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Original article

Novel Cu(II) quinoxaline N^1,N^4-dioxide complexes as selective hypoxic cytotoxins

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Abstract

As an effort to develop novel selective hypoxia-cytotoxins and to improve bioavailability and pharmacological and toxicological properties of quinoxaline N^1,N^4-dioxide derivatives (L1 = 3-amino-6(7)-chloroquinoxaline-2-carbonitrile N^1,N^4-dioxide, L2 = 3-amino-6(7)-bromoquinoxaline-2-carbonitrile N^1,N^4-dioxide and L3 = 3-amino-6(7)-methylquinoxaline-2-carbonitrile N^1,N^4-dioxide) and to get a synergism among metals and these type of bioreductive agents, L2 and three novel Cu(II) complexes of general formulae $[Cu^{II}(H_2O)_x(L - H)_2]$, where L = L1 ($x = 1$), L2 ($x = 0$) or L3 ($x = 2$) were developed. L2 and complexes were synthesized and structurally characterized by elemental and thermal analyses, and FTIR, electronic, MS, NMR, and EPR spectroscopies. The new compounds were subjected to cytotoxic evaluation in V79 cells in hypoxic and aerobic conditions. The complexes showed excellent selective cytotoxicity in hypoxia, being their cytotoxicity similar to or higher than that of the ligands L1–L3. Besides, the copper complexes were so poorly cytotoxic in oxia as the free ligands. In addition, for the first time Cu(II)-quinoxaline complexes are reported as a family of hypoxic cytotoxins.

Keywords: Copper complexes; Antitumoral agents; Hypoxic selective cytotoxins

1. Introduction

Due to their rapid growth, cancerous cells can become relatively isolated from the blood supply, turning increasingly difficult the diffusion of oxygen and resulting, frequently, in hypoxia. Hypoxic cells of these so called solid tumors are an important target for cancer chemotherapy, being not accessible for conventional cytotoxic drugs in adequate concentrations. In addition, they are also more resistant to ionizing radiation therapies than their aerobic counterparts. Bioreductive prodrugs, able to be selectively bioactivated in the absence of oxygen within tumor tissue by their metabolism to active cytotoxic species, have been developed as selective antitumoral agents [1,2]. Several types of compounds that are cytotoxic only under hypoxic conditions are known [1]. Among the organic compounds, several N-oxides have been described as cytotoxic agents having some of them entered clinical trials. In particular, having the hypoxic cytotoxin tirapazamine (3-amino-1,2,4-benzotriazine-1,4-dioxide) as structural antecedent [3], the capacity of a series of quinoxaline N^1,N^4-dioxide derivatives to act as bioreductive drugs has been previously described [1,4–6]. The best in vitro biological results were obtained with the 3-amino-2-carbonitrile derivatives. In

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order to improve the delivery properties 3-alkylamino derivatives were developed. Although some of them showed the best in vitro activity, they were not useful for therapy owing to too short in vivo half lives [7–10]. On the other hand, attempts towards selectively targeting transition metal complexes to hypoxic cells by making use of the redox characteristics of the metal center have been described [11]. The proper selection of the metal center is of fundamental importance and it is done on the basis of the redox properties and the lability of the resulting in vivo reduced species. So, the reduced species formed releases the ligand, i.e. the bioactive drug, of the complex. For this purpose the complexes may have reduction potentials in the appropriate range to undergo reduction by cellular reductases. This process is inhibited in oxygenated cells apparently by competition for cellular reductants between the metal complex and oxygen [2]. Complexes of Co(III), Ru(III), Cu(II), Re(V) and Tc(V) with different organic ligands (i.e. nitrogen mustards and bis(thiosemicarbazones)) have been tested for this purpose [11–17]. This approach can lead to the selective delivery of the resulting metal complex as a cytotoxin for therapy or to the delivery of a radionuclide for radiodiagnosis or radiotherapy [14,17]. In particular, the hypoxic selectivity of certain copper bis(thiosemicarbazones) and their use as vehicles for the delivery of radioactive copper isotopes to tumors has attracted much recent interest ([15,16] and references therein). The chemistry of copper is specially attractive for the development of hypoxic selective metal complexes. On one hand, this metal has two oxidation states and, usually, the reduction potential of the Cu(II) complexes in biological conditions ($E^{0'}(Cu^{2+}/Cu^{+})$) is accessible within the cellular potential range [11]. Both cations prefer different donor atoms according to the soft and hard Pearson classification. So, Cu(II) complexes may be reduced by cellular reductases leading to Cu(I) complexes of low stability, that could liberate the ligands (bioreductive prodrugs) of their coordination sphere [18]. In addition, copper has a number of radioactive isotopes which decay with combinations of beta, positron and gamma emission processes, useful for imaging and therapeutic applications in medicine [11,19]. So, combination in the same molecule of radioactive copper isotopes and hypoxic cytotoxins can be an adequate way to develop new agents for tumor therapy bearing a dual mechanism of action. For example, coordination of copper to tirapazamine resulted in a potential agent for the treatment of hepatic tumors using combined therapeutic strategies (bioreduction and radiotherapy) [20].

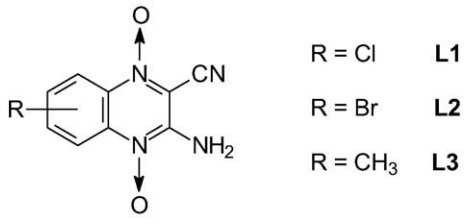

Fig. 1. Selected ligands L: **L1** = 3-amino-6(7)-chloroquinoxaline-2-carbonitrile N^1,N^4-dioxide, **L2** = 3-amino-6(7)-bromoquinoxaline-2-carbonitrile N^1,N^4-dioxide and **L3** = 3-amino-6(7)-methylquinoxaline-2-carbonitrile N^1,N^4-dioxide.

Owing to our interest in the development of novel hypoxia-cytotoxic agents and trying to improve the bioavailability and the pharmacological and toxicological properties of the quinoxaline N^1,N^4-dioxide derivatives previously developed and to get a synergism among the metal and these type of compounds, we synthesized and physicochemically characterized three novel Cu(II) complexes of general formulae $[Cu^{II}(H_2O)_x(L-H)_2]$, where L = L1 ($x = 1$), L2 ($x = 0$) or L3 ($x = 2$) (Fig. 1). The new compounds were subjected to cytotoxic evaluation in V79 cells in hypoxic and aerobic conditions.

2. Chemistry

The quinoxaline ligands **L1**–**L3**, having suitable donor atoms, resulted interesting tools for the development of the desired complexes due to their different electronic and lipophilic properties that could lead to different biological responses. They were prepared with excellent yields by reaction between the corresponding benzofuroxan and malononitrile (Fig. 2). **L1**–**L3** were generated as a mixture of six- and seven-substituted isomers that were not possible to separate neither by chromatography nor by crystallization [4–10]. The complexes **1**–**3** were synthesized by reaction of **L1**–**L3** with $CuSO_4$ in ethanol for derivatives **1** and **2** and water for derivative **3**, at room temperature with good yields (Fig. 2) [21,22].

The composition of the complexes was confirmed by C, N, H and Cu analyses. The structure of the complexes was determined by thermal analysis, conductivity measurements, IR, electronic spectroscopy, FAB-MS, NMR, and EPR. Thermal analysis showed that water molecules in derivatives **1** and **3** were not coordinated to the metal, since a rapid initial mass loss was observed at low temperatures [23]. Conductimetric measurements performed to 10^{-4}–10^{-3} M DMF solutions of the complexes showed no significant conductivity over that of the pure solvent. These results indicate that the complexes are non-charged, in agreement with the formula assigned. Characteristic IR bands of the ligands **L1**–**L3** and

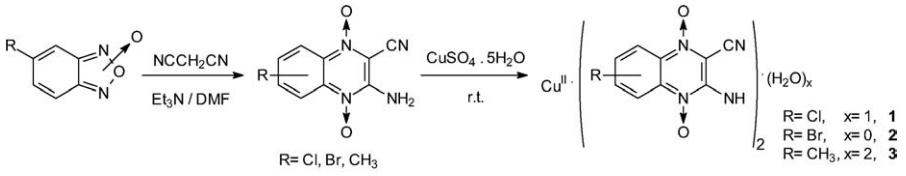

Fig. 2. Synthetic pathways for the preparation of **L1**–**L3** and copper complexes **1**–**3**.

derivatives **1**–**3** are shown in Table 1. The IR spectra of all the complexes are similar. After coordination the two bands corresponding to $\nu_{as}(NH_2)$ and $\nu_s(NH_2)$ of the amino group disappeared and only one band was observed according to the presence of a secondary amine [24]. This behavior supported the coordination of the ligand to copper through the amino group with the corresponding deprotonation. Furthermore, the disappearance of the $\nu(N \rightarrow O)$ band showed that all the N → O groups participate in the coordination. The dimeric molecule presented in Fig. 7(a and b) shows the coordination of four quinoxaline molecules to two copper atoms through six of their eight N → O groups. The other two N → O groups possibly present additional interactions with copper atoms of other neighboring molecules, forming intermolecular bonds, as it was previously observed for Cu-aminoacids complexes (Fig. 7c) [25,26]. This behavior could explain the complete disappearance of the N → O bands. The $\nu(C{\equiv}N)$ suffered only minor changes in agreement with the fact that this group was not coordinated to the metal. The electronic spectra could be characteristic of Cu(II) in a distorted octahedral environment. The spectra of **2** and **3** showed broad bands at 594 and 562 nm and a shoulder at 547 and 546 nm, respectively, suggesting the presence of unresolved low-energy components. Compound **1** presented two poorly defined bands at 610 and 547 nm [27]. Each complex had its own fragmentation pattern in FAB-MS spectroscopy (Table 1 and Fig. 3) that confirmed the existence of ligand–metal species.

The NMR experiments showed broad signals typical for Cu(II) paramagnetic complexes. EPR experiments were then performed in order to get insight into the structure of the complexes. The EPR spectra of polycrystalline samples of **1**–**3** are shown in Fig. 4.

The EPR spectra were characteristic of magnetically non-diluted Cu(II) complexes with the usual line in the region $g = 2$ (H_0 around 3300 G, which corresponds to the $\Delta M_s = \pm 1$ transition) and an additional weak peak in the half-field region (H_0 around 1650 G, see inset in Fig. 4) for all the solid-state samples. This weak peak was assigned to the $\Delta M_s = 2$ transition between the $M_s = -1$ and $M_s = +1$ states of the triplet ($S = 1$) in a system containing two coupled spins of

Table 1
Characteristic spectroscopic data for ligands **L1**–**L3** and complexes **1**–**3** in IR and FAB-MS

References	IR					FAB-MS	
	ν_{as}N–H (cm^{-1})	ν_sN–H (cm^{-1})	νC≡N (cm^{-1})	νN–O (cm^{-1})	νC=N→O (cm^{-1})	Relative intensity (%)	
						[Cu(L – H)L]$^+$	Other ions
L1	3430	3295	2237	1337	1614, 1649	–	–
1	3358 (broad)		2225	–	1589, 1561	1.99/2.97/1.20[a]	[Cu(H_2O)(L – H)L]$^+$ – Cl: 0.95/0.40[b]
L2	3436	3295	2237	1335	1613, 1648	–	–
2	3364		2221	–	1586, 1561	0.25/1.11/0.76[c]	[Cu(L – H)L]$^+$ – O: 0.59/1.38/0.95[c]
L3	3329	3264	2233	1333	1617, 1629, 1644	–	–
3	3356 (broad)		2225	–	1610, 1592, 1558	0.76	[L + H]$^+$: 3.00

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

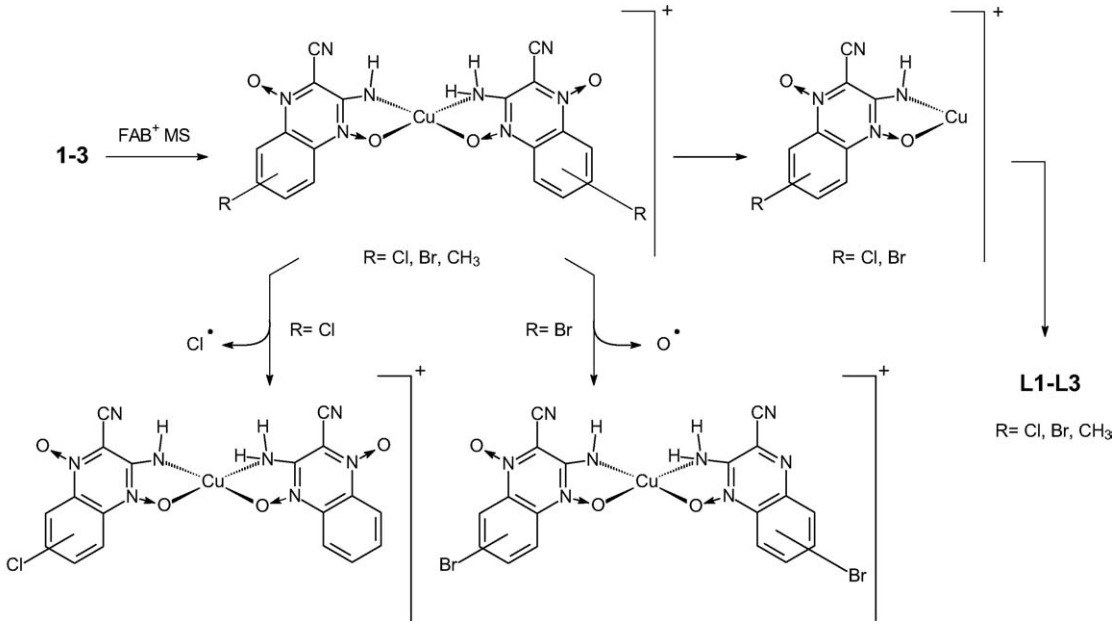

Fig. 3. Schematic patterns of fragmentation for **1**–**3** in FAB$^+$ MS.

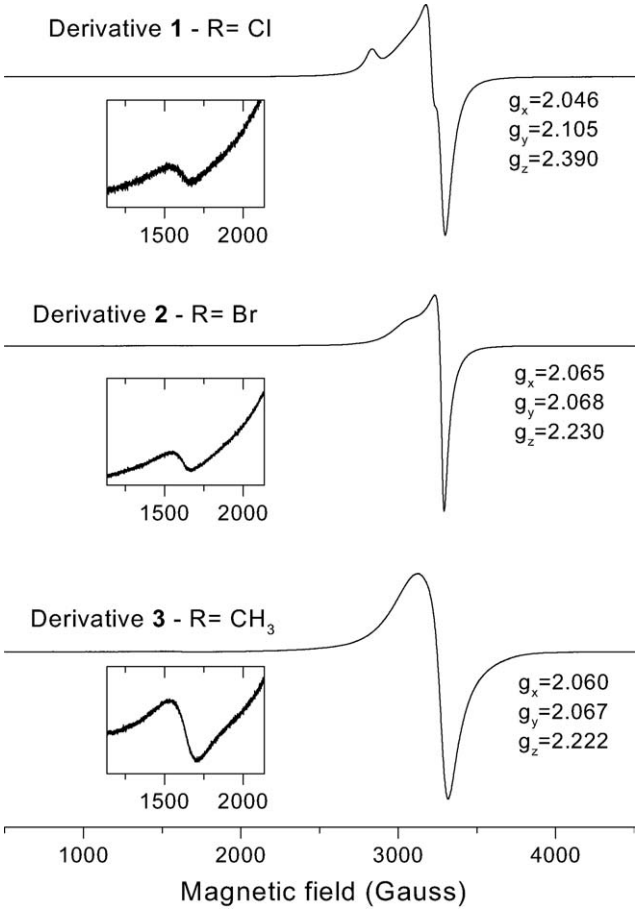

Fig. 4. X-band EPR spectra of polycrystalline samples of derivatives **1**, **2**, and **3** at 4 K. The insets show the half-field transition observed in systems containing coupled spins. The *g*-values were obtained by means of spectral simulation using the program WINEPR SinFonia [30].

1/2 [28]. The existence of two copper ions coupled by superexchange pathways in all polycrystalline samples are thus supported by the EPR results. No hyperfine structure is observed and this also serves as an indication of the coupling between the Cu(II) ions. It is well-known that exchange couplings average out interactions such as the hyperfine interactions [26]. The *g*-values (g_x, g_y, g_z) for the $g \approx 2$ line (see inset in Fig. 4) indicated a slightly distorted axial symmetry for the Cu(II) ion in the derivatives **2** and **3**, whereas a rhombic microenvironment around the Cu(II) existed in the complex **1**. EPR spectra of frozen solution samples (Fig. 5) did not show a half-field line characteristic of exchange couplings between paramagnetic ions, indicating that in this case the Cu(II)-containing molecules exist as monomers.

3. Pharmacology

3.1. Cytotoxic studies

Compound **L1**–**L3** and complexes **1**–**3** were subjected to preliminary cytotoxic evaluation on V79 cells under hypoxic and aerobic conditions using a cloning assay as previously

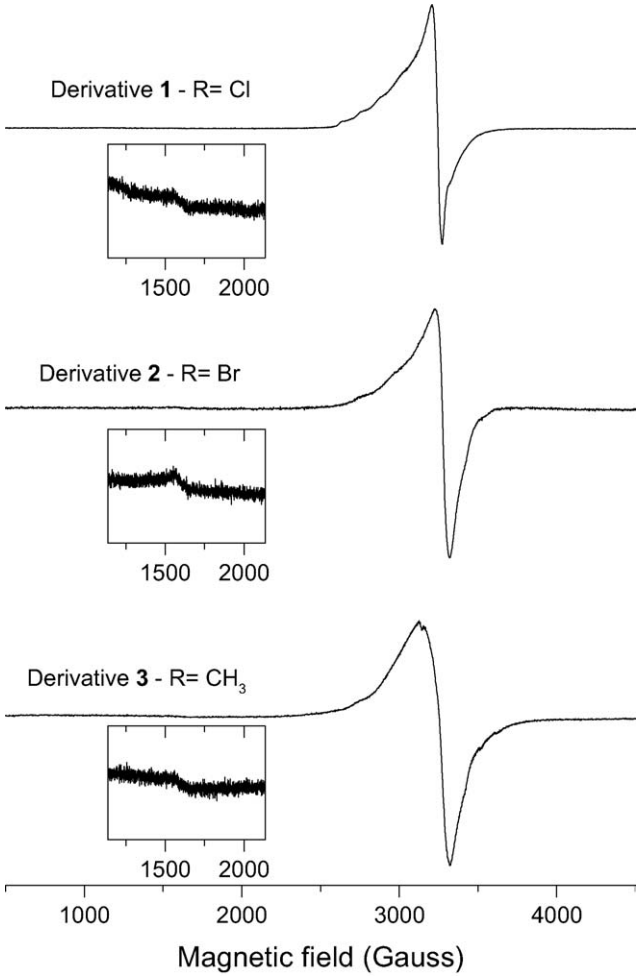

Fig. 5. X-band EPR spectra of frozen solution samples of derivatives **1**, **2**, and **3** at 4 K. The insets show the half-field line is no longer observed, thus suggesting that the complexes exist as monomers. The *g*-values are not much different from those presented in Fig. 4.

described [4–10]. Initially, all the complexes were tested at 20 μM. The survival fraction in both conditions (SFair and SFhypox) was determined. The obtained results are summarized in Table 2. The compounds were toxic in hypoxia and inactive in air at this dose. Therefore, they were tested at different doses to obtain a dose–response curve in air and hypoxia (Fig. 6).

Table 2
Hypoxic and oxic cytotoxicity on V79 cells for ligands **L1**–**L3** and complexes **1**–**3**, using 2 h exposure time and 20 μM drug concentration, and its lipophilicity expressed as RM

References	Sfair[a,c]	Sfhypox[b,c]	R_M[d]
L1	100	0	–0.43
L2	90	0	–0.63
L3	82	22	–0.05
1	100	1	–0.75
2	91	5	–0.43
3	100	10	–0.23

[a] Survival fraction in air.
[b] Survival fraction in hypoxia.
[c] Positive control: 7-chloro-3-[3-(*N*,*N*-dimethylamino)propylamino]-2-quinoxalinecarbonitrile N^1,N^4-dioxide hydrochloride.
[d] $R_M = \log[(1/R_f) - 1]$.

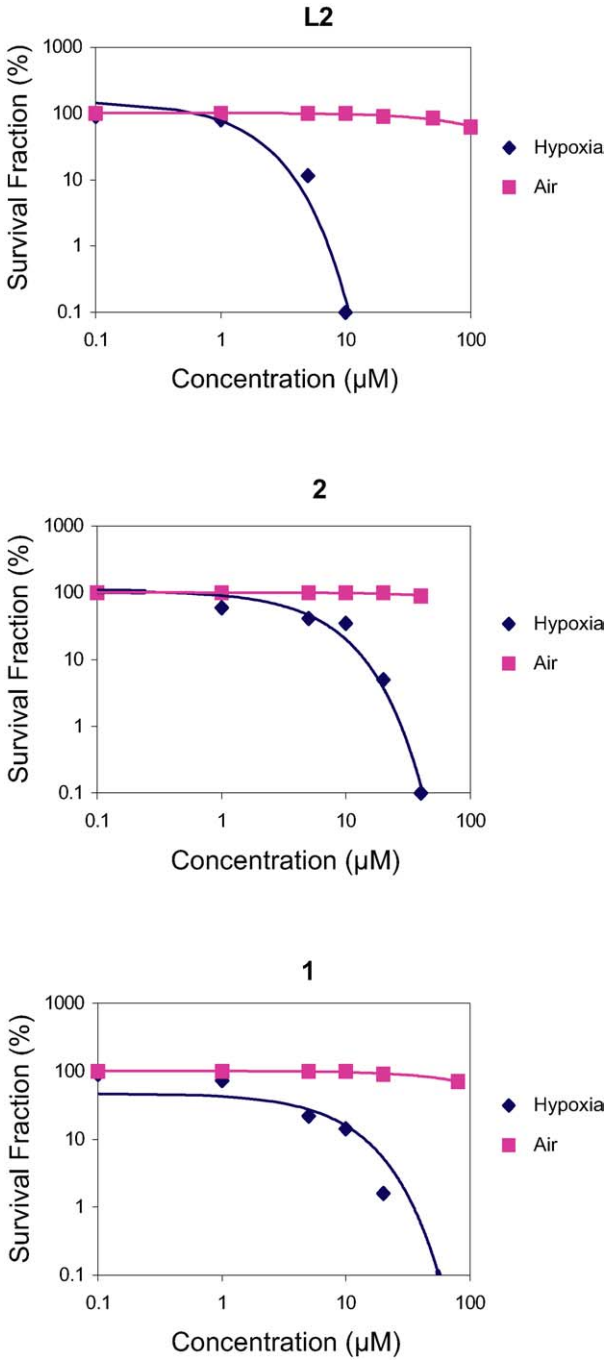

Fig. 6. Comparative dose–response curves in air and hypoxia of complexes **1** and **2** and **L2**. Mean survival percentages of cells treated with the compounds at different concentrations (μM) with respect to the untreated control cells are represented.

3.2. Lipophilicity studies

Lipophilicity was experimentally determined in order to study the change on ligands' physicochemical properties with the complexation to copper and their relationship with activity. Reversed-phase TLC experiments were performed for all the derivatives on precoated TLC-C_{18} and eluted with DMSO/physiological serum (75:25, v/v). The R_f values were converted into R_M values via the relationship: $R_M = \log [(1/R_f) - 1]$ [29]. Table 2 summarizes R_M for each compound.

3.3. Stability of the complexes in aqueous medium

The stability of complexes **1–3** was followed during 24 h, at 10^{-4} M 1% DMSO solutions (buffer phosphate, pH 7.4) at 37 °C, monitoring by TLC. Free ligand was not detected under these conditions.

4. Results and discussion

The quinoxaline derivative **L2** and the three new Cu-quinoxaline complexes **1–3** were synthesized by efficient procedures, spectroscopical characterized and biologically evaluated as selective hypoxic cytotoxins.

Compounds **1–3** are Cu(II) complexes coordinated with two deprotonated 3-aminoquinoxaline-2-carbonitrile dioxides, with general formulae $[Cu^{II}(H_2O)_x(L - H)_2]$ (when L = **L1**, $x = 1$; L = **L2**, $x = 0$; and L = **L3**, $x = 2$). According to EPR studies, they exist in solid state as dimeric forms, whereas in solution one copper is coordinated with two quinoxaline ligands. Due to the lack of adequate crystals for X-ray analysis, we theoretically studied the spatial distribution of **1–3** in solid state. A possible stereodistribution of derivative **2** in solid state, simulated at PM3 level, is shown in Fig. 7 [30].

L2 displayed similar biological behavior as the other quinoxaline N^1,N^4-dioxide derivatives previously studied showing a potency (P) similar to **L1** (7.2 versus 9.0 μM) [6–10]. **L2** and the complexes **1–3** showed excellent selective cytotoxicity in hypoxia against V79 cells. The cytotoxicity in hypoxia of the complexes **1–2** was maintained respect to the free ligands **L1–L2** (P in hypoxia: **L1**, 9.0; **1**, 35.1; **L2**, 7.2; **2**, 27.9 μM), whereas the selectivity was very high because they were very poorly cytotoxic under well oxygenated conditions (i.e. % cell survival in oxia 40 μM for **L2**, 87%, and for **2**, 91%; Fig. 6).

The novel compounds **1–3** resulted excellent bioreductive species showing that in this case the complexation did not affect the ligand bio-response. Moreover, this complexation improved the selectivity towards low oxygenated cells.

In relation to physicochemical properties relevant to biological activity, the lipophilicity of the ligands did not change significantly when they were bonded to Cu(II). So, transport of the complexes through the cell membrane may be similar to that of the free ligands. The complexes were stable in aqueous medium in the concentrations of the biological assays. So, they would be stable enough to promote cytotoxic effects. Although the in vitro biological response improved by coordination of the quinoxaline derivatives to copper, the structural and electronic modifications due to coordination did not improve the poor solubility in physiological conditions of these ligands.

5. Conclusions

The assayed Cu(II)-quinoxaline complexes showed cytotoxic selectivity in hypoxia similar to that of the free ligands.

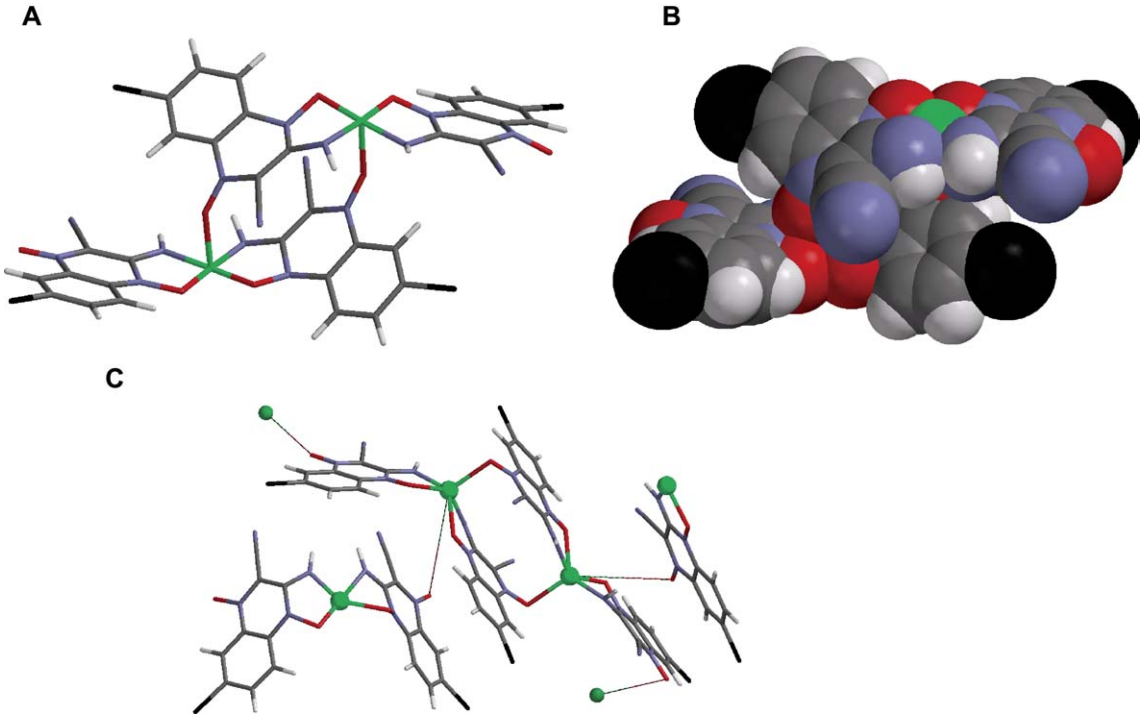

Fig. 7. Stereospatial structure of **2** in solid state (**a**) tube and (**b**) space filling representations. (**c**) Simulation of interactions between neighboring molecules, forming intermolecular bonds (some atoms were omitted in the representation).

For the first time this kind of compounds is reported as a family of hypoxic selective cytotoxins. Although the copper complexation of the chosen ligands did not improve neither their potency nor their selectivity towards hypoxic V79 cells, the information obtained in this work suggests that compounds **1**–**3** are reasonable starting points for a drug discovery effort. This information will let us to re-design new structures to improve the bioavailability and, as a consequence, the desired activity, by modifying the ligands and/or the metal and its oxidation state. Synthetic attempts in this way are currently in progress. These results along with the possibility of combining in the same molecule a radioactive copper isotope and hypoxic cytotoxins could be of interest for further in vitro and in vivo studies.

6. Experimental protocols

6.1. Chemistry

All starting materials were commercially available research-grade chemicals and were used without further purification. Quinoxaline (**L1** and **L3**) and benzofuroxan derivatives were prepared as previously reported [7]. For the organic procedures the solvents were dried and distilled prior to use, and the reactions were carried out in a nitrogen atmosphere. Elemental analyses were performed on a Fisons EA 1108 CHNS-O analyzer and the copper determinations were performed using iodimetric analysis. Thermogravimetric measurements were done 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}. Conductimetric measurements were performed at 25 °C in 10^{-4} M (complex **2**) or 10^{-3} M (complexes **1** and **3**) DMF solutions using a Conductivity Meter 4310 Jenway [31]. Electronic spectra in the visible range were obtained through a Nujol suspension with a Shimadzu UV-1603 equipment. IR spectra were recorded on a FTIR Bomen MB 102 spectrophotometer in the range 4000–200 cm^{-1}, using the KBr pellet technique. Routine FAB+ spectra of the metal complexes **1**–**3** 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. Mass spectra of ligands **L1**–**L3** were recorded on a Shimadzu GC–MS QP 1100 EX instrument at 70 eV. ^{1}H-NMR and ^{13}C-NMR spectra were recorded on a Bruker DPX-400 instrument in DMSO-d_6. X-band (9.5 GHz) EPR experiments were performed using a Bruker ELEXSYS E580 spectrometer equipped with a rectangular cavity and 100 kHz field modulation. The samples were measured at liquid He temperatures (ca. 4 K) using a commercial Oxford cryostat. The *g*-values were obtained by means of spectral simulation using the program WINEPR SimFonia [32].

*6.1.1. 3-Amino-6(7)-bromoquinoxaline-2-carbonitrile N^1,N^4-dioxide (**L2**)*

A mixture of 5-bromobenzofuroxane (700 mg, 3.3 mmol), malononitrile (320 mg, 4.8 mmol), triethylamine (0.1 mL) and dimethylformamide (1.4 mL) as solvent was stirred at room temperature during 24 h. The resulting precipitate was collected by filtration and washed with petroleum ether, and ethyl ether. Red–orange solid (780 mg, 84%); m.p. > 270 °C.

(Found (%): C, 38.40; H, 1.55; N, 19.86. $C_9H_5BrN_4O_2$ requires C, 38.46; H, 1.79; N, 19.93); δ_H (400 MHz , DMSO-d_6) 7.77 (dd, 0.3H, J = 9.1 Hz, J = 1.8 Hz), 8.03 (dd, 0.7H, J = 9.1 Hz, J = 1.8 Hz), 8.15 (bs, 2H, $-NH_2$), 8.19 (d, 1H, J = 9.1 Hz), 8.37 (d, 0.7H, J = 1.7 Hz), and 8.39 (d, 0.3H, J = 1.7 Hz); δ_C (100 MHz, DMSO-d_6) 110.01, 111.08, 111.18, 120.48, 122.44, 128.98, 131.22, 131.61, 133.00, 136.79, 137.69, and 147.04. *m/z* (EI) 280 (M^+, 11%), 264 (43), and 239 (61).

*6.1.2. General procedure for the synthesis of copper complexes **1** and **2***

L1 or **L2** (0.5 mmol) was dissolved in 500.0 mL of dried ethanol by sonication and mixed with 120.0 mL of an ethanolic solution containing $CuSO_4{\cdot}5H_2O$ (0.25 mmol). A purple solid was isolated by centrifugation and washed with small portions of ethanol. Finally, it was dried in air at room temperature.

*6.1.2.1. Cu(**L1**)$_2{\cdot}H_2O$.* Yield 47.7%. Found (%): C 39.78, H 2.31, N 20.49, Cu 11.73. $C_{18}H_{12}Cl_2N_8O_5$ requires: C 39.09, H 1.81, N 20.27, Cu 11.49. Thermogravimetric measurements: water release began around 44 °C and extended up to 59 °C, after 275 °C a rapid degradation was observed. FAB^+ MS: *m/z* 534/536/538 (1.99/2.97/1.20%), 518/520 (0.95/0.40), 298/300 (3.01/2.04) and 236/238 (2.96/2.50).

*6.1.2.2. Cu(**L2**)$_2$.* Yield 53.6%. Found (%): C 34.15, H 2.17, N 17.25, Cu 9.72. $C_{18}H_{10}Br_2N_8O_4$ requires: C 34.54, H 1.26, N 17.91, Cu 10.15. Thermogravimetric measurements: after 275 °C a rapid degradation was observed. FAB^+ MS: *m/z* 622/624/626 (0.25/1.11/0.76%), 606/608/610 (0.59/1.38/0.95), 342/344 (0.54/0.84) and 278/280 (96.46/100.00).

6.1.3. Cu(L3)$_2{\cdot}2H_2O$

L3 (3.0 mmol) was dissolved in 800.0 mL of distilled water and mixed with 800.0 mL of an aqueous solution containing $CuSO_4{\cdot}5H_2O$ (1.5 mmol), under continuous stirring. The red precipitate was briefly digested, separated by centrifugation and washed with small portions of water. Finally, it was dried in air at room temperature. Yield 54.8%; Found (%): C 45.47, H 3.13, N 21.17, Cu 12.52. $C_{20}H_{20}N_8O_4$ requires: C 45.47, H 3.17, N 21.17, Cu 11.99. Thermogravimetric measurements: water release began at around 46 °C and extended up to 65 °C, after 270 °C a rapid degradation was observed. FAB^+ MS: *m/z* 494 (0.76%), 217 (3.00) and 201 (2.00).

6.2. Pharmacology

6.2.1. Bioreductive activity

6.2.1.1. Cells. V79 cells (Chinese hamster lung fibroblasts) were obtained from European Collection of Animal Cell Cultures (ECACC) and maintained in logarithmic growth as subconfluent monolayers by trypsinization and subculture to (1–2) × 10^4 cells per cm^2 twice weekly. The growth medium was Eagle's minimal essential medium (EMEM), containing 10% (v/v) fetal bovine serum (FBS) and penicillin/streptomycin at 100 U/100 μg mL^{-1}.

6.2.1.2. Aerobic and hypoxic cytotoxicity. Suspension cultures. Monolayers of V79 cells in exponential growth were trypsinized, and suspension cultures were set up in 50 mL glass flasks: 2 × 10^4 cells per 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.

6.2.1.3. Treatment. Compounds solutions were prepared just before dosing. Stock solutions, 150-fold more concentrated, were prepared in pure DMSO (Aldrich) or sterilized distilled water. Thirty minutes 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).

6.2.1.4. Cloning. 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 hemocytometer and 10^2–10^3 cells were plated in six-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 SFhipox) was calculated as $PE_{treated}/PE_{control}$ × 100. The compounds were tested at 20 μM in duplicate flasks both in aerobic and hypoxic conditions.

For dose–response assays, the compounds were tested under the same conditions than in the screening assay but at five different concentrations in air and in hypoxia. For each compound, two dose–response curves were obtained by plotting the SF in air and hypoxia at each concentration tested. Then, potency (P) under hypoxia was calculated. P was defined as the dose in μM which gives 1% of cell survival with respect to the control.

6.3. Physicochemical properties

6.3.1. Lipophilicity studies

Reversed-phase TLC experiments were performed on precoated TLC plates SIL RP-18W/UV_{254} and eluted with DMSO/physiological serum (75:25, v/v). Stock solutions were prepared in pure DMSO (Aldrich) prior to use. The plates were developed in a closed chromatographic tank, dried and the spots were located under UV light. The R_f values were averaged from two to three determinations, and converted into R_M.

6.3.2. Stability studies

Thin layer chromatography was performed on precoated silica gel 60 F 254 TLC plates (Aldrich), and eluted with dichloromethane/methanol (95:5, v/v). The copper complexes were dissolved in DMSO and diluted with buffer phosphate pH 7.4 at 37 °C until 10^{-4} M (1% DMSO). Solutions were kept during 24 h at room temperature before application on the plates. The plates were developed in a closed chromatography tank, dried and the spots were located under UV light. The spots of the complexes were compared with those of the free ligands.

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References

[1] H. Cerecetto, M. González, Mini Rev. Med. Chem. 1 (2001) 219–231.
[2] W.A. Denny, Eur. J. Med. Chem. 36 (2001) 577–595.
[3] J. Scott, T. Daniels, R.R. Chatterji, K.S. MacGillivray, Gates, J. Org. Chem. 63 (1998) 10027–10030.
[4] A. Monge, J.A. Palop, A. Piñol, F.J. Martínez-Crespo, S. Narro, M. González, et al., J. Heterocyclic Chem. (1994) 1135–1139.
[5] A. Monge, J.A. Palop, M. González, F.J. Martínez-Crespo, A. López de Ceráin, Y. Sáinz, et al., J. Heterocyclic Chem. (1995) 1213–1217.
[6] M.A. Ortega, M.J. Morancho, F.J. Martínez-Crespo, Y. Sáinz, M.E. Montoya, A. López de Ceráin, et al., Eur. J. Med. Chem. 35 (2000) 21–30.
[7] A. Monge, F.J. Martínez-Crespo, A. López de Ceráin, J.A. Palop, S. Narro, V. Senador, et al., J. Med. Chem. 38 (1995) 4488–4494.
[8] E. Zamalloa, I. Aldana, C.M. Bachiller, A. Monge, Arzneimittelforschung 47 (1997) 873–878.
[9] E. Zamalloa, C. Dios-Viéitez, E. González-Peña, A. Monge, Arzneimittelforschung 47 (1997) 1044–1046.
[10] A. Monge, J.A. Palop, A. López de Ceráin, V. Senador, F.J. Martínez-Crespo, Y. Sáinz, et al., J. Med. Chem. 38 (1995) 1786–1792.
[11] P.J. Blower, J.R. Dilworth, R.I. Maurer, G.D. Mullen, C.A. Reynolds, Y. Zheng, J. Inorg. Biochem. 85 (2001) 15–22.
[12] M.J. Clarke, Coord. Chem. Rev. 232 (2002) 69–93.
[13] M.J. Clarke, F. Zhu, D.R. Frasca, Chem. Rev. 99 (1999) 2511–2533.
[14] J.L.J. Dearling, J.S. Lewis, G.E. Mullen, M.T. Rae, J. Zweit, P.J. Blower, Eur. J. Nucl. Med. 25 (1998) 788–792.
[15] A.R. Cowley, J.R. Dilworth, P.-S. Donnelly, E. Labisbal, A. Sousa, J. Am. Chem. Soc. 124 (2002) 5270–5271.
[16] C.J. Andersen, M.J. Welch, Chem. Rev. 99 (1999) 2219–2234.
[17] C.M. Archer, B. Edwards, N.A. Powell, Current directions, in: S.J. Mather (Ed.), Radiopharmaceutical Research and Development, Kluwer Academic Publishers, Dodrecht, The Netherlands, 1996, pp. 81–88.
[18] G. Wulfsberg, in: Principles of Descriptive Inorganic Chemistry, University Science Books, Sausalito, CA, 1991, pp. 266–305.
[19] P.J. Blower, J.S. Lewis, J.S. Zweit, J. Nucl. Med. Biol. 23 (1996) 957–980.
[20] P. Lin, K. Ho, J. Nucl. Med. 39 (1998) 677–678.
[21] 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.
[22] M.H. Torre, G. Facchin, E. Kremer, E.E. Castellano, O.E. Piro, E.J. Baran, J. Inorg. Biochem. 94 (2003) 200–204.
[23] M.E. Brown, Introduction to Thermal Analysis, Chapman and Hall, New York, 1988.
[24] R.T. Conley, J. Calderón Martínez, R. Avendaño Ruíz, Espectroscopía Infrarroja, Alhambra, Madrid, 1979.
[25] C.M. Weeks, A. Cooper, D.S. Norton, Acta Crystallogr. Sect. B 25 (1969) 443–450.
[26] T.G Fawcett, M. Ushay, J.P. Rose, R.A. Lalancette, J.A. Potenza, H.J. Schugar, Inorg. Chem. 18 (1979) 327–332.
[27] A.B.P. Lever, Inorganic Electronic Spectroscopy, Elsevier, Amsterdam, 1986.
[28] A. Bencini, D. Gatteschi, in: EPR of Exchange Coupled Systems, Springer, Berlin, 1990, pp. 176 (chapter 7).
[29] H. Cerecetto, R. Di Maio, M. González, M. Risso, P. Saenz, G. Seoane, A. Denicola, G. Peluffo, C. Quijano, C. Olea-Azar, J. Med. Chem. 42 (1999) 1941–1950.
[30] Firstly the compounds were built with standard bond lengths and angles using the PC SPARTAN Pro molecular modelling program (PC SPARTAN Pro, Wavefunction, Inc., 18401 Von Karman Avenue, Suite 370, Irvine CA 92612, USA) and the energy of each compound was minimized by molecular mechanics (MM) methods (using SYBYL molecular mechanics force fields implemented in PC SPARTAN Pro package). Compounds minimized by MM were geometry fully optimised at RHF level using semi empirical PM3 method in gas phase. PC SPARTAN Pro, Wavefunction, Inc. 18401 Von Karman Avenue, Suite 370. Irvine, CA 92612, USA.
[31] W.J. Geary, Coord. Chem. Rev. 7 (1971) 81–122.
[32] W.I.N.E.P.R. Program, SimFonia version 1.25, Bruker Analytische Messtechnik GmbH, 1996.