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ROS-mediated autophagy was involved in cancer cell death induced by novel copper(II) complex

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ABSTRACT

In this study, we investigated autophagy induced in HeLa cells by copper(II) complex of ethyl 2-[bis(2-pyridylmethyl)amino] propionate ligand (ETDPA) (formula: [(ETDPA)Cu(phen)](ClO4)2 (abbreviated as LCu),a novel synthetic copper(II) complex whose DNA binding activity has been proved. Cell viability, autophagic levels and generation of ROS were evaluated following the exposure to LCu. LCu-induced cell death in a dose- and time-dependent manner, which was demonstrated by enhanced fluorescence intensity of monodansylcadervarine (MDC), as well as elevated expression of autophagy-related protein MAP-LC3. These phenomena were all attenuated after pretreatment with autophagy inhibitors 3-MA or NH_4Cl. Furthermore, our data indicated that LCu-triggered autophagy through ROS: cellular ROS levels were increased after LCu treatment, which was reversed by ROS scavenger NAC (N-acetylcysteine). As a consequence, Lcu-mediated autophagy was partly blocked by NAC. In summary, we synthezied a novel copper(II) complex and showed that this compound was effective in killing HeLa cells via ROS-triggered autophagic pathway.

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Introduction

One of the most rapidly developing areas of pharmaceutical research is the discovery of metal-based anticancer drugs. Since the success of Cisplatin [cis-diamminedichlorideplatinum(II)], more and more attention has been paid to metal complexes (Zhang and Lippard, 2003). Among all metals, copper (Cu) is an essential trace element that plays a central role in the biochemistry and physiology of every living organism (Harris and Gitlin, 1996). Cu is required for normal cellular activity as a co-factor for many enzymes, and it was transported into cells mainly by Copper Transport Protein 1 (Prohaska, 2008), lacking of copper will cause certain diseases e.g. neurological disorders including Alzheimer's disease (Macreadie, 2008). However, the role of copper is much more complex because copper can also promote nucleic acid cleavage and therefore can be used as metallodrugs to cause DNA damages (Hammud et al., 2008; Katsarou et al., 2008; Marzano et al., 2008; Urquiola et al., 2008). Published data demonstrate that one reason for toxicity of copper(II) complexes is their ability to bind and cleave DNA, which leads to cell cycle arrest and apoptotic cell death. And this the same strategy as of Cisplatin (Jamieson and Lippard,1999).

Another mechanism for copper(II) complexes-mediated cytotoxicity is through generation of ROS, which in turn leads to cell death (Tardito et al., 2007). Copper participates in a Haber–Weiss reaction, through which it catalyses the formation of ROS, and then ROS leads to peroxidation of membrane lipids (Britton, 1996). Liang and Zhou (2007) found out that ROS played an important role in copper-induced toxicity in yeast. The elevated levels of copper (both in serum and tumor) and copper-mediated oxidative stress in cancer cells can be used as a base for potential cancer treatment (Gupte and Mumper, 2009). And combining copper with other functional molecules is a new strategy to develop potent antitumor compounds. Ligands can significantly alter the biological properties by limiting the adverse effects of metal ion overload and facilitate metal ion re-distribution (Zhao et al., 2007), and ethyl 2-[bis(2-pyridylmethyl)amino]propionate (ETDPA) was used in our complex (Chen et al., 2009).

Autophagy is a physiological cellular mechanism in which cytosol and organelles are sequestered within double-membrane vesicles that deliver the contents to the lysosome vacuole for degradation and recycling macromolecules during nutritional starvation of the cell, contributing to the maintenance of cellular homeostasis. It is classified as Type II program cell death (Galluzzi et al., 2007). Within its basic role in the recycle of proteins and organelles sustaining survival, autophagy can also lead to cell death. Many chemicals such as arsenic trioxide (Kanzawa et al., 2003), avicin D (Xu et al., 2007), Sodium selenite (Kim et al., 2007) can induce autophagic cell death. Many cellular stresses can also

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cause autophagy such as ROS and mitochondria dysfunction (Scherz-Shouval and Elazar, 2007). Currently inducing autophagic cell death has been studied as a potential method for cancer therapy.

We have reported previously that newly synthesized copper(II) complex LCu exhibited good antitumor activities through its DNA binding and clipping activity, but whether there is any other mechanism remains unclear (Chen et al., 2009). The aim of the present study was to determine the antitumor effect of this novel synthetic copper(II) complex, the effect of the copper(II) complex on DNA synthesis and its contribution to ROS production. Furthermore, since ROS may be involved in both apoptotic and autophagic cell death, we wanted to identify the type of cell death triggered by copper(II) complex and confirm the role of ROS in this process. We expect that this complex can kill tumor cells through multi-pathways.

Materials and methods

Materials

Copper(II) complex LCu was synthesized by Prof. Chen Qiuyun, 3-[4, 5-dimehyl-2-thiazolyl]-2, 5-diphenyl-2H-tetrazolium bromide (MTT) were purchased from Amresco, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide(JC-1), Fluo-3AM, Propidium Iodide(PI) were bought from Invitrigene. Culture Medium (MEM/DMEM/1640), trypsin and EDTA-2⋯Na were purchased from Gibco. Fetal bovine serum was obtained from Sijiqing Biological Engineering Materials (Hang Zhou, PR China). DCFH-DA was bought from Beyotime institute of biotechnology (Nantong, PR China). Antibodies against LC3 and β-actin were from Abcam (Cambridge, UK). All the other chemicals were of high purity from commercial sources.

Cell culture

Four human cancer cell lines including HeLa, ECA109, HepG2 and A549 were obtained from Cancer Cell Repository (Shanghai cell bank). Cells were maintained in RPMI-1640 medium and DMEM medium (Gibco, USA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum, antibiotics (100 U/ml penicillin and 100 U/ml streptomycin), at 37 °C in a humidified atmosphere of 5% CO_2.

Cell proliferation assay

The cells were plated at a density of approximately 4×10^3 viable cells per well in 96-well microtiter plates. Various concentrations of compound were used to treat cells in triplicates. After incubation for the indicated time, MTT assay was performed to measure cell viability by a 96-well plate reader (Spectra MAX 190, Molecular Devices Corporation).

Cell cycle analyses

Into 6-well plates 1×10^5 HeLa cells were seeded, and harvested following different treatments with LCu. Cells were collected and washed with PBS then resuspended in 1 ml DNA staining solution (20 μg/ml of Propidium Iodide (PI) and 100 μg/ml of RnaseA in PBS) for 30 min on ice. DNA content was analyzed by FACS (Becton Dickinson, USA). The resulting DNA histograms were quantified using the Cell Quest Pro software.

Nuclei observation on H_2B-GFP-labled HeLa cells

H_2B-labled HeLa cells (stable cell line) were generously provided by Prof. Li Chaojun. Cells were plated in 24-well plate at the density of 2.4×10^4. After incubating for the indicated time with the compound, nuclei change was observed by fluorescence microscope (Nikon TE2000 inverted microscope).

Acidic vesicular organelles labeled by monodansylcadervarine (MDC)

HeLa cells (2.4×10^4) were seeded into 24-well plates and cultured for 24 h and then compounds were added. After a different time treatment, the cells were incubated with 50 μmol/L MDC at 37 °C for 15 min and then washed with PBS 3 times, 5 min every time. The cellular fluorescent changes were observed using fluorescence microscope.

GFP-LC3 plasmid transfection

HeLa cells were transfected with 1 μg of GFP-LC3 expressing plasmid in each well of 6-well plates using liptofectamine as per the manufacturer's instructions (Invitrogen). After 4 h, cells were treated with LCu and the fluorescence of GFP or GFP-LC3 was viewed under fluorescent microscope.

Western blot analysis for LC3

Intracellular LC3 protein level was detected. First, after incubating by LCu, 2×10^6 cells were collected and centrifuged at 1000 g, washed by PBS, and then precipitation was lysed in 100 μL precooling lysate buffer containing 0.5% Triton X100, 100 mM Tris–HCl, 150 mM NaCl, 0.1 U/ml aprotinin for 30 min on ice and centrifuged at 12,000 g for 2 min. The supernatant was collected and this was followed by protein concentration determination using the NJJC protein assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) with bovine serum albumin (BSA) as standard control. The supernatant was mixed with double volume of sample buffer (62.5 mM Tris, pH 6.8, 2% SDS, 5% mercaptoethanol, 1% bromophenol blue, and 25% glycerol) and boiled for 5 min. On a 10% SDS-PAGE, 20 μg protein was separated. Proteins were transferred from the gel to a polyvinylidene-difluoride (PVDF) membrane (Bio-Rad). The membrane was blocked using 5% skimmed milk for 2 h at room temperature, incubated overnight at 4 °C with primary antibody against the LC3 protein in PBS+Tween 20 (PBST) at a dilution of 1:3000, using β-Actin (primary antibody dilution, 1:5000) as loading control. After washing six times, 10 min each wash, with PBST, the membrane was incubated in the appropriate HRP-conjugated secondary antibody (diluted 1:3000 in PBST) at room temperature for 2 h. The membrane was then incubated with enhanced chemiluminescence reagent (ECL) solution for 3 min. Visualization of the immunolabeled bands was carried out by autoradiography.

Determination of intracellular reactive oxygen species (ROS)

ROS was measured with the non-fluorescent probe 2, 7-dichlorofluorescein diacetate (DCFH-DA). DCFH-DA can passively diffuse into cells and be deacetylated by esterase to form non-fluorescent 2, 7-dichlorofluorescein (DCFH). In the presence of ROS, DCFH reacts with ROS to form the fluorescent product DCF, which is trapped inside the cells. The cells were washed with ice-cold 1x PBS and incubated with DCFH-DA at 37 °C for 20 min. Then DCF fluorescence intensity was detected by fluorescence spectrometry (Spectra MAX GEMINI, Molecular Devices, USA)

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at excitation wavelength 488 nm and at emission wavelength 535 nm. The results were expressed as relative fluorescence intensity per 10^4 cells.

Statistical analysis

Comparisons were made by one-way analysis of variance (ANOVA). Differences were considered to be significant when $P<0.05$. All experiments were repeated at least three times.

Results

Inhibitory effect of the LCu on proliferation of cancer cells

To evaluate the effect of the copper(II) complex on cell proliferation of human cancer cell lines, we treated four cancer cell lines including HeLa, A549, HepG2, ECA109 with various concentrations for 48 h, and MTT assay was then performed. The 50% inhibitory concentration (IC_{50}) was calculated (see in Table 1). Values of IC_{50} of LCu on four cancer cell lines were between 5 and 20 μmol/L while IC_{50} was only 5.1 μmol/L on HeLa cells. Then HeLa cells were treated with various doses of the complex for 12, 24, 36, 48 h, and the MTT assay results show that copper(II) complex inhibited HeLa cell proliferation not only in a dose-dependent manner, but also in a time-dependent way (Fig. 1).

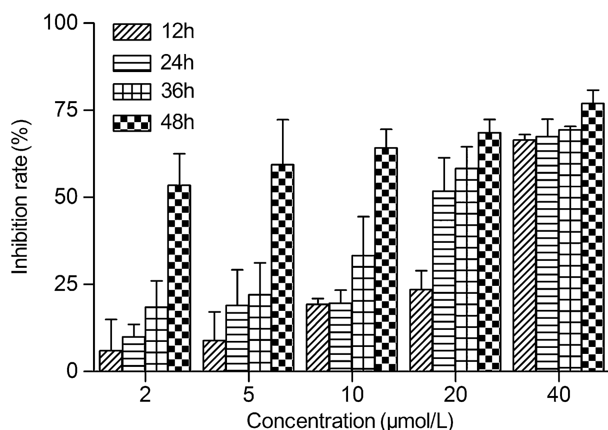

Fig. 1. Dose- and time-dependent antitumor effect of Lcu. HeLa cells were exposed to the compound (0–40 μmol/L). Cell proliferation was determined by MTT assay every 12 h. Each column represents the mean of the data from three independent experiments.

LCu-induced G0/G1 cycle arrest

To identify whether LCu exhibits an effect on DNA synthesis, the effect of LCu on cell cycle distribution of HeLa cells was analyzed (Fig. 2). HeLa cells were incubated with 4 μmol/L LCu for different hours and fixed for FACS analysis. A significant reduction of cells in G2/M phase was detected at 24 h, while after 48 h only 2.7% cells remained in S or G2/M phase. Meanwhile, the G1 population increased from 58% in control cells to 68.5% and 80.5% at 24 and 48 h post-treatment, respectively.

LCu-induced autophagic cell death

Next we were interested to identify which cell death pathway was employed when cells were treated with LCu. The possibility of autophagy was analyzed by AVO (autophagic vacuole organelles) formation of green fluorescent protein (GFP)-LC3 vacuoles, expression of LC3 and conversion from cytoplasmic form of LC3 (LC3-I, 18 kDa) to the preautophagosomal and autophagosomal membrane-bound form of LC3 (LC3-II, 16 kDa) by western blot. AVO formation was detected and measured by staining of MDC (Biederbick et al., 1995). The LCu-treated group showed higher fluorescent density and more MDC-labeled particles in HeLa cells compared with the control group (Fig. 3A), indicating that LCu increased MDC recruitment to autophagosomes in the cytoplasm. Since LC3 is a specific marker for autophagosome formation, detection of LC3 vacuoles formation and expression were carried out (Kabeya et al., 2000). GFP-LC3 plasmid was transfected into HeLa cells, and cells with GFP-LC3 vacuoles (dots) were observed using a fluorescent microscope. Formation of GFP-LC3 vacuoles (dots) was induced 12 h after treatment (Fig. 3B). Consistent with the results of MDC staining and GFP-LC3 vacuoles formation, conversion of LC3-I to LC3-II after treatment was also detected (Fig. 3C). Furthermore, we found that after pretreated with 3-MA

Table 1
IC_{50} of LCu on A549, HeLa, MCF-7 and ECA109 cells.

Cancer cells	IC50 (μmol/L)
A549	19.3 ± 1.2
HeLa	5.1 ± 0.5
MCF-7	6.5 ± 0.8
ECA109	5.2 ± 0.5

Data represent mean values ± SD of three independent experiments.
IC_{50} values were determined graphically from the growth inhibition curves obtained after a 48-h exposure of the cells to each drug, by using the software from China Pharmaceutical University.

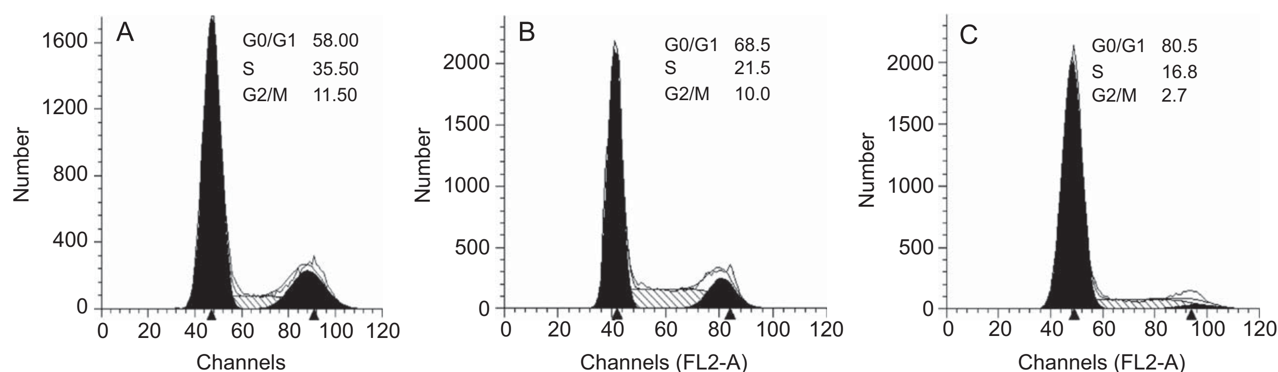

Fig. 2. LCu-induced G0/GI cell cycle arrest in HeLa cells. Changes in the cell cycle distribution of HeLa cells treated with 4 μmol/L LCu for the indicated times (A: 0 h, B: 24 h and C: 48 h) were analysed by PI staining by FACS. Percentages of cells in G0/G1, S and G2/M phase are indicated.

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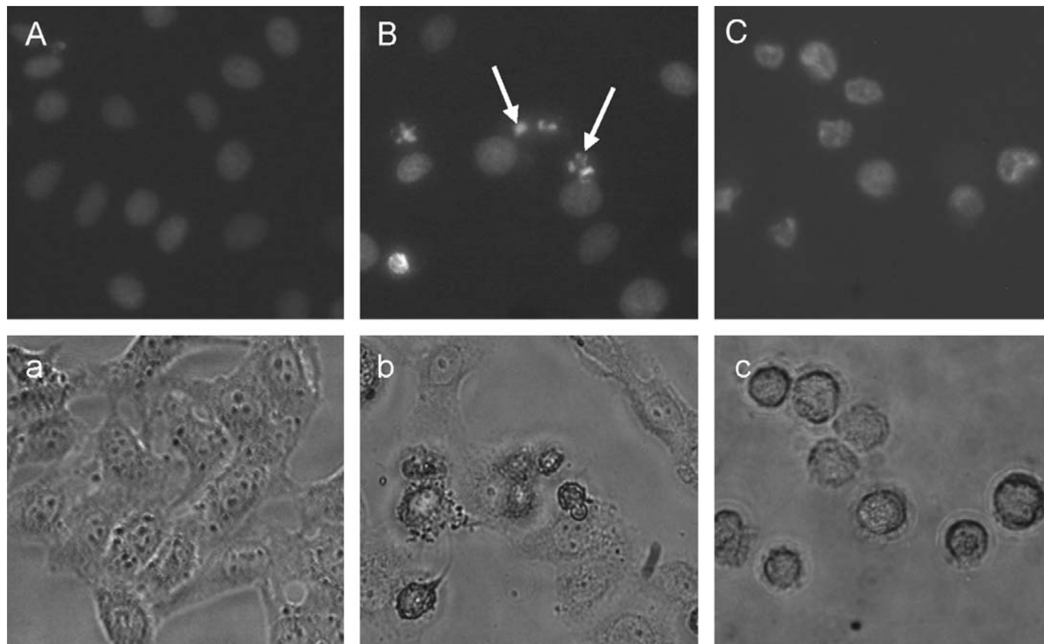

Fig. 3. LCu-induced cell death but without nuclei condensation(200×). H_2B-labled HeLa cells were exposed to LCu; nuclei and morphology change was observed, Cisplatinum was used as positive control. A/a: Control; B/b: Cisplatinum 30 μmol/L 16 h and C/c: LCu 10 μmol/L.

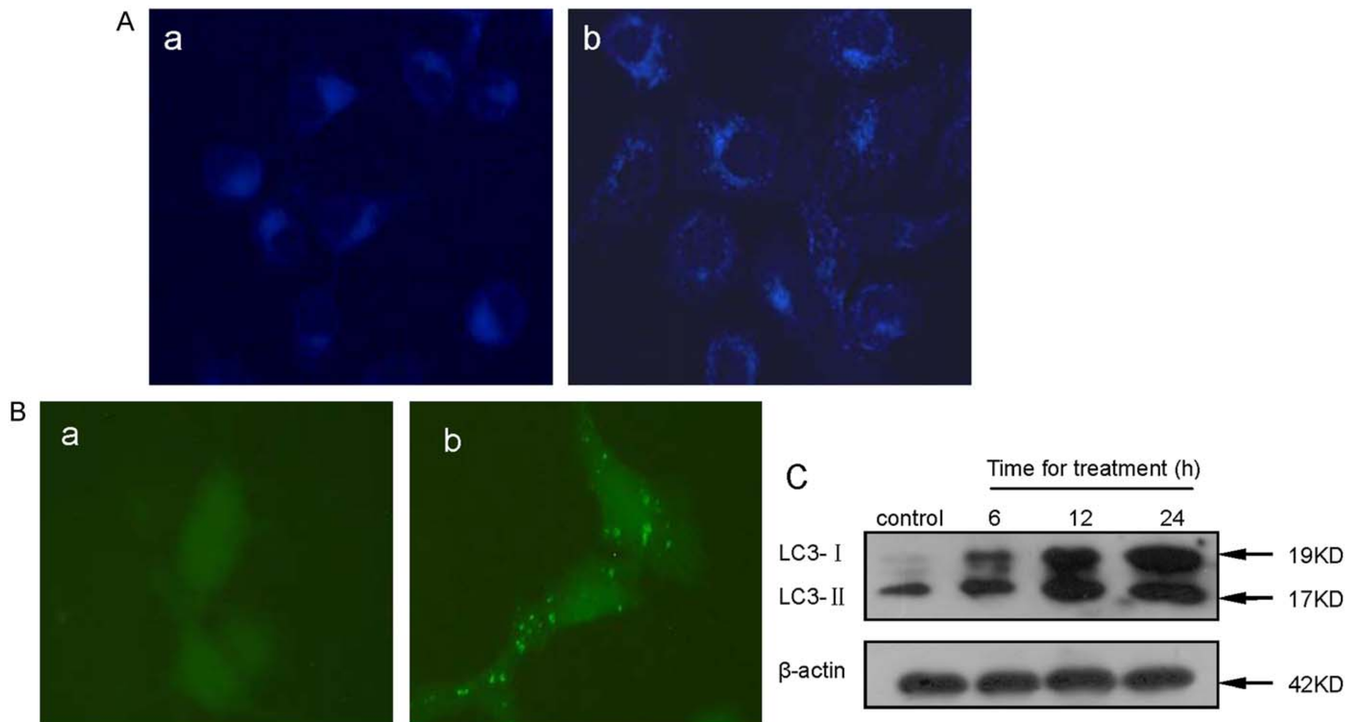

Fig. 4. . Autophagy induced by LCu(200×). A: MDC staining enhanced by LCu treatment(200×) a: control, b: 10 μmol/L for 6 h; B: GFP-LC3 vacuoles (dots) formed after treated by LCu(200×) a: control, b: 10 μmol/L for 6h; C: Expression of LC3-I to LC3-II in HeLa cells treated with 10 μmol/L Lcu.

or NH_4Cl, two inhibitors of autophagy, cell death and LC3-II expression were reduced (Fig. 5). In addition, we found no or little characteristics of apoptosis such as nuclei condensation, apoptotic body emerged in H_2B-labled HeLa cells treated with LCu. In Fig. 4, we that after treatment by Cisplatinum, nuclei condensed and divided to several parts, while after treatment by LCu, cells shrinked and died; but the change of its nuclei was not the same as that treated by Cisplatinum. These results suggest that HeLa cells underwent autophagy rather than apoptosis when exposed to LCu.

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Elevated intracellular reactive oxygen species (ROS) induced by LCu

In order to detect the changes of intracellular ROS, we used fluorescence spectrometry to measure the fluorescence of DCF after LCu treatment. As shown in Fig. 6, treatment with 10 μmol/L of LCu for 6 h led to an increase of ROS in HeLa cells. ROS formation increased significantly as detected by higher fluorescence intensity compared to control group. The concentration of ROS increased in a dose- and time-dependent manner, suggesting that the continuous generation of ROS was involved in the whole process.

LCu-induced autophagy was blocked ny NAC

To confirm whether ROS is essential for Lcu-induced cell death, ROS scavenger NAC (N-acetylcysteine) was used to block ROS generation. Cells were pretreated with NAC for 2 h and then treated with LCu for different periods. We found that inhibition rate reduced, suggesting that the toxcity of LCu reduced (Fig. 7A). Furthermore, we confirmed that LC3 exppresion also reduced (Fig. 7B). These results suggest that Lcu-mediated autophagy may be dependent on its ability to generate ROS.

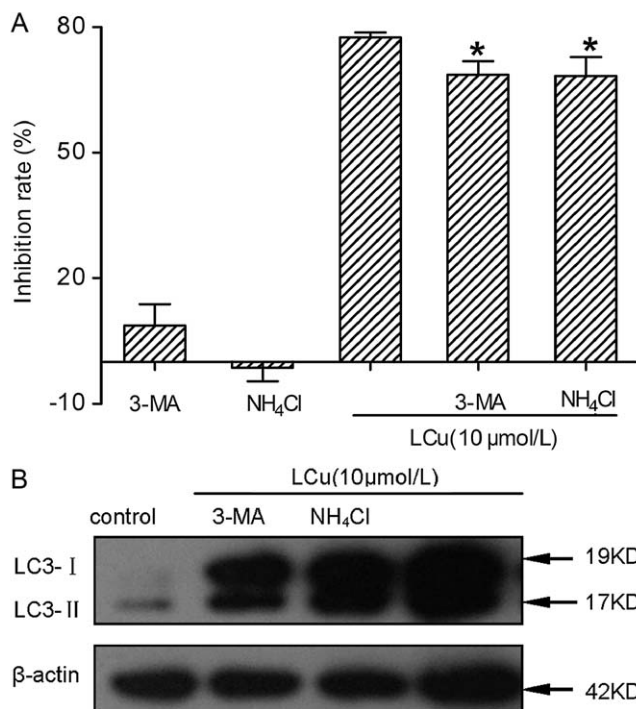

Fig. 5. . 3-MA, NH4Cl pretreatment reduced cell death and autophagy induced by Lcu. Cells were pretreated by 3-MA (10 mmol/L), NH_4Cl (20 mmol/L) for 2 h, then treated with 10 μmol/L LCu for 24 h; MTT was used to evaluate the cell viability(A), and protein was collected for western blot (B). * $P<0.05$ vs. control.

Disscusion

The success of Cisplatin in the treatment of cancer patients led to the suggestion that other metal complexes might also be potential drugs in future chemotherapy regimens. In the present study, we have demonstrated that compound LCu, a novel copper(II) complex, effectively inhibited the proliferation of four human cancer cell lines in a dose- and time-dependent way.

Most of the copper(II) complexes have shown the activity for DNA binding and cleavage and the ability to induce cell cycle arrest and apoptosis (Rajendiran et al., 2007; Selvakumar et al., 2006) The novel complex described in our paper showed DNA binding, cleavage activity and also G0/G1 cell cycle arrest (Chen et al., 2009). It exhibited high toxicity on cancer cell lines. Furthermore, it triggered autophagy rather than apoptotic cell death. The characteristics of apoptosis include nuclei condensation. Apoptotic body emerged, which was not obvious in our treated cells. Meanwhile, the following characteristics of autophagy was observed: AVO formation, formation of green fluorescent protein (GFP)-LC3 vacuoles, expression of LC3 and conversion from LC3-I to LC3-II. And after pretreatment by 3-MA and NH_4Cl, LC3-II expression weakened. Therefore, we believe that LCu-induced autophagic cell death.

Although till now the precise mechanism of autophagy remains unclear, the reactive oxygen species (ROS) that originated from mitochondrial oxidative stress seems to play a central role (Chen et al., 2008; Scherz-Shouval and Elazar, 2007). Our present data clearly demonstrated that LCu stimulated production of ROS, which shows that these oxygen radicals were partly toxic and causes loss of cell viability and it is the main source for autophagy. Thus NAC, the ROS scavenge, significantly protected against Cu-induced cell death and it also blocked autophagy.

Indeed, ROS play many roles in cellular processes including DNA damage, mitochondrial dysfunction, activation of signaling pathways and activation of transcription factors leading to upregulation of genes (Schumacker, 2006). In our present work, we found that ROS was caused by LCu-induced autophagy in HeLa cells. Our ongoing research suggests there are also changes in mitochondria functions. Therefore, the effects of LCu on mitochondria function and apoptotic process warrant further investigation.

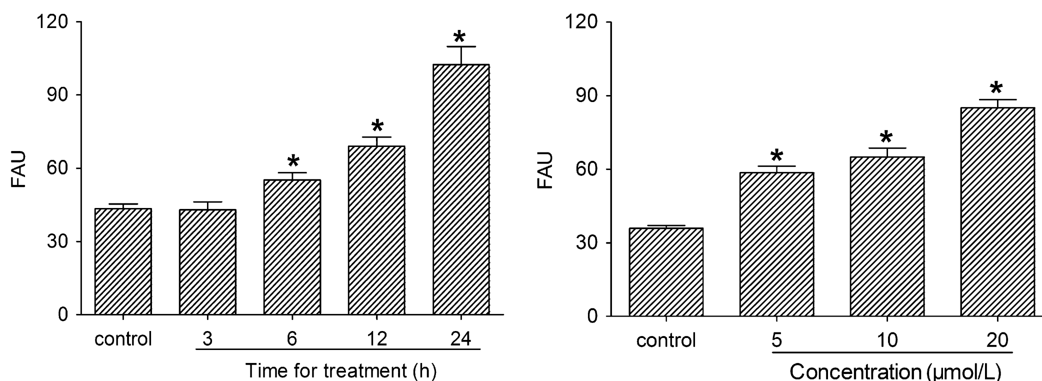

Fig. 6. . LCu triggers ROS generation. A: Cells were exposed to LCu at 10 μmol/L for different time; B: Dose-dependent ROS generation after treatment with LCu for 24 h. The relative levels of fluorescence were quantified by fluorescence spectrometry. Values are the mean ± S.D. ($n = 3$) of fluorescence intensity for 10^4 cells (FAU: fluorescence arbitrary units). *$P<0.05$ vs. control.

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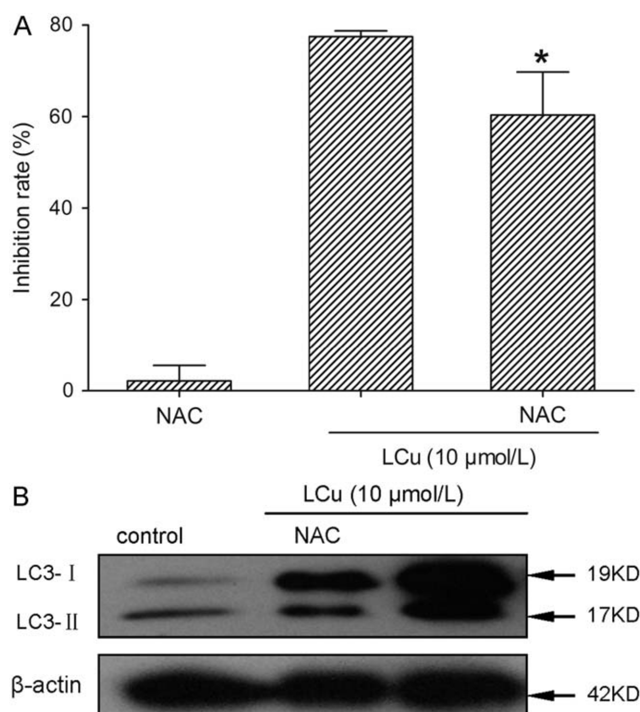

Fig. 7. NAC pretreatment reduced cell death and autophagy induced by Lcu. Cells were pretreated by NAC (5 mmol/L) for 2 h, then treated with 10 μmol/L LCu for 24 h, MTT was used to evaluate the cell viability(A), and protein was collected for western blot (B). $^{*}P<0.05$ vs. control.

Based on the above analysis, we demonstrated that LCu was able to inhibit proliferation of cancer cells and increase intracellular ROS levels. ROS triggered by copper(II) complex may be a key to cell death. On the one hand, LCu can directly damage DNA and cause cell death; on the other hand, LCu can trigger ROS production which also leads to cell death. In conclusion, LCu exhibits the effect of antitumor activity via multi-mechanisms; therefore, LCu may kill tumor cells through different pathways. It less likely that tumor cells develop drug resistance. Therefore, further investigations such as *in vivo* anticancer study, toxicity observation in normal cells, tissues and animals are needed to develop it into a new agent for chemotherapy.

Acknowledgement

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