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Complete List of Authors:	Nunes, Patrique; Universidade de Lisboa, Centro de Química Estrutural, Instituto Superior Técnico; Instituto Superior Técnico, Departamento de Engenharia Química; Universidade de Lisboa Centro de Química Estrutural Correia, Isabel; Universidade de Lisboa, Centro de Química Estrutural, Instituto Superior Técnico; Universidade de Lisboa Departamento de Engenharia Química; Universidade de Lisboa Centro de Química Estrutural Marques, Fernanda; Universidade de Lisboa, Instituto Superior Técnico; Universidade de Lisboa Departamento de Engenharia e Ciências Nucleares; Centro de Ciências e Tecnologias Nucleares Matos, António; Centro de Investigação Interdisciplinar Egas Moniz Correia dos Santos, Margarida; Universidade de Lisboa, Centro de Quimica Estrutural, Departamento de Engenharia Química, Instituto Superior Técnico; Universidade de Lisboa Departamento de Engenharia Química; Universidade de Lisboa Centro de Química Estrutural Azevedo, Cristina; Universidade de Lisboa, Centro de Quimica Estrutural, Departamento de Engenharia Química, Instituto Superior Técnico; Universidade de Lisboa Departamento de Engenharia Química; Universidade de Lisboa Centro de Química Estrutural Capelo-Martínez, José-Luis; Universidade Nova de Lisboa, LAVQ, REQUIMTE, Departamento de Química, Faculdade de Ciências e Tecnologia; Universidade Nova de Lisboa, PROTEOMASS Scientific Society, Santos, Hugo; Universidade Nova de Lisboa, LAVQ, REQUIMTE, Departamento de Química, Faculdade de Ciências e Tecnologia, ; Universidade Nova de Lisboa, PROTEOMASS Scientific Society Gama, Sofia; University of Białystok, Faculty of Chemistry Pinheiro, Teresa; Universidade de Lisboa, Institute for Bioengineering and Biosciences and Departamento de Engenharia e Ciências Nucleares, Instituto Superior Técnico, ; Universidade de Lisboa Departamento de Engenharia e Ciências Nucleares; University of Lisbon Institute for Bioengineering and Biosciences Cavaco, Isabel; Universidade do Algarve - Campus de Gambelas; Universidade de Lisboa Centro de Química Estrutural Pessoa, João; Universidade de Lisboa, Centro Química Estrutural; Universidade de Lisboa Instituto Superior Técnico; Universidade de Lisboa Departamento de Engenharia Química; Universidade de Lisboa Centro de Química Estrutural

Copper complexes with 1,10-phenanthroline derivatives: underlying factors affecting their cytotoxicity

Patrique Nunes[a], Isabel Correia*[a], Fernanda Marques[b], António Pedro Matos[c], Margarida M.C. dos Santos[a], Cristina G. Azevedo[a], José-Luis Capelo,[d,e] Hugo M. Santos,[d,e] Sofia Gama[f], Teresa Pinheiro[g], Isabel Cavaco[a,h] João Costa Pessoa* [a]

Patrique Nunes, Dr. Isabel Correia, Prof. M. M. C. dos Santos, Prof. C. G. Azevedo, Prof. J. C. Pessoa

[a] Centro de Química Estrutural and Departamento de Engenharia Química, Instituto Superior Técnico, Universidade de Lisboa, Av Rovisco Pais, 1049-001 Lisboa, Portugal.

Dr. F. Marques
[b] Centro de Ciências e Tecnologias Nucleares and Departamento de Ciências e Engenharia Nucleares, Instituto Superior Técnico, Universidade de Lisboa, Estrada Nacional 10, 2695-066 Bobadela LRS, Portugal.

Dr. A.P. Marques
[c] Centro de Investigação Interdisciplinar Egas Moniz, Campus Universitário, Quinta da Granja, Monte de Caparica, 2829-511 Caparica, Portugal.

Prof. J. L. Capelo, Dr. H. M. Santos
[d] LAVQ, REQUIMTE, Departamento de Química, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa, 2829-516 Caparica (Portugal).
[e] PROTEOMASS Scientific Society, Madan Park, Rua dos Inventores, 2825-152 Caparica (Portugal).

Dr. S. Gama
[f] Department of Analytical Chemistry, Faculty of Chemistry, University of Białystok, ul. Ciołkowskiego 1K, 15-245 Białystok, Poland.

Dr. T. Pinheiro
[g] Institute for Bioengineering and Biosciences and Departamento de Engenharia e Ciências Nucleares, Instituto Superior Técnico, Universidade de Lisboa, Av. Rovisco Pais 1, 1049-001 Lisboa, Portugal.

Prof. I. Cavaco
[h] Departamento de Química e Farmácia, Universidade do Algarve, Campus de Gambelas, 8005-139 Faro, Portugal

*Corresponding authors:

icorreia@tecnico.ulisboa.pt; joao.pessoa@tecnico.ulisboa.pt

ABSTRACT

The interpretation of *in vitro* cytotoxicity data of Cu(II)-1,10-phenanthroline (phen) complexes normally does not take into account the speciation that complexes undergo in cell incubation media, and its implications in cellular uptake and mechanisms of action. We synthesize and test the activity of several distinct Cu(II)-phen compounds; up to 24 h of incubation the cytotoxic activity differs for the Cu-complexes and the corresponding free ligands, but for longer incubation times (*e.g.,* 72 h) all compounds display similar activity. Combining the use of several spectroscopic, spectrometric and electrochemical techniques, the speciation of Cu-phen compounds in cell incubation media is evaluated, indicating that the originally added complex almost totally decomposed, and that Cu(II) and phen are mainly bound to bovine serum albumin. Several methods are used to disclose relationships between structure, activity, speciation in incubation media, cellular uptake, distribution of Cu in cells and cytotoxicity. Contrary to what is reported in most studies, we conclude that interaction with cell components and cell death involves the separate action of Cu-ions and phen molecules, not [Cu(phen)$_n$] species. This conclusion should similarly apply to many other Cu-ligand systems reported to date.

Introduction

1,10-Phenanthroline (phen) is a bioactive molecule and a versatile building block, and as a ligand it yields complexes with interesting biological and physiological properties. [1] Phen and derivatives have been an extensively used class of ligands in medicinal inorganic chemistry, and metal complexes with phen ligands have also shown potential utility as fluorescent probes and characteristic features for DNA binding. [2] [3] [4] [5] [6] Copper, as a naturally occurring and essential element, has been considered attractive in medicinal practices; the biological properties of its complexes depend on its oxidation state, on the structure of the complex and on the nature of ligands.[7] On the other hand, inside cells Cu(II) is normally reduced to Cu(I), a process expected to proceed more efficiently in cancer cells, due to their anoxic character. [8] [9] Additionally, Cu ions stimulate the proliferation and migration of endothelial cells and are required in several angiogenic factors and in the formation of new blood vessels by tumor cells, which enable tumor growth, invasion and

metastasis. Noteworthy, it has also been shown, mostly in cell-free experiments, that copper chelation affects the action of many of these factors.[10]

The majority of Cu-compounds developed so far as prospective anticancer or/and as antimicrobial agents are mononuclear species,[11 12 13 14] and their suggested mode of action is miscellaneous but differs from that of cisplatin. Proposed mechanisms have included DNA interaction, mitochondrial impairment, proteosome inhibition and ROS generation.[12, 13, 15-17] A great variety of Cu-complexes has been tested as cytotoxic agents and showed antitumor activity in several *in vitro* tests and a few in *in vivo* experiments,[16, 18] namely 2,2′-bipyridine (bipy) and phen-containing complexes. [19 20 21 22 23 24] Remarkably, a Cu-complex containing 4,7-dimethyl-1,10-phenanthroline (Me_2phen) as co-ligand, $[Cu(Gly)(Me_2phen)]NO_3$, from the group of compounds designated as Casiopeinas, is in clinical trials.[25, 26] A few other Cu-compounds have also reached clinical trials. [9 27 28 29 30]

Complexes containing phen have been reported to bind DNA by intercalative and non-intercalative interactions, either as free ligands or included in metal complexes. [11 21 31 32] Overall, they have been mostly proposed as agents for binding, cleavage and oxidative modification of DNA [19 26 33 34 35 36 37 38 39 40 41] and many of them depict cytotoxic activity against several cancer and non-cancer cells. [11 42 43 44 45] DNA has often been suggested as one of the main targets for this class of Cu-complexes, [46] and several mechanisms have been suggested.[11 35 47] The activities of phen, $[Cu(phen)_2]^{2+}$ and $CuC1_2$ on Ehrlich ascites tumor cells, their membrane permeability, and DNA cleavage activity were compared in a relevant work by Byrnes and coworkers.[42] Both DNA damage and cytotoxicity were observed with cells treated with $[Cu(phen)_2]^{2+}$, possibly due to multiple mechanisms of oxidative damage. In contrast to $[Cu(phen)_2]^{2+}$, phen alone depicted no double strand and very modest levels of DNA single strand breaks. [42] However, for mechanisms assuming DNA as main target, Cu-phen species must reach the nucleus of cells, which is questionable to occur. In fact, most studies of nuclease activity were done in cell-free conditions, with the Cu-phen complexes in direct contact with solutions containing DNA molecules. [41]

It has become clear that Cu-complexes can act by different mechanisms other than DNA binding; [18 48 49 50] namely, superoxide dismutase (SOD) mimetic activity has been attributed to some complexes as well as the ability to target intracellular redox processes and modulate ROS. [14, 32, 51-55] Topoisomerase and proteosome are also probable targets of Cu-complexes. [15 16] Proteosome inhibition or interactions with proteins involved in the cell cycle have been

reported, [15, 49] e.g. with tubulin, or in the cytoskeleton formation, such as integrin, or those present in the extracellular medium, like fibronectin. [56] Cu-complexes show matrix metalloproteinase inhibition properties [57] and the generation of ROS may also disrupt enzyme pathways and induce changes in structural proteins. [58] However, the underlying mechanisms affecting the anticancer properties of most Cu-phen complexes remain generally unknown or, at least, incompletely explored. [58, 59]

In most experiments where the Cu-complexes were added to cell incubation media, the possibility of their decomposition and speciation before cellular uptake was normally not evaluated. To expand our understanding on the mode of action of these biological relevant compounds, three Cu(II) complexes with phen derivatives are considered herein: $[Cu(phen)_2(H_2O)](NO_3)_2$ (**1**),[60] $[Cu(Me_2phen)_2(NO_3)](NO_3)$ (**2**)[44] and $[Cu(amphen)_2(H_2O)](NO_3)_2$ (**3**) (amphen = 5-amino-1,10-phenanthroline). To clarify if cytotoxicity of Cu(II)-complexes with phen and related ligands is solely due to the free polypyridyl ligand or if there are other effects (*e.g.* synergistic), we investigated the stability and speciation of **1** in cell incubation medium, and carried out studies with A2780 cell lines at several incubation times, as well as cellular uptake/distribution determinations of Cu. The anticancer properties of these Cu-phen complexes, and how differences in the ligands can affect their activity are also discussed based on data from mass spectrometry and micro-analytical approaches such as electron microscopy. The rational to select the A2780 cells to evaluate uptake and cellular distribution by inductively coupled plasma mass spectrometry (ICP-MS), and the ultrastructural changes induced by Cu-complexes by transmission electron microscopy (TEM), was to allow comparison of our results with other related metal based complexes previously synthesized in our group. [19, 61, 62]

We demonstrate in this study that for the correct understanding of their mechanisms of action the speciation of Cu-complexes in incubation media, determining which species are present, must be evaluated. To do this it is important to clarify the binding of each of the several Cu- and phen-species present to bovine serum albumin (BSA), the most important protein present in cell incubation media. The determination of which species form in the Cu-phen-BSA system and of the corresponding binding constants is required, this being also one of the objectives of this work.

Results and discussion

Characterization

The three Cu(II)-complexes were synthesized by reaction of $Cu(NO_3)_2$ with 1,10-phenanthroline and its 5-amino and 4,7-dimethyl derivatives. The complexes were characterized by elemental analysis and spectroscopic techniques (see Supporting Information SI-1). The molecular structure of $[Cu(phen)_2(H_2O)](NO_3)_2$ (**1**) was determined by single-crystal X-ray diffraction (SC-XRD), and the structural formula is schematically shown in Figure 1. [60] [63] The crystal structure of $[Cu(Me_2phen)_2(NO_3)]NO_3$ (**2**) was also previously determined. [44] A few other related complexes were also structurally characterized, namely $[Cu(phen)_3](ClO_4)_2$, [64] $[Cu(phen)_2(X)]C(CN)_3$ (X = Cl, Br, MeCOO), [65] several $[Cu(phen)_2(OOCMe)](X)_2$, (X = NO_3, ClO_4, BF_4) with fluxional CuN_4O_2 structures, [66] [67] $[Cu(phendione)_2(H_2O)(OClO_3)]ClO_4$ (phendione = 1,10-phenathroline-5,6-dione) [68] and $[Cu(bipy)_2(NO_3)]NO_3$. [69] We expect complex **3** in the solid state to have either a NO_3^- ion coordinated as in **2**, or a water molecule as in **1**. Compounds **1-3** show cytotoxic activity (Table 1) and show a d-d transition in the visible range at 680-710 nm and EPR spectra at 100 K that could be simulated assuming axial symmetry (see Figure S1 and Table 2 for details). To the best of our knowledge complex **3**, containing amphen, is new.

Hereafter, when globally addressing the phen compounds, not one in particular, we will designate them as Xphen; similarly we will represent complexes **1**-**3** in solution either as $[Cu(Xphen)_2]^{2+}$ or as $[Cu(Xphen)_2]$.

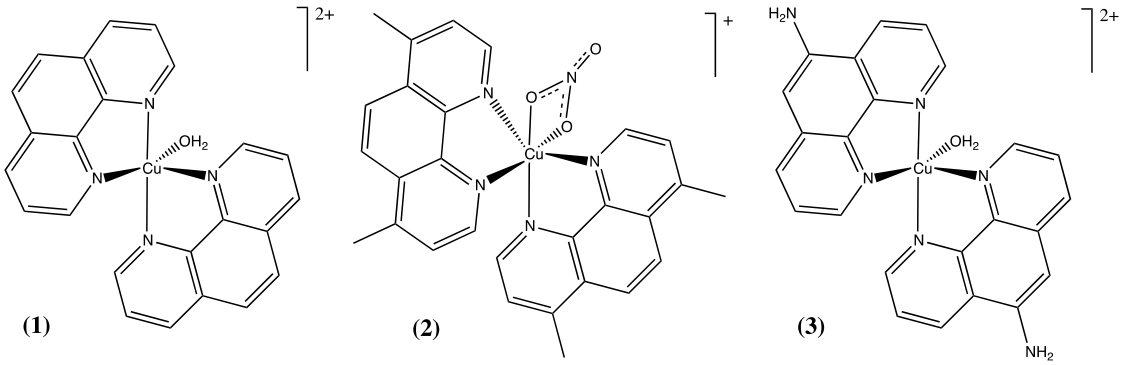


Figure 1. Molecular structures expected for Cu-complexes **1**, **2** and **3**. The molecular structures of **1** and **2** were previously determined by SC-XRD. [44] [60]

Cytotoxicity

The cytotoxic activity of **1-3** against the A2780 cells, as given by their IC_{50} values, is presented in Figure 2 and Table 1. Noteworthy, while upon 3 h of incubation, phen and amphen present no relevant cytotoxicity (IC_{50} >100 μM), Me_2phen depicts moderate activity (IC_{50}, 37±8 μM). It is clear that the IC_{50} values of complexes and corresponding ligands depend on the incubation time. At 3 h, almost all differ, but at 48 h and 72 h they are all approximately equal (except bipy) and most of them are in the sub-micromolar range. Noteworthy, at 72 h, all IC_{50} values are also equal to the corresponding phen, amphen and Me_2phen complexes of Zn(II) [2] and of Fe(III), [70] tested in the same cell line. The IC_{50} values at 3 and 24 h for **1-3** differ from those of the respective free ligands and these differences cannot be solely attributed to the presence of two polypyridine molecules in each complex. Cisplatin, used for comparison (Table 1), has a different cytotoxic profile, in particular for exposure times longer than 3 h. Enhancement of activity of $[Cu(phen)_2]$ compared to the respective free ligand was reported before with different cancer cell systems. [22 71 72]

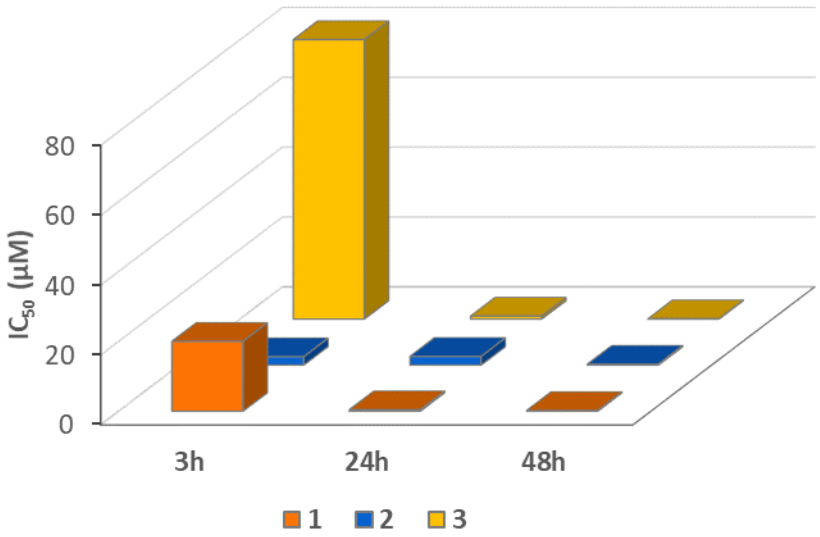


Figure 2. Effect of exposure time on the cytotoxic activity of the Cu(II)-complexes **1-3** in the A2780 cells upon 3, 24 and 48 h of incubation.

Table 1. Cytotoxic activity of the compounds and of the reference drug cisplatin in the A2780 cells as measured at several incubation times. Free ligands and corresponding complexes are compared. Data are mean±SD of two or more independent experiments done with at least 6 replicates per condition.

IC_{50} / μM A2780	3 h	24 h	48 h	72 h
bipy	> 100	> 100	52 ± 25	---
$[Cu(bipy)_2]$	> 100	3.8 ± 2.2	0.27 ± 0.12	---
phen	> 100	> 100	5.8 ± 2.3	1.7 ± 0.6
$[Cu(phen)_2]$ (**1**)	20.9 ± 4.6	0.4 ± 0.15	0.19 ± 0.07	0.08 ± 0.03
Me_2phen	37 ± 8	14 ± 5	1.4 ± 0.4	0.19 ± 0.13
$[Cu(Me_2phen)_2]$ (**2**)	2.4 ± 1.3	2.5 ± 1.0	0.26 ± 0.04	0.09 ± 0.02
amphen	> 100	41 ± 29	1.8 ± 0.6	0.26 ± 0.11
$[Cu(amphen)_2]$ (**3**)	80 ± 32	0.87 ± 0.27	0.08 ± 0.03	0.05 ± 0.03
Cisplatin	> 100	43 ± 10	14 ± 5.5	1.9 ± 0.2

Uptake studies by ICP-MS

ICP-MS was used to provide quantitative information on complexes' subcellular localization. [73] Figure 3 depicts the results obtained for the amount of Cu found in the cytosol, membranes, nucleus and cytoskeletal fractions of A2780 cells in terms of ng Cu/ 10^6 A2780 cells, as well as in % of total Cu in each cell fraction. In terms of % of the total cellular uptake, the complexes present a similar profile and major retention in the cytoskeletal fraction.

We emphasize that at 3 h incubation time, the amount of Cu uptaken is not correlated with the total Cu amount in the cell incubation media, but depends on the ligand present. It is clear in Figure 3A that although the total amount in the cell incubation media was 21 μM (for **1**) and 80 μM (for **3**), the amount of Cu uptaken was much higher in the case of complex **1** than of **3**. Moreover, the amounts of Cu uptaken in the cases of **2** and **3** are similar, however their concentrations in the cell incubation media were quite different: 2.4 μM (for **2**) and 80 μM (for **3**). It is also interesting to compare $[Cu(phen)_2]$ (**1**) with the related ternary [Cu(sal-Gly)(phen)] complex (sal-Gly = N-salicylideneglycinate),

which was previously reported by some of us. [19] The difference between **1** and [Cu(sal-Gly)(phen)] is the presence of one Sal-Gly ligand and only one phen ligand. In the A2780 cells, the cytotoxicity of both complexes differs for the short and long incubation times; in fact for 3 h, the IC_{50} values are 2.4 μM *vs* 26 μM and for 72 h, 80 nM *vs* 3 μM for **1** and [Cu(sal-Gly)(phen)], respectively. The additional phen ligand, changes the activity of the complex, but in a way that cannot be easily anticipated. One of the striking differences is that upon uptake, the cellular distribution of Cu differs (Figure 3 and S2 in SI section) *i.e.*, upon incubation with **1**, Cu is mainly found in the cytoskeleton (45%), followed by the membranes (29 %), while upon incubation with [Cu(sal-Gly)(phen)] Cu is mainly found in the membranes (48 %) followed by the cytoskeleton (24 %).

Another important aspect is that the cellular uptake of Cu is not correlated with the cytotoxic activity. The uptake of Cu upon 3 h incubation with **2** and **3** is the same (62 ng Cu/10^6 cells), but **2** is much more active; in fact complex **2** has an IC_{50} much lower than **3** (2.4 *vs* 80 μM). The higher uptake of Cu with **1** (218 ng Cu/10^6 cells) did not result in higher activity; a similar comment can be made for [Cu(sal-Gly)(phen)], with a total Cu uptake of 221 ng Cu/10^6 cells.

The basal levels of copper in the A2780 cells was also quantified. For these cells, the total copper found by ICP-MS was 2.2 ng/million cells. The copper amount distributed in each cellular fraction was below the detection limit of the instrument used, therefore it could not be accurately measured.

For kinetically labile metal complexes the determination of lipophilicity requires the knowledge of formation constants of $[Cu(Xphen)]^{2+}$, $[Cu(Xphen)_2]^{2+}$ and $[Cu(Xphen)_3]^{2+}$, and of the partition coefficients of the free Xphen ligands,[74] and for positively charged metal complexes the values determined depend on the anion that is transferred to the organic phase with the $[Cu(Xphen)_n]^{2+}$ complexes. We determined very rough values for the distribution coefficients (*D*) of complexes **1-3** by the simple saturation shake flask method, obtaining 0.40, 1.1 and 0.54 for **1**, **2** and **3**, respectively. The uptake of complexes does not correlate with the *D* values for complexes **1-3**, as e.g. **1** is more hydrophilic but is the one that shows the highest uptake. The sequence of *D* values of the Cu-Xphen complexes also differs from those of the free ligands as obtained using *Molinspiration*: [75] amphen ($10^{1.61}$) < phen ($10^{1.90}$) < Me_2phen ($10^{3.26}$).

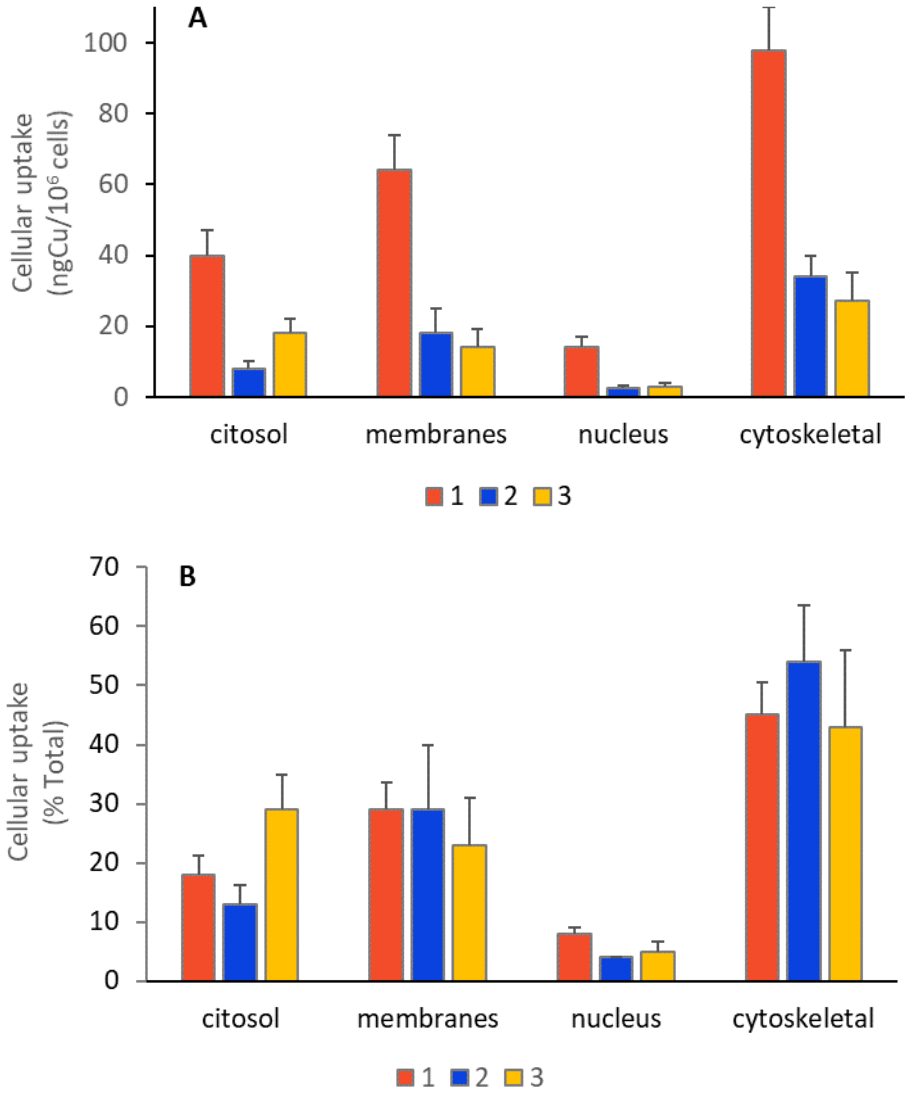


Figure 3. Uptake of Cu obtained in A2780 cellular fractions as measured by ICP-MS after 3 h incubation. Cells were incubated with 21 μM of $[Cu(phen)_2]$ (**1**), 2.4 μM of $[Cu(Me_2phen)_2]$ (**2**) and 80 μM of $[Cu(amphen)_2]$ (**3**), concentrations corresponding to their IC_{50} values at 3 h. A) Copper content as ng Cu/10^6 A2780 cells; B) copper content as percentage of total Cu uptake. For complexes **2** and **3**, the amount of Cu found at the nucleus fraction is close to the limit of detection of the technique.

Based on these results and others by some of us with similar findings,[76] we can consider that different cellular targets may be involved for Cu-complexes. Important evidence among complexes **1-3** is the high amount of Cu found in the cytoskeleton, approximately 50% of total Cu uptake at 3 h incubation, regardless of the concentration used in the assays (which

was a concentration equivalent to the IC_{50} at 3 h incubation). This is a noteworthy result if we consider the central role of cytoskeletal proteins in tumor cell migration, metastasis and chemoresistance. [77] DNA has been considered a target for this type of compounds as they have intercalating properties; however, these uptake studies show that Cu ions do not easily reach the nuclear fraction.

Transmission electron microscopy (TEM)

Transmission electron microscopy (TEM) allowed the observation of the structural integrity of the cells as well as the effects down to cellular organelle level in the A2780 cells after treatment with **1** and **2** at a concentration equivalent to their IC_{50} values at 24 h. TEM studies confirm higher cytotoxic effects of **2** when compared to **1**. Cellular degenerative changes resulting in multinucleated cells with disruption of the nuclear membrane were seen for both complexes, but more frequently in **2**. Mitochondrial alterations and lipid droplets were also observed. Apoptotic blebs, although present in all treated samples, were also more evident in **2** (Figure 4). Some of these effects, were also described for the reference drug cisplatin, namely, the deterioration of the mitochondrial structure and appearance of apoptotic cells found in early and late stages of apoptosis, which seemed to be the major mechanism of cell death following exposure to cisplatin. [78] [79] However, the effects described for cisplatin vary according to the type of cancer cells and the drug concentration used. [80]

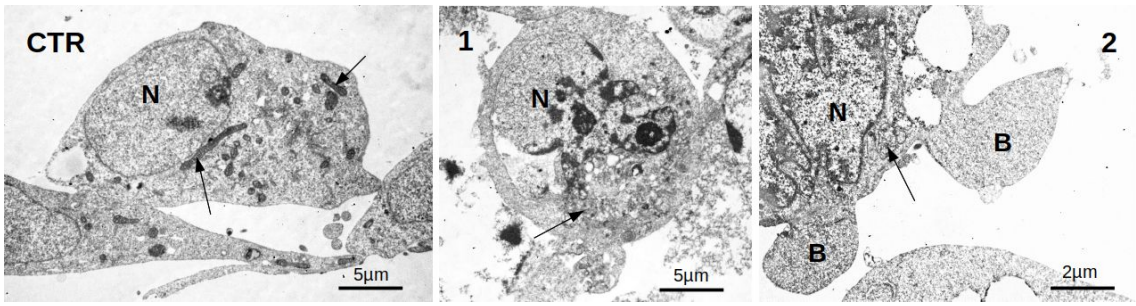


Figure 4. Thin section transmission electron microscopy images of A2780 cells treated for 24 h with $[Cu(phen)_2]$ (**1**) and $[Cu(Me_2phen)_2]$ (**2**) (see Experimental Section for details). CTR: control (no treatment); cells treated with **2** showing mitochondrial alterations and apoptotic blebs more evident than **1**. N: nucleus; B: apoptotic blebs; Arrows: mitochondria.

Cytotoxicity with varying % of FBS

It is important to highlight that, due to the presence of several Cu-binders, once the [Cu(Xphen)$_2$] complexes **1**, **2** or **3** are added to the incubation media, they will no longer be present in its original formulation.

In a typical incubation media for mammalian cells, as RPMI used here, there are several low molecular weight potential binders for metal ions, such as chloride (100-110 mM), glucose (10-25 mM), pyruvate (*ca*. 1 mM), amino acids (*ca*. 0.01 to 1 mM), vitamins and redox mediators (*ca*. 1 to 40 μM each). [81] The relevance of each of these binders depends on the metal ion considered and on the range of concentration of the metal complex being evaluated.

Regarding high molecular weight binders, albumins are the main compounds to consider. FBS is typically present in cell incubation media in the range 2 to 10 % and is the main source of albumin present in contact with cells. The FBS used in our cytotoxicity measurements contained ~26 g/L of BSA. When added in 10 % proportion, as recommended, this represents a significant amount of bovine serum albumin (ca. 40 μM).

The effect of BSA on the cytotoxic activity was evaluated by varying the amount of FBS (1-20 %) in the incubation medium (Figure 5). For this purpose, the complexes were evaluated at a concentration equivalent to their IC_{50} values in standard conditions (3 h exposure, medium containing 10% FBS). For each FBS concentration, a control (untreated cells) was included to evaluate any changes in the cellular viability induced by the different FBS amounts used. As seen in Figure 5, the activity of [Cu(Me$_2$phen)$_2$] (**2**) and [Cu(amphen)$_2$] (**3**) against the A2780 cells appears not to be much affected by the change of the % of FBS. In contrast, the activity of **1** increased with increasing % of FBS in culture medium. Data from Figure 5 suggest that, in the conditions used, only the cytotoxicity of complex **1** is altered in the presence of FBS.

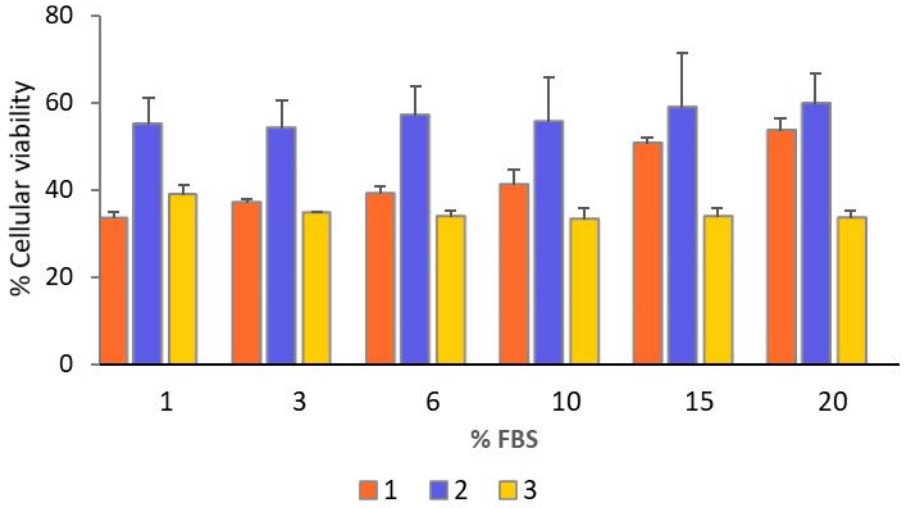


Figure 5. The effect of serum proteins content in the cell's incubation media on the cytotoxic activity of [Cu(phen)$_2$] (**1**, orange), [Cu(Me$_2$phen)$_2$] (**2**, purple) and [Cu(amphen)$_2$] (**3**, yellow), against the A2780 cells, as measured by the % of FBS added to the incubation medium. The complexes were evaluated at a concentration equivalent to their IC_{50} in standard conditions (3 h exposure, medium containing 10% FBS): **1** - 20 μM; **2** – 2.4 μM and **3** - 80 μM. The total concentrations of BSA, in the incubation media varies in the range ca. 4 - 80 μM, corresponding to 1 - 20 % FBS, respectively. Data are mean±SD of two independent experiments done with at least 6 replicates per condition.

One noteworthy result from these experiments (Figure 5) is the contrasting insensitivity of **2** and **3** to the variation of the FBS % when compared to **1**. This result may be rationalized by the lower concentration of **2** or the higher concentration of **3** used in these assays. In fact, the concentrations were 20 μM for **1**, 2.4 μM for **2** and 80 μM for **3**, corresponding to the IC_{50} values at 3 h incubation. Therefore, in the assays with different % of FBS, the total concentration of [Cu(Me$_2$phen)$_2$] is moderate to low and that of [Cu(amphen)$_2$] is in excess when compared to the albumin concentrations.

The importance of several potential binders present in the incubation media was also considered in section SI-3. After making several speciation calculations, taking the relevant concentrations and formation constants into account, we concluded that besides the phenanthrolines, the main potential binder of Cu(II) is BSA, particularly in the experiments with lower concentrations of Cu-phen complexes.

These assays show that the presence of BSA may have implications on cytotoxic activity of complexes. Therefore, we set out to evaluate the binding of BSA to [Cu(Xphen)$_2$] by several methodologies, firstly (a) qualitatively by confirming their binding by using mass spectrometric (Matrix-Assisted Laser Desorption/Ionization – Time of Flight mass spectrometry, MALDI-TOF MS), spectroscopic (EPR and circular dichroism) and electrochemical techniques, and then (b) quantitatively by using circular dichroism (CD) measurements. Most of this data is presented either at the end of this text, or in the Supporting Information.

Binding of Cu-complexes to BSA

MALDI-TOF mass spectrometric measurements. To evaluate the formation of Cu-BSA and Cu-phen-BSA species, several MALDI-TOF MS experiments were carried out. Samples were prepared with (i) [BSA]:[Cu^{2+}] molar ratios of 1:5, (ii) [BSA]:[Cu(Xphen)$_2$] of 1:2 and 1:3, (iii) [BSA]:[Xphen] of 1:3, iv) [BSA]:[Cu^{2+}]:[phen] of 1:1:1, 1:2:1 1:2:2 and 1:2:4, by mixing different volumes of stock solutions with buffer.

In the experiments with samples of BSA + Cu(II), the binding of the first Cu(II) atom to BSA (at the high affinity site, site I) probably corresponds to an increase in mass of 63.5 Da, as well as the binding of the second Cu(II) to BSA (at site II). However, further binding of Cu(II) ions forming [Cu_n(BSA)] with n>2 may each correspond to higher mass increases because water molecules may also be included in the Cu(II) coordination sphere. In the experiments with samples containing BSA, Cu(II) and Xphen, we expect that masses corresponding to Cu(II), Xphen and/or Cu(Xphen) might be found.

Some MS spectra are depicted in Supporting Information (Figures S3-S5) and Table S1 summarizes the most relevant results and the tentative assignments done. The increase in mass observed for the samples of BSA + copper salt, BSA + [Cu(Xphen)$_2$] complexes, BSA + Xphen and BSA + copper salt + Xphen, provided clear evidence of the binding of Cu(II) to BSA, as well as of Cu+Xphen species to BSA. Overall, we found evidence that suggests the binding of (a) at least four Cu(II) ions to BSA (samples of BSA:Cu(II) with 1:5 molar ratio), (b) of Cu+Xphen species to BSA (in several samples containing BSA, Cu(II) and Xphen) and (c) of [Cu_2(BSA)(phen)] (in several samples containing BSA, Cu(II) and phen).

EPR. EPR spectra of the complexes in cell incubation media (CM) in the presence and absence of FBS (10% v/v) were measured. Table 2 includes the spin Hamiltonian parameters determined by simulation (see section SI-5 and Fig. S6 for further details). The g_z and $|A_z|$ values determined for **1-3** in MeOH differ significantly from those in cell media containing or not FBS.

It is known that BSA has a high affinity site for Cu(II) and the spin Hamiltonian parameters for the [Cu^{II}(BSA)] species are: $g_x = g_y = 2.055$; $g_z = 2.177$; $|A_x|,|A_y| = 16\times10^{-4}$ cm^{-1}, $|A_z| = 217\times10^{-4}$ cm^{-1}.[82] The spin Hamiltonian parameters obtained for complexes **1-3** in the cell media containing or not containing FBS are quite similar, but they differ significantly from those measured for [Cu^{II}(BSA)] and for the complexes in MeOH. In the experiments corresponding to the data presented in Table 2 the concentration of BSA in the cell culture media + 10% FBS assays was *ca.* 40 μM, thus being around one order of magnitude lower than the total concentration of Cu-phen compound initially dissolved (*ca.* 500 μM; for significantly lower concentrations the EPR spectra would be too weak). On the other hand, the spin Hamiltonian parameters expected for the ternary BSA-Cu-phen species, which presumably may also form in these samples, are expected to be similar to those measured for HSA-Cu-phen complexes.[56] Therefore, we could not distinguish/detect significant amounts of Cu-BSA or Cu-BSA-phen species in the conditions used, but their formation cannot be ruled out (see also below).

EPR studies were also done with the ovarian cells. Two different assays were carried out (see experimental section): (i) A2780 cells were incubated with **1** or $Cu(NO_3)_2$ (1 mM) for 30 min and then the cellular suspensions and the incubation medium were separated and analysed by EPR; and (ii) A2780 cells were first fractionated in their components: membranes, cytosol, nucleus and cytoskeleton, and then, to each fraction controlled amounts of $Cu(NO_3)_2$ or [$Cu(phen)_2$] (**1**) were added, so that the $[Cu(II)]_{total} \approx 0.5$ mM.

In the first assay (Figure 6) it is very clear that the Cu(II)-species present in the incubation media somewhat differ. Thus, as expected, adding Cu(II) ions or [$Cu(phen)_2$] to cell incubation media yields different speciation of Cu-species. The corresponding spin Hamiltonian parameters are presented in Table 3. No EPR spectra could be measured with the fraction containing only the cell pellet, probably because most of the copper inside cells is present as Cu(I).

The spin Hamiltonian parameters measured for **1** in contact with the cell incubation medium also containing 10 % FBS ([BSA] ~40 μM), which was in contact with the A2780 cells for 30 min are g_z = 2.26, $|A_z|$ = 169.5×10^4 cm^{-1}. The hyperfine coupling constant differs by 3.5×10^4 cm^{-1} from those shown in Table 2 (g_z = 2.262, $|A_z|$ = 173×10^4 cm^{-1}), where the relative ratio **1**:BSA is half of that of the sample of the black spectrum in Figure 6. In both samples complex **1** is in high excess in relation to BSA, the main Cu-species present being [Cu_n(BSA)] and [$Cu(phen)_n$] (with n≥2); therefore, the higher the molar ratio **1**:BSA, the higher is the relative amount of [$Cu(phen)_n$] species present.

Table 2. Spin Hamiltonian parameters obtained for complexes **1-3** by simulation of the monomeric species observed. CM - cell culture media: CM+FBS – cell culture media + 10% (v/v) FBS.

	$g_{x,y}$	g_z	$\|A_x\|,\|A_y\|$ (×10^{-4} cm^{-1})	$\|A_z\|$ (×10^{-4} cm^{-1})	$g_z/\|A_z\|$ (×10^4 cm)
[$Cu(phen)_2(H_2O)$](NO_3)$_2$ (**1**)					
MeOH	2.074	2.296	21	153	149
CM	2.059	2.262	18	173	131
CM + FBS	2.058	2.262	19	173	131
[$Cu(Me_2phen)_2(NO_3)$](NO_3) (**2**)					
MeOH	2.074	2.292	24	150	150
CM	2.059	2.262	20	167	135
CM + FBS	2.060	2.264	23	166	136
[$Cu(amphen)_2(H_2O)$](NO_3)$_2$ (**3**)					
MeOH	2.074	2.296	21	153	150
CM	2.062	2.264	16	171	131
CM + FBS	2.060	2.265	18	169	134

Byrnes and coworkers [42] measured the EPR spectra of $CuCl_2$ and [$Cu(phen)_2$] complexes in contact with Ehrlich ascites tumor cells in PBS buffer. The pattern of both spectra resemble that of the grey spectrum depicted in Figure 6 for $Cu(NO_3)_2$. The authors stated that the spin Hamiltonian parameters are indistinguishable from those of Cu-histidine [$Cu(His)_2$] complexes (g_z = 2.220, $|A_z|$ = 183×10^4 cm^{-1}), and conclude that these Cu-His complexes

formed externally to the cells. These EPR parameters are indeed close to those measured in this work for $Cu(NO_3)_2$ added to incubation medium, but differ significantly from those we similarly obtained with complex **1**, either in contact with A2780 cells (Table 3, $g_z = 2.264$, $|A_z| = 169.5\times10^4$ cm^{-1}), or in contact with the incubation media containing FBS (Table 2, $g_z = 2.262$, $|A_z| = 173\times10^4$ cm^{-1}), or in aqueous solutions. [56] Probably the main reasons for the difference in the measured spin Hamiltonian parameters are (i) the presence of BSA (and other potential ligands) in our samples and (ii) differences in the concentrations of $[Cu(phen)_2]$ (**1**) added. However, it is clear that when $[Cu(Xphen)_2]$ complexes are added to the incubation media, the EPR spectra are not due to $[Cu(His)_2]$ complexes.

Table 3. Spin Hamiltonian parameters obtained from the EPR spectra depicted in Figure 4 (cell medium, CM) and Figure 6 (membrane, cytosol and nucleus fractions of A2780 cells).

Compound	Medium	Source	g_x,g_y	g_z	$\|A_x\|$,$\|A_y\|$ ($\times10^4$ cm^{-1})	$\|A_z\|$ ($\times10^4$ cm^{-1})	g_z / $\|A_z\|$
$Cu(NO_3)_2$	CM	Fig. 6	2.055	2.260	12.9	184.3	123
$[Cu(phen)_2]$	CM	Fig. 6	2.066	2.264	2.7	169.5	134
$Cu(NO_3)_2$	Membrane	Fig. 7A	2.054	2.265	12.9	182.8	124
$[Cu(phen)_2]$	Membrane	Fig. 7B	2.055	2.265	17.7	161.6	140
$Cu(NO_3)_2$	Cytosol	Fig. 7A	2.062	2.292	11.0	155.1	148
$[Cu(phen)_2]$	Cytosol	Fig. 7B	2.068	2.271	8.5	165.5	137
$Cu(NO_3)_2$	Nucleus	Fig. 7A	2.057	2.262	12.5	184.9	122
$[Cu(phen)_2]$	Nucleus	Fig. 7B	2.061	2.270	11.5	162.4	140

The EPR spectra measured in the second assay carried out by adding the Cu-compounds to the fractionated A2780 cells are shown in Figure 7 and Figures S7-S9 (Supporting Information). It was not feasible to introduce the cytoskeleton fraction in EPR tubes, so these fractions were discarded. It should be noted that there is no way of predicting if the amount of Cu(II) added is in excess or not to Cu(II)-binders present in cell fractions. The first relevant remarks are that the EPR spectra for all fractions containing $Cu(NO_3)_2$ or

[Cu(phen)$_2$] (**1**) significantly differ (see Figures S7-S9) and the spin Hamiltonian parameters, collected in Table 3, and particularly the $|A_z|$, have quite different values. This means that distinct Cu(II)-species were formed in the distinct fractions analysed. Comparing the spectra measured for Cu(NO$_3$)$_2$ (Figure 7A), all spectra somewhat differ, but those measured with the cytosol and nucleus fractions have some similarities, and the same global comment applies to the spectra measured for **1** depicted in Figure 7B.

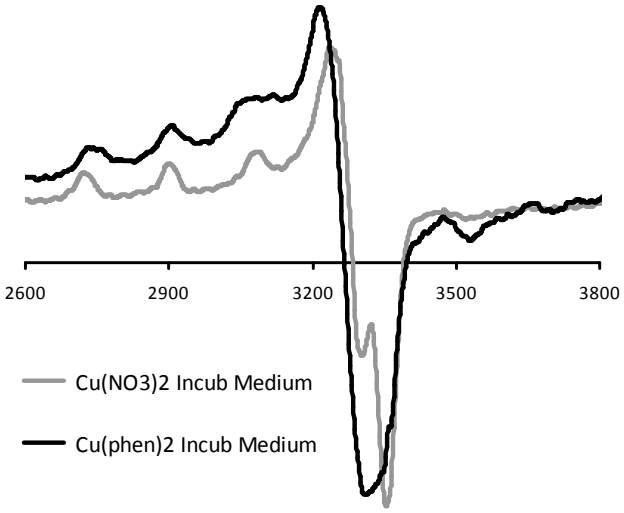
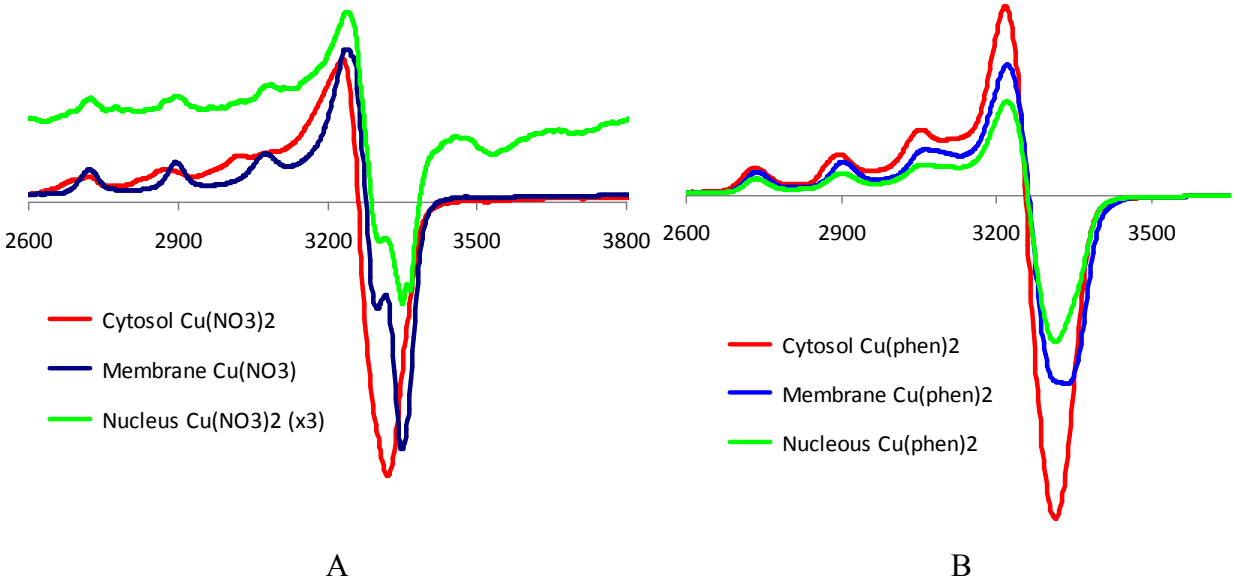


Figure 6. First derivative EPR spectra measured at ~100 K of samples of either [Cu(phen)$_2$] (**1**) or Cu(NO$_3$)$_2$ (1 mM) in cell incubation medium containing 10 % FBS, after contact for 30 min with A2780 cells.



A B

Figure 7. First derivative EPR spectra measured at ~100 K of samples of cell fractions of A2780 cells upon adding 0.5 mM solutions of either $Cu(NO_3)_2$ or $[Cu(phen)_2]$. The spectrum for $Cu(NO_3)_2$ in the nuclear fraction shown in A is multiplied by 3.

Determination of binding constants of Cu(II)- and Cu-phen species to BSA.

Cell incubation media (CM) are very complex solutions and contain many potential ligands for Cu(II). Bovine serum albumin is present at relatively high concentration (ca. 40 μM when 10 % FBS is added), and may bind Cu(II) ions. [33] [32] [56] [83] [84] [85] Moreover, from the experiments described above, and other EPR and circular dichroism measurements carried out (see below), it is clear that Cu-phen species may also bind to BSA. Considering the composition of the cell media used, [81] upon doing several modeling calculations (see section SI-3) we concluded that the relevant Cu(II)-binders are phen and BSA, and that all low molecular weight components do not contribute significantly to binding of Cu ions.

To determine the binding constants of Cu-phen species to BSA we carried out extensive electrochemical and circular dichroism measurements. These are described at the end of the Materials and Methods and in the SI section. Scheme 1 summarizes the processes taking place in solutions containing Cu(II), BSA and phen that are relevant in these systems.

For the quantitative determination of the relevant conditional binding constants, several CD spectra were recorded for the systems Cu-BSA and Cu-BSA-phen, at different concentrations (4×10^{-4} M ≤ [BSA] ≤ 6×10^{-4} M, 8×10^{-5} M ≤ [Cu] ≤ 3×10^{-3} M, 8×10^{-5} M ≤ [phen] ≤ 2×10^{-3} M) and molar ratios (BSA:Cu and BSA:Cu:phen of 1:n and 1:1:n and 1:2:n, with 0.2 ≤ n ≤ 6), in the wavelength range 350 ≤ λ ≤ 800 nm. The obtained data (*ca*. 70 spectra were selected) was processed using HypSpec [86] computer program, which uses a least-squares refinement procedure, to determine the log values of the conditional binding constants (β) for the species $[Cu_p(BSA)]$ and $[(Cu)_p(phen)_q(BSA)_r]$, according to the equilibria represented in equation 1. The obtained values of log β_{pqr} (with r=1) are reported in Table 4. In Figure 8 graphical representations of the different BSA-containing species formed are depicted.

$$p\ Cu^{2+} + q\ phen + r\ BSA \leftrightarrows (Cu)_p(phen)_q(BSA)_r \quad (1)$$

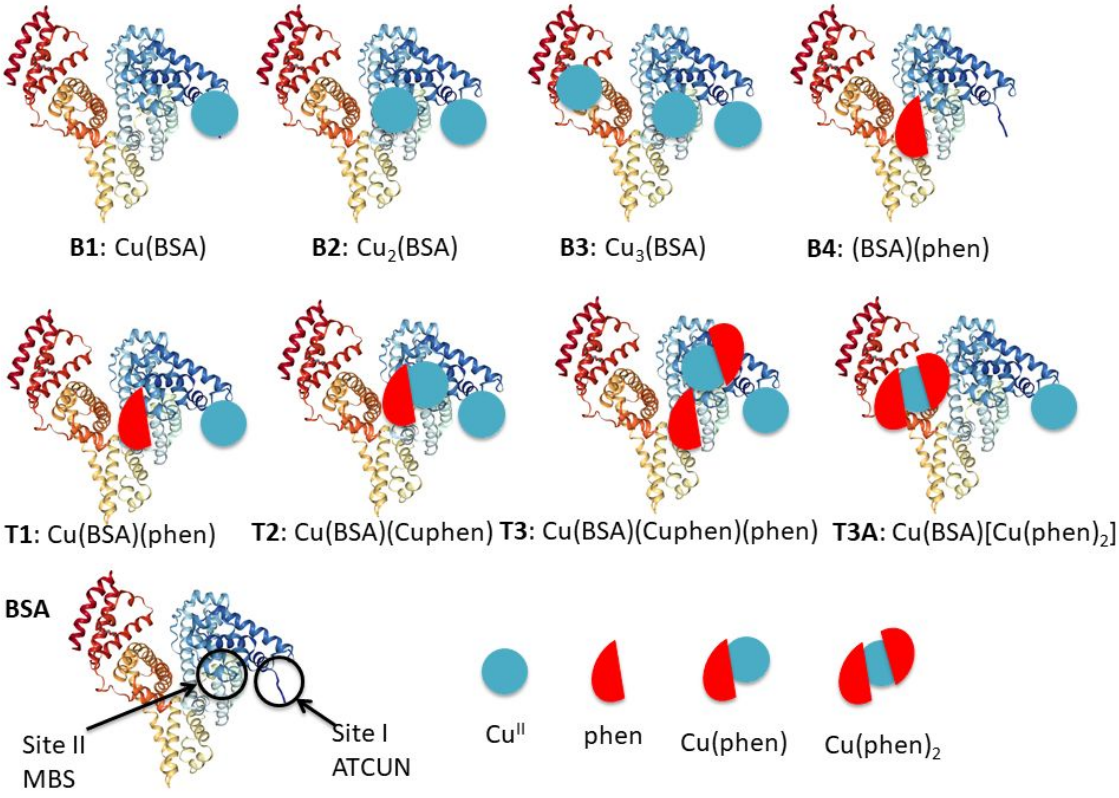
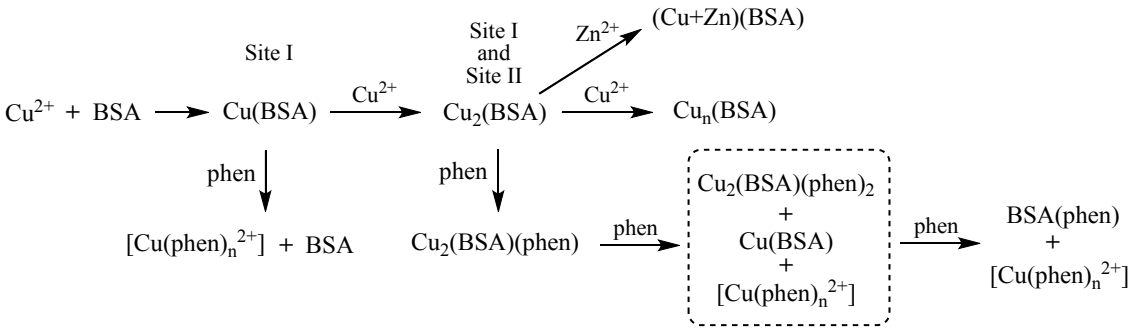


Figure 8. Schematic representation of the BSA-containing species that may form in solutions of $Cu(NO_3)_2$, BSA and phen. The BSA-phen (**B4**) species are barely visible in CD spectra in the range 350-800 nm,[56] but its formation and binding constant were determined by fluorimetric measurements.[2] Note that in **T1** the Cu ion and the phen molecule are bound at different locations, and the corresponding log β(**T1**) ≈ log β(**B1**) + log β(**B4**).



Scheme 1. Processes taking place in buffered solutions containing BSA to which $Cu(NO_3)_2$ is added, followed by additions of phen (and of $Zn(NO_3)_2$).

Table 4. Conditional binding constants obtained from the CD spectra measured for the systems Cu-BSA and Cu-BSA-phen, at pH 7.4 ± 0.1.[a] [b]

Species	log β_{pq1} [c]	log K[c]
B1 Cu(BSA)	12.5 ± 0.5	12.5
B2 Cu_2(BSA)	21.5 ± 2.0	9.0
B3 Cu_3(BSA)	25.4 ± 2.0	3.9
T1 Cu(BSA)(phen)	17.9 ± 1.5	5.4
T2 Cu_2(BSA)(phen)	29.0 ± 1.5	7.5
T3 Cu_2(BSA)$(phen)_2$	32.5 ± 1.0	3.5

[a] The following stability constants were assumed for $[Cu(phen)_n]^{2+}$ complexes: log β_{110} = 9.1, log β_{120} = 15.85 and log β_{130} = 20.9[87] (log β_{pqr} defined by eq. 1, with r=0) and $Cu(OH)^+$ (log β = -6.5).[88]

[b] For the calculations using the HypSpec [86] computer program, 70 CD spectra in the range 350-800 nm, each included with a 0.5 nm step, were used. The SD values included are not those obtained from individual calculations, but were established considering the range of values obtained in several distinct calculations.

[c] The K values correspond to the stepwise binding constants (see below).

Speciation in incubation media and relevance for cytotoxicity

A recent study by Sendzik et al [50] highlighted that Cu(II) bound to human serum albumin (HSA) may be reduced to Cu(I) by ascorbate, even in aerobic conditions, Cu(I) ions then binding strongly to this protein. It is plausible that the same may happen with bovine serum albumin (BSA). However, the typical incubation media used do not contain enough amounts of reducing agents to produce and maintain significant amounts of Cu^I(BSA) species, thus we will restrict our discussion to Cu(II)-containing species.

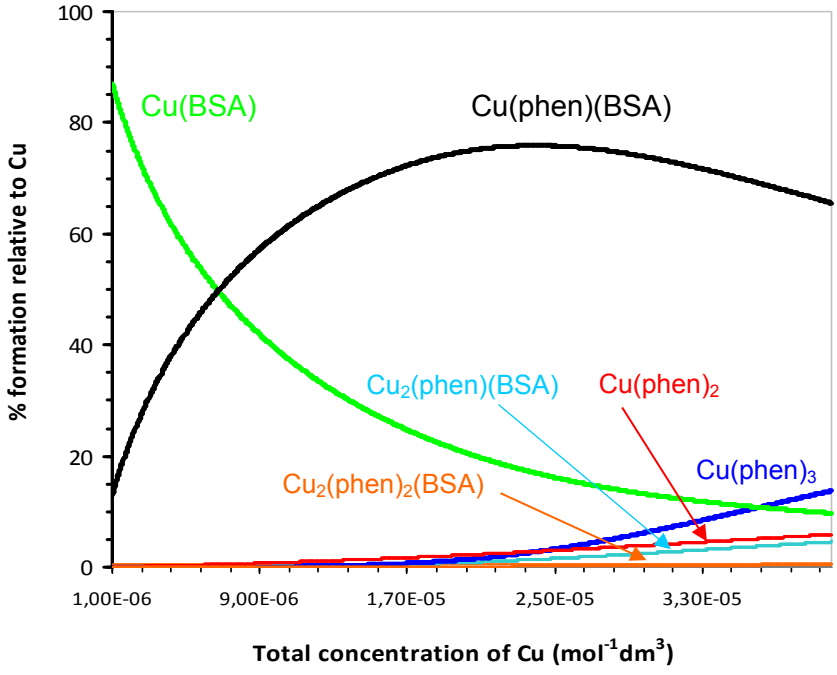
Once the most relevant binding constants are known, it is possible to calculate the speciation diagrams for the conditions prevailing in the several media used in our studies. These results are presented below and in the Supporting Information SI-11. Figure 9 depicts speciation diagrams of (a) % Cu and (b) % phen for the Cu-BSA-phen system, assuming the formation of $[Cu(phen)_q]$ (q = 1 to 3) and $[Cu_n(BSA)]$ (n = 1 to 3) and $[Cu_p(phen)_q(BSA)]$ complexes with binding constants as shown in Table 4.

Regarding Cu-containing species it can be seen that, up to a total concentration of Cu(II) of ~25 μM, most of Cu(II) is in the form of [Cu(BSA)] and [Cu(BSA)(phen)] complexes. Noteworthy, $[Cu(phen)_n]$ (n = 0-3) species are not present in much relevant concentrations

in this cell incubation media, except for $[Cu]_{total}$ > 25 μM, much higher than the IC_{50} values for complex **1** at 24, 48 and 72 h. The relative importance of [Cu(BSA)(phen)] (**T1** in Figure 8) progressively increases up to ~75 % when $[Cu]_{total}$ ~25 μM (higher than the IC_{50} value), and $[Cu_2(BSA)(phen)_2]$ is never relevant. Regarding phen-containing species, up to a $[Cu]_{total}$ ~40 μM, (phen)BSA, free phen and [Cu(BSA)(phen)] are dominant. Namely, [Cu(BSA)(phen)] corresponds to ca. 30-38 % of total phen in the range of $[Cu]_{total}$ 10-40 μM. Regarding BSA-containing species (see Figure S33 in Supporting Information), up to a $[Cu]_{total}$ ~40 μM, BSA, [Cu(BSA)], (phen)BSA and [Cu(BSA)(phen)] are the relevant species present.

As the total amount of BSA increases in the incubation media, globally the relative % of Cu-species bound to BSA increases and that of Cu-phen species decreases. Figure S34 shows speciation diagrams equivalent to those of Figure 9, but with a $[BSA]_{total}$ of 20 μM, and Figure S35 depicts the relative importance of the several species as the $[BSA]_{total}$ varies in the range 4 to 80 μM for $[Cu(phen)_2]_{total}$ = constant = 20 μM. Globally the cellular viability, depicted in Figure 5 for $[Cu(phen)_2]$ (**1**), increases as the relative importance of Cu-phen and phen decrease, and [Cu(BSA)] and (phen)BSA increase. This suggests that BSA may be sequestering Cu ions and phen molecules, the higher its concentration, the lower their uptake by cells and the higher the cellular viability. Decrease of copper uptake *in vivo* by albumin has been previously suggested. [89]

(A)



(B)

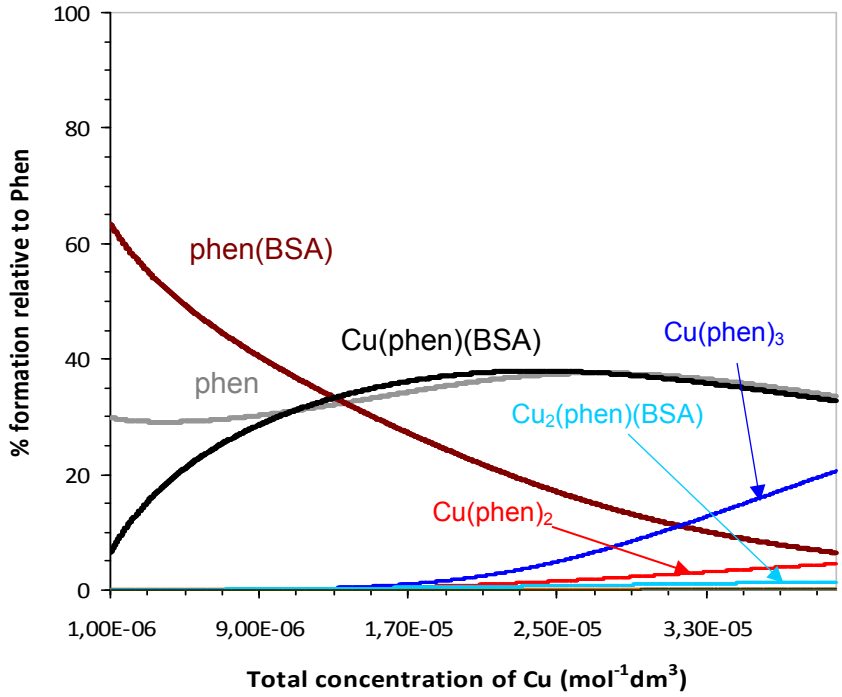


Figure 9. Species distribution diagrams for the Cu(II)-BSA-phen system at pH=7.4. Speciation diagrams assuming the formation of $[Cu(phen)_n]$ complexes (n = 1-3), the formation of $[Cu_n(BSA)]$ (n = 1-3) and of Cu-BSA-phen species, as a function of the total concentration of Cu(II) (added as $[Cu(phen)_2]^{2+}$), in the range 1-40 μM. The $[BSA]_{total}$ = 40 μM, which approximately corresponds to having 10% FBS in the incubation media of cells. The figure depicts the formation of A) Cu-containing species, and B) phen-containing species. The corresponding representation of BSA-containing species is depicted in Figure S33. The formation constants used for calculations with HySS [90] computer program are those of Table 4.

It is clear that once the $[Cu(Xphen)_2]$ complexes **1-3** are added to the incubation media of cells, the complexes dissolved will no longer be present in their original form, thus the straightforward assignment of active species is not possible. This has been normally disregarded in most publications addressing cytotoxicity of Cu-complexes.

We highlighted that the cytotoxicity, particularly for shorter incubation times, differs for phen and $[Cu(phen)_2]$. The uptake of Cu depends on the ligand, as clearly shown above, thus addition of $[Cu(Xphen)_2]$ complexes, instead of Cu(II) salts or Xphen molecules, somewhat affects the efficiency of the uptake of Cu(II) ions and of Xphen molecules by cells. This was previously suggested in the case of 2,9-dimethyl-1,10-phenanthroline (2,9-Me_2phen), [91] [72] as well as with phen [15] [48] and phendione (1,10-phenanthroline-5,6-dione). [48]

The higher the amount of Cu-phen complex added to the incubation media, the higher the relative proportion of [Cu(phen)$_n$] species present, therefore, the higher the possibility of uptake of Cu-phen species by the so-called 'passive diffusion'. In these conditions it may be anticipated that the cytotoxicity of Cu-phen complexes and corresponding free ligands should differ, and that is what is observed for the lower incubation times (e.g. at 3 h incubation). At higher incubation times ($\geq$ 24 h), the IC_{50} values for the Cu-Xphen complexes are lower than ca. 3 μM, and in these conditions, in the cell media most of Cu(II) and phen are bound to BSA.

Although at this stage it is not possible to assign the cytotoxicity of **1** to a particular species, most probably it is not predominantly due to either $[Cu(phen)]^{2+}$ or $[Cu(phen)_2]^{2+}$; besides these species not being much relevant in the incubation media, BSA in [Cu(BSA)(phen)] has Cu(II) and phen bound at distinct sites (Figure 8). Thus, although Cu-phen species might be uptaken, the cytotoxicity will most probably be due to the separate action of Cu ions and phen molecules.

Copper is an essential trace element in mammalian cells and because it is toxic, in humans its homeostasis is tightly regulated through a system of protein transporters. [92] [93] Cu(II) is reduced to Cu(I) by plasma membrane reductases, and the copper transporter 1 (CTR1) makes the high affinity Cu uptake, [94] [95] and the presence of Cu-chelators such as phen may possibly cause CTR1 up-regulation. [48] [96] Cu ions are strictly handled inside cells in such a way that virtually no free Cu ions exist. [94] [95] [97] [98] In fact, cells have specific Cu(I) chaperones which deliver Cu to proteins, and the intracellular copper concentration is rigorously controlled so that it is only provided to the essential enzymes. [99] Thus, once inside cells and copper present as Cu(I), it binds to strong ligands such as proteins containing adequate sequences of peptides, and/or small molecules such as GSH. [100] [101] [102] [103] [104] It is plausible that in contact with the incubation media containing much higher concentrations than the normal ones this controlled uptake might be disrupted, excess Cu ions uptaken and becoming toxic.[48] For Cu, our ICP-MS studies with the ovarian A2780 cancer cells indicate that the cytoskeleton is a plausible target, as copper accumulates mostly in this fraction after incubation of cells with complex **1**. This result contrasts with that found for cisplatin in the A2780 cells, which showed a different cellular uptake profile. The distribution was diffuse, and accumulation (Pt amount) was observed mainly in the cytoplasm and the nucleus. [105]

The toxicity of phen has been attributed to its action as a chelator, namely of Zn(II), inhibiting nucleotidyl transferases[106] and DNA synthesis;[107] nuclear fragmentation and impairment of mitochondrial function, leading to apoptosis, have also been reported. [22] [71]

Therefore, even if some Cu-phen species are uptaken by cells (according to the speciations obtained, the higher the concentration of Cu-complex added to the cell media, the higher the possibility of this actually occurring), the phen ligand will be lost and globally metal ion and phen will probably target distinct sites in cells, and the biological activity will result mostly from the separate action of both the Cu(I) species formed and phen molecules. A few published studies already suggested this possibility. [91] [108]

Addition of Cu(II) ions to the cell incubation media have been reported to increase the cytotoxicity of *e.g.,* 2,9-Me_2phen [72] and of phen.[71] In the case of 2,9-Me_2phen this was ascribed to the high stability of the respective Cu(I) complex $[Cu^I(2,9\text{-}Me_2phen)_2]$, which has a formation constant higher than that of $[Cu^I(phen)_2]$ [109] by ~$10^{3.3}$, thus, while complexes with ligands that bind strongly to Cu(I), such as 2,9-Me_2phen, might remain partly intact inside cells, for $[Cu^I(phen)_n]$ (n = 1 or 2) that is not plausible.

Conclusions

In this work three Cu(II) complexes, $[Cu(Xphen)_2]^{2+}$, with three different phenanthroline derivatives, were synthesized and studied to assess their stability in cell incubation media, as well as their cytotoxic activity as a function of incubation time and in the presence of varying concentrations of bovine serum albumin. Their cellular uptake and cellular effects in the ovarian A2780 cancer cells were also evaluated.

Upon 3 h incubation with complexes **1-3** the cellular uptake displayed a common trend for the three complexes, with a similar distribution profile of copper with high accumulation in the cytoskeleton. Up to 24 h incubation times, the cytotoxic activity differed for the complexes and corresponding free ligands. Complex $[Cu(Me_2phen)_2]$ (**2**) is the most active but this effect is not correlated with the cellular Cu uptake, since the higher uptake was observed for $[Cu(phen)_2]$ (**1**). At cellular organelle level, TEM studies indicated that disruption of the nuclear membrane and apoptotic blebs were observed in ovarian cells treated with **1**, but were more pronounced with **2**, the most cytotoxic complex. Cellular

degenerative changes resulting in multinucleated cells were seen for both complexes although more frequently in **2**.

To evaluate speciation of [Cu(phen)$_2$] (**1**) in cell incubation medium, several spectroscopic, spectrometric and electrochemical studies were used to sort out which are the relevant Cu- and phen-containing species. Mainly based on the measurement of circular dichroism spectra of solutions containing BSA and Cu(II) and/or phen, the binding constants of the relevant Cu-BSA and Cu-BSA-phen species were determined. For low total concentrations of Cu(II), ca. < 30 μM, BSA is the main binder of Cu(II) and phen molecules in the cell incubation medium, the formation of [Cu(phen)$_n$] species only being relevant when the BSA:Cu(II) molar ratios are low. The other potential ligands present are either not relevant binders or are present in very low concentrations.

Among the main conclusions we highlight that as soon as the Cu-phenanthroline complexes **1-3** are added to cell incubation media, they undergo speciation, the main species present not being [Cu(Xphen)$_2$]. Moreover, once uptaken and inside the cells, the Cu-phen complexes also no longer exist as such and most probably the ligand and the metal ion are directed to distinct targets. Notwithstanding, at incubation times up to 24 h the added [Cu(Xphen)$_2$] complexes are more cytotoxic than the corresponding free ligands, while upon 48 and 72 h incubation all complexes and free ligands display almost identical and high cytotoxicity. When higher amounts of complexes **1-3** are added to cell media, the relative concentrations of [Cu(Xphen)$_n$] species present are higher, their uptake by "passive diffusion" becomes more relevant, this accounting for the higher relative cytotoxicity compared to when only the free Xphen ligands are added.

In this work we present and discuss data that highlights the relevance of evaluating speciation of the Cu-complexes complexes in the cell media, so that the mechanisms of cell death might be understood. We emphasize the influence that the composition of the cell incubation media, namely the amount of bovine serum albumin, might have in the cellular viability when evaluating metal-based prospective drugs, particularly with complexes with labile metal ions that bind strongly to albumins, as is the case of Cu(II). We also highlight that the binding of the Cu(II) ion and of the Cu+phen species to BSA may affect the IC_{50} values determined. BSA sequesters Cu(II) ions and phen molecules, decreasing their uptake by the A2780 cells.

Most studies probing the biological action of Cu-phen complexes on specific targets were carried out with cell-free systems. Although they cannot be definitely ruled out, it is not probable that mechanisms of cell death involving the interaction of Cu-phen complexes with DNA might be involved. Use of Cu-phen complexes yield a more efficient uptake of Cu and phen, but concerning cell death, plausible mechanisms should involve the separate action of Cu ions and phen molecules. Possible exceptions are those phenanthroline ligands where the respective Cu(I)-Xphen complex is highly stable, as is the case of $[Cu^{I}(2,9\text{-}Me_2phen)_2]$, which might be able to compete with the strong intracellular Cu-binders.

Overall, the reported results constitute a valuable contribution for future studies addressing cytotoxicity of copper complexes and assessment of their mechanisms of action.

Materials and Methods

1,10-phenanthroline and 4,7-dimethyl-1,10-phenanthroline were from Alpha Aesar and 5-amino-1,10-phenanthroline from Fluorochem. Care must be taken during its manipulation and disposal, as all compounds are toxic if swallowed; phen is very toxic to aquatic life; and amphen may cause allergic skin reactions and serious eye damage. $[Cu(NO_3)_2]3H_2O$ was from May & Baker. All materials used were either p.a. or reagent grade. Phosphate buffered saline (PBS) and HEPES were purchased from Sigma-Aldrich. Fat free bovine and human serum albumin for spectroscopic and electrochemical experiments were also from Sigma-Aldrich. For the biological experiments, RPMI-1640 (RPMI = Roswell Park Memorial Institute) medium, as well as fetal bovine serum (FBS), penicillin/streptomycin mixture, trypsin/EDTA and PBS were purchased from Gibco, Thermo Fisher Scientific. A2780 cancer cells (ovarian, cisplatin sensitive) were acquired from Sigma-Aldrich. The RPMI-1640 medium contains ~5×10^{-9} M of Cu(II),[110] and upon addition of 10 % FBS [Cu(II)] typically ~1.5 μM;[111] these Cu(II) ions should be bound to BSA.

Elemental analysis for C, H and N were obtained on a FISONS EA 1108 CHNS-O apparatus. The infrared spectra were recorded on a JASCO FT/IR 4100 spectrophotometer and the UV-Visible absorption spectra were recorded on a Perkin Elmer Lambda 35 UV-Vis spectrophotometer with 10.0 mm quartz cuvettes. Circular dichroism (CD) measurements were carried out with a Jasco 720 Spectropolarimeter coupled with a UV-Vis detector, in the

350-800 nm range. The first derivative X-band EPR spectra of the frozen solutions at ~100 K were recorded either on Bruker ESP 300E or Bruker Elexys III E580 spectrometers. These were operated at ~ 9.51 GHz with a frequency modulation of 100 KHz. A 500-MS Varian Ion Trap Mass Spectrometer was used to measure electro spray ionization mass spectra (ESI-MS spectra) of methanolic solutions of the complexes in both positive and negative modes. Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectra were obtained using a Bruker Daltonics Ultraflex MALDI TOF/TOF Mass Spectrometer operating in linear mode with positive ion extracting at 25 000 V and a pulsed ion extraction of 480 ns. Each final spectrum was the accumulated result of at least 1000 laser shots that were obtained from 10 different manually selected regions of the same sample, over a range of 14 000–100 000 Da. Voltammetric measurements were carried out with a potentiostat/galvanostat from ECO Chemie, Autolab PSTAT10 connected to the Metrohm 663 VA Stand featuring a conventional three-electrode configuration: a Static Mercury Drop Electrode (SMDE) as the working electrode, an Ag/AgCl/KCl(sat) as the reference electrode and a carbon rod auxiliary electrode.

Methods and Determination of Binding Constants

Synthesis. The complexes were prepared according to literature procedures used for Cu-phen complexes:[34, 112-114] Details are given in the Supporting Information section.

Distribution coefficients. Octanol/water partition coefficients of complexes **1-3** were determined by the shake flask method in PBS buffer, according reported procedures.[115] The solvents used in the assays and calibration curves were previously saturated in each other, by stirring the mixtures for 48 h. Equal volumes (5 mL) of solutions of the complexes in PBS (pH 7.4) were mixed with *n*-octanol and placed in 15 mL screw-cap vials. [Complex **1**] = 1.4 mM, [Complex **2**] = 0.47 mM and [Complex **3**] = 0.91 mM. The mixtures were vigorously stirred for 22 h, allowed to stand for 2 h, and the phases separated. After adequate dilution of the samples their UV absorption spectra were measured, and the concentration determined from calibration curves obtained for each phase. The following wavelengths were used in the determination: 267, 270 and 287 nm, for **1**, **2** and **3**, respectively. The distribution coefficients, *D*, were obtained as the ratio between the concentrations in the organic and aqueous phases.

Cytotoxicity analysis against cancer cells. A2780 cells were cultured in RPMI medium supplemented with 10% (v/v) FBS and 1% antibiotics at 37 °C in a 5% CO_2/95% humidified atmosphere. The cytotoxic effects of the complexes were assessed by the MTT (MTT = 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay as previously described.[2] To evaluate the effect of serum proteins on the cytotoxic activity of the complexes, A2780 cells were incubated in media containing different concentrations of serum (FBS) from 1 to 20% (v/v) and the complexes at concentrations equivalent to their IC_{50} found at 3 h. Controls for each concentration of FBS were included in the assays. After incubation, the same procedure mentioned above was followed.

Cellular Uptake by ICP-MS. A2780 cells (approx. 10^6 cells/5mL) were incubated with the complexes in RPMI medium at a concentration equivalent to their IC_{50} values at 3 h incubation. The cellular fractions cytosol, membranes, nucleus and cytoskeletal were obtained from the cellular pellet using a cell fractionation kit (FractionPREP™, Biovision). These fractions were digested with nitric acid (0.5 mL, 65%) at 100 ºC during 12 h. The solution was diluted in MilliQ water to 10 mL. The Cu content (^{64}Cu) was measured by a Thermo X-Series Quadrupole ICP-MS (Thermo Scientific) equipped with Ni cones and a glass concentric nebulizer (Meinhard, 1.0 mL min^{-1}) refrigerated with a Peltier system. Indium (^{115}In) was used as internal standard at a concentration of 10 µg/L. Standard solutions were prepared from ICP-MS 71 A (Inorganic Venture) with a final concentration of 5.0% nitric acid. The following quality control was applied: internal standard accepted variation between 80 and 120%; blank's concentrations inferior to the limit of detection (1 µg/L) and sample duplicates not differing more than 10%. The Cu levels in each fraction were expressed as ng Cu/10^6 A2780 cells.

Morphological analysis by transmission electron microscopy (TEM). A2780 cells at approx. 70% confluence were treated with the Cu(II)-complexes, **1** and **2** at concentrations equivalent to their IC_{50} values found at 24 h incubation. Non-treated cells were used as controls. After incubation, cells were processed following a standard procedure previously reported.[76] The final thin sections of samples, which were stained with 2% aqueous uranyl acetate and Reynold's lead citrate. These stained sections were analyzed and photographed in a JEOL 1200-EX electron microscope.

MALDI-TOF mass spectrometric measurements. Cu(II) stock solutions were prepared in deionized H_2O and those of Cu-phenanthroline compounds in DMSO, all with ca. 3 mM immediately before mixing. Solutions of BSA were prepared with *ca.* 300 mM by dissolving the protein in NH_4HCO_3 buffer (pH 7.4, 25 mM) and allowed to stand overnight to allow equilibration. The concentration of the BSA stock solution was determined by spectrophotometry (see below). [116]

Samples for MALDI-TOF MS were prepared varying the BSA:Cu(II):Xphen molar ratios by mixing different volumes of the stock solutions with buffer. The % of organic solvent was < 5 % (v/v) and the BSA concentration was ~50 μM. Samples were prepared by the layer-by-layer preparation method. A thin layer of a saturated solution of sinapinic acid (SA) in acetone is spotted onto the MALDI-target. [117] [118] After acetone evaporation, 0.5 μL of the analyte is hand-spotted over the SA thin layer and allowed to evaporate. Finally, 1 μL of a saturated solution of SA in 30% (v/v) in acetonitrile is hand-spotted on top of the analyte and allowed to dry. At least two samples were prepared for each solution to probe, and m/z values were obtained from the z = +1 and z= +2 peaks. The present experiments yielded a significantly higher dispersion of m/z values than in studies with human serum transferrin, [117] [118] thus the SDs in the mass measured are sometimes relatively high. The solution of BSA used for calibration gave a MW = 66431 ± 20 Da (see e.g. Figure S3).

Circular dichroism measurements with solutions containing bovine serum albumin (BSA). The measurements were done at ~23 °C in a room with controlled temperature using quartz Suprasil® cells of 0.2, 0.5, 1.0 or 2.0 cm optical path. One or three accumulations were made for each spectrum. The differential absorption values (ΔA values) measured correspond to $\Delta A = \Delta\varepsilon \times b \times C$, where b is the optical path, C is the total concentration of BSA and Δε the differential absorptivity per mole of BSA.

The solutions of BSA, with concentrations varying in the range 200 to 600 μM were normally prepared in PBS buffer of pH = 7.4 and left with very gentle stirring for ~3 h. Their exact concentrations were established by measuring the absorbance at 279 nm and taking $\varepsilon = 44309\ cm^{-1}\ M^{-1}$. [116] Normally 5.00 mL of these solutions were transferred to a 2.0 cm optical path quartz cell and accurately known volumes of solutions of $Cu(NO_3)_2$, phen or $Zn(NO_3)_2$ (with known concentrations) were added. After each addition the sample was left with very gentle stirring inside the cell compartment of the CD apparatus, for about

5-10 min, before the measurement of the spectrum. Each CD spectrum obtained was processed with the Jasco 32 software available with the CD instrument, was converted into Delta Epsilon (Δε) values expressed in M^{-1} cm^{-1} taking the total concentration of albumin in the solution under consideration. CD data was also evaluated with HypSpec software[86] for determination of complex formation constants.

Electrochemical Studies. Differential pulse polarography (DPP) and cyclic voltammetry (CV) were used. In DDP, differential pulse amplitude, $E_{DP,}$ of $|25|$ mV, step height, $E_{SH,}$ of 5 mV, pulse amplitude, t_p, of 0.05 s and repetition period (drop time) of 0.5 s were used. In most experiments, the total Cu(II) concentration, was 9×10^{-6} M, the BSA concentration varied between 0 and 2×10^{-6} M, and the phen concentration between 0 and 2×10^{-5} M. The electrolyte medium was 0.1 M $NaNO_3$, pH = 7.0±0.1 (phosphate buffer). The voltammetric response of Cu was calibrated in the absence of BSA and/or phen in the same medium.

In CV, in most experiments, the initial potential, E_i was set equal to the final potential, E_f, and equal to 0.2 V and the direction of the scan was reversed at the switching potential E_λ = –1.25 V. The scan rate (v) varied between 0.01 and 0.20 V/s. Similar total concentrations were used as in the DPP experiments.

Throughout the work potential (E) values are referred to the Ag/AgCl/KCl(sat) electrode and are affected by an error of 5-10 mV. Before each voltammetric measurement, solutions were purged with N_2 during ~15 minutes and a continuous flow was kept on top of the solutions during experiments. All measurements were done at least in duplicate and in a room with temperature controlled at 25±2 °C.

Standard solution of 1000 mg L^{-1} (*Trace*CERT, Fluka) of $Cu(NO_3)_2$ was used directly or to prepare daily diluted Cu(II) standards when necessary.

EPR spectra of A2780 cell fractions after incubation of the cells with [Cu(phen)$_2$] or Cu(NO$_3$)$_2$. Accurately measured amounts of $[Cu(phen)_2]$ or $Cu(NO_3)_2$ were each added to about 10^6 A2780 cells in plaques of six wells (2 mL of cell suspension per well), so that Cu would have a final concentration of 0.5 mM in the incubation medium containing 10 % FBS. After ~30 min of incubation the cells were trypsinized and the cellular suspension centrifuged to separate the cells from the incubation medium. The cells were washed with

PBS, placed inside an EPR tube and frozen. The corresponding incubation medium was also placed inside EPR tubes and frozen.

In another experiment around 5×10^6 A2780 cells were placed in a 25 mL culture flask (T25) and were fractionated in their components: membranes, cytosol, nucleus and cytoskeleton, using a Fraction PREP™ Cell Fractionation Kit from Bio Vision. To each fraction controlled amounts of (i) solution of $Cu(NO_3)_2$ or (ii) solution of $[Cu^{II}(phen)_2]$ (**1**) were added so that the copper would have a total concentration of 0.5 mM. Then each fraction was placed inside an EPR tube, DMSO added (so that it would be ~5% v/v) and were frozen at 77 K.

Stability in cell media. The stability of the Cu-complexes in phosphate-buffered saline (PBS, 0.01 M, pH=7.4) and in Minimum Essential Medium Eagle (MEM) without phenol red, in the absence and the presence of 10% fetal bovine serum (FBS) was evaluated by UV-Vis and EPR spectroscopy. Stock solutions of the complexes were prepared in DMSO (*ca.* 1.2 mM) and diluted with the aqueous media to obtain final solutions with 50 μM concentration and 4% DMSO (v/v) for UV-Vis studies. For the EPR spectroscopic assays solutions with ca. 500 μM and 5% DMSO were prepared. Samples were taken and frozen in liquid nitrogen immediately after preparation and after 3, 24 and 48 h. The EPR spectra were measured by keeping the acquisition parameters constant. Spin Hamiltonian parameters were obtained by simulation of the spectra. [119]

Determination of binding constants of Cu(II)- and Cu-phen species to BSA (and to HSA)

We start by defining the conditional partial (K_n) and global (β_n) binding constants of Cu(II) with these proteins at fixed pH as:

$$K_1: \quad Cu^{2+} + \text{protein} \leftrightarrows (Cu)_1(\text{protein}) \qquad (2A)$$

$$K_2: \quad (Cu)_1(\text{protein}) + Cu^{2+} \leftrightarrows (Cu)_2(\text{protein}) \qquad (2B)$$

$$K_n: \quad (Cu)_{n-1}(\text{protein}) + Cu(II) \leftrightarrows (Cu)_n(\text{protein}) \qquad (2C)$$

$$K_1 = \frac{[(Cu^{2+})_1(protein)]}{[Cu^{2+}][protein]} \quad (3A) \qquad K_2 = \frac{[(Cu^{2+})_2(protein)]}{[(Cu^{2+})_1(protein)][Cu^{2+}]} \quad (3B)$$

For the system also including phen, the binding constants β_{pqr} , with r=1, were defined by eq. 1 and correspond to:

$$\beta_{pq1} = \frac{[(Cu^{2+})_p(phen)_q(protein)]}{[Cu^{2+}]^p[Phen]^q[protein]} \quad (4)$$

The K_n and β_{pq1} values depend on the pH.

Human and bovine serum albumin contain a primary strong binding site for Cu(II), the N-terminal site (site I),[83, 120 121] and a secondary site, the multimetal binding site (MBS or site II) located at the interface between domains I and II of HSA. [83] The MBS is the primary binding site for Zn(II) ions.[83] As covalent bonds are established between donor atoms of BSA (or HSA) and the Cu(II) ions, the chirality of the protein amino acids is effectively transferred to copper(II) and circular dichroism may be observed for metal-centered bands in the visible range due to d-d transitions. The binding at site I is much stronger than at site II, and conditional binding constants (see eqs. 2-4) at pH = 7.4 have been determined from CD data for HSA as log β_1(Site I) = 12.0±0.5 and log β_2(Site I+II) = 18.0±1.0 (partial binding constant: log K_2(Site II) ≈6); [56 83 84] In bovine serum albumin, binding site I is also much stronger than site II and conditional binding constants for Cu(II) at pH = 7.4 have been reported in the range: (a) log β_1 (Site I) 11-13.5 [83 122 123 124] and (b) log K_2 (Site II) in the range 6-8.[83, 123, 124] Other weaker binding sites with log K_n ≈5-7 (n ≥ 3) were also determined, [123 124] namely by gel chromatography with Chelex-100 columns.[123]

Electrochemical Studies.

To properly address these systems, we started by evaluating the Cu(II)+BSA and Cu(II)+phen systems. These are included in detail in sections SI-8 and SI-9, as well as the study of the system Cu(II)+HSA by electrochemistry (section SI-7, Figs. S10 and S11). A conditional formation constant for 1:1 Cu:phen complex of log K_1 = 6.3±0.1 was obtained. For the Cu(II)+BSA system the K value determined (log K = 6.2±0.1) corresponds to a Cu-binding site in BSA with moderate affinity (Site II, K_2) and agrees fairly well with

previous determined values (see above),[123] although it is somewhat lower than the value obtained by us from CD measurements (see below).

The binding in the copper(II)-phen-BSA system was studied by DPP in 0.1 M $NaNO_3$ at pH = 7.0±0.1 (phosphate buffer). It was concluded that the voltammetric behavior of BSA and phen remains unchanged in terms of peak potentials as shown in Table 5, which also summarizes all other peak potentials.

The interaction of Cu(II)-phen complexes with BSA was analyzed by DPP, adding BSA to the electrolyte containing Cu(II) and phen in concentrations such that 0.5≤[Cu]:[Phen]≤1. The DP polarograms of Figure 10 show that the peak due to Cu(II) reduction, which was seen in solutions containing BSA and Cu(II) (peak 1), is no longer seen in the presence of phen (see also section SI-9). As to the signal due to phen reduction (peak 2), its potential and height remain the same in the absence and presence of BSA. The signal due to BSA reduction (peak 3) increases with the increase of protein in solution. These results agree with the data (e.g. Figs. S12 and S13) and discussion included in sections SI-8 to SI-10. Peak currents due to the reduction of the Cu(II)-phen complex (peak 4) decrease, till they vanish with BSA additions.

Table 5. Peak Potentials, (E_p±0.01) V, from DP polarograms. Medium: 0.1 M $NaNO_3$, pH = 7.0±0.1 (phosphate buffer, 0.01 M). See sections SI-8 to SI-10 for details.

Reduction	**E_p (V)**
Cu(II)	0.03
phen	-0.97
phen (in the presence of BSA)	-0.98
phen (in the presence of Cu)	-0.97
BSA	-0.56
BSA (in the presence of phen)	–0.55
BSA (in the presence of Cu)	-0.68
BSA + phen + Cu	-0.68
Complex Cu-phen	-0.40 V (phen, 1.58×10^{-5} M)
Complex Cu-phen (in the presence of BSA)	-0.42 (phen 1.47×10^{-5} M)

The results indicate that BSA interacts with the $[Cu(II)(phen)]^{2+}$ complex. The decrease in peak current associated with its reduction can be interpreted in terms of the binding of the complex to the bulky protein according to the scheme (omitting charges):

$$[Cu(II)(phen)] + BSA \rightarrow [Cu(II)(phen)(BSA)] \quad (5)$$

In fact this corresponds to the process:

$$[Cu(phen)] + [Cu(BSA)] \rightarrow [Cu_2(phen)(BSA)] \quad (6)$$

as the first Cu(II) ion binds very strongly to BSA, Cu(II) is in excess and [Cu(phen)] binds at a site distinct from the N-terminal of BSA. A binding constant, K=[Cu(phen)(BSA)]/([BSA][Cu(phen)]), can be estimated from the peak currents of signal 4, in the absence of BSA, ip_{AbsBSA}, and upon protein additions, $ip_{PresBSA}$, according to: [125] [126]

$$\log (1/[BSA]) = \log K + \log (ip_{AbsBSA}/(ip_{AbsBSA}-ip_{PresBSA}) \quad (7)$$

Figure S23 depicts the plot of log(1/[BSA]) as a function of log $(ip_{AbsBSA}/(ip_{AbsBSA}-ip_{PresBSA})$. A good linear relationship was obtained with a slope close to 1, and log K = 5.9±0.1 was obtained from the intercept. Considering the accepted formation constant of [Cu(phen)] (log K_1=9.1)[87] and that of [Cu(BSA)] calculated in section SI-10 by CD measurements at pH = 7.4 (log K_1=12.5), using eq. 7 this corresponds to log β(**T2**) = 27.1 (as defined by eq. 1), which agrees reasonably well with the value included in Table 4 (29.0 ± 1.5).

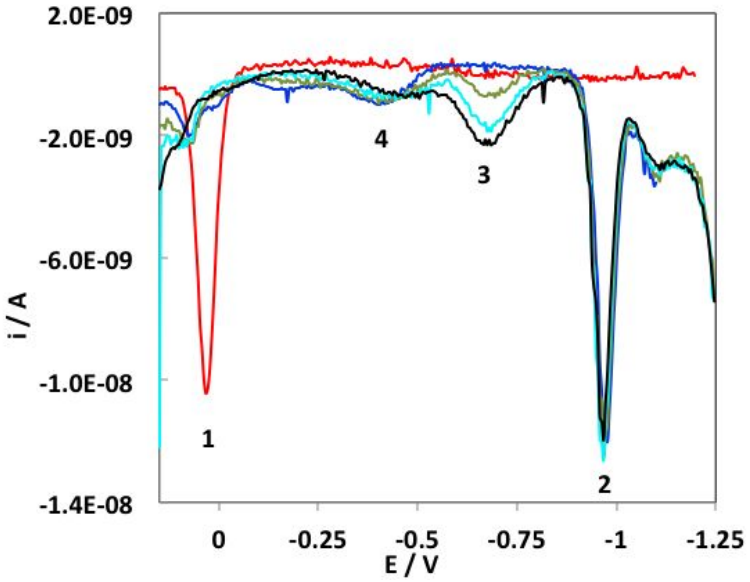


Figure 10. Differential pulse polarograms of solutions containing Cu(II) (9.00×10^{-6} M) and phen (1.45×10^{-5} M). Only Cu(II) (red); Cu(II)+phen (blue); upon additions of BSA: [BSA] (M): 3.16×10^{-7} (green); 6.31×10^{-7} (cyan); 1.26×10^{-6} (black). Voltammetric conditions: E_i = 0.15 V; E_f = −1.40 V; E_{DP} = 25 mV; E_{SH} = 5 mV; t_p = 50 ms. Medium: 0.1 M $NaNO_3$, pH = 7.0±0.1 (phosphate buffer 0.01 M). Peak 1: Cu(II)→Cu(0) reduction; Peak 2: $phen_{adsorbed}$ reduction; peak 3: $BSA_{adsorbed}$ reduction; peak 4: Cu(II)(phen)→ Cu(I)(phen) reduction.

Circular Dichroism spectrophotometry

As previously highlighted by some of us,[33, 117] conditional binding constants of labile metal complexes with most bidentate or tridentate ligands with proteins such as BSA or HSA, cannot be determined by using simple fluorescence quenching methods, because at the low concentrations used in these assays these metal complexes are extensively hydrolysed and the quenching of Trp fluorescence is due to several distinct species, and not to a single one (see Figures S31 and S32 in Supporting Information). This is particularly relevant for metal ions, such as Cu(II) or Zn(II), that bind strongly to serum albumins. Additionally, using conditional binding constants in the range 10^5-10^7 for the binding of $[Cu(phen)_n]$ to BSA, to calculate the species distribution diagram, these do not yield to the formation of relevant amounts of [Cu(phen)(BSA)] species, as is clear from the speciation shown in Figure S32, thus the quenching observed is not due to this complex. Therefore, other spectroscopic methods are required to determine conditional binding constants of Cu-phen species to BSA or HSA, and circular dichroism spectrophotometry is an adequate one.

Solutions containing bovine serum albumin, Cu(II) and phen. In solutions containing Cu(II)-compounds and chiral ligands, in this case the BSA protein, several complex species may form. The ΔA values may be due to one or more types of species. Therefore, the Δε values correspond to: [127, 128] $\Delta\varepsilon = \Sigma\, \Delta\varepsilon_i \times C_i$, where $\Delta\varepsilon_i$ is the differential molar absorptivity of the chiral species i of molar concentration C_i. The Σ includes all chiral species present. The Cu(II)+BSA+phen system was studied by recording CD spectra in the 350-800 nm range to enable the determination of the species formed and the corresponding binding constants. Considering the binding constants β_{pq1} defined as shown in eq. 4, several sets of CD (and EPR) spectra were measured at pH = 7.4 for the systems Cu(II)+BSA (Figs. S14 to S22) and Cu(II)+BSA+phen (Figs. 11, 12 and S14 to S29), at various molar ratios. Most of these data

are included in sections SI-9 and SI-10. Figure 11 emphasizes some relevant aspects observed for solutions containing Cu:BSA:phen molar ratios of 1:1:n.

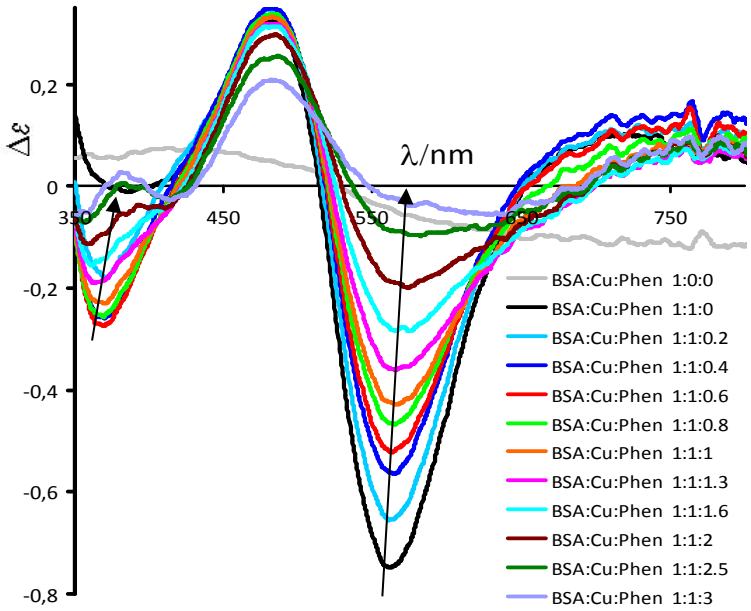


Figure 11. CD spectra in the 350-800 nm range of solutions containing 0.4 mM BSA in PBS, upon addition of a solution of $Cu(NO_3)_2$, so that the molar Cu:BSA ratios was 1:1, followed by additions of a phen solution up to a final molar ratio of Cu:BSA:phen of 1:1:3.

When $Cu(NO_3)_2$ is added to BSA up to a molar ratio of 1:1, Cu(II) binds at site I and the CD spectrum depicts the corresponding typical spectrum with negative bands at ~365 and ~560 nm, and a positive band at ~480 nm. In Figure 11 a positive band appears at 700-740 nm, indicating that a significant amount of $[Cu_2(BSA)]$ also formed. Upon additions of phen globally the $|\Delta\varepsilon|$ values decrease in the 350-750 range, this meaning that the Cu(II) ion is being progressively removed from BSA by the competing phen ligand, forming mostly $[Cu(phen)_n]^{2+}$ species in the bulk solution, which are CD silent. Some more detailed remarks may also be done: (i) the band with λ_{max} at 560 nm decreases continuously its intensity upon adding phen (and the λ_{max} slightly shifts to higher wavelengths), (ii) at ca. 365 nm, there is no clearly visible band for the solution 1:1:0 (of BSA:Cu:phen); as phen is added, there is an increase in the (negative) intensity up to 1:1:0.6 (of BSA:Cu:phen), then there is a decrease in intensity, apparently due to the formation of another positive band at ca. 380 nm; (iii) in the range ca. 650-850 nm, the band due to the binding of Cu(II) to BSA at ~720 nm has

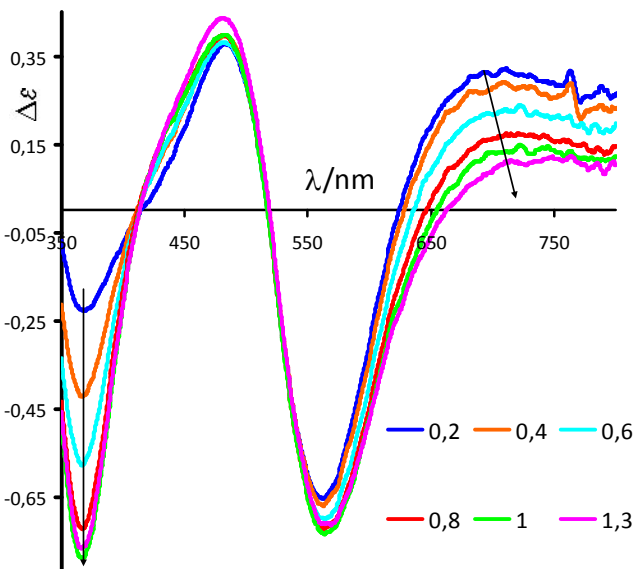
some modifications, namely, apparently a positive band forms with λ_{max} at 760 nm, and at ca. 690 nm the Δε values decrease.

Figure S25 depicts CD spectra of some of the solutions corresponding to the spectra of Figure 11 and after progressive additions of a solution of $Zn(NO_3)_2$ till Cu:BSA:phen:Zn molar ratios of 1:1:3:4. Globally the addition of Zn(II) ions, which compete with Cu(II) for the binding to phen, has an effect of increasing the |Δε| values in the 350-750 range, as the Cu(II) (from $[Cu(phen)_n]$ species in the bulk solution) is progressively released by phen and binds at Site I of BSA.

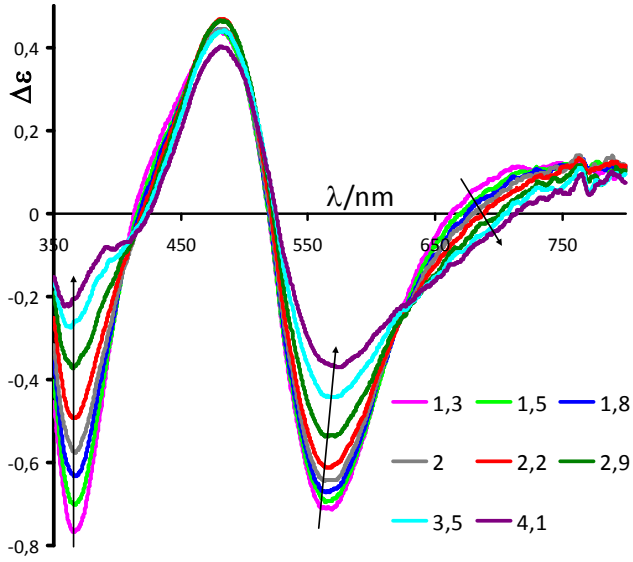
Figure 12 depicts some relevant aspects observed for solutions containing Cu:BSA:phen molar ratios of 1:2:n. It is clear that when phen is added to the solution containing $[Cu_2(BSA)]$, the |Δε| values at ~365 nm increase significantly; this can only be explained by the progressive formation of a $[Cu_2(BSA)(phen)]$ species (e.g. **T2**, Figure 8), this band corresponding to a ligand to metal charge transfer transition. The bands at ~475 and ~562 nm remain without much changes, this meaning that the Cu(II) bound at Site I of BSA is not being affected. The band at ca. 660-800 nm decreases its intensity, this indicating that the added phen ligand is binding to the Cu(II) ions bound at Site II. In Figure 12B it is clear that the band at ~365 nm decreases intensity, the band at ca. 475 nm is not much affected, and the band at ~562 nm decreases intensity probably due to the formation of a band with $\Delta\varepsilon > 0$ at ca. 700 nm, due to the presence of a Cu(II)-BSA-phen complex, distinct from the initially formed $[Cu_2(BSA)(phen)]$. A possible formulation for this species is $[Cu_2(BSA)(phen)_2]$; this formulation may correspond to more than one species (see e.g. **T3**, **T3A** in Figure 8). Further additions of phen up to a molar ration Cu:BSA:phen of 2:1:10.6 globally decrease the |Δε| values, and as more phen is added to the solution, more Cu(II) ions are displaced from BSA to form CD silent $[Cu(phen)_n]$ species in the bulk solution.

Figure S28 depicts the CD spectrum of a solution containing $Cu(NO_3)_2$ and BSA with Cu:BSA molar ratio of 2:1 at pH 7.4, and after additions of phen up to a Cu:BSA:phen molar ratio of 2:1:4. This was followed by the addition of a solution of $Zn(NO_3)_2$. While the addition of phen globally decreases the |Δε| values, due to partial removal of Cu(II) from Site I (due to formation of $[Cu(phen)_n]$ complexes in the bulk solution), upon the addition of Zn(II) ions, which competes with Cu^{2+} in the binding to phen, globally the |Δε| values in the visible range increase. Figure S29 depicts EPR spectra in a similar experiment: upon

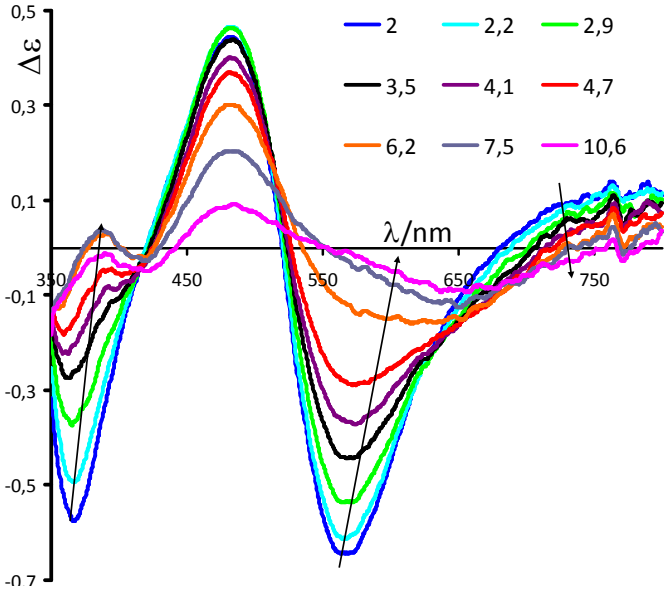
addition of a solution of $Cu(NO_3)_2$, so that the molar Cu:BSA ratios was 2:1, followed by additions of a phen solution up to a final molar ratio of Cu:BSA:phen of 2:1:8. Then a solution of $Zn(NO_3)_2$ was progressively added up to a Cu:BSA:phen:Zn molar ratio of 2:1:8:3. This last spectrum is very similar to that of a 2:1:1:0 molar ratio, indicating that the Zn(II) ion, besides competing with Cu(II) for the binding to Site II of BSA, it also competes with Cu(II) for the binding to phen, also forming $[Zn(phen)_n^{2+}]$ complexes in the bulk solution.



A



B



C

Figure 12. CD spectra in the 350-800 nm range of solutions containing 0.4 mM BSA in PBS at pH = 7.4, upon addition of a solution of $Cu(NO_3)_2$, so that the molar Cu:BSA ratios was 2:1, followed by additions of a phen solution up to the indicated Cu:BSA:phen molar ratios.

Scheme 1 summarizes the processes taking place in the solutions.

As expected, the additions of Cu(II) or phen yield significant changes in the visible wavelength range of CD spectra and several distinct Cu-BSA and Cu-BSA-phen species are detectable in the spectra. Thus, CD spectrophotometry gives a good insight on the formation of the different species. Taking advantage of this, the several measurements done at pH 7.4 for the systems Cu-BSA and Cu-BSA-phen at various Cu(II):BSA:phen molar ratios were used to determine the corresponding conditional binding constants. For this purpose important aspects needed to be taken into account. In particular: (a) BSA, phen and BSA(phen) do not absorb, thus are not spectroscopically relevant in the measured wavelength range; (b) The species phen(BSA) was considered in the speciation model and its log $\beta_{Cu(BSA)}$ maintained constant and equal to 4.75;[2] (c) The system Cu-phen was studied in several conditions, and the reported formation constants vary significantly with the media. [87 109, 129 130] In our calculations we considered the values reported from Banks *et al.*[87] (Table 4). These complexes were included in the speciation model and their formation constants were kept fixed. (d) Most probably the CD spectrum of [Cu(BSA)(phen)] will not differ much from that of [Cu(BSA)]. In fact, we expect (see also Figure 8 and Figure S14 in Supporting Information) that the additional phen will bind to BSA at a site distinct from the N-terminal site, so it will not be interacting with the Cu(II) ion. Therefore we expect that the log K value for the process [Cu(BSA)] + phen $\rightarrow$ [Cu(BSA)(phen)] will be of the same order of magnitude as that of: BSA + phen $\rightarrow$ BSA(phen).

Taking all this into account, the HypSpec program [86] was used to process the set of CD spectra measured. Complexes of $[Cu_n(BSA)]$ with n = 1-3 and $[(Cu)_p(phen)_q(BSA)]$ were those with conditional binding constants to be determined taking 70 CD spectra measured in the range 350-800 nm. The measured spectra are significantly noisy; furthermore, the error in the concentrations of the different components is relatively high due to the difficulty in determining the exact concentration of BSA and the changes occurring in the solution while titration experiments are being done, like pH (± 0.1) and solvent composition, among others. Nevertheless, different models could be fitted (see Table S6), the final obtained vales are

depicted in Table 4 and the CD spectrum calculated for each species depicted in Fig. S30. The log K value for the process [Cu(BSA)] + phen → [Cu(BSA)(phen)] (*ca.* 5) is of the same order of magnitude than that of BSA + phen → BSA(phen) (4.75).[2] This is in agreement with the expected binding of phen to BSA, in the [Cu(BSA)(phen)], in a site distinct from the N-terminal (see (d) above), without interaction with the Cu(II) ion, resulting in a log $\beta_{Cu(BSA)(phen)} \approx \log \beta_{Cu(BSA)} + \log K_{(BSA)(phen)}$. The values of log β of the species [Cu(BSA)(phen)] and [Cu_2(BSA)(phen)$_2$] are in good agreement with the corresponding log β value for [Cu(HSA)(phen)] and [Cu_2(HSA)(phen)$_2$] (*ca.* 17 and 31 respectively).[33, 56]

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Supporting Information. Synthesis and characterization; Cellular uptake of Cu; Potential ligands in incubation media for mammalian cells; MALDI-TOF mass spectral data; Stability in cell media; EPR spectra in cell fractions; Binding of Cu(II) to HSA; Binding of Cu(II) to 1,10-phenanthroline (electrochemical studies); Binding of Cu(II) to BSA; Binding of Cu(II)-phen species to BSA; Speciation diagrams.

Conflict of interest

The authors declare no conflict of interest.

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Table of Contents graphic

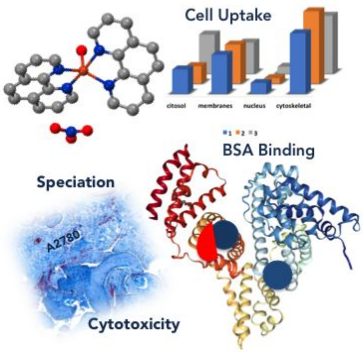

Table of Contents Synopsis

The interpretation of cytotoxicity, cellular uptake and mechanism of action of Cu(II)-1,10-phenanthroline complexes is correlated with their speciation in cell culture media and binding to albumin. The Cu-phenanthroline complexes undergo speciation and once up-taken by cells, the complexes no longer exist as such and most probably the ligand and metal-ion are directed towards distinct targets.

Keywords: copper; phenanthrolines; cytotoxicity; cell incubation media; speciation.