Antitumor Agents

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Characterization of a Ruthenium(III)/NAMI-A Adduct with Bovine Serum Albumin that Exhibits a High Anti-Metastatic Activity**

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Interest in ruthenium-based anti-metastatic drugs with low cytotoxicity is rapidly growing,[1–3] for example, NAMI-A (**1**, Figure 1)[2] has completed phase I clinical trials.[4] Although its anti-metastatic mechanism and those of related Ru complexes remain unclear, growing evidence points to the crucial role of Ru interactions with the cell surface, or extracellular matrix components (e.g., collagen), rather than Ru penetration into cells.[2,3,5] Stepwise aquation of **1** (Cl^- substitution) occurs in minutes at pH ≈ 7.4 and 310 K,[5d] as does protein binding.[5b,6] In human clinical trials, intravenous delivery of **1** and other Ru anti-cancer drugs results in extensive binding to blood proteins,[4,7] with ≥ 90% of total Ru bound to albumin and ≤ 1% to other proteins, such as transferrin.[7] Such binding is generally thought to be detrimental for the cytotoxic activity of metal-based anticancer drugs (owing to the decrease in cellular uptake),[1,8] but its role in the specific anti-metastatic activity of **1** and its analogues is controversial. An animal model study of Sava and co-workers[9] suggested that the binding of **1** to albumin or transferrin reduced both its bioavailability and its anti-metastatic activity, but they also showed[10] that the anti-metastatic activity of **1** increased in the presence of biological reductants (ascorbate or glutathione). These conditions accelerate the aquation and protein binding of **1**.[11]

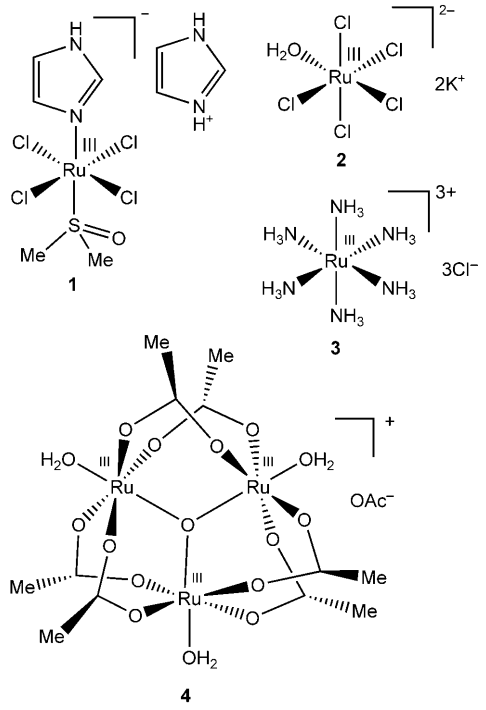

Figure 1. Structures of NAMI-A (**1**) and model Ru^{III} complexes (**2**–**4**).

Ru^{III}–BSA (bovine serum albumin) adducts from the reaction of **1** with BSA in neutral aqueous solutions were studied by UV/Vis and circular dichroism (CD) spectroscopy,[12] electrochemistry,[13] and X-ray absorption spectroscopy (XAS and X-ray absorption near-edge structure (XANES)).[14] Importantly, a combination of Ru L- and K-edge spectra (XANES) and Cl and S K-edge spectra was used to cross-reference changes in the chemical environments of the metal and the ligands.[14] Nevertheless, the mode of Ru^{III} binding in the BSA adduct remains uncertain, apart from the facts that Cl^- ligands are most perturbed during the binding,[14] S-donor groups of BSA are unlikely to take a major part in the binding,[14] and Ru^{III} is likely to bind to various surface donor groups of the protein, rather than specific binding sites.[12] A detailed mass-spectrometric study of the reactions of Ru^{II}–arene anti-cancer drugs with human serum albumin showed Ru binding mainly to surface histidine and methionine residues.[15] Studies of changes in the coordination spheres of complexes in biological media involve empirical analysis of the near-edge (XANES) region of the XAS from spectral libraries of model compounds.[16] In the case of Ru^{III} complexes, the XAFS (X-ray absorption fine structure) region was also included,[1] which was also used to study the Ru environment in a Ru^{III}–BSA adduct. The potential anti-metastatic activity of this adduct was compared with **1** in a range of cell assays targeting substrate adhesion,[5,17] motility,[18] and invasion,[5,19] using a well-established invasive human lung adenocarcinoma (A549) cell line.[20]

Samples of Ru^{III}–BSA adducts were prepared[12,14] by the reactions of BSA (25–150 μM) with **1** (25–600 μM) in buffered saline (20 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 140 mM NaCl, pH 7.4, 4 h, 310 K), purified

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Supporting information for this article (full details of the used reagents, experimental conditions, and instrumental techniques) is available on the WWW under http://dx.doi.org/10.1002/anie.200906079.

by gel-filtration chromatography (molecular weight cutoff, 30 kDa), and freeze-dried for Ru K-edge XAS (at 10–15 K, fluorescence detection mode) at the Australian National Beamline Facility (ANBF; Supporting Information). Figure 2 shows a comparison of XAS data for a Ru:BSA adduct (1:1 mol mol^{-1}) with those for the parent complex, **1**, and for model Ru^{III} complexes containing predominantly Cl^-, NH_3, or CH_3COO^- ligands (**2**–**4**). As observed previously,[1] the pre-edge and edge spectral regions for the Ru^{III} complexes were similar, hence, only post-edge areas are shown in Figure 2a,b. The spectrum of Ru^{III}–BSA was significantly different from those of **1** and **2** (Figure 2a) and closer to those of **3** and **4** (Figure 2b). Multiple linear regression analysis[16a,b] led to an excellent fit of the Ru^{III}–BSA XAS with models **3** and **4** (65% and 35% mol, respectively, Figure 2c and Supporting Information Table S1). Models **1** and **2** had no spectral contributions (negative correlation coefficients).[16a]

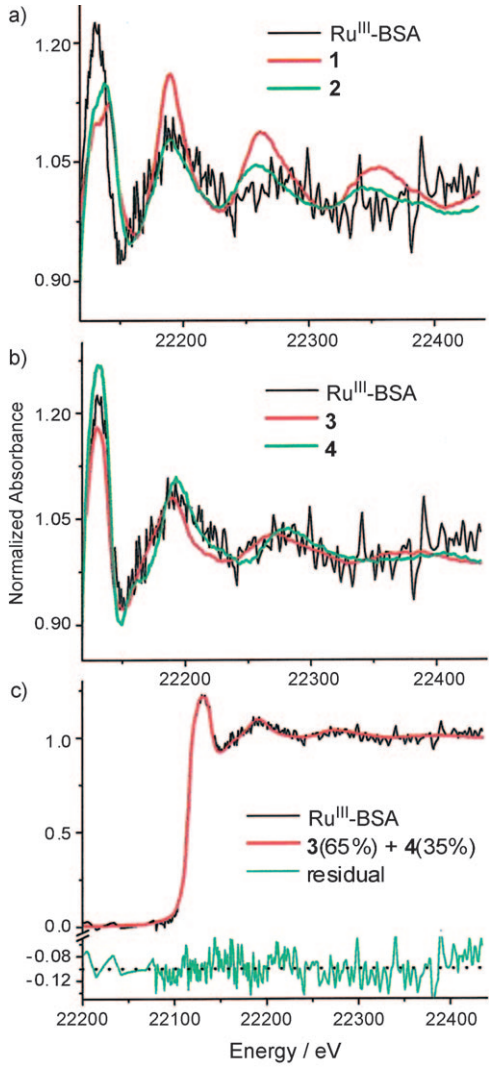

Figure 2. A comparison of post-edge areas of Ru K-edge XAS of a Ru^{III}–BSA adduct (1:1 molar ratio, freeze-dried solid) with those of model complexes **1**–**4** (a, b; solid mixtures with boron nitride); and the results of multiple linear regression analysis for Ru^{III}–BSA (c; full spectra).

These findings are in accord with an earlier study of the Ru^{III}–BSA (1:1) adduct,[14] but the hypothesis that only Cl^- ligands of **1** are significantly perturbed upon BSA binding[14] provides an incomplete picture. As shown in Figure 2c and Table S1 in the Supporting Information, the Cl^- ligands of **1** were completely replaced with protein N-donor (amine or imine) and carboxylato residues. The use of XAFS fitting and of a wide range of model compounds (Figure 1 and 2) revealed a profound change in the coordination environment of **1** caused by BSA binding, which was not obvious from the previous study.[14]

In addition to the Ru^{III}–BSA (1:1) adduct, other reaction products of **1** with biological media were studied: 1) a Ru^{III}–BSA (4:1) adduct using a higher concentration of **1**; 2) **1** (0.50 mM) treated with undiluted bovine serum (ca. 0.6 mM BSA,[8] 4 h, 310 K) and freeze-dried without further separation; and 3) **1** (0.50 mM) treated with cell culture medium supplemented with 2% v/v fetal calf serum, as used in subsequent cell assays (4 h at 310 K, freeze-dried without further separation). The spectra from (1) and (2) were similar to that of the Ru^{III}–BSA (1:1) adduct (Supporting Information, Figure S1), but multiple linear regression analyses (Supporting Information, Table S1, Figure S2) revealed significant differences, including higher proportions of model **4** (59% for (1) and 51% for (2)) and the inclusion of model **2** (20% for (1) and 12% for (2)). The spectrum from (3) was significantly different from all the others (Supporting Information, Figure S1), and its best fit (Supporting Information, Table S1, Figure S2) was a combination of models **3** (84%) and **2** (16%). The difference in fits between (2) and (3) is likely to reflect the binding of Ru^{III} to low-molecular-weight ligands, such as amino acids, in cell culture medium. The presence of model **2** in the fits for (1)–(3) (unlike the Ru^{III}–BSA (1:1) adduct, Figure 2c) indicated either incomplete Cl^- ligand substitution from **1** during BSA binding (for (1)) or binding of Ru^{III} to Cl^- ions present in the medium (ca. 150 mM for both (2) and (3)).[8] These results emphasize the diverse modes of binding of Ru^{III} to biological ligands, and the need for the studies on the links between the coordination sphere and the biological activities of Ru reaction products with biological media.[1] Replacement of mainly Cl/S-donor ligands in **1** (Figure 1) with mainly N/O-donor ligands in its reaction products in biological media (Supporting Information, Table S1) was confirmed by the analysis of Fourier-transformed XAS data[21] (Supporting Information, Figure S3), and it was also in agreement with the data on the reactions of Ru^{III} and Ru^{II} complexes with BSA.[12–15]

The purified Ru^{III}–BSA (1:1) adduct or **1** were added to cell-culture medium ($[Ru]_{final} = 0.10$–10 μM) to test cell adhesion, motility, and invasion, using a type I bovine collagen substrate (Figure 3, Figure 4, and Supporting Information, Figure S4).[17–19] In agreement with published kinetic data,[5d,12] when freshly prepared aqueous stock solutions of **1** ($[Ru]_{final} = 0.10$–10 μM) were added to cell-culture medium immediately prior to cell treatments, decomposition of **1** (10 μM, 310 K) was complete within approximately 1 h, and ≥90% of Ru products were protein-bound (UV/Vis spectroscopy, gel-filtration chromatography, and atomic absorption spectroscopy, Supporting Information). This result shows

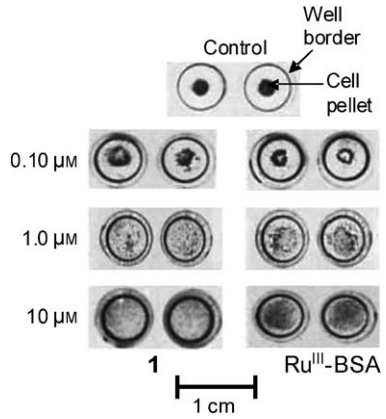

Figure 3. Typical results of substrate adhesion assays[5,17] for A549 cells in the presence of absence of Ru^{III} (0.10–10 μM). See Supporting Information for experimental details.

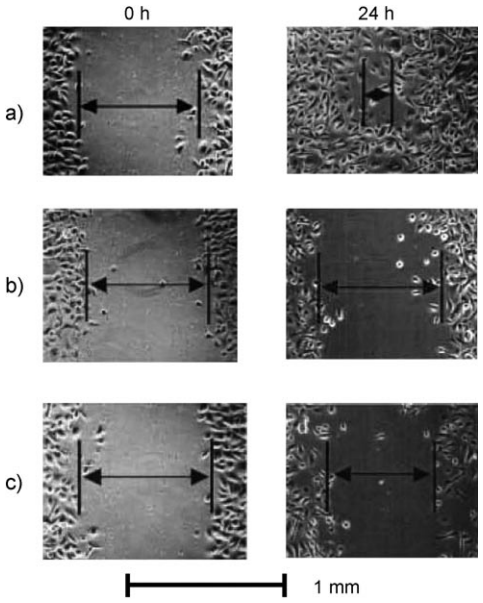

Figure 4. Typical results of cell motility assays[18] (phase contrast light microscopy, ×20 objective) for A549 cells in the absence of Ru (a), or with 10 μM 1 (b) or Ru^{III}–BSA (1:1) adduct (c; Supporting Information). Average gap widths[18a] are indicated by the arrows.

that reactions of low concentrations (≤ 10 μM) of **1** with cell-culture medium (containing ca. 12 μM BSA from serum supplementation)[8] led to a Ru^{III}–BSA (ca. 1:1) adduct, similar to that isolated and characterized by XAS (Figure 2, Supporting Information, Table S1). In agreement with the literature data on **1**,[22] no obvious toxic effects (cell rounding or detachment, ≤ 100 μM Ru^{III}) were observed in any cell assays. To obtain reproducible results, it was imperative that the cells had intact surface proteoglycan layers, which enabled their interactions with the collagen substrate.[17] Therefore, cells were collected from monolayers by gentle scrapping in saline after treatment with Na_2EDTA (disodium *N*,*N*,*N*′,*N*′-ethanediaminetetraacetate, 0.50 mM, 273 K), rather than by trypsinization, which damages the proteoglycan coating.[17]

For substrate adhesion assays (Figure 3),[5,17] approximately 5×10^3 of A549 cells per well were seeded into a collagen-coated, U-bottom 96-well plate and allowed to attach for 24 h, then the medium was replaced with ruthenium-containing medium for 4 h. The cell layers were carefully washed with saline to remove external Ru and treated with a trypsin-like enzyme solution (5 min, 310 K, Supporting Information). After removal of the enzyme solution and washing the wells with saline, the plates were gently centrifuged (10 min at 120 *g*), the cells were fixed with glutaraldehyde, stained with Trypan blue, and photographed without magnification. Control cells were affected by the trypsin-like enzyme and formed a compact pellet at the bottom of the well, but Ru^{III} treatments (**1** or Ru^{III}–BSA (1:1) adduct) led to a concentration-dependent increase in trypsin resistance,[5c,d] and the cells remained spread over the wells (Figure 3).

For cell motility assays, each well of a collagen-coated 12-well culture plate was seeded with approximately 2.5×10^5 of A549 cells and allowed to reach approximately 90% confluence within 48 h, then approximately 1 mm wide gaps in cell layers were made with pipette tips. The medium was immediately replaced with that containing Ru compounds (10 μM), and cell movement across the gaps was observed for 24 h. In the absence of Ru, the gap was completely closed over that time (Figure 4a), while little cell movement was observed in the presence of either **1** (Figure 4b) or Ru^{III}-BSA (1:1) adduct (Figure 4c). In order to minimize cell proliferation during the assay, the amount of fetal calf serum added to the medium was reduced to 0.8% (v/v).[18]

For collagen invasion assays[5,19] approximately 5×10^4 A549 cells were seeded onto approximately 5 mm-thick collagen gel layers in a 24-well cell culture plate in media without or with Ru (10 μM), and the penetration of cells into the gel was monitored for up to 38 h. Many more cells remained on the surface of the gel in the presence of either of the Ru compounds than in the controls (Supporting Information, Figure S4). Those cells that penetrated into the gel in the presence of Ru stayed just beneath the surface, while in the absence of Ru, some cells migrated as far as 2 mm into the gel. Quantitative results were obtained by first counting the cells collected from the surface of the gel (by trypsinization and mild collagenase digestion), then those recovered from the bulk of the gel (harsh collagenase digestion).[19] The proportions of invasive cells (three parallel experiments) were (32 ± 3)% in the absence of Ru and (2.0 ± 0.5)% in the presence of either **1** or Ru^{III}–BSA adduct.

Three independent assays showed a striking ability of nontoxic Ru^{III} concentrations to affect A549 lung cancer cell interactions with collagen, that is, increased cell adhesion to the substrate (Figure 3), reduced cell motility (Figure 4), and decreased the ability of cells to penetrate into collagen gels (Supporting Information, Figure S4). These changes are likely to be essential for the in vivo anti-metastatic activity of Ru drugs, often applied against lung cancer.[2,3,9,10] Although the abilities of **1** to increase cell adhesion and to reduce invasiveness were reported previously,[5] the improved methodologies reported herein convincingly demonstrated these effects at physiologically relevant Ru concentrations (1.0–

10 μM, up to 100 μM Ru was used previously[5]). Most importantly, the results demonstrated that a purified Ru^{III}–BSA (1:1) adduct has similar activity in all assays to that of **1** freshly added to the cell medium (which is expected to form a Ru^{III}–BSA adduct within minutes).[12] The XAS studies showed that the coordination environment in the Ru^{III}–BSA adduct is completely different from that of the parent complex, which is highly biologically relevant, since $\geq 90\%$ of **1** binds to blood albumin following intravenous delivery.[4,7,8] This result explains both the high anti-metastatic efficacy and low toxicity of NAMI-A (**1**). These findings emphasize the fact that **1** acts as a pro-drug, that is, its reactions in biological environments are essential for its pharmacological activity.[1] In general, reaction rates of Ru pro-drugs with biological environments are likely to classify them as being cytotoxins, anti-metastatic agents, or having both activities.[1] For example, formation of ruthenium–protein adducts may also account for the anti-metastatic activity of some Ru^{II} arene complexes that were previously characterized as only cytotoxins.[3]

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