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In vitro and *in vivo* biological activity screening of Ru(III) complexes involving 6-benzylaminopurine derivatives with higher pro-apoptotic activity than NAMI-A

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ABSTRACT

A series of novel octahedral ruthenium(III) complexes involving 6-benzylaminopurine (L) derivatives as N-donor ligands has been prepared by the reaction of $[(DMSO)_2H][trans\text{-}RuCl_4(DMSO)_2]$ with the corresponding L derivative. The complexes **1–12** have the general compositions *trans*-$[RuCl_4(DMSO)(n\text{-}Cl\text{-}LH)]\cdot x$Sol (**1–3**), *trans*-$[RuCl_4(DMSO)(n\text{-}Br\text{-}LH)]\cdot x$Sol (**4–6**), *trans*-$[RuCl_4(DMSO)(n\text{-}OMe\text{-}LH)]\cdot x$Sol (**7–9**) and *trans*-$[RuCl_4(DMSO)(n\text{-}OH\text{-}LH)]\cdot x$Sol (**10–12**); n = 2, 3, and 4, $x = 0$–1.5; and Sol = H_2O, DMSO, EtOH and/or $(Me)_2CO$. The complexes have been thoroughly characterized by elemental analysis, UV–visible, FTIR, Raman, and EPR spectroscopy, ES + (positive ionization electrospray) mass spectrometry, thermal analysis, cyclic voltammetry, magnetic and conductivity measurements. The X-ray molecular structure of *trans*-$[RuCl_4(DMSO)(3\text{-}Br\text{-}LH)]\cdot(Me)_2CO$ (**5**) revealed the distorted octahedral coordination in the vicinity of the central atom, and also confirmed that the 3-*Br*-L ligand is present as the N3-protonated N7-H tautomer and is coordinated to Ru(III) through the N9 atom of the purine moiety. The tested complexes have been found to be *in vitro* non-cytotoxic against K562, G361, HOS and MCF7 human cancer cell lines with $IC_{50} > 100$ μM in contrast to the moderate results regarding the antiradical activity with $IC_{50} \approx 10^{-3}$ M. On the contrary, *in vivo* antitumor activity screening showed that the prepared Ru(III) complexes possess higher pro-apoptotic activity than NAMI-A. The reduction of Ru(III) to Ru(II) and Ru(II)-species formation in tumor tissues was confirmed by means of a simple method of detection and visualization of intracellular Ru(II) by fluorescence microscopy. The originality of this method is based on the preparation of a Ru(II)-bipyridine complex *in situ*.

1. Introduction

A great number of coordination compounds have been used in the therapy of various diseases up to now. The successful development of metal-containing anticancer drugs clearly started with *cis*-$[Pt(NH_3)_2Cl_2]$, referred to as cisplatin [1,2]. In these days, cisplatin and its analogs are still ones of the most effective chemotherapeutic agents in clinical use. However, their high toxicity and acquired or intrinsic drug resistance remain the main drawbacks in their clinical applications. These limitations have prompted the search for alternative chemotherapeutic strategies [3–5]. Therefore, nowadays, anticancer research is focused on the investigation of either "non-classical" platinum-containing complexes or compounds involving other suitable transition metals, *e.g.* Ru, Rh, Ti, and V [1,2,6]. Among the "non-platinum" complexes, the ruthenium compounds have attracted significant attention, since they offer many suitable features for becoming new metallopharmaceuticals and a promising alternative to platinum-based substances. Ruthenium complexes can exhibit more advantageous physico-chemical properties compared to Pt-involving drugs, *e.g.* coordination satiation in connection with the octahedral geometry and, as a consequence of this, another mode of interactions with DNA; and a range of oxidation states accessible under physiological conditions [2,7–9]. Additionally, ruthenium compounds are generally less toxic than their platinum counterparts, which is believed to be due to the ability of ruthenium to mimic iron in binding to biomolecules, including serum proteins (*e.g.* transferrin and albumin) [2,9]. Indeed, the selectivity of Ru species might be derived from the capacity of these compounds to be transported with transferrin into tumor cells, which in their higher iron requirements often over-express transferrin-receptors [10,11]. Moreover, the selectivity against solid tumors and lower toxicity of some ruthenium compounds (with biologically accessible reduction potentials and substitutable ligands) is often connected with the "activation-by-reduction" hypothesis, when ruthenium(III) complexes remain in their relatively inactive Ru(III) oxidation states until they reach the tumor site. In this environment, with its lower oxygen content and pH than in normal tissues, reduction to the more reactive Ru(II) oxidation state takes place [2,7,12].

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In the past few decades, a large number of Ru(II/III) complexes have been prepared and characterized, while some of them have already demonstrated the ability to control tumor proliferation, growth and metastasis in preclinical models [2,7,12–14]. Among these complexes, the Ru(III) complexes involving N-donor heterocycles as ligands have been intensively studied both due to their antitumor activity, especially against metastatic cancers [15,16], and in connection with their ability to bind to imine sites of biomolecules with a relatively high affinity [17,18]. To date, two ruthenium agents involving N-donor heterocyclic ligands, NAMI-A {[ImH][*trans*-$RuCl_4(DMSO)(Im)$] (Im = imidazole)} and KP1019 {[IndH][*trans*-$RuCl_4(Ind)_2$] (Ind = indazole)}, have entered human clinical trials [14]. Despite their structural and chemical similarities, these two Ru(III) complexes show distinct antitumor behaviors. In preclinical studies, NAMI-A has demonstrated inhibitory effects against the formation of cancer metastases in a variety of tumor animal models but appears to lack direct cytotoxic effects, while KP1019 has shown direct antitumor activity against a wide range of primary human tumors by inducing apoptosis. These differences derive from diverse modes of action of these two complexes. KP1019 is a new tumor-inhibiting drug acting by a mechanism involving accumulation in transferrin receptor-(over)expressing tumor cells as well as subsequent reduction to Ru(II) species in reductive tumor environment (the "activation-by-reduction" mechanism) [19]. On the other hand, in the case of the selective antimetastatic activity of NAMI-A, the "activation-by-reduction" mechanism has been challenged by recent findings. In fact, although reduction of NAMI-A is likely to occur *in vivo* (reducing agents might be glutathione or ascorbic acid), it seems that it is not specifically needed for the activation and consequent biological action. Due to NAMI-A relative lability at physiological pH, activation may occur by an aquation (*i.e.* chlorido ligands are substituted by aqua ligands) [10,20,21]. Additionally, the studies on the NAMI-A mechanism of action seem to exclude DNA as the primary cellular target. It likely binds to molecules (probably proteins) expressed or relevant only in the metastatic tumor cells [11,22]. Overall, the mechanism of metastasis control seems to be attributable to the combined effects of anti-angiogenic and anti-invasive properties of NAMI-A on tumor cells and blood vessels [23]. Anyway, for both NAMI-A and KP1019, a phase I study has been recently reported. A phase II study evaluating KP1019 in patients with advanced colorectal cancer is now being planned [14].

The experience acquired in the last years in the area of ruthenium compounds indicates the relationship between the structure and activity for compounds that appear to function by the mechanisms including the transport into the cell via the transferrin cycle and activation by reduction. The nature of the N-donor ligand and/or presence of DMSO in the coordination sphere of the central atom influence redox properties and antitumor activity of such complexes [24]. For instance, due to π-acceptor properties of S-bonded DMSO, the reduction potential values, E_{red}, of such complexes are shifted to higher values compared to those without the coordinated DMSO molecule {*e.g.* E_{red} for NAMI-A equals 253 mV, and for [ImH][*trans*-$RuCl_4(Im)_2$] equals −160 mV, both vs normal hydrogen electrode (NHE), measured in water} [25]. The higher values of E_{red} are more suitable for biological application due to similarity of the electrode potential between the tumor and normal tissues [24].

The organic compounds containing the 6-benzylaminopurine (L) skeleton belong to the group of aromatic cytokinins which affect a variety of important physiological processes such as cell division, differentiation and senescence [26]. A suitable modification of the purine skeleton at the C2 and/or N9 positions can dramatically change and extend the spectrum of biological effects of these compounds. The most promising representative of such derivatives, *(R)*-Roscovitine, *i.e.* 2-[(*R*)-(1-ethyl-2-(hydroxyethyl)amino)]-6-benzylamino-9-isopropylpurine, can be classified among cyclin-dependent kinase inhibitors (*i.e.* enzymes being able to regulate the human cell cycle and showing a significant antineoplastic activity) [27], and has successfully passed *in vivo* and preclinical testing and it is currently being tested (named as Seliciclib or CYC202) in the IIb-phase of clinical evaluations on patients with non-small cell lung cancer (NSCLC) [28]. To date, only one Ru-complex bearing unsubstituted L with the composition of [$RuCl_4(DMSO)(LH)$] has been prepared [29]. The interaction study of [$RuCl_4(DMSO)(LH)$] with plasmidic DNA was performed and the results showed different morphological changes in the DNA forms. In our previous results, we have already shown that coordination of the L derivatives to suitable transition metal ions (*e.g.* Pt, Pd, Cu *etc.*) can lead to the formation of complexes with notable biological activity (*in vitro* cytotoxicity, SOD-like activity) [30,31].

On the basis of the above-mentioned facts, we decided to prepare, fully characterize a series of novel Ru(III)-complexes involving the variously benzyl-substituted 6-benzylaminopurine derivatives, and study their biological activity (*in vitro* cytotoxicity, antiradical SOD-mimic activity, *in vivo* antitumor activity), and moreover, based on the obtained findings, try to explain the possible mechanism of their biological action.

2. Experimental section

2.1. Materials

$RuCl_3 \cdot xH_2O$, 6-chloropurine, 2-methoxybenzylamine, 3-methoxybenzylamine, 4-methoxybenzylamine, 2-hydroxybenzylamine hydrochloride, 3-hydroxybenzylamine, 4-hydroxybenzylamine, 2-chlorobenzylamine, 3-chlorobenzylamine, 4-chlorobenzylamine, 2-bromobenzylamine hydrochloride, 3-bromobenzylamine, 4-bromobenzylamine, 2,2′-bipyridine and solvents used were purchased from Aldrich Co. or Acros Co. and used as received.

2.2. Cell line for in vivo testing

The cell line L1210—Mouse DBA/2 lymphocytic leukemia was obtained from ECACC (European Collection of Cell Cultures), through Sigma-Aldrich Co. The frozen cells were thawed, reconstituted, and cultivated according to the standard procedure recommended by ECACC in the culture medium containing the Fischer's medium (Invitrogen), supplemented with 2 mM glutamine (Sigma-Aldrich Co.), and 10% horse serum (from controlled herd, Sigma-Aldrich Co.).

2.3. Physical measurements

Elemental analyses (C, H, N) were performed on a Fisons EA-1108 CHNS-O Elemental Analyzer (Thermo Scientific). Infrared spectra were obtained on a Nexus 670 FTIR spectrometer (Thermo Nicolet) using the KBr (4000–400 cm^{-1}) and Nujol (600–200 cm^{-1}) techniques. Raman spectroscopy measurements were performed using an NXR FT-Raman Module (Thermo Nicolet) in the 3750–150 cm^{-1} region. Electronic absorption spectra were recorded on a Lambda40 spectrometer (Perkin-Elmer) in the 45,000–10,000 cm^{-1} range. Conductivity measurements of the complexes in the *N,N′*-dimethylformamide (DMF) solutions (10^{-3} M) were performed on a Cond340i/SET conductometer (WTW) at 25 °C. Variable temperature magnetization measurements were carried out in the temperature range of 2–300 K at an applied magnetic field of 1 T on a grounded polycrystalline sample with a Quantum Design MPMS-XL-7 SQUID magnetometer. Isothermal magnetization measurements were performed at 2 K with magnetic fields up to 7 T. The magnetic data were corrected for the sample holder and for the diamagnetic contributions estimated using Pascal constant [32]. The EPR spectra were recorded on a MiniScope MS100 spectrometer (Magnettech GmbH, Germany) at the X-band frequency for the powder samples at room temperature. TG and DTA (thermogravimetric and differential thermal analysis) measurements of complexes **2**, **3**, **4** and **8** were made in air in the temperature range of 25–1000 °C using a TG/DTA 6200 Exstar thermal analyser (Seiko Instruments, USA) with a sample weight of 5–15 mg and thermal gradient of 5 °C min^{-1}. X-ray