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Original article

New transition metal ion complexes with benzimidazole-5-carboxylic acid hydrazides with antitumor activity

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ABSTRACT

Metal complexes of 2-methyl-1*H*-benzimidazole-5-carboxylic acid hydrazide (**4a**; L_1) and its Schiff base 2-methyl-*N*-(propan-2-ylidene)-1*H*-benzimidazole-5-carbohydrazide (**5a**; L_2) with transition metal ions e.g., copper, silver, nickel, iron and manganese were prepared. The complexes formed were 1:1 or 1:2 M:L complexes and have the structural formulae $[Cu(L_1)Cl(H_2O)]Cl\cdot 3H_2O$ (**6**), $[Ag(L_1)NO_3(H_2O)]$ (**7**), $[Ni(L_1)Cl_2(H_2O)_2]\cdot H_2O$ (**8**), $[Fe(L_1)Cl_3(H_2O)]\cdot 3H_2O$ (**9**) and $[Mn(L_1)_2Cl(H_2O)]Cl\cdot 3H_2O$ (**10**) for ligand L_1, and $[Cu(L_2)Cl_2(H_2O)_2]\cdot H_2O$ (**11**), $[Ag(L_2)_2]NO_3\cdot H_2O$ (**12**), $[Ni(L_2)_2Cl_2]\cdot 5H_2O$ (**13**), $[Fe(L_2)_2Cl_2]Cl\cdot 2H_2O$ (**14**) and $[Mn(L_2)Cl_2(H_2O)_2]\cdot H_2O$ (**15**) for ligand L_2. The antitumor activity of the synthesized compounds has been studied. The silver complex **7** was found to display cytotoxicity ($IC_{50} = 2$ μM) against both human lung cancer cell line A549 and human breast cancer cell line MCF-7.

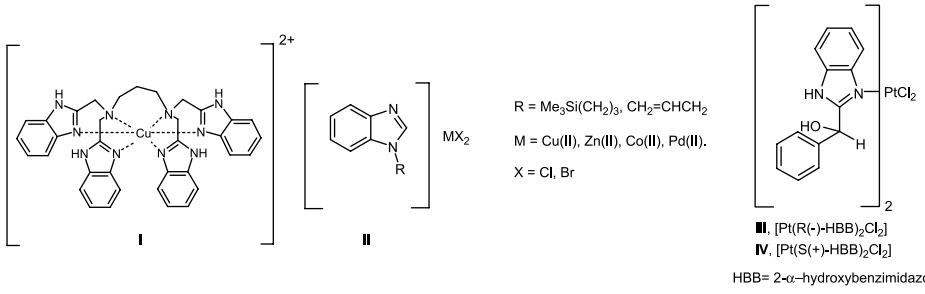
1. Introduction

Benzimidazole ring acts as ligand toward transition metal ions in a variety of biologically important molecules [1].

Cu(II) complex of $CuL(ClO_4)_2$ (L = *N*,*N*,*N*′,*N*′-tetrakis[2-benzimidazolyl)methyl]-1,3-diaminopropane (**I**) can electrostatically bind to the phosphate groups of DNA backbone and partially intercalate into the double helix of DNA [2].

N-Trimethylsilylpropylbenzimidazole (**II**) metal complexes exhibit cytotoxic activity on four monolayer tumor cell lines: MG-22A (mouse hepatoma), HT-1080 (human fibrosarcoma), B16 (mouse melanoma, Neuro 2A (mouse neuroblastoma)) and normal mouse fibroblast cells [3].

It was reported that some of the 2-substituted benzimidazole Pt(II) complexes have in vitro cytotoxic activities on different cancer cell lines. *cis*-[Pt (R(−) and S(+)-HBB)$_2$Cl$_2$] (HBB = 2-α-hydroxybenzylbenzimidazole) **III** and **IV**, respectively, were synthesized and have in vitro cytotoxic activities on the human MCF-7 breast cancer and HeLa cervix cancer cell lines [4–8].



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2. Results and discussion

2.1. Chemistry

2-Methyl-1*H*-benzimidazole-5-carboxylic acid (**1**) was prepared from 3,4-diaminobenzoic acid with acetic acid in hydrochloric acid according to Philips method [9,10]. Esterification of **1** was performed with ethanol and sulfuric acid to obtain the ester **2** which was nitrated to yield the 6(5)-nitro derivative **3**. The absence of the *meta* coupling between the two aromatic protons besides the presence of two singlets in the ^{1}H NMR spectrum gave the evidence of the presence of the nitro group in the 5 or 6 position.

The acid hydrazides **4**(**a**, **b**) were formed by the reaction of **2** or **3** with hydrazine hydrate [11]. Compound **4a** was condensed with acetone to afford the hydrazone **5a** while **4b** condensed with acetone, furan-2-carboxaldehyde, 5-methylfuran-2-carboxaldehyde, pyridine-2-carboxaldehyde, indole-3-carboxaldehyde and 4-methylbenzaldehyde to afford **5**(**b**–**g**), respectively (cf. Scheme 1).

The metal complexes **6**–**10** of the hydrazide **4a** and the metal complexes **11**–**15** of the Schiff base **5a** with $CuCl_2$, $AgNO_3$, $NiCl_2$, $FeCl_3$ and $MnCl_2$, respectively, were synthesized according to the reported methods by Merchán et al. [12] to form 1:1 or 1:2 (metal:ligand) complexes. These complexes were characterized by elemental analyses, IR, ^{1}H NMR (for diamagnetic complexes), electronic and mass spectra, magnetic moments and conductance measurements as well as thermo-gravimetric analyses. Based upon this characterization, the prepared complexes have the structural formulae: $[Cu(L_1)Cl(H_2O)]Cl\cdot 3H_2O$ (**6**), $[Ag(L_1)NO_3(H_2O)]$ (**7**), $[Ni(L_1)Cl_2(H_2O)_2]\cdot H_2O$ (**8**), $[Fe(L_1)Cl_3(H_2O)]\cdot 3H_2O$ (**9**) and $[Mn(L_1)_2Cl(H_2O)]Cl\cdot 3H_2O$ (**10**) for ligand **4a**; L_1 (cf. Scheme 2). On the other hand, $[Cu(L_2)Cl_2(H_2O)_2]\cdot H_2O$ (**11**), $[Ag(L_2)_2]NO_3\cdot H_2O$ (**12**), $[Ni(L_2)_2Cl_2]\cdot 5H_2O$ (**13**), $[Fe(L_2)_2Cl_2]Cl\cdot 2H_2O$ (**14**) and $[Mn(L_2)Cl_2(H_2O)_2]\cdot H_2O$ (**15**) for ligand **5a**; **L_2** (cf. Scheme 3).

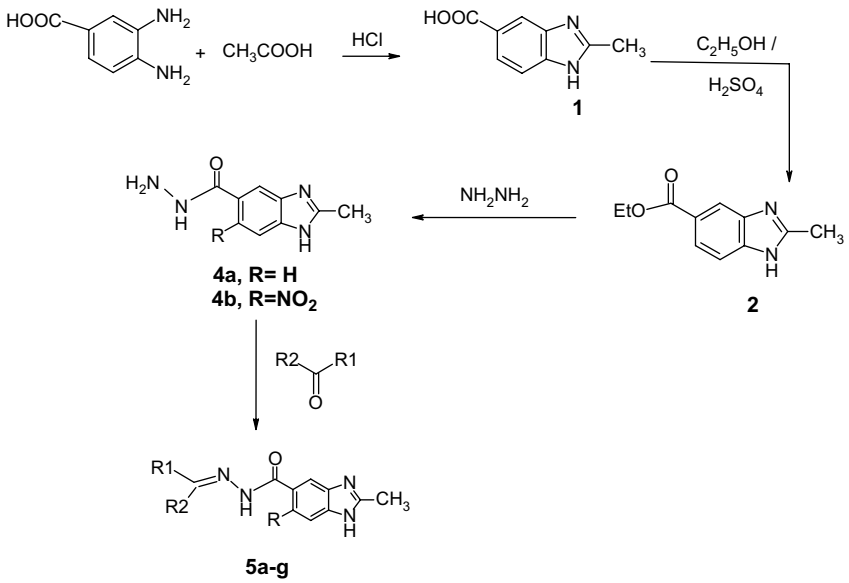
The molar conductance values of the prepared metal complexes **7**–**9**, **11**, **13** and **15** range from 12.3 to 28.9 $\Omega\,cm^2\,mol^{-1}$, which indicated their non-ionic nature [13]. Complexes **6**, **10**, **12** and **14** behaved as ionic compounds which gave molar conductance 67, 78, 65 and 76 $\Omega\,cm^2\,mol^{-1}$, respectively.

The results obtained from thermo-gravimetric analyses [14,15] were in agreement with the suggested theoretical formulae from the elemental analyses. For example, for complexes **7** and **10**, the mass loss in the temperature range 60–145 °C may be due to the loss of hydrated water in complex **10**, while the mass loss in the temperature range from 160 to 244 °C, 145 to 271 °C may be due to the loss of coordinated water molecules in **7** and **10** complexes, respectively.

The two complexes under investigation have two decomposition steps: the first step for **7** and **10** was at 244–401 °C, 271–756 °C, respectively, to form unstable intermediates, then finally decomposition in the range 401–1000 °C, 756–1050 °C, respectively, took place to form Ag and Mn_2O_3. The amounts of residue were in agreement with the calculated values.

For complexes **11**–**13** the mass loss in the temperature range 70–120 °C, 70–110 °C and 70–135 °C may be due to the loss of hydrated water in complexes **11**–**13**, respectively, while at 150–280 °C the mass loss may be due to coordinated water in complex **11**.

Also, the first decomposition took place at 292–439 °C, 208–387 °C and 290–439 °C to form unstable intermediates for **11**–**13**, respectively, finally, the metal residues CuO, Ag_2O and NiO were



5a, R = H, $R_1 = R_2 = CH_3$

5b, R = NO_2, $R_1 = R_2 = CH_3$

5c, R = NO_2, R_1 = H, R_2 = 2-furyl

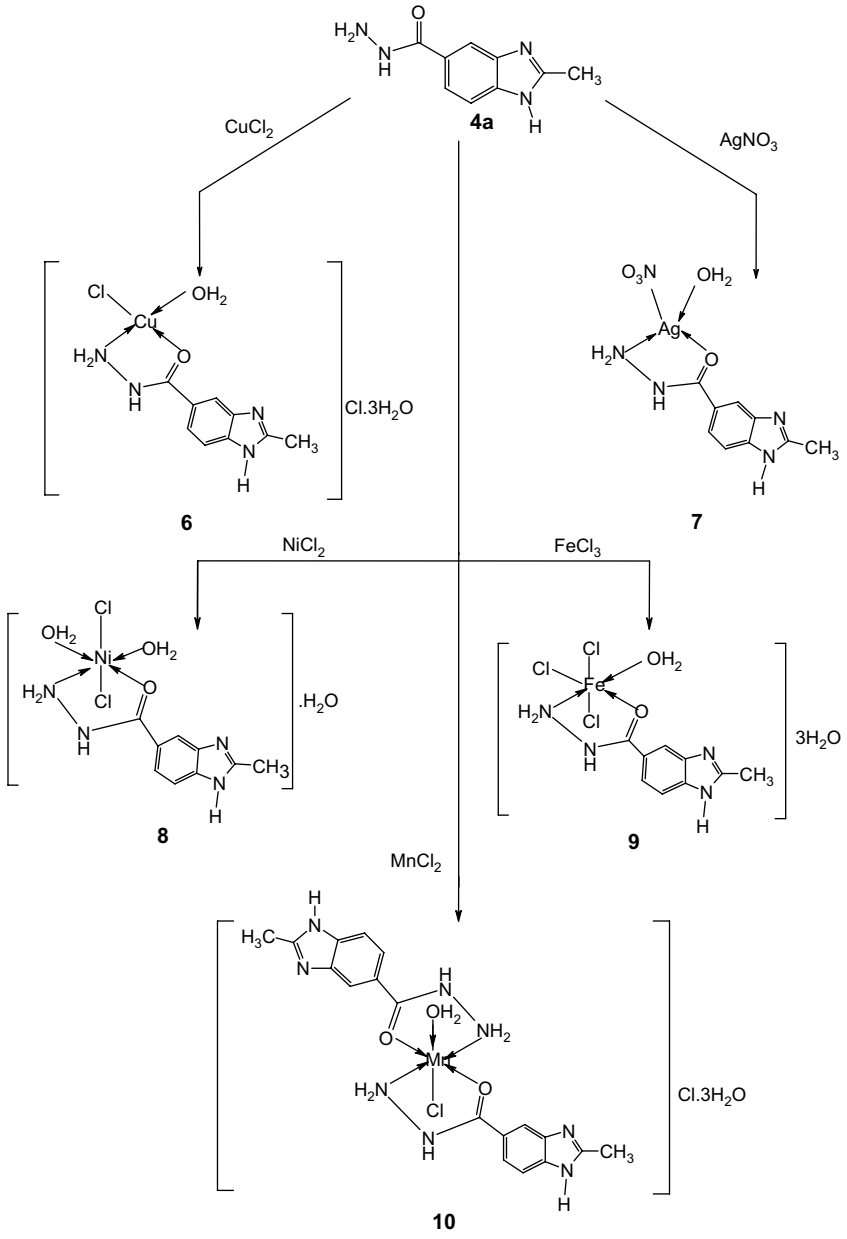
5d, R = NO_2, R_1 = H, R_2 = 5-methyl-2-furyl

5e, R = NO_2, R_1 = H, R_2 = 2-pyridyl

5f, R = NO_2, R_1 = H, R_2 = 3-indolyl

5g, R = NO_2, R_1 = H, R_2 = 4-methylphenyl

Scheme 1.



Scheme 2.

formed at 439–637 °C, 387–580 °C and 439–880 °C, respectively. The amounts of residue were in agreement with the calculated values (cf. Table 1).

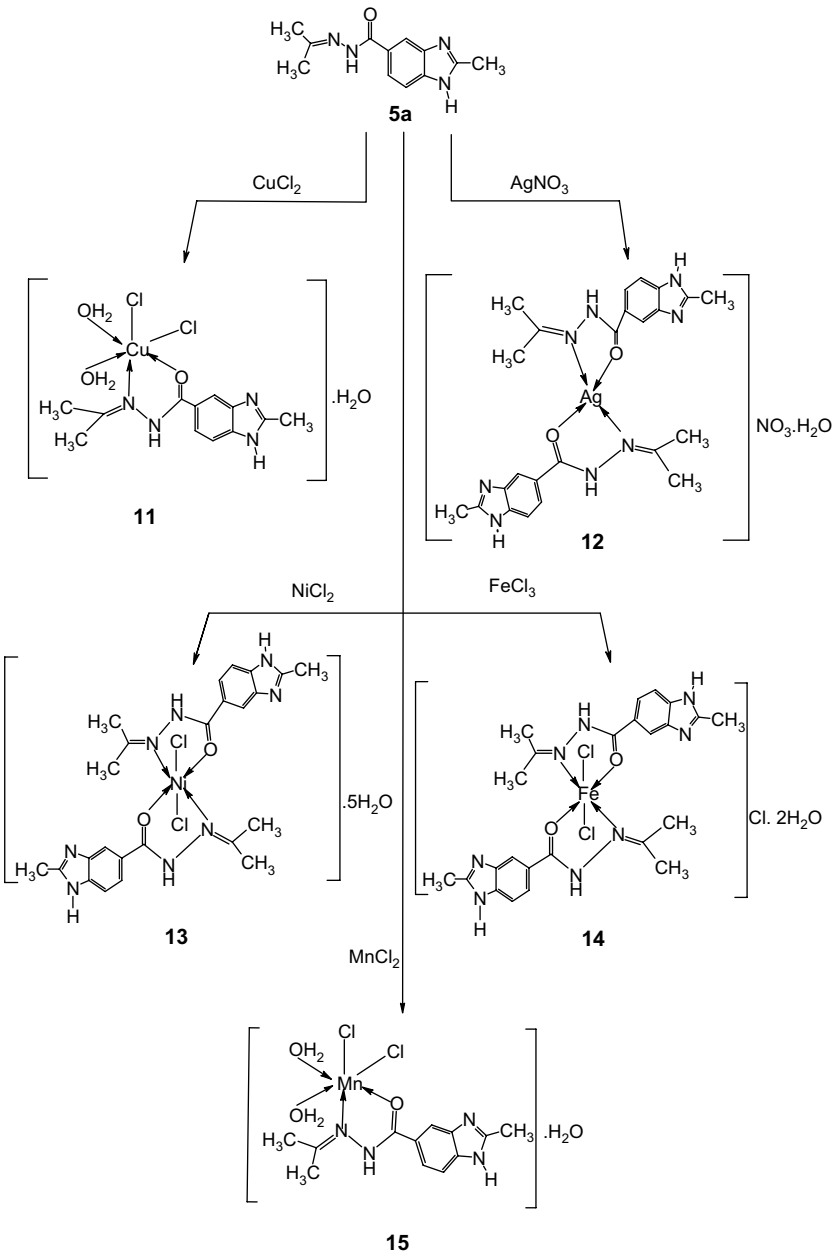
The IR spectra of complexes **6**–**15** showed a broad band in the range of 3587–3386 cm^{-1}, due to ν(OH), suggesting the presence of water molecules. The retention of broad feature at 2800–2600 cm^{-1} and only slight shift of ν(C═N) (ring) in these complexes suggested the involvement of –NH nitrogen (ring) in hydrogen bonding and non-involvement of tertiary nitrogen (ring) in coordination [16–19].

The IR spectra of complexes **6**–**10** showed weakness of broad bands of $\nu(NH_2)$ and considerable lower frequency shift of ν(C═O) indicated the involvement of carbonyl oxygen in coordination. Furthermore the presence of ν(M–N) and ν(M–O) in the FT-IR spectra of these complexes indicated the bidentate nature of the ligand **4a**. For complexes **11**–**15**, the IR spectra exhibited a considerable lower frequency shift of both ν(C═O) and ν(C═N) indicating the involvement of carbonyl oxygen and azomethine nitrogen in coordination [20–22]. The FT-IR spectra of all the complexes exhibited new bands in all the spectra due to the presence of M–N bands and M–O bands for **11**–**15**, metal ion complexes, respectively. Ligand **5a** acted as a bidentate ligand.

The analyses by mass spectrometry at 20 eV for compounds **6**, **7**, **9**, **12** and **14** showed the presence of molecular ion peaks $M^+ + 1$, $M^+ - 2$, M^+, $M^+ + 2$ and $M^+ + 2$, respectively. This behavior confirmed that both ligands are strongly attached to metal ion. In compound **14** the water molecule was lost and peak at $M^+ - H_2O$ was observed. Compounds **8**, **10**, **11** and **15** decomposed without melting and their mass spectra were not recorded.

The presence of molecular ion peaks was consistent with IR spectra of complexes which were in agreement with a strong bonding from ligands toward the metal ions.

The ^{1}H NMR spectra of the diamagnetic complexes **7** and **12** exhibited the presence of only a slight shift of δ (CH_3 of benzimidazole) compared to free ligands in these complexes. The presence of exchangeable proton (NH of ring) signal was in agreement with IR spectra of complexes where imidazole ring wasn't involved in complex formation. The downfield shift of



Scheme 3.

Table 1
Thermo-gravimetric analyses' results of the investigated metal complexes **6**–**15** of ligands **4a** and **5a**

Compound	Structural formula	M:L	Water elimination				Decomposition stages (°C)	Residue (%) (metal oxide or metal)	
			Temperature (°C)	Hydrated water mass loss % Calcd (Found)	Temperature (°C)	Coordinated water mass loss % Calcd (Found)		Temperature (°C)	Calcd (Found)
7	$Ag(L_1)NO_3(H_2O)$	1:1	–	–	160–244	4.77 (3.68)	244–401, 401–1000	1000	28.54 (28.00)
10	$Mn(L_1)_2Cl(H_2O)Cl\cdot 3H_2O$	1:2	60–145	9.36 (9.68)	145–271	3.12 (3.68)	271–756, 756–1050	1050	27.36 (28.12)
11	$[Cu(L_2)Cl_2(H_2O)_2]\cdot H_2O$	1:1	70–120	4.32 (4.50)	150–280	8.63 (8.82)	292–439, 439–637	637	10.99 (10.62)
12	$[Ag(L_2)_2]NO_3\cdot H_2O$	1:2	70–110	2.78 (2.69)	–	–	208–387, 387–580	580	35.76 (35.97)
13	$[Ni(L_2)_2Cl_2]\cdot 5H_2O$	1:2	70–135	13.27 (13.00)	–	–	290–439, 439–880	880	11.01 (10.93)

δ (NH_2 of hydrazide of complex **7**) compared to free ligand was consistent with IR spectra.

The electronic spectrum of complex **4a** exhibited bands at 277, 325 nm and that of complex **5a** showed bands at 260, 280 nm which were assigned to $\pi \rightarrow \pi^*$ and $n-\pi^*$ transitions. Complex **6** had two strong bands at 277, 325 nm assignable to metal–ligand charge transfer (MLCT) and only one band at 790 nm (d–d transition). The magnetic moment at 1.87 B.M. is characteristic of typical square-planar geometry [23]. The diamagnetic complexes such as **7** and **12** displayed strong bands at 260, 320 nm and 270, 285 nm, respectively, indicating a tetrahedral geometry of these complexes. Complexes of **4a** and **5a** showed charge transfer bands at range 255–325 nm. Complexes **11** and **14** displayed bands at 350, 345 nm, respectively. Also, complex **8** displayed three bands at 330, 422 and 830 nm assignable to d–d transitions. Complexes **10**, **13** and **15** did not show the characteristic band in the d–d region. All complexes have magnetic moments in the range 1.68–1.93 B.M. (low spin complexes) characteristic of the octahedral geometry [24] (cf. Table 2).

2.2. Cytotoxicity

The cytotoxicity of the synthetic intermediates **1**–**3**, the ligands **4a**–**b** and **5a**–**g**, and the metal ion complexes **6**–**15** was tested using the AlamarBlue assay in breast cancer cell line MCF-7 and lung cancer cell line A549. For comparison UK-1 and Mitomycin C were also tested (cf. Table 3). All of the synthetic intermediates and ligands were non-cytotoxic at the highest concentration examined (100 μM) against A549 cell line, with the exception of the acyl hydrazone ligand **5g**, which has an IC_{50} of 73 μM. Ligand **5g** was also cytotoxic against MCF-7 cells ($IC_{50} = 3$ μM), as were **5c** ($IC_{50} = 81$ μM) and ethyl ester **2** ($IC_{50} = 62$ μM). The only metal ion complex examined here that displayed cytotoxicity was the silver complex **7**, with IC_{50} values of 2 μM against both A549 and MCF-7 cell lines. However, a number of these metal ion complexes derived from ligand **5a** (e.g., **12**–**15**) were not sufficiently soluble in either DMSO or water to perform cytotoxicity assays. Other complexes could only be evaluated at concentrations less than 25 μM (complex **9**), 20 μM (complexes **6** and **10**), or 7 μM (complex **11**), due to limited solubility.

The pronounced cytotoxicity of silver complex **7**, relative to the free ligand **4a** and the other metal ion complexes derived from **4a** was striking. While a variety of Ag(I) complexes have been identified as potential anticancer agents [25], compound **7** was the first example of a hydrazide-derived Ag(I) complex with cytotoxic activity in human cancer cell lines.

3. Conclusion

IR spectra showed that the ligand **4a** behaved as a bidentate ligand in its 1:1 M:L complexes with $[Cu(L_1)Cl(H_2O)]Cl\cdot 3H_2O$ (**6**), $[Ag(L_1)NO_3(H_2O)]$ (**7**), $[Ni(L_1)Cl_2(H_2O)_2]\cdot H_2O$ (**8**), also with $[Fe(L_1)Cl_3(H_2O)]\cdot 3H_2O$ (**9**) and $[Mn(L_1)_2Cl(H_2O)]Cl\cdot 3H_2O$ (**10**) (1:2 M:L). Also, the ligand **5a** behaved as a bidentate ligand in its 1:1 M:L complexes $[Cu(L_2)Cl_2(H_2O)_2]\cdot H_2O$ (**11**) and $[Mn(L_2)Cl_2(H_2O)_2]\cdot H_2O$ (**15**), respectively, but behaved as bidentate ligand in its 1:2 M:L complexes $[Ag(L_2)_2]NO_3\cdot H_2O$ (**12**), $[Ni(L_2)_2Cl_2]\cdot 5H_2O$ (**13**) and $[Fe(L_2)_2Cl_2]Cl\cdot 2H_2O$ (**14**), respectively. The molar conductance showed the ionic nature of **6**, **10**, **12** and **14**.

Electronic spectra of **8**–**11**, **13**–**15** were in agreement with the octahedral environment around the metal ion, but **7** and **12** were in favor of the tetrahedral one. Moreover, the spectral data together with the magnetic moment showed a square-planar geometry of complex **6**.

Of the complexes examined, the Ag(I) complex **7** displayed the most promising anticancer activity against two human cancer cell lines. The other complexes derived from ligands **4a** and **5a** were not cytotoxic, besides the limited solubility of these complexes limited their evaluation.

4. Experimental

4.1. Physical measurements

Microanalyses of the ligands and complexes were performed in the Microanalytical Laboratory Center, Faculty of Science, Cairo University, Egypt. Molar conductance was measured at room temperature on electronic conductivity model 19000-05 (USA). The IR spectra (4000–400 cm^{-1}) were recorded using KBr pellets in a Jasco FT/IR 300 E Fourier transform infrared spectrophotometer and in the 500–100 cm^{-1} region using polyethylene-sandwiched Nujol mulls on a Perkin Elmer FT-IR 1650 (spectrophotometer).

The ^1H and ^{13}C NMR spectra were recorded using Joel EX-200, 270 and 500 MHz NMR spectrophotometers. The electronic absorption spectra were recorded using a Shimadzu UV-240 UV–visible recording spectrophotometer. The mass spectroscopy was carried out using Finnigan mat SSQ 7000 (Thermo. Inst. Sys. Inc., USA) spectrophotometer at 70 and 20 eV. Thermo-gravimetric analyses were carried out using a DTA-7 and TGA-7 Perkin Elmer 7 series thermal analyses system. The magnetic moments were measured using Gouy method with $Hg[Co(SCN)_4]$ as calibrant.

4.1.1. Preparation of 2-methyl-1H-benzimidazole-5-carboxylic acid (1) [9,10]

To a solution of 3,4-diaminobenzoic acid (40 mmol) in concentrated hydrochloric acid (30 mL) was added glacial acetic acid (60 mmol). The reaction mixture was heated under reflux for 4 h, diluted with water (500 mL) and brought to pH 4.5 with aq. ammonia where the precipitate was formed, it was filtered off and crystallized from ethanol/water as a brown powder. $R_f = 0.43$ (chloroform/ethyl acetate/methanol, 1:1:1/5). Yield: 85%, m.p.

Table 2
Electronic absorption spectral bands and magnetic moment of the ligands **4a**, **5a** and their complexes **6**–**15**

Compound	Ligand and its complexes	Electronic absorption bands, λ_{max} (nm)			Magnetic moment B/M
		Mode	Intraligand and charge transfer bands	d–d Bands	
4a	$L_1 = C_9H_{10}N_4O$	DMSO	277, 325	–	–
5a	$L_2 = C_{12}H_{14}N_4O$	DMSO	260, 280	–	–
6	$[Cu(L_1)Cl(H_2O)]Cl\cdot 3H_2O$	DMSO	275, 320	790	1.87
7	$Ag(L_1)NO_3(H_2O)$	DMSO	260, 275, 320	–	Diamagnetic
8	$[Ni(L_1)Cl_2(H_2O)_2]\cdot H_2O$	DMSO	255, 277	330, 422, 830	2.88
9	$[Fe(L_1)Cl_3(H_2O)]\cdot 3H_2O$	DMSO	260, 277, 325	–	1.68
10	$[Mn(L_1)_2Cl(H_2O)]Cl\cdot 3H_2O$	DMSO	260, 285, 325	–	1.93
11	$[Cu(L_2)Cl_2(H_2O)_2]\cdot H_2O$	DMSO	270, 280, 285	350	1.93
12	$[Ag(L_2)_2]NO_3\cdot H_2O$	DMSO	270, 285	–	Diamagnetic
13	$[Ni(L_2)_2Cl_2]\cdot 5H_2O$	DMSO	265, 285	–	2.87
14	$[Fe(L_2)_2Cl_2]Cl\cdot 2H_2O$	DMSO	260, 290	345	1.63
15	$[Mn(L_2)Cl_2(H_2O)_2]\cdot H_2O$	DMSO	260, 280, 285	–	1.98

Table 3
Cytotoxicity of the synthetic intermediates **1**–**3**, the ligands **4a**–**b** and **5a**–**g**, and the metal ion complexes **6**–**15** against MCF-7 and A549 cell lines

Compound	IC_{50} for MCF-7[a] (μM)	IC_{50} for A549[a] (μM)
1	>100	>100
2	62 ± 18	>100
3	>100	>100
4a	>100	>100
4b	>100	>100
5a	>100	>100
5b	>100	>100
5c	81 ± 12	>100
5d	>100	>100
5e	>100	>100
5f	>100	>100
5g	3 ± 0.3	73 ± 20
6	>20[b]	>20[b]
7	2 ± 0.3	2 ± 1
8	>100	>100
9	>25[c]	>25[c]
10	>20[b]	>20[b]
11	>7[d]	>7[d]
12	nd[e]	nd[e]
13	nd[e]	nd[e]
14	nd[e]	nd[e]
15	nd[e]	nd[e]
UK-1	1.4 ± 0.9	2.0 ± 0.5
Mitomycin C	0.2 ± 0.07	0.4 ± 0.1

[a] Determined using the AlamarBlue assay. See Section 4.2 for details.
[b] Highest concentration tested was 20 μM.
[c] Highest concentration tested was 25 μM.
[d] Highest concentration tested was 7 μM.
[e] Not determined due to limited solubility in DMSO or aqueous solutions.

>300 °C. ^{1}H NMR (270 MHz, DMSO-d_6) δ 2.5 (s, 3H, CH_3), 7.5 (d, J = 8.5 Hz, 1H, H7), 7.8 (d, J = 8.5 Hz, 1H, H6), 8.1 (s, 1H, H4). ^{13}C NMR (DMSO-d_6) δ 14.74 (CH_3), 113.766, 116.372, 122.895, 123.883, 138.797 and 141.978 (Ar–C), 154.11 (C=N), 168.12 (carboxylic C=O). IR (cm^{-1}): 3425.13 (NH), 3200.51–2400.35 (intermolecular H-bonded OH), 1700.02 (carboxylic C=O), 1624.12 (C=N). MS: m/z 176 (M^+, 100%). Anal. Calcd for $C_9H_8N_2O_2$: C, 61.36; H, 4.58; N, 15.90. Found: C, 61.30; H, 4.52; N, 15.86.

4.1.2. Preparation of ethyl 2-methyl-1H-benzimidazole-5-carboxylate (**2**)

Compound **1** (8 mmol) in absolute ethanol (50 mL) and few drops of concentrated sulfuric acid were refluxed for 20 h, diluted with water (500 mL) and brought to pH 7 with $NaHCO_3$ solution (5%). The precipitate formed was filtered off and crystallized from ethanol/water as a buff powder. R_f = 0.76 (petroleum ether/ethyl acetate/methanol, 1:1:1/4). Yield: 79%, m.p. 170–172 °C. ^{1}H NMR (270 MHz, DMSO-d_6) δ 1.35 (t, J = 6.92 Hz, 3H, CH_3 ester), 2.55 (s, 3H, CH_3 benzimidazole), 4.30 (q, J = 6.92, 2H, CH_2 ester), 7.5 (d, J = 8.5 Hz, 1H, H7), 7.75 (d, J = 8.5 Hz, 1H, H6), 8.05 (s, 1H, H4), 12.5 (br, NH, D_2O exchangeable). IR (cm^{-1}): 3468.75 (NH), 2800–2600 (intermolecular H-bonded NH), 1712.94 (C=O), 1626.2 (C=N). MS: m/z 204.1 (M^+, 62%). Anal. Calcd for $C_{11}H_{12}N_2O_2$: C, 64.69; H, 5.92; N, 13.72. Found: C, 64.77; H, 5.88; N, 13.78.

4.1.3. Preparation of ethyl 2-methyl-6-nitro-1H-benzimidazole-5-carboxylate (**3**)

In an ice/NaCl bath, concentrated sulfuric acid (2 mL) was added to compound **2** (5 mmol). The solution was stirred for 30 min, then a mixture of concentrated nitric acid (0.4 mL) and concentrated sulfuric acid (0.6 mL) was added dropwise to this solution. The reaction mixture was stirred for 20 min. The temperature of the reaction was kept within 0–5 °C range during the reaction. The reaction then was brought to pH 6–7 with aqueous ammonia at room temperature. The precipitate formed was filtered off and crystallized from ethyl acetate as a yellow powder. R_f = 0.78 (chloroform/ethyl acetate/methanol, 1:1:1/5). Yield: 98%, m.p. 100–102 °C. ^{1}H NMR (270 MHz, DMSO-d_6) δ 1.35 (t, J = 6.92 Hz, 3H, CH_3 ester), 2.8 (s, 3H, CH_3 benzimidazole), 4.35 (q, J = 6.92 Hz, 2H, CH_2 ester), 8.15 (s, 1H, H4), 8.5 (s, 1H, H7), 9.45 (br, NH, D_2O exchangeable). ^{13}C NMR (270 MHz, DMSO-d_6) δ 13.278 (CH_3 ester), 13.861 (CH_3 benzimidazole), 62.720 (CH_2 ester), 111.546, 115.831, 123.941, 132.767, 133.843 and 145.339 (Ar–C), 157.722 (C=N), 164.537 (carboxylic ester C=O). IR (cm^{-1}): 3453.33 (NH), 1736.92 (C=O), 2800–2600 (intermolecular H-bonded NH), 1629.96 (C=N), 1545.96 and 1383.28 (NO_2). MS: m/z 249 (M^+, 10.05%). Anal. Calcd for $C_{11}H_{11}N_3O_4$: C, 53.01; H, 4.45; N, 16.86. Found: C, 53.13; H, 4.39; N, 16.90.

4.1.4. Preparation of 2-methyl-1H-benzimidazole-5-carbohydrazide (**4a**) and 2-methyl-6-nitro-1H-benzimidazole-5-carbohydrazide (**4b**)

General procedure. Compound **2** or **3** (7 mmol) and hydrazine monohydrate 98% (10 mL) were stirred at room temperature for 15 h. The reaction mixture was then concentrated under reduced pressure and triturated with methanol several times to get rid of the excess hydrazine hydrate, the solid was recrystallized from ethanol as a brown powder.

*4.1.4.1. Compound **4a**.* R_f = 0.44 (petroleum ether/methanol, 2:3). Yield: 80%, m.p. 260–262 °C. ^{1}H NMR (500 MHz, DMSO-d_6) δ 2.45 (s, 3H, CH_3), 4.4 (br, NH_2, D_2O exchangeable), 7.41 (d, J = 8.5 Hz, 1H, H7), 7.59 (d, J = 8.5 Hz, 1H, H6), 7.91 (s, 1H, H4), 9.63 (s, 1H, NH hydrazide, D_2O exchangeable), 10.32 (br, NH benzimidazole, D_2O exchangeable). IR (cm^{-1}): 3410.87 (NH benzimidazole), 3331.11, 3296.87 and 3265 (NH_2, NH), 2800–2600 (intermolecular H-bonded NH), 1666.00 (C=O), 1625.62 (C=N benzimidazole). MS: m/z 190 (M^+, 100%). Anal. Calcd for $C_9H_{10}N_4O$: C, 56.83; H, 5.30; N, 29.46. Found: C, 56.75; H, 5.20; N, 29.35.

*4.1.4.2. Compound **4b**.* R_f = 0.48 (petroleum ether/methanol, 2:3). Yield: 86%, m.p. 188–190 °C. ^{1}H NMR (270 MHz, DMSO-d_6) δ 2.49 (s, 3H, CH_3), 4.46 (s, NH_2 hydrazide, D_2O exchangeable), 7.53 (s, 1H, H4), 8.16 (s, 1H, H7), 8.95 (br, NH benzimidazole, D_2O exchangeable), 9.70 (s, 1H, NH hydrazide, D_2O exchangeable). IR (cm^{-1}): 3406.2 (NH benzimidazole), 3325.4–3265.6 (NH_2, NH), 1677.2 (C=O), 1626.7 (C=N benzimidazole), 1524.2 and 1331.2 (NO_2). MS: m/z 235 (M^+, 12.25%). Anal. Calcd for $C_9H_9N_5O_3$: C, 45.96; H, 3.86; N, 29.78. Found: C, 45.83; H, 3.89; N, 29.74.

4.1.5. Preparation of compounds **5a**–**g**

General procedure. The metal complexes of the studied ligands were prepared by mixing a hot ethanolic solution containing the transition metal ion with the required amount of an ethanolic solution of the corresponding ligands to form 1:1, 1:2 M:L complexes. The reaction mixture was stirred at 50 °C, for a time depending on the transition metal salt used. The resulting precipitates were filtered off, washed with diethyl ether followed by warm ethanol and then dried in a vacuum desiccator over anhydrous $CaCl_2$.

4.1.6. 2-Methyl-N-(propan-2-ylidene)-1H-benzimidazole-5-carbohydrazide (**5a**)

Buff solid. R_f = 0.47 (ethyl acetate/methanol, 4:1/4). Yield: 91%, m.p. 203–205 °C. ^{1}H NMR (200 MHz, DMSO-d_6) δ 1.97 (s, 3H, CH_3 isopropylidine), 2.01 (s, 3H, CH_3 isopropylidine), 2.52 (s, 3H, CH_3 benzimidazole), 7.30 (s, 1H, NH hydrazide, D_2O exchangeable), 7.49 (d, J = 8.5 Hz, 1H, H7), 7.66 (d, J = 8.5 Hz, 1H, H6), 7.99 (s, 1H, H4), 10.28 (br, NH benzimidazole, D_2O exchangeable). IR (cm^{-1}): 3410.72 (NH benzimidazole), 3308.73 (NH hydrazide), 2800–2600 (intermolecular H-bonded NH), 1659.99 (C=O), 1627.82 (C=N benzimidazole), 1619.00 (C=N Schiff base). MS: m/z 230 (M^+, 9%).

Anal. Calcd for $C_{12}H_{14}N_4O$: C, 62.59; H, 6.13; N, 24.33. Found: C, 62.55; H, 6.18; N, 24.27.

*4.1.7. 2-Methyl-6-nitro-*N*-(propan-2-ylidene)-1*H*-benzimidazole-5-carbohydrazide (**5b**)*

Buff solid. $R_f = 0.49$ (ethyl acetate/methanol, 4:1/4). Yield: 92%, m.p. 289–290 °C. ^{1}H NMR (500 MHz, DMSO-d_6) δ 1.85 (s, 3H, CH_3), 1.90 (s, 3H, CH_3), 2.55 (s, 3H, CH_3 benzimidazole), 7.30 (s, 1H, NH hydrazide, D_2O exchangeable), 7.51 (s, 1H, H4), 8.19 (s, 1H, H7), 10.54 (br, NH benzimidazole, D_2O exchangeable). IR (cm^{-1}): 3420.3 (NH benzimidazole), 3343.75 (NH hydrazide), 1670.62 (C=O), 1635.7 (C=N Schiff base), 1627.82 (C=N benzimidazole), 1524 and 1329.7 (NO_2). MS: *m*/*z* 275 (M^+, 12%). Anal. Calcd for $C_{12}H_{13}N_5O_3$: C, 52.36; H, 4.76; N, 25.44. Found: C, 52.41; H, 4.83; N, 25.35.

4.1.8. N*-((Furan-2-yl)methylene)-2-methyl-6-nitro-1*H*-benzimidazole-5-carbohydrazide (**5c**)*

Green solid. $R_f = 0.46$ (ethyl acetate/methanol, 4:1/4). Yield: 85%, m.p. 285–287 °C. ^{1}H NMR (500 MHz, DMSO-d_6) δ 2.45 (s, 3H, CH_3 benzimidazole), 6.3 (m, 1H, H4′ furan), 6.71 (m, 1H, H3′ furan), 7.2 (m, 1H, H5′ furan), 7.53 (s, 1H, NH hydrazide, D_2O exchangeable), 7.75 (s, 1H, CH=N), 8.03 (s, 1H, H4 benzimidazole), 8.61 (s, 1H, H7 benzimidazole), 9.34 (br, NH benzimidazole, D_2O exchangeable). IR (cm^{-1}): 3437.50 (NH benzimidazole), 3201.87 (NH hydrazide), 1664.07 (C=O), 1630.7 (C=N Schiff base), 1625.86 (C=N benzimidazole), 1518.25 and 1331.93 (NO_2). MS: *m*/*z* 312 ($M^+ - 1$, 50%). Anal. Calcd for $C_{14}H_{11}N_5O_4$: C, 53.68; H, 3.54; N, 22.36. Found: C, 53.60; H, 3.50; N, 22.31.

*4.1.9. 2-Methyl-*N*-(5-methylfuran-2-yl)methylene-6-nitro-1*H*-benzimidazole-5-carbohydrazide (**5d**)*

Green solid. $R_f = 0.44$ (ethyl acetate/methanol, 4:1/4). Yield: 93%, m.p. 159–161 °C. ^{1}H NMR (500 MHz, DMSO-d_6) δ 2.09 (s, 3H, CH_3 furan), 2.49 (s, 3H, CH_3 benzimidazole), 6.03 (d, $J = 8.1$ Hz, 1H, H4′ furan), 6.44 (d, $J = 8.1$ Hz, 1H, H3′ furan), 7.50 (s, 1H, NH hydrazide, D_2O exchangeable), 7.65 (s, 1H, CH=N), 7.71 (s, 1H, H4 benzimidazole), 8.28 (s, 1H, H7 benzimidazole), 9.36 (br, NH benzimidazole, D_2O exchangeable). IR (cm^{-1}): 3421.87 (NH benzimidazole), 3328.12 (NH hydrazide), 1653.18 (C=O), 1633.98 (C=N Schiff base), 1625.86 (C=N benzimidazole), 1521.45 and 1337.45 (NO_2). MS: *m*/*z* 327 (M^+, 10%). Anal. Calcd for $C_{15}H_{13}N_5O_4$: C, 55.05; H, 4.00; N, 21.40. Found: C, 55.08; H, 4.12; N, 21.30.

*4.1.10. 2-Methyl-6-nitro-*N*-((pyridin-2-yl)methylene)-1*H*-benzimidazole-5-carbohydrazide (**5e**)*

Yellow solid. $R_f = 0.43$ (ethyl acetate/methanol, 4:1/4). Yield: 83%, m.p. 230–232 °C. ^{1}H NMR (500 MHz, DMSO-d_6) δ 2.43 (s, 3H, CH_3 benzimidazole), 7.32 (s, 1H, NH hydrazide, D_2O exchangeable), 7.60 (s, 1H, CH=N), 7.65 (m, 1H, H5′ pyrimidine), 7.71 (s, 1H, H4 benzimidazole), 7.86 (m, 1H, H4′ pyrimidine), 8.00 (m, 1H, H3′ pyrimidine), 8.28 (s, 1H, H7 benzimidazole), 8.95 (m, 1H, H6′ pyrimidine), 9.53 (br, NH benzimidazole, D_2O exchangeable). IR (cm^{-1}): 3418.33 (NH benzimidazole), 3223.38 (NH hydrazide), 1650.77 (C=O), 1632.98 (C=N Schiff base), 1625 (C=N), 1518.91 and 1384.32 (NO_2). MS: *m*/*z* 324 (M^+, 20%). Anal. Calcd for $C_{15}H_{12}N_6O_3$: C, 55.55; H, 3.73; N, 25.91. Found: C, 55.60; H, 3.82; N, 25.79.

4.1.11. N*-((1*H*-Indol-3-yl)methylene)-2-methyl-6-nitro-1*H*-benzimidazole-5-carbohydrazide (**5f**)*

Yellow solid. $R_f = 0.42$ (ethyl acetate/methanol, 4:1/4). Yield: 87%, m.p. 268–270 °C. ^{1}H NMR (270 MHz, DMSO-d_6) δ 2.59 (s, 3H, CH_3), 7.18–7.23 (m, 2H, H5′ + H6′ indole), 7.28–7.31 (m, 2H, H4′ + H7′ indole), 7.66 (s, 1H, H4 benzimidazole), 7.67 (d, $J = 2.14$ Hz, 1H, H2′ indole), 7.77 (s, 1H, NH hydrazide, D_2O exchangeable), 7.83 (d, $J = 2.14$ Hz, 1H, CH=N), 8.12 (s, 1H, H7 benzimidazole), 12.14 and 13.05 (br, NH benzimidazole, indole, D_2O exchangeable). IR (cm^{-1}): 3489.37, 3431.38, 3359.37 (NH benzimidazole, indole, hydrazide), 1660.44 (C=O), 1640.62 (C=N Schiff base), 1625 (C=N), 1519.53 and 1334.28 (NO_2). MS: *m*/*z* 362 (M^+, 18%). Anal. Calcd for $C_{18}H_{14}N_6O_3$: C, 59.67; H, 3.89; N, 23.19. Found: C, 59.70; H, 3.99; N, 23.10.

*4.1.12. 2-Methyl-*N*-(4-methylbenzylidine)-6-nitro-1*H*-benzimidazole-5-carbohydrazide (**5g**)*

Green solid. $R_f = 0.46$ (ethyl acetate/methanol, 4:1/4). Yield: 90%, m.p. 140–142 °C. ^{1}H NMR (270 MHz, DMSO-d_6) δ 2.1 (s, 3H, CH_3 phenyl), 2.48 (s, 3H, CH_3 benzimidazole), 6.95 (d, $J = 8.01$ Hz, 2H, H3′ + H5′ phenyl), 7.31 (d, $J = 8.01$ Hz, 2H, H2′ + H6′ phenyl), 7.78 (s, 1H, NH hydrazide, D_2O exchangeable), 8.07 (s, 1H, CH=N), 8.31 (s, 1H, H4 benzimidazole), 8.50 (s, 1H, H7), 9.03 (br, NH benzimidazole, D_2O exchangeable). IR (cm^{-1}): 3486.75 (NH benzimidazole), 3375 (NH hydrazide), 1666.64 (C=O), 1635.62 (C=N Schiff base), 1625 (C=N), 1525.42 and 1333.57 (NO_2). MS: *m*/*z* 337 (M^+, 15%). Anal. Calcd for $C_{17}H_{15}N_5O_3$: C, 60.53; H, 4.48; N, 20.76. Found: C, 60.48; H, 4.40; N, 20.80.

*4.1.13. Preparation of the metal complexes (**6**–**15**)*

General procedure. A solution of the metal ion in ethanol was added to a solution of the corresponding ligand in ethanol to form 1:1 or 1:2 M:L complexes. The reaction mixture was stirred at 50 °C, for a time depending on the transition metal salt used. The resulting precipitates were filtered off, washed with diethyl ether followed by warm ethanol. The formed complexes were kept under dry conditions.

*4.1.14. Cu–L_1 complex [Cu(L_1)Cl(H_2O)]Cl·3H_2O (**6**)*

Green solid. Yield: 65%, m.p. 286–288 °C, molar conductance Λ_m (Ω^{-1} cm^2 mol^{-1}) = 67. IR (cm^{-1}): 3580–3300 (OH water molecules, NH benzimidazole), 3307.61–3155.80 (NH_2, NH hydrazide), 2800–2600 (intermolecular H-bonded NH), 1626.59 (C=N benzimidazole), 1608.00 (C=O coordinated). FT-IR (cm^{-1}): 691 (Cu–N), 691 (Cu–O). MS: *m*/*z* 396 ($M^+ + 1$). Anal. Calcd for $C_9H_{18}Cl_2CuN_4O_5$: C, 27.25; H, 4.57; Cl, 17.87; Cu, 16.02; N, 14.12. Found: C, 27.21; H, 4.41; Cl, 17.91; Cu, 15.99; N, 14.09.

*4.1.15. Ag–L_1 complex [Ag(L_1)NO_3(H_2O)] (**7**)*

Pale brown solid. Yield: 75%, m.p. 234–235 °C, molar conductance Λ_m (Ω^{-1} cm^2 mol^{-1}) = 23. ^{1}H NMR (500 MHz, DMSO-d_6) δ 2.7 (s, 3H, CH_3), 3.66 (br, NH_2, D_2O exchangeable), 7.66 (d, $J = 8.5$ Hz, 1H, H7), 7.79 (d, $J = 8.5$ Hz, 1H, H6), 8.3 (s, 1H, H4), 9.63 (s, 1H, NH hydrazide, D_2O exchangeable), 10.3 (br, NH, D_2O exchangeable). IR (cm^{-1}): 3587–3386 (OH water molecules, NH benzimidazole), 3307.61–3143.12 (NH_2, NH hydrazide), 2800–2600 (intermolecular H-bonded NH), 1648.00 (C=O coordinated), 1625.36 (C=N). FT-IR (cm^{-1}): 360 (Ag–N), 315 (Ag–O). MS: *m*/*z* 375 ($M^+ - 2$). Anal. Calcd for $C_9H_{12}AgN_5O_5$: C, 28.59; H, 3.20; Ag, 28.53; N, 18.52. Found: C, 28.52; H, 3.17; Ag, 28.47; N, 18.49.

*4.1.16. Ni–L_1 complex [Ni(L_1)Cl_2(H_2O)$_2$]·H_2O (**8**)*

Dark brown solid. Yield: 82%, m.p. >300 °C (decomposed without melting), molar conductance Λ_m (Ω^{-1} cm^2 mol^{-1}) = 8.7. IR (cm^{-1}): 3570–3350 (OH water molecules, NH benzimidazole), 3300–3164.12 (NH_2, NH hydrazide), 2800–2600 (intermolecular H-bonded NH), 1630.00 (C=O coordinated), 1625.37 (C=N). FT-IR (cm^{-1}): 713 (Ni–N), 554 (Ni–O). Anal. Calcd for $C_9H_{16}Cl_2N_4NiO_4$: C, 28.91; H, 4.31; Cl, 18.97; N, 14.99; Ni, 15.70. Found: C, 28.80; H, 4.33; Cl, 18.85; N, 14.82; Ni, 15.79.

*4.1.17. Fe–L_1 complex [Fe(L_1)Cl_3(H_2O)]·3H_2O (**9**)*

Yellow solid. Yield: 71%, m.p. 218–219 °C, molar conductance Λ_m (Ω^{-1} cm^2 mol^{-1}) = 28. IR (cm^{-1}): 3592–3375 (OH water molecules,

NH benzimidazole), 3203.12–3155.80 (NH_2, NH hydrazide), 2800–2600 (intermolecular H-bonded NH), 1625 (C=N), 1613.12 (C=O coordinated). FT-IR (cm^{-1}): 500 (Fe–N), 485 (Fe–O). MS: *m/z* 425 ($M^+ + 2$). Anal. Calcd for $C_9H_{18}Cl_3FeN_4O_5$: C, 25.47; H, 4.27; Cl, 25.06; Fe, 13.16; N, 13.20. Found: C, 25.43; H, 4.37; Cl, 25.00; Fe, 13.29; N, 13.11.

*4.1.18. Mn–L_1 complex $[Mn(L_1)_2Cl(H_2O)]Cl\cdot 3H_2O$ (**10**)*

Buff solid. Yield: 63%, m.p. >300 °C (decomposed without melting), molar conductance Λ_m ($\Omega^{-1}\,cm^2\,mol^{-1}$) = 78. IR ($cm^{-1}$): 3587–3386 (OH water molecules, NH benzimidazole), 3287–3146.25 (NH_2, NH hydrazide), 2800–2600 (intermolecular H-bonded NH), 1639.76 (C=O coordinated), 1625.00 (C=N). FT-IR (cm^{-1}): 465 (Mn–N), 385 (Mn–O). Anal. Calcd for $C_{18}H_{28}Cl_2MnN_8O_6$: C, 37.38; H, 4.88; Cl, 12.26; Mn, 9.50; N, 19.38. Found: C, 37.45; H, 4.92; Cl, 12.31; Mn, 9.40; N, 19.35.

*4.1.19. Cu–L_2 complex $[Cu(L_2)Cl_2(H_2O)_2]\cdot H_2O$ (**11**)*

Dark brown solid. Yield: 65%, m.p. 276–278 °C (decomposed without melting), molar conductance Λ_m ($\Omega^{-1}\,cm^2\,mol^{-1}$) = 15.8. IR ($cm^{-1}$): 3589–3310 (OH water molecules, NH benzimidazole, hydrazide), 2800–2600 (intermolecular H-bonded NH), 1635.86 (C=O coordinated), 1625.37 (C=N benzimidazole), 1578.00 (C=N Schiff base). FT-IR (cm^{-1}): 711 (Cu–N), 563 (Cu–O). Anal. Calcd for $C_{12}H_{20}Cl_2CuN_4O_4$: C, 34.42; H, 4.81; Cl, 16.93; Cu, 15.17; N, 13.38. Found: C, 34.41; H, 4.76; Cl, 16.91; Cu, 15.29; N, 13.29.

*4.1.20. Ag–L_2 complex $[Ag(L_2)_2]NO_3\cdot H_2O$ (**12**)*

Pale brown solid. Yield: 60%, m.p. 219–220 °C, molar conductance Λ_m ($\Omega^{-1}\,cm^2\,mol^{-1}$) = 65. ^1H NMR (200 MHz, DMSO-d_6) δ 2.10 (s, 6H, $2CH_3$ isopropylidine), 2.14 (s, 6H, $2CH_3$ isopropylidine), 2.73 (s, 6H, $2CH_3$ benzimidazole), 7.40 (br, NH hydrazide, D_2O exchangeable), 7.75 (d, J = 8.5 Hz, 2H, H7 + H7′), 7.90 (d, J = 8.5 Hz, 2H, H6 + H6′), 8.45 (s, 2H, H4 + H4′), 10.35 (br, NH benzimidazole, D_2O exchangeable). IR (cm^{-1}): 3580–3377 (OH water molecules, NH benzimidazole), 3303.00 (NH hydrazide), 2800–2600 (intermolecular H-bonded NH), 1639.62 (C=O coordinated), 1627.39 (C=N benzimidazole), 1577.37 (C=N Schiff base). FT-IR (cm^{-1}): 356.86 (Ag–N), 318 (Ag–O). MS: *m/z* 649 ($M^+ + 2$). Anal. Calcd for $C_{24}H_{30}AgN_9O_6$: C, 44.46; H, 4.66; Ag, 16.64; N, 19.44. Found: C, 44.60; H, 4.57; Ag, 16.55; N, 19.40.

*4.1.21. Ni–L_2 complex $[Ni(L_2)_2Cl_2]\cdot 5H_2O$ (**13**)*

Green solid. Yield: 61%, m.p. >300 °C (decomposed without melting), molar conductance Λ_m ($\Omega^{-1}\,cm^2\,mol^{-1}$) = 12.3. IR ($cm^{-1}$): 3570–3350 (OH water molecules, NH benzimidazole), 3305.25 (NH hydrazide), 2800–2600 (intermolecular H-bonded NH), 1638.00 (C=O coordinated), 1625.00 (C=N benzimidazole), 1576.83 (C=N Schiff base). FT-IR (cm^{-1}): 720.12 (Ni–N), 561.85 (Ni–O). Anal. Calcd for $C_{24}H_{38}Cl_2N_8NiO_7$: C, 42.38; H, 5.63; Cl, 10.42; N, 16.47; Ni, 8.63. Found: C, 42.29; H, 5.57; Cl, 10.35; N, 16.41; Ni, 8.60.

*4.1.22. Fe–L_2 complex $[Fe(L_2)_2Cl_2]Cl\cdot 2H_2O$ (**14**)*

Brown solid. Yield: 67%, m.p. >300 °C, molar conductance Λ_m ($\Omega^{-1}\,cm^2\,mol^{-1}$) = 76. IR ($cm^{-1}$): 3590–3366 (OH water molecules, NH benzimidazole), 3238.14 (NH hydrazide), 2800–2600 (intermolecular H-bonded NH), 1638.47 (C=O coordinated), 1625.00 (C=N benzimidazole), 1583.75 (C=N Schiff base). FT-IR (cm^{-1}): 520 (Fe–N), 472 (Fe–O). MS: *m/z* 639 ($M^+ - H_2O$). Anal. Calcd for $C_{24}H_{32}Cl_3FeN_8O_4$: C, 43.76; H, 4.90; Cl, 16.15; Fe, 8.48; N, 17.01. Found: C, 43.70; H, 4.93; Cl, 16.09; Fe, 8.40; N, 17.07.

*4.1.23. Mn–L_2 complex $[Mn(L_2)Cl_2(H_2O)_2]\cdot H_2O$ (**15**)*

Brown solid. Yield: 59%, m.p. >300 °C (decomposed without melting), molar conductance Λ_m ($\Omega^{-1}\,cm^2\,mol^{-1}$) = 26. IR ($cm^{-1}$): 3587–3386 (OH water molecules, NH benzimidazole), 2800–2600 (intermolecular H-bonded NH), 1633.44 (C=O coordinated), 1623.44 (C=N benzimidazole), 1578.12 (C=N Schiff base). FT-IR (cm^{-1}): 450 (Mn–N), 380 (Mn–O). Anal. Calcd for $C_{12}H_{20}Cl_2MnN_4O_4$: C, 35.14; H, 4.91; Cl, 17.29; Mn, 13.39; N, 13.66. Found: C, 35.12; H, 4.81; Cl, 17.11; Mn, 13.44; N, 13.55.

4.2. Cytotoxicity assays

Cell culture cytotoxicity assays were carried out as described previously [26]. Briefly, aliquots of 100 μL cell suspension (1–3 × 10^3 cells) were placed in microtiter plates in an atmosphere of 5% CO_2 at 37 °C. After 24 h, 100 μL of culture media and 2 μL of the compound in DMSO were added to each well in duplicate, and the plates incubated for an additional 72 h at 37 °C. There was no effect on the growth of cells compared to that of cells in culture media alone at this DMSO concentration. Compounds, along with mitomycin C as a positive control, were evaluated at final concentrations ranging from 0.001 to 50 μM.

After the 72 h incubation, culture media were removed from each well, and 200 μL of fresh media and 20 μL of AlamarBlue reagent were added, followed by additional 6 h incubation. Cell viability was detected by fluorescent intensity using a Beckman Coulter DTX880 plate reader with excitation at 530 nm and emission at 590 nm.

The fluorescence data obtained from the cytotoxicity studies were used to calculate the percent growth according to the following equation:

$$\%\ \text{Growth} = 100 \times (F_{\text{test}} - F_{\text{time0}})/(F_{\text{ctrl}} - F_{\text{time0}}) \quad (1)$$

where mean F_{time0} = the averaged measured fluorescent intensities of AlamarBlue reagent at the time just before the exposure of the cells to the test substance, mean F_{test} = the averaged measured fluorescent intensities of AlamarBlue reagent after 72 h exposure of the cells to the test substance at a particular concentration, mean F_{ctrl} = the averaged measured fluorescent intensities of AlamarBlue reagent after 72 h exposure of the cells to the vehicle without the test substance.

The IC_{50}, the compound concentration for which the growth of treated cells from time 0 was only 50% as much as the vehicle-control was determined by non-linear regression fitting the data to equation [2]:

$$y = \min + (\max - \min)/\left(1 + 10^{((x - \log IC_{50}) \times n)}\right) \quad (2)$$

where min = the minimum response plateau (0% growth), max = the maximum response plateau (100% growth), y = % growth at each test compound concentration, and n is a fitted parameter (the Hill slope coefficient).

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Appendix Supplementary data

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.ejmech.2008.07.013.

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