 and 1346 cm^{-1} in the free ligand undergo a positive shift of 36 and 14 cm^{-1}, respectively, in the complex, suggesting the involvement of thione sulfur in bonding. Absence of ν(NH) and ν(OH) bands in the spectrum of $[Fe(Sotbh-H)(H_2O)_2]$ suggests the involvement of these group as bonding site, by loss of both the hydrazinic protons via enolization and thioenolization and one phenolic proton. Presence of new bands at 1619 and 850 cm^{-1} assigned to the ν(C=N) of NCO and ν(C–S) further supports the above observation. [Co(Sotbh)] displays a band at 3435 cm^{-1} assigned to ν(OH), ruling out the involvement of phenolic OH as bonding site. Absence of bands due to ν(NH), ν(C=O) and ν(C=S) and appearance of a new band at 1597 cm^{-1} due to the ν(C=N) of NCO suggests that both NH protons are lost via enolization and thioenolization. The bonding of the resulting enolic oxygen and thiolato sulfur takes place with the metal ion. The thioamide I and II bands at 1491 and 1370 cm^{-1} undergo a positive shift of 52 and 25 cm^{-1}, respectively, in the complex suggesting the involvement of these groups as bonding sites. $[Ni(Sotbh)(H_2O)_2]$ shows the presence of a band at 3404 cm^{-1}, assigned to ν(OH) and absence of ν(NH), ν(C=O) and ν(C=S) and appearance of new bands at 1606 and 860 cm^{-1} suggest that both the hydrazinic protons are lost via enolization and thioenolization and the bonding of the resulting enolic oxygen and the thiolato sulfur takes place with the metal ion. Thioamide I and II bands at 1489 and 1412 cm^{-1} undergo a positive shift of 50 and 66 cm^{-1}, respectively, thereby supporting the involvement of these groups as bonding sites.

The spectra of [Cu(Sotbh)] and [Zn(Sotbh)] display bands at 3450 and 3427 cm^{-1}, respectively, for ν(OH). There is significant change in the bands due to thioamide I, II and ν(C=S) as compared to the free ligand in the spectra of [Cu(Sotbh)] and [Zn(Sotbh)]. The presence of new bands at 1597 cm^{-1} for ν(C=N) of NCO in place of ν(C=O) and at 840 cm^{-1} for ν(C–S), suggest bonding through enolic oxygen and thiolato sulfur to the metal ion.

NMR spectra

The ^1H NMR spectrum of H_2Sotbh exhibits signals at δ13.0 and 12.2 ppm for the OH protons and at δ 5.6–4.0 (br) ppm for the NH protons, which disappear on D_2O exchange. The protons due to the two aromatic rings appear as multiplets between δ 8.66 and 7.0 ppm. The diamagnetic [Zn(Sotbh)] shows a broad signal at δ 12.6–11.86 ppm for both OH protons and a multiplet between δ 8.4 and 6.6 ppm for the aromatic ring protons. Absence of NH signals further support bonding through the enolic oxygen and thiolato sulfur via enolization and thioenolization. The low solubility and paramagnetic nature of other metal complexes precluded the recording of their NMR spectra.

Mössbauer spectra

The Mössbauer spectra (Fig. 2a and b) of $[Fe(Sotbh-H)(H_2O)_2]$ at room and liquid nitrogen temperatures were studied. The Mössbauer spectra of the complex show three lines at both the temperatures. The two sites observed at room and liquid nitrogen temperature reveals the spin-cross over phenomenon exhibited by Fe(III) complex. The Mössbauer parameters at room temperature are isomer shift, $\delta = 0.35$ mm s^{-1} and quadrupole splitting, $\Delta = 0.48$ mm s^{-1} for site 1 and the corresponding values for site 2 are $\delta = 0.47$ mm s^{-1} and $\Delta = 1.29$ mm s^{-1}. The MB parameters for site 1 are characteristic of high-spin state $S = 5/2$ and for site 2 are characteristics of low spin state $S = 3/2$. They are present in 3:1 ratio. On lowering the temperature to 78 K, the corresponding values for site 1 are found to be $\delta = 0.51$ mm s^{-1} $\Delta = 0.51$ mm s^{-1}, whereas, for site 2, $\delta = 0.53$ mm s^{-1} $\Delta = 1.13$ mm s^{-1} in 1:1 ratio. These values suggest the quantum admixture of $S = 5/2$ and $S = 3/2$ spin states. They also follow the usual trend of high-spin Fe(III) ion going into more of low spin state on lowering the temperature as suggested by the data that both iron species are present in equal quantity at 78 K.

Antitumor studies

To investigate the antitumor activity of H_2Sotbh and its metal complexes their effect on the growth of Dalton's lymphoma (DL) cells in vitro was measured by MTT assay. It was observed that treatment of tumor cells with $[Mn(HSotbh)_2]$, [Cu(Sotbh)] and [Ni(Sotbh)

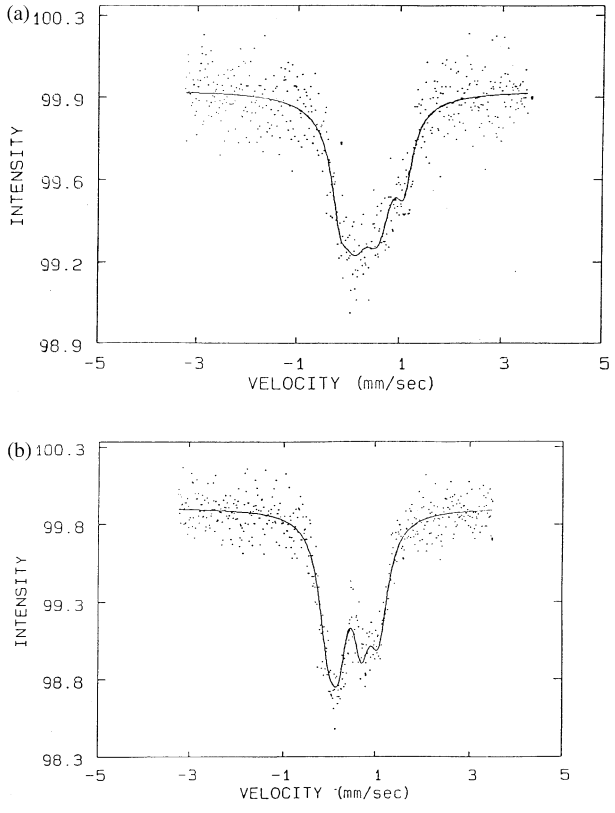


Figure 2. (a) Mössbauer spectrum of $[Fe(Sotbh-H)(H_2O_2]$ at room temperature; (b) Mössbauer spectrum of $[Fe(Sotbh-H)(H_2O)_2$ at 78 K.

$(H_2O)_2$] caused maximum growth inhibition followed by [Fe(Sotbh-H)$(H_2O)_2$] and the ligand (Table 2). The reason for the observed inhibition of tumor cell growth by the metal complexes is unclear however, several possibilities could be considered. The cytostatic activity of the metal complexes could be a direct result of the interaction of the metal complexes with DNA, thus interfering with the process of DNA replication. Indeed, DNA binding of metal complexes has been documented.[33] Furthermore, inhibition/activation of various enzymes directly/indirectly involved in DNA replication is not ruled out. Interaction of metal complexes with protein components of viable cells has been reported.[34,35] Binding of metal complexes with protein may cause alterations in the structural and functional organization of proteins. However, more studies will be needed to confirm the existence of one or more of such possibilities in our system. These results may also indicate a possible decline of the overall metabolic activity of the tumor cells with a concomitant inhibition of the activity of various enzymes involved in respiration.

Although the metal complexes showed cytostatic effects on the tumor cells in vitro, these results do not necessarily indicate if these cells are actually killed by the direct action of the metal complexes. To check this in the next part of the investigation we studied the effect of the metal complexes on the tumor cell killing with respect to the mode of cell death. Cisplatin has been reported to induce apoptotic cell death in the tumor cells.[36,37] It was observed that the tumor cells were killed by the induction of apoptosis (Fig. 3a). [Mn(HSotbh)$_2$] and [Cu(Sotbh)] were found to be most effective in the induction of tumor cell apoptosis. The mechanism of the induction of apoptosis remains poorly understood and is thought to be dependent on multiple mechanism(s) ultimately culminating in the activation of DNA cleaving endonucleases.[36,38] As shown in Figure 3b, [Mn(HSotbh)$_2$] and [Cu(Sotbh)] cause an increase in% specific DNA fragmentation, it is probable that these metal complexes may act via the activation of endonucleases to kill the tumor cells by inducing apoptotic cell death.

Furthermore, the metal complexes at the concentration checked did not inhibit the growth of normal spleenocyte and bone marrow cells (data not shown), which comprise a major proportion of proliferating cells indicate that the cytostatic effect of the metal complexes was limited to the tumor phenotype and not on all proliferating cells. The killing of tumor cells specifically could be attributed to the susceptibility of tumor cell DNA to a damage by metal complexes as compared to the normal cells. Indeed, in case of cisplatin, it has been shown that the tumor cells are killed specifically because they are unable to overcome the genetic load of mutations caused by the drug because of their defective DNA repair mechanism.[39]

Table 2. Effect of H_2Sotbh and its metal complexes on tumor cell growth in vitro (ID_{50} values in μg/mL)

Compounds	ID_{50}
H_2Sotbh	24.7
[Mn(Sotbh)$_2$]	0.92
[Ni(Sotbh)$(H_2O)_2$]	1.35
[Fe(Sotbh-H)$(H_2O)_2$]	15.6
[Cu(Sotbh)]	0.91
Cisplatin	0.71

ID50 = average drug concentration (μg/mL) for 50% inhibition of tumor cell growth. Values are mean ± SD of three experiments. $\rho < 0.05$ with respect to values of ID_{50} of H_2Sotbh alone.

In the next part of the investigation we checked the life prolonging effect in DL bearing mice administered with phosphate buffer saline (PBS) alone or containing ligand or the metal complexes as indicated in the Experimental. As shown in Table 3, minimal % T/C was observed in mice administered with ligand alone as compared to that of mice administered with metal complexes. Maximum% T/C was found for [Cu(Sotbh)] followed by that of [Mn(HSotbh)$_2$] and [Ni(Sotbh)$(H_2O)_2$]. Our observations show that H_2Sotbh, [Fe(Sotbh-H)$(H_2O)_2$] and [Zn(Sotbh)] did not have antitumor activity whereas, significantly higher life prolonging ability in tumor bearing mice were observed for [Mn(HSotbh)$_2$], [Cu(Sotbh)] and [Ni(Sotbh)$(H_2O)_2$]. Increase in the value of% T/C, an indicator of the effect of drug administration on the survival of tumor bearing mice, suggests that such effect could either result from the direct cytotoxic/cytostatic action of the complexes on tumor cells or due to the activation of certain host derived mechanism resulting in a decrease of tumor load.

Progression of growth of various tumors including DL is invariably associated with the onset of immunosuppression in tumor bearing host,[40–43] one of the reasons being induction of apoptosis and inhibition of proliferation of hematopoietic precursor cells.[44,45] Since in vivo administration of these metal complexes prolonged survival of tumor bearing animals and these metal complexes did not show cytotoxicity against normal cells in vitro, we were interested to investigate if the administration of metal complexes could reverse tumor growth associated induction of apoptosis in various hematopoietic cells. For this DL bearing mice were administered with metal complexes, and the% of apoptotic thymocyte, spleenocyte and bone marrow cells were enumerated. As shown in Figure 4a, administration of metal complexes in tumor bearing mice resulted in the inhibition of tumor associated apoptosis of thymocyte, splenocyte and bone marrow cells. Similar results were obtained for % DNA fragmentation as well (Fig. 4b). The reversal of tumor growth associated induction of apoptosis of hematopoietic cells by metal complexes is predicted to be due to two reasons: (1) reduction of tumor load resulting due to the cytotoxic effect of metal complexes on tumor cells, leading to a decrease in the tumor associated concentration of apoptotic factors; (2) direct protective effect of metal complexes on the hematopoietic cells. Although not very clear, the probability of the latter could be due to the fact that metal complexes can bind to DNA and several proteins in cells, which could result in the protective effect.

Although more investigations will be required to confirm the mechanism of action of metal complexes on tumor and normal cells, the study suggests that $[Mn(HSotbh)_2]$ and [Cu(Sotbh)] can cause prolongation of survival in tumor bearing animals by:

1. directly killing tumor cells;
2. reversing tumor associated immunosuppression.

The finding of this investigation may have long lasting clinical implication with the novel proposition that metal complexes of *N*-salicyloyl-*N*′-*o*-hydroxythiobenzhydrazide may have dual mechanism of action in tumor regression.

Experimental

Preparation of H_2Sotbh

Sodium salt of *o*-hydroxydithiobenzoate. A solution of *o*-hydroxybenzaldehyde (21 mL ~ 25 g) in 60 mL of ethanol was heated to 65 °C and 135 mL of the filtered ammonium polysulfide was added in 10 mL portions during 10 min keeping temperature at 65 °C. The reaction mixture was boiled to reflux for 1 h, immediately cooled in ice, 100 mL of ether was added and the solution was acidified with concd HCl. The dithioacid which separated as red oil, was filtered through suction to remove the precipitated sulfur. The filtrate was transferred into a separating funnel, 100 mL of ether was also added and the ethereal layer containing the dithioacid

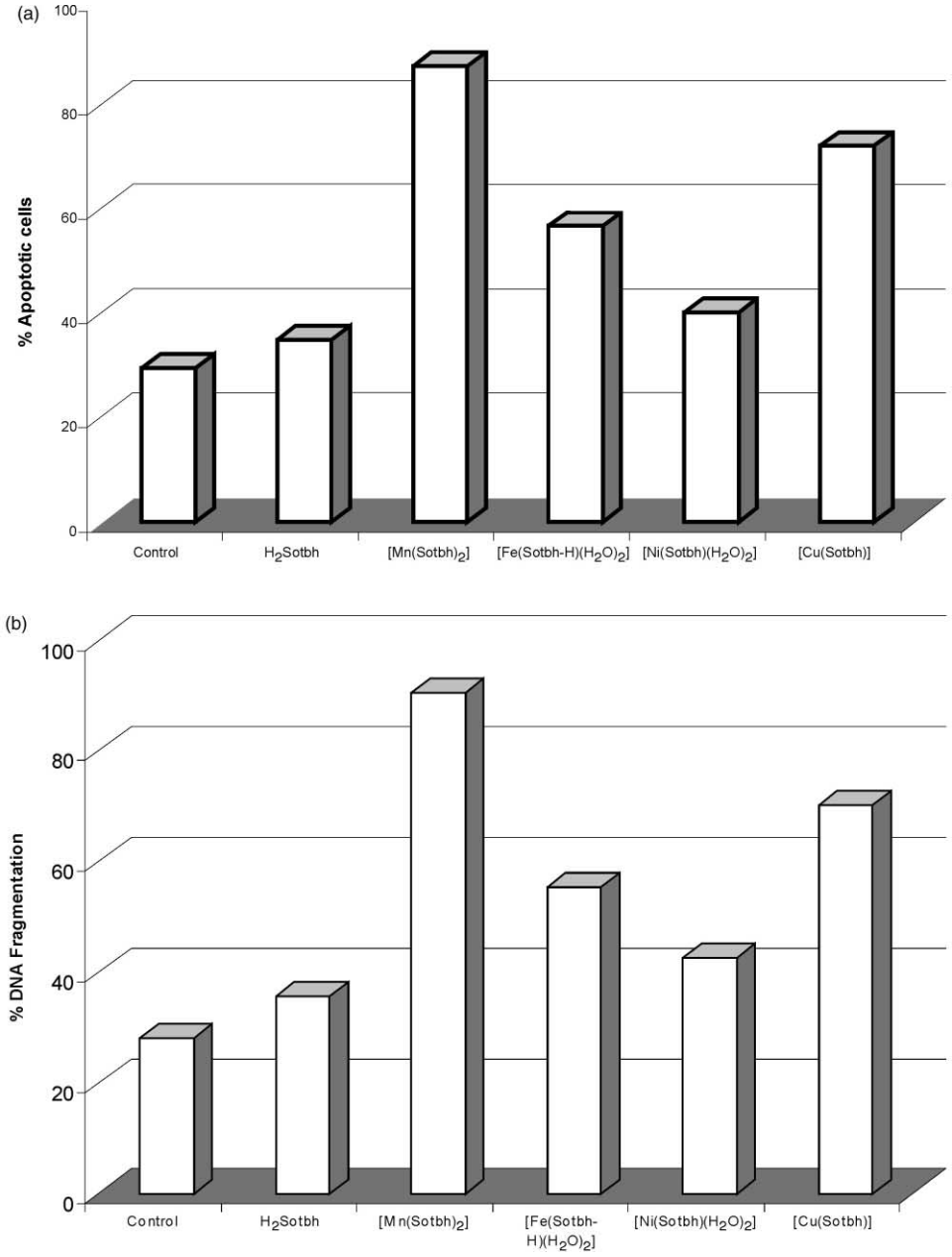


Figure 3. (a) Effect of H_2Sotbh and its metal complexes on the induction of apoptosis in tumor cells. DL cells were incubated in medium alone or containing H_2Sotbh or its metal complexes (10 μg/mL) for 24 h and the number of cell showing apoptotic morphology was enumerated. Values are mean of three experiments. (b) Effect of H_2Sotbh and its metal complexes on % DNA fragmentation of tumor cells. DL cells were incubated in medium alone or containing H_2Sotbh or its metal complexes (10 μg/mL) for 24 h and the % DNA fragmentation was evaluated. Values are mean of three experiments.

was separated and washed with distilled water. The red colored sodium salt of the dithioacid was extracted by shaking the ethereal solution of the dithioacid twice with aqueous NaOH solution (8 g in 100 mL).

Carboxymethyl-*o*-hydroxydithiobenzoate. To the sodium salt of *o*-hydroxydithiobenzoate was added a solution of chloroacetic acid (20 g) neutralized with sodium carbonate (pH of solution 7). After standing the reaction

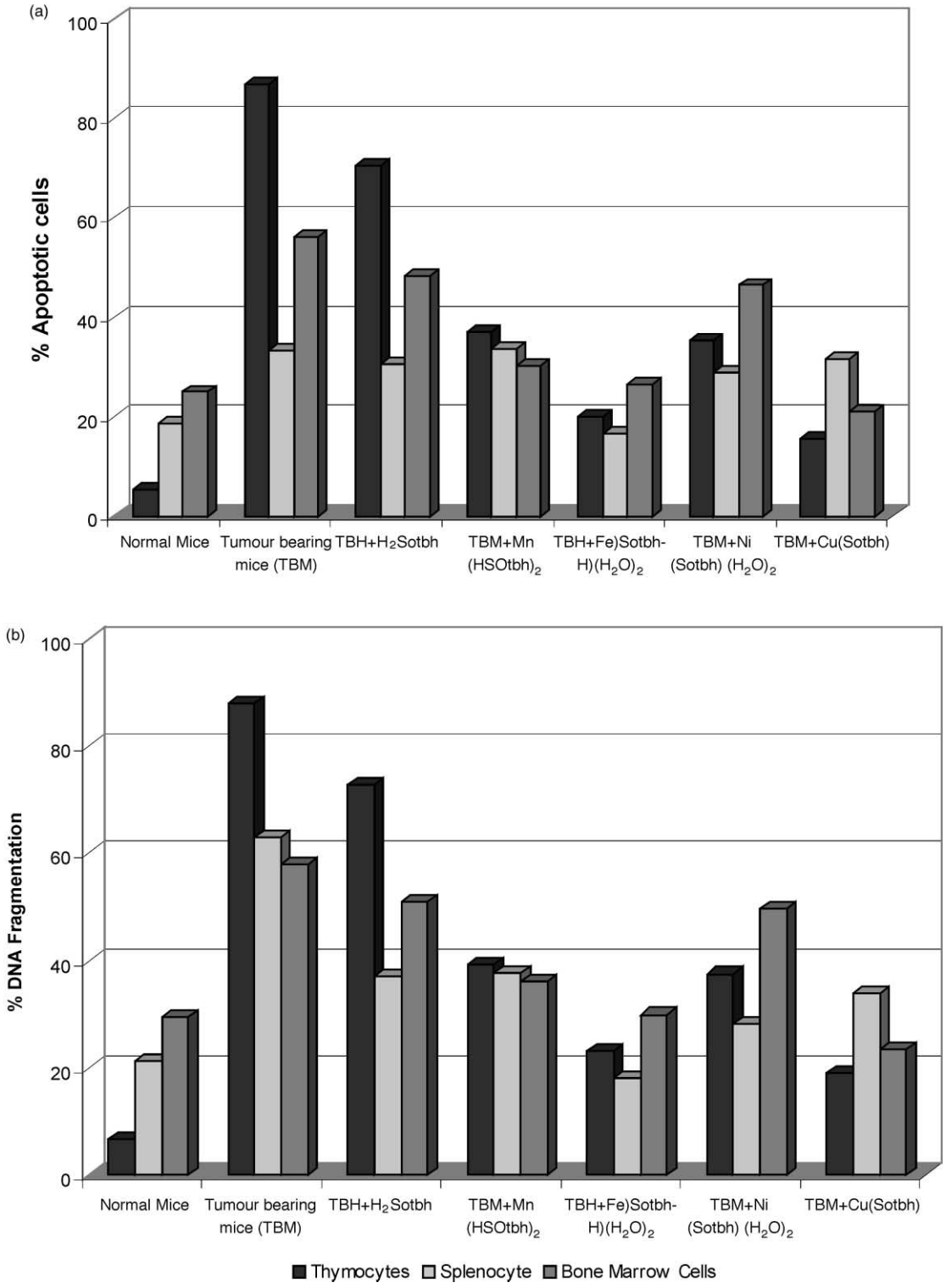


Figure 4. (a) Effect of in vivo administration of H_2Sotbh or its metal complexes on the induction of apoptosis in thymocyte, splenocyte and bone marrow cell of normal or tumor bearing mice treated with ligand or its metal complexes. Thymocytes, splenocytes and bone marrow cells of normal or tumor bearing mice or tumor bearing mice treated with ligand or its metal complexes were enumerated for the number of cell showing apoptotic morphology. Values are mean of three experiments. (b) Effect of in vivo administration of H_2Sotbh or its metal complexes on % DNA fragmentation of thymocyte, splenocyte and bone marrow cell of normal or tumor bearing mice treated with ligand or its metal complexes. Thymocytes, splenocytes and bone marrow cells of normal or tumor bearing mice or tumor bearing mice treated with ligand or its metal complexes and checked for % DNA fragmentation. Values are mean of three experiments.

mixture overnight at room temperature, the dark solution was acidified with concd HCl and the ester which separated on cooling was filtered off, washed with cold water and recrystallized from hot ethanol in the presence of animal charcoal, mp 120 °C.

N-Salicyloyl-*N*′-*o*-hydroxythiobenzhydrazide (H_2Sotbh) was prepared by mixing an equivalent quantity of carboxymethyl-*o*-hydroxydithiobenzoate and salicylic acid hydrazide, each dissolved separately in one equivalent of 1 N NaOH solution and adding acetic acid dropwise to the above ice cooled solution, after standing the solution for ~2 h. The product thus obtained was suction filtered, washed with water and recrystallized from ethanol which yielded white compound, mp 220 °C.

Metal complexes of H_2Sotbh. [Mn(HSotbh)$_2$], [Fe(Sotbh-H)(H_2O)$_2$], [M(Sotbh)] [M = Co(II), Cu(II) and Zn(II)], [Ni(Sotbh)(H_2O)$_2$] were prepared by adding a DMF solution (10 mL) of H_2Sotbh (1.2 g, 5.2 mmol) dropwise to the ethanolic solution (20 mL) of the respective metal(II) acetate or to an aqueous solution of $FeSO_4 \cdot 7H_20$ in an 1:1 molar ratio. Colored precipitate of the complexes started separating out after 5 min of stirring at room temperature but the reaction mixture was further stirred at room temperature for 2 h to ensure the completion of the reaction. The insoluble complexes thus obtained were filtered off, washed with ethanol and dried in vacuo. The products obtained were characterized by various physico-chemical methods.

Physical measurements

The complexes were analysed for their metal and sulfur contents as described elsewhere.[46] Carbon, hydrogen and nitrogen contents were estimated on a Perkin-Elmer 240C microanalyzer. The electronic spectra were recorded on a Cary-2390 UV–vis. spectrophotmeter in DMSO solutions and as Nujol mulls.[47] Room temperature magnetic susceptibility measurements were made on a Cahn Faraday electrobalance using cobalt mercury tetrathiocyanate as a calibrant and the experimental magnetic susceptibilities were corrected for diamagnetism by using the procedure of Figgis and Lewis.[48] The ^{1}H NMR spectra were obtained in DMSO-d_6 on JEOL FX-90Q spectrometer using TMS as internal reference.

Table 3. Effect of in vivo administration of H_2Sotbh and its metal complexes on the survival of tumor bearing mice

Compounds	Post inoculation life span (% *T*/*C*)
H_2Sotbh	148
[Mn(Sotbh)$_2$]	248
[Ni(Sotbh)(H_2O)$_2$]	209
[Fe(Sotbh-H)(H_2O)$_2$]	168
[Cu(Sotbh)]	267
Cisplatin	250

Treatment responses (six mice per treatment group) presented as % *T*/*C*, was calculated according to the equation: mean life span of treated mice/mean life of control mice by 100. A % $T/C \geq 125$ is considered biologically significant. $C = 20 \pm 2$ days, experiment terminated after 50 days. Values are mean ± SD of three experiments. $\rho < 0.05$ with respect to values of H_2Sotbh alone.

The Mössbauer spectra were collected using a Cryophysics MS-1 microprocessor controlled spectrometer operating in the constant acceleration mode.

Antitumor screening

Mice. Inbred populations of BALB/c mice of either sex of 8–12 weeks were used for the study. The mice were fed food and water ad libitum under pathogen-free conditions and were treated with utmost human care.

Tumor systems

DL (a spontaneous murine T cell lymphoma) were maintained in culture in vitro as well as in ascites by serial transplantation in BALB/c mice by an intraperitoneal injection of 5×10^5 cells/mouse.

Thymocyte preparation and culture

Thymuses obtained from normal and tumor bearing mice with or without administration of complexes were weighed on a chilled watch glass, diced on ice and passed through a stainless steel screen using a syringe plunger. These cells, after washing with phosphate buffered saline (PBS) by centrifugation at 200*g* for 10 min at 4 °C, were used directly for thymocyte counts. Cell viability in the thymocyte preparation was determined by mixing 10 mL sample with an equal volume of 0.4% trypan blue-PBS solution[49] and counting the cells on a hemocytometer under light microscope. Cells that did not excude trypan blue were considered nonviable. For culturing thymocytes in vitro, thymocytes were maintained in complete RPMI 1640 medium at 37 °C in humidified atmosphere of 5% CO_2 in air.

Splenocyte preparation and culture

Spleens obtained from normal and tumor bearing mice with or without administration of complexes were weighed on a chilled watch glass, diced on ice and passed through a stainless steel screen using a syringe plunger. These cells, after washing with phosphate buffered saline (PBS) by centrifugation at 200*g* for 10 min at 4 °C, RBC were depleted by treatment with 0.84% ammonium chloride for 10 min at room temperature. Cells were again washed in PBS and then cultured in a humidified atmosphere at 5% CO_2, to remove adherent cells. Non-adherent cells were collected and used for assay.

Bone marrow cell preparation and culture

Bone marrow cells (BMC) were obtained from the femurs of normal and tumor bearing mice with or without administration of complexes, as described elsewhere.[50] Briefly, the mice were killed by cervical dislocation and the BMC were obtained from the femoral shafts by flushing it with serum-free medium. BMC were then agitated gently to prepare a single cell suspension and then washed thrice with serum-free medium by centrifugation at 200*g* at 4 °C. BMC were then incubated in plastic tissue culture flask for 2 h at 37 °C to

remove the adherent macrophage. The non adherent BMC were then used for proliferation.

Proliferation assay

Different cells obtained from normal or tumor bearing mice treated with or without complexes were incubated at a concentration of 1.5×10^6 cells per well in a 96-well plastic tissue culture plate with medium containing submitogenic doses of concanavalian-A (1 μg/mL). Cultures were then incubated at 37 °C in CO_2 incubator for 48 h and assayed for proliferation and growth inhibition using MTT assay.

In vitro growth inhibitory assay

The MTT assay was used to measure the cytotoxic effect of the ligand and the complexes. The procedures employed the pale yellow tetrazolium salt [3-(4,5-dimethylthiazol)-2-yl-2,5-diphenyl-2H-tetrazolium bromide] (MTT), which was cleaved by active mitochondria to form a dark blue formazon product that can be completely solubilized in acidic isopropanol.[51] The assay provides a simple way to detect living and growing cells without use of radioactivity. Briefly, 5×10^4 tumor cells were plated in triplicate in 96-well flat bottom tissue culture plates, and treated with different concentrations of drugs for the time indicated. MTT (0.005 g cm^{-3} in PBS) was added to the cell culture and incubated for 4 h in a 37 °C, 5% CO_2 humidified incubator. The formazon crystals formed during the reaction were dissolved in 100 mL of 0.04 N HCl in isopropanol and absorbance was read at 570 nm. The average drug concentration (μg/mL) for 50% inhibition (ID_{50}) of tumor cell-growth was determined by plotting the log of drug concentration versus the growth rate (% control).

Morphological evaluation of apoptotic cells

Cells were air dried, fixed in methanol, stained with Wright staining solution, mounted in glycerine and analyzed under light microscope at 450× magnification. Apoptotic cells were identified on the basis of morphological features that included contracted cell bodies, condensed, uniformly circumscribed and densely stained chromatin, or membrane-bound apoptotic bodies containing one or more nuclear fragments.[52] The percentage of apoptotic cells was determined by counting more than 300 cells in at least three separate visions.

Quantitation of percent DNA fragmentation

Percent DNA fragmentation was quantified following a method described by Sellins and Cohen[53] with slight modification. Cells (5×10^5 cells/mL) were suspended in 0.5 mL of lysis buffer (Tris–EDTA buffer, pH 7.4 containing 0.2% Triton X-100) and were centrifuged for 15 min at 13,000*g* at 4 °C in a microfuge tube (labeled as B). Supernatant was transferred to another tube (labeled as T). 0.5 mL of 25% trichloroacetic acid was added to T and B tubes, which were then vortexed vigorously. Tubes were kept overnight at 4 °C for precipitation. Supernatant was discarded after centrifugation at 13,000*g* for 10 min and then DNA in each pellet was hydrolyzed with 80 μL of 5% trichloroacetic acid by heating on water bath at 90 °C for 15 min and 160 μL of freshly prepared diphenylamine (150 mg diphenylamine in 10 mL glacial acetic acid, 150 μL concd H_2SO_4 and 50 mL of acetaldehyde solution) was added and the tubes were allowed to stand overnight at room temperature to develop color. 100 μL of this colored solution was transferred to a 96-well flat bottom ELISA plate (NUNC, Denmark) and absorbance at 600 nm noted on Biorad plate reader (Australia). Percent fragmented DNA was calculated using formula:

$$\%\ \text{fragmented DNA} = \frac{T}{T+B}100$$

where T = absorbance of fragmented DNA, T + B = absorbance of total DNA.

In vivo studies

In order to aassess the antitumor activity of the compounds, 6–8 groups of BALB/c mice were inoculated intraperitoneally with DL (10^6) cells followed by treatment with the metal complexes (10 mg/kg body weight) in a single ip injection on days 1, 5.9 and 12 after tumor transplantation. This treatment protocol was selected for administration, as various previous studies[54] have shown that metal complexes of sulfur donor ligands have shown optimal antitumor activity at a dose of 10 mg/kg. The antitumor efficacy of each agent is expressed as % T/C and is given by,

$$\%\ T-C = \frac{\text{Mean life span of treated mice}}{100\ \text{Mean life span of control mice}} \times 100$$

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