BioMetals **16:** 311–320, 2003.

Synthesis, Characterization and Antitumor Studies of Mn(II), Ni(II), Cu(II) and Zn(II) Complexes of N-Nicotinoyl-N′-*o*-Hydroxythiobenzhydrazide

Anuraag Shrivastav[1], Nand Kishore Singh[1,*] & Sukh Mahendra Singh[2]
[1]*Department of Chemistry, Faculty of Science, Banaras Hindu University, Varanasi, 221005, India;* [2]*School of Biotechnology, Faculty of Science, Banaras Hindu University, Varanasi, 221005, India;* *Author for correspondence: (Tel: +91-542-307320; E-mail: nksingh@banaras.ernet.in)*

Received 25 February 2002; Accepted 26 April 2002; published on line September 2002

Key words: antitumor activity, N-nicotinoyl-N′-*o*-hydroxythiobenzhydrazide, 3d-metal complexes

Abstract

A new ligand N-Nicotinoyl-N′-*o*-hydroxythiobenzhydrazide (H_2Notbh) forms complexes [Mn(Notbh)(H_2O)], [M(Notbh)] [M = Ni(II) Cu(II) and Zn(II)] which were characterized by various physico-chemical techniques. All the metal complexes were observed to inhibit the growth of tumor *in vitro*, whereas, ligand did not. *In vivo* administration of these complexes resulted in prolongation of survival of tumor bearing mice. Tumor bearing mice administered with metal complexes showed reversal of tumor growth associated induction of apoptosis in lymphocytes. The paper discusses the possible mechanisms and therapeutic implication of the H_2Notbh and its metal complexes in tumor regression and tumor growth associated immunosuppression.

Introduction

The coordination chemistry of nitrogen-sulfur donor ligands such as substituted thiosemicarbazide (Campbell 1975), thiosemicarbazones (Valdes-Martinez *et al.* 1996; West *et al.* 1996, 1997; Belrado *et al.* 1998) and dithiocarbazates(Ali & Livingstone 1974) is well documented in literature. A few papers have appeared on the synthetic and structural aspects of the 3d-metal complexes of thiohydrazides (Singh *et al.* 1996, 1996, 1997, 1999; Gabel *et al.* 1977). The hydrazones derived from the condensation of pyridine hydrazides with aldehydes and ketones exhibit bidentate or tridentate ligational behaviour (Singh *et al.* 1984, 1986) and show antibacterial (Dave *et al.* 1981) and antitumor (Antholine *et al.* 1976) activities. In addition to the antifungal (Singh *et al.* 1981) and antibacterial properties, some of the transition metal complexes of nitrogen-sulfur donor ligands also exhibit antitumor activity against a variety of tumor cells (Das & Livingstone 1978; Liu *et al.* 1992). Thiosemicarbazones such as 3-ethoxy-2-oxobutyraldehyde bis(thiosemicarbazonato) copper(II) complexes (CuKTS) have been found to exhibit antitumor activity (Minkel *et al.* 1978, 1978) due to their binding with DNA. Thiosemicarbazones of 1-formylisoquinoline and 2-formyl pyridine and their derivatives were also demonstrated to be effective against animal tumor by inhibiting ribonucleoside diphosphate reductase activity (Blanz *et al.* 1970, 1974) and synthesis of DNA (Sartorelli *et al.* 1976). In addition, the copper complexes of thiosemicarbazide inactivate lambda phage infectivity and transfection by lambda DNA (Levinson 1976), and inhibit the activity of RNA dependent DNA polymerase of Rous sarcoma virus. We have reported that transition metal complexes of N-salicyloyl-N′-2-furanthiocarbohydrazide have antitumor activity against different tumor cells (Agrawal *et al.* 1986; Singh *et al.* 1997). Many chemotherapeutic agents have also been reported to possess immunomodulatory properties (Shrivastav *et al.* 2002; Kleinerman *et al.* 1980; Lichtenstein *et al.* 1986; Ogura *et al.*1982). However, the immunomodulatory potential of the metal complexes with an-

titumor 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 study the such activity of transition metal complexes of newly synthesized ligand N-nicotinoyl-N′-*o*-hydroxythiobenzhydrazide. In the present investigation we report the synthesis, characterization and antitumor activity of 3d-metal complexes of N-nicotinoyl-N′-*o*-hydroxythiobenzhydrazide. Further we also investigated the immunomodulatory action of these complexes on tumor bearing hosts.

$(3\text{-}C_5H_4N)C(O)NHNHC(S)C_6H_4\text{-}2\text{-}OH$

N-nicotinoyl-N′-*o*-hydroxythiobenzhydrazide (H_2Notbh)

Materials and methods

Physical measurements

The complexes were analysed for their metal and sulfur contents as described elsewhere (Jeffery *et al.* 1989). Carbon, hydrogen and nitrogen contents were estimated on a Perkin-Elmer 240C microanalyzer. The infrared spectra of the ligand and its complexes were recorded on a JASCO FT/IR-5300 spectrophotometer in KBr in the 4000–400 cm^{-1} region. The electronic spectra were recorded on a Cary-2390 UV-Vis. spectrophotmeter in DMSO solutions. The ^1H NMR spectra were obtained in DMSO-d_6 on JEOL FX-90Q spectrometer using TMS as internal reference.

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. Dalton's Lymphoma (a spontaneous murine T cell lymphoma) was 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 (Shanker *et al.* 2000) and counting the cells on a hemocytometer under light microscope. Cells that did not exude 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 (Parajuli *et al.* 1995). 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 sub-mitogenic doses of concanavalin-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 formazan product that can be completely solubilized in acidic isopropanol (Mossmann *et al.* 1986). 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 formazan crystals formed during the reaction were dissolved in 100 μl of 0.04N 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 45× 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 (Shanker *et al.* 2000). 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 (1987) 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 conc. H_2SO_4 and 50 ml of acetaldehyde solution) was added and the tubes were allowed to stand overnight at room temperature to develop colour. 100 μl of this coloured solution was transferred to a 96 well flat bottom ELISA plate (NUNC, Denmark) and absorbance at 600 nm noted on an ELISA plate reader (BioRad, Australia). Percent fragmented DNA was calculated using formula:

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

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

In vivo *studies*

In order to assess 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 i.p. injection on days 1, 5, 9 and 12 after tumor transplantation. This treatment protocol was selected for administration, as various previous studies 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

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

Compounds

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 conc. 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 was separated and washed with distilled water. The red coloured sodium salt of the dithioacid

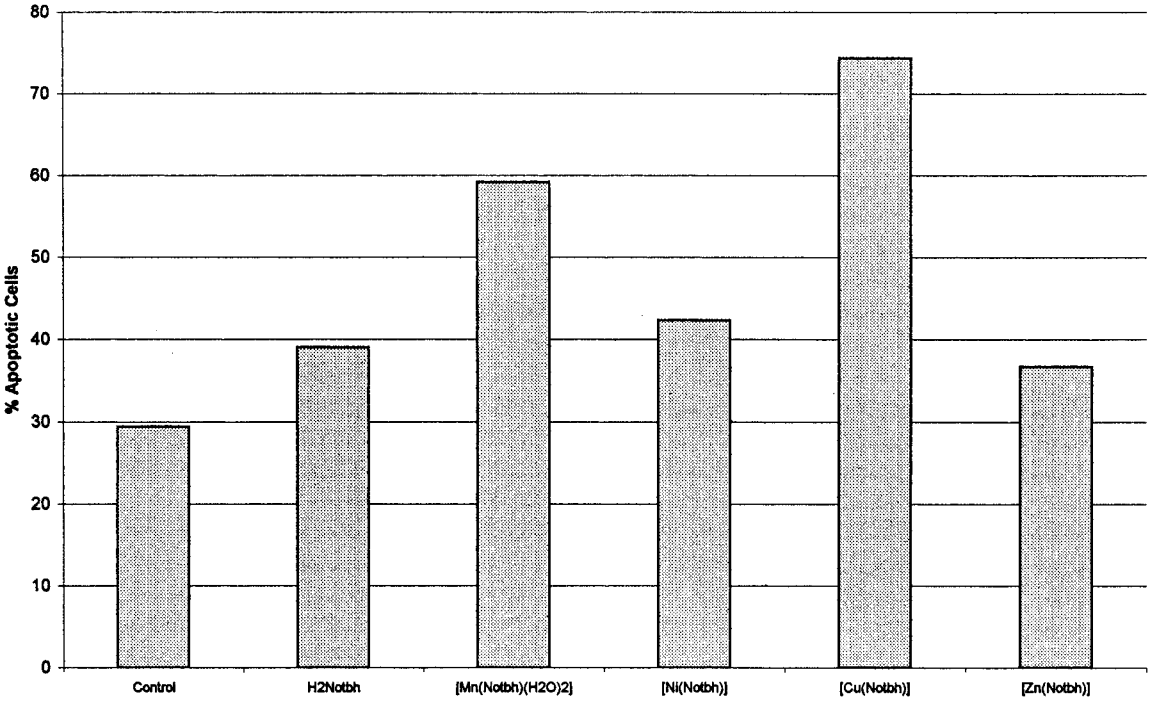

Fig. 1. Effect of H_2Notbh and its metal complexes on the induction of apoptosis in tumor cells. DL cells were incubated in medium alone or containing H_2Notbh or its metal complexes (10 μg/ml) for 24 h and the number of cell showing apoptotic morphology was enumerated. Values are mean of three experiments.

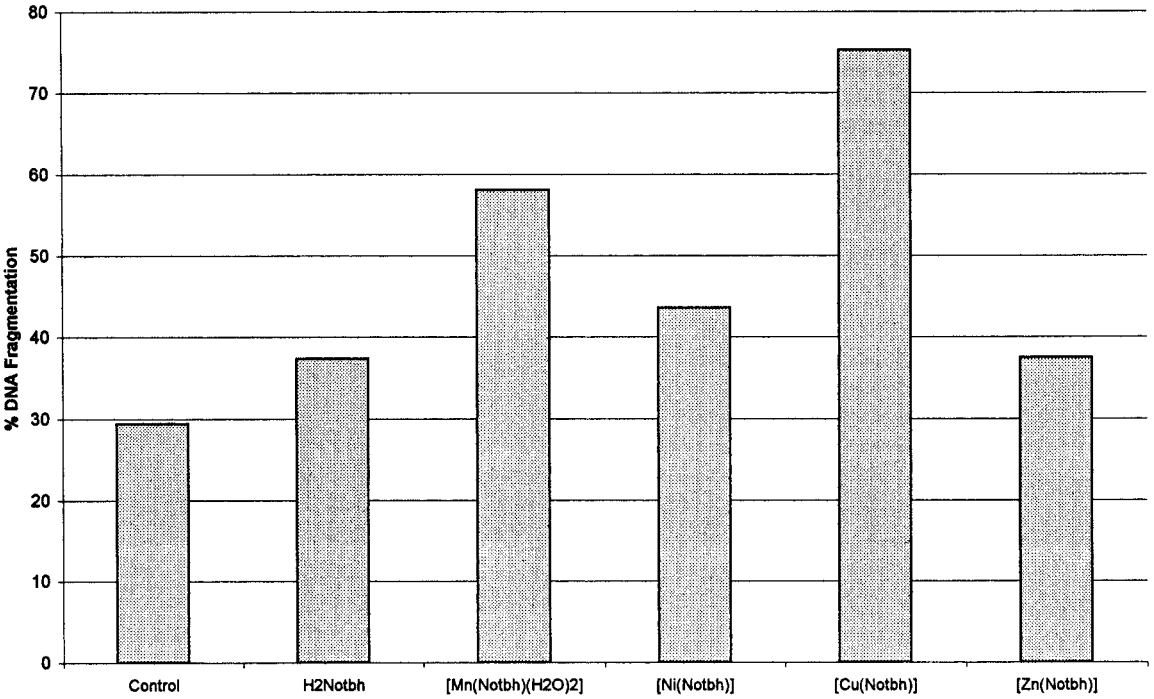

Fig. 2. Effect of H_2Notbh and its metal complexes on % DNA fragmentation of tumor cells. DL cells were incubated in medium alone or containing H_2Notbh or its metal complexes (10 μg/ml) for 24 h and the % DNA fragmentation was evaluated. Values are mean of three experiments.

Table 1. Analytical data and physical properties of H_2Notbh and its metal complexes.

Compound	Colour	m.p. (°C)	Yield (%)	Found (Calcd) %					μ_{eff} (B.M.)
				M	S	C	H	N	
H_2Notbh	Yellow	238	60	–	11.9 (11.7)	57.2 (57.1)	3.8 (4.0)	15.8 (15.4)	–
[Mn(Notbh)(H_2O)$_2$]	Maroon	>300	57	15.6 (15.2)	9.2 (8.8)	42.8 (43.1)	3.4 (3.6)	11.8 (11.6)	5.9
[Ni(Notbh)]	Brown	>300	61	17.6 (17.8)	9.4 (9.7)	47.4 (47.3)	2.6 (2.7)	12.3 (12.7)	Dia[a]
[Cu(Notbh)]	Black	305	70	19.2 (19.0)	9.8 (9.6)	46.3 (46.6)	2.8 (2.7)	12.4 (12.6)	2.11
[Zn(Notbh)]	Light Yellow	295	72	19.7 (19.4)	9.1 (9.5)	46.4 (46.4)	2.8 (2.7)	11.9 (12.5)	Dia[a]

[a]Diamagnetic

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 mixture overnight at room temperature, the dark solution was acidified with conc. 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, m.p. 120 °C.

N-nicotinoyl-N′-o-hydroxythiobenzhydrazide (H_2Notbh). N-nicotinoyl-N′-*o*-hydroxythiobenzhydrazide (H_2Notbh) was prepared by mixing an equivalent quantity of carboxymethyl-*o*-hydroxydithiobenzoate and nicotinic acid hydrazide, each dissolved separately in one equivalent of 1N 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 yellow compound.

[Mn(Notbh)(H_2O)$_2$], [M(Notbh)] [M = Ni(II), Cu(II) and Zn(II)]. [Mn(Notbh)(H_2O)$_2$], [M(Notbh)] [M = Ni(II), Cu(II) and Zn(II)] were prepared by adding a DMF solution (10 ml) of H_2Notbh (1.2 g, 5.2 mmol) dropwise to the ethanolic solution (20 ml) of the respective metal(II) acetate in an 1:1 molar ratio. Coloured 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.

Table 2. Effect of *in vivo* administration of H_2Notbh and its metal complexes on the survival of tumor bearing mice.

Compounds	Post inoculation life span (% T/C)
H_2Notbh	115
[Mn(Notbh)(H_2O)$_2$]	228
[Ni(Notbh)]	209
[Cu(Notbh)]	248
[Zn(Notbh)]	135
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 ≥ 125 is considered biologically significant. C = 20 ± 2 days, experiment terminated after 50 days.

Results and discussion

Chemistry

The analytical data correspond to the 1:1 metal to ligand stoichiometry, and the complexes are coloured. These complexes were formed by loss of two protons from the ligand. The complexes are soluble in polar coordinating solvent, DMSO, and melt above 300 °C, except [Cu(Notbh)] and [Zn(Notbh)] which

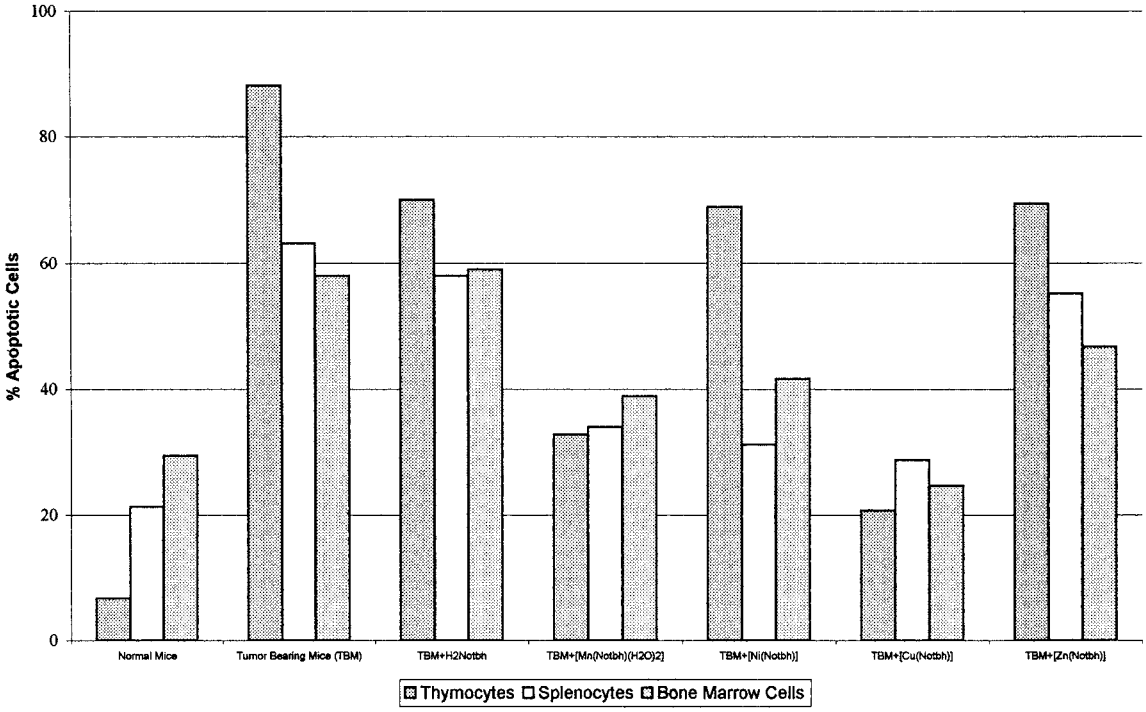

Fig. 3. Effect of *in vivo* administration of H_2Notbh or its metal complexes on the induction of apoptosis of thymocyte, splenocyte and bone marrow cell of normal or tumor bearing mice treated with ligand or its metal complexes. Thymocytes, splenocytes and bone marrow cells of normal or tumor bearing mice or tumor bearing mice treated with ligand or its metal complexes were enumerated for and the number of cell showing apoptotic morphology. Values are mean of three experiments.

Table 3. Effect of H_2Notbh and its metal complexes on tumor cell growth *in vitro* (ID_{50} values in μg/ml).

Compounds	DL
H_2Notbh	25.71
[Mn(Notbh)(H_2O)$_2$]	0.88
[Ni(Notbh)]	1.68
[Cu(Notbh)]	0.82
[Zn(Notbh)]	17.10
Cisplatin	0.71

ID_{50} = Average drug concentration (μg/ml) for 50% inhibition of tumor cell growth. Values are mean ± SD of three experiments. $P < 0.05$ with respect to values of ID_{50} of H_2Notbh alone.

melt at 305 and 295 °C, respectively. The composition of the complexes along with colour, m.p. magnetic susceptibility and elemental analyses are given in Table 1.

The magnetic moment of [Mn(Notbh)(H_2O)$_2$] suggests high-spin Mn(II) and the UV-Vis spectrum shows two d-d bands at 17,985 and 19,380 cm^{-1} assigned to the $^6A_{1g} \rightarrow {}^4T_{1g}$, $^4T_{2g}$ transitions, respectively for six coordinate high-spin Mn(II) centre possessing distorted octahedral geometry. Another band observed at 24,630 cm^{-1} may be assigned to a charge-transfer transition. The magnetic moments of [Ni(Notbh)] and [Cu(Notbh)] suggest a square-planar geometry around Ni(II) and Cu(II). [Ni(Notbh)] is diamagnetic and displays bands at 15,520 and 20,660 cm^{-1} which may be assigned to the $^1A_{1g} \rightarrow {}^1B_{2g}$, $^1B_{1g}$ transitions, respectively, for a square-planar geometry around Ni(II). A magnetic moment of 2.11 B.M. for [Cu(Notbh)] indicates its square-planar geometry, which is further supported by the presence of three d-d bands at 11,790, 13,510 and 15,060 cm^{-1} assigned to the $^2B_{1g} \rightarrow {}^2A_{1g}$, $^2B_{2g}$, ^2E_g transitions, respectively (Lever 1984).

The IR spectrum of H_2Notbh shows a band at 3396 cm^{-1} assigned to ν(OH). The bands at 3117 and 3059 cm^{-1} are assigned to ν(NH). A band at 1664 cm^{-1} is observed for ν(C=O). The bands occurring at 1431, 1334 and 956 cm^{-1} are assigned to thioamide I [β(NH) + ν(CN)], thioamide II [ν(CN) + β(NH)] ν(N-N) and ν(C=S), respectively. A broad band at 3441 cm^{-1} observed in the spectrum

of [Mn(Notbh)(H_2O)$_2$] may be assigned to ν(OH) of the ligand as well as of water molecules. The ν(OH) band for [Cu(Notbh)] is observed at 3447 cm^{-1}. Bands due to ν(NH), ν(C=O) and ν(C=S) of the ligand are absent in both the complexes and in place of these, two new bands appear in 1604–1620 and 827–830 cm^{-1} regions for [Mn(Notbh)(H_2O)$_2$] and [Cu(Notbh)] due to the ν(C=N) of NCO mode, suggesting that both the hydrazinic protons are lost *via* enolisation and thioenolisation leading to bonding of the resulting enolic oxygen and thiolato sulfur with the metal ion (Geetharani *et al.* 1977). The thioamide I and ν(N-N) bands undergo positive shifts of 44 and 43 cm^{-1} in [Mn(Notbh)(H_2O)$_2$] and, 60 and 63 cm^{-1} in [Cu(Notbh)], respectively, suggesting the involvement of these groups as bonding sites. [Ni(Notbh)] and [Zn(Notbh)] exhibit bands for ν(OH) at 3422 cm^{-1}. Absence of bands due to ν(NH), ν(C=O) and ν(C=S) in the spectra of [Ni(Notbh)] and [Zn(Notbh)] suggest the bonding of enolic oxygen and thiolato sulfur by loss of both the hydrazinic protons *via* enolisation and thioenolisation, with the metal ions. The appearance of new bands at 1606 and 1597 cm^{-1}, due to ν(C=N) of NCO in Ni(II) and Zn(II) complexes, respectively, further suggest bonding through the enolic oxygen. The presence of a band due to ν(C–S) at 817 and 819 cm^{-1} in the spectra of Ni(II) and Zn(II) complexes, respectively, suggests bonding through the thiolato sulfur. Further, thioamide I, II and ν(N-N) bands undergo a positive shift of 44, 29 and 64 cm^{-1} in Ni(II) complex, 25, 51 and 43 cm^{-1} in Zn(II) complex, respectively, suggesting the involvement of these groups in bonding.

The ^{1}H NMR spectrum of H_2Notbh exhibits signals at δ 12.0 ppm for the phenolic proton and at δ 6.2 ppm for the hydrazinic protons, which are lost on D_2O exchange. The signals due to the aromatic rings appear as multiplet between δ9.6-7.1 ppm. The low solubility of the Zn(II) complex precluded the recording of its NMR spectra.

Antitumor studies

To investigate the antitumor activity of H_2Notbh and its metal complexes, DL (Dalton's Lymphoma) bearing mice were administered with PBS (phosphate buffer saline) alone or containing ligand or the metal complexes as indicated in the experimental section. As shown in Table 2 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(Notbh)] followed by those of [Mn(Notbh)(H_2O)$_2$] and [Ni(Notbh)]. Our observations show that H_2Notbh, and [Zn(Notbh)] did not have antitumor activity whereas, the life of tumor bearing mice administered with [Mn(Notbh)(H_2O)$_2$], [Cu(Notbh)] and [Ni(Notbh)(H_2O)$_2$] was significantly prolonged. Increase in the value of % T/C indicates a prolongation of the life of tumor bearing mice and 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 antitumor defense mechanism(s). Therefore, in the next part of the study we carried out investigations to understand the mechanism(s) underlying the prolongation of the life span of tumor bearing mice treated with these metal complexes.

One of the evidence comes from the experiments in which tumor cells, incubated in presence or absence of metal complexes were checked for their effect on viability by MTT assay in which 3,(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) is metabolized to an insoluble coloured formazan salt by mitochondrial enzyme activity of SDH in living cells. As shown in Table 3, treatment of tumor cells with [Mn(Notbh)(H_2O)$_2$], [Cu(Notbh)] and [Ni(Notbh)(H_2O)$_2$] caused maximum growth inhibition followed by [Zn(Notbh)] and the ligand indicating an inhibition of the SDH activity. These results thus indicate a possible decline of the overall metabolic activity of the tumor cells with a concomitant inhibition of the activity of the enzymes involved in halting the process of 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 tumor cell killing to identify the mode of cell death. The results suggest that apoptosis was induced in tumor cells treated with metal complexes. [Cu(Notbh)] and [Mn(Notbh)(H_2O)$_2$] were found to be most effective in the induction of tumor cell apoptosis (Figure 1). 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 (Ranjan *et al.* 1998). Indeed results presented in Figure 2 show that [Mn(Notbh)(H_2O)$_2$] and [Cu(Notbh)] cause an increase in the percentage of specific DNA fragmentation, a hallmark feature of apoptosis, indicating that

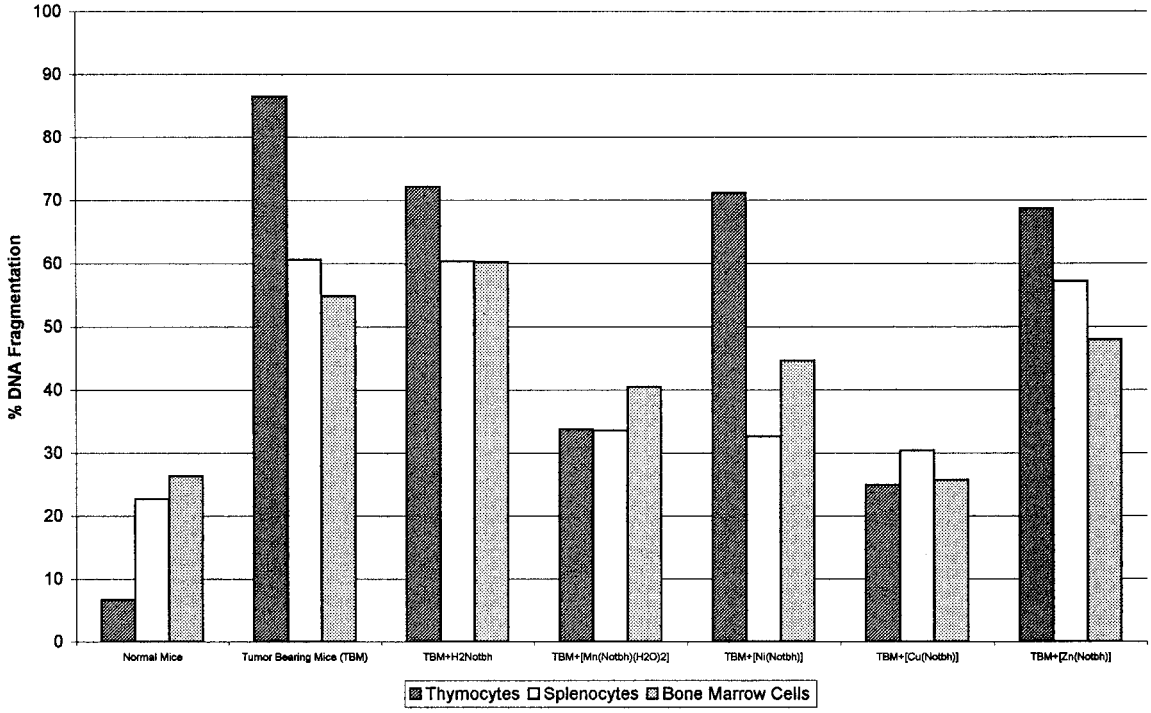

Fig. 4. Effect of *in vivo* administration of H_2Notbh or its metal complexes on % DNA fragmentation of thymocyte, splenocyte and bone marrow cell of normal or tumor bearing mice treated with ligand or its metal complexes. Thymocytes, splenocytes and bone marrow cells of normal or tumor bearing mice or tumor bearing mice treated with ligand or its metal complexes and checked for % DNA fragmentation. Values are mean of three experiments.

these metal complexes may induce apoptosis culminating in the activation of endonucleases causing DNA fragmentation. Although the reasons for the higher activity of Mn(II) and Cu(II) complexes remain unclear, we speculate that these complexes may directly or indirectly induce/activate biomolecules related to the apoptotic machinery of tumor cells. Indeed, reports suggest that Mn(II) may induce apoptosis by, caspase-3 activation, cleavage of poly(ADP) ribose and condensation of DNA (Oubrahim *et al.* 1995). The same reason may or may not apply for Cu(II) complex, but some earlier report have shown that Cu(II) complexes bind with DNA (Minkel *et al.* 1978) and various cellular proteins and thus may induce apoptosis. Cu(II) complexes of thiosemicarbazones were found to be potent inducers of apoptosis in tumor cells (Easmon *et al.* 2001). However, more confirmatory studies with respect to structure activity relationship will be essential to decipher exact mechanism of action of these complexes.

Furthermore, the metal complexes at the concentration checked did not inhibit the proliferation of normal splenocyte and bone marrow cells (data not shown), indicating that the cytostatic effect of the metal complexes was restricted to the tumor phenotype alone. Selective killing of tumor cells could be attributed to the fact that tumor cells are unable to counter the load of mutations owing to their defective DNA repair mechanism (Rosenberg 1985).

Progression of growth of various tumors including DL is invariably associated with the onset of immunosuppression in tumor bearing host (Singh *et al.* 1997; Deckers *et al.* 1973; Loeffler *et al.* 1992) one of the reasons being induction of apoptosis and inhibition of proliferation of hematopoietic precursor cells (Handy *et al.* 1985; Kumar *et al.* 1995). 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 3. admin-

istration 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 (Figure 4). 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 $[Mn(Notbh)(H_2O)_2]$ and [Cu(Notbh)] 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-nicotinoyl- N′-*o*-hydroxythiobenzhydrazide may have dual mechanism of action in tumor regression.

References

Agrawal S, Singh NK, Aggarwal RC, Sodhi A, Tandon P. 1986 Synthesis, structure and antitumor activity of N-salicyloyl-N′-(2-furylthiocarbonyl)hydrazine and its copper(II) complex. *J Med Chem* **29**, 199–202.

Ali MA, Livingstone SE. 1974 Metal complexes of sulfur-nitrogen chelating agents. *Coord Chem Rev* **13**, 101–132.

Antholine WE, Knight JM, Petering DH. 1976 Some properties of copper and zinc complexes of 2-formylpyridine thiosemicarbazone. *J Med Chem* **19**, 339–341.

Blanz EJ, French FA, DoAmaral JR, French DA. 1970 Carcinostatic activity of thiosemicarbazones of formylheteroaromatic compounds VII: 2-formylpyridine derivatives bearing additional ring substituents. *J Med Chem* **13**, 1124–1130.

Campbell MJM. 1975 Transition metal compounds of thiosemicarbazide and thiosemicarbazones. *Coord Chem Rev* **15**, 279–.

Das M, Livingstone SE. 1978 Cytotoxic action of some transition metal chelates of schiff bases derived from 5-methyldithiocarbazates. *Br J Cancer* **37**, 466–469.

Dave LD Thampy SK, Shelat YA. 1981 Antimicrobial activity of o-hydroxythiosemicarbazones and their Ni(II), Cu(II) and Co(II) chelates. *J Institution Of Chemists (India)* **53**, 169–172; 237–241.

Deckers PJ, Davis RC, Parker GA, Mannick JA. 1973 The effect of tumor on concomitant tumor immunity. *Cancer Res* **33**, 33–39.

Easmon J, Purstinger G, Heinisch G, Roth T, Fiebig HH, Holzer W, Jager W, Jenny M, Hofmann J. 2001 Synthesis, cytoxicity and antitumor activity of copper(II) and iron(II) complexes of (4)N-azabicyclo[3.2.2]nonane thiosemicarbazones derived from acyl diazines. *J Med Chem* **44**, 2164–2171.

Eastmen A. 1990 Activation of programmed cell death by anticancer agents: cisplatin as a model system. *Cancer Cells* **2**, 275–280.

French FA, Blanz EJ, Shaddix SC, Brockman RW. 1974 Alpha-N-formylheteroaromatic thiosemicarbazones. Inhibition of tumor derived ribonucleoside diphosphate reductase and correlation with *in vivo* antitumor activity. *J Med Chem* **17**, 172–181.

Gabel J, Lassen E, Trinderup P. 1977 Nickel(II) complexes of thiohydrazonates. *Acta Chem Scand* **31**, 657–665.

Geetharani K, Sathyanarayana DN. 1977 metal complexes of thiosemicarbazides: vibrational analysis of Ni(II) thiosemicarbazide complexes and their deutero isotopomers. *Austr J Chem* **30**, 1617–.

Hardy CL, Balducci L. 1985 Hemopoietic alterations of cancer. *Amer J Med Sci* **290**, 196–205.

Jeffery GH, Bassett J, Mendham J, Denney RC. 1989 *Vogel's Text Book of Quantitative Inorganic Analysis'* ELBS, Longman, Singapore, 5th ed.

Kleinerman ES, Zwelling LA, Muchmore AV. 1980 Enhancement of naturally occurring human spontaneous monocyte mediated cytotoxicity by cis-diaminedichloroplatnum(II). *Cancer Res* **40**, 3099–3102.

Kumar A, Singh SM. 1995 Effect of cisplatin-administration on the proliferation and differentiation of bone marrow cells of tumor bearing mice. *Immunol Cell Biol* **73**, 220–225.

Lever ABP. 1984 *'Inorganic Electronic Spectroscopy.'* 2nd Edn. Elsevier, Amsterdam.

Levison W, Helling R. 1976 *'Antitumor Agents and Chemotherapy'*, **9**, 160–.

Lichtenstein AK, Pende D. 1986 Enhancement of natural killer cytotoxicity by cis-diamminedichloroplatnum(II) *in vivo* and *in vitro*. *Cancer Res* **46**, 639–644.

Liu MC, Lin TS, Sartorelli AC. 1992 Synthesis and antitumor activity of amino deivatives of pyridine-2-carboxaldehydethiosemicarbazone. *J Med Chem* **20**, 3672–3677.

Loeffler CM, Smyth MJ, Longo DL, Kopp WC, Harvey LK, Tribble HR, Tase JE, Urba WJ, Leonard AS, Young HA, Ochoa C. 1973 Immunoirregulation in cancer bearing host. Down regulation of gene expression and cytotoxic function of $CD8^+$ T-Cells. *J Immunol* **149**, 949–956.

Minkel DT, Petering DH. 1978 Initial reaction of 3-ethoxy-2-oxobutyraldehyde bis(thiosemicarbazonato)copper(II) with Ehrlich ascites tumor cells. *Cancer Res* **38**, 117–123.

Minkel DT, Saryan LA, Petering DH. 1978 Structure-function correlation in the reaction of bis(thiosemicarbazonato)copper(II) complexes with Ehrlich ascites tumor cells. *Cancer Res* **38**, 124–129.

Mossmann TR, Cherwinski H, Bond MV, Giedliv MA, Coffmann R. 1986 Two types of murine helper T-cell clones. I. Definition according to profiles of lymphokine activities and secreted protiens *J Immunol* **13**, 2348–2357.

Ogura T, Shindo H, Shinzato O, Namba M, Yamamura Y. 1982 *In vitro* tumor cell killing by peritoneal macrophages from mitomycin c treated rats. *Cancer Immunol Immunother* **13**, 112–117.

Oubrahim H, Stadtman ER, Chock PB. 2001 Mitochondria play no roles in Mn(II)-induced apoptosis in HeLa cells. *Proc Natl Acad Sci USA* **98**, 9505–9510.

Parajuli P, Singh SM, Kumar A. 1995 Effect of cisplatin and FK-565 on the activation of tumor associated bone marrow derived macrophages by Dalton's lymphoma. *Int J Immunopharmac* **17**, 1–7.

Ranjan P, Sodhi A, Singh SM. 1998 Murine peritoneal macrophages treated with cisplatin and interferron-gamma undergo NO-mediated apoptosis via activation of endonucleases. *Anticancer Drugs* **9**, 333–342.

Rosenberg B. 1985 Fundamenta studies with cisplatin. *Cancer* **55**, 2303–2316.

Satorelli AC, Agrawal KC, Mooney PD. 1976 Potential antitumor agents. 13. 4-Methyl-5-amino-1-formyl isoquinoline thiosemicarbazone. *J Med Chem* **19**, 970–972.

Sellins KS, Cohen JJ. 1987 Gene induction by γ-irradiation leads to DNA fragmentation in lymphocytes. *J Immunol* **139**, 3199–3206.

Shanker A, Singh SM, Sodhi A. 2000 Ascitic growth of a transplantable T-cell lymphoma of spontaneous origin induces thymic involution: induction of apoptosis in thymocytes. *Tumor Biol* **21**, 315–327.

Shanker A, Singh SM. 2000 Characterization of factors inducing apoptosis in thymocytes of mice bearing a transplantable T-cell lymphoma of spontaneous origin. *Neoplasma* **47**, 90–95.

Shrivastav A, Singh NK, Singh SM. 2002 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. *Bioorg Med Chem* **10**, 887–895.

Singh M, Yadav LDS, Mishra SBS. 1981 Studies on some antifungal transition metal chelates of N-(5-phenyl-1,3,4-thiadiazole-2-yl)dithiocarbamic acid. *J Inorg Nucl Chem* **43**, 1701–1706.

Singh NK, Agrawal N, Aggarwal RC. 1984 Synthesis, characterization and antibacterial studies of Fe(II), Co(II), Cu(II) and Zn(II) complexes of pyridine-4-carboxaldehyde isonicotinoyl hydrazone. *Ind J Chem* **23A**, 1011–1015.

Singh NK, Agrawal N, Aggarwal RC. 1986 Synthetic and physicochemical studies on Co(II), Ni(II), Cu(II) and Zn(II) complexes of pyridine-4-carboxaldehyde thioisonicotinoyl hydrazone. *Synth React Inorg Met-Org Chem* **16**, 361–376.

Singh NK, Singh N, Prasad GC. 1996 Complexes of N-aroyl-N'-thiobenzhydrazide with Co(II), Ni(II) and Zn(II). *Transition Met Chem* **21**, 296–299.

Singh NK, Singh N, Prasad GC, Sodhi A, Shrivastava A. 1996 Synthesis, characterization and antitumor studies on N-salicyloyl-N'-thiobenzhydrazide and its Copper(II) complex. *Transition Met Chem* **21**, 556–559.

Singh NK, Singh N, Prasad GC, Sodhi A, Shrivastava A. 1997 Antiutumor activity studies of newly synthesized N-salicyloyl-N′-(p-hydroxybenzthioyl)hydrazine and its copper(II) complex both *in vivo* and *in vitro*. *Bioorg Med Chem* **5**, 245–251.

Singh NK, Shrivastava A, Thomas MJK. 1999 Synthesis and spectroscopic characterization of binary and mixed ligand complexes of N-furoyl-N'-thiobenzhydrazide with some 3d-metal ions. *Synth React Inorg Met-Org Chem* **29**, 1427–1449.

Singh SM, Parajuli P, Shrivastava A, Sodhi A. 1997 Alteration in immune response by tumor cells: underlying mechanisms. *Int J Immunopathol Pharmacol* **1**, 27–35.

Valdes-Martinez J, Toscano RA, Zenstella Deheasa A, Salberg MM, Bain GA, West DX. 1996 Synthesis, crystal and molecular structure of 5-bromo-salicylaldehyde-2-methylthiosemicarbazonato(nitrato)copper(II) monohydrate. *Polyhedron* **15**, 427–431.

West DX, Bain GA, Butcher RJ, Jasinski JP, Pozdniakiv RY, Valdis-Martinez J, Toscano RA, Ortega SH. 1996 Strctural studies of three isomeric forms of heterocyclic N(4) substituted thiosemicarbazones and two Ni(II) complexes. *Polyhedron* **15**, 665–674.

West DX, Padhye SB, Sonawane PB. 1991 Electrochemical studies of copper(II) 2-acetylpyridine ^{4}N-dialkyl-thiosemicarbazones, relation to their spectral, magnetic and biological properties. *Structure and Bonding* **76**, 1–50.

West DX, Saleda JS. 1992 Chemical and antifungal properties of Ni(II) complexes of 2-formylpyridine, ^{4}N-methyl, -dimethyl,-diethyl and -dipropylthiosemicarbazones. *Transition Met Chem* **17**, 568–572.