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Short Communication

Ruthenium (II) nitrofurylsemicarbazone complexes: new DNA binding agents

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Abstract

Complexes of the type [Ru(II)Cl_2(DMSO)$_2$L], where L are 5-nitrofurylsemicarbazone derivatives, were prepared in an effort to combine the potential anti-tumor activity of the metal and the free ligands. The new complexes are excellent DNA binding agents for calf thymus DNA. So, their in vitro anti-tumor activity was tested in cellular models and the complexes were found to be non-cytotoxic on the tumor cell lines assayed, neither in aerobic conditions nor in the bio-reductive assay performed. Redox behavior, lipophilicity and stability were studied in order to explain the lack of cellular cytotoxic effects. The complexes resulted 10–100 times more hydrophilic than the parent ligands thus the bio-activity of these compounds would be compromised by their inadequate lipophilic properties.

Keywords: Ruthenium complexes; DNA binding evaluation; Anti-tumor activity; Nitrofurylsemicarbazone

1. Introduction

These last decades have seen a growing interest in transition metal complexes as potential anti-neoplastic agents [1]. Some promising results have been obtained with derivatives of different metals, like Pt, Ti, Rh, Au and Ru. In particular, several ruthenium complexes have exhibited good to excellent anti-tumor activity in some tumor screens and Ru(II)-dimethylsulfoxide complexes containing imidazole ligands have shown anti-metastatic activity against some murine tumor models [2]. Although it has been claimed that DNA-independent mechanisms are also responsible for the anti-tumor activity, it is accepted that DNA is an important target for Ru drugs. On the other hand, several types of organic compounds have been described as hypoxia-selective cytotoxic agents. These agents belong to the classes of nitro, quinone, nitrogen mustard and *N*-oxides derivatives [3]. In particular, some 5-nitrofuryl derivatives have demonstrated selective hypoxia cytotoxicity [4]. As part of our research program, *N*-oxide derivatives have been synthesized and biologically evaluated as hypoxia selective cytotoxins [5] and the 5-nitrofuryl moiety has been used as pharmacophore to create different anti-trypanosomal agents [6].

In this work, we have incorporated the 5-nitrofuryl pharmacophore and Ru in the same molecule, in order to obtain complexes with a potential dual mechanism of cytotoxic action (see Fig. 1).

The ability of a series of Ru(II)-5-nitrofuryl complexes to bind to calf thymus DNA (CT DNA) was studied. Owing to the high DNA binding levels observed, their in vitro cytotoxicity using three different kinds of mammalian tumor cells (mammary, kidney and colon) was evaluated. In addition, their selective cytotoxic effects under hypoxic conditions were evaluated against V79 cells. In order to

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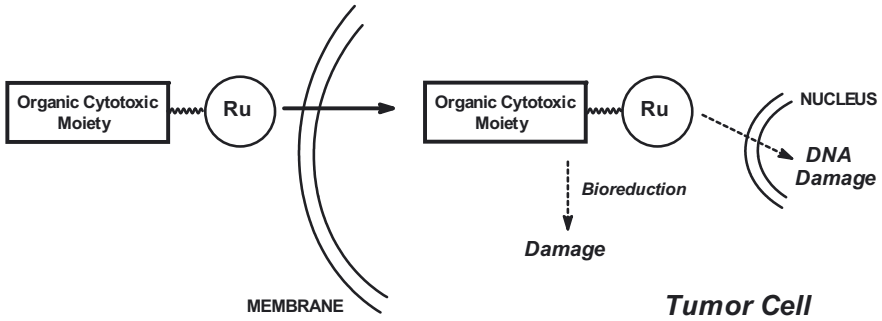

Fig. 1. Speculative dual mechanism of action of ruthenium complexes **4–6**.

explain the complexes' lack of cellular activity, physicochemical properties—redox behavior, lipophilicity and stability—were studied.

2. Chemistry

The semicarbazone moiety, having suitable donor atoms, allowed us to include the Ru atom and the 5-nitrofuryl pharmacophore in the same molecule. Ru coordination chemistry of this bidentate ligand has been poorly described [7]. Semicarbazones **1–3** [6b,8] (Fig. 2) resulted interesting tools for the development of the desired compounds, due to their different electronic and lipophilic properties that could lead to different biological responses. The complexes **4–6** were obtained by reaction of 1 equiv. of the precursor $[Ru(II)Cl_2(DMSO)_4]$ [9] with 2 equiv. of **1, 2**, or **3** in ethanol (for derivatives **4** and **5)** or toluene (for derivative **6)** at reflux, through a ligand substitution reaction where DMSO acts as leaving group [8]. The complexes were identified by microanalysis (C, H, N, S), IR, electronic spectra, ^{1}H-NMR, ^{13}C-NMR and HETCOR experiments.

3. Pharmacology

3.1. DNA interaction studies

The complexes **4–6** were tested for their DNA interaction ability. Each complex was mixed with native CT DNA in water solution as indicated in Section 6, and after an exhaustive washing, the amount of Ru was determined by atomic absorption spectroscopy [10,11]. After incubation and washing, the DNA retains a noticeably orange color, similar to that of the complexes. The study gives clear evidence that complexes are capable of binding to DNA. Binding levels, collected in Table 1, were determined combining atomic absorption determinations (for the metal) and electronic absorption measurements for DNA quantification.

3.2. Cytotoxic studies

Compounds **1–3** and complexes **4–6** were tested at 10^{-4} M doses in aerobic conditions against MCF-7 (human mammary adenocarcinoma) (ATCC HTB-38), TK-10 (human kidney carcinoma) (NCI), and HT-29 (human colon adenocarcinoma) (ATCC HTB-38) tumor lines, according to previously described procedures [12]. Survival percentage (SP), growth percentage (GP) and cytotoxicity were determined. The results are collected in Table 1.

On the other hand, complexes **4–6** were subjected to preliminary cytotoxic evaluation on V79 cells under hypoxic and aerobic conditions using a cloning assay as previously described [13]. All the complexes were tested at 20 μM. The survival fraction in both conditions (SFair and SFhipox) was determined. The obtained results are summarized in Table 1.

Derivatives **4–6** showed no cytotoxicity on tumor cells in oxia and neither cytotoxicity nor selectivity in the bioreductive assay.

3.3. Electrochemical studies

In order to explain the lack of activity of complexes **4–6**, the electrochemical behavior of ligand **1** and the corresponding complex **4** was studied. Cyclic voltammetric experiments were performed, in organic medium, in order to determine the peak potentials of the nitro moiety in both compounds. In both cases, when the potential was scanned in a negative direction, at all scan rates investigated, a peak corresponding to the reduction of the nitro group was observed (see Fig. 3). Table 2 lists the values of the voltammetric peaks.

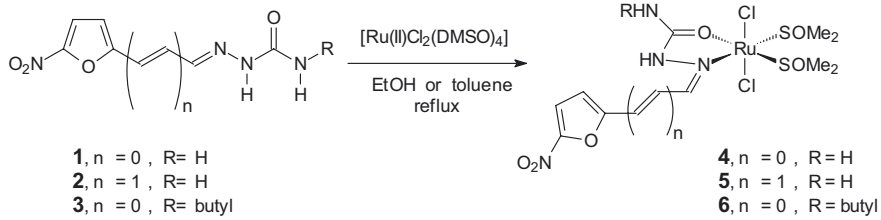

Fig. 2. Preparation of compounds **4–6**.

Table 1
DNA binding levels, cytotoxicity results and bio-reductive evaluation of compounds **1–6**

Compound	DNA binding results [a, b]			Tumor cell cytotoxicities [c]									Bio-reductive evaluation [c]	
	Nmol per mg	Metal per base	Base per metal	MCF-7 SP (%) [f]	GP (%) [g]	CP (%) [h]	TK-10 SP (%)	GP (%)	CP (%)	HT-29 SP (%)	GP (%)	CP (%)	SFair [d]	SFhipox [e]
1	ND [i]	ND	ND	10.0	–	57.2	94.0	85.0	–	96.0	88.7	–	ND	ND
2	ND	ND	ND	22.0	–	7.5	0.0	–	100.0	37.0	–	43.5	ND	ND
3	ND	ND	ND	61.0	48.3	–	100.0	100.8	–	103.0	109.3	–	ND	ND
4	128	0.042	24	106.0	141.0	–	84.0	16.4	–	118.0	179.3	–	100.0	72.0
5	261	0.086	12	100.0	99.5	–	81.0	0.3	–	118.0	177.9	–	100.0	79.0
6	164	0.054	19	101.0	107.6	–	89.0	43.7	–	124.0	207.8	–	100.0	90.0
							Reference for bio-reductive assay [j]						100.0 [j]	0.0 [j]

[a] DNA base:compound ratio=5.
[b] Data are averages of triplicate runs.
[c] The tests were carried in duplicate.
[d] Survival fraction in air.
[e] Survival fraction in hypoxia.
[f] Survival percentage.
[g] Growth percentage.
[h] Cytotoxicity percentage.
[i] ND: no determined.
[j] Positive control: 7-chloro-3-[3-(*N,N*-dimethylamino)propylamino]-2-quinoxalinecarbonitrile 1,4-dioxide hydrochloride.

Ligand **1** and complex **4** showed similar electrochemical behavior in the reduction process with $E_{1/2}$ = –0.89 and –0.96 V (scan rate: 0.5 V s^{-1}), respectively.

3.4. Partition coefficient determination

The partition coefficients, P_{OW}, were determined for the ligands and the corresponding complexes using electronic spectroscopy in the visible region, *n*-octanol as non-polar phase and physiological serum as polar one [14]. Table 2 lists the values of the P_{OW}.

3.5. Stability of the complexes in aqueous medium

The stability of complexes **4–6** was followed during 24 h, at 10^{-4} M 1% DMSO solutions (buffer phosphate, pH 7.4) at 37 °C, using electronic spectroscopy in the visible region. Liberation of the ligand was not observed under these conditions.

4. Results and discussion

The new ruthenium complexes **4–6**, synthesized by an efficient procedure [8], showed excellent DNA binding properties. The observed ruthenium DNA binding levels were similar or higher than that of other previously reported antitumor metal complexes [15,16]. However, they were very poor or non-cytotoxic agents in both cellular conditions assayed. At the studied doses, complexes showed no cytotoxic activity in tumoral mammalian cells, while ligands **1** and **2** were very cytotoxic at 10^{-4} M. On the other hand, the results of the bio-reductive assay showed that the developed complexes were non-cytotoxic neither in oxic nor in hypoxic conditions, at 20 μM, where the reference compound (positive control) is a potent and selective bio-reductive agent.

The physicochemical properties of the complexes could be indicative of the reasons for their lack of activity. The redox potential of the ligands is adequate [4b,17] for a selective reductive metabolism in tumoral tissue, effected by en-

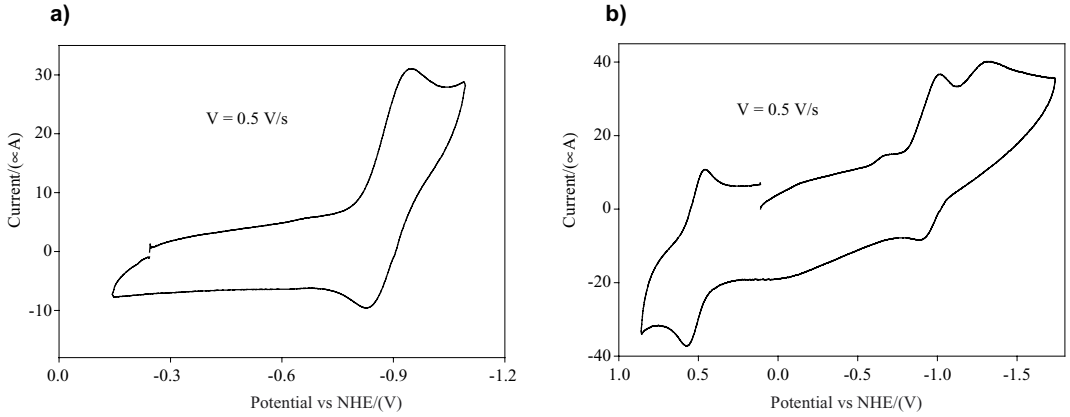

Fig. 3. Selected cyclic voltammograms at 25 °C of 10^{-3} M solutions of **1** (a) and **4** (b) in DMSO/0.1 M (TBA)PF_6 at 0.5 V s^{-1}.

Table 2
Physicochemical characterization of developed compounds

Compound	P_{OW}	$E_{1/2}$ [a]
1	1.93	–0.89
4	0.07	–0.96
2	9.33	
5	0.13	
3	2.25	
6	0.32	

[a] Scan rate: 0.5 V s^{-1}.

dogenous enzymes such as cytochrome P450 reductase. The reduction potential for the nitro moiety slightly changed when the ligand was coordinated to ruthenium, shifting from –0.89 to –0.96 V. This moderate change could not be responsible for the great change in the biological response between ligands and complexes.

The complexes did not liberate the ligand in aqueous medium in the same concentration as that of the biological assays. So, the ligands probably remain bound to the metal when interacting with tumor cells.

However, the poor lipophilicity of the developed complexes could be the key for the lack of cytotoxic activity. When the ligands **1–3**, with P_{OW} values ranging between 1.93 and 9.33, coordinate to ruthenium, the corresponding P_{OW} decreased drastically (0.07–0.32). As it is well known, the lipophilicity of a drug plays a significant role in numerous biological responses [14b]. Our complexes resulted 10–100 times more hydrophilic than the parent ligands, and thus the transport of the complexes through the cell membrane could be compromised by their inadequate lipophilic properties.

5. Conclusions

The information obtained in this work gives a guide to design more lipophilic ruthenium complexes with this family of ligands, that could show the desired anti-tumor activity maintaining or improving the DNA binding levels. Synthetic attempts in this direction are currently in progress. In addition, further studies in order to understand the detailed mechanism of DNA interaction are being performed.

6. Experimental protocols

6.1. Chemistry

All starting materials and compound **1** were commercially available research-grade chemicals and were used without further purification. The compounds **2–6** and $[Ru(II)Cl_2(DMSO)_4]$ were prepared as previously reported [6b,8,9]. 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 Carlo Erba EA 1108 CHNS-O analyzer. Infrared spectra were reordered on a Perkin Elmer 1310 or a Bomen MB 102 apparatus, using potassium bromide tablets. ^{1}H-NMR, ^{13}C-NMR spectra and HETCOR experiments were recorded on a Bruker DPX-400 (at 400 and 100 MHz) instrument.

6.2. Pharmacology

6.2.1. Anti-tumor activity

6.2.1.1. Cells. An adequate number of cells (MCF-7, TK-10 or HT-29) was maintained in 225 μl of RPMI medium, supplemented with L-glutamine (1%), penicillin/streptomycin (1%), non-essential amino acids (1%) and 10% (v/v) fetal bovine serum (FBS). The cultures were maintained at 37 °C and 5% CO_2 for 48 h. The absorbance at 540 nm before the treatment was determined.

6.2.1.2. Treatment. Compound solutions were prepared just before dosing. Stock solutions, 1 mM, were prepared in 10% DMSO (Aldrich) and 25 μl (final concentration 10^{-4} M) were added to each well. The cells were exposed for 24 h at 37 °C in 5% CO_2 atmosphere.

6.2.1.3. Measurement. After exposure to the compound, the medium was eliminated and the cells were washed with PBS. The cells were fixed with 50 μl of TCA (50%) and 200 μl of culture medium (without FBS) for 1 h at 4 °C. Then the cells were washed with purified water and treated with Sulforhodamine B (0.4% w/v in 1% acetic acid) for 10 min at room temperature. Then plates were washed with 1% acetic acid and dried overnight. Finally, 100 μl of Tris buffer (pH 10.0) was added and absorbance at 540 nm was determined. The cell SP was calculated for all of the compounds as [(absorbance of cells post-treatment with product – absorbance of blank)/(absorbance of cells post-treatment with solvent – absorbance of blank)] × 100. For the compounds which promote cell growth with respect to untreated cells, the GP was calculated as [(absorbance of cells post-treatment with product – absorbance of cell pre-treatment)/(absorbance of cells post-treatment with solvent – absorbance of cell pre-treatment)] × 100. For the compounds that killed cells, the cytotoxicity percentage (CP) was calculated as [(absorbance of cells post-treatment with product – absorbance of cell pre-treatment)/(absorbance of cell pre-treatment)] × 100.

6.2.2. Bio-reductive activity

6.2.2.1. 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) FBS and penicillin/streptomycin at 100 U/100 μg ml^{-1}.

6.2.2.2. 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.

6.2.2.3. 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).

6.2.2.4. 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 per 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.

6.2.3. DNA binding assay

CT DNA (Type I) was from Sigma Chemical Co. UV absorption spectra were recorded using a HP 8453 diode array UV–vis spectrophotometer. Quantification of ruthenium was done by ICP spectrometry on a Perkin Elmer Optima 3000 emission spectrometer. Standards were prepared in 10% HCl by diluting a ruthenium standard solution.

CT DNA was dissolved in water (overnight). CT DNA concentration per nucleotide was determined by UV absorption spectroscopy using the molar absorption coefficient of 6000 mol^{-1} dm^3 cm^{-1} at 260 nm. Complexes' solutions in DMSO (10^{-3} M) were incubated at 37 °C with CT DNA during 96 h. DNA–complexes mixtures were exhaustively washed to eliminate the unreacted complex. Interaction levels were determined as nmol of Ru bound per mg of DNA base or as mol of Ru bound per mol of DNA base.

6.3. Physicochemical properties

6.3.1. Cyclic voltammetry

DMSO (spectroscopy grade) was obtained from Aldrich. Tetrabutylammonium hexafluorophosphate ($TBAPF_6$) (0.1 M), used as supporting electrolyte, was obtained from Fluka. Cyclic voltammetry was carried out with a computer controlled Princeton Applied Research (PAR) Potentiostat/Galvanostat model 263A. A standard three-electrode cell was used with a glassy carbon electrode as the working electrode, a platinum wire as the counter electrode and a Ag/(10^{-3} M) $AgNO_3$ in CH_3CN as the reference electrode. Measurements were performed in oxygen purged 10^{-3} M DMSO solutions. Cyclic voltammograms were recorded at different scan rates (0.030–3 V s^{-1}) and at 25 °C. During the measurements a continuous gas stream was passed over the solutions.

6.3.2. P_{OW} *determination*

Lipophilicity tests were performed by the "shake flask" methodology determining the partition coefficient, P_{OW}, of the complexes in physiological solution/*n*-octanol. Concentration of Ru complexes in both phases was determined spectrophotometrically by measuring the absorbance at the maximum of each complex.

6.3.3. Stability studies

The stability studies were performed in a mixture of DMSO (1%)–phosphate buffer (pH 7.4) at 10^{-4} M concentration. The complexes were kept at 37 °C and the variation of the spectrum in the range 300–800 nm was studied during 24 h.

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