New copper-based complexes with quinoxaline N-1,N-4-dioxide derivatives, potential antitumoral agents

ARTICLE *in* JOURNAL OF INORGANIC BIOCHEMISTRY · FEBRUARY 2008

Impact Factor: 3.44 · DOI: 10.1016/j.jinorgbio.2007.07.028 · Source: PubMed

10 AUTHORS, INCLUDING:

Hugo Cerecetto
University of the Republic, Uruguay
235 PUBLICATIONS **4,116** CITATIONS
SEE PROFILE

Mercedes Gonzalez
University of the Republic, Uruguay
234 PUBLICATIONS **4,104** CITATIONS
SEE PROFILE

Antonio J Costa-Filho
University of São Paulo
83 PUBLICATIONS **998** CITATIONS
SEE PROFILE

María Torre
University of the Republic, Uruguay
71 PUBLICATIONS **867** CITATIONS
SEE PROFILE

Available online at www.sciencedirect.com
ScienceDirect
Journal of Inorganic Biochemistry 102 (2008) 119–126
Journal of Inorganic Biochemistry
www.elsevier.com/locate/jinorgbio

New copper-based complexes with quinoxaline N^1,N^4-dioxide derivatives, potential antitumoral agents

Carolina Urquiola [a], Dinorah Gambino [a], Mauricio Cabrera [b], María Laura Lavaggi [b], Hugo Cerecetto [b], Mercedes González [b], Adela López de Cerain [c], Antonio Monge [c], Antonio J. Costa-Filho [d], María H. Torre [a,*]

[a] *Cátedra de Química Inorgánica, Facultad de Química, Universidad de la República, Gral Flores 2124, C.C. 1157, 11800 Montevideo, Uruguay*
[b] *Departamento de Química Orgánica, Facultad de Química – Facultad de Ciencias, Universidad de la República, Iguá 4225, 11400 Montevideo, Uruguay*
[c] *CIFA, Universidad de Navarra, Pamplona, Spain*
[d] *Instituto de Física, Universidade de São Paulo, C.P. 369, 135600 São Carlos, Brazil*

Received 11 April 2007; received in revised form 12 July 2007; accepted 20 July 2007
Available online 1 August 2007

Abstract

Taking into account our previous studies on cytotoxic metal compounds, new copper complexes with 3-aminoquinoxaline-2-carbonitrile N^1,N^4-dioxide derivatives as ligands were synthesized and characterized by different spectroscopic methods. The hypoxic selective cytotoxicity towards V79 cells and the superoxide dismutase-like activity of the complexes were determined and related to physicochemical properties of the compounds. In particular, the copper(II) complex with 3-amino-6-chloro-7-fluoroquinoxaline-2-carbonitrile N^1,N^4-dioxide showed cytotoxic selectivity in hypoxia being the most lipophilic compound of the series. On the contrary, the complex with 3-aminoquinoxaline-2-carbonitrile N^1,N^4-dioxide was cytotoxic but not selective and that with 3-amino-7-chloro-6-methoxy-quinoxaline-2-carbonitrile N^1,N^4-dioxide was not cytotoxic towards V79 cells neither in oxia nor in hypoxia in the assayed conditions. The σ_m Hammett substituent electronic descriptor was related to the effect in hypoxic conditions and the SOD-like activity was correlated to the effect in normoxia.

Keywords: Copper complexes; Quinoxaline N^1,N^4-dioxides; Hypoxia selective cytotoxins; Antitumor agents

1. Introduction

One general strategy for the research on new anticancer agents has been the therapeutic use of metal containing compounds [1,2]. Specially, in the search of less toxic metal-based antineoplastic drugs, essential metal complexes such as copper-based drugs have been developed [3].

The cytotoxicity of these complexes can be explained by different mechanisms of action. In particular, the report by Oberley and Buettner showed that cancer cells have a less than normal superoxide dismutase (SOD) activity and the treatment with bovine native Cu-SOD decreased the growth of several solid tumors [4]. As a consequence a variety of copper complexes presenting SOD-like activity were examined for anticancer activity [3]. For instance, [Cu(II)(3,5-diisopropyl salicylate)$_2$] shows important SOD-like activity and can be used for the differentiation of cultured cancer cells from normal cells at concentrations that do not cause cell death [5,6]. Besides, it has been reported that complexes with SOD-mimetic action activate H_2O_2 to give reactive radical species such as hydroxyl radical ($OH^{\cdot}$) that present high biocidal potency [7,8].

On the other hand, copper(II) complexes with amino acids [9], casiopeines [10], oligopeptides [11,12], mono- and bis-thiosemicarbazones [5,13], among others, have

* Corresponding author. Tel.: +598 2 9249739; fax: +598 2 9241906.
E-mail address: mtorre@fq.edu.uy (M.H. Torre).

0162-0134/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.jinorgbio.2007.07.028

been reported to be effective cytotoxins in tumor models as well as in tumor cell cultures.

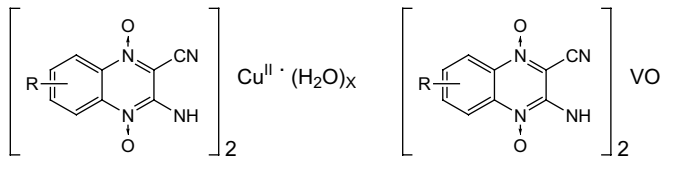
Most of these copper complexes have biologically inactive ligands but several of them contain bioactive organic compounds that act in a different way. Another advantage of using copper ion as central metal is the possibility of employing radioactive isotopes which can provide an additional antitumoral mechanism of action [14].

Looking for new strategies for the design of cytotoxic molecules, well known bioreductive compounds that are selectively activated under hypoxic conditions by cellular reductases were developed. The hypoxic cells in solid tumors live in a more favorable environment for reductive reactions than those which are well oxygenated. In these conditions bioreductive prodrugs would be activated through a reductive mechanism in the absence of oxygen and could become significantly more toxic for hypoxic cells than for well-oxygenated ones. In particular, several heterocyclic *N*-oxides and related compounds such as quinoxaline *N*-dioxide derivatives can be reduced in hypoxic conditions forming cytotoxic species that have the DNA as main biological target [15–17].

As part of our research program involving metal complexes with potential activity as chemotherapeutics, we have previously developed new copper(II) and vanadyl complexes as selective hypoxic cytotoxins (Fig. 1) trying to improve the bioavailability and the pharmacological and toxicological properties of the 3-aminoquinoxaline-2-carbonitrile N^1,N^4-dioxide derivatives, ligands which have proved to act as bioreductive compounds [18–20].

In these first series, the cytotoxicity in hypoxia of these complexes was similar or slightly better than that of the free ligands whereas under well-oxygenated conditions the complexes were almost non cytotoxic [20,21].

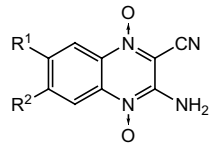
Taking into account these antecedents, in this work we describe the development of a new series of Cu(II) complexes but with non- and di-substituted 3-aminoquinoxaline-2-carbonitrile N^1,N^4-dioxide derivatives, trying to improve bioactive profile of these hypoxia selective cytotoxins and to get a metal–ligand synergism. Besides, we present the SOD-like activity of the complexes with the aim of detecting potential correlations with cytotoxicity in normoxia. Consequently, we performed the synthesis, analytical and spectroscopic characterization, and biochemical and biological evaluation of three new Cu(II) complexes of general formulae $[Cu^{II}(H_2O)_x(L\text{-}H)_2]$, where L are the 3-aminoquinoxaline-2-carbonitrile N^1,N^4-dioxide derivatives shown in Fig. 2 and L–H the deprotonated ligands.



1, R=Cl, x=1
2, R=Br, x=0
3, R=Me, x=2

4, R=Cl
5, R=Br
6, R=Me

Fig. 1. Hypoxia selective metal compounds with quinoxaline N^1,N^4-dioxide derivatives where one of the aryl substituent is a H atom and the other is those specified in the figure.



L1, -R^1=-R^2= -H
L2, -R^1= -F, -R^2= -Cl
L3, -R^1= -Cl, -R^2= -OMe

Fig. 2. 3-aminoquinoxaline-2-carbonitrile N^1,N^4-dioxides used as ligands.

2. Experimental

2.1. Synthesis

All starting materials were commercially available research-grade chemicals and were used without further purification.

Each ligand (L) (0.15 mmol) was sonicated in dried ethanol until total dissolution. To the resulting solution 20.0 mL of an ethanolic solution of $CuSO_4 \cdot 5H_2O$ (0.075 mmol) were added. After stirring for half an hour, a purple precipitate was formed, then filtered, washed with small portions of ethanol and dried at room temperature.

The stoichiometries of the compounds were $[Cu(L1\text{–}H)_2]$ (code Cu–L1), $[Cu(L2\text{–}H)_2]$ (code Cu–L2) and $[Cu(L3\text{–}H)_2(H_2O)]$ (code Cu–L3) (L–H are the deprotonated ligands) and the yields were 53.2%, 41.2% and 39.4%, respectively. The results of the elemental analysis (%) obtained with a Carlo Erba EA 1108 analyzer were: Cu–L1 ($C_{18}N_8H_{10}O_4Cu$) Found/Calc.: C, 46.17/46.41; N, 23.94/24.05; H, 2.49/2.16; Cu–L2 ($C_{18}H_6F_2Cl_2N_8O_4Cu$) Found/Calc.: C, 37.98/37.88; N, 19.35/19.63; H, 1.08/1.06; Cu–L3 ($C_{20}N_8H_{14}O_7Cl_2Cu$) Found/Calc.: C, 39.62/39.18; N, 17.92/18.28; H, 1.96/2.29. IR (KBr) (ν/cm^{-1} for Cu–L1, Cu–L2 and Cu–L3, respectively): 3386, 3362 and 3386 (N–H); 1575 and 1593, 1573 and 1595, and 1562 and 1584 (C=N → O), 2221, 2231 and 2228 (C≡N). For Cu–L3: 1340 (N → O).

2.2. Conductimetric and thermogravimetric measurements

Conductimetric measurements using a Conductivity Meter 4310 Jenway were performed at 25 °C in a DMF solution (10^{-4} M), solvent that allow to determine the electrolyte type of conducting complex (number of ions per molecule of complex) [22] and in a DMSO solution (10^{-4} M) for studying the stability of the complexes in the solvent used in the cytotoxic studies.

Thermogravimetric measurements were obtained with a Shimadzu TGA 50 thermobalance, with a platinum cell, working under flowing air (50 mL min^{-1}) and at a heating rate of 6 °C min^{-1} and 0.5 °C min^{-1}.

2.3. Spectroscopic measurements

Low-temperature (120 K) EPR experiments were performed using a Varian E109 spectrometer equipped with a rectangular cavity. To perform the experiments on solution-state samples, powder samples of the titled complexes were dissolved in DMSO. The solution-state sample was then centrifuged, and the supernatant was drawn in a quartz sample tube for low-temperature EPR experiments. General experimental conditions were: microwave power, 40 mW; modulation amplitude, 4.0 G; modulation frequency, 100 kHz. The *g*-values were obtained by means of spectral simulation using the software package EasySpin [23].

IR spectra, in the range between 4000 and 200 cm^{-1}, were recorded on a Bomem M 102 FTIR spectrophotometer using the KBr pellet technique. Electronic spectra of the complexes were registered on a Shimadzu UV-1603 spectrophotometer in nujol suspension and DMSO solution.

Routine FAB+ spectra of the metal complexes have been measured with a TSQ spectrometer (Finnigan) with nitrobenzylalcohol as matrix. The ion gun was operated at 8 kV and 100 μA (probe temperature: 30 °C). Xenon was used as primary beam gas.

2.4. Lipophilicity test

Lipophilicity tests were performed determining the partition coefficient between *n*-octanol and physiological solution [24]. The concentration of the compounds in the physiological solution before and after the contact with *n*-octanol was determined by atomic absorption spectrometry using a Perkin Elmer 5000 equipment with an iron Photron lamp, $\lambda = 324.8$ nm and acetylene/air flame. All tests were run in duplicate.

2.5. Stability test

Thin layer chromatography was performed on precoated silica gel 60 F 254 TLC plates (Aldrich). The copper complexes were dissolved in DMSO and diluted with phosphate buffer (NaH_2PO_4, 50 mM), pH = 7.4 at 37 °C until the concentration reached 10^{-4} M (1% DMSO). These solutions were kept at room temperature during 24 h before application on the plates. The elution was performed with chloroform for Cu–L1, chloroform:methanol (90:10, v/v) for Cu–L2, and dichloromethane:methanol (95:5, v/v) for Cu–L3 and the spots were located under UV light and compared with those of the free ligands.

2.6. Cytotoxic studies

The complexes were tested for cytotoxicity to V79 cells (Chinese hamster lung fibroblasts) under hypoxic and aerobic conditions using a cloning assay, at a single dose, as previously described [20,21,25–28].

The cells were obtained from ECACC (European Collection of Animal Cell Cultures) and maintained in logarithmic growth as subconfluent monolayers by trypsinization and subculture to $(1–2) \times 10^4$ cells/cm^2. These operations were performed twice weekly. The growth medium was EMEM (Eagle's Minimal Essential Medium), containing 10% (v/v) fetal bovine serum (FBS) and penicillin/streptomycin at 100 U/100 μg/mL. Monolayers of V79 cells were trypsinized, and suspension cultures were set up in 50 mL glass flasks: 2×10^4 cells/mL in 30 mL of EMEM containing 10% (v/v) FBS and HEPES (10 mM). The glass flasks were submerged and stirred in a water bath at 37 °C, where they were gassed with humidified air or pure nitrogen. Compound solutions were prepared just before dosing. Stock solutions, 150-fold more concentrated, were prepared in pure DMSO (Aldrich) or sterilized distilled water. Thirty min after the start of gassing, 0.2 mL of the stock compound solution was added to each flask (two flasks per dose). In every assay there was one flask with 0.2 mL of DMSO (negative control) and another with 7-chloro-3-[3-(*N,N*-dimethylamino)propylamino]-2-quinoxalinecarbonitrile 1,4-dioxide hydrochloride (positive control). After 2 h exposure to the compound, the cells were centrifuged and resuspended in plating medium (EMEM plus 10% (v/v) FBS and penicillin/streptomycin). Cell numbers were determined with a haemocytometer and 10^2–10^3 cells were plated in 6-well plates to give a final volume of 2 mL/30 mm of well. Plates were incubated at 37 °C in 5% CO_2 for 7 days and then stained with aqueous crystal violet. Colonies with more than 64 cells were counted. The plating efficiency (PE) was calculated by dividing the number of colonies by the number of cells seeded. The percent of control-cell survival for the compound-treated cultures (SFair and SFhypox) was calculated as $PE_{treated}/PE_{control} \times 100$. The compounds were tested at 20.0 μM in duplicate flasks, both in aerobic and hypoxic conditions.

The compound which gave a low surviving fraction in hypoxia was tested at different doses in normoxia and hypoxia. Two dose–response curves were obtained by plotting the Sfair and Sfhypox at each concentration tested. The potency values P (dose which gives 1% cell survival in hypoxia or normoxia), and the hypoxic cytotoxicity ratio HCR (ratio between doses in air and hypoxia giving the same cell survival (1%)), were calculated.

2.7. Determination of SOD-like activity

Superoxide dismutase (SOD)-like activity was investigated using Beauchamp and Fridovich's method – as improved by Imanari et al. [29]. This method is based on the inhibitory effect of SOD on the reduction of nitrobluetetrazolium (NBT) by the superoxide anion generated by the xanthine/xanthine oxidase system. The solutions of the complexes were prepared in DMSO before adding the carbonate–bicarbonate buffer (Na_2CO_3, 33 mM; $NaHCO_3$, 17 mM), pH = 10.2 at 37 °C. For comparative purposes, the activity of native superoxide dismutase from bovine erythrocytes has also been determined. All the reagents used in this assay were purchased from SIGMA.

3. Results and discussion

3.1. Spectroscopic measurements

3.1.1. EPR spectra

X-band (9.1 GHz) EPR spectra of Cu–L1, Cu–L2, and Cu–L3 in polycrystalline samples at 120 K are shown in Fig. 3. These spectra are characteristic of exchange coupled Cu(II) pairs showing, besides the usual resonance around 320 mT ($g \sim 2$), an extra peak at ca. 160 mT (see insets in Fig. 3), which is assigned to the so-called half-field transition. The latter corresponds to $\Delta M_s = 2$ transition between the $M_s = -1$ and $M_s = +1$ states of the triplet ($S = 1$) in a system containing two $S = 1/2$ spins [30]. The absence of a hyperfine structure, due to the interaction of the electron spin $S = 1/2$ with the nuclear spin $I = 3/2$ of Cu(II) ions, is also indicative of an exchange coupled regime between the metal ions. The g-values determined from spectral simulations (Fig. 3) indicate a slightly distorted axial symmetry and a similar structural environment around the Cu(II) center in all complexes. Besides, linewidhts are somewhat larger for Cu–L3. This increase in the widths leads to broad lines and thus lower resolution for the shoulder around 300 mT, which cannot be distinguished from the resonance at ca. 315 mT (g_x and g_y) for Cu–L3 complex.

The low-temperature EPR spectra of the complexes dissolved in DMSO were also measured (Fig. 4). In all cases, a feature worth mentioning is the lack of the resonance associated with the half-field transition (around 160 mT – see inset in Fig. 4). This is indicative that in the solution state the complexes exist as monomers as previously observed for other copper compounds [21,31]. Simulations of the solution-state spectra yielded somewhat different g-values for Cu–L1 ($g_x = 2.035$, $g_y = 2.105$, $g_z = 2.300$) when compared to the values obtained from the solid-state sample. There is a change in the local symmetry experienced by the paramagnetic center that is now fully rhombic for the Cu–L1 complex in the solution state. This is probably due to the addition of DMSO in the copper environment. The g-values calculated for compounds Cu–L2 and Cu–L3 were approximately equal in both solid- and solution-state (Cu–L2: $g_x = 2.050$, $g_y = 2.055$, $g_z = 2.208$; Cu–L3: $g_x = 2.050$, $g_y = 2.050$, $g_z = 2.250$) indicating similar local environments around the copper ions in these cases. The main difference found in the solid-state as compared to the solution-state EPR spectra of Cu–L2 and Cu–L3 resided in the narrow linewidths needed to achieve a reasonable fit of the experimental data in the solution-state. This is expected if, upon dilution, we change from an intermediate exchange coupling regime, which exists in solid-state and leads to a broadening phenomenon, to a situation where the copper ions are no longer coupled to each other

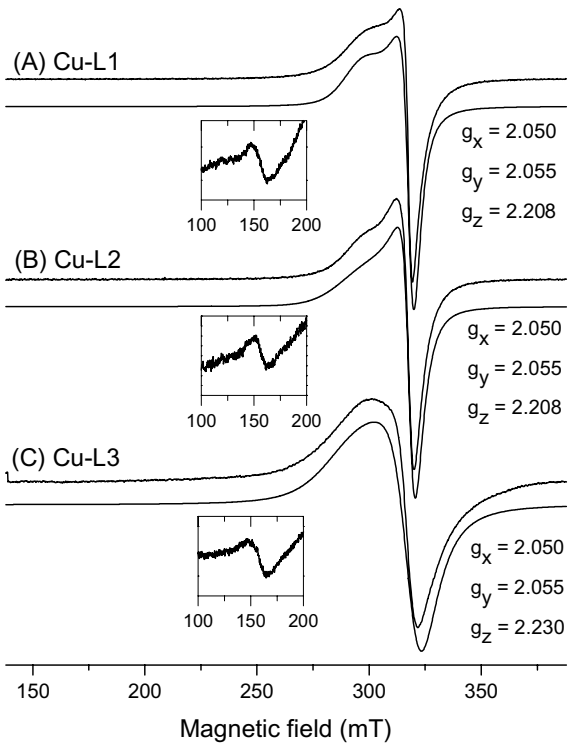


Fig. 3. X-band EPR spectra (upper traces) of polycrystalline sample of Cu–L1, Cu–L2 and Cu–L3 and their respective simulations (lower traces) obtained using the EasySpin software. The insets show the resonance associated with the half-field transition (see text for discussion). The g-values calculated from the simulations are also shown.

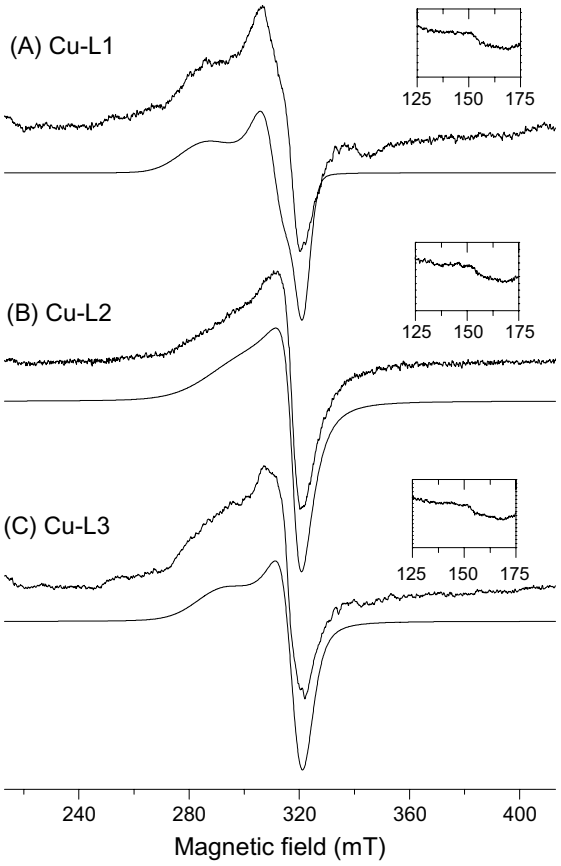


Fig. 4. X-band EPR spectra (upper traces) of frozen solution samples of Cu–L1, Cu–L2 and Cu–L3 and their respective simulations (lower traces) obtained using the EasySpin software.

as suggested by the lack of the half-field transition in the insets of Fig. 4. The same behavior concerning the linewidths is also observed for Cu–L1 complex.

3.1.2. Infrared spectra

The IR spectra of the complexes were compared with those of the free ligands and previously reported complexes [18–21,32].

All the complexes showed a similar spectral pattern. After the coordination the ligand bands corresponding to ν_a (NH_2)(In 3353–3380 cm^{-1} range) and ν_s (NH_2)(In 3228–3288 cm^{-1} range) of the amino group disappeared and only one band was observed (3362–3386 cm^{-1}). This is in accordance with the presence of a secondary amine formed by deprotonation of the primary amine as a consequence of the coordination with copper ion. This behavior was previously observed for vanadyl and copper complexes with this family of ligands, and for metal chelates with aromatic ligands involving the amino group in ortho position to the *N*-oxide [18–21,33]. Furthermore, the strong $\nu(N \rightarrow O)$ band for the free ligands (1338–1350 cm^{-1}) turned weak upon complexation for Cu–L3 and shifted in the case of Cu–L1 and Cu–L2. In the first case this behavior could be explained by the coordination of one $N \rightarrow O$ group per ligand molecule keeping the other uncoordinated as shown in Fig. 5. In the case of Cu–L1 and Cu–L2 the disappearance in the corresponding range of the strong $\nu(N \rightarrow O)$ band was probably due to the fact that the uncoordinated $N \rightarrow O$ groups have additional interactions with other copper atoms from neighboring molecules, which form intermolecular bonds in the solid state. This behavior was previously observed in related complexes [21]. The $\nu(C{\equiv}N)$ (2221–2238 cm^{-1}) suffered only minor changes upon complexation in agreement with the fact that this group did not coordinate to copper ion.

3.1.3. Electronic spectra

All ligands' nujol spectra showed one band in the 480–490 nm range. Upon coordination, these bands shifted to higher wavelength: Cu–L1 (536 nm), Cu–L2 (526 nm) and Cu–L3 (533 nm). Besides, all the complexes showed a d–d broad band in the 572–582 nm range characteristic of Cu(II) in a distorted octahedral geometry [34]. Possibly, this behavior is due to the fact that in the crystal packing there is another quinoxaline molecule as sixth ligand interacting with the copper ion, in addition to those shown in Fig. 5. This behavior was previously reported for similar complexes [21]. In DMSO solutions these two bands were not resolved and only a very broad band was observed in the range 502–602 nm. This result does not allow us to obtain information about the decomposition of the dimeric unit observed by EPR measurements in solution.

3.1.4. FAB-MS spectra

Each complex had its own fragmentation pattern in FAB-MS spectroscopy; Table 1 collects relevant data that confirmed the existence of ligand–metal species.

Only for Cu–L3 the molecular ion species ($[Cu(L–H)_2]^+$) can be observed showing the corresponding pattern of two chlorine atoms and one copper in the molecule. Besides, it is observed the loss of oxygen, typical behavior for *N*-oxide containing heterocycles. All three complexes present a fragment that corresponds to one copper coordinated to one ligand ($[Cu(L–H)]^+$, as well as another fragment corresponding to the ligand. Furthermore, in the case of Cu–L1 and Cu–L2 some fragments confirm the coordination through the $N \rightarrow O$ group, in accordance with the IR data and the proposed structure shown in Fig. 5.

3.2. Conductimetric and thermogravimetric measurements

Conductimetric measurements in DMF solution of the complexes showed no significant change in the conductivity in comparison with that of the pure solvent. This behavior is in agreement with the neutrality of the complexes, according to the formula assigned. In addition, the conductivity of DMF and DMSO solutions was measured during a week and no major changes were observed in either solvent, showing the stability of the complexes in these solvents.

Thermogravimetric measurements performed with Cu–L3 at different heating rates showed that water release began at 122 °C simultaneously with the rapid degradation of the ligand. The temperature of the water release is in

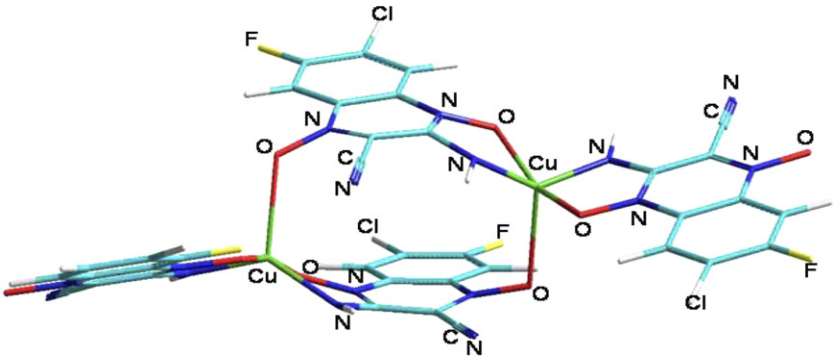


Fig. 5. Proposed three-dimensional structure of dimeric complex Cu–L2 calculated with HyperChem (TM) Professional 7.51.

Table 1
Characteristic fragments observed in FAB-MS spectroscopy of the complexes

Compound	Relative intensity (%)			
	$[Cu(L–H)_2]^+$	$[Cu(L–H)_2–X)]^+$	$[Cu(L–H)]^+$	L^+
Cu–L1	–	–X=CN, O 2.0/1.0	3.0	24.0
Cu–L2	–	–X = F 3.0/4.0/1.4[a] –X=3 O, CN 2.0/1.0/0.5[a]	6.0/3.5/2.5[b]	10.0
Cu–L3	2.0/2.0/0.5[c]	–X=O 2.0/2.0/0.5[c] –X=OCH_3 4.0/4.0/1.5[c] –X=CN, O 8.0/4.0/2.0[c]	9.0/7.0/1.5[b]	15.0/10.0[d]

[a] Pattern for one copper and two chlorines in the ion's structure.
[b] Pattern for one copper and one chlorine in the ion's structure.
[c] Pattern for one copper and two chlorines in the ion's structure.
[d] Pattern for one chlorine in the ion's structure.

agreement with the presence of a coordinated water molecule.

3.3. Stability

The complexes were stable under the experimental conditions. No free ligand spots were observed in any chromatogram of the three complexes. Besides, no major changes on the conductivity of DMSO solutions were observed. For these reasons we assumed that the complexes remain stable during the cytotoxic studies.

3.4. Cytotoxic studies

Table 2 shows the results of the cytotoxic studies for Cu–L1, Cu–L2 and Cu–L3 at 20.0 μM. The three complexes presented significantly different cytotoxic activities and consequently these compounds might act with a different mechanism of action. Cu–L1 is cytotoxic in normoxia and hypoxia, therefore the mode of action is independent from the concentration of oxygen in the cell. On the contrary, Cu–L2 is only cytotoxic in hypoxia, therefore the activity is related to the low level of oxygen concentration. Cu–L3 is inactive in both conditions probably because it might not be able to penetrate through the cell membrane due to its low lipophilicity (see in Section 3.6) or it might be inactive inside the cell.

According to these results, only Cu–L2 presented selectivity in hypoxia at 20.0 μM. For this reason it was tested at different doses in hypoxic conditions. Data for potency in hypoxia (P) and hypoxic cytotoxicity ratio (HCR) for Cu–L2 and the corresponding ligand, L2, are shown in Table 3. The low solubility of Cu–L2 did not allow us to obtain the exact value of potency in normoxia. The potency in normoxia is higher than 40.0 μM while in hypoxic conditions is 20.0 μM.

Although, Cu–L2 showed selective activity in hypoxia it is less potent than the free ligand. Nevertheless, the potency in hypoxia of this complex resulted at least similar to that of Tirapazamine, the first bioreductive cytotoxic drug introduced into clinical trials.

Cu–L2 complex resulted to be as potent as the previously reported Cu(II) complexes **1** and **2** (Fig. 1) ($P_1 = 35.1$, $P_2 = 27.9$ μM, [21]). In addition, the HCR value for this compound is of the same order than the corresponding for related vanadyl complexes **4** and **5** previously described ($HCR_4 = 15$, $HCR_5 = 15$, [20]). In spite of the fact that there are organic molecules with higher cytotoxicity in hypoxia [15], Cu–L2 presents a promising pharmacological profile that could be an interesting starting point for future chemical modifications in order to improve the activity.

Another interesting observation is that the electronic properties of quinoxaline substituents (H, F, Cl, OMe, Fig. 2) could be related to the potency in hypoxia of the complexes bearing selective hypoxic cytotoxicity. Trying

Table 2
Partition coefficient octanol/physiological serum (P), SOD-like activity (IC_{50}), and hypoxic and normoxic cytotoxicity on V79 cells for the new developed copper complexes

Compound	P/log P	IC_{50} (μM)	Sfnormox[c]/SD[d]	Sfhypox[e]/SD[d]
Cu–L1	7.17/0.86	18.0	18/6	1/0
Cu–L2	[a]	89.0	77/10	1/2
Cu–L3	1.25/0.10	>149.0[b]	100/1	100/0

[a] Cu–L2 low physiological serum solubility did not allow determining this value.
[b] The complex Cu–L3 was not soluble in the assayed conditions at higher concentrations than 149.0 μM.
[c] Sfnormox: survival fraction in normoxia.
[d] SD: standard deviation.
[e] Sfhypox: survival fraction in hypoxia.

Table 3
Data of potency in hypoxia (P) and hypoxic citotoxicity ratio (HCR) of Cu–L2 and L2

Compound	P (μM)	HCR
L2	2.0	10
Cu–L2	20.0	>2
Tirapazamine[a]	30.0	75

[a] Refs. [20,21,25–28].

to find out correlations between selective activity in hypoxia and structural data, we compared Cu–L2 with complexes **1** and **2** which were also selective in hypoxia. For example, the potency for complexes **1**, **2** and Cu–L2 can be correlated with the sum of Hammett σ_m constants (electronic descriptors of the substituents depending on their donor–acceptor characteristics) [35,36]. The sum of Hammet constants was calculated using $\sigma_{m,Cl} = 0.37$, $\sigma_{m,Br} = 0.39$ and $\sigma_{m,H} = 0.00$ for complexes 1 and 2, and $\sigma_{m,F} = 0.34$ and $\sigma_{p,Cl} = 0.23$ for complex Cu–L2 and taking into account the substituent relative position in the quinoxaline moiety. The $\Sigma\sigma$ are 0.37, 0.39 and 0.57 for complexes 1, 2 and Cu–L2, respectively. As it can be seen, compounds with better hypoxic potency, $P_1 = 35.1$, $P_2 = 27.9$, $P_{Cu\text{-}L2} = 20.0$ μM (lower doses producing 1% survival fraction in hypoxia) have higher σ values due to the halogen substituents in aromatic cycles.

3.5. Lipophilicity test

Table 2 shows the values of the partition coefficient octanol/physiological serum (P) for the three complexes. Cu–L3 presented the lower lipophilicity and it was the less cytotoxic compound. Probably it could not penetrate through the cell membrane. The complex Cu–L1 and Cu–L2 are more lipophilic than Cu–L3 and at the same time more active.

3.6. SOD-like activity

The Cu(II) concentration required to produce a 50% inhibition(IC_{50}) of NBT reduction is shown in Table 2. IC_{50} for Cu–L3 was not exactly determined due to the fact that this complex is insoluble in higher concentrations.

IC_{50} values for Cu–L1 and Cu–L2 were of the same order than those obtained from copper complexes showing pharmacological properties associated with the dismutation of the superoxide radical. For example, pharmacologically active copper(II) complexes with aminoacids [9] showed IC_{50} values in the range 30.3–37.2 μM and Cu(II)-sulfonamides presented values between 38 and 73 μM, all of which were determined with the same experimental technique [37,38]. An interesting aspect is the relationship found between the cytotoxic effect in normoxia and the SOD-like activities (Table 2). The complex Cu–L1 with the lowest IC_{50} presented the highest cytotoxicity in normoxia. This behavior is in accordance with the hypothesis proposed by Ramadan for several copper complexes where the H_2O_2 formed by the dismutation of O_2^- can be related to the production of reactive radical species such as the cytotoxic hydroxyl radical $OH^{\cdot}$. Cu–L2 (good cytotoxic compound in hypoxia) presented lower SOD-like activity and consequently lower activity in normoxia while the Cu–L3 was inactive. Obviously no relationship was observed between hypoxic selectivity and SOD-like activity.

4. Conclusions

New copper complexes with quinoxaline N^1,N^4-dioxide derivatives were synthesized and characterized by different spectroscopic methods and tested for cytotoxicity to V79 cells under hypoxic and aerobic conditions. As the EPR spectra showed the complexes presented dimeric units in solid state and in DMSO they became monomeric species. According to the stability tests the ligands remained coordinated with the copper ion in the DMSO solutions. Although the complexes are similar only Cu–L2 showed cytotoxic selectivity in hypoxia and presented the highest lipophilicity. On the contrary, Cu–L1 was not selective but showed good profile as cytotoxic agent in normoxia, being this effect related to its SOD like activity. Cu–L3 was not cytotoxic in the assayed conditions probably due to its insolubility or its low lipophilicity. In addition, the potency for the hypoxic selective complexes Cu–L2 and the previously reported **1** and **2** could be correlated with the sum of Hammett σ_m constants, showing that compounds with better hypoxic potency have higher σ values due to the halogen substituents in aromatic rings.

Acknowledgements

This work was supported by CHLCC-Uruguay, CSIC and PEDECIBA of Uruguay. We thank Prof. Dr. Ulrich Abram (Institut für Chemie, Anorganische und Analytische Chemie, Freie Universität Berlin) for performing the FAB-MS experiments.

References

[1] G. Natile, M. Coluccia, Coord. Chem. Rev. 216–217 (2001) 383–410.
[2] C. Orvig, M.J. Abrams, Chem. Rev. 99 (1999) 2201–2203.
[3] J.R.J. Sorenson, Biology of Copper Complexes, The Humana Press Inc., Clifton, 1987, pp. 361– 455.
[4] L.W. Oberley, G.R. Buettner, Cancer Res. 39 (1979) 1141–1149.
[5] N.P. Farrell, Uses of Inorganic Chemistry in Medicine, The Royal Society of Chemistry, Cambridge, 1999, pp. 133–153.
[6] J.R.J. Sorenson, Chem. Brit. (1984) 1110–1113.
[7] A.M. Ramadan, J. Inorg. Biochem. 65 (3) (1997) 83–189.
[8] J. Ueda, T. Ozawa, M. Miyazaki, Y. Fujiwara, J. Inorg. Biochem. 55 (1994) 123–130.
[9] E.J. Baran, Acta Farm. Bonaerense 4 (1985) 125–133.
[10] A. Marín-Hernández, I. Gracia-Mora, L. Ruiz-Ramírez, R. Moreno-Sánchez, Biochem. Pharmacol. 65 (2003) 1979–1989.
[11] L. Pickart, M. Thaler, Nature 288 (1980) 715–717.
[12] E. Kimoto, H. Tanaka, J. Gyotoku, F. Morishige, Cancer Res. 43 (2) (1983) 824–828.
[13] M. Baldini, M. Belicchi-Ferrari, F. Bisceglie, P.P. Dall' Aglio, G. Pelosi, S. Pinelli, P. Tarasconi, Inorg. Chem. 43 (2004) 7170–7179.
[14] P.S. Lin, K.C. Ho, J. Nucl. Med. 39 (1998) 677–678.
[15] H. Cerecetto, M. González, Minirev. Med. Chem. 1 (2001) 219–231.
[16] H. Cerecetto, M. González, M.L. Lavaggi, Med. Chem. 2 (2006) 315–327.
[17] B. Ganley, G. Chowdhury, J. Bhansali, J.S. Daniels, K.S. Gates, Bioorg. Med. Chem. 9 (2001) 2395–2401.
[18] C. Urquiola, M. Vieites, G. Aguirre, A. Marín, B. Solano, G. Arrambide, P. Noblía, M.L. Lavaggi, M.H. Torre, M. González, A.

Monge, D. Gambino, H. Cerecetto, Bioorg. Med. Chem 14 (2006) 5503–5509.
[19] P. Noblía, M. Vieites, M.H. Torre, A.J.C. Filho, H. Cerecetto, M. González, M.L. Lavaggi, Y. Adachi, H. Sakurai, D. Gambino, J. Inorg. Biochem. 100 (2006) 281–287.
[20] M. Vieites, P. Noblía, M.H. Torre, H. Cerecetto, M. González, M.L. Lavaggi, A.J.C. Filho, A.L. de Cerain, A. Monge, B. Parajón-Costa, D. Gambino, J. Inorg. Biochem, in press, doi: 10.1016/j.jinorgbio.2006.03.012.
[21] M.H. Torre, D. Gambino, J. Araujo, H. Cerecetto, M. González, M.L. Lavaggi, A. Azqueta, A.L. de Cerain, A.M. Vega, U. Abram, A.J. Costa-Filho, Eur. J. Med. Chem. 40 (2005) 473–480.
[22] W.J. Geary, Coord. Chem. Rev. 7 (1971) 81–122.
[23] S. Stoll, A. Schweiger, J. Magn. Reson. 178 (2006) 42–55.
[24] A. Leo, C. Hansch, D. Elkins, J. Chem. Soc. 71 (1971) 525–554.
[25] A. Monge, J.A. Palop, A.L. de Ceráin, V. Senador, F.J. Martínez-Crespo, Y. Sáinz, S. Narro, E. García, C.D. Miguel, M. González, E. Hamilton, A.J. Barker, E.D. Clarke, D.T. Greenhow, J. Med. Chem. 38 (1995) 1786–1792.
[26] A. Monge, F.J. Martínez-Crespo, A.L. de Ceráin, J.A. Palop, S. Narro, V. Senador, A. Marín, Y. Sáinz, M. González, E. Hamilton, A.J. Barker, J. Med. Chem. 38 (1995) 4488–4494.
[27] M. Boiani, H. Cerecetto, M. González, M. Risso, C. Olea-Azar, O. Ezpeleta, A.L. de Ceráin, A. Monge, Eur. J. Med. Chem. 36 (2001) 771–782.
[28] H. Cerecetto, M. González, M.L. Lavaggi, A. Azqueta, A.L. de Cerain, A. Monge, J. Med. Chem. 48 (2005) 21–23.
[29] Y. Iwamoto, J. Mifuchi, Chem. Pharm. Bull. 30 (1982) 237–241.
[30] A. Bencini, D. Gatteschi, EPR of Exchange Coupled System, Springer-Verlag, Berlin, 1990.
[31] G. Facchin, M.H. Torre, E. Kremer, E.J. Baran, A. Mombrú, H. Pardo, M.P. Araujo, A.A. Batista, A.J. Costa-Filho, Inorg. Chim. Acta 355 (2003) 408–413.
[32] A. Monge, J.A. Palop, A. Piñol, F.J. Martínez-Crespo, S. Narro, M. González, Y. Sainz, A.L. de Ceráin, J. Heterocyclic Chem. 31 (1994) 1135–1139.
[33] N.M. Karayannis, L.L. Pytlewski, C.M. Mikulski, Coord. Chem. Rev. 11 (1973) 93.
[34] A.B.P. Lever, Inorganic Electronic Spectroscopy, Elsevier, Amsterdam, 1984, pp. 554–572.
[35] F. Ruff, I.G. Csizmadia, Organic Reactions Equilibria, Kinetics and Mechanism, Elsevier, Amsterdam, 1994, pp. 161–187.
[36] C. Hansch, A. Leo, D. Hoekman, Exploring QSAR. Hydrophobic, Electronic, and Steric Constants, A.C.S. Professional Reference Book, Washington, DC, 1995.
[37] R.M. Tótaro. Torre, E. Friet, I. Viera, E. Kremer, E.J. Baran, Acta Farm. Bonaerense 12 (1993) 73–78.
[38] E. Kremer, G. Facchin, E. Estévez, P. Alborés, E.J. Baran, J. Ellena, M.H. Torre, J. Inorg. Biochem. 100 (2006) 1167–1175.