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Anticancer activity of ruthenium(II) polypyridine complexes bearing pyrrolidine substituents

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ABSTRACT

A set of homoleptic ruthenium polypyridine (bipyridine and terpyridine) complexes flanked with several electron-donating pyrrolidine moieties has been characterized and the anticancer activity was evaluated toward human lung adenocarcinoma epithelial (A549) and murine colon carcinoma (CT26). Good antiproliferative effects were observed with an IC_{50} ranging from ca. 4 to 21 μM against both cell lines. Dependence was found between the cytotoxicity, the lipophilicity and the Ru^{II}/Ru^{III} oxidation potential. All the studied compounds interacted quite well with albumin while the interaction with DNA was marginal. The biological studies revealed that the ruthenium complexes induced the ROS overproduction which might be one but not the only way of cell death induction.

1. Introduction

Metal complexes display unique properties in terms of tuneable geometry, electrochemical and photophysical properties and biological activity [1–3]. In this context, ruthenium complexes have been the focus of much attention during the past decade as promising alternatives to platinum-based anti-cancer agents [1,2]. To date tetrachlororuthenates NKP1339 [4] and NAMI-A [5,6] are lead compounds that have entered clinical trials for respectively colorectal and non-small cell lung cancer treatment. Besides these "reactive" complexes that can create covalent bonds with biomolecules, attention has also been paid to "inert" ruthenium polypyridine complexes as DNA binding agents. The luminescent $[Ru(phen)_2(dppz)]^{2+}$ complex with (dppz = dipyrido [3,2-a:2′,3′-c] phenazine) is a relevant example of "light switch" for DNA since its luminescence is efficiently increased upon DNA intercalation by the dppz ligand [7–9].

Ruthenium polypyridine complexes can also display toxicity against some cancer cell lines without exhibiting any specific interaction with DNA. Starting from $Ru(bpy)_3^{2+}$ that is known to be inactive [10], works have focused on the modification of the ligands around the metal in order to tune the complexes properties. The introduction of cyclometallating ligands i.e. by replacing an N–Ru bond by a C–Ru one was reported by Pfeffer to promote a good antitumor activity. The variations in cytotoxicity where closely related to several factors such as the redox potential and the lipophilicity [11–13]. Besides cyclometallation, another approach is to modify the substitution on bipyridine ligands [14–17]. Our group has reported that pyrrolidine moieties deeply affected the electronic and electrochemical properties of the corresponding homoleptic ruthenium polypyridine complexes [18]. This feature could be of interest for the biological applications and we decided to examine their potential role as anti-cancer agents. Herein we report our investigations along this direction with complexes **1–4** designed containing pyrrolidine-based bipyridine and terpyridine ligands (Fig. 1).

2. Experimental

2.1. Synthesis

Complexes **1–4** were prepared according to the published procedures [18] and general procedures are described in Supplementary information.

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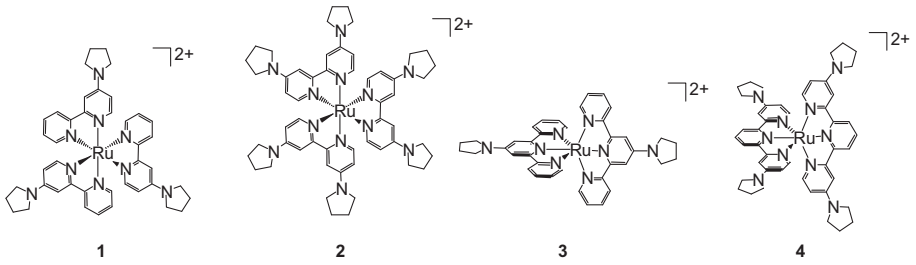

Fig. 1. Complexes studied in this work. The counter anion is PF_6^-.

2.2. Measurements

Cyclic voltammetry has been performed with a Radiometer PST006 potensiostat galvanostat using a conventional three-electrodes cell purged under argon. The scan rate was 100 mV/s, the solution was CH_3CN containing 0.1 M Bu_4NPF_6 as supporting electrolyte or deionized water containing 0.3% of DMSO v/v for better solubilization of the complexes in this case 0.1 M KCl was used as supporting electrolyte.

UV–Vis absorption spectra of Ru complexes were recorded at room temperature in water solution with a small amount of DMSO (<0.5% v/v) using Perkin Elmer Lambda 35 spectrophotometer. Luminescence measurements were registered on Perkin Elmer LS55 spectrofluorimeter in the range 465–900 nm upon excitation at a maximum of charge transfer band for each Ruthenium complex. The average of three scans was subjected to smoothing.

For determination of the quantum yield of luminescence, a water solution of $[Ru(bpy)_3]Cl_2$ was used as a standard [19]. Spectra were recorded with concentrations that gave less than 0.05 absorbance units at the excitation wavelength. Reference measurement was performed with the same parameters as corresponding ruthenium complex. Values were calculated according to the following equation [20]:

$$\Phi = \Phi_{ref} \times \left[\frac{A_{ref}}{A}\right] \times \left[\frac{I}{I_{ref}}\right] \times \left[\frac{n^2}{n_{ref}^2}\right]$$

where I is the integrated intensity of luminescence, A is the optical density, and n is the refractive index, *ref* refers to the values for reference. The mean value from minimum three independent experiments was calculated.

2.3. Binding constant determination

The experimental details concerning interaction with calf thymus DNA are described in Supplementary information. Human serum albumin (HSA) was dissolved in water to prepare a stock solution. Concentration of this solution was determined spectrophotometrically using a molar absorption coefficient of 44 000 cm^{-1} M^{-1} at 280 nm [21]. Emission spectra were recorded between 300 and 550 nm upon excitation at 295 nm (selective excitation of tryptophan residue in HSA). Three scans were used to smooth the average spectra. Spectra were corrected due to dilution and internal filter effect (multiply by 10^((A_{ex} + A_{em})/2) where A_{ex} is the absorption at extinction wavelength (295 nm) and A_{em} is absorption at emission wavelength (355 nm)). Protein binding experiments were performed by recording fluorescence spectra of protein (concentration 0.5 μM) in the presence of increasing Ru complexes concentration (up to 2 μM) in TRIS–HCl buffer pH 7.4 at 37 °C. The ruthenium-protein solution was incubated for 5 min in 37 °C before recording spectra.

2.4. In vitro studies

Cytotoxicity was tested using murine colon carcinoma (CT26) and human lung adenocarcinoma epithelial (A549) cell lines. Cells were cultured in DMEM medium supplemented with ferine serum (10%) and antibiotics: penicillin (100 units/mL) and streptomycin (100 μg/mL) at atmosphere of 5% CO_2 at 37 °C. Cells were seeded on 96 wells plates with a seeding density of 5000 cells per well and incubated overnight. Next cells were incubated with various concentrations of Ru compounds (freshly diluted in DMSO and then added to the appropriate medium, final DMSO concentration varied from 0.3% to 0.7% (v/v)) or cisplatin in medium without serum for 24 h in the dark. Next cells were washed with PBS and their viability was measured using Alamar Blue assay [13,22]. Experiments were performed in triplicates and each experiment was performed at least three times to get the mean values ± standard deviation.

For cell imaging Olympus fluorescence microscope IX51 equipped with XC10 camera was used. A549 cells were seeded into 6 wells plate with density 200 000 cells/well. After cultivation for 24 h, the cells were incubated with **1** (20 μM). After incubation (24 h at 37 °C), the Ru complexes were washed out and the cells were washed with PBS three times. Next, keeping cells in PBS buffer, images of the studied Ru complexes embedded in A549 cells were taken using a 470–495 nm excitation filter. For co-localization experiments, A549 cells were seeded with a density of 15 000 cells per well 24 h prior the staining. LysoTracker Blue, ER-Tracker™ Blue-White DPX and Mitotracker Green (Life Technologies) were used to image lysosomes, ER and mitochondria according to the manufacture manuals.

The cyto-ID Hypoxia/Oxidative stress detection kit was used for detection of the total reactive oxygen species (ROS) produced in the cells upon addition of Ru complexes. As a positive control pyocyanin was used. The level of oxidative stress was determined in A549 and CT26 cells using Infinite 200 microplate reader (Tecan) at 525 nm using 490 nm excitation wavelength. Cells were seeded in a black 96 wells plate with a density 5000 cells/well. After overnight incubation ruthenium complexes were added in serum free medium at concentration $IC_{50}/4$ and incubated for another 24 h. Then cells were washed twice with PBS and detection of ROS was performed according to manufacture protocol. Experiment was performed in triplicates and results were calculated versus untreated cells after background subtraction while as a positive control pyocyanin was applied. For ROS inhibitors assay, Tiron (4,5-dihydroxy-1,3-benzenedisulfonic acid disodium salt, 5 mM) and NAC (N-acetylcysteine, 10 mM) were applied 1 h before the treatment with the Ru complexes and kept in the medium during Ru complexes treatment until the cells were analyzed.

3. Results and discussion

The complexes have been prepared quickly and efficiently by reaction of the appropriate stoichiometry of ligands with