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THE ANTIPROLIFERATIVE, ANTIOXIDANT ACTIVITIES AND TOXICITY OF MIXED-LIGAND AMINE-CONTAINING COPPER(II) COORDINATION COMPOUNDS WITH 2-(2-HYDROXYBENZYLIDENE)-N-(PROP-2-EN-1-YL)HYDRAZINECARBOTHIOAMIDE

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Abstract. Five compounds 2-(2-Hydroxybenzylidene)-N-(prop-2-en1-yl)hydrazinecarbothioamide (H2L), bis[μ2-2-({2-[(prop-2-en-1-yl) carbamothioyl] hydrazinylidene}methyl) phenolato-S,N,O:O] diaquadicopper (II) nitrate (1), bis [μ2-2-({2-[(prop-2-en-1-yl) carbamothioyl] hydrazinylidene}methyl)phenolato-S,N,O:O]d iimidazoldicopper(II) nitrate (2), bis[μ2-2-({2-[(prop-2-en-1-yl)carbamothioyl]-hydrazinylidene}methyl)phenolato-S,N,O:O]bis-(3,5-dibromopyridine)dicopper(II) nitrate hexahydrate (3), bis[μ2-2-({2-[(prop-2-en-1-yl)carbamothioyl]-hydrazinylidene}methyl) phenolato-S,N,O:O]bis(4-methylpyridine)dicopper(II) nitrate hexahydrate (4) were synthesized. The antiproliferative properties of these compounds towards cancer cell lines HeLa, RD and normal cell line MDCK have been investigated. The tested compounds demonstrated high antioxidant and antiproliferative, selective activities towards cancer cells. Direct toxic evaluation of compounds was performed by Daphnia magna bioassay.

Introduction

Thiosemicarbazones and coordination compounds of transition metals with them are biologically active compounds. Many of them exhibit antiproliferative and antioxidant activities [1, 2]. In most cases, the coordination of thiosemicarbazones to copper(II) ions leads to the most significant increase in biological activity compared to ions of other 3d metals. A number of studies have shown that the introduction of various amines into the inner sphere of copper(II) thiosemicarbazonates leads to a change in their biological properties. In this regard, it is of interest to study synthesized mixed-ligand amino-containing copper (II) coordination compounds with thiosemicarbazones [1].

Material and methods

1.1. In vitro antiproliferative activity assay

Cells lines RD (human muscle rhabdomyosarcoma spindle and large multinucleated cells, ATCC CCL-136), MDCK (Madin Darby Canine Kidney epithelial normal cells, ATCC CCL-34) for experiments were taken after cryopreservation, in liquid nitrogen vapor phase at −180°C to −196°C in freeze medium: complete growth medium supplemented with 5% (v/v) DMSO. For the formation of a healthy monolayer on the substrate, cells were cultured for at least three weeks, passaged every 2-3 days, followed by trypsinization of adhesive cell clusters and replacement of growth media, inactivated fetal bovine serum was added as a growth factor. Cells

in logarithmic growth phase were used for experiments. Viability of cells was assessed by dye 0.2% trypan blue.

Investigation of the antiproliferative activity of the synthesized compounds in relation to cells lines HeLa and MDCK was carried out by resazurin assay. Resazurin is a non-fluorescent indicator dye, which is converted to highly red fluorescent resorufin via reduction reactions of metabolically active cells. The amount of fluorescence produced is proportional to the number of living cells. Resazurin was dissolved in physiological buffers (resulting in a deep blue colored solution) and added directly to cells in culture in a homogeneous format. Usually, in the presence of NADPH dehydrogenase or NADH dehydrogenase as the enzyme, NADPH or NADH is the reductant that converts resazurin. Cells of lines were trypsinized from subconfluent cultures by adding 3 ml of trypsin-EDTA 0.05% (Invitrogen) to 50 ml of culture flasks with confluent cells followed by 5-15 min incubation at 37°C with regular gentle shaking and counted under an inverted microscope. The trypsin reaction was stopped by adding 10 ml of appropriate culture medium containing 10% FBS. The cell suspension was centrifuged at 750 rpm for 10 min at 25°C. The cell pellet was suspended in 2 ml of medium with 10% FBS and thoroughly mixed. Cells were counted and brought to a concentration of 1×105 cells/ml. The resulting cell suspension was seeded into triplicate wells of a 96-well microtiter plat (100µL/well) and incubated at 37°C, 5% CO2. After an initial 2-3 h period to allow cell attachment, 10 µL of the tested compounds and reference controls were directly added to the medium resulting. The plate was further incubated for 24 h at 37°C, 5% CO2. The tested compounds and reference controls were dissolved in DMSO to prepare the stock solution of 10 mM, which were used as reference at final concentrations ranging from 10, 1, 0.1 µM in medium. The compounds were incubated with cell suspension at 37°C, 5% CO2 for 24 h. Following each treatment, 20 µL resazurin indicator solution was added to each well and incubated at 37°C, 5% CO2 for 4 h. Subsequently, the absorbance was read by hybrid reader (Synergy H1, BioTek) with 570 nm and 600 nm filters. The percentage of cell proliferation inhibition was calculated according to the formula: $\% \text{ inhibition} = 100 - \frac{Abs_{570\text{ nm (sample)}} - Abs_{600\text{ nm (sample)}}}{Abs_{570\text{ nm (control)}} - Abs_{600\text{ nm (control)}}} \times 100$

1.2. In vitro antioxidant activity assay

The antioxidant activity by the ABTS•+ method was assessed according to the method described by Re et al. [9] with modifications. ABTS•+ assay is an excellent tool for determining the antioxidant activity of hydrogen-donating antioxidants and of chain-breaking antioxidants. The ABTS•+ radical was formed through the reaction of ABTS (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)) solution 7 mM with potassium persulfate (K2S2O8) solution 140 mM, incubated at 25°C in the dark for 12-20 hours at room temperature. The resulting solution was further diluted by mixing with acetate buffered saline (0.02 M, pH 6.5) to obtain an absorbance of 0.70 ± 0.01 units at 734 nm.

The dilutions of the tested compounds were prepared in DMSO at concentrations ranging

from 1 to 100 μM. After that, 20 μL of each tested compound dilution were transferred in a 96-wells microtiter plate and 180 μL of working solution of ABTS•+ were dispensed with dispense module of hybrid reader (BioTek). The decrease in absorbance at 734 nm was measured exactly after 30 min of incubation at 25°C. DMSO was used as negative control. Blank samples were run by solvent without ABTS•+. All tests were performed in triplicate and the obtained results were averaged. The percent of inhibition (I %) of free radical cation production of ABTS•+ was calculated by using the following equation:

$$I\,(\%) = \frac{Abs_{734\ nm_0} - Abs_{734\ nm_1}}{Abs_{734\ nm_0}} \times 100, \text{ where}$$

Abs_{734nm0} is the absorbance of the control solution; $Abs_{734\ nm1}$ is the absorbance in the presence of sample solutions or standards for positive controls.

1.3. In vivo acute toxicity assay

The general toxicity of the new tested compounds was evaluated using *Subphylum: Crustacea Order: Cladocera. Species: Daphnia magna* (Straus, 1820). The *Daphnia magna* originated from a culture maintained parthenogenetically at the Institute of Zoology, Center of Research of Biological Invasions, Laboratory of Systematics and Molecular Phylogeny. The test design was based on GOST R 56236-2014 (ISO 6341: 2012). This International Standard specifies a procedure for the determination of the acute toxicity of chemical substances to the water flea *Daphnia magna* (Straus, 1820). This method is applicable to chemical substances, which are soluble under the conditions of the test, or can be maintained as a stable suspension or dispersion under the conditions of the test. The test specified in this International Standard involves the determination of the immobilization of the *Daphnia magna* after 24 h and 48 h exposure to the test sample under the conditions specified in this International Standard. The Daphnia magna acute mobility inhibition assay was performed using juvenile individuals of *Daphnia magna* aged up to 24 h, originating from ephippia.

The test-organisms *Daphnia magna* were fed with *Chlorella vulgaris* (Beijerinck 1890). These unicellular algae were grown using aseptic technology to exclude contamination of the culture by bacteria, algae or protozoa. The Chlorella vulgaris were cultivated in Prat's growth medium containing KNO_3 (1 Mm), $MgSO_4.7H_2O$ (40 μM), $K_2HPO_4.3H_2O$ (400 μM), $FeCl_3.6H_2O$ (3.6 μM) in H_2O distilled (adjusted the pH to 7.0, autoclaved and stored at 5°C).

The *Daphnia magna* were maintained in aerated aqueous straw infusion growth media supplemented with $CaCl_2$ (11.76 g/l), $NaHCO_3$ (2.59 g/L), KCl (0.23 g/L), $MgSO_4.7H_2O$ (4.93 g/L). (pH~7.5±0.2; O2 ≥6.0 mg/L)

Juveniles were selected according to their size and kept in fresh medium for 24 h. The *Daphnia magna* were cultured in Costar® 24-well culture clear sterile multiple well plates covered by a lid to prevent the possibility of contamination and evaporation but at the same time to allow gaseous exchange between air and culture medium. Each well contained 10 daphnids in 1000 μL final volume of each dilution of the tested compounds.

The bioassay was then repeated at the concentrations ranging from 0.1 to 100 Mm (0.1, 1, 10, and 100 μM) in order to determine LC50 for each compound, including the positive

control. The final test solutions contained up to 0.1% DMSO and had a final volume of 1 mL. A 0.1% solution of DMSO in aerated medium (pH~7.5±0.2; O2 ≥6.0 mg/L) was used as a negative control and compounds were used as positive controls. Throughout the experiment, the juvenile daphnids were incubated at 22±20C, using a 16 h /8 h light/dark cycle (500–1000 lx). The mobility (viability) of the test organisms was observed after the 24 h and 48 h exposure. The experiment was performed in triplicate. The daphnids were considered immobilized only if they did not swim during the 15 s which follow gentle agitation of the test and control solutions, even if they could still move their antennae. The percentage of viability (V (%)) of *Daphnia magna* was calculated according to the formula: $V(\%) = \frac{N_{(sample)}}{N_{(control)}} \times 100$, where N - Number of viability of *Daphnia magna.*

1.3.Statistical analysis

The cell proliferation assay results were reported as the percent inhibition of the test and control substances. As an indicator of efficiency of the experimental compounds on proliferation of cell lines, the half maximal inhibitory concentration was used. According to FDA documents, IC50 is an indicator of the concentration of medicinal substance required for 50% inhibition of the tested reaction in vitro. The toxicity activity of compounds was evaluated as the median lethal concentration values (LC50) were calculated from the dose-response equation determined by the least squares fit method, using the GraphPad Prism software. All data are presented as means ± standard deviation (SD).

Results and discussion

The 2- (2- Hydroxybenzylidene) -N- (prop -2 - en1 -yl) hydrazinecarbothioamide (**H_2L**), bis [μ2-2- ({2-[(prop-2-en-1-yl) carbamothioyl]-hydrazinylidene }methyl)phenolato-S,N,O:O]-diaquadicopper (II) nitrate (**1**), bis[μ2-2-({2-[(prop-2-en-1-yl)carbamothioyl] hydrazinylidene}-methyl)phenolato-S,N,O:O]diimidazoldicopper (II) Nnitrate (**2**), bis [μ2-2-({2-[(prop-2-en-1-yl)carbamothioyl]hydrazinylidene}methyl) phenolato-S,N,O:O]bis-(3,5-dibromopyridine)-dicopper(II)Nnitrate hexahydrate (**3**), bis [μ2-2-({2-[(prop-2-en-1-yl)carbamothioyl]-hydrazinylidene}methyl)phenolato-S,N,O:O] bis-(4 -methylpyridine)dicopper(II) nitrate hexahydrate (**4**) were synthesized in Research Laboratory of Advanced Materials in Biopharmaceutics and Technics of the Moldova State University by acad. A. Gulea et al.

The thiosemicarbazone **H_2L** and complexes **1-4** were synthesized as described in the literature [1]. The thiosemicarbazone **H_2L** was characterized by NMR (1H and 13C) spectroscopy. The complexes **1-4** were characterized by electronic, FT-IR and EPR spectroscopy, molar conductivity, magnetic susceptibility measurements and elemental analysis. Also, the crystal structure of complexes were determined by single-crystal X-ray diffraction analysis. Melting points, IR, and NMR spectra of the tested compounds correspond to the literature data [1].

The antiproliferative activity experiments were displayed in a dose-dependent manner and showed concentration dependence between the inhibitory effects of the tested compounds at the micromolar concentration range. It is known from the literature that, as a rule, anticancer

chemotherapeutic drugs have a high cytotoxic effect on normal cells, which leads to serious side effects that can be fatal. On this basis, we have exploited normal kidney epithelial cells of line MDCK for selective cytotoxicity evaluation.

Table 1 shows concentrations IC50 of half-maximal inhibition, which represent a measure of efficiency of antiproliferative action of the investigated substances in relation to human muscle rhabdomyosarcoma spindle and large multinucleated cells of line RD and to model line of normal mammalian cells MDCK, and also indices of selectivity of anticancer activity, indicating how many times the activity against cancer cells is higher than in normal cells MDCK.

Study of the antiproliferative activity of the **H_2L** ligand and compounds 1-4 against the cancer cells RD showed that the **H_2L** ligand shows almost no activity, suppressing the growth and reproduction of cancer cells by 14.0±1.2 % at a concentration of 10 µM. The tested complexes 1-4 and the FDA-approved anticancer drug doxorubicin (DOXO) possess antiproliferative activity on RD cells with IC50 values of 0.6±0.1, 0.8±0.3, 1.03±0.03, 1.3±0.2 and 16.2±0.3 µM, respectively. Thus, the inhibitory activity of the tested complexes on RD cells proliferation manifest higher than the corresponding values of the DOXO.

The tested compounds **H_2L, 1-4** and DOXO inhibit the formation and growth of MDCK cells, with IC50 values of ≥10, 6.0±0.4, 12.0±0.6, 9.0±0.9, 3.5±0.5 and 7.1±0.5 µM, respectively.

The selectivity index SI of complexes **1-4** and DOXO are 10, 15, 9, 3 and 0.4, respectively. Thus, the complexes **1-4** are 12.5–27 times more active than doxorubicin against RD cells. In addition, complexes **1-4** are superior to doxorubicin in selectivity for this cancer cell line.

In order to exclude the eventual presence of concomitant adverse effects associated with oxidative stress, the tested compounds were tested by ABTS•+ assay. The antioxidant potency of the tested compounds was compared to the DOXO and the reference antioxidant control trolox. It is known, that doxorubicin-induced cardiomyopathy carries a poor prognosis and is frequently fatal. Doxorubicin induces toxic damage to the mitochondria of cardiomyocytes contributing to increased oxidative stress.

Table 1. Antproliferative activity of the tested compounds H2L, 1-4 and the positive control DOXO on cells of lines RD, MDCK and selectivity activity

Compound	**MDCK**	**RD**	
	IC_{50} (µM)	**IC_{50} (µM)**	**SI**
DOXO	7.1±0.5	16.2±0.3	0.4
H_2L	≥10	≥10	-
1	6.0±0.4	0.6±0.1	10
2	12.0±0.6	0.8±0.3	15
3	9.0±0.9	1.03±0.03	9
4	3.5±0.5	1.3±0.2	3

The results of studying the antioxidant activity of trolox, DOXO, **H_2L** ligand and complexes **1–4** against ABTS•+ cation radicals are presented in figure 1 in the form of semi-maximal inhibition concentrations IC50. The uncoordinated thiosemicarbazone H_2L shows higher activity than trolox. Coordination of **H_2L** to the copper(II) ion (complex 1) leads to a decrease in antioxidant activity, whereas the introduction of amines into the inner sphere (complexes **2–4**) re-increases antioxidant activity, and the activity of complexes with amines exceeds not only that of complex 1, but also the activity of the initial thiosemicarbazone **H_2L**. Analyzing the results of ABTS•+ method, it was observed that the tested compounds **2–4** showed the best antioxidant activity compared with trolox and DOXO.

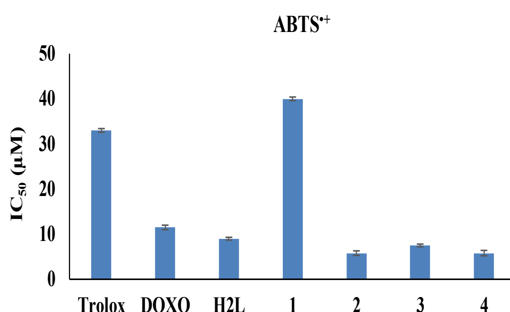

Figure 1. The influence of the tested compounds and reference controls for ABTS•+

Toxicity studies are an important stage in the development of drugs, being a prerequisite before starting their use in preclinical and clinical trials. Since the fundamental principle of toxicity studies is the protection of animals, including those participating in studies, it is currently recommended that in all possible cases, studies should be conducted on in vivo invertebrate organisms, avoiding the inclusion of laboratory animals in studies. Daphnia magna is a cladoceran organism used frequently along with Artemia salina (Linnaeus, 1758) in the cytotoxicity and biological activity evaluation of synthesis compounds [2].

The toxicity of the tested compounds was evaluated using the *Daphnia magna* bioassay in the Institute of Zoology, Center of Research of Biological Invasions by acad. I. Toderas et al. The results of *Daphnia magna* bioassay are given in table 2. The tested compounds have manifested the general toxicity against *Daphnia magna* after 24h and 48h of exposure, according to the sequence: **3 ≥ 1 ≥ 4 ≥ 2 and 1 ≥ 3 ≥ 2 ≥ 4**, respectively. The complexes 1–4 are less toxic for Daphnia magna than for cells of lines RD, HeLa [1] and MDCK. The tested copper complexes showed promising antiproliferative activity and low toxicity on *Daphnia magna*.

Table 2. Toxicity on Daphnia magna of complexes 1–4.

Compound	**Incubation Period**	
	24 h	**48 h**
	LC_{50} (μM)	**LC_{50}** (μM)
1	12.8±4.1	8.9±1.3
2	3.3±0.1	3.5±2.7
3	14.0±3.2	4.4±0.1
4	7.9±4.2	3.1±0.1

The above experimental data indicate the prospects for further search for selective anticancer substances with antioxidant activity and low toxicity among copper mixed-ligand amino-containing coordination compounds.

Conclusions

In conclusion, our results may be useful in designing novel antiproliferative agents.The ligand **H_2L** and the complexes **1–4** have been screened for their in vitro antiproliferative, antioxidant activities and toxicity. Inhibitors of cancer cell proliferation complexes **1–4** characterized by high selective activity, low toxicity and higher efficiency compared to DOXO have been identified, which opens up prospect of their employment as anticancer agents.The tested compounds **H_2L** and **1–4** have manifested higher antioxidant activity against ABTS•+ radical compared to the reference compounds DOXO and trolox. The synthesized complexes **1–4** are less toxic for Daphnia magna than for cells of lines RD and MDCK. Thus, the copper complexes **1–4** showed promising antiproliferative activity against cancer cells and low toxicity on Daphnia magna.

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