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Anti-Cancer Agents in Medicinal Chemistry, 2016, *16*, 000-000

RESEARCH ARTICLE

Copper(II) Complexes with Saccharinate and Glutamine as Antitumor Agents: Cyto- and Genotoxicity in Human Osteosarcoma Cells

J.F. Cadavid-Vargas[a,b], I.E. León[a,b], S.B. Etcheverry[a,b], E. Santi[c], M.H. Torre[c] and A.L. Di Virgilio[a,b,*]

[a]*CEQUINOR (Centro de Química Inorgánica), CONICET- Facultad de Ciencias Exactas, UNLP. 47 y 115 (1900) La Plata, Argentina;* [b]*Cátedra de Bioquímica Patológica, Facultad de Ciencias Exactas, UNLP. 47 y 115 (1900) La Plata, Argentina;* [c]*Química Inorgánica, Facultad de Química, Universidad de la República, Gral. Flores 2124, Montevideo, Uruguay*

ARTICLE HISTORY

Received: December 10, 2015
Revised: March 31, 2016
Accepted: May 11, 2016

DOI: 10.2174/1871520616666160513130204

Abstract: We report herein the antitumor actions of three copper(II) complexes on MG-63 human osteosarcoma cells. The three complexes: Cu-sac, Cu-gln and Cu-sac-gln (sac= saccharinate, gln= glutamine) caused a decline in cell viability. The half-maximal inhibitory concentration in MG-63 cells for Cu-sac-gln is 170 μM, showing the strongest antiproliferative effect. Moreover, only Cu-sac-gln caused a decrease in the mitochondrial activity from 100 μM. Our results indicate that the copper(II) complexes studied here produce DNA damage and suggest that the rise of reactive oxygen species (ROS) is the central mechanism action. Genotoxicity studied by the Cytokinesis-block micronucleus (MN) assay and the Single cell gel electrophoresis (comet assay) could be observed in MG-63 cells treated with Cu-sac-gln from 100 and 50 μM, respectively. Cu-sac and Cu-gln also induced DNA damage; however their effect was definitively weaker. The generation of reactive oxygen species increased from 50 μM of Cu-sac-gln and Cu-sac and only from 250 μM of Cu-gln, as well as a reduction of the GSH/GSSG ratio from 50 μM. When cells were treated with several concentrations of the complexes in addition to a combination of 50 μM of vitamin C plus 50 μM of vitamin E, a total recovery in cell survival was obtained for Cu-gln in the whole range of tested concentrations while only a partial viability recovery was obtained from 250 μM of Cu-sac and Cu-sac-gln. Overall, our results point to a differential cyto- and genotoxicity of the three copper(II) complexes and demonstrate that the complexation with both ligands confers the most potent antitumor action in human osteosarcoma cells.

A.L. Di Virgilio

Keywords: Copper(II) complexes; antitumor drugs; cytotoxicity; genotoxicity; human osteosarcoma cells.

1. INTRODUCTION

Copper is a ubiquitous trace element vital in growth and development [1-3]. It acts as a cofactor for proteins since it has the ability to present different redox states in a variety of biological reactions under physiological conditions [4-6].

Nevertheless, significant amounts of Cu were found in many types of human cancers [7-11]. The reason seems to be related to the critical role that copper plays in angiogenesis [9], which is essential for growth, invasion and metastasis [10]. Therefore, therapeutic use of copper chelators has been postulated for numerous sorts of tumors as antiangiogenic compounds [11, 12].

On the other hand, as a result of its anti-inflammatory attribute, copper has shown to be useful in disorders with an inflammatory origin such as cancer [13-15]. It has previously been shown that Casiopeínas® interacts with DNA and promotes disruption by a mechanism related to an increase in the level of free radicals [16-18] which confers antineoplastic potential. In fact, many copper compounds have been enlisted for clinical trials [16, 19]. In addition, copper compounds have been proposed to induce selective tumor cell apoptosis by several mechanisms related to DNA intercalation, tumor specific proteasome inhibitory activity and topoisomerases inhibition [20]. On the other hand, numerous enzymes have been recently targeted by therapeutic drugs in clinical and preclinical use [21]. Protein tyrosine phosphatases (PTPs) are a large family of signaling enzymes which play an important role in signal transduction and regulation of cellular processes. The inhibition of some of the PTPs delays tumor onset, and prevents the growth and metastasis of cancer cells. Because of the role of PTPs in the disease onset and progression, PTP inhibitors are therefore expected to be promising therapeutic drugs [22-27].

Recently, it has been indicated that copper complexes are very potent PTPs inhibitors. The strong PTPs inhibition activity of copper complexes suggests that they may regulate cell process by interfering with cellular signaling pathways *via* inhibiting PTPs [21].

In order to offer valuable and innocuous pharmaceutical compositions, substances have to be incorporated into the formula to conserve the properties. These substances include antioxidants, antimicrobial, chelating agents and sweeteners such as saccharine (1,1-dioxo-1,2-benzothiazol-3-one). Saccharinate anion is a very interesting and versatile ligand, which coordinates to metallic centers through one N, one O (carbonylic) and two O (sulfonic) atoms [28-31]. Polyfunctional ligands -ligands of a predominantly organic nature that possess several functional groups- are capable of binding to metal centers. At the same time, they can form relatively strong interactions with a crystalline environment, mostly through

*Address correspondence to this author at the CEQUINOR and Cátedra de Bioquímica Patológica, Facultad de Ciencias Exactas, UNLP. 47 y 115 (1900) La Plata, Argentina; Tel: +54 221 4235333 int. 49; Fax: +54 221 4259485; E-mail: aldivirgilio@biol.unlp.edu.ar

1871-5206/16 $58.00+.00

hydrogen bonding [32] making saccharinate an outstandingly valuable ligand to be incorporated principally to maintain the stability of the complex. Moreover, there is other aspect that can be related with health: it can form complexes with metals coming from food [33] or with toxic metals inside the body. For example, saccharinate has been found to present potential use as antidote for metal poisoning [34]. For these reasons, there have been numerous synthesis of mixed copper complexes with saccharin [32, 35-39] including essential amino acids [28].

On the other hand, metal ions can result harmful at high doses since they can interact with biomolecules disrupting the biological functions, however insufficient data regarding the toxicity of these metals has been provided and only pieces of information are given considering mostly the cytotoxicity of different copper(II) complexes on several cell lines or the effect on DNA. Teams of cancer biologists made an effort to identify individual chemicals that are carcinogenic or to determine whether or not chronic lifetime exposures to mixtures of noncarcinogenic chemicals in the environment (at low-dose levels) have carcinogenic potential [40]. Copper has been reported to show relevant cancer-associated dysregulation in metabolism [41]; however, toxicological data that are available for many suspected or known environmental disruptors, generally lack mechanistic information [40]. The present study deals with the effects of a series of Cu(II) complexes with saccharinate (sac) and glutamine (gln): $[Cu(sac)_2(H_2O)_4].2H_2O$ (Cu-sac), $[Cu(gln)_2]$ (Cu-gln) and $Na_2[Cu(sac)_2(gln)_2].H_2O$ (Cu-sac-gln) (Fig. **1** shows a scheme of the complexes). We have investigated the action of these compounds on cell viability on human osteosarcoma cell line MG-63, which is one of the most employed cell line to study bone tumors [39, 42]. In particular, we pay special attention to the cyto and genotoxicity actions of these complexes and to the association to oxidative stress.

2. EXPERIMENTAL

2.1. Materials

Tissue culture materials were purchased from Corning (Princeton, NJ, USA), Dulbecco´s Modified Eagles Medium (DMEM), TrypLE™ from Gibco (Gaithersburg, MD, USA), and fetal bovine serum (FBS) from Internegocios SA (Argentina). MTT, Trypan Blue, Crystal Violet, cytochalasin B from *Dreschslera dematioidea* and vitamin E were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Vitamin C and Giemsa were from Merck (Buenos Aires, Argentina). Dihydrorhodamine 123 (DHR) was purchased from Molecular Probes (Eugene, OR, USA). Bleomycin (*BLM*) (Blocamycin®) was kindly provided by Gador S.A. (Buenos Aires, Argentina). SYBR® Green and low-melting-point agarose were purchased from Invitrogen Corporation (Buenos Aires, Argentina).

2.2. Methods

2.2.1. Synthesis of Cu(II) Complexes

The copper(II) complexes were synthesized and characterized as previously reported [43, 44]. The heteroleptic copper(II) complex with saccharinate and glutamine $Na_2[Cu(sac)_2(gln)_2].H_2O$ (Cu-sac-gln) was synthesized and identified as in a previous report [28]. The yield was 89% for Cu-sac, 74% for Cu-gln and 65% for Cu-sac-gln.

The stability of the complexes in aqueous solutions was studied at 37°C for 24 h by UV-Vis spectral studies (Cu-sac 752 nm, Cu-gln 616 nm, Cu-sac-gln 625 nm). During a 24 h period at 37°C spectral changes were untraceable.

2.2.2. Preparation of Cu(II) Complexes Solutions

Fresh stock solutions of Cu(II) complexes were prepared in dimethyl sulfoxide (DMSO) at 100 mM, vortexed for 10 min and stored at 4ºC in the dark. To obtain test solutions, stock solutions were diluted with DMEM at the concentrations indicated in the legends of the figures. The maximum DMSO concentration was 0.5% in order to avoid toxic effects.

2.2.3. Cell Culture and Growth Conditions

MG-63 and MC3T3-E1 cells were purchased from ATCC (CRL1427™ and CRL2594™, respectively) and grown as the supplier recommends. For experiments, cells were grown in multi-well plates and incubated under the conditions described below.

2.2.4. Cell Viability: Crystal Violet Assay

Cells were treated with different concentrations (50–500 µM) of the complexes for 24 h at 37 °C. Besides, incubations were also carried out with different concentrations of the complex plus a mixture of vitamins C and E (50 µM each) during 24 h. After treatment, the assay was carried out as described by Okajima *et al.* [35-36].

2.2.5. Neutral Red (NR) Uptake Assay

The NR accumulation assay was performed according to Borenfreund and Puerner [47] after treating MG-63 cells with different concentrations of the complexes for 24 h at 37 °C in 5% CO_2 in air. The absorbance of the solution was measured in a Microplate Reader (7530, Cambridge technology, Inc, USA) at 540 nm, and compared with the control wells (untreated cells). Cell viability was plotted as the percentage of the control value.

2.2.6. MTT (Methyl Tetrazolium) Assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed according to Mosmann [48] after exposing cells to different concentrations of the complexes at 37 °C

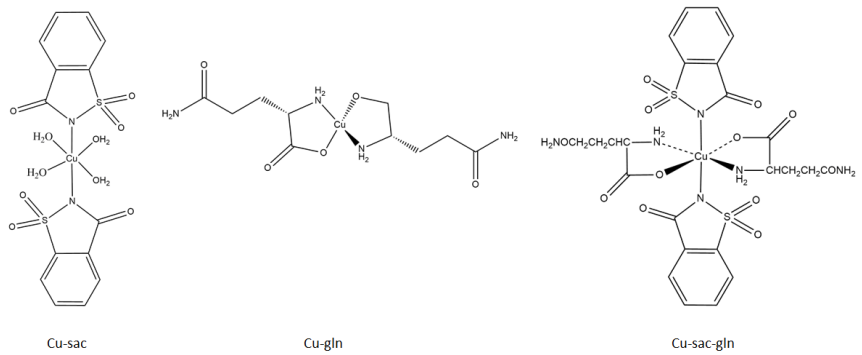

Fig. (1). Chemical structure of $[Cu(sac)_2(H_2O)_4].2H_2O$ (Cu-sac), $[Cu(gln)_2]$ (Cu-gln) and $Na_2[Cu(sac)_2(gln)_2].H_2O$ (Cu-sac-gln).

for 24 h. Color development was measured spectrophotometrically at 570 nm. Cell viability was plotted as the percentage of the control value.

2.2.7. Cytokinesis-block Micronucleus (MN) Assay

MG-63 cells were treated with Cu-sac, Cu-sac-gln or Cu-gln along with cytochalasin B (4.5 µg/mL). After 24 h, the MN assay was performed as reported by Fenech [49].

2.2.8. Single Cell Gel Electrophoresis (Comet Assay)

For detection of DNA damage, the comet assay was employed based on the method of Singh *et al*. [50]. Analysis was performed in an Olympus BX50 fluorescence microscope. Randomly captured cells, 100 per experimental point, were used to determine the tail moment, using Comet Score version 1.5 software.

2.2.9. Determination of Reactive Oxygen Species (ROS) Level

ROS level was determined by oxidation of dihydrorhodamine-123 (DHR-123) to rhodamine by spectrofluorescence as previously reported [51] after the incubation with Cu-sac, Cu-sac-gln or Cu-gln. Results were corrected for protein content, measured with the Pierce™ BCA Protein Assay Kit.

2.2.10. Fluorometric Determination of Cellular GSH/GSSG Ratio

GSH and GSSG levels were determined in MG-63cells as described by Hissin and Hilf [52]. The GSH/GSSG ratio was calculated as % basal for all experimental conditions.

2.2.11. Statistical Analysis

Results are expressed as the mean of three independent experiments and plotted as mean ± standard error of the mean (SEM). The total number of repeats (n) is specified in the legends of the figures. Statistical analysis of the data was carried out by ANOVA, followed by the Fisher´s Least Significant Difference (LSD) procedure to discriminate among the means. The statistical analyses were performed using STATGRAPHICS Centurion XVI.I. In the comet assay, Mann-Whitney Rank Sum Test was carried out to compare treated cells group against the control group.

3. RESULTS

3.1. Effect of Copper(II) Complexes on Cell Viability

Cell viability of three copper(II) complexes was assessed in MG-63 cells after 24 h treatment by determining the incorporation of crystal violet dye. Fig. **2** (parts **A**, **B** and **C**) shows the modification of cell viability (as percentage of control absorbance) with complexes concentration. It can be observed that, after exposing MG-63 cells to different concentrations (5–500 µM) of Cu(II) complexes, the three complexes induced a significant expansion in cell viability at lower concentrations, however at higher concentrations cell viability decreases reaching a reduction of 80% comparing to control values ($p<0.001$). It is important to consider that Cu-sac-gln was the only that reached that effect at a lower concentration (200 µM, Fig **2A**). Moreover, the half-maximal inhibitory concentrations in MG-63 cells are 170 µM, 270 µM and 280 µM for Cu-sac-gln, Cu-sac and Cu-gln, respectively. According to these results, the following potency order can be established for the antitumor action: Cu-sac-gln> Cu-sac ≈ Cu-gln.

To understand the real potential of copper(II) complexes and to address their selectivity for cancer cells, we investigated their effects on the viability of MC3T3-E1, an osteoblastic mouse calvaria cell line, which shows a normal phenotype [53]. Fig. **2** (parts **A**, **B** and **C**) shows the effect of Cu-sac, Cu-sac-gln and Cu-gln on the viability of MC3T3-E1. As can be seen, the three complexes induced a decrease in cell viability along with the concentration; however, the reducing rate is lower than for the

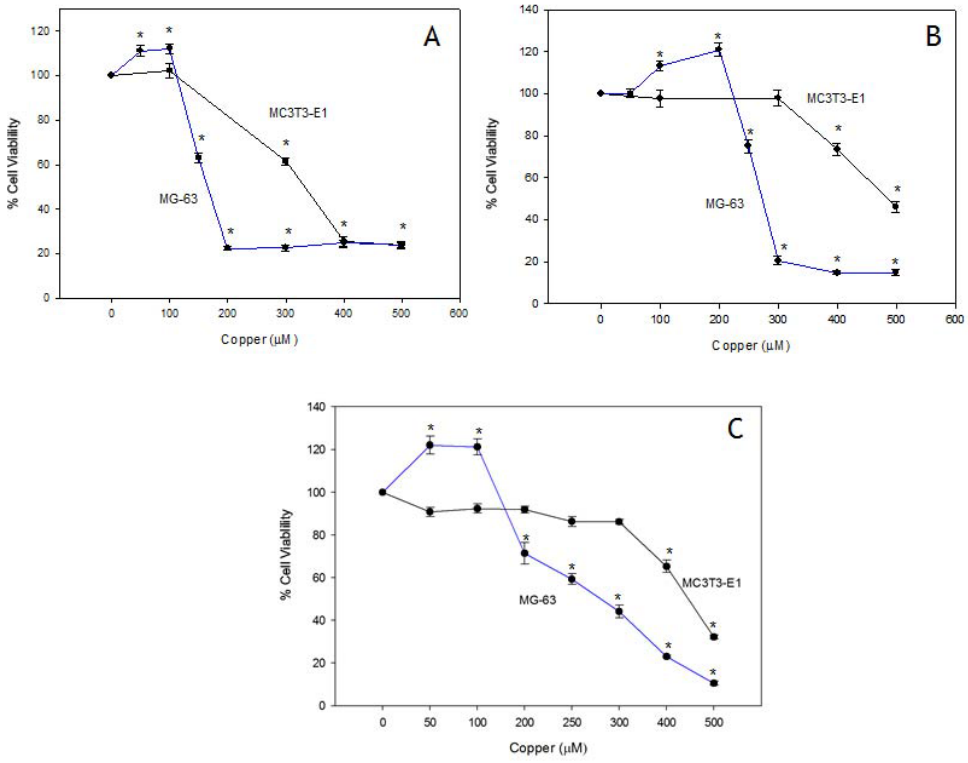

Fig. (2). Effects of (A) Cu-sac-gln, (B) Cu-sac and (C) Cu-gln on MG-63 or MC3T3-E1 cell viability. Cells were incubated in Dulbecco's modified Eagle's medium (DMEM) alone (control) or with different concentrations of the complexes at 37°C for 24 h. The results are expressed as the percentage of the basal level and represent the mean ± s (standard error of the mean) (n = 18), * significant differences versus control, $p < 0.001$.

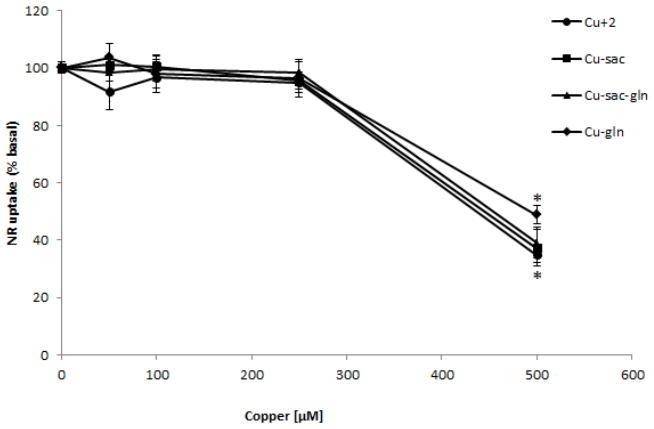

Fig. (3). Neutral red (NR) uptake by MG-63 osteosarcoma cells in culture. Tumor cells were incubated with different concentrations of the complexes for 24 h at 37°C. After incubation, cell viability was determined by the uptake of NR. The dye taken up by the cells was extracted and the absorbance was read at 540 nm. The results are expressed as the percentage of the basal level and represent the mean ± s (standard error of the mean) (n = 18), * significant differences versus control, $p < 0.001$.

tumor cell line. In particular, Cu-sac-gln showed only a decrease of c.a. 20 % at 200 µM (Fig. **2A**) compared with control values ($p<0.001$).

3.2. Cytotoxicity Induction in Osteosarcoma MG-63 Cells in Culture

3.2.1. Effects of the Complexes on the Lysosomal Metabolism: NR Assay

Lysosomes of viable cells have great affinity for the vital dye NR. When cellular metabolism is altered by different factors, the ability of the lysosomes to uptake NR is diminished. For this reason, the NR assay is a way of measuring living cells. The cytotoxic effects of the three complexes affected the function of lysosomes in MG-63 cells. As can be seen in Fig. **3**, a statistically significant decrease in the incorporation of the NR into the lysosomes could be determined only at 500 µM ($p<0.001$) for the three Cu(II) complexes.

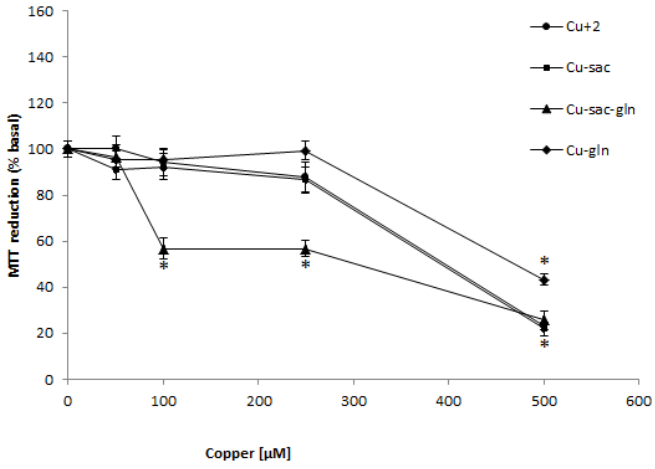

Fig. (4). MTT assay in MG-63 cells. After incubation with different concentrations of the complexes, mitochondrial activity was determined by the conversion of the tetrazolium salt to a colored formazan by mitochondrial dehydrogenases. Color development was measured at 570 nm after cell lysis in DMSO. Results are expressed as % basal and represent the mean ± s (standard error of the mean) (n = 18), * significant differences versus control, $p < 0.001$.

3.2.2. MTT Reduction. Effect of Chemical Complexation in MG-63 Cells

The cytotoxicity of Cu(II) complexes was also assayed after 24 h incubation of MG-63 cells by evaluating the reduction of the methyl tetrazolium. Fig. **4** shows the correlation between cell viability (as percentage of control absorbance) and complex concentration. Cytotoxicity is extremely related to the decreased ability of mitochondria to reduce MTT to an insoluble violet compound (formasan). The treatment of MG-63 cells with different concentrations (50–500 µM) showed that only Cu-sac-gln significantly diminished the capability of cells to develop the insoluble product, from 100 µM ($p < 0.001$).

MG-63 cells were also exposed to Cu(II) cation in order to analyze the effect of chemical complexation. Fig. **4** shows the reducing effect of copper(II) cation only at 500 µM, as Cu-sac and Cu-gln. Therefore, the heteroleptic copper(II) compound produced the strongest antiproliferative effect in the range from 100 to 250 µM ($p<0.05$) according to the MTT assay, whereas at 500 µM, the inhibition effect of the three complexes and copper(II) cation did not show significant differences.

3.3. Genotoxicity Studies

In order to study how copper(II) complexes induced genotoxicity, we assessed the rise of MN incidence and the generation of DNA damage by the comet assay. MN production in binucleated cells was shown after exposure to Cu-sac, Cu-sac-gln or Cu-gln in comparison to $CuCl_2$ for 24 h (Fig. **5**). We could assessed an increase in MN frequency at concentrations higher than 50, 100 and 250 µM of Cu-sac, Cu-sac-gln and Cu-gln, respectively($p<0.01$). Only Cu-sac started its effect at a lower concentration than the copper ion. Moreover, even though the three complexes displayed a concentration-related induction of MN frequency, only Cu-sac-gln overcame the effect of bleomycin (positive control) uncovering the strongest and statistically different genotoxic action in comparison with the other two complexes from 100 µM ($p<0.01$).

The Single Cell Gel Electrophoresis assay measures single- and double-strand DNA breaks. The alkaline version also detects abasic sites. As it is shown in Fig. **6**, all the tested complexes caused a significant genotoxic effect in human osteosarcoma cells from 50 µM ($p<0.001$). Moreover, the genotoxicity in Cu-gln treated cells increases with the concentration; however Cu-sac-gln and Cu-sac reached a plateau at 50 µM with *ca.* 50% of damaged cells. In those cells exposed to 50 µM Cu-sac-gln, the highest genotoxic effect was observed reaching almost 60% of damaged cells.

3.4. Mechanism of Action

We evaluated the effect of oxidative stress through the assessment of ROS level and the quantification of the GSH/GSSG ratio. DHR-123 changes to rhodamine123 which can be detected by fluorescence. Incubation of MG-63 cells with the Cu(II) complexes raised ROS level from 50 µM Cu-sac-gln and Cu-sac (Fig. **7**). Cu-sac-gln showed a more pronounced effect at 100 µM (causing a 250% ROS increase over the basal level) and statistically different from the other two complexes. Besides, Cu-sac-gln showed a concentration-related increase between 100 and 250 µM (indicated by # in Fig. **7**) and no modification at 500 µM. However, Cu-sac showed a plateau from 50 to 250 µM, no concentration-related effect was registered, i.e. there are no statistically significant differences between the concentrations tested until 500 µM. On the other hand, Cu-gln showed a statistically significant effect only from 250 µM.

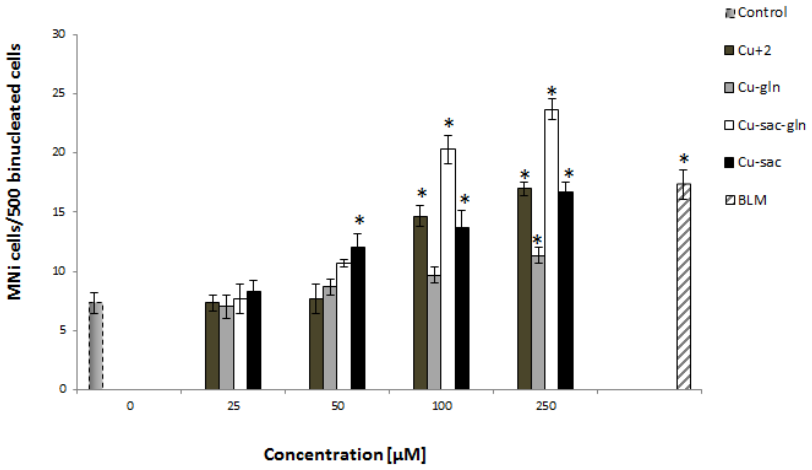

Fig. (5). Micronuclei induction in MG-63 cells after 24 h exposure to Cu(II) complexes, * significant differences versus control, p<0.01. BLM (0.5 µg/mL) stands for Bleomycin used as a positive control (pulse of 30 minutes at the beginning of treatment).

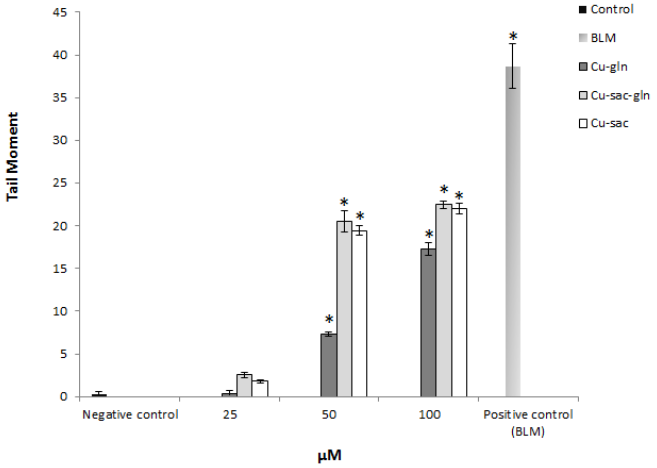

Fig. (6). Tail moment. Analysis of DNA damage measured by comet assay in MG-63 cells exposed to different concentrations of Cu(II) complexes. BLM (10 µg/mL) stands for Bleomycin used as a positive control (pulse of 20 minutes at the end of treatment).

At the highest tested concentration, ROS level did not differ between the three studied complexes reaching *ca.* 350% increase over the control values.

These findings triggered new studies to determine the effect of ROS level on the antiproliferative action of the copper complexes. In the presence of a mixture of 50 µM of vitamin C plus 50 µM of vitamin E (ROS scavengers), a total recovery of cell survival was obtained for Cu-gln in the whole range of concentrations while only a partial viability recovery was obtained from 250 µM of Cu-sac and Cu-sac-gln (p<0.001; Fig. **8**, parts **A**, **B** and **C**).

All the three complexes decreased the GSH/GSSG ratio in MG-63 cells in a concentration-dependent manner above 50 µM (p<0.001; Fig. **9**). It is most likely that an increase in ROS level may cause a reduction in GSH levels and/or an accumulation of GSSG inside the cells.

4. DISCUSSION

The occurrence of innovative metal compounds as antitumor agents is currently undergoing a dramatic expansion. Cisplatin is an effective Pt-based drug, which is employed in different sarcomas

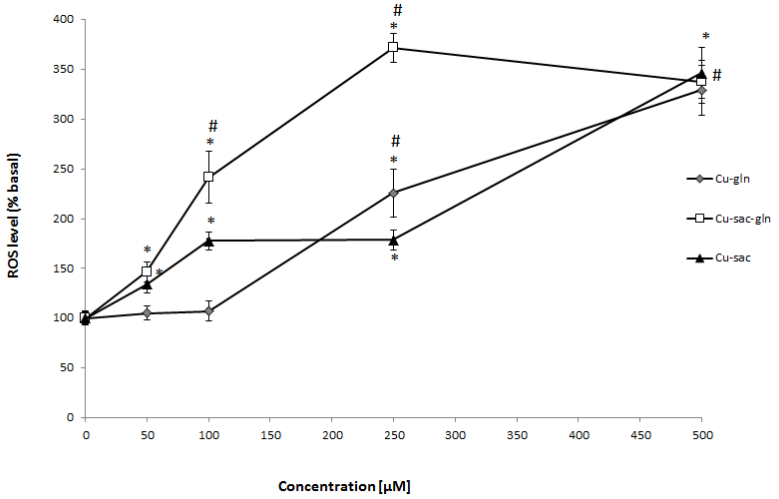

Fig. (7). Induction of ROS by Cu(II) complexes in MG-63 cell line. Cells were incubated with growing concentrations of Cu-sac, Cu-gln and Cu-sac-gln at 37 °C for 24 h. ROS production in the cells was evaluated through the oxidation of DHR-123 to Rhodamine123. Results represent the mean ± s (standard error of the mean) (n= 12), * significant differences versus control, p<0.01. # significant differences versus previous concentration, p< 0.05 (at 500 µM, the # corresponds only for Cu-sac).

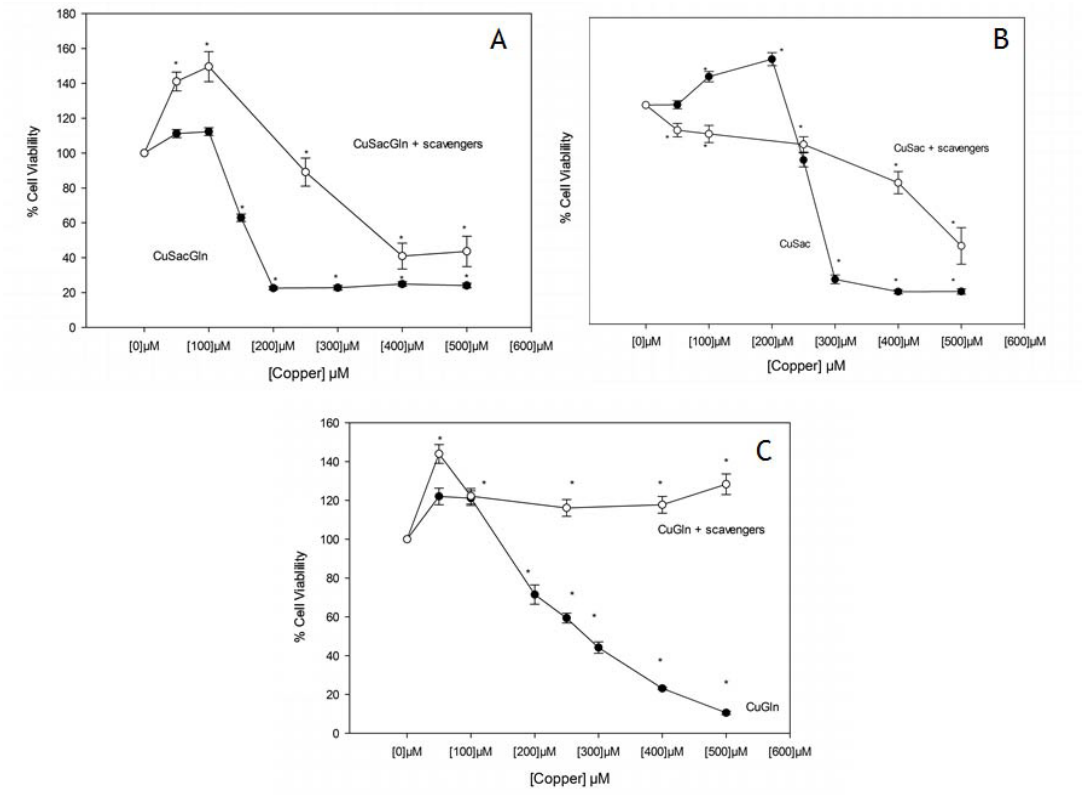

Fig. (8). Effects of (A) Cu-sac-gln, (B) Cu-sac and (C) Cu-gln on MG-63 cell viability. Cells were incubated with different concentrations of the complexes at 37°C for 24 h in the presence or not of a mixture of vitamins C and E (50 µM). The results are expressed as the percentage of the basal level and represent the mean ± s (standard error of the mean) (n = 18), * significant differences versus control, $p < 0.001$.

and carcinomas. However, it has been reported severe toxicity and drug resistance [54]. Thus, much of the scientific community pursue the development of new metal based antitumor drugs to reduce toxicity and to increase clinical efficiency [55]. In addition to non-Pt compounds, copper compounds are potentially good candidates as anticancer drugs in medicinal chemistry [19, 56-58].

Assuming that endogenous metals produce less damage and the interaction with many ligands may change the pharmacological and toxicological properties of both components, we present here the analysis of the cyto- and genotoxicity of a novel heteroleptic copper(II) complex with saccharinate and glutamine (Cu-sac-gln)

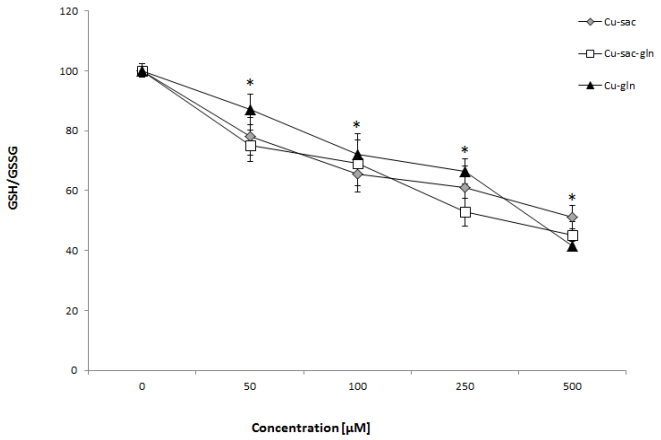

Fig. (9). Alteration of GSH/GSSG ratio by Cu(II) complexes in MG-63 cells. Results are expressed as % basal and represent the mean ± s (standard error of the mean) (n= 9), * significant differences versus control, $p<0.001$.

evaluated in human osteosarcoma MG-63 cells and compare with two related copper(II) complexes (Cu-sac and Cu-gln). Moreover, underlying mechanisms of toxicity were studied.

Cell viability studied in MG-63 cell line by the Crystal Violet assay showed a biphasic response after exposure to the three copper(II) complexes. There was a steep stimulation of cell viability at lower concentrations producing an increase of 20 % higher than the control values. At higher concentrations (*ca.* 100 µM), a statistically significant harmful effect was seen. This inverted U-shaped concentration-response curve was previously observed for copper ions in bacteria, in human cells and in earthworms [59-61]. Nevertheless, this phenomenon was not observed in the osteoblasts of non-cancerous phenotype. The three complexes induced a decrease in cell viability in MC3T3-E1 cells in the whole range of concentrations; however, the effect was weaker than that for the tumor cell line. As a whole, these results indicate that these Cu(II) complexes are excellent candidates for further study their mechanisms of action because they are less noxious to the normal phenotype osteoblasts than for the osteosarcoma cells.

To better understand the cytotoxicity of the complexes on the MG-63 cell line, next we investigated the effects on two parameters: the metabolism of lysosomes and mitochondria which are key organelles for the cells. Normally, cells incorporate the neutral red (NR) dye and actively transport it to the lysosomes. If a drug impairs this metabolic activity, the capacity of the lysosomes to uptake NR decreases. The obtained results with NR dye do not agree with the effects of the complexes on the MG-63 osteoblast viability making evident that lysosomes disturbance is not the mechanism of action employed by these complexes.

On the other hand, cytotoxicity of Cu(II) complexes was assessed by measuring the capacity of mitochondria to reduce the methyl tetrazolium. The data presented here show that only the

heteroleptic copper(II) complex significantly reduced the capability to form the formazan from 100 μM. These results demonstrate the beneficial effect of chemical complexation on cell viability, since the complexation with both ligands (saccharinate and glutamine) produced the strongest effect in comparison with the homoleptic complexes.

These results agree with previous reports where the cytotoxic and pro-apoptotic activity of copper(II) complexes were evaluated against different cancer cell lines [62-64]. Several reports have shown the synthesis, structure and characterization of copper(II) complexes with α amino acids, which showed deleterious effects on cancerous cell lines [65]. Ternary copper(II)-dipeptide-phenanthroline complexes induced cytotoxicity in several tumor cells [66]. Interestingly, it has also been described a differential behavior between tumor and non-cancerous human cells exerted by a series of copper(II) complexes [67, 68]. The advantage of chemical complexation was also previously observed by one of us with a copper(II) complex of Losartan assayed on two osteoblast-like cells in culture (UMR106, murine osteosarcoma cells and MC3T3-E1, calvaria-derived cells). It has been demonstrated that the free cation Cu(II) was more deleterious for the normal-phenotype osteoblast, however the complex exerted similar injurious action on both cell lines. The greater antiproliferative effect of the complex in comparison with the free ligand and the copper ion was supported by the higher lipophilicity of the neutral complex [69]. Moreover, another copper(II) complex with santonic acid also displayed a differential behavior between normal-phenotype and tumor cells. The complex showed stronger inhibition on cell proliferation of Caco-2, TC7 and UMR106 than for MC3T3-E1 cells. Besides, the complex with santonic acid is considered a potential candidate for antineoplastic studies since the antiproliferative effect is much more manifest than the effect of the free copper ion [70]. Previously, we have also shown the inhibitory effect of a copper(II) complex with Norfloxacin, a fluoroquinolone antibiotic, on UMR106 and MC3T3-E1 demonstrating a stronger decrease in cell viability of the tumoral cell line UMR106 than in MC3T3-E1 cells. Moreover an advantageous effect in comparison to free copper ion could also be observed [71].

Since DNA injury plays an important part in the development of tumors, we studied the genotoxicity of copper(II) complexes induced in human osteosarcoma cells *in vitro*. Genetic damage was detected with high MN frequency of binucleated cells exposed to the three copper(II) complexes. However, Cu-sac-gln induced a stronger and statistically different reaction than the other two complexes from 100 μM, even though the genotoxic effect started at a higher concentration than Cu-sac. Another biomarker of genetic damage is the induction of 'comets'. The alkaline version determines single- and double-strand breaks in addition to places of breakages and repair. We demonstrated that the three complexes induced a significant genotoxic effect starting at a lower concentration than the induction of MN. On the whole, these findings showed that the three complexes generated DNA damage in MG-63 cells, producing comet and MN induction. However the mixed Cu(II) complex with saccharinate and glutamine induced the strongest genotoxic effect. It has previously reported that copper(II) complexes interact with DNA sequences and induce cleavages in plasmids [67, 72]. These effects are strictly associated to the increase in MN frequency as it has been previously reported for organic copper compounds [73, 74]. There is convincing evidence that copper(II) complexes induce genotoxic effects by DNA fragmentation and base oxidation correlated to high levels of ROS [75].

Oxidative stress is the major mode of action for different antitumor compounds. In fact, a high oxidative state makes tumor cells more susceptible to elevated ROS amount [76]. Thus, we evaluated the effect of the copper(II) complexes with saccharinate and aminoacids on oxidative stress through the oxidation of the probe DHR-123 which measures levels of peroxynitrite, H_2O_2, and OH• [77] and the ratio GSH/GSSG, where GSH is the reducing agent that reduces free radicals [78]. Our results show that the three complexes induced an increase in ROS levels and a decrease in the GSH/GSSG ratio in MG-63 human osteosarcoma cell line, in spite of their superoxide dismutase-like activity previously measured [28, 79]. Moreover, the copper(II) complex containing glutamine and saccharinate moieties showed the strongest oxidative effect from 50 μM, which may explain its harmful effect related to mitochondrial damage. This is consistent with the results in the Cristal Violet assay, which show the lower capacity for cell viability recovery when cells were treated with the complexes along with a mixture of vitamins E and C. So, it can be expected that copper(II) complexes-exposed cells become more vulnerable to oxidative injury because the balance between oxidants and antioxidants is disturbed and that leads to cytotoxicity.

Since copper(II) complexes generate ROS-mediated genotoxic damage in bacteria and in mammalian cells [75, 80], we can attribute the genetic damage to increased levels of oxidative stress induced by the three copper(II) complexes from 50 μM. The elicitation of genetic damage might be caused by an elevated production of ROS or by interaction with DNA, that is, a direct binding or intercalation upon entry into the nucleus [67, 81]. In fact, since the discovery of the DNA intercalation process [82], many copper complexes are tested as DNA-targeting compounds [19, 83, 84].

Recently, *in vitro* DNA binding studies of saccharinate and its copper(II) complex were carried out [85]. The results showed that saccharinate binds to DNA by noncovalent interactions preferentially *via* minor groove binding, while the mode of binding of the Cu-sac complex is intercalation [85]. Although glutamine is not a DNA intercalator, it clearly contributes to the reactivity of the complex towards DNA. This could explain the high efficacy of Cu-sac-gln in the MN and Comet assay in MG-63 cells. But in this case it would remain to be demonstrated the nuclease activity of the heteroleptic complex.

5. CONCLUSION

In this paper we have thoroughly investigated the mode of action of three copper(II) complexes with very attractive and versatile ligands as the saccharinate anion (sac) and glutamine (gln).

The antitumor facet of Cu-sac, Cu-gln and Cu-sac-gln in a human osteosarcoma cell line has been examined, since osteosarcoma is the most frequent bone tumor. We have demonstrated that the complexation of Cu(II) with both saccharinate and glutamine to obtain a heteroleptic complex, improved the antitumor activity in comparison to the homoleptic complexes. Moreover, the human osteosarcoma cells were highly more sensitive than the non-cancerous bone related cells in relation to the antiproliferative effects. The three copper(II) complexes studied here caused a cyto- and genotoxic effect in a concentration dependent fashion. However, the heteloleptic compound interacted with mitochondria producing the strongest cell viability reduction and DNA damage, associated with an increase in oxidative stress.

Given all the properties of these compounds, it would be very interesting to test them in *in vivo* studies for cancer treatments.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

ACKNOWLEDGEMENTS

This work was partly supported by UNLP (11X/690), by ANPCyT (PICT 2014-2223) and by CONICET (PIP 1125) from

Argentina. Etcheverry S.B. and Di Virgilio A.L. are members of the Carrera del Investigador, CONICET, Argentina. Cadavid-Vargas J.F. and León I.E. are fellowship from CONICET, Argentina. Santi E. and Torre M.H. thank PEDECIBA Química from Uruguay.

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