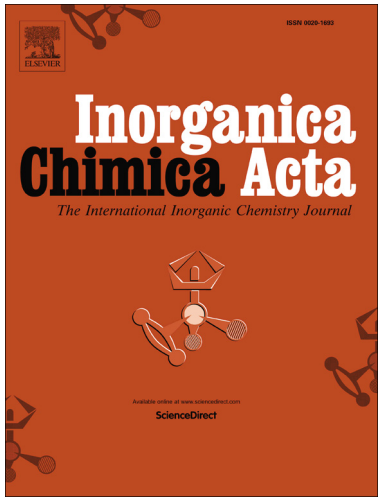

Synthesis, structural characterization and cytotoxic activity against tumor cells of heteroleptic copper (I) complexes with aromatic diimines and phosphines.

Natalia Alvarez, Carolina Noble, María H. Torre, Eduardo Kremer, Javier Ellena, Marcio Peres de Araujo, Antonio J. Costa-Filho, Luis Felipe Mendes, M. Gabriela Kramer, Gianella Facchin

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Synthesis, structural characterization and cytotoxic activity against tumor cells of heteroleptic copper (I) complexes with aromatic diimines and phosphines.

Revised version

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Key words

Cu(I) coordination compounds, X-ray structure, cytotoxic activity.

Abstract

Copper coordination complexes are increasingly recognized as potential drugs for therapeutic use in various diseases, especially in cancer. In an attempt to find new leaders for the development of antitumor Cu compounds, heteroleptic Cu(I) complexes with triphenylphosphine (PPh_3) and dimethylbipyridine (dmbpy), phenanthroline (phen) or neocuproine (neo) were prepared and characterized. The compounds [CuCl(dmbpy)(PPh_3)] (**1**), [CuCl(phen)(PPh_3)]·0,25H_2O (**2**) and [CuCl(neo)(PPh_3)] (**3**) were obtained. The crystal structures of compounds **2** and **3** were determined. In both complexes, the Cu(I) centre presents distorted tetrahedral coordination. All complexes present cytotoxic activity, against tumor cell lines, being more active than Cisplatin, with IC_{50} in the low micromolar range. This activity augments in the order **1**<**2**<**3**, following the same order as the lipophilicity.

1 Introduction

Copper coordination complexes are increasingly recognized as potential drugs for therapeutic use in various diseases. In particular, triggered by the success of Cisplatin for the treatment of cancer, and under the hypothesis that endogenous metals may be less toxic than Pt, several copper complexes were studied looking for cytotoxic activity [1-3]. As a consequence, a variety of Cu(II)-complexes were found to present good antitumor activity, as tested *in vitro* on several cancer cell lines, and a few of them on *in vivo* experiments including ongoing preclinical studies [1-5].

Cu(I) is the chemical form of Cu accepted to be relevant in the active internalization of copper in mammalians [1-3]. In spite of that, relatively few studies report on the antitumor activity of Cu(I) complexes, probably due to the difficulty in obtaining stable Cu(I) complexes in aqueous media. Cu(I)-phosphine complexes were studied

following the discovery of the *in vivo* antitumor activity of the anti-arthritic Au(I)-phosphine complex Auranophine, and of $[Au(dppe)_2][Cl]$ (dppe: 1,2-bis(diphenylphosphino)ethane). Cu(I) complexes with phosphine ligands that present cytotoxic activity have been reported [4, 6-8]. An outstanding example is $[Cu(thp)_4][PF_6]$ (thp= tris(hydroxymethyl)phosphine), which has strong cytotoxic effects. It is more active than Cisplatin and presents activity even against Cisplatin resistant cells with high selectivity to tumor cells. The proposed mechanism of action for the latter is parapoptotic cell death [9, 10]. Recently a Cu(I) compound with both cytotoxic and antiangiogenic activity was also reported [11]. Review studies suggest that the presence of a labile ligand is necessary for Cu complexes to exhibit cytotoxic activity [1].

Heteroleptic Cu(I) complexes with general formula $[Cu(N\text{-}N)(PPh_3)_2]X$ [12-19] and $[Cu(N\text{-}N)(PPh_3)X]$ [20-23], where N-N represents an aromatic di-imine, P a monodentate phosphine and X an halogen, were previously studied due to the luminescent properties of these complexes or as catalysts [24, 25].

Our research group has developed several Cu(II) complexes with different sets of ligands looking for compounds with antitumor activity [26-41]. Special attention has been given to ternary complexes [Cu(L-dipeptide)(phenanthroline)] with dipeptides and different phen derivatives, finding compounds with elevated cytotoxic activity, as [Cu(L-ala-phe)(phen)] [40]. In an attempt to obtain complexes with stronger activity, we widened our studies to phosphine-Cu(I) compounds, keeping the Cu-di-imine core which proved active to the date, therefore preparing Cu-di-imine-phosphine (where di-imine: dimethylbiyridine, phenanthroline or neocuproine) mixed ligand complexes. In this paper we report the synthesis and characterization of

[CuCl(dmbpy)(PPh_3)], [CuCl(phen)(PPh_3)]·0,25H_2O and [CuCl(neo)(PPh_3)] which to the best of our knowledge is the first report on the cytotoxic activity of this group of complexes.

2 Experimental

Reagents for synthesis and biochemical studies were used as commercially available: $CuSO_4·5H_2O$, NaCl and Na_2SO_3 (Fluka); ligands: dimethylbiyridine (dmbpy), 1,10-phenanthroline (phen), neocuproine (neo) and triphenylphosphine (PPh_3) (SIGMA).

2.1 Synthesis of the complexes and analytical characterization

The complexes were synthesized under Ar atmosphere using standard Schlenck techniques: 0.15 mmol of CuCl (previously synthesized according to Jolly [42]) , 0.15 mmol of di-imine ligand (where di-imine: dmbpy, phen or neo) and 0.15 mmol PPh_3 were dissolved in dichloromethane, to a final volume of 5 mL. The yellow resulting solution was further stirred for 1 h at room temperature. A yellow amorphous solid was obtained after addition of hexane. Yield: 50-60 %.

Small crystals were obtained by slow evaporation of a dichloromethane solution of the compounds in dichloromethane at -5 °C for **2** and **3**.

Elemental analysis: **[CuCl(dmbpy)(PPh_3)] (1)** Calc. for $CuN_2C_{30}H_{27}PCl$: %C 66.05%, %N 5.13%, H 4.99 found: 66.67%, N 4.78%, H 5.42%, **[CuCl(phen)(PPh_3)]·0,25H_2O (2)** Calc. for $CuN_2C_{30}H_{23}PClO_{0,25}$: %C 66.99%, %N 5.13%, H 4.34 found: 66.16%, N 4.83%, H 4.57 %, **[CuCl(neo)(PPh_3)] (3).** Calc. for $CuN_2C_{32}H_{27}PCl$: %C 67.48%, %N 4.92%, H 4.78 found: 67.31%, N 4.83%, H 5.08%.

2.2 Physical measurements

Chemical analyses for carbon, nitrogen, hydrogen and sulfur were performed with a Thermo Scientific Flash 2000 analyzer. Infrared spectra were recorded with a Shimadzu IR Prestige 21 spectrophotometer from 4000 to 400 cm−1 using KBr disks.

UV–visible (UV–vis) spectra of the complexes´ solutions were carried out with a Thermo Scientific Evolution 60 spectrophotometer, using 1 cm path length quartz cells.

X-band (9.5 GHz) EPR measurements were carried out on DMSO/aqueous solutions (3:1) using a JEOL JES-FA200 spectrometer and a cavity with 100 kHz field modulation. Measurements were performed at room temperature and experimental parameters were adjusted to avoid signal saturation and line shape distortions.

2.3 Crystal structure determination

Data from suitable single crystals were collected at 293.0(2) K for complexes **2** and **3** on an Enraf-Nonius FR590 Kappa-CCD diffractometer using graphite monochromated MoKα radiation (0.71073 Å) and a Bruker D8 Venture diffractometer using mirror-layered monochromated CuKα radiation (1.54178 Å), respectively. Data collection and processing software used for **2** were Bruker AXS Collect software and HKL Denzo-Scalepack program suite. Whereas for **3** Bruker Apex2 was used for data collection and processing. The structures were solved by direct methods using the SIR-92 [43] in the case of complex **2** and intrinsic phasing within Apex2 for **3**. Model refinement was performed with SHELXL [44] within Shelxle [45]. Gaussian absorption correction was applied for complex **2** and semi-empirical from equivalents for **3** [46, 47]. Molecular structure graphics were prepared using Mercury [48].

All non-hydrogen atoms were refined using anisotropic displacement parameters. Hydrogen atoms of C-H groups were stereochemically positioned and refined isotropically with the *riding-model* setting their thermal parameter as 1.2 times the equivalent isotropic displacement parameter of the C atom they are bonded to. Water hydrogen atoms were found in the difference Fourier map, positionally fixed and their thermal parameters set to 1.5 times the equivalent U_{iso}. Summary of the crystallographic data, experimental details and refinement results are listed in Table 1.

Table 1. Summary of crystallographic data, experimental details and refinement results.

Identification code	CuClphenPPh3	CuClneoPPh3
Empirical formula	C120 H94 Cl4 Cu4 N8 O P4	C32 H27 Cl Cu N2 P
Formula weight	2183.87	569.51
Temperature	293(2) K	293(2) K
Crystal system	Triclinic	Monoclinic
Space group	P-1	C 2/c
Unit cell dimensions	a = 9.7780(5) Å	a = 23.9819(6) Å
	b = 15.6430(6) Å	b = 9.3905(2) Å
	c = 18.3840(8) Å	c = 24.6018(6) Å
	α= 109.140(3)°	α= 90°
	β= 103.430(2)°	β= 98.1830(10)°
	γ = 92.068(3)°	γ = 90°
Volume	2564.5(2) Å$_3$	5484.0(2) Å$_3$
Z	1	8
Density (calculated)	1.414 Mg/m$_3$	1.380 Mg/m$_3$
Absorption coefficient	1.041 mm^{-1}	2.747 mm^{-1}
F(000)	1122	2352
Crystal size	0.272 x 0.108 x 0.094 mm$_3$	0.820 x 0.198 x 0.066 mm$_3$
Theta range for data collection	2.991 to 25.355°.	3.63 to 74.79°.
Index ranges	-11<=h<=11, -18<=k<=17, -20<=l<=22	-29<=h<=29, -10<=k<=11, -30<=l<=30
Reflections collected	24557	64609
Independent reflections	9325 [R(int) = 0.0769]	5615 [R(int) = 0.0510]
Completeness to theta = 25.242°	99.4 %	99.8 %
Max. and min. transmission	0.949 and 0.825	0.84 and 0.55
Refinement method	Full-matrix least-squares on F^2	
Data / restraints /	9325 / 0 / 640	5615 / 0 / 337

parameters		
Goodness-of-fit on F_2	1.013	1.145
Final R indices [I>2sigma(I)]	R1 = 0.0529, wR2 = 0.1003	R1 = 0.0414, wR2 = 0.1021
R indices (all data)	R1 = 0.1143, wR2 = 0.1193	R1 = 0.0511, wR2 = 0.1065
Largest diff. peak and hole	0.413 and -0.348 e.Å^{-3}	0.380 and -0.255 e.Å^{-3}

2.4 Lipophilicity

Lipophilicity was determined by reverse phase thin layer chromatography, using ALUGRAM® RP-18W/UV254 plates. Solutions of the complexes were prepared in methanol. Samples were run in methanol: aqueous buffer (Tris pH=7.4 buffer) 9:1. UV-light was used to reveal the complexes. Caffeine was used as a control in all runs. Lipophilicity is expressed as R_M (where R_M=log10[(1/Rf)-1]).

2.5 Cytotoxic activity studies

Cell lines were obtained from the American Type Culture Collection (ATCC): HeLa (CCL-2™, human cervical adenocarcinoma), MDA MB 231 (HTB-22™, human metastatic breast adenocarcinoma) and A549 (CCL185 ™, human lung epithelial carcinoma). HeLa cells were grown in high glucose (4.5 g/L) Dulbecco's Modified Eagle Medium (DMEM), A549 and MDA-MB-231cells were grown in RPMI Medium containing stable L-Glutamine and supplemented with 10% Fetal Bovine Serum (FBS, GE Healthcare). Cells were removed enzymatically from flasks using 0.01% Trypsin-EDTA solution. Cultured cells were incubated at 37°C on 5% CO_2 atmosphere for 48 h, containing aqueous solutions of the Cu complexes (final concentrations ranging from 1 to 50 μM). Cell viability in response to the complexes was determined by colorimetric assay using Cell Counting Kit – 8 (Fulka) on 15.000 cells grown in 96-well plates. The kit utilizes a water-soluble tetrazolium salt that is reduced by cells to give a colored product (formazan). Therefore, formazan concentration is directly proportional to the number of living cells. Absorbance of

converted dye was measured at 450 nm using a microplate reader. The IC_{50} was estimated from the semi logarithmic dose-response curves. Cu-complexes solutions were prepared by dissolving the corresponding solid complexes, in DMSO and then sterilized by filtration.

3 Results

3.1 Crystal structures

In both complexes, the Cu(I) ion is in the centre of a distorted tetrahedral coordination polyhedron, bonded to two N atoms from phen or neocuproine, one P from the phosphine and one Cl^- (Figures 1 and 2). Bonding distances and angles are similar to those reported for related compounds as confirmed by statistical analysis using Mogul [49]. Selected bond lengths and angles are presented in Table 2.

Figure 1. ORTEP representation of the asymmetric unit of $[CuCl(phen)(PPh_3)]\cdot 0{,}25H_2O$ **(2)**.

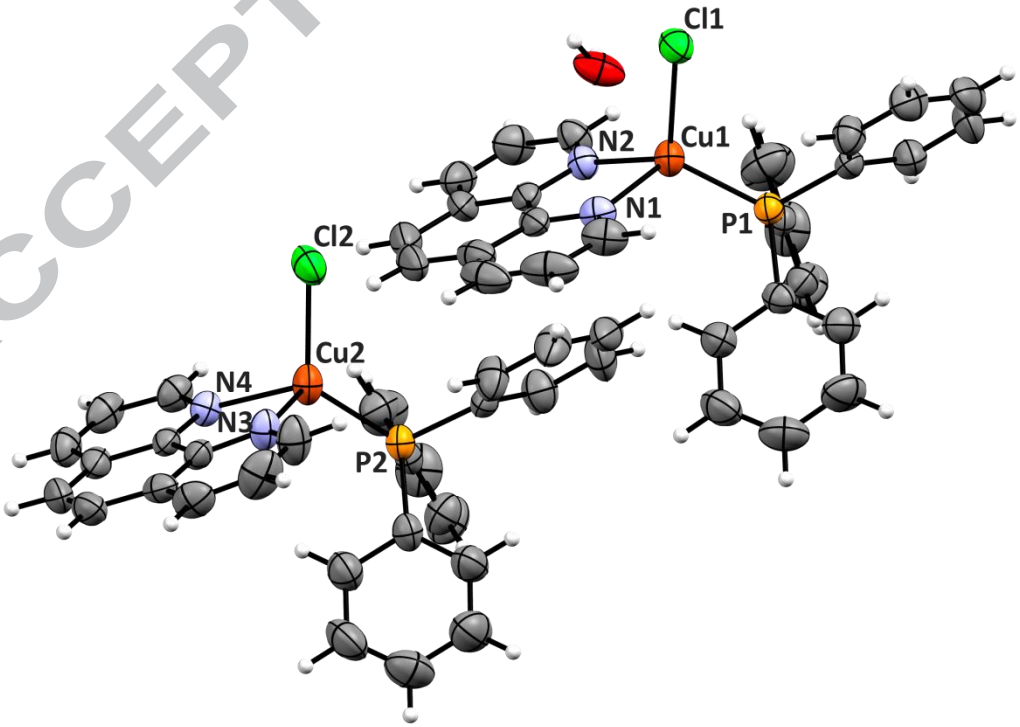

Figure 2. ORTEP representation of the asymmetric unit of [CuCl(neocuproine)(PPh_3)] (**3**).

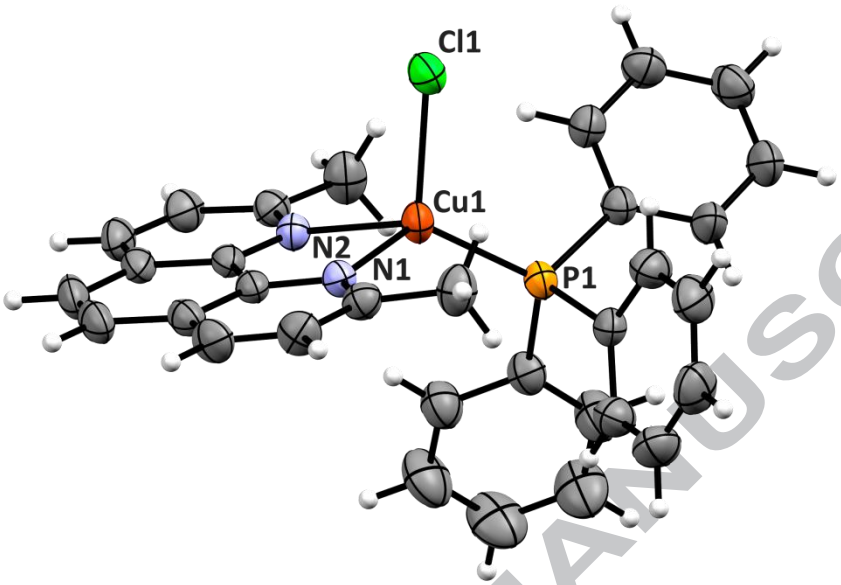

Table 2. Selected bond distances (Å) and angles (°) for [CuClphenPPh_3] and [CuClneoPPh_3].

[CuClphenPPh_3]				[CuClneoPPh_3]	
Bond distances					
Cu1-N1	2.086(3)	Cu2-N3	2.102(3)	Cu1-N1	2.137(2)
Cu1-N2	2.091(3)	Cu2-N4	2.094(3)	Cu1-N2	2.115(2)
Cu1-P1	2.1813(11)	Cu2-P1	2.1902(11)	Cu1-P1	2.2070(8)
Cu1-Cl1	2.2998(11)	Cu2-Cl2	2.2713(13)	Cu1-Cl1	2.3258(8)
Bond angles					
N1-Cu1-N2	80.34(13)	N3-Cu2-N4	79.77(12)	N1-Cu1-N2	79.34(8)
N1-Cu1-P1	118.65(9)	N3-Cu2-P2	111.45(9)	N1-Cu1-P1	124.99(6)
N2-Cu1-P1	120.39(9)	N4-Cu2-P2	121.78(9)	N2-Cu1-P1	123.61(6)
N1-Cu1-CL1	106.53(9)	N3-Cu2-Cl2	109.15(10)	N1-Cu1-Cl1	103.60(6)
N2-Cu1-CL1	108.38(9)	N4-Cu2-Cl2	105.15(9)	N2-Cu1-Cl1	103.71(6)
P1-Cu1-CL1	116.68(4)	P2-Cu2-Cl2	121.61(4)	P1-Cu1-Cl1	115.07(3)

The distortion of the coordination tetrahedron was analyzed using the α, β and δ angles, as defined by Starosta *et. al* [23] and explained in Fig. 3.

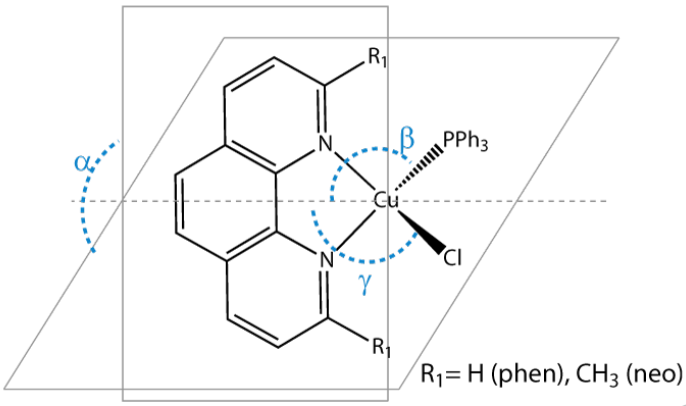

Figure 3. Definition of α, β and δ angles as tetrahedral distortion evaluation parameters, where α is the angle between the di-imine plane and the plane defined by the CuCl and CuP bonds. For a perfect tetrahedron α = 90°, and deviation from this value is considered a flattening distortion. β and δ are the angles between the line of intersection of the former planes and the Cu-Cl and Cu-P bonds, respectively. The difference β - δ for a perfect tetrahedron is 0°, deviations are considered a rocking distortion [23].

Values of α, β and δ angles for the presented structures are presented in Table 3.

Table 3. α, β and δ angles as defined in Fig. 3.

Compound	α (°)	β (°)	δ (°)
2 (Cu1/Cu2)	88,33/82,31	131,02/112,84	112,30/125,45
3	89,49	146,21	98,72

From the parameters listed in Table 3 it can be inferred that neither **2** nor **3** present significant flattening distortion. However, the addition of the methyl groups in the 2 and 9 positions of the phenanthroline in **3** results in an increased rocking distortion. Intramolecular C-H···π interactions (distance 2,70 Å) and a weak C-H···Cl (distance 3.11 Å) hydrogen bond constrain the tetrahedral geometry in **3**. As a consequence, **3** presents only one molecule per asymmetric unit as opposed to two molecules in **2**.

Crystal packing in **2** presents a unidimensional chain along the a axis with molecules bound through O-H···Cl (H···Cl distance 2.35 Å), C-H···O (H···O distance 2.53 Å)

and C-H···Cl (H···Cl distances 2.86-2.92 Å) hydrogen bonds, as well as, C-H···π interactions between the phenyl groups in the triphenylphoshpine ligand. These chains are arranged in an antiparallel fashion bound by π stacking through the phen moiety (interplanar distance 3.5 Å). Even though the same intermolecular interactions are observed for **3**, the packing diagram in this case shows intercalated zig zag chains, with C-H···Cl (H···Cl distances 2,87-3,28 Å) hydrogen bonding and weak π stacking (interplanar distance 3.7 Å) within and C-H···π (H···π distance 3.09 Å) bonds between adjacent chains.

Hirshfeld surfaces and their corresponding 2D fingerprint plots were constructed using CrystalExplorer software [50] and used to interpret intermolecular interactions semiquantitatively [51, 52]. Fingerprint plots are included as supplementary material. The shape of the fingerprint plots confirms the presence of the interactions discussed above. It is worth highlighting that the most polar intermolecular bonds found in these structures are non classic hydrogen bonds. Particularly, in the fingerprint plot H···Cl contacts correspond to 10.5% and 8.9% of the total contacts in **2** and **3**, respectively. In **2** there is also a contribution of 2.3% due to H···O contacts. Being **2** the structure with the most and the shorter polar contacts, it could be expected to be more polar than **3**.

3.2 Infrared spectra

The infrared spectra of the complexes present similar features, as shown in figure 4. Vibrations in the range of 2900-3500 cm^{-1} are attributed to C-H stretching vibrations in the ligands for all complexes. Other distinctive bands, like the ones due to C-N, C-C stretching and C-H rocking appear around 1585 and 1150-1260 cm^{-1}, respectively [53]. Absorption bands typical of the triphenylphosphine ligand appear in the range 500-525 cm^{-1} [54]. These common features of the infrared spectra of the complexes suggest that **1** presents a coordination scheme similar to that observed for **2** and **3**.

Figure 4. Infrared Spectra of **1**, **2** and **3**.

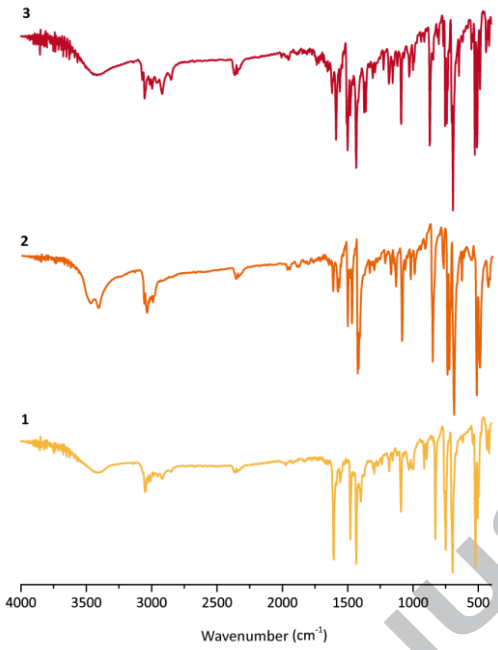

Table 4. Wavenumber (cm^{-1}) of representative absorption bands in the complexes, as well as their tentative assignment.

	v (O–H)	v (C–H)	v (C–C), v (C–N)	v(P-C), ρ(C-H)	ρ(C-H), δ(C–C–C), δ(C–C–N)
1		3428	1585	1091	851
2	3476	3414	1584	1092	858
3		3431	1588	1090	845

3.3 Stability of the Cu(I) compounds in solution.

EPR was used to check whether copper compounds remained as Cu(I) or were rapidly oxidized to Cu(II) in solution. DMSO solutions present no EPR signal, even after several weeks. As an approach to biological studies conditions, water was added to the DMSO solution and the EPR spectrum was repeatedly registered during 48h. Compounds **1** and **2** show a slow apparition of the characteristic Cu(II) signal in 48 h, meanwhile compound **3** shows no EPR signal in 48 hours. Therefore Cu remains mainly in the Cu (I) oxidation state in DMSO/water solution in the biological assays time frame. EPR spectra of the solutions are included as supplementary material.

3.4 Lipophilicity

Lipophilicity is a relevant parameter as it influences the pharmacodinamic of a drug, specially its ability to permeate cellular membranes. Table 5 shows the lipophilicity (expressed as R_M) of the complexes. All complexes are more lipophilic than both ligands. This fact is in agreement with the observed coordination, where the hydrophilic groups are bonded to the Cu(I) and hindered from the solution. The lipophilicity of the complexes follows the same order found for the di-imine ligand.

Table 5.

Compound	R_M
PPh3	-0,09
dmb	-0,25
phen	-0,19
neo	-0,14
[CuCl(PPh_3)(dmb)]	0,27
[CuCl(PPh_3)(phen)]	0,30
[CuCl(PPh_3)(neo)]	0,45

3.5 Cytotoxic activity

Table 6 presents the IC_{50} values for the studied complexes. All the complexes present higher cytotoxic activity (lower IC_{50}) on HeLa, MDA MB 231 and A459 cells than Cisplatin and according to *Santini et al*.[1] may be classified as very active (IC_{50} in the low micromolar range). The cytotoxic activity increases in the same order as observed for the lipophilicity, being [CuCl(neo)(PPh_3)] the most lipophilic and active. This observation is in agreement with the hypothesis that the complexes act as "copper ionophores" that deliver copper inside the cell [10, 55].

In other series of Cu(II) mixed ligand complexes containing di-imines, a similar relation between the aromatic di-imine ligand and the cytotoxic activity was observed, being the Cu-neocuproine-coligand system the most active of the series [56]. This highlights the pharmacological potential of the Cu-neocuproine moiety.

With relation to the mechanisms underlying the biological activity of Cu-complexes they are, to the date, not completely known [11, 57]. Taken into account our results and the information in the literature it can be proposed that the cytotoxic activity of Cu-di-imine -phosphine complexes is due to:

- DNA interaction in an alternative way than observed for Cisplatin. We have previously study several Cu-phen containing complexes that intercalate isolated DNA though the di-imine moiety [40, 41] . It has been also observed in several Cu-phen-derivatives containing complexes [1].
- Generation of reactive oxygen species (ROS) which oxidize and degrade DNA [58]. ROS production in vitro has been observed for related complexes. Our complexes are likely to degrade DNA. We tried to determine its DNA binding but this measurement was hampered as we systematically had evidence of DNA degradation (data not published).
- Mitochondrial toxicity as observed in the literature for related Cu-complexes [59].
- Cellular death by an apoptotic mechanism induced by the molecular events previously described as observed for Cu-bi-imine complexes [1, 59]. In spite of that cellular death by a parapoptotic mechanism can´t be ruled out as it has been reported for Cu-phosphine complexes [9, 10].

Table 6. Cytotoxic activity (expressed by IC_{50}) of the studied complexes against HeLa (human cervical adenocarcinoma), MDA MB 231 (human metastatic breast adenocarcinoma) and A459 (human lung epithelial carcinoma) cell lines.

Compound	IC_{50} HeLa (μM)	IC_{50} MDA MDB 231(μM)	IC_{50} A459

			(μM)
[CuCl(dmbpy)(PPh_3)]	4	8.4	4.5
[CuCl(phen)(PPh_3)]·0,25H2O	3.6	4.3	3.5
[CuCl(neo)(PPh_3)]	2.8	1.4	1.3
Cisplatin	30	50	50

Conclusions

Three new Cu(I)-complexes were prepared an characterized, including two new crystal structures. Complex [CuCl(neo)(PPh_3)] is particularly redox-stable in DMSO/water solution.

The complexes present high cytotoxic activity on the studied tumor cells, being the [CuCl(neo)(PPh_3)] the most active.

Supplementary material

Supplementary crystallographic data can be obtained free of charge from The Cambridge Crystallographic Data on request, quoting deposit numbers: CCDC 1479605 (C2), and CCDC 1479606 (C3). Fingerprint plots and EPR spectra are presented in Figs. S1 and S2 respectively.

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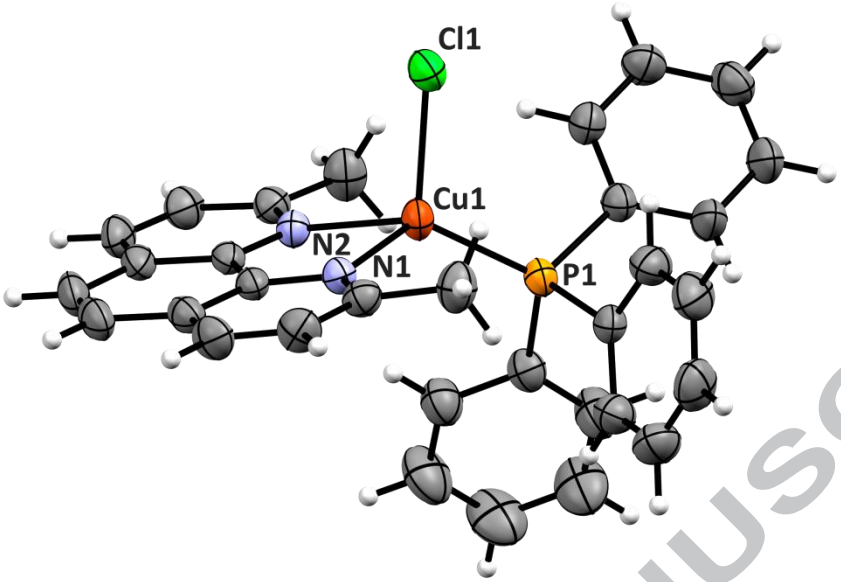

To find new leaders for the development of antitumor Cu compounds, heteroleptic Cu(I) complexes with triphenylphosphine (PPh_3) and dimethylbipyridine (dmbpy), phenanthroline (phen) or neocuproine (neo) were prepared and characterized. The crystal structures of compounds **2** and **3** were determined. [CuCl(neo)(PPh_3)] (figure) presents high cytotoxic activity against tumor cell lines, being more active than Cisplatin.

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