Pharmacological Reports 68 (2016) 1221–1224

Contents lists available at ScienceDirect

Pharmacological Reports

journal homepage: www.elsevier.com/locate/pharep

Original article

Autophagy in MCF-7 cancer cells induced by copper complexes

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ARTICLE INFO

Article history:
Received 3 June 2016
Received in revised form 25 July 2016
Accepted 29 July 2016
Available online 30 July 2016

Keywords:
Schiff base copper complexes
Autophagy
Cell death
Cancer cell lines

ABSTRACT

Background: Autophagy plays an important role in cancer cells. Targeting autophagy in cancer can provide new opportunities for drug development.
Methods: In this study we tested four Schiff base Cu(II) complexes against human breast cancer cells (MCF-7) and human non-cancerous cells (HEK-293T). We have tested their cytotoxic effect by evaluating IC_{50} using MTT test. To detect morphological changes of the actin fibers we have used fluorescent microscopy. To determine the type of cell death we used electrophoretic analysis and western blot analysis (protein LC3).
Results: IC_{50} values of the complexes increased with time of their influence, indicating acquired resistance of MCF-7 to the complexes. Healthy cells HEK-293T were not sensitive to the Cu(II) complexes. Compared with the control cells (cells without Cu(II) complexes) which were without morphological changes of actin fibers, Cu(II) complexes induced condensation and asymmetric conformational changes in actin filaments. To examine the type of cell death induced by the Cu(II) complexes we treated MCF-7 cells with Cu(II) complexes (1, 10, 50 and 100 μmol/L) during a 72 h incubation period. By electrophoresis we have not detected any DNA fragmentation. To determine whether Cu(II) complexes induced autophagy or necrotic cell death we used the western blot analysis. MCF-7 cells influenced with tested Cu(II) complexes produced LC3 protein after their 72 h incubation indicating autophagy in MCF-7 cancer cells.
Conclusions: Tested Schiff base copper (II) complexes have antiproliferative activity against cancer cells but not against healthy cells. They have induced autophagy in the cancer cell line MCF-7.

Introduction

In the last few years basic cancer research has made remarkable advances in the understanding of cancer molecular biology. One of the most important advances was the finding that the malignant phenotype is profoundly affected by apoptosis [1].

Metal-based drugs with large applications in medicine are of increasing clinical and commercial importance to the development of novel anticancer agents. In the last few decades, Schiff bases and their complexes have become well known for their extensive biological potential [2].

Copper-based complexes have been investigated on the assumption that endogenous metals may be less toxic to normal cells compared to cancer cells and can induce apoptotic cell death [3] or autophagy [4]. Apoptotic cell death and autophagy are programmed cell deaths without inflammation of the surrounding healthy tissue [5].

In this study we specify the effects and a type of cell death induced by four Cu(II) complexes containing the Schiff base derived from salicylaldehyde, glutamic acid and additional molecular *O*- or *N*- donor ligands in the breast cancer cell line MCF-7.

Materials and methods

Cell cultures

Human breast cancer cells (MCF-7), human embryonic kidney non-cancerous cells (HEK-293T) and mouse leukemia cells (L1210) were purchased from the American Type Culture Collection (Manassas, VA, USA) and maintained in Dulbecco's Modified Eagle

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http://dx.doi.org/10.1016/j.pharep.2016.07.011
1734-1140/

Medium (DMEM, Life Technologies, Inc., Rockville, MD, USA) 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.

Schiff base Cu(II) complexes

Schiff base Cu(II) complexes of composition $[Cu_2(sal\text{-}_{D,L}\text{-}glu)_2(isoquinoline)_2]\cdot 2C_2H_5OH$ (**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**); 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 were prepared. The synthesis and the structure of the complexes are described in publications by Langer et al. [6–9]. Schiff base Cu(II) complexes were dissolved in distilled water for the preparation of stock solutions with a concentration of 10 mmol/L.

Cytotoxic analysis

We have determined the cytotoxic effects of Cu(II) complexes on carcinoma and non-cancerous cells by using an MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] colorimetric technique. Cells were seeded (1×10^4 cells/200 μL well) in individual wells of 96-multiwell plates. We added different concentrations of Cu(II) complexes (0.001, 0.01, 0.1, 1, 10, 50 and 100 μmol/L) to the cells and incubated them for 24, 48 and 72 h at 37 °C (humidified atmosphere of 5% CO_2/95% air). After exposure was finished, the cells were treated with the MTT solution (5 mg/mL) in PBS (phosphate-buffered saline) (20 μL) for 4 h. The dark crystals of formazan formed in intact cells were dissolved in DMSO (dimethyl sulfoxide) (200 μL). The plates were shaken for 15 min and the optical density was determined at 595 nm using a MicroPlate Reader (Biotek, USA). All dye exclusion tests were performed three times.

Staining of actin filaments

Cell suspension (2×10^3 cells/well) was seeded into 6-well chamber plates on slides (20/20 mm). Cells were then treated with Cu(II) complexes (IC_{50} concentration) for 24 h. Non-treated cells were set as controls (C). To stain actin filaments, the following procedures were all carried out at room temperature. Cells were washed with 2 mL of PBS 3 times and fixed with 1 mL of 4% formaldehyde in PBS for 20 min. Then cells were washed with 2 mL of PBS 3 times and stained with 40 μL/slide Phalloidin-Atto 488 (Sigma Aldrich, USA) (1 μg/mL) for 30 min in the dark. After incubation cells were washed with distilled water. Actin fibers were visualised by a fluorescence microscope (ZEISS, Germany).

Detection of cell death

Untreated (control) carcinoma cells MCF-7 (1×10^6), Cu(II) complexes (1, 2, 3, 4) – treated (0.1–100 μmol/L) carcinoma cells MCF-7 and untreated (control) non-cancerous cells HEK-293T (1×10^6), Cu(II) complexes (1, 2, 3, 4) – treated (0.1–100 μmol/L) cells HEK-293T were incubated for 24, 48 and 72 h, then harvested, washed in PBS and lysed with 50 μL of solution (10 mmol/L Tris, 10 mmol/L EDTA (ethylenediaminetetraacetic acid), 0.5% Triton X-100) supplemented with proteinase K (1 mg/mL). Samples were incubated at 37 °C for 1 h and then heated at 70 °C for 10 min. Following lysis, 2.5 μL of RNase (200 μg/mL) was added followed by repeated incubation at 37 °C for 1 h. The samples were subjected to electrophoresis at 40 V for 2.5 h in a 2% agarose gel containing the ethidium bromide. Separated DNA fragments were visualised using a UV transilluminator (254 nm, Ultra-Lum Electronic UV Transilluminator, USA). As a positive control we used L1210 with 6 μmol/L *cis*-platin (Sigma Aldrich, 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 a lysing buffer and boiled for 3 min at 100 °C. Cell lysates were separated by SDS–PAGE (polyacrylamide gel electrophoresis) and blotted onto a nitrocellulose membrane (Bio-Rad, USA). After blocking (5% milk), the membranes were incubated at 4 °C with rabbit antibodies against LC3 (1:500, Santa Cruz Biotechnologies). The blots were then washed and incubated for 1 h with anti-rabbit secondary antibody (HRP – Horseradish Peroxidase-Conjugated Antibodies) (1:5000, Santa Cruz Biotechnologies). Immunoreactive bands were visualised with a SuperSignal Wast Femto (Thermo Scientific, USA).

Results

Cytotoxicity analysis of cancerous and non-cancerous cell lines

Cells (1×10^4 cells per well) were treated with several concentrations of Cu(II) complexes (0.001, 0.01, 0.1, 1, 10, 50 and 100 μmol/L) for 24, 48 and 72 h and cytotoxicity was evaluated by an MTT test. Cu(II) complexes (1–4) depending on type of the ligand in their structure induced different degrees of growth inhibition in MCF-7 cells (Table 1). IC_{50} values increased with time of complexes' influence. We compared antiproliferative/cytotoxic effects of Cu(II) complexes against carcinoma cells with effects against noncarcinoma cells (HEK-293T). As indicated in Table 1, HEK-293T cells were not sensitive to the Cu(II) complexes.

In order to ascertain the influence of Cu(II) ions and free ligands on cell proliferation we also tested $CuSO_4 \cdot 5H_2O$ and the free ligands (isoquinoline, imidazole, salicylaldehyde and L-glutamic acid (0.001–100 μmol/L)). All tested compounds showed $IC_{50} >$ 100 μmol/L (results are not shown).

Visualisation of actin fibers by fluorescence microscopy

Cells (MCF-7, HEK-293T) were cultured on glass slides to subconfluency with/without Cu(II) complexes at IC_{50}

Table 1
Growth inhibitory concentrations IC50 (μmol/L) of Cu(II) complexes for the MCF-7 and HEK-293T cells.

	MCF-7 (IC_{50})			HEK-293T (IC_{50})		
	24 h	48 h	72 h	24 h	48 h	72 h
1	1.00 ± 0.23	13.00 ± 0.35	21.00 ± 0.61	80.50 ± 0.30	>100	>100
2	25.00 ± 0.33	48.00 ± 0.74	>100	>100	>100	>100
3	0.010 ± 0.003	3.00 ± 0.48	18.50 ± 0.80	>100	26.50 ± 0.33	70.00 ± 9.90
4	75.00 ± 11.00	83.00 ± 14.00	91.50 ± 6.20	>100	68.00 ± 17.00	76.00 ± 11.00

Cells were treated with complexes for 24, 48 and 72 h. The values represent means ± SD of three independent experiments.