Ruthenium-based complex nanocarriers for cancer therapy

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ABSTRACT

A new organometallic ruthenium complex, named AziRu, along with three amphiphilic nucleoside-based ruthenium complexes, ToThyRu, HoThyRu and DoHuRu, incorporating AziRu in their skeleton, have been synthesized, stabilized in POPC phospholipid formulations and studied for their antineoplastic activity. Self-aggregation behavior of these complexes was investigated, showing that the three synthesized AziRu derivatives able to form liposomes and, under specific conditions, elongated micelles. The formulations prepared in POPC proved to be stable for months and showed high in vitro antiproliferative activity. The here described results open new scenarios in the design of innovative transition metal-based supramolecular systems for anticancer drugs vectorization.

1. Introduction

In the last decades, research has proposed a growing number of antineoplastic agents. Among these, transition metal-based complexes represent, nowadays, a very important class of chemotherapeutics, intensively used for clinical treatments. The antineoplastic activity of the most important of these, Cisplatin, was discovered in 1969 [1] and in thirty years this complex has become one of the most used drugs in the treatment of some tumoral diseases, such as testicular, breast, uterine and ovarian cancers [2]. Since ever, some analogues of Cisplatin have been tested and then approved as drugs, such as Carboplatin and Oxaliplatin [3].

In addition to extremely high and selective anti-proliferative activity, novel anticancer drugs should exhibit specific efficacy toward the formation and growth of metastases. In fact, many tumors can develop metastases already very extended at the diagnosis time, making scarcely effective the surgical treatment [4]. If the primary tumor can be surgically removed, the pharmacological therapy seems to be the best choice for the metastases treatment, because of their non specific localization in the body [5]. On the other hand, unlike the primary tumor cells, metastases are not responsive to several chemotherapeutics, probably because of their different proliferation kinetics [6,7]. These limitations have prompted researchers operating in this field to develop more effective and less toxic metal-based anticancer agents.

In this context, ruthenium complexes have attracted much interest as a promising alternative to platinum, showing a remarkable antitumoral and antimetastatic activity, associated with lower toxicity. Among Ru-based complexes, the most promising compounds have been found and studied by Sava and co-workers [8−13]. Since the early 90's, Sava has been a pioneer in studying transition-metal complexes, developing, among others, the complex named NAMI-A, endowed with relevant anticancer activity. This compound, along with KP1019, has gone over the phase I clinical trial with good outcome [14,15]. Despite these encouraging results, a possible drawback of Ru complexes has also been highlighted. In fact, it has been shown that, under physiological conditions, the chloride ligands of the Ru complexes are replaced by hydroxide ligands in relatively few hours. This leads to partial hydrolysis of the complex and to poly-oxo species formation [16,17]. Although it is claimed that the formation of poly-oxo species does not really alter the Ru complexes anticancer activity [18], it is to consider that, generally speaking, the premature aquation and hydrolysis of other anticancer drugs, as in the case of Cisplatin, can deactivate or activate too early most of the administered complex [19,20]. Thus, the design of longlife Ru-based antineoplastc agents is still a mandatory goal.

A large number of alternative Ru-based complexes have been published in the recent years [21]. In this frame, some of us recently

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proposed a new concept for Ru-based anticancer therapy, centered on the design of amphiphilic nanovectors carrying ruthenium complexes [22,23].

As widely known, self-assembled amphiphiles allow an efficient bottom-up strategy in order to obtain nanosized aggregates whose size and shape are quite easily tunable. Further benefits related to the use of nanostructures containing Ru complexes concern their capability: *i*) to transport a larger amount of the metal inside the blood stream; *ii*) to be "stealth" to the human immune system, specifically increasing the complex permanence time in the blood; *iii*) to make the aggregates selective toward cancer cells by inserting within the ruthenium complexes some "marker" molecules, recognized by protein receptors specifically over-expressed by cancer tissues, *iv*) to tune the shape and size of the aggregates by acting either on their molecular structure or on external physico-chemical parameters like pH and ionic strength. These last effects may be very useful in a *stimuli-responsive* scenario [24,25].

With these aims we have synthesized and characterized a series of amphiphilic Ru complexes able to form supramolecular aggregates: the structures of these complexes, baptized ToThyRu, HoThyRu and DoHuRu, are shown in Fig. 1 and are based on a pyrimidine deoxyribo-(Thymidine, as in the case of ToThyRu and HoThyRu) or ribonucleoside (Uridine, for DoHuRu). In the general design of these complexes, the nucleoside, chosen as the starting poly-functional scaffold, was decorated with one or two oleic acid chains, attached at the ribose secondary hydroxy groups, as well as with an oligo-(ethylene oxide) chain at the 5′-end, so to confer the desired amphiphilicity to the nucleolipids, and to make the resulting aggregates resistant toward the enzymatic degradation. Finally, a pyridine residue was attached to the nucleobase as a chelating moiety able to complex Ru(III) ions. The minimal structure incorporating the Ru(III)-complex, with one pyridine ligand, named AziRu, reported in Fig. 1, was also studied for comparison.

Nucleolipids have been selected as scaffold for building up the amphiphilic Ru complexes because of their capability to mime the molecular organizations of the biological systems, as well as for the possibility to form a wide variety of supramolecular systems such as liposomes/vesicles, cubic phases, ribbons, *etc.* [26–28], that have found an increasing application in the biomedical field.

The synthesized molecules have been studied as pure aggregates, as well as in mixture with palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), at selected POPC/Ru molar ratios. Indeed, the combination of Ru complexes with phospholipids can allow a fine tuning of the metal amount to be administered, as well as protection from degradation, since the ruthenium complex is lodged in the liposome bilayer. Among phospholipids, POPC is of particular interest because it is one of the components of natural membranes [29].

The aggregation behavior of the prepared nanoaggregates has been investigated through an experimental strategy which has been proved to be extremely informative [30,31]. It combines dynamic light scattering (DLS) to estimate aggregate dimensions, small angle neutron scattering (SANS) to analyze the aggregate morphology and to determine their geometrical characteristics, and electron paramagnetic resonance (EPR) to get information on the dynamics of the lipid hydrophobic tail in the bilayer. Finally, testing of the antiproliferative activity of the aggregates has been carried out.

2. Materials and methods

2.1. General procedure for the synthesis of the nucleolipids Ru(III) complexes ToThyRu, HoThyRu, DoHuRu

The selected nucleolipid (ToThy, HoThy or DoHu, 0.033 mmol), synthesized in our laboratories as previously described [32], was dissolved in 1.0 ml of the appropriate anhydrous solvent (CH$_3$CN for ToThy; CH$_2$Cl$_2$ for HoThy and DoHu) and

then [*trans*-RuCl$_4$(DMSO)$_2$]$^-$ Na$^+$ (0.033 mmol) was added. The mixture was stirred at 40 °C and the reaction was monitored by TLC on alumina. After 4 h, TLC showed in all cases the total disappearance of the starting material, with concomitant formation of the desired salt in almost quantitative yields, and the solvent was removed *in vacuo*. The obtained Ru(III) complexes were then fully characterized by ^{1}H and ^{13}C NMR spectroscopy and ESI-MS analysis. All the collected spectral data are reported in the Supplementary Material Section.

2.2. Sample preparation

Ru-containing samples have been prepared by dissolving a suitable amount of the complex in pure chloroform, in order to get a concentration of ∼1 mg ml^{-1}. For pseudo-ternary systems containing POPC, an appropriate amount of this phospholipid is added to the Ru-complex solution, in order to have the pre-fixed molar ratio. The dissolution has been favored by a slight warming (∼40 °C) and a very short sonication treatment (∼5 min). Subsequently, an appropriate amount of this solution has been transferred in round-bottom glass tubes. A thin film was obtained through evaporation of the solvent and vacuum desiccation. The samples have been then hydrated with different media, namely, pure H$_2$O (or D$_2$O), a 0.9% wt NaCl solution and, finally, a pH 7.4 buffer for miming physiological conditions. This buffer has been prepared by dissolving sodium dihydrogenphosphate (NaH$_2$PO$_4$) and disodium hydrogenphosphate (Na$_2$HPO$_4$) in D$_2$O or H$_2$O at concentrations equal to 0.0773 mol dm^{-3} and 0.123 mol dm^{-3}, respectively. The pH has been checked to be within 0.1 pH units by means of a Radiometer pHM220 pH-meter, equipped with a AgCl/Ag electrode and a glass electrode previously calibrated with IUPAC standard buffer solutions [33]. All the solutions were vortexed, and the suspensions were then sonicated and repeatedly extruded through polycarbonate membranes of 100 nm pore size, for at least 11 times. The final amount of Ru complex was 0.1 mmol kg^{-1} in both binary and ternary systems; POPC concentration, in systems containing this component, was chosen accordingly to the pre-fixed Ru/POPC molar ratio.

Samples prepared for EPR experiments also included 1% (w/w) of spin-labeled phosphatidylcholine (1-palmitoyl-2-[n-(4,4-dimethyloxazolidine-N-oxyl)]stearoyl-sn-glycero-3-phosphocholine, n–PCSL, n = 7,14), purchased from Avanti Polar Lipids and stored at −20 °C in ethanol solutions.

Samples prepared for fluorescence microscopy were prepared as reported above by adding 2% mol of 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) ammonium salt, here abbreviated as Rhod, purchased from Avanti Polar Lipids and used as received.

2.3. Dynamic light scattering (DLS)

DLS measurements were performed with a home-made instrument composed by a Photocor compact goniometer, an SMD 6000 Laser Quantum 50 mW light source operating at 5325 Å, a photomultiplier (PMT-120-OP/B) and a correlator (Flex02-01D) from Correlator.com. All the measurements were performed at (25.00 ± 0.05) °C with the temperature controlled through the use of a thermostat bath. In DLS, the intensity autocorrelation function $g^{(2)}(t)$ is measured and related to the electric field *autocorrelation function* $g^{(1)}(t)$ by the Siegert relation [34]. This latter function can be written as the Laplace transform of the distribution of the relaxation rate Γ used to calculate the translational diffusion coefficient D:

$$g^{(1)}(t) = \int_{-\infty}^{+\infty} \tau A(\tau) \exp\left(-\frac{t}{\tau}\right) d\ln \tau \qquad (1)$$

where $\tau = 1/\Gamma$. Laplace transforms were performed using a variation of CONTIN algorithm incorporated in Precision Deconvolve software. From the relaxation rates, the z−average of the diffusion coefficient D may be obtained as:

$$D = \lim_{q \to 0} \frac{\Gamma}{q^2} \qquad (2)$$

$q = 4\pi n_0/\lambda \sin(\theta/2)$ is the modulus of the scattering vector, n_0 is the refractive index of the solution, λ is the incident wavelength and θ represents the scattering angle. Thus D is obtained from the limit slope of Γ as a function of q^2, where Γ is measured at different scattering angles. For each sample, relaxation times were acquired at five to six angles at least, and three or more measurements per angle were performed in order to improve statistical accuracy.

For spheres diffusing in a continuum medium at infinite dilution, the diffusion coefficient D_∞ is dependent on the sphere radius R_H, called *hydrodynamic radius*, through the Stokes−Einstein equation [35]:

$$R_H = \frac{kT}{6\pi\eta_0 D_\infty} \qquad (3)$$

where k is the Boltzmann constant, T is the absolute temperature and n_0 is the solvent viscosity. For not spherical particles, R_H represents the radius of equivalent spherical aggregates. Due to the high dilution, for non interacting species it is possible to make the approximation: $D \cong D_\infty$ and $\eta \cong \eta_0$, where η represents the solution viscosity. In this hypothesis, equation (3) can be reasonably used to estimate the hydrodynamic radius of the aggregates [36].