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Cytotoxic, pro-apoptotic, pro-oxidant, and non-genotoxic activities of a novel copper(II) complex against human cervical cancer

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ABSTRACT

Cisplatin remains one of the most effective current chemotherapeutic agents; however, metal complexes synthesis has increased in order to produce new anti-neoplastic drugs with DNA binding and apoptotic activities in tumor cells and less toxicity for patients. In this study, we evaluated the cytotoxic activity of a novel copper(II) complex (LQM402) against cervical cancer cell lines and found that LQM402 exhibited selective cytotoxicity against HeLa and Ca Ski cells. FITC-annexin assay and DNA fragmentation indicated that apoptosis could be involved in HeLa cell death. Caspase 3/7 and cytochrome c analysis by immunoblotting suggest the intrinsic pathway. LQM402 is a lipid peroxidation inductor according to TBARS production. Additionally, the Ames and micronucleus tests demonstrated non-genotoxic activity for this compound in *Salmonella typhimurium* and CD1 mice, respectively. Therefore, LQM402 may be a promising and safe anti-cervical cancer compound.

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1. Introduction

Cervical cancer (CeCa) is a common malignancy around the world. This disease is a public health problem in Latin America, where it causes approximately 32,000 deaths in women per year (Almonte et al., 2010). Because currently used anti-tumor drugs induce toxic side effects and resistance, it is necessary to research and synthesize new, effective products for the treatment of advanced tumors that induce fewer negative effects (Zou et al., 2005). Since the discovery of the standard anti-proliferative drug cisplatin, derivatives such as carboplatin, oxaliplatin, and other metal complexes have been synthesized and tested for antitumor activities *in vitro* (Chitambar and Purpi, 2010; Laine and Passirani, 2012) and *in vivo* (Chen et al., 2007; Milacic et al., 2006). The participation of copper in the electron transfer reactions of many cellular processes and its function as a component of metalloproteinases and enzymes has been considered during the development of chemotherapeutic drugs (Marzano et al., 2009; Wang and Guo, 2006; Ruiz-Azuara and Bravo-Gomez, 2010).

Copper, like other trace metals, is essential to proteins that are involved in several biological processes, including respiration, metabolism, DNA synthesis, and oxidation–reduction reactions (Marzano et al., 2009). Copper deficiency or imbalance is associated with severe neurological disorders, including Alzheimer's, Parkinson's (Donnelly et al., 2008), Wilson's, and Menkes' diseases (Wang and Guo, 2006). The chemical characteristics of transition metals provide opportunities to develop novel metal-based anticancer drugs with different mechanisms of action; thus, complexes that contain copper or other metals represent a new generation of alternative antitumor agents (Ruiz-Azuara and Bravo-Gomez, 2010). Casiopeínas® are copper complexes that were designed on the basis of the antitumor activities of cisplatin and other series of metal compounds. These compounds were found to be several times more cytotoxic than cisplatin against some cervical cancer cell lines (Gracia-Mora et al., 2001). Various copper complexes are also cytotoxic against other types of human cancer cells. A ternary copper(II) complex of amino-coumarins with phenanthroline inhibited the growth of PC3 human prostate cancer cells and HL-60 human myeloid leukemia cancer cells (Jia et al., 2010). Benzene-1,2-dithiol-modified, cisplatin-based copper(II) and zinc(II) complexes were also shown to be cytotoxic

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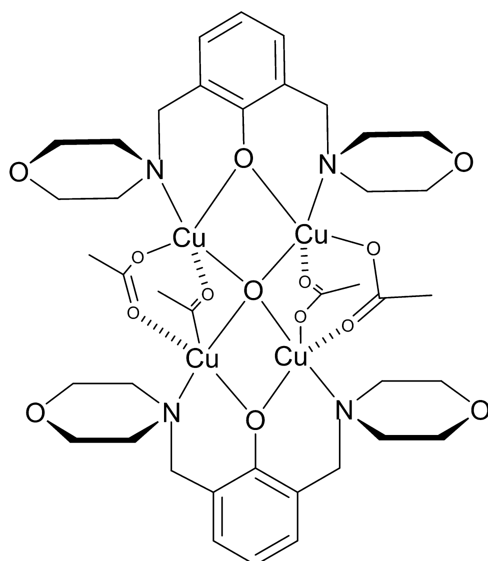

Fig. 1. Proposed structure for the novel copper(II) complex LQM402.

against human cancer cell lines, including HeLa (human cervical cancer), Hep2 (human laryngeal epithelial cancer), HepG2 (human liver cancer), and MCF-7 (human breast cancer cells) (Raman et al., 2010). Treatment with10-deacetylbaccatin thiosemicarbazone alone only arrested the growth of viable MCF-7 breast cancer cells, while the addition of copper to the same compound induced notable cell lethality (Murugkar et al., 1999). In recent years, reports on cytotoxic copper complexes have shown that the mechanism of action is based on DNA intercalation and cleavage activity (Krishnamoorthy et al., 2011; Lakshmipraba et al., 2011) or apoptosis induction activity (Boulsourani et al., 2011; Tardito et al., 2009). In this paper, we evaluated the *in vitro* cytotoxic abilities of the copper(II) complex LQM402 against four human cervical cancer cell lines, of which HeLa and Ca Ski cells were the most sensitive. DNA ladder and TUNEL fragmentation assays showed that LQM402 induced DNA cleavage in HeLa cells in a time-dependent manner. We hypothesize that the cytotoxic effects are related to the pro-apoptotic activity of LQM402, as detected by annexin-FITC staining and the diminished expression of pro-caspases 3 and 7, as well as the presence of cytochrome c in cytosolic extracts. Moreover, LQM402 did not induce mutations in *Salmonella typhimurium* and CD1 mice.

2. Materials and methods

2.1. Chemistry

The complex (μ_4-Oxo)tetra-μ_4-acetate{4-hydroxi-3,5-bis(morpholinomethyl)}tetra copper(II) (LQM402; Fig. 1) was prepared in ethanol with 1.602 g of the ligand 2,6-Bis(morpholinomethyl)phenol (Velazquez et al., 2008), 1.081 g Cu(II) acetate and 0.1 ml NH_4OH. The solution was stirred for 18 h at room temperature. The solvent was discarded and the precipitate was collected and crystallized (yield: 86%). The resulting green crystals displayed the following characteristics: formula, $C_{44}H_{72}Cu_4O_{14}N_4$; molecular weight, 1132 g/mol; and melting point, 198–200 °C (unpublished data; in patent processing).

2.2. Culture of cell lines

Four human cervical carcinoma (CeCa) cell lines, HeLa, SiHa, Ca Ski, and C-33 A, and primary non-tumoral human fibroblasts (FB) were used. HeLa cells were kindly donated by Teresa Ramírez-Apan, M.Sc. from the Instituto de Química at Universidad Nacional Autónoma de México (UNAM). SiHa, Ca Ski, and C-33 cell lines were kindly donated by Juan Carlos Gomora, Ph.D. from the Instituto de Fisiología at UNAM. The cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (HyClone, Thermo Scientific, Logan, UT, USA) and 1% antibiotic–antimycotic solution (Gibco, Carlsbad, CA, USA) in culture flasks at 37 °C in a 5% humidified CO_2 incubator. Semi-confluent cells (70–80%) were removed with 0.1% trypsin (Sigma–Aldrich, St. Louis, MO, USA), washed twice with phosphate-buffered saline solution (PBS) that contained 0.2 g KCl, 0.2 g KH_2PO_4, 8 g NaCl, and 2.16 g $Na_2HPO_4 \cdot 7H_2O$ per liter, pH 7.4, centrifuged, and resuspended in media. Separately, 5×10^3 FB, HeLa, or SiHa cells and 8×10^3 Ca Ski or C-33 A cells per well were deposited into 96-well microplates. The cells were incubated overnight before additional treatments.

2.3. Cell treatment and MTT assay

Cytotoxicity was determined with a standard MTT assay as described by Mosmann (1983). This assay is based on the reduction of yellow MTT (3-(4,5-dimethythiazol-2-yl)-2,5-diphenyltetrazolium bromide) to formazan by mitochondrial succinate dehydrogenase in living cells. The resulting purple formazan crystals are dissolved in DMSO and subsequently the absorbance is measured and related to the absorbance value of formazan produced by untreated cells. The absorbance value of formazan is directly proportional to the number of viable cells (Cory et al., 1991). Briefly, CeCa cells that had been seeded into 96-well microplates were treated for 24 h with different concentrations of LQM402 compound (12.5–100 μM). Cisplatin (Sigma–Aldrich, St. Louis, MO, USA) at the same concentrations was used as the positive control, and media plus 1% DMSO was used as the negative control. Additionally, HeLa cells were treated for 48 and 72 h. After treatment, the media was aspirated from the wells and replaced with medium containing MTT (1 mg/ml), and the cells were incubated for an additional 4 h at 37 °C. After incubation and the removal of the MTT media, the formazan crystals were dissolved with DMSO and the plates were read in a microplate reader (ELx808, Bio-Tek, Winooski, VT, USA) at 570 nm. All treatments were performed at least three times in triplicate. Mean values were obtained and used to calculate the percent viability according to the formula: % viability = (mean OD treated cells × 100)/mean OD control cells.

The concentration values that caused a 50% cell growth inhibition (IC_{50}) were calculated from the dose–effect relationship of each cell line by non-linear regression with Prism 5.0 software (GraphPad, La Jolla, CA, USA).

2.4. Phosphatidylserine detection by flow cytometry assay

The apoptotic activity of LQM402 was assessed by FITC-labeled annexin V binding and PI uptake with the Annexin-V FITC kit (BioVision, Milpitas, CA, USA). According to the manufacturer's instructions, 5×10^4 HeLa cells were seeded in DMEM media with 10% FBS and allowed to adhere for 24 h. On the following day, the media was replaced with media that contained LQM402 at 74.74 μM and the cells were incubated for an additional 6, 12, or 24 h. After the treatment, the cells were harvested, washed twice with PBS, resuspended in 500 μl binding buffer plus 5 μl annexin V-FITC and 5 μl PI (provided within the kit). The tubes were gently stirred, kept in the dark on ice for 10 min, and analyzed on a FACScan flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) (Vermes et al., 1995).

2.5. Cell Death detection by TUNEL assay

The HeLa programmed cell death was detected using the *In Situ* Cell Death Detection kit, AP version 11.0 from Roche according to the manufacturer instructions with minimal modifications. Briefly, HeLa cells were seeded in coverslips inside 6 well plates and treated with 74.74 μM LQM402 for 24, 48, and 72 h. 1% DMSO and 20 μM cisplatin-treated cells (48 h) were the vehicle and positive control, respectively. After treatments, cells were washed twice with PBS and fixed with 4% paraformaldehyde in PBS pH7.4 and permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate for 20 min at 2–8 °C. After permeabilization, cells were washed twice with PBS and covered with 50 μl of the TUNEL reaction mixture and incubated at 37 °C for 60 min in a humidified atmosphere in the dark. Finally, coverslips with cells were rinsed three times with PBS and placed on slides using the Vectashield® Mounting Media for fluorescence (Vector Labs) for their observation under a fluorescence microscope in the range of 515–565 nm (green light).

2.6. Ladder assay (detection of DNA fragmentation)

DNA isolation from HeLa cells treated with 74.74 μM for 24, 48, and 72 h was performed using the new DMSO-SDS-TE method according to Suman et al., 2012. Floating and attached cells seeded and treated in 100 mm petri dishes were collected and washed with PBS. 100 μl of DMSO was added directly to the cell pellet and mixed well followed by vortexing. 100 μl of SDS (2% sodium dodecyl sulphate)-TE (Tris–EDTA) buffer (pH 7.4) was added followed by mixing and vortexing. The resulting solution was centrifuged at 13,000 rpm at 4 °C and supernatant was collected in a new tube. 25 μl of the supernatant for each treatment was loaded on 1.5% agarose gel and running for 60 min at 100 V. 1% DMSO and 20 μM cisplatin-treated cells (48 h) were the negative and positive control, respectively.

2.7. Western blotting analysis

The expression levels of caspases 3, 7, and 9 and cytochrome c were determined by immunoassays. Control and 74.74 μM LQM402-treated HeLa cells from different time points (3, 6, 12, 18, and 24 h) were washed twice with PBS and total or

cytoplasmic extracts were obtained with RIPA buffer (0.5 M Tris–HCl, 1.5 M NaCl, 2.5% deoxycholic acid, 10% NP-40, 10 mM EDTA) or digitonin, respectively (all from Sigma–Aldrich) at pH 7.4, in the presence of a Complete Protease Inhibitor Cocktail Tablet (Roche Diagnostics, Mannheim, Germany). All lysis reactions were performed on an ice bath. Total protein concentrations were determined according to the Bradford method (BioRad, Hercules, CA, USA). Equal amounts of protein from each lysate (30 μg) were loaded and run on 12% SDS-polyacrylamide gels. The proteins were then transferred to a nitrocellulose membrane that was blocked with 5% non-fat milk in 0.1% Tween 20-Tris buffered saline at pH 7.5. After washing, hybridization was performed overnight with primary antibodies against caspases 3, 7, and 9 and cytochrome c (1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4 °C. Horseradish peroxidase-conjugated IgG secondary antibodies were also used (Santa Cruz Biotechnology), followed by detection with an enhanced chemiluminescence kit (Pierce, Rockford, IL, USA) and autoradiography developer film (Kodak, Rochester, NY, USA). Anti-β-actin (1:500) was used to ensure equal loading of proteins on the gel (Montes-Sanchez et al., 2009).

2.8. Ames test

Mutagenicity tests are biological assays used to assess the mutagenic potential of chemical compounds (Hong et al., 2011). We performed the Ames test on the following set of histidine auxotrophic *S. typhimurium* strains: TA100 (*hisG46, rfa uvrB* pKm101), which can detect base-pair substitution mutations; TA98 (hisD3502, *rfa uvrB* pKm101), which can detect frameshift mutations; and TA102 (*hisG428*, rfa, pQ1, pKm101), which can detect reactive ROS-induced DNA damage. The mutagenicity of LQM402 was evaluated in these three strains, with or without Arochlor 1254-induced rat liver homogenate (S9 mixture), with an incorporation method as described by Maron and Ames (1983). Three concentrations of LQM402 (50, 100, and 200 μM/plate) were used in the presence or absence of 500 μl of the S9 mixture. LQM402 was dissolved in DMSO at the indicated concentrations and mixed with 100 μl of bacteria culture ($1–2 \times 10^9$ CFU/ml) in 2 ml molten agar that was subsequently added to minimal agar of Vogel Bonner plates with 0.5 mM histidine/biotin. The plates were incubated for 48 h at 37 °C and the histidine (His^+) revertant colonies formed were counted with a Fisher colony-counter. For a positive Ames test result, the tested chemical must induce twice as many His^+ revertants as are obtained by spontaneous reversion (Ames et al., 1975; Szyba et al., 1992). The positive controls used in this test were picrolonic acid (PA) at 50 μg/plate, N-methyl-N′-nitro-N-nitrosoguanidine (NMNG) at 10 μg/plate, mitomycin C (Mit C) at 10 ng/plate, 2-Aminoanthracene (2AA) at 10 μg/plate, and cyclophosphamide (CP) at 500 μg/plate.

2.9. In vivo micronucleus test

Micronucleus tests detect undamaged chromosomes that were not pulled to the appropriate pole of the spindle due to a missing centromere and chromosomal fragments that were not incorporated into daughter cell nuclei at mitosis (Schmid, 1975). For this test, we used CD1 male mice (25–30 g) that were obtained from Rismart, Mexico. Mice were maintained according to the Mexican office of regulations for the production, care, and use of laboratory animals (NOM-062-ZOO-1999) and the protocol approved by the Ethics Committee of the National School of Medicine and Homeopathy of Mexico (Registration No. ENMH-CB-010–2011). The mice were kept in an animal room at 25 ± 2 °C, 50 ± 5% humidity, and under a 12 h:12 h light–dark cycle. Mice were fed a standard diet (Purina, Cuautitlan, Mexico) and water *ad libitum*. Treatment groups consisted of five mice that were each subjected to one intraperitoneal injection. Mice in the control group (vehicle) received sterile water, mice in the positive control group received 60 mg/kg Mit C, and mice in the treatment groups received 5, 10, or 15 mg/kg LQM402 dissolved in sterile water. The same doses of LQM402 were also administered to mice that had received 60 mg/kg Mit C 30 min earlier with the objective of determining the anti-mutagenic potential of LQM402. Peripheral blood smears from the mouse tails were obtained before the treatments and at 24, 48, and 72 h after the treatments. The smears were stained with Giemsa solution (Sigma–Aldrich) in phosphate buffer solution (0.6 M, pH 6.8). To estimate the number of micronucleated polychromatic erythrocytes (MNPCE), 1000 polychromatic erythrocytes (PCE) were scored per smear (Diaz Barriga et al., 1999) and the ratio of PCE was estimated from 2000 erythrocytes. The values obtained from LQM402 or Mit C treated animals were compared to those obtained from control animals.

2.10. Lipid peroxidation induction

Evaluation of reactive species formation is frequently used to measure the antioxidant or pro-oxidant activities in several types of samples. Because the malondialdehyde and similar compounds produced by lipid peroxidation reactions with thiobarbituric acid, this test is named thiobarbituric acid reactive species (TBARS) (Cathcart et al., 1991). For this assay, we used adult male Wistar rats (200 ± 50 g) that were provided by the Institute of Cellular Physiology-UNAM and maintained at 25 ± 2 °C and 50 ± 5% humidity under a 12 h:12 h light–dark cycle. The rats were fed with a standard diet and water *ad libitum*. For rat brain homogenate preparation, the animals were sacrificed with CO_2 according to the official protocols previously mentioned to avoid unnecessary pain. Whole brains were rapidly dissected and homogenized in PBS to produce a 1/10 (w/v) homogenate. The homogenate was subsequently centrifuged for 10 min at 3400 rpm. The protein content in brain supernatant solutions was measured with Folin and Ciocalteu's phenol reagent and the solutions were adjusted with PBS solution to 2.66 mg/ml protein (Dominguez et al., 2005).

All lipid peroxidation experiments were conducted in an ice bath. Briefly, 375 μl of brain supernatant solution and 50 μl of 20 μM EDTA were added to 1.5 ml tubes. Next, 25 μl of LQM402 dilutions in DMSO to different concentrations (1–100 μM) or DMSO only for control were added to the tubes. The samples were incubated at 37 °C for 3 h with constant shaking. After the incubation, 500 μl of TBA solution (0.5% TBA in 0.05 N NaOH and 30% trichloroacetic acid at a 1:1 ratio) was added to each mixture and the samples were cooled on ice for 10 min, centrifuged at 10,000 rpm for 5 min, and incubated at 90 °C for 30 min. After cooling at room temperature, the sample absorbances were measured at 540 nm in a microplate reader (ELx808, Bio-Tek, Winooski, VT). Additionally, control experiments to test the induction of lipid peroxidation were conducted in the presence of rat brain homogenate to give a basal reading or 20 mM 2,2′-azobis(2-methyl-propionamidine) dihydrochloride (AAPH) or 10 μM ferrous sulfate ($FeSO_4$) to give positive control readings; the latter condition was incubated for 1 h.

2.11. Antioxidant assay

The scavenging activity of LQM402 was measured by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method. DPPH is a stable free radical that can be reduced in the presence of free radical scavengers, upon which it converts from its original violet color to yellow (Waffo Teguo et al., 1998). Separately, three concentrations of LQM402 (1, 10, and 100 μM) that had been dissolved in ethanol from a 20 mM stock DMSO solution were allowed to react with the stable free radical DPPH (100 μM) for 30 min at room temperature in the dark. Each dilution was tested in triplicate. The reaction absorbances were measured at 515 nm with a microplate reader (ELx808, Bio-Tek, Winooski, VT). The percent radical scavenging activity (%RSA) for each sample was calculated in comparison with the DMSO-treated control group according to the following equation:

$$\%\,\mathrm{RSA} = 100 - \left[\left(\frac{\text{absorbance of test compound}}{\text{absorbance of control}}\right) \times 100\right]$$

2.12. Statistical analysis

Data were presented as the mean ± standard error. For comparisons of treated groups against a control group, two-way ANOVA and Bonferroni post-tests were conducted for data analyses when applicable. Significance was set at $p < 0.05$, $p < 0.01$, or $p < 0.001$, depending on the experiment.

3. Results and discussion

3.1. LQM402 is cytotoxic against CeCa cells in a concentration-dependent manner

The anti-neoplastic drugs that are generally used to treat CeCa cause serious side effects and generate resistance, indicating a need for safe alternatives to treat this disease. Cis-diaminedichloroplatinum (II) (Cisplatin) is still one of the most effective current chemotherapeutic anti-tumor agents. In response, several metal complexes have been synthesized with the intent to produce new anti-neoplastic compounds that include metals such as gallium, gold, nickel, copper, and zinc (Frezza et al., 2010; Laine and Passirani, 2012). Herein we tested the new copper(II) complex LQM402 against the HeLa, Ca Ski, SiHa, and C-33 A cervical cancer cell lines to determine the potential anti-tumor effects. Our data in Fig. 2 show differential concentration-dependent cytotoxic activities in the cell lines at 24 h. At the highest concentration (100 μM), viability decreased as a function of the tumor cell line as follows: SiHa (57.75%), C-33 A (51.44%), Ca Ski (36.62%), and HeLa (19.45%); these data indicate that HeLa cells were the most sensitive to LQM402. In all of the cell lines, cytotoxic activity was not observed at LQM402 concentrations below 25 μM; however, at concentrations ranging from 50 to 100 μM, it caused a dramatic loss in viability of all CeCa cell lines except for the SiHa line. The LQM402 IC_{50} values were significantly lower for HeLa and Ca Ski cells (<100 μM; $p < 0.05$) than the other cell lines (>100 μM; Table 1), suggesting likely differences in membrane permeability or metabolism of the copper(II) complex among the cell lines. The

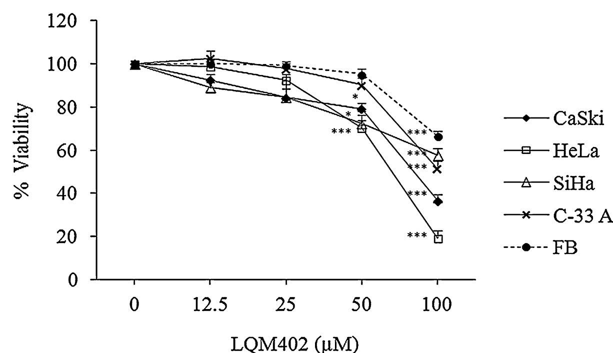

Fig. 2. LQM402 inhibits the viability of cervical cancer cell lines. The cervical cancer cell lines HeLa, SiHa, Ca Ski, and C-33 A were treated with different concentrations of LQM402 for 24 h and analyzed by MTT assay. CeCa cell viability decreased in a concentration-dependent manner while normal fibroblasts (FB) were less affected. The graph shows the means and standard errors of at least three independent experiments, performed in triplicate. $^{*}p < 0.05$ and $^{***}p < 0.001$ represent significant differences compared with control (vehicle-treated cells).

cytotoxic activity of our compound is in agreement with reports describing the cytotoxic activities of copper complexes introduced into HeLa cells by nanoparticles delivery vectors (Harris et al., 2011), as well as Casiopeínas® evaluated by sulforhodamine b assay in the same CeCa cell lines used in our study (Gracia-Mora et al., 2001; Krishnamoorthy et al., 2011, 2012). Other tested copper complexes have showed being cytotoxic to cell lines from different types of cancer such as prostate, liver, and myeloid leukemia (Jia et al., 2010; Lakshmipraba et al., 2011; Hammud et al., 2008). In this study, fibroblasts were used as a non-malignant human control cells and were found to be less affected by LQM402 than tumor cells, suggesting an advantage for this complex as a potential selective anticancer drug. This result is comparable to those obtained in another study of macrocyclic copper(II) complexes that were evaluated at similar concentrations (1–100 μM) and by the same method in normal V79 Chinese hamster lung fibroblasts; that study reported cell survival rates above 80% at 50 μM in a 24 h incubation (Fernandes et al., 2007), while our complex induced 95.14% survival at the same concentration and incubation time. On the other hand, compared with the antitumor drug cisplatin, LQM402 was less cytotoxic against HeLa cells; however, the IC_{50} value of LQM402 for non-tumoral fibroblasts was higher than that of cisplatin, which suggests that LQM402 was less toxic against these normal cells (Table 1). Because of the selectivity of LQM402 for HeLa cells, we tested the cytotoxicity of copper complex in this cell line at 48 and 72 h and compared with cisplatin treatment at the same times. The obtained IC_{50} values are showed in Table 2.

3.2. LQM402 induces phosphatidylserine residue externalization on HeLa cells

Most anticancer drugs exert their effect by apoptotic induction, which is the recommended action mechanism due to the lack of

Table 1
Inhibitory concentration (IC_{50}) values obtained in cervical cancer cell lines for LQM402.

Cell line	IC_{50} (μM) ± S.E.
HeLa	74.74 ± 3.34
Ca Ski	88.53 ± 2.64
SiHa	182.05 ± 16.65
C-33 A	192.55 ± 12.10
Fibroblast	199.47 ± 14.32

Each data is given as the mean and standard error (S.E.) of at least three independent experiments in triplicate. Cisplatin IC_{50}: HeLa 18.9 ± 0.99 μM, Ca Ski 66.6 ± 8.5 μM, SiHa 69.92 ± 1.25 μM, C-33 A 64.98 ± 6.88 μM, FB 185.23 ± 28.6 μM.

Table 2
Inhibitory concentration (IC_{50}) values obtained in HeLa for LQM402 and cisplatin at 48 and 72 h.

Compound	IC_{50} (μM)	
	48 h	72 h
LQM402	53.90	39.61
Cisplatin	17.28	15.83

Each data is given as the mean of three independent experiments in triplicate.

an inflammatory response (Mizutani, 2007; Kerr et al., 1994). Apoptosis is an important mechanism of cell death that is involved in many physiologic processes related to homeostasis and is a central regulator of pathophysiological conditions. Cancer cells have been reported to develop several mechanisms with which to resist apoptotic cell death, and resistance to apoptosis is considered a hallmark of cancer (Hanahan and Weinberg, 2000, 2011; Ocker and Hopfner, 2012). To determine whether the inhibitory effects of LQM402 on HeLa cell viability resulted from apoptosis, we measured apoptotic processes by annexin V-FITC staining and propidium iodide (PI) accumulation, based on the early apoptotic phosphatidylserine residue flip from the inner to outer surface of the cytoplasmic membrane, and analyzed cell staining by flow cytometry (van Engeland et al., 1998; Vermes et al., 1995). Fig. 3 shows that a concentration of 74.74 μM LQM402 was able to induce time-dependent apoptosis in HeLa cells, with frequencies of 10.96% and 51.44% apoptotic cells at 6 and 24 h, respectively, compared with 5.86% apoptotic cells at 24 h in cells treated with 0.1% DMSO-treated; this finding was consistent with our previous IC_{50} data. At 12 h, nearly half of the LQM402-treated apoptotic HeLa cells were in early apoptosis, with the remainder in late apoptosis. At 24 h, 3.77% of the treated cells were labeled only by propidium iodide (necrosis) and the rest of cells were stained by both annexin V and PI (late apoptosis), which indicates that the cells have lost membrane integrity (Cai et al., 2002). Because most of the treated cells were in early apoptosis, we can say that LQM402 has a similar potential as other copper complexes to induce apoptosis in HeLa cells at early time points. For example, human ovarian carcinoma (CH1) and murine leukemia (L1210) cells exhibited typical morphological changes in the cytoplasm (shrinkage), chromatin (condensation), and nuclei (DNA fragmentation) after treatment with Casiopeína® II for 24 h (De Vizcaya-Ruiz et al., 2000). A Schiff base copper(II) also induced apoptosis in MCF-7 cells at concentrations of 20 μM or less in a 48 h incubation, as determined with flow cytometry (Ma et al., 2012), and a study of the cell cycle in HCT116 cells demonstrated that copper bis(thiosemicarbazone) complexes induced both apoptosis and DNA cleavage in those cells (Palanimuthu et al., 2013). These and many other studies that were published in the last decade confirm that copper complexes are potential cytotoxic compounds.

3.3. LQM402 induces DNA fragmentation in HeLa cells

It is known that programmed cell death or apoptosis is the most common form of cell death and that this process is associated with DNA cleavage (Kroemer et al., 2009). Therefore, to evaluate if the copper complex LQM402 was able to induce DNA cleavage, we perform TUNEL and DNA-ladder assays with LQM402-treated HeLa cells at the IC_{50} value obtained in these cells at 24 h (74.74 μM) for 24, 48 and 72 h incubation times. In the TUNEL assay, we found DNA cleavage labeling in a time-dependent manner (Fig. 4). The amount of cells showing DNA cleavage induced by LQM402 at 72 h was similar that the induced by the positive control cisplatin; however, it seems that the morphology or DNA fragmentation pattern observed in some LQM402-treated cells is different from the cisplatin-treated cells. The TUNEL reaction preferentially labels DNA strand breaks generated during apoptosis; nevertheless, DNA

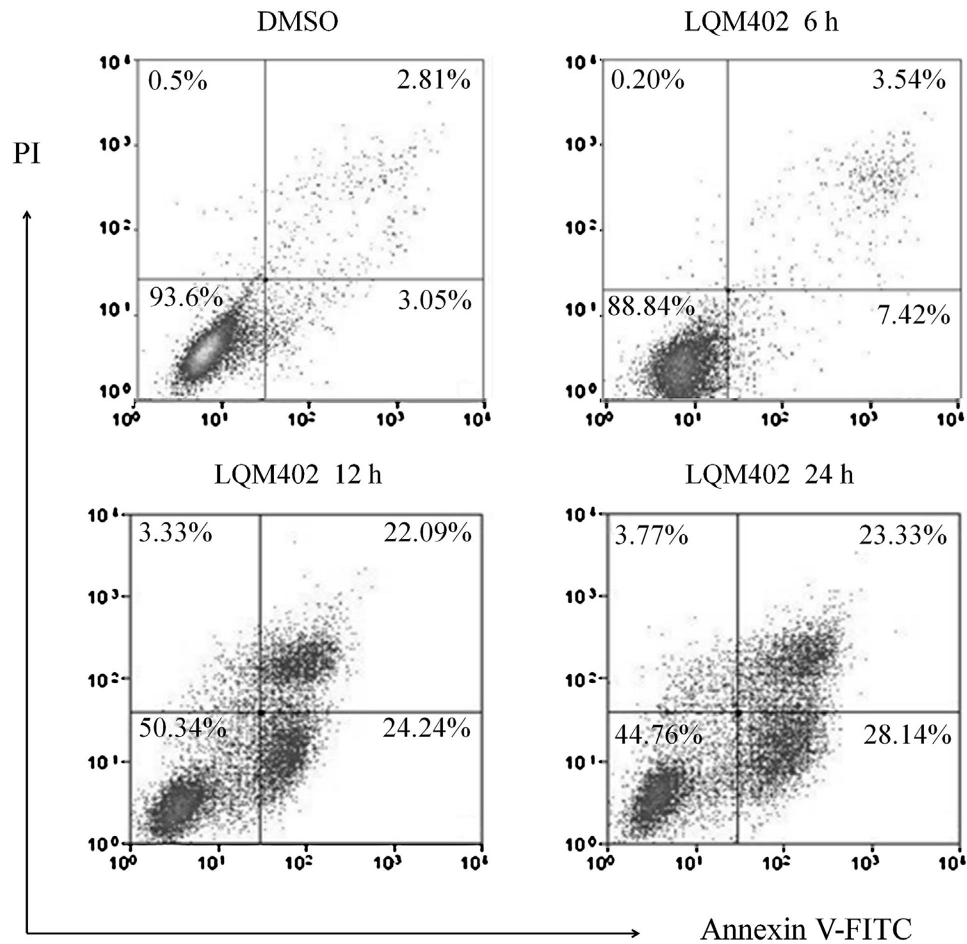

Fig. 3. LQM402 promotes apoptosis. HeLa cells treated with 1% DMSO for 24 h or 74.74 μM LQM402 for 6, 12, and 24 h were analyzed by flow cytometry to detect annexin V-FITC staining. The percentage of apoptotic cells increased in a time-dependent manner in LQM402-treated cells HeLa cells when compared to DMSO-treated cells. Graphs are representative of three independent experiments, each with 10,000 analyzed events.

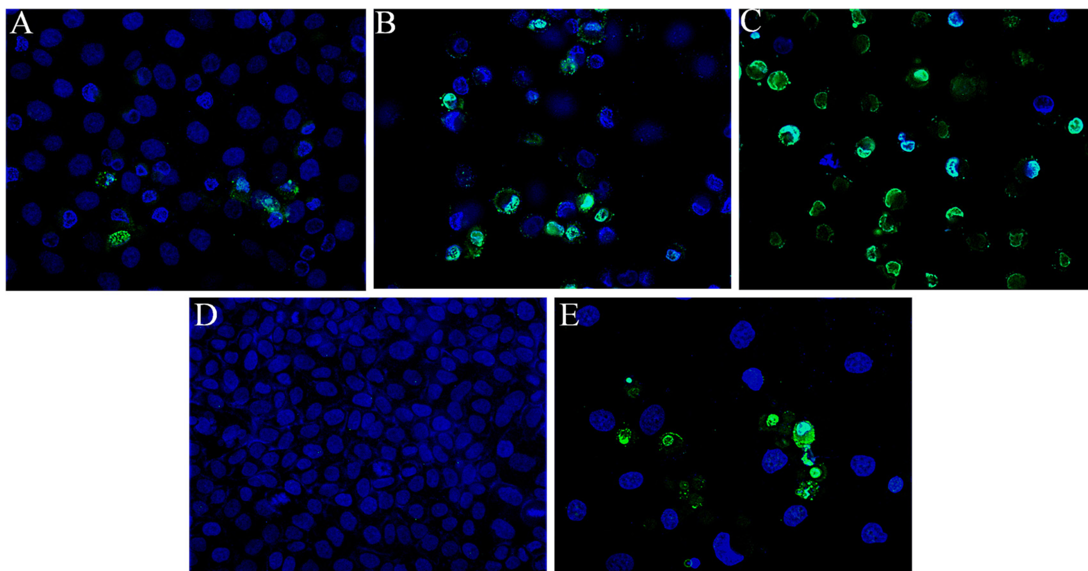

Fig. 4. LQM402 induces DNA fragmentation in a time-dependent manner. HeLa cells were treated with 74.74 μM LQM402 for (A) 24, (B) 48, and (C) 72 h and DNA strand breaks were labeled with fluorescein by TUNEL assay showing a time-dependent increase of cells in apoptosis. (D) 1% DMSO and (E) 20 μM cisplatin for 48 h were used as negative and positive controls, respectively.

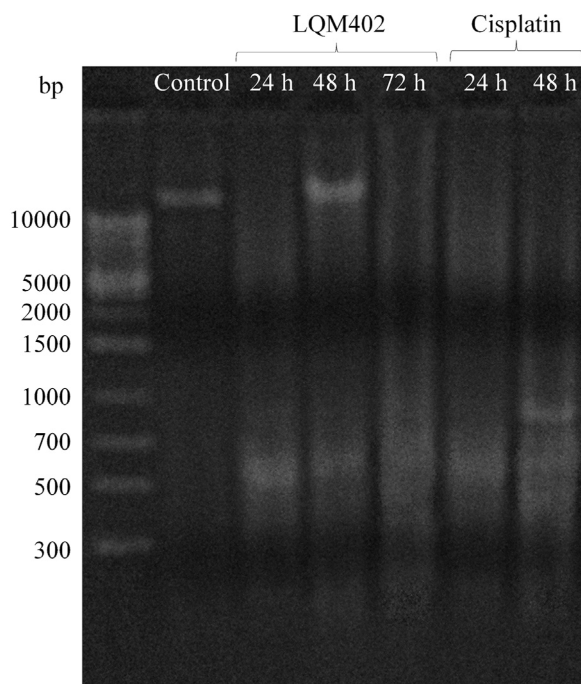

Fig. 5. LQM402 treatment in HeLa cells induces DNA degradation. HeLa cells were treated with 74.74 μM LQM402 at different time points and DNA was obtained with DMSO-SDS-TE method. All of the LQM402 treatments generated DNA fragments of approximately 600 bp as 20 μM cisplatin did at 24 h. Only cisplatin treatment generated several size DNA fragments.

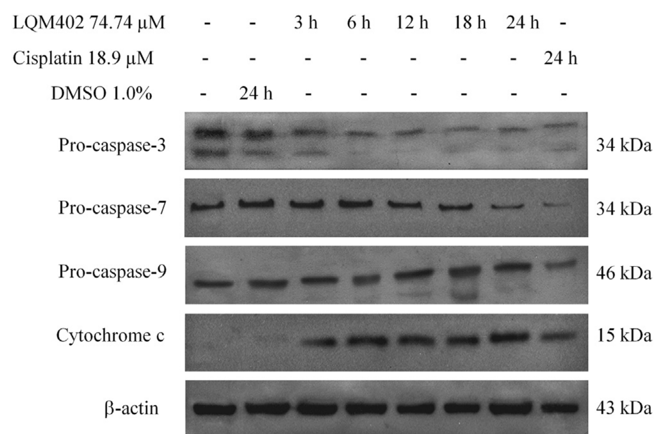

Fig. 6. LQM402 induces changes in the expression of apoptosis-associated proteins. HeLa cells were treated with 74.74 μM LQM402 at different time points and the lysates were analyzed by immunoblotting. Expression of pro-caspases 3 and 7 was diminished in a time-dependent manner. Cytosolic cytochrome c levels increased in an incubation time-dependent manner. Representative blots from repeated experiments are shown.

cleavage can be incomplete in some forms of cell death or may occur in random form in late stages of necrosis (Zong and Thompson, 2006). In the DNA-ladder assay, we observed DNA degradation in LQM402-treated cells at all the times tested (aprox. 600 bp) compared with the control cells. Cisplatin treatment at 24 h induced similar DNA fragments (600 bp) as LQM402 treatment, meanwhile approximately 1000, 600, and 400 bp fragments were generated at 48 h (Fig. 5). Several copper complexes with different structural features have been shown to bind double-helical DNA and to promote double-strand DNA damage, this activity is mainly associated to their ability to induce or to generate free radicals (Liu et al., 1999).

3.4. Western blotting

Apoptosis and necrosis are the major types of cell death. Each is characterized by morphological and biochemical features and currently, genetics contribute to specifically define those processes (Kroemer et al., 2009; Ouyang et al., 2012). However, there are also less studied cell death mechanisms such as anoikis, cornification, entosis, mitotic catastrophe, necroptosis, netosis, parthanatos, and pyroptosis (Galluzzi et al., 2012) that are less commonly observed.

The apoptotic process depends on the activation of caspases that are involved in both the extrinsic and intrinsic pathways. To determine if LQM402 induced caspase activation, we obtained lysates from HeLa cultures that were treated with the complex at different time points and examined the expression of caspase-3, caspase-7, and caspase-9. The immunoblotting experiments showed the depletion of procaspase-3 in HeLa cells treated with 74.74 μM LQM402 for 18 and 24 h, compared to control cells ($p < 0.01$ and $p < 0.001$, respectively), and similar results after treatment with 20 μM cisplatin for 24 h ($p < 0.05$). Although a densitometric analysis did not show significant differences between the treatments, both procaspase-7 and procaspase-9 showed trends toward reduced expression, which suggested the formation of active forms of these zymogens; however, the active forms of these caspases were not detected in our assays. Cytochrome c was detected in cytoplasmic lysates from HeLa cells treated with LQM402 for 6, 12, 18, and 24 h or with cisplatin for 24 h, but not in lysates from control (untreated) and 1% DMSO-treated cells (Fig. 6). Initially, our data indicate that the intrinsic pathway could be involved in LQM402-induced apoptosis, but the possibility of other routes or cell death types should not be dismissed because other cell death processes have been observed in response to different Cu(II) complexes. For instance, the thioxotriazole copper(II) complex (A0) studied by Tardito and coworkers induced cell death after caspase-3 activation, but the cells did not display the known characteristics of classic caspase-dependent apoptosis. The authors reported that A0-induced cell death involved endoplasmic reticulum stress and paraptotic death (Tardito et al., 2009). Furthermore, apoptotic cell death was reported in C6 glioma cells that were exposed for 24 h to concentrations of [(4,4′-dimethyl-2,2′bipiridine)(acetylacetonate) Copper(II) nitrate] (Cas III-ia) ranging from 11.23 to 22.4 μM. However, treatment with 33.7–44.9 μM of that compound induced ultrastructural findings that suggested autophagic cell death (Trejo-Solis et al., 2012). These results opened approaches to different mechanisms of action for new copper or other metal-based compounds designed for anticancer therapy.

On the other hand, while 74.74 μM LQM402 diminished the expression of procaspase-3 and procaspase-9 at 24 h, 50 μM of the Isatin-diimine copper(II) complexes bis-[(2-ox-indol-3-yl-imino)-1,3-diaminopropane-N,N′,O,O′]copper(II)perchlorate ([Cu(isapn)]$(ClO_4)_2$) and [bis-(2-oxindol-3-yl-imino)-2-(2-aminoethyl)pyridine-N,N′]copper(II) perchlorate([Cu(isaepy)2]$(ClO_4)_2$), also known as Cu(isapn) and Cu(isaepy), induced the expression of the active forms of these caspases after 24 and 48 h of treatment in the SH-SY5Y neuroblastoma cell line (Filomeni et al., 2007). Additionally, 11.23–22.4 μM of Cas III-ia induced mitochondrial cytochrome c liberation, which was found in the cytoplasm (Trejo-Solis et al., 2012). These results are similar to our findings in LQM402-treated HeLa cells; however, these data represent initial investigations into the pathway of LQM402-induced HeLa cell death and further studies are required.

Table 3
Mutagenic potency of LQM402 on *S. typhimurium* in the Ames test (plate incorporation assay) expressed as the number of His$^+$ revertants ± standard error (S.E.).

Metabolic activation system	Test substance	Conc. μM/plate	His$^+$ revertants (Mean ± S.E.)		
Type culture strain			TA98	TA100	TA102
	DMSO	10 μl/plate	20.33 ± 1.0	144.3 ± 7.2	208.7 ± 37
		200	29.16 ± 2.0	136.1 ± 9.6	302.1 ± 73
	LQM402	100	25.00 ± 3.3	152.5 ± 9.4	299.3 ± 68
S9 (−)		50	19.33 ± 1.8	143.5 ± 7.1	297.4 ± 72
	PA	1.89×10^{-3}	77.3 ± 18.1	–	–
	NMNG	6.79×10^{-6}	–	8033 ± 535	–
	Mit C	2.99×10^{-8}	–	–	1891 ± 154
	DMSO	10 μl/plate	30.1 ± 2.77	142.1 ± 11	245.1 ± 24
		200	27.6 ± 2.21	158.6 ± 7.0	307.2 ± 64
S9 (+)	LQM402	100	32.3 ± 5.04	129.0 ± 9.1	306.0 ± 67
		50	29.3 ± 2.75	145.0 ± 13	326.4 ± 72
	2AA	5.17×10^{-6}	2919 ± 425	–	1253 ± 44
	CP	0.0179	–	507 ± 22.8	–

The results correspond to the mean of three independent experiments performed in triplicate and its standard deviation (S.D.). Arochlor 1254-induced rat liver homogenate (S9) was used as the metabolic activation system. PA, picrolonic acid; NMNG, N-methyl-N′-nitro-N-nitrosoguanidine; Mit C, Mitomycin C; 2AA, 2-Aminoanthracene; and CP, Cyclophosphamide were used as positive for mutagenic drugs.

3.5. LQM402 is a non-mutagenic agent in the Ames test

Because potential anti-neoplastic drugs should be non-toxic to non-malignant cells, we measured the genotoxic effects of LQM402 on histidine mutants of the *S. typhimurium* strains TA 98, TA 100, and TA 102. The compound was tested with or without the addition of the S9 activator because many carcinogenic agents must be activated (Ames et al., 1973). Table 3 shows the numbers of His$^+$ revertants of *S. typhimurium* after at 48 h incubation with the DMSO vehicle control or different concentrations of LQM402. LQM402 was not mutagenic in any of the three tested strains at 50, 100, and 200 μM when compared to all of the employed positive controls (PA, NMNG, Mit C, 2AA, and CP) with or without S9 mixture, all of which induced mutation rates that were several times higher (3–64) than the control spontaneous reversion (DMSO; Table 2). Our results indicate that these doses of LQM402 are not mutagenic to the strains assayed by the Ames test and are similar to results obtained in an Ames study of novel copper(II) quinolinone Schiff base complexes that were cytotoxic against human hepatocarcinoma cells (Hep-G2) and non-mutagenic in the *S. typhimurium* strains TA98 and TA100 (Duff et al., 2012). Other copper complexes evaluated by Ames tests were $[Cu(phendione)_3](ClO_4)_2 \cdot 4H_2O$ and $[Cu(phen)_2(mal)] \cdot 2H_2O$, which were also cytotoxic against Hep-G2 and a human kidney adenocarcinoma (A-498) cell line but non-mutagenic in the *S. typhimurium* strains TA98 and TA102 (Deegan et al., 2006, 2007). These results suggest that some copper complexes can effectively kill cancer cells without mutagenic activity.

3.6. LQM402 does not induce micronucleus formation in mice peripheral blood

To assess the genotoxic activity of LQM402 in an *in vivo* model, the number of micronucleated polychromatic erythrocytes (MNPCE) was scored in 1000 polychromatic erythrocytes (PCE), according to previously published criteria (Fenech, 2000). The ability of LQM402 to induce MNPCE is shown in Fig. 7, where it is observed that only mice treated with the positive control Mit C had statistically significant increases in the numbers of MNPCE at 48 and 72 h with respect to the mice treated with the vehicle (distilled water). It is usually possible to observe initial mutagenic effects from micronucleus-inducing agents at 24 h; the highest amount of MN is observed at 48 h, and the level decreases at 72 h. This was observed with our positive control Mit C, but not with LQM402. Fig. 7 also shows the inhibitory effect of LQM402 on Mit C-induced MNPCE when the copper complex was administered in addition to Mit C. All LQM402-treated groups had similar numbers of MNPCE both before (0 h) and after treatment (24, 48, and 72 h), with mean values ranging from 2 to 3.6, and no significant differences were observed among the groups or with respect to the control. These results suggest that LQM402 has anti-mutagenic properties.

In the micronucleus test, an increase in the ratio of polychromatic erythrocytes (PCE) with respect to normal chromatic erythrocytes (NCE) means that proliferative activity is stimulated, likely due to an early phase of cell depletion (Ecobichon, 1997). The LQM402-induced PCE/NCE ratios are expressed in Table 4. None of the concentrations and time points for LQM402 showed an increase in PCE/NCE, compared with the ratio exhibited for the control group. At 24 and 48 h after administration of Mit C, the ratio for PCE/NCE was significantly different ($p < 0.05$) from the control ratio at the same times and at 72 h, the ratio decreased to almost the initial value (0 h). When LQM402 at 5 mg/kg (C1), 10 mg/kg (C2), and 15 mg/kg (C3) concentrations was administered 30 min after the administration of 60 mg/kg Mit C, it inhibited increases in the PCE/NCE ratio, indicating an anti-cytotoxic effect of LQM402 at the tested concentrations against Mit C-induced toxicity in murine blood cells. There are few studies of copper complex genotoxicity that were evaluated by the micronuclei assay. A micronuclei evaluation of the copper complexes $CuL(ClO_4)_2$ and $CuL(NO_3)$ for the macrocyclic ligand 1,1′-bis(bis-(6,6′-oxymethylenyl-2,2′-bipyridine) binaphthyl (L) in cultured human lymphocytes indicated genotoxicity by the induction of micronucleated cells at a dose of 0.15 mg/kg for 24 h and cytotoxicity by a reduction in cell number at that same dose and time (Beynek et al., 2007); these effects were not observed with our copper complex even at 33 to 100-fold higher doses. In reports of the genotoxic activities of other copper complexes, researchers commonly used DNA fragmentation assays. Most of these reports

Table 4
Ratio of polychromatic erythrocytes (PCE) with respect to 2000 normochromatic erythrocytes (NCE) induced by LQM402 in mice.

Treatment	Ratio of PCE/NCE			
	0 h	24 h	48 h	72 h
Control[a]	0.06	0.05	0.05	0.05
Mit C 60 mg/kg	0.07	0.11*	0.12*	0.09
LQM402 5 mg/kg	0.07	0.06	0.06	0.06
LQM402 10 mg/kg	0.06	0.06	0.04	0.03
LQM402 15 mg/kg	0.06	0.07	0.04	0.06
Mit C + LQM402 5 mg/kg	0.06	0.06	0.07	0.08
Mit C + LQM402 10 mg/kg	0.06	0.06	0.04	0.05
Mit C + LQM402 15 mg/kg	0.06	0.06	0.04	0.05

[a] Distilled water.
* Statistically significant difference with respect to control, $p < 0.05$.

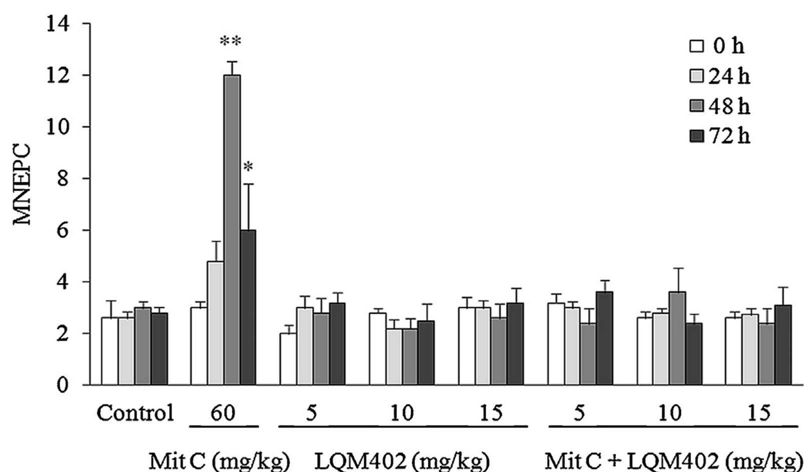

Fig. 7. LQM402 does not induce micronuclei formation. Three concentrations of LQM402 were administered intraperitoneally in single doses to CD1 mice. The numbers of micronucleated polychromatic erythrocytes (MNPCE) in 1000 counted polychromatic erythrocytes were analyzed at 0, 24, 48, and 72 h in Giemsa-stained tail blood smears. The same concentrations of LQM402 were also administered 30 min after the administration of 60 mg/kg Mit C. The genotoxic effect of Mit C was observed only when it was administered alone. The graph shows the means and standard errors of 5 mice per experimental group. Significant differences from the control are indicated by $^{**}p < 0.01$ and $^{*}p < 0.05$.

suggested possible clastogenic effects or reactive oxygen species as the mechanisms for the mutagenic effects of copper complexes (Beynek et al., 2007; Szyba et al., 1992; Serment-Guerrero et al., 2011).

3.7. LQM402 induces lipid peroxidation of rat brain homogenate

Cytotoxicity and apoptosis occur in response to several causes, including excesses of free radicals or other lipid peroxidation products (Barrera, 2012). As for most of the essential elements, an imbalance in endogenous copper could alter the redox system in a cell and cause systemic damages and pathologies (Wang and Guo, 2006). On the other hand, while copper chelation can be used as an anti-angiogenic therapy (Hancock et al., 2011; Lowndes and Harris, 2004; Lowndes et al., 2008), copper complexes such as casiopeínas® can induce the overproduction of ROS and lead to mitochondrial toxicity (Serment-Guerrero et al., 2011). In our Ames test of the TA102 strain, the number of His^+ revertants induced by incubation with LQM402 was nearly double the number of spontaneous His^+ revertants, which could indicate oxidative stress in the absence of genotoxicity. Therefore, to test the pro-oxidant effects of LQM402, we evaluated its ability to induce lipid peroxidation in a rat brain homogenate. We found that in a concentration range of 5.62–17.7 μM, LQM402 induced significant increases in lipid peroxidation ($p < 0.01$) above the basal level; these increases were not observed at concentrations below 5.62 μM or in the range of 31.62–100 μM (Fig. 8). LQM402 10 μM induced the maximum level of lipid peroxidation (6.7 nmol TBARS/mg proteins), which was higher than the level induced by the 20 mM AAPH positive control (3.7 nmol TBARS/mg protein) and similar to that induced by the 10 μM $FeSO_4$ positive control (7.4 nmol TBARS/mg protein). These data suggest that LQM402 is able to peroxidize lipids and therefore to induce related biological processes. Thus, LQM402-induced cell death and apoptosis in HeLa cells could be due to lipid peroxidation and/or lipid peroxidation products. The metals Cu(II), Cr(II), Co(II), Pb(II), and Fe(II) play important roles in redox processes in biological systems and some of them are inductors of lipid peroxidation by the oxidative features of the Fenton reaction (Benedet and Shibamoto, 2008); thus, it is not unusual that metal-based compounds that include these metals have such activities. Some casiopeínas® and other copper complexes can generate reactive oxygen species (Trejo-Solis et al., 2012; Serment-Guerrero et al., 2011) and this activity has been assumed to be the major mode of action behind the cytotoxicity of copper complexes (Kowol et al., 2012).

Cu(II) and other transition metals such as (Mn(II), Mn(III), and Fe(III)) display antioxidant properties. The superoxide scavenger activities of macrocyclic copper(II) complexes were determined by a xanthine–xanthine oxidase system (Fernandes et al., 2007). Thus, we assayed the capability of LQM402 to inhibit $FeSO_4$-induced lipid peroxidation in an attempt to determine whether LQM402 had antioxidant activity. LQM402 was found to have an additive effect on $FeSO_4$-induced TBARS production at concentrations from 5.62 μM to 13.34 μM in rat brain homogenate; however, LQM402 displayed inhibitory activity in the concentration range of 23.71–56.23 μM (Fig. 9). Together, our results indicate that LQM402 has both inductive and inhibitory activities in lipid peroxidation.

3.8. LQM402 is not a scavenging agent for the DPPH radical

The DPPH radical scavenging assay was used as an additional test to measure the antioxidant activity of LQM402. We tested LQM402 at three concentrations, 1, 10, and 100 μM, in a 100 μM DPPH solution. The highest concentration of LQM402 achieved a maximum value of 5.18% reduction of the DPPH solution, from which we could not determine the effective concentration required to reduce 50% of the DPPH solution (EC_{50}). This activity was very low compared to the activities of known antioxidants such as α-tocopherol (85.79% DPPH reduction at 74.13 μM) or quercetin (85.53% at 23.71 μM). LQM402 could inhibit the pro-oxidant activity of $FeSO_4$ but could not act as a scavenger of the DPPH free radical. This finding implied a different potential mechanism from the superoxide scavenger activity presented by macrocyclic copper(II) complexes, which displayed EC_{50} values of scavenging activity at concentrations below 30.82 μM (Fernandes et al., 2007). Nevertheless, in other studies, the inflammatory drug mefenamic acid and the compounds azo ligand (E)-4-((1H-1,2,4-triazol-3-yl)diazenyl)benzene-1,3-diol and (E)-4-((5-mercapto-1,3,4-thiadiazol-2-yl)diazenyl)benzene-1,3-diol did not exert antioxidant properties when complexed with Cu(II) (Kovala-Demertzi et al., 2009; Gaber et al., 2013), which reveals roles for ligands besides the bonded metal.

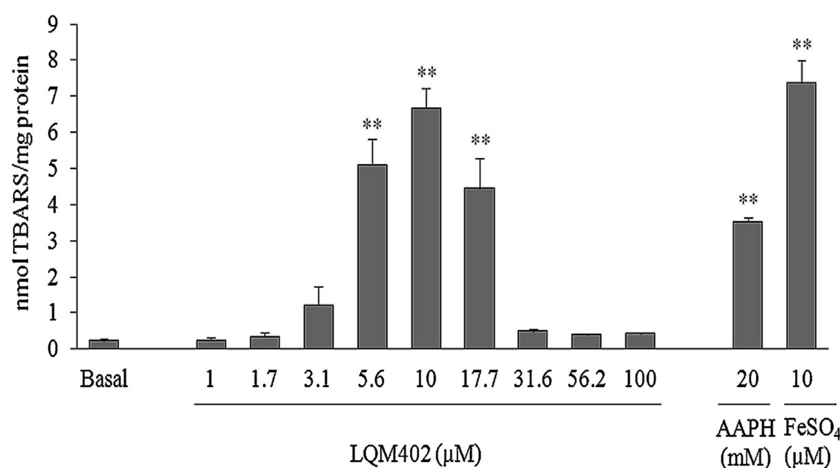

Fig. 8. LQM402 is an inductor of lipid peroxidation. Rat brain homogenate (2 mg/ml protein) was incubated with LQM402 at several concentrations (1–100 μM) at 37 °C for 3 h. TBARS production was measured at 540 nm. Significant TBARS production, indicated by $^{*}p < 0.01$, was only observed at concentrations from 5.62 μM to 17.78 μM. At 10 μM, LQM402 is a more potent lipid peroxidation inductor than AAPH (20 mM) and is similar to $FeSO_4$ (10 μM). The graph shows the means and standard errors of three independent experiments.

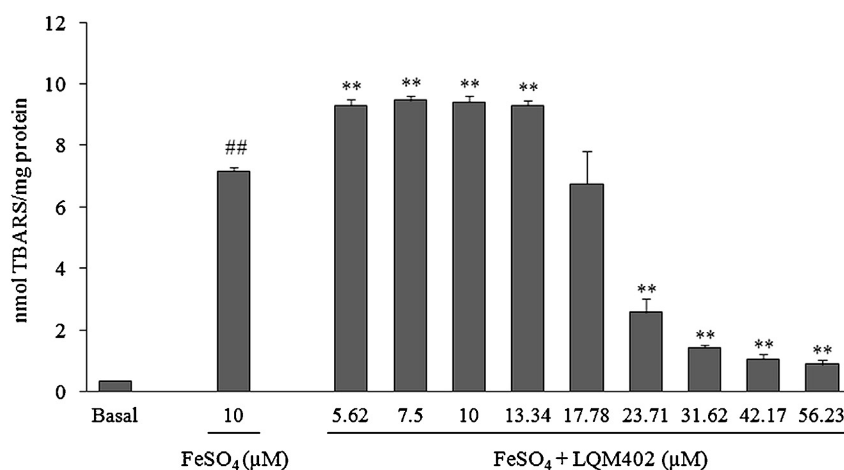

Fig. 9. LQM402 enhances and inhibits $FeSO_4$-induced TBARS production. Lipid peroxidation was induced in rat brain homogenate by 10 μM $FeSO_4$ alone or simultaneously adding LQM402 at different concentrations (5.62–56.23 μM). TBARS production was measured at 540 nm. TBARS induced by $FeSO_4$ was enhanced with 5.62–13.34 μM LQM402 and was inhibited in a concentration-dependent manner at concentrations of 23.71 μM and higher. The graph shows the mean and standard errors of three independent experiments. $^{\#\#}p < 0.01$ indicates significant difference of LQM402-treated groups compared to the basal level and $^{**}p < 0.01$ represents significant differences compared with $FeSO_4$.

4. Conclusion

This study demonstrated that the copper complex LQM402 is cytotoxic against CeCa cell lines, with selectivity for HeLa and Ca Ski cells, and it displays less cytotoxicity against normal fibroblasts. This compound induced the translocation of membrane phosphatidylserine in HeLa cells, a feature of apoptotic death, as detected by annexin V-FITC staining. Initially, the LQM402-induced cell death indicated the activation of the intrinsic apoptotic pathway as determined by the presence of cytochrome c in treated HeLa cell cytoplasmic extracts and by the decreased expression of procaspases 3 and 7. It is possible that the cytotoxic activity that led to apoptotic death in HeLa cells was due to the activity of LQM402 as a lipid peroxidation inductor and it also may be associated to the DNA degradation observed in TUNEL and DNA ladder assays. Although there are reports of copper complexes with antioxidant or reactive oxygen species inductor activities, the novel copper(II) complex LQM402 displayed both inhibitory and inductive effects on lipid peroxidation. Therefore, because LQM402 has no genotoxic activity, it might be used as a potential therapeutic agent against cervical cancer. Currently, our research is focused on the effects of this copper(II) complex on tumor growth in a nude mouse model of cervical cancer, as well as on the expression of genes related to cancer progression.

Conflict of interest

The authors declare that there is no conflict of interest concerning this manuscript.

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