

# COPPER COORDINATION COMPOUNDS WITH THIOSEMICARBAZONES: IN VITRO ASSESSMENT OF THEIR POTENTIAL IN INHIBITING GLIOMA VIABILITY AND PROLIFERATION

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## ABSTRACT

**Introduction.** Glioblastoma is the most aggressive brain tumour with very low survival rate, the development of newer therapeutic agents or strategies against it being an ongoing and intensively researched issue.

**The aim of the study** was to investigate in vitro the potential of 9 copper coordination compounds with benzothiazole, phenyl, and allyl thiosemicarbazones (CCT) to inhibit the viability and proliferation of rat C6 glioma cells compared to doxorubicin.

**Materials and methods.** We used the rat C6 glioma cell line from the American Type Culture Collection

## RÉSUMÉ

**Composés de coordination du cuivre avec des thiosemicarbazones: évaluation *in vitro* de leur potentiel d'inhibition de la viabilité et de la prolifération du glioblastome**

**Introduction.** Le glioblastome est la tumeur cérébrale la plus agressive, avec un taux de survie très faible. Le développement de nouveaux agents thérapeutiques ou stratégies pour le combattre est un sujet de recherche intensif.

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(ref CCL-107). Rat C6 glioma cells were grown in 200 mL cell culture flasks and maintained in a 3.5% CO<sub>2</sub> incubator (ico50 Memmert GmbH Germany) at 37°C. The compounds were administered in doses of 1 µM and 10 µM. The viability was evaluated by MTT test and proliferation by resazurin one.

**Results.** The study results show that the compounds have selective action on *in vitro* viability and proliferation of C6 glioma cells that depends on the chemical structure and concentration. **Conclusions.** Phenyl derivatives showed a more promising action on C6 glioma cells viability and proliferation compared with benzothiazole and allyl thiosemicarbazones. Also, a lower concentration (1 µM) was more efficient than the higher one (10 µM).

**Keywords:** copper coordination compounds with thiosemicarbazones, glioma C6 cell line, viability, proliferation, resazurin test, MTT test

#### List of abbreviations:

COX-2 – cyclooxygenase-2  
DOXO – Doxorubicin hydrochloride  
DMEM – Dulbecco's Modified Eagle's Medium  
MGMT – O6-methylguanine DNA methyltransferase  
NDRG1 – N-myc downstream regulated gene-1  
Pgp – P-glycoprotein  
ROS – reactive oxygen species  
RR – ribonucleotide reductase  
Topo2α – topoisomerase 2α  
TSCs – thiosemicarbazones

## INTRODUCTION

Gliomas are the most common primary malignant brain tumours in adults (about 81%)<sup>1</sup>. They have an extremely unfavourable prognosis, with a survival rate of about 5% at 5-year, despite the various types of treatment available<sup>1,2</sup>. The surgical treatment of gliomas is compromised by the invasive nature of the tumour cells. The infiltration of the surrounding brain tissue limits or even impedes the surgical resection. Chemotherapy, designed to kill dividing neoplastic cells, remains ineffective for non-dividing invading cells, that eventually form secondary tumours at sites distant from the primary tumour mass<sup>3</sup>. Meanwhile, the drug dose sufficient to achieve the desired anticancer effect, killing of the tumour cells, is often toxic to healthy cells and leads to many side effects, which, in turn, limit the usefulness and effectiveness of the treatment.

The last decades have been characterized by targeted research activities on the discovery/development of new effective and selective anticancer agents,

**L'objectif de l'étude** était d'investiguer *in vitro* le potentiel de 9 composés de coordination de cuivre avec la benzothiazole, la phénylthiosemicarbazone et l'allylthiosemicarbazone (CCT) pour inhiber la viabilité et la prolifération des cellules de gliome C6 du rat par rapport à la doxorubicine.

**Matériel et méthodes.** La lignée cellulaire de gliome C6 du rat provenant de la Collection de Culture de Type Américain (réf CCL-107) a été utilisée. Les cellules de gliome C6 du rat ont été cultivées dans des flacons de culture cellulaire de 200 mL et maintenues dans un incubateur à 3,5 % de CO<sub>2</sub> (ico50 Memmert GmbH Allemagne) à 37 °C. Les composés ont été administrés à des doses de 1 µM et 10 µM. La viabilité a été évaluée par le test MTT et la prolifération par le test de rézaurine.

**Résultats.** Les résultats de l'étude montrent que les composés ont une action sélective sur la viabilité et la prolifération *in vitro* des cellules de gliome C6 qui dépend de la structure chimique et de la concentration.

**Conclusions.** Les dérivés phényl ont montré une action plus prometteuse sur la viabilité et la prolifération des cellules de gliome C6 par rapport aux benzothiazoles et aux thiosemicarbazones allyles. De plus, la concentration plus faible (1 µM) s'est avérée plus efficace que la concentration plus élevée (10 µM).

**Mots-clés:** composés de coordination de cuivre avec des thiosemicarbazones, lignée cellulaire de gliome C6, viabilité, prolifération, test de rézaurine.

but devoid of the side effects of conventional anticancer agents<sup>4,6</sup>. Thiosemicarbazones are powerful intermediates for the synthesis of pharmaceutical and bioactive substances and are widely used in medicinal chemistry. The coordination compounds of thiosemicarbazone derivatives are of increasing interest as potential anticancer drugs, due to their various pharmacological antitumour action, such as inhibition of cell growth and tumour invasion and promotion of tumour regression<sup>7,9</sup>. Thiosemicarbazones (TSCs), Schiff base ligands, are considered some of the most important scaffolds and are incorporated into many anticancer agents. Various synthesized aliphatic, aromatic, and heteroaromatic carbaldehyde TSCs have been evaluated for their antitumour activity<sup>10,11</sup>.

In this context, investigations focusing on the directed synthesis of coordination compounds containing 3d metals, and in particular, compounds consisting of polydentate chelators and macrocyclic ligands, assembled by condensation of thiosemicarbazone with aldehydes and ketones, are particularly noteworthy<sup>12,13</sup>. Over time, studies have shown that

these compounds exhibit a diverse range of biological activities, including antimicrobial, antifungal, and antitumour ones<sup>12-14</sup>.

Cu(II) complexes derived from Schiff base ligands obtained from 2-hydroxybenzaldehyde or terephthalic aldehyde and 4-amino-antipyrine exhibit potent antimicrobial activity against various bacterial and fungal strains, including *Ps. aeruginosa*, *A. Baumannii*, *E. coli*, and *S. aureus*<sup>12</sup>. Antimicrobial activity, including bacteriostatic and bactericidal action against both Gram-positive and Gram-negative bacteria, as well as antifungal activity was identified for the Cu(II), but also for the Ni(II) and Zn(II) complexes of salicylidene thiosemicarbazones<sup>13</sup>. New copper(II) complexes ([Cu(1,10-Phen)(L)] (1), [Cu(2,2'-Bpy)(L)] (2), [Cu(3,4-Lut)(L)] (3)) showed enhanced antimicrobial, antifungal, with complex 3 demonstrating superior inhibitory activity against *Enterobacter cloacae* and *Candida parapsilosis*, attributed to the monodentate N-heteroaromatic base (3,4-dimethylpyridine)<sup>14</sup>.

Copper, nickel, cobalt, and iron complexes with 2-hydroxybenzaldehyde 4,S-diallylthiosemicarbazone demonstrated a potent antioxidant activity<sup>14,15</sup>. It was found that highly active 2-hydroxybenzaldehyde-4-allyl-S-methylthiosemicarbazone complexes with Cu(II), Co(III), Fe(III), and Cr(III) displayed multiple biological activities, Cr(III), Fe(III), and Co(III) complexes demonstrating remarkable antioxidant effect exceeding that of Trolox<sup>16</sup>, while N4,S-diallylthiosemicarbazones copper(II) coordination compounds having a better antiradical activity than trolox<sup>17</sup>.

Also, the synthesized coordination compounds were tested for their antitumor activity. It was established that 2-hydroxybenzaldehyde-4-allyl-S-methylthiosemicarbazone complexes with Cu(II), Co(III), Fe(III), and Cr(III) exhibited antiproliferative activities

(human leukemia HL-60 cells; human cervical epithelial HeLa cells; human epithelial pancreatic adenocarcinoma BxPC-3 cells; human muscle rhabdomyosarcoma spindle and large multinucleated RD cells) with the copper coordination compound 2 exhibiting higher selectivity<sup>16</sup>. Zinc, copper, nickel, and cobalt complexes containing N4-allylthiosemicarbazone ligands exhibited powerful anti-cancer properties against different types of cancer cells. The nickel and zinc complexes showed exceptional selectivity, while the copper complexes had strong inhibitory effects but lower selectivity and higher toxicity<sup>18</sup>. Selective inhibition of BxPC-3 cancer cells was identified for some new N4,S-diallylthiosemicarbazones and copper(II) coordination compounds, surpassing the effectiveness of doxorubicin<sup>17</sup>.

An important conclusion from the previous studies of the anticancer action of coordination complexes of transition metals compared to the effects of ligands is that the results are more promising in the case of complexes with transition metals, but the presence of certain groups in the ligand influences the activity of the complexes<sup>13,19,21</sup>.

Many of the synthesized substances show antitumour properties clearly superior to doxorubicin – a drug currently widely used in oncology<sup>17,22</sup>, but their ability to influence glioma cells has not been elucidated.

**THE OBJECTIVE OF THE STUDY** was to investigate *in vitro* the influence of new CCTs on cell viability and cytotoxicity on rat C6 glioma cell culture.

## MATERIALS AND METHODS

The study protocol was approved by the Institutional Research Ethics Committee of Nicolae Testemitanu State University of Medicine and

**Table 1.** The copper coordination compounds with thiosemicarbazones tested *in vitro*.

Code	Chemical Name of the Substance
<b>Benzothiazole derivatives of thiosemicarbazone</b>	
CMA-18	Chloro-{1-(1,2-benzothiazol-3-yl)-2-[1-(pyridin-2-yl) ethylidene] diazanido} copper
CMD-8	Chloro-{4-ethyl-2-[phenyl (pyridin-2-yl)methylidene] hydrazine-1- carbothioamido} copper
MG-22	Chloro-{N'-(4-methoxyphenyl)-N,N-dimethylcarbmimido thioato} copper
<b>Phenyl thiosemicarbazone derivatives</b>	
CMC-34	Chloro-{N'-[phenyl(pyridin-2-yl)methylidene]-N- pyridin- 2- ylcarbamo-hydrazonothioato}copper
CMJ-33	Chloro-{4-(3-methoxyphenyl)-2-[1-(pyridin-2-yl)ethylidene] hydrazine-1-carbothioamido} copper
CMT-67	Nitrato-{N-phenyl-N'-(pyridin-2-ylmethylidene) carbamo-hydrazonothioato}copper
<b>Allyl thiosemicarbazone derivatives</b>	
CMG-41	Nitrato-{N'-[phenyl(pyridin-2-yl)methylidene]-N-prop-2- en-1- ylcarbamo-hydrazonothioato}copper
TIA-123	Chloro-{N'-[phenyl(pyridin-2-yl)methylidene]-N- prop-2-en- 1-ylcarbamo-hydrazonothioato}copper
TIA1-160	Acetato- { 2-(((methylsulfanyl)(prop-2-en-1-ylamino)methylidene) hydrazinylidene) methyl} phenolato}copper

Pharmacy of Republic of Moldova (approval no. 73/26.04.2017).

### Tested Copper Coordination Compounds with Thiosemicarbazones

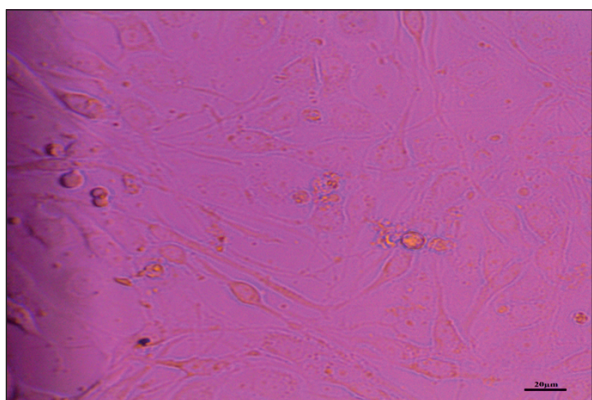
Nine copper coordination compounds with thiosemicarbazones (CCT) coded as CMA-18, CMD-8, MG-22, CMC-34, CMJ-33, CMT-67, CMG-41, TIA-123 and TIA-160, were tested in the study (Table 1). CCT were synthesized in the Laboratory of Advanced Materials in Biopharmaceutics and Technology of the State University of Moldova. As reference compound, it was utilized Doxorubicin hydrochloride (DOXO), extensively applied in cancer treatment (10 mg per vial, Naprod Life Sciences Pvt. Ltd, India).

### Rat C6 glioma cells

The rat C6 glioma cell line from the American Type Culture Collection (ref CCL-107) was used (Figure 1). This particular cell line was chosen due to its morphological resemblance to glioblastoma multiforme. When implanted in rats, these cells generate



**Figure 1.** Rat C6 glioma cells visualized under an inverted phase contrast microscope (x100) in Dulbecco's Modified Eagle's Medium (DMEM).



**Figure 2.** Control cells visualized during the MTT assay.

tumours that exhibit morphological features and vascularization levels similar to human glioblastomas<sup>23, 24</sup>.

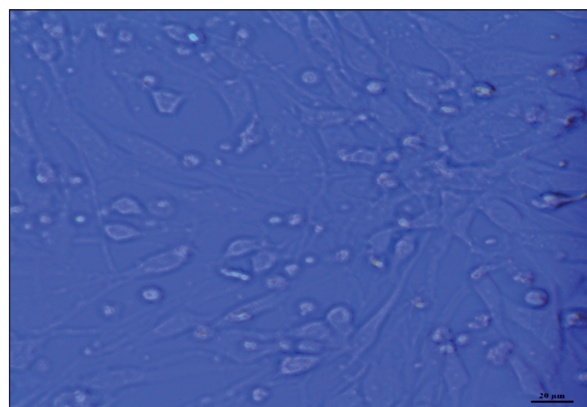
### Cell culture

Rat C6 glioma cells were grown in 200 mL cell culture flasks and maintained in a 3.5% CO<sub>2</sub> incubator (ico50 Memmert GmbH Germany) at 37°C. The culture medium was DMEM (Sigma Aldrich, UK) supplemented with 10% fetal bovine serum (Sigma Aldrich, UK), 2 mM L-glutamine (Sigma Aldrich, UK), 1% non-essential amino acids (Sigma Aldrich, UK), gentamicin (PC «Здоров'я», Kharkov, Ukraine) and fluconazole (100 μg/L) (PC Darnitsa, Kiev, Ukraina). The medium was refreshed every 2 days. After reaching the confluence, cells were washed with PBS (Sigma Aldrich, UK), dissociated with 0.05% trypsin-EDTA (HiMedia Laboratories Pvt. Ltd, India), and subsequently centrifuged at 1500 rpm for 3 min. After counting, cells were resuspended in culture medium and used for MTT and resazurin assays.

### Assessment of the action of CCT on C6 glioma cell viability with the MTT Assay

The cell viability was assessed using the colorimetric assay with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich, UK), following a modified version of the method proposed by Mosmann<sup>25</sup>. The method relies on the reduction of the yellow MTT tetrazolium salt in the metabolically active cells, resulting in formation of violet formazan crystals. The amount of formazan produced is directly proportional to the number of viable cells and was determined by measuring the absorbance at 570 nm after solubilizing the formazan crystals. The results were presented as a percentage compared to the control cells (untreated with CCT) (Figure 2).

To perform the method, 100 μL of cell suspension at a density of 1×10<sup>4</sup> cells/well were added to each



**Figure 3.** C6 glioma control cells visualized during the resazurin assay.



well of a 96-well flat-bottom microplate and allowed to adhere for 24 hours at 37°C in a 3.5% CO<sub>2</sub> incubator. After 24 hours of incubation, the culture medium was replaced with fresh medium (90 µL). Subsequently, 10 µL of CCT at concentrations of 1.0 µM and 10.0 µM were added to the cells and incubated in a CO<sub>2</sub> incubator for 24 hours at 37°C. DOXO (Naprod Life Sciences Pvt. Ltd., India) was used as a reference substance at the same concentrations, and untreated C6 glioma cells were used as the control.

After incubation, 10 µL of MTT working solution (5 mg/mL in phosphate-buffered saline) was added to each well of the plate, and it was further incubated until the intracellular violet formazan crystals were visible under a microscope (4 hours at 37°C). The medium was then removed, and the formed formazan crystals were dissolved by adding 100 µL of isopropanol (Neo Froxx GmbH, Germany) per well (30 minutes at 37°C).

The colour intensity of the solution was measured spectrophotometrically at 570 nm using the Multimode Synergy H1 Hybrid Reader (BioTek Instruments, USA).

## 2.5. CCT Cytotoxicity Assessment on C6 glioma cells using the Resazurin Assay

The resazurin assay is an *in vitro* cytotoxicity test that is based on the conversion of resazurin to fluorescent resorufin by mitochondrial enzymes and enzymes located in the cytoplasm of living cells (Figure 3). Thus, the amount of resorufin generated is directly correlated with the number of living cells. The resazurin test was performed according to the method described by Riss et al.<sup>26</sup>.

The cell suspension was adjusted to a concentration of 5x10<sup>4</sup> cells/mL. In a 96-well microplate, 90 µL of C6 cell culture was placed. To allow cell adhesion, the plate was incubated in a 3.5% CO<sub>2</sub> incubator at 37°C for 3 hours. Then, 10 µL of the tested compound solutions (1.0 µM and 10.0 µM) were added to the wells of the microplate. Parallel samples of the reference compound, DOXO, were prepared at the same concentrations, and control samples containing cells not exposed to the test compound were also included. The plate was incubated for 24 hours in a 3.5% CO<sub>2</sub> incubator at 37°C.

After incubation, 20 µL of a 0.15 g/L resazurin solution (resazurin sodium salt, Sigma-Aldrich) was added to all wells of the microplate, and the mixture was further incubated in a 3.5% CO<sub>2</sub> incubator at 37°C for 4 hours. The absorbance was measured at 570 nm and 600 nm using a Power Wave HT microplate spectrophotometer (BioTek Instruments, USA).

To assess cytotoxicity, the percentage difference in resazurin reduction between CCT-treated cells and control cells was calculated using the following formula (1):

$$\frac{((O2 \times A1) - (O1 \times A2))}{(O2 \times C1) - (O1 \times C2)} \times 100 \quad (1)$$

In the formula, O1 - molar absorption coefficient (ε) of oxidized resazurin at 570 nm; O2 - molar absorption coefficient (ε) of oxidized resazurin at 600 nm; A1 - absorbance of the samples to be investigated at 570 nm; A2 - absorbance of the samples to be investigated at 600 nm; C1 - absorbance of the positive control at 570 nm; C2 - absorbance of the positive control at 600 nm.

## Statistical analysis

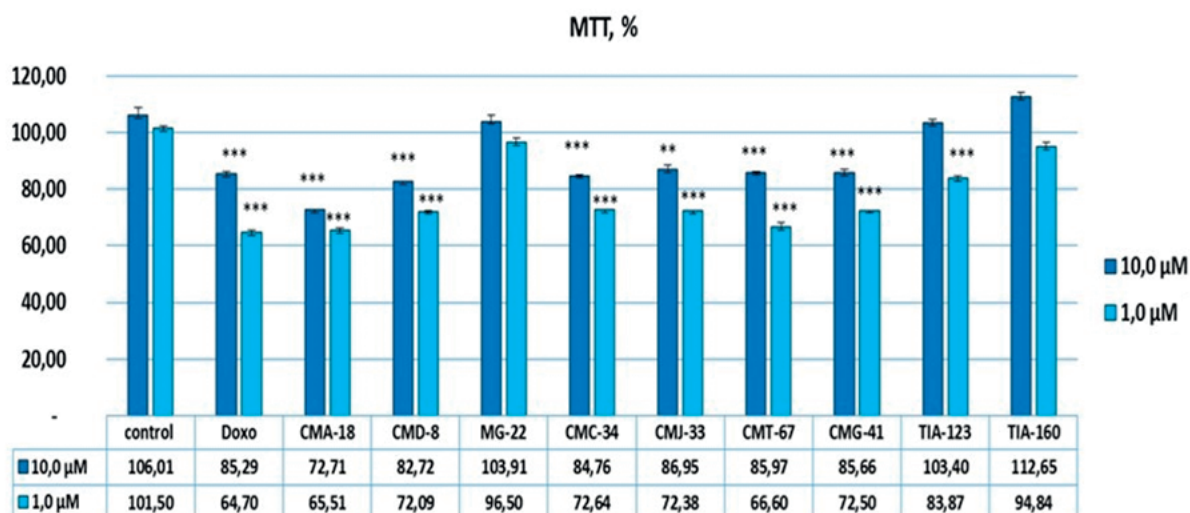
The statistical evaluation of the obtained data was performed using the Statistical Package for the Social Sciences (SPSS) software (version 23, SPSS Inc., Chicago, IL, USA). The arithmetic mean ± standard error (X ± m) were calculated. To test the significance of the difference between the studied indices of the compared groups, post-hoc tests for multiple comparisons were applied: Games-Howell after One-Way ANOVA and Welch's ANOVA. The significance threshold "p" (p < 0.05) was considered as the criterion for establishing the validity of the differences.

## RESULTS

During the incubation period, a non-significant increase in cell viability according to the MTT test was observed in the control samples (by 1 - 6%, p > 0.05). A significant decrease was observed with DOXO at both concentrations of 1.0 µM (approximately by 35%, p < 0.001) and 10.0 µM (approximately by 15%, p < 0.001) (Figure 4).

The results of the MTT assay highlighted a different influence of the CCT, on the viability of the rat C6 glioma cells in both doses of the tested compounds - 10.0 µM and 1.0 µM (Figure 4).

CCT at the dose of 1.0 µM exerted a more pronounced effect compared to the effect obtained at the dose of 10.0 µM on the viability of rat C6 glioma cells, assessed by the MTT assay (Figure 4). All studied CCT compounds, except MG-22 (3%, p > 0.05) and TIA-160 (5%, p > 0.05), at the concentration of 1.0 µM, induced a statistically significant decrease in the viability of C6 glioma cells to varying extents. The most pronounced inhibitory effect, similar to DOXO, was observed with CMA-18 (65.51%, p < 0.001) and CMT-67 (66.6%, p < 0.001). CMD-8,



**Figure 4.** The influence of copper coordination compounds with thiosemicarbazones at doses of 10.0  $\mu\text{M}$  and 1.0  $\mu\text{M}$  on the viability of rat C6 glioma cells (CCL-107) tested *in vitro* using the MTT assay. Note: statistical significance in comparison with the control – \*\* –  $p < 0.01$ ; \*\*\* –  $p < 0.001$ .

CMC-34, CMJ-33, and CMG-41 reduced glioma cell viability by approximately 28% ( $p < 0.001$  in all cases), while the effect of TIA-123 was only of 16% ( $p < 0.001$ ). At the concentration of 10.0  $\mu\text{M}$ , CMA-18 decreased the viability of C6 glioma cells by 28% ( $p < 0.001$ ), CMD-8, CMC-34, CMJ-33, and CMG-41 diminished it by approximately 13-17% ( $p < 0.001$  in all cases). MG-22, TIA-123, and TIA-160 did not affect glioma C6 cells ( $p > 0.05$ ).

The resazurin assay determined that the control samples exhibited a statistically inconclusive increase in cell proliferation (Figure 5). Administration of DOXO at the dose of 1  $\mu\text{M}$  resulted in an 8.8% increase in C6 cell proliferation ( $p < 0.0001$ ), while at the dose of 10  $\mu\text{M}$ , it increased by 25.2% ( $p < 0.0001$ ).

Treatment with CCT at the dose of 1  $\mu\text{M}$  yielded varied responses. Four of the tested compounds induced statistically significant enhancement in glioma C6 cell proliferation – CMD-8 by 11%, CMJ-33 by 7.7%, TIA-123 by 5.5% ( $p < 0.001$  in all cases), and MG-22 by 7.4% ( $p < 0.01$ ). CMA-18, CMC-34, CMT-67, CMG-41, and TIA-160 did not exert any influence on the proliferative capacity of C6 glioma cells.

Increasing the dose up to 10  $\mu\text{M}$  significantly enhances the effect of the CCT on C6 glioma cells. Significant statistical effects on C6 cell proliferation were observed at this concentration for CMA-18 (+ 20.8%,  $p < 0.0001$ ), CMD-8 (+ 23%,  $p < 0.0001$ ), MG-22 (+ 25.5%,  $p < 0.0001$ ), CMC-34 (+ 23.8%,  $p < 0.0001$ ), CMT-67 (+ 27.8%,  $p < 0.0001$ ), CMG-41 (+ 25.3%,  $p < 0.0001$ ), TIA-123 (+ 26.8%,  $p < 0.0001$ ), and TIA-160 (+ 23%,  $p < 0.0001$ ), while the effect of CMJ-33 was not statistically conclusive.

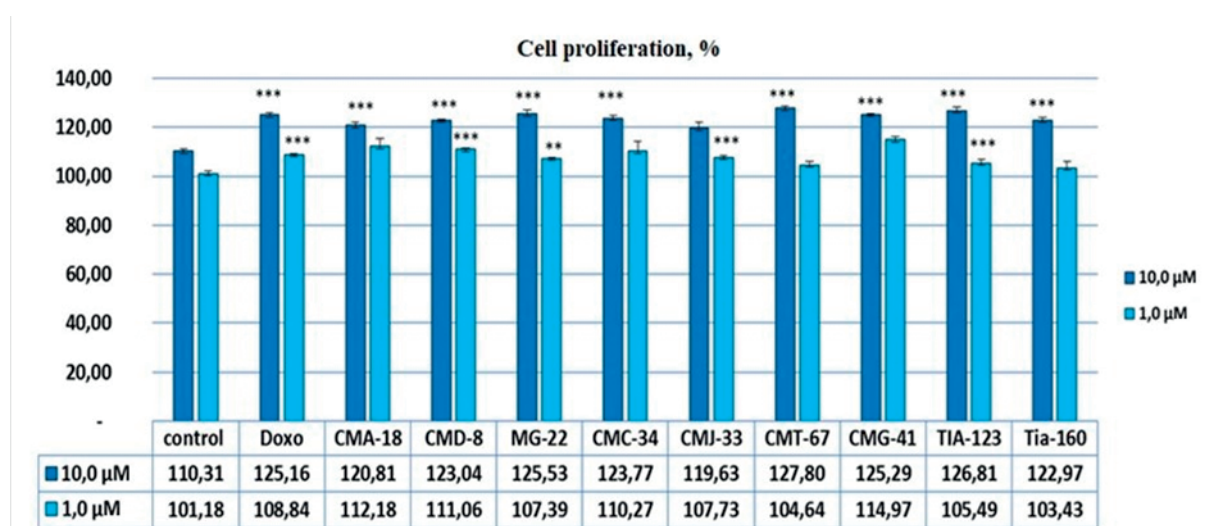
## DISCUSSION

MTT and resazurin assays are widely used to evaluate the effects of potential drug compounds on cells in *in vitro* studies.

The ability of MTT to penetrate biological membranes due to its lipophilic properties, as well as the presence of multiple enzymes and compounds within the cell that can reduce MTT, has led to the extensive use of the method to assess cell viability based on the evaluation of metabolic activity intensity<sup>27,28</sup>. Coordination compounds derived from thiosemicarbazones can intervene through various mechanisms in metabolic processes<sup>29-31</sup>, which can impact cellular function and viability. The mentioned effects have been reported in both normal and cancer cells<sup>32-36</sup>.

Resazurin is used for the *in vitro* assessment of cell proliferation. There is a direct correlation between the reduction of resazurin in the growth medium and the quantity/proliferation of living organisms, ranging from bacteria to mammalian cells<sup>37,38</sup>. The reliability of the method for assessing cytotoxicity on fibroblasts<sup>39</sup>, cancer cell lines<sup>40</sup>, as well as evaluating cell proliferation in lymphocytes<sup>41</sup>, hepatocyte cultures<sup>34</sup>, and primary neuronal cells<sup>42</sup> has been confirmed.

The impact of coordination compounds with thiosemicarbazones on the viability and proliferation of cancer cells *in vitro* has been reported by numerous research groups. For instance, Pitucha et al. (2021) reported the effects on melanoma cell cultures (G361, A375, SK-MEL-28)<sup>35</sup>, Ma et al. (2020) conducted experiments on human hepatocellular carcinoma (HepG2), human breast carcinoma (MCF-7), human colorectal carcinoma (HCT-116), and human lung



**Figure 5.** The influence of copper coordination compounds with thiosemicarbazones at doses of 10.0 μM and 1.0 μM on the proliferation of rat C6 glioma cells (CCL-107) tested *in vitro* using the resazurin assay. Note: statistical significance in comparison with the control – \*\* –  $p < 0.01$ ; \*\*\* –  $p < 0.001$ .

carcinoma (A549) cell lines<sup>43</sup>, Niso et al. (2021) studied various forms of human pancreatic cancer cell cultures (BxPC3, AspC1, MiaPaCa2, PANC-1), among others<sup>44</sup>.

Some studies have been conducted on the action of thiosemicarbazones on glioblastoma cell lines. For example, Turan-Zitouni et al. (2016) reported the antitumour effectiveness of the compound 3,3'-dimethoxy-N(4),N(4')-bis(4-(4-bromophenyl)thiazol-2-yl)-[1,1'-biphenyl]-4,4'-diamine (IC<sub>50</sub> of 11.3 ± 1.2 μg/mL), showing significant inhibition of the viability of the glioblastoma C6 cell line<sup>45</sup>. Tumosiene et al. reported modest efficacy in reducing the viability of human U-87 glioblastoma cell cultures (American Type Culture Collection, Manassas, VA, USA) for 39 newly synthesized compounds administered at a concentration of 100 μM, possibly due to the presence of specific drug efflux systems in brain tumour cells<sup>46,47</sup>. The U-87 glioblastoma culture is resistant to temozolomide, typically used for the treatment of this tumour, which reduces cell viability by up to 60% at a concentration of 100 μM after a 48-hour incubation<sup>48</sup>. Juszczak et al. highlighted the efficacy of two thiosemicarbazone-derived compounds in micromolar concentrations on tumour cells from nervous system cancers (rhabdomyosarcoma/medulloblastoma, glioma)<sup>33</sup>, while Soares et al. (2012) tested N4-Phenyl 2-acetylpyridine thiosemicarbazone (H2Ac4Ph; N-(phenyl)-2-(1-(pyridin-2-yl)ethylidene)hydrazinecarbothioamide) and four derivatives of the compound, demonstrating significant cytotoxic capacity on T98G glioma cell lines (IC<sub>50</sub>: 140–1.0 nM) and U87 (IC<sub>50</sub>: 160–1.4 nM) after a 48-hour exposure<sup>49</sup>.

The coordination compounds with thiosemicarbazones tested in our study showed a decrease in

the viability of C6 glioblastoma cells after a 24-hour incubation, as assessed by the MTT assay, ranging between 15-27% at the concentration of 10 μM and 3-35% at the concentration of 1 μM.

Considering the shorter duration of action and significantly lower concentration of the compounds, we could suggest that the investigated coordination compounds with thiosemicarbazones have a significant capacity to inhibit the viability of glioblastoma cells. A comparison of the compounds' actions revealed that all three phenyl derivatives (CMC-34, CMJ-33, and CMT-67) of thiosemicarbazones demonstrated a diminishing effect on the viability of C6 cells compared to the benzothiazole derivatives (CMA-18, CMD-8, and MG-22) and allyl derivatives (CMG-41, NIA-123, and TIA-160), among which only two exhibited activities.

In our study, the resazurin test revealed that the administration of DOXO at doses of 1 μM and 10 μM did not have a major negative impact on the proliferation of C6 cells, which increased compared to control cultures by 8.8% and 25.2%, respectively ( $p < 0.001$  in both cases). The observation that DOXO enhances the proliferation of C6 glioma cells compared to the control can be justified based on the characteristics of glioma cells.

Glioma cells, including C6 glioma cells, are highly proliferative and aggressive tumour cells derived from glial cells in the brain. They often exhibit dysregulated cell cycle control and signaling pathways, which can contribute to increased proliferation. Moreover, the observation that DOXO enhances proliferation may be time and dose-dependent, prolonged exposure to DOXO can trigger compensatory responses, such



as upregulation of growth factors or survival pathways, which can promote cell proliferation.

Also, DOXO generates reactive oxygen species (ROS) as a byproduct of its metabolism<sup>50</sup>. ROS can act as signaling molecules that regulate cellular processes, including cell proliferation. In some cases, moderate levels of ROS can promote cell proliferation by activating pro-survival signaling pathways or stimulating growth factor production<sup>51</sup>.

The pro-proliferative effect of DOXO observed in this study should be further investigated to understand the underlying mechanisms and confirm the reproducibility of the results. Additionally, considering the potential adverse effects and clinical implications, the balance between the cytotoxic and pro-proliferative effects of DOXO should be carefully evaluated in the context of glioma treatment.

The tested CCT compounds exhibited a similar to DOXO impact on proliferation rate of the C6 glioma cells *in vitro* when administered at the dose of 10  $\mu$ M. However, at the low dose of 1  $\mu$ M, five of the tested substances (CMA-18, CMC-34, CMT-67, CMG-41, and TIA-160) did not induce cell proliferation in the glioma culture.

Analysing the cumulative effects of CCT on the viability (MTT test) and proliferation (resazurin test) of C6 glioma cells, we found that the compounds CMA-18, CMC-34, CMT-67, and CMG-41 significantly decreased viability while not stimulating cell proliferation. Therefore, we can conclude that the phenyl derivatives tested in the current study exhibited the most promising actions among the three groups of substances included in the study.

Our research has **limitations** as it did not include the study and identification of the mechanisms of action of the tested CCT on the viability and proliferation of C6 glioma cells. This would require further comprehensive research.

In this context, we can consider the results of other scientists who have established several molecular mechanisms through which TSC exert their action on cancer cells, such as induction of oxygen free radical generation with membrane damage and subsequent cell disruption<sup>52</sup>, apoptosis induction, cell cycle modulation and cell arrest in the G2/M phase, the suppression of Bcl-XL and Bcl-2 expression and the hyper expression of caspase-3<sup>32</sup>, the amplification of the expression of the metastasis suppressor protein - N-myc downstream regulated gene-1 (NDRG1)<sup>53</sup> etc.

The ability of thiosemicarbazones to chelate metal ions has been recognized as a major mechanism of antiproliferative effects<sup>54</sup>, determined by the formation of biologically active complexes, including coordination with metal centers in enzymes<sup>55</sup>. TSCs possess an additional functional group that is not

coordinated to their "primary" metal ion, thus suggesting that biological activity may also depend on non-coordinated groups<sup>53</sup>.

The antineoplastic activity of TSC is most often attributed to the ability of the compounds to inhibit mammalian ribonucleotide reductase (RR). RR is responsible for reducing ribonucleotides to deoxyribonucleotides, which are the building blocks for DNA replication and repair in all living cells. TSCs, known iron chelators, can destabilize or damage the tyrosyl free radical stabilized with non-heme iron and thus inhibit the catalytic function of RR<sup>56</sup>. A strong correlation was found between tumour growth rate and ribonucleotide reductase activity<sup>30</sup>.

The ability to inhibit *in vitro* the proliferation of erythroleukemic cells (K562), melanoma cells (SK-Mel-28) and breast cancer cells (MCF-7) specific to several forms of TSC with iron chelating properties<sup>57</sup>, as well as pronounced and selective activity against human lung cancer xenografts *in vivo*<sup>58</sup> were reported.

There are also some studies regarding the mechanisms of action of thiosemicarbazones on glioma and/or other brain tumours: (a) cross the blood-brain barrier and penetrate glioblastoma tumour cells, enhancing the passage of DOXO into these cells, increasing the accumulation of DOXO in the tumour and subsequent toxicity in glioblastoma, with greater effects on blood-brain barrier cells and P-glycoprotein (Pgp)-rich cancer stem cell clones compared to differentiated clones derived from the same tumor<sup>59</sup>; (b) inhibit DNA synthesis in C6 cells in a dose-dependent manner, inducing apoptotic effects and depolarization of mitochondrial membranes<sup>60,61</sup>; (c) down-regulate the expression of epidermal growth factor receptor, platelet-derived growth factor receptor, and insulin-like growth factor 1, leading to a strong induction of apoptosis in neuroblastoma cell lines<sup>62</sup>; (d) affect the expression and activity of genes encoding cyclooxygenase-2 (COX-2), O6-methylguanine DNA methyltransferase (MGMT), and topoisomerase 2 $\alpha$  (Topo2 $\alpha$ ) in medulloblastoma (Daoy) and neuroblastoma (SH-SY5Y) cell lines<sup>63</sup>, etc.

## CONCLUSIONS

Nine copper coordinating compounds with thiosemicarbazones (CMA-18, CMD-8, MG-22, CMC-34, CMJ-33, CMT-67, CMG-41, TIA-123 and TIA-160) were studied in order to select the most effective one in decreasing the viability and inhibiting the proliferation of the rat C6 glioma cells. The study results show that the compounds have selective action on viability and proliferation that depends on the chemical structure and concentration, the phenyl derivatives in lower concentration (1  $\mu$ M) being more promising.



The data obtained indicate the need to continue studies of the effect of TCC on glioma cells, to establish the mechanisms of the revealed effects, as well as the optimal concentration for their anticancer action.

#### Author Contributions:

Conceptualization, O.T., V.G.; methodology, V.P., L.A., A.G., P.G., V.G., O.T.; validation, V.P., L.A., P.G., E.P., A.G., O.T., V.G.; formal analysis, V.P., O.T.; data curation, V.P., L.A.; writing – original draft preparation, V.P., L.A.; writing – review and editing, O.T., E.P., V.G.; supervision, V.G.; project administration, O.T., V.G.; funding acquisition, V.G., A.G. All authors have read and agreed to the published version of the manuscript. All the authors have read and agreed with the final version of the article.

#### Compliance with Ethics Requirements:

“The authors declare no conflict of interest regarding this article”

“The authors declare that the study protocol was approved by the Institutional Research Ethics Committee of Nicolae Testemitanu State University of Medicine and Pharmacy of Republic of Moldova (approval no. 73/26.04.2017).”

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