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EXPERIMENTAL STUDY

Schiff base Cu(II) complexes as inhibitors of proteasome in human cancer cells

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ABSTRACT

BACKGROUND: It has been demonstrated that proteasome inhibitors might be potential anticancer drugs. The copper complexes can be used as specific proteasome inhibitors in tumor cells able to induce apoptosis by the ubiquitin-proteasome pathway. The goal of our study was to test the cytotoxic and proteasome inhibitory effects of five Schiff base Cu(II) complexes - $[Cu_2(sal\text{-}D,L\text{-}glu)_2(isoquinoline)_2] \cdot 2C_2H_5OH$ (1), $[Cu(sal\text{-}5\text{-}met\text{-}L\text{-}glu)(H_2O)]\cdot H_2O$ (2), $[Cu(ethanol)_2(imidazole)_4][Cu_2(sal\text{-}D,L\text{-}glu)_2(imidazole)_2]$ (3), [Cu(sal-D,L-glu)(2-methylimidazole)] (4) on human lung carcinoma cells A549, cervix carcinoma cells HeLa and glioblastoma cells U-118MG.

MATERIAL AND METHODS: For the cytotoxic analysis we used MTT test and for monitoring the proteasome inhibition western blot analysis.

RESULTS: We have observed different cytotoxic effects of tested complexes on human cancer cells depending on the ligand present in their structure. Cu(II) complexes 4 and 5 were the most effective against A549 cells; all complexes were cytotoxic against HeLa cells and the complex 4 was the most effective against U-118MG. Moreover, we have detected the inhibition of the proteasome activity in human cancer cells A549 by Cu(II) complexes 1, 2 and 4 at IC_{50} concentration.

CONCLUSION: Results of our study suggest that isoquinoline- and imidazole-based copper complexes could be used as inhibitors of the proteasome system in cancer cells A549 *(Tab. 1, Fig. 1, Ref. 26).* Text in PDF *www.elis.sk.*

KEY WORDS: proteasome, copper complexes, Schiff base, cancer.

Introduction

Proteasome is the multienzyme complex present in the nucleus and the cytoplasm of all eukaryotic cells. It clears approximately 90 % of damaged intracellular proteins, including proteins regulating the cell's basic functions such as proliferation, apoptosis, angiogenesis and metastasis, and is the primary component of the ubiquitin-proteasome system (UPS) (1–5).

New findings, as well as the identification of new protein ligases involved in UPS dysregulation, open up the possibility of treating several diseases just through UPS regulation. Due to degradation of the tumor suppressor, which is a key regulatory factor, dysregulation or inhibition of proteasome activity plays an important role in the treatment of cancer (6), also in connection with the finding that in cancer cells, proteasome activity is increased and cells are more susceptible to inhibition (7). Medicines containing metals have been available for several years. Cisplatin, a platinum-containing compound, is known to be one of the most effective antitumor drugs. Cisplatin-based chemotherapy, however, leads to serious side effects which makes it more difficult for clinical use (8). In addition to platinum analogues, attention is also drawn to other complexes containing metal ions such as zinc, copper, gold and chelating agents (9). In addition to anti-inflammatory effects, recent studies show other properties of copper and copper complexes. For example, ^{64}Cu radionuclides are becoming widely used as potential diagnostic and therapeutic agents in radiation oncology (10).

The literature also mentions antitumor activity of disulfide (DSF), a drug used to treat alcoholism. However, the Cu-DSF combination becomes a powerful inducer of radical oxygen species and opens up new therapies (11). Recent studies suggest that Cu-DSF may have strong anti-tumor activity through inhibition of the proteasome. The disadvantage, however, is that the Cu-DSF complex is poorly soluble in biological fluid and unstable (12). Copper and its complexes in combination with other substances are also capable of affecting a wide variety of tumor cells, including cells that are resistant to a particular type of drug (13).

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We have studied effects of Cu(II) complexes with different ligands in their structure (isoquinoline, imidazole, methylimidazole, glutamate) against various types of human cancer cells. In our previous work we demonstrated their ability to inhibit the proteasome activity in human colon carcinoma cells (14). Following the previous study we used five copper complexes (Schiff base Cu(II) complexes) with different ligands in their structure to study their ability to dysregulate the UPS in human cancer cells - human lung carcinoma cells A549, the human cervix carcinoma cells HeLa and the human glioblastoma cells U-118MG. The development of novel proteasome inhibitors as potential anticancer drugs has been shown to be a good way to increase survival and improve the lives of patients with cancer (15). Therefore, great attention is now being paid to the development of drugs that could be efficient inhibitors of proteasome (16).

Materials and methods

Cell cultures

Human lung cancer cells (A549), human cervix cancer cells (HeLa) and human glioblastoma cells (U-118MG) were purchased from the American Type Culture Collection (Manassas, VA, USA) and maintained in Dulbecco's Modified Eagle Medium (DMEM, Life Technologies, Inc., Rockville, MD) containing 10% fetal bovine serum, 100 µg/mL streptomycin, and 100 U/mL penicillin G at 37 °C in a humidified atmosphere of 5 % CO_2/95 % air.

Cu(II) complexes

Complexes of composition [Cu(sal-L-glu)(H_2O)$_2$].H_2O (1), [Cu_2(sal-D,L-glu)$_2$(isoquinoline)$_2$] . 2C_2H_5OH (2), [Cu(sal-5-met-L-glu)(H_2O)].H_2O (3), [Cu(ethanol)$_2$(imidazole)$_4$][Cu_2(sal-D,L-glu)$_2$(imidazole)$_2$] (4), [Cu(sal-D,L-glu)(2-methylimidazole)] (5) were prepared, where (sal-D,L-glu) or (sal-L-glu) is N-salicylidene-D,L- or L-glutamate and (sal-5-met-L-glu) is N-salicylidene-5-methylester-L-glutamate. The synthesis and the structure of the complexes are described in the publication by Langer et al. (17). Schiff base Cu(II) complexes were dissolved in distilled water for the preparation of stock solution with a concentration of 10 mmol/L.

Cytotoxicity analysis

Effects of Cu(II) complexes on viability of human carcinoma cells A549, HeLa and U-118MG were determined by using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] colorimetric technique. Cells (8×10^3 cells/200 µL well) were placed in individual wells of 96-multiwell plates. Each concentration was tested four times. Cu(II) complexes were diluted with distilled water. Final concentrations of the complexes added to the cells were 0.001, 0.01, 0.1, 1, 10, 50 and 100 µmol/L. After 72h exposure to seven concentrations of Cu(II) complexes (37°C, humidified atmosphere of 5 % CO_2/95 % air), cells were treated with MTT solution (5 mg/mL in PBS, 20 µL) for 4h. The dark crystals of formazan formed in intact cells were dissolved in DMSO (200 µL). The plates were shaken for 15 min and the optical density was determined at 595 nm using a MicroPlate Reader (Biotek, USA).

Western blotting

Cells were grown in 6-well microplates and treated with Cu(II) complexes (IC_{50} concentration) for different time periods. After the treatment, cells were resuspended in lysing buffer and boiled for 3 min at 100 °C. Cell lysates were separated by SDS–PAGE gel and blotted onto nitrocellulose (NC) membrane (Bio-Rad, USA). After blocking, the membranes were incubated with rabbit antibodies against LC3 (1:500, Santa Cruz Biotechnologies) at 4 °C. The blots were then washed and incubated for 1h with anti-rabbit secondary antibody (HRP – Horseradish Peroxidase-Conjugated Antibodies) (1:5000, Santa Cruz Biotechnologies). Immunoreactive bands were visualized with a SuperSignal Wast Femto (Thermo Scientific,U.S.).

DNA constructs

pCMVHAUbiquitin construct (Ubiquitin DNA sequence ligated into pCMV-Ha vector (Clontech, CA) and pCMVcMycUbiquitin construct (Ubiquitin DNA sequence ligated into pCMV-Myc vector (Clontech, CA) prepared at the Institute of Chemical Technology in Prague by Markéta Landová and Anna Lounková, allowed to express ubiquitin fused to either the c-Myc or hemagglutinin (HA) epitope tag for detection with appropriate antibodies. pRK5-HAUbiquitin construct was provided by ADDGENE (Parkin mediates nonclassical), proteosomal-independent ubiquitination of synphilin-1: implications for Lewy body formation by Chung et al (18) and Lim et al. (19). c-Myc and HA are well-characterized and highly immunoreactive tags, and thus are easily detected via western blot.

Transfection and the proteasome activity

Human lung cancer cells A549, human cervix cancer cells HeLa and human glioblastoma cells U-118MG were seeded in a single 6-well cell culture plate. Transfection with the plasmids was carried out by FuGene HD (Promega, USA) according to manufacturer´s instructions. After 4–5 h the medium was changed and Cu(II) complexes were added to the final concentration of IC50. After next 24 hours cells were washed with PBS. Non-treated cells were used as a negative control and cells treated with the commercially available inhibitor of proteasome MG132 (2.5 mol/L) (Sigma Aldrich, USA) were used as a positive control.

Statistical analysis

Results are expressed as arithmetic mean ± standard deviation (SD) of the mean of three separate experiments (each experiment was done with three parallels).

Results

Cytotoxicity analysis

Effects of tested Cu(II) complexes (1, 2, 3, 4, 5) on the human lung carcinoma cells A549, human cervix carcinoma cells HeLa and human glioblastoma cells U-118MG were evaluated using MTT assay during 72h treatment (Table 1). Concentration range of the complexes was 0.001-100 µmol/L.

Tab. 1. Inhibitory effects of Cu (II) complexes on the proliferation of human cancer cells A549, HeLa and U-118MG. Cells were treated with copper complexes (1–5) at the concentration range of 0.001–100 µmol/mL for 24, 48 and 72h and the cell death was detected by the MTT test. Results are expressed as the mean ± standard deviation from three independent experiments.

complex	A549 (IC_{50})			HeLa (IC_{50})			U-118MG (IC_{50})		
	24h	48h	72h	24h	48h	72h	24h	48h	72h
1	> 100	> 100	> 100	> 100	0.005	> 100	>100	> 100	> 100
2	> 100	> 100	> 100	> 100	0.01	> 100	>100	> 100	> 100
3	> 100	> 100	> 100	> 100	0.57	97	>100	> 100	> 100
4	0.055± 0.013	45±1.527	49±6.506	75.5±3.122	0.05± 0.010	30±5.246	>100	62.5±1.524	72.5±5.742
5	95±12.124	34±4.509	100	0.9±0.208	6±0.929	99±7.247	>100	> 100	> 100

We have found cytotoxic effects of two Cu(II) complexes (4 and 5) on A549 cell line (Tab. 1), while the complex 4 was more effective. IC_{50} values of the complex 4 increased after 72h influence. On the other hand, IC_{50} values of the complex 5 decreased after 48h treatment but increased after 72h treatment.

The human cervix carcinoma cells HeLa (Tab. 1) were the most sensitive to all Cu(II) complexes after 48h influence. Compared to 48h incubation, after 72h incubation we have observed increased IC_{50} values for all complexes used.

The glioblastoma cells U-118MG (Tab. 1) were more sensitive only to the complex 4 during 48 h and 72 h treatment. IC_{50} values decreased after 48h treatment but increased after 72 h treatment.

Tested Cu(II) complexes have not antiproliferative activity against healthy cells (20).

In order to ascertain the influence of copper(II) ions and free ligands on cell proliferation we tested also individual constituents of our Cu(II) complexes separately, in the form of $CuSO_4.5H_2O$, salicylaldehyde and L-glutamic acid (0.001–100 µmol/L). All tested compounds showed IC_{50} > 100 µmol/L.

Inhibitory effects of Cu(II) complexes on proteasome activity

We investigated the ability of Cu(II) complexes to inhibit proteasome in the human lung cancer cells A549, human cervix cancer cells HeLa and human glioblastoma cells U-118MG. The proteasome inhibition we monitored by western blot analysis (Fig. 1). Cells were transfected with plasmid DNA (pCMVHAUbiquitin) and treated with the Cu(II) complexes at the concentration of IC_{50} (µmol/L) for 24h. We detected no ubiquitin bands (10 kDa) in human cervix carcinoma cells (HeLa) and human glioblastoma cells (U-118MG) (results not shown) and detected ubiquitin bands in human lung cancer cells (A549) (Fig. 1). Cu(II) complexes inhibited proteasome in A549 cells, we detected ubiquitin band (10 kDa) in the samples containing Cu(II) complexes 1, 2, 4. Ubiquitin levels in the cells treated with Cu(II) complexes 1 and 2 were comparable or higher (complex 4) than cells incubated with the commercially used inhibitor of proteasome MG132 (line C3).

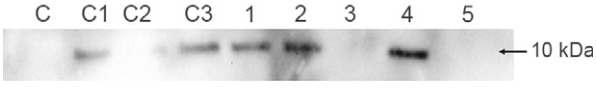

Fig. 1. Detection of proteasome inhibition in A549 cells by western blot analysis after the treatment with Cu(II) complexes at IC_{50} concentration for 24h. C – cells without transfection, without commercial inhibitor MG123, without treatment with Cu(II) complexes, C1 – cells without transfection, with commercial inhibitor MG123, without treatment with Cu(II) complexes, C2 – cells with transfection, without commercial inhibitor MG123, without treatment with Cu(II) complexes, C3 – cells with transfection, with commercial inhibitor MG123, without treatment with Cu(II) complexes, 1, 2, 3, 4 and 5 – cells with transfection, treated with Cu(II) complexes 1–5 at IC_{50} concentration for 24 h.

Discussion

We have found different biological effects of our five copper complexes on the human cancer cells based on the type of ligand in their structure. The most effective cytotoxic effect had the complex with imidazole (complex 3) and methylimidazole (complex 4) ligands in their structure on all human cancer cell lines used (A549, HeLa and U-118MG). This is in agreement with other studies reporting that the imidazole-based compounds are effective against various types of cancer cells by inhibiting the PI3K/Akt/mTOR signaling pathway (21). Imidazole-based compounds can reduce breast cancer cell proliferation, phosphorylation of PDK, Akt, mTOR, cell invasion regulation and PARP cleavage, resulting in induction of apoptosis. To determine the mechanism of their cytotoxicity we examined their ability to inhibit the proteasome activity (22).

To detect proteasome inhibition we used western blot analysis. In case of proteasome inhibition, ubiquitin accumulates, which can be seen as a pronounced band on the nitrocellulose membrane. Ubiquitin is a small protein used to label proteins destined for degradation.

Based on our results (Fig. 1) we assume the inhibition of proteasome in human lung carcinoma cells A549 after the treatment with complexes $[Cu(sal\text{-}L\text{-}glu)(H_2O)_2].H_2O$ (**1**), $[Cu_2(sal\text{-}D,L\text{-}glu)_2(isoquinoline)_2].2C_2H_5OH$ (2) and $[Cu(ethanol)_2(imidazole)_4]$ $[Cu_2(sal\text{-}D,L\text{-}glu)_2(imidazole)_2]$ (**4**). Their inhibitory activity is the same or even higher than that of commercially available inhibitor MG123.

Due to the fact that we found no ubiquitin bands after the treatment of HeLa and U-118MG cells with our copper complexes, we cannot clearly state whether our tested copper complexes are/are not proteasome inhibitors in those cells. The proteasome inhibitory action of different copper complexes in HeLa cells was observed by Santoro et al. (2016). They found that although ubiquitine dependent proteolysis was inhibited by copper complexes in a concentration-dependent manner, a moderate recovery of proteasome activity in HeLa cells at 80 µmol/L compared to samples exposed to a concentration of 40 µmol/L was observed. The authors ex-

plain this effect by the increased presence of ROS in HeLa cells (at higher copper concentration) and subsequent activation of the antioxidant defense of the cell, which activates inhibited proteasome (23). This fact indicates that HeLa cells are less sensitive to the proteasome inhibition by copper complexes.

Glioblastoma is generally regarded as one of the most resistant tumors. It rapidly infiltrates and proliferates and is therefore a therapy challenge (24). Numerous studies explain this effect as the problem of drug transfer through the blood-brain barrier (25). Influence of copper complexes on proteasome in gliomas on the level of *in vitro* models has not been examined yet and therefore opens up new possibilities of research (26).

Conclusion

Results of our study suggest that isoquinoline- and imidazole-based copper complexes could be used as inhibitors of the proteasome system in cancer cells A549.

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