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NEW INORGANIC AND ORGANIC MOLECULAR INHIBITORS OF CANCER CELLS PROLIFERATION THE MECHANISMS OF ACTION

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CONCEPTUAL MILESTONES OF RESEARCH

Relevance and importance of the problem. According to a recent report by the World Health Organization, there are now more than 10 million cases of cancer per year worldwide. Cancer refers to a diversity of diseases, characterized by the uncontrolled proliferation of cells. The continuous proliferation of cancer cells develops into tumor tissues and may spread across to other organs. The principal need in the chemoprevention of cancer remains the discovery of new agents that are effective and safe.

It is known that a wide variety of genes are involved in the development of tumors and many cell processes are deregulated, including mechanisms for controlling cell proliferation, DNA repair, chromosome stability, cell-cell interactions, cell-matrix interactions, angiogenesis, cell aging and apoptosis. In this regard, it is necessary to take into account the basic cellular processes for correct prescriptions of anticancer drugs and understanding their mechanism of action.

It is known that many of the antitumor drugs act due to DNA damage and are most active in the S phase, when DNA replication occurs, while taxanes disrupt mitosis, preventing the formation of a spindle. Such drugs act only on dividing cells, therefore tumors with a high growth fraction are most sensitive to chemotherapy.

High systemic toxicity and drug resistance remain a major challenge for modern medicine in the management of cancer despite the significant progress made in the anticancer therapy. Chemotherapy can produce severe side effects caused by its cytotoxic effect on normal cells. This limits their use and it is an indication to reduce the drug dose, interrupt and even cease the treatment. Therefore, it is important that the anticancer drugs exert antiproliferative and cytotoxic activity in tumor cells without affecting normal tissues, so the principal need in the chemoprevention of cancer remains the discovery of new agents that are effective and safe.

So in the present study, we have compared the antiproliferative and antioxidant activities of 2-formilpyridine *N*(4)-phenylthiosemicarbazone (CMT-22), complex copper(II) [Cu(L)Cl] with 2-formilpyridine *N*(4)-phenylthiosemicarbazone ligand (CMT-67), copper(II) mixed-ligand complex chloro(*N*-phenyl-*N*'-[(pyridin-2-yl)methylidene]carbamohydrazonothioato)(4-aminobenzene-1-sulfonamide)copper (CMT-68), as well as (CMJ-23) and copper(II) complex (CMJ-33) with the FDA approved anticancer drugs doxorubicin and cisplatin, using various cancer cell lines as well as normal mortal cells *in vitro*. Further, hemolysis and formation of methemoglobin (MetHb) in human RBSs was also tested *in vitro*. Finally, we have also evaluated the toxicity of the tested compounds on *Paramecium caudatum in vivo*.

The aim of this work is to establish the effect of new molecular organic and inorganic (organometallic) inhibitors on the proliferative activity of human cancer cell lines in comparison with the frequently employed anticancer drugs doxorubicin and *cis*-dichlorodiammineplatinum; identification of the mechanism of the inhibitor action.

The following **objectives** were set for this: detection of antiproliferative activity of the tested substances CMT-22, CMT-67, CMT-68, CMJ-23, CMJ-33 on the cancer cells MeW-164 (human malignant melanoma), HeLa (human cervix adenocarcinoma), BxPC-3 (human primary pancreatic adenocarcinoma), RD (human rhabdomyosarcoma); testing substances against normal kidney epithelial cells of MDCK line in order to detect selective cytotoxicity; identification of the inhibition mechanism in cancer cell proliferation by the test substances; *in vitro* assessment of the development probability and the nature of possible side effects of the tested substances

associated with hemolysis and the formation of methemoglobin in human erythrocytes; determination of the toxicity of the tested substances

Research hypothesis. Based on the literary analysis and molecular modelling, we suggested that the 2-formylpyridine *N*(4)-phenylthiosemicarbazone (*N*-phenyl-2-(pyridin-2-ylmethylidene) hydrazinecarbothioamide, CMT-22, HL), complex copper(II) [Cu(L)Cl] with CMT-22 ligand (chloro(*N*-phenyl-*N*'-[(pyridin-2-yl)methylidene]carbamohydrazonothioato copper, CMT-67), mixed-ligand copper (II) complex [Cu(Str)(L)Cl] with CMT-22 and 4-aminobenzenesulfonamide (Str) ligands (chloro(*N*-phenyl-*N*'-[(pyridin-2-yl)methylidene]carbamohydrazonothioato)(4-aminobenzene-1-sulfonamide)copper, CMT-68), as well as coded organic compound CMJ-23 and copper(II) complex CMJ-33 with ligand CMJ-23 are potent inhibitors of cancer cell proliferation with high selective activity.

Research methodology. *In vitro* antiproliferative activity of the tested compounds was investigated using the flow fluorescence cytometry, cell proliferation MTT and resazurin assays. DNA fragmentation was determined by electrophoresis method. Antioxidant activity was estimated using $ABTS^{\bullet+}$, $DPPH^{\bullet}$, ORAC-Fl and LOX methods. *In vitro* assessment of the development probability and the nature of possible side effects of the tested compounds associated with hemolysis and the formation of methemoglobin in human erythrocytes was investigated using spectrophotometric assays. The screening of direct toxicity of the tested compounds was performed with the aid the NR method for determining toxicity using *Paramecium caudatum*.

Novelty and relevance of the study: methods for studying the biological activity of substances (method for detection of lipoxygenase activity; method for determining the antioxidant capacity; procedure for determining the induction capacity of hemolysis; *in vitro* method of determination of cell viability and cytotoxicity) have been adapted; antiproliferative and antioxidant activities of 5 synthetic compounds (tiosemicarbazones and Cu(lI) coordination compounds with tiosemicarbazones) have been assessed; the investigated substances have been established to not cause the formation of methemoglobin and to not increase the index of hemolysis in human erythrocytes; a method for assessment of direct toxicity using *Paramecium caudatum* has been developed, and the direct toxicity of the substances under study has been estimated; the mechanism of the compound action on the proliferation of cancer cells associated with apoptosis has been elucidated by the following research methods: NMR spectroscopy, X-ray diffraction, electrophoretic separation of DNA fragments, flux fluorescence and microscopy; the tested compounds (2-formylpyridine *N*(4)-phenylthiosemicarbazone (*N*-phenyl-2-(pyridin-2-ylmethylidene)hydrazinecarbothioamide, CMT-22, HL), complex copper(II) [Cu(L)Cl] with CMT-22 ligand (chloro(*N*-phenyl-*N*'-[(pyridin-2-yl)methylidene]carbamohydrazonothioato copper, CMT-67), mixed-ligand copper (II) complex [Cu(Str)(L)Cl] with CMT-22 and 4-aminobenzenesulfonamide (Str) ligands (chloro(*N*-phenyl-*N*'-[(pyridin-2-yl)methylidene]carbamohydrazonothioato)(4-aminobenze-ne-1-sulfonamide)copper, CMT-68), as well as coded organic compound CMJ-23 and copper(II) complex CMJ-33 with ligand CMJ-23) are of interest from the point of view of their employment as less toxic and more effective anticancer agents.

Scientific problem solved in this thesis is the identification of new inhibitors of cancer cells proliferation with high selective activity and lower toxicity compared to FDA-approved reference anticancer compounds (DOXO and CDDP), as well as the elucidation of the

antiproliferative mechanism of action of the tested compounds. The antioxidant action of organic and inorganic molecular inhibitors on radicals ($ABTS^{\bullet+}$, $DPPH^{\bullet}$, $HO_2^{\bullet}$) has been determined. The compounds under study have been found to not cause the formation of methemoglobin and to not increase the index of hemolysis in human erythrocytes.

The theoretical importance and potential application value of the work. New inhibitors of cancer cell proliferation with high selective activity and low toxicity have been identified which made it possible to propose them for preclinical studies. A method for determination of the dependence of toxicity on the concentration of substances using *Paramecium cadatum* has been developed. The use of this method allows to accelerate and reduce the cost of biotesting.

The mechanism of the action of the antiproliferative activity of the tested compounds has been found to associate with apoptosis. NMR spectroscopy and X-ray diffraction analyses have demonstrated that thiosemicarbazones interact with the DNA fragment (guanine), forming hydrogen bonds, which causes DNA fragmentation and finally apoptosis.

The findings are of scientific significance and can be used for special training courses in Biopharmaceutical Chemistry and Biochemistry.

Principal scientific results proposed for support: studies on the anticancer activity of synthesized compounds; detection of compounds characterized by high antiproliferative activity in cancer cells and low toxicity in normal cells; identification of the antioxidant activity in the compounds under study; studies on the toxicity of the compounds tested *in vitro* and *in vivo.*

Implementation of scientific results. A method for determination of direct toxicity of substances using *Paramecium caudatum* has been developed and patented. Two molecular inhibitors of cancer cell proliferation and one substance with antioxidant activity have been patented. Modification and adaptation of methods for studying the biological activity of substances have resulted in the implementation of three innovations. The obtained results are of scientific significance and can be used when reading special courses in Biopharmaceutical Chemistry and Biochemistry.

Dissemination and publication of the research findings. The main results of the thesis were presented in the form of 3 communication and 11 posters at national and international scientific conferences: 3rd French-Romanian Colloquium on Medicinal Chemistry, Iaşi, Roumanie, 2014; The XVIII-th International Conference „Physical Methods in Coordination and Supramolecular Chemistry" Chişinău, Republica Moldova, 2015; Conferinţa ştiinţifică anuală a colaboratorilor şi studenţilor, Chişinău, Republica Moldova 2015; International Scientific Conference on Microbial Biotechnology (3rd edition) Chişinău, Republica Moldova, 2016; The 6th International Conference Ecological & Environmental Chemistry, Chisinau, Republic of Moldova, 2017; The 4th French-Romanian Colloquium on Medicinal Chemistry, Iasi, Romania, 2017; Conferința științifică națională cu participare internațională „Integrare prin Cercetare și Inovare", USM, 2017; Simpozionul international, „Ecologia funcțională a animalelor", consacrat aniversării a 70 de ani de la nașterea academicianului Ion Toderaș, Republica Moldova, 2018; International Conference Achievements and perspectives of modern chemistry, Chisinau, Republic of Moldova, 2019.

The structure and scope of the thesis. The thesis consists of introduction, four chapters, general conclusions and recommendations, 204 references, 5 annexes, 129 pages, 41 figures, 8

schemes, 6 tables. The results are published in 28 scientific publications (7 articles, 4 patents, 14 theses at conferences, 3 innovations).

Keywords: anticancer compound, antiproliferative activity, human cancer cell line, selective activity, antioxidant activity, hemolysis.

THESIS CONTENT

The Introduction describes the relevance and importance of the discussed issue, identifies main goals and objectives of the thesis, and describes scientific novelty and originality of the results, theoretical and practical significance, dissemination and publication of the research findings, thesis overview, keywords.

1. CANCER AND GENERAL ASPECTS OF CHEMOTHERAPY

In chapter 1, the literature review is carried out. The respective chapter is comprised of six main subchapters that describes epidemiology, etiology, pathogenesis of cancer, as well utilization of chemotherapy in view of the cell cycle in cancer, side effects associated with oxidative stress and resistance. In "Literature review", characterization of the antitumor drugs (naturally-occurring, semi-synthetic, synthetic) and description of the mechanism of actions is presented. The analysis of the situation in this domain helped to find out and identify research problems. The chapter ends with conclusions.

2. CHARACTERISTICS OF THE OBJECTS OF STUDY AND RESEARCH METHODS

The study of this work was implemented over the period 2013-2020 in the Research Laboratory of Advanced Materials in Biopharmaceutics and Technics of the Moldova State University, Research Biochemical Laboratory of the *Nicolae Testemitanu* State University of Medicine and Pharmacy, Institute of Zoology and Medical Research Center of the Polish Academy of Sciences.

This chapter begins with a description of the characteristics of the tested compounds (tiosemicarbazones and Cu(lI) coordination compounds with tiosemicarbazones). The 2-formylpyridine *N*(4)-phenylthiosemicarbazone (*N*-phenyl-2-(pyridin-2-ylmethylidene)hydrazinecarbothioamide, CMT-22, HL), complex copper(II) [Cu(L)Cl] with CMT-22 ligand (chloro(*N*-phenyl-*N*'-[(pyridin-2-yl)methylidene]carbamohydrazonothioato copper, CMT-67), mixed-ligand copper (II) complex [Cu(Str)(L)Cl] with CMT-22 and 4-aminobenzenesulfonamide (Str) ligands (chloro(*N*-phenyl-*N*'-[(pyridin-2-yl)methylidene]carbamohydrazonothioato) (4-aminobenzene-1-sulfonamide)copper, CMT-68), , as well as coded organic compound CMJ-23 and copper(II) complex CMJ-33 with ligand CMJ-23 were synthesized in Research Laboratory of Advanced Materials in Biopharmaceutics and Technics of the Moldova State University by acad. A. Gulea et al. Since compounds CMJ-23 and CMJ-33 are not patented, their structural formula is not disclosed [1-3].

The thiosemicarbazone CMT-22 and copper(II) complex CMT-67 were synthesized as described in the literature [4]. The thiosemicarbazone CMT-22 was characterized by NMR (^{1}H and ^{13}C) spectroscopy. The complex CMT-67 was characterized by electronic, FT-IR and EPR

spectroscopy, molar conductivity, magnetic susceptibility measurements and elemental analysis. Also, the crystal structure of CMT-67 was determined by single-crystal X-ray diffraction analysis. Melting points, IR, and NMR spectra of the tested compounds correspond to the literature data [4]. The copper(II) mixed-ligand complex CMT-68 was synthesized by reaction between 2-formylpyridine*N*(4)-phenylthiosemicarbazone (CMT-22) with $CuCl_2 \cdot 2H_2O$ and 4-aminobenzenesulfonamide (Str) [5, 6].

Biological activities of the tested compounds were compared with the FDA approved reference anticancer compounds such as doxorubicin ((7*S*,9*S*)-7-[(2*R*,4*S*,5*S*,6*S*)-4-amino-5-hydroxy-6-methyloxan-2-yl]oxy-6,9,11-trihydroxy-9-(2-hydroxyacetyl)-4-methoxy-8,10-dihydro-7*H*-tetracene-5,12-dione) [7] cicplatin (*cis*-dichlorodiammineplatinum) and the used in biological or biochemical applications antioxidants such as trolox (6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) and rutin (2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-3-[α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranosyloxy]-4*H*-chromen-4-one).

Antiproliferative activity of the tested compounds against human melanoma cells of line MeW-164 was investigated in the Medical Research Center of the Polish Academy of Sciences using the MTT (3-(4,5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) assay and flow fluorescence cytometry method. The line MeW-164 was derived from melanoma cell line collection established in culture from melanoma metastases, surgically removed from patients in the Warsaw Cancer Center. Investigation of the antiproliferative activity of the synthesized compounds in relation to HeLa (human cervix adenocarcinoma, ATCC CCL-2), BxPC-3 (human primary pancreatic adenocarcinoma, ATCC CRL-1687), RD (human rhabdomyosarcoma, ATCC CCL-136), MDCK (Madin Darby Canine Kidney epithelial normal cells, ATCC CCL-34) was carried out in the research Biochemical Laboratory of the *Nicolae Testemițanu* State University of Medicine and Pharmacy by resazurin assay.

Cells lines 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 ((3Z,3'Z)-3,3'-[(3,3'-dimethylbiphenyl-4,4'-diyl)di(1Z)hydrazin-2-yl-1-ylidene]bis(5-amino-4-oxo-3,4-dihydronaphthalene-2,7-disulfonic acid) Euroclone)

To determine the mechanism of action of the tested compounds associated with a direct effect on the genomic DNA of the cells, the electrophoretic DNA fragmentation method is represented.

In addition, antioxidant activity and free radical-scavenging capacity *in vitro* is described as important assays ($ABTS^{\bullet+}$, $DPPH^{\bullet}$, ORAC-Fluorescein, LOX) to determine antioxidant properties of the tested compounds potentially useful to prevent cancer.

The fourth and fifth sections present toxic red blood cells (RBCs) hemolysis and formation of methemoglobin (metHb) in intact erythrocytes assays *in vitro* to determine the side effects of the tested compounds associated with tissue hypoxia and hemolysis in human blood.

Last in this chapter is subchapter devoted to toxicity NR-assay *in vivo* with *Paramecium caudatum*, as the test-object.

The necessary calculations are extensively described for each method.

3. ANTIPROLIFERATIVE ACTIVITY OF THE TESTED COMPOUNDS

3.1. *In vitro* antiproliferative activity of the tested compounds CMT-22, CMT-67 and CMT-68 on different lines cancer cells

The antiproliferative activity of the tested compounds CMT-22, CMT-67 and CMT-68 on MeW-164 cells was tested, using the MTT assay. The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) dye is used as an index of the integrity of the internal mitochondrial membrane in living cells. The substance is a yellow tetrazolium salt which is used to measure cell activity and viability due to the breakdown caused by the reduction of the ring of the tetrazolium salt MTT by the action of mitochondrial enzyme dehydrogenases [8].

The antiproliferative activity experiments were displayed in a dose-dependent manner and showed concentration dependence between the inhibitory effects of the tested compounds CMT-22, CMT-67 and CMT-68 at the micromolar concentration range.

The tested compounds CMT-22, CMT-67, CMT-68 and the referent control DOXO possess antiproliferative activity on melanoma cells of line MeW-164 with IC_{50} values 2.5±0.1 μM, 1.0±0.1 μM, 1.0±0.1 μM and 7.3±0.3 μM, respectively (Figure 1). Thus, all compounds showed high antiproliferative activity against line MeW-164.

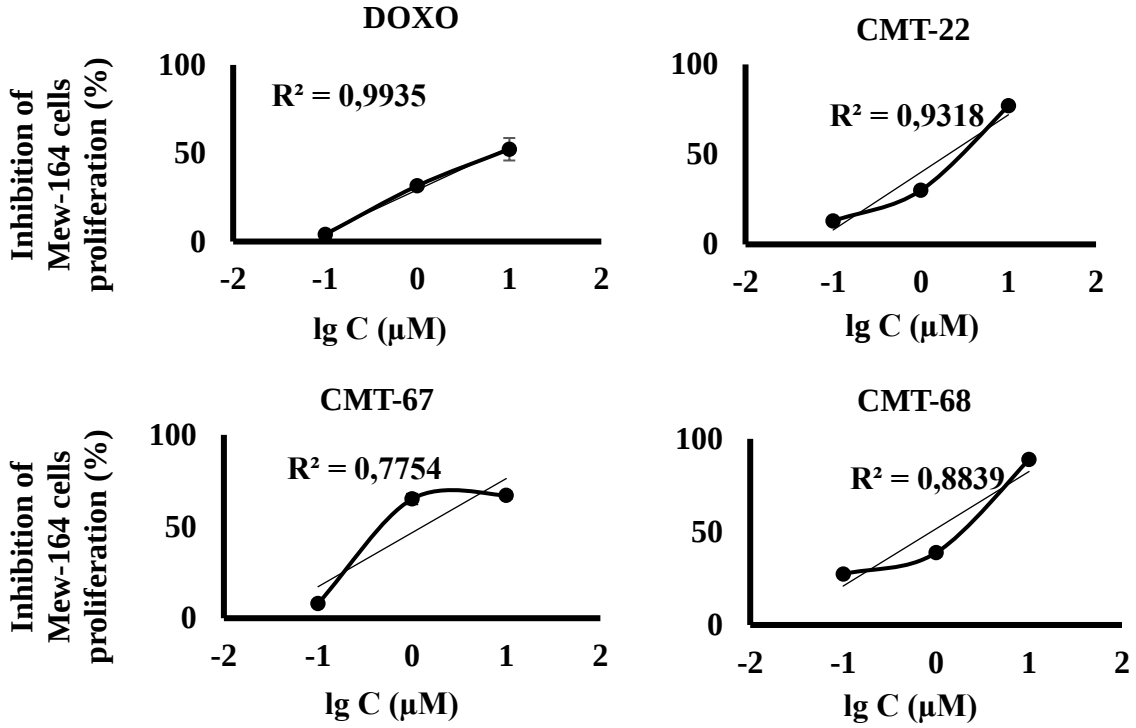


Fig. 1. Antiproliferative activity of the positive control DOXO and the tested compounds CMT-22, CMT-67, CMT-68 on human melanoma cells of line MeW -164. Mew-164 cells were treated with DOXO, CMT-22, CMT-67 and CMT-68 at concentrations 0.1 μM, 1 μM, 10 μM for 24 h. Values are represented as mean ± SD of 3 replicates.

The viability of cells HeLa, BxPC-3, RD and MDCK were determined by the resazurin assay [9,10]. 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 (7-hydroxy-3H-phenoxazin-3-one-10-oxide sodium salt) to resorufin (7-Hydroxy-3H-phenoxazin-3-one).

The comparative study and concentration ranges identification of cytotoxic activity of the tested compounds CMT-22, CMT-67, CMT-68 and the referent controls DOXO, CDDP in regard to human cancer cells of HeLa line are shown in figure 2.

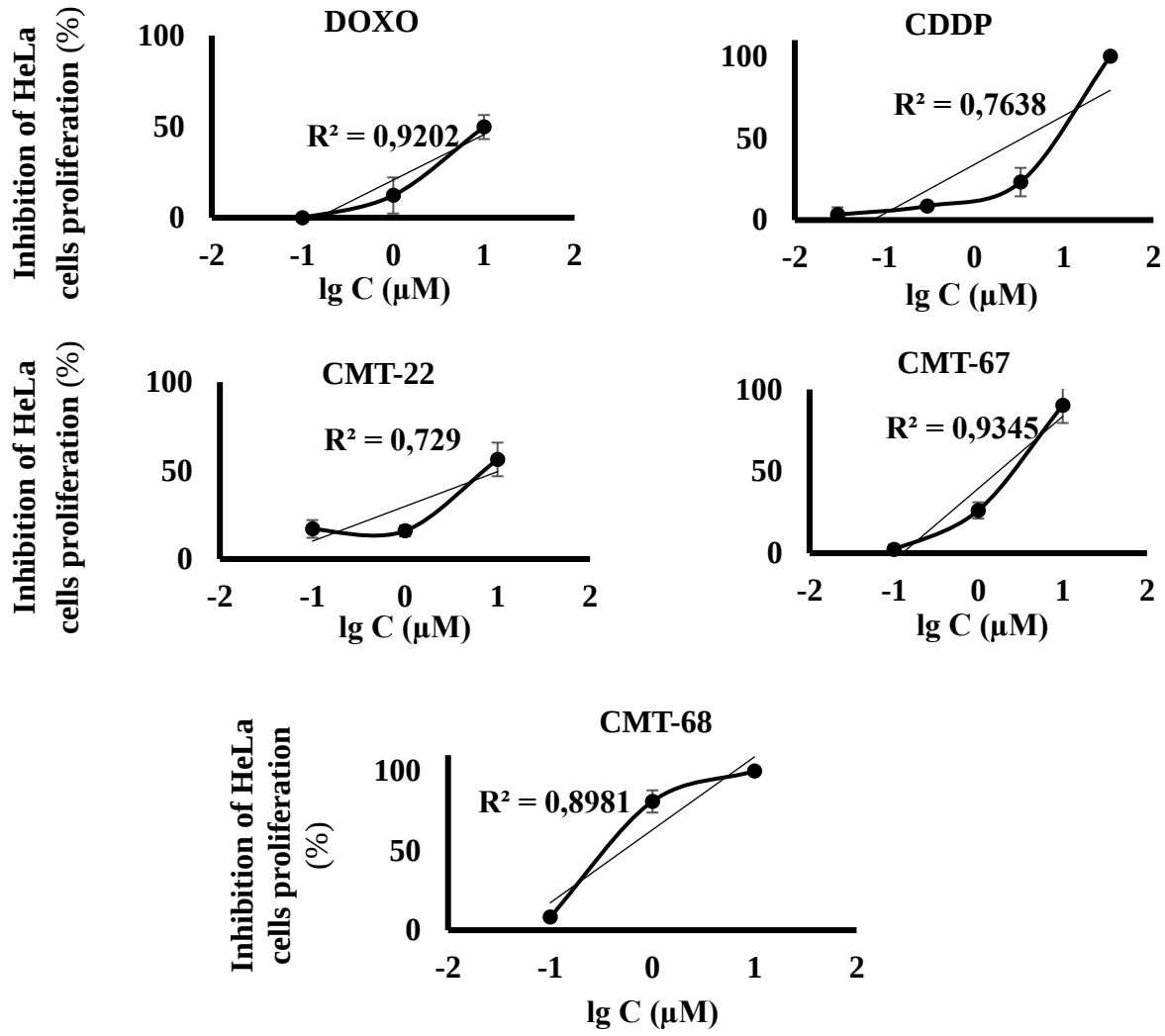


Fig. 2. Antiproliferative activity of the positive controls (DOXO, CDDP) and the tested compounds (CMT-22, CMT-67 CMT-68) on the cervical epithelial cells HeLa. HeLa cells were treated with DOXO, CMT-22, CMT-67 and CMT-68 at 0.1, 1 and 10 μM and CDDP at 0.03, 0.3, 3.3 and 33 μM for 24 h. Values are represented as mean ± SD of 3 replicates.

It was founded, that the tested compounds CMT-22, CMT-67 and CMT-68 exhibited inhibitory activity against cells HeLa, with IC_{50} values of 8.3±0.2 μM, 2.1±0.4 μM, and 0.40±0.04 μM, respectively.

The IC_{50} values of reference drugs DOXO and CDDP were found to be 10.0±0.4 μM and 4.0±0.3 μM, respectively.

Thus, it was established that the tested compounds copper complexes (CMT-67 and CMT-68) exhibit stronger inhibitory activity on HeLa cells proliferation than DOXO and CDDP. The inhibitory activity of CMT-22 is comparable to that of the clinically used anticancer drugs (DOXO and CDDP).

The tested compounds CMT-22, CMT-67 and CMT-68 inhibit the formation and growth of the pancreatic adenocarcinoma cells of line BxPC-3, which demonstrates the capacity of experimental substances to inhibit the process of metastasis.

It was found that the IC_{50} values of adenocarcinoma cells BxPC-3 are ≥10 μM for CMT-22; 0.6±0.1 μM for CMT-67; 1.7±0.2 μM for CMT-68; 5.24±0.03 μM for DOXO and 11.2±1.2 μM for CDDP (Figure 3).

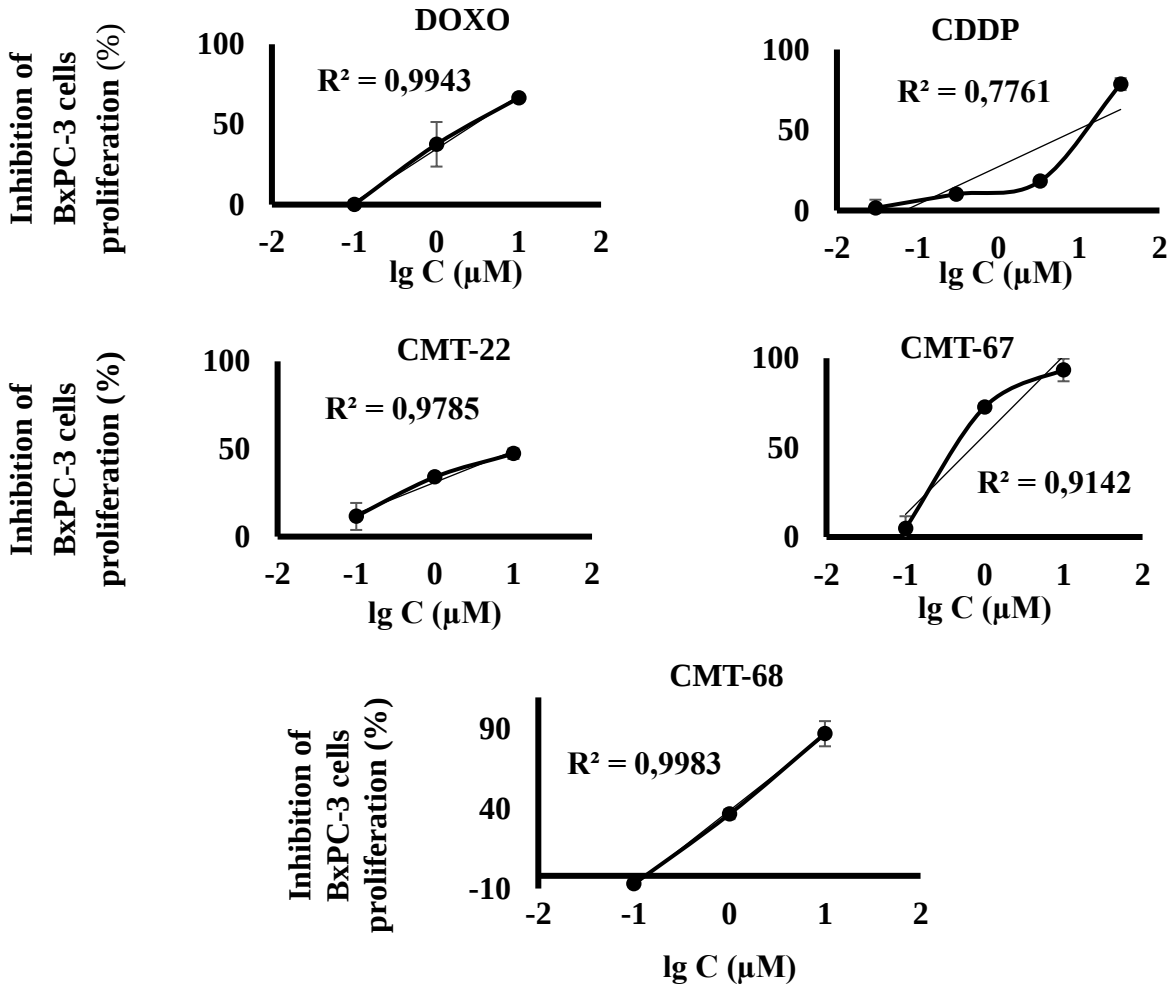


Fig. 3. Antiproliferative activity of the positive controls (DOXO, cisplatin) and the tested compounds CMT-22, CMT-67 and CMT-68 on the pancreatic adenocarcinoma cells of line BxPC-3. BxPC-3 cells were treated with DOXO, CMT-22, CMT-67 and CMT-68 at 0.1, 1 and 10 μM and cisplatin at 0.03, 0.3, 3.3 μM and 33 μM for 24 h. Values are represented as mean ± SD of 3 replicates.

Thus, the experiment showed that thiosemicarbazone CMT-22 inhibits proliferation of BxPC-3 cells only at concentration of 10 μM by 47.3%, but also it was established that the

copper(II) complex CMT-67 and copper(II) mixed-ligand complex CMT-68 exhibit stronger inhibitory activity on BxPC-3 cells proliferation than DOXO and cisplatin.

It was established that the tested compounds CMT-22, CMT-67 and CMT-68 exhibit stronger inhibitory activity on line RD proliferation than DOXO. Thus, the IC_{50} values of RD are 1.1±0.1 μM for CMT-22; 0.27±0.02 μM for CMT-67; 1.3±0.3 μM for CMT-68 and 2.3±0.9 μM for DOXO (Figure 4).

The inhibitory rates of the tested compounds copper complexes (CMT-67 and CMT-68) on cancer cells MeW-164, HeLa, BxPC-3, RD proliferation and the inhibitory rates of tested thiosemicarbazone CMT-22 on the cells MeW-164, HeLa, RD proliferation manifest higher than the corresponding values of the positive controls (cisplatin and DOXO) [11].

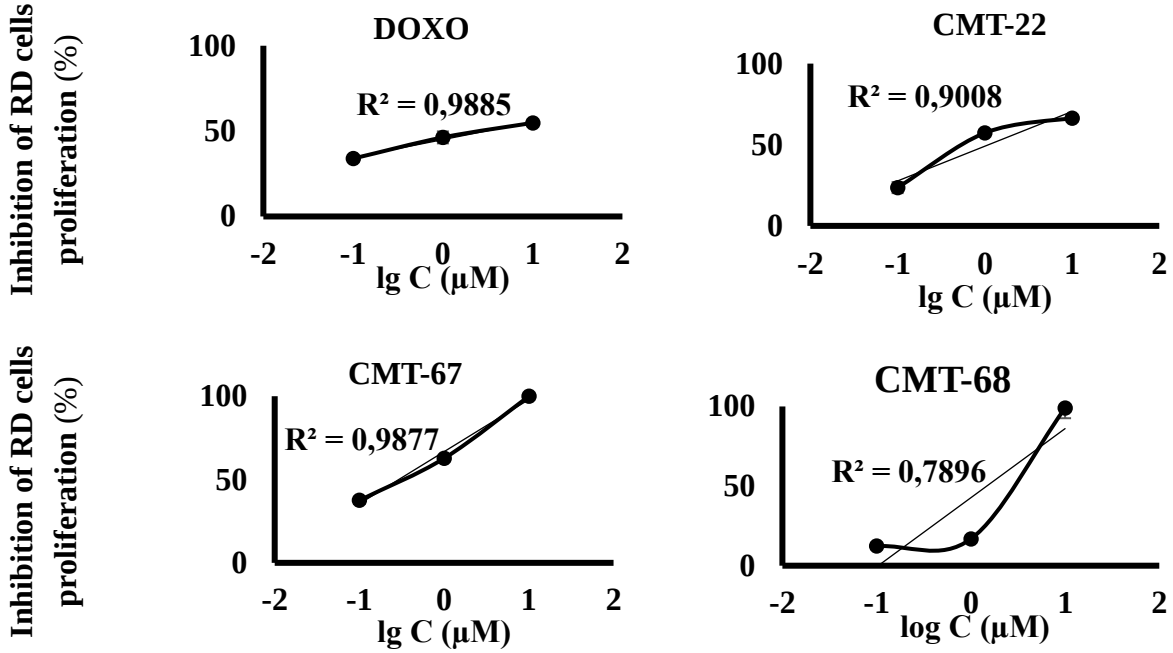


Fig. 4. Antiproliferative activity of the positive controls DOXO, cisplatin and the tested compounds CMT-22, CMT-67, CMT-68 on human muscle rhabdomyosarcoma spindle and large multinucleated cells of line RD. RD cells were treated with DOXO, CMT-22, CMT-67 and CMT-68 at 0.1 μM, 1 μM and 10 μM for 24 h. Values are represented as mean ± SD of 3 replicates.

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 MDCK line for selective cytotoxicity evaluation.

As it can be seen from figure 3.10, the thiosemicarbazone CMT-22 in the concentration range from 0.1 μM to 100 μM does not show inhibitory activity on cell proliferation, but, on the contrary, enhances the proliferative effect, which indicates its non-toxic properties with respect to the MDCK cell line. The proliferation effect of MDCK cells was manifested at low concentrations of the thiosemicarbazone CMT-22, possibly due to the degree of dissociation, which is enhanced at lower concentrations. An increase in the concentration of the compound CMT-22 to 100 μM leads to an inhibitory effect with 12.5±1.8%.

The tested complexes CMT-67 and CMT-68 inhibit the formation and growth of MDCK cells, with values IC_{50}=4.0±0.1 μM and 12.0±0.9 μM, respectively. The positive control DOXO demonstrated cytotoxic effect against MDCK cell line, with values IC_{50} =7.0±0.3 μM.

The search for new compounds that exhibit selectivity for cancer cells but not for normal cells is a major challenge in anticancer drug research [12].

Thus, selectivity indices (SI) of the tested compounds were estimated (Table 1). For comparison purposes, the cytotoxicity of DOXO as a reference anticancer drug was also evaluated under the same conditions. The SI of DOXO, CMT-67 and CMT-68 vary in the range of 0.7–3.0, 1.9–14.8, and 7.1–30.0, respectively. Thiosemicarbazone CMT-22 showed selective cytotoxicity to the cancer lines BxPC-3, RD, HeLa and MeW-164, with a selectivity index of ≥10, ≥91, ≥12 and ≥40, respectively [13].

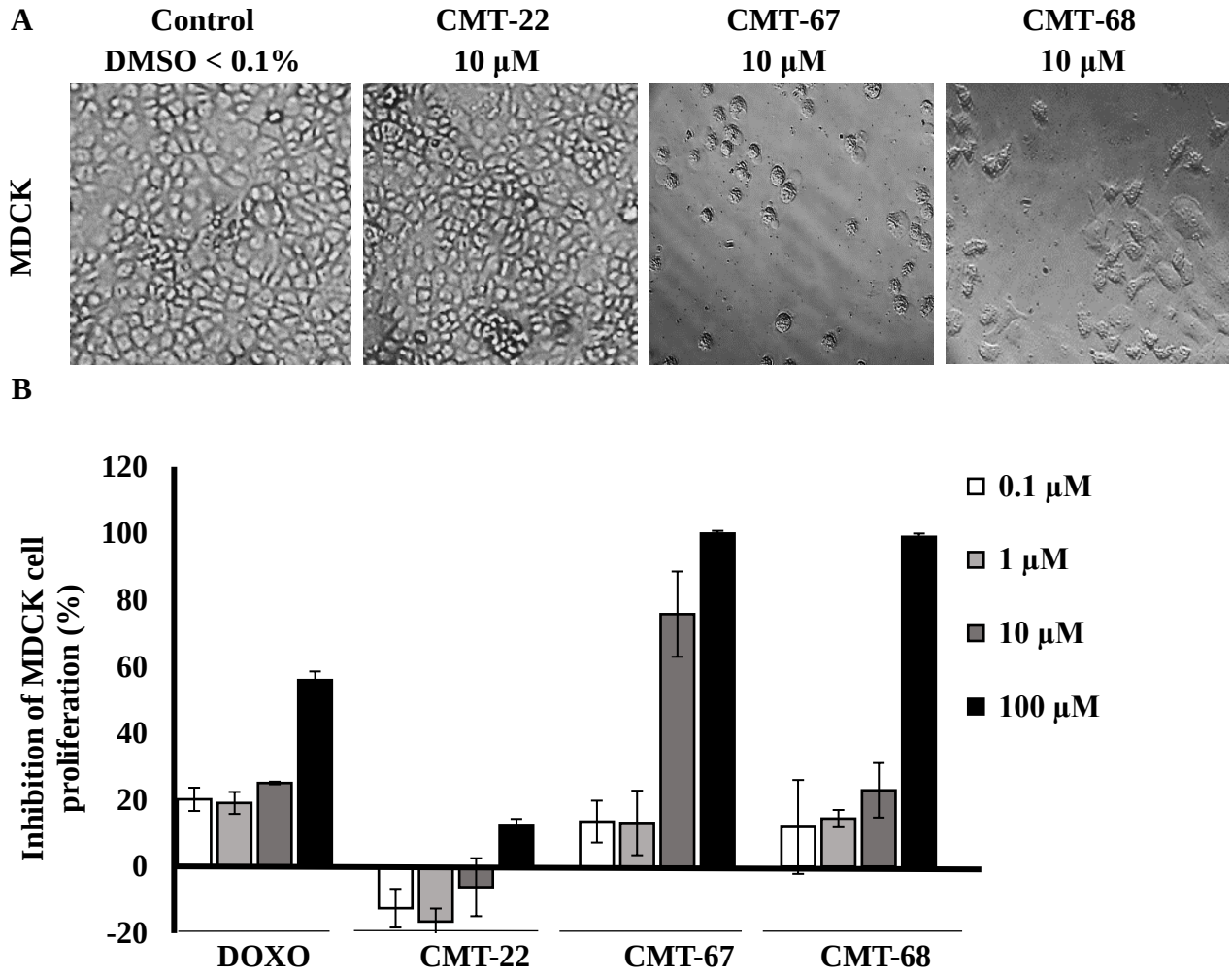


Fig. 5. A: Phase-contrast images of MDCK cells after 24 h treatment with the tested compounds at concentration 10 μM. Untreated MDCK cells served as a control. B: Nephrocitotoxic effect *in vitro* of DOXO, CMT-22, CMT-67, CMT-68 on MDCK cells after 24 h exposure. MDCK cells were treated with CMT-22, CMT-67, CMT-68 and DOXO at concentrations 100 μM, 10 μM, 1 μM and 0.1 μM. Values are represented as mean ± SD of 3 replicates.

Importantly, all tested compounds demonstrate a more selective activity than DOXO in the tested cell lines. It was concomitantly found that CMT-22 possesses the most selective cytotoxicity, what is an important aspect in personalized chemotherapy. Obviously, coordination of thiosemicarbazone CMT-22 to the metal center leads to a marked enhancement of its antiproliferative activity.

It should be mentioned that thiosemicarbazone CMT-22 slightly inhibits the proliferation of cancer cells only at a concentration of 10 μM. Its addition into the complex compounds leads to an increase in anticancer activity. The main influence on the anticancer activity of the complex is exerted by the nature of the central atom. Thus, coordination to the copper(II) ion leads to inhibition of the growth and division of cancer cells at a concentration range of 10–0.4 μM. It is known from the literature, that the introduction of amines into the inner sphere of copper(II) complexes with various azomethines results in an increase in their anticancer activity; copper(II) mixed-ligand complex CMT-68 behaves as described above [3].

Table 1. The selectivity index (SI) of the tested compounds CMT-22, CMT-67, CMT-68 and the positive control DOXO on cancer cell of lines BxPC-3, RD, HeLa, MeW-164 and normal kidney epithelial cells of MDCK line

Compound	SI_1	SI_2	SI_3	SI_4
DOXO	1.3	3.0	0.7	0.95
CMT-22	≥10	≥91	≥12	≥40
CMT-67	6.7	14.8	1.9	4.0
CMT-68	7.1	9.2	30.0	12

Notes: SI valium is selectivity index. The SI_1 of each compound was calculated as the ratio of the IC_{50} for MDCK cells / IC_{50} for cancer cells of line BxPC-3; $SI_2=IC_{50}$ MDCK / IC_{50} RD; $SI_3=IC_{50}$ MDCK / IC_{50} HeLa; $SI_4=IC_{50}$ MDCK / IC_{50} MeW-164. Substances with a SI > 3 are considered to be promising.

3.2. *In vitro* antiproliferative activity of the tested compounds CMJ-23 and CMJ-33 on different lines of cancer cells

The antiproliferative activity of the tested compounds CMJ-23 and CMJ-33 on human melanoma cells of line MeW-164 was determined, using the fluorescent flow cytometry assay by Nucleo Counter [14].

It was found that the IC_{50} values are 0.40±0.02 μM for CMJ-23 and 0.20±0.01 μM for CMJ-33 on the tested cells MeW-164. Thus, both compounds showed high antiproliferative activity against cells MeW-164, but copper(II) complex CMJ-33 is 50% more active than its ligand CMJ-23 (Table 2).

Next, the comparative study and concentration ranges identification of cytotoxic activity of the tested compounds CMJ-23, CMJ-33 and the positive control DOXO, in regard to human epithelioid cervix carcinoma cells of HeLa line, human epithelial pancreatic adenocarcinoma cells of BxPC-3 line and human muscle rhabdomyosarcoma cells of RD line are shown in table 2. The viability of cancer cells HeLa, BxPC-3, sarcoma cells RD and and normal epithelial cells of MDCK line was assessed by the redox indicator of resazurin, which allowed us to measure the number of viable cells.

The tested compound CMJ-23 cytotoxicity against cancer cells BxPC-3 and sarcoma cells RD yielded respective IC_{50} values of 2.5±0.7 μM and 0.30±0.04 μM. However, the compound CMJ-23 was significantly less toxic giving IC_{50} value of 18.7±1.0 μM against HeLa cells. In contrast, the tested thiosemicarbazone CMJ-33 demonstrated potent toxicity to the cell lines BxPC-3, RD, HeLa with IC_{50} values of 0.10±0.04, 0.20±0.03, 0.40±0.02 μM, respectively. The reference control DOXO exhibited cytotoxic activity against cell lines BxPC-3, RD and HeLa, with IC_{50} values of 6.0±0.8 μM, 2.3±0.9 μM and 6.2±1.0 μM, respectively.

Thus, copper(II) complex CMJ-33 exhibits stronger inhibitory activity on cancer cells proliferation than the reference control DOXO. However, the antiproliferative activity of CMJ-23 is comparable to that of the DOXO. Obviously, coordination of organic molecules to the metal center leads to a marked enhancement of their biological activity.

Table 2. Antiproliferative activity of the positive control DOXO and the tested compounds CMJ-23, CMJ-33 on the cancer lines BxPC-3, RD, HeLa and MeW -164

	IC_{50} (μM)			
Compound	**BxPC-3**	**RD**	**HeLa**	**MeW -164**
DOXO	6.0±0.8	2.3±0.9	6.2±1.0	7.3±0.3
CMJ-23	2.5±0.7	0.30±0.04	18.7±1.0	0.40±0.02
CMJ-33	0.10±0.04	0.20±0.03	0.40±0.02	0.20±0.01

As the main drawback of compounds with anticancer properties is their toxicity, thus for the tested compounds it is necessary to determine the selectivity of cytotoxic action. For this purpose, the cytotoxic effect of these substances was studied on cells MDCK. The comparative study and concentration ranges identification of proliferative activity of the tested compounds CMJ-23, CMJ-33 and the reference control DOXO in regard to normal epithelial cells MDCK are shown in figure 6.

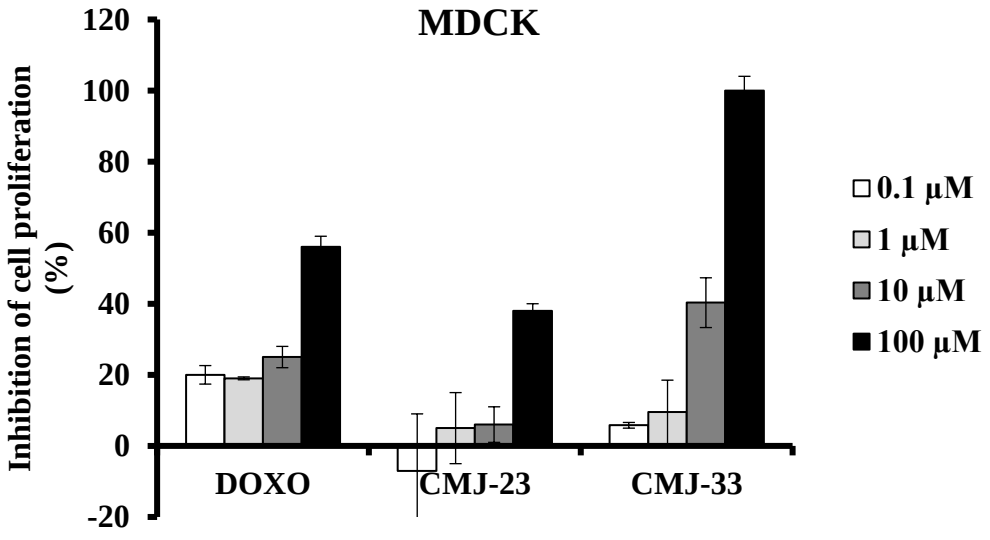


Fig. 6. Antiproliferative activity of the positive control DOXO and the tested compounds CMJ-23, CMJ-33 on normal kidney epithelial cells of MDCK line. Values presented were mean± SD of 3 replicates.

It was established that the tested compounds CMJ-23 and CMJ-33 showed low cytotoxic activity against normal kidney epithelial cells of MDCK line, with IC_{50}≥100 μM and IC_{50}=11.0±1.0 μM, respectively. The positive control DOXO exhibited cytotoxicity against normal kidney epithelial cells of MDCK line with IC_{50} of 7.0±0.3 μM.

It was concomitantly found that the cytotoxic activity of the tested compounds CMJ-23 and CMJ-33 on normal cells line MDCK is significantly lower than that exerted on the cancer cells, and lower than that exerted by DOXO is shown in table 3.

Table 3. The selectivity index (SI) of the tested compounds CMJ-23, CMJ-33 and the positive control DOXO on cancer cell of lines BxPC-3, RD, HeLa, MeW-164 and normal kidney epithelial cells of MDCK line

Compound	SI_1	SI_2	SI_3	SI_4
DOXO	1.2	3.04	1.13	0,96
CMJ-23	≥40	≥330	≥5.3	≥250
CMJ-33	110	55	28	55

Notes: SI valium is selectivity index. The SI_1 of each compound was calculated as the ratio of the IC_{50} for MDCK cells / IC_{50} for cancer cells of line BxPC-3; $SI_2=IC_{50}$ MDCK / IC_{50} RD; $SI_3=IC_{50}$ MDCK / IC_{50} HeLa; $SI_4=IC_{50}$ MDCK / IC_{50} MeW-164.

The selectivity index (SI) that is the ratio between the IC_{50} value for the normal cells (MDCK line) and IC50 values for the cancer cells varies in the range of 0.96–3.04 for DOXO and 28-110 for CMJ-33. Thiosemicarbazone CMJ-23 showed selective cytotoxicity to the cancer lines BxPC-3, RD, HeLa and MeW-164 with a selectivity index of ≥40, ≥330, ≥5.3 and ≥250 respectively. Importantly, all tested compounds demonstrate more selective activity than DOXO in all tested cell lines. An additional experiment aiming at the evaluation of the nephrocytotoxic effect on normal kidney epithelial cells line MDCK revealed that compound CMJ-23 is significantly less toxic than compound CMJ-33, what is an important aspect in personalized chemotherapy.

3.3. The ability of the tested compounds to induce DNA fragmentation *in vitro*

Currently, the used anticancer drugs have been shown to induce apoptosis in susceptible cells. Apoptosis is an important process of many pathological conditions. The principle of apoptosis was described by Vogt which shows it as a programmed death of cells, which may even occur in multicellular organisms. Various biochemical changes such as loss of cell membrane asymmetry and attachment, cell shrinkage, nuclear fragmentation, chromatin condensation, and chromosomal DNA fragmentation take place during apoptosis. DNA fragmentation occurs at an end stage of apoptosis, which includes activation of calcium and magnesium dependent nucleases that degrade genomic DNA.

Human cells of line HEp-2 (ATCC CCL-23) were treated with CMT-22, CMT-67, CMT-68, CMJ-23 and CMJ-33 at 5 μM for 24 h. Cells of this line contain HeLa marker chromosomes, and were derived via HeLa contamination. This line was originally thought to be derived from an epidermoid carcinoma of the larynx, but was subsequently found, based on isoenzyme analysis, HeLa marker chromosomes, and DNA fingerprinting, to have been established via HeLa cell contamination. The cells are positive for keratin by immunoperoxidase staining.

The tested compounds CMJ-23 and CMJ-33 have demonstrated promising ability toward cleavage of genomic DNA. Thus, they have shown enhanced antiproliferative activity associated with increased induction of apoptosis by breaking the structures of the genomic DNA in the cell nucleus. It is very important, because cellular death is the underlying pharmacological purpose for chemotherapy. Disruption of the apoptotic pathways is the hallmark of cancer, being a major obstacle in chemotherapy. [7].

A magnetochemical study of the tested substances showed that they have a magnetic moment characteristic of one unpaired electron, which indicates their monomeric structure. It is known from the literature that compounds with a monomeric structure are able to intercalate between the nitrogenous bases of nucleic acids, which causes apoptosis in cells [7].

Probably, the ability to induce DNA fragmentation of the mixed-ligand complex CMT-68 at concentration 5 μM has resulted from its inner ligand environment properties, so the presence of an additional amino group 4-aminobenzenesulfonamide in the internal sphere of the copper(II) mixed-ligand complex, characterized by the presence of a lone electron pair on the nitrogen atom, makes it easier to interact with the atoms of the grooves of DNA molecules at the moment of replication or transcription.

Possibly, a much higher antiproliferative activity of copper coordination compounds can be caused by coordination of CMT-22 with the copper central atom, which leads to a change of electron density in the thiosemicarbazone moiety. So, the copper atom in these coordination compounds is able to coordinate DNA molecules.

Electron density distribution in the 4-allylthiosemicarbazone 2-formylpyridine molecule, which also manifests high antiproliferative activity against human promyelocytic cell line HL-60, has been studied based on the X-ray diffraction data provided by the Research Laboratory of Advanced Materials in Biopharmaceutics and Technics of the Moldova State University in collaboration with the Institute of Applied Physics.

The presence of high electron density in the nitrogen atom of the pyridine ring has been found to allow this molecule to form hydrogen bonds with a DNA molecule.

In addition, NMR spectroscopic studies have allowed the values of the energies of hydrogen bonds to be calculated, arising from the interaction of thiosemicarbazone CMT-22 with the guanine fragment of the DNA molecule in the range of 8 - 13 kJ/mol.

Thus, the interaction of the molecular inhibitors of cancer cell proliferation with a DNA molecule has been found to occur due to the formation of hydrogen bonds, which triggers the process of cell death by apoptosis.

4. STUDIES OF THE MECHANISM OF ACTION AND SIDE EFFECTS OF NEW ORGANIC AND ORGANOMETALLIC CANCER CELL INHIBITORS ASSOCIATED WITH OXIDATIVE STRESS

Free radicals have been implicated in the causation of several oxidative damage diseases such as liver cirrhosis, atherosclerosis, cancer, diabetes, ageing. An antioxidant can be defined as any substance that when present at low concentrations compared with those of an oxidizable substrate can inhibit the oxidation of lipids, proteins or other molecules by preventing the initiation or propagation of oxidative chain reactions and can thus prevent or repair the damage done to the body’s cells by oxygen.

Thiosemicarbazones and its metal complexes antioxidants have gained attention recently for their capacity to protect organisms and cells from damage induced by oxidative stress or scavenge free radicals [15]. These compounds, which show considerable biological activity, may represent an interesting approach for designing new anticancer drugs [16].

In order to exclude the eventual presence of concomitant adverse effects associated with oxidative stress, the tested compounds: thiosemicarbazone CMT-22, copper(II) complex CMT-67, copper(II) mixed-ligand complex CMT-68 and copper(II) complex CMJ-33 with its ligand CMJ-23 were tested by several antioxidant-capacity (AC) assays, such as $ABTS^{\bullet+}$, $DPPH^{\bullet}$, ORAC and LOX [17]. The antioxidant potency of the tested compounds was compared to the FDA-approved anticancer drug doxorubicin (DOXO) and the reference antioxidant controls trolox and rutin. 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.

Drug-induced hemolysis and methemoglobin formation is a relatively rare but serious toxicity liability caused by oxidative stress, so CMT-22, CMT-67, CMT-68, CMJ-33 and CMJ-23 were performed to screen for toxic hemolysis and methemoglobin formation in human red blood cells (RBCs).

Finally, direct toxic evaluation of CMT-22, CMT-67, CMT-68, CMJ-33 and CMJ-23 and anticancer drug doxorubicin was studied by NR-colorimetric assay of the quantification of the membrane permeability and lysosomal activity of *Paramecium caudatum*, which is one of the most commonly used test-objects in laboratory research aimed at directly determining the toxicity of chemical compounds, which are used in toxicological medicine [18].

4.1. $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ radical scavenging activity of the tested compounds

The $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ assays are widely used methods for the assessment of the total antioxidant capacities of the anticancer compounds *in vitro.*

According to this work, the tested compounds were capable of scavenging $ABTS^{\bullet+}$ radical in a concentration dependent manner. The coordination of metal ions to thiosemicarbazone CMT-22 resulted in a wider spectrum of activity comparable to those of the used reference antioxidant compounds (trolox, rutin) and reference anticancer compound DOXO. The tested compounds exhibited better scavenging activity than the reference antioxidant compounds rutin and trolox at the lowest concentration 1 μM. The IC_{50} values of the tested compounds are listed in table 4, with CMT-68 possessing the highest antioxidant potency (IC_{50} = 0.67±0.01 μM), followed by CMT-67, CMJ-23, CMJ-33, CMT-22 with IC_{50} values of 4.9±0.1μM, 6.20±0.01μM, 11.4±0.4μM, 14.9±1.4μM, respectively.

It was found that the reference compounds trolox, rutin, and DOXO exhibited antioxidant activity, with IC_{50} values of 26.9±0.7 μM, 20.7±0.1 μM, 11.5±0.6 μM, respectively. The $ABTS^{\bullet+}$ radical cation scavenging ability of the tested compounds and reference compounds can be ranked in the order CMT-68 ≥ CMT-67 ≥ CMJ-23 ≥ CMJ-33 ≥ DOXO ≥ CMT-22 ≥ rutin ≥ trolox. Analyzing the results of $ABTS^{\bullet+}$ method, it was observed that the tested compounds showed the best antioxidant activity compared with trolox and rutin.

The examined and compared changes in the $DPPH^{\bullet}$ free radical scavenging ability of the thiosemicarbazone CMT-22, copper(II) complex CMT-67, copper(II) mixed-ligand complex CMT-68 and copper(II) complex CMJ-33 with its ligand CMJ-23 as well as the anticancer drug

DOXO and the reference antioxidant compounds trolox, rutin on the basis of percent inhibition are represented in figure 4. It was observed that the free ligands CMT-22 and CMJ-23 have higher activity than that of the copper(II) complexes. At the highest concentration 100 μM, the antioxidant activity of the free ligands was found to be 57.1±0.5 % for CMT-22 and 61.7±0.8 % for CMJ-23, but upon complexation they changed in the range of 32.4 %–54.1 %.

Table 4. The influence of the tested compounds and reference controls for $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ free radicals

Compound	$ABTS^{\bullet+}$ radical cation scavenging activity	$DPPH^{\bullet}$ radical scavenging activity
	IC_{50} (μM) ±SD	IC_{50} (μM) ±SD
Trolox	26.9±0.7	48.9±0.8
Rutin	20.7±0.1	64.8±2.1
DOXO	11.5±0.6	≥100
CMT-22	14.9±1.4	72.7±0.5
CMT-67	4.9±0.1	83.1±1.3
CMT-68	0.67±0.01	133.0±2.5
CMJ-23	6.20±0.01	48.3±0.8
CMJ-33	11.4±0.4	139.0±2.5

It was found that the inhibitory effect of the compounds on percentage $DPPH^{\bullet}$ scavenging activity was in a concentration dependent manner. The antioxidant activity of the tested compounds CMT-22, CMT-67, CMT-68, CMJ-33, CMJ-23 can be attributed to the effect of release of hydrogen to reduce the $DPPH^{\bullet}$ radical. This proton release was pronounced in thiosemicarbazone CMT-22, with IC_{50} value of 72.7±0.5 μM, followed by CMT-67 with IC_{50} of 83.1±1.3 μM, CMT-68 with IC_{50} of 133.0±2.5 μM, CMJ-23 with IC_{50} of 48.3±0.8 μM, CMJ-33 with IC_{50} of 139.0±2.5 μM. For the comparative analysis of the tested compounds, the anticancer drug DOXO and the reference antioxidant compounds trolox, rutin were determined with the IC_{50} values of ≥100 μM, 48.9±0.8 μM, and 64.8±2.1μM, respectively. The $DPPH^{\bullet}$ radical scavenging ability of the compounds can thus be ranked in the order CMJ-23 ≥ trolox ≥ rutin ≥ CMT-22 ≥ CMT-67 ≥ CMT-68 ≥ CMJ-33 ≥ DOXO.

The scavenging of the $DPPH^{\bullet}$ radical by the tested compounds was found to be moderate compared to that of $ABTS^{\bullet+}$ radical cation. The enhanced inhibition displayed on the $ABTS^{\bullet+}$ radical cation by the tested samples shows that the compounds are capable of donating electrons to neutralize free radicals, which indicates their potentials as chemotherapeutic agents for radical chains terminator.

4.2. Oxygen Radical Absorption Capacity (Fl) activity of the tested compounds

The oxygen radical absorbance capacity (ORAC) assay measures the radical chain breaking ability of antioxidants by monitoring the inhibition of peroxyl radical induced oxidation. Peroxyl radicals are the predominant free radicals found in lipid oxidation in biological systems under physiological conditions. Hence, ORAC values are considered by some to be of biological relevance as a reference for antioxidant effectiveness. In this assay, the peroxyl radical produced by a generator reacts with a fluorescent probe resulting in the loss of fluorescence, which is recorded with a fluorescence microplate reader. A set of fluorescence

decay curves can be constructed in the absence or presence of antioxidants, and the net integrated area under the decay curves (area gain in the presence of antioxidants compared to that of a blank run without antioxidants) can be calculated as an indicator of the peroxyl radical scavenging capacity of the antioxidants.

The antioxidant property for the tested compounds, anticancer drug DOXO, and the reference antioxidant control trolox was determined by the ratio of the slope (m) of the linear regression curve. Slope (m) values are 3.4 for CMT-22, 3.5 for CMT-67, 3.6 for CMT-68, 3.7 for CMJ-23, 3.6 for CMJ-33, 0.5 for DOXO and 1.2 for trolox. The calculated trolox equivalents (TE) were used for comparative analysis of the antioxidant capacity of the tested compounds and anticancer drug DOXO. Analyzing the ORAC results, it was observed that TE (trolox equivalent) values are 2.8 for CMT-22, 2.9 for CMT-67, 3.0 for CMT-68, 3.1 for CMJ-23, 3.0 for CMJ-33, 0.4 for DOXO [19].

The oxygen antioxidant capacity of the compounds can thus be ranked in the order CMJ-23 ≥ CMJ-23 = CMT-68 ≥ CMT-67 ≥ CMT-22 ≥ DOXO ≥ trolox. Thus, it was found that the tested compounds showed the highest oxygen radical absorbance capacity compared with DOXO.

4.3. Antilipoxygenase activity of the tested compounds

Lipoxygenases (LOX) enzymes are reported to convert the arachidonic, linoleic, and other polyunsaturated fatty acid into biologically active metabolites that are involved in the inflammatory and immune responses. LOX also play a significant role in cancer cell growth, metastasis, invasiveness, cell survival and induction of cancer necrosis factor alpha (TNF-α) (Arfan et al., 2010). Inflammation is favorable in most cases, because it is a kind of body`s defensive response to external stimuli; sometimes, inflammation is also harmful, such as attacks on the body's own tissues. It is likely that inflammation is a unified process of injury and resistance to injury. Inflammation brings extreme pain to patients, showing symptoms of rubor, swelling, fever, pain and dysfunction. In these aspects, the medicinal properties of the tested compounds should be investigated on biological activities to counteract the inflammatory process, being with no side effects and with high economic viability.

In this regard, in this study, the ability of the tested compounds CMT-22, CMT-67, CMT-68, CMJ-33, CMJ-23 as well as the anticancer drug DOXO to inhibit the activity of lipoxygenases was evaluated [20].

The antioxidant quercetin was used as positive control. The tested compounds CMT-22, CMT-67, CMT-68, CMJ-33 and CMJ-23 were able to induce inhibition of soybean LOX in a dose-dependent manner with the IC_{50} values of 0.20±0.02 μM, 0.40±0.05 μM, 0.30±0.06 μM, 0.30±0.02 μM and 0.30±0.01 μM, respectively. In contrast, the IC_{50} values for the assay positive control quercetin and anticancer drug DOXO reached values of 15.6±1.6 μM and 5.6±0.3 μM. Thus, these data demonstrate that the tested compounds are potent inhibitors of LOX activity (Table 5).

Analyzing the antioxidant properties of the tested compounds CMT-22, CMT-67, CMT-68, CMJ-23 and CMJ-33 by LOX assays, we can see that they are potent reductive inhibitors and showed good results, comparing to DOXO and quercetin.

Table 5. The percentage of inhibition of the tested compounds CMT-22, CMT-67, CMT-68, CMJ-33, CMJ-23 and positive control quercetin and anticancer drug DOXO on the inhibition of the lipoxygenase activity.

Compound	% inhibition mean ± SD			IC_{50}
	0.05 µM	0.5 µM	5 µM	
Quercetin	20±1.5	28±2.1	50±0.8	15.6±1.6
DOXO	34.5±0.5	37.0±1.0	40.0±1.0	5.6±0.3
CMT-22	48.5±0.5	52.5±2.5	61.0±1.0	0.20±0.02
CMT-67	44.2±1.5	53.1±0.5	77.6±1.9	0.40±0.05
CMT-68	48.4±0.5	53.1±0.4	58.1±0.9	0.30±0.06
CMJ-33	46±1.0	54±0.4	61±1.2	0.30±0.02
CMJ-23	42.0±0.2	54±0.9	70±0.7	0.30±0.01

Lipoxygenase plays a major role in many inflammatory lung diseases including chronic obstructive pulmonary disease (COPD), asthma and chronic bronchitis. Over-expression of LOX is related with some specific carcinomas including pancreatic, gastric and brain tumor. Therefore, novel potent inhibitors of LOX are required to enable the drug discovery efforts. The tested compound CMT-22 showed an excellent inhibitory potential for LOX. The tested compounds showed a strong potential to be developed as new anti-inflammatory drugs.

4.4. Impacts of the tested compounds on methemoglobin formation

Studies on the mechanisms underlying the biochemical processes of disturbance of the oxygen transport function of the blood is an urgent task of modern biology and medicine.

One of the main links in the chain of metabolic disorders of the blood oxygen transport system is the reaction of the hemoglobin transformation into its inactive form - methemoglobin. In this regard, there is a need to search for methods and means of correcting such lesions that increase the efficiency of redox processes of methemoglobin reduction.

In the blood of a healthy person, the content of methemoglobin does not exceed 3-4%, which is achieved by the equilibrium between the reactions of its formation and the reactions of methemoglobin reduction. If the content of methemoglobin in the blood exceeds 3%, this is methemoglobinemia. Many medicinal substances, especially with prolonged use in large doses, can cause methemoglobinemia. However, in most people metHb in Hb is restored after drug withdrawal under the influence of methemoglobin reductase [21]. Any methemoglobinemia is based on acute or chronic hypoxia due to a decrease in oxygen saturation of arterial blood. Moreover, methemoglobin not only does not participate in oxygen transport, but also worsens the transport function of the existing oxyhemoglobin.

The severity of symptoms depends on the content of methemoglobin in the blood. Cyanosis occurs at around 15-30 % metHb and tissue hypoxia can occur as levels rise further - metHb levels of 70 % can be fatal [22].

Erythrocytes contain endogenous enzymatic and non-enzymatic methemoglobin reductase systems. The main system of protection against oxidizing agents, which allows maintaining the hemoglobin fraction in healthy subjects at the level of 1.0-1.5%, includes three components: reduced nicotinamidine nucleotide (NAD-H), heme-containing hemoproteincytochrome b5 and the enzyme cytochrome b5 reductase. The electron donor is the glycolysis product NAD-H. An electron is transferred from NAD-H to cytochrome b5 and ultimately to methemoglobin. Electron transport is catalyzed by the enzyme cytochrome b5 reductase. This mechanism is

responsible for the recovery of 99 % of hemoglobin from methemoglobin. Another way to restore hemoglobin, associated with the activity of NADP-methemoglobin reductase, under normal conditions, has little effect. Its role increases in the event of a deficiency of cytochrome b5 reductase.

Thus, the tested compounds were subjected to screening of methemoglobin formation in human RBCs, because it is a serious toxic effect. This study of the tested compounds CMT-22, CMT-67, CMT-68, CMJ-23 and CMJ-33 showed good results, which did not exceed the permissible values in the 1-10 μM concentration range (Figure 7).

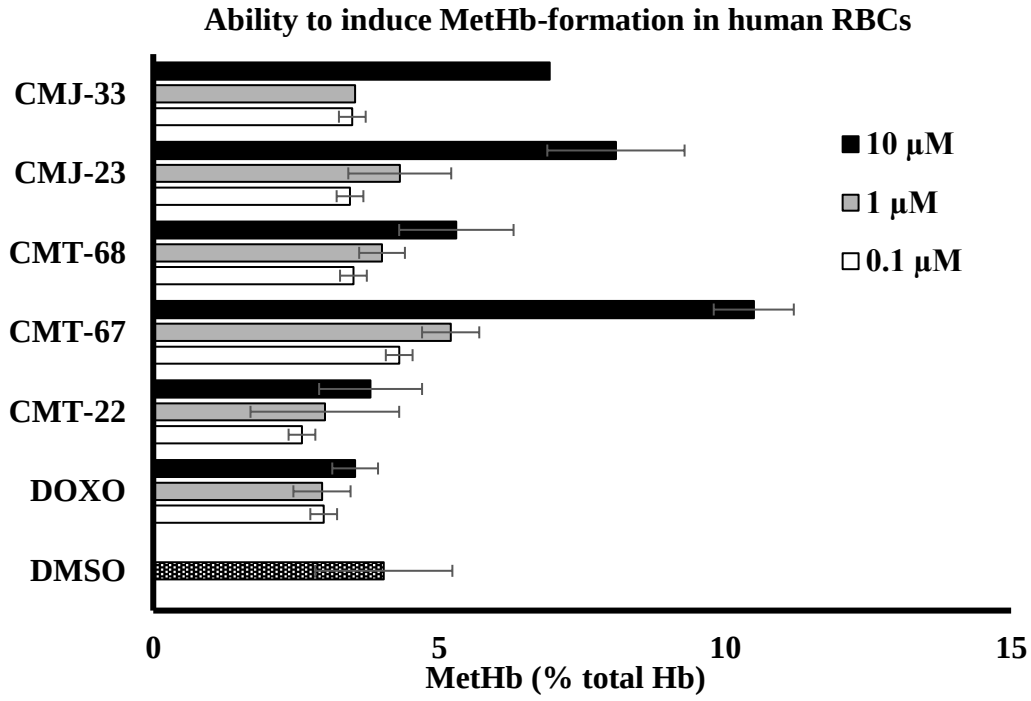


Fig. 7. Effect of the tested compounds CMT-22, CMT-67, CMT-68, CMJ-23, CMJ-33 and the anticancer control DOXO on metHb formation *in vitro.* Values are represented as mean ± SD of 3 replicates.

No significant fluctuations in metHb formation were observed after exposure of RBCs to various concentrations of the tested compounds. Thus, the application of 10 μM of CMT-22, CMT-67, CMT-68, CMJ-23, CMJ-33 and DOXO induced the formation of metHb in 3,8±0.5 %, 10.5±0.6 %, 5.3±0.8 %, 8.09±1.2 %, 6.9±0.6 %, 3.0±0.5 % of cases, respectively.

This study of the tested compounds showed results which did not exceed the permissible values in the therapeutic concentration range [23].

4.5. Impacts of the tested compounds on RBCs hemolysis

The hemolysis process is characterized by a rupture or a sharp increase in the permeability of the erythrocyte membrane and the release of hemoglobin into the plasma. Because lysis of RBCs is one of the major side effect caused by thiosemicarbazones, the ability of CMT-22, CMT-67, CMT-68, CMJ-23, CMJ-33 and DOXO was compared to induce human RBCs hemolysis.

The *in vitro* hemolysis assay evaluates hemoglobin release in the plasma as an indicator of red blood cell lysis, following test agent exposure (tested compounds). Formulations with a hemolysis value of <10 % were considered nonhemolytic while values > 25 % were considered regarded as for hemolysis.

Additionally, hypotonic 0.1 % and isotonic 0.9 % solution of NaCl were used as positive and negative controls, respectively. As expected, application of negative and positive controls have induced 100% and < 10% of RBCs hemolysis [24]. In contrast, various concentrations of the tested compounds demonstrated low hemolytic activity reaching maximum values of 8.2±0.1 %, 10.2±0.2 %, 10.6±0.1 %, 14.2±0.6 % and 10.3±0.2 %, respectively, after exposure of RBCs to 10 μM of CMT-22, CMT-67, CMT-68, CMJ-23 and CMJ-33, respectively. Incubation of RBCs with DOXO drug promotes hemolysis of 15.0±0.2 % (Figure 8).

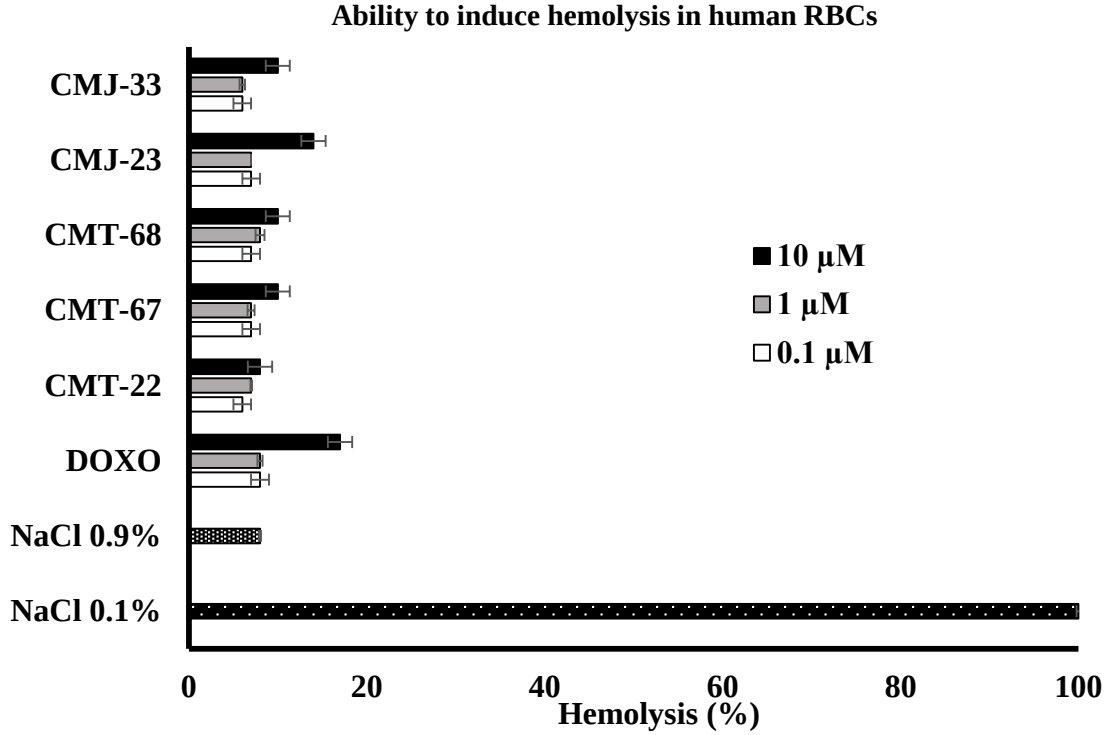


Fig. 8. Percentage of hemolysis activity of the tested compounds CMT-22, CMT-67, CMT-68, CMJ-23, CMJ-33 and anticancer control DOXO as well as NaCl 0.1 %, NaCl 0.95%. Values are represented as mean ± SD of 3 replicates.

These results indicate, that the tested compounds have a low ability to induce RBCs hemolysis, as compared with DOXO. So, induced hemolysis study of the tested compounds showed results, which did not exceed the permissible values in the therapeutic concentration range

4.6. Toxicity activity of the tested compounds

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 vitro* cell lines or *in vivo* unicellular organisms, avoiding the inclusion

of laboratory animals in studies. It should also be taken into account the problems of contamination of control animals with the test substance [25,26].

In this work, unicellular organisms *Paramecium caudatum* were used as test objects to detect the toxicity of active substances. *Paramecium caudatum*, subkingdom *Protozoa*, class *Ciliophora*, a species of highly organized protozoa that combine all the properties of a single cell and an entire organism, is one of the most widespread inhabitants of continental freshwater basins.

Biological screening of substances was carried out by two main methodological approaches using microscopic and spectrophotometric methods. As a result, in the course of experiments related to the assessment of the toxicity of a substance with respect to *Paramecium caudatum*, a new spectrophotometric method was developed using neutral red dye (NR), which allows accelerating biotesting in order to most accurately determine the dependence of toxicity on the concentration of the substance [27]. The mechanism of the method consists in the ability of the intravital NR dye (3-amino-7-dimethylamino-2-methylphenazine hydrochloride) to be absorbed and retained in the lysosomes of living *Paramecium* [28].

The advantage of the method using *Paramecium caudatum* consists in its humane approach, rapid investigation, besides, it needs significantly lower volumes of the substances to be studied. This method does not substitute the methods using test animals,still it should me mentioned that earlier we found, in most cases, a correlation of the data on the toxicity of biologically active substances in rats with the data obtained using *Paramecium Caudatum*. This fact makes it possible to employ this method in preliminary screening and evaluation of substance toxicity with the aim of narrowing down the concentration range while identifying letal doses in rats for further preclinical studies.

It was found that the LC_{50} values for 24 h are 4.9±0.5 μM for CMT-22, 10.0±0.2 μM for CMT-67, 25.5±3.8 μM for CMT-68, 44.5±1.5 μM for CMJ-23, 24.3±1.1 μM for CMJ-33 and 1.0±0.4 μM for DOXO. LC_{50} values for 48 h are 12.1±2.5 μM for CMT-22, 5.1±0.6 μM for CMT-67, 6.9±0.9 μM for CMT-68, 11.6±0.5 μM for CMJ-23, 13.3±0.2 μM for CMJ-33 and 1.10±0.01 μM for DOXO.

Direct toxic evaluation of compounds, performing *Paramecium caudatum* colorimetric bioassay demonstrated that the LC_{50} after 24 h treatment for CMT-22 is 5 times less, for CMT-67 is 10 times less, for CMT-68 is 26 time less, for CMJ-23 is 45 time less and for CMJ-33 is 24 time less than DOXO. The LC_{50} after 48 h treatment for CMT-22 is 11 times less, for CMT-67 is 5 times less, for CMT-68 is 6 time less, for CMJ-23 is 11 time less and for CMJ-33 is 12 time less than DOXO.

Thus, these results have demonstrated that the tested compounds have lower toxicity for 24 and 48 hours than that exerted by DOXO. The highest toxicity activity on *Paramecium caudatum* was exhibited by DOXO.

GENERAL CONCLUSIONS

1. For the first time, new inhibitors of cancer cell proliferation (CMT-22, CMT-67, CMT-68, CMJ-23 and CMJ-33) characterized by high selective activity, low toxicity and higher efficiency compared to DOXO and CDDP have been identified, which opens up prospect of their employment as anticancer agents.

2. For the first time in the Republic of Moldova, local compounds have been tested on various cancer cell lines. The tested compounds have been found to manifest a high antiproliferative activity towards a series of cancer cells i.e. MeW-164 (human malignant melanoma, Warsaw Cancer Center), HeLa (human cervix adenocarcinoma, ATCC CCL-2), BxPC-3 (human primary pancreatic adenocarcinoma, ATCC CRL-1687), RD (human rhabdomyosarcoma, ATCC CCL-136) that in most cases is by 1.2-60 times higher than that of DOXO and also by 2.7-260 times more selective towards cancer cells compared to DOXO. The tested copper(II) complexes exhibit a higher antiproliferative activity, while the corresponding thiosemicarbazones CMT-22 and CMJ-23 in most cases are more selective [3, 10, 11, 13, 23, 13, 14, 16, 23, 28, 31].

3. It was revealed that the mechanism of action of antiproliferative activity of the tested compounds is associated with apoptosis of cells. NMR spectroscopy and X-ray analyses have demonstrated that thiosemicarbazones interact with the DNA fragment (guanine) forming hydrogen bonds, which causes DNA fragmentation and finally apoptosis.

4. The tested compounds have manifested higher antioxidant activity against $ABTS^{\bullet+}$ and peroxyl radicals compared to the reference compounds, according to the sequence: CMT-68 ≥ CMT-67 ≥ CMJ-23 ≥ CMJ-33 ≥ DOXO ≥ CMT-22 ≥ rutin ≥ trolox and CMJ-23 ≥ CMJ-23 = CMT-68 ≥ CMT-67 ≥ CMT-22 ≥ DOXO ≥ trolox, respectively. The ability of the tested compounds to inhibit the LOX activity in comparison with quercetin and DOXO is more essential according to the rank order CMT-22 ≥ CMJ-23 = CMJ-33 = CMT-67 = CMT-68 ≥ DOXO ≥ quercetin [10, 14, 17, 23, 16, 19, 31].

5. The tested compounds have been revealed to not induce hemolysis growth and the formation of metHb, which indicates the absence of the known side effects associated with the utilization of anticancer drugs. [23].

6. The methods developed to study the biological activity of substances have been adapted [20, 21, 24]. A method for assessment of the direct toxicity *in vivo* using *Paramecium caudatum* has been developed and patented, which has allowed evaluation of the toxic effect of the chemical compounds studied and their concentration range in order to assess the toxicity in preclinical investigation [27, 18, 28,25]. The LC_{50} (μM) toxicity values of the compounds have been found to be lower that those of DOXO by 5-45 times

RECOMMENDATIONS

1. To recommend utilization of the biochemical research methods adapted and modified, as well as the patented method for studies on the toxicity of molecular inorganic and organic inhibitors using *Paramecium caudatum*, as a cost-effective, rapid, and humane one.

2. It is proposed to use these substances for further preclinical and clinical studies as highly effective low-toxic selective molecular inhibitors of cancer cells.

3. To continue further search for substances with high antiproliferative activity and high selectivity among substituted 2-formylpyridine 4-phenylthiosemicarbazones.

4. It is recommended to further continue in-depth study on the mechanism of the antiproliferative activity of the proposed substances in view of ruling out genotoxic, mutagenic, and teratogenic effects *in vivo*.

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ADNOTARE

Garbuz Olga, „Noi inhibitori moleculari anorganici şi organici ai proliferării celulelor canceroase, mecanisme de acțiune", teză de doctor în științe biologice, Chișinău, 2021.

Teza constă din: introducere, patru capitole, concluzii generale şi recomandări, 204 de referințe bibliografice, 5 anexe, 129 de pagini de text de bază (până la Bibliografie), 41 de figuri, 8 scheme, 6 tabele şi 5 anexe. Rezultatele obţinute sunt publicate în 28 de lucrări ştiinţifice (7 articole, 14 teze la conferinţe, 4 brevete de invenţie, 3 inovații).

Cuvinte-cheie: compuși anticancer, activitate antiproliferativă, celule canceroase umane, activitate selectivă, activitate antioxidantă, hemoliză.

Scopul lucrări: elucidarea efectului noilor inhibitori moleculari pe bază de compuși organici și complecși asupra proliferării liniilor celulare ale cancerului uman în comparație cu medicamentele anticanceroase doxorubicina și *cis*-diclorodiamminplatinum; determinarea mecanismului de acțiune a inhibitorilor.

Obiectivele cercetării: detectarea activității antiproliferative a substanțelor testate CMT-22, CMT-67, CMT-68, CMJ-23, CMJ-33 împotriva celulelor canceroase HeLa, BxPC-3, RD, MeW-164; testarea acțiuniii substanțelor investigate asupra celulelor renale epiteliale normale de linia MDCK pentru a detecta citotoxicitatea selectivă; stabilirea mecanismului de inhibare a proliferării celulelor canceroase de către substanțele testate; evaluarea *in vitro* a probabilității de dezvoltare și a naturii posibilelor efecte secundare ale substanțelor testate asociate cu hemoliza și formarea methemoglobinei în eritrocitele umane; determinarea toxicității substanțelor testate.

Noutatea şi originalitatea ştiinţifică: au fost adaptate metode de studiu al activității biologice a substanțelor testate; a fost determinată activitatea antiproliferativă şi antioxidantă a cinci compuşi autohtoni (tiosemicarbazone şi compuşi coordinativi ai Cu(lI) cu tiosemicarbazone); a fost elaborată o metodă nouă pentru determinarea toxicităţii directe, utilizând *Paramecium caudatum* şi evaluată toxicitatea directă a substanţelor investigate; a fost elucidat mecanismul efectului substanțelor asupra proliferării celulelor canceroase; a fost stabilit că compușii testați prezintă interes din punctul de vedere al utilizării lor ca agenți anticancer mai puțin toxici și mai eficienți.

Problema ştiinţifică importantă soluţionată constă în identificarea unor inhibitori noi de proliferare a celulelor canceroase cu activitate selectivă înaltă şi toxicitate mai scăzută, comparativ cu compuşii anticancer de referinţă aprobaţi de FDA (DOXO şi CDDP), precum şi elucidarea mecanismului de acţiune antiproliferativă a compuşilor autohtoni testaţi. A prezentat interes şi acţiunea antioxidativă a inhibitorilor moleculari organici şi anorganici asupra radicalilor ($ABTS^{\bullet+}$, $DPPH^{\bullet}$, $HO_2^{\bullet}$),comparativ cu standardele de referinţă, precum şi evaluarea impactului compuşilor asupra indicilor sistemului de hemoliză a eritrocitelor *in vitro,* în vederea evaluării impactului lor.

Semnificația teoretică a lucrării și valoarea aplicativă. Au fost identificați noi inhibitori ai proliferării celulelor canceroase cu activitate selectivă ridicată și toxicitate scăzută, ceea ce a făcut posibilă propunerea acestora pentru studii preclinice. A fost propusă o metodă directă de biotestare a toxicității cu un cost redus, folosind cantități mici de substanță A fost stabilit mecanismul de acțiune a substanțelor asupra proliferării celulelor canceroase. Rezultatele obținute au semnificație științifică și științifico-didactică și pot fi utilizate la predarea cursurilor speciale de Chimie biofarmaceutică și Biochimie.

Implementarea rezultatelor științifice obținute. A fost elaborată și brevetată o metodă de determinare a toxicității directe a substanțelor, folosind *Paramecium caudatum*. Au fost brevetați doi inhibitori moleculari de proliferare a celulelor de cancer și o substanță cu activitate antioxidantă. În rezultatul modificării și adaptării metodelor de studiu a activității biologice a substanțelor au fost impliementate trei inovații.

ANNOTATION

Garbuz Olga, „New inorganic and organic molecular inhibitors of cancer cells proliferation, the mechanisms of action", thesis for PhD in biological sciences, Chisinau, 2021.

The thesis consists of introduction, four chapters, general conclusions and recommendations, 204 references, 5 annexes, 129 pages, 41 figures, 8 schemes, 6 tables. The results are published in 28 scientific publications (7 articles, 4 patents, 14 theses at conferences, 3 innovations).

Keywords: anticancer compound, antiproliferative activity, human cancer cell line, selective activity, antioxidant activity, hemolysis.

Field of study: Nature Sciences

The aim of the thesis: elucidation of the effect of the new molecular inhibitors based on organic and complex compounds on the proliferation of human cancer cell lines in comparison with such anticancer drugs as doxorubicin and cis-dichlorodiammineplatinum; determination of the inhibitors action mechanism.

The objectives of the thesis: detection of the antiproliferative activity of the tested substances CMT-22, CMT-67, CMT-68, CMJ-23, CMJ-33 on the cancer cells HeLa, BxPC-3, MeW-164, RD; testing substances against normal kidney epithelial cell line MDCK in order to detect selective cytotoxicity; identification of the mechanism of inhibition of the cancer cell proliferation by the test substances; *in vitro* assessment of the probability of development and the nature of possible side effects of the tested substances associated with hemolysis and the formation of methemoglobin in human erythrocytes; determination of the toxicity of substances.

Novelty and relevance of the study: methods for studying the biological activity of substances were adapted; antiproliferative and antioxidant activities of five synthetic compounds (tiosemicarbazones and Cu(lI) coordination compounds with tiosemicarbazones) were determined; it was established *in vitro* that the investigated substances do not cause the formation of methemoglobin and do not increase the index of hemolysis in human erythrocytes; a method for determining direct toxicity using *Paramecium* was developed, and the direct toxicity of the investigated substances was assessed; the mechanism of the effect of the substances on the proliferation of cancer cells was revealed; the tested compounds are of interest from the point of view of their use as less toxic and more effective anticancer agents.

Scientific problem solved in this thesis is the identification of new inhibitors of cancer cells proliferation with high selective activity and lower toxicity compared to FDA-approved reference anticancer compounds (DOXO and CDDP), as well as the elucidation of the mechanism of antiproliferative action of the tested compounds. The antioxidant action of organic and inorganic molecular inhibitors on radicals ($ABTS^{\bullet+}$, $DPPH^{\bullet}$, $HO_2^{\bullet}$) was determined. It has been found that the tested compounds do not cause the formation of methemoglobin and do not increase the index of hemolysis in human erythrocytes.

The theoretical importance and potential application value of the work. New inhibitors of cancer cell proliferation with high selective activity and low toxicity have been identified which made it possible to propose them for preclinical studies. A method for evaluation of the substances toxicity using *Paramecium* has been developed which allows to accelerate and reduce the cost of biotesting. The mechanism of the substances effect on the cancer cells proliferation has been determined. The findings are of scientific interest and can be used for special training courses in Biopharmaceutical Chemistry, and Biochemistry.

Implementation of scientific results. A method for determination of direct toxicity of substances using *Paramecium caudatum* has been developed and patented. Two molecular inhibitors of cancer cell proliferation and one substance with antioxidant activity have been patented. Three innovations have been implemented as a result of modification and adaptation of methods for studying the biological activity of substances.

АННОТАЦИЯ

Гарбуз Ольга: «Новые молекулярные неорганические и органические ингибиторы пролиферации раковых клеток, механизм действия», диссертация доктора биологических наук, Кишинёв, 2021.

Диссертация состоит из: введения, 4-х глав, общих выводов и рекомендаций, библиографии из 204 наименований, 129 страниц, 5 приложений, 41 рисунков, 8 схем и 6 таблиц. Полученные результаты опубликованы в 28 научных работах (7 статей, 14 тезисов докладов на конференциях, 4 патента, 3 инновации).

Ключевые слова: противораковое вещество, антипролиферативная активность, раковые клетки человека, селективная активность, антиоксидантная активность, гемолиз.

Область исследования: естественные науки.

Цель: выявление влияния новых молекулярных ингибиторов на основе органических и комплексных соединений на пролиферацию линий раковых клеток человека в сравнении с используемыми противораковыми препаратами доксорубицином и *цис*-дихлородиамминплатиной; определение механизма действия ингибиторов.

Задачи исследования: выявление антипролиферативной активности тестируемых веществ CMT-22, CMT-67, CMT-68, CMJ-23, CMJ-33 в отношении клеток раковых линий HeLa, BxPC-3, MeW-164, RD; тестирование веществ в отношении линии MDCK эпителиальных нормальных клеток почки собаки с целью выявления селективной цитотоксичности; выявление механизма ингибирования пролиферации раковых клеток исследуемыми веществами; оценка *in vitro* вероятности развития и характер возможных побочных эффектов тестируемых веществ, связанных с гемолизом и образованием метгемоглобина в эритроцитах человека; определение токсичности тестируемых веществ.

Научная новизна и оригинальность: адаптированы методики исследования биологической активности веществ; определены антипролиферативная и антиоксидантная активности 5 синтетических соединений; установлено *in vitro*, что исследуемые вещества не вызывают образование метгемоглобина и не повышают индекс систем в эритроцитах крови человека; разработан метод определения прямой токсичности с использованием *Paramecium caudatum*, и произведена оценка прямой токсичности исследованных веществ; выявлен механизм воздействия веществ на пролиферацию раковых клеток; тестируемые соединения представляют интерес с точки зрения их применения в качестве менее токсичных и более эффективных противораковых веществ.

Решенная научная проблема. Выявлены новые молекулярные ингибиторы пролиферации раковых клеток, обладающие высокой селективной активностью и низкой токсичностью. Установлен механизм действия ингибиторов в отношении радикалов $ABTS^{\bullet+}$, $DPPH^{\bullet}$, $HO_2^{\bullet}$. Обнаружено, что исследуемые вещества не вызывают образования метгемоглобина и гемолиза в эритроцитах человека.

Теоретическая и практическая значимости работы. Выявлены новые ингибиторы пролиферации раковых клеток, обладающие высокой селективной активностью и низкой токсичностью, что позволило предложить их для предклинических исследований. Разработан метод установления зависимости токсичности от концентрации веществ, позволяющий ускорить и удешевить биотестирование. Выявлен механизм действия веществ на пролиферацию раковых клеток. Полученные результаты имеют научную значимость и могут быть использованы при чтении спецкурсов по Биофармацевтической химии и Биохимии.

Внедрение полученных научных результатов. Разработан и запатентован метод определения прямой токсичности веществ с использованием *Paramecium caudatum*. Запатентованы 2 молекулярных ингибитора пролиферации раковых клеток и одно вещество с антиоксидантной активностью. В результате модифицирования и адаптации методик исследования биологической активности веществ были внедрены 3 инновации.

GARBUZ OLGA

NEW INORGANIC AND ORGANIC MOLECULAR INHIBITORS OF CANCER CELLS PROLIFERATION THE MECHANISMS OF ACTION

163.02 – BIOCHEMISTRY

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