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Co(II), Ni(II), Cu(II) and Zn(II) complexes of isatinyl-2-aminobenzoylhydrazone: synthesis, characterization and anticancer activity

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This article describes the synthesis, structural aspects and biological studies of Co(II), Ni(II), Cu(II) and Zn(II) complexes of a new hydrazone derived from the condensation of isatin and 2-aminobenzoylhydrazide. The ligand is well characterized using ^{1}H NMR, ^{13}C NMR, 2D HETCOR, mass and IR spectral studies. The chelating tendency of the ligand towards transition metal ions is established using analytical and spectral studies, which reveal the monobasic tridentate nature of the ligand. Octahedral geometry for Co(II), Cu(II) and Zn(II) and tetrahedral geometry for Ni(II) are tentatively proposed. All the synthesized compounds were screened for *in vitro* anticancer activity against Ehrlich ascites carcinoma and human cancer cell lines (adenocarcinoma HT29, kidney cancer cell line K293 and breast cancer cell line MDA231) using tryphan blue exclusion method and MTT assay. Copyright © 2014 John Wiley & Sons, Ltd.

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Keywords: isatin; hydrazone; transition metal complex; 2-aminobenzoylhydrazone; anticancer activity

Introduction

Hydrazones derived from organic acid hydrazides are azomethines characterized by the presence of the triatomic group >C=N–N< and form an interesting class of compounds which finds extensive applications in biological, clinical, analytical and various other fields.[1–4] The hydrazone functional group >C=N–N< is very important for a compound to exhibit biological activity since this group forms hydrogen bonds with lipid layers and thereby increases the lipophilic nature of the compound. It has also been reported that the introduction of a primary amino group on an aromatic acid hydrazide at the *ortho* position makes it strongly active against tuberculosis mycobacteria, and metal complexes of 2-aminobenzoylhydrazide have shown significant anti-tubercular activity compared to the parent ligand.[5] A great deal of work has been done on the metal complexes of 2-aminobenzoylhydrazone due to their enhanced activity compared with the parent ligand.[6–8]

Nitrogen- and oxygen-containing ligands have always fascinated coordination chemists. Isatin (indole-2,3-dione; or 2,3-dioxoindoline; or indoline-2,3-dione) is a most important derivative of oxindoles and its derivatives are known for a broad range of biological activities, including antibacterial, antifungal, anticonvulsant, antiviral, antiproliferative and anticancer activities.[9–13] Isatin is also present in biological systems whose metabolic pathways are still unknown.[14,15] A variety of metal complexes of isatin hydrazones have been described in the literature. Many of them did not reveal a biological relevance, but as soon as pharmacological activities started to be demonstrated for these compounds, their metal complexes gained biological importance and were investigated for various activities.[16] Hydrazones of isatin and their transition metal complexes have been extensively studied for their anticancer activity[17–21] and have proven to be promising candidates. This prompted us to synthesize and characterize them and evaluate their preliminary cytotoxicity against Ehrlich ascites carcinoma (EAC) and human cancer cell lines using tryphan blue exclusion method and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

Keeping in mind the wide spectrum of pharmacological activities exhibited by indole-2,3-dione and 2-aminobenzoylhydrazide, we have designed a new hydrazine, namely isatinyl-2-aminobenzoylhydrazone (ISABH, **L**) with a free amino group at the *ortho* position of the hydrazide moiety, to enhance the biological activity. Considering the role of transition metals in the enhancement of biological activity of the ligand, metal complexes of ISABH have been synthesized, thoroughly characterized and screened for their anticancer activity.

Material and Methods

Physical Measurements

Methyl anthranilate (SD Fine Chemicals, India), hydrazine hydrate (SD Fine Chemicals, India) and isatin (Spectrochem, India) were of AR grade and used without further purification. Solvents were distilled before use. 2-Aminobenzoylhydrazide was prepared

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following earlier reports.[22] After wet ashing with HCl and $HClO_4$, cobalt, nickel and copper were determined gravimetrically; zinc was determined volumetrically.[23] Chloride content was determined as silver chloride after decomposition with dilute HNO_3.[23] Elemental analysis (C, H, N) was carried out with a TruSpec CHN/CHNS analyser. The mass spectrum of the ligand was obtained with a Shimadzu GCMS-QP2010S. Magnetic susceptibility measurements were made at room temperature with a Gouy balance using $Hg[Co(SCN)_4]$ as the calibrant and diamagnetic corrections were made using Pascal's constants. Electronic spectra were recorded using a Varian CARY 50 Bio UV–visible spectrophotometer with DMF as solvent. The IR spectra of ligand and complexes were recorded as KBr pellets in the region 400–4000 cm^{-1} with a Nicolet 170SX FT-IR spectrometer. ^1H NMR and ^{13}C NMR spectra of the ligand and its Zn(II) complex and 2D HETCOR of the ligand were recorded in DMSO-d_6 with a Bruker Avance 400 MHz spectrometer using tetramethylsilane (TMS) as the internal standard. Conductivity measurements of 10^{-3} M solutions of complexes in DMF were made using an ELICO-CM82 conductivity bridge provided with a cell having a cell constant of 0.51. Simultaneous thermogravimetric (TG) and differential thermal analysis (DTA) curves were recorded with a PerkinElmer TGA7 analyser at a heating rate of 10°C min^{-1} and maximum temperature of 1000°C in nitrogen atmosphere. The electron paramagnetic resonance (EPR) spectra of a polycrystalline Cu(II) complex were recorded at room temperature and liquid nitrogen temperature with a Varian E-4 X-band spectrometer using tetracyanoethylene as the calibrant.

Cell Culture

EAC-bearing Balb/C mice were obtained from Central Animal House, HSK College of Pharmacy, Bagalkot, Karnataka, India. The animal experiments were performed according to the rules and regulations of the Institutional Animal Ethical Committee (IAEC/Clearance/2007/1-8), HSK College of Pharmacy, Bagalkot, Karnataka.

The cell lines were maintained in RPMI-1640 medium supplemented with 10% heat-inactivated foetal calf serum containing 5% of a mixture of gentamycin (3 μg ml^{-1}), penicillin (100 units ml^{-1}) and streptomycin (100 μg ml^{-1}) in the presence of 5% CO_2 in air at 37°C and routinely sub-cultured using a 0.25% trypsin–0.02% ethylenediaminetetraacetic acid solution. Cells were collected on 12–14 days of post-transplantation suspended in phosphate buffer saline (pH = 7.4), centrifuged and washed with cold phosphate buffer saline.

Determination of Cytotoxicity of Compounds to EAC Cells (*In Vitro* Studies) using Tryphan Blue Exclusion Method (Cell Viability Test)

In order to transfer EAC cells from *in vivo* conditions to *in vitro* medium, they were drawn with a sterile injector from the peritoneal cavity of mouse and by diluting with HBSS; concentrations of 10^7 cells ml^{-1} were thus obtained. Later these cells were transferred to test tubes at a final concentration of 375 000 cells ml^{-1}. The test tube contained Minimal Essential Medium (MEM; Gibco), 2.5% foetal calf serum and 10 mM Hepes. After cells were brought to in the *in vitro* method in this way, they were propagated at 37°C.

The cells (1×10^6 cells in 0.1 ml of MEM) were incubated in clean sterile tubes with the test compounds (0.01 ml) for 3 h at 37°C, keeping the final volume at 0.9 ml. The compounds were tested at concentrations of 5, 10, 25, 50, 100, 150, 300, 600 and 1200 μg ml^{-1}, and these solutions were prepared in dimethylsulfoxide (DMSO). The volume of DMSO was pegged below 0.1% of the total volume. The control tube had 10 μl of solvent. The final volume was made up to 0.9 ml with MEM. To each tube, 100 μl of tryphan blue solution was added. The live (without stain) and dead (with blue stain) cells were counted using a haemocytometer and percentage inhibition of viable cells was calculated using the formula

$$\text{Inhibition of viable cells } (\%) = \frac{\text{Number of viable cells}}{\text{Total number of cells}} \times 100.$$

Cytotoxicity Assay using MTT Method (Effect on Cell Proliferation)

Cells (adenocarcinoma, breast and kidney cancer cell lines) were seeded at a density of 2×10^5 cells ml^{-1} per well into a sterile 96-well plate and allowed to adhere overnight. The solutions of compounds of concentrations 10, 25, 50, 100 and 200 μg ml^{-1} were prepared in DMSO and 20 μl of the test compound solutions was added to different wells. Cells were incubated with the test compounds for a period of 24, 48, 72 or 96 h. After the required incubation period, 50 μl of MTT was added to each well and the plates were incubated at 37°C in a humid atmosphere with 5% CO_2 and 95% air for 4 h. The medium was then gently aspirated, and 150 μl of DMSO was added to dissolve the formazan crystals. The amount of formazan product was measured spectrophotometrically at 570 nm using an ELISA microplate reader (Biotech, USA). Each compound concentration had three replicates per assay, and each experiment was carried out on three separate occasions. The IC_{50} value, defined as the drug concentration causing a 50% reduction in cellular viability, was calculated for each test compound at 96 h incubation. This value was used as a means for comparing the cytotoxicity of the compounds for each of the three cell lines used in this experiment.

Results and Discussion

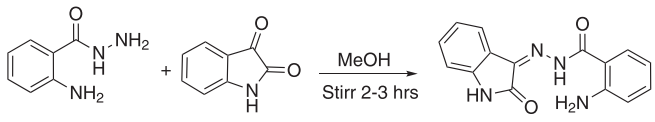
Chemistry

*Synthesis of ISABH (**L**)*

To a methanolic solution (20 ml) of 2-aminobenzoylhydrazide (0.151g, 1 mmol), isatin (0.147g, 1 mmol) was added and stirred for 3 h; a yellowish-orange solid separates (Scheme 1). The solid was filtered, washed with ethanol and dried in air and recrystallized from methanol. Yield 92%; m.p. 230–232°C.

*Synthesis of Co(II), Ni(II), Cu(II) and Zn(II) complexes of ISABH (**C1–C4**)*

To 15 ml of an ethanolic suspension of **L** (0.280g, 1 mmol), 5 ml of an ethanolic solution of equimolar quantity of metal salt (1 mmol) was slowly added with constant stirring. The pH was adjusted to 6–7 by adding 2–3 drops of ammonia, followed by refluxing for about an hour. The precipitates thus obtained were filtered, washed repeatedly with ethanol and acetone and dried in air. Yield 60–65%.



Scheme 1. Synthetic route for the preparation of ISABH.

Table 1. Analytical and physicochemical data for ISABH and its metal complexes

Compound	Compound code	Molecular formula	Elemental analysis (%): found (calc.)					μ_{eff} (BM)	λ_m [a]	Electronic spectra, λ_{max} (nm)
			C	H	N	M	Cl			
ISABH	**L**	$C_{15}N_4H_{12}O_2$	64.31 (64.28)	4.44 (4.28)	20.14 (20)		—	—	—	401, 708
[Co(ISAB)$_2$]	**C1**	[Co($C_{15}H_{11}N_4O_2$)$_2$]	58.50 (58.35)	3.48 (3.56)	18.30 (18.15)	9.59 (9.59)	—	4.72	2.5	455, 372, 663
[Ni(ISAB)Cl]·H_2O	**C2**	[Ni($C_{15}H_{11}N_4O_2$)Cl]·H_2O	58.38 (58.37)	3.59 (3.56)	18.25 (18.16)	9.80 (9.51)	9.40 (9.07)	2.84	2.1	387, 446, 613, 678
[Cu(ISAB)$_2$]	**C3**	[Cu($C_{15}H_{11}N_4O_2$)$_2$]	58.01 (57.92)	3.57 (3.53)	18.10 (18.01)	10.34 (10.22)	—	1.98	3.7	390, 455, 870, 874
[Zn(ISAB)$_2$]	**C4**	[Zn($C_{15}H_{11}N_4O_2$)$_2$]	57.80 (57.74)	3.60 (3.52)	17.90 (17.96)	10.57 (10.48)	—	Dia [b]	0.4	381, 462

[a] Ω^{-1} cm^{-2} mol^{-1}.
[b] Dia = diamagnetic.

Physical measurements

The elemental analysis of **L** is consistent with the molecular formula $C_{15}N_4H_{12}O_2$ and its mass spectrum shows *m/z* peak at 280 that corresponds to its molecular weight. The transition metal complexes of ISABH are stable at room temperature and non-hygroscopic in nature. Complexes are insoluble in common organic solvents but soluble in coordinating solvents like DMF and DMSO. Formulation of these complexes has been done by elemental analysis and molar conductance. Analytical data for the complexes are given in Table 1. Lower molar conductance values of the complexes measured in DMF (10^{-3} M solutions) adequately confirm the non-electrolytic nature of the complexes.

Infrared spectral studies

Infrared spectral data for the ligand and its complexes are presented in Table 2 along with assignments. Sharp bands of medium intensity at 3443 and 3349 cm^{-1} in the ligand spectrum are due to asymmetric and symmetric stretching vibrations of –NH_2 group.[24] A sharp absorption band at 3197 cm^{-1} is attributed to ν(–NH) stretching. The absorption bands characteristic of ν(C=O) vibrations of isatinic and 2-aminobenzoylhydrazide fragments appear at 1716 cm^{-1}[25] and 1648 cm^{-1},[26] respectively, and the absorption band at 1629 cm^{-1} is assigned to azomethine ν(C=N) stretching. The carbonyl stretching frequency of hydrazide is observed at lower frequency compared with ν(C=O) of aromatic acid hydrazides without –NH_2 group at the *ortho* position in which it has appeared at 1673 cm^{-1}.[25] This may be due to the presence of intramolecular hydrogen bonding between carbonyl oxygen and hydrogen of –NH_2 group, which is clear from the crystal structure of 2-aminobenzoylhydrazide.[27] In the IR spectra of all the complexes, the stretching frequency of lactam carbonyl of isatin has decreased by 36–66 cm^{-1}, indicating the involvement of oxygen of lactam carbonyl in the coordination. On complexation the ν(C=O) band of hydrazide disappears and a new band appears at 1614–1618 cm^{-1}, which is attributed to ligation of carbonyl oxygen via enolization and deprotonation.[24] Moreover in the IR spectra of the complexes, new absorption bands at 1186–1189 cm^{-1} are observed and are assigned to the vibrations of C–O–M bonds. These data indicate that the ligand is in the enolic form in the complexes. This is also supported by the appearance of a new absorption band at 1278–1308 cm^{-1} due to ν(C–O) stretching in all the complexes. The azomethine stretching frequency in all the complexes has shifted to lower frequencies, which indicates the involvement of the nitrogen atom of azomethine group in the coordination with metal ions.

NMR spectral studies

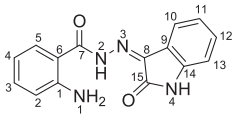
The ^{1}H NMR spectral assignments were done by comparing with isatin and 2-aminobenzoylhydrazide.[26] The numbering system for the assignments of protons is given in Scheme 2. Chemical shift assignments of ^{1}H NMR and ^{13}C NMR spectra of **L** and Zn(II) complex are given in Table 3.

The ^{1}H NMR spectrum of **L** shows singlets at 13.84 and 11.3 ppm integrating for one proton and 6.74 ppm integrating for two protons assigned to N2H, N4H and N1H_2, respectively, and all are D_2O exchangeable. N1H_2 protons appear downfield to TMS, due to the presence of intramolecular hydrogen bonding between N1H_2 proton and C7 carbonyl oxygen, whereas N2H is sandwiched between C7 carbonyl and N3=C8 azomethine nitrogen and also intramolecular hydrogen bonding with C15 carbonyl oxygen. Hence, N2H proton is observed downfield to TMS owing to its electron

Table 2. Diagnostic IR bands of ISABH and its metal complexes

Compound code	ν(–NH_2)		ν(C=O)		ν(C=N)	ν(C=N) (new)	ν(C–O)	ν(N–H)
	ν_{symm}	ν_{sym}	Lactone	Hydrazide				
L8	3472m	3349m	1716s	1648s	1629s	—	—	3197s
C29	3406m	3291m	1675s	n.o.	1580m	1614s	1283s	3180s
C30	3407m	3303m	1656s	n.o.	1518s	1615s	1278s	3157m
C31	3471s	3362s	1698m	n.o.	1577s	1615s	1308s	3254w
C32	3469s	3329s	1663s	n.o.	1581s	1618s	1293m	3213s

m = medium; s = strong; w = weak; n.o. = not observed.



Scheme 2. Numbering scheme for ISABH.

deficiency. Four doublets at 6.97, 7.60, 7.46 and 6.85 ppm each integrating for one proton are assigned to C2H, C5H, C13H and C10H, respectively. Four triplets at 7.28, 6.63, 7.11 and 7.38 ppm are attributed to C3H, C4H, C11H and C12H, respectively. The ^{13}C NMR spectrum of ISABH shows 15 signals corresponding to the total number of carbon atoms present in **L**. Thus the ^{1}H NMR and ^{13}C NMR analyses indicate the formation of the ligand. Carbon signals due to carbonyl carbon C7, azomethine carbon C8 and lactonyl carbon C15 are observed at 165.72, 137.89 and 163.91 ppm, respectively. Remaining aromatic carbons are observed in the anticipated region.

The ^{1}H NMR spectrum of Zn(II) complex shows the disappearance of N2H indicating the deprotonation of N2H proton via enolization and supporting the coordination of the ligand in the enol form. N1H has shifted upfield by 0.28 ppm indicating the breakdown of intramolecular bonding between N1H_2 proton and carbonyl oxygen on complexation. C2H and C5H show downfield shifts of 0.53 and 0.11 ppm due to coordination through carbonyl oxygen. No considerable change is observed in the chemical shifts of remaining aromatic protons. In the ^{13}C NMR spectrum of Zn(II) complex, signals corresponding to carbonyl carbon C7, lactam carbon C15 and azomethine carbon C8 have shifted downfield to 13.64, 3.05 and 4.94 ppm indicating coordination through carbonyl oxygen, lactam oxygen and azomethine nitrogen.

Electronic spectral studies and magnetic properties

The electronic spectrum of the ligand shows two prominent absorptions at 401 and 708 nm. The band at 401 nm correspond to the azomethine group and the broad band at 708 nm corresponds to the $n \rightarrow \pi^*$ transition from amide oxygen of isatin to azomethine group.[28,29] The spectra of all the complexes show $\pi \rightarrow \pi^*$ transitions in the range 372–390 nm. In addition, they also display bands at 455, 446, 455 and 462 nm in the case of Co(II), Ni(II), Cu(II) and Zn(II) complexes, respectively, and are assigned to ligand-to-metal charge transfer transitions. A broad band around 870–894 nm appearing as an envelope in the spectrum of the Cu(II) complex is assigned to $^{2}E_g \rightarrow {}^{2}T_{2g}$ transition which reveals octahedral geometry.[28,29] The Ni(II) complex exhibits an

Table 3. ^{13}C NMR and ^{1}H NMR chemical shifts (ppm) of ISABH and its Zn(II) complex

Position	^{13}C NMR		^{1}H NMR	
	ISABH	Zn(II) complex	ISABH	Zn(II) complex
C1	152.05	151.22	—	—
C2	112.01	111.83	6.97 (d, 1H) $J_{2,3}$ = 7.6 Hz	7.08 (d, 1H) $J_{2,3}$ = 7.6 Hz
C3	134.44	133.54	7.28 (dd, 1H) $J_{2,3}$ = 7.6 Hz, $J_{3,4}$ = 7.5 Hz	7.22 (dd, 1H) $J_{2,3}$ = 7.6 Hz, $J_{3,4}$ = 7.5 Hz
C4	116.16	116.20	6.63 (dd, 1H) $J_{3,4}$ = 7.5 Hz, $J_{4,5}$ = 7.5 Hz	6.57 (dd, 1H) $J_{3,4}$ = 7.5 Hz, $J_{4,5}$ = 7.5 Hz
C5	121.54	123.10	7.60 (d, 1H) $J_{4,5}$ = 7.5 Hz	8.13 (d, 1H) $J_{4,5}$ = 7.5 Hz
C6	116.16	114.89	—	—
C7	165.72	179.36	—	—
C8	137.89	142.83	—	—
C9	120.89	116.64	—	—
C10	127.98	131.01	7.46 (d, 1H) $J_{10,11}$ = 7.6 Hz	8.05 (d, 1H) $J_{10,11}$ = 7.6 Hz
C11	123.54	125.21	7.11 (dd, 1H) $J_{10,11}$ = 7.6 Hz, $J_{11,12}$ = 7.7 Hz	7.22 (t, 1H) $J_{10,11}$ = 7.6 Hz, $J_{11,12}$ = 7.7 Hz
C12	132.27	133.40	7.38 (dd, 1H) $J_{11,12}$ = 7.7 Hz, $J_{12,13}$ = 7.5 Hz	7.43 (t, 1H) $J_{11,12}$ = 7.7 Hz, $J_{12,13}$ = 7.5 Hz
C13	118.11	113.82	6.85 (d, 1H) $J_{12,13}$ = 7.5 Hz	6.77(d, 1H) $J_{12,13}$ = 7.5 Hz
C14	143.02	131.98	—	—
C15	163.91	166.96	—	—
N1	—		6.74 (s, 2H)	7.02 (s, 2H)
N2	—		13.84 (s, 1H)	n.o.
N4	—		11.32 (s, 1H)	11.22 (s, 1H)

s = singlet, d = doublet, dd = doublet of doublets, n.o. = not observed.

absorption at 613–678 nm and this absorption is observed as a split band, which is evidence of spin–orbit coupling in mixing a spin singlet ^1E_g with $^3T_{1g}(F)$ thereby allowing the spin-forbidden transition to gain intensity from the spin-allowed one. The low intensities of the d–d bands compared with intra-ligand and metal-to-ligand charge transfer absorptions in DMF solution prevent an accurate assignment for the Co(II) complex.

The corrected magnetic moments at room temperature for diamagnetism are given in Table 1. The magnetic moment of the Co(II) complex is 4.72 BM which is greater than the spin-only value (3.46 BM) indicating the high-spin state of the complex and an octahedral geometry.[28] The magnetic moment observed for the Ni(II) complex of 2.84 BM reveals the high-spin state of the complex. The magnetic moment value for the Cu(II) complex is well within the range corresponding to a spin-only value for one unpaired electron with an octahedral geometry and indicating no metal–metal interaction.[30]

EPR spectral studies

The EPR spectra of a polycrystalline sample of Cu(II) complex at room temperature (300 K) and liquid nitrogen temperature (77 K) exhibit similar features with $g_1 = 2.04$, $g_2 = 2.06$ and $g_3 = 2.13$ suggesting a rhombic pattern. The observed g values are less than 2.3 indicating the covalent nature of the metal–ligand bonding.[30,31]

Thermal studies

The Ni(II) complex shows a weight loss of 4.02% (calc. 4.60%) between 50 and 100°C indicating the presence of one uncoordinated water molecule. Weight loss of 9.40% (calc. 9.25%) around 300–350°C corresponds to the loss of one coordinated chlorine atom. The plateau obtained after heating the Ni(II) complex above 900°C corresponds to the formation of stable NiO, and the metal content calculated from this residue (15.08%) tallies with the metal analysis (15.27%). In the case of the Zn(II) complex, the residue weight obtained after heating the complex above 900°C corresponds to the formation of stable ZnO, and the metal content thus calculated (11.03%) is in good agreement with the metal analysis (10.85%). No weight loss is observed in the temperature range 30–200°C, indicating the absence of lattice-held and coordinated solvent molecules. Thus thermal studies support the suggested compositions for the complexes.

Fast atom bombardment mass spectral studies

The fast atom bombardment mass spectra of complexes **C2** and **C4** show peaks with *m/z* values of 391 and 621 corresponding to the molecular weight of the respective complexes. These values are in good agreement with the proposed compositions for the complexes.

Pharmacology

Tryphan blue exclusion method (cell viability test)

The compounds were tested using the short-term *in vitro* cytotoxicity towards EAC cells as a preliminary screening technique of tryphan blue exclusion method (cell viability test) for their cytotoxic potential.[32] This is one of the methods for assessing the cytotoxicity of anticancer compounds. This test is based on the principle that a living cell membrane has the ability to prevent the entry of a dye. Hence, the cells remain unstained and can be easily distinguished from dead cells that take up the dye. The percentage of viable cells was determined. Results for the short-term *in vitro* cytotoxicity of the compounds are shown in Table 4. These preliminary experiments were carried out at nine different concentrations of the compounds. The ligand and all the complexes are found to exhibit higher cytotoxicity at higher concentration. The ligand is found to be moderately active and to exhibit the highest percentage of inhibition (56.55%) at 1200 μg ml^{-1}. All the complexes exhibit higher cytotoxicity compared to the parent ligand. The Ni(II) and Zn(II) complexes (**C2** and **C4**) are found to exhibit moderate activity with highest percentage of inhibition of 57.41 and 66.76%, respectively, at the highest concentration. The Co(II) and Cu(II) complexes show very good activity. The Cu(II) complex exhibits promising activity causing 52.08% cell death at 10 μg ml^{-1} and percentage of inhibition of viable cells increases with increasing concentration. At 1200 μg ml^{-1}, the percentage inhibition is found to be 99.52% which is the highest among all the tested compounds.

The IC_{50} values of all the compounds show that the Cu(II) complex exhibits the highest activity, showing 50% inhibition of EAC cells at 15.35 μg ml^{-1} (Fig. 1). The next highest activity is observed for Co(II) complex with IC_{50} of 32.87 μg ml^{-1}. The order of cytotoxicity is found to be **C3** > **C1** > **C4** > **C2** > **L**.

MTT assay (cell viability test)

The effects of ISABH and its Co(II), Ni(II), Cu(II) and Zn(II) complexes on cell proliferation were tested against various human cancer cell lines, namely human adenocarcinoma HT29, kidney cancer cell line K293 and breast cancer cell line MDA231, at five concentrations, determined following incubation of model cells using the MTT assay.[33] This is a colorimetric determination to test the *in vitro* growth inhibition effect of the test compounds in which MTT is converted into formazan blue by living cells. The assay uses a tetrazolium salt, MTT, to assess cellular metabolism and hence viability. In metabolically active cells, MTT is reduced by the mitochondrial enzyme succinate dehydrogenase with the formation of insoluble purple formazan crystals. These are then solubilized, and the absorbance measured spectrophotometrically at 570 nm.[34] A concentrated stock solution of each test compound was prepared in DMSO and stored at −20°C until required for use. Prior to the

Table 4. Short-term *in vitro* cytotoxicity of compounds towards EAC cells

Compound	Percentage cell death at different concentrations after 3 h									IC_{50} (μg ml^{-1})
	5 μg ml^{-1}	10 μg ml^{-1}	25 μg ml^{-1}	50 μg ml^{-1}	100 μg ml^{-1}	150 μg ml^{-1}	300 μg ml^{-1}	600 μg ml^{-1}	1200 μg ml^{-1}	
L	23.65	32.21	36.63	36.78	42.68	41.78	50.81	49.73	56.55	263.66
C1	27.87	29.15	48.81	61.57	96.42	97.12	95.49	98.31	99.404	32.87
C2	23.17	23.86	24.44	25.64	30.87	46.78	53.68	54.33	57.41	246.96
C3	40.71	52.08	52.30	53.25	94.60	97.48	97.69	98.85	99.52	15.35
C4	27.23	28.05	43.48	45.64	51.36	57.46	60.67	67.78	66.76	82.44

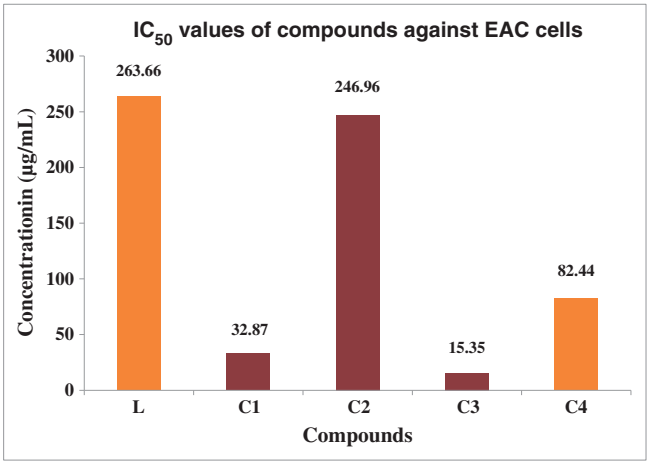


Figure 1. *In vitro* cytotoxicity against EAC cells.

assay, the stock solution was diluted to the required dilutions using blank RPMI-1640 media. The maximum percentage of DMSO present in the wells was 0.5% (v/v) and this was incorporated as a negative control in all the experiments. Compounds were tested against a panel of human tumour cell lines of different origins (breast, kidney) at five concentrations: 10, 25, 50, 100 and 200 $\mu g\ ml^{-1}$ (Table 5).

All the complexes exhibit concentration-dependent cytotoxicity. The ligand is found to exhibit very good activity against all the cancer cell lines and its cytotoxicity against human adenocarcinoma HT29 is significant with 76.49% inhibition. Its activity against kidney cancer K293 is not notable but considerable inhibitory activity is observed against breast cancer MDA231 (67.85%). The IC_{50} of the ligand (Table 6) against HT29, K293 and MDA231 clearly illustrates that the ligand is potent against HT29 with IC_{50} of 41.42 $\mu g\ ml^{-1}$. The Co(II) complex exhibits promising cytotoxicity against HT29 with inhibiting proliferation rate of 91.69% which is also obvious from its IC_{50} of 56.03 $\mu g\ ml^{-1}$. Its cytotoxicity against K293 and MDA231 is found to be poor. All the other complexes are found to be least potent against K293 and MDA231 while they exhibit promising activity against HT29. The Cu(II) complex exhibits noteworthy cytotoxicity against HT29 with IC_{50} of 60.87 $\mu g\ ml^{-1}$. The activity of the Zn(II) complex is lower against all the strains.

Table 6. IC_{50} values of compounds against human cancer cell lines

Compound	IC_{50} ($\mu g\ ml^{-1}$)		
	HT29 (adenocarcinoma)	K293 (kidney cancer cell line)	MDA231 (breast cancer cell line)
L	41.42	261.42	106.34
C1	56.03	307.25	324.18
C2	104.18	160.99	145.93
C3	60.87	192.53	158.06
C4	148.38	570.95	150.34

Table 5. Anticancer activity of test compounds from MTT assay

Compound	Concentration ($\mu g\ ml^{-1}$)	Absorbance (percentage inhibition)		
		HT29 (human adenocarcinoma)	K293 (human kidney cancer cell line)	MDA231 (human breast cancer cell line)
L	200	0.732 (00.00)	0.729 (40.71)	0.152 (67.85)
	100	0.132 (76.49)	0.921 (25.08)	0.198 (58.13)
	50	0.221 (60.68)	1.125 (08.49)	0.261 (44.84)
	25	0.312 (44.42)	1.021 (16.96)	0.351 (25.78)
	10	0.368 (34.40)	1.112 (09.57)	0.398 (15.89)
C1	200	0.047 (91.69)	0.824 (32.99)	0.310 (34.42)
	100	0.167 (70.27)	0.982 (20.15)	0.350 (25.95)
	50	0.256 (54.42)	1.024 (16.73)	0.382 (19.25)
	25	0.324 (42.34)	1.135 (07.75)	0.412 (12.89)
	10	0.422 (24.96)	1.201 (02.33)	0.414 (13.03)
C2	200	0.115 (79.50)	0.526 (57.11)	0.198 (58.07)
	100	0.256 (54.43)	0.719 (41.51)	0.268 (43.27)
	50	0.368 (34.51)	0.985 (19.93)	0.302 (36.15)
	25	0.455 (19.21)	1.027 (16.53)	0.324 (31.50)
	10	0.467 (16.90)	1.125 (08.56)	0.350 (26.01)
C3	200	0.062 (88.95)	0.648 (47.29)	0.202 (57.41)
	100	0.152 (72.22)	0.802 (34.75)	0.285 (39.87)
	50	0.149 (73.52)	0.925 (24.75)	0.332 (29.81)
	25	0.436 (22.41)	1.162 (05.48)	0.362 (23.45)
	10	0.446 (20.61)	1.212 (01.43)	0.389 (17.70)
C4	200	0.231 (58.86)	1.022 (16.97)	0.212 (55.11)
	100	0.322 (42.64)	1.142 (07.10)	0.254 (46.30)
	50	0.368 (34.51)	1.192 (03.04)	0.291 (38.41)
	25	0.456 (18.85)	1.225 (0.439)	0.352 (25.52)
	10	0.524 (06.76)	1.212 (01.43)	0.421 (11.01)
Growth control	—	0.562	1.2299	0.473

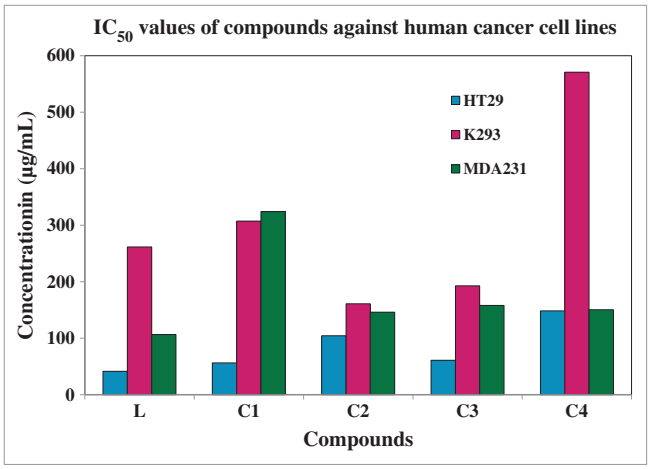


Figure 2. Cytotoxicity of **L** and its transition metal complexes against human cancer cell lines.

Among all the complexes, **C1** and **C3** exhibit impressive potency with IC_{50} of 56.03 and 60.87 $\mu g\ ml^{-1}$ against HT29. For remaining strains **C2** is found to exhibit better activity compared to the other complexes. The ligand is found be a good potential inhibitor of cell proliferation of HT29, K293 and MDA231 cell lines compared to its complexes. The *in vitro* cytotoxicity of the compounds against human cancer cell lines is displayed in Fig. 2 showing their IC_{50} values.

Anticancer drugs exert their action by various mechanisms. The isatin hydrazones and their metal complexes, in particular copper complexes, were reported to exert their action through antiproliferative and pro-apoptotic action. The protein kinases are critical components of signalling pathways in control of cell proliferation of many human cancers. The isatin derivatives selectively inhibit this class of proteins and effects on cell kinase activity, cell proliferation, cell cycle progression and apoptosis. These have been reported as potent inhibitors of vascular endothelial growth factor (VEGF) that stimulates angiogenesis. The oxindole derivatives have been reported to act as angiogenesis inhibitors. Angiogenesis inhibitors stop the growth of blood vessels from surrounding tumour tissues and VEGF exerts its action by binding to cell surface receptors.

Compounds in the present investigation being derivatives of isatin (**L**, **C1–C4**) exhibit their cytotoxicity activity against EAC and human cancer cell lines possibly through the abovementioned mechanisms, i.e. through pro-apoptosis, or through inhibition of protein kinase or through inhibition of cyclin-dependent kinases. Another possible mechanism could be through angiogenesis inhibitory activity. Thus the preliminary cytotoxicity results of ISABH and its transition metal complexes show that these can be a potent class of anticancer agents. Though the mode of action is unclear, it is envisaged that further molecular-level studies could elaborate on their exact mode of action.

Conclusions

The mode of coordination of ISABH in metal complexes is well established from elemental analysis, molar conductivity, IR, NMR and electronic spectral and thermal studies. These studies indicate that the ligand essentially coordinates through carbonyl oxygen of isatin fragment, azomethine nitrogen and carbonyl oxygen of hydrazide fragment via deprotonation and acts as a monobasic tridentate ligand. The mode of coordination in complexes is

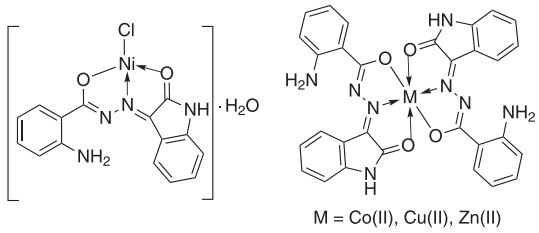


Figure 3. Proposed structure of the complexes.

presented in Fig. 3. The preliminary *in vitro* cytotoxicity activity of the compounds is impressive and further studies are necessary to elucidate their exact mechanism of action.

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