Journal of Inorganic Biochemistry 104 (2010) 379–384

Contents lists available at ScienceDirect

Journal of Inorganic Biochemistry

journal homepage: www.elsevier.com/locate/jinorgbio

Syntheses, characterization and antitumor activities of transition metal complexes with isoflavone

Xiang Chen, Li-Jun Tang, Yu-Na Sun, Pei-Hong Qiu *, Guang Liang

School of Pharmacy, Wenzhou Medical College, Wenzhou 325 035, China

ARTICLE INFO

Article history:
Received 13 July 2009
Received in revised form 17 November 2009
Accepted 18 November 2009
Available online 26 November 2009

Keywords:
Isoflavone
Metal complexes
Antitumor activities
Flow cytometry

ABSTRACT

Five new complexes were synthesized by the reaction of 4′-methoxy-5,7-dihydroxy-isoflavone ligand (**a**) with transition metal ions zinc (Zn^{2+}) (complex **b**), manganese (Mn^{2+}) (complex **c**), copper (Cu^{2+}) (complex **d**), cobalt (Co^{2+}) (complex **e**) and nickel (Ni^{2+}) (complex **f**). The composition of the complexes has been characterized by elemental analysis, IR, mass spectrometry (MS) and ^1H NMR spectrometric techniques. Their antitumor properties were evaluated against five human cancer cell lines using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and flow cytometry. The results indicated that the complexes possess higher growth inhibitory effects than free isoflavone and corresponding metal ions. Complex **c** and **f** showed greater antitumor activity and selectivity than other described complexes, even more effective than the positive control cisplatin against the selected cell lines. In addition, DNA flow cytometric analysis demonstrated that complexes display a significant G_2/M phase arrest, which then progressed to early apoptosis as detected by flow cytometry after double-staining with annexin V and propidium iodide (PI).

1. Introduction

With the discovery of cisplatin in 1965 by Rosenberg et al. [1], numerous platinum complexes were synthesized and their applications as antineoplastic agents were established, whereas the impressive clinical effectiveness of cisplatin is limited by significant side effects including nephrotoxicity, emetogenesis, neurotoxicity and the emergence of drug resistance [2–4]. Since then numerous non-platinum metal complexes were studied, including ruthenium, iron, and cobalt complexes with different ligands [5–8].

Flavonoids are important natural anti-oxidant and have been extensively studied because of their numerous biological activities [9–13]. Most of flavonoids are good metal chelators which can chelate many metal ions to form different complexes due to their high superdelocalizability and conjugated system. It has been extensively reported that the interaction of metals with flavonoids contributes to the anti-oxidant activity [14–23].

Isoflavones belong to one of subclasses of flavonoids and have been shown extensive biological activities with low toxicity, such as antiviral, antiinflammatory, anti-oxidant and antitumoral actions [24–26]. Several researchers has reported the interaction of metals with some isoflavones [27,28], while the composition characterized and the biological activities of these complexes have been not described. The aim of this study was to obtain Zn^{2+}, Mn^{2+}, Cu^{2+}, Co^{2+} and Ni^{2+} complexes (**b**, **c**, **d**, **e**, **f**) with the 4′-methoxy-5,7-dihydroxy-isoflavone (**a**). All compounds were tested against human lung cancer A549 cells, human adenocarcinoma Hela cells, human hepatoma carcinoma HepG2 cells, human colon carcinoma SW620 cells and human breast carcinoma MDA-MB-435 cells with the aim of assessing the activity and selectivity in the antitumor action and evaluated whether the complexes possess higher anti-proliferative activities than the parent isoflavone to develop promising anti-cancer candidates.

2. Experimental

2.1. Materials and methods

Zinc acetate, manganese acetate, cupric chloride, nickel chloride and cobalt chloride were purchased from standard chemical suppliers and used without further purification. All reagents were of analytical grade. The ligand 4′-methoxy-5,7-dihydroxy-isoflavone, was prepared as previously reported [29].

Melting points were recorded on a XP4 Electrothermal melting point apparatus and are uncorrected. Infrared spectra were measured on a 670 FT-IR spectrophotometer using the KBr pellet technique (4000–400 cm^{-1}). The electrospray mass spectrum, in negative mode (ESI^-), of the complexes dissolved in DMSO:methanol (1:1) were obtained from an Esquire HCT instrument. ^1H NMR spectra were recorded on a Bruker Avance-600 using dimethyl sulfoxide-d_6 (DMSO-d_6) as solvent. Elemental analyses were

* Corresponding author. Tel./fax: +86 577 86699218.
E-mail address: wzqph@163.com (P.-H. Qiu).

0162-0134/$ - see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.jinorgbio.2009.11.008

performed on a PE-2400-II Elemental Analyzer. Molar conductivity (Λ) were measured on DDS-11A model digital conductivity meter based on the measurements designed by Geary [30]. A long-term UV–visible (UV–VIS) study was carried to verify the stability of new complexes in solution.

2.2. Synthesis of ligand 4′-methoxy-5,7-dihydroxy-isoflavone, **a**

4′-Methoxy-5,7-dihydroxy-isoflavone (**a**) was prepared by using the reaction previously described [29,31,32]. Phloroglucinol (12.6 g, 0.1 mol) and *p*-methoxy benzacetonitril (15 mL) in ether (50 mL) were cooled in an ice bath and saturated with a stream of HCl gas (HCl was produced by reacting NaCl and concentrated H_2SO_4). The reaction mixture was refrigerated for 12 h, saturated again with HCl gas, and refrigerated for another 12 h. The precipitate obtained was washed with ether twice and refluxed with 1% aqueous H_2SO_4 (250 mL) for 3 h. A yellow precipitate was formed, filtered off to give the intermediate deoxybenzoin (12.7 g, 46.3%): m.p. 194–196 °C [32].

A mixture of deoxybenzoin (6.8 g, 0.025 mol) and boron trifluoride ether ($BF_3{\cdot}Et_2O$) (20 mL) was cooled to 10 °C and N,N-dimethylformamide (DMF) (60 mL) was added dropwise. In another flask, DMF (60 mL) was cooled to 10 °C and methylsulfonyl chloride ($MeSO_2Cl$) (30 mL) was added in small portions. The mixture was then allowed to stand at 55 °C for 20 min and added to the above reaction mixture slowly. During the addition, the temperature of the reaction mixture was maintained below 27 °C. The mixture was then stirred at room temperature for 4 h. The workup was carried out by pouring the reaction mixture into methanolic HCl (0.1 M) followed by heating at 70 °C for 20 min and extracting the product by acetoacetate after removing the methanol and most of DMF. The organic layer was washed with water and dried with $MgSO_4$. The solvent was removed under vacuum using a rotary evaporator and product was purified by recrystallization.

a: Yield 6.1 g, 86.1%, m.p. 213–215 °C. IR (KBr) υ (cm^{-1}): 3388 (υ_{O-H}), 1653 ($\upsilon_{C=O}$), 1609–1440 ($\upsilon_{C=C}$), 1248 (υ_{C-O-H}), 1175 (υ_{C-O-C}). ^{1}H NMR δ (600 MHz, DMSO-d_6, ppm, s: singlet, d: doublet, t: triplet, m: multiplet): 3.77 (3H, s, OCH_3), 6.21 (1H, d, $^3J_{HH}$ = 2.1 Hz, CH-6), 6.38 (1H, d, $^3J_{HH}$ = 2.1 Hz, CH-8), 6.98 (2H, d, $^3J_{HH}$ = 8.7 Hz, CH-3′, CH-5′), 7.48 (2H, d, $^3J_{HH}$ = 8.7 Hz, CH-2′, CH-6′), 8.36 (1H, s, CH-2), 10.90 (1H, s, OH-5), 12.92 (1H, s, OH-7). EIMS *m*/*z*: 282.7 [M^-].

2.3. Synthesis of complexes

Ligand **a** (0.284 g, 0.001 mol) was dissolved in ethanol (30 mL) adjusted to pH 7–8 by addition of triethylamine, stirring for 1 h at 40 °C followed by hydrated metal (II) salt (acetate or chloride) (0.001 mol) in ethanol (10 mL) which were introduced slowly via a syringe. The reaction mixture were stirred at 60 °C for 12 h, standing at room temperature for 2 days. Powered solids were yielded by filtration, rinsed with ethanol and dried under vacuum.

2.3.1. Zn(a)$_2$, **b**

Yield 0.13 g, 40.3%. m.p. >300 °C. Anal. Calcd. for **b**: $C_{32}H_{22}O_{10}Zn$ (%): C, 60.86; H, 3.49; Zn, 10.30. Found (%): C, 60.56; H, 3.57; Zn, 10.48. IR (KBr) υ (cm^{-1}): 3369 (υ_{O-H}), 1645 ($\upsilon_{C=O}$), 1611–1411 ($\upsilon_{C=C}$), 1280 (υ_{C-O-H}), 1187 (υ_{C-O-C}), 562 (υ_{Zn-O}) cm^{-1}. ^{1}H NMR δ (600 MHz, DMSO-d_6, ppm): 3.77 (6H, s, OCH_3), 6.21 (2H, d, $^3J_{HH}$ = 4.2 Hz, CH-6, CH-L6), 6.37 (2H, d, $^3J_{HH}$ = 4.2 Hz, CH-8, CH-L8), 6.98 (4H, d, $^3J_{HH}$ = 8.3 Hz, CH-3′, CH-5′, CH-L3′, CH-L5′), 7.47 (4H, d, $^3J_{HH}$ = 8.3 Hz, CH-2′, CH-6′, CH-L2′, CH-L6′), 8.35 (2H, s, CH-2, CH-L2), 12.92 (2H, s, OH-7, OH-L7). EIMS calcd. for **b** (*m*/*z*): 631. Found (*m*/*z*): 631.2 [M^-]. Λ = 5.3 S cm^2 mol^{-1}.

2.3.2. Mn(a)$_2$, **c**

Yield 0.13 g, 42.1%. m.p. >300 °C. Anal. calcd. for **c**: $C_{32}H_{22}O_{10}Mn$ (%): C, 61.84; H, 3.54; Mn, 8.86. Found (%): C, 62.16; H, 3.37; Mn, 9.28. IR (KBr) υ (cm^{-1}): 3383 (υ_{O-H}), 1621 ($\upsilon_{C=O}$), 1609–1417 ($\upsilon_{C=C}$), 1248 (υ_{C-O-H}), 1179 (υ_{C-O-C}), 532 (υ_{Mn-O}). ^{1}HNMR δ (600 MHz, DMSO-d_6, ppm): 3.71 (6H, s, OCH_3), 5.32 (2H, d, $^3J_{HH}$ = 4.2 Hz, CH-6, CH-L6), 5.88 (2H, d, $^3J_{HH}$ = 4.2 Hz, CH-8, CH-L8), 6.49 (4H, d, $^3J_{HH}$ = 8.3 Hz, CH-3′, CH-5′, CH-L3′, CH-L5′), 7.48 (4H, d, $^3J_{HH}$ = 8.3 Hz, CH-2′, CH-6′, CH-L2′, CH-L6′), 8.34 (2H, s, CH-2, CH-L2), 11.81 (2H, s, OH-7, OH-L7). EIMS calcd. for **c** (*m*/*z*): 621. Found (*m*/*z*): 619.8 [M^-]. Λ = 4.5 S cm^2 mol^{-1}.

2.3.3. Cu(a)$_2$, **d**

Yield 0.25 g, 80.3%. m.p. >300 °C. Anal. calcd. for **d**: $C_{32}H_{22}O_{10}Cu$ (%): C, 60.95; H, 3.49; Cu, 10.16. Found (%): C, 62.46; H, 3.38; Cu, 9.78. Although the elemental analysis value for "C" is not satisfactory, the chemical formula for this compound, Cu(a)$_2$, **d** is well supported with the other analysis data including IR, ^{1}H NMR and especially EIMS as shown below. IR (KBr) υ (cm^{-1}): 3375 (υ_{O-H}), 1626 ($\upsilon_{C=O}$), 1611–1415 ($\upsilon_{C=C}$), 1256 (υ_{C-O-H}), 1179 (υ_{C-O-C}), 582 (υ_{Cu-O}). ^{1}H NMR δ (600 MHz, DMSO-d_6, ppm): 3.71 (6H, s, OCH_3), 5.81 (2H, d, $^3J_{HH}$ = 4.2 Hz, CH-6, CH-L6), 6.38 (2H, d, $^3J_{HH}$ = 4.2 Hz, CH-8, CH-L8), 6.98 (4H, d, $^3J_{HH}$ = 8.3 Hz, CH-3′, CH-5′, CH-L3′, CH-L5′), 7.48 (4H, d, $^3J_{HH}$ = 8.3 Hz, CH-2′, CH-6′, CH-L2′, CH-L6′), 8.45 (2H, s, CH-2, CH-L2), 12.89 (2H, s, OH-7, OH-L7). EIMS calcd. for **d** (*m*/*z*): 630. Found (*m*/*z*): 628.1 [M^-]. Λ = 3.2 S cm^2 mol^{-1}.

2.3.4. Co(a)$_2$, **e**

Yield 0.21 g, 68.2%. m.p. >300 °C. Anal. calcd. for **e**: $C_{32}H_{22}O_{10}Co$ (%): C, 61.44; H, 3.52; Co, 9.44. Found (%): C, 61.28; H, 3.68; Co, 9.12. IR (KBr) υ (cm^{-1}): 3385 (υ_{O-H}), 1629 ($\upsilon_{C=O}$), 1610–1437 ($\upsilon_{C=C}$), 1246 (υ_{C-O-H}), 1180 (υ_{C-O-C}), 783 (υ_{Co-O}). ^{1}H NMR δ (600 MHz, DMSO-d_6, ppm): 3.71 (6H, s, OCH_3), 5.81 (2H, d, $^3J_{HH}$ = 4.2 Hz, CH-6, CH-L6), 6.08 (4H, d, $^3J_{HH}$ = 8.3 Hz, CH-3′, CH-5′, CH-L3′, CH-L5′), 6.98 (2H, d, $^3J_{HH}$ = 4.2 Hz, CH-8, CH-L8), 7.48 (4H, d, $^3J_{HH}$ = 8.3 Hz, CH-2′, CH-6′, CH-L2′, CH-L6′), 8.65 (2H, s, CH-2, CH-L2), 12.18 (2H, s, OH-7, OH-L7). EIMS calcd. for **e** (*m*/*z*): 625. Found (*m*/*z*): 625.7 [M^-]. Λ = 8.9 S cm^2 mol^{-1}.

2.3.5. Ni(a)$_2$, **f**

Yield 0.24 g, 76.6%. m.p. >300 °C. Anal. calcd. for **f**: $C_{32}H_{22}O_{10}Ni$ (%):C, 61.44; H, 3.52; Ni, 9.44. Found (%): C, 61.96; H, 3.35; Ni, 9.18. IR (KBr) υ (cm^{-1}): 3394 (υ_{O-H}), 1628 ($\upsilon_{C=O}$), 1609–1441 ($\upsilon_{C=C}$), 1245 (υ_{C-O-H}), 1179 (υ_{C-O-C}), 535 (υ_{Ni-O}). ^{1}H NMR δ (600 MHz, DMSO-d_6, ppm): 4.11 (6H, s, OCH_3), 5.31 (2H, d, $^3J_{HH}$ = 4.2 Hz, CH-6, CH-L6), 5.48 (2H, d, $^3J_{HH}$ = 4.2 Hz, CH-8, CH-L8), 7.00 (4H, d, $^3J_{HH}$ = 8.3 Hz, CH-3′, CH-5′, CH-L3′, CH-L5′), 7.48 (4H, d, $^3J_{HH}$ = 8.3 Hz, CH-2′, CH-6′, CH-L2′, CH-L6′), 8.34 (2H, s, CH-2, CH-L2), 12.36 (2H, s, OH-7, OH-L7). EIMS calcd. for **f** (*m*/*z*): 625. Found (*m*/*z*): 623.4 [M^-]. Λ = 9.1 S cm^2 mol^{-1}.

2.4. Biological materials and methods

All reagents were purchased from Sigma and have an analytic purity.

2.4.1. Cell culture

For routine testing, five cancer cell lines were used in this study: A549 (human lung cancer cell line), Hela (human cervix cancer cell line), HepG2 (human hepatoma carcinoma cell line), SW620 (human colon carcinoma cell line) and MDA-MB-435 (human breast carcinoma cell line), purchased from Chinese Academy of Sciences cell bank, China. Cells were grown at 37 °C in a humidified atmosphere containing 5% CO_2, in RPMI 1640 medium supplemented with 10% heat inactivated (56 °C for 30 min) fetal bovine serum (FBS), 100 IU/mL of penicillin and 100 μg/mL of streptomycin.

2.4.2. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

MTT assay was carried out based on the method described by Mosmann [33]. Briefly, cells were seeded in 96-well plates at initial seeding densities of 4000 cells per well with 180 μL of culture media and incubated in an atmosphere of 5% CO_2 for 24 h to allow attachment onto the wells. All compounds were dissolved immediately in DMSO, and diluted with the growth medium. The final concentrations were reached to 600 μM, 300 μM, 100 μM, 50 μM, 10 μM, 1 μM and 0.1 μM, respectively. In addition, the final DMSO concentration in the culture medium, <1%, showed no obvious cytostatic effect in preliminary tests. Twenty microliters of each compound dilution was added into the appropriate wells in three replicates. Culture medium only containing different concentrations of DMSO was added to the cells in the control wells. Culture medium with the corresponding concentrations of compounds, but void of cells was used as blank. Following incubation at 37 °C for 48 h, 20 μL of 0.1% MTT was added into each well. The plates were re-incubated for 4 h and 150 μL DMSO was introduced to dissolve the insoluble blue formazan precipitate produced by MTT reduction. The plates were shaken for 10 min and the optical density of each well was measured at 570 nm on a microplate spectrophotometer (Bio-Tek Instruments, ELx808zu, USA).

Assays were performed in triplicate in three independent experiments. The concentration required for 50% inhibition of cell viability (IC_{50}) was calculated using the software "Dose–Effect Analysis with Microcomputers". Data were analyzed using Student unpaired *t*-test.

2.4.3. Cell cycle analysis

Flow cytometry was used to evaluate the number of cells in the particular phases of the cell cycle. The selected cells (MDA-MB-435, SW620, HepG2 and A549 cells) were seeded in six-well plates containing 5×10^6 cells per well in their respective culture media and pre-incubated for 24 h to induce cell cycle in exponential growth. The cells were then treated with 10 μM of compounds **a** and **c** that were dissolved in DMSO and diluted with growth medium. Growth medium only containing DMSO was used as control. After 24 h incubation, the cells were trypsinised, washed twice in PBS, centrifuged at 800*g* for 5 min at 4 °C, fixed with ice-cold 70% ethanol for 30 min and treated with 50 μg/mL of RNase A at 37 °C for 30 min. To determine their DNA contents, cells were stained with 50 μg/mL propidium iodide (PI) in the dark at 4 °C for 30 min and analyzed using a FACS scan flow cytometer (Elite ESP, Beckman Coulter, Brea, CA).

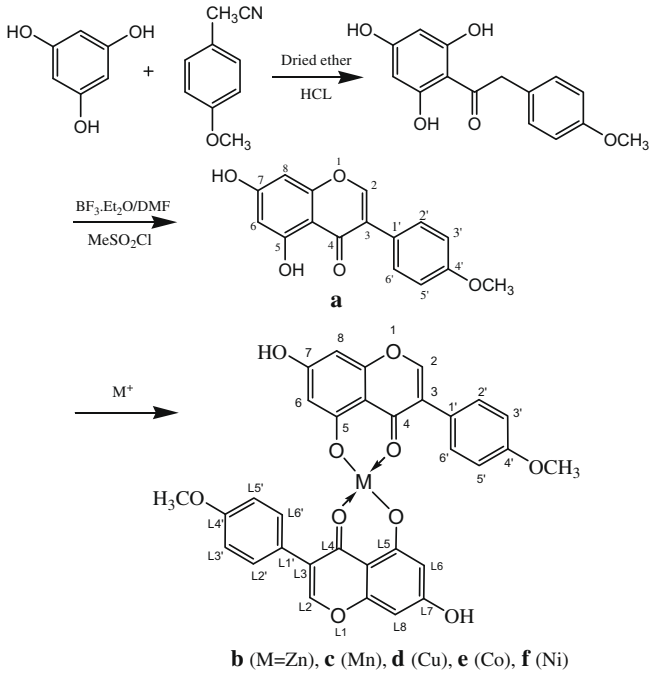
2.4.4. Annexin V and PI binding assay

To assess the simultaneous observation of early phase of apoptotic and necrotic features, SW620 cells were treated with ligand **a** and complex **c** at 10 μM final concentration for 24 h, then cells were measured using flow cytometry by adding annexin V-FITC to 10^6 cells per sample according to the manufacturer's specifications (Bender MedSystems, Vienna, Austria). Simultaneously, the cells were stained with PI.

3. Results and discussion

3.1. Synthesis

Ligand **a** was produced by the reaction of phloroglucinol and *p*-methoxy benzacetonitril, in the presence of $BF_3{\cdot}Et_2O$, DMF and $MeSO_2Cl$ for the synthesis of corresponding Zn^{2+}, Mn^{2+}, Cu^{2+}, Co^{2+} and Ni^{2+} complexes (Scheme 1). The complexes were obtained by titrating an ethanolic solution of hydrated metal(II) salt (chloride or acetate) to an ethanol solution of ligand **a** (pH 7–8). The



Scheme 1. Synthesis of 4′-methoxy-5,7-dihydroxy-isoflavone and Zn^{2+}, Mn^{2+}, Cu^{2+}, Co^{2+} and Ni^{2+} complexes.

complexes were isolated as powder solids in different yields, the purity of which was confirmed by thin-layer chromatography (TLC) and elemental analysis. The results of molar conductivity showed that all complexes were nonelectrolyte in DMSO [30]. In addition, all the complexes were insoluble in water, slightly soluble in methanol and soluble in DMSO or mixture of water/DMSO. The complexes were characterized by IR, ^1H NMR, mass spectroscopies, elemental analysis and melting point determination.

3.2. Spectroscopic data

The IR spectra of the ligand, **a** showed the characteristic absorption for υ (C=O) at 1653 cm^{-1}. The broad band at 3380 cm^{-1}, a strong band at 1248 cm^{-1} and a medium band around 1175 cm^{-1} were assigned to hydrogen bond stretching, phenolic υ(C–O) vibrations and υ (C–O–C), respectively. The bands arising from υ (C=C) were observed at 1440–1621 cm^{-1}. Compared with the IR spectra of ligand **a**, the absorption bands of complexes **b–f** exhibited downward shifts (8–22 cm^{-1}) for the C=O groups and showed extremely slightly shifts for other groups, indicating that coordination occurred *via* oxygen atom of the carbonyl oxygen group. For all the complexes, new bands in the region of 500–600 cm^{-1} detected were assigned to stretching frequencies of υ (M–O).

The ^1H NMR spectrum of synthesized ligand **a** was consistent with the reported data in literature [29]. Resonances of hydrogen atoms belonging to 5,7-hydroxyl groups appeared at 12.92 and 10.90 ppm, respectively. For the corresponding complexes **b–f**, the resonances of the 7,L7-hydroxyl group protons were found at 12.92, 11.81, 12.89, 12.18 and 12.36 ppm, respectively, and the signals of the benzopyran and aromatic protons displayed extremely slightly shifts either in comparison with ligand **a**. However, it is worth to noted that the complete disappearance of signals arising from the 5,L5-hydroxyl group protons was expressed, pointing out that the coordination of oxygen atoms from the 5-hydroxyl group.

All compounds have been investigated by positive/negative mass spectrometric measurements, giving valuable structural information. The major structurally informative molecular ions

Table 1
Main MS data of compounds **a**–**f** (*m*/*z*).

Compd.	Molecular ions (-MS)	Fragment ions (-MS2)
a	282.7	–
b	631.2	593.1, 346.8, 309.0, 282.9
c	619.8	589.4, 336.1, 282.8
d	628.1	612.8, 344.8, 301.0, 282.9
e	625.7	341.7, 284.9
f	623.4	607.0, 590.0, 341.6, 280.8, 262.8

Table 2
Long-term UV–VIS data (λ_{max}) of compounds **a**–**f** in DMSO/water solution during 30 days.

λ_{max} (nm)	Time (days)				
	0	3	7	15	30
a	262	263	262	262	262
b	277	276	276	277	277
c	286	286	284	286	286
d	271	269	269	268	268
e	296	296	296	297	295
f	298	297	291	293	293

and fragment ions in the negative-ion mode of compounds **a**–**f** are presented in Table 1. As expected, the molecular ions peak of complexes **b**–**f** was found at *m*/*z* 631.2 $[M]^-$, 619.8 $[M]^-$, 628.1 $[M]^-$, 625.7 $[M]^-$ and 623.4 $[M]^-$, respectively, indicating that each complex containing one metal ion and two molecule ligands was formed. The appearance of the characteristic cluster of isotopic peaks of the corresponding metal ion isotopes demonstrated that metal ions have been successfully coordinated to the ligand **a**. In addition, the predominant peaks corresponding to ions containing Zn, Mn, Cu, Co and Ni in the complexes are those with metal bonded to one ligand molecule at *m*/*z* 346.8, 336.1, 344.8, 341.7 and 341.6, respectively. The intact negative ion of ligand molecule as a dominant fragment ion appeared at *m*/*z* 283. Complexes **c** and **f** showed ions obtained by loss of a methoxyl, which was observed at *m*/*z* 589.4 and 590.0. It is worth noting that the spectrum of complex **b** showed sodium adduct ion based on addition of sodium ion presumably to oxygen atom from hydroxyl at *m*/*z* 593.1. In complex **d**, the loss of a methyl yielded a fragment ion at *m*/*z* 612.8 and another important fragment ion was that of small abundance obtained by sequential loss of a ligand, a methoxyl as well as a methenyl from the 2-position at *m*/*z* 301.0.

The results of the long-term UV–VIS study are showed in Table 2. The band of ligand **a** was observed at 262 nm. Compared with ligand **a**, the absorption wavelength of new complexes (**b**–**f**) exhibited blue shift. In addition, it is significant to note that the λ_{max} (nm) values of new compounds were hardly changed during one month, meaning that new complexes were stable in solution.

The data of elemental analysis, IR, Ms and ^{1}H NMR spectrum confirmed the bonding of ligand **a** to metal ions and the metals

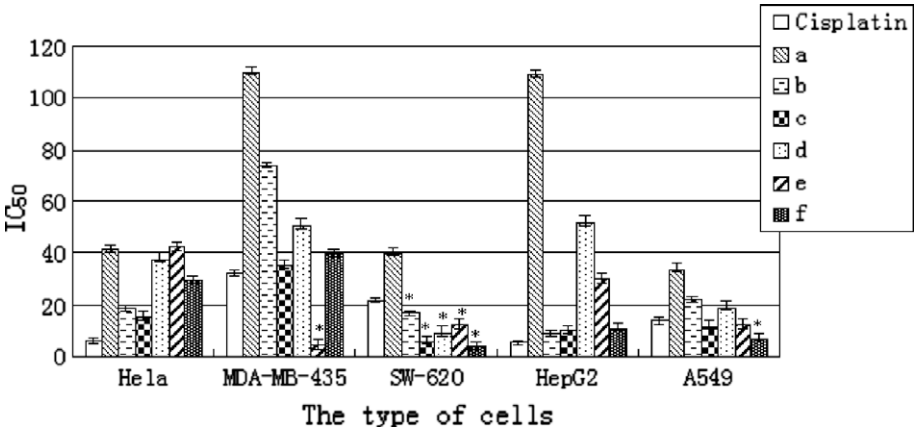


Fig. 1. Five selected human cancer cell lines were incubated in complete cell growth medium with and without different concentrations (600 μM, 300 μM, 100 μM, 50 μM, 10 μM, 1 μM and 0.1 μM) of new compounds (**a**–**f**) for 48 h. The IC_{50} values were calculated by the software "Dose–Effect Analysis with Microcomputers". Horizontal and vertical axes indicate relative cell types and IC_{50} (μM) values, respectively. (*p<0.05, Complexes compared with the ligand **a** and cisplatin.).

Table 3
Cell cycle distribution analyzed by flow cytometry after 24 h treatment with ligand **a** and complex **c** at 10 μM final concentration and the corresponding DMSO control.

Cell line	Compd.	Apoptosis rate (%)	Cell cycle distribution (%)		
			G_1	S	G_2/M
MDA-MB-435	Control	10.2	51.3	30.4	19.1
	Ligand **a**	18.4	63.6	22.5	14.1
	Complex **c**	21.2	81.0	7.2	12.1
SW-620	Control	9.1	53.7	21.4	25.5
	Ligand **a**	28.7	46.2	22.8	31.3
	Complex **c**	62.2	40.9	14.2	45.3
HepG2	Control	4.9	57.9	28.1	14.3
	Ligand **a**	25.4	52.1	21.2	27.0
	Complex **c**	68.8	42.1	19.9	38.4
A549	Control	11.2	57.5	32.3	10.4
	Ligand **a**	32.4	48.2	28.4	23.6
	Complex **c**	47.7	37.4	25.0	38.5

are bridged by the position of 5-deprotonated phenolic oxygen and the oxygen atoms of carbonyl groups. The elemental analysis and mass spectrum agreed well with the formation of 1:2 (metal/ligand) stoichiometry.

3.3. Antitumor activities

The ligand **a** and corresponding metal complexes (**b**, **c**, **d**, **e**, **f**) were tested for antitumor activity using the MTT assay at concentrations ranging from 0.1 to 600 μM against five human cancer cell lines, consisting of A549 (human lung carcinoma cell line), Hela (human uterine cervix cancer cell line), HepG2 (human liver cancer cell line), SW620 (human colon carcinoma cell line) and MDA-MB-435 (human breast carcinoma cell line).

The results, expressed as concentrations of the compounds required to inhibit the tumor cell growth by 50% (IC_{50}), are showed in Fig. 1. Ligand **a**, metal ions and cisplatin were given for comparative purposes. Metal ions had only low activity ($IC_{50} > 50$ μM) against the investigated cell lines. In general, SW620 and A549 cells showed higher sensitivities to the antiproliferative effect of these complexes, while MDA-MB-435 and Hela cell lines were found to be more resistant. In SW620 cell line, it was shown that complexes **c** and **f** induced more than three times stronger response than ligand **a** and the positive control cisplatin. Moreover, complexes **b**, **d**, and **e** exhibited slightly more effective, indicating that the described complexes improved antitumor activity. In HepG2 cells, the IC_{50} values of all complexes displayed at least two times more effective compared with that of the ligand **a**. It is interesting to note that in A549 cells, complex **f** showed two times more effective than cisplatin, three times more effective than ligand **a**. As shown in Fig. 1, complex **e** and **f** exhibited the lowest IC_{50} values against the MDA-MB-435 and SW620 cell lines, respectively. All of these results demonstrated that this newly synthesized complexes have elevated anti-proliferative activities in comparision with ligand **a** and corresponding metal ions, and some were even more effective than the positive control cisplatin.

To verify the possibility that inhibition of cell proliferation is associated with induction of apoptosis and cell cycle phase arrested after compounds treatment, the cell lines of MDA-MB-435, SW620, HepG2 and A549 were treated with ligand **a** and complex **c** at 10 μM final concentration for 24 h. The results revealed that complex **c** induced blocks in the G_2/M phase of the cell cycle in the SW620, HepG2 and A549 cell lines (Table 3), with concomitant reductions in the percentages of cells in the G_1 phase of the cell cycle. However, the majority of the cell population was arrested at the G_1 phase accompanying reduction in the S and G_2/M phase following the complex **c** treatment in the MDA-MB-435 cells (Table 3). Furthermore, complex **c** was more effective than the ligand **a**.

Fluorescein isothiocyanate (FITC)-conjugated annexin V has been utilized to detect the externalization of phosphatidylserine that occurs at an early stage of apoptosis. Propidium iodide (PI) is used as a marker of necrosis due to cell membrane destruction [34]. To elucidate whether complexes-induced cell death involved apoptosis or necrosis, a biparametric cytofluorimetric analysis using annexin V and PI double-staining was performed. The cells were treated with ligand **a** and complex **c** at 10 μM final concentration for 24 h. As showed in Table 3, the apoptosis rate of complex **c** treated were substantially higher on SW620, HepG2 and A549 cell lines, and slightly higher on the MDA-MB-435 cells, relative to their respective controls (DMSO) and the ligand **a**. The representative pictures of compound **a** and **c** treated on SW620 cells and the control were presented in Fig. 2. Annexin V^+/PI^- (Q_4) population represented cells undergoing apoptosis, and annexin V^+/PI^+ (Q_2) population corresponded

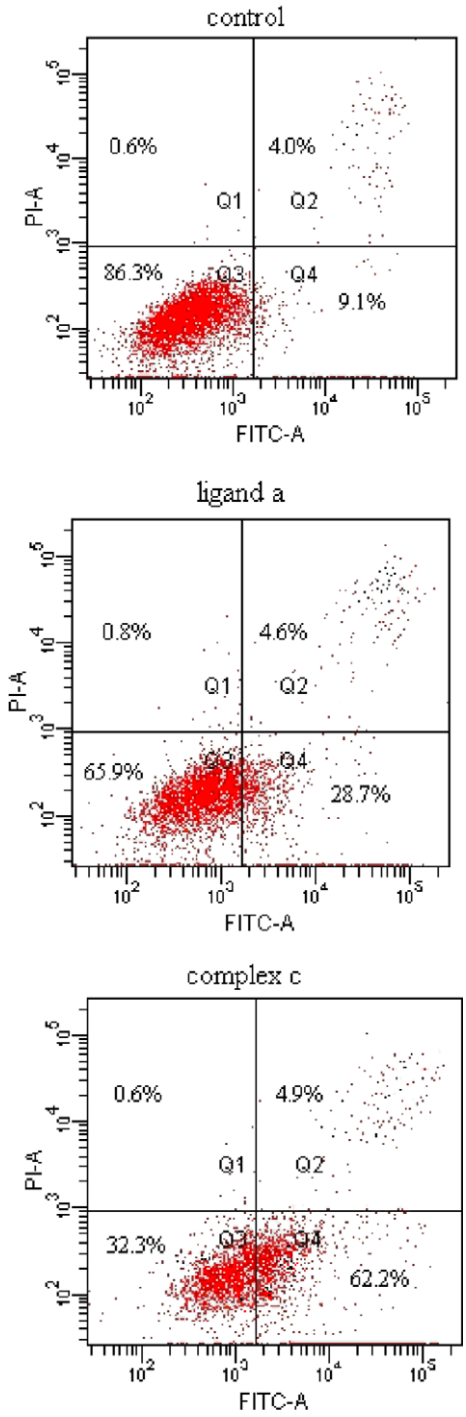


Fig. 2. Results of flow cytometric cellular apoptosis of untreated SW620 control cells and cells treated with ligand **a** and complex **c** at 10 μM final concentration for 24 h. The Q4 region represents signals from cells undergoing apoptosis, and the Q2 region corresponds to necrotic cells. Scattergram of the treated cells were compared with those of the untreated control cells.

to necrotic cells. As showed in Fig. 2, both ligand **a** and complex **c** could induced cell apoptosis compared with that of control. In addition, complex **c** provoked two times more apoptotic cells in comparision with ligand **a** when treating on SW620 cells at 10 μM final concentration for 24 h. In contrast, there was no obvious change of necrotic cells between these two compounds.

4. Conclusion

Ligand 4′-methoxy-5,7-dihydroxy-isoflavone (**a**) and five new complexes (**b**–**f**) were synthesized. New compounds were characterized by IR, ^{1}H NMR, Mass spectroscopy and elemental analysis.

All of the investigated compounds were tested for *in vitro* antitumor activities against five human cancer cell lines consisting of A549, Hela, HepG2, SW620 and MDA-MB-435. Complex **c** and **f** possessed greater activity and selectivity than the other described complexes in the antitumor action against the selected cancer cell lines. The possible mechanisms by which complex **c** can exert an anti-profliferative effect on MDA-MB-435, SW620, HepG2 and A549 cells were also investigated. Flow cytometry analysis showed that complex **c** acted on non-specific phase of the cell cycle arrest and induced cell apoptosis in MDA-MB-435, SW620, HepG2 and A549 cells treated at the concentration of 10 μM. The findings indicated that the anti-proliferative effect of complex **c** is associated with cellular apoptosis and cell cycle arrest and profits to the development of a new anti-cancer drug.

Acknowledgement

This work was supported by Zhejiang Province Extremely Key Subject Building Funding "Pharmacology and Biochemical Pharmaceutics 2008 ".

References

[1] B. Rosenberg, L. Van Camp, T. Krigas, Nature 205 (1965) 698–699.
[2] A. Alama, B. Tasso, F. Novelli, Drug Discov. Today 14 (2009) 500–508.
[3] M.A. Jakupec, M. Galanski, B.K. Keppler, Rev. Physiol. Biochem. Pharmacol. 146 (2003) 1–53.
[4] D. Wang, S.J. Lippard, Nature Rev. Drug Discov. 4 (2005) 307–320.
[5] I. Kostova, Curr. Med. Chem. 13 (2006) 1085–1107.
[6] P. Köpf-Maier, H. Kopf, E.W. Neuse, J. Cancer Res. Clin. Oncol. 108 (1984) 336–340.
[7] I. Ott, B. Kircher, P. Schumacher, K. Schmidt, R. Gust, J. Med. Chem. 48 (2005) 622–629.
[8] M.A. Yakupec, Dalton Trans. (2008) 183–194.
[9] M. Leopoldini, T. Marino, N. Russo, M. Toscano, J. Phys. Chem. A 108 (2004) 4916–4923.
[10] Y.J. Kim, Y.C. Bae, K.T. Suh, J.S. Jung, Biochem. Pharmacol. 72 (2006) 1268–1276.
[11] A.I. Morales, C. Vicente-Sánchez, J.M. Santiago Sandoval, J. Egido, P. Mayoral, M.A. Arévalo, M. Fernández-Tagarro, J.M. López-Novoa, F. Pérez-Barriocanal, Food Chem. Toxicol. 44 (2006) 2092–2100.
[12] S. Luangaram, U. Kukongviriyapan, P. Pakdeechote, V. Kukongviriyapan, P. Pannangpetch, Food Chem. Toxicol. 45 (2007) 448–456.
[13] A. Torreggiani, M. Tamba, A. Trinchero, S. Bonora, J. Mol. Struct. 759 (2005) 744–751.
[14] S.B. Bukhari, S. Memon, M.M. Tahir, M.I. Bhanger, J. Mol. Struct. 892 (2008) 39–46.
[15] S.B. Bukhari, S. Memon, M.M. Tahir, M.I. Bhanger, Spectrochim. Acta Part A 71 (2009) 1901–1906.
[16] W.J. Chen, S.F. Sun, W. Cao, Y. Liang, J.R. Song, J. Mol. Struct. 918 (2009) 194–197.
[17] F.B.A. El Amrani, L. Perello, JA. Real, J. Inorg. Biochem. 100 (2006) 1208–1218.
[18] P. Ryan, M.J. Hynes, J. Inorg. Biochem. 102 (2008) 127–136.
[19] Y.N. Ni, S. Du, S. Kokot, Anal. Chim. Acta 584 (2007) 19–27.
[20] J. Tan, B.C. Wang, L.C. Zhu, Colloids Surf. B Biointerfaces 55 (2007) 149–152.
[21] J.P. Cornard, L. Dangleterre, C. Lapouge, J. Phys. Chem. A 109 (2005) 10044–10051.
[22] F.V. De Souza Rubens, F. De Giovani Wagner, Redox Rep. 9 (2004) 97–104.
[23] M. Leopoldini, N. Russo, S. Chiodo, M. Toscano, J. Agric. Food Chem. 54 (2006) 6343–6351.
[24] Y. Sanchez, D. Amran, E. de Blas, P. Aller, Biochem. Pharmacol. 77 (2009) 384–396.
[25] M. Privat, C. Aubel, S. Arnould, Y. Communal, M. Ferrara, Y. Bignon, Biochem. Biophy. Res. Commun. 379 (2009) 785–789.
[26] A. Yokosuka, M. Haraguchi, T. Usui, S. Kazami, V.C. Blank, C. Poli, M. Marder, L.P. Roguin, Bioorg. Med. Chem. Lett. 17 (2007) 3091–3094.
[27] G.M. Saxena, T.R. Seshadri, Proc. Math. Sci. 46 (1957) 218–223.
[28] R.R. Priya, K.G. Kumar, M.C. Narayanan, Asian J. Sci. Res. 1 (2008) 176–179.
[29] S. Balasubramanian, M.G. Nair, Syn. Commun. 30 (2000) 469–484.
[30] W.J. Geary, Coordin. Chem. Rev. 7 (1971) 81–122.
[31] Y.C. Chang, M.G. Nair, R.C. Santell, W.G. Helferich, J. Agric. Food Chem. 42 (1994) 1869–1871.
[32] K. Wahala, T.A. Hase, J. Chem. Soc. Perkin Trans. 1 (1991) 3005–3008.
[33] T. Mosmann, Immunol. J. Meth. 65 (1983) 55–63.
[34] S. Pervaiz, M.A. Seyed, J.L. Hirpara, M.V. Clement, K.W. Loh, Blood 93 (1999) 4096–4108.