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

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Doctoral thesis in biological sciences

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NOI INHIBITORI MOLECULARI ANORGANICI ŞI ORGANICI AI PROLIFERĂRII CELULELOR DE CANCER, MECANISME DE ACȚIUNE

163.02 – BIOCHIMIE

Teză de doctor în ştiinţe biologice

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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.

Domeniul de studiu: ştiinţe ale naturii.

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 audatum* 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.

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ABBREVIATIONS

AAPH	2,2′-azobis(2-methylpropionamidine) dihydrochloride
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
AC	antioxidant capacity
ATCC	American Type Culture Collection
AUC	area under curve
BxPC-3	human epithelial pancreatic adenocarcinoma cells line
bFGF	basic fibroblast growth factor
CDDP	cisplatin
CMJ-23	coded organic compound
CMJ-33	copper(II) complex
CMT-22	with 2-formylpyridine N(4)-phenylthiosemicarbazone ligand
CMT-67	complex copper(II) with 2-formylpyridine N(4)-phenylthiosemicarbazone ligand
CMT-68	chloro(*N*-phenyl-*N*'-[(pyridin-2-yl)methylidene] carbamohydrazonothioato)(4-aminobenzene-1-sulfonamide)copper
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethylsulfoxide
DOXO	doxorubicin ((7S,9S)-7-[(2R,4S,5S,6S)-4-amino-5-hydroxy-6-methyloxan-2-yl]oxy-6,9,11-trihydroxy-9-(2-hydroxyacetyl)-4-methoxy-8,10-dihydro-7H-tetracene-5,12-dione)
DPPH	1,1-diphenyl-2-picrylhydrazyl
EDTA	Ethylenediaminetetraacetic acid
FBS	foetal bovine serum
FDA	Food and Drug Administration
FTC	ferrothiocyanate
HAT	hydrogen atom transfer
HeLa	human cervical epithelial cells of line
HEPES	N-2 hydroxyethylpiperazine-N'-2-ethane sulfonic acid
HEp-2	human epithelial type 2 cells line
HL	2-formylpyridine N(4)-phenylthiosemicarbazone
IC_{50}	half maximal inhibitory concentration
I %	percent of inhibition

LC_{50}	median lethal concentration
LOX	lipoxygenase
LTs	leukotrienes
MDCK	normal kidney epithelial cells of Madin Darby dog's line
MeW-164	human melanoma cell line
MEM	Minimum Essential Medium
metHb	methemoglobin
MTT	(3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide)
NR	neutral red (3-Amino-7-dimethylamino-2-methylphenazine hydrochloride)
ORAC	Oxygen Radical Absorbance Capacity
PBS	phosphate buffered saline
Pgp	P-glycoproteid
PI	propidium iodide (3,8-Diamino-5-[3-(diethylmethylammonio)propyl]-6-phenylphenanthridinium diiodide)
RBC	red blood cell
RD	human muscle rhabdomyosarcoma spindle and large multinucleated cells line
RMS	rhabdomyosarcoma stages
RPMI	roswell park memorial institute
SD	standard deviation
TE	trolox equivalents
TGFb	transforming growth factor b
TNF	tumor necrosis factor
V%	percentage of viability
VEGF	vascular endothelial growth factor

INTRODUCTION

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 [1].

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 [2].

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.

Antineoplastic agents are divided into cytotoxic and cytostatic. Doxorubicin, CDDP, fluorouracil, hydroxyurea, cyclophosphamide are the most known among the cytostatic drugs.

Doxorubicin, a frontline drug regarded as one of the most potent of the Food and Drug Administration (FDA) approved chemotherapeutic agents, has been used in cancer treatment for more than 30 years. Doxorubicin causes toxicity, especially cardiotoxicity while providing a cure in select cases, which forces the treatment to become dose-limiting. Doxorubicin (DOXO) cardiomyopathy is known to have a poor prognosis and is frequently fatal. DOXO causes toxic damage to the mitochondria of cardiomyocytes contributing to enhanced oxidative stress [3, 4].

Platinum-based anticancer drugs also play a leading role in the treatment of various malignant tumors, but severe side effects such as nephrotoxicity, neurotoxicity, and drug resistance have limited their wide range of clinical applications [5, 6]. This has stimulated extensive research and has promoted chemists to establish alternative approaches on the basis of using endogenous metals to improve the pharmacological properties. Among the many bio-essential metals, copper complexes are regarded as promising alternatives to platinum complexes as anticancer drugs because copper is biocompatible and exhibits many significant roles in biological systems. Also, copper shows the altered metabolism of cancer cells and differential response between normal and tumor cells. It is proven that the concentration of copper in cancerous tissues exceeds that of normal tissue, and the sequestration of copper can prevent the establishment of new blood vessels. Therefore, cancer cells may represent a suitable, selective target for copper-based agents [7, 8].

In recent years, a large number of synthetic copper(II) complexes of thiosemicarbazones ligands has been reported to act as pharmacological agents and as potential anticancer and cancer-inhibiting agents, and they have been found to be active both *in vitro* and *in vivo*.

Thiosemicarbazone is a class of organic compounds that possesses a wide spectrum of biological activities and medical properties. Thiosemicarbazones contain a wide range of donor atoms and, therefore, can form coordination compounds with transition metal ions [9].

Different models, such as cancer cell lines, explants of tumor and normal tissues, enzyme systems are used to develop and test anticancer substances, but the selection of anti-tumor substances is carried out mainly on various cancer cell lines.

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* [10]

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).

In **CHAPTER 1 „CANCER AND GENERAL ASPECTS OF CHEMOTHERAPY”**, 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.

In **CHAPTER 2 „CHARACTERISTICS OF THE OBJECTS OF STUDY AND RESEARCH METHODS”**, the characteristics of the tested compounds or the objects of study, each research method, data on method characteristics and methodological quality is described extensively.

This chapter begins with a description of the characteristics of the investigated compounds (2-formilpyridine *N*(4)-phenylthiosemicarbazone (CMT-22), complex copper(II) with ligand 2-formilpyridine *N*(4)-phenylthiosemicarbazone (CMT-67), complex copper(II) mixed-ligand chloro(*N*-phenyl-*N*'-[(pyridin-2-yl)methylidene]carbamohydrazonothioato)(4-aminobenzene-1-sulfonamide)copper (CMT-68), as well as an organic substance CMJ-23, copper complex CMJ-33 and the FDA approved anticancer drugs doxorubicin, cisplatin), followed by the representing of culture conditions of cell lines, such as 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), MDCK (Madin Darby Canine Kidney epithelial normal cells, ATCC CCL-34).

This section is devoted to the discussion of cell proliferation/viability assay methods (MTT, resazurin, fluorescence cytometr). 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 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 neutral red (NR) - assay *in vivo* with *Paramecium caudatum,* as the test-object. The necessary calculations are extensively described for each method.

CHAPTER 3 „ANTIPROLIFERATIVE ACTIVITY OF THE TESTED COMPOUNDS” contains four main subchapters: 1) *In vitro* antiproliferative activity of the tested compounds CMT-22, CMT-67 and CMT-68 on different lines cancer cells; 2) *In vitro* antiproliferative activity of the tested compounds CMJ-23 and CMJ-33 on different lines cancer cells; 3) The ability of the tested compounds to induce DNA fragmentation *in vitro*; 4) Conclusion.

This chapter is reported to the results of study, emphasizing data on anticancer activity of the tested compounds in comparison with their cytotoxic effect in regard to the normal cells.

The results of our study demonstrate that all tested compounds exhibited an antiproliferative activity in relation to the tested cancer cells of lines. The tested compounds for normal cells showed selective cytotoxicity. It was determined that the tested compounds show better selectivity towards cancer cells than DOXO.

Electrophoresis method of DNA fragmentation confirms the mechanism of action of the studied substances linked with a direct effect on cell genomic DNA. The research findings identified a possible mechanism of action of the tested cpmpounds associated with cellular apoptosis.

CHAPTER 4 „STUDIES OF THE MECHANISM OF ACTION AND SIDE EFFECTS OF NEW ORGANIC AND ORGANOMETALLIC CANCER CELL INHIBITORS ASSOCIATED WITH OXIDATIVE STRESS” contains seven subchapters: 1) $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ radical scavenging activity of the tested compounds; 2) Oxygen Radical Absorption Capacity (FL) activity of the tested compounds; 3) Antilipoxygenase activity of the

tested compounds; 4) Impacts of the tested compounds on methemoglobin formation; 5) Impacts of the tested compounds on RBCs hemolysis; 6) *Paramecium* toxicity activity of the tested compounds; 7) Conclusion.

The respective chapter begins with a description of the results on the antioxidant activity of the tested compounds, followed by reporting of experimental results on the tested compounds screening including toxic hemolysis and methemoglobin formation in human RBCs observations, which do not exceed the permissible values in the therapeutic concentration range. The following subchapter presents data on direct toxic evaluation that demonstrates lower toxicity for 24 h and 48 h of the compounds under study in comparison with that of doxorubicin.

1. CANCER AND GENERAL ASPECTS OF CHEMOTHERAPY

1.1. Cancer epidemiology

The problem of cancer remains a priority for modern society. The World Health Organization has recently demonstrated that more than 18 million new cases of cancer are recorded all over the world annually; while 9.6 million deaths were reported in 2018 [1]. The International Agency for Research on Cancer released the latest estimates on the global burden of cancer in September 2018 [11, 12]. They reveal a large geographical diversity in cancer occurrence and variations in the number and profile of the disease between and within regions worldwide. Every fifth male and every sixth female develop cancer during their lifetime in the world, and every eighth male and every eleventh female dies from the disease. It has been estimated worldwide that the total number of people diagnosed of cancer who are alive within five years, called the five-year prevalence, constitutes 43.8 million. Though, Europe's population makes only 9%, the continent accounts for 23.4% of the global cancer cases and 20.3% of the cancer deaths. The Americas account for 21.0% of incidence and 14.4% of mortality due to cancer having 13.3% of the global population. The proportions of cancer deaths in Asia and in Africa (57.3% and 7.3%, respectively) are higher than those of incident cases (48.4% and 5.8%, respectively) in contrast to other world regions because of a higher frequency of certain cancer types connected with poorer prognosis and higher mortality rates; moreover, the access to timely diagnosis and treatment is limited in many countries.

Worldwide, the leading types in terms of the number of new cases include lung and female breast cancers; around 2.1 million diagnoses were estimated for each of these types in 2018, contributing to about 11.6% of the total cancer incidence burden. Colorectal cancer (1.8 million cases, 10.2% of the total), prostate cancer (1.3 million cases, 7.1%), and stomach cancer (1.0 million cases, 5.7%) are the most commonly diagnosed ones (Figure 1) [11, 12].

Lung cancer also accounts for the largest number of deaths (1.8 million deaths, 18.4% of the total), because of its poor prognosis worldwide, followed by colorectal cancer (881,000 deaths, 9.2%), stomach cancer (783,000 deaths, 8.2%), and liver cancer (782,000 deaths, 8.2%). Female breast cancer ranks as the fifth main cause of death (627,000 deaths, 6.6%) due to relatively favorable prognosis, at least in more developed countries (Figure 2) [11, 12].

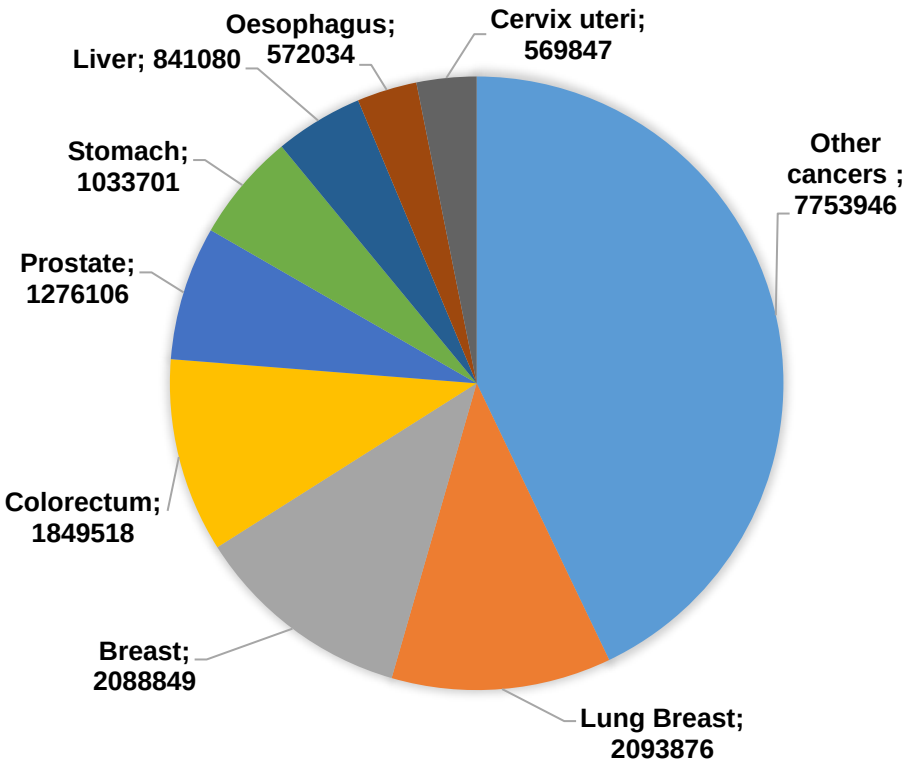


Fig. 1.1. Number of new cases in 2018, both sexes, all ages [11, 12].

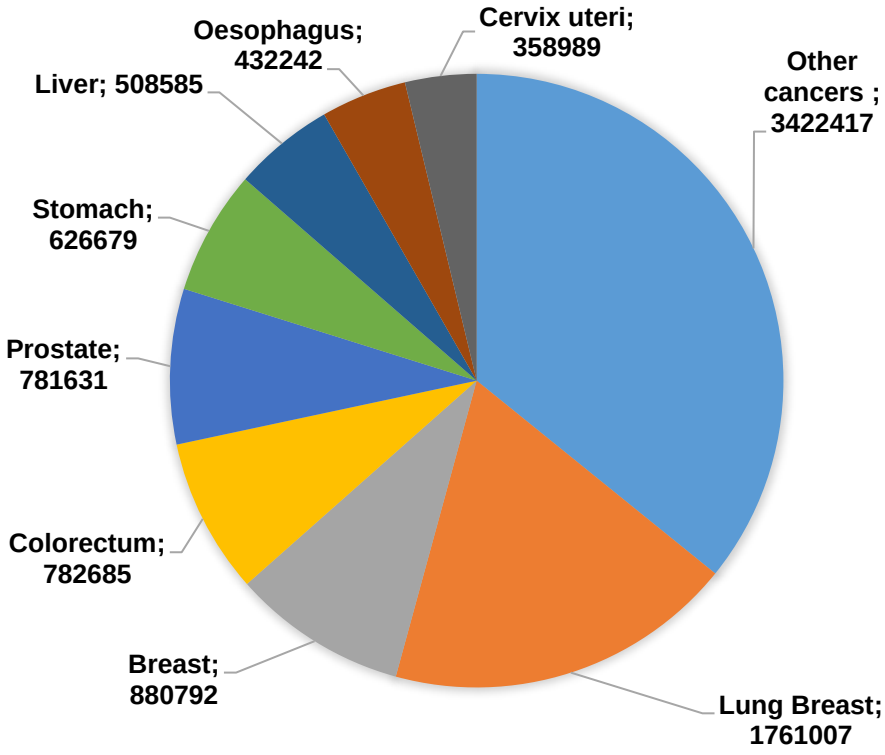


Fig. 1.2. Number of deaths in 2018, both sexes, all ages [11, 12].

1.2. Cancer etiology and pathogenesis

Cancerous tumors are characterized by uncontrollable, not regulated by external signals proliferation of cells and their capacity to penetrate into surrounding tissues and organs destroying them and to spread by blood or lymth flow with formation of secondary foci - metastases [13].

The tumor cell transformation occurs as a result of accumulation of mutations affecting various regulatory mechanisms. Frequently, the cell cycle is disrupted when the cell is going through restriction points. Usually, telomerase expression and excess expression of growth factors and their receptors are observed in tumor cells. Tumor cells are characterized by changes in cytoskeleton and loss of sensitivity to contact inhibition of proliferation. The proteins involved in apoptosis are frequently not expressed in them. As a rule, mutations disturb different paths of signal transmission. The high mutation frequency in tumor cells facilitates their adaptation to environment, and one of the manifestations of such adaptation is the development of the resistance to drugs. With the tumor growth, the cells start to secrete proteases that promote invasive growth and metastasis development. At a certain stage, cells start to secrete factors that contribute to vessel proliferation and improvement of blood supply to the tumor [14]. Some mutations give a possibility to tumor cells to remain invulnerable to the immune system; for example, in some tumors, the expression of HLA class I antigens is reduced and the cells are not recognized by T-lymphocytes [15, 16].

A large variety of genes is involved in tumor development [17]. Deregulation of multitude of cell functions, including the mechanisms of control over cell proliferation [18], DNA repair, [19] chromosome stability, intercellular interactions, cell-matrix interactions, angiogenesis, cell aging, apoptosis etc. [20, 21].

Hyperplasia, adenoma, dysplasia *in situ* cancer, invasive cancer are observed as genetic disorders accumulated in the epithelial tissues. Tumor transformation is practically always caused by genetic disorders, however, in the experiment, tumor cells may be produced without gene damage. Thus, the cells of the primary ectoderm of the mouse embryo proliferate uncontrollably in the culture. After such cells are introduced to animals, they develop a tumor, teratocarcinoma. However if teratocarcinoma cells are reintroduced into the early embryo, under the influence of neighboring cells they differentiate into cells of normal organs and tissues according to the injection site. Thus, external factors influence the gene expression in target cells. Receiving many different signals, the cell responds to them by activating a specific set of transcription factors. It depends on this set whether the cell will proliferate, differentiate, or die.

In *in vitro* cultures, human fibroblasts and epithelial cells after 50-60 divisions of the "Hayflick number" [22] irreversibly stop in the G1- and G2 phases of the cell cycle. The mechanism is based on a progressive shortening of the telomere length as a result of incomplete replication of the chromosome terminal sections in each of the mitotic cycles. The stop of the cell cycle is conditioned by the formation of "sticky" chromosome ends, which causes them to connect and trigger reactions similar to those observed under the action of DNA-damaging agents [23]. However, in cells with active telomerase, which elongates DNA telomere repeats or when other, so called "alternative mechanisms of telomere elongation" are activated based particularly, on non-reciprocal recombination of their sites, the restriction of the division number - immortalization - can be lifted [24].

This is evidenced by the fact that in contrast to normal human tissues, in the cells of most tumors, as well as in stem cells, telomerase is active and transduction of the vectors expressing the catalytic telomerase subunit (TERT) increases the life span of normal human cells of some lines by as many as 20 divisions. The interconnectedness of the processes of apoptosis inhibition and telomerase activation in cancer cells is evidenced by the overproduction of the Bcl-2 protein in cancer cells accompanied by an elevation of the telomerase activity level.

It has been found that telomerase activity is regulated by the Myc oncoprotein that increases the transcription of the TERT subunit gene, the expression level of which determines the enzyme activity in normal cells [25, 26].

It is known from literature that a number of other cellular and viral oncoproteins (activated Ras, Mdm2, cycline D1, Cdc25A, E7 HPV) do not activate telomerase [27], while HPV16 E6 possesses this capacity, it being linked precisely with the capacity of this viral protein to boost Myc expression [27]. It is not excluded that telomerase activation in mutagen-stimulated lymphocytes, as well as in the proliferative zones of hair follicles and intestinal crypts, is also conditioned by the expression of the Myc protein in them [28, 29].

It is noteworthy that in the lines that proliferate normal cells, telomerase, as a rule, is inactive. However, the activation alone of the mechanisms preventing telomere shortening is not sufficient for cell immortalization. Thus, TERT or E6 transduction by removing restriction on the number of cell divisions in the cells of some lines does not result in immortalization of fibroblasts IMR-90, keratinocytes and breast epithelial cells, although it causes activation of telomerase and telomere elongation in them. Immortalization in such cells occurs only in the case of supplementary inactivation of the function of certain tumor suppressers. Moreover, inactivation of different suppressors is required for different types of cells. Thus, in human kerotinocytes and mammary epithelial cells immortalization is observed upon transduction of

TERT and simultaneous inactivation of either pRb or p16INK4a, while elimination of p53 [30] or p19ARF does not induce such effect [31]. On the other side, in mouse cells, in the majority of which, unlike human ones telomerase is constitutively activated [32], immortalization, as a rule, is induced by just inactivation of p53 or p19ARF [33, 34].

The data accumulated during the last decade point to the necessity of angiogenesis for the growth of the vast majority of malignant tumors. Having reached a certain stage of development, a malignant tumor penetrates through the basal membrane and invades neighboring connective tissue. Angiogenesis is regulated by angiogenic stimulating factors such as angiogenin, vascular endothelial growth factor [VEGF] [35], basic fibroblast growth factor [bFGF], transforming growth factor b [TGFb] and cytokines (interleukin [IL]-1, 6 and 8) as well as through inhibitors angiostatin and endostatin.

Malignant cells express collagenases, heparanases and plasminogen activators and advance through supporting tissues along the path of least resistance. Angiogenesis plays an important role in the delivery of nutrients to a proliferating tumor [36]. The synthesis of collagen, vitronectin and fibronectin by malignant cells forms the basis for the development of tumor cells and contributes to its engraftment in metastasis sites. Capillary vessels first develop in the adjacent tissues, which are later substituted by tumor cells. The capillary vessels surrounding the tumor are formed from the cells of the normal endothelium, however they differ considerably from the normal ones in terms of morphology density and vascular permeability. A dense network of capillaries supplies the developing tumor with oxygen, required nutrients and allows the elimination of toxic waste products of tumor cells. The presence of a capillary network also facilitates the introduction and spread of metastatic tumor cells. As a result of angiogenesis, „dormant" metastases are capable to initiate exponentially progressively growing tumor [37].

1.3. Utilization of chemotherapy in view of the cell cycle in cancer

To correctly prescribe antitumor drugs and understand their mechanism of action, it is necessary to take into account the basic processes of cell proliferation. Many of the antitumor drugs act at the expense of DNA damage and are most active in the S phase when DNA replication occurs, while taxans disrupt mitosis preventing the formation of a division spindle. Such drugs act only on dividing cells, therefore tumors with a high growing fraction are most sensitive to chemotherapy. However, most antitumor agents also attack normal rapidly dividing cells (in bone marrow, hair follicles, gastrointestinal mucosa), which limits the utilization of

these products. At the same time, tumors with a low growth fraction (colon or lung cancer) often are resistant to chemotherapy.

The duration of the cell cycle varies with the cell type, the division of all cells occurs according to the general scheme. The phases of the cell cycle are as follows: (1) G1 presynthetic phase; (2) S synthetic phase; (3) G2 postsynthetic phase, and (4) mitosis. As a result of mitosis, two daughter cells are formed from the G2 cell, which immediately start a new cell period, entering the G1 phase or proceed to a dormant state (G0). The cells of some tissues at the G0 phase differentiate and lose their capacity to continue division. Many other cells especially in slowly growing tumors, after a durable dormant state can again start to divide. Reaching a control point at the boundary of G1 and S phases, cells with damaged DNA are exposed to apoptosis, if they retain the TP53 gene and the p53 protein encoded by it accomplishes its control functions. With mutation of this gene, cells manage to avoid apoptosis: they proceed to the S phase, divide and some of them can give rise to a population of drug-resistant cells.

The growth of the population of tumor cells is characterized by a slow linear growth of the tumor at its minimum and maximum size and by exponential growth at the intermediate stage. A low growth rate is characteristic of very small and very large tumors, while the growth is exponential with an intermediate tumor size.

The basic principles of cancer therapy are determined by the kinetics of cell proliferation. The log-kill hypothesis postulates that a certain dose of drug the kills a constant fraction of tumor cells (expressed in cell log number), and not a constant cell number. It means that after a course of treatment a part of the tumor cells accidentally survives, without acquiring specific resistance to the drug. In this connection, healing is more probable when a combined therapy isemployed. Log-killing varies with the speed of the cell growth, its progressive decrease being observed in the late stages of tumor growth, when the cells no longer proceed to the cell cycle. Early relapses of slowly growing tumors result from their slight log-killing. Late relapses of rapidly growing tumors can appear notwithstanding the efficient treatment if the number of therapy courses was too low during the so-called risk period.

Slowdown of tumor growth and death of malignant cells correlate with cytological and spatial factors. Proximity to blood vessels and access to oxygen are significant factors determining the vital capacity and growth of a malignant cell, which leads to important therapeutic consequences in the case of chemotherapy when many antitumor drugs specifically target dividing cells [38].

1.4. Side effects associated with oxidative stress and resistance in anticancer chemotherapy

In the management of cancer, high systemic toxicity and drug resistance remain a major challenge for modern medicine despite the significant progress made in the anticancer therapy. Chemotherapy can have severe side effects because of its cytotoxic action on normal cells. This limits its employment and it is indicated to reduce the drug dose, interrupt and even cease the treatment. It is important, therefore, that the anticancer drugs induce antiproliferative and cytotoxic activity in tumor cells without affecting normal tissues [39, 40].

Hemolysis induction is one of the severe side effects of chemotherapy. Two mechanisms are involved in this process. Toxic hemolysis implies direct toxicity of the drug, its metabolite, or an excipient in the formulation; allergic hemolysis implies toxicity caused by an immunological reaction in patients pre-sensitised to a drug [41]. For most drugs, toxic hemolysis involves lower doses given to individuals who are genetically predisposed to hemolysis although the majority of normal individuals may suffer toxic hemolysis at sufficiently high concentrations of hemolytic drugs. To test the excipients intended for injectable use for hemolytic potential, it is strongly recommended that an *in vitro* hemolysis study be performed at a concentration intended for administration. The *in vitro* hemolysis assay evaluates hemoglobin release in the plasma, which is as an indicator of red blood cell lysis after test agent exposure. Compounds with a hemolysis value of <10% are considered nonhemolytic while the values of >25% are considered fit for hemolysis [42].

A major dose-limiting toxicity was symptomatic dyspnea in early clinical trials due to treatment-related methemoglobinemia [43, 44]. Methemoglobinemia is a reversible condition in which more than 15% of a patient's hemoglobin is incapable of carrying oxygen because of iron oxidation [45, 46]. Erythrocyte hemoglobin carries oxygen in reversible connection with iron in a reduced, ferrous Fe^{2+}state. Fe^{2+} iron hemoglobin oxidizes to Fe^{3+} methemoglobin at a daily rate of approximately 3%. Methemoglobin returns to hemoglobin due to the action of cytochrome b5 reductase and cytochrome b5 [47]. This pathway is responsible for 94% of the conversion of methemoglobin to hemoglobin and normally maintains methemoglobin levels below 1% of total hemoglobin. Dyspnea occurs when blood methemoglobin levels reach 25% [48, 49, 50, 51, 52]

Induced toxic hemolysis and elevated index of methemoglobin are the result of the effect of oxidative stress of free-radical nature caused by chemotherapy [53].

Free radicals (FR) are highly reactive molecules, containing one or several unpaired electrons on the outer electron membrane. Cellular damage occurs when FR meet a molecule and try to extract an electron pair from it for the odd electron. Unstable due to the electronic deficiency, such FR will capture the missing electron of a neighboring molecule, which leads, in its turn, to transformation of that molecule into a FR. The new FR will capture an electron from the following molecule in a chain chemical reaction that produces radicals. In such reactions, the newly formed FR will ultimately capture an electron from a modified molecule without being able to further function. Such an event results in the destruction of the molecule and the containing it cell. As occurs when the reaction involves paired basic molecules in a DNA strand, the triggered chain reaction may lead to atomic structures cross-linking. DNA cross-linking can cause various aspects of aging, especially that of cancer. An anticancer compound should not be prooxidant as it may produce adverse modifications to cell components [54, 55].

It is known from literature that the biological action of the anticancer anthracyclines doxorubicin and daunorubicin is frequently connected with their ability to induce oxidative stress through RF generation and reactive oxygen species (ROS) [56, 57]. This property is due to the presence of a quinone moiety in the drugs' structures, which may cause metabolic reduction generating superoxide via aerobic redox cycling and, subsequently, other more reactive oxygen forms. ROS are believed to possibly play a role in cardiotoxicity induced by anthracycline. A few studies have recently revealed that anthracyclines have also reducing capacities as they react with oxidants. This is not surprising, as the drugs contain an electron-donating hydroquinone moiety in their chromophores [58, 4].

In cancer treatment, development of multidrug resistance to chemotherapy remains a major challenge. Resistance exists against every effective anticancer drug and can develop using numerous mechanisms that include decreased drug uptake, increased drug efflux, activation of detoxifying systems, activation of DNA repair mechanisms, evasion of drug-induced apoptosis, etc [59, 60, 61]**.**

Drug metabolism enzymes are the second line of cellular resistance once in the intracellular compartment. The process employs phase I and II enzymes. It is cytochrome P450 enzymes (CYPs) and epoxide hydrolases that mainly mediate phase I or oxidative metabolism [62]. CYPs belong to a superfamily of hemoproteins that comprises, based on their degree of sequence homology, 57 genes classified in 18 families and 44 subfamilies. CYPs are locatedd in mitochondria and the endoplasmic reticulum (ER) and, by incorporating one atom of molecular oxygen into the substrate and one into water, catalyze the monooxygenase reaction. This reaction also demands a source of electrons that are supplied by the NADPH cytochrome P450 reductase

and ferredoxin in ER and mitochondria, respectively. Microsomal CYPs metabolize both endogenous and exogenous compounds while mitochondrial CYPs are involved in the metabolism of endogenous substrates [63, 64]. Therapeutic drugs are therefore metabolized by microsomal CYP and epoxide hydrolases, which convert highly mutagenic aromatic metabolites (epoxide) created from the CYP metabolism in a metabolite that can be conjugated by the phase II enzymes and then effluxed by transporters such as the members of the ABCC transporter family. Although mainly expressed in the liver, extrahepatic expression of CYPs has been shown in both normal and tumor tissues. The CYP3A, 2D6, and 2C families metabolize most chemotherapeutic drugs [65, 66]**.**

Drug detoxification is mainly realized by phase I reactions. However, a prodrugbased strategy may employ these enzymes. Hence, it is particularly important to understand the polymorphism effect on CYP enzyme activity. This is well illustrated by the findings referring to 516G>T polymorphism in CYP2B6, which proves that depending on the drug administered, the effect of polymorphisms can vary dramatically [67, 68]. Studies have singled out the synergism between CYP enzymes and ABC transporters that occurs when CYP enzyme produced metabolites, especially CYP3A4, are better substrates for ABCB1 than the parent compound or when ABCB1 prevents the saturation of CYP enzymes by requiring a subsequent entry of the drug into the cell. This process enhances exposure to CYP enzymes and can dramatically reduce the efficiency of chemotherapeutic treatment [69]. Phase II enzymes are included in conjugation reactions involving glutathionylation, glucuronidation, and sulfation [70]. These enzymes comprise UDP-glucuronosyltransferases (UGT), arylamine N-acetyltransferases (NAT), sulfotransferases, and glutathione-S-transferase (GST), which transform the reactive species into hydrophilic nontoxic metabolite conjugates. Members of the ABCC family of ABC transporters efflux afterwards these conjugated metabolites. Overall survival in cancer patients have also been related to genetic polymorphisms in these families of genes. Ekhart et al. demonstrated the impact of genetic polymorphisms in both these enzymes and in phase I enzymes including ABC transporters on survival after chemotherapy [71]**.**

P-glycoproteid (Pgp) encoded by the ABCB1 gene transports drugs from the cell ensuring the efflux of cytostatics, in particular, anthracyclines, which facilitates, when it is expressed, development of multiresistance to antitumor drugs [72].

The activity of Pgp determines the resistance of tumor cells to many antitumor drugs. The basic issue of PGp biochemistry has not been elucidated up to now, how one protein ensures the resistance to a wide range of various compounds and how a single protein transports various substances.

It is suggested that twelve transmembranous domains form pores and canals through which Pgp actively removes substances, and the energy of ATP hydrolysis is somehow transferred from two ATP-binding domains, ensuing the operation of the efflux pump. It is unclear how the ATP energy is transformed into drug transfer, however, it is well known that ATP analogs are linked to Pgp and that a purified Pgp molecule possesses ATP-ase activity. Evidence has been obtained in favor of the fact that Pgp functions as a flippase transferring the substrates bound by it from the inner lipid layer of the cell membrane to the outer one or into external ambient [73, 74].

An increase in Pgp expression was founded by biopsy in tumors sampled both before and after chemotherapy. This protein was detected in all types of neoplasms [73].

Alkaloids of rosy periwinkle (*Catharanthus roseus),* anthracyclins, dactinomycin, epipodophyllotoxins and colchicines are eliminated from the cell by P-glycoproteid and induce its synthesis. Under the action of one of them, the tumor acquires resistance to the rest of them but remains sensitive to other groups of cytostatics (alkylating agents, antimetabolites) [75, 76, 77].

To monitor the integrity of a cell's DNA and govern proper responses to any genetic damage a complex network of interacting pathways has evolved. This network consists of sensor complexes that discover DNA breaks. Mre11-Rad50-Nbs1 (MRN) has been shown to recognize or sense doublestrand breaks (DSBs) in DNA, while the RPA-ATRIP complex binds to single strand breaks (SSBs). MRN and RPA-ATRIP involve then kinases ATM and ATR, respectively to phosphorylate/activate a multitude of other proteins including the checkpoint kinases Chk1 and 2, initiating a cascade that leads to cell-cycle arrest and DNA repair. However, if the damage is too extensive, rather than repair itself, the cell will enter one of these states: senescence, which is characterized by an irreversible growth arrest, apoptosis, or necrosis.

A complex network of interacting pathways has evolved to monitor the integrity of a cell's DNA and govern proper responses to any genetic damage [78]. This network includes sensor complexes that detect DNA breaks. It has been shown that Mre11-Rad50-Nbs1 (MRN) recognizes or senses DNA doublestrand breaks (DSBs), while the RPA-ATRIP complex binds to single strand breaks (SSBs). Kinases such as ATM and ATR are then recruited by MRN and RPA-ATRIP, respectively, and phosphorylate/activate a myriad of other proteins including the checkpoint kinases Chk1 and 2, initiating a cascade that results in cell-cycle arrest and DNA repair [79]. However, the cell will enter one of these states: senescence, which is characterized by an irreversible growth arrest, apoptosis, or necrosis if the damage is too extensive, rather than repair itself. Many chemotherapeutic drugs have been used to kill proliferating cells, which

causes extensive DNA damage that ultimately results in cell cycle arrest and cell death. However, the efficiency of such therapeutic agents as platinum drugs and alkylating agents can be considerably diminished by the ability of cells to repair DNA [80, 81]. DNA repair involves an intricate network of repair systems, each of them targeting a specific subset of lesions. These pathways include the nucleotide excision repair (NER) pathway, the base excision repair (BER) pathway, the direct reversal pathway (MGMT, ABH2, ABH3), the homologous recombination (HR) pathway, the nonhomologous end joining (NHEJ) pathway, and the mismatch repair (MMR) pathway. It has been reported that there is an inverse correlation of ERCC1 (NER pathways) with either response to platinum therapy or survival in colorectal, non-small cell lung cancer, and ovarian cancers [81]. It has also been demonstrated that MMR deficiency is connected with cisplatin resistance. The MMR mechanism removes the newly inserted intact base instead of the damaged one, thereby triggering subsequent rounds of futile repairs, which can result in cell death [82].

1.5. Antitumor drugs

The first antitumor drug was registered in 1946. That was embikhin developed on the basis of poisonous mustard gas used during the WWI. A focused screening for antitumor drug has started since the 1940-ies [83].

Presently, more than 100 drugs are known that are capable to cause necrosis of cancer cells when membrane, nucleus and other their components are damaged which results in cell death (cytotoxic effect) or trigger the apoptosis process (cytostatic effect) of a malignant cell.

In this connection, antitumor drugs are divided into cytotoxic and cytostatic (antimitotic and karyoclast) ones. Among cytostatic drugs the most known include doxorubicin, cisplatin, fluorouracil, hydroxyurea, and cyclophosphamide [84].

However, antitumor drugs do not have a high selective capacity and along with cancer cells, they damage the cells of normal proliferating tissues (cells of lymphoid tissue, bone marrow. gastrointestinal tract, genital glands, skin etc.) [85].

Different models are used to develop and test anticancer drugs (cancer cell lines, explants of tumor and normal tissues, enzyme systems), but the main selection of antitumor substances is carried out on experimental tumors (usually on cancer cell lines and various strains of transplanted tumors of mice or rats). Attempts are also being made to use animals with tumors caused by oncogenic agents and animals with spontaneous tumors. No distinct correlations in the sensitivity to antitumor agents of experimental neoplasms and human tumors have not been

established. Therefore, the question of the spectrum of antitumor activity, i.e., the question of which tumors the selected antitumor agent will act on, is finally resolved only in clinical trials.

1.5.1. Naturally-occurring and semi-synthetic antineoplastic drugs, their mechanism of action

Alkaloids. Alkaloids of rosy periwinkle, vinblastine, vincristine, vindesine, and vinorelbine are asymmetric dimeric compounds. A slight difference in their structure results in distinctive differences in the range of antitumor activity and toxicity. The alkaloids of rosy periwinkle act on dividing cells and block mitosis. This effect is explained by binding of products with r-tubulins and polymerization disorder in a- and r-tubulins in microtubules. When cells are incubated with vinblastine, microtubules dissolution occurs to produce crystalline structures containing tubulins and vinblastine in a molar ratio of 1:1. Due to the destruction of fission spindle microtubules, the cell ceases dividing in metaphase. At the same time, chromosomes randomly disperse in the cytoplasm or accumulate in unusual structures. It is suggested that abnormal divergence of chromosome during mitosis results in cell death. Changes characteristic of apoptosis are observed in both tumor and normal cells in the presence of alkaloids of rosy periwinkle [86]. Besides division, microtubules are necessary for cell movement, phagocytosis and axonal transport. Side effects of rosy periwinkle, for example neuropathy, are associated with the disturbance of these processes [87].

Alkaloids of rosy periwinkle possess a powerful and selective antitumor action but strongly vary in their effect on normal tissues. Vincristine is included in standard chemotherapy regimens for leukemia and solid tumors in children and are frequently used for lymphomas in adults.

Due to weak inhibition of blood formation, it is very important for polychemotherapy of leukemia and lymphomas. Vinblastine is indicated for testicular tumors and lymphomas, as well as second line chemotherapy for solid tumors. The lack of neurotoxicity makes vinblastine a valuable drug for testicular tumors (in combination with cisplastin) and recurrent lymphoma. Non-small cell lung and breast cancers are sensitive to vinorelbine; indications for vinorelbine employment expand with new tests performed. Vinorelbine possesses low neurotoxicity and moderately inhibits blood formation [88].

Complete cross-resistance to different rosy periwinkle alkaloids is not observed despite their structural similarity. However, after treatment with only one naturally-occurring cytostatic agent, the cells can become multiresistant, that is, insensitive immediately to many drugs of

different structures. This is how cross-resistance to different rosy periwinkle alkaloids, epipodophyllotoxins, taxanes and anthracyclines develops.

All rosy periwinkle alkaloids are neurotoxic, and vincristine has a characteristic cumulative effect. The first and most common symptoms are numbness and tingling in the limbs and loss of tendon reflexes, followed by muscle weakness. Sensitivity disorders usually do not require an immediate dose reduction, however, when motor impairment is observed, the drug must be discontinued in most cases.

Paclitaxel was isolated from the bark of Pacific yew (*Taxus brevifolia*). Paclitaxel attracted attention due to its capacity to induce microtubules assembly at low temperature and in the absence of GTP. The product binds with P-tubulin and impede disassembly of microtubules because of which microtubule bundles and their conglomerates of a quaint shape accumulate in the cell, and the cell cycle stops in mitosis. The cytotoxicity of paclitaxel depends on its concentration and duration of action [89].

Paclitaxel cytotoxicity depends on its concentration and action duration. Its effect is diminished by the products that block the cell cycle in the S phase and impede mitosis onset. The search for optimal combinations of paclitaxel with other antitumor drugs including doxorubicin and cisplatin is in progress. It has been noticed that administration of paclitaxel after cisplatin slows down the elimination of paclitaxel and enhances toxicity compared with the inverse order of administration.

It has been shown that resistance to paclitaxel of some lines of tumor cells is associated with amplified expression of the ABCBI gene that encodes P-glycoproteid and with the mutations of the P-tubulin gene, such mutations being able to enhance sensitivity to alkaloids of rosy periwinkle. The clinical significance of these findings is unclear. Palitaxel causes apoptosis but its activity against test tumors does not require the presence of a normal protein p53.

Podophyllotoxin isolated from *Podophyllum* peltatum was used by North American Indians and the first colonists as a vomitive, purgative and anthelmintic drug. Two semi-synthetic podophyllotoxin glycosides, etoposide and teniposide are active for a number of malignant neoplasms, including leukmia in children, lymphogranulomatosis, large-cell lymphomas, testicle tumors and small cell lung cancer [90].

Etoposide and teniposide are close in the action mechanism and the range of antitumor activity. In contrast to podophyllotoxin, they do not block mitosis, but form a triple complex with DNA topoisomerase II and DNA. In this case, the enzyme cuts both DNA strands, however the unwinding of the DNA supercoil followed by elimination of disruption do not proceed. The enzyme remains bound to the free DNA end, therefore DNA breaks accumulate in the cell

resulting in its death. Epipodophyllotoxins are most active in the S and G2 phases. The reasons for the resistance to these products are as follows: amplification of the ABCB1 gene that encodes P-glycoproteid, mutations or activity decline of DNA-topoisomerase II, as well as mutations of the TP53 gene required for apoptosis [91].

Enzymes. It was shown in 1953 that guinea pig serum causes regression of lymphomas in test animals, which is associated with the presence of asparaginase in it (Kidd, 1953). Fifteen years later, this enzyme found application as an antitumor product, the action of which is based on the differences in the metabolism of tumor and normal cells (Broome, 1981).

Normal cells usually develop asparagine in the amount sufficient for protein synthesis, but many tumor cells, especially in acute lymphocytic leukemia, are in need of exogenous asparagine. Hydrolyzing asparagine in the blood to aspartate and ammonium ions, asparaginase discontinues its supply into neoplastic cells, disrupts protein synthesis in them and causes their death. The combination of asparaginase, methotrexate, doxorubicin, vincistine and prednisone frequently used in acute lymphocytic leukemia. The order of the medicine administration is crucial: for example, administration of asparaginase after methotrexate enhances the effect, while the inverse order of administration reduces it. The fact is that asparagenase disturbs protein synthesis, whereupon interferes with the action of methotrexate, that is most active in the S phase. The cell death under the action of asparaginase occurs through apoptosis. The development of resistance is linked with the acquisition of the capacity to synthesize asparagine.

Escherichia coli produces two isoenzymes of asparaginase, but just one of them possesses antitumor activity. This isoenzyme in purified form is employed in clinical practice. Pegaspargase (an enzyme conjugated with polyethylene glycol) or asparagenase produced by *Erwinia chrysanthemi* are prescribed in case of allergy to the product isolated from *Escherichia coli* [92].

Hormonal products, their synthetic analogs, as well as antihormonal medicines are widely used in oncologic practice for hormone-dependent tumors. Ch.Huggins and col. first started to use hormones in the treatment of prostate cancer in 1941. Estrogens, gestagens (progestins), androgens and corticosteroids are used for hormonel therapy of malignant tumors. Synthetic products of chloditan capable to reduce the corticosteroid level and tamoxifen having an antiestrogenic action are employed of the antihormones.

Steroid hormones form complexes with protein molecules (receptors), bind to chromatin and disrupt the synthesis of nucleic acids of target cells (cells, sensitive to this hormone) and, in certain doses, inhibit proliferative processes in the mammary gland (androgens, estrogens), prostate gland (estrogen), blood-forming organs (corticosteroids), endometrium (gestagens).

Antitumor antibiotics. Actinomycins are chromopeptides; they usually contain the same chromophore – actinocine (a flat derivative of phenoxazone) giving them an orange-red color. Naturally-occurring actinomycins are distinguished by the structure of the amino acids included in peptide chains. Addition of different amino acids into nutrient medium can alter the type of actinomycin and its biological activity (Crooke, 1983). The actinomycin cytotoxicity is conditioned by the capacity to bind to the double helix of DNA. The crystallographic study of its complex with deoxyguanosine made it possible to explain the dactinomycin action. A flat phenoxasone nucleus interposes between the neighboring pairs of guanine – cytosine and cytosine – guanine, while peptide chains are settled in small DNA grooves. This results in the formation of a very stable complex of the product with DNA, which blocks primarily transcription. RNA-polymerases are significantly more sensitive to dactinomycin than DNA-polymerases. Besides, dactinomycin causes single-stranded DNA breaks, which may be connected with the formation of free radicals or the inhibition of DNA-topoisomerases [93, 94].

Dactinomycin acts on rapidly dividing normal and tumor cells; this is one of the most powerful antitumor drugs. In test animals, it leads to atrophy of thymus, spleen and other lymphoid organs. Dactinomycin causes alopecia. Contact with tissues leads to a pronounced inflammatory reaction. Sometimes erythema and even necrosis may develop on the skin areas irradiated before, during or after treatment with dactinomycin.

Anthracyclines and their derivatives belong to the most powerful antitumor drugs. Daunorubicin and doxorubicin are isolated from *Streptomyces peucetius caesius* cultures, idarubicin is produced synthetically. These products don't differ significantly n the structure, but daunorubicin and idarubicin are mostly administered for acute leukemia, while the activity range of doxorubicin is broader and includes many solid tumors. A significant drawback of anthracyclines is their cardiotoxicity, resulting in the development of dilatation cardiomyopathy that is frequently irreversible [3, 95, 96]. This atypical for antitumor drugs side effect depends on the total dose of the drug. Hundreds of anthracyclines and close for the structure substances have been synthesized in search for more active and less cardiotoxic drugs. Some of them have been clinically employed: idarubicin for leukemia, epirubicin for solid tumor, mitoxantrone for prostate cancer, leukemia and high-dose chemotherapy [97, 98].

Anthracyclines contain a tetracyclic core and daunosamine monosaccharide connected to the nucleus through a glycosidic bond. Due to the conjugated quinone and hydroquinone rings, it is easily oxidized and reduced. Doxorubicin and daunorubicin vary strongly in the activity range, but their formulas differ only by one hydroxy group at the C-14 atom.

Anthracyclines and their analogues wedge into the DNA molecule causing disruption of replication and transcription, single and double stranded DNA breaks, sister chromatid exchanges, having thereby a mutagenic and carcinogenic action on the cell. DNA breaks occur at the expense of binding of antitumor drugs to a complex consisting of DNA-topoisomerase II and DNA, which prevents the elimination of DNA breaks formed by the enzyme. Due to quinone groups, anthracyclines form free radicals in solution, as well as in normal and tumor cells. In the presence of NADPH they are reduced by NADPH-oxidase into unstable semiquinones which rapidly react with oxygen to produce superoxide radicals. The latter can transform into hydrogen peroxide and hydroxyl radicals, which oxidize the nitrogenous bases of DNA. The production of free radicals is significantly accelerated by the interaction of doxorubicin with iron ions. Besides, intramolecular transfer of electrons in semiquinone radicals contributes to formation of lipid peroxides, nitric oxide and other highly reactive compounds. Enzymes, in particular superoxide dismutase and catalase, play an important role in protecting cells from the toxic effects of anthracyclines. The action of these enzymes is enhanced by exogenous antioxidants (a-coferol) and the iron-binding substances, for example, dexrasoxane, a product that diminishes cardiotoxicity of anthracyclines. Finally, anthracyclines react with the cell membrane, disrupting its function at the expense of the formation of lipid peroxides, this mechanism may be important for both antitumor activity and cardiotoxicity of these drugs [99].

Anthracyclines initiate apoptosis through activation of the p53 protein and caspas (intracellular proteases); ceramide (a breakdown product of sfingolipids) and CD95 receptor (Fas protein) are also involved in this process.

As mentioned before, under the action of anthracyclines tumor cells may acquire multiresistance. It is caused by the amplification of the ABCB1 gene, which encodes P-glycoproteid removing these and other antitumor drugs from the cell. Anthracylines are also removed from the cell by carrier protein of the MRP family. Additional resistance mechanisms include enhancement of glutathione peroxidase activity and decrease in the DNA-topoisomerase II activity [100].

Daunorubicin is quite effective in the cases of acute lymphopaecosis and myeloid leukemia. The combination of daunorubicin or idarubicin with cytarabine is the principle chemotherapy regimen for scheme acute myeloid leukemia. Daunorubicin and adarubiin cause inhibition of blood formation, stomatitis, alopecia, lesion of gastrointestinal tract and skin. A particular side effect of these drugs is myocardial damage accompanied by the development of tachycardia, arrhythmia, dyspnea, arterial hypotension, pericardial effusion and cardiac insufficiency resistant to digoxin.

Doxorubicin is indicated for lymphomas and acute leukemia, but, in contrast to doxorubicin it is also active against solid tumors, primarily, breast cancer. In combination with cyclophosphamide, vincristine, procarbosine and other drugs, it contributes to healing of lymphogranulomatosis and lymphoma. Doxorubicin is an important component in the chemotherapy regimens for breast cancer and small cell lung cancer. This drug is active in sarcomas, as well as cancer of the body and cervix, prostate cancer, tumors of the head and neck, tumors of the testis and myeloma [101, 102, 103].

Bleomycins are a group of antibiotics damaging DNA. Umezawa and col. first isolated them from the Streptomycesverticillus culture. Bleomycin contains a mixture of bleomycins A2 and B2, which are copper binding glycopeptides. Bleomycins differ only in the structure of end carboxamide, which can be modified through supplementing the nutrient medium with different amines.

Bleomycins attracted interest for their high activity in squamous cell carcinoma of the lung, cervical cancer, head and neck tumors, as well as lymphomas and testicular tumors. These drugs have a slight inhibitory action on blood formation and immune system, but cause unusual lesions to skin and lungs. Since bleomycins differ from other antitumor drugs in terms of toxicity and mechanism of action, they play an important role in polychemotherapy.

Bleomycins are water-soluble major glycopeptides containing a pyrimidine nucleus coupled with propionamide, p-aminoalaninamide, L-glucose, and 3-O-carbamoyl-O-mannose. This structure binds metal ions in a qui-molar ratio. A tripeptide chain, two thiazole rings and a substituted carboxamide (a DNA binding fragment) are sequentially attached to the nucleus [104].

Bleomycin has a number of interesting biochemical properties, but its cytotoxic effect is associated with the oxidation of deoxyribose of cTMP and other nucleotides with the formation of single and double-stranded DNA breaks. *In vitro*, bleomycin causes cell retention in the G2 phase, and chromosomal aberrations including breaks, deletions, fragmentation, and translocations occur in many of them. Activation of bleomycin can also occur with the participation of the flavoproteid NADPH oxidase. Another part of the bleomycin molecule binds to DNA; thus, the resulting free radicals oxidize deoxyribose, breaking DNA chains. The main side effect of bleomycin is lung damage, including life-threatening pneumosclerosis [105].

In 1958, Wakaki et al. isolated mitomycin (mitomycin C) from Streptomyces caespitosus culture. Mitomycin is activated in the cell through enzymatic or spontaneous reduction of the quinone ring and the cleavage of the methoxy group and becomes a mono-or bifunctional alkylating agent. *In vitro*, recovery was observed mainly under conditions of hypoxia.

Mitomycin forms DNA cross-links between the N-6 adenine atom and 0-6 or N-7 guanine atoms, disrupts replication, and causes single and double-stranded DNA breaks. It has a strong radiosensitizing effect, and in rodents it has a teratogenic and carcinogenic effect. Stability mechanisms include impaired activation, inactivation of the reduced quinone ring and excretion from the cell by the action of a P-glycoproteid [106, 107].

Various combinations of mitomycin with fluorouracil, cisplatin and doxorubicin are employed for cancer of the cervix, stomach, colon, breast, bladder, lung, and head and neck tumors [108].

1.5.2. Synthetic antitumor drugs, their mechanism of action

Alkylating agents are substances the molecule of which contain chloroethylamine, ethyleneimine, epoxy groups or methanesulfonic acid residues, and constitute the most extensive group of antitumor agents. Currently, five main subclasses of alkylating agents are used: (1) chloroethylamines, (2) ethylenimines, (3) alkyl sulfonates, (4) nitrosourea derivatives and (5) triazenes.

All alkylating agents have strong electrophilic groups which, through the formation of carbcations or transition complexes, interact with nucleophilic groups to form a covalent bond. The cytotoxic effect of these drugs is directly related to DNA alkylation. Nitrogen base atoms (especially *N*-7 guanine, *N*-1 and *N*-3 adenine, *N*-3 cytosine and 0-6 guanine) are alkylated, as well as phosphate groups of DNA, amino groups and sulfhydryl groups of proteins [109].

The main pharmacological effect of alkylating agents is a disturbance of DNA replication and cell division. The ability of these drugs to damage DNA in rapidly dividing cells explains both their antitumor effects and many side effects. Tissues with a high proliferative index are primarily affected, but some drugs damage those tissues where this index is small (liver, kidneys), as well as mature lymphocytes. Although resting cells are also alkylated, cytotoxicity increases dramatically if DNA is damaged during the preparation of the cell for division. If the reparation systems manage to repair DNA damage before division begins, then cell death does not occur.

Unlike many other antitumor drugs, alkylating agents act on cells in all phases of the cell cycle. Nevertheless, this action is usually manifested when the cell enters the S phase - the cell cycle stops. In a synchronized cell culture, it can be found that cells are slightly more sensitive to chloroethylamines at the end of the G1 phase and in the S phase than in mitosis, in the G2 phase and at the beginning of the G1 phase. The fact is that unpaired nucleotides are more susceptible

to alkylation than nucleotides in helical DNA, and DNA strands in individual regions are unlaced during replication.

The mechanism of cell death during DNA alkylation has not been studied in detail. It was shown that in normal cells of the bone marrow and gastrointestinal mucosa, DNA damage causes the cell cycle to stop at the boundary of the G1 and S phases, after which DNA repair occurs or, if the DNA is damaged too much, apoptosis develops. The p53 protein controls these processes, and with mutation or deletion of the TP53 gene, tumor cells avoid apoptosis and continue to proliferate (Fisher, 1994).

Although DNA is the primary target of all alkylating agents, it should be mentioned that there are important differences between bifunctional and monofunctional drugs. The former develop cross-links of DNA chains and have mainly a cytotoxic effect, while the latter (procarbazine, temozolomide) methylate DNA and, along with cytotoxicity, exhibit pronounced mutagenicity and carcinogenicity. Obviously, crosslinking of DNA chains is more detrimental to a cell than methylation of individual purine bases with their subsequent cleavage and breaking of the DNA chain. Methylation is compatible with cell life, but it is fraught with mutations that, transmitted to daughter cells, can cause malignant degeneration [110].

Most cells are capable of DNA repair; apparently, repair plays an important role in the low sensitivity of dormant cells to alkylating agents, in the selectivity of the action of these agents on various types of cells, and in the development of resistance. Alkylation of one DNA strand is usually eliminated quite easily, but chain crosslinking, which occurs, for example, under the action of chloroethylamines, requires more complex repair systems. Against the background of low doses of these drugs, the cell can eliminate some of the cross-links, but with an increase in the dose, their number increases dramatically, causing DNA breakdown. Repair enzymes were found(Matijasevic et al., 1993), that cleave alkyl groups from the 0-6 guanine atom (methylguanine DNA methyltransferase), the N-3 adenine and N-7 atoms of guanine (3-methyladenine DNA glycosylase). The high activity of methylguanine DNA methyltransferase provides the cell with resistance to nitrosourea derivatives and triazenes (Pegg, 1990).

Antimetabolites compete with metabolites and lead to disruption of the activity of tumor cells. Methotrexate - an antagonist of folic acid, which is its chemical analogue; mercaptopurine - an analogue of purine; fluorouracil, fluoroafur and cytarabine, that are pyrimidine analogues present the greatest interest [111].

Methotrexate forms a strong complex with dihydrofolate reductase and thereby blocks the recovery of dihydrofolic acid into its tetrahydroform and its further conversion to formide tetrafolic acid (leucovorin, citrovorum factor) and other metabolically active derivatives of folic

acid involved in the synthesis of purines, methine, thymine, thymine histidine. Previously used aminopterin also belongs to antifolium products. Methotrexate, therefore, inhibits the synthesis of DNA, RNA, thymidylates, and proteins [112, 113, 114]. Previously used aminopterin also belongs to antifolium products. A specific feature of the antitumor effect of other antimetabolites, which are analogues of purines and pyrimidines, is that they turn into abnormal biologically active nucleotides, leading to metabolic disorders.

Mercaptopurine inhibits purine nucleotide synthesis and metabolism by inhibiting an enzyme called phosphoribosyl pyrophosphate amidotransferase. Since this enzyme is the rate limiting factor for purine synthesis, this alters the synthesis and function of RNA and DNA. Mercaptopurine interferes with nucleotide interconversion and glycoprotein synthesis [115, 116].

Fluorouracil inhibits the process of cell division by blocking the synthesis of DNA and the formation of structurally incompatible RNA [117].

One of the mechanisms of action of cytarabine is its incorporation into DNA during its synthesis, which leads to disruption of this process and to cell death. In addition, cytarabine inhibits the activity of the DNA-polymerase, which inhibits DNA replication [118].

1.5.3. Other synthetic anticancer drugs

Hydroxyurea was synthesized by Dresler and Stein in 1869, but its biological activity was discovered only in 1928, when hydroxyurea turned out to cause leukopenia and megaloblastic anemia in experimental animals. In the 1950s its activity against various tumors of mice, including leukemia and solid tumors was discovered; clinical trials started in the 1960s.

Hydroxyurea belongs to ribonucleoside diphosphate reductase inhibitors. A clear relationship was found between the activity of this enzyme and the growth rate of liver cancer in rats. Ribonucleoside diphosphate reductase is a good target for anticancer drugs, as it catalyzes the reduction of ribonucleotides to deoxyribonucleotides - a limiting reaction in DNA replication. Hydroxyurea inactivates the tyrosine free radical formed in the active center of the enzyme. It acts in the S phase, when the enzyme activity is maximum, and causes the cell cycle to stop at the border of the G1 and S phases. Since the cells in the G1 phase are highly sensitive to radiation, the drug has a radiosensitizing effect. Hydroxyurea can also enhance the effect of DNA damaging drugs: cisplatin, alkylating agents, DNA topoisomerase II inhibitors. But, it is more interesting the increased effect of antimetabolites, especially nucleoside analogues, since a decrease in the supply of deoxyribonucleotides under the influence of hydroxyurea promotes the inclusion of cytarabine, gemcitabine and fludarabine in the DNA. This interaction is also

important for antiretroviral therapy: inhibition of ribonucleoside diphosphate reductase in the host cell facilitates the incorporation of nucleoside reverse transcriptase inhibitors into the viral DNA. It was shown, that hydroxyurea is converted *in vivo* into nitric oxide, thereby enhancing the expression of genes encoding TNF-α, IL-6, p-chain globin and other proteins, as well as accelerating the destruction of double microchromosomes containing numerous copies of genes. However, the clinical significance of these observations is not clear [119, 120].

The main mechanism of resistance to hydroxyurea is an increase in the activity of ribonucleoside diphosphate reductase, for example, due to amplification of the gene encoding it or to enhanced translation. In addition, resistance can be caused by synthesis of an enzyme with reduced affinity for hydroxyurea [121].

The main side effect is inhibition of blood formation (leukopenia, megaloblastic anemia, rarely thrombocytopenia); when the drug is cancelled for several days, these phenomena quickly disappear. Gastrointestinal disorders and mild skin reactions are also possible, stomatitis, alopecia, and neurological disorders are less common.

Platinum compounds. In 1965, Rosenberg and co-workers discovered the cytotoxic effect of platinum complex compounds, showing that the current passed between platinum electrodes immersed in a nutrient medium disrupts the proliferation of Escherichia coli. It soon became clear that this was due to the formation of inorganic compounds of platinum in the presence of chlorine and ammonium ions. The most active of these compounds was cisplatin (*cis*-diamindichloroplatin), which has found a wide clinical application (Rosenberg, 1973). Subsequently, more than 100 different platinum preparations were obtained. One of them, carboplatin was approved by the FDA in 1989 for the treatment of ovarian cancer, while other drugs are being tested. The range of cisplatin activity is very wide, its effect on epithelial tumors being of particular value. It serves as the basis for chemotherapy regimens that can treat testicular tumors; the drug showed high activity in cancer of the ovaries, bladder, esophagus, lungs, as well as tumors of the head and neck.

Cisplatin is a water-soluble inorganic compound of divalent platinum. Clinical trials of other platinum preparations are being carried out, namely tetraplatin, ormiplatin, iproplatin, oxaliplatin; cisplatin-resistant tumors remain sensitive to some of them. Complex compounds of divalent or tetravalent platinum with various organic radicals are more stable and less nephrotoxic than cisplatin, but they have not yet shown a high antitumor activity. In the carboplatin molecule, platinum is also bound to a complex organic radical [122].

The chlorine atoms contained in cisplatin are easily replaced by nucleophiles, for example, thiols or water. In the latter case, the molecule acquires a positive charge, due to which,

apparently, the drug is activated, which then reacts with nucleic acids and proteins. Hydrolysis is promoted by a low concentration of chlorine ions. Otherwise, cisplatin stabilizes, so the creation of a high concentration of chlorine ions in the urine prevents the toxic effect of the drug on the kidneys. Carboplatin hydrolysis is slower than that of cisplatin, while the dibasic butane dicarboxylic acid is cleaved off. Reacting with DNA, primarily with the N-7 atom of guanine, platinum products form cross-links in the same DNA chain between neighboring guanines or between guanine and adenine. Crosslinking between chains occurs slower and in smaller quantities. By binding to DNA, cisplatin disrupts replication and transcription, and also causes mutations and chain breaks. Although a clear relationship between the binding of platinum products to DNA and their activity has not been established, the antitumor effect seems to depend on the ability of cells to form and accumulate platinum complexes with DNA. A quantitative assessment of the cytotoxic effect of these complexes is difficult to make, since it is not easy to determine the role of genetic characteristics and environmental factors acting on normal and tumor cells, and in addition, the role of other antitumor drugs, usually prescribed together with platinum preparations. However, according to experimental studies, crosslinking between guanine and adenine is crucial for cytotoxicity [5].

The activity of cisplatin in different phases of the cell cycle depends on the type of cells, however, crosslinking of DNA chains is manifested primarily in the S phase. Cisplatin possesses a mutagenic, carcinogenic and teratogenic effect. Cases of secondary leukemia are described in particular, treatment of ovarian cancer with cisplatin or carboplatin increases the risk of leukemia by four times.

Cisplatin-resistant test tumors may remain sensitive to its analogues. Resistance to cisplatin and carboplatin is usually cross, but at the same time, sensitivity to oxaliplatin and derivatives of tetravalent platinum remains, which attracts interest to these drugs. Sensitivity to cisplatin is affected by a number of factors - the accumulation of the drug in the tumor cells, the intracellular concentration of glutathione and other thiols, for example metallothionein, which bind and inactivate the drug, as well as the rate of DNA repair. Binding to cisplatin causes the DNA helix to bend, which is recognized by special proteins with highly mobile domains; they are believed to inhibit repair. The complex of cisplatin with DNA is eliminated in the process of excision repair of nucleotides. The repair enzymes recognize the altered nucleotide, remove the damaged fragment of the chain around it and restore the integrity of the DNA chain. Inhibition of excision repair may increase sensitivity to cisplatin.

Obviously, cisplatin resistance is also associated with the repair system of unpaired DNA nucleotides. Proteins entering this system, especially MLH1 and MSH6, probably play an

important role in recognizing platinum-linked nucleotides and triggering apoptosis. Deficiency of these proteins causes resistance to cisplatin *in vitro*. Recognition by the MLH1 protein of the cisplatin DNA complex enhances the synthesis of p73 protein (p53 analog) and AY1 tyrosine kinase, which ultimately leads to apoptosis. With a deficiency of proteins involved in the repair of unpaired nucleotides, or with inability to activate tyrosine kinase A1, cells do not undergo apoptosis under the action of cisplatin.

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Ultrastructural analysis of platinum-based antineoplastic medication showed that cisplatin treated renal tubular cells of mouse kidney, which demonstrates disruption of cristae, and extensive mitochondrial swelling, supporting the involvement of mitochondria in cisplatin induced nephrotoxicity [124, 6]. Moreover, cisplatin considerably impairs kidney mitochondrial bioenergetic functions. Cisplatin inhibits complexes I to IV and decreases intracellular ATP in proximal tubular cells [5, 125].

Thus, cisplatin demonstrates multifactorial resistance and high toxicity for normal cells, which limits its broad application in medical practice. Therefore, it is crucial to develop new transition metal ion-based compounds as potential anti-cancer drugs with low toxicity for non-cancer mammalian cells [126].

Copper thiosemicarbazone complexes. Since copper is biocompatible and displays many important roles in biological systems, its complexes are considered to be promising alternatives as anticancer drugs to platinum ones among the many bio-essential metals. Also, copper promotes the altered metabolism in cancer cells and differential response between normal and tumor ones. The concentration of copper has been demonstrated to be higher in cancerous tissues in comparison with that in normal tissue, and the sequestration of copper may prevent the appearance of new blood vessels [127].

It is known from literature that copper possesses pro-angiogenic properties and it has been found that copper salts and copper extracted from tumors caused migration of endothelial cells, an early step of angiogenesis. It is noteworthy that introducing copper to the cornea of

rabbits induced the devlopment of new blood vessels and copper contributed to increased proliferation of human endothelial cells in the absence of serum and growth factors. On the contrary, copper had little effect on the proliferation of both arterial smooth muscle cells and fibroblasts in humans [128]. Moreover, zinc or iron employed at the same concentration as copper diminished endothelial cell growth [7]. No doubt, the findings have demonstrated that copper is a powerful inducer of the angiogenic process [129].

High copper concentrations in serum are linked with various types of cancers including reticulum cell sarcoma, bronchogenic and laryngeal squamous cell carcinomas, lymphoma, breast, cervical, lung and stomach cancers. Startlingly, increased copper amounts in serum correlated with the phase of the disease and its progression in breast and colorectal cancers. Serum copper levels were clearly linked to drug resistance in patients with advanced breast, lung or colon cancer and in the ones who were administered various chemotherapeutics (e.g. doxorubicin, etoposide or 5-fluorouracil) as single agents or in combination. Non-responders had by about 130–160% more copper in their serum [130, 131]. The observations led to the hypothesis that the copper level in serum may be considered a biomarker of cancer recurrence and may be measured to monitor treatment efficacy. Noteworthy, the levels of iron, zinc and selenium are frequently lower in the serum of cancer patients in contrast to copper. Copper is a redox active metal that may increase the ROS production, which afterwards can damage most biomolecules [132].

The molecular pathways through which copper acts to induce a pro-angiogenic response vary. Copper can directly bind to angiogenin and the angiogenic growth factor, as well as can increase its affinity for endothelial cells [133, 134]. Copper can also regulate the secretion of such angiogenic molecules as FGF and IL-1a. FGF-1 and IL-1a are secreted only after copper-dependent development of a multi-protein complex. The signal sequence for endoplasmic reticulum (ER)-Golgi mediated secretion is absent in FGF-1 and IL-1a. For example, copper deficit inhibits the activity of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB), which in turn lowers expression of five pro-angiogenic mediators (VEGF, bFGF, IL-1a, IL-6 and IL-8) [8]. Copper is also transported into the cells nuclei by the copper chaperone CCS, where it can regulate development of the hypoxia-inducible factor 1 (HIF-1) transcriptional complex and thereby regulate expression of VEGF, a powerful angiogenic factor [135, 136]. Likewise, ATOX1 can enter the cell nuclei as a copper-dependent transcription factor. It has been shown to regulate platelet-derived growth factor (PDGF) signaling and, hence, potentially malignant angiogenesis and vascular remodeling [137, 138].

Indeed, studies have demonstrated that copper chelation can impede cancer growth and progression, which is compelling evidence that copper is crucial for malignant angiogenesis. An obvious role that copper plays in metastasis consists in regulating angiogenesis, which is a basic process necessary for metastatic potential. However, there is growing evidence that copper has also a direct influence on the capacity of tumor cells to invade and metastasize.

Due to their high antitumor activity and advantages relative to other metal anticancer products, copper-based complexes have been extensively synthesized and investigated as anticancer agents up to the present. As mentioned before, platinum-based anticancer drugs play a major role in the treatment of various malignant tumors, however, their wide range of clinical applications has been limited because of severe side effects such as neurotoxicity, nephrotoxicity, and drug resistance. This has stimulated extensive studies and forced chemists to search for alternative approaches to using endogenous metals with the aim of improving the pharmacological properties. Because copper is biocompatible and exhibits many significant roles in biological systems among many bio-essential metals, its complexes are believed to be promising alternatives to platinum ones as anticancer drugs. Besides, copper is associated with differential response between normal and tumor cells and altered metabolism of cancer cells. Hence, cancer cells can be chosen as a suitable, selective target for copper-based agents. It has been reported recently that a large number of synthetic copper(II) complexes of thiosemicarbazones ligands act as pharmacological agents and as potential anticancer and cancer-inhibiting agents, and they turned out to be active both *in vitro* and *in vivo* [139, 140, 141].

Thiosemicarbazone is a class of organic compounds that possesses a broad range of biological activities and medical properties. Thiosemicarbazones can form coordination compounds with transition metal ions since they contain a wide spectrum of donor atoms [142]. Thiosemicarbazones are produced through a condensation reaction of thiosemicarbazide and aldehydesor ketones. Pharmacological studies have shown that thiosemicarbazones are characterized by various biological activities. Thiosemicarbazones contain nitrogen and sulphur atoms with powerful coordination capacity, and are able to form various complexes with new structures [143]. Metal complexes are produced from thiosemicarbazone ligands and exhibit high anticancer activity [144, 145]. Nowadays, thiosemicarbazide derived copper complexes have become a potential research hotspot. Copper thiosemicarbazone complexes may provide reactive oxygen species and act against topoisomerase II, DNA, and other potential targets in the nucleus [146]. Therefore, the copper thiosemicarbazone complex is a promising candidate for creating ideal non-platinum and multi-target antitumor drugs.

1.6. Conclusions to chapter 1

1. Since cancer represents one of the most serious health problems and major causes of death around the world, the development of novel efficient and safe products has become a hotspot in the chemotherapy of tumor diseases.

2. Despite the significant progress made in the anticancer therapy, high systemic toxicity and drug resistance remain a major challenge for modern medicine in the management of cancer. Chemotherapy can produce severe side effects caused by its cytotoxic effect on normal cells, and this limits its use.

3. 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.

4. Doxorubicin causes toxic damage to the mitochondria of cardiomyocytes contributing to enhanced oxidative stress.

5. Platinum-based anticancer drugs play a leading role in the treatment of various malignant tumors, but severe side effects such as nephrotoxicity, neurotoxicity, and drug resistance have limited their wide range of clinical applications.

6. Among the many bio-essential metals, copper complexes are regarded as promising alternatives to platinum complexes as anticancer drugs because copper is biocompatible and exhibits many significant roles in biological systems.

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.

2.1. Characterization of the tested compounds

In this work anticancer activity of tiosemicarbazones and Cu(lI) coordination compounds with tiosemicarbazones is described. 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) [147], 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.

The thiosemicarbazone CMT-22 (Figure 2.1. A), and copper (II) complex CMT-67 (Figure 2.1. B), were synthesized as described in the literature [148].

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 [148].

The copper (II) mixed-ligand complex CMT-68 (Figure 2.2.) was synthesized by reaction between 2-formylpyridine*N* (4)-phenylthiosemicarbazone (CMT-22) with $CuCl_2 \cdot 2H_2O$ and 4-aminobenzenesulfonamide (Str) [149, 150].

Biological activities of the tested compounds were compared with the Food and Drug Administration (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) [151], cicplatin (*cis*-dichlorodiammineplatinum) [5] and the used in biological or biochemical applications antioxidants such as trolox (6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), rutin (2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-3-[α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranosyloxy]-4*H*-chromen-4-one) (Figure 2.3. A, B, C, D).

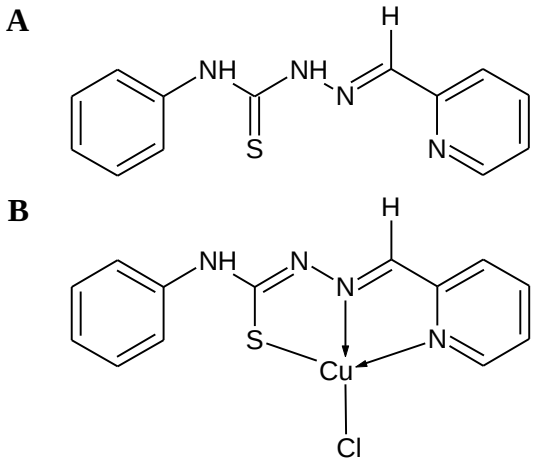


Fig. 2.1. Structural formula of the 2-formylpyridine *N*(4)-phenylthiosemicarbazone (*N*-phenyl-2-(pyridin-2-ylmethylidene)hydrazinecarbothioamide, CMT-22, HL) (A) and copper(II) complex [Cu(L)Cl] (B).

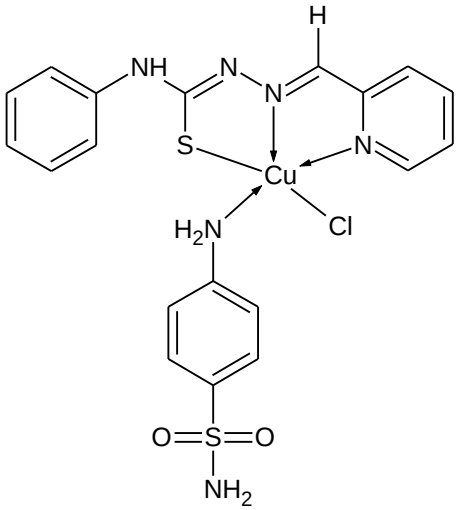


Fig. 2.2. Structural formula of the mixed-ligand complex chloro(*N*-phenyl-*N*'-[(pyridin-2-yl)methylidene]carbamohydrazonothioato)(4-aminobenzene-1-sulfonamide)copper.

The tested compounds and the reference controls were each dissolved in dimethylsulfoxide (DMSO) to create 10 mM stock solutions that were stored at a prescribed temperature of 7°C. The stock solutions were further diluted with cell culture medium or physiological saline solution to appropriate concentrations before use. The maximum final concentration of DMSO (<0.1%) did not affect cell proliferation and did not induce cytotoxicity on the tested cell lines.

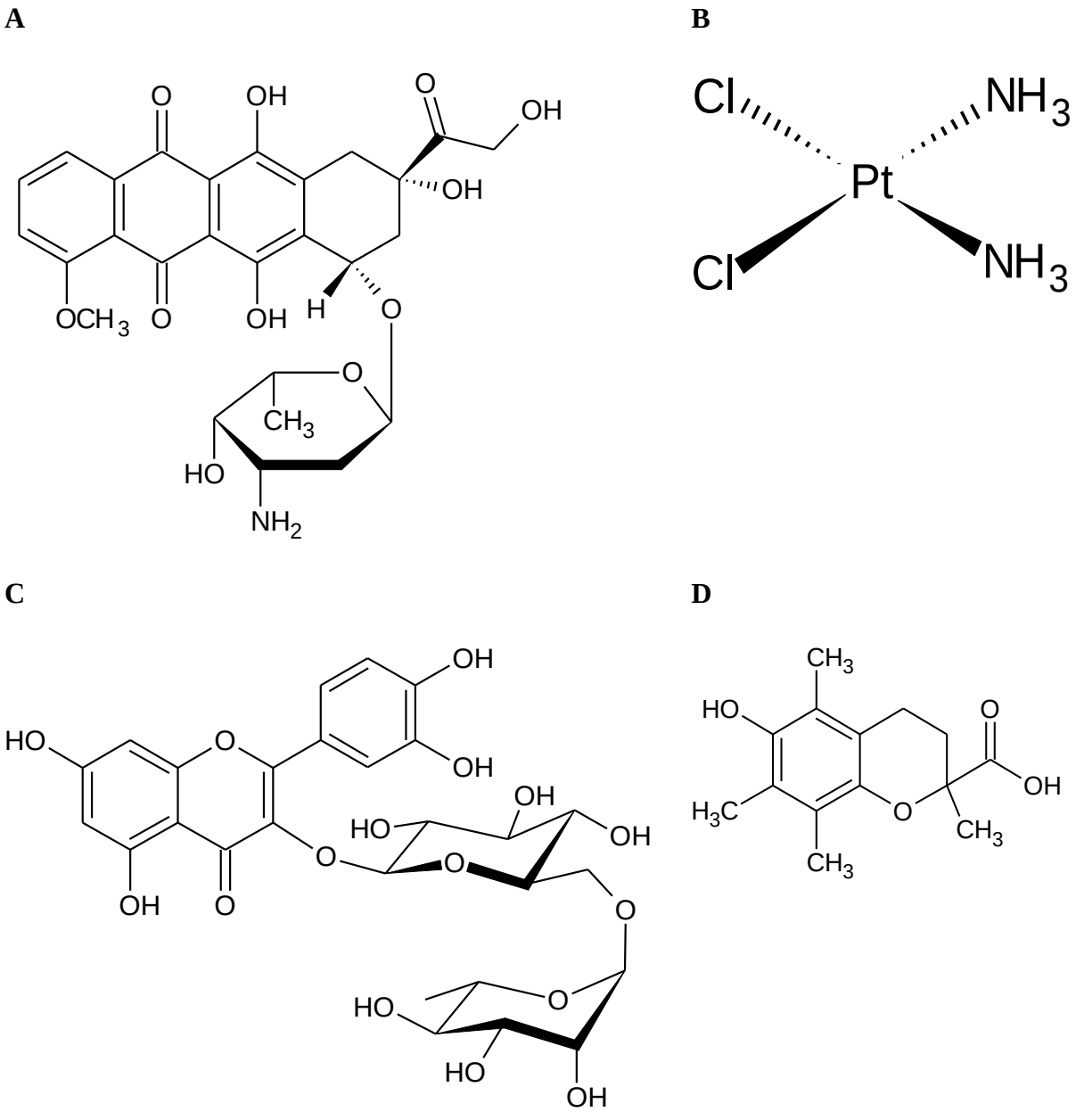


Fig. 2.3. Structural formula of the reference compounds:
A – doxorubicin; B – cicplatin; C – rutin; D – trolox

2.2. *In vitro* antiproliferative activity

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.

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 temperatures ranging from −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) (Figure 2.4).

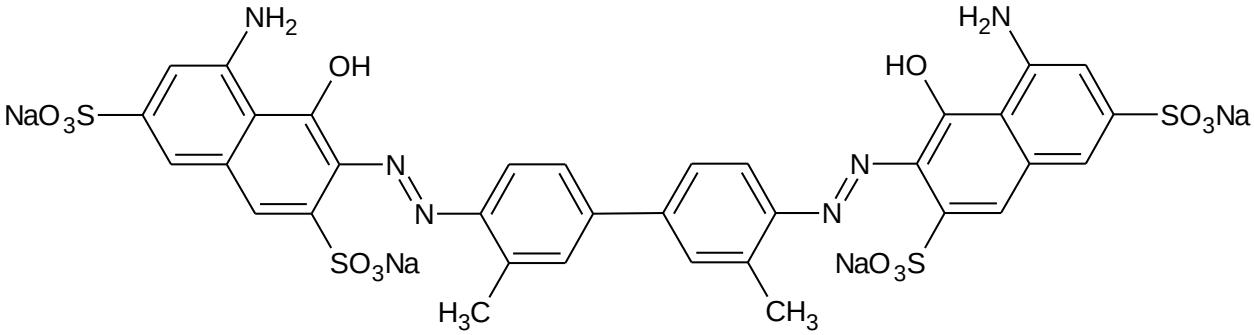


Fig. 2.4. Structural formula of the trypan blue ((3Z,3'Z)-3,3'-[(3,3'-dimethylbiphenyl-4,4'-diyl) di(1Z) hydrazin-2-yl-1-ylidene] bis (5-amino-4-oxo-3,4-dihydronaphthalene-2,7-disulfonic acid)).

2.2.1. Cell culture

In order to study, human melanoma MEW-164 cell line, human cervical epithelial HeLa cell line, human epithelial pancreatic adenocarcinoma BxPC-3 cell line, human muscle rhabdomyosarcoma RD cell line and normal kidney epithelial MDCK cell line were used. All cells were tested in the middle log – phase of growth. The log – phase (sometimes called the logarithmic phase or the exponential phase) is a period characterized by cell doubling. Cells culture doubling continues at a constant rate so both the number of cells increase doubles with each consecutive time period. For this type of exponential growth, plotting the natural logarithm of cell number against time produces a straight line. The slope of this line is the specific growth rate of the cell, which is a measure of the number of divisions per cell per unit time. The actual rate of this growth depends upon the growth conditions, which affect the frequency of cell division events and the probability of both daughter cells surviving. Exponential growth cannot continue indefinitely, however, because the medium is soon depleted of nutrients and enriched with wastes.

Human melanoma cell line MeW-164 culture conditions.

Human melanoma cell line MeW-164, passage 8 was used (Figure 2.5). 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 [152].

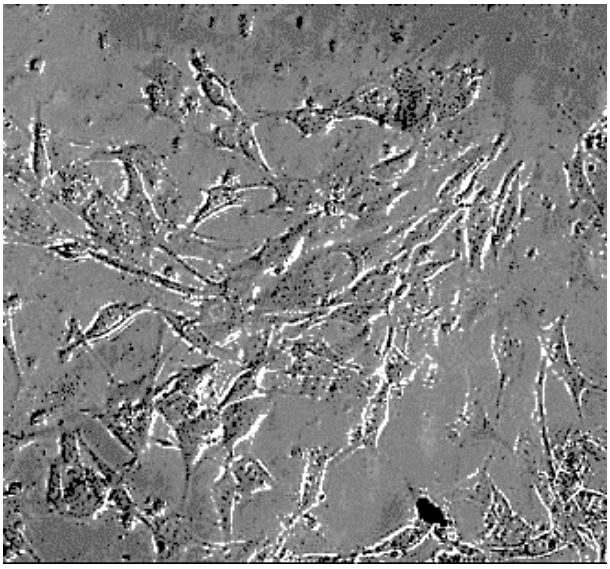


Fig. 2.5. Monolayer culture of human melanoma MEW-164 cell line.

Human melanoma cells were grown in suspension in minimum essential medium (MEM) (Biomed-Lublin) 90% v/v containing L-glutamine and supplemented with foetal bovine serum

(FBS) (Invitrogen) 10% v/v and penicillin/streptomycin 1% v/v in 75 cm^2 cell culture flasks (CellStar) and incubated at 37^0C in a 5% CO_2 humidified atmosphere, with fresh culture medium changes occurring every 2 to 3 days.

Human cervical epithelial cell lines HeLa (ATCC CCL-2) and HEp-2 (ATCC CCL-23) culture conditions.

The HeLa line is an immortal cell line used in scientific research. It is the oldest and most commonly used human cell line. The HeLa line was derived from cervical cancer cells taken on February 8, 1951 from Henrietta Lacks. HeLa cells have been reported to contain human papilloma virus 18 (HPV-18) sequences. P53 expression was reported to be low. Epithelioid cervix carcinoma cell line HeLa, passages 7-8 were used (Figure 2.6). Human epithelial cell line HEp-2 contain HeLa marker chromosomes, and were derived by 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.

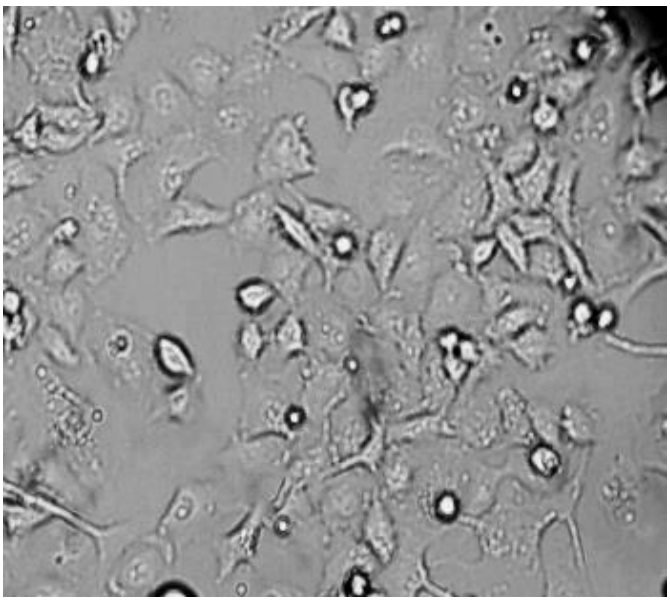


Fig. 2.6. Monolayer culture of human cervical epithelial HeLa cell line.

The HeLa and HEp-2 cells were cultured as monolayer in Dulbecco's Modified Eagle Medium (DMEM) high glucose (Invitrogen) containing L-glutamine, bovine albumin fraction (V 7.5%) 0.2% v/v (Invitrogen), buffer (*N*-2 hydroxyethylpiperazine-*N*'-2-ethane sulfonic acid) (HEPES) 20mM (Invitrogen), antibiotics penicillin-streptomycin (final concentration penicillin 100 U/mL and streptomycin sulfate100 μg/mL) (Invitrogen) and supplemented with FBS-

irradiated 10% v/v (Cambrex) in 75 cm^2 falcon culture flasks (CellStar) and incubated in 5% carbon dioxide (CO_2), 78% air in humidified atmosphere at 37^0C. The culture medium was changed every 2-3 days.

The cell layer was briefly rinsed with trypsin 0.25% w/v and ethylenediaminetetraacetic acid (EDTA) 0.53 mM solution to remove all traces of serum which contains trypsin inhibitor. After that, 2.0 mL of trypsin-EDTA solution was added to the flask and the cells were observed under an inverted microscope until cell layer was dispersed usually within 5 to 15 min. In order to avoid the formation of cell aggregates, 8.0 mL of complete growth medium Dulbecco's Modified Eagle Medium (DMEM) and aspirate cells were added by gently pipetting, and appropriate aliquots of the cell suspension was added to new falcon culture flasks. Viability of cells was assessed by dye exclusion assay with trypan blue (Euroclone) 0.2%.

Human epithelial pancreatic adenocarcinoma cell line BxPC-3 (ATCC CRL-1687) culture conditions.

The BxPC-3 line is a human pancreatic cancer cell line used in the study of pancreatic adenocarcinomas and treatments thereof. BxPC-3 cells were derived from a 61-year-old female. The cells produce mucin, and exhibit an epithelial morphology. Human epithelial pancreatic adenocarcinoma cell of line BxPC-3, passage 8 was used (Figure 2.7).



Fig. 2.7. Monolayer culture of human epithelial pancreatic adenocarcinoma BxPC-3 cell line.

Cell line BxPC-3 was cultured as monolayer in Roswell Park Memorial Institute (RPMI) 1640 medium (ATCC), supplemented with fetal bovine serum (ATCC) 10% v/v and

antibiotics penicillin-streptomycin (final concentration penicillin 100 U/mL and streptomycin sulfate100 μg/mL). Cells were grown in 75-cm^2 cell culture flasks (Thermo Scientific) at 37°C and 5% CO_2, with fresh culture medium changes occurring every 2 to 3 days.

The cell layer was briefly rinsed with Trypsin 0.25% w/v and EDTA 0.53 mM solution to remove all traces of serum which contains trypsin inhibitor. Then, 2.0 mL of trypsin-EDTA solution was added to the flask and the cells were observed under an inverted microscope until cell layer is dispersed (usually within 3 to 5 min). In order to avoid the formation of cell aggregates, 8.0 mL of complete growth medium RPMI-1640 and aspirate cells were added by gently pipetting, and appropriate aliquots of the cell suspension were added to new cell culture flasks. Viability of cells was assessed by dye exclusion assay with trypan blue 0.2% (Euroclone).

Human muscle rhabdomyosarcoma cell line RD (ATCC CCL-136) culture conditions.

The RD (rhabdomyosarcoma) cells were derived directly from biopsy specimens of a 7-year-old female with a pelvic rhabdomyosarcoma stages (RMS) previously treated with cyclophosphamide and radiation and found to have refractory disease.

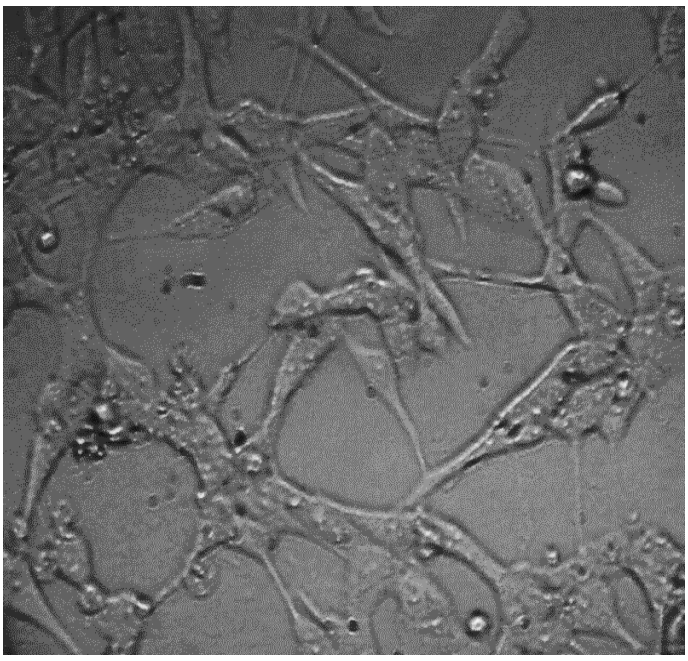


Fig. 2.8. Monolayer culture of human muscle rhabdomyosarcoma RD cell line.

Human muscle rhabdomyosarcoma spindle and large multinucleated cell line RD, passage 8 was used (Figure 2.8). Cells were cultured and grown in the Dulbecco's Modified Essential Medium (Invitrogen) with 4 mM L-glutamine, glucose 4.5 g/L, bovine albumin fraction 0.2% v/v, HEPES buffer 20 mM, antibiotics penicillin-streptomycin (final concentration

penicillin 100 U/mL and streptomycin sulfate 100 μg/mL) and supplemented with heat-inactivated FBS 10% v/v. Cells were maintained at 37ºC in a humidified 5% CO_2 atmosphere in the incubator in 25-cm^2 cell culture flasks (culture dishes). The culture medium was changed every 3 days.

The cell layer was briefly rinsed with trypsin 0.25% w/v and EDTA 0.53 mM solution to remove all traces of serum which contains trypsin inhibitor. After that, 2.0 to 3.0 ml of trypsin-EDTA solution was added to flask and the cells were observed under an inverted microscope until cell layer is dispersed (usually within 5 to 10 min). In order to avoid the formation of cell aggregates, 8.0 ml of complete growth medium and aspirate cells were added by gently pipetting, and appropriate aliquots of the cell suspension were added to new cell culture flasks. Viability of cells was assessed by dye exclusion assay with trypan blue 0.2%.

Culture conditions for normal kidney epithelial cell line MDCK (ATCC CCL-34).

The MDCK cell line was derived from a kidney of an apparently normal adult female cocker spaniel, 1958, by S.H. Madin and N.B. Darby.

Normal kidney epithelial cells MDCK were maintained at passage numbers 7–8 (Figure 2.9) in the (DMEM) high glucose (Invitrogen) containing L-glutamine, bovine albumin fraction (V 7.5%) 0,2% v/v (Invitrogen), HEPES buffer (N-2 hydroxyethylpiperazine-N'-2-ethane sulfonic acid) 20mM (Invitrogen), antibiotics penicillin-streptomycin (final concentration penicillin 100 U/ml and streptomycin sulfate100 μg/ml) (Invitrogen) and supplemented with fetal bovine serum (irradiated) 10% v/v (Cambrex) in 75-cm^2 culture flasks (CellStar) and incubated in 2% CO_2, 78% of air in humidified atmosphere at 37°C, with medium renewal every 2–3 days.

The cell layer was briefly rinsed with Trypsin 0.25% w/v and EDTA 0.53 mM solution to remove all traces of serum which contains trypsin inhibitor. After that, 3.0 ml of trypsin-EDTA solution was added to flask and the cells were observed under an inverted microscope until cell layer is dispersed (usually within 10 to 15 min). In order to avoid the formation of cell aggregates, 8.0 ml of complete growth medium (DMEM) and aspirate cells were added by gently pipetting, and appropriate aliquots of the cell suspension were added to new cell culture flasks. Cells in logarithmic growth phase were used for experiments. Viability of cells was assessed by trypan blue 0.2%.

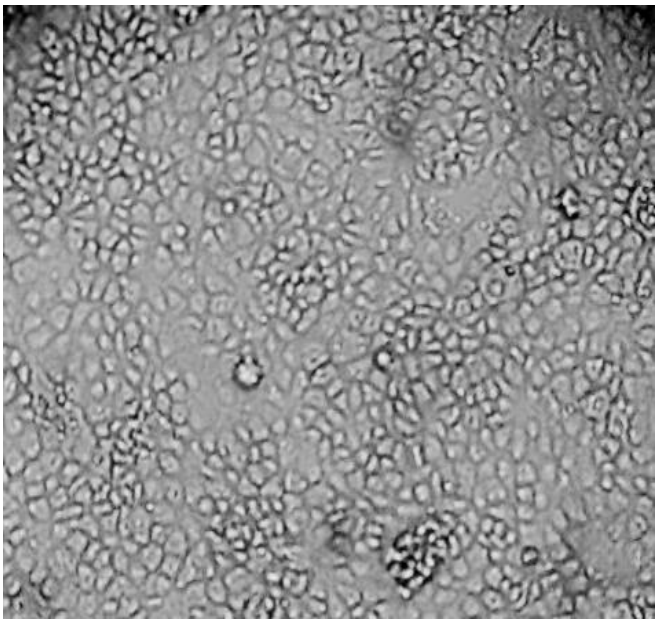


Fig. 2.9. Monolayer culture of normal kidney epithelial cells of MDCK line.

2.2.2. Seeding cells on plate

Taking the culture flasks from the CO_2 incubator, the culture medium was removed. The cells were washed twice with a warm trypsin-EDTA 0.25% disintegrator with a volume of 2 ml. With the residual volume of the disintegrator, the flask was placed in a CO_2 - incubator for 7 minutes.

A **B**

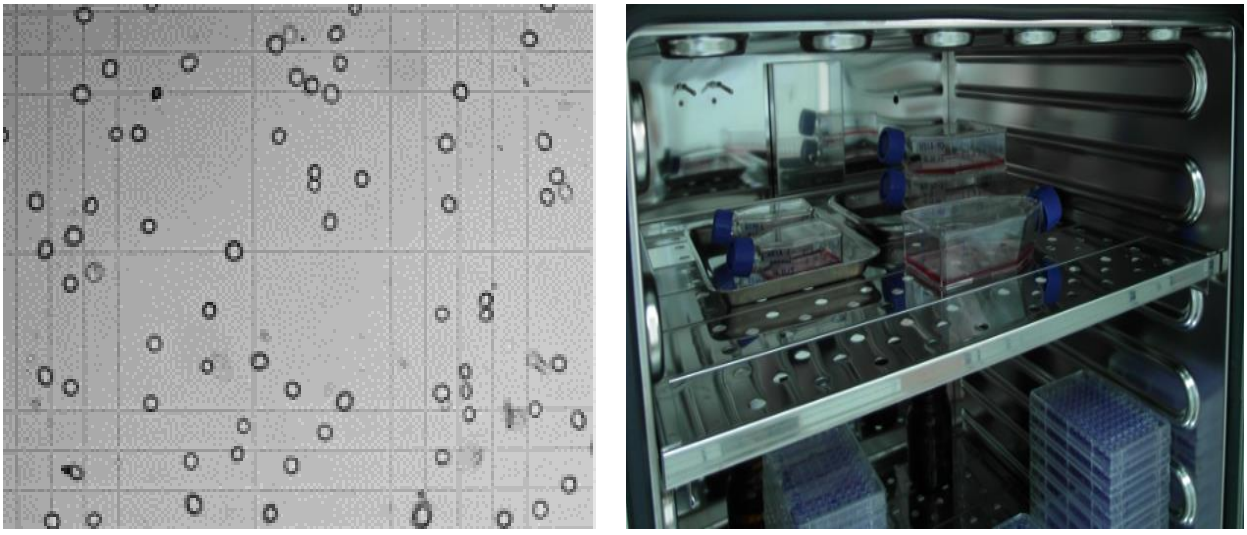


Fig. 2.10. Seeding cells on the culture plate.

A: A Goryaev's chamber with cell suspension.

B: Cultivation of the tested cell lines in a CO_2 – incubator.

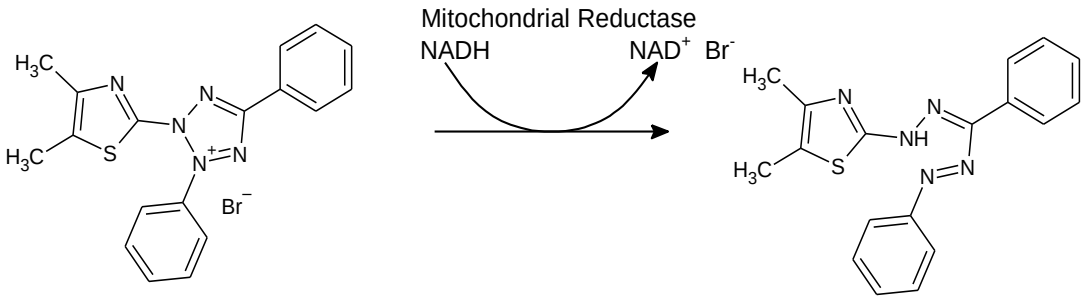
The detached cell monolayer was transferred to a centrifuge tube with suspension for centrifugation for 5 minutes at 1000 rpm, after which the supernatant was removed. After resuspending the pellet in 2 ml of complete nutrient medium, the number of cells was calculated using the Goryaev chamber (Figure 2.10. A).

Preparing a cell suspension (1 million cells in 10 ml complete nutrient medium), the cells were seeded in a 96-well plate (100 μL of cell suspension per well) and placed in a CO_2 - incubator (Figure 2.10. B).

2.2.3. Cell Proliferation MTT Assay

The MTT assay is used to measure cellular metabolic activity as an indicator of cell viability, proliferation and cytotoxicity. It was used to determine the possible cytotoxic effect of the tested compounds on cancer cell line MeW-164.

This colorimetric assay is based on the reduction of a yellow tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide or methyl thiazole tetrazolium bromide (MTT) to purple formazan crystals by metabolically active cells (Scheme 2.1). The viable cells contain NAD(P)H-dependent oxidoreductase enzymes which reduce the methyl thiazole tetrazolium bromide to formazan. The insoluble formazan crystals are dissolved using a solubilization solution and the resulting colored solution is quantified by measuring absorbance using a multi-well spectrophotometer. The darker the solution, the greater the number of viable, metabolically active cells.



3-(4,5-dimethylthiazol-2-yl)-3,5-diphenyltetrazolium bromide

(MTT)

(*E*,*Z*)-5-(4,5-dimethylthiazol-2-yl)-1,3-diphenylformazan

(Formazan)

Scheme 2.1. Chemical scheme of the metabolism of yellow MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and purple formazan product in living cells.

The functional activity there of is interpreted as a measure of cellular viability. It is important to mention that mitochondria belonging to dead cells are not able to respire. Determination of the ability of cells to reduce MTT to formazan after exposure to a compound allows obtaining information about the toxicity of the compound being evaluated.

The tested compounds were dissolved in a physiological saline solution and DMSO, in which DMSO concentration did not exceed 0.1%. The experiment was performed according to the method described by Mosmann [153].

Suspension cells were harvested, using centrifugation method (MPW 370 centrifuge). Adherent cells were released from their substrate by trypsinization (EDTA 0.53mM, Lonza, Belgium). The dilutions of cells $5x10^4$ cells per ml were prepared in culture medium (RPMI 1640 medium 90% (Sigma), containing fetal bovine serum 10% (Invitrogen)). $5x10^3$ cells/100 μL of the dilutions were plated out in 96 wells of a microtiter plate. All determinations were done in triplicate. The cells were incubated for 2-3 hours. The tested compounds 10 μL at three concentrations of 0.1, 1 and 10 μM (three tests were performed for each concentration) were added and incubated for 24 h at 37^0C, 5% CO_2 (SANYO, CO_2 - incubator). After that, 10 μL of MTT reagent was added to each well, including controls (cells without treatment). The plates were returned to cell culture incubator for another 24 h, periodically viewing the cells under an inverted microscope (OLYMPUS CK40) for presence of intra cellular punctuate purple precipitate. When the purple precipitate was clearly visible under the microscope, 100 μL of detergent reagent was added in all wells, including controls. The plates with cover were left in the dark for 4 hours at room temperature. The optical absorbance was measured at 540 nm on a Synergy multi-mode microplate reader (BioTek).

The results were reported as the percentage of cell proliferation inhibition compared to the control (basal cell proliferation = 100%) [154]. Absorbance values greater than the control indicate cell proliferation, while lower values suggest cell death or inhibition of proliferation.

$$\%\ \text{inhibition} = 100 - \frac{\text{Abs}_{540\ \text{nm}(\text{sample})}}{\text{Abs}_{540\ \text{nm}(\text{control})}} \times 100 \qquad (2.1)$$

As an indicator of efficiency of the experimental compounds on proliferation of cancer cells, the half maximal inhibitory concentration (IC_{50}) was used, which is a quantitative indicator of the activity of antagonists test reactions *in vitro* in the pharmacological studies. The IC_{50} values were calculated according to the Hill equation, using the software.

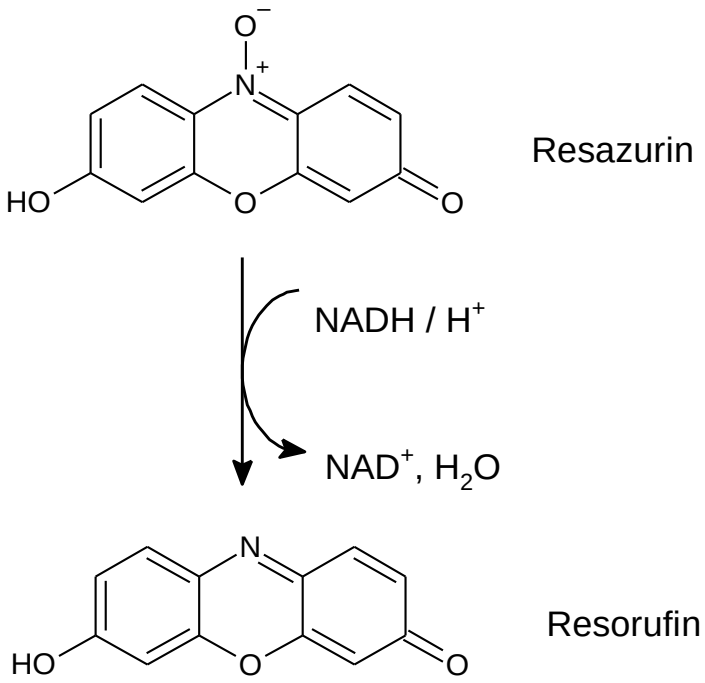
2.2.4. Cell proliferation resazurin assay

Resazurin cell viability assay is a rapid, reliable, sensitive and safe measurement of cell viability [155]. Resazurin detects cell metabolism by converting from a nonfluorescent dye to the highly red fluorescent dye resorufin in response to chemical reduction of growth medium resulting from cell growth. The continued cell growth maintains a reduced environment while inhibition of growth maintains an oxidized environment. The reduction related to growth causes the redox indicator to change from oxidized (nonfluorescent, blue) form to reduced (fluorescent, red) form.

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) (Scheme 2.2)

Cells of lines HeLa, BxPC-3, RD, MDCK 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 (OLYMPUS). 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 1×10^5 cells/ml. The resulting cell suspension was seeded into triplicate wells of a 96-well microtiter plat (Becton Dickinson and Company, Lincoln Park, NJ, USA) (100µL/well) and incubated at 37°C, 5% CO_2. 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% CO_2.

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% CO_2 for 24 h. Following each treatment, 20 µL resazurin indicator solution was added to each well and incubated at 37°C, 5% CO_2 for 4 h.



Scheme 2.2. Reduction of resazurin (7-hydroxy-3H-phenoxazin-3-one-10-oxide sodium salt) to resorufin (7-Hydroxy-3H-phenoxazin-3-one) in living cells with NADH.

Subsequently, the absorbance was read by hybrid reader (Synergy H1, BioTek) with 570 nm and 600 nm filters [156, 157]. The percentage of cell proliferation inhibition was calculated according to the formula:

$$\%\,\text{inhibition} = 100 - \frac{\text{Abs}_{570\,\text{nm}(\text{sample})} - \text{Abs}_{600\,\text{nm}(\text{sample})}}{\text{Abs}_{570\,\text{nm}(\text{control})} - \text{Abs}_{600\,\text{nm}(\text{control})}} \times 100 \qquad (2.2)$$

As an indicator of efficiency of the experimental compounds on proliferation of cancer cells, the half maximal inhibitory concentration was used, which is a quantitative indicator of the activity of antagonists test reactions *in vitro* in the pharmacological studies. The IC_{50} values were calculated according to the Hill equation, using the software.

2.2.5. Cell proliferation assay by flow fluorescence cytometry

The suspension cells MeW-164 were harvested, using centrifugation method (MPW 370 centrifuge). Adherent cells were released from their substrate by trypsinization (EDTA 0.53 mM, Lonza, Belgium). Cells were counted and brought to a concentration of 5×10^4 cells/mL. The resulting cell suspension was seeded into wells of a 24-well plat 0.9 mL/well and incubated at

37°C, 5% CO_2. After an initial 4 hrs period to allow cell attachment, tested compounds were added at final concentrations ranging from 10, 1, 0.1 μM (for each concentration three tests were performed). The cell suspension was treated by lysis buffer for cell lysis and stabilizing buffer for optimizing the pH of the lysed cell suspension. After incubation, the total cells count and viability of cells in suspension were measured by flow cytometry using integrated fluorescence microscope (NucleoCounter NC-100), and nucleo cassettes with fluorescent intercalating dye propidium iodide (3,8-Diamino-5-[3-(diethylmethylammonio)propyl]-6-phenylphenanthridinium diiodide) (Sigma).

Propidium iodide (PI) is a red-fluorescent cell viability dye which is excluded from live cells with intact membranes, but penetrates dead or damaged cells and binds to DNA and RNA by intercalating between the bases. It is widely used as a counterstain to differentiate and exclude non-viable cells in flow cytometric analyses, and can be excited using blue (488 nm), green (532 nm), or yellow-green (561 nm) laser lines, with detection in the FL2 or FL3 channels. PI is used in DNA fluorescence imaging applications to discriminate early and late stages of apoptosis, to study cell-mediated cytotoxicity.

The results from the NucleoCounter represent either total or nonviable cell concentration, depending on the sample preparation. The percent of cell proliferation inhibition was calculated using NucleoView computer software.

2.2.6. DNA fragmentation

DNA fragmentation analysis reveals the ability of the tested compounds to induce apoptosis in cells. It was carried out by the methodology of Kumar et al. [158], where HEp-2 cells (1×10^5) were treated with 10 μM tested compounds for 24 h and then lysed with 250 μl of lysis buffer. After incubation at 37°C for 90min, 200 mg/mL of proteinase K and lithium chloride 0.2% w/v were added and incubated again for 60 min at 50°C. After incubation was over, suspension was centrifuged at 13000 rpm for 3min; the aqueous phase was transferred to fresh tube containing deproteinizing mixture of phenol, chloroform, and isoamyl alcohol (25:24:1) and again centrifuged at 13000 rpm for 3 min. DNA was precipitated from the aqueous phase with 3 volumes of chilled ethanol containing 0.3 M sodium acetate at 4°C. The purity and concentration of nucleic acids were determined by spectrophotometer (ScanDrop) at wavelengths of 260 nm and 280 nm. The samples were subjected to electrophoresis in 0.8% w/v agarose gel using TBE buffer (Sigma) with ethidium bromide (3,8-diamino-5-ethyl-6-

phenylphenanthridinium bromide) (Sigma) (Figure 2.11) at 90V for 35 min and visualised on a UV transilluminator.

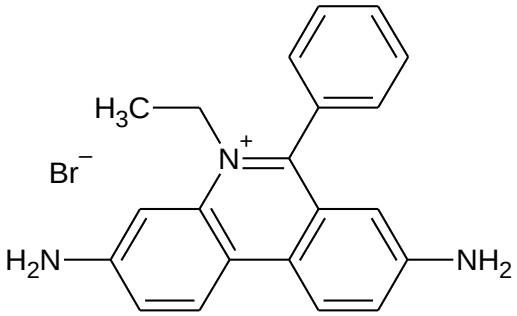


Fig. 2.11. Structural formula of ethidium bromide (3,8-Diamino-5-ethyl-6-phenylphenanthridinium bromide).

2.3. Antioxidant and antilipoxygenase activity assays

2.3.1. ABTS$^{\bullet+}$ radical cation scavenging assay

The antioxidant activity by the ABTS$^{\bullet+}$ method was assessed according to the method described by Re et al [159] with modifications. ABTS$^{\bullet+}$ assay is an excellent tool for determining the antioxidant activity of hydrogen-donating antioxidants and of chain-breaking antioxidants.

The ABTS$^{\bullet+}$ radical was formed through the reaction of ABTS (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)) (Sigma) solution 7 mM with potassium persulfate ($K_2S_2O_8$) (Sigma) 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$^{\bullet+}$ 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$^{\bullet+}$ [160].

The measurement was made by hybrid reader (Synergy H1, BioTek). All tests were performed in triplicate and the obtained results were averaged. The percent of inhibition (I %) of free radical cation production of ABTS$^{\bullet+}$ was calculated by using the following equation:

$$I\ (\%)\ = \frac{Abs_{734\ nm_0} - Abs_{734\ nm_1}}{Abs_{734\ nm_0}} \times 100\ \ \text{, where} \qquad (2.3)$$

$Abs_{734\ nm0}$ is the absorbance of the control solution;

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

The IC_{50} values were calculated according to the Hill equation, using the software.

2.3.2. DPPH• radical scavenging assay

The DPPH• assay was done according to the method of Brand-Williams et al. [161] with some modifications. The effect of antioxidants on DPPH• radical scavenging is due to the hydrogen donating ability or radical scavenging activity of the samples. The scavenging reaction between (DPPH) and an antioxidant (H-D) can be written as: (DPPH) + (H-D) → DPPH-H + (D). The DPPH reagent was diluted with methanolic solution to give an absorbance of 0.7 ± 0.01 at 517 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 microtitre plate and 180 µL of DPPH• (1,1-diphenyl-2-picrylhydrazyl) reagent 0.002% w/v (Sigma) with methanolic solution were dispensed with dispense module of hybrid reader (Synergy H1, BioTek), and were shaken for 10 s. The decrease in absorbance at 515 nm was measured exactly after 30 min of incubation at 25°C (Hybrid reader, Synergy H1, BioTek) [162].

All determinations were done in triplicate. DMSO was used as negative control. Blank samples were run by solvent without DPPH• radical. The measurement was made by hybrid reader (Synergy H1, BioTek). The percent of inhibition (I %) of free radical production of DPPH• was calculated by using the following equation:

$$I\ (\%)\ = \frac{Abs_{517\ nm_0} - Abs_{517\ nm_1}}{Abs_{517\ nm_0}} \times 100\ \ \text{, where} \qquad (2.4)$$

$Abs_{517\ nm\ 0}$ is the absorbance of the control solution;

$Abs_{517\ nm\ 1}$ is the absorbance in the presence of sample solutions or standards for positive controls.

The IC_{50} values were calculated according to the Hill equation, using the software.

2.3.3. Oxygen radical absorbance capacity (ORAC-Fluorescein) assay

The Oxygen Radical Absorbance Capacity (ORAC) assay is a sensitive method based on the detection of chemical damage to fluorescein through the decrease in its fluorescence emission by peroxyl radicals that are generated *in situ* by the thermal decomposition of the free radical initiator 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AAPH). The ORAC procedure used an automated plate reader (Synergy H1, BioTek, USA) with 96-well plates (Prior et al) [163]. The ORAC assay is unique in that its reactive oxygen species (ROS) generator, AAPH produces a peroxyl free radical upon thermal decomposition that is commonly found in the body, making the reaction biological relevant. Furthermore, since AAPH is reactive with both water and lipid soluble substances it can be used to measure the total antioxidant potential. The radical can oxidize fluorescein to generate a product without fluorescence.

The automated ORAC assay was performed on a microplate reader with fluorescence filters FP (485 nm / 528 nm). The experiment was conducted at 37^0 C under pH 7.4 condition with a blank sample in parallel. The tested compounds were prepared in DMSO for use as a stock solution. Trolox (2 mM methanolic stock solution) was used as reference at dilution concentrations ranging from 3,125 to 100 μM in phosphate-buffered saline (PBS, sigma) (pH 7.4). Phosphate buffer was used as blank, instead of the antioxidant solution.

The reaction was carried out in phosphate buffered saline (PBS) (10 mM, pH 7.4) and the final reaction mixture was 200 μL. 25 μL of each tested compounds dilution were transferred in a 96-wells black microtitre plate (Nunc black microwell, Denmark) and 150 μL of working solution of fluorescein disodium (3',6'-dihydroxy-spiro[isobenzofuran-1[3H], 9'[9H]-xanthen]-3-one) (sigma) (15 nM final concentration) were dispensed with dispense module of hybrid reader (Synergy H1, BioTek). The mixture was preincubated for 30 min at 37°C prior to shaking at 700 rpm (Shaker-thermostat, Sky Line, Elmi ST-3L). After that, 25 μL of (2,2'-Azobis (2-methylpropionamidine) dihydrochloride) (Sigma) solution (240 mM final concentration) were dispensed with dispense module of hybrid reader (Synergy H1, BioTek). The plate was immediately placed in the hybrid reader (Synergy H1, BioTek) and the fluorescence was recorded every minute for 100 min. The black microtitre plate was automatically agitated prior to each reading [164].

The antioxidant capacity, expressed as the area under curve (AUC), was calculated by a statistical program, using the following formula:

$$\mathrm{AUC} = 1 + \frac{\mathrm{RFU_1}}{\mathrm{RFU_0}} + \frac{\mathrm{RFU_2}}{\mathrm{RFU_0}} + \frac{\mathrm{RFU_3}}{\mathrm{RFU_0}} + \ldots + \frac{\mathrm{RFU_n}}{\mathrm{RFU_0}} \quad \text{, where} \tag{2.5}$$

RFU_0 is the relative fluorescence units at time point zero;

RFU_n is the relative fluorescence units at time points.

The Net AUC was calculated by subtracting the blank AUC from the AUC of each sample, the standards, and the positive control:

$$\text{Net AUC} = \text{AUC (sample)} - \text{AUC(blank)} \tag{2.6}$$

The slope (m) was calculated as follows:

$$\text{Slope (m)} = \frac{\mathrm{dy}}{\mathrm{dx}} \tag{2.7}$$

To determine trolox equivalents (TE) of each sample range, the ratio of the slope (m) of the linear regression analysis of the compound to the slope of the linear regression of trolox was used:

$$\mathrm{TE} = \frac{\mathrm{m_{(compound)}}}{\mathrm{m_{(trolox)}}} \tag{2.8}$$

2.3.4. Colorimetric assay for lipoxygenase activity

Lipoxygenases (LOX) comprise a class of non-heme iron-containing dioxygenases that stereospecifically insert molecular oxygen into *cis*-*cis*-1,4-pentadiene-containing polyunsaturated fatty acids. Among the six identified functional LOX gene isoforms in humans, 5-lipoxygenase (5-LOX), platelet 12-lipoxygenase (p 12-LOX), and 15-lipoxygenase (15-LOX) were originally discovered in leukocytes, platelets, and reticulocytes, respectively. Linoleic or arachidonic, the substrate of LOX, is an essential constituent of cellular membranes that is released by tightly regulated phospholipase cleavage.

The LOX assay [165] system is widely employed to determine the antioxidant activity of the tested compounds. In the LOX assay, LOX-derived lipid hydroperoxides oxidize the ferrous ion (Fe^{2+}) to the ferric ion (Fe^{3+}), the latter of which binds with thiocyanate $[SCN]^-$ to generate a red ferrothiocyanate (FTC) complex.

The LOX method was carried with some modifications. The LOX colorimetric assay was performed in 96-well flat-bottom plates in a total assay volume of 210 µL.

Various concentrations of the tested compounds, DOXO as well as the positive assay control Quercetin were premixed with soybean lipoxidase 500-1000 U/mL in Tris–HCl buffer (50 mM, pH 7.5) and were incubated for 5 min at room temperature, shaking at 1000 rpm (Shaker-thermostat, Sky Line, Elmi ST-3L). Then, a solution of 2 mM linoleic acid in MeOH was dispensed with dispense module of hybrid reader (Synergy H1, BioTek) to induce the enzymatic reaction. After 6 min of the incubation at room temperature the enzymatic reaction was stopped by adding 100 μL of ferrithiocyanate complex (FTC; chromogen) with dispense module (MultiFlo, Biotek). The FTC was prepared *ex tempore* by mixing the equivalent volumes of 4.5 mM $FeSO_4$ in 0.2 M HCl with 3% NH_4SCN methanolic solution [166].

The absorbance at 500 nm was measured, using a hybrid reader (Synergy H1, BioTek). The percentage inhibition of lipoxygenase activity was calculated according to the formula:

$$\%\ \text{inhibition} = \frac{\text{Abs}_{500\ \text{nm}_0} - \text{Abs}_{500\ \text{nm}_1}}{\text{AAbs}_{500\ \text{nm}_0}} \times 100 \quad \text{, where} \tag{2.9}$$

$\text{Abs}_{500\ \text{nm}0}$ is the absorbance of the control (100% activity LOX)

$\text{Abs}_{500\ \text{nm}1}$ is the absorbance of the sample.

IC_{50} was calculated, using statistical program.

2.4. *In vitro* toxic red blood cells (RBCs) hemolysis assay

To determine the resistance of red blood cell to oxidative stress (hemolysis), human blood was used (cod.140080991, Balti). The hemolytic activity was carried out by detection of hemoglobin release from erythrocyte in response to various concentrations of the tested compounds and DOXO. Human venous blood was incubated at room temperature with gentle shaking at 500 rpm for 30 min. Then, the samples were centrifuged at 1000 rpm for 10 min. The haemoglobin absorbance of the supernatant was measured at 540 nm. For negative (0% lysis) and positive (100% lysis) controls, isotonic solution (0.9%) and hypotonic solution (0.1%) of NaCl were used, respectively. The positive absorbance value should be 0.8 ± 0.3, while the negative one should be less than 0.03 [167]. The measurement was obtained by a plate reader (BioTek) [168]. The hemolytic rates of the compounds were calculated as the following equation:

$$\text{Hemolytic rate}\ (\%) = \frac{\text{A}_\text{t}}{\text{A}_\text{c}} \times 100, \text{where} \tag{2.10}$$

A_t is the absorbance value of the tested compound;

A_c is the absorbance value of the positive control.

2.5. *In vitro* formation of methemoglobin (metHb) in intact erythrocytes assay

The assay was performed as described with minor modifications. Human venous blood was used (cod.140080991, Balti). The blood sample methemoglobin concentration was measured using the method below, which is based on Evelyn and Malloy method.

Serum was removed after centrifugation at 1000g for 5 min, and the red blood cells (RBCs) were washed in cold phosphate-buffered saline (PBS) at 1000g for 5 min. The washed erythrocytes were diluted in physiological saline (1:1) with various concentrations of the tested compound as well as DOXO were incubated at 37^0C with gentle shaking at 500 rpm for 3 h.

After that, solution of 2% digitonin was used to induce haemolysis. The measurement was made by hybrid reader (BioTek), in absorbance at 540 and 630 nm. Then, the hemolysate was divided into two parts. The absorbance of the first part was read at 630 nm and then read again at 630 nm after addition of 2% potassium cyanide (KCN), to convert the metHb into cyanmethemoglobin. The change in the intensity of light absorption after addition of potassium cyanide is directly proportional to the concentration of metHb [169].

Finally, 10 μL of 2% potassium hexacyanoferrate(III) ($K_3[Fe(CN)_6]$) (causes translation remaining oxyhemoglobin to methemoglobin) were applied and the absorbance was measured at 540 nm. The measurement was obtained by a plate reader (BioTek) at 540/630 nm [168].

Ability to induce MetHb formation in human RBCs was calculated using the following equation:

$$\text{MetHb (mM)} = \frac{A_1 - A_2}{4.3 - 0.71}, \text{where} \qquad (2.11)$$

A_1 – the maximum absorbance value of MetHb at 630 nm (pH 6.8);

A_2 - the absorbance value of test sample at 630 nm, where cyanmethemoglobin is directly proportional to the concentration of MetHb

4.30 - millimolar absorbance coefficient of MetHb at 630 nm (pH 6.8)

0.71 - millimolar absorbance coefficient of cyanmethemoglobin at 630 nm (pH 6.8).

The Hb (mM) was calculated by equation:

$$\text{Hb (mM)} = \frac{A_t}{11} \times 100, \text{where} \quad (2.12)$$

A_t - absorbance value of test sample at 540 nm;

11- millimolar absorbance coefficient of cyanmethemoglobin at 540 nm.

The MetHb (percentage total Hb) was calculated by equation:

$$\text{Total Hb (\%)} = \frac{\text{MetHb}}{\text{Hb}} \times 100 \quad (2.13)$$

2.6. *In vivo* toxicity assay

A new spectrophotometric method for determination of toxicity regarding test objects *Paramecium caudatum* has been developed and patented [170]. The use of this method accelerates biotesting and gives possibility accurately establish the dependence of toxicity on the substance concentration.

Taxonomic Hierarchy (NCBI BLAST)

Kingdom: *Protozoa*

Phylum: *Ciliophora*

Class: *Ciliatea*

Subclass: *Rhabdophorina*

Order: *Hymenostomatida*

Suborder: *Peniculina*

Family: *Parameciidae*

Genus: *Paramecium*

Species: *Paramecium caudatum* (Ehrenberg, 1838)

Because some *Paramecium* species are readily cultivated and easily induced to conjugate and divide, they have been widely used for the toxicity screening.

Paramecium caudatum were maintained in aqueous straw infusion growth media supplemented with NaCl (1.0 g/l), KCl (0.1 g/L), $NaHCO_3$ (0.2 g/L), $MgSO_4$ (0.1 g/L), and $CaCl_2$ (0.1 g/L) at 23–25 °C in the dark. *Protozoans* were fed with *Saccharomyces cerevisiae* yeast that was added to growth media. *Paramecium caudatum* were cultured in a growth chamber 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 [171].

To run the assay, live *Paramecium caudatum* (3×10^3 cells/mL) cells were exposed to various concentrations of the tested compounds for 24 hours. The cells were then incubated with Neutral red dye (NR) (3-Amino-7-dimethylamino-2-methylphenazine hydrochloride) for 12 hours, which is a weak cationic dye that easily penetrates the cell membrane and accumulates intracellularly in lysosomes, where it binds with anionic sites to the lysosomal matrix. Afterwards, the cells of *Paramecium caudatum* were washed twice with H_2O and centrifuged at 1000 rpm for 5 min.

Finally, the NR dye was extracted by NaOH (3M). The absorbance was measured at 690 nm and 540 nm using a microplate reader (BioTek). The percentage of viability of *Paramecium caudatum* (V (%)) was calculated according to the formula:

$$V(\%) = \frac{Abs_{540\ nm(sample)} - Abs_{690\ nm(sample)}}{Abs_{540\ nm(control)} - Abs_{690\ nm(control)}} \times 100, \text{where} \quad (2.14)$$

The median lethal concentration (LC_{50}) values were calculated, using GraphPad.

2.7. 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, IC_{50} 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 (LC_{50}).

All data are presented as means ± standard deviation (SD). The formula for the sample standard deviation is:

$$s = \sqrt{\frac{1}{N-1}\sum_{i=1}^{N}(x_i - \overline{x})^2} \quad (2.15)$$

$\{x_1, x_2, \ldots, x_N\}$ are the observed values of the sample items;

$\overline{x}$ is the mean value of these observations;

N is the number of observations in the sample.

The Trendline Analysis in Excel is a linear least squares regression tool that can be employed to provide some correlation to data points. The accuracy of the fit can be interpreted using the R-squared. R-squared is the proportion of the variance in the dependent variable that is predictable from the independent variables. R-squared measures the strength of the relationship between model and the dependent variable on a convenient 0 – 100% scale. As the R-squared value approaches 1, the accuracy of the fit approaches 100%.

R-squared provides a measure of how well observed outcomes are replicated by the model, based on the proportion of total variation of outcomes explained by the model [172, 173].

The most general definition of the coefficient of determination is:

$$R^2 = \frac{SS_{res}}{SS_{tot}} = 1 - \frac{SS_{err}}{SS_{tot}} \quad (2.16)$$

SS_{res} - Regression Sum of Squares

SS_{tot} - Total Sum of Squares

SS_{err} - Error Sum of Squares

In the best case, the modeled values exactly match the observed values, which results in $SSR_{res} = 0$ and $R^2 = 1$.

2.8. Conclusions to chapter 2

1. Based on the known methods and their modifications, 5 compounds were synthesized: 2-formylpyridine N(4)-phenylthiosemicarbazone (HL), complex copper(II) [Cu(L)Cl] with 2-formylpyridine N(4)-phenylthiosemicarbazone ligand, copper(II) mixed-ligand complex chloro(*N*-phenyl-*N*'-[(pyridin-2-yl)methylidene] carbamohydrazonothioato)(4-aminobenzene-1-sulfonamide)copper [Cu(Str)(L)Cl], as well as coded organic compound (CMJ-23) and copper(II) complex (CMJ-33) with ligand (CMJ-23). The purity, composition and structure of the synthesized substances were established on the basis of electronic, FT-IR and EPR spectroscopy, molar conductivity, magnetic susceptibility measurements and elemental analysis and for the previously described substances, are consistent with published data.

2. Cell lines such as 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) and

normal kidney epithelial cells of MDCK line (ATCC CCL-34) were cultured to study the inhibitory effect of the tested compounds.

3. Antiproliferative properties were studied for cancer and normal cell lines using the methods described for the tested compounds.

4. The electrophoretic DNA fragmentation method was used to identify the mechanism of action of the studied substances associated with a direct effect on the genomic DNA of the cell.

5. Antioxidant properties were determined using the above described methods for the tested substances.

6. The studied substances were tested to determine the side effects associated with tissue hypoxia and hemolysis in human blood *in vitro*.

7. *Paramecium caudatum* test objects were used to determine direct toxicity and the absence of resistance of the tested substances.

3. ANTIPROLIFERATIVE ACTIVITY OF THE TESTED COMPOUNDS

This work represents a series of comparative antiproliferative studies on several cancer cells of the thiosemicarbazone HL (CMT-22), copper(II) complex (CMT-67), copper(II) mixed-ligand complex (CMT-68) , and complex copper(II) (CMJ-33) with its ligand (CMJ-23).

To test the anticancer properties of the tested compounds, a series of suitable for preclinical studies cancer cell lines was used. So, the antiproliferative effect of the tested compounds was determined using human melanoma cell line MeW-164 [174], human epithelioid cervix carcinoma cell line HeLa, human epithelial pancreatic adenocarcinoma cell line BxPC-3, human muscle rhabdomyosarcoma spindle and large multinucleated cell line RD. An additional experiment aiming at the evaluation of the nephrocytotoxic effect of the tested compounds on normal kidney epithelial cells of MDCK line was used in this work [175].

It was mentioned before that many of the antitumor drugs act due to DNA damage and are most active in the period S; therefore, cells in the middle phase of exponential growth were used for research.

To estimate the results on the cytotoxic effect of the cell lines, the tested compounds CMT-22, CMT-67, CMT-68, CMJ-23 and CMJ-33 were compared to doxorubicin (DOXO) and cisplatin (CDDP) as the referent controls, which is used in the clinical management of a wide range of cancers.

For a comparative assessment of the results, IC_{50} (μM) values were used as indices, which are quantitative indicators of the antagonist substance efficiency when choosing the optimal therapeutic dose.

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

The morphological examinations of cells were photographed using the inverted microscope (LOMO). Because cytoplasmic vacuolation is a future of cellular death, the ability of the tested compounds to induce formation of cellular vacuoles was microscopically examined on a variety of human cancer cell types, including MeW-164, HeLa, BxPC-3 and sarcoma cells RD, as well as on normal kidney epithelial cell line MDCK [176]. The morphological changes in MeW-164, HeLa, BxPC-3 and RD cancer cell lines after 24 h treatment with CMT-22, CMT-67 and CMT-68 at concentration 10 μM are represented in figures 3.1, 3.3, 3.5, 3.7. As expected, no cellular vacuoles formation was microscopically observed in the control (non-chemical

compounds exposed cells). Thus, no vacuoles were identified in BxPC-3, and MDCK cells after incubation for 24 hours with concentration 10 μM of CMT-22 (Figure 3.5, 3.9). In contrast, cellular vacuolization was observed in MeW-164, HeLa, RD cancer cells in response to concentration 10 μM of CMT-22 (Figure 3.1, 3.3, 3.7). Further, intensive cellular vacuolization was identified in all cancer cells as well as MDCK cells after exposure to 10 μM of complex CMT-67 (Figure 3.1- 3.9). Intensive cellular vacuolization was also detected in all cancer cells, but not for normal kidney epithelial cell line MDCK after exposure to 10 μM of the mixed-ligand complex CMT-68 (Figure 3.1- 3.9).

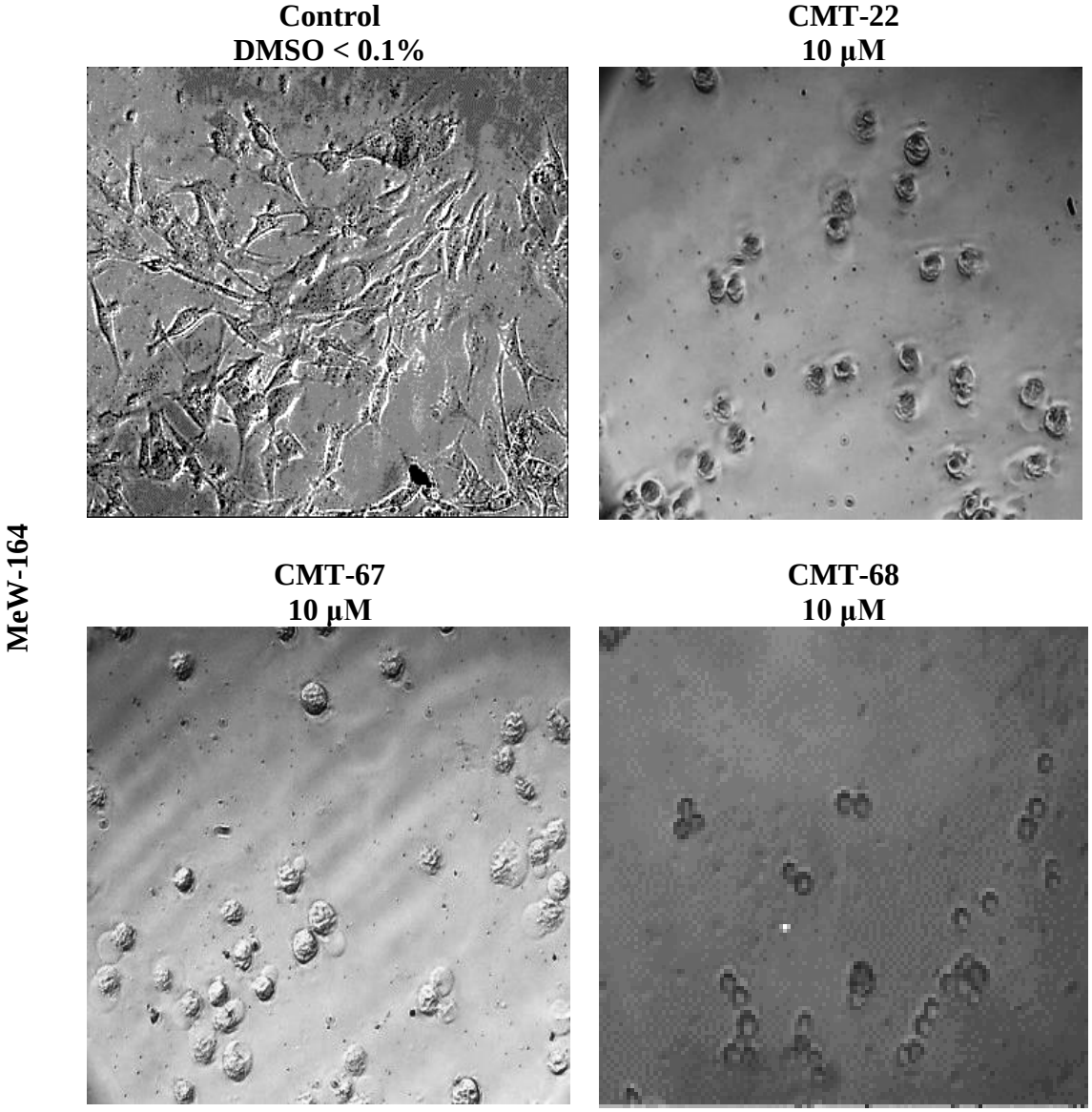


Fig. 3.1. Phase-contrast images of MeW-164 cells after 24 h treatment with CMT-22, CMT-67, CMT-68 at concentration 10 μM. Untreated MeW-164 cells served as a control.

The compounds have been tested for antiproliferative activity on the following cancer cells: human melanoma cell line (MeW-164), human cervical epithelial cell line (HeLa), human epithelial pancreatic adenocarcinoma cell line (BxPC-3), human muscle rhabdomyosarcoma spindle and large multinucleated cell line (RD).

The human melanoma cells of line (MeW-164) were incubated for 24 h in the presence of synthesized compounds and the number of viable cells was measured using the MTT assay. The results are expressed as the percentage of cell growth inhibition at three concentrations (Figure 3.2) [154].

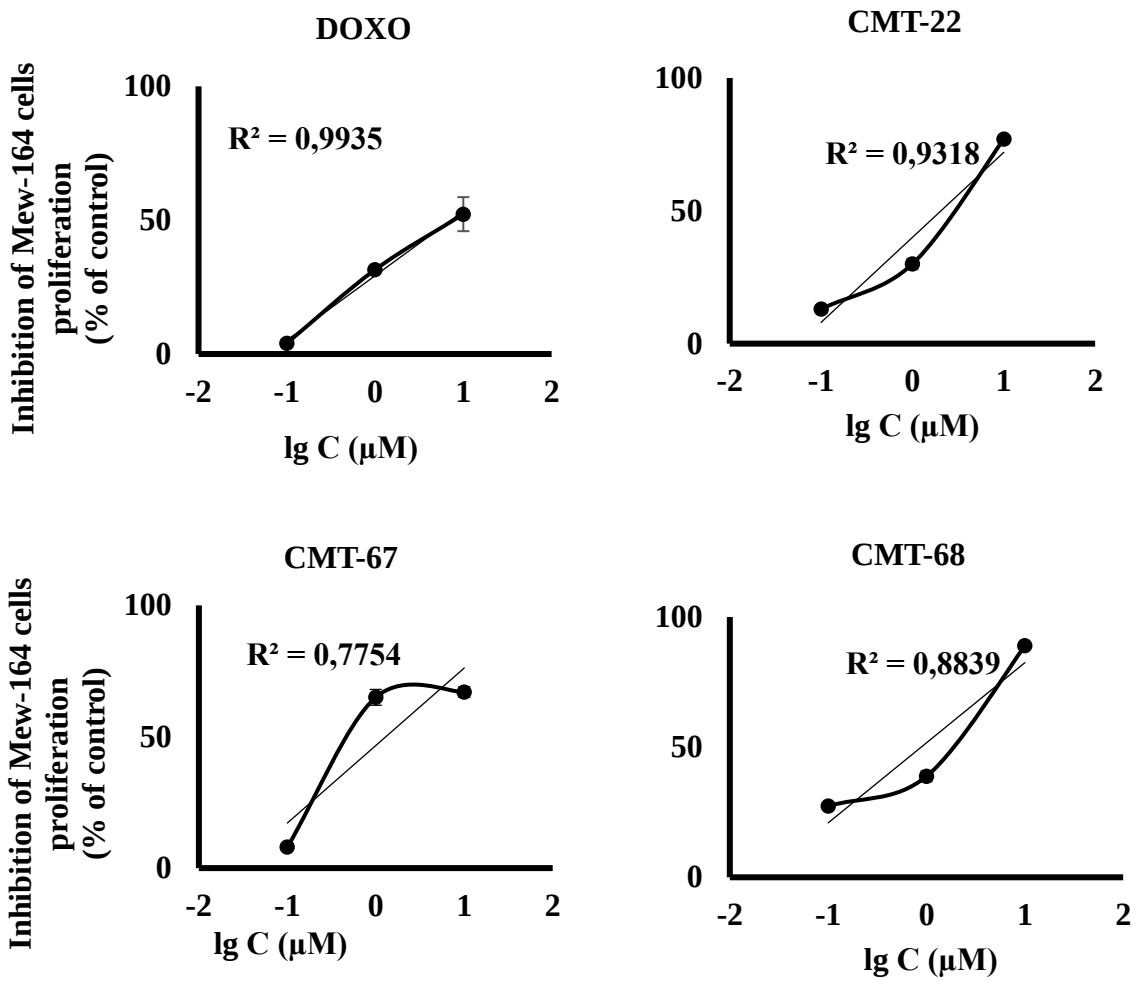


Fig. 3.2. Antiproliferative activity of the positive control DOXO and the tested compounds CMT-22, CMT-67, CMT-68 on human melanoma cells of line MeW -164. Mew-164 cells were treated with DOXO, CMT-22, CMT-67 and CMT-68 at concentrations 0.1 μM, 1 μM, 10 μM for 24 h. Values are represented as mean ± SD of 3 replicates.

The 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. The curves were characterized by the coefficient of determination values (R^2) of 0.93, 0.78, 0.88, 0.99 for CMT-22, CMT-67, CMT-68 and DOXO, respectively (Figure 3.2). Thus, all compounds showed high antiproliferative activity against line MeW-164.

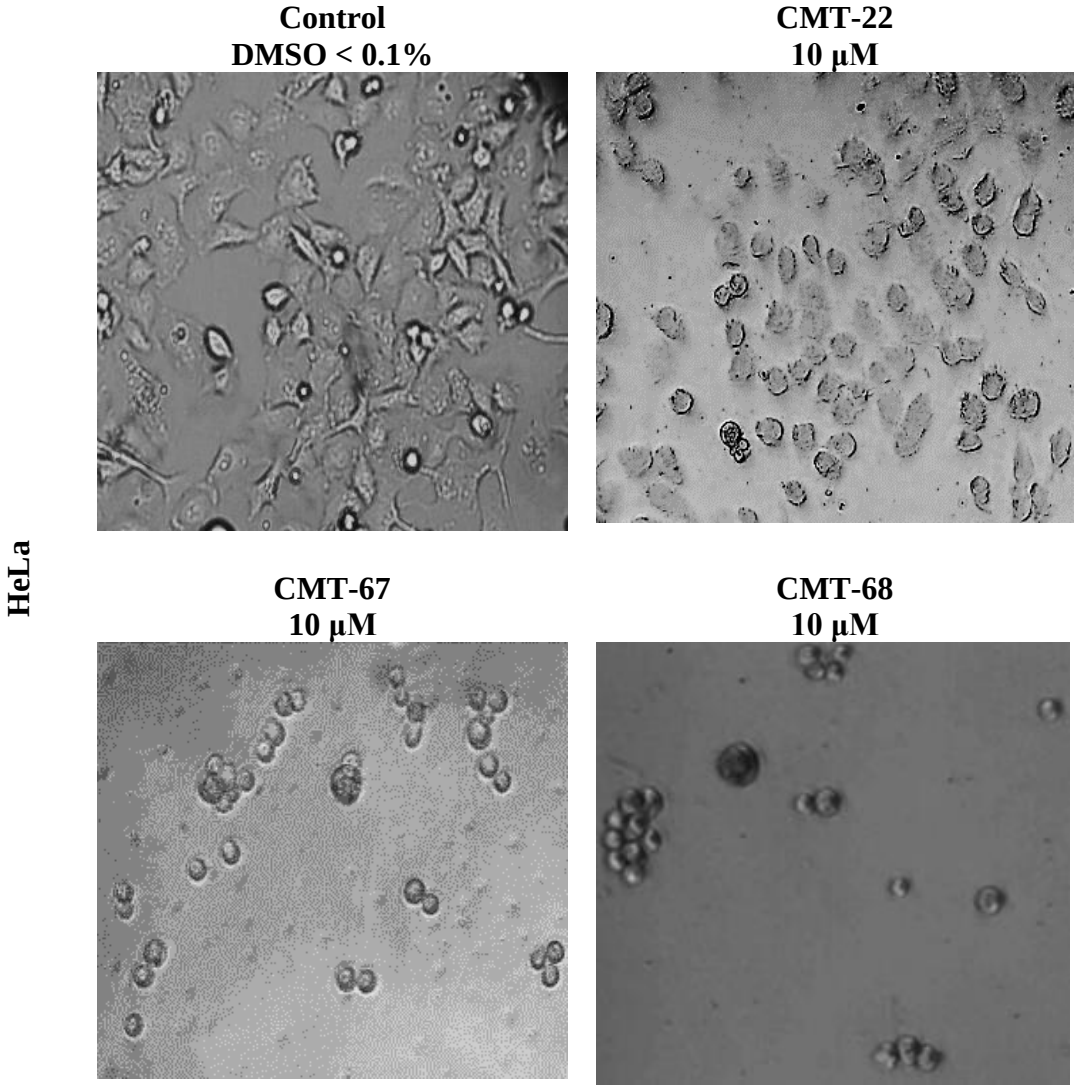


Fig. 3.3. Phase-contrast images of HeLa cells after 24 h treatment with CMT-22, CMT-67 and CMT-68 at concentration 10 μM. Untreated HeLa cells served as a control.

The viability of cells HeLa, BxPC-3, RD and MDCK were determined by the resazurin assay [156, 157]. 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 3.4.

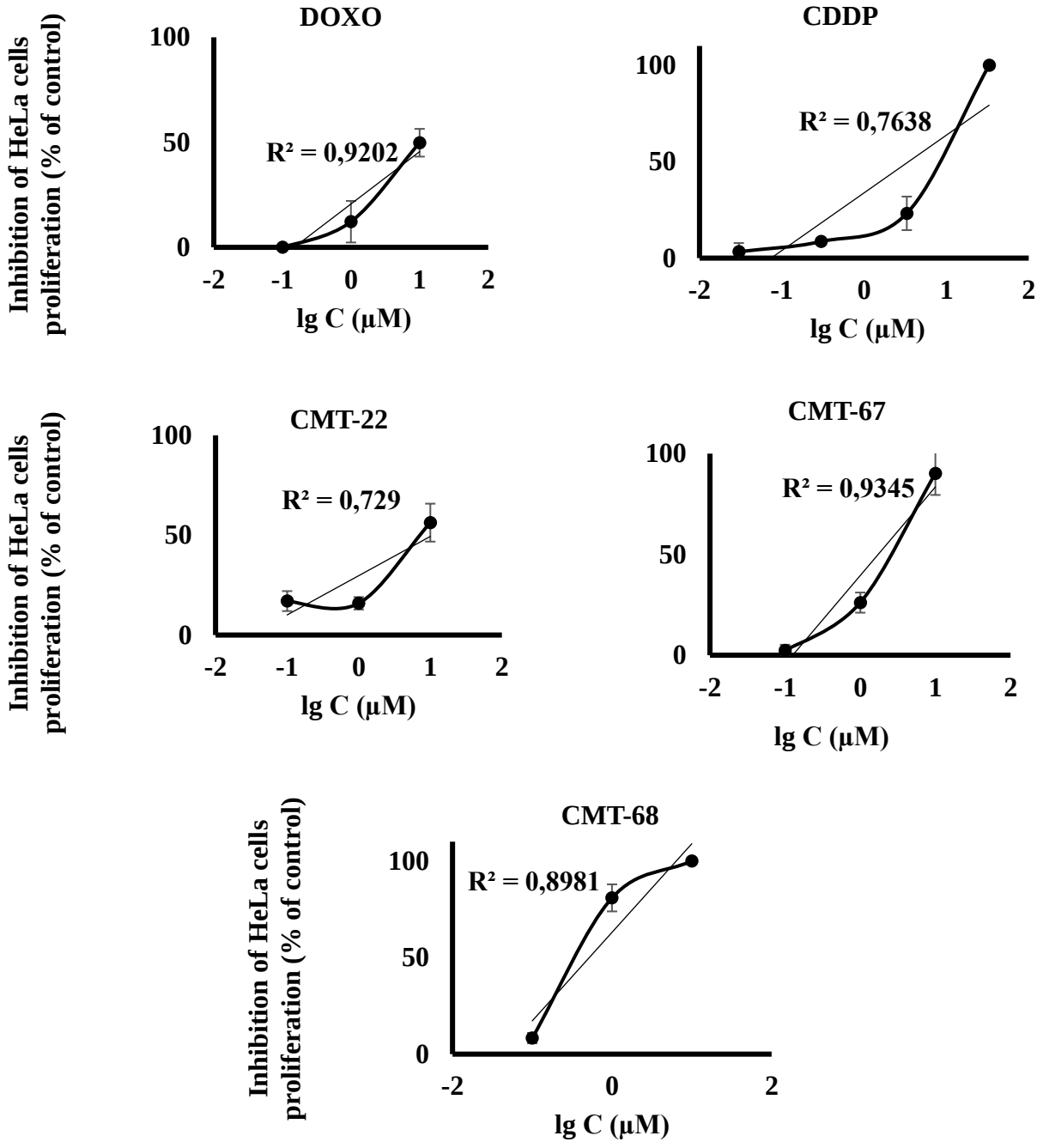


Fig. 3.4. Antiproliferative activity of the positive controls (DOXO, CDDP) and the tested compounds (CMT-22, CMT-67 CMT-68) on the cervical epithelial cells HeLa. HeLa cells were treated with DOXO, CMT-22, CMT-67 and CMT-68 at 0.1, 1 and 10 μM and CDDP at 0.03, 0.3, 3.3 and 33 μM for 24 h. Values are represented as mean ± SD of 3 replicates.

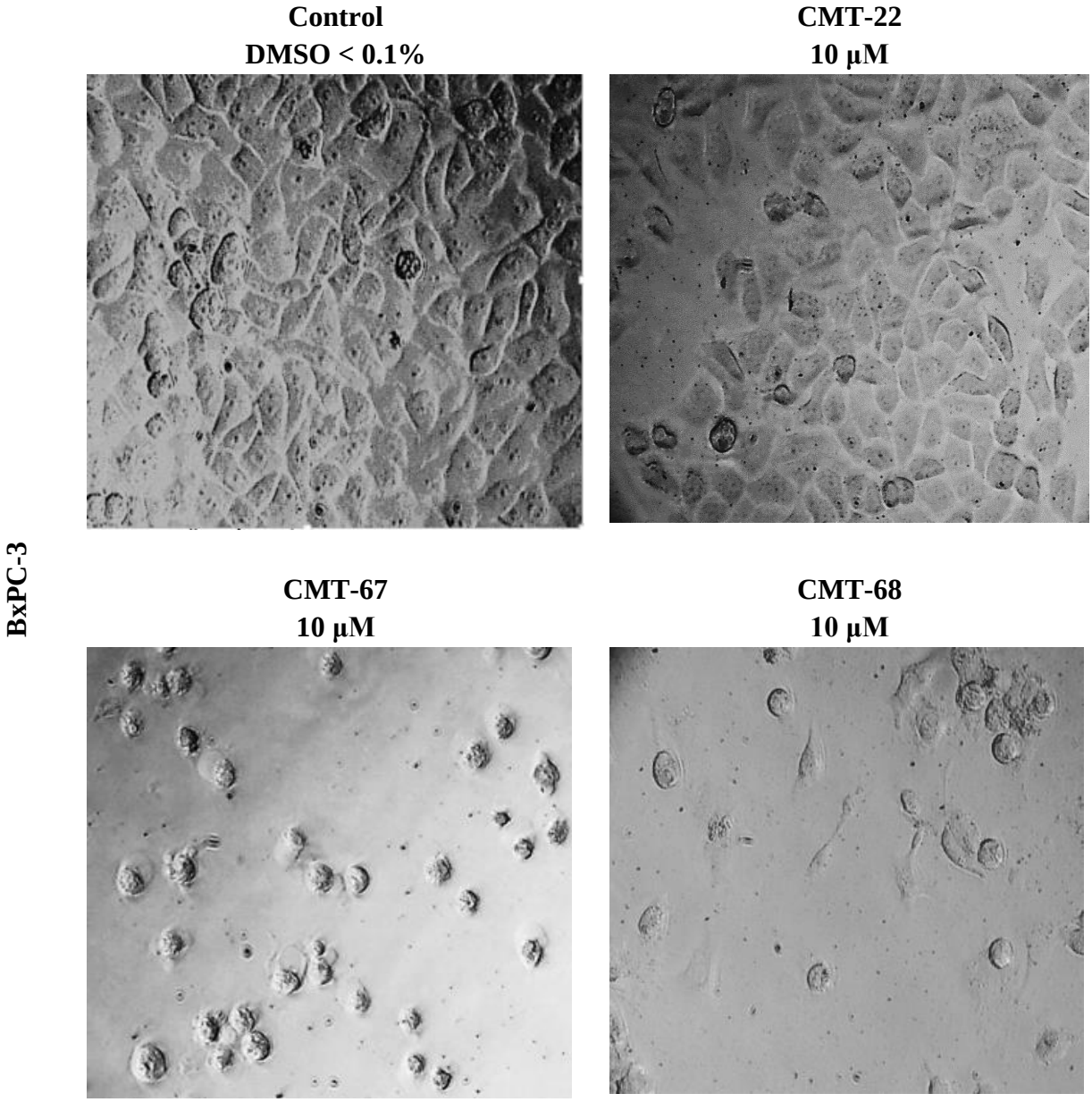


Fig. 3.5. Phase-contrast images of BxPC-3 cells after 24 h treatment with CMT-22, CMT-67 and CMT-68 at concentration 10 μM. Untreated BxPC-3 cells served as a control.

It was founded, that the tested compounds CMT-22, CMT-67 and CMT-68 exhibited inhibitory activity against human epithelioid cervix carcinoma cells of HeLa line, with IC_{50} values of 8.3±0.2 μM, 2.1±0.4 μM, and 0.40±0.04 μM, respectively. The curves were characterized by the determination coefficients (R^2) values of 0.73, 0.94, 0.93 and regression slope values (m) of 0.51, 1.73, and 1.41 for CMT-22, CMT-67 and CMT-68, 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, with the coefficients of determination R^2 = 0.94, 0.76, respectively.

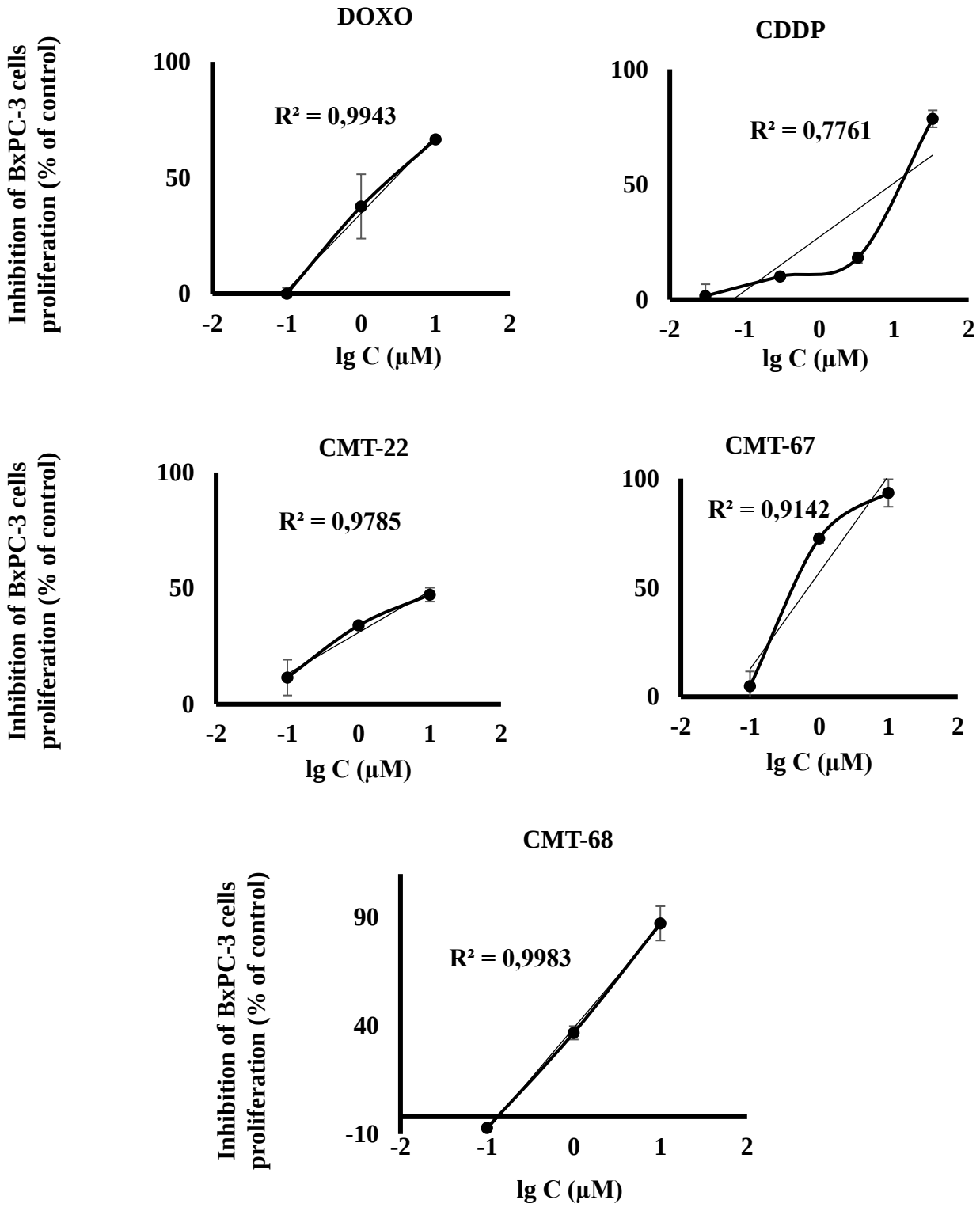


Fig. 3.6. Antiproliferative activity of the positive controls (DOXO, CDDP) and the tested compounds CMT-22, CMT-67 and CMT-68 on the pancreatic adenocarcinoma cells of line BxPC-3. BxPC-3 cells were treated with DOXO, CMT-22, CMT-67 and CMT-68 at 0.1, 1 and 10 µM and CDDP at 0.03, 0.3, 3.3 µM and 33 µM for 24 h. Values are represented as mean ± SD of 3 replicates.

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).

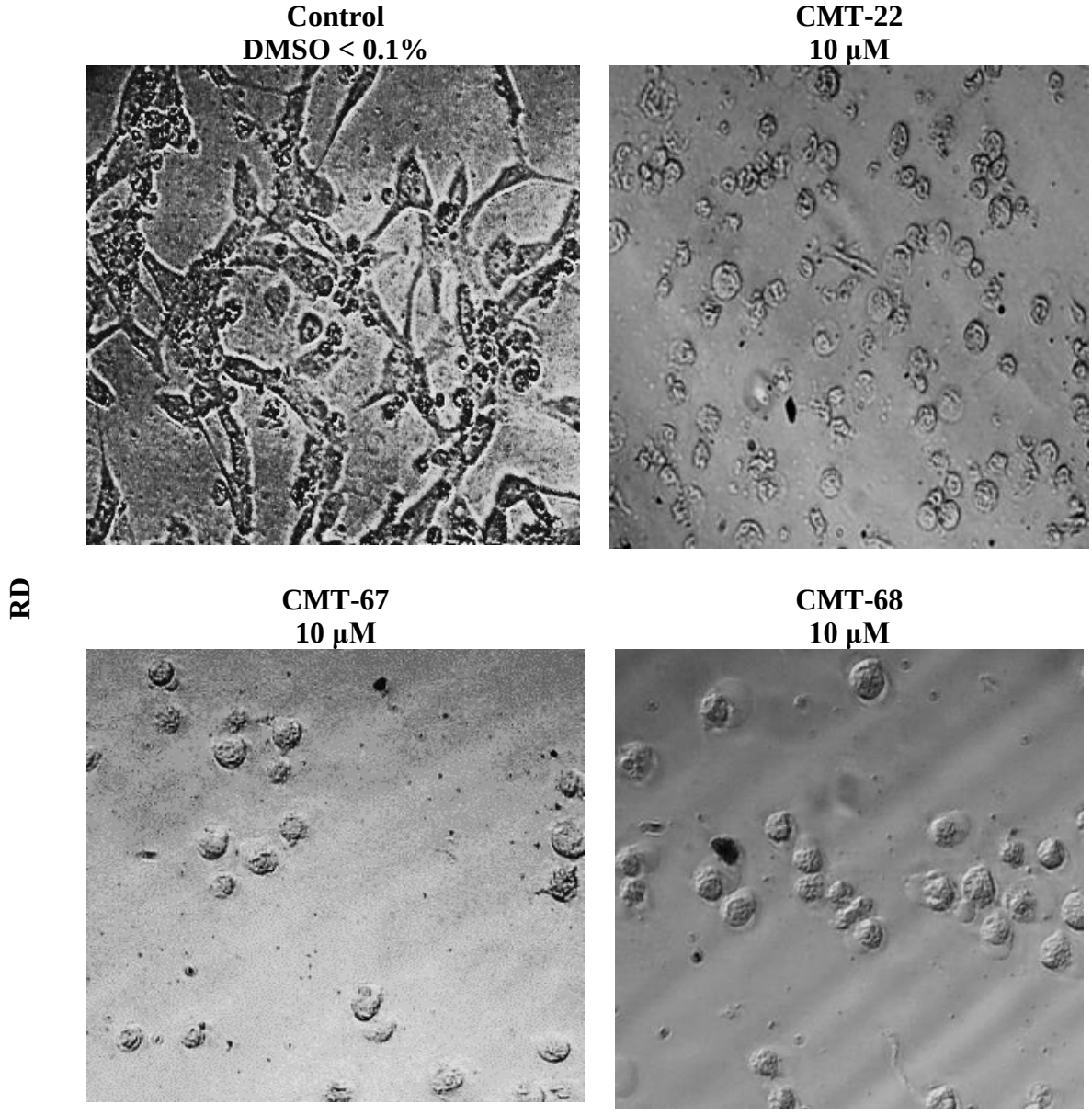


Fig. 3.7. Phase-contrast images of the human muscle rhabdomyosarcoma spindle and large multinucleated cells of line RD after 24 h treatment with CMT-22, CMT-67 and CMT-68 at concentration 10 µM. Untreated RD cells served as a control.

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 BxPC-3 cells 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, with determination coefficients (R^2 = 0.98, 0.91, 0.998, 0.99, 0.78), respectively (Figure 3.6).

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 CDDP.

It was established that the tested compounds CMT-22, CMT-67 and CMT-68 exhibit stronger inhibitory activity on human muscle rhabdomyosarcoma spindle and large multinucleated cells of 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, with the coefficient of determination values (R^2 = 0.9, 0.98, 0.79, 0.99), respectively (Figure 3.8).

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 (CDDP and DOXO) [164].

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.

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%.

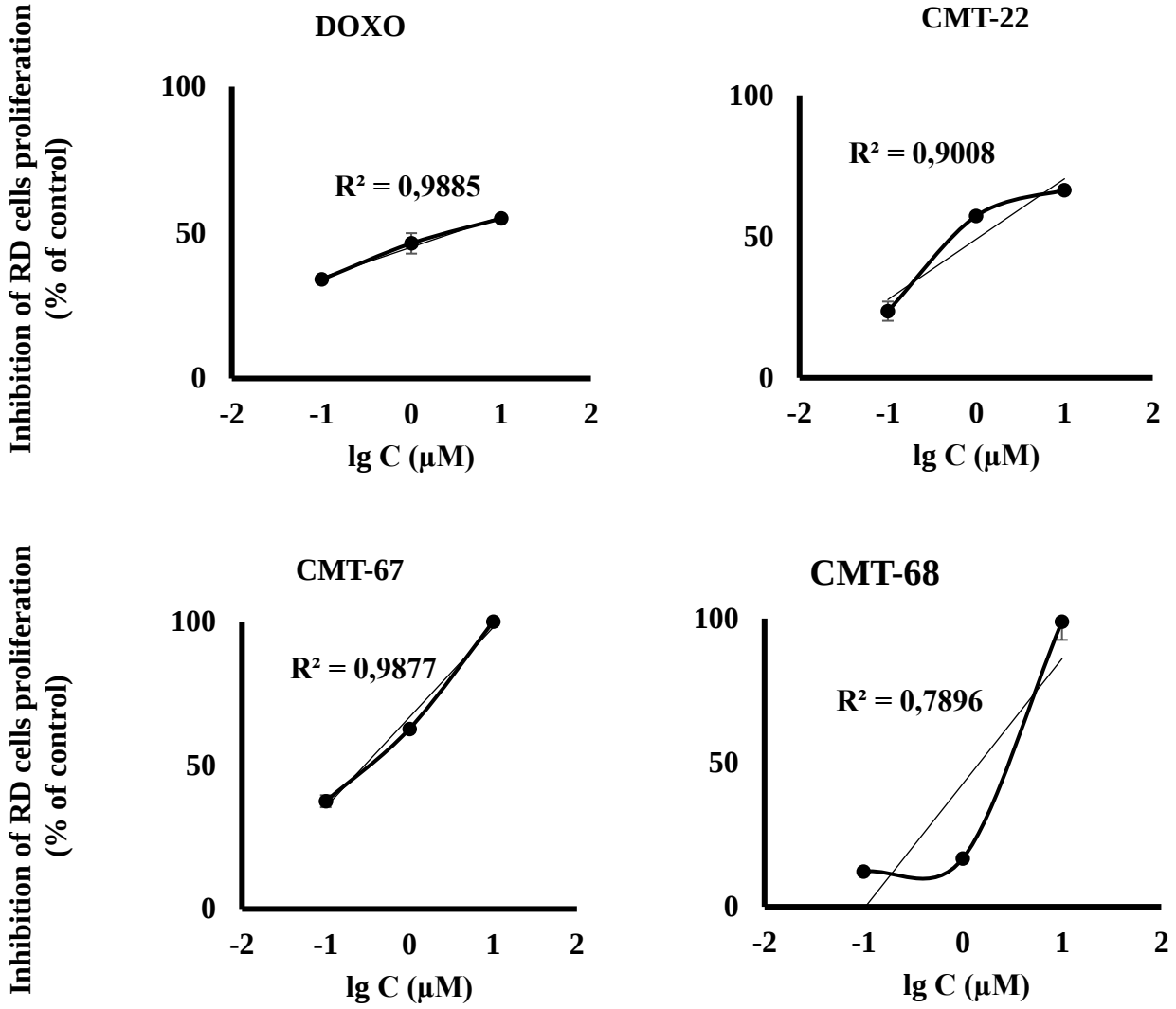


Fig. 3.8. Antiproliferative activity of the positive controls DOXO, CDDP and the tested compounds CMT-22, CMT-67, CMT-68 on human muscle rhabdomyosarcoma spindle and large multinucleated cells of line RD. RD cells were treated with DOXO, CMT-22, CMT-67 and CMT-68 at 0.1 μM, 1 μM and 10 μM for 24 h. Values are represented as mean ± SD of 3 replicates.

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, with the coefficient of determination values (R^2 = 0.7, 0.7), respectively. The positive control DOXO demonstrated cytotoxic effect against MDCK cell line, with values IC_{50} = 7.0±0.3 μM, with the coefficient of determination value 0.7.

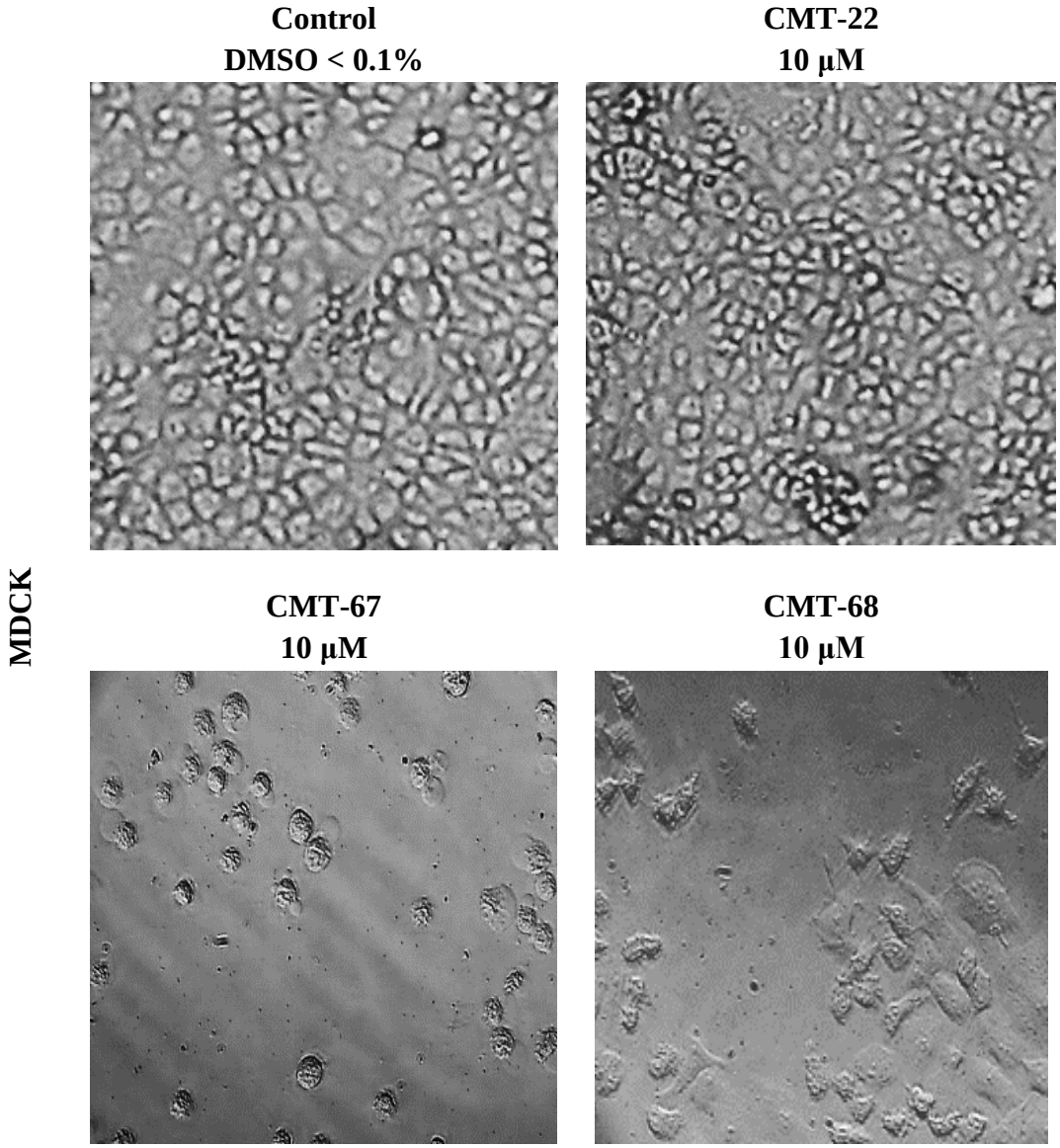


Fig. 3.9. Phase-contrast images of MDCK cells after 24 h treatment with the tested compounds at concentration 10 μM. Untreated MDCK cells served as a control.

The search for new compounds that exhibit selectivity for cancer cells but not for normal cells is a major challenge in anticancer drug research [177, 176].

Thus, selectivity index (SI) of the tested compounds were estimated (Table 3.2). 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 (SI) of ≥10, ≥91, ≥12 and ≥40, respectively [178].

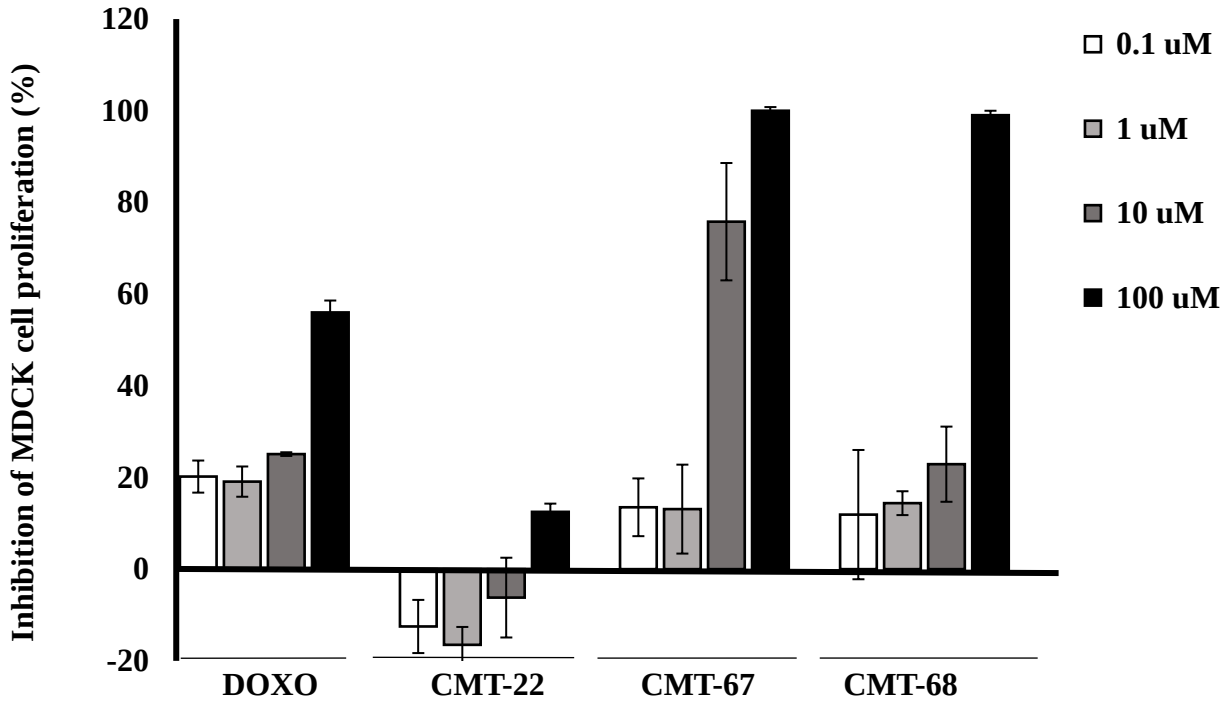


Fig. 3.10. Nephrocitotoxic effect *in vitro* of DOXO, CMT-22, CMT-67, CMT-68 on MDCK cells after 24 h exposure. MDCK cells were treated with CMT-22, CMT-67, CMT-68 and DOXO at concentrations 100 μM, 10 μM, 1 μM and 0.1 μM. Values are represented as mean ± SD of 3 replicates.

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 [179], that the introduction of amines into the inner sphere of copper(II) complexes with various azomethines results in an increase in their anticancer activity, and copper(II) mixed-ligand complex CMT-68 behaves as described above [147].

Table 3.1. Antproliferative activity of the tested compounds CMT-22, CMT-67, CMT-68 and the positive control DOXO on cancer cells of lines BxPC-3, RD, HeLa, MeW-164 and normal kidney epithelial cell line MDCK

	MDCK	BxPC-3		RD		HeLa		MeW-164	
Compound	**IC_{50} (µM)**	**IC_{50} (µM)**	**SI_1**	**IC_{50} (µM)**	**SI_2**	**IC_{50} (µM)**	**SI_3**	**IC_{50} (µM)**	**SI_4**
DOXO	7.0±0.3	5.24±0.03	1.3	2.3±0.9	3.0	10.0± 4.0	0.7	7.3± 0.3	0.95
CMT-22	≥100	≥10	≥10	1.1±0.1	≥91	8.3±0.2	≥12	2.5± 0.1	≥40
CMT-67	4.0±0.1	0.6 ± 0.1	6.7	0.27± 0.02	14.8	2.1±0.4	1.9	1.0± 0.1	4.0
CMT-68	12.0± 0.9	1.7 ± 0.2	7.1	1.3±0.3	9.2	0.40±0.04	30.0	1.0± 0.1	12

Notes: IC_{50} valium is a concentration inhibiting cellular proliferation by 50%. SI valium is selectivity index.

Substances with a SI > 3 are considered to be promising.

The SI_1, SI_2, SI_3, SI_4 of each compound were calculated as the ratio of the IC_{50} for MDCK cells / IC_{50} for cancer cell lines BxPC-3, RD, HeLa, MeW-164, respectively.

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

The morphological examinations of the cancer cells were observed and photographed using the phase contrast inverted microscope (OLYMPUS). The vacuolization of the cancer cell membranes, as well as small and round cells were observed. It is likely that the tested compounds significantly induced apoptosis in the cancer cell lines, such as MeW-164, HeLa, BxPC-3 and sarcoma cells RD [180]. As expected, no cellular vacuoles formation was microscopically observed in the control (non-chemical compound exposed cells) (Figures 3.11, 3.12).

Thus, no vacuoles were identified in MDCK cells after incubation for 24 h with concentration 10 μM of CMJ-23 (Figure 3.12). In contrast, cellular vacuolization was observed in cancer cells MeW-164, HeLa and sarcoma cells RD in response to concentration 10 μM of CMJ-23. Further, intensive cellular vacuolization was identified in all cancer and sarcoma cells as well as MDCK cells after exposure to concentration 10 μM of complex CMJ-33.

Study of antiproliferative activity showed that there is concentration dependence between inhibitory effects of the tested compounds at the micromolar concentration range. The inhibitory effect was displayed in a dose-dependent manner.

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.

Figure 3.13 shows the percent inhibition of the compounds on the tested cell line MeW-164. 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. The curves were characterized by the coefficient of determination values (R^2) of 0.97 and 1 for CMJ-23 and CMJ-33, respectively. 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.

Next, the viability of cancer cells HeLa, BxPC-3 and sarcoma cells RD and normal cells of line MDCK was assessed by the redox indicator of resazurin, which allowed us to measure the number of viable cells.

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 figures 3.14 – 3.16.

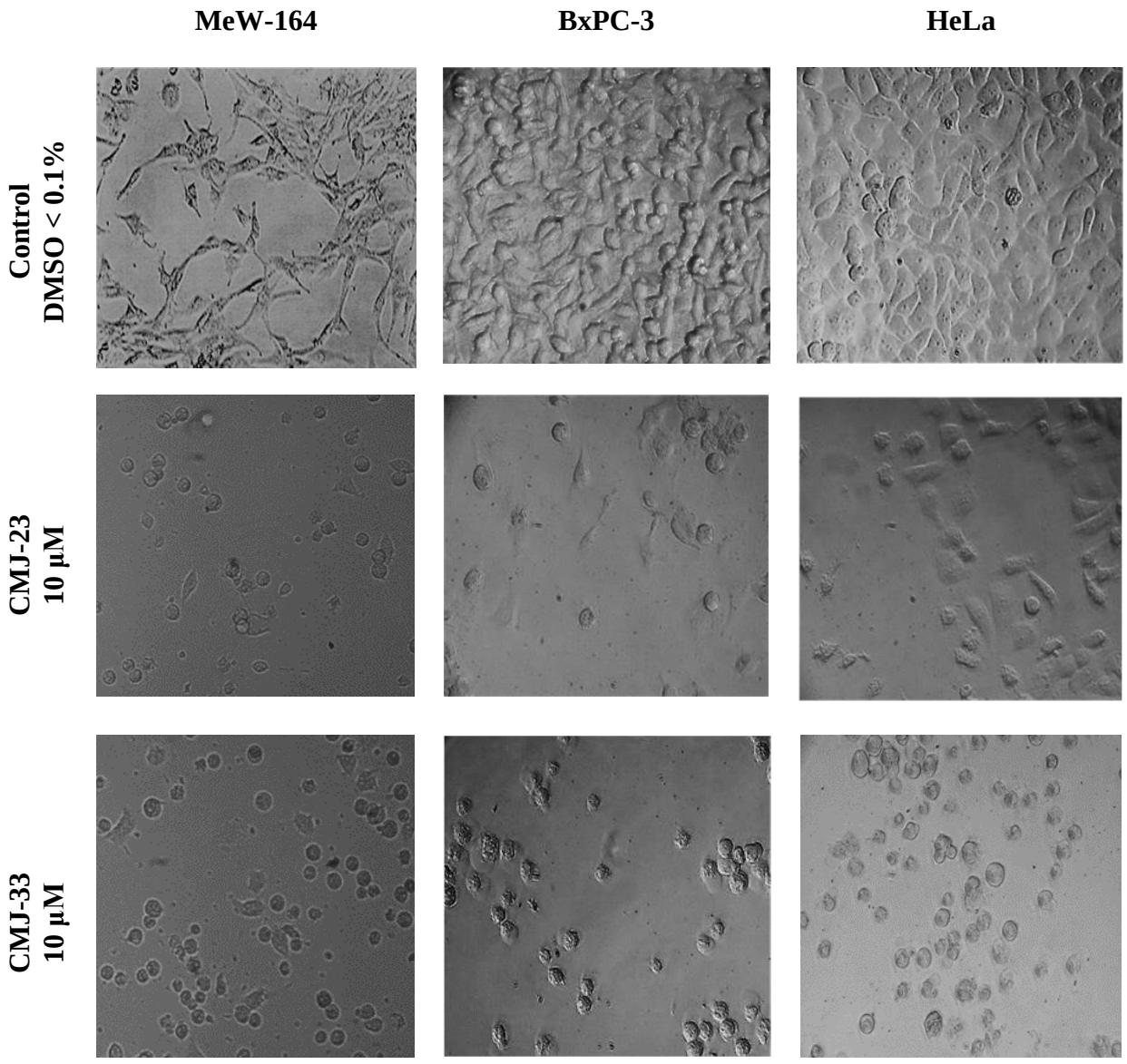


Fig. 3.11. Phase-contrast images of cells MeW-164, BxPC-3 and HeLa after 24 hours treatment with the tested compounds CMJ-23 and CMJ-33 at concentration 10 µM. Untreated cells served as a control

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, with the coefficient of determination values (R^2) 0.9, 0.7, respectively. However, the compound CMJ-23 was significantly less toxic giving IC_{50} value of 18.7±1.0 µM against HeLa cells with the coefficient of determination values (R^2) 0.9.

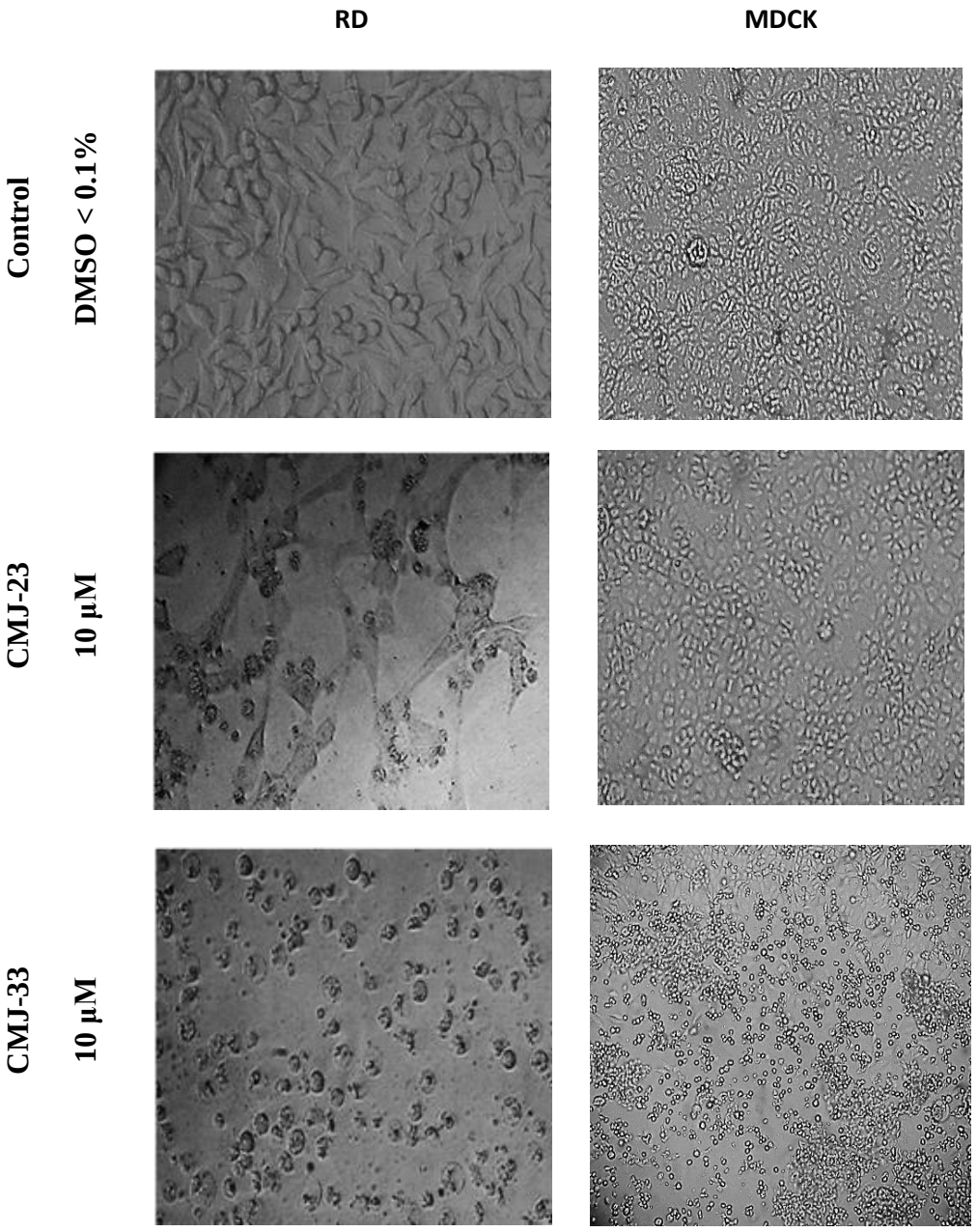


Fig. 3.12. Phase-contrast images of cells RD and MDCK after 24 hours treatment with the tested compounds CMJ-23 and CMJ-33 at concentration 10 μM. Untreated cells served as a control

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, and with the coefficient of determination values 1.0, 0.9 and 0.8, 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 [180], and coefficient of determination values are 0.9, 1.0 and 0.8, respectively.

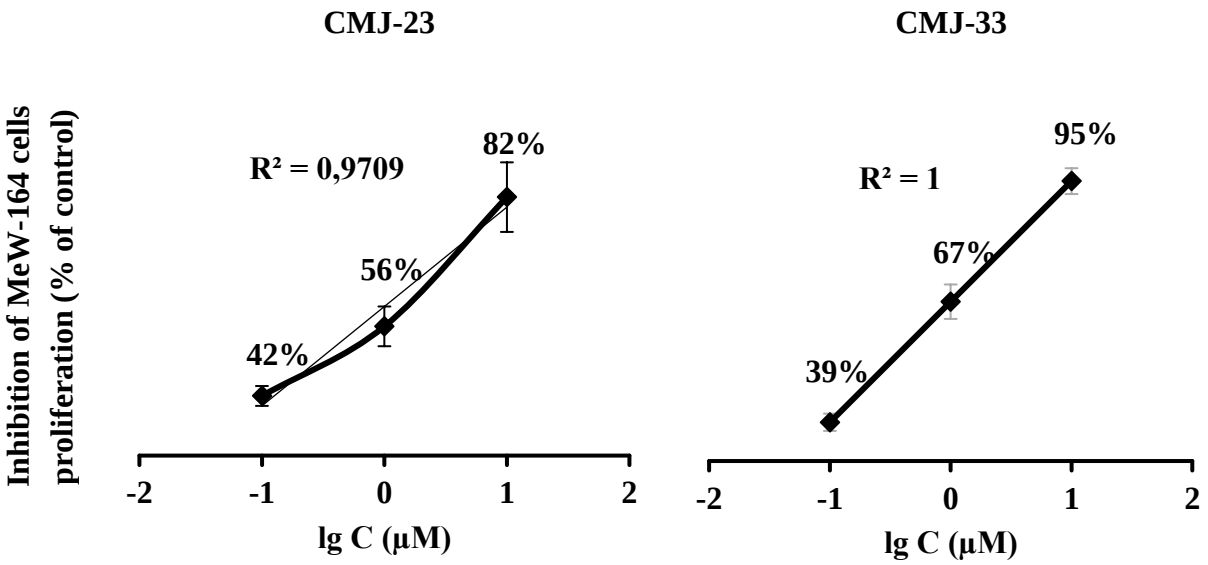


Fig. 3.13. Antiproliferative activity of the tested compounds CMJ-23 and CMJ-33 on human melanoma cells of line MeW -164. MeW -164 cells were treated with CMJ-23 and CMJ-33 at 0.1 µM, 1 µM or 10 µM for 24 h Values are represented as mean ± SD of 3 replicates.

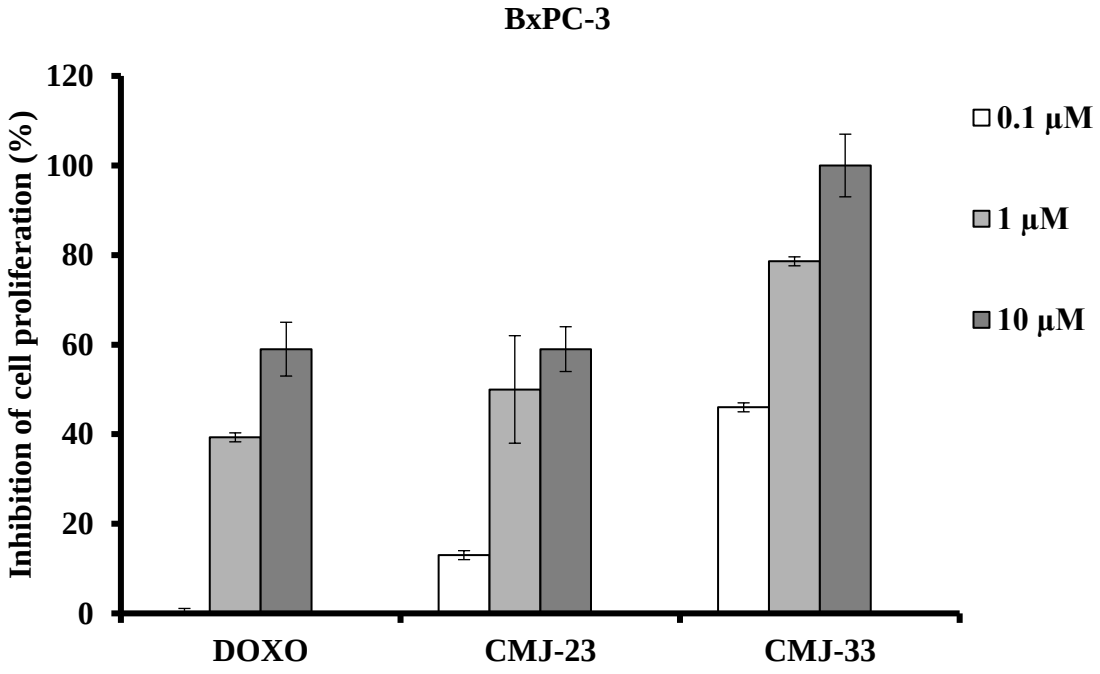


Fig. 3.14. Antiproliferative activity of the positive control DOXO and the tested compounds CMJ-23, CMJ-33 on human epithelial pancreatic adenocarcinoma cells of line BxPC-3. Values are represented as mean ± SD of 3 replicates.

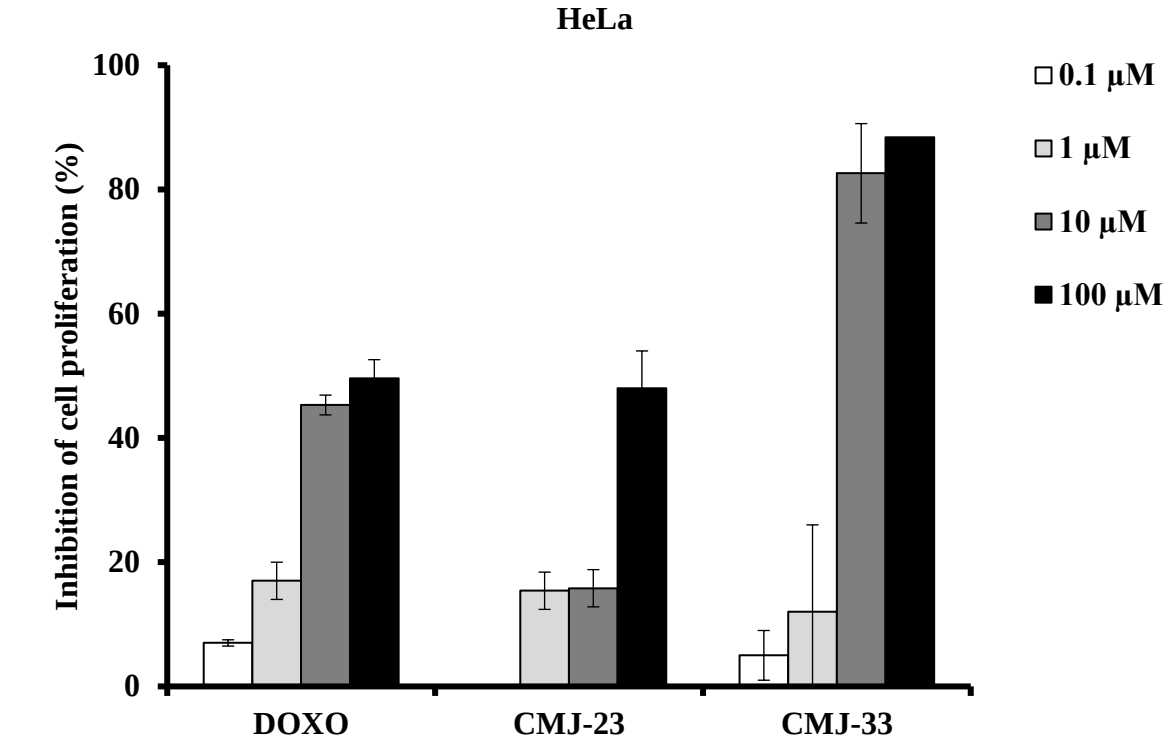


Fig. 3.15. Antiproliferative activity of the positive control DOXO and the tested compounds CMJ-23, CMJ-33 on human epithelioid cervix carcinoma cells of line HeLa. Values are represented as mean ± SD of 3 replicates.

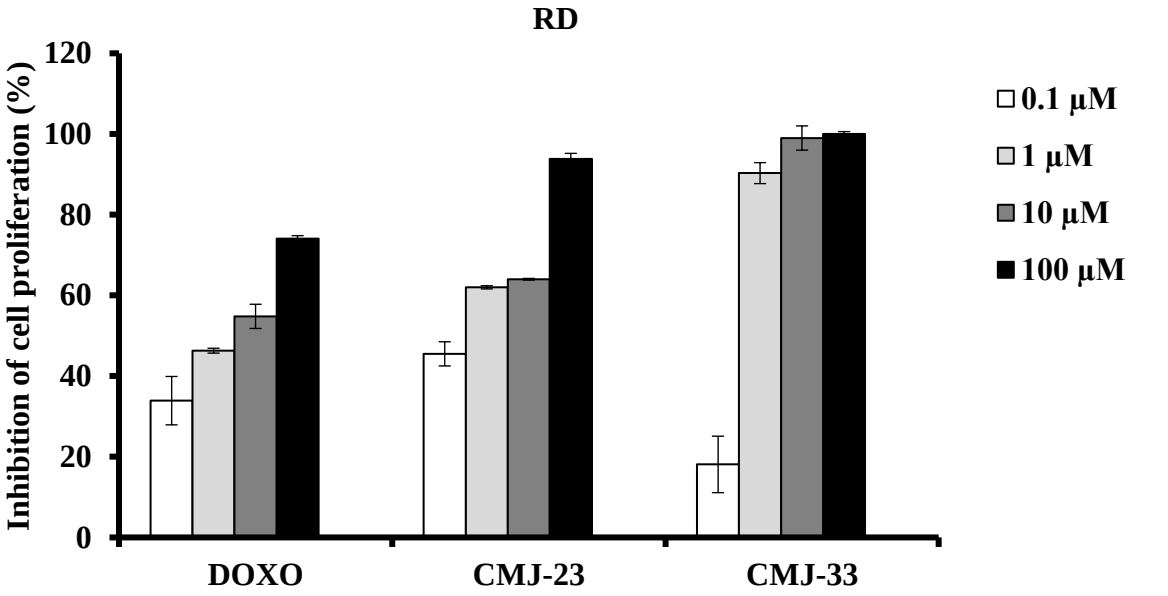


Fig. 3.16. Antiproliferative activity of the positive control DOXO and the tested compounds CMJ-23, CMJ-33 on human muscle rhabdomyosarcoma spindle and large multinucleated cells of line RD. Values are represented as mean ± SD of 3 replicates.

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.

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 normal kidney epithelial cells of MDCK line. 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 kidney epithelial cells of line MDCK are shown in figure 3.17.

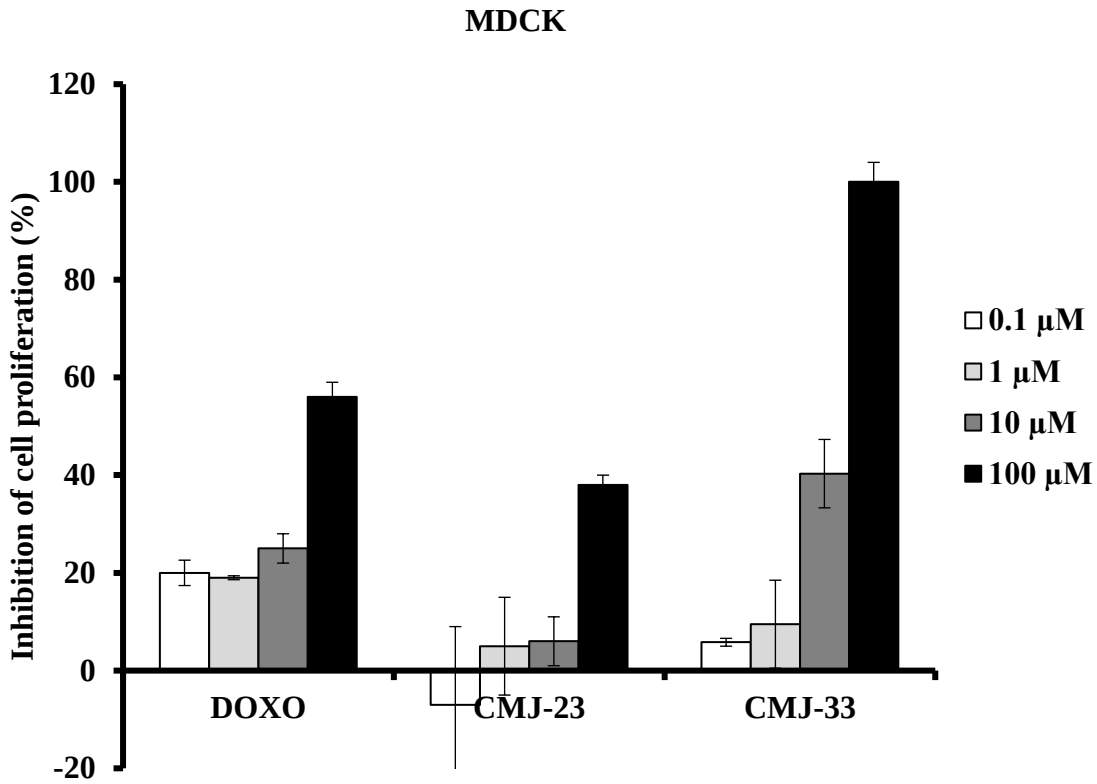


Fig. 3.17. Antiproliferative activity of the positive control DOXO and the tested compounds CMJ-23, CMJ-33 on normal kidney epithelial cells of MDCK line. Values presented were mean± SD of 3 replicates.

It was established that the tested compounds CMJ-23 and CMJ-33 showed low cytotoxic activity against MDCK line, with IC_{50} ≥100 µM, R^2 =0.8 and IC_{50} = 11.0±1.0 µM, R^2 = 0.8, respectively. The positive control DOXO exhibited cytotoxicity against MDCK line with IC_{50} of 7.0±0.3 µM and with the coefficient of determination value 0.7

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 3.4.

The selectivity index that is the ratio between the IC_{50} value for the normal cells (MDCK line) and IC_{50} 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 (SI) 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 MDCK line revealed that compound CMJ-23 is significantly less toxic than compound CMJ-33, what is an important aspect in personalized chemotherapy.

Table 3.2. Antiproliferative activity 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 cell line MDCK

	MDCK	**BxPC-3**		**RD**		**HeLa**		**MeW -164**	
Compound	**IC_{50} (µM)**	**IC_{50} (µM)**	**SI_1**	**IC_{50} (µM)**	**SI_2**	**IC_{50} (µM)**	**SI_3**	**IC_{50} (µM)**	**SI_4**
DOXO	7.0±0.3	6.0±0.8	1.2	2.3±0.9	3.04	6.2±1.0	1.13	7.3±0.3	0,96
CMJ-23	≥100	2.5±0.7	≥40.0	0.30±0.04	≥330	18.7±1.0	≥5.3	0.40±0.02	≥250.0
CMJ-33	11±1	0.10±0.04	110.0	0.20±0.03	55.0	0.40±0.02	28	0.20±0.01	55.0

Notes: IC_{50} valium is a concentration inhibiting cellular proliferation by 50%. SI valium is selectivity index.

Substances with a SI > 3 are considered to be promising.

The SI_1, SI_2, SI_3, SI_4 of each compound were calculated as the ratio of the IC50 for MDCK cells / IC50 for cancer cell lines BxPC-3, RD, HeLa, MeW-164, respectively.

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.

The tested compounds induced apoptosis of celss were examined by DNA fragmentation of agarose gel electrophoresis and flow cytometric analysis [181]. It was carried out as per DNA fragmentation analysis on agarose gel, containing ethidium bromide, which is an intercalating agent commonly used as a fluorescent tag (nucleic acid stain).

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.

In order to study the influence of the tested substances on DNA fragmentation, a concentration of 5 μM was used, which is average IC_{50} (μM) of all tested compounds against HeLa cells. As it can be obsered form figure 3.18, the tested compounds CMT-22, CMT-67, CMT-68, CMJ-23 and CMJ-33 induce DNA fragmentation in cancer cells because the light intensities of tracks of DNA molecules in cases 1-5 (Figure 3.18) are considerably lower than in case of control (Figure 3.18, lane C). These results as well as the results of flow fluorescent cytometry provide evidence that the tested compounds CMT-22, CMT-67, CMT-68, CMJ-23 and CMJ-33 induce cell-cycle arrest and apoptosis of cells [182, 183, 184].

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.

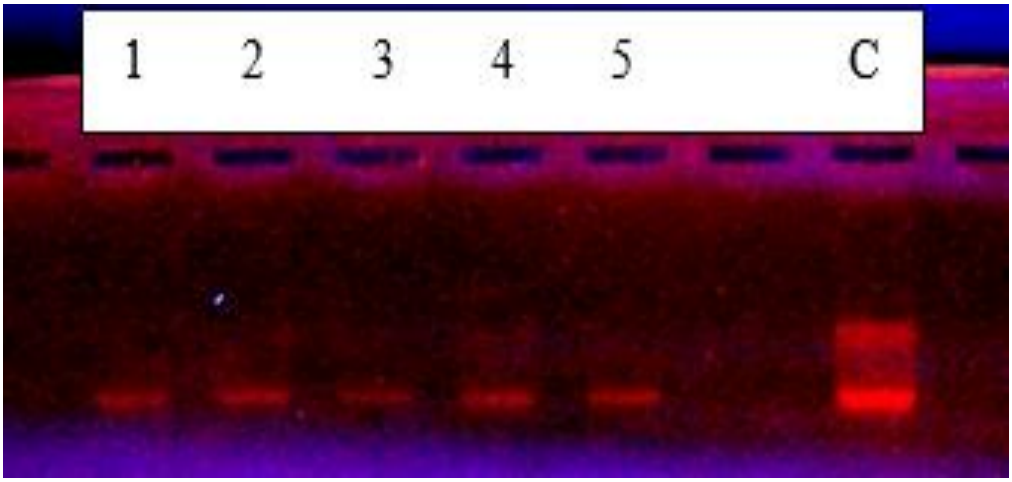


Fig. 3.18. The degradation effect of the tested compounds on the genomic DNA isolated from HEp-2 cells. *Notes:* Lane 1: fragmented DNA of cells after 24 h treatment with CMT-67 at 5 µM. Lane 2: fragmented DNA of cells after 24 h treatment with CMT-22 at 5 µM. Lane 3:fragmented DNA of cells after 24 h treatment with CMT-68 at 5 µM. Lane 4: fragmented DNA of cells after 24 h treatment with CMJ-33 at 5 µM. Lane 5: fragmented DNA of cells after 24 h treatment with CMJ-23 at 5 µM. Lane C: control - genomic DNA of cells without treatment.

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 [109].

Probably, the ability to induce DNA fragmentation of the mixed-ligand complex CMT-68 at concentration 5 µM (Figure 3.18, lane 3) 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 DNA molecules grooves during 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 (Figure 3.19).

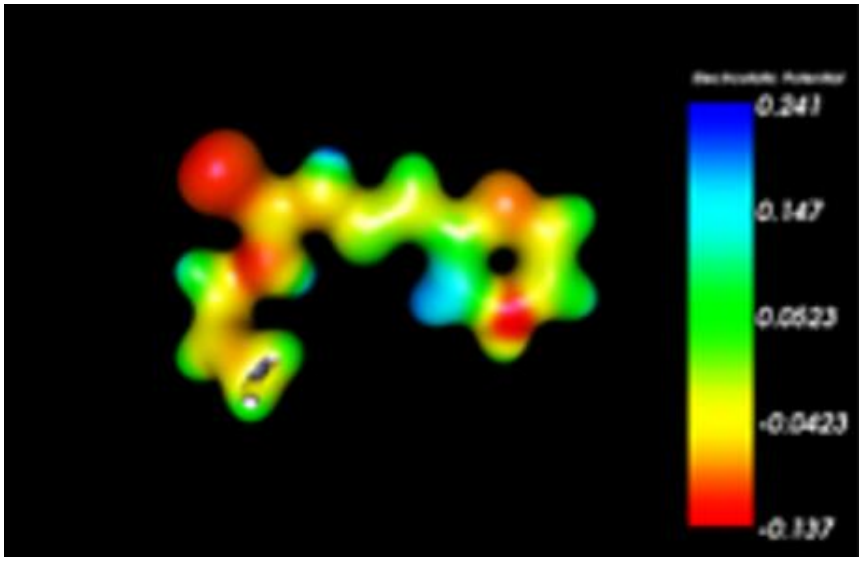
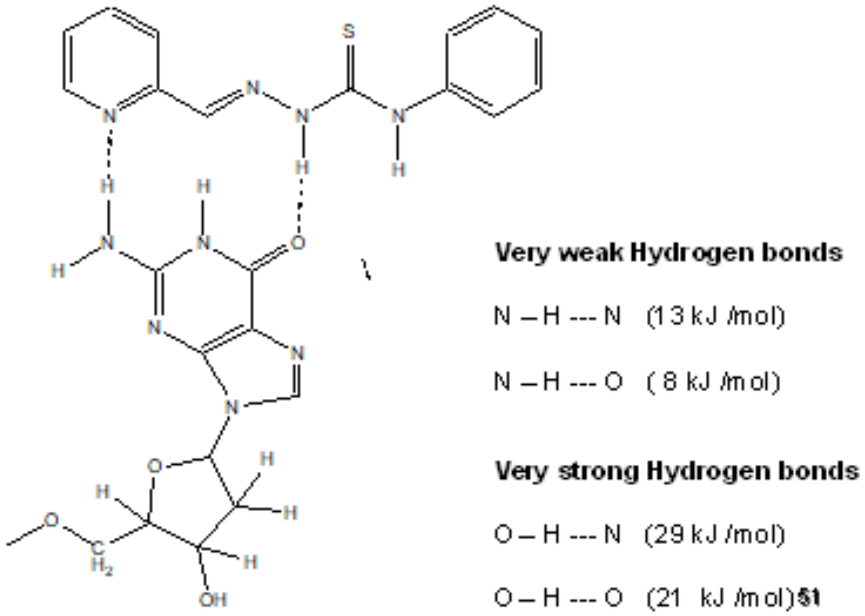


Fig. 3.19. Structure – molecular electrostatic surface charge. Azot from Py ring has electronegative charge and the capacity to form H-bonds with DNA and to inhibit cells proliferation is high.



Scheme 3.1. Interaction of thiosemicarbazone CMT-22 with guanine fragment of DNA molecule

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 (Scheme 3.1).

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.

3.4. Conclusions to chapter 3

1. All tested compounds exhibited an antiproliferative activity in relation to the tested cancer cells of lines.

2. Analyzing the results of the antiproliferative activity, it was established that the tested compounds exhibited high inhibitory activity on the tested cell of lines, such as MeW-164 (humanmalignantmelanoma), HeLa (humancervixadenocarcinoma), BxPC-3 (humanprimarypancreaticadenocarcinoma), RD (humanrhabdomyosarcoma) proliferation.

3. The tested compounds demonstrated selective cytotoxicity towards cancer cells. It was detected that the tested compounds have higher values of selectivity index than DOXO, while the tested thiosemicarbazones showed the highest values of selectivity index compared to DOXO and the tested complexes, which is an important aspect in personalized medicine.

4. Based on NMR and X-ray analysis, it was established that thiosemicarbazones interact with the DNA fragment (guanine) forming hydrogen bonds, which causes DNA fragmentation and finally apoptosis.

5. Electrophoresis method of DNA defragmentation confirms the mechanism of action of the studied substances linked with a direct effect on cell genomic DNA. The research findings identified a possible mechanism of action of the tested compounds associated with cellular apoptosis. Thus, the tested compounds showed enhanced antiproliferative activity, linked with increased induction of apoptosis, breaking structures of genomic DNA in the cell nucleus.

6. Altogether, these study observations indicated that the tested compounds may be potential anticancer drugs.

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

Active forms of oxygen (ROS), which include free radicals, peroxides of inorganic and organic origin and oxygen ions are products of normal oxygen metabolism in a living cell. Only 5% of the oxygen consumed by the cell as a result, as a rule, of enzymatic reactions, are converted to ROS. Cells are protected from reactive oxygen species by low molecular weight antioxidants, for example, vitamin C, glutathione, uric acid, as well as antioxidant enzymes such as superoxide dismutase, catalase, and glutathione peroxidase. The presence of various chemical pollutants in the environment stimulates increased ROS formation in the body, which leads to oxidative stress and a decrease in antioxidant protection. This, in turn, contributes to the development of various pathologies, and ultimately to cell death and tissue necrosis.

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 [185].

Synthetic antioxidants are widely used to inhibit oxidative processes. Most currently used antioxidant drugs are direct-acting antioxidants. Primary screening of direct-acting antioxidants is carried out on *in vitro* model systems, i.e. when the efficiency of their antioxidant action is determined primarily by the chemical structure of the substance and does not depend, in any way, on the general homeostasis of the body. Hydrophilic (ascorbic acid, uric acid, cysteine) and lipophilic (tocopherols, retinol, bilirubin) are distinguished among direct-acting antioxidants.

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 [186]. These compounds, which show considerable biological activity, may represent an interesting approach for designing new anticancer drugs [187].

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 [188], such as $ABTS^{\bullet+}$, $DPPH^{\bullet}$, ORAC and LOX [189]. The antioxidant potency of the tested compounds was compared to the

FDA-approved anticancer drug doxorubicin 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 [3, 164].

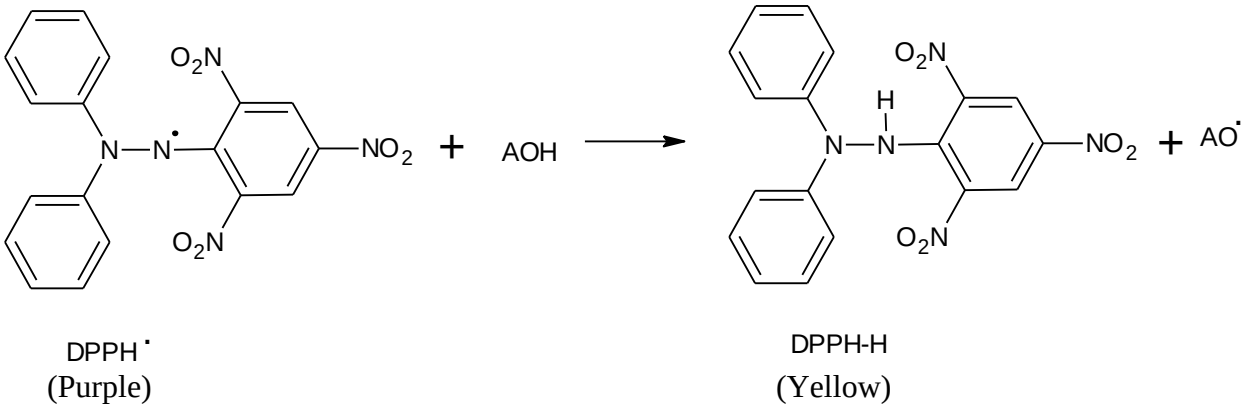
Drug-induced hemolysis and methemoglobin formation is a relatively rare but serious toxicity liability caused by oxidative stress, so *in vitro* assessment of the probability of development and the nature of possible side effects of the tested substances (CMT-22, CMT-67, CMT-68, CMJ-33 and CMJ-23) associated with hemolysis and the formation of methemoglobin in human red blood cells (RBCs) was investigaited.

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 [171].

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

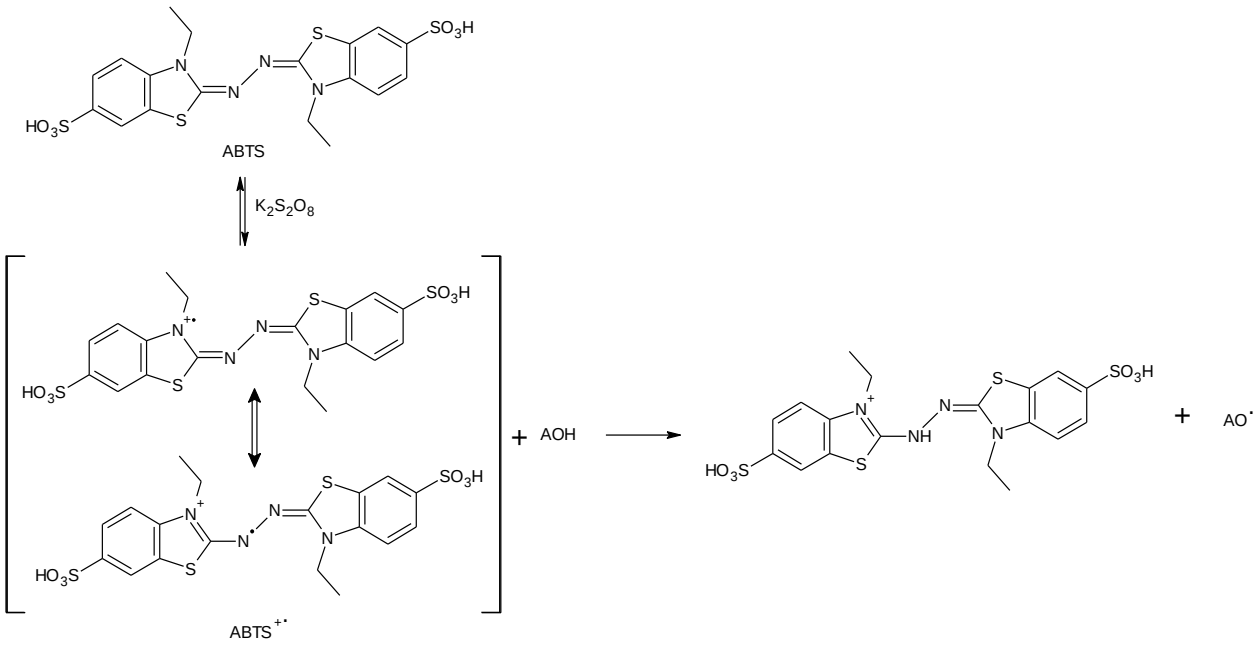
The $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ assays are widely used methods for the assessment of the total antioxidant capacities of the anticancer compound *in vitro* [162].

The effect of antioxidants on $DPPH^{\bullet}$ radical scavenging is due to the hydrogen donating ability or radical scavenging activity of the samples. The scavenging reaction between ($DPPH^{\bullet}$) and an antioxidant (AOH) can be depicted as scheme 4.1.



Scheme 4.1. Reaction of stable radical $DPPH^{\bullet}$ with an antiradical compound (AOH)

The $ABTS^{\bullet+}$ method is based on the variation in the long wavelength absorption of a stable radical, the radical cation $ABTS^{\bullet+}$ (2,2'-azobis-(3-ethylbenzothiazoline-6-sulfonate)) [160]. Unlike the commercially available stable free radical $DPPH^{\bullet}$, $ABTS^{\bullet+}$ is generated by the oxidation of ABTS, commonly using potassium persulfate (Scheme 4.2). Antioxidants inhibit the oxidation of $ABTS^{\bullet+}$ by electron transfer radical scavenging and hydrogen donating [159, 190, 189].



Scheme 4.2. Oxidation of $ABTS^{\bullet+}$ by potassium persulfate to generate radical cation $ABTS^{\bullet+}$ and its reaction with an antiradical compound (AOH)

Thus, the tested compounds CMT-22, CMT-67, CMT-68, CMJ-33, CMJ-23, as well as the FDA-approved anticancer drug doxorubicin (DOXO) and the reference antioxidant compounds trolox and rutin were screened for free radical scavenging activity by the $ABTS^{\bullet+}$ method. The percentage inhibition results of free radical scavenging activity of the tested compounds are shown in figure 4.1.

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 4.1, 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±0.1 μM, 6.20±0.01 μM, 11.4±0.4 μM, 14.9±1.4 μM, respectively (Table 4.1). The coefficients of determination (R^2) values are 0.99, 0.99, 0.99, 1, 1 for the tested compounds CMT-22, CMT-67, CMT-68, CMJ-33, CMJ-23, respectively.

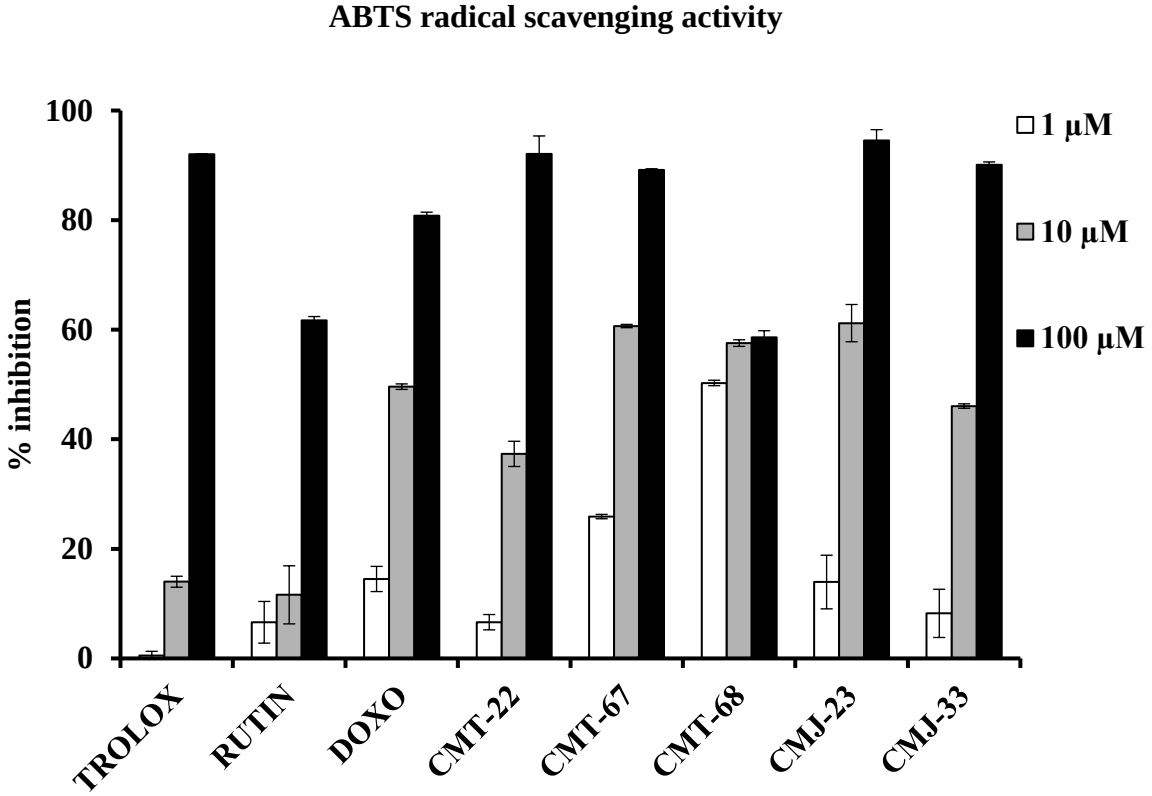


Fig. 4.1. $ABTS^{\bullet+}$ scavenging activity of the tested compounds CMT-22, CMT-67, CMT-68, CMJ-33, CMJ-23, as well as the anticancer drug doxorubicin and the reference antioxidant compounds (trolox, rutin). Values are represented as mean ± SD of 3 replicates.

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 increased antioxidant activity of CMT-68 and CMT-67 complexes (Table 4.1) can be attributed to the electron withdrawing effect of the Cu(II) ion which facilitates the release of hydrogen to reduce the $ABTS^{\bullet+}$ radical.

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 4.2.

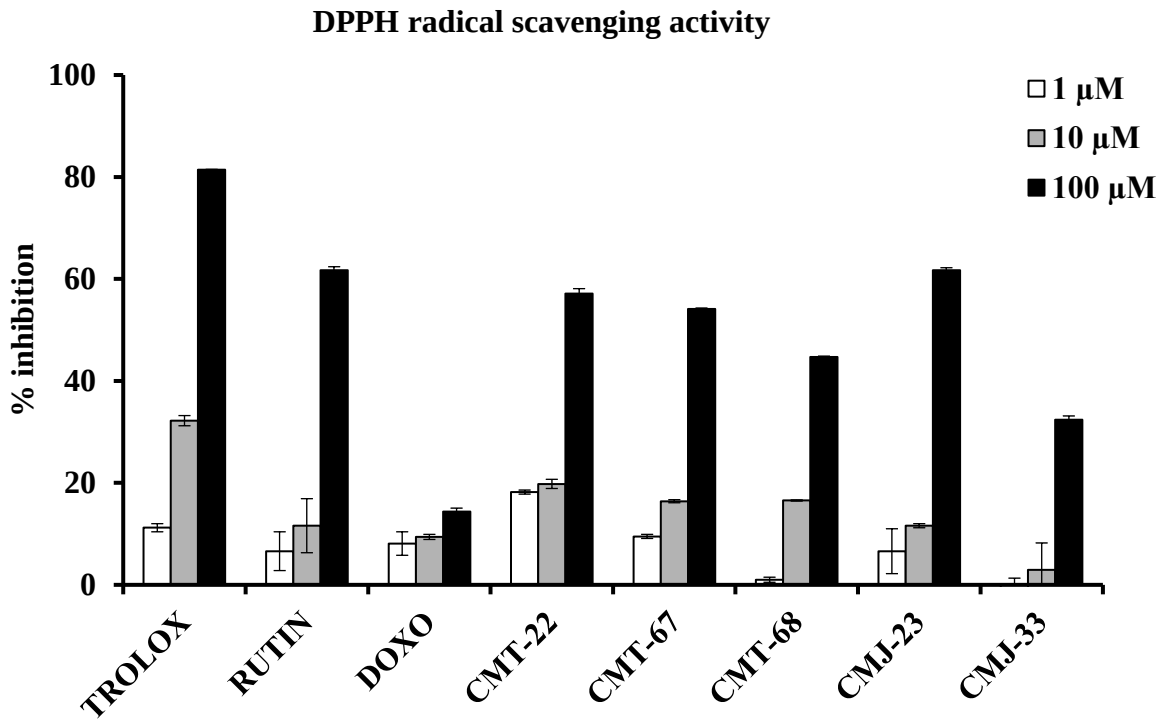


Fig. 4.2. $DPPH^{\bullet}$ scavenging activity of the tested compounds CMT-22, CMT-67, CMT-68, CMJ-33, CMJ-23, as well as the anticancer drug doxorubicin and the reference antioxidant compounds (trolox, rutin). Values are represented as mean ± SD of 3 replicates.

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 %.

It was found that the inhibitory effect of the compounds on percentage DPPH$^{\bullet}$ scavenging activity was in a concentration dependent manner. The IC_{50} values of the tested compounds and the reference controls are represented in table 4.1, along with the coefficient of determination values. 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.

Table 4.1. 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

Note: Dose-response plots of percent inhibition versus concentration were obtained from triplicate samples and adjusted to sigmoidal curves, from which values of the 50% inhibitory concentration (IC_{50}) were calculated.

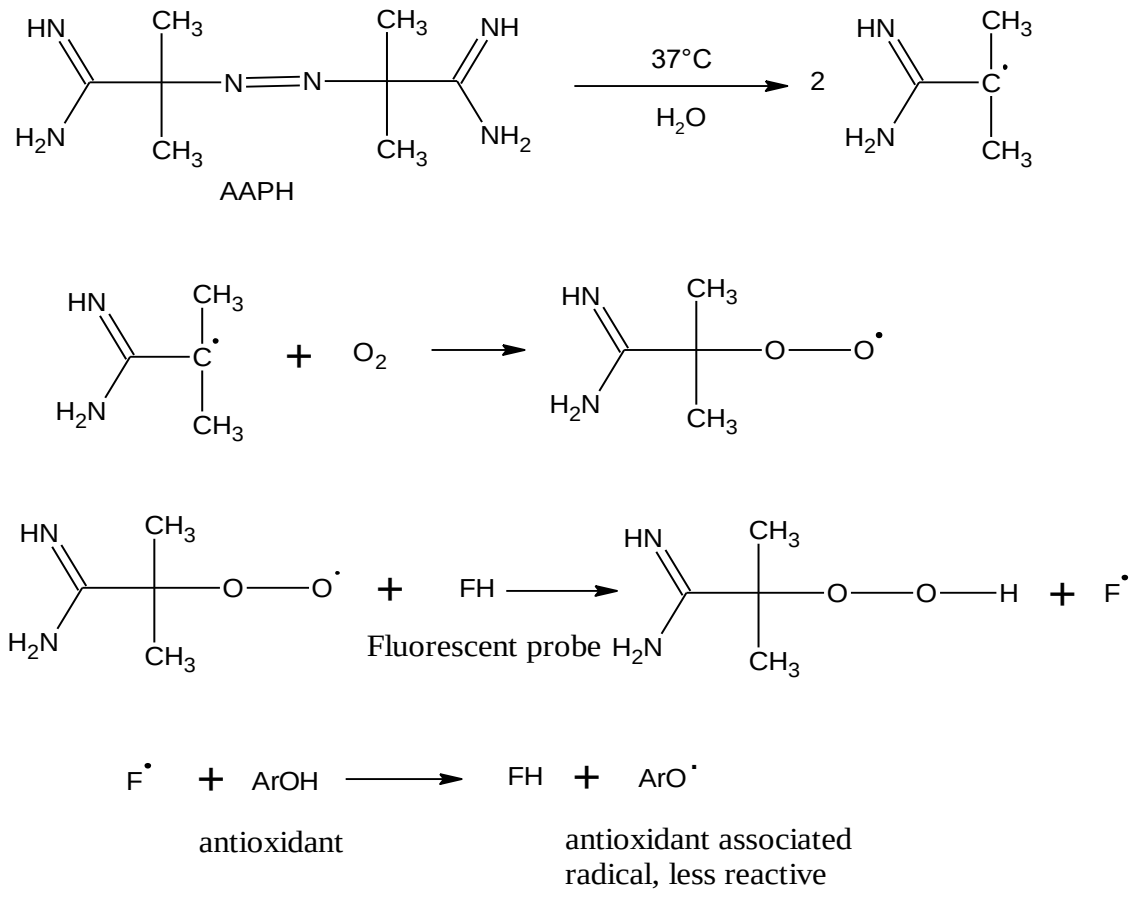
The scavenging of the DPPH$^{\bullet}$ radical by the tested compounds was found to be moderate compared to that of ABTS$^{\bullet+}$ radical cation. The enhanced inhibition displayed on the ABTS$^{\bullet+}$

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 antioxidant capacity of the tested compounds such as 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 measured by ORAC assay and their potency was compared with that of the reference antioxidant control trolox, a water-soluble vitamin E analogue.

The ORAC assay is based on *in situ* production of peroxyl free radicals generated via an azo-compound, 2,2,-azobis(2-methylpropionamidine) dihydrochloride, according to the reaction presented in scheme 4.3.



Scheme 4.3. Oxygen Radical Absorption Capacity (ORAC) assay measuring principle

An azo-compound, 2,2,-azobis(2-methylpropionamidine) dihydrochloride (AAPH), thermally generates C centred free radicals, which in the presence of oxygen generates the peroxy free radicals interacting with the fluorescent probe.

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.

A standard antioxidant trolox (Figure 4.3), was used as reference, and ORAC values of the tested compounds are reported as trolox equivalents. The ORAC method measures hydrogen atom donating ability of antioxidants, and is, therefore, a hydrogen atom transfer (HAT) - based method.

The oxygen antioxidant capacity of the tested compounds CMT-22, CMT-67, CMT-68, CMJ-33, CMJ-23 were estimated by comparison to the standard curve of trolox. Figure 4.3 depicting the kinetic curves of trolox demonstrates the concentration dependent protection of fluorescein against the oxidative degradation by AAPH. The progress of each reaction was followed in real time using the current state option [164].

The Net AUC calculated by equation is graphically indicated in figure 4.4. The curves were characterized by the coefficient of determination values (R^2): 0.99 for trolox, 0.98 for DOXO, 0.88 for CMT-22, 0.87 for CMT-67, 0.67 for CMT-68, 0.94 for CMJ-23 and 0.78 for CMJ-33.

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 (Table 4.2).

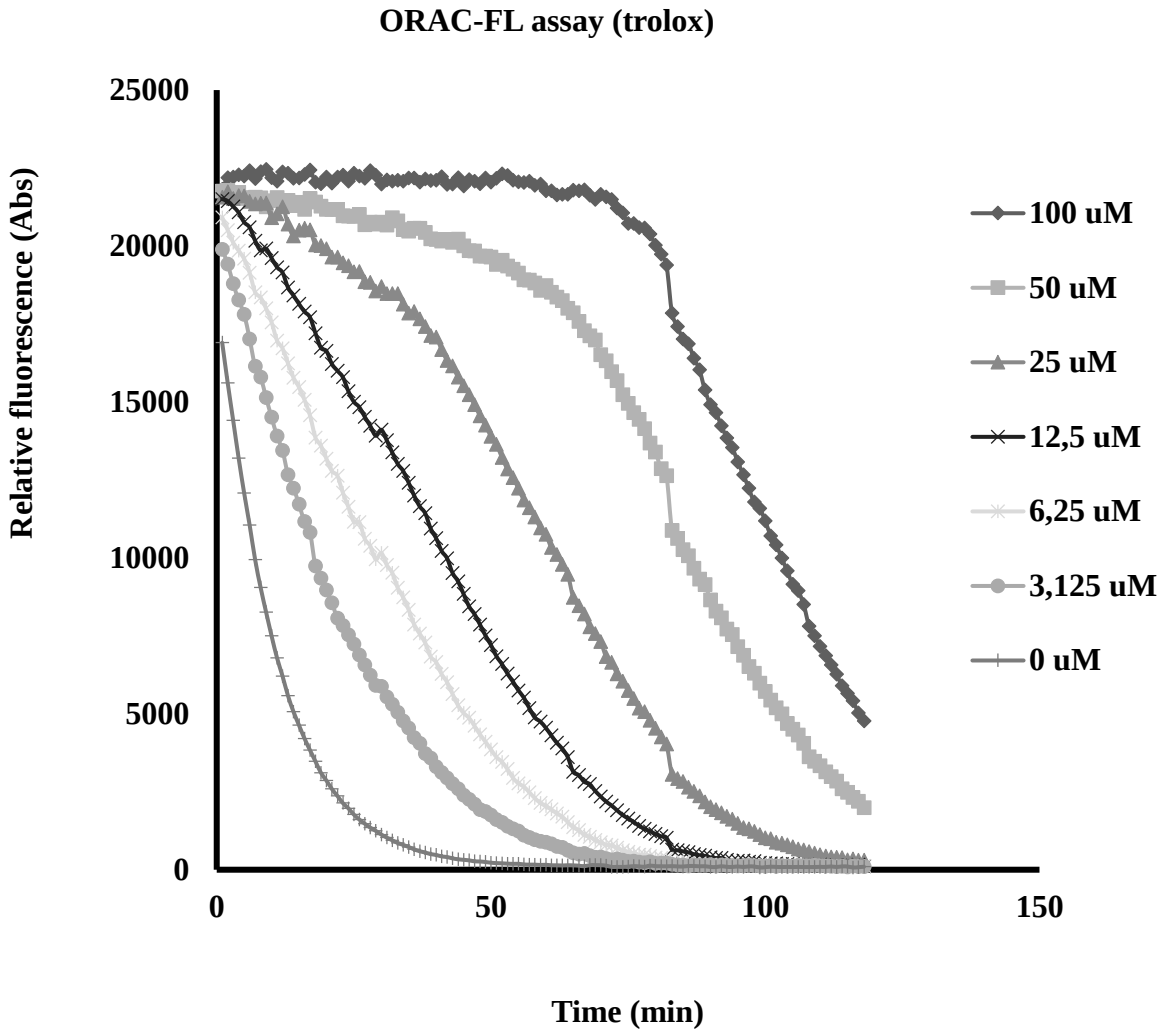


Fig. 4.3. Plots of trolox kinetic curves. The representative curves from ORAC assay of varying concentrations of trolox antioxidant standards ranging from 0 to 100 µM. The automated ORAC assay was performed on a microplate reader with fluorescence filters FP (485 nm / 528 nm). The experiment was conducted at 37^0 C under pH 7.4 condition with a blank sample in parallel. The fluorescence was recorded every minute for 100 min.

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 [191].

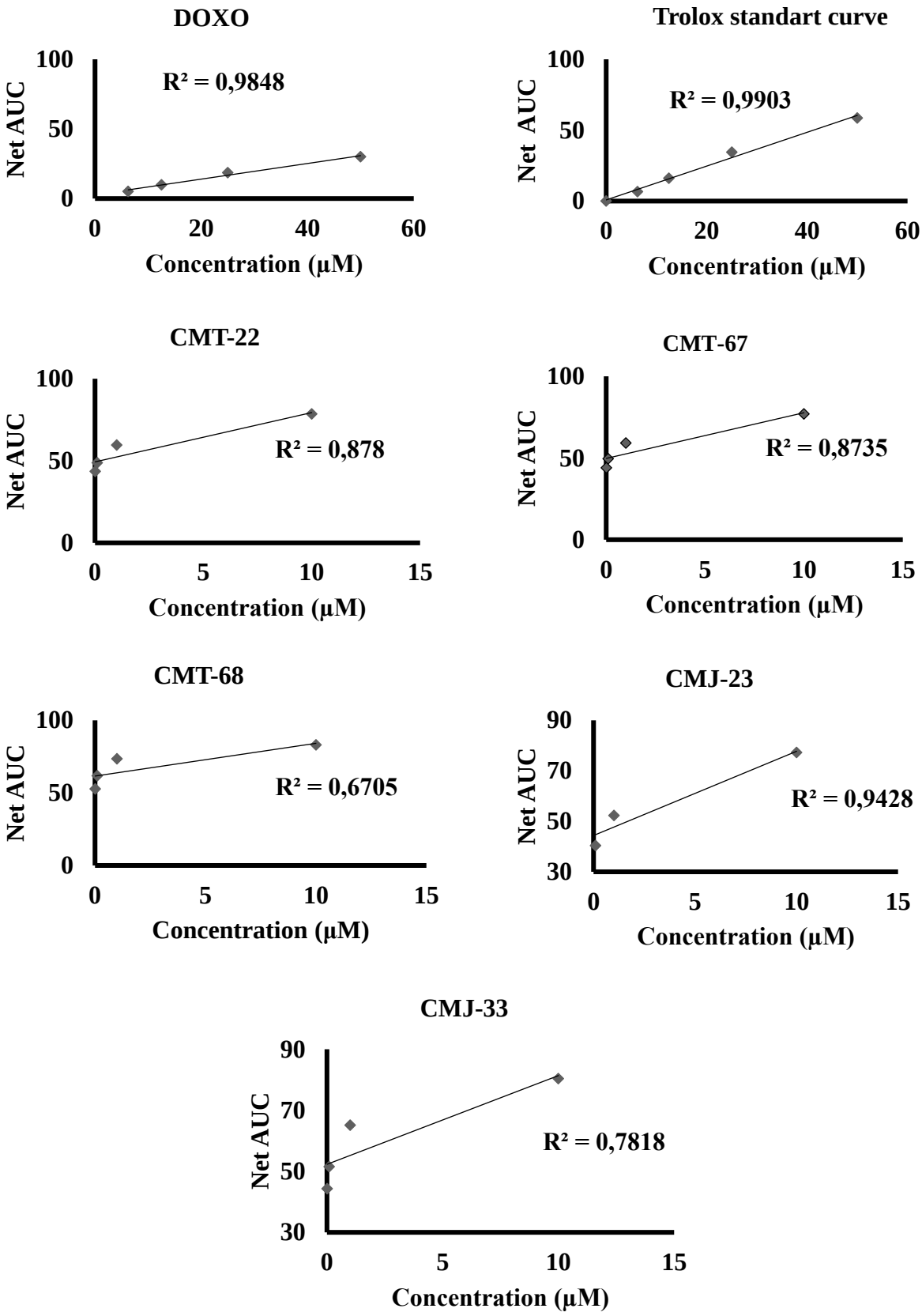


Fig. 4.4. The Net AUC of varying concentrations of the reference antioxidant control trolox, the anticancer drug doxorubicin and the tested compounds CMT-22, CMT-67, CMT-68, CMJ-33 and CMJ-23. Values are represented as mean ± SD of 3 replicates.

Table 4.2. Antioxidant activity of the tested compounds, reference antioxidant control trolox and anticancer drug doxorubicin (DOXO)

Oxygen Radical Absorbance Capacity (ORAC-FR)		
Compound	**m**	**TE**
Trolox	1,2	1
DOXO	0,5	0.4
CMT-22	3.4	2.8
CMT-67	3.5	2.9
CMT-68	3.6	3.0
CMJ-23	3.7	3.1
CMJ-33	3.6	3.0

Note: trolox equivalent (TE), slope (m)

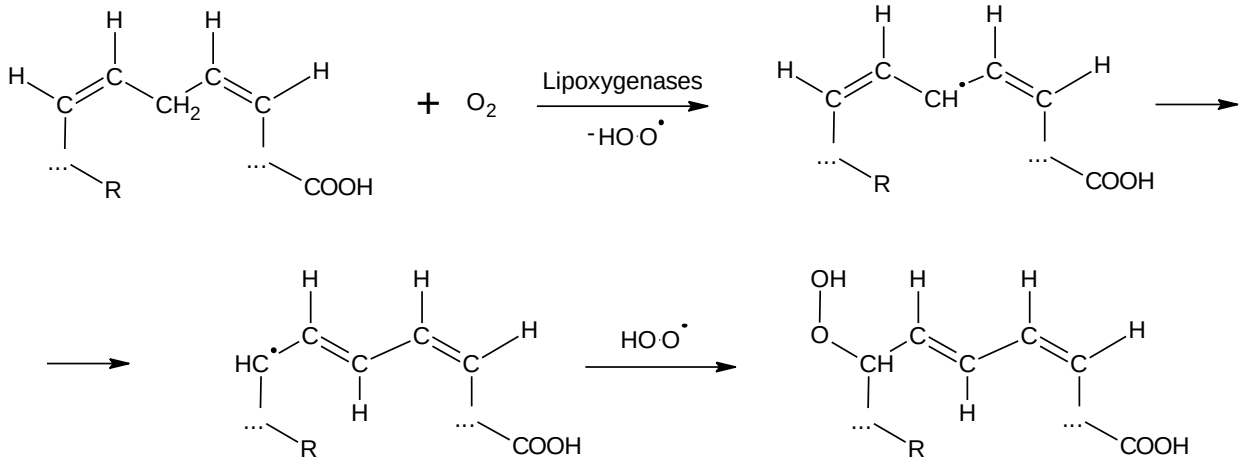
The oxygen antioxidant capacity of the compounds can thus be ranked in the order CMJ-23 ≥ CMJ-23 = CMT-68 ≥ CMT-67 ≥ CMT-22 ≥ DOXO ≥ trolox. Thus, it was found that the tested compounds showed the highest oxygen radical absorbance capacity compared with DOXO.

4.3. Antilipoxygenase activity of the tested compounds

Lipoxygenase constitutes a family of non-heme iron containing enzymes, as versatile biocatalysts are capable of catalyzing many reactions involved in the xenobiotic metabolism. The lipoxygenase enzyme catalyzes the reaction: unsaturated fatty acid + O_2 = unsaturated fatty acid hydroperoxide. It is believed that only unsaturated fatty acids that contain a *cis*-*cis*-1,4-pentadiene group are exposed to the enzyme, as shown in scheme 4.4.

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).

The intermediate formation of radical results in peroxide, one double bond moves to the adjacent (conjugated) position, and the unsaturated fatty acid passes into the *cis*-trans isomer. Linoleic, linolenic and arachidonic acids are oxidized by the enzyme at the same rate. Fatty acids with trans-configuration of double bonds are not oxidized by the enzyme.



Scheme 4.4. Oxidation reaction of polyunsaturated fatty acid by lipoxygenase enzyme.

Lipoxygenases are the key enzymes in the biosynthesis of leukotrienes (LTs) that play an important role in several inflammation-related diseases such as arthritis, asthma, cancer, and allergic diseases. High levels of LTs could be observed in the case of asthma, psoriasis, allergic rhinitis, rheumatoid arthritis, and ulcerative colitis. Therefore, it is of the view that the production of LTs can be prevented via inhibition of the LOX pathway and targeting LOX with inhibitors is of a promising therapeutic target for treating a wide spectrum of human diseases. Pidgeon et al., also suggested that LOX inhibitors may lead to the design of biologically and pharmacologically targeted therapeutic strategies inhibiting LOX isoforms and their biologically active metabolites which may be useful in cancer treatment. Many COX-2 or 5-LOX inhibitors have been developed as drugs to treat inflammation [166].

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 [192] was evaluated (Table 4.3).

Table 4.3. 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 by FTC-based LOX activity method.

Compound	% inhibition mean ± SD			R^2
	0.05 μM	0.5 μM	5 μM	
Quercetin	20.0±1.5	28.0±2.1	50.0±0.8	0.94
DOXO	34.5±0.5	37.0±1.0	40.0±1.0	0.99
CMT-22	48.5±0.5	52.5±2.5	61.0±1.0	0.95
CMT-67	44.2±1.5	53.1±0.5	77.6±1.9	0.93
CMT-68	48.4±0.5	53.1±0.4	58.1±0.9	0.91
CMJ-33	46.0±1.0	54.0±0.4	61.0±1.2	0.95
CMJ-23	42.0±0.2	54.0±0.9	70.0±0.7	0.97

Note: Values are represented as mean ± SD of 3 replicates.

The antioxidant quercetin was used as positive control. Quercetin is a plant flavonol from the flavonoid group of polyphenols, the most abundant of the flavonoid molecules, are widely distributed in plants.

The tested compounds CMT-22, CMT-67, CMT-68, CMJ-33 and CMJ-23 were able to induce inhibition of 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 (Figure 4.5).

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. 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 [193].

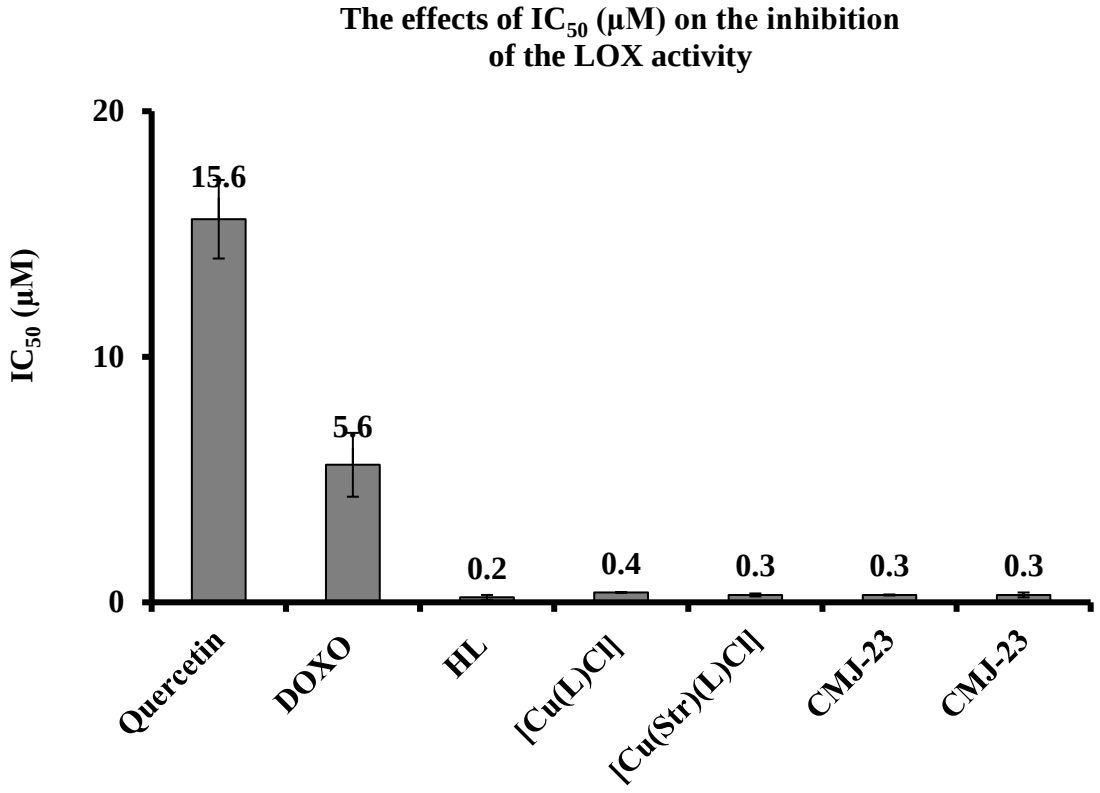


Fig. 4.5. The effects of IC_{50} of the positive control quercetin, anticancer drug DOXO and the tested compounds CMT-22, CMT-67, CMT-68, CMJ-33 and CMJ-23 on the inhibition of the lipoxygenase activity.

Therefore, novel potent inhibitors of LOX are required to enable the drug discovery efforts [194]. 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. Impact 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.

The spatial structure of the hemoglobin molecule was studied in detail by X-ray diffraction analysis as early as the end of the 1940s, mainly due to the works of the English biophysicist M. Perutz. Hemoglobin is a complex protein represented by two parts: a non-protein planar part — heme (4% of the hemoglobin molecule) and a protein oligomeric globule (96%).

Heme (protogem) is the tetrapyrrole aromatic structure of protoporphyrin IX, which necessarily contains the Fe^{2+} ion. The SP^2 hybrid atoms of the C and N π- electronic systems of the porphyrin oligand provide its planar structure [195]. Heme is a prosthetic group of heme-containing proteins that ensures their main functions: oxygen binding and transport, participation in the electron transfer chain, oxygen reduction to water, microsomal oxidation, decomposition of peroxides, etc. The presence of Fe^{2+} ion and the aromatic nature of the tetrapyrrole core provide intense heme color, which allows assigning of heme-containing proteins to the class of chromoproteins.

Globin, a protein component of hemoglobin, belongs to the group of histones. The number of amino acid residues in the polypeptide chain of different types ranges from 140 to 150, and numbers 141, 146, 153 being more common. Human hemoglobin protomers contain either 141 (α-chain) or 146 (β-, γ-, δ -chains) of amino acid residues.

The secondary structure of hemoglobins is represented by an α-helix. Polypeptide chains in a hemoglobin molecule are represented by more than 70% twisted into α-helix spirals, fragments of which are separated by sections that form the bends of the secondary chain. Tertiary ovoid globules are formed as a result of this stacking. The number of helical regions varies in different Hb chains. The α-chain has 7 such domains, the β-chain - 8. Spiralized hemoglobin fragments are denoted in Latin letters, starting at the O end of the polypeptide chain (for example, in the α-subunit: A, B, C, D, E, F, H). The spatial layout of the tertiary structure of hemoglobin subunits corresponds to the principles of tertiary folding of most globular proteins: maximum compactness, hydrophobic amino acid radicals are located inside the globule, most hydrophilic amino acid residues are located on the surface of the protomer. The physiological form of the quaternary structure of human hemoglobins is tetrameric. All four hemoglobin protomers are spatially located in a certain ratio of „quaternary structure", forming a tetrahedral configuration with respect to each other. The hemoglobin tetramer is a spheroid 64A long, 55A wide and 50A high. Each Swedberg unit is in contact with three other Swedberg units, loosely linked by non-covalent bonds (hydrogen, salt and, mainly, hydrophobic). Nonpolar hydrophobic

bonds, being turned inside the protein, play a major role in stabilizing the quaternary hemoglobin structure.

O_2 addition is ensured by the content of the Fe^{2+} atom in the heme. This reaction is reversible and depends on the partial O_2 pressure. Besides transporting O_2 to tissues, Hb carries out the reverse transport of the main final gas metabolite of tissues - carbon dioxide. Approximately 15-20 % of carbon dioxide and H^+ ions present in the blood are carried by Hb molecules. Hemoglobin binds two protons for every four released oxygen molecules.

Typically, up to 1 % methemoglobin is accumulated in red blood cells per day, which is associated with the normal process of autooxidation of normal hemoglobin. Methemoglobin is formed as a result of iron oxidation in hemoglobin heme, i.e. methemoglobin formation occurs under the influence of any substances. This is facilitated by a change in the conformation of the protein part of the hemoglobin molecule due to the oxidation of a number of functional groups of the protein.

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 [169].

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.

Toxic methemoglobinemia of exogenous origin is known to occur when exposed to chemical agents, such as analgesics (acetanilide, phenacetin), nitrobenzenes / nitrobenzoates, nitroglycerin, nitrofuragin, trinitrotoluene, hydroxylamine, dimethylamine, local anesthetics (lidocaine, prilocadone, prilocadone, prilocainum, prilocadone, dlocadone, dlocadone, dlocadone, metoclopramide (Cerucal), sulfamethoxazole, sulfonamides, menadione (vitamin K3), naphthoquinone, phenazopuridine (Puridium), antibiotics (ampicillin, amikacin, gentamicin, carbenicillin). Some of them directly oxidize Hb, others form intermediate aggressive forms. Acute poisoning poses a threat to life, therefore, those who exhibit atypical cyanosis or cyanosis, combined with a normal blood gas content, need to measure methemoglobin content.

The severity of symptoms depends on the content of methemoglobin in the blood (Table 4.4) [45]. Cyanosis occurs at around 15-30 % metHb and tissue hypoxia can occur as levels rise further - metHb levels of 70 % can be fatal [45].

Table 4.4. Relationship of clinical symptoms with the proportion of methemoglobin fraction in the blood [45].

MetHb (%)	Symptomatology
<3	Lack of clinical manifestations
3—15	Grayish skin tone
15—30	Cyanosis, brown staining of blood
30—50	Dyspnea, headache, weakness, dizziness, fainting
50—70	Tachypnea, metabolic acidosis, arrhythmias, convulsions, coma
>70	Hypoxia, death

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.

However, in conditions of massive toxic methemoglobin formation, the enzymatic efficiency is sharply reduced. Therefore, this requires the presence of drugs in the cell that can activate endogenous methemoglobin reductase systems or restore methemoglobin directly. Substances with redox properties can be considered the most promising among the drugs studied to date. The most active antidote for toxic methemoglobinemia is methylene blue, which has a

high redox potential. However, methylene blue has negative properties. The most dangerous of them is intravascular hemolysis. Currently, methylene blue is not used in medical practice.

The biomedical significance of metal-catalyzed HbO_2 oxidation is through acquired methemoglobinemia. There has been serious concern about hypoxia and methemoglobinemia side effects during anticancer therapies using the iron chelator 3-amino-2-pyridinecarboxaldehyde thiosemicarbazone (3-AP, triapine), which is being examined in phase I, II and phase III clinical trials for the treatment of various cancers. 3-AP has been shown to induce the formation of metHb (methemoglobinemia) and hypoxia in patients, limiting its usefulness [196].

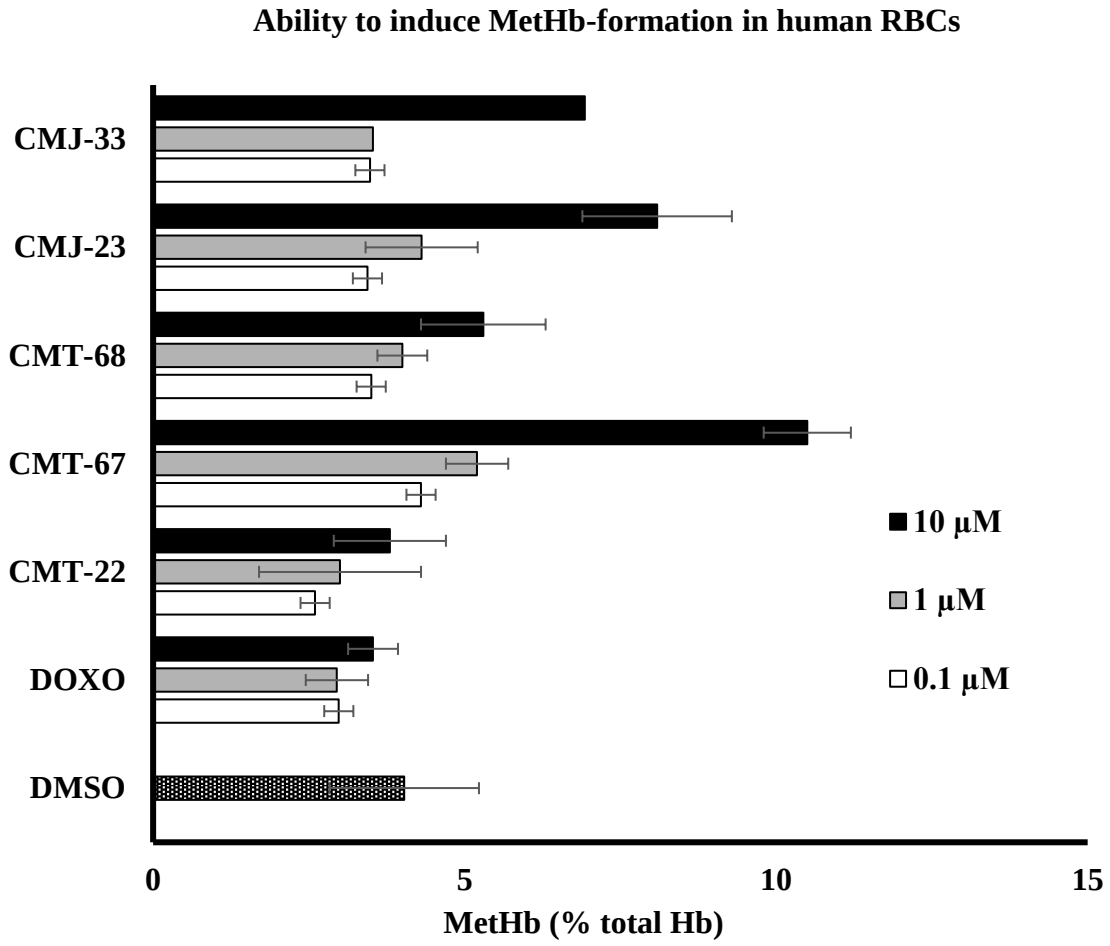


Fig. 4.6. Effect of the tested compounds CMT-22, CMT-67, CMT-68, CMJ-23, CMJ-33 and the anticancer control DOXO on metHb formation *in vitro*. The blood sample methemoglobin concentration was measured using the method below, which is based on Evelyn and Malloy method. Double dilutions of the tested compounds were prepared in DMSO at three concentrations (0.1 µM, 1 µM, 10 µM). Values are represented as mean ± SD of 3 replicates.

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 4.6).

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 [168].

The study protocol was approved by the Research Ethics Committee of the *Nicolae Testemitanu* State University of Medicine and Pharmacy (18.06.2015).

4.5. Impact of the tested compounds on RBCs hemolysis

Toxic ROS (reactive oxygen species) constantly appear in small amounts from O_2. These compounds are strong oxidizing agents or extremely reactive free radicals that destroy cellular structures and functional molecules.

The oxygen molecule (O_2) contains two unpaired electrons and, therefore, is a biradical. However, unpaired electrons are arranged so that the O_2 molecule remains relatively stable. Nevertheless, if the molecule attaches an additional electron, a highly reactive superoxide radical ($^{\bullet}O_2^-$) is formed. The next stage of reduction leads to the peroxide anion ($^{\bullet}O_2^-$), which easily binds protons and, as a result, transfers to hydrogen peroxide (H_2O_2). The addition of the third electron leads to the splitting of the molecule into O_2^- and O^- ions. While O_2^- forms water by the addition of two protons, the protonation of O^- leads to a particularly dangerous hydroxyl radical.

The addition of the fourth electron and the final protonation of O^- end with the formation of water is depicted below (Scheme 4.5).

$$\begin{array}{l} Hb\left(Fe^{2+}\right) \\ \quad\downarrow \longrightarrow \bar{e} + O_2 \longrightarrow O_2^- \xrightarrow[\bar{e},2H^+]{} H_2O_2 \xrightarrow[\bar{e},H^+]{} H_2O + OH^{\bullet} \\ MetHb\left(Fe^{3+}\right) \end{array}$$

Scheme 4.5. Formation of reactive oxygen forms and possible pathways for electron loss.

Red blood cells are especially susceptible to ROS damage, which, due to their transport function, are characterized by a high oxygen concentration. ROS can cause erythrocyte hemolysis. Red blood cells contain an enzyme system that prevents the toxic effects of oxygen radicals and the destruction of red blood cell membranes.

To protect against ROS and other radicals, all cells contain antioxidants. The latter are reducing agents that readily react with oxidizing substances and therefore protect the more important molecules from oxidation. Biological antioxidants include vitamins C and E, coenzyme Q and some carotenoids. The bilirubin formed during the destruction of heme also serves as protection against oxidation.

Glutathione, the Glu-Cys-Gly tripeptide, which is found in almost all cells in high concentration, is especially important. The thiol group of the cysteine residue is the reducing agent here. Two molecules of the reduced form (GSH) develop a disulfide upon oxidation. Red blood cells also have a system (superoxide dismutase, catalase, GSH) that can inactivate ROS and repair the damage they cause. For this, substances are needed that ensure the maintenance of normal metabolism in red blood cells. The metabolism in red blood cells is, in essence, limited by anaerobic glycolysis and the pentose phosphate pathway.

The ATP formed during glycolysis serves primarily as a substrate of Na^+, K^+, ATPase, which supports the membrane potential of red blood cells. NADPH + H^+ is formed in the pentose phosphate pathway, which supplies H^+ to regenerate reduced glutathione (GSH) from glutathione disulfide (GSSG) using glutathione reductase. Reduced glutathione is the most important red blood cell antioxidant; it serves as a coenzyme in the reduction of methemoglobin into functionally active hemoglobin. Selenium-containing glutathione peroxidase is also an important protective enzyme.

H_2O_2 detoxication occurs with the aid of reduced glutathione which detoxifies, as well as hydroperoxides, which arise during the reaction of ROS with unsaturated fatty acids of the erythrocyte membrane.

When taking certain medicinal substances, which are strong oxidizing agents, the potential of glutathione protection may not be sufficient. This leads to an increase in the content of ROS in cells that cause the oxidation of SH groups of hemoglobin molecules. The formation of disulfide bonds between the hemoglobin and methemoglobin protomers results in their aggregation - the formation of Heinz bodies. The latter contribute to the destruction of red blood cells (hemolysis) when they penetrate into small capillaries. 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 effects caused by thiosemicarbazones [197], the ability of CMT-22, CMT-67, CMT-68, CMJ-23, CMJ-33 and DOXO was compared to induce human RBCs hemolysis (Figure 4.7).

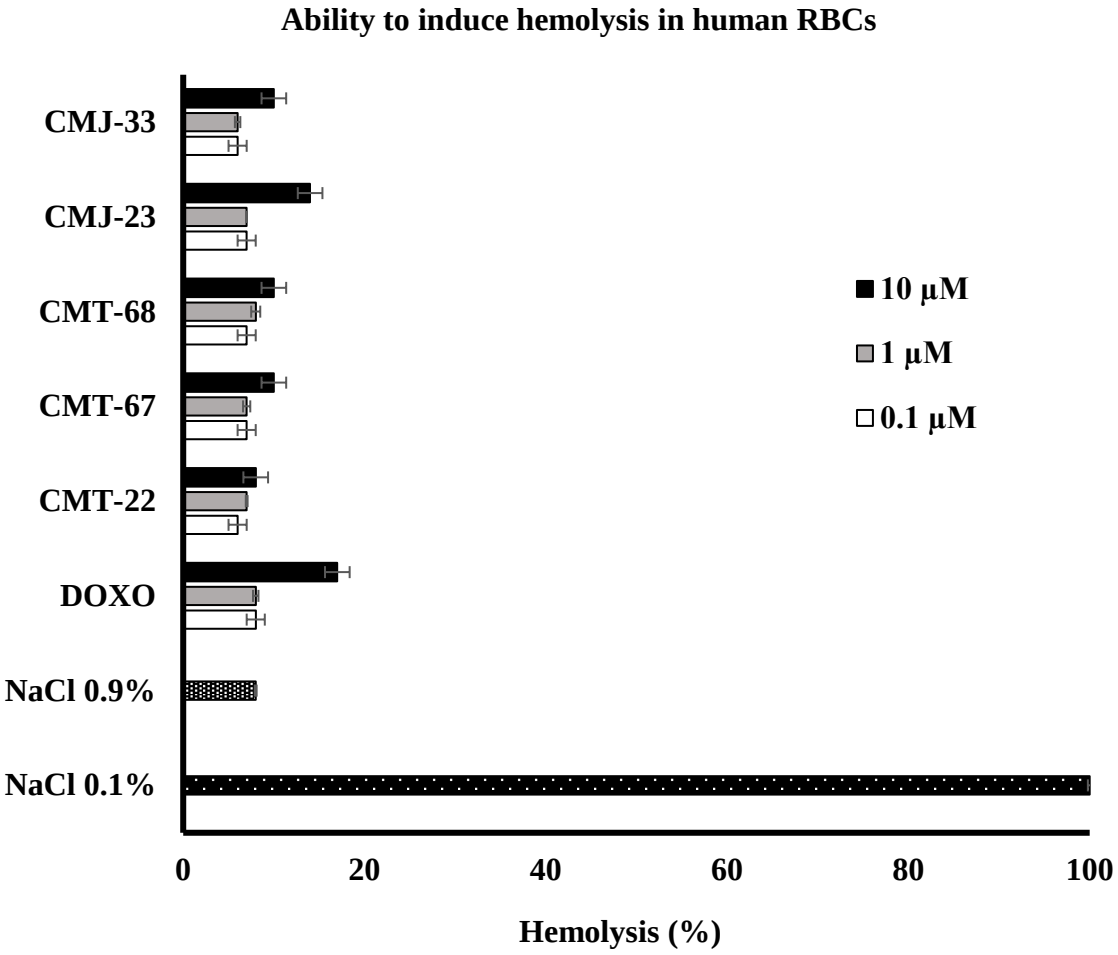


Fig. 4.7. Percentage of hemolysis activity of the tested compounds CMT-22, CMT-67, CMT-68, CMJ-23, CMJ-33 and anticancer control DOXO as well as NaCl 0.1 %, NaCl 0.95%. Hemolytic activity was evaluated by determining hemoglobin release from erythrocyte after incubation with compounds at three concentrations (0.1 µM, 1 µM, 10 µM). Values are represented as mean ± SD of 3 replicates.

Although the majority of normal individuals may suffer toxic hemolysis at sufficiently high concentrations of hemolytic drugs, for most drugs toxic hemolysis involves lower doses given to individuals who are genetically predisposed to hemolysis. It is strongly recommended for excipients intended for injectable use, that an *in vitro* hemolysis study should be performed at the intended concentration for administration to test for hemolytic potential. 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 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, respectively [167].

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 4.7).

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 [168].

The study protocol was approved by the Research Ethics Committee of the *Nicolae Testemitanu* State University of Medicine and Pharmacy (18.06.2015).

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.

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 [202, 203, 204]. These unicellular organisms are close to higher animals in their biochemical parameters, so the results of such biotesting can be extrapolated to humans.

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 [170]. 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*.

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.

The survival rate of *Paramecium caudatum* and median lethal concentration (LC_{50}), which are quantitative indicators of the efficiency of the antagonist substance in choosing the optimal therapeutic dose, were used as indicators for a comparative evaluation of the findings [198-200].

The effect of the tested compounds CMT-22, CMT-67, CMT-68, CMJ-23, CMJ-33 and the anticancer control DOXO at a single concentration 100 μM on the locomotor behavior of *Paramecium caudatum* was investigated for 30 min by an inverted microscope (LOMO) with microcamera, and compared with controls, organisms *Paramecium caudatum* without treatment (Figure 4.8).

The test objects with complex CMT-67, CMT-68, and CMJ-33 showed the reduced activity, cellular volume initially decreased and the zygote nucleus was formed by fusion of the migratory and stationary nucleus as well as cyst formation. In test objects with CMT-22, CMJ-23 and DOXO the following changes were observed: ciliates actively moved, almost did not form a cluster, there were single fixed specimens, also the cellular volume initially increased followed by the disintegration of protoplasm and internal membranes. The DOXO was found more potent for lysis of *Paramecium caudatum* among the tested compounds.

After 24 h *Paramecium caudatum* were in the bottom of wells. The light microscope revealed that most of the *Protozoa* slowly moved. Some organisms in this period were motionless, contractile vacuoles were ruptured and their contents were thoroughly mixed up with protoplasm, it appeared as coagulation of proteins.

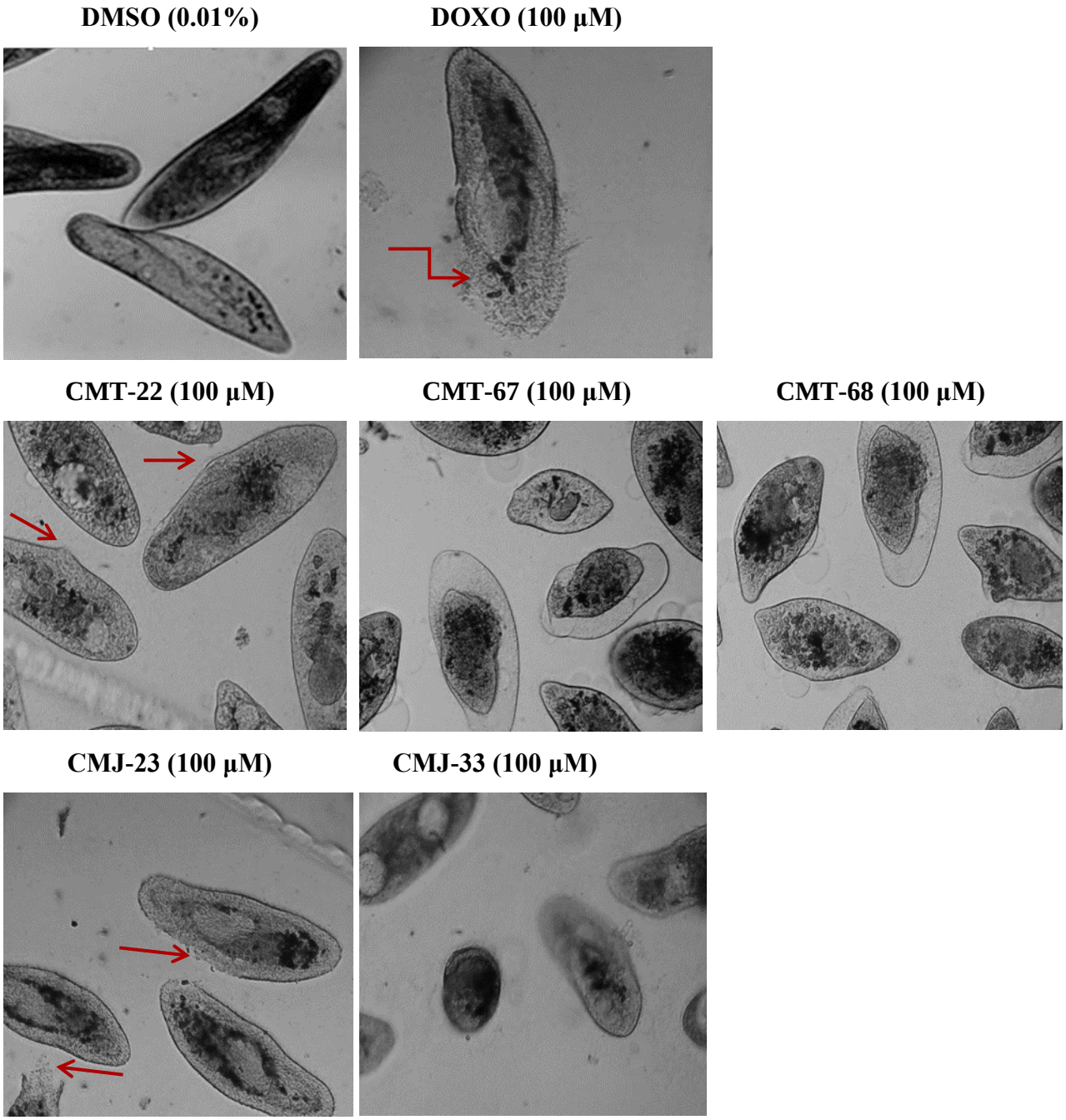


Fig. 4.8. *In vivo* direct toxic evaluation of the tested compounds CMT-22, CMT-67, CMT-68, CMJ-23, CMJ-33 and the positive control DOXO. Effects of compounds on *Paramecium caudatum* exposed to high concentration of 100 µM after 30 min of treatment. Schematic pictures showing organisms without treatment as control; lysis by DOXO; beginning of lysis by CMT-22, cyst formation by CMT-67, cyst formation by CMT-68, beginning of lysis by CMJ-23 and cyst formation by CMJ-23. Arrows indicate the rupture of the outer membrane.

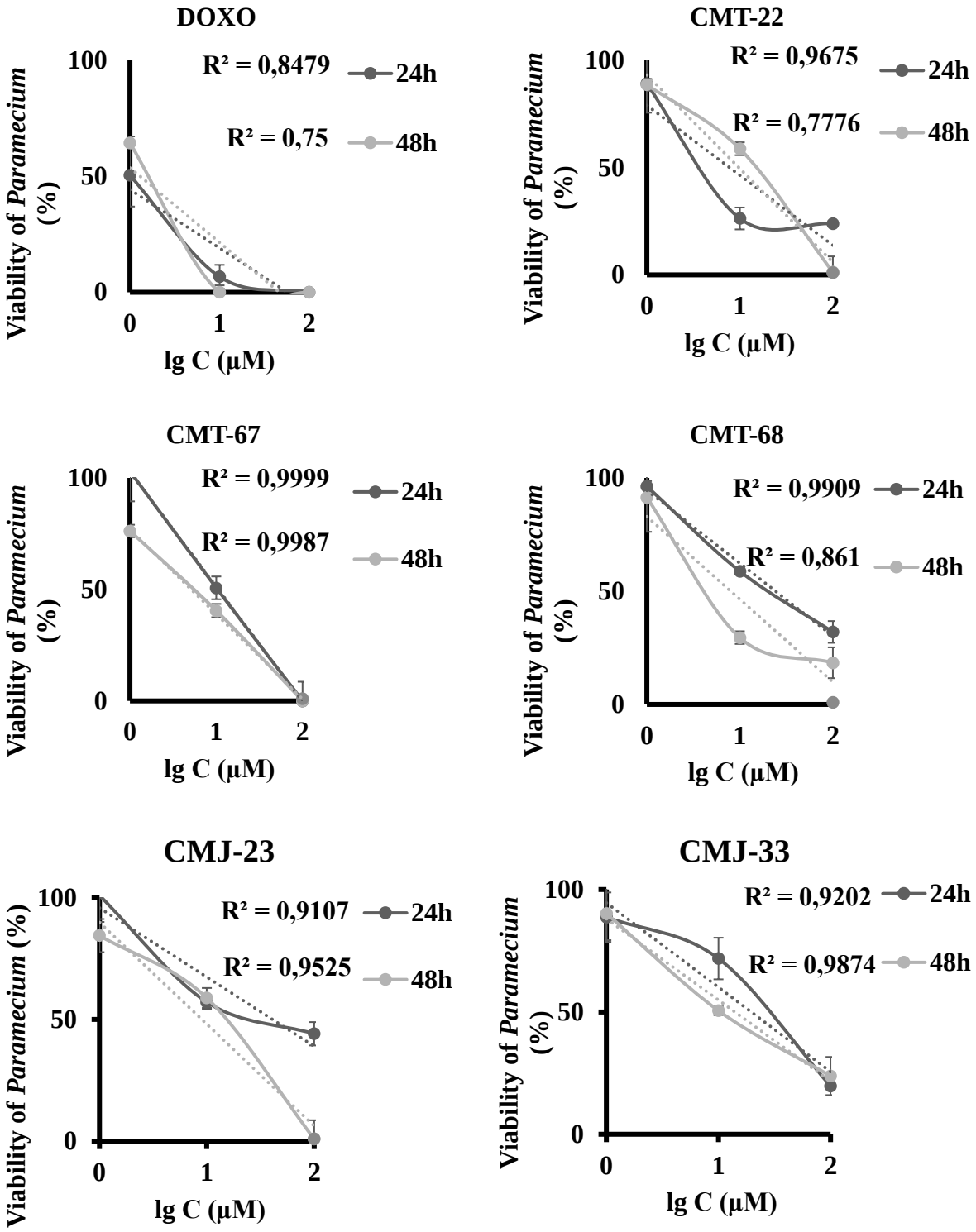


Fig. 4.9. Effect of the tested compounds and DOXO on growth of *Paramecium caudatum* exposed to three concentrations (1, 10, 100 µM) after 24 and 48 h of treatment. Direct toxic evaluation was performed by NR bioassay. NR was used for 12 h, which is a weak cationic dye that easily penetrates the cell membrane and accumulates intracellularly in lysosomes, where it binds with anionic sites to the lysosomal matrix. Values are represented as mean ± SD of 3 replicates.

Subsequently, the outer membrane was also affected and the cell contents oozed out due to internal pressure. In the represented experiment, we observed that the compounds affected the test organism, *Paramecium caudatum* in a concentration dependent manner.

The percent of viability of *Paramecium* after 24 and 48 hours treatment with the tested compounds CMT-22, CMT-67, CMT-68, CMJ-23, CMJ-33 and control DOXO was determined by the NR-method, and is graphically indicated in figure 4.9.

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. The coefficient of determination values (R^2) are all fairly high: 0.97 for CMT-22, 0.99 for CMT-67, 0.99 for CMT-68, 0.91 for CMJ-23; 0.92 for CMJ-33; 0.85 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. The coefficient of determination values (R^2) are all fairly high: 0.78 for CMT-22, 0.99 for CMT-67, 0.86 for CMT-68, 0.95 for CMJ-23, 0.99 for CMJ-33, 0.75 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.

4.7. Conclusions to chapter 4

1. The tested compounds showed antioxidant activity by several antioxidant-capacity assays.

2. Selective $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ radical scavenging ability of the tested compounds and the standards can be ranked in the order CMT-68 ≥ CMT-67 ≥ CMJ-23 ≥ CMJ-33 ≥ DOXO ≥ CMT-22 ≥ rutin ≥ trolox and CMJ-23 ≥ trolox ≥ rutin ≥ CMT-22 ≥ CMT-67 ≥ CMT-68 ≥ CMJ-33 ≥ DOXO, respectively. The enhanced inhibition displayed on the $ABTS^{\bullet+}$ radicals shows that

the compounds are capable of donating electrons to neutralize free radicals, which indicates their potentials as chemotherapeutic agents for radical's chains terminator.

3. It was established that the tested compounds showed the highest antioxidant capacity in the oxygen radical absorbance capacity (ORAC) compared with anticancer drug doxorubicin, and the reference antioxidant compound trolox.

4. The tested compounds CMT-22, CMT-67, CMT-68, CMJ-23 and CMJ-33 proved that they are potent reductive inhibitors and showed good results, comparing to DOXO and quercetin by LOX assay.

5. The tested substances exhibit antioxidant activity against $ABTS^{\bullet+}$ and peroxyl radicals, as well as inhibit the activity of LOX. Moreover, the activity of the tested substances exceeds the activity of the corresponding standards by 1.4-40 times.

6. The screening for toxic hemolysis and methemoglobin formation in human RBCs showed results, which did not exceed the permissible values in the therapeutic concentration range. The tested compounds have low ability to induce RBCs hemolysis as well as formation of the toxic metHb, as compared with DOXO.

7. Direct toxic evaluation of compounds was performed by *Paramecium* colorimetric bioassay. It was found that the tested compounds have a lower toxicity than DOXO.

Altogether, these study observations indicated that the tested compounds might be potential anticancer drugs, which have less side effects associated with oxidative stress than DOXO and cicplatin.

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 [167, 147, 157, 160, 164, 168, 174, 178, 180, 187, 201].

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 [141, 160, 162, 180, 186, 187, 201].

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. [168].

6. The methods developed to study the biological activity of substances have been adapted [167, 169, 188, 192]. 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 [170]. The LC_{50} (μM) toxicity values of the compounds have been found to be lower that those of DOXO by 5-45 times [171, 200].

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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ANNEXE

Annexe 1. Patents

MD 4349 B1 2015.05.31

REPUBLICA MOLDOVA

(19) Agenția de Stat
pentru Proprietatea Intelectuală

(11) **4349** (13) **B1**
(51) Int.Cl: *C07C 337/08* (2006.01)
C07D 213/48 (2006.01)
C07C 49/543 (2006.01)
A61K 31/175 (2006.01)
A61P 35/00 (2006.01)

(12) BREVET DE INVENȚIE

In termen de 6 luni de la data publicării mențiunii privind hotărârea de acordare a brevetului de invenție, orice persoană poate face opoziție la acordarea brevetului

(21) Nr. depozit: a 2014 0062
(22) Data depozit: 2014.06.23

(45) Data publicării hotărârii de acordare a brevetului:
2015.05.31, BOPI nr. 5/2015

(71) Solicitant: UNIVERSITATEA DE STAT DIN MOLDOVA, MD
(72) Inventatori: GULEA Aurelian, MD; LIPKOWSKI Andrzej, PL; GARBUZ Olga, MD; MATALINSKA Joanna, PL; ȚAPCOV Victor, MD
(73) Titular: UNIVERSITATEA DE STAT DIN MOLDOVA, MD

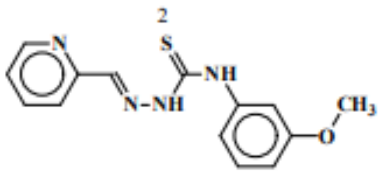
MD 4349 B1 2015.05.31

(54) Compusul N-(3-metoxifenil)-2-(piridin-2-ilmetilen)-hidrazincarbotioamidă - inhibitor al proliferării celulelor MeW-164 ale melanomului uman

(57) Rezumat:

Invenția se referă la chimie și medicină, și anume la un compus organic din clasa tioamidelor care poate găsi aplicare în medicină în calitate de preparat citostatic la profilaxia și tratamentul melanomului uman.

Esența invenției constă în sinteza compusului N-(3-metoxifenil)-2-(piridin-2-ilmetilen)-hidrazincarbotioamidă cu formula:



care manifestă proprietatea de inhibare a proliferării celulelor MeW-164 ale melanomului uman.

Revendicări: 2
Figuri: 6

MD 1279 Y 2018.08.31

REPUBLICA MOLDOVA

(19) Agenţia de Stat
pentru Proprietatea Intelectuală

(11) **1279** (13) **Y**

(51) **Int.Cl:** ***G01N 1/28*** (2006.01)
G01N 1/30 (2006.01)
G01N 21/00 (2006.01)
C12N 1/10 (2006.01)

(12) BREVET DE INVENŢIE DE SCURTĂ DURATĂ

În termen de 6 luni de la data publicării menţiunii privind hotărârea de acordare a brevetului de invenţie de scurtă durată, orice persoană poate face opoziţie la acordarea brevetului

(21) Nr. depozit: s 2017 0067
(22) Data depozit: 2017.05.23

(45) Data publicării hotărârii de acordare a brevetului:
2018.08.31, BOPI nr. 8/2018

(71) Solicitant: INSTITUTUL DE ZOOLOGIE AL ACADEMIEI DE ŞTIINŢE A MOLDOVEI, MD
(72) Inventatori: TODERAŞ Ion, MD; GULEA Aurelian, MD; GUDUMAC Valentin, MD; ROŞCOV Elena, MD; GARBUZ Olga, MD
(73) Titular: INSTITUTUL DE ZOOLOGIE AL ACADEMIEI DE ŞTIINŢE A MOLDOVEI, MD

(54) Metodă de apreciere a toxicităţii substanţelor chimice

(57) Rezumat:

1

Invenţia se referă la metode de apreciere a toxicităţii substanţelor chimice.

Metoda, conform invenţiei, include pregătirea culturii de *Paramecium caudatum*, adăugarea în probele de cercetat a substanţelor chimice testate în diverse concentraţii, incubarea probelor de cercetat şi de control, adăugarea colorantului 3-amino-7-dimetilamino-2-metilfenazin clorhidrat, incubarea cu adăugarea ulterioară a soluţiei de formalină, centrifugarea probelor, înlăturarea

2

supernatantului, adăugarea soluţiei de hidroxid de natriu, determinarea absorbanţei cu ajutorul unui spectrofotometru, după care se calculează procentul de paramecii vii şi se determină concentraţia letală (LC_{50}), totodată cu cât valoarea concentraţiei LC_{50} este mai mică, cu atât toxicitatea substanţei chimice testate este mai mare.

Revendicări: 1
Figuri: 1

MD 1279 Y 2018.08.31

MD 4644 B1 2019.08.31

REPUBLICA MOLDOVA

(19) Agenţia de Stat pentru Proprietatea Intelectuală

(11) **4644** (13) **B1**

(51) **Int.Cl:** ***C07F 11/00*** (2006.01)
C01G 39/00 (2006.01)
A61K 31/724 (2006.01)
A61P 39/06 (2006.01)

(12) BREVET DE INVENŢIE

In termen de 6 luni de la data publicării menţiunii privind hotărârea de acordare a brevetului de invenţie, orice persoană poate face opoziţie la acordarea brevetului

(21) Nr. depozit: a 2018 0020
(22) Data depozit: 2018.03.22

(45) Data publicării hotărârii de acordare a brevetului:
2019.08.31, BOPI nr. 8/2019

(71) Solicitant: UNIVERSITATEA DE STAT DIN MOLDOVA, MD

(72) Inventatori: FUIOR Arcadie, MD; FLOQUET Sebastien, FR; CADOT Emmanuel, FR; GARBUZ Olga, MD; ŢAPCOV Victor, MD; TODERAŞ Ion, MD; GULEA Aurelian, MD

(73) Titular: UNIVERSITATEA DE STAT DIN MOLDOVA, MD

MD 4644 B1 2019.08.31

(54) Ansamblu supramolecular $(NMe_4)(C_{42}H_{70}O_{35})[Mo_{10}O_{10}S_{10}(OH)_{11}(H_2O)_4]\cdot 27H_2O$ care manifestă activitate antioxidantă

(57) Rezumat:

1

Invenţia se referă la chimie şi medicină, şi anume la un ansamblu supramolecular biologic activ al ciclului anorganic monoanionic $[Mo_{10}O_{10}S_{10}(OH)_{11}(H_2O)_4]^-$ cu beta-ciclodextrină din clasa polioxotiomolibdaţilor. Ansamblul supramolecular manifestă activitate antioxidantă înaltă şi poate găsi aplicare în medicină în calitate de substanţă, care inhibă procesele de oxidare ale moleculelor organice in organismul uman.

Esenţa invenţiei constă în obţinerea antioxidantului sintetic – ansamblului

2

supramolecular cu formula $(NMe_4)(C_{42}H_{70}O_{35})[Mo_{10}O_{10}S_{10}(OH)_{11}(H_2O)_4]\cdot 27H_2O$, unde $(NMe_4)^+$ este cation de tetrametilamoniu, iar $C_{42}H_{70}O_{35}$ – beta-ciclodextrină.

Ansamblul supramolecular revendicat extinde arsenalul de antioxidanţi sintetici cu activitate biologică înaltă.

Revendicări: 2

Figuri: 1

MD 4620 B1 2019.02.28

REPUBLICA MOLDOVA

(19) Agenţia de Stat
pentru Proprietatea Intelectuală

(11) **4620** (13) **B1**

(51) **Int.Cl:** ***C07F 1/08*** (2006.01)
C07C 337/08 (2006.01)
C07D 213/48 (2006.01)
C07C 311/38 (2006.01)
C07C 311/39 (2006.01)
C07C 311/43 (2006.01)
A61K 31/30 (2006.01)
A61K 31/63 (2006.01)
A61P 35/00 (2006.01)
A61P 35/02 (2006.01)

(12) BREVET DE INVENŢIE

In termen de 6 luni de la data publicării menţiunii privind hotărârea de acordare a brevetului de invenţie, orice persoană poate face opoziţie la acordarea brevetului

(21) Nr. depozit: a 2018 0027
(22) Data depozit: 2018.04.11

(45) Data publicării hotărârii de acordare a brevetului:
2019.02.28, BOPI nr. 2/2019

(71) Solicitant: UNIVERSITATEA DE STAT DIN MOLDOVA, MD

(72) Inventatori: GULEA Aurelian, MD; ISTRATI Dorin, MD; ŢAPCOV Victor, MD; GARBUZ Olga, MD; GUDUMAC Valentin, MD; GROPPA Stanislav, MD

(73) Titular: UNIVERSITATEA DE STAT DIN MOLDOVA, MD

(54) Utilizare a di(μ-S)-bis{(4-aminobenzensulfamid)-cloro-{N-[fenil-2-(piridin-2-ilmetiliden)hidrazin-1-carbotioamido(1-)]}}cupru in calitate de inhibitor al proliferării celulelor cancerigene

MD 4620 B1 2019.02.28

(57) Rezumat:

1

Invenţia se referă la chimie şi medicină, şi anume la utilizarea unui compus coorinativ biologic activ de cupru, din clasa tiosemicarbazonaţilor metalelor de tranziţie în calitate de inhibitor al proliferării celulelor cancerigene.

Esenţa invenţiei constă în utilizarea di(μ-S)-bis{(4-aminobenzensulfamid)-cloro-{N-

2

[fenil-2-(piridin-2-ilmetiliden)hidrazin-1-carbotioamido(1-)]}cupru in calitate de inhibitor al proliferării celulelor cancerigene de leucemie mieloidă umană, de cancer de col uterin, de cancer pancreatic şi de rabdomiosarcom embrionar.

Revendicări: 2

Annexe 2. Innovations



Republica Moldova
Ministerul Sănătăţii

CERTIFICAT DE INOVATOR

Nr. 5319

Pentru inovaţia cu titlul

Metodă de determinare a activităţii lipoxigenazei

Inovaţia a fost înregistrată pe data de 24.02.2014
la Universitatea de Stat de Medicină şi Farmacie
"Nicolae Testemiţanu"

Se recunoaşte calitatea de autor(i)

**Ştîrba Olga, Andronache Lilia,
Garbuz Olga**

Data eliberării 13.03.2014

L.Ş.

(Semnătura autorizată)



Republica Moldova
Ministerul Sănătăţii

CERTIFICAT DE INOVATOR

Nr. 5465

Pentru inovaţia cu titlul

Procedeu de dozare a nivelului de oxihemoglobină methemoglobină şi sulfohemoglobină în aceiaşi probă

Inovaţia a fost înregistrată pe data de 20.05.2015
la Universitatea de Stat de Medicină şi Farmacie
"Nicolae Testemiţanu"

Se recunoaşte calitatea de autor(i)
**Pantea Valeriana ,Tagadiuc Olga,
Andronache Lilia , Ştîrba Olga,
Garbuz Olga, Gudumac Valentin**

Data eliberării 05.06.2015

L.Ş.

(Semnătura autorizată)



Republica Moldova
Ministerul Sănătăţii

CERTIFICAT DE INOVATOR

Nr. *5505*

Pentru inovaţia cu titlul

Metoda de determinare *in vitro* a viabilităţii şi citotoxicităţii celulare

Inovaţia a fost înregistrată pe data de *17.05.2016*
la Universitatea de Stat de Medicină şi Farmacie "Nicolae Testemiţanu"

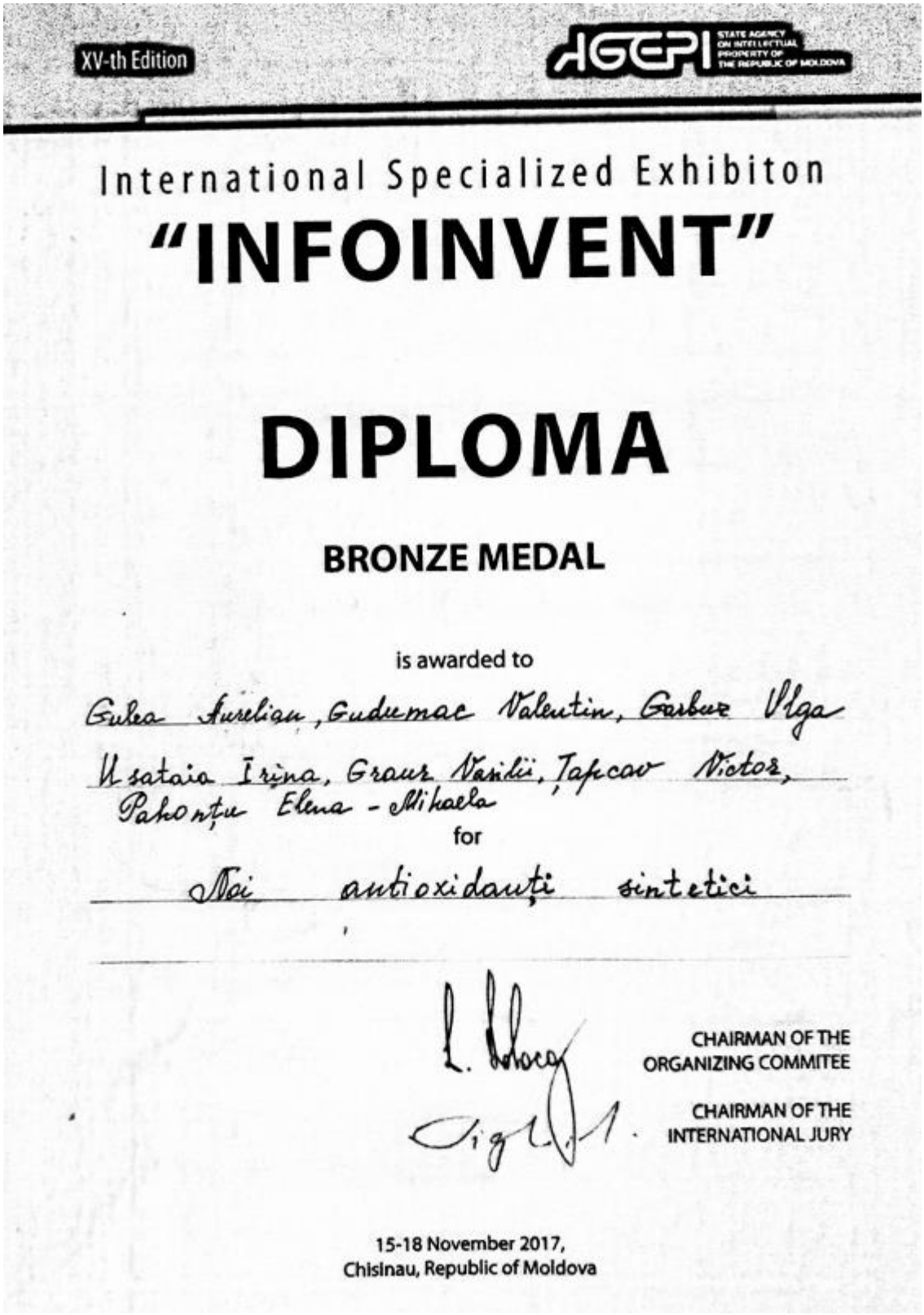
Se recunoaşte calitatea de autor(i)
Andronache Lilia, Garbuz Olga, Svet Inna, Gudumac Valentin

L.Ş.

Data eliberării *25.05.2016*

(Semnătura autorizată)

Annexe 3. Diplomas



XV-th Edition

AGEPI STATE AGENCY ON INTELLECTUAL PROPERTY OF THE REPUBLIC OF MOLDOVA

International Specialized Exhibiton

"INFOINVENT"

DIPLOMA

BRONZE MEDAL

is awarded to

Gulea Aurelian, Gudumac Valentin, Garbuz Olga
Usataia Irina, Graur Vasilii, Tapcov Victor,
Pahontu Elena - Mihaela

for

Noi antioxidanți sintetici

CHAIRMAN OF THE ORGANIZING COMMITEE

CHAIRMAN OF THE INTERNATIONAL JURY

15-18 November 2017,
Chisinau, Republic of Moldova

DIPLÔME

Inventions Geneva

SALON INTERNATIONAL DES INVENTIONS

GENÈVE

Après examen, le Jury International a décidé

de remettre à: **Fuior Arcadie, Sébastien Floquet, Emmanuel Cadot, Garbuz Olga, Tapcov Victor, Toderas Ion, Gulea Aurelian**

pour l'invention: **Nouveau composé supra moleculair en tant qu'antioxydant très fort**

MÉDAILLE D'ARGENT
SILVER MEDAL
SILBERMEDAILLE

Genève, le 13 avril 2018

Le Président du Jury: David Taji

Le Président du Salon: Jean-Luc Vincent



DIPLÔME

Inventions Geneva

SALON INTERNATIONAL DES INVENTIONS

GENÈVE

Après examen, le Jury International a décidé

de remettre à: Istrati Dorin, Ţapcov Victor, Garbuz Olga., Gudumac Valentin, Groppa Stanislav, Gulea Aurelian

pour l'invention: Inhibiteur de la prolifération de cellules de cancer avec un large champ d'action

MÉDAILLE D'OR
GOLD MEDAL
GOLDMEDAILLE

Genève, le 13 avril 2018

Le Président du Jury: David Taji

Le Président du Salon: Jean-Luc Vincent

ROMANIAN ASSOCIATION FOR NONCONVENTIONAL TECHNOLOGIES

Bucharest, ROMANIA

SPECIAL AWARD FOR THE INVENTION

Inhibitor of cancer cells proliferation with wide range of action

awarded to

D. Istrati, V. Tapcov, O. Garbuz, V. Gudumac, S. Groppa, A. Gulea

Presented at

46th International Exhibition of Inventions Geneva

President,

Prof. Niculae Ion MARINESCU, PhD. Eng.

Scientific Secretary,

Prof. Liviu Daniel GHICULESCU, PhD. Eng.

Geneva, April 11 -15, 2018

MOLDOVA

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Expoziția Internațională Specializată

„INFOINVENT"

Ediția a XVI-a

DIPLOMĂ

MEDALIA DE AUR

se acordă

ARCADIE FUIOR, SÉBASTIEN FLOQUET, EMMANUEL CADOT,
OLGA GARBUZ, VICTOR ŢAPCOV, ION TODERAŞ, AURELIAN GULEA

pentru

COMPUS SUPRAMOLECULAR NOU
CA ANTIOXIDANT FOARTE PUTERNIC

PREŞEDINTELE
COMITETULUI ORGANIZATORIC

PREŞEDINTELE JURIULUI

20-23 noiembrie 2019,
Chişinău, Republica Moldova

MOLDOVA

AGEPI AGENȚIA DE STAT PENTRU PROPRIETATEA INTELECTUALĂ A REPUBLICII MOLDOVA

Expoziția Internațională Specializată

„INFOINVENT"

Ediția a XVI-a

DIPLOMĂ

MEDALIA DE ARGINT

se acordă

DORIN ISTRATI, VICTOR ȚAPCOV, OLGA GARBUZ, VALENTIN GUDUMAC, STANISLAV GROPPA, AURELIAN GULEA

pentru

INHIBITOR AL PROLIFERĂRII CELULELOR CANCEROASE CU O GAMĂ LARGĂ DE ACȚIUNI

PREȘEDINTELE COMITETULUI ORGANIZATORIC

PREȘEDINTELE JURIULUI

20-23 noiembrie 2019,
Chișinău, Republica Moldova

Annexe 4. Conferences

CONFERINȚA ȘTIINȚIFICO-PRACTICĂ
ÎN DOMENIUL MEDICINEI DE LABORATOR

CERTIFICAT DE PARTICIPARE

Seria ML Cod XV Nr.3233

Eliberat Dnei/Dlui Carbuz Olga

participant la sesiunile Conferinței din 9 septembrie 2015 MoldMedicin&MoldDent 2015- Chișinău

Manifestarea este creditată cu 10 credite

MANAGEMENTUL CALITATII INVESTIGATIILOR DE LABORATOR –
VIZIUNI MODERNE ASUPRA PROBLEMEI SI CAILE DE PERFECTIONARE

Președintele Asociației Medicilor de laborator RM dr.hab.med., profesor universitar

ANATOL VISMEVSCHI

ASOCIAȚIA C.L.D.s

Președintele Comisiei de specialitate în medicina de laborator MS RM dr.hab.med., profesor universitar

VALENTIN GUDUMAC

Centrul Republican al Controlului Extern al Calității Cercetărilor de Laborator RM, dr.st.biol.

SVETLANA CARAGIA



UNIVERSITATEA DE STAT DE MEDICINĂ ŞI FARMACIE
"NICOLAE TESTEMIŢANU" DIN REPUBLICA MOLDOVA

CERTIFICAT

DE PARTICIPARE

Se acordă Dnei/Dlui Olga Cerbuz

pentru participare activă la Zilele Universităţii
şi Conferinţa ştiinţifică aniversară a cadrelor ştiinţifico-didactice,
doctoranzilor, masteranzilor, rezidenţilor şi studenţilor
5 octombrie 2015

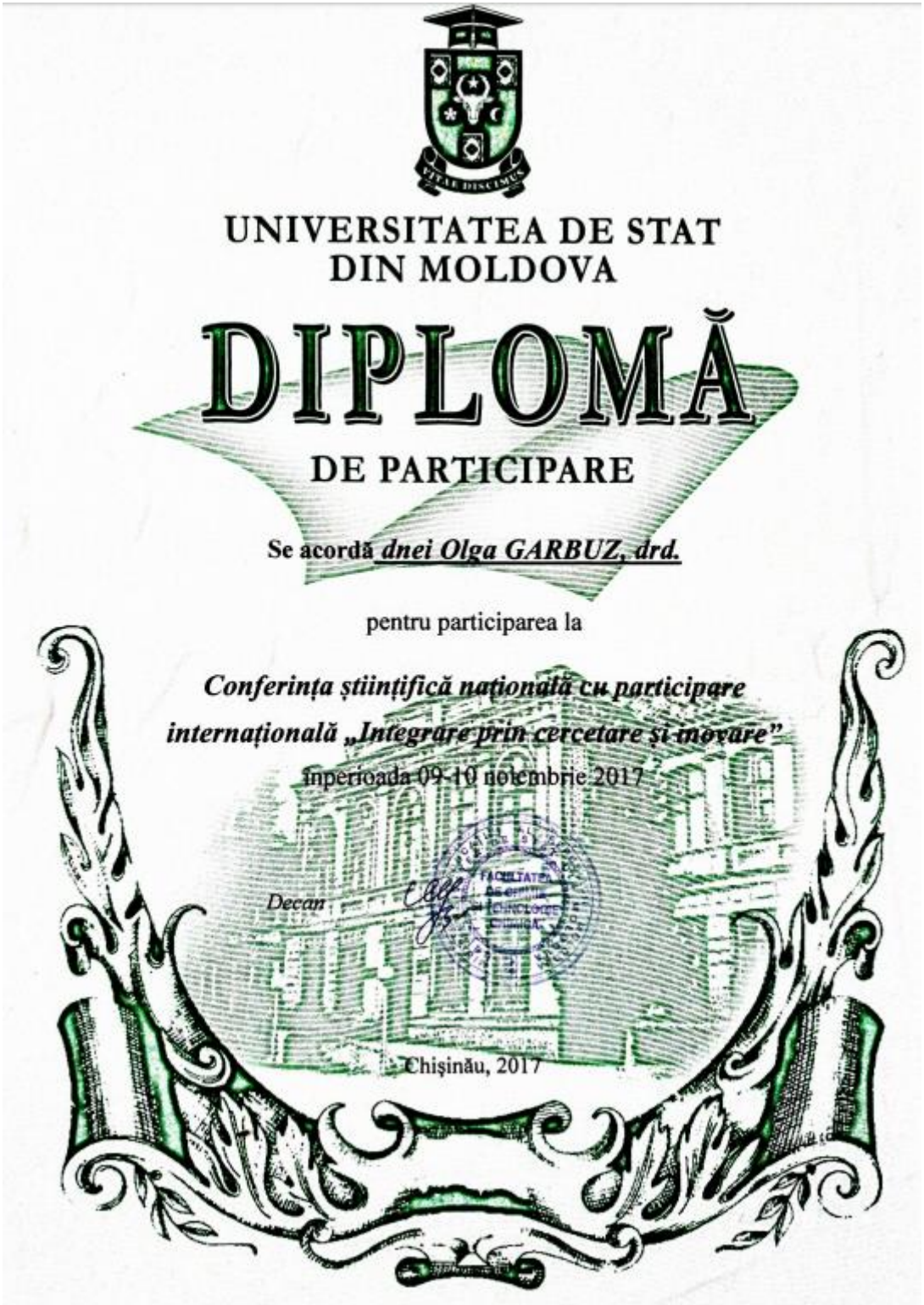
70 1945 2015

12 ore
de educaţie medicală continuă

DEPARTAMENTUL ŞTIINŢĂ

Dorin Goncearenco,
prorector pentru activitate ştiinţifică,
profesor universitar, dr. hab. şt. med.


ACADEMIA DE ȘTIINȚE A MOLDOVEI
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International Scientific Conference
on Microbial Biotechnology 3rd edition
dedicated to the 70th anniversary of foundation of first research institutions and the 55th anniversary of the inauguration of the Academy of Sciences of Moldova
Diplomă
de participare
CERTIFICATE OF PARTICIPATION
Conferința științifică internațională
International Scientific Conference
Biotehnologie Microbiană
Microbial Biotechnology
Se decernează
Awarded to
Olga Sarbuz
Președinte al Comitetului Științific Internațional
President of the International Scientific Committee
Prof. RUDIC Valeriu
CHISINAU, MOLDOVA
OCTOBER 12–13, 2016



UNIVERSITATEA DE STAT
DIN MOLDOVA

DIPLOMĂ

DE PARTICIPARE

Se acordă *dnei Olga GARBUZ, drd.*

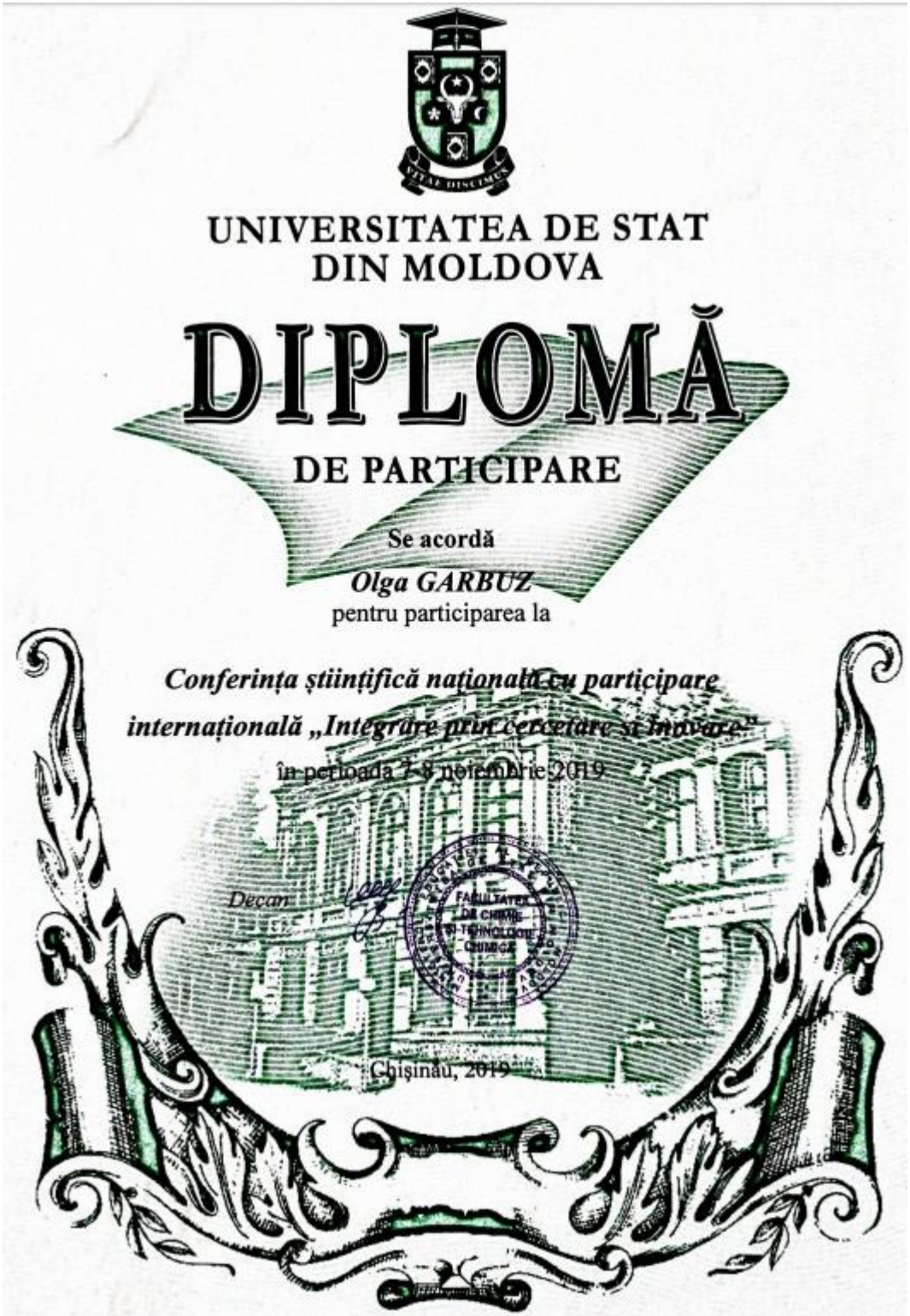
pentru participarea la

Conferința științifică națională cu participare internațională „Integrare prin cercetare și inovare”

în perioada 09-10 noiembrie 2017

Decan

Chișinău, 2017



UNIVERSITATEA DE STAT
DIN MOLDOVA

DIPLOMĂ

DE PARTICIPARE

Se acordă

Olga GARBUZ

pentru participarea la

Conferința științifică națională cu participare internațională „Integrare prin cercetare și inovare"

în perioada 7-8 noiembrie 2019

Decan

FACULTATEA DE CHIMIE ȘI TEHNOLOGIE CHIMICĂ

Chișinău, 2019

Annexe 5. Ethical approval

MINISTERUL SĂNĂTĂȚII AL REPUBLICII MOLDOVA

INSTITUȚIA PUBLICĂ
UNIVERSITATEA
DE STAT DE MEDICINĂ ȘI FARMACIE
«NICOLAE TESTEMIȚANU»
DIN REPUBLICA MOLDOVA

MINISTRY OF HEALTH OF THE REPUBLIC OF MOLDOVA

PUBLIC INSTITUTION
NICOLAE TESTEMITANU
STATE UNIVERSITY
OF MEDICINE AND PHARMACY
OF THE REPUBLIC OF MOLDOVA

MD 2004, Chișinău, bd. Ștefan cel Mare și Sfânt, 165; tel. (+37322) 20-57-01, fax. (+37322) 24-23-44; rector@usmf.md; www.usmf.md

nr. 43
la nr. 68 din 18.06.2015

Aviz favorabil al Comitetului de Etică a Cercetării

La Proiectul Instituțional cu titlul ***„Identificarea mecanismelor celulare și moleculare ale acțiunii compușilor bioactivi autohtoni noi și argumentarea folosirii lor în chimioprevenția și tratamentul unor procese tumorale”,*** investigatorul principal: Gudumac Valentin - doctor habilitat în științe medicale, profesor universitar.

Comitetul de Etică a Cercetării USMF „Nicolae Testemițanu” , examinând la ședința din 06 aprilie 2015 următoarele documente:

1. Forma de solicitare pentru evaluare etică a cercetării.
2. Protocolul proiectului.
3. Acordul informat.
4. Fișa de informare a participantului.
5. CV-ul investigatorului principal.

A decis că proiectul de cercetare ***„Identificarea mecanismelor celulare și moleculare ale acțiunii compușilor bioactivi autohtoni noi și argumentarea folosirii lor în chimioprevenția și tratamentul unor procese tumorale”,*** corespunde exigențelor etice.

Conflict de interese : Doamna Tagadiuc Olga, fiind în conflict de interese, în conformitate cu decizia Comitetului de Etică a Cercetării s-a aflat în sala ședinței, fără a avea drept de vot cu privire la proiectul de cercetare examinat.

Lista nominală a membrilor CEC prezenți în ședință: Curocichin Ghenadie, Gramma Rodica, Nacu Viorel, Bețiu Mircea, Diug Eugen, Tagadiuc Olga, Paulescu Andrei, Rusu Natalia, Nemerenco Ala, Spinei Larisa, Gavriliuc Mihail.

Președintele
Comitetului de Etică a Cercetării Mihail Gavriliuc

DECLARATION ON THE ASSUMPTION OF RESPONSIBILITY

I, the undersigned, Garbuz Olga, declare on my own responsibility that the materials presented in the thesis are the result of my own research and scientific achievements. I realize that otherwise will suffer the consequences in accordance with the legislation in force.

Garbuz Olga

Data:

CURRICULUM VITAE

INFORMAŢII PERSONALE Garbuz Olga

63/27, Dimineții, Chișinău, Republica Moldova

069-320770

olhamos@mail.ru

Data naşteri 25.01.1978

EXPERIENŢA PROFESIONALĂ

01.01.2020– prezent	Universitatea de Stat din Moldova, Laboratorul Materiale avansate în Biofarmaceutică şi Tehnică Cercetător științific
05.08.2019–20.01.2020	I.P. „Laboratorul central de testare a băuturilor alcoolice/nealcoolice si a produselor conservate”, Chișinău Specialist coordonator, locțiitor al șefului în sectorul de încercări instrumentale al Laboratorului de Încercări
05.11.2018– 28.07.2019	Î.S. “Centrul National de Verificare si Certificare a Productiei Vegetale şi Solului”, Chișinău Specialist principal
01.01.2016–prezent	Institutul de Zoologie, Laboratorul Sistematica si Filogenie Moleculara , Chișinău Cercetător științific
01.02.2015–01.10.2018	Universitatea de Stat din Moldova, Laboratorul Materiale avansate în Biofarmaceutică şi Tehnică Cercetător științific
01.02.2013–01.10.2015	Universitatea de Stat din Moldova, Chimie Coordinativa Cercetător științific
15.06.2009 – 20.03.2013	Federația Rusă, S.A. „Prioskolie” Inginer – chimist
11.09.2007 – 09.07.2009	Federația Rusă, Departamentul Sănătății regiunii Belgorod, Instituția municipală de asistență medicală „Spitalul central raional - Novyj Oskol” Biolog în laboratorul de diagnostic clinic
25.09.2000 – 27.05.2002	Federația Rusă, S.A. „OSKOLMYASO” Laborant - chimist

EDUCAŢIE ŞI FORMARE

2014- 2020	Universitatea de Stat din Moldova Biochimie Doctorand
2005 – 2007	Universitatea Aristotel de Tesalonic, Școala de Greaca Modernă Limba Greacă Modernă
2003 –2004	Universitatea de Stat din Tiraspol (Chișinău, Republica Moldova) Biologie Magistru în Biologie
1995 – 2000	Universitatea de Stat din Tiraspol, Republica Moldova Biologie si chimie, Diplomă de licență. Seria AL nr. 0033234
1985 – 1995	Școala Generală nr.1, Orhei

Limba(i) maternă(e) Rusă

Alte limbi străine cunoscute

	INTELEGERE		VORBIRE		SCRIERE
	Ascultare	Citire	Participare la conversație	Discurs oral	
Română	B2	B2	B1	B1	B1
Greacă	B2	B2	B1	B1	B2
Engleza	B2	B2	B1	B1	B2

Niveluri: A1/A2: Utilizator elementar - B1/B2: Utilizator independent - C1/C2: Utilizator experimentat
Cadrul european comun de referință pentru limbi străine