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Cytotoxic palladium complexes of bioreductive quinoxaline N^1,N^4-dioxide prodrugs

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ABSTRACT

Four new palladium(II) complexes with the formula Pd(L)$_2$, where L are quinoxaline-2-carbonitrile N^1,N^4-dioxide derivatives, were synthesized as a contribution to the chemistry and pharmacology of metal compounds with this class of pharmacologically interesting bioreductive prodrugs. Compounds were characterized by elemental, conductometric and thermogravimetric analyses, fast atom bombardment mass spectrometry (FAB-MS) and electronic, Fourier transform infrared (FTIR) and ^{1}H-nuclear magnetic resonance spectroscopies. The complexes were subjected to cytotoxic evaluation on V79 cells in hypoxic and aerobic conditions. In addition, a preliminary study on interaction with plasmid DNA in normoxia was performed. Complexes showed different in vitro biological behavior depending on the nature of the substituent on the quinoxaline ring. Pd(**L1**)$_2$ and Pd(**L2**)$_2$, where **L1** is 3-aminoquinoxaline-2-carbonitrile N^1,N^4-dioxide and **L2** is 3-amino-6(7)-methylquinoxaline-2-carbonitrile N^1,N^4-dioxide, showed non selective cytotoxicity, being cytotoxic either in hypoxic or in aerobic conditions. On the other hand, Pd(**L3**)$_2$, where **L3** is 3-amino-6(7)-chloroquinoxaline-2-carbonitrile N^1,N^4-dioxide, resulted in vitro more potent cytotoxin in hypoxia (P = 5.0 μM) than the corresponding free ligand (P = 9.0 μM) and tirapazamine (P = 30.0 μM), the first bioreductive cytotoxic drug introduced into clinical trials. In addition, it showed a very good selective cytotoxicity in hypoxic conditions, being non-cytotoxic in normoxia. Its hypoxic cytotoxicity relationship value, HCR, was of the same order than those of other hypoxia selective cytotoxins (i.e., Mitomycine C, Misonidazole and the N-oxide RB90740). Interaction of the complexes with plasmid DNA in normoxia showed dose dependent ability to relax the negative supercoiled forms via different mechanisms. Pd(**L2**)$_2$ introduced a scission event in supercoiled DNA yielding the circular relaxed form. Meanwhile, both Pd(**L1**)$_2$ and Pd(**L3**)$_2$ produced the loss of negative supercoils rendering a family of topoisomers with reduced electrophoretic mobility. Pd(**L3**)$_2$ showed a more marked effect than Pd(**L1**)$_2$. Indeed, for the highest doses assayed, Pd(**L3**)$_2$ was even able to introduce positive supercoils on the plasmid DNA.

1. Introduction

Clinically used anticancer drugs are mainly systemic anti-proliferative cytotoxins that preferentially kill rapidly dividing cells. Due to their mechanism of action they usually produce simultaneous damage on proliferating normal cells that leads to narrow therapeutic indexes. In addition, tumor cells can become relatively isolated from blood supply due to their rapid growth, turning increasingly difficult the diffusion of oxygen and resulting, frequently, in hypoxia. Ordinary anti-cancer drugs show low therapeutic efficacy for the treatment of these hypoxic solid tumors, since the majority of these cells are not rapidly dividing ones. In addition, solid tumor cells are not reached by conventional cytotoxic drugs in adequate concentrations and are more resistant to ionizing radiation therapies than well-oxygenated ones.[1] Environmental differences between hypoxic tumor cells and normal tissue might be exploitable in the design of novel drugs for the selective treatment of these solid tumors. Compounds able to be selectively bioactivated by their metabolism in the cell have been developed as selective anti-tumor prodrugs.[2,3] Although the enzymes that catalyze the reductive activation occur in all cells, selectivity is achieved by back-oxidation of

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the transient reduction product in normal cells by molecular oxygen.[1] Different organic compounds and transition metal complexes can be recognized as hypoxia-selective tumor activated prodrugs.[3] After initial work by Denny et al. on Co(III) complexes with nitrogen mustards as ligands,[3] further attempts towards selectively targeting metal-based bioreductive prodrugs to hypoxic cells by making use of the redox characteristics of the metal center have mainly involved Co(III), Cu(II), Ru(III), Re(III/V), Tc(III/V) and Pt(IV) coordination compounds.[3–10] Among carbon-based compounds that are bioactivated under hypoxic conditions, a number of heterocyclic N-oxides have been developed. Through a single-electron reduction process nitroxide radical is generated as cytotoxic metabolite, which is suggested to initiate radical-mediated oxidative DNA strand cleavage.[1,11,12] In particular, having the hypoxic-cytotoxin 3-amino-1,2,4-benzotriazine-1,4-dioxide (tirapazamine) as structural antecedent,[13] the capacity of a series of quinoxaline N^1,N^4-dioxide derivatives to act as bioreductive drugs has been previously described by part of our team.[2,14–16] Although excellent in vitro biological results have been obtained with some 3-aminoquinoxaline-2-carbonitrile N^1,N^4-dioxide derivatives, they were not useful for therapy owing to too short in vivo half lives and low solubility in physiological media.[17–20]

In spite of the fact that it is widely recognized that many organic bioactive compounds used in medicine exert their action by bioactivation or biotransformation through chelation, in most cases little is known about the effect of metal binding on organic drugs' activity. Among other effects, metal coordination can assist targeting organic drugs to specific tissues. Relevant physicochemical properties of the organic compound can be modified, leading to improved bioavailability and/or increased time of residence. For instance, lipophilicity, which may control the rate of entry of a drug into the cell, is commonly modified by coordination.[21–25] Therefore, we have focused our current research on the development of metal complexes of bioactive quinoxaline N^1,N^4-dioxide derivatives. Novel copper(II) and vanadium(IV) complexes with 3-aminoquinoxaline-2-carbonitrile N^1,N^4-dioxide derivatives have been synthesized, characterized and in vitro biologically evaluated, trying to assess the effect of metal coordination on cytotoxicity and selectivity towards hypoxic tissue.[26–28] Since some of those complexes have shown excellent selective cytotoxicity in hypoxia, coordination compounds of other metals are currently under development. In particular, palladium was selected as metal center since it has been extensively studied for the development of novel metal-based therapeutic agents.[29]

In the present work we studied the effect of palladium coordination to 3-aminoquinoxaline-2-carbonitrile N^1,N^4-dioxide derivatives (L shown in Fig. 1) on bioactivity and selectivity of these bioactive compounds. Four new palladium(II) complexes, $Pd(L)_2$, were synthesized and characterized by elemental, conductometric and thermogravimetric analyses, FAB-MS and electronic, FTIR and ^{1}H-NMR spectroscopies. The complexes were subjected to cytotoxic evaluation on V79 cells in hypoxic and aerobic conditions. In addition, a preliminary study on interaction with plasmid DNA under normoxia was performed in order to get further insight into the biological behavior of the complexes.

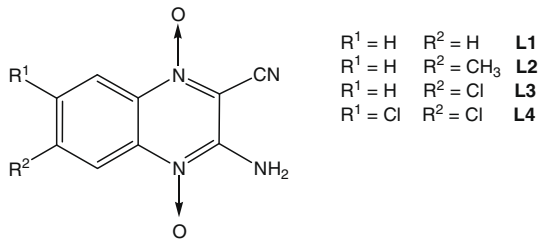


Figure 1. Selected ligands L: **L1** = 3-aminoquinoxaline-2-carbonitrile N^1,N^4-dioxide, **L2** = 3-amino-6(7)-methylquinoxaline-2-carbonitrile N^1,N^4-dioxide, **L3** = 3-amino-6(7)-chloroquinoxaline-2-carbonitrile N^1,N^4-dioxide and **L4** = 3-amino-6,7-dichloroquinoxaline-2-carbonitrile N^1,N^4-dioxide.

2. Results and discussion

2.1. Chemistry

Four novel complexes, $Pd(L)_2$ (Fig. 2), with 3-aminoquinoxaline-2-carbonitrile N^1,N^4-dioxide derivatives (L) as ligands were synthesized in good yield and high purity by substitution onto $[PdCl_4]^{2-}$. Unfortunately, it was impossible to obtain single crystals adequate for a crystallographic study. Nevertheless, complexes could be adequately characterized in the same way as previously done for the reported copper and vanadium compounds of this family of ligands by using a combination of several physicochemical methods.[26–28,30,31] Analytical data and conductometric, thermogravimetric and FAB-MS results were in agreement with the proposed analytical and structural formula. Conductometric measurements performed to 10^{-4}–10^{-3} M dimethylformamide (DMF) solutions of the complexes showed that they behave as non electrolytes, in agreement with the non charged formula proposed. In addition, it was demonstrated by conductometry that the compounds are stable in DMF solution for at least 6 days at 30 °C and in DMSO for at least 24 h. In both solvents the compounds remained non-conducting demonstrating that substitution of the negatively charged L by solvent molecules does not occur during the period studied. Additionally, stability of the complexes in DMSO–water solutions during the time involved in the biological tests was stated by TLC, as described under Section 4 (Cytotoxic studies).

Thermogravimetic measurements (TGA) performed on palladium **L1**–**L3** complexes showed the release of crystallization water molecules beginning at around 50 °C. TGA performed on palladium **L4** complex showed the presence of two methanol molecules of crystallization. Percentages of mass release were in agreement with the proposed formula (mass release calcd/found (%): $Pd(\mathbf{L1})_2{\cdot}H_2O$ 3.4/3.2; $Pd(\mathbf{L2})_2{\cdot}H_2O$ 3.2/3.3; $Pd(\mathbf{L3})_2{\cdot}3H_2O$ 8.5/8.3; $Pd(\mathbf{L4})_2{\cdot}2CH_3OH$ 8.9/8.5). As a representative example, TGA curve depicting the course of thermal decomposition of $Pd(\mathbf{L2})_2{\cdot}H_2O$ was included as Supplementary data.

FAB-MS experiments showed the presence of a characteristic fragmentation pattern for each complex (Scheme 1S, Supplemen-

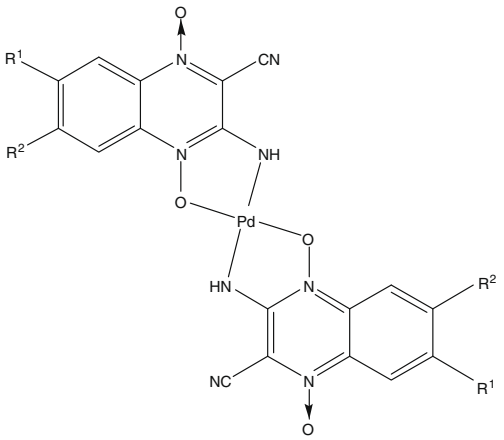


Figure 2. Proposed structure of $Pd(L)_2$ complexes.

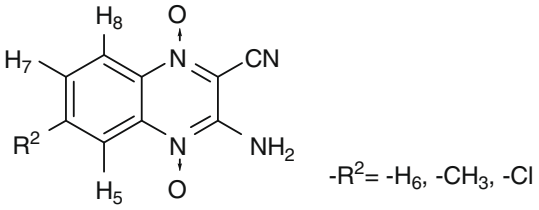
tary data), as previously observed for copper and vanadium complexes with this family of ligands.[26–28,30,31] The isotopic distribution of some ions agreed with the presence of Pd, or Pd and chlorine in the case of Pd(**L3**)$_2$·3H$_2$O, allowing the unequivocal assignment of these fragments. In addition, the presence of associated water molecules was observed in some of the spectra, that is, for Pd(**L3**)$_2$·3H$_2$O the fragment corresponding to Pd(**L3**)$_2$·2H$_2$O was observed.

Complexes are dark violet solids and their DMF solutions showed intense absorption bands ($\varepsilon = 10^3$–10^4 M^{-1} cm^{-1}) around 500–550 nm due to the presence of the coordinated quinoxaline derivatives. The bands of the free ligands shifted to higher wavelengths due to coordination to Pd(II).

FTIR spectra of the new complexes showed a similar pattern with those previously reported for Cu(II) and V(IV)O^{2+} complexes with this family of ligands.[26–28,30,31] Main vibration bands related with coordination are given in Table 1. Both strong bands corresponding to $\nu_{as}(NH_2)$ and $\nu_s(NH_2)$ of the amino group, located for the free ligands in the 3450 and 3250 cm^{-1} region, disappeared after coordination and only one band (ν(NH)) of medium to weak intensity around 3350–3400 cm^{-1} was observed, in agreement with the presence of a secondary amine. This behaviour supports the coordination of the ligand to palladium through the deprotonated amino group.[26–28,30,31] The deprotonation of the aromatic amine group has been previously reported for vanadium and copper complexes of this family of ligands and for metal chelates of quinoline *N*-oxides and other aromatic amine N-oxides involving an amine substituent in *ortho* position to the N-oxide.[32] The strong ν(N→O) stretching mode, located near 1300–1350 cm^{-1} for the free ligands, turned to weak or almost disappeared in the palladium complexes without a significant displacement. As previously reported, this effect could be explained by the coordination of only one of the N→O groups per ligand molecule, remaining the other N→O group uncoordinated. Although the band related to the ν(NO) motion should be displaced due to coordination to the metal of part of the NO groups, the complexity of the spectra in this frequency region did not allow us to tentatively assign this new band.[31] IR spectra showed that the ν(C≡N) suffered only minor changes in agreement with the fact that this group was not coordinated to the metal.

^{1}H NMR integrations and signal multiplicities were in agreement with the proposed formula (Table 2). The experiments showed the signals expected for the ligandś protons but very slightly shifted due to coordination to palladium, as previously observed for other palladium complexes.[33] In addition, the experiments showed that the complexes were stable in DMSO. Substitution process by the solvent, coordination of DMSO to palladium, was not detected by ^{1}H NMR in DMSO-d_6 during the time involved in the experiments and at least during 24 h at 30 °C. This stability to substitution is in agreement with the previously demonstrated coordination characteristics of the bidentate 3-aminoquinoxaline-2-carbonitrile N^1,N^4-dioxide ligands that lead to vanadium and copper complexes of high stability in DMSO medium.[26–28] Thus, dissolution of the palladium compounds in such media allowed studying their biological behavior. The low solubility of the complexes in DMSO-d_6 did not allow us to perform adequate ^{13}C NMR spectra, obtaining very weak signals after 24 h of accumulation. Instead of these spectra, we performed the corresponding HSQC (Heteronuclear Single Quantum Correlation) and HMBC (Heteronuclear Multiple Bond Correlation) experiments. HSQC enhanced the signal to noise ratio providing a factor of two of improvement in sensitivity for some specific carbons. However, carbons 2, 3 and CN could not be identified in these experiments.

Table 2
^{1}H NMR chemical shift values (δ) in ppm of [Pd(L)$_2$] complexes at 30 °C



Proton	δ_{L1}	δ_{L2}	δ_{L3}
–CH$_3$	–	2.43	–
–H$_5$	8.30	8.10	8.27
–H$_6$	7.94	–	–
–H$_7$	7.67	7.80	7.95
–H$_8$	8.29	8.03	8.29
–NH	8.04	7.96	8.16

The results of the analytical and physicochemical characterization of the palladium complexes confirmed that the new compounds are neutral complexes with two molecules of deprotonated 3-aminoquinoxaline-2-carbonitrile N^1,N^4-dioxide ligands coordinated through the amine and the neighboring N→O group to the palladium(II) central atom. Proposed structure is shown in Figure 2.

2.2. Cytotoxicity studies

Pd(L)$_2$, were L = **L1**, **L2**, **L3** and **L4**, were subjected to preliminary cytotoxic evaluation on V79 cells under hypoxic and aerobic conditions using a cloning assay.[14–20] Hypoxic and oxic cytotoxicity was evaluated using 2 h exposure time and initial 20 μM drug concentration. The survival fraction values in both conditions (SFair and SFhypox) for the complexes are shown in Table 3.

Table 1
Main IR bands of the new palladium complexes, Pd(L)$_2$ and the free ligands **L1**-**L4**[27,30,31]

	IR (cm^{-1})					
	ν_{as} NH$_2$	ν_s NH$_2$	νN–H	νN–O[a]	νC=N→O	νC≡N
L1	3353 s	3262 s	–	1343 vs	1604 s, 1626 s	2237 w
Pd(L1)$_2$	–	–	3400 vw	1345 w	1561 s, 1618 w	2235 w
L2	3329 s	3264 m	–	1333 vs	1617 s, 1644 s	2233 w
Pd(L2)$_2$	–	–	3406 w	–	1561 s, 1618 w	2230 w
L3	3430 s	3295 s	–	1343 vs	1604 s, 1626 s	2237 w
Pd(L3)$_2$	–	–	3396 vw	1337 w	1614 s, 1664 w	2242 w
L4	3370 s	3282 s	–	1330 s	1596 m, 1628 s	2240 m
Pd(L4)$_2$	–	–	3347 vw	–	1561 s, 1579 s	2232 vw

ν: stretching; ν_{as}: asymmetric stretching, ν_s: symmetric stretching, s: strong, m: medium, w: weak, vw: very weak.
[a] See text. Crystallization molecules of the complexes were omitted for simplicity.

Table 3
Hypoxic and oxic cytotoxicity on V79 cells for the $Pd(L)_2$ complexes at 20 μM (experimental conditions see text)

Compound	SFair[a]	SFhypox[b]
Pd(**L1**)2	0	0
Pd(**L2**)2	2	0
Pd(**L3**)2	92	0
Pd(**L4**)2	62	48

[a] Survival fraction in air.
[b] Survival fraction in hypoxia. 7-chloro-3-[3-(*N*,*N*-dimethylamino)propylamino]quinoxaline-2-carbonitrile N^1,N^4-dioxide hydrochloride was used as positive control. Crystallization molecules of the complexes were omitted for simplicity.

Complexes showed different behaviors depending on the nature of the substituent on the aromatic ring. Pd(**L4**)$_2$ showed simultaneously the lowest activity in hypoxia and very low activity in oxia at the assayed dose, probably due to its very low solubility. Pd(**L1**)$_2$ and Pd(**L2**)$_2$ were cytotoxic in both conditions, showing none selectivity. Both complexes could be considered unspecific cytotoxins and potential anti-tumor compounds in oxia. On the other hand, Pd(**L3**)$_2$ resulted highly toxic in hypoxia and inactive in air at this dose. Therefore, only Pd(**L3**)$_2$ was tested at different doses to obtain dose-response curves in air and hypoxia. Potencies (P) and hypoxic cytotoxicity relationship (HCR) values were determined (Table 4), being P the dose which gives 1% of cell survival with respect to the control, either in hypoxia or in air (P in hypoxia and P in normoxia, respectively), and HCR the relationship between concentration of drug in air and concentration of drug in hypoxia that produce the same level of cell killing (1%). As shown in Table 4, the new complex showed a lower potency value in hypoxia than the corresponding free ligand and tirapazamine, the first bioreductive cytotoxic drug introduced into clinical trials. In addition, it showed a high potency value in normoxia and consequently a high selectivity, resulting in a good HCR value. When comparing its in vitro activity with those of previously reported copper and vanadyl complexes, Pd(**L3**)$_2$ showed better P value than the copper complexes and almost as good value as those of the best vanadyl complexes.[26–28] In addition, the HCR value for the new palladium compound is of the same order than those of other hypoxia selective cytotoxins (i.e., Mitomycine C, Misonidazole and the N-oxide RB90740[2]).

Table 4
Potency (P) in normoxia and in hypoxia and hypoxic cytotoxicity relationship (HCR) for Pd(**L3**)$_2$

Compound	P in normoxia (μM)[a]	P in hypoxia (μM)[a]	HCR[b]
L3	—	9.0	150 [20]
$Pd(L3)_2$	>40[c]	5.0	>8
$VO(L3)_2$	—	3.0	15 [27]
$Cu(L)_2$[d]		20.0	>2 [28]
Positive control[e]	—	0.4	250 [17]
Tirapazamine	—	30.0	75 [20]

Potency in hypoxia and HCR of **L3**, Tirapazamine, VO(**L3**)$_2$, a $Cu(L)_2$ complex and the quinoxalinecarbonitrile dioxide derivative used as positive control were included for comparison.
[a] P (potency) is the dose which gives 1% of hypoxic (P in hypoxia) or normoxic (P in normoxia) cell survival with respect to the control.
[b] HCR (hypoxic cytotoxicity relationship) is defined as the dose in air divided by the dose in hypoxia giving 1% of control cell survival.
[c] Low solubility did not allow determining the exact value since the complex was not soluble in the assayed conditions at higher concentrations.
[d] L: 3-amino-6(7)-chloro-7(6)-fluoroquinoxaline-2-carbonitrile N^1,N^4-dioxide.
[e] 7-chloro-3-[3-(*N*,*N*-dimethylamino)propylamino]quinoxaline-2-carbonitrile N^1,N^4-dioxide hydrochloride. Crystallization molecules of the complexes were omitted for simplicity.

2.3. Plasmid DNA interaction studies

Trying to get insight into possible reasons for the quite different biological behavior of Pd(**L3**)$_2$ in respect to **L1** and **L2** analogous complexes in normoxia (SFair 92%, 0% and 2%, respectively, Table 3), a preliminary plasmid DNA interaction study was performed. Similar studies with Pd(**L4**)$_2$ were not performed due to its low solubility.

In the assayed conditions, plasmid DNA is visualized as two forms: supercoiled DNA (higher mobility) and circular relaxed DNA (lower mobility). The corresponding linear DNA would have had an intermediate mobility. Since the gel was run in the absence of ethidium bromide (or other intercalating compounds), it was possible to evaluate the effect that incubation with the complexes had on DNA tertiary structure. Interaction of the three complexes with plasmid DNA showed dose dependent ability to relax the negative supercoiled forms via different mechanisms. Pd(**L2**)$_2$ introduced a scission event in supercoiled DNA yielding an increase of the circular relaxed form (Fig. 3A). For a better visualization, the intensity of the bands was quantified and plotted as percentage of each band with respect to total band intensity in the lane (Fig. 3B). Meanwhile, both Pd(**L1**)$_2$ and Pd(**L3**)$_2$ produced the loss of negative supercoils, seen as a decrease in mobility for the supercoiled form, rendering a family of topoisomers with reduced electrophoretic mobility (Fig. 3C and D). Pd(**L3**)$_2$ had a more marked effect than Pd(**L1**)$_2$. Indeed, for Pd(**L1**)$_2$ a complete relaxation of the supercoiled plasmid was not reached even for the highest assayed dose (r_i = 6) (Fig. 3D). In the case of Pd(**L3**)$_2$ a complete relaxation of supercoiled plasmid was observed at r_i = 1, as in this condition all DNA migrated like the circular form. For higher Pd(**L3**)$_2$ doses supercoiled form was again observed. This pattern can be explained by the introduction of positive supercoils on the plasmid DNA (Fig. 3C). The dose dependent effect of both Pd(**L1**)$_2$ and Pd(**L3**)$_2$ on the migration of supercoiled DNA plasmid was measured and is graphically shown in Figure 3E. In addition, the distortion of the DNA conformation produced by the highest doses of Pd(**L3**)$_2$ was such, that even the circular relaxed form was affected leading to a dose dependent increase in the electrophoretic mobility for the nicked form (Fig. 3C). The alteration of supercoiled plasmid DNA migration is typically caused by compounds that alter DNA topology such as intercalators. It has also been reported that non-intercalators such as minor groove binders could have a similar performance.[34] This phenomenon could also explain the observed increase in the mobility of the nicked form since the interaction with the metal complex promoting anomalous structures, such as bends, could introduce some degree of compactation.

Globally, these results show that the three complexes interact with DNA. While Pd(**L2**)$_2$ relaxes the supercoiled form by a cleavage mechanism, both other complexes do not introduce nicks but drastically distort the DNA conformation, being Pd(**L3**)$_2$ notably more active than Pd(**L1**)$_2$.

3. Conclusions

Four new palladium(II) complexes with 3-aminoquinoxaline-2-carbonitrile N^1,N^4-dioxide derivatives as ligands were synthesized and characterized as a contribution to the chemistry and pharmacology of metal compounds with this kind of pharmacologically interesting ligands. Different biological behavior between the different complexes and between each complex and the corresponding ligand was observed. Cytotoxicity evaluation of the compounds on V79 cells in hypoxic and aerobic conditions showed different in vitro biological behavior depending on the nature of the substituent on the aromatic ring. **L1** and **L2** complexes showed non selective cytotoxicity, being cytotoxic either in hypoxic or in oxic conditions. On the other hand, Pd(**L3**)$_2$ showed a lower potency value in hypoxia than the corresponding free ligand and tirapaz-

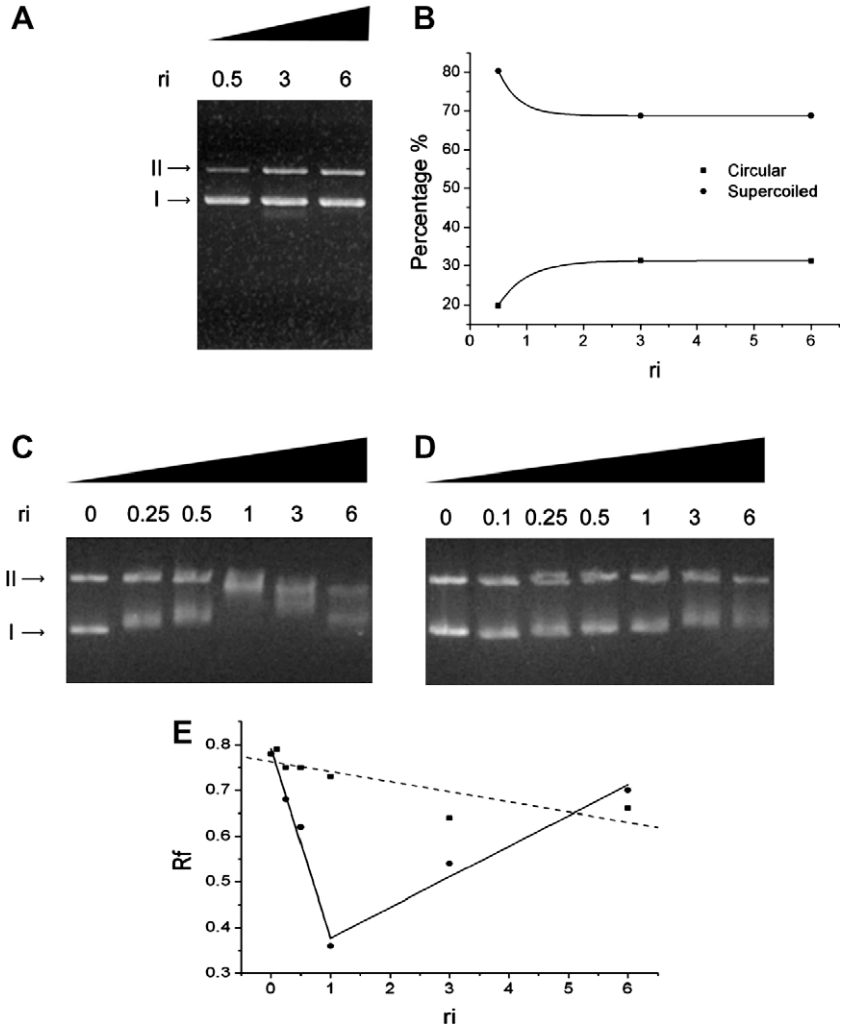


Figure 3. Plasmid DNA interaction with $Pd(L)_2$ complexes (Crystallization molecules of the complexes were omitted for simplicity). Reactions were incubated in 10 mM Tris–HCl pH 7.4 in a final volume of 20 μL for 24 h at 37 °C using pBSK II BlueScript, Stratagene (300 ng per reaction) at different r_i (mol of metal complex/mol of DNA base pair). Electrophoresis was carried out in the absence of ethidium bromide (EtBr). Gel was stained after run. (A) $Pd(\mathbf{L2})_2$, (C) $Pd(\mathbf{L3})_2$, (D) $Pd(\mathbf{L1})_2$. The r_i values are indicated at the top of each lane. Supercoiled (I) and circular (II) plasmid DNA forms are indicated at the left. (B) Quantification of the effect of the incubation with $Pd(\mathbf{L2})_2$—from A—on percentage of the circular relaxed and supercoiled plasmid DNA. (E) Quantification of the effect of the incubation with $Pd(\mathbf{L3})_2$ (●)—from C—and $Pd(\mathbf{L1})_2$ (■)—from D—on the electrophoretic mobility of the supercoiled form at different r_i values.

amine and a very good selective cytotoxicity in hypoxic conditions, being non cytotoxic in normoxia. Trying to understand the reasons of the different biological behaviours that depend on the nature of the substituent on the quinoxaline moiety, DNA interaction studies were performed. Results showed that the complexes interact with DNA in aerobic conditions producing dose dependent effects but via different mechanisms. Nevertheless, results do not allow explaining the differences in cytotoxicity under normoxia. Further studies are currently in progress to get a deeper insight into the mechanism of action of these complexes.

4. Experimental

4.1. Materials

All common laboratory chemicals were purchased from commercial sources and used without further purification. The ligands, 3-amino-quinoxaline-2-carbonitrile N^1,N^4-dioxide (**L1**), 3-amino-6(7)-methylquinoxaline-2-carbonitrile N^1,N^4-dioxide (**L2**), 3-amino-6(7)-chloroquinoxaline-2-carbonitrile N^1,N^4-dioxide (**L3**) and 3-amino-6,7-dichloroquinoxaline-2-carbonitrile N^1,N^4-dioxide (**L4**) were synthesized by reaction of the corresponding benzofuroxan and malonitrile and characterized, as previously described.[26] **L1**-**L3** were obtained as mixtures of 6- and 7-substituted isomers.[26] C, H and N analyses were performed with a Carlo Erba Model EA1108 elemental analyzer. Thermogravimetric measurements were obtained with a Shimadzu TGA 50 thermobalance, with a platinum cell, working under flowing nitrogen (50 mL min^{-1}) and at a heating rate of 6 °C min^{-1} (rt −80 °C range) and 0.5 °C min^{-1} (80–350 °C range). Routine FAB+ spectra of the metal complexes were measured up to m/z = 1500 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. Conductometric measurements were performed at 25 °C in 10^{-3}–10^{-4} M DMF solutions using a Conductivity Meter 4310 Jenway.[35] Electronic spectra were recorded on a Shimadzu UV-1603 spectrophotometer in DMF solution. FTIR spectra (4000–400 cm^{-1}) were measured as KBr pellets on a Bomen M102 instrument. ^{1}H NMR spectra and HSQC and HMBC experiments of the complexes were recorded on a Bruker DPX-400 instrument at 30 °C. ^{1}H NMR experiments were performed immediately after dissolution in DMSO-d_6 and repeated after 24 h.

4.2. Syntheses of the complexes

Complexes Pd(**L1**)$_2$·H$_2$O, Pd(**L2**)$_2$·H$_2$O and Pd(**L3**)$_2$·3H$_2$O were prepared by refluxing $Na_2[PdCl_4]$ (40 mg, 0.136 mmol) with L (0.372 mmol) (L = **L1**, **L2** or **L3**) in methanol (18 mL) during 24 h. Palladium complexes were filtered off from the hot solution as dark violet solids and washed with small portions of methanol. Pd(**L4**)$_2$·2CH$_3$OH was synthesized by a similar procedure by refluxing $Na_2[PdCl_4]$ (8.0 mg, 0.027 mmol) with **L4** (14.0 mg, 0.054 mmol) in 24 mL methanol–DMF (1:1) during 24 h. A dark solid was isolated by centrifugation and washed with small portions of methanol. Finally, compounds were dried in air at room temperature.

4.2.1. [Pd(3-aminoquinoxaline-2-carbonitrile N^1,N^4-dioxide)$_2$]·H$_2$O, Pd^{II}(**L1**)$_2$·H$_2$O

Yield: 37 mg, 52%. Anal. Calcd for Pd($C_9H_5N_4O_2$)$_2$·H$_2$O: C, 41.20; H, 2.30; N, 21.35. Found: C, 41.41; H, 2.52; N, 21.34. λ_{max} (DMF): 550 nm. FAB$^+$- MS, *m/z* (assignment, abundance %): 509/507/506 ($M^{+\cdot}$−H$_2$O−H, 3/4/3), 397/395/394 ($M^{+\cdot}$−H$_2$O−2CN−4O+3H, 1/2/1), 365/366/368 ($M^{+\cdot}$−H$_2$O−2CN−2O−2NO+2H, 6/8/6), 203 (L+H, 24). ^{1}H NMR (DMSO-d_6): 7.67 (t, 2H, *J* = 7.9 Hz), 7.87 (br s, 2H), 7.94 (t, 2H, *J* = 7.9 Hz), 8.04 (br s, 2H), 8.29 (d, 2H *J* = 7.5 Hz), 8.30 (d, 2H, *J* = 7.8 Hz). ^{13}C NMR (DMSO-d_6) from HSQC and HMBC experiments: 119.0, 120.5, 128.5, 133.0, 135.0, 138.0.

4.2.2. [Pd(3-amino-6(7)-methylquinoxaline-2-carbonitrile N^1,N^4-dioxide)$_2$]·H$_2$O, Pd^{II}(**L2**)$_2$·H$_2$O

Yield: 46 mg, 61%. Anal. Calcd for Pd($C_{10}H_7N_4O_2$)$_2$·H$_2$O: C, 43.30; H, 2.91; N, 20.20. Found: C, 43.23; H, 2.95; N, 20.04. λ_{max} (DMF): 549 nm. FAB-MS, *m/z* (assignment, abundance %): 540/538/537 ($M^{+\cdot}$−O·, 1/2/1), 445/443/442 ($M^{+\cdot}$−2CN−O−NO+3H, 1/2/1), 372/370/369 ($M^{+\cdot}$−H$_2$O−2CN−4NO+4H, 1/2/1), 217 (L+H·, 73). ^{1}H NMR (DMSO-d_6): 2.43 (s, 6H), 7.77-7.84 (m, 2H), 7.90 (br s, 2H), 7.96–8.10 (m, 6H).

4.2.3. [Pd(3-amino-6(7)-chloroquinoxaline-2-carbonitrile N^1,N^4-dioxide)$_2$]·3H$_2$O, Pd^{II}(**L3**)$_2$·3H$_2$O

Yield: 39 mg, 45%. Anal. Calcd for Pd($C_9H_4N_4O_2Cl$)$_2$·3H$_2$O: C, 34.23; H, 2.23; N, 17.74. Found: C, 34.36; H, 2.20; N, 17.65. λ_{max} (DMF): 500 nm. FAB-MS, *m/z* (assignment, abundance %): 615/613/611 ($M^{+\cdot}$−H$_2$O−H, 1/2/2), 578 ($M^{+\cdot}$−2 H$_2$O−OH, 1), 495 ($M^{+\cdot}$−4O−2Cl−H, 1), 391 (L+OH+Pd+NO, 1), 237 (L+H., 12). ^{1}H NMR (DMSO-d_6): 7.95 (dd, 2H, J_1 = 9.1, J_2 = 2.1 Hz), 8.16 (br s, 8 H), 8.27 (d, 2H *J* = 2.0 Hz), 8.29 (d, 2H, *J* = 9.2 Hz). ^{13}C NMR (DMSO-d_6) from HSQC and HMBC experiments: 119.5, 121.0, 132.5, 133.0, 135.5, 137.0.

4.2.4. [Pd(3-amino-6,7-dichloroquinoxaline-2-carbonitrile N^1,N^4-dioxide)$_2$]·2CH$_3$OH, Pd^{II}(**L4**)$_2$·2CH$_3$OH

Yield: 12 mg, 67%. Anal. Calcd for Pd($C_9H_3N_4O_2Cl_2$)$_2$·2CH$_3$OH: C, 33.80; H, 1.99; N, 15.77. Found: C, 33.75; H, 2.06; N, 15.67. The low solubility in DMSO-d_6 did not allow us to acquire the corresponding NMR spectra.

The **L1**–**L3** complexes are very slightly soluble in methanol and soluble in DMF and DMSO.

4.3. Cytotoxic studies

The four Pd(L)$_2$ complexes were subjected to preliminary cytotoxic evaluation on V79 cells under hypoxic and aerobic conditions using a cloning assay as previously described.[14–20] At a first stage the complexes were tested at 20 μM. The survival fraction in both conditions (SFair and SFhypox) was determined. The compound showing hypoxia selectivity was tested at different doses to obtain a dose-response curve in air and in hypoxia.

4.4. Bio-reductive activity

Cells: V79 cells (Chinese hamster lung fibroblasts) 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) × 10^4 cells/cm^2 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. *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/mL in 30 mL of EMEM containing 10% (v/v) FBS and *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) (10 mM) (pH 7.2). The glass flasks were submerged and stirred in a water bath at 37 °C, where they were gassed with humidified air or pure nitrogen. *Treatment*: Compounds solutions were prepared just before dosing. Stock solutions, 150-fold more concentrated, were prepared in pure DMSO (Aldrich) (Stability of the complexes at 10^{-4}–10^{-5} M concentration in a similar medium (DMSO–phosphate buffer pH 7.4) was assessed by thin layer chromatography (TLC) during more than 2 h by a procedure previously described.[26] Loss of free ligand from the complex was not detected. In addition, through ^{1}H NMR spectroscopy it was shown that the complexes were stable in DMSO for at least 24 h, since new signals due to DMSO coordination were not detected). Thirty min after the start of gassing, 0.2 mL of the stock compound solution was added to each flask, two flasks per dose (pH 7.2). 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]quinoxaline-2-carbonitrile 1,4-dioxide hydrochloride (positive control). *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 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 SFhipox) was calculated as $PE_{treated}/PE_{control} \times 100$. The compounds were tested at 20 μM in duplicate flasks both in aerobic and hypoxic conditions. For dose-response assays the compound Pd(**L3**)$_2$ was tested under the same conditions than in the screening assay but at five different concentrations in air and in hypoxia. Two dose–response curves were obtained by plotting the SF in air and hypoxia at each concentration tested. Then, potencies (P) under hypoxia and oxia were calculated (dose which gives 1% cell survival in hypoxia or normoxia with respect to the control), and the hypoxic cytotoxicity ratio HCR (ratio between doses in air and hypoxia giving the same cell survival (1%)), were calculated.

4.5. Plasmid DNA interaction studies

Plasmid DNA interaction studies for Pd(**L1**)$_2$, Pd(**L2**)$_2$ and Pd(**L3**)$_2$ were performed using a procedure previously described.[33] Plasmid DNA (pBSK II BlueScript, Stratagene; 300 ng per reaction) was obtained and purified according to standard techniques.[36] Briefly, *Escherichia coli* XL1 cells were transformed with pBSK II. Transformation was verified by PCR (Polymerase chain reaction) and plasmidic DNA was purified (Qiagen Plasmid Maxi Kit). Spectrophotometric DNA quantification was carried out assuming an absorptivity at 260 nm of 0.02 mL/μg cm. Complexes were dissolved in 1% DMSO–H$_2$O. The purified DNA was incubated in the presence of the complexes for 24 h at 37 °C (final volume: 20 μL,

reaction buffer: Tris (tris(hydroxymethyl)aminomethane hydrochloride) 10 mM, EDTA (ethylenediaminotetraacetic acid disodium salt) 0.1 mM. No effect on DNA due to DMSO addition was observed even for higher concentrations than the used for dissolution purposes.[33] Molar ratios r_i (r_i = mol of complex:mol of base pair) in the range 0.1–6.0 were assayed. After incubation, reactions were stopped by adding of loading buffer (25% bromophenol blue, 50% glycerol, 25 mM EDTA pH 8.0). In all cases, samples were electrophoresed in 0.7% agarose buffered with TB (90 mM Tris-borate) at 70 V for 1 h. The gel was subsequently stained with an ethidium bromide solution (0.5 μg/mL) for 30 min and destained in water for 20 min. Bands were visualized under UV light and quantified using OneDSCAN.

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Supplementary data

TGA curve depicting the course of thermal decomposition of Pd(**L2**)$_2$·H_2O and FAB-MS fragmentation scheme for each complex are provided. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2008.12.064.

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