

Effect of a Copper (II) Complex on The Induction of Apoptosis in Human Hepatocellular Carcinoma Cells

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Abstract

Objectives: In the present study, we aimed to identify the anti-proliferative potential of [Cu(L)(2imi)] complex [L = 2-(((5-chloro-2-oxyphenyl)imino)methyl)phenolato) and 2imi = 2-methyl imidazole] against HepG2 cells as an in vitro model of human hepatocellular carcinoma and normal mouse fibroblast L929 cells. **Methods:** The cytotoxic and apoptotic effects of [Cu(L)(2imi)] complex on HepG2 cells and normal fibroblasts (L929) were examined by MTT assay and flow cytometry, respectively. **Results:** Cytotoxicity induced by [Cu(L)(2imi)] complex was time dependent. Also, there was a positive correlation between cytotoxicity and an increase in Cu complex concentration. For HepG2 cells, the cell viability percentage was 50% at 58 µg/mL after 24 h treatment, whereas in the same concentration and conditions, the viability percentage was surprisingly higher (about 100%) for L929 cells. Also, after 48 h treatment, the viability percentage of HepG2 cells at 55 µg/mL concentration was 50% in contrast with 89.3% for L929 cells in the same conditions. Flow cytometry findings suggest that [Cu(L)(2imi)] complex is capable of decreasing cancer cell viability through apoptosis and did not efficiently activate the necrosis process. **Conclusions:** Finally, we found that [Cu(L)(2imi)] complex possess the potential for development as an anti-cancer drug for human hepatocellular carcinoma.

Keywords: Apoptosis- [Cu(L)(2imi)] complex- cytotoxicity- hepatocellular carcinoma- mouse fibroblast L929 cells

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Introduction

Cancer is one of the most deadly diseases (Chen and Hu, 2009) and is a major health problem of global concern that afflicts a significant proportion of the world's population in all generations (Atawodi, 2011). Reports of the American Cancer Society show that Deaths due to cancer and new cancer cases will increase to approximately 13.2 and 21.4 million patients by 2030, respectively (Mi et al., 2013). Liver cancer is the seventh most common cancer in women and the fifth most common cancer in men worldwide (El-Serag, 2012; Hosseini et al., 2017).

Primary liver cancer, especially hepatocellular carcinoma (HCC) is one of the most common and deadly cancers in the world (El-Serag and Rudolph, 2007), and many efforts have been made to treat the disease (Abid-Essefi et al., 2003; Franke et al., 2003). The apoptosis induction is an effective way to kill cancer cells (Karimabad et al., 2017; Ramezani et al., 2017; Sheikhezadei et al., 2018)

It has been shown in many reports that metal

complexes are used as anticancer agents in many drugs (Fricker, 1994). Presumably, the most well-known of these drugs is cisplatin [cis-diamminedichloroplatinum(II)] (Marzano et al., 2002), although cisplatin is applied as an anti-cancer drug to treat many types of cancer. However, severe side effects and resistance induced by long-term treatment with this drug has attracted attention to the development of alternative drugs with the increase in morbidity (Kim et al., 2011).

Several therapeutic approaches have been introduced to treatment of HCC including, chemotherapy, radiotherapy and immunotherapy. Chemotherapy is a famous approach which is used for treatment of several cancers including HCC world-widely. However, the current drugs which are applied for chemotherapy are associated with several side effects which are derived from their effects on the non-cancerous normal cells. Therefore, investigators are trying to find new therapeutic strategies for cancer treatment with the lowest side effects (Zainodini et al., 2018; Bagrezaei et al., 2018). Based on the fact that HCC is a prevalent cancer world-wide, hence, several studies are designed

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to introduce new chemotherapy strategies to overcome the disease. The use of metallic complexes as anticancer drugs attracted many attentions of researchers in the field of pharmaceutical chemistry (van Rijt and Sadler, 2009; Barry and Sadler, 2013; Santini et al., 2013; Munteanu and Suntharalingam, 2015). Copper-based complexes are one of these compounds that have shown promising anticancer activities (Santini et al., 2013; Mohammadzadeh et al., 2018). Copper is an essential element involved in critical biological functions such as energy metabolism, oxygen transport, enzyme activity, and cell signaling. Moreover, this metal is a necessary cofactor for the tumor angiogenesis (Brem, 1999; Brewer, 2001; Theophanides and Anastassopoulou, 2002; Tisato et al., 2010).

The main aim of this study was to evaluate the anti-cancerous effects of a Cu(II) complex [Cu(L)(2imi)] derived from 2-(((5-chloro-2-oxyphenyl)imino) methyl) phenolato (L) and 2-methylimidazole (2imi) on the HepG2 cell line. On the other hand, due to the various side effects of chemotherapy on the normal cells, another aim of this study was to explore the effects of the [Cu(L)(2imi)] complex on the survival and apoptosis of mouse fibroblast L929 cells, as normal cells. The in vitro anti-cancer activity of [Cu(L)(2imi)] complex was evaluated by MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) assay and apoptosis was studied using flow cytometry.

Materials and Methods

Materials and instrumentation

MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) and dimethyl sulfoxide (DMSO) were prepared from Roche (Mannheim, Germany). Fetal bovine serum (FBS), RPMI-1640, trypsin enzyme and penicillin–streptomycin were purchased from Gibco-BRL (Grand Island, NY, USA). Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit was purchased from Ebioscience (San Diego, CA, USA).

All reagents and solvents for synthesis and analysis were commercially available and purchased from Merck or Sigma and used as received without further purifications. Elemental analyses were performed on a Thermo Finnigan Flash Elemental Analyzer 1112EA. Melting points were measured on an Electrothermal-9100 apparatus and uncorrected. FT-IR spectra were recorded on an FT-IR Tensor 27 infrared spectrophotometer as KBr discs in the range of 400–4,000 cm^{-1} .

Synthesis of 6-(((5-chloro-2-hydroxyphenyl)amino) methylene)cyclohexa-2,4-dien-1-one [H2L]

[H2L] was prepared according to our previous report (Takjoo et al., 2014). Briefly, 5 mL ethanol containing 0.3 g (2 mmol) of 2-amino-4-chlorophenol was added to an ethanolic solution (5 mL) of 2-hydroxy-benzaldehyde (0.2 g, 2 mmol). The obtained mixture was vigorously stirred and simultaneously heated for 15 min. After cooling the mixture, an orange precipitate was observed that was separated, washed with cold ethanol and dried in a desiccator over anhydrous CaCl_2 . Yield:

84%. M.p.: 156 °C. Anal. Calcd for $\text{C}_{13}\text{H}_{10}\text{ClNO}_2$ (247.68 g mol⁻¹): C, 63.04; H, 4.07; N, 5.66. Found: C, 63.07; H, 4.08; N, 5.64%. FT-IR (KBr) cm^{-1} : $\nu(\text{OH})$ 3045, $\nu(\text{N-H})$ 2097 $\nu(\text{C=N})$ 1626, $\nu(\text{C=Cring})$ 1494, $\nu(\text{C-O})$ 1313, $\nu(\text{C-Cl})$ 653 (Figure 1).

Synthesis of (2-Methylimidazole)(2-(((5-chloro-2-oxyphenyl)imino)methyl)phenolato)) copper(II) [Cu(L)(2imi)]

[Cu(L)(2imi)] was synthesized according to the procedure reported previously (Ebrahimipour et al., 2015). Briefly, a 4-mL solution of $\text{Cu}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ (0.02 g, 0.1 mmol) in methanol was added to 3 mL methanol containing H_2L (0.1 mmol, 0.03 g) and NaOH (0.2 mmol, 0.01 g). The mixture was refluxed in a water for 5 min while stirred at a constant speed. After addition of 4 mL methanolic solution of 2-methyl imidazole (0.3 mmol, 0.02 g) to the reaction mixture, the reflux was continued for 2 h. Then, the solvent of the mixture was slowly evaporated, resulted in the precipitation of red crystals which were separated, dried in air at room temperature and stored in a CaCl_2 desiccator. Yield: 58%. M.p.: 206°C. Molar conductance (10^{-3} M, DMSO) 21.0 Ω^{-1} cm^2 mol⁻¹. Anal. Calcd for $\text{C}_{17}\text{H}_{14}\text{ClCuN}_3\text{O}_2$ (391.31 g mol⁻¹): C, 52.18; H, 3.61; N, 10.74. Found: C, 52.16; H, 3.49; N, 10.76%. FT-IR (KBr) cm^{-1} : $\nu(\text{NH})$ 3420, $\nu(\text{C=N})$ 1610, $\nu(\text{C=Cring})$ 1471, $\nu(\text{C-O})$ 1280, $\nu(\text{C-Cl})$ 654 (Figure 1).

Cell culture

Mouse fibroblast L929 cells and Human hepatocellular carcinoma cell line HepG2 were obtained from Pasteur Institute (Tehran, Iran). Cells were maintained in a humidified atmosphere containing 5% CO_2 at 37°C in RPMI 1640 medium supplemented with streptomycin (100 $\mu\text{g}/\text{mL}$), Penicillin (100 IU/mL) and 10% fetal bovine serum (FBS). Culture medium was exchanged with fresh medium every 1–2 days, when the cells occupied 70–80% of the flask, the cells were treated with different concentrations (0.03–100 $\mu\text{g}/\text{mL}$) of copper (II) complex [Cu(L)(2imi)] for 24 and 48 hours.

Cytotoxicity evaluation

MTT assay

Both HepG2 and L929 Cells were cultured in 96-well plates in triplicate and incubated at 37 °C and 5% CO_2 for 24 h. Then, graded amounts of [Cu(L)(2imi)] complex were added to the wells in 10 μL of FBS free culture medium and the plates were incubated in a 5% CO_2 humidified atmosphere for 24 h and 48 h. Six replica wells were used for controls. The [Cu(L)(2imi)] complex were tested at concentrations ranging between 0.03 and 100 μM . After that, 0.1 mg of MTT (in 20 μL of PBS) was added to each well, and cells were incubated at 37°C for 4h. The formed formazan crystals were then dissolved in 100 μL of DMSO and the absorbance was read at 570 nm using an Enzyme-linked Immunosorbent Assay (ELISA) reader. The sensitivity of HepG2 and L929 to [Cu(L)(2imi)] complex treatment was expressed in terms of IC50 (drug concentration producing 50% inhibition of cell growth, calculated on the regression line in which absorbance values at 570 nm were plotted against the logarithm of

drug concentration).

The viability of HepG2 and L929 cells were determined by trypan blue exclusion (TBE) assay and counting in a hemocytometer after 24 h and 48 h treatment with [Cu(L)(2imi)] complex at IC₅₀, concentrations and Morphological changes on cells were examined using an inverted microscope under 400× magnification.

Apoptosis evaluation by flow cytometry

Double staining with Annexin V-FITC and PI for flow cytometry analyses was performed using Annexin V-FITC/PI apoptosis detection kit (Ebioscience, USA). HepG2 cells were incubated with concentrations 0.58 µg/mL and 113 µg/mL of [Cu(L)(2imi)] complex for 24 h and 0.55 µg/mL and 113 µg/mL for 48 h. L929 cells were also treated with concentrations 105.3 µg/mL and 250 µg/mL of [Cu(L)(2imi)] complex for 24 h and 0.83 µg/mL and 200 µg/mL for 48 h.

First, 5×10^5 of treated and/or untreated cells were harvested after 24 h or 48 h and washed twice with sterile and cold PBS (Phosphate saline buffer), then re-suspended in the binding buffer (calcium buffer, 200 µL). Annexin V-FITC (5 µL) was added to the cells and incubated in the dark at room temperature for 10 minutes. After washing the cells with PBS, the remaining cell pellet was dissolved in 190 µL binding buffers. After addition of 10 µL propidium iodide (PI), the samples were then incubated for 5 min in the dark at 4°C and were examined under a Cyflow® space flow cytometer (Partec, Münster, Germany). Flowmax software (Partec) was used for data analysis. The measurements were performed in at least 3 repetitions.

Statistical analysis

SPSS software, version 19 was used for this analysis. Descriptive analysis (mean values and standard error) of cells viability was performed. Whole experiments were carried out for three times in each individual sample and all of obtained results were presented as the mean value of those three. Statistical analysis for compare differences

among groups was performed by one-way ANOVA and Dunnett's procedure for multiple comparisons with a single control group. P value of less than 0.05 was considered statistically significant.

Results

Effect of [Cu(L)(2imi)] complex on cell viability

The MTT method was used to evaluate the inhibitory effects of the [Cu(L)(2imi)] complex on the growth of HepG2 as in vitro model of hepatocellular carcinoma cells and mouse fibroblast L929 as normal cells. HepG2 cells were incubated with different concentrations of [Cu(L)(2imi)] complex (0.03–110 µg/mL) for 24 h and 48 h. The results demonstrated that [Cu(L)(2imi)] complex decreased the viability of HepG2 cells, in a dose- and time-dependent manner ($p < 0.05$) (Figure 2). In the MTT assay, the statistical analysis revealed that [Cu(L)(2imi)] complex significantly inhibited the proliferation of HepG2 cells ($p < 0.05$) (Figure 3). At higher concentrations of [Cu(L)(2imi)], the number of colony-forming cells was reduced in culture when observed under inverted microscope. HepG2 cells stick firmly to the floor of the culture flask and create an overlapping layer of cells, but after treatment with [Cu(L)(2imi)] complex, the morphology of HepG2 cells has changed dramatically. The cells were separated from the culture plates after 24 h of treatment. Also nuclear condensation, apoptotic body formation and cell shrinkage were observed under inverted microscope (Figure 4). The IC₅₀ values obtained for HepG2 cells treated with the

Table 1. IC₅₀ Values (µg/mL) of [Cu(L)(2imi)] Ccomplex on HepG2 and L929 Cells after 24 h and 48 h treatment

Cell line	IC ₅₀ (µg/mL) for [Cu(L)(2imi)] complex	
	24 h	48 h
HepG2	58	55
L929	105.3	83

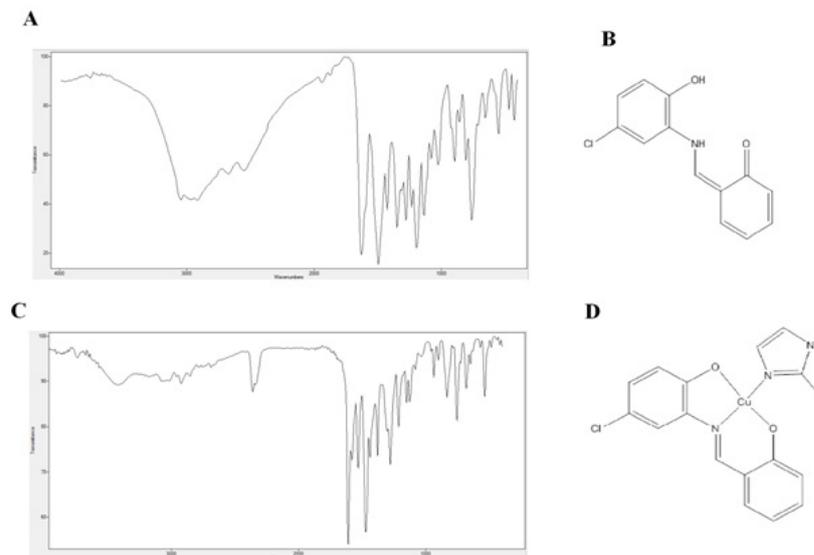


Figure 1. Demonstrates FTIR Spectrum of H2L (A), chemical structure of H2L (B), FTIR spectrum of [Cu(L)(2imi)] (C) and chemical structure of [Cu(L)(2imi)] (D).

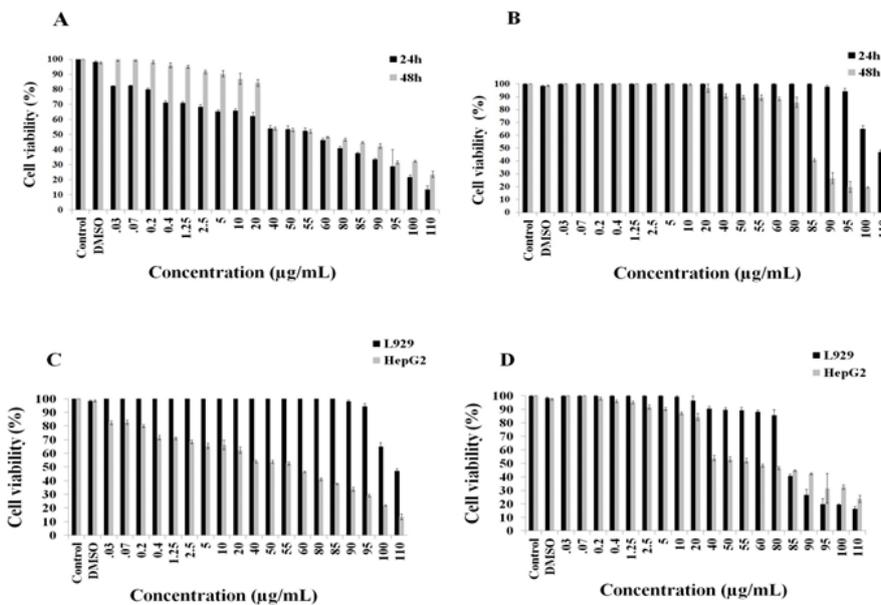


Figure 2. Demonstrates the Changes in Viability of HepG2 Cells Treated with Different Concentrations of [Cu(L)(2imi)] after 24 h and 48 h, Using MTT Method. All changes observed in the cell viability are significant ($P < 0.05$) except those observed for concentrations 0.03-1.25 $\mu\text{g/mL}$ for 48 h (A). Demonstrates the changes in viability of L929 cells treated with different concentrations of [Cu(L)(2imi)] after 24 h and 48 h, using MTT method. All changes observed in the cell viability are significant ($P < 0.05$) except those observed for concentrations 0.03-85 $\mu\text{g/mL}$ for 24 h and 0.03-20 $\mu\text{g/mL}$ for 48 h (B). Comparison of the changes in viability of HepG2 with L929 cells treated in different concentrations of [Cu(L)(2imi)] after 24 h (C). Comparison of the changes in viability of HepG2 with L929 cells treated in different concentrations of [Cu(L)(2imi)] after 48 h (D).

Cu(II) complex for 24 h and 48 h were 58 $\mu\text{g/mL}$ and 55 $\mu\text{g/mL}$, respectively ($p < 0.05$) (Figure 2 and Figure 3). Furthermore, the cytotoxicity of [Cu(L)(2imi)] complex against L929 cells was also investigated using MTT assay (Figure 2 and Figure 3). L929 cells were also incubated with different concentrations of [Cu(L)(2imi)] complex

(0.03–110 $\mu\text{g/mL}$) for 24 h and 48 h (Figure 2 and Figure 3). The IC50 values of [Cu(L)(2imi)] complex against L929 cells were calculated to be 105.3 $\mu\text{g/mL}$ and 83 $\mu\text{g/mL}$ respectively after 24 h and 48 h treatment with the Cu complex ($p < 0.05$) (Figure 2, Table 1).

The results showed that the [Cu(L)(2imi)] complex

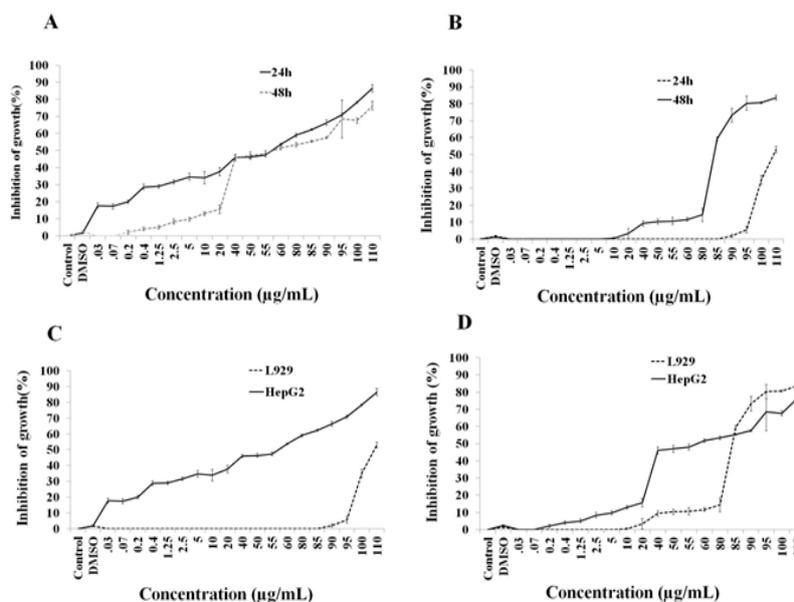


Figure 3. Demonstrates the Growth Inhibition of HepG2 Cells Treated with Different Concentrations of [Cu(L)(2imi)] after 24, 48 h. All changes observed in the cell inhibition are significant ($P < 0.05$) except those observed for concentrations 0.03-1.25 $\mu\text{g/mL}$ for 48 h (A). Demonstrates the growth inhibition of L929 cells treated with different concentrations of [Cu(L)(2imi)] after 24 h and 48 h. All changes observed in the inhibition are significant ($P < 0.05$) except those observed for concentrations 0.03-85 $\mu\text{g/mL}$ for 24 h and 0.03-20 $\mu\text{g/mL}$ for 48 h (B). Demonstrates the growth inhibition of HepG2 cells in comparison with L929 cells treated to different concentrations of [Cu(L)(2imi)] after 24 h (C) and 48 h (D).

Table 2. Apoptosis Rates in HepG2 and L929 Cells after 24 h and 48 h Treatment with [Cu(L)(2imi)] Complex at IC50 Concentrations

Apoptosis rates	Apoptosis percentage at IC50 concentration of [Cu(L)(2imi)] complex ($\mu\text{g/mL}$)			
	HepG2		L929	
	24 h (58 $\mu\text{g/mL}$)	48 h (55 $\mu\text{g/mL}$)	24 h (105.3 $\mu\text{g/mL}$)	48 h (83 $\mu\text{g/mL}$)
Normal live cells	50.31%	49.75%	51.59%	56.68%
Early apoptosis cells	42.13%	42.48%	40.78%	37.12%
Late apoptosis cells	0.60%	3.39%	1.74%	0.23%
Necrosis cells	6.96%	4.39%	5.88%	2.97%

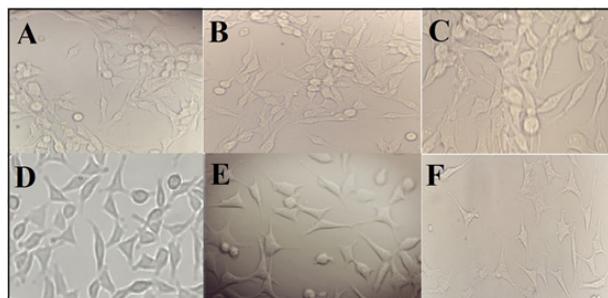


Figure 4. Morphological Changes of HepG2 and L929 Cells after Exposure with [Cu(L)(2imi)] Complex. Un-Treated HepG2 Cells (A), HepG2 cells after treated with [Cu(L)(2imi)] complex at $\frac{1}{2}$ IC-50 concentration (29 $\mu\text{g/mL}$) for 24 h (B), HepG2 cells after treated with [Cu(L)(2imi)] complex at IC-50 concentration (58 $\mu\text{g/mL}$) for 24 h (C), Un-treated L929 cells (D), L929 cells after treated with [Cu(L)(2imi)] complex at $\frac{1}{2}$ IC-50 concentration (52.65 $\mu\text{g/mL}$) for 24 h (E), L929 cells after treated with [Cu(L)(2imi)] complex at IC-50 concentration (105.3 $\mu\text{g/mL}$) for 24 h (F) that were observed with an inverted microscope under 400 \times magnification.

inhibited the growth of L929 cells in higher concentration than HepG2 cells and the growth inhibition of both L929 and HepG2 cells had a dose-and time-dependent manner ($p < 0.05$) (Figure 3)

[Cu(L)(2imi)] complex induces apoptosis in HepG2 cells

The HepG2 cells were treated at IC50 concentration of [Cu(L)(2imi)] complex (58 $\mu\text{g/mL}$) for 24 h and (55 $\mu\text{g/mL}$) for 48 h. Then, the treated cells were stained with annexin V and propidium iodide and the apoptotic effect of [Cu(L)(2imi)] complex on HepG2 cells were detected by flow cytometry. The results of flow cytometry analysis revealed that the [Cu(L)(2imi)] complex induced apoptosis in treated HepG2 cells compared to control (un-treated cells), indicating that apoptotic cell death is involved in [Cu(L)(2imi)] complex induced toxicity (Figure 5). The percentages of early apoptotic HepG2 cells following treatment at IC50 concentration (58 $\mu\text{g/mL}$) for 24 h and 55 $\mu\text{g/mL}$ for 48 h were 42.13% and 42.48%, respectively (Figure 5, Table 2). The percentages of necrotic and late

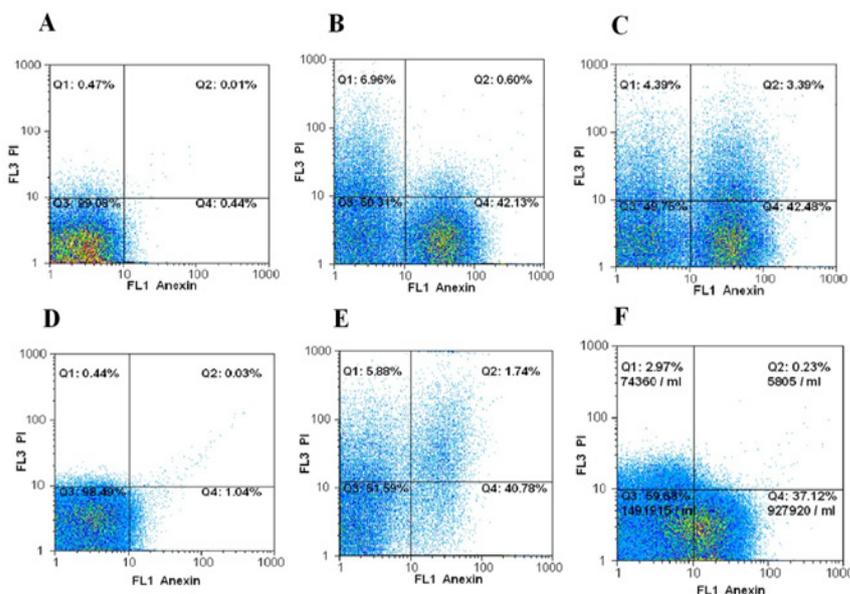


Figure 5. Flow Cytometry Analysis of HepG2 and L929 Cells Treated with [Cu(L)(2imi)] Complex. Un-treated HepG2 cells (A), HepG2 cells treated at IC50 concentration (58 $\mu\text{g/mL}$) of [Cu(L)(2imi)] for 24 h (B), HepG2 cells treated at IC50 concentration (55 $\mu\text{g/mL}$) of [Cu(L)(2imi)] for 48 h (C), Un-treated L929 cells (D) L929 cells treated at IC50 concentration (105.3 $\mu\text{g/mL}$) of [Cu(L)(2imi)] for 24 h (E) and L929 cells treated at IC50 concentration (83 $\mu\text{g/mL}$) of [Cu(L)(2imi)] for 48 h (F) stained with Annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI). Subsequently, apoptotic and necrotic cells were quantified by flow cytometry. The different subpopulations were defined as Q1 Annexin V-negative but PI-positive, i.e. necrotic cells; Q2 Annexin V/PI-double positive, i.e. late apoptotic cells; Q3 Annexin V/PI-double negative, i.e. normal live cells; and Q4 Annexin V-positive but PI-negative, i.e. early apoptotic cells.

apoptotic cells were about 6.96% and 0.60% after 24 h treatment and 4.39% and 3.39% after 48 h treatment, respectively (Figure 5, Table 2).

The test for L929 was also performed as normal cells with concentration of IC₅₀, 105.3 µg/mL for 24 h and 83 µg/mL for 48 h. The percentages of early apoptotic L929 cells following treatment with IC₅₀ concentrations for 24 h and 48 h (105.3 µg/mL and 83 µg/mL) were 40.78% and 37.12%, respectively (Figure 5, Table 2). The percentages of necrotic and late apoptotic cells were about 5.88%, 1.74% after 24 h and 2.97%, 0.23%, after 48 h treatment with [Cu(L)(2imi)] complex, respectively (Figure 5, Table 2).

Normal living cells were observed in Q3 region (Figure 5). The early apoptotic cells were determined by the sum of the cells in Q4 and late apoptosis and necrosis cells appeared mainly in the Q2 and Q1 regions, respectively (Figure 5)

Discussion

[Cu(L)(2imi)] was synthesized according to the procedure reported previously (Ebrahimipour et al., 2015) and was characterized. The primary objective of this study was to identify the anti-cancer properties of the [Cu(L)(2imi)] complex. We also attempted to determine whether this complex could selectively kill human cancer cells. This is the first study on the biological activity of this complex as an anti-cancer agent.

To evaluate the anti-cancer potential of the [Cu(L)(2imi)] complex synthesized, the compound was initially tested for cytotoxicity against HepG2 and L929 cells. The cytotoxic activities, expressed as IC₅₀ values, were determined by the MTT assay. This experiment is based on MTT conversion into formazan with water insoluble crystals via dehydrogenases in the mitochondria of alive cells with a dose-dependent fashion (Van Tonder et al., 2015). In previous studies have been reported that copper (II) complexes have anti-cancer activities against several human cancer cell lines (González et al., 2013; Hajrezaie et al., 2014; Ma et al., 2015; Gan et al., 2016; Yu et al., 2016). Thati et al., 2007 reported that a copper (II) complex ([Cu(4-Mecdoa)(phen)₂]) had a dose- and time-dependent anti-proliferative effect on non-neoplastic hepatic (CHANG) and HepG2 cells. However, it should be noted that the complex was more effective against HepG2 cells compared to CHANG cells (Thati et al., 2007).

Additionally, copper (II) complexes have been found to inhibit cell growth in HepG2 cell line. For example, a copper (II) complex of anthracenyl terpyridine showed cytotoxicity towards different cancer cell lines such as HepG2 (Kumar et al., 2011).

The IC₅₀ values of [Cu(L)(2imi)] complex in the HepG2 cell line after 24 h and 48 h treatment were compared with the IC₅₀ values of this complex against L929 fibroblast mouse cells, as normal cells. The results were displayed that in HepG2 cells, the IC₅₀ value after 24 h treatment (58 µg/mL) was very close to this observed after 48 h treatment (55 µg/mL). Similarly, in L929 cells, the IC₅₀ values after 24 h and 48 h treatment with the complex were not close to each other about 105.3 µg/

mL and 83 µg/mL, respectively ($p < 0.05$). Altogether data indicate that the IC₅₀ values for L929 cells were about 2-fold over those observed in HepG2 cells after 24 h treatment. On the other hand, surprisingly, in the concentration of 58 µg/mL (IC₅₀ obtained for HepG2 cells after 24 h treatment) and 55 µg/mL (IC₅₀ obtained for HepG2 cells after 48 h treatment) approximately 100 % and 89.3% of L929 cells are alive, respectively. As one can see, the IC₅₀ of the Cu (II) complex against HepG2 cell line was higher than that against L929 cell line. It can be concluded that hepatocellular carcinoma cells are much more sensitive to the complex compared with L929 normal cells. On the other words, [Cu(L)(2imi)] can selectively inhibit the growth of the HepG2 cancerous cells. The results of the flow cytometry measurements also demonstrated that the [Cu(L)(2imi)] complex induced apoptosis in vitro. Several mechanisms exist in cells to promote cell death. Necrosis and Apoptosis are two redundant and distinct forms of cell death occurring in response to physical and chemical stresses.

The apoptosis regulates a tightly cell death program and plays an essential role in many biological processes (Jänicke et al., 1998), while in necrosis process the plasma membrane was destructed and cofactors and cytosolic enzymes was released into the external medium (Mignani et al., 2017). The anti-cancer activity reflected the number of apoptotic and necrotic cells after exposure to chemicals. The results of present study revealed that the IC₅₀ concentration of Cu complex reduced the overall number of viable HepG2 cells after 24 h and 48 h (Figure 2). But in the same conditions, this complex acted differently on L929 cells and had low effect on the viability of normal L929 cells in contrast with cancerous HepG2 cells. This means that this complex surprisingly kills the cancerous cells, selectively, therefore its side effects will probably reduce on normal cells. To determine the correlation between anti-proliferative activity and apoptosis induction capability of [Cu(L)(2imi)] complex, the flow cytometry analysis was also carried out. Flow cytometry is a rapid and reliable method to demonstrate the entry of cells into the programmed cell death process. Flow cytometry analysis is based on the use of two fluorescent dyes for intact cells, including annexin-V and propidium iodide (PI). Accordingly to the fluorescence monitored in flow cytometry analysis, cells can be classified as alive or normal, in early apoptosis, in late apoptosis and necrosis or dead cells (Baskić et al., 2006; Hosseini et al., 2017).

Many studies have shown that copper (II) complexes are able to induce apoptosis in HepG2 cells (Pravin et al., 2017). Interestingly, Fan et al., 2016 demonstrated that metam/copper (II) led to cell death by induction of apoptosis, while metam/zinc (II) cell death mode was necrosis (Fan et al., 2016).

As expected DMSO had a negligible effect on the cell viability at 24 and 48 h (Figure 2 and Figure 3), whereas IC₅₀ concentrations of [Cu(L)(2imi)] complex had a considerable increase of cells in early apoptosis at 24 h (42.13%) and 48 h (42.48%), together with a minor number of cells in late apoptosis and necrosis.

For evaluation of the anti-proliferative effect of [Cu(L)(2imi)] complex on HepG2 and L929 cells, initially

cells were exposed for 24 h and 48 h to a different concentrations of this complex. As shown in Fig. 5 and Table 2, the number of alive cells was reduced in both cell lines while the percentage of cells in early apoptosis increased and cells remained at the early stage of the apoptotic pathway with only a low percentage of cells reaching the late apoptotic stage. Therefore, it is possible to conclude that [Cu(L)(2imi)] complex induced cell death through the activation of the apoptotic process. Therefore, [Cu(L)(2imi)] complex is a more potent inducer of apoptosis, especially at IC50 concentration.

It was possible to report a dual effect of [Cu(L)(2imi)] complex relative to the range of concentration used in cellular testing. At a low concentration, this complex was toxic and induced apoptosis in HepG2 cells without considerable effects on L929 cells. At higher concentrations, the toxic effect of [Cu(L)(2imi)] complex was observed on normal cells that were not affected in lower concentration. Also, flow cytometry analysis indicates that necrosis does not probably initiate cell death, but rather is a consequence of the activation of the apoptotic process.

In conclusion, the present study confirmed that [Cu(L)(2imi)] complex exerted inhibitory effects on HepG2 cells. Furthermore, the results demonstrated that [Cu(L)(2imi)] complex enhanced apoptosis using flow cytometry measurements. The result of the present study revealed that L929 (normal cells) was less sensitive to the complex. Data was shown that Cu complex triggered cell death via apoptosis and did not efficiently activate the necrosis process. Finally, we found that the [Cu(L)(2imi)] complex possess the potential for development as an anti-cancer drug for human hepatocellular carcinoma. Moreover, future studies are required to investigate DNA interaction, cleavage and molecular mechanisms involved in apoptosis induction of [Cu(L)(2imi)] complex.

Abbreviations

DMSO, dimethyl sulfoxide; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; HCC, hepatocellular carcinoma; HepG2, human hepatocellular liver carcinoma cell line; IC50, the half maximal inhibitory concentration; MTT, 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide; PBS, phosphate buffered saline; PI, propidium iodide; SPSS, statistical package for the social sciences

Conflict of Interest

The authors declare that they have no conflict of interest.

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