Journal of Inorganic Biochemistry 247 (2023) 112334

Contents lists available at ScienceDirect

Journal of Inorganic Biochemistry

journal homepage: www.elsevier.com/locate/jinorgbio

Bioactive copper(II) agents and their potential involvement in the treatment of copper deficiency-related orphan diseases

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ARTICLE INFO

Keywords:
Amino acid
Copper:Histidine complexes
Copper transporters
Cytochrome c oxidase activity
Paracellular transport, Caco-2 differentiated cells

ABSTRACT

The deregulation of copper homoeostasis can promote various diseases such as Menkes disease or hypertrophic cardioencephalomyopathy. We have recently synthesized solid copper(II) complexes ($[Cu(His)_2Cl_2]$ and $[Cu(Ser)_2]$), stable in physiological media and with potential as therapeutic agents. This report describes: ***i)*** the biocompatibility of these complexes at concentrations up to 100 μM using a differentiated Caco-2 cells model; ***ii)*** their transport across the intestinal epithelium using a transepithelial resistance assay and monitoring the amount of copper complexes at the apical and basolateral sides of the cells. The results suggest that the flow occurs through paracellular routes. The intracellular copper retention was <2.7% with no significant differences in intracellular copper content between 6 h and 48 h, suggesting an early copper retention process. Furthermore, this is the first evidence that demonstrates $[Cu(His)_2Cl_2]$ and $[Cu(Ser)_2]$ induce transcriptional downregulation of the four major copper transporters (*CTR1, DMT1, ATP7A, ATP7B*), and the upregulation of the metallothionein gene expression. A remarkable finding was the increase in cytochrome *c* oxidase activity observed after the treatment of differentiated Caco-2 cells with copper(II) complexes at concentrations of 50–100 μM. The understanding of the transport mechanisms of these copper(II) complexes across the intestinal epithelium and of their subsequent biological activities could contribute to the development of optimal pharmaceutical formulations for the therapy of copper deficiency-related diseases.

1. Introduction

Metal ions are involved in a wide range of biological functions in both intra- and extracellular contexts [1]. Among these, copper plays an essential role in living organisms as a structural component of several enzymes including laccase, ascorbate oxidase, ceruloplasmin, amine oxidases, superoxide dismutase, cytochrome *c* oxidase [2,3]. It is well known that the functions of these proteins are mainly due to the presence of this metal in their structures [2]. Moreover, copper bound to small biologically active molecules such as amino acids, have various physiological functions and may exert therapeutic properties [4]. In mammals, free copper ions are fortunately almost absent due to their reactivity and capacity to generate free radicals [5]. A complex system of molecules is involved in the uptake, distribution and delivery to their targets these essential but also potentially toxic metal ions [6]. For most of the cells, copper absorption is mainly mediated by the high affinity copper uptake protein 1 (CTR1) [7,8]. In addition, it is not excluded that divalent metal transporter 1 (DMT1) can be a vehicle for the entrance of copper into the cells [9,10]. The ATP7A and ATP7B proteins are responsible for transporting extracellular copper into the secretory pathway [6,8]. Numerous metallochaperones are involved in the cellular trafficking of this metal [6,7]. In particular, metallothionein (MT) has been largely studied mainly for its role in metal detoxification [8,11].

The deregulation of copper homoeostasis leads to various diseases such as neutropenia, thrombocytopenia, anemia, or encephalopathy

Abbreviations: AP, Apical; ATP7A, ATPase copper transporting alpha; ATP7B, ATPase copper transporting beta; BL, Basolateral; BM, Copper-free buffer medium; CM, Complete medium; COX, Cytochrome c oxidase; CTR1, Copper uptake protein 1; DMT1, Divalent metal transporter 1; H_2O_2, Hydrogen peroxide; HA, Histamine; HCEM, Hypertrophic cardioencephalomyopathy; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; His, L-Histidine; Im, Imidazol; MD, Menkes disease; MT, Metallothionein; NR, Neutral red; PBS, Phosphate buffer saline; ROS, Reactive oxygen species; Ser, L-Serine; SGF, Simulated gastric fluid; SIF, Simulated intestinal fluid; TEER, Transepithelial electrical resistance.

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https://doi.org/10.1016/j.jinorgbio.2023.112334
Received 31 May 2023; Received in revised form 9 July 2023; Accepted 18 July 2023
Available online 19 July 2023

[12,13]. Menkes disease (MD) is a severe multi-systemic disease caused by deficient absorption of copper(II) due to an inherited genetic dysfunction of the gene encoding the intestinal ATP7A protein [14,15]. Copper(II) complexed with L-Histidine (His), when administered parenterally, has been used to treat MD [14]. However, daily injectable administration of this complex complicates its use, and alternative ways of administration are desirable. The decrease in cytochrome *c* oxidase (COX) activity has been shown in copper-deficiency diseases, including MD and hypertrophic cardioencephalomyopathy (HCEM) [13,16,17].

HCEM is a mitochondrial disease characterized by a large clinically severe multi-systemic disorder [17]. This disease is caused by mutations in the *SCO2* gene that encodes the metallochaperone Sco2 involved in the assembly of copper to the subunit I of COX [17–19]. In contrast to MD, the intestinal absorption of copper induced by ATP7A is not affected in HCEM [17,19] and oral administration of copper may be beneficial, provided gastro-protection during the stomach transit. Indeed, copper supplementation was found to reverse the cardiac dysfunction in patients affected by HCEM [18].

Copper(II) complexes have recently received increasing attention, especially for their potential in biomedical applications [14,20,21]. Copper bound to His was found to play a key role in the transport and availability of copper to cells and tissues [14]. L-Serine (Ser) is a non-essential amino acid that can be synthesized by many cells [22,23] and that forms mixed copper(II) complexes also containing His ligand [14]. The copper(II)-L-amino acid complexes may be absorbed via the peptide or amino acid transport pathways, which could be increased when compared to commonly used inorganic sources [22]. Recently, we have synthesized and characterized the solid complexes $[Cu(His)_2Cl_2]$ and $[Cu(Ser)_2]$ and found a good biocompatibility (neuronal viability around 90%) of copper complexes at concentrations below 100 μM [24]. These observations open new perspectives for the possible use of these complexes as therapeutic agents for the treatment of various diseases related to copper deficiency.

The present study was first aimed to evaluate the biocompatibility of $[Cu(His)_2Cl_2]$ and $[Cu(Ser)_2]$ in differentiated Caco-2 cells: the most common and extensively characterized cell-based model for the assessment of absorption of drugs via the intestinal membrane enterocytes [25]. The flow of copper(II) complexes through the intestinal epithelium and their possible intracellular accumulation were investigated, as well as their transcriptional effect on the expression of metal transporters and metallothionein. To the best of our knowledge, this work is the first to investigate the impact of $[Cu(His)_2Cl_2]$ and $[Cu(Ser)_2]$ complexes on enhance of cytochrome *c* oxidase activity in enterocytes. This will contribute to increase our understanding of the mechanisms regulating the transport of copper(II) complexes across the intestinal epithelium and of their biological activity, in view of their oral administration as pharmaceutical forms for the therapy of copper deficiency related serious diseases such as HCEM.

2. Materials and methods

2.1. Synthesis of copper(II) complexes

The copper(II) complexes were prepared as described by Esmaili et al., [24]. Briefly, a cold solution (400 mL) of $CuCl_2$ (1.93×10^{-3} mol/L) in isopropanol was added dropwise to an aqueous solution (8 mL) of His (3.84×10^{-3} mol/L) at pH 6.8. The mixture was stirred continuously on ice for 45 min. The complexation was carried out in isopropanol to facilitate the recovery of $[Cu(His)_2Cl_2]$ which precipitates. The solid was collected by filtration, washed with cold isopropanol, and dried at room temperature. For $[Cu(Ser)_2]$ complex, a cold solution (15 mL) of $CuCl_2$ (2.64×10^{-2} mol/L) in ethanol was added dropwise to a solution of 30 mL of Ser (5.19×10^{-2} mol/L) in ethanol containing NaOH (0.01 mol/L). The mixture was stirred on ice for 30 min and the solid was filtered, washed with ethanol, and dried at room temperature. The copper(II) histamine ($[Cu(HA)_2Cl_2]$) and copper(II) imidazol ($[Cu(Im)_2Cl_2]$) complexes were prepared using a 1:5 M ratio of $CuCl_2$:ligand (HA; Histamine or Im; Imidazole) [26,27]. Practically, an aqueous solution (10 mL) of $CuCl_2$ (0.5 mol/L) was added dropwise to a methanolic solution (0.1 mol/L; 10 mL) of HA or Im. The reaction was carried out at 40 °C for 4 h with constant stirring. The final precipitated product was retained by filtration on a Büchner funnel, washed with ethanol and the powder was dried at room temperature. All chemicals purchased from Sigma-Aldrich (Oakville, ON, Canada) were of analytical grade and they were used as received, without any further purification.

2.2. Culture, differentiation, and treatment of Caco-2 cells

Human Caco-2 cells were provided by Dr. A. Zweibaum [28]. Stock cultures were maintained at 37 °C in a humidified atmosphere at 5% CO_2 in complete medium [CM; Dulbecco's Modified Eagle (DMEM; ThermoFisher Scientific, ON, Canada), supplemented with 15% *v*/v inactivated (56 °C for 30 min) fetal bovine serum (FBS; Wisent Bioproducts, St-Bruno, QC, Canada), 0.1 mM non-essential amino acids, 19 mM $NaHCO_3$ (Sigma-Aldrich, Oakville, ON, Canada) and 50,000 U/mL-50 μg/L penicillin-streptomycin (Gibco™, ThermoFisher Scientific)] [29]. The CM was changed every two days until 70–80% of confluence; then cells were passaged by trypsinization using 0.05% *v*/v trypsin-1 mM EDTA solution (Sigma Aldrich, Oakville, ON, Canada) and seeded in 75 cm^2 flasks (Corning Life Sciences, Corning, NY, USA) at 10^6 cells/flask. For viability assay, cells were seeded in 96-well plates (5×10^3 cells/well) and in 60 mm diameter Petri dishes (3×10^5 cells) for RT-qPCR analysis and COX activity. As the differentiation of Caco-2 cells takes place spontaneously fourteen days after the confluence [30], the cells were incubated (37 °C, 5% CO_2) for 21 days and the CM was changed three times per week. After differentiation, the CM was removed and cells were washed three times with phosphate buffered saline [PBS: NaCl 0.8% (*w/v*), KCl 0.02% (w/v), KH_2PO_4 0.02% (w/v), Na_2HPO_4 0.12% (w/v), pH 7.3)]. Subsequently, the medium was replaced by a copper-free buffer medium [BM; 137 mM NaCl, 5.9 mM KCl, 4 mM D-glucose, 1.2 mM $MgCl_2$, 2.5 mM $CaCl_2$ and 10 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), pH 7.4] containing copper(II) complexes or ligands (His, Ser, HA, Im) or $CuCl_2$ at defined concentrations and incubated (37 °C, 5% CO_2) at the times indicated in the figures. The control cultures contained only vehicle-treated cells. The viability of cells treated with ligands (His, Ser, HA or Im) and $CuCl_2$ was included for comparison. The treatments were done using stock solutions freshly prepared in BM and filtered through 0.2 μm Millex™ sterile syringe filters (Sigma-Aldrich, Oakville, ON, Canada).

For transepithelial transport, Caco-2 cells were seeded in polycarbonate filter cell culture chamber inserts (Corning® Transwell®, 12 mm Transwell with 0.4 μm pore diameter; Sigma-Aldrich, ON, Canada) at a density of 12×10^3 cells/cm^2 and were allowed to differentiate for 21 days changing the CM three times a week. This culture system is composed of two compartments physically divided: the upper or apical (AP) and the lower or basolateral (BL) layers that is particularly useful to study the transepithelial transport and the integrity of cells monolayers. After differentiation, the CM was removed, and cells were washed three times with PBS. Then, 0.5 mL of BM supplemented with $[Cu(His)_2Cl_2]$ or $[Cu(Ser)_2]$ at 50 or 100 μM were added into the AP compartment. In the BL compartments, 1.5 mL of BM were added. Similarly, the AP exposure of cells to His, Ser or $CuCl_2$ (50 or 100 μM) was included for comparison.

2.3. Viability of Caco-2 cells

Following the treatments, the viability of cells was determined using the neutral red (NR) assay which measures the accumulation of NR dye via active transport in the lysosomes of living cells [31]. In short, 0.2 mL of freshly prepared NR solution (138 μM in 20 mM HEPES, 140 mM NaCl, 4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgCl_2$ and 20 mM D-glucose, pH 7.4) was added to each culture well prewashed with PBS. After 2 h of

incubation (37 °C, 5% CO_2), the NR solution was removed, and the cultures were washed quickly with 1% formaldehyde in 1% $CaCl_2$. Then 0.2 mL of NR eluents (EtOH: H_2O: HAc 50:49:1) was added to extract NR from cells. After 10 min of stirring at room temperature, the absorbance was read at 540 nm using a microplate reader (Molecular SpectraMax EM Microplate Reader, CA, USA).

2.4. Effect of the copper(II) complexes on cell monolayers TEER

The transepithelial electrical resistance (TEER) was established as an indicator of the integrity of the cell monolayers [32]. Prior, the intrinsic resistance (ohms, Ω) of the filters (without cells) and BM (blank resistance) was measured using a Millicel ERS-2 Voltohmmeter (Millipore, Bedford, MA, USA). In addition, the integrity of cell monolayers was verified before various treatments of differentiated cells by measuring the TEER. The Caco-2 cells monolayers grown on filters displayed resistance values $\leq$250 Ω × cm^2 and were considered for this study. For the effect of various treatments on TEER of differentiated Caco-2 cell monolayers, the readings were taken at different times up to 48 h. All measures were taken at three different places on the cell monolayer. The TEER was calculated using the following formula: TEER (Ω × cm^2) = (Total resistance − blank resistance) (Ω) × Area (cm^2) and expressed in percentages referring to initial values of TEER before cell treatments.

2.5. Efflux and retention of copper(II) complexes in cells

Differentiated Caco-2 cells are an in vitro model commonly used to assess the retention of bioactive molecules [25]. After treatment of differentiated Caco-2 cells seeded in 12-well polycarbonate filter cell culture chamber inserts, the AP and the BL medium was harvested for further analysis. Untreated differentiated cells were included as control. Cells were harvested with 1 mL of PBS and centrifuged at 4000 rpm for 5 min. The cell pellets were stored at −80 °C overnight before lyophilisation. For standard curves, the solutions were prepared in BM alone (blank) or containing 0.01 to 10 ppm of copper(II). Subsequently, an acidic digestion with 500 µL of nitric acid (HNO_3) was carried overnight to lyophilized products and standard solutions. Then, 300 µL of H_2O_2 was added for others 24 h. Finally, the volume was adjusted for a final concentration of 5% of HNO_3. The concentration of Cu(II) was determined by inductively coupled plasma optical emission spectrometry (ICP/OES, Agilent 5100, Agilent Technologies, Santa Clara, CA, USA). The values are presented as the percentage of Cu(II) in samples relative to the initial amount of Cu(II) added as $[Cu(His)_2Cl_2]$ or $[Cu(Ser)_2]$. For copper(II) cell retention, the results are expressed in nmol/ mg protein. The protein content was determined by the Bio-Rad assay with bovine serum albumin (BSA) as the standard.

2.6. RT-qPCR assay of copper transporters and metallothionenin gene expression

After differentiation, Caco-2 cells were treated with $[Cu(His)_2Cl_2]$ or $[Cu(Ser)_2]$ at concentrations of 50 or 100 µM for 6 h. Total RNA was extracted from the cell pellets using 1 mL TRizol reagent per well (Life Technologies, Gaithersburg, MD, USA). Next, 1–2 µg of total RNA was reverse-transcribed into cDNA using the high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA) and following the manufacturer instructions. Real-time quantitative polymerase chain reaction (RT-qPCR) was then performed to determine the gene expression level of the copper transporters *ATP7A, ATP7B, CTR1, DMT1*, and of metallothionein (MT) using the SsoFast EvaGreen Supermix (Bio-Rad, Hercules, CA, USA). Amplification was performed in a CFX Connect Real-Time System (Bio-Rad, version 2.1). The resulting amplicons were detected by measuring the fluorescence elicited by the binding of the SYBR Green dye to the double strand DNA. QuantiTect primers were purchased from Qiagen (Montreal, QC, CA): *ATP7A* (Hs_ATP7A_QT00075852), *ATP7B* (Hs_ATP7B_QT00075782), *CTR1* (Hs_SLC31A1_QT00099267), *DMT1* (Hs_SLC11A2_QT00070308) and *MT* (Hs_MT1A_QT01004591). *18S ribosomal RNA* (Hs_RRN18S_QT00199367) and *Peptidylprolyl Isomerase A* (Hs_PPIA_4_SG_QT01866137) were included as housekeeping genes and their relative gene expression was used to normalize the expression of genes of interest. Quantification was performed following the ΔCT method, and the relative quantified value (RQV) was expressed as $2^{-\Delta CT}$. Finally, to confirm the specificity of product amplification, amplicons were electrophoresed on a 2% agarose gel, visualized with GreenGlow™ (Denville Scientific Inc., Saint-Laurent, QC, Canada) and using a ChemiDoc MP Imaging System (Fig. S1) Bio-Rad, Hercules, CA, USA). The expected PCR product sizes are as follows: *ATP7A* (72 bp), *ATP7B* (111 bp), *CTR1* (90 bp), *DMT1* (72 bp) and *MT* (101 bp).

2.7. Cytochrome c oxidase activity

Cytochrome c oxidase (COX) activity was determined using a colorimetric assay by following the oxidation of reduced cytochrome *c* through the decrease of absorbance at 550 nm. Briefly, sodium dithionite ($Na_2S_2O_4$) is added to a solution of Cytochrome c (0.09 mM) in phosphate buffer (0.1 M; pH 7) to ensure the total reduction of Cytochrome c. After the treatments, the differentiated Caco-2 cells were harvested with 1 mL of PBS and centrifuged at 4000 rpm for 5 min. Subsequently, 20 µL of lysis buffer (Triton X 0.1%; Tris 25 mM; pH 7.4) are added to the pellet to permeabilize the membranes. After 5 min of incubation, 300 µL of Tris buffer (25 mM; pH 7.4) were added and the mixture was homogenized using a scraper. The whole procedure is carried out at 4 °C and 50 µL of the homogenate was used for the protein assay by the Bio-Rad assay with BSA as the standard. In a 96-well plate, 10 µL of the cell homogenate and 190 µL of the reduced Cytochrome c solution were added to each well. The absorbance was measured at 550 nm every 30 s for a total time of 10 min at 25 °C. The enzymatic activity was calculated from the dilution of the fraction and the molar extinction coefficient at 25 °C and pH 7 of ferrocytochrome ($\varepsilon = 19.1\ mM^{-1}\ cm^{-1}$). One unit of COX is the amount of enzyme that oxidizes 1 µmol of reduced Cytochrome c per minute at 25 °C and pH 7. The results were expressed in percentages as (%) of the values obtained for untreated (control) cultured cells.

3. Results and discussion

3.1. Biocompatibility of copper(II) complexes

For biocompatibility assessment, the NR assay was used as a convenient method to measure cell viability when exposed to copper compounds at higher concentration than those of physiological copper levels [31]. As shown in Fig. 1 (A,C and D), His, HA and Im ligands exhibited a good biocompatibility at concentration up to 300 µM. This result fit well the previous ones showing that the treatment of differentiated Caco-2 cells with His, HA ($LC_{50} \cong$ 10 mM) and Im, did not affect cell viability at concentrations up to 1 mM [29]. Differently, Ser concentration-dependently decreased cell viability until a plateau of remaining viability of ~60% at 300 µM Ser (Fig. 1B). Interestingly, Ser was the only ligand toxic to cells in contrast to its copper(II) complex $[Cu(Ser)_2]$. Such cytotoxicity could be attributed to racemization by a serine racemase of L-Serine to D-Serine, a process known to be toxic to cells [33,34]. The present work is focused on $[Cu(His)_2Cl_2]$ and $[Cu(Ser)_2]$ as potential therapeutic agents for the treatment of various diseases related to copper deficiency.

It is well known that free copper can be highly toxic due to its ability to promote the generation of reactive oxygen species (ROS), cell apoptosis and DNA damage [7,35]. A drastic decrease in cell viability was observed when Caco-2 cells were exposed to $CuCl_2$ at concentrations between 50 and 300 µM (Fig. 1). The copper(II) complexes showed less cytotoxic effects than $CuCl_2$ at similar concentrations.

As shown in Fig. 1, copper(II) complexes induced a moderate loss of

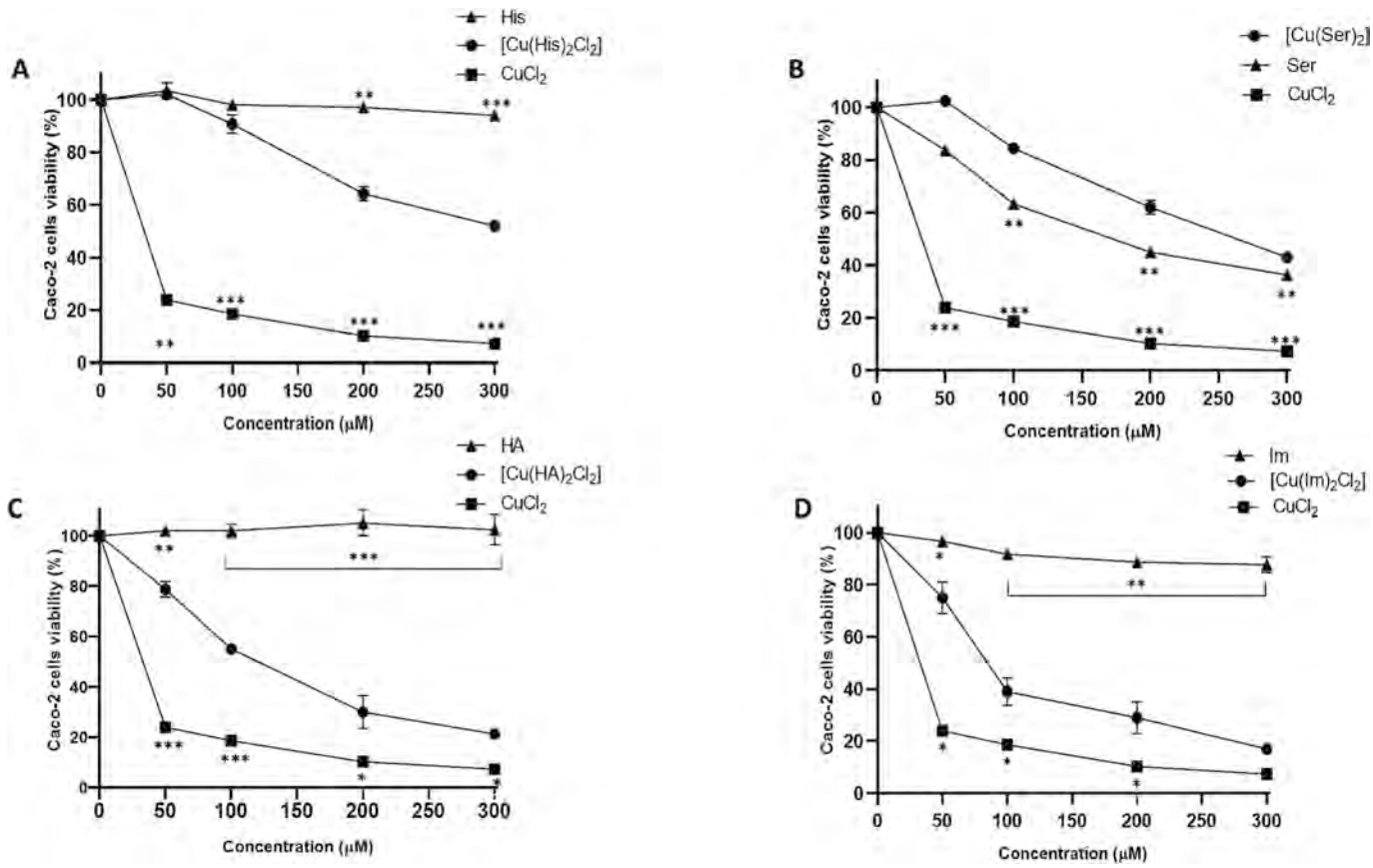

Fig. 1. Effect of copper(II) complexes and ligands on the viability of differentiated Caco-2 cells. Following differentiation, Caco-2 cells were treated with A) [Cu $(His)_2Cl_2$], B) $[Cu(Ser)_2]$, C) $[Cu(HA)_2Cl_2]$, or D) $[Cu(Im)_2Cl_2]$ for 24 h. Cell viability was also assessed upon treatment with the corresponding His, Ser, HA or Im ligands, and with $CuCl_2$ for comparison. Data are mean ± SD and expressed as percentages of untreated control cultures ($n = 3$; $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$; two-way ANOVA multiple comparisons).

cell viability in a concentration-dependent manner. The tridentate [Cu $(His)_2Cl_2$] ligation (Fig. 2S) confers greater stability compared to bidentate and monodentate ligands [14]. The structural characterization of $[Cu(Ser)_2]$ revealed two serine molecules bound to the copper ion in a bidentate manner (Fig. 2S-A) and showed that two amino nitrogen and two carboxyl oxygen are coordinated to the copper [24]. The [Cu $(His)_2$] was described as neutral five-coordinated complex with a distorted square pyramidal geometry with bidentate and tridentate L-histidine ligands (Fig. 2S-B). The tridentate chelation of one of the L-histidine ligands provides an additional stability over the binary [Cu (Ser)2] complex. Moreover, the enhanced stability of $[Cu(His)_2]$ versus $[Cu(Ser)_2]$ is evidenced when comparing the stoichiometric stability constant value of both complexes (log β ~ 18 and 14 respectively) [14,36]. This fact can also explain the negligible concentrations of the $[Cu(Ser)_2]$ complex detected in human blood [14]. Consequently, the $[Cu(His)_2Cl_2]$ complex demonstrated a better biocompatibility than the bidentate $[Cu(Ser)_2]$ and $[Cu(HA)_2Cl_2]$ or the monodentate [Cu $(Im)_2Cl_2$] complexes, which can release copper ions into the culture medium. Among all copper(II) complexes, the $[Cu(His)_2Cl_2]$ complexes, closely followed by $[Cu(Ser)_2]$, were significantly more biocompatible (viability ≥90%) at concentration up to 100 μM. Similar results were previously obtained in a P19 neurons model [24].

The understanding of copper(II) complexes retention and flow by intestinal cells is important for the design and development of therapeutic strategies, and for the use of these potential bioactive copper(II) agents in the treatment of copper deficiency disorders. It is worth noting that non-toxic concentrations (50–100 μM) of copper(II) complexes (Fig. 1) were chosen for the rest of investigation. These concentrations are comparable or slightly higher than physiological concentrations are not impacting on cell permeability. Indeed, the range of normal physiological copper concentrations is between 1.6 μM in plasma [37] to 70 μM in the brain parenchyma. In contrast, in patients affected by Wilson's disease, higher concentrations up to 1300 μM copper(II) were found [38].

3.2. *Effects of copper(II) complexes on transepithelial electrical resistance (TEER)*

Caco-2 cells have been described as a useful model for predicting drug transport through the intestinal epithelium comparable to in vivo models [25]. They can spontaneously differentiate into cell monolayers reflecting mature intestinal enterocytes that exhibit a paracellular permeability close to physiological human conditions [25,30]. The measure of transepithelial electrical resistance (TEER) in Caco-2 cells is a strong indicator of tight junction integrity and is a widely used technique to assess the paracellular drug transport pathway [25]. Fig. 2 shows the impact of $[Cu(His)_2Cl_2]$ and $[Cu(Ser)_2]$ on TEER of differentiated Caco-2 cell monolayers measured at up to 48 h and expressed as a percentage related to initial values of TEER. The exposure of cells to these copper(II) complexes did not trigger a significant change in TEER values compared to untreated control cultures for the first 6 h of treatment. These bioactive copper(II) agents are not stable in pH ≤ 5 [24]. Thus, it will be necessary to use more convenient pharmaceutical formulations for its release in the intestinal tract region with pH 7. The acidic pH (1.5–3.5) in the stomach increases in the small intestine to about pH 5–6 in the duodenum and pH 7–8 in the distal jejunum and ileum while in colon it decreases to pH 6 [39]. As the estimated time for the transit of drugs through the small intestine is not longer than about

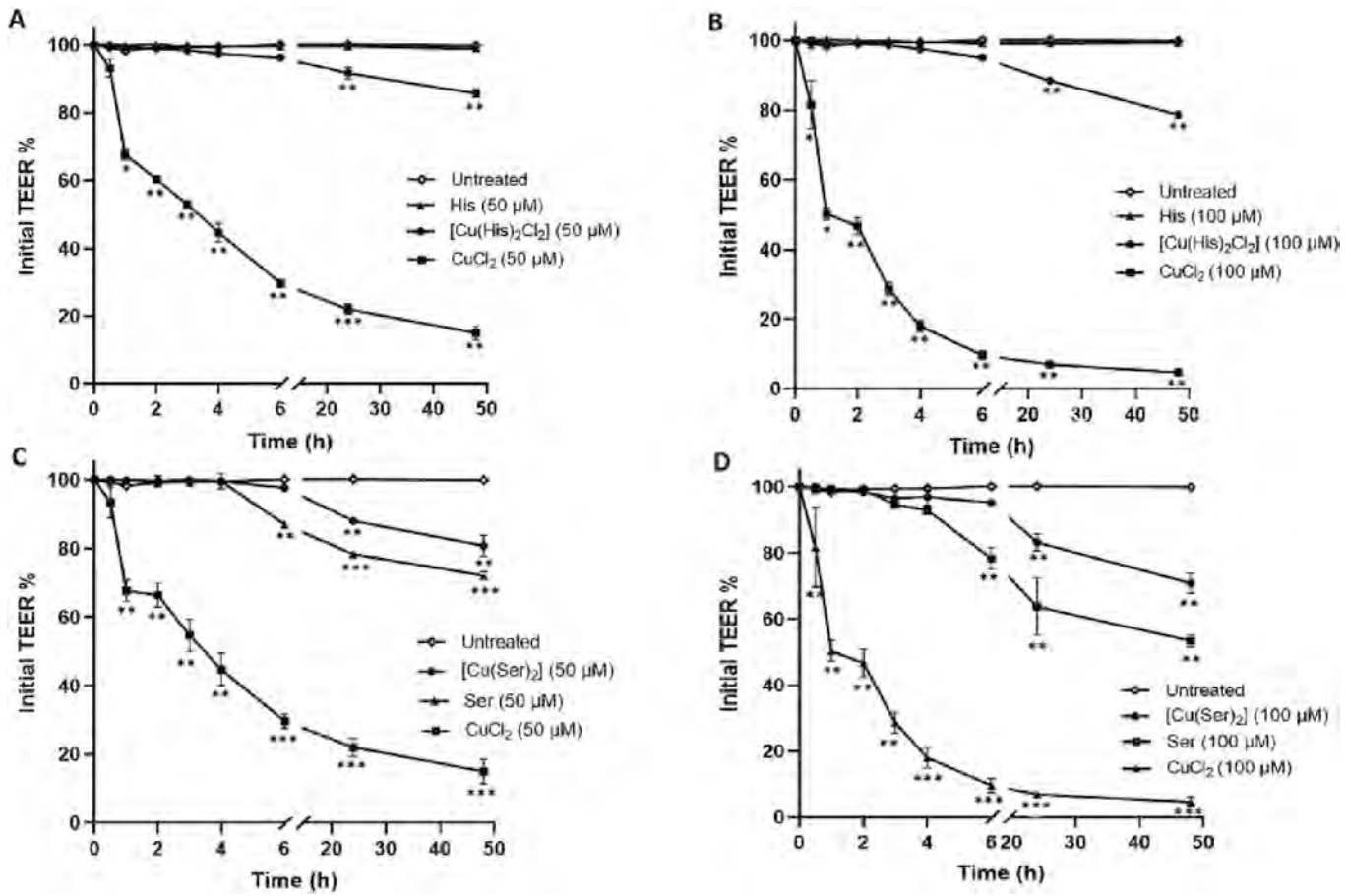

Fig. 2. Effect of copper(II) complexes on transepithelial electric resistance (TEER) in differentiated Caco 2 cell monolayers. Cells were exposed to $[Cu(His)_2Cl_2]$, His, $CuCl_2$ (A,B) and to$[Cu(Ser)_2]$, Ser, $CuCl_2$ (C, D) at concentrations of 50 μM (A, C) or 100 μM (B, D). The TEER values were measured for the times indicated in the figure. Data are expressed as mean of a percentage of TEER ± SD ($n = 3$) related to its initial values. The TEER values are calculated after subtracting the intrinsic resistance of the filters without cells (135 ± 7 Ω•cm^2). The values of cells treated with $CuCl_2$ were included for comparison. The significant differences compared to untreated (control) cultures were estimated as statistically significant for P values lower than 0.05 (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$; two way ANOVA multiple comparisons).

4.5 h [39], the results showing unmodified TEER for at least 6 h (Fig. 2), suggest that intestinal cell membranes would not be affected during the intestinal transit of these complexes. Differently, both copper complexes promoted a significant time-dependent decrease of TEER after 24 up to 48 h. These results point to the loss of the integrity of the cell monolayers and a related increased cell permeability after a long exposure time (Fig. 2). Also, for $[Cu(Ser)_2]$ at 100 μM, the TEER results could be correlated with the 15.3 ± 1.5% of loss on cell viability (Fig. 1B) after 24 h of treatment. The presence of copper in the structure might be affecting the integrity of cell monolayers by damages caused to the membranes or to the proteins involved in tight junctions [7]. The decreased TEER values could be directly related to the use of paracellular route by drugs [32]. However, the transcellular passage or the active uptake via intestinal transporters of $[Cu(His)_2Cl_2]$ or $[Cu(Ser)_2]$ cannot be excluded.

His did not affect the TEER values but Ser, at both concentrations, caused a time-dependent decrease of TEER. As discussed above, this amino acid has cytotoxic effects on differentiated Caco-2 cells (Fig. 1B) which may have a direct influence on the increase of the cell monolayer permeation with moderate effect at 24–48 h. Contrary to copper(II) complexes, the $CuCl_2$ caused a marked decrease in TEER values in the first 6 h. This clearly suggest for the copper(II) complexes with lesser deleterious effects on cell monolayers integrity than $CuCl_2$ at similar concentrations.

3.3. Flow and retention of copper(II) complexes in Caco-2 cells

The flow time course of $[Cu(His)_2Cl_2]$ or $[Cu(Ser)_2]$ through AP to BL compartments was next estimated from the amounts of Cu(II) determined by ICP/OES. Fig. 3 shows a time-dependent decrease of copper(II) concentrations, in AP compartments while an increase in BL compartments was observed.

From the initial amount of $[Cu(His)_2Cl_2]$ added to AP compartments at 50 or 100 μM, a decrease was found in both cases, and was more pronounced at 100 μM (from 6.5 ppm to 3.69 ± 0.04 ppm) after 48 h of treatment (Fig. 3A and B). On the contrary, Cu(II) was not detected in BL compartments at the beginning of experiments but the amount was constantly increase until the end of exposure. The amounts of copper(II) quantified were up to 2.49 ± 0.03 ppm during the exposure to $[Cu(His)_2Cl_2]$. Similar flow effect were found when cells were exposed to $[Cu(Ser)_2]$ (Fig. 3C and D).

Cu(II) content was also quantified in differentiated Caco-2 cells exposed to 100 μM of copper(II) complexes. A rapid increase of the intracellular Cu(II) content was observed until 6 h, followed by a plateau (Fig. 4). The intracellular concentration of copper(II) was increased 4.5-fold, compared to the initial intracellular copper content. Similar patterns were obtained when cells were treated with 50 μM $[Cu(His)_2Cl_2]$ or $[Cu(Ser)_2]$ but with a moderate rise in the concentration of Cu(II) up to 2.5 folds after 48 h.

The percentages of Cu(II) from the initial concentration of this cation as $[Cu(His)_2Cl_2]$ or $[Cu(Ser)_2]$ in AP and BL compartments and on

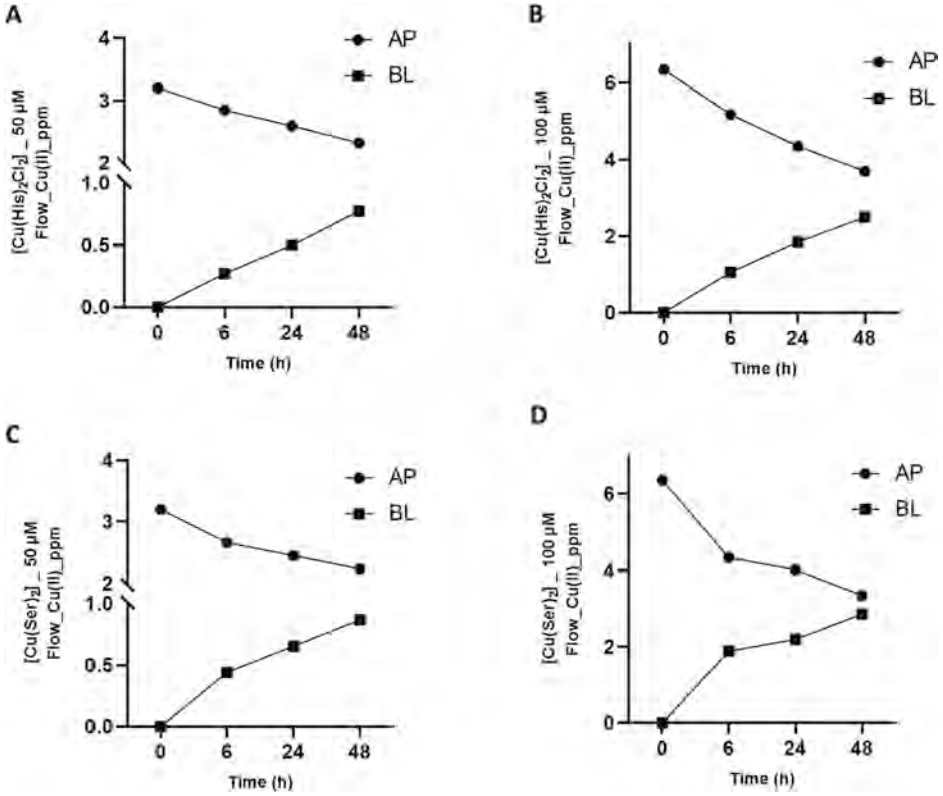

Fig. 3. Time course of copper(II) flow through the apical (AP) to the basolateral (BL) compartments. After differentiation, cells were exposed to $[Cu(His)_2Cl_2]$ (A, B) or $[Cu(Ser)_2]$ (C, D) at concentrations of 50 μM (A, C) or 100 μM (B, D) for 6, 24 and 48 h. Data are means ± SD of $n = 3$ independent experiments performed in duplicate.

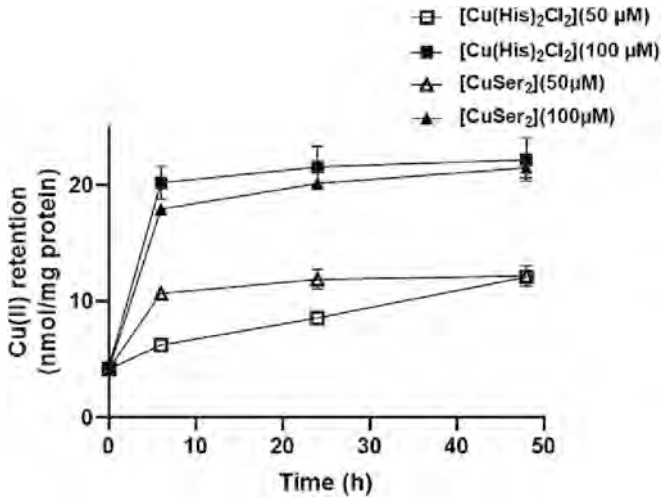

Fig. 4. Time course of copper(II) retention (nmol/mg protein) in differentiated Caco-2 cells exposed to $[Cu(His)_2Cl_2]$ or $[Cu(Ser)_2]$ at concentrations of 50 μM or 100 μM for 6, 24 and 48 h. Data are mean ± SD of $n = 3$ independent experiments in duplicate.

differentiated Caco-2 cells increased in a time-dependent manner on BL sides while they decreased on AP compartments. Worth to note, the relative percentage of copper(II) complexes were significantly different in the time for each compartment. Whereas, no significant differences between the percentage of copper(II) were found in cells (2.3–2.7%). The highest percentages in the BL compartments were found after 48 h of treatment of cells with 100 μM of $[Cu(His)_2Cl_2]$ or $[Cu(Ser)_2]$ reaching up to 39.4 ± 0.2% and 44.9 ± 0.8% respectively (Fig. 5B, D). These results are agreement with the capacity of absorption of human gastrointestinal system estimated in 30–40% of ingested copper from the typical diets [40].

However, these could also be related to the loss of the integrity of the cellular monolayers demonstrated by the decrease in TEER (Fig. 2B, D) and in the case of $[Cu(Ser)_2]$ by the moderate cytotoxic effect observed on cellular viability (Fig. 1 B). These results support the novel concept of orally administered copper(II) complexes. During the first 6 h of treatment (Fig. 5), the percentages of copper(II) found in BL compartment from the total added as complexes were in the range of 8.5 ± 0.2% (50 μM $[Cu(His)_2Cl_2]$) to 29.7 ± 0.8% (100 μM $[Cu(Ser)_2]$).

These results are important considering that the $[Cu(His)_2Cl_2]$ and $[Cu(Ser)_2]$ complexes will be properly formulated for their release in the region of the small intestine with pH 7 and that the time of intestinal transit from pylorus to the colon is estimated at a maximum of 4.5 h [35]. Another valuable result is the fact that there is no significant retention of copper(II) at the level of intestinal cells suggesting a good absorption of the administered copper(II) complexes (Fig. 5).

Furthermore, the results shown in Fig. 2-5 indicated that these copper(II) complexes could be using the paracellular route for their passage across the intestinal epithelium. Actually, the entrance by paracellular pathway of hydrophilic molecules with low molecular weight, like $[Cu(His)_2Cl_2]$ and $[Cu(Ser)_2]$, was previously described [32]. Moreover, considering that tight junctions restrict the paracellular transport of drugs [32], the design of a pharmaceutical formulation to enhance the transit of complexes by this route will be required.

3.4. Effects of copper(II) complexes on the expression of metal transporters and metallothionein

The transport pathway of substances across the intestinal epithelium may occur through different routes [25,39]. Free copper ions can

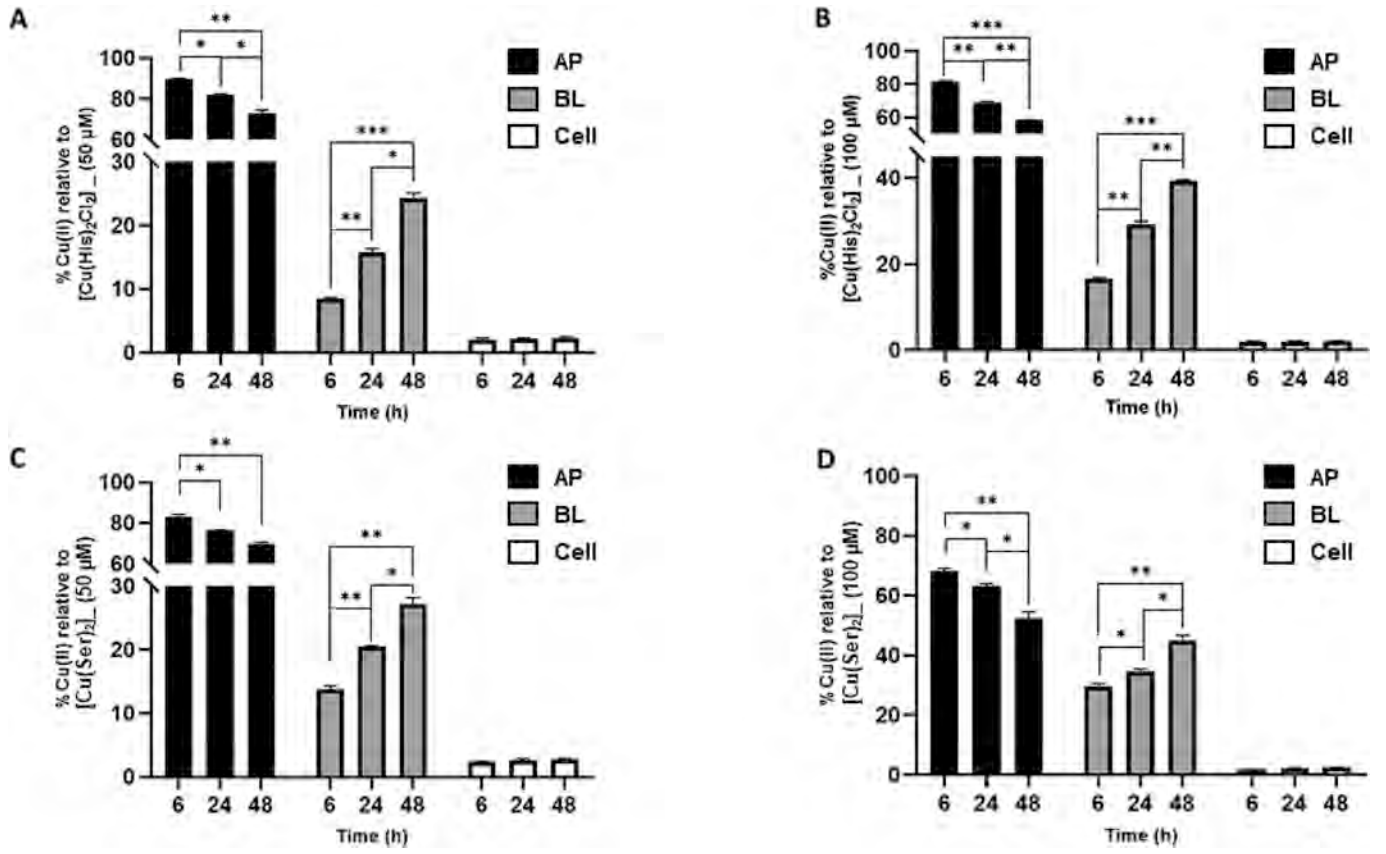

Fig. 5. Percentages of copper(II) from the corresponding initial concentration of copper complexes on apical (AP) and basolateral (BL) compartments, and on differentiated Caco-2 cells exposed to (A, B) $[Cu(His)_2Cl_2]$ or (C, D) $[Cu(Ser)_2]$ at concentrations of 50 µM (A, C) or 100 µM (B, D) for 6 h, 24 h and 48 h. The concentrations of Cu(II) were determined by ICP-OES. Data (mean ± SD) are expressed as percentages related to each concentration of copper(II) complexes used in the experiments ($n = 3$; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$; two-way ANOVA multiple comparisons).

generate free radicals via Fenton and Haber-Weiss reactions [5,35] and its homoeostasis in biological systems is controlled by copper transport proteins [7,41]. It was then of interest to evaluate whether $[Cu(His)_2Cl_2]$ or $[Cu(Ser)_2]$ promote changes in mRNA levels of copper transporters. The results shown in Figs. 4 and 5 suggested copper-transport processes in differentiated Caco-2 cells.

Fig. 6 A and B shows that the treatment with copper(II) complexes decreases significantly the relative *CTR1* and *DMT1* transcript levels. *CTR1* is a major transporter of cuprous (Cu^{+}) ions across the apical membrane of the intestinal cells [6,7], whereas *DMT1* could be involved in the uptake of divalent ions like cupric (Cu^{2+}) ions [41]. It was previously shown that the intra and extracellular copper amounts could modify the gene expression of both copper transporter [6,41]. It was also found that the increase of copper concentration in the lumen reduces the absorption of copper through downregulation of *CTR1* expression in enterocytes [10]. Similarly, reduced mRNA levels of *DMT1*, when Caco-2 cells were exposed to a high-copper environment, were observed elsewhere [42,43]. It is widely accepted that regulating the expression of these transporters contributes to cell survival and to maintaining copper levels in the organism [41].

In line with the previous observation, the RT-qPCR results reported here showed that the treatment with copper(II) complexes, at both

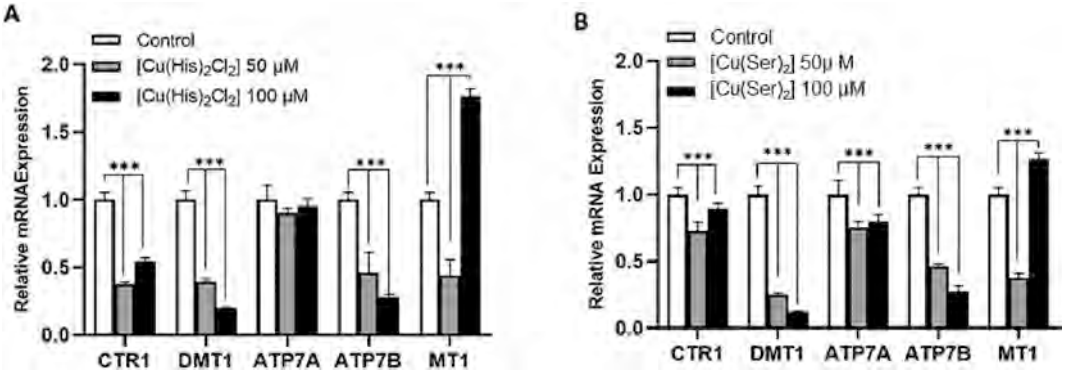

Fig. 6. The relative gene expression of copper transporters *CTR1*, *DMT1*, *ATP7A* and *ATP7B*, and of metallothionein (*MT*) in differentiated Caco-2 cells treated with: A) $[Cu(His)_2Cl_2]$ or B) $[Cu(Ser)_2]$ at concentrations of 50 or 100 µM. The gene expression data evaluated by RT-qPCR. are mean ± SEM of three independent experiments in duplicate ($n = 3$; $^{***}P < 0.001$; two-way ANOVA multiple comparisons).

concentrations, decreased the relative *ATP7A* and *ATP7B* transcript levels in differentiated Caco-2 cells compared to untreated control cells. Both ATPases participate in the transfer of copper across the BL membrane into the circulation [6,44]. The under-expression of the relative mRNA levels of these four major copper transporters suggests that the copper(II) found in the BL compartments (Figs. 3 and 5) might not originate from the copper trafficking machinery and supports the use of paracellular pathway by $[Cu(His)_2Cl_2]$ and $[Cu(Ser)_2]$ complexes. It cannot be excluded that these results are also related to the increase of intracellular copper observed (Fig. 4), suggesting that differentiated Caco-2 cells can modulate their uptake of copper, thus preventing further cell damage.

On the other hand, a higher expression of *MT* transcripts was observed in cells treated with 100 μM of $[Cu(His)_2Cl_2]$ or of $[Cu(Ser)_2]$. It is well known that the gene expression of *MT* is closely related to the metal detoxification [11,45]. Various copper chaperones are involved in copper delivery to its specific target [7] whereas others, such as *MT*, are involved in maintaining the amount of intracellular copper relatively constant [46]. *MT* diminishes the amount of copper ions available for delivery to the portal vein by binding the copper irreversibly as one of the mechanisms in which the cell is protected from copper toxicity [44]. This opposite responses in *MT* gene expression could be easily correlated with the differences in moderate cytotoxicity of $[Cu(His)_2Cl_2]$ and $[Cu(Ser)_2]$ at 100 μM shown in Fig. 1. Differently, the treatment with 50 μM of copper(II) complexes significantly decreased the relative *MT* transcript level of mRNA in differentiated Caco-2 cells.

3.5. Effects of copper(II) complexes on the COX activity

Cytochrome c oxidase (COX or complex IV) is the terminal enzyme of the electron transport chain. Its catalytic activity requires the insertion of two copper sites [47]. The availability of copper may be directly linked to the electron transfer chain and to the energy demands in the aerobics organisms, through the la modulation of the assembly of COX protein. It was shown that copper deficiency reduces the expression and the activity of complex IV [48], but not the other respiratory complexes [49]. More recent studies showed that the metalation of COX depends on the levels of a copper complex with a yet-unidentified ligand stored in the mitochondrial matrix [50]. Hence, it was of interest to evaluate whether the copper(II) complexes here reported can increase the activity of the COX. After the treatment of Caco-2 cells with $[Cu(His)_2Cl_2]$ and $[Cu(Ser)_2]$ (10–100 μM) for 24 h, the COX activity was determined by spectrophotometry following the decrease of absorbance at 550 nm. Fig. 7 shows that exposure of cells to copper(II) complexes at concentration higher than 50 μM induced an increase in COX activity. The enzyme activity was raised about 2-fold when cells were treated with 50 μM of $[Cu(His)_2Cl_2]$ and $[Cu(Ser)_2]$ and about 3-fold at 100 μM of these complexes. It appears that the $[Cu(Ser)_2]$ complex induced more change in enzymatic activity compared to $[Cu(His)_2Cl_2]$. These results could be related to the fact that bidentate complexes like $[Cu(Ser)_2]$ can exchange copper more easily than tridentate complexes like $[Cu(His)_2Cl_2]$. Moreover, they also fit well the previous studies showing that non-cytotoxic copper treatment was able to improve mitochondrial function by stimulating the biogenesis of COX and of the complex IV assembly in erythropoietin cell lines K562 or in mutant mouse deficient in COA6 cytochrome assembly factor [51,52].

The decrease in COX activity has been shown in copper-deficiency diseases such as Menkes disease and hypertrophic cardioencephalomyopathy [15,16]. Consequently, the treatment with copper supplements could markedly improve the conditions of these patients [14,17]. Therefore, the capacity of $[Cu(His)_2Cl_2]$ and of $[Cu(Ser)_2]$ at 50–100 μM to rescue the activity of COX clearly supports the possible use of these complexes in the therapy of copper deficiency diseases. However, a further report will be aimed to the proper gastroprotective formulation of the describe copper complexes. Also, more fundamental research is still needed to understand how these copper(II) complexes may impact on mitochondrial copper machinery.

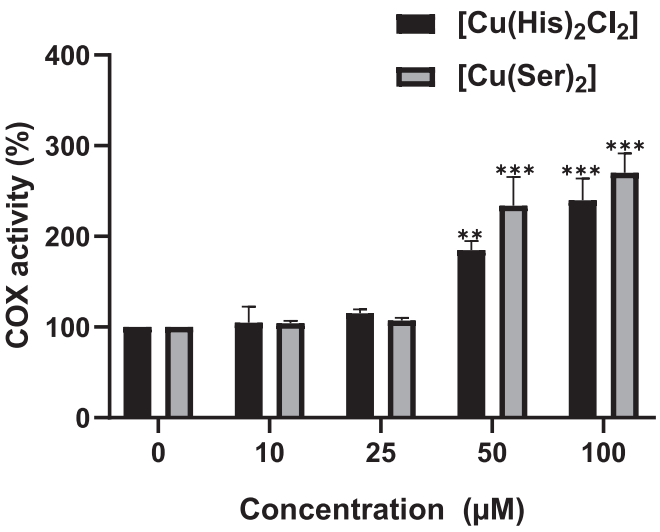

Fig. 7. Effect of copper(II) complexes on Cytochrome c oxidase (COX) activity in differentiated Caco-2 cells treated with $[Cu(His)_2Cl_2]$ or $[Cu(Ser)_2]$ at concentrations up to 100 μM. Data are mean ± SD and expressed the percentages related to COX activity of untreated (control) cultures ($n = 3$; $^{**}P < 0.01$, $^{***}P < 0.001$; two-way ANOVA multiple comparisons).

4. Conclusion

The data here describe show a good biocompatibility of $[Cu(His)_2Cl_2]$ and $[Cu(Ser)_2]$ complexes at concentrations up to 100 μM. The copper(II) complexes were less cytotoxic than $CuCl_2$ at similar concentrations. The exposure of cells at biocompatible concentrations of copper(II) complexes did not induce significant changes in TEER for the first 6 h of treatment. These results suggest that $[Cu(His)_2Cl_2]$ and $[Cu(Ser)_2]$ would not affect the intestinal epithelium during their intestinal transit (same duration of 4.5–6 h). Additionally, the amount of $[Cu(His)_2Cl_2]$ and $[Cu(Ser)_2]$ complexes increased in a time-dependent manner on BL sides while decreased on AP compartments suggesting the use of a paracellular route by the copper(II) complexes. These results are particularly important for the future development of a pharmaceutical formulation designed to enhance the transit of copper complexes through the tight junctions (that restrict the paracellular transport of drugs). No significant differences in intracellular copper(II) were observed between 6 h and 48 h. The $[Cu(His)_2Cl_2]$ and $[Cu(Ser)_2]$ reduced the transcript levels of the four major copper transporters *CTR1*, *DMT1*, *ATP7A*, and *ATP7B* after 6 h. The increase in *MT* transcript levels could be correlated with the moderate cytotoxicity shown by $[Cu(His)_2Cl_2]$ and $[Cu(Ser)_2]$ at 100 μM. Finally, the exposure of cells to copper(II) complexes at concentrations higher than 50 μM induced an increase in COX activity. Indeed, the rescue of COX activity is particularly useful as approach for the use of these complexes as therapeutic agents to treat copper deficiency diseases such as Menkes disease and hypertrophic cardioencephalomyopathy.

Declaration of Competing Interest

The authors declare that they have no competing interests.

Data availability

Data will be made available on request.

Acknowledgments

The investigation was supported by the Natural Sciences and Engineering Research Council (NSERC) of Canada to MAM (Grant No. 06919) and to BA (Grant No 06651) and by the "Fondation Courtois" (QC, Canada) to MAM. BA holds an Institutional Research Chair in Cancer Prevention and Treatment. MGP was granted a studentship from CERMO-FC. NGS is supported by a doctoral Fellowship from the Fonds de recherche du Québec - Nature et technologies (FRQNT).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jinorgbio.2023.112334.

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