Metallo-organic Copper(II) Complex in Nano Size as a New Smart Therapeutic Bomb for Hepatocellular Carcinoma

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ABSTRACT

Copper is a micronutrient essential to all organisms and is a critical impact in redox Chemistry, growth and development. In biology copper has a crucial role for the function of several enzymes and proteins involved energy metabolism, respiration and DNA synthesis, notably cytochrome oxidase, super dismutase, ascorbate oxidase and tyrosinase. Chemotherapeutic study of copper complex nanoparticles both in vitro and in vivo in the treatment of hepatocellular carcinoma induced in rats have been done. The treatment efficacy of copper complex nanoparticles was evaluated by measuring antioxidant activities against oxidative stress caused by diethylnitrosamine in liver tissue. The measurements included reduced glutathione content and superoxide dismutase activity, total antioxidant capacity, as well as malondialdehyde level. Liver and kidney function tests were also determined, in addition to the evaluation of serum alpha-fetoprotein in liver tissue. Histopathological examination was also performed for liver tissue. Results showed that copper nanoparticles has a high potency in the treatment of hepatocellular carcinoma which induced by diethylnitrosamine in rats as it ameliorated from the investigated parameters toward normal control animals. These findings were well appreciated with histopathological studies of diethylnitrosamine group treated with copper complex nanoparticles. It was found that Cu(II) nanoparticles can interact with antioxidant to form cancer-specific proteasome inhibitors and apoptosis inducers in liver cancer cell.
INTRODUCTION

The liver is a vital organ that fulfills a wide range of functions including detoxification of various metabolites, protein synthesis, and the regulation of immune responses. Hepatocellular carcinoma (HCC) is a malignant tumor that is the fifth most common type of cancer and the third leading cause of cancer-related death globally. The rate of HCC has been increasing in Egypt with a doubling in the incidence rate in the past years. This has been attributed to several biological and environmental factors. Other factors such as cigarette smoking, occupational exposure to chemicals such as pesticides, and endemic infections in the community, such as schistosomiasis, may have additional roles in the etiology or progression of the disease. Diethylnitrosamine (DEN) is found in a wide variety of foods such as cheese; soybeans; smoked, salted, and dried fish; cured meat; and alcoholic beverages, as well as in ground water having a high level of nitrates. In rats, DEN is a potent hepatocarcinogen influencing the initiation stage of carcinogenesis during a period of enhanced cell proliferation accompanied by hepatocellular necrosis and induces DNA carcinogen adducts, DNA-strand breaks, and in turn HCC without cirrhosis through the development of putative pre-neoplastic focal lesions. It was Barnett Rosenberg in 1965 who accidentally discovered the biological activity of cis-platin, which was recognized as an anticancer drug. The therapeutic activity of cis-platin is achieved by binding with DNA to form crosslinks as major lesions, thus inhibits replication and transcription processes and finally the cell’s repair mechanism and leads to cellular apoptosis. Despite of good clinical success of cis-platin, it lacks tumor tissue selectivity leading to some severe side effects. Advances in nanotechnology and growing needs in biomedical applications have driven the development of multifunctional nanoparticles. Nanoparticles have the potential to be ideal carriers for delivering anticancer and other therapeutics to diseased sites with minimal collateral damage to normal tissues. Functional copper nanoparticles (Cu NPs) have evoked keen interest in recent decades owing to their size- and shape-dependent optical, catalytic, and therapeutic properties. copper-based nanomaterial have been notable for excellent therapeutic applications. Functional Cu NPs have shown apoptosis-inducing properties through target specific pathways. Copper complexes are used as very effective anticancer agents. This property is associated with the inhibition of DNA replication and mitosis by the addition of Cu complex NPs to DNA strand. This study aimed to evaluate the antitumor activity of Cu complex NPs in the treatment of hepatocellular carcinoma both in vitro and in vivo induced in rats.

METHODS

Chemicals

Diethylnitrosamine (DEN) and carbon tetra chloride (CCl₄) were purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA).
Animals

60 healthy male albino rats 8 weeks old (180 - 200 g) were purchased from Institute of Ophthalmic Disease Research, Cairo, Egypt. Rats were housed in cages at regulated temperature (22- 25 °C). They were kept under good ventilation under a photoperiod of 12-h light/12-h darkness schedule with lights-on from 06:00 to 18:00. They all received a standard laboratory diet (60% ground corn meal, 10% bran, 15% ground beans, 10% corn oil, 3% casein, 1% mineral mixture and 1% vitamins mixture), purchased from Meladco Feed Company (Aubor City, Cairo, Egypt) and supplied with water ad libitum throughout the experimental period. Animals received humane care and the present study complies with the animal care guidelines.

Hepatocellular rats model

Experimental hepatocellular carcinoma rats were subjected by a single intra peritoneal injection of freshly prepared DEN (200 mg/kg body weight), then 2 weeks later received a subcutaneous injection of CCl₄ once every week (3 mL/kg body weight/day) for 10 weeks to promote the carcinogenic effect of DEN as shown in figure 1.

Figure 1. Subcutaneous and intraperitoneal injection

In vitro studies

The chemotherapeutic effect was measured in vitro for the synthesized complex using the Sulfo-Rhodamine-B-stain (SRB) assay using the published methods. Cells were plated in 96-multiwell plate (10⁴ cells/well) for 24 hrs before treatment with the compounds to allow attachment of cell to the wall of the plate. Different concentrations of the compounds in DMSO under test (0, 1.56, 3.125, 6.5, 12.5, 25 and 50 µg/ml) were added to the cell monolayer triplicate wells were prepared for each individual dose. Monolayer cells were incubated with the compounds for 48 hrs at 37°C and under atmosphere of 5% CO₂. After 48 hrs, cells were fixed, washed and stained with Sulfo-Rhodamine-B-stain. Excess stain was washed with acetic acid and attached stain was recovered with Tris EDTA buffer (10 mM Tris HCl, 1 mM disodium EDTA, pH=7.5-8). Color intensity was measured in an ELISA reader. The relation between surviving fraction and drug concentration is plotted to get the survival curve of each tumor cell line after the specified compound.
**In vivo studies**

*Determination of LD₅₀ using experimental animals.* In screening drugs, determination of LD₅₀ is usually an initial step in the assessment and evaluation of the toxic characteristics of a substance. The LD₅₀ of the studied compounds was determined as described by Akhila et al.¹³

**EXPERIMENTAL DESIGN**

Animals were allowed 10 days for adaptation. They were then randomly distributed into six equal groups, 10 rats each. The animal groups were recognized as follows:

1. Group 1 (Control). Normal healthy control animals.
2. Group 2 (Cu complex NPs): Each animal was injected intra peritoneal with 10% of LD₅₀ of Cu complex NPs for 6 weeks.
3. Group 3 (Cu complex NPs): Each animal was injected intra peritoneal with 10% of LD₅₀ of Cu complex NPs for 12 weeks.
4. Group 4 (DEN): Each animal was subjected by a single intra peritoneal injection of freshly prepared DEN (200 mg/kg body weight), then 2 weeks later received a subcutaneous injection of CCl₄ once every week (3 mL/kg body weight/day) for 10 weeks to promote the carcinogenic effect of DEN.
5. Group 5 (DEN + Cu complex NPs): Rats received DEN as in group 4 and then treated with Cu complex NPs for 6 weeks as in group 2 after induction.
6. Group 6 (DEN + Cu complex NPs): Rats received DEN as in group 4 and then treated with Cu complex NPs for 12 weeks as in group 2 after induction.

**Blood collection and tissue preparation**

At the end of the experimental period (6 and 12 weeks), At the end of the treatment period, animals were fasted overnight prior to dissection under light ether anesthesia. Blood was drawn from the venacava and centrifuged at 3000g for 10 min. Immediately after blood collection, (liver, kidney, brain, lung, spleen and testes) removed and preserved in 10% formalin solution for further histopathological and immunohistopathological studies.

**Biochemical analysis**

Alpha-fetoprotein (AFP) level was estimated by immunoenzymatic colorimetric method according to Acosta. Aspartate transaminase (AST) activity, alanine transaminase (ALT) activity and alkaline phosphatase (ALP) were measured using kinetic kits purchased by Human Diagnostic Kits, Total protein, Albumin, Bilirubin, Renal functions, Creatinine was determined, Antioxidants "Malondialdehyde (MDA), Glutathione (GSH), Super oxide dismutase (SOD) and Total antioxidants) was determined for each rat.

**Statistical analysis**

Data were subjected to statistical significance tests using one-way analysis of variance (ANOVA), followed by Duncan’s multiple range test. The statistical analysis was carried out using SPSS 12.00 software. The results were expressed as mean ± SD and the differences were considered significant at P ≤ 0.05.
RESULTS

Transmission electron microscopy characterization (TEM)

TEM for colloidal Cu complex nanoparticles were obtained using a JEOL 1230 transmission electron microscope (120 kV). TEM samples were prepared by dropping the colloids onto carbon-coated TEM grids (carbon coated Cu grids, Ted Pella, Redding, CA, USA) and allowing the liquid carrier to evaporate in air.

The average diameter of these spherical Cu nanoparticles was determined to be 55.51 nm with a standard deviation of 17.41 nm as shown in A,B and C.

\[\begin{array}{ccc}
A & B & C \\
\end{array}\]

In vitro studies

Microscopic picture of cell culture of hepatocellular carcinoma

The chemotherapeutic activity of the tested Cu complex NPs by comparing it with the standard drug (Vinblastine Sulfate) as shown below. There is decreasing in the number of available cells. Most of the remaining observed degeneration changes in the form of irregulatory cell membrane opaque and not well formed chromatin regulated of swelling cytoplasm , other showed optatic change in the formed of chunked cells and increase eosinophilia cells, and picknitoic nucleus as shown in figures 2-5.
Figure 2. Histogram showed non-treated HepG-2 cells

Figure 3. Histogram showed HepG-2 cells treated with standard drug at 500 µg/ml

Figure 4. Histogram showed HepG-2 cells treated with copper complex nanoparticles at 0.5 µg/ml
In vivo studies

Determination of liver superoxide dismutase, glutathione, and malondialdehyde levels. The data represented in Table 2 indicated that liver superoxide dismutase (SOD) and glutathione (GSH) were significantly decreased in DEN rats than control animals. DEN groups subjected to Cu complex NPs resulted in significant increase in antioxidants levels compared to DEN group. DEN animals also showed significant increase in malondialdehyde (MDA) level than normal rats. Treatment with Cu complex NPs showed significant decrease in MDA level than DEN rats. More pronounced increase in SOD and GSH levels associated with reduction in MDA level was observed in DEN group treated with Cu complex NPs compared to non-treated ones. Liver function tests. A significant decrease was recorded in albumin and protein levels in DEN animals than control group as shown in Table 3. Results in Table 1 also indicated significant increase in AFP, total bilirubin level, ALT, AST, and ALP activities in DEN rats than normal rats. Amelioration in liver function tests was shown in DEN animals subjected to different treatments toward control animals in comparison to DEN group especially in Cu complex NPs–treated DEN rats. Serum AFP level showed significant increase in DEN rats compared to control animals. Treatment of DEN-induced group with Cu complex NPs showed a significant decrease in AFP level.

Table 1. Statistical analysis (ANOVA) for liver and kidney function tests in the different groups.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Group 1 (Control)</th>
<th>Group 2 Cu NPs (6 Weeks)</th>
<th>Group 3 Cu NPs (12 Weeks)</th>
<th>Group 4 DEN</th>
<th>Group 5 Cu NPs+ DEN (6 Weeks)</th>
<th>Group 6 Cu NPs+ DEN (12 Weeks)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AFP</td>
<td>0.94±0.049</td>
<td>0.90±0.027</td>
<td>0.93±0.036</td>
<td>2.72±0.301</td>
<td>1.16±0.141</td>
<td>1.23±0.665</td>
</tr>
<tr>
<td>AST</td>
<td>46.90±1.930</td>
<td>40.50±1.448</td>
<td>40.50±1.347</td>
<td>133.04±5.160</td>
<td>74.22±2.875</td>
<td>62.14±3.904</td>
</tr>
<tr>
<td>ALT</td>
<td>34.14±1.335</td>
<td>29.93±0.920</td>
<td>31.80±1.700</td>
<td>133.04±5.160</td>
<td>43.28±1.074</td>
<td>39.574±1.542</td>
</tr>
<tr>
<td>Alb</td>
<td>3.706±0.434</td>
<td>4.008±0.188</td>
<td>4.100±0.316</td>
<td>2.237±0.212</td>
<td>3.224±0.182</td>
<td>3.768±0.085</td>
</tr>
<tr>
<td>ALP</td>
<td>146.74±21.221</td>
<td>99.70±0.418</td>
<td>96.98±0.192</td>
<td>262.98±0.715</td>
<td>146.04±0.743</td>
<td>141.06±2.262</td>
</tr>
<tr>
<td>T. Bilirubin</td>
<td>0.48±0.011</td>
<td>0.50±0.031</td>
<td>0.51±0.015</td>
<td>2.25±0.015</td>
<td>0.96±0.031</td>
<td>0.61±0.015</td>
</tr>
<tr>
<td>Creatinine</td>
<td>0.50±0.019</td>
<td>0.51±0.021</td>
<td>0.55±0.026</td>
<td>0.56±0.023</td>
<td>0.59±0.021</td>
<td>0.49±0.043</td>
</tr>
</tbody>
</table>

ANOVA: analysis of variance; ALP: alkaline phosphatase; DEN: diethylnitrosamine;

SD: standard deviation; Alb: albumin; AST: aspartate aminotransferase; ALT: alanine aminotransferase.

Each value is represented as mean ± SD. Data with different superscripts are significantly different at $p \leq 0.05$.

*Significance versus group 1 (control).

+Significance versus group 2 (Cu NPs after 6 weeks).

+Significance versus group 3 (Cu NPs after 12 weeks).

+Significance versus group 4 (DEN).

+Significance versus group 5 (Cu NPs+ DEN after 6 weeks).

+Significance versus group 6 (Cu NPs+ DEN after 12 weeks).

**Table 2. Statistical analysis (ANOVA) for liver antioxidant levels in the different groups**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Group 1 (Control)</th>
<th>Group 2 Cu NPs (6 Weeks)</th>
<th>Group 3 Cu NPs (12 Weeks)</th>
<th>Group 4 DEN</th>
<th>Group 5 Cu NPs+ DEN (6 Weeks)</th>
<th>Group 6 Cu NPs+ DEN (12 Weeks)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA</td>
<td>4.084±0.089&lt;sub&gt;&lt;sup&gt;abc&lt;/sup&gt;&lt;/sub&gt;</td>
<td>4.038±0.038&lt;sub&gt;&lt;sup&gt;abc&lt;/sup&gt;&lt;/sub&gt;</td>
<td>4.102±0.071&lt;sub&gt;&lt;sup&gt;abc&lt;/sup&gt;&lt;/sub&gt;</td>
<td>5.972±0.088&lt;sup&gt;d&lt;/sup&gt;</td>
<td>4.610±0.133&lt;sup&gt;e&lt;/sup&gt;</td>
<td>4.394±0.348&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>GSH</td>
<td>2011.540±1.023&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2088.300±0.277&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2037.100±0.252&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1112.000±0.710&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1840.300±0.331&lt;sup&gt;e&lt;/sup&gt;</td>
<td>1896.900±4.017&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>SOD</td>
<td>866.240±0.792&lt;sup&gt;b&lt;/sup&gt;</td>
<td>880.660±1.094&lt;sup&gt;c&lt;/sup&gt;</td>
<td>867.860±1.716&lt;sup&gt;c&lt;/sup&gt;</td>
<td>407.140±1.346&lt;sup&gt;d&lt;/sup&gt;</td>
<td>751.360±1.161&lt;sup&gt;e&lt;/sup&gt;</td>
<td>808.840±0.890&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>T.Antioxidants</td>
<td>6.796±0.055&lt;sup&gt;*&lt;/sup&gt;</td>
<td>6.228±0.258&lt;sup&gt;ef&lt;/sup&gt;</td>
<td>6.292±0.139&lt;sup&gt;ef&lt;/sup&gt;</td>
<td>2.596±0.126&lt;sup&gt;e&lt;/sup&gt;</td>
<td>3.330±0.0435&lt;sup&gt;e&lt;/sup&gt;</td>
<td>6.188±0.188&lt;sup&gt;ef&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

ANOVA: analysis of variance; SOD: superoxide dismutase; DEN: diethylnitrosamine; MDA: malondialdehyde; GSH: glutathione; SD: standard deviation.

Each value is represented as mean ± SD. Data with different superscripts are significantly different at $p \leq 0.05$.

*Significance versus group 1 (control).

+Significance versus group 2 (Cu NPs after 6 weeks).

+Significance versus group 3 (Cu NPs after 12 weeks).

+Significance versus group 4 (DEN).

+Significance versus group 5 (Cu NPs+ DEN after 6 weeks).

+Significance versus group 6 (Cu NPs+ DEN after 12 weeks).

**Figure 6.** (A) Liver of rats in normal control, (B) Liver of rats in DEN group showing fine fibrosis dividing the degenerated and necrotic dysplastic hepatocytes into nodules (H&E 16×). (C) Liver of rat in DEN + Cu NPs group (12 weeks) showing dilatation of central vein with very fine fibroblastic cell proliferation dividing the vacuolar degenerated focal areas of hepatocytes into few nodules with no signs of dysplasia (H&E 16×). (D) Cu NP group (12 weeks) showing normal histological structure of the central vein with surrounding hepatocytes in the parenchyma (H&E 40×).
Histopathological studies

There was no histopathological alteration, and the normal histological structure of the central vein and surrounding hepatocytes in the parenchyma was observed in control, Cu NPs group as shown in Figure 4 (A), (D), respectively. Fine fibroblastic cell proliferation dividing the degenerated, necrotic, and dysplastic hepatocytes into nodules was seen in liver of DEN animals (Figure 4 (B)). In the liver of DEN rats administered with Cu NPs, dilatation was noticed in the central veins associated with fine fibroblastic cell proliferation dividing the focal vacuolar degenerated hepatocytes into few nodules with no signs of dysplasia (Figure 4 (C)).

DISCUSSION

Reports indicate that Cu complex NPs might be useful as therapeutics in cancer therapy, and Cu complex NPs in combination with Hadron therapy led to an enhancement of strongly lethal DNA damage caused by double-strand breaks.\(^1\) This study was conducted to evaluate the efficiency of biologically synthesized Cu complex NPs in the treatment of HCC both in vitro and in vivo. Accordingly, the cytotoxic effects and the biological activity of Cu complex NPs as antitumor agents were examined in vitro against human liver carcinoma cell line (HepG-2 cells) using crystal violet cytotoxicity assay. Results showed potent effect of Cu complex NPs more than standard drug (Vinblastine Sulfate) in a dose-dependent manner, where increasing concentration of Cu complex NPs resulted in increased percentage of dead cells. This result is in harmony with Alshatwi et al.\(^1\) who reported that Cu complex NPs inhibit cell proliferation via induction of apoptotic cell death. In addition, Cu complex NPs produce a cytotoxic effect by reducing cell viability and causing inter-nucleosomal DNA fragmentation, G2/M cell-cycle arrest, and hypo-diploid accumulation emphasizing that the Cu complex NPs have potential anticancer properties and can be applied as cancer therapeutics. A previous study showed that Cu complex NPs’ uptake by the cells endocytosis and emphasized intracellular release of Cu\(^{+2}\) ions from Cu complex NPs (that) blocks cell division by binding to DNA causing DNA damage and contributed to the cytotoxicity and metabolic stress activating cell death via apoptosis. Also, down regulation of proliferating cell nuclear antigen, a factor critical for DNA replication and repair following Cu NP treatment, supports the anti-proliferative effects of Cu complex NPs. In this study, injection of DEN in male albino rats induced significant deleterious changes in antioxidant status. The results revealed a marked depletion in serum GSH content, total antioxidants and SOD activity with a significant increase in MDA level compared to the control group. These findings were in agreement with other studies which reported that DEN confers its hepatocarcinogenicity through the metabolic activation in the hepatic microsomes, resulting in the release of ethylcarbionium ions that bind to the DNA, producing adducts and generating superoxide radicals through lipid peroxidation of phospholipid membrane fatty acids. MDA, a product of lipid peroxidation of polyunsaturated fatty acid metabolism and degradation, has been established as a mutagenic and carcinogenic entity. Lowering of MDA level and the increase in levels of GSH and SOD in DEN rats treated with Cu complex NPs indicate their
potential as inhibitors of DEN-induced intracellular oxidative stress. Pt NPs have unveiled antioxidant properties that scavenge reactive oxygen species (ROS), including superoxide anion (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), and free radicals. The present biochemical results revealed a decrease in total protein and albumin levels, while significant increase was shown in total bilirubin level and ALT, AST, and ALP activities in DEN group than control rats, which agrees with previous studies. This may be attributed to DEN induced oxidative stress which induces liver tissue damage and impairment of liver function. In this study, post treatment of DEN animals with Cu complex NPs resulted in improvement in liver function tests compared to DEN rats in agreement with others. The DEN rats also showed significant increase in AFP level compared to control animals. This result is in line with previous studies that indicated that increase in serum AFP level upon DEN induction is associated with increase in tumor growth. Administration of Cu complex NPs resulted in lowering of AFP level compared with DEN rats, which indicates the antitumor activity of Cu complex NPs and also the decrease in the production rate of tumor as indicated by Yamada et al., who claimed that Cu complex NPs are thought to serve as a reservoir for Cu ions that can induce DNA damage in cancer cells. In this study, histopathological finding revealed that liver of DEN rats showed fibroblastic cell proliferation dividing the degenerated, necrotic, and dysplastic hepatocytes into nodules. This result correlates with others, and they found that liver tissue of DEN-treated rats showed hydropic degeneration, and focal areas of necrosis, portal inflammation, and hepatocytes showed partial loss of architecture and significant tumor nests. Cu complex NPs treatment showed histopathological improvement. All sections showed more or less normal architecture. Collectively, most of the histological manifestations observed in cancer control were greatly reduced especially when Cu complex NPs were used in treatment.

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REFERENCES


