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Biological evaluation of nitrile containing Ru(II) polypyridyl complexes as potential photodynamic therapy agents

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ABSTRACT

The use of Ru complexes in light-mediated treatment of cancer (i.e. Photodynamic Therapy – PDT) has recently become extremely relevant with the entry into clinical trials of the first complex of this class against bladder cancer in the very near future. Herein, we report on the potential application as PDT agents of two inert Ru(II) polypyridyl complexes bearing a nitrile containing dppz ligand and two bipy or phen ancillary ligands for **1** and **2**, respectively (dppz = dipyrido[3,2-a:2′,3′-c]phenazine, bipy = 2,2′-bipyridine, phen = 1,10-phenathroline). More specifically, a full characterization of the novel compound **2** was first performed. The distribution coefficients (log *D*) and $^1O_2$ quantum yields in two solvent systems and at two irradiation wavelengths were then determined for both compounds. The phototoxicity of complexes **1** and **2** was evaluated on cervical cancer HeLa cells and on non tumorigenic retinal pigment epithelial (RPE1-hTERT) cells. None of the complexes was found to be phototoxic. *In vitro* fluorescence microscopy indicated a scarce cellular uptake for **2**. The lack of biological activity for complexes **1** and **2** highlights that more investigations are required in order to understand the relationship between structure and biological activity for this class of compounds.

1. Introduction

The application of ruthenium complexes as biologically active compounds was thoroughly investigated in the last decades. Two Ru(III) complexes are currently undergoing Phase II clinical trials as anticancer agents (i.e. NAMI-A and KP-1339) and a Ru(II) organometallic complex is under clinical optimization (i.e. RAPTA-C) [1–4]. The complexes which are under evaluation are characterized by a mechanism of cytotoxicity based on ligand exchange. Another class of Ru compounds, namely substitutionally inert Ru(II) polypyridyl complexes, is characterized by favourable photophysical properties that make these complexes attractive for applications such as photosensitizers (PSs) in Photodynamic Therapy (PDT) and Photoactivated Chemotherapy (PACT) [5]. The use of such complexes for light-mediated treatment of cancer has thoroughly been investigated in the last years [5]. As a highlight of this research, a Ru(II) polypyridyl compound will be soon tested as a PDT agent in the clinics against non-muscle invasive bladder cancer (TLD-1433, Fig. 1) [6]. Several reasons make this type of compounds useful in this field of research. For example, they display a strong absorbance in the visible region of the electromagnetic spectrum. Moreover, they are characterized by a reasonably long-lived triplet excited state. This allows for energy transfer to triplet oxygen ($^3O_2$) to form the toxic singlet oxygen ($^1O_2$). However, in this recent and promising research area, there is still a great room for optimization. For example, it would be highly desirable to have novel PSs with enhanced biological activity and/or activation wavelengths in the PDT window (650–900 nm) [23,24] and/or possibly O_2-independent mechanisms of action [25–27]. Our group has investigated the potential of Ru(II) polypyridyl compounds as drug candidates over the last years. More specifically, were ported on the activity of Ru(II) polypyridyl complexes as anticancer drug candidates [7,8] as well as PSs in antibacterial PDT [9] and as PDT [9,10] or PACT [11,12] agents in cancer treatment. Herein, we report on the biological evaluation of two Ru(II) complexes of the $[Ru(II)(L)_2dppz\text{-}7\text{-}CN]^{2+}$ class as potential PDT agents, where **1**: L = bipy and **2**: L = phen (see Fig. 1; dppz = dipyrido[3,2-a:2′,3′-c]phenazine, bipy = 2,2′-bipyridine, phen = 1,10-phenathroline). **1** was previously synthesized by Gordon *et al.*, who performed photophysical studies and DFT calculations on this complex [13]. The biological activity of this compound was, however, never investigated. **2** is a novel parent complex of **1**, bearing two extended aromatic phen groups as ancillary ligands. To characterize the compounds, we measured the distribution coefficients (log *D*) for both complexes to obtain

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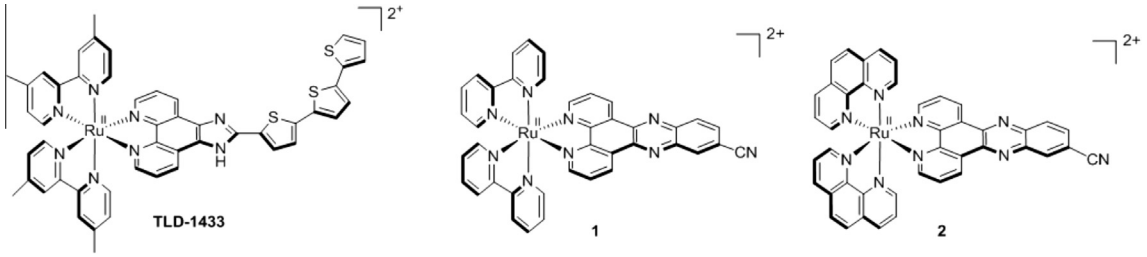

Fig. 1. Structures of TLD-1433 and of the two complexes examined in this study $[Ru(bipy)_2dppz\text{-}7\text{-}CN]^{2+}$ **1** and $[Ru(phen)_2dppz\text{-}7\text{-}CN]^{2+}$ **2**. The complexes **1** and **2** were isolated as PF_6^- salt and racemic mixture of the isomers.

information on their behavior in biological environments. The production of $^1O_2$ in two different media (i.e., acetonitrile and PBS solution buffer) upon irradiation at 420 and 575 nm was also evaluated. Finally, the cytotoxic activity of the complexes was investigated on two different cell lines, namely non tumorigenic retinal pigment epithelial (RPE1-hTERT) cells and cervical cancer (HeLa) cells. In addition, the phototoxicity of complexes **1** and **2** was tested on HeLa cells upon irradiation at 420 nm. Due to the higher luminescence quantum yields, the cellular localization of **2** was also studied when incubated in HeLa cells.

2. Experimental part

2.1. Instruments and methods

All commercial chemicals were used without further purification. ^1H and ^{13}C NMR measurements were carried out on Bruker 500 spectrometers and referenced to residual solvent peaks. ESI-MS and R.P. UPLC-MS were performed using a Bruker Daltonics HCT 6000 mass spectrometer. R.P. UPLC-MS spectra were recorded on a Waters Acquity™ system equipped with a PDA detector and an auto-sampler. R.P. UPLC-MS was performed on an Acquity UPLC BEH C18 column (21.50 × 1.7 mm). The UPLC run (flow rate: 0.6 mL min^{-1}) was carried out with a linear gradient of A (double distilled water containing 0.1% v/v formic acid) and B (acetonitrile): t = 0 min, 5% B; t = 0.25 min, 5% B; t = 1.5 min, 100% B; t = 2.5 min, 100% B. High resolution ESI-MS spectra were recorded using a Bruker ESQUIRE-LC quadrupole ion trap instrument. UV spectra were recorded on a Varian Cary 100 spectrophotometer. Elemental microanalyses were performed on a LecoCHNS-932 elemental analyser. UV-A irradiation was performed using a Rayonet RPR-200 photochemical reactor with 6 (for biological evaluations) or 12 (for singlet oxygen measurements) bulbs (14 W each) and maximum intensity output at 420 nm and 575 nm. Samples were irradiated in a Starna GmbH 3.5 mL fluorescence quartz cuvette (width 1 cm) placed in the centre of the reactor. The light intensity at that spot, measured with an X11optometer (Gigahertz-Optik), was 77.2 W m^{-2} at 420 nm and 69.3 W m^{-2} at 575 nm. The temperature inside the reactor was 30 °C. *In vitro* luminescence experiments were performed using a CLSM Leica SP5 microscope.

2.2. Synthesis of **1**

1 was synthesized as previously reported. All analytical data matched what previously reported [13].

2.3. Synthesis of **2**

dppz-7-CN [13] (107 mg, 0.350 mmol, 1.0 eq) and $[Ru(phen)_2Cl_2]$ [14] (185 mg, 0.355 mmol, 1.1 eq) were suspended in 100 mL of degassed EtOH/H_2O 1/1 solution. The mixture which was refluxed for 3 h turned red during the reaction. The organic solvent was removed by rotary evaporation and the crude product purified by column chromatography on silica gel with CH_3CN/aq. KNO_3 0.4 M (10/1) as the eluent. The fractions containing the product were combined and the eluent was removed on a rotary evaporator. Workup of the reunited fractions after the column: CH_3CN (30 mL) was added to the solid residue to dissolve the red product. The white, insoluble solid (excess of KNO_3) was removed by filtration. The solvent was removed by rotary evaporation and the residue redissolved in water (40 mL). NH_4PF_6 was then added to make the complex precipitate as a PF_6 salt. The precipitate was collected by filtration, washed with water (20 mL) and diethyl ether (25 mL) and dried on a vacuum pump, to obtain **2** as a red powder in 62% yield. ^1H NMR (500 MHz, CD_3CN) δ (ppm): 7.62–7.68 (m, 4H), 7.77–7.80 (m, 2H), 8.01–8.02 (d, 2H), 8.13–8.15 (m, 2H), 8.22–8.23 (m, 2H), 8.25–8.27, (m, 5H), 5.57–8.59 (d, 1H), 8.60–8.64, (t, 4H), 8.93–8.94 (d, 1H), 9.58–9.63 (m, 2H). ^{13}C NMR (126 MHz, CD_3CN): 116.13, 118.67, 126.84, 126.90, 128.38, 128.42, 129.03, 129.04, 131.27, 132.02, 132.04, 132.23, 133.33, 134.59, 134.79, 137.06, 137.92, 137.98, 142.45, 142.61, 143.10, 144.61, 148.74, 148.81, 152.27, 152.46, 153.91, 154.22, 155.78, 155.92. ESI-MS (pos. detection mode): m/z $[M\text{-}2PF_6]^{2+}$ 384.7, $[M\text{-}2PF_6{+}TFA]^{2+}$ 882.2. ESI HR-MS calcd. for $[C_{43}H_{25}N_9Ru]/z$ $[M\text{-}2PF_6]^{2+}$ 384.5633, found 384.5644; Elem. Anal. Calc. for $C_{43}H_{25}F_{12}N_9P_2Ru + CH_3COCH_3$: C, 49.47; H, 2.80; N, 11.29. Found: C, 49.37; H, 2.61; N, 11.50%. UPLC r.t.:1.05 min.

2.4. Luminescence quantum yields and lifetimes

For the luminescence quantum yields measurements, emission spectra were recorded with a Varian Cary Eclipse Fluorescence Spectrophotometer equipped with a Hamamatsu R3896 photomultiplier tube as detector, where the sample temperature can be controlled by a Peltier thermostatic system. Emission spectra for excitation at 440 nm were corrected for the spectral sensitivity of the detection system by standard correction curves. The emission intensities were normalized to a nominal absorption value of 0.1. The quantum yield in aerated CH_3CN was determined by comparison with the emission of $[Ru(bipy)_3]Cl_2$ in aerated water (Φ = 0.042) [15].

Luminescence lifetime measurements were recorded in degassed CH_3CN using an Edinburgh LP920 Laser Flash Photolysis transient absorption spectrometer with a flash lamp pumped Q-switched Nd:Yag laser (355 nm) as excitation source.

2.5. Distribution coefficients

The distribution coefficient of each complex was experimentally determined by using the "shake-flask" method as previously reported in our group [10]. Briefly, the complexes were dissolved in a 10 mM phosphate buffer (pH 7.01) previously saturated with *n*-octanol to give about 1 mL of a solution with a concentration of 50 μM. The same volume of octanol (previously saturated with