Received: 17 November 2018 | Revised: 18 December 2018 | Accepted: 2 January 2019

DOI: 10.1002/jcb.28491

RESEARCH ARTICLE

WILEY Journal of Cellular Biochemistry

A novel copper (II) complex activated both extrinsic and intrinsic apoptotic pathways in liver cancerous cells

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Abstract

Recent advances have put fundamental focus on the application of copper (II) (Cu [II]) complexes as agents for fighting against cancer. To determine whether [Cu(L)(2imi)] complex as a novel Cu complex can induce apoptosis in HepG2 as cancerous cells and L929 as normal cells via extrinsic or intrinsic apoptotic pathways, both cell lines were treated for 24 and 48 hours at IC_{50} concentrations of [Cu(L)(2imi)] complex. Then, the expression of some apoptosis-related genes including p53, caspase-8, bcl-2, and bax were assayed by real-time polymerase chain reaction. The [Cu(L)(2imi)] complex seems to inhibit the expression of bcl-2 in complex-treated HepG2 cancerous cells following the 24- and 48-hour treatment. The complex upregulated the p53, bax, and caspase-8 genes, therefore treatment of HepG2 cancerous cells with [Cu(L)(2imi)] complex induces programmed cell death via the upregulation of relative bax/bcl-2 ratio. Finally, this copper complex triggered apoptosis in HepG2 cells via both intrinsic and extrinsic pathway, whereas treatment of normal L929 cells with this complex induce apoptosis only via intrinsic pathway with the upregulation of relative bax/bcl-2 ratio and does not affect the expression level of caspase-8 gene and does not trigger the extrinsic pathway. Finally, these results obtained from present study confirm the role of a novel Cu complex on the induction of apoptosis process in HepG2 and L929 cells by overexpression of bax, inhibition of bcl-2 and increase of the relative bax/bcl-2 ratio. These results support that the [Cu(L)(2imi)] complex is able to induce apoptosis in cancerous cells, therefore, it has a potential for development as a novel anticancer drug.

KEYWORDS

bax, bcl-2, caspase-8, [Cu(L)(2imi)] complex, hepatocellular carcinoma, p53

1 | INTRODUCTION

Nowadays, the major focus of research on chemotherapy for cancer includes the identification, characterization, and development of new cancer chemopreventive agents.[1] Accordingly, manufacturing medications to fight against cancer is a very time-consuming and costly process, and a solution to expand the abundance of such medications might be integrating or reusing already-approved medications for anticarcinogenic purposes.[2] Metallodrugs have come recently to be used for curing various diseases and disorders, including cancer, inflammation, and bacterial

infection; however, understanding the molecular mechanism of some metallodrugs requires further research and experimentation.[3,4] Anticancer drugs which have platinum basis, including cisplatin, oxaliplatin and carboplatin, played a leading role in anticancer activity.[5,6] However, all these platinum-based agents can be put to limited use due to having side effects or negative repercussions, including toxic side effects, general toxicity, and multidrug resistance when the subject is undergoing clinical chemotherapy, a topic which motivated researchers to try to synthesize and study the nonplatinum transition metal complexes, which have shown anticancer activities, less toxicities, and specific antitumor mechanism different from platinum-based anticancer drugs.[6-8] Recent advances have put fundamental focus on the application of copper (II) (Cu [II]) complexes as agents for fighting against cancer.[9-11] Due to some characteristics and properties, such as being bioessential, less toxic, and being endogenous, copper has become quite significant within the field of anticancer studies.[12,13] Due to having considerable antibacterial and anticancer applications, Schiff bases have become a coral class of compounds within the field of medicinal chemistry.[14,15] A group of Schiff-based Cu (II) complexes have indicated considerable antibacterial and antitumor activities, a property which was supposed to be due to central Cu (II) or the specific structure or electronic properties of the coordination.[16,17] Several Cu (II) complexes have been studied for their potential pharmacological and anticancer agents over the past few years which further revealed that these copper complexes can generate cancer cell death by apoptotic and nonapoptotic mechanism.[18] Reactive oxygen species (ROS) upregulation and the ROS are some of the most common molecular effects of exposure to Cu the reactive oxygen species,[19] DNA cleavage,[20] Bax upregulation,[21] and the induction of p53 expression.[22] Apoptosis, which is defined as a genetically controlled and evolutionary-conserved form of active cell death, is the most probable consequence of all above-mentioned circumstances. This phenomenon takes place, primarily, through caspase-dependent or caspase-independent pathways. Depending on the stimulus, this pathway may trigger two different paths that converge on the activation of effector capsizes in mammals.[23] In intrinsic pathway mode, cell death signals are derived directly from mitochondria.[24,25] In extrinsic pathway mode, the activation is initiated in the tumor necrosis factor family receptors.[26,27] Caspase-8 and -9 are involved in extrinsic and intrinsic pathway, in order.[24,28] It has also been reported that incubation of a human hepatic cell line (HepG2) with $[Cu(phen)2]^{2+}$ resulted in internucleosomal DNA fragmentation.[29] In previous study, we indicated that [Cu(L)(2imi)] complex-exerted inhibitory effects on HepG2 cells. Furthermore, the results demonstrated that [Cu(L)(2imi)] complex-enhanced cell death via apoptosis and did not efficiently activate the necrosis process. The results revealed that L929 cells (as normal cells) were less sensitive to the complex.[30] Our aim in the present study is to investigate the molecular mechanism involved in induction of apoptosis by [Cu(L)(2imi)] complex on HepG2 as cancerous and L929 as normal cells. Therefore, in the present study the expression of some apoptosis-related genes including p53, caspase-8, bcl-2, and bax were assayed by real-time polymerase chain reaction (RT-PCR).

2 | MATERIALS AND METHODS

2.1 | Cell culture and [Cu(L)(2(imi)] complex synthesis

Human carcinoma cell line (HepG2) and Mouse embryo Fibroblast Cells (L929) were obtained from Pasteur Institute (Tehran, Iran). Cells were maintained in a humidified atmosphere containing 5% CO_2 at 37°C in Rosewell Park Memorial Iinstitute (RPMI) 1640 medium supplemented with 100 units/mL penicillin, 100 μg/mL streptomycin and 10% fetal bovine serum. [Cu(L)(2(imi)] complex was synthesized according to the procedure reported previously.[31]

2.2 | Cytotoxicity evaluation

The Trypan blue assay was performed for determination of the cell viability. Initially, cell density of cell line suspension was determined using a hemacytometer. Then, the 0.4% solution of Trypan blue was prepared in 1× phosphate-buffered saline (pH = 7.3) and 0.1 mL of this stock solution was added to 0.1 mL of treated HepG2 or L929 cells with [Cu(L)(2imi)] complex and allowed for 7 minutes before the observation under a optical microscope at low magnification. Then, the number of blue-staining cells and total cells were counted and the percentage of cell viability was calculated by the formula below:

$$\% \text{ viable cells} = (1.00 - [\text{Number of blue cells} \div \text{Number of total cells}]) \times 100$$

Also, after treatment of HepG2 and L929 cells with [Cu(L)(2imi)] complex at IC_{50} concentrations the morphological analysis was investigated under an inverted microscope.

2.3 | Apoptotic genes transcription exposed to [Cu(L)(2imi)] complex

To evaluate The messenger RNA (mRNA) expression of apoptosis regulatory genes p53, caspase-8, bcl-2, and bax were detected after treating the HepG2 and L929 cells with copper complex (II) at IC_{50} concentration for- 24 hours.

Treated and untreated HepG2 and L929 cells were harvested and washed with phosphate-buffered saline (PBS) at 4°C. Total RNA was extracted using total RNA extraction Kit (Bioneer, Daejeon, Korea) following instructions presented in the protocol. Total RNA was eluted in 40 L of RNase-free water. The concentration of RNA was estimated using the absorbance values at 260 nm, while the purity of each sample was determined calculating the 260/280 ratio. RNA integrity was assessed by inspection of the 28S and 18S ribosomal RNA bands in a 1% agarose gel. Total RNA, 500 ng, was reverse transcribed to produce the first strain of complementary DNA (cDNA) (Bioneer, Korea), according to the manufacturer's instructions. The RT-PCR was prepared to a final volume of 20 μL: 2 μL of cDNA, 2 μL of the forward and reverse primers (Table 1), 6 μL of Dnase-free water, and 10 μL SYBR Green with 0.4 ROX (Takara, Japan). The following procedure was implemented: 95°C for 1 minute (1 cycle) and 95°C for 3 seconds, and 30 seconds at the annealing temperatures (54°C for β-actin, p53, and caspase-8 genes; 58°C for bax gene and 60°C for bcl-2 gene), and 72°C for 20 seconds (40 cycles). All procedures were performed in a Applied Biosystem's Step One Plus Real-time PCR machine (Applied Biosystem, Foster City, CA). Then, the melting curve was analyzed to demarcate the specificity of the reaction. Melting curves were analyzed after amplification reactions and single amplification products were present in all reactions. RT-PCR reactions were carried out in triplicate and only comparative threshold cycle (C_t) values leading to a C_t mean with a standard deviation below 0.2 were considered a five-fold dilution series of cDNA, prepared from a mix of the samples, was used as reference for construction of standard curves, reaction efficiencies and r^2 determination. The $\Delta\Delta C_t$ method was used to calculate relative mRNA expression levels of genes with β-actin gene as a housekeeping gene for normalization and values were normalized to the control average value. Those values which had been obtained from the exposed groups were compared in terms of statistics.

TABLE 1 Primer sequences used in the study

Genes	Primer sequences (5′ → 3′)
Bcl-2	F: CTTCTTTGAGTTCGGTGGGG R: AAATCAAACAGAGGCCGCAT
p53	F: TGAAGCTCCCAGAATGCCAG R: GCTGCCCTGGTAGGTTTTCT
Caspase-8	F: ATTAGGGACAGGAATGGAACAC R: GGAGAGGATACAGCAGATGAAG
Bax	F: TGCCTCAGGATGCGTCCACCAA R: CCCCAGTTGAAGTTGCCGTCAG
β-Actin	F: GGGCATGGGTCAGAAGGATT R: CGCAGCTCATTGTAGAAGGT

Abbreviations: F, forward; R, reverse.

2.4 | DNA fragmentation assay

DNA from treated and untreated HepG2 and L929 cells with [Cu(L)(2imi)] complex at IC_{50} concentrations for 24 hours were extracted using a DNA extraction kit (CinnaPure DNA; catalog no. PR881613; SinaClon, Tehran, Iran) according to the manufacturer's instructions.

2.4.1 | Cells preparation for DNA extraction

Cells were cultured in 75-cm flask and treated with [Cu(L)(2imi)] complex at IC_{50} concentrations for 24 hours. Then, about 6×10^6 cells were collected by centrifugation (5 minutes at 3000*g*) and the pellet, washed twice using sterile PBS and the supernatant, was discarded completely by pipetting. Then, 100 μL of prelysis buffer and 20 μL of ributinase were added to the tube and the reaction was vortexed completely and then incubated at 55°C for 30 minutes.

2.4.2 | DNA extraction

About 100 μL of prepared cells was transferred to a sterile 1.5-mL tube and 400 μL lysis buffer was added and vortexed at maximum speed for 20 seconds in the following 300 μL precipitation solution was added and vortexed completely. Then, the solution was transferred to a spin column with collection tube by pipetting and centrifuged at 13 000*g* for 1 minute. The collection tube was discard and spin column was placed in new collection tube and 400 μL wash buffer I was added to spin column and centrifuged at 13 000*g* for 1 minute and discard supernatant. Then, the spin column was washed with 400 μL of wash buffer II and centrifuged at 13 000*g* for 1 minute for discarding supernatant (this step was repeated twice). Finally, to resolve completely, the extracted DNA was incubated in 30 μL 65°C preheated elution buffer for 3 to 5 minutes at 65°C.

2.4.3 | DNA gel electrophoresis

About 5 μL of extracted DNA from L929 and HepG2 cells before and after treatment with [Cu(L)(2imi)] complex at IC_{50} concentrations for 24 hours was loaded onto a 2% agarose gel, and electrophoresis was conducted at 50 V for 2 hours in 0.5 3 Tris–borate–EDTA (TBE) buffer (one 3TBE buffer contains 90 mM Tris–borate/2 mM ethylenediaminetetraacetic acid). The gel was stained with 2 mg/mL ethidium bromide for 15 minutes, distained with water for 1 hour, and visualized under UV light gel documentation (Bio-Rad, Hercules, CA).

2.4.4 | Statistical analysis

After the evaluation of the normal distribution of the data, one-way analysis of variance under SPSS software (SPSS Inc., Chicago, IL), version 17, was used to analyze the data. A level of $P < 0.05$ was accepted as statistically significant.

3 | RESULTS

3.1 | Induction of apoptosis with [Cu(L)(2imi)] complex

Apoptosis is specialized by some characterization such as DNA fragmentation, chromatin condensation, and cell shrinkage. To determine whether apoptosis could be induced in both cell lines, they were treated for 24 and 48 hours at IC_{50} concentrations of [Cu(L)(2imi)] complex.

After treatment, cells were analyzed under inverted microscope for apoptotic morphological feature and by staining with Trypan blue uptake for cell death as well as DNA fragmentation was performed using electrophoresis of DNA on 2% agarose gel.

3.1.1 | DNA fragmentation in HepG2 and L929 cells after 24- and 48-h treatment with [Cu(L)(2imi)] complex in IC_{50} concentration

Analysis of DNA fragmentation was performed by electrophoresis of extracted DNA from untreated and treated (for 24 and 48 hours) cells with [Cu(L)(2imi)] complex on 2% agarose gel. As shown in Figure 1, DNA fragmentation was observed in both L929 and HepG2 cells after treatment with [Cu(L)(2imi)] complex at IC_{50} concentrations. Cleavage and fragmentation of DNA is one of the evidence that showed morphological features of programmed cell death (Figure 1).

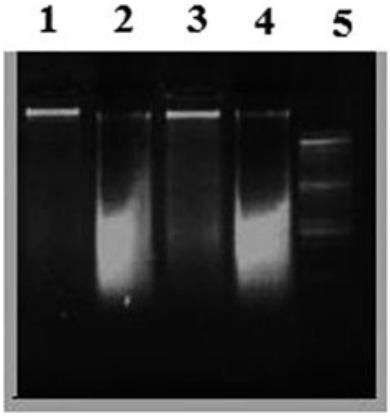

FIGURE 1 DNA fragmentation assay for detection of apoptosis. Lane 1, Gel electrophoresis of DNA isolated from untreated HepG2 cells. Lane 2, DNA from HepG2 cells treated with [Cu(L)(2imi)] complex at IC_{50} concentration (58 μg/mL) after 24 hours. Lane 3, DNA isolated from untreated L929 cells. Lane 4, DNA from L929 cells treated with [Cu(L)(2imi)] complex at IC_{50} concentration (105.3 μg/mL) after 24 hours. Lane 5, DNA ladder

3.1.2 | Evaluation of viability using the Trypan blue dye exclusion assay

To investigate the cell viability, the Trypan blue dye exclusion and 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide assay was performed in HepG2 and L929 cells after 24- and 48-hour treatment with [Cu(L)(2imi)] complex using a hemocytometer. The Trypan blue exclusion assay is commonly used for investigation of the integrity of the biological membranes. After the staining of cells using Trypan blue, the normal or healthy cells exclude dye and remain uncolored, while only death cells take up this reagent, therefore all nonviable cells appear blue-purple (Figure 2). Death cells were counted to determine the cell viability of HepG2 and L929 cells in different concentrations of [Cu(L)(2imi)] complex after the 24- and 48-hour treatment. The results of the present study revealed that the viability of HepG2 and L929 cells were significantly decreased after 24- and 48-hour treatment with [Cu(L)(2imi)] complex in a manner which was quite dependent on time and dose. In previous study, the IC_{50} concentrations for HepG2 cells were obtained 58 and 55 μg/mL after 24- and 48-hour treatment, respectively, while the IC_{50} concentrations for L929 cells were 105.3 and 83 μg/mL, respectively (Figure 3). The results of the present study showed that the treatment of L929 cells with IC_{50} concentration of HepG2 cells in 24 and 48 hours was not significantly decreased the viability of L929 cells (Figure 2).

3.1.3 | Morphological analyses for evaluation of viability and apoptotic cell death using inverted microscopy

The apoptotic and necrotic cells were characterized using special morphological features. In previous study, we indicated that after treatment of HepG2 and L929 cells with [Cu(L)(2imi)] complex at IC_{50} concentrations, the apoptotic and necrotic morphological features were observed under the inverted microscopy with ×40 magnification. It seemed that [Cu(L)(2imi)] complex could not only inhibit the growth of HepG2 and L929 cells in IC_{50} concentrations, but also induce some apoptotic and necrotic cells based on the morphological analyses. Morphological observation revealed that [Cu(L)(2imi)] complex could significantly induced apoptosis in HepG2 and L929 cells compared with untreated cells at IC_{50} concentrations. The percentage of apoptotic cells

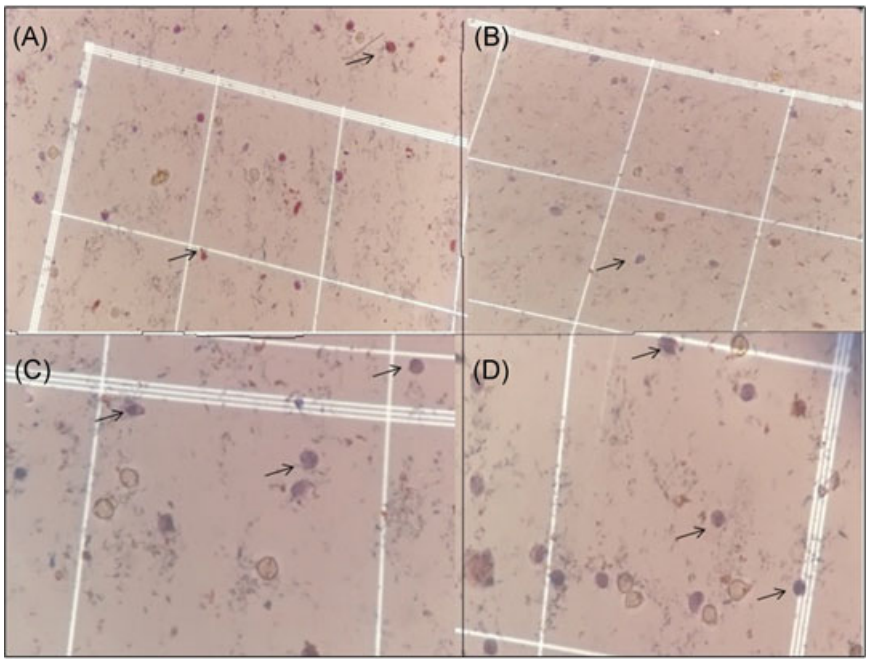

FIGURE 2 The Trypan blue exclusion assay for detection of death cells. Illustration of optical microscopic images of the HepG2 cells treated with [Cu(L)(2imi)] complex at IC_{50} concentration (58 μg/mL) after (A) 24 and (B) 48 hours. The L929 cells treated with [Cu(L)(2imi)] complex at IC_{50} concentration (105.3 μg/mL) after (C) 24 and (D) 48 hous. The cells were stained using Trypan blue dye after treatment with [Cu(L)(2imi)] complex to examine cell death that indicated by arrows

induced by [Cu(L)(2imi)] complex was significantly increased gradually with the increase of the concentration of the complex.

3.2 | Evaluation of some apoptotic gene expression in HepG2 and L929 cells

To investigate whether [Cu(L)(2imi)] complex is able to induce apoptosis in HepG2 and L929 cells via intrinsic and/or extrinsic apoptotic pathway, we performed the SYBR Green-based RT-PCR analyses. To evaluate the molecular mechanisms involved in the apoptosis induction after exposition of HepG2 cancerous cells and L929 as normal cells with [Cu(L)(2imi)] complex, we measured the expression levels of bcl-2, bax, caspase-8, and p53 genes in cells after a 24- and 48-hour treatment at IC_{50} concentrations (Figure 4).

The results showed a dramatic increase (2-fold) in the expression level of bax gene HepG2 cells after a 24-hour copper complex treatment ($P < 0.05$) (Figure 4A), without a significant effect following the 48-hour treatment (Figure 4C). In L929 cells, we interestingly observed that the expression level of bax gene decreased after 48-hour treatment ($P < 0.05$) (Figure 4D), while a two-fold increase in the expression level of bax gene following the 24-hour copper complex treatment was observed ($P < 0.05$) (Figure 4B).

Many studies have been shown that cell survival promotes with the expression of bcl-2 because this gene inhibits the apoptosis process. Bcl-2 protects cancerous cells from death induced by apoptosis therefore many researchers try to apply a strategy for development of a variety of anticancer drugs for the inhibition of the bcl-2 expression.

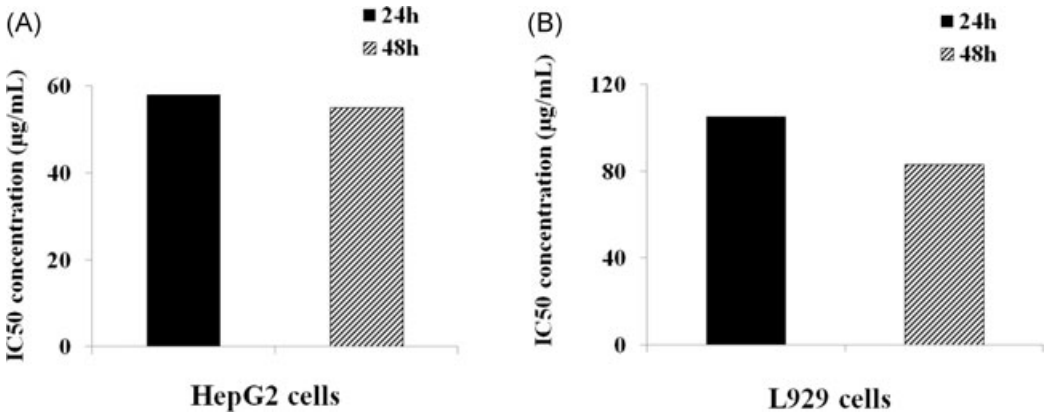

FIGURE 3 Demonstrates IC_{50} concentrations of [Cu(L)(2imi)] complex in (A) HepG2 and (B) L929 cells after the 24- and 48-hours treatment

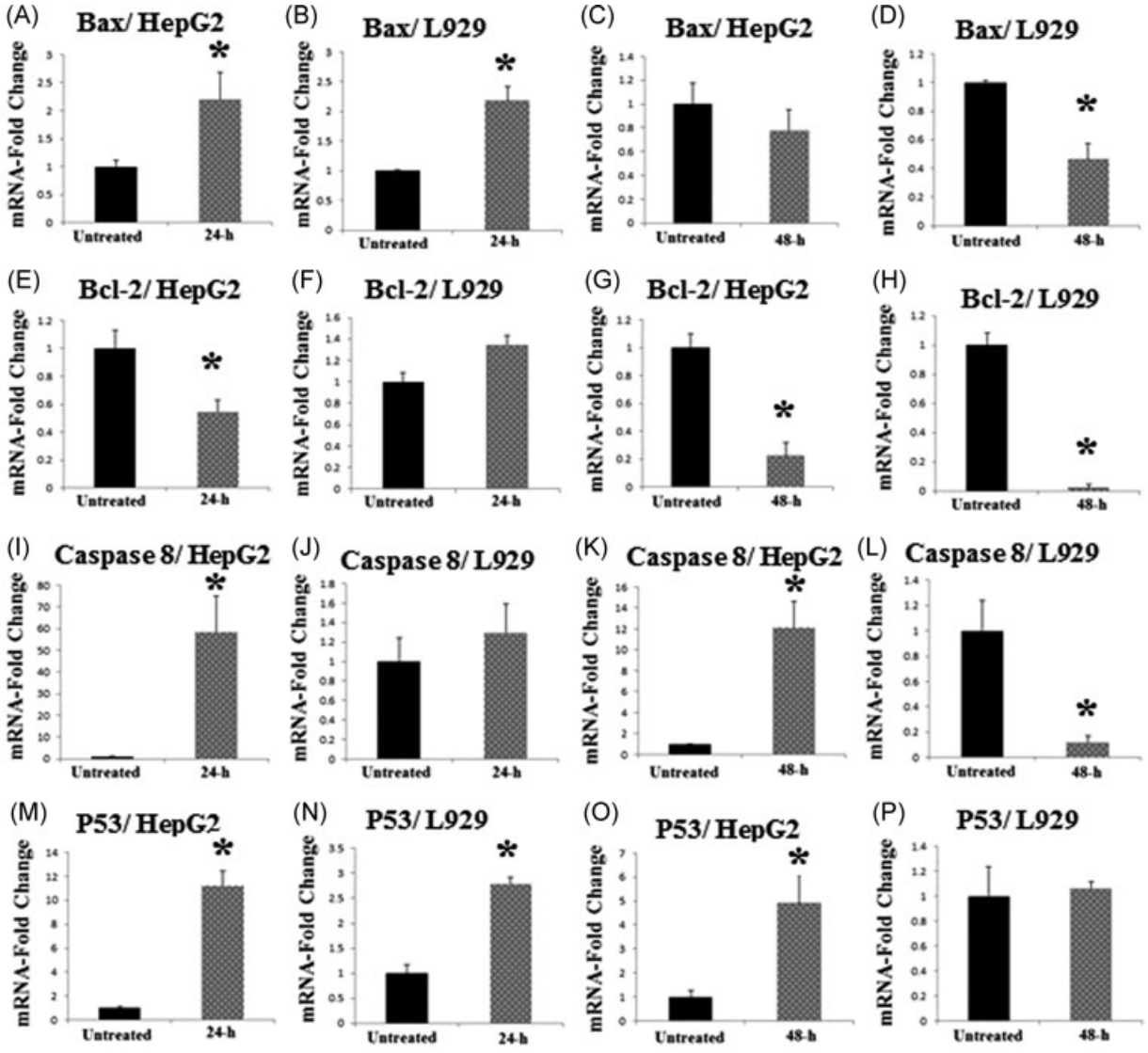

FIGURE 4 The effect of [Cu(L)(2imi)] complex on some genes expressions involved in extrinsic and intrinsic apoptotic pathways. Real-time PCR analysis detected the mRNA expression levels of bax, bcl-2, caspase-8, and p53 in HepG2 and L929 cells at IC_{50} concentration after a 24- and 48-hour treatment with [Cu(L)(2imi)] complex. β-Actin served as an internal control. Data are represented as mean ± SD. Each data point is an average of results obtained from three independent experiments performed in triplicate. *$P < 0.05$ indicates a significant difference with the untreated cells. mRNA, messenger RNA; PCR, polymerase chain reaction

In the present study, the expression level of bcl-2 gene was significantly decreased after both 24- (2-fold) (Figure 4E) and 48-hour (Figure 4G) (3-fold) treatment of HepG2 cells with the complex ($P < 0.05$). In L929 cells, the bcl-2 was also downregulated after the 48-hour treatment (Figure 4H) ($P < 0.05$), while any significant effect was not observed in the expression level of this gene after the 24-hour treatment (Figure 4F).

In HepG2 treated cells the expression levels of caspase-8 were significantly overexpressed under both treatment-time conditions ($P < 0.05$) (Figure 4I and Figure 4K).

In L929 cells, the expression level of caspase-8 gene after 48-hour treatment was significantly downregulated ($P < 0.05$) (Figure 4L), while it was unchanged after 24-hour treatment with [Cu(L)(2imi)] complex (Figure 4J).

The p53 gene was upregulated following both 24- (Figure 4M) and 48-hour (Figure 4O) complex treatment of HepG2 cells and showed an increase approximately two- and three-fold in the expression level of this gene, respectively ($P < 0.05$). The expression level of p53 gene was significantly increased about three-fold in L929 cells after 24-hour (Figure 4N) ($P < 0.05$), without any significant increase in its expression level after the 48-h treatment (Figure 4P).

We evaluated the relative bax/bcl-2 ratios in HepG2 and L929 cells after 24- and 48-hour treatment with [Cu(L)(2imi)] complex at IC_{50} concentrations (Figure 5).

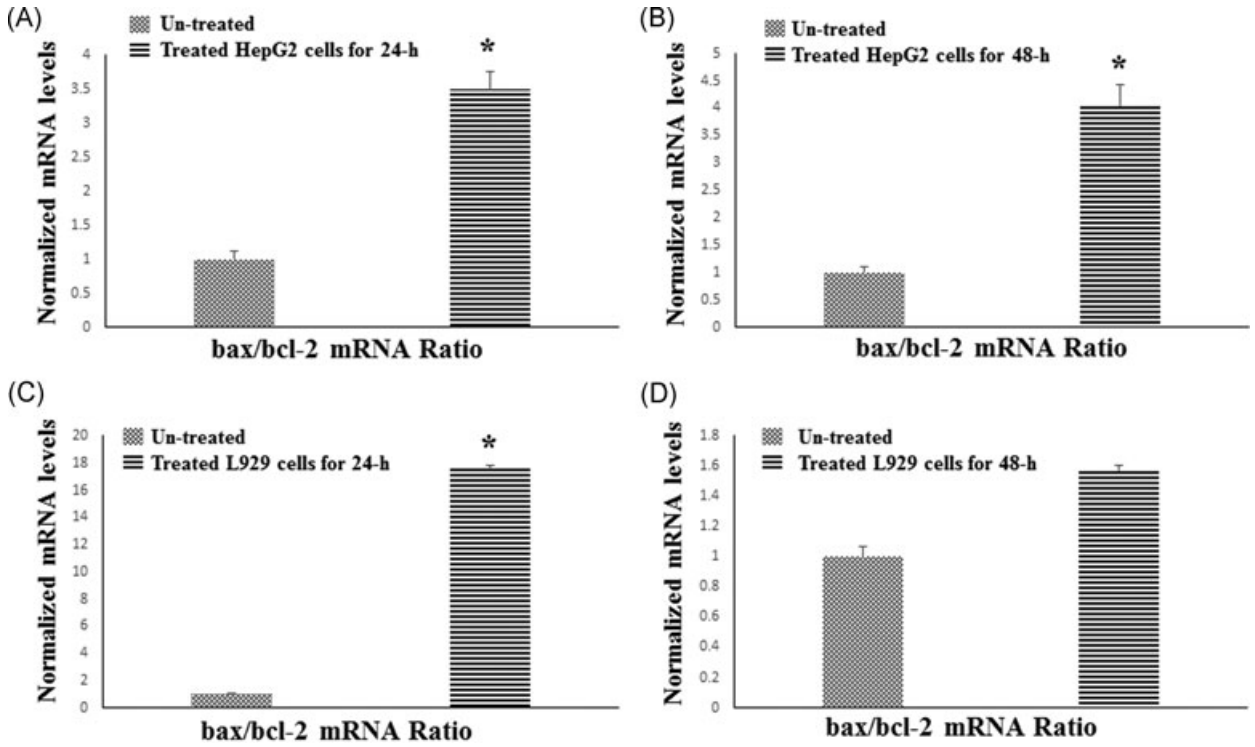

FIGURE 5 The effect of [Cu(L)(2imi)] complex on bax/bcl-2 mRNA ratio. Real-time PCR analysis detected the expression levels of bax/bcl-2 mRNA in (A,B) HepG2 and (C,D) L929 cells at IC_{50} concentrations after the 24- and 48-hour treatment with [Cu(L)(2imi)] complex, respectively. β-Actin served as an internal control. Data are represented as mean ± SD. Each data point is an average of results obtained from three independent experiments performed in triplicate. $^*P < 0.05$ indicates a significant difference with the untreated cells. mRNA, messenger RNA; PCR, polymerase chain reaction

We observed a 3.5- and four-fold decrease in bax/bcl-2 ratio in HepG2 cells after the 24- (Figure 5A) and 48-hour (Figure 5B) treatment with [Cu(L)(2imi)] complex at IC_{50} concentrations, respectively ($P < 0.05$). In L929 cells, this ratio was 17-fold after 24-hour treatment with the complex (Figure 5C) ($P < 0.05$), while no significant increase was observed in bax/bcl-2 ratio after 48-hour treatment (Figure 5D).

4 | DISCUSSION

Some anticancer drugs are derived from natural resources such as herbal medicines[32] or bacteria.[33] However, the majority of these drugs were synthetized chemically from different compounds and were used as chemotherapeutic agents for treatment of different cancers for example vanadium[34] and Cu[35] complexes, indole derivatives,[36,37] proline-derived compounds,[38] and so forth. But because of the change in the nature of cancer cells and their escape from death and resistance to anticancer drugs, research to find new compounds with new anticancer properties has always been one of the major concerns of researchers.

In the present study, a new Cu (II) complex, [Cu(L)(2imi)] was synthesized and structurally characterized according to our previously reported procedure.[31] In our previous study, we showed that this complex was able to exert cytotoxic effects and induce apoptosis in HepG2 cell line. Since the liver is responsible for detoxification in the human body, it is in charge of absorbing, distributing, metabolizing, and eliminating those complexes which contain metal. HepG2 lines, which were used as a classic liver cell model in our study, are functioning polarized hepatocyte cells. Unlike other trace metals in the body, copper is a critical element and a cofactor, which is quite required for tumor angiogenesis processes.[39] Cellular copper has been shown not to be all protein-bound and it has also been indicated to be storable in membranes.[40] Given its role as a cofactor of enzymes and angiopoiesis, copper shows it is capable of several biological processes and reactions. In one of the recommended usages for copper, it has been introduced as an element within depletion therapy to prevent cancer.[41] Despite significant anticancer activities of cooper complexes,[42,43] the main mechanism through which these elements function has not come under light quite well yet. Research findings have shown that copper complexes trigger cell death through generating double-strand DNA breakage.[44]

Inhibition of cytotoxic-drug based proliferation of cancer cells might occur due to three factors:cycle arrest, induction

of apoptosis, or a combination of these factors.[45] Apoptosis is a type of programmed cell death involving the activation of an interior mechanism followed by multiple pathways of signal transmission to terminate the cell under pathological and physiological conditions.[46,47] There are two major apoptotic pathways: the cell death receptor-mediated (extrinsic) apoptotic pathway and the mitochondria-mediated (intrinsic) apoptotic pathway. Bcl-2 family members are the major apoptosis-regulating proteins. They are primarily located at the mitochondrion and are associated with the release of cytochrome *c*.[48]

In the present study, we observed that the expression of mRNA levels of tumor-suppressor gene p53 and apoptotic genes (bax and caspase-8) were upregulated while the expression of antiapoptotic gene bcl-2 was downregulated in HepG2 cells treated with Cu (II) complex. Upregulation of p53 leads to activation of proapoptotic members of bcl-2 family, such as bax induces permeabilization of the outer mitochondrial membrane, which releases soluble proteins from the intermembrane space into the cytosol, where they promote caspase activation.[49]

Fan et al[39] investigated the effect of Metam/Cu (II) on HepG2 cells. The results showed that Metam/Cu (II) increased the protein expression ratio of bax/bcl-2. Several studies demonstrated that different Cu (II) complexes can increase the expression of bax/bcl-2 ratio in other cancer cell lines such as MCF-7 and BEL-7404 cells.[50,51] These findings indicate that the bcl-2 family of proteins may be involved in the induction of apoptosis by Cu (II) complexes. In agreement with our results, Zhu et al[52] showed that a Cu (II) complex of a coumarin derivative upregulated p53 and Bax and downregulated bcl-2. The activation of p53 plays a proapoptotic role by inducing and downregulating many fundamental cell death effectors, which ultimately leads to apoptotic cell death.[53] Interestingly, a Schiff base Cu (II) complex has been reported to activate caspase-8 in HT-29 cells.[54]

Previous studies have been reported that p53 promotes the expression of the bax gene that is one of the proapoptotic members of bcl-2 family and represses the bcl-2 expression, an antiapoptotic gene from this family. Therefore, in the present study, we determined whether the expression levels of bcl-2 and bax genes were regulated in the HepG2 and L929 cells following [Cu(L)(2imi)] complex treatment. The expression levels of p53 and caspase-8 were also determined. IC_{50} concentrations of [Cu(L)(2imi)] complex were used and cells were exposed to the complex for 24 and 48 hours.

Surprisingly, the expression level of bax gene was significantly downregulated in L929 and unchanged in HepG2 cells after the 48-hour treatment. While the bax expression was significantly increased after 24-hour treatment in both HepG2 and L929 cells (Figure 4). The expression levels of bcl-2 gene in HepG2 cells were significantly decreased after the 24- and 48-hour treatment while caspase-8 was significantly overexpressed under both treatment-time conditions.

In L929 cells, the expression levels of both caspase-8 and bcl-2 genes after the 48-hour treatment were significantly downregulated, while they were unchanged after the 24-hour treatment with [Cu(L)(2imi)] complex (Figure 4).

Bax gene is a proapoptotic member of Bcl-2 family that it was expressed in cytosol after exposure of cells to apoptotic stresses such as cytotoxic drugs. The bax protein that is a mediator of the programmed cell death process translocates from cytosol to mitochondria and triggers apoptosis following the release of cytochrome *c* from mitochondria.

In this present study, we demonstrated that bax/bcl-2 ratio was increased in HepG2 and L929 cells exposed to [Cu(L)(2imi)] complex for 24 hours. Bax and bcl-2 were involved in intrinsic apoptosis pathway that is independent of caspase activity or extrinsic pathway of programmed cell death. The inhibition of bcl-2 induces redistribution of Bax to the mitochondria and triggers intrinsic apoptotic pathway following exposure of HepG2 and L929 cells with the complex at IC_{50} concentration.

However, our complex did not upregulate caspase-8 in L929 normal cells as control.

The results of the present study revealed that [Cu(L)(2imi)] complex triggered apoptosis in HepG2 cells via both extrinsic and intrinsic apoptotic pathways while in L929 cells, this complex induced apoptosis via only the intrinsic pathway.

In conclusion, the results from the current study in addition to our previous study suggest that Schiff base [Cu(L)(2imi)] complex induced apoptosis. Therefore, this complex has potential for development as a novel anticancer drug and there is a need for more investigation on the effect of this complex on animals and human.

ACKNOWLEDGMENT

Authors would like to offer their thanks to the staff of the Molecular Medicine Research Center, Research Institute of Basic Medical Sciences, Rafsanjan University of Medical Sciences, for their real assistance.

CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

AUTHOR CONTRIBUTIONS

AR participated in the collection of data and drafted the manuscript; M Mohamadi participated in the study design

and edited the manuscript; FM participated in the collection of data and statistical analysis. M Mohamadi participated in the statistical analysis and revised the manuscript. MRH and MRM participated in the study design and interpretation of data. SKF participated in the interpretation of data, study design, and revised the manuscript. All authors approved the final version of the manuscript.

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How to cite this article: Rezaei A, Mahmoodi M, Mohammadizadeh F, et al. A novel copper (II) complex activated both extrinsic and intrinsic apoptotic pathways in liver cancerous cells. *J Cell Biochem*. 2019;1-10.
https://doi.org/10.1002/jcb.28491