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New polydentate Ru(III)-Salan complexes: Synthesis, characterization, anti-tumour activity and interaction with human serum proteins

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ABSTRACT

Two new Ru(III) complexes bearing tetradentate N_2O_2 bis(aminophenolate) ligands (*i.e.* Salan-type ligands), were synthesized and characterized. The paramagnetism of the new [Ru^{III}(Salan)Cl(PPh_3)] (Salan = 4-methoxy/5-methoxy derivatives of 1,4-bis(salycilidene)cyclohexanediamine, PPh_3 = triphenylphosphane) was proved by spectroscopic studies. These complexes exhibit a $4d^5$ low-spin distorted octahedral geometry. The anti-tumour activity of ligands and complexes was screened *in vitro* against A2780, MCF7 and MDAMB231 human cancer cell lines. Both ligands and complexes exhibit moderate to high cytotoxicity against all investigated cell lines, in some cases surpassing that of *Cisplatin*. Coordination to the Ru(III) center enhanced the cytotoxicity of each bis(aminophenolate) ligand by at least twofold.

Binding of both Ru(III)-Salan complexes to human serum albumin is strong, as evaluated by steady-state and time-resolved fluorescence spectroscopy, suggesting that this protein might be a transport vehicle in the blood serum for these agents. The cytotoxicity of the protein-bound Ru(III)-Salan complex was assessed, as well as the effect of serum albumin binding on the activity of these complexes.

These new Ru(III)-Salan are the first compounds of this class studied for anti-tumour proposes reported in the literature.

1. Introduction

The discovery of *cisplatin*'s anti-tumour activity was a landmark in the field of chemotherapy [1]. *Cisplatin* and two other platinum(II)-based drugs (carboplatin and oxaliplatin) are the only metallodrugs approved worldwide in cancer chemotherapy, and integrate to date many anti-cancer therapies [2,3]. Despite their relevance and great success in clinic, platinum drugs present some limitations such as innate or acquired tumour resistance, severe side-effects and limited spectra of action [4,5]. The need to overcome these drawbacks and to expand the scope of action of metallodrugs has powered research in the pursuit for alternative efficient therapies. This is the case of ruthenium complexes, which correspond to the class of most widely studied non-platinum compounds, showing considerable potential use as metallodrugs for cancer treatment [6,7], some of them being reported as selective anti-tumour agents with low toxicity [8]. These alternative non-platinum agents afford mechanisms of action quite distinct from those of *cisplatin* and its analogs, with some of the ruthenium complexes reported directly acting on *cisplatin* unresponsive primary solid tumours, while some other Ru-compounds act on the metastatic process and prevent the tumour from spreading [4]. Our group has been engaged in the development of "$Ru^{II}Cp$" (Cp = η^5-cyclopentadienyl) compounds as anti-cancer agents [9–12], and have recently reported a family of organometallic "$Ru^{II}Cp$" complexes with N-heteroaromatic co-ligands exhibiting anti-proliferative activity in the nanomolar range against human colon adenocarcinoma, pancreatic cancer and leukemia [11,12], reinforcing the great potential of Ru^{II} compounds as anti-tumour agents. Structured "Piano-stool" η^6-C_6H_6– ruthenium complexes are potential anticancer agents, as they present themselves to be versatile scaffolds/templates for rational drug design [13–18].

Ruthenium(III) complexes are less explored in this frame. NAMI-A ([HIm][*trans*-$Ru^{III}Cl_4$(DMSO)Im], Im = imidazole) [19] and KP1019 ([HInd][*trans*-$Ru^{III}Cl_4(Ind)_2$], Ind = indazole) [6] are octahedral ruthenium(III) complexes that constitute the sole examples of ruthenium compounds currently in advanced phases of clinical trials [20]. Thus, the exploration of complexes involving Ru(III) seems

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to be a pertinent area for the research of potential candidates for chemotherapy.

Due to the cytotoxicity already reported for platinum-based drugs [21,22] there has been a considerable growing interest on the use of ligands containing (O,N), (O,S), (N,S) and (S,S) donor atoms. Tetradentate N_2O_2 salen ligands (salen = bis(salicylaldehyde) ethylenediimine) are known for their versatility in terms of ease of preparation allowing steric and electronic modifications. Salen metal complexes are well known, mostly due to their use in catalysis [23–25]. The imine-reduced variant, tetradentate bis(aminophenolate) ligands (also known as tetrahydrosalen or Salan) also offering the N_2O_2 binding motif are much less studied, probably due to a less straightforward synthesis. However, when compared to salens, Salan compounds offer increased flexibility and are stronger nitrogen donors, which makes them quite attractive ligands to yield stable complexes [26–32]. In addition, the phenolate oxygen is a recognized hard donor and is suitable for stabilizing the +3 oxidation state of a coordinated ruthenium ion [33].

Recent reports regarding the anticancer activity of titanium complexes with salen- or Salan-type ligands showed remarkable results towards a diverse array of human tumour cell lines [34–38]. Ru-Salan complexes are virtually unexplored, apart from one report on the synthesis of a $[Ru^{II}(R_2\text{-Salan})(CH_3CN)_2]$ (R = *tert*-butyl) for catalytic applications [26].

Herein we present the synthesis and characterization of two new ruthenium(III) complexes with Salan-type ligand, as well as the evaluation of their cytotoxic activity in A2780 ovarian (*cisplatin* sensitive) and MCF7 and MDAMB231 breast human tumour cell lines. To the best of our knowledge these are the very first Ru^{III}-Salan compounds reported. Since drug binding to plasma proteins can exert a significant effect on its distribution, activity and pharmacokinetics[39,40], we have also investigated the interaction of the new Ru^{III}-Salan complexes with human serum albumin as a vehicle for the transport in the blood plasma and possible passive targeting, and assessed the activity of the {protein-complex} adduct(s) formed.

2. Experimental

2.1. Materials and methods

The Salan ligands were synthesized in aerobic conditions, and solvents used were p.a. grade with no further treatment prior to use. 1R,2R-cyclohexane-1,2-diamine tartrate, 4-methoxysalicylaldehyde and 5-methoxysalicylaldehyde were purchased and used without further purification.

The synthesis of the metal complexes was carried out under nitrogen atmosphere using standard *Schlenk* techniques. The solvents used were dried following standard methods [41]. Starting material tris(triphenylphosphane)ruthenium(II) dichloride $[\{(C_6H_5)_3P\}_3RuCl_2]$ was purchased and used without further purification. Human serum albumin (fatty acid free, A3782) and apotransferrin (Iron-free human apo-transferrin, T4382) were purchased from Sigma–Aldrich. Millipore® water was used for the preparation of 10 mM Hepes (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, Sigma Aldrich) buffer solutions in all experiments involving fluorescence spectroscopy, adjusted to pH 7.4 with KOH and HCl.

^{1}H NMR spectra were recorded on a Bruker Avance 400 MHz spectrometer or Bruker Avance II$^+$ at probe temperature. The ^{1}H chemical shifts (δ) are reported in parts per million (ppm) downfield from internal Me_4Si. Elemental analyses were obtained at *Laboratório de Análises,* (*Instituto Superior Técnico,* Lisboa), using a Fisons Instruments EA1108 system. Data acquisition, integration and handling were done using a PC with the software package EAGER-200 (Carlo Erba Instruments). FT-IR spectra were recorded in the 4000–400 cm^{-1} range from KBr pellets in a Mattson Satellite FT-IR spectrophotometer; only significant bands are cited in text. Isotropic electronic spectra were recorded on a Jasco V-560 spectrometer in the range 240/270–900 nm (in dichloromethane/DMSO) with 1 cm path quartz Suprasil® cuvettes at room temperature. Circular dichroism (CD) spectra were recorded on a Jasco J-720 spectropolarimeter (JASCO, Hiroshima, Japan) with a 175–800 nm photomultiplier (EXEL-308) using quartz Suprasil® CD cuvettes (with 1 cm, 0.5 cm or 0.1 cm path length according to the signal intensity) at $t = (25 \pm 2)$°C. Each CD spectrum measured was the result of three accumulations originally recorded in ellipticity (millidegrees), and converted to $\Delta\varepsilon = \varepsilon_L - \varepsilon_R$ = {differential absorption} with the Jasco spectropolarimeter software. CD spectra were represented as $\Delta\varepsilon_m$ *vs.* λ with $\Delta\varepsilon_m$ = {differential absorption}/(bC) where b is the optical path and (unless otherwise stated) C is the total concentration of complex in the cuvette. Samples of appropriate concentration of each complex ($C \sim 10^{-4}$–10^{-6} M for UV–Vis, $C \sim 0.5 \times 10^{-4}$ M for CD) were prepared in dichloromethane and DMSO, and spectra recorded in the range 270–800 nm or 240–800 in the case of CH_2Cl_2 or DMSO, respectively. EPR spectra were recorded at 77 K with a Bruker ESP 300E X-band spectrometer coupled to a Bruker ER 041 XK X-band frequency meter (9.45 GHz) from DMSO and DMF solutions of each complex (previously frozen in liquid nitrogen). The electrochemical experiments were performed on an EG&G Princeton Applied Research Model 273A potentiostat/galvanostat and monitored with a personal computer loaded with Electrochemistry PowerSuite v2.51 software from Princeton Applied Research. Cyclic voltammograms were obtained in 0.2 or 0.1 M solutions of $[NBu_4][PF_6]$ in CH_2Cl_2 or CH_3CN respectively, using a three-electrode configuration with a platinum-disk working electrode (1.0 mm diameter), a silver-wire pseudo-reference electrode and a Pt wire auxiliary electrode. The electrochemical experiments were carried out under a N_2 atmosphere at room temperature. The redox potentials of the complexes were measured in the presence of ferrocene as the internal standard and the redox potential values are normally quoted relative to the SCE (saturated calomel electrode) by using the ferrocenium/ferrocene redox couple (Ep/2 = 0.46 or 0.40 V *versus* SCE for CH_2Cl_2 or CH_3CN, respectively) [42]. The supporting electrolyte was purchased from Aldrich Chemical Co., recrystallized from ethanol, washed with diethyl ether and dried under vacuum at 110 °C for 24 h. Reagent grade acetonitrile and dichloromethane were dried over P_2O_5 and CaH_2, respectively, and distilled under nitrogen atmosphere before use.

Magnetic susceptibility was estimated using the Evan's method. A 3.56 mM solution of complex **1** in $CDCl_3$ was sealed in a capillary tube, and the solvent shift at 295.5 K was recorded on a 400 MHz Bruker Avance II$^+$ spectrometer. μ_{eff} (μ_B) was calculated using procedures reported in the literature [43,44]. The solvent density correction was not necessary considering the high dilution of the sample. Diamagnetic corrections were then applied to χ_M using Pascal's constants [45].

2.2. Synthesis

2.2.1. Synthesis of the ligands

2.2.1.1. Ligand 4-MeO-Sal-Chan, ***L1****.* 1R,2R-cyclohexane-1,2-diamine tartrate (1.0 g, 3.9 mmol) was suspended in 25 mL of MeOH. Potassium hydroxide (1.2 g, 7.8 mmol) was dissolved in 5 mL of water and this solution was added to the diamine suspension and stirred for ca. 10 min 4-methoxysalicylaldehyde (1.2 g, 7.8 mmol) was added and the mixture was stirred for an additional 30 min period. Sodium borohydride was added carefully to the bright yellow mixture until it became nearly colorless. The