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Influence of redox activation of NAMI-A on affinity to serum proteins: transferrin and albumin

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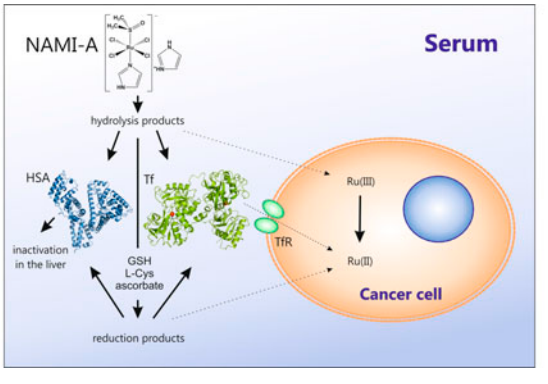

Imidazolium *trans*-tetrachloridodimethylsulfoxideimidazoleruthenate(III), NAMI-A, is a ruthenium drug exhibiting unique properties under clinical studies such as ability to inhibit the process of metastases without exerting tumor toxicity. Its place of action is concentrated at the extracellular level, therefore, the transformation and fate of this drug upon injection is of great importance. This study focuses on evaluation of the reducing potency of blood stream on interaction with two serum proteins: albumin and transferrin. It was investigated by applying various simplified serum models preserving physiological concentration of proteins and the amount of Ru complex as found in patients. It was shown that ruthenation of albumin is slightly increased while transferrin decreased upon addition of reductant in blood stream (ascorbate, glutathione, and cysteine) at physiological concentration. Interestingly, in serum models comprising low-molecular-mass components the amount of the reductant in the solution had a pronounced effect on the Ru content, in particular in transferrin. Supplementation of serum models with glutathione at concentration enough for complete reduction of NAMI-A selectively enhanced the binding of Ru complex to transferrin while ruthenation of albumin was almost unchanged. Spectrofluorimetric studies revealed that reduction has a marginal effect on binding affinity, therefore, both Ru(III) and (II) derivatives equally can compete for binding to transferrin.

Keywords: NAMI-A; Transferrin; Albumin; Reducing agents; ICP-MS; ELISA

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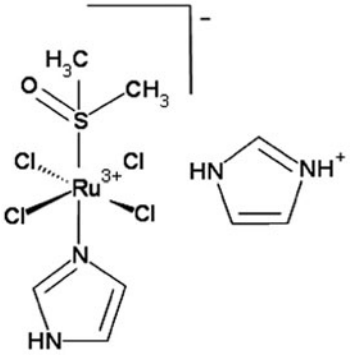
Dedicated to Professor Rudi van Eldik on the occasion of his 70th birthday.

1. Introduction

Ruthenium(III) compound shown in scheme 1, so called NAMI-A, discovered by Sava and co-workers has become one of the most intriguing ruthenium complexes tested against cancer so far [1]. This drug, currently undergoing phase II clinical trial, exhibited the antimetastatic properties while not affecting the primary tumor unlike most tested ruthenium complexes [2–5]. The origin of such activity is still not clear. It was suggested that inhibition of metastasis can arise from enhanced cell adhesion, integrin-dependent inhibition of cancer cell motility and invasiveness as well as inhibition of neo-angiogenesis in the tumor tissue [2, 6–8]. Recently, by applying X-ray fluorescence imaging of single cells, it has been proved that NAMI-A is not internalized by cells supporting the hypothesis that this compound operates at the extracellular level [9]. The clinical studies showed that after intravenous administration of NAMI-A most of the ruthenium was bound to serum proteins (ca. 98–99%) [10]. Consequently, the interaction of this compound with serum proteins is of importance and can strongly influence its pharmacokinetics and the overall pharmacological profile. In particular, human serum albumin (HSA, ca. 0.6 mM [11]) and transferrin (Tf, 25–50 μM [12]), the most abundant plasma proteins, are considered as the first fate of NAMI-A complex. It was proved that in spite of much higher concentration of HSA in human plasma over Tf, the ruthenated Tf was still detected under physiological conditions [13]. These proteins can act as transport, delivery, or storage systems for ruthenium complex, however, more specific role directly related to mode of action cannot be excluded.

NAMI-A at physiological pH 7.4 is not stable and undergoes advanced hydrolysis leading to release of chloride and DMSO forming a number of potentially active species [14–17]. Furthermore, it is possible to modify its properties by redox activation. It was proposed that reduction of NAMI-A prior to injection improves pharmacological activity against tumor metastases [18]. The binding of NAMI-A to serum albumin was facilitated when the drug was in its reduced form [18]. Due to relatively high-reduction potential (+235 mV) [19] NAMI-A can be reduced by biological reductants *in vivo*. Human serum contains non-enzymatic antioxidants like ascorbate ($E_0 \approx 60$ mV [20, 21], 50 μM [22]), glutathione (GSH, E_0 from −240 to −170 mV [21, 23], 3.4 μM [24]), and cysteine (Cys, E_0 from −230 to −210 mV [25], 33 μM [26]). It was shown that both ascorbate and cysteine reduce NAMI-A rapidly and efficiently [27–29], there are no such data available for



Scheme 1. The structure of NAMI-A.

glutathione; however, in some reports it was claimed that it can easily reduce NAMI-A [29, 30]. Taking into account that Ru concentration in the blood of patients within 24 h after treatment is ca. 0.4–0.8 mM [10] one can expect that the reducing environment of blood can induce reduction of Ru(III) to Ru(II) at least partially. In this investigation, the effect of such reduction on NAMI-A derivatives binding to albumin and transferrin was studied by evaluation of the percentage of Ru content in proteins. To get insight into possible interactions, several serum models were applied. The ruthenium content was determined for mineralized samples by inductively coupled plasma mass spectrometry (ICP-MS). Transferrin concentration was assessed by applying the enzyme-linked immunosorbent assay (ELISA), while albumin level was detected using absorption spectroscopy. Alternatively, the supplementation with GSH during treatment can elevate reducing agent concentration to promote the complete reduction of NAMI-A in blood. During treatment of patients with ovarian cancer with cisplatin, the addition of GSH decreases the observed toxicity and results in improved quality of life for patients [31]. To this end, we have additionally applied the serum models with increased concentration of GSH (enough for complete reduction of NAMI-A ca. 1.5 equivalent) to check the influence of redox activation of NAMI-A on affinity to both serum proteins.

2. Experimental procedures

2.1. *Reagents*

NAMI-A, (HIm)[*trans*-$RuCl_4$(dmso)(Im)], was synthesized according to reported procedure [1] and its purity was checked by elemental analysis. The analysis calculated for NAMI-A ($M = 458.17$): C, 20.95; H, 3.30; N, 12.23; and S, 6.99. Found: C, 21.37; H, 3.33; N, 12.03; and S, 6.90. Human apotransferrin (apo-Tf) and holotransferrin (holo-Tf) (powder, BioReagent, suitable for cell culture, ≥98%) as well as human serum albumin (HSA) (powder, fatty acid free, globulin free, ≥99%) were purchased from Sigma-Aldrich (Germany). Human Tf ELISA (Immunoperoxidase Assay for Determination of Transferrin in Sera) was obtained from ICL (Portland, USA). All other chemicals were obtained in the highest available purity and all solutions were prepared in MiliQ quality water (18 MΩ); eluents were filtered through 0.45 μm membrane filters and degassed.

2.2. *Preparation of stock solution and serum models*

NAMI-A was dissolved in MilliQ water and its concentration was determined spectrophotometrically using molar absorptivity at 390 nm of 3644 M^{-1} cm^{-1} [19]. All protein stock solutions were prepared by dissolving in buffers and the concentration was measured spectrophotometrically applying absorption coefficients at 280 nm of 86,400, 84,000, and 42,000 M^{-1} cm^{-1} for holo-, apo-Tf, and HSA, respectively [32, 33]. In most experiments, NAMI-A concentration was kept constant at 0.70 mM that relates to the typical Ru concentration in the blood of patients within 24 h after treatment (0.4–0.8 mM Ru) [10]. Serum model 0 was prepared by dissolving proteins at concentrations found in serum (see figure 1) in 50 mM Tris/HCl buffer pH 7.4 containing 0.1 M NaCl, 25 mM $NaHCO_3$ and supplemented with a mixture of reducing agents such as L-cysteine (33 μM), ascorbic acid (50 μM), and glutathione (3.5 μM). In Serum model 0* as the solely reducing agent,

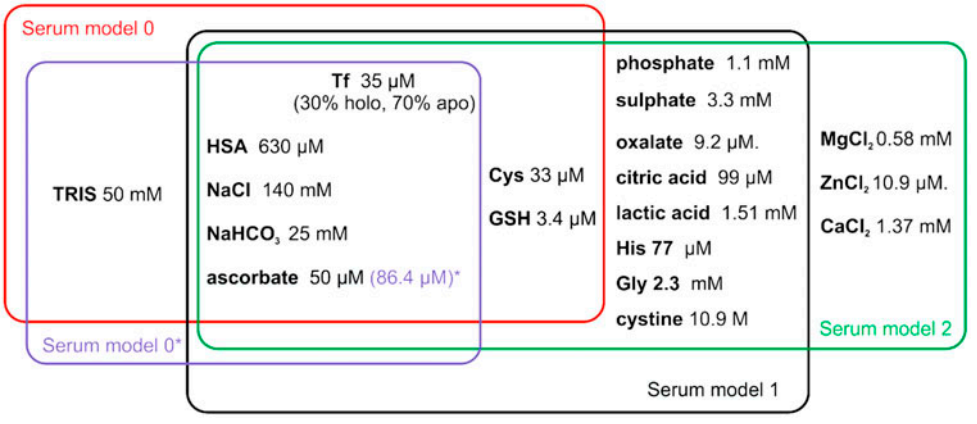


Figure 1. The composition of the applied serum models to study the Ru distribution between Tf and HSA (pH 7.4).

ascorbate was used preserving the same overall reductant concentration (86.4 µM) as in model 0. Serum models 1 and 2 were prepared by mixing components as depicted in figure 1, adjusting the pH to 7.4, followed by addition of the same amount of proteins as in models 0 and 0*. In serum models 1 and 2 with elevated GSH additionally the GSH was added to reach the value of 0.105 mM.

NAMI-A was added into each serum model by addition of small volume of concentrated stock solution, about 2–5 µL. models mixed with NAMI-A were incubated for 24 h at 37 °C.

2.3. *Spectrofluorimetric titration*

A Perkin Elmer LS55 equipped with the PolyScience circulation flow was used for fluorescence studies. Measurements were carried out in a quartz cell with a 1 cm path length. The emission spectra upon excitation at 295 nm were measured from 305 to 500 nm. The fluorescence intensities were corrected due to dilution effects as previously described [28].

2.4. *Stopped-flow measurements*

Stopped-flow kinetics measurements were performed on an Applied Photophysics stopped-flow apparatus SX20 equipped with PolyScience thermostat. In a typical experiment, water solution of NAMI-A was rapidly mixed with buffered solution of GSH in the absence or presence of a mixture of proteins. The changes in absorbance were recorded at 390 nm.

2.5. *Separation of protein fractions*

Fast protein liquid chromatography has been employed to separate the mixture of the unbound ruthenium species, Tf–Ru adducts and HSA–Ru adducts. The chromatographic system ÄKTA Pure (GE Pharmacia) with injection loop of 500 µL and fraction collector F9-R were used for separation. The Mono Q 5/50 GL column (Tricorn, GE Healthcare Life Science; 5 × 50 mm I.D., 10 µm particle diameter) was applied for separation of proteins.

The chromatographic runs were monitored spectrophotometrically at 280 nm (Monitor UV-900, GE Pharmacia) as well as by measuring the conductivity (Monitor pH/C-900, GE Pharmacia). 0.02 M Bis–Tris/HCl pH 7.0, A, and 0.02 M Bis–Tris/HCl, 1 M NaCl pH 7.0, B, were used as eluents. models were diluted 4-fold with buffer A immediately before separation and filtered through 0.22 μm syringe filter. Chromatographic run conditions were used as followed: injection volume: 400 μL, target concentration of B: 50%, gradient length: 20 column volumes (CV), flow rate: 1 mL min^{-1}. After separation, the column was equilibrated with 5 CV of eluent B and re-equilibrated with eluent A, 5 CV.

2.6. *Determination of protein and ruthenium concentration*

The Tf and HSA concentrations in the models were analyzed using human Tf ELISA kit or HSA absorption coefficient at 280 nm. Standard curve for Tf was prepared with the supplied standards with concentration ranging from 9.375 to 600 ng mL^{-1} for Tf. The protein fractions were diluted appropriately in a sample diluent buffer and the tests were performed according to the manufacturer's protocol. ELISA 96-well plates were read using Tecan Infinite 200 Reader plate at 450 nm for Tf. HSA concentration was measured without dilution.

The total Ru concentration in samples was measured by application of ICP-MS using an ELAN 6100 Perkin Elmer spectrometer. Before the determination of Ru content, 200 μL of the samples were mineralized using 500 μL of UltraPUR concentrated nitric acid and then diluted with ultrapure water.

The experiments were carried out at least in triplicate.

3. Results and discussion

3.1. *Impact of NAMI-A reduction on binding affinity towards holo-Tf*

NAMI-A upon getting into the blood stream can undergo rapid reduction by ascorbate or cysteine (Cys) forming $[Ru^{II}Cl_4(DMSO)(Im)]^{2-}$ [15]. To examine the influence of this transformation on the binding to holo-Tf, changes in the fluorescence intensity of protein were monitored as a function of time at various NAMI-A : Cys ratios. This reductant was chosen for spectrofluorimetric studies since both cysteine and its oxidized form cystine had marginal influence on holo-Tf fluorescence. Two other important low-molecular reducing agents like ascorbic acid and glutathione were not applied since both influenced the emission intensity of protein making the interpretation of quenching studies difficult [28]. After addition of the reductant to the NAMI-A solution the mixture was kept 5 min and next the holo-Tf was added. As shown in figure 2, the fluorescence of holo-Tf was readily quenched and after ca. 25 min the reaction was completed. This was at least four times longer reaction time than found for the unreduced form of NAMI-A [13]. However, the relative fluorescence intensity of holo-Tf-NAMI-A adduct was much higher [13] than for the corresponding adduct with the reduced formed, in particular at NAMI-A : Cys ratio of 1 : 1. This points out that Trp residues are better accessible for the reduced NAMI-A so that the quenching is higher in comparison with non-reduced.

The interaction between the reduced NAMI-A and holo-Tf was further examined by measuring the fluorescence quenching as a function of the ruthenium complex concentration. For each experiment, NAMI-A was freshly reduced and kept with the reductant for 5 min

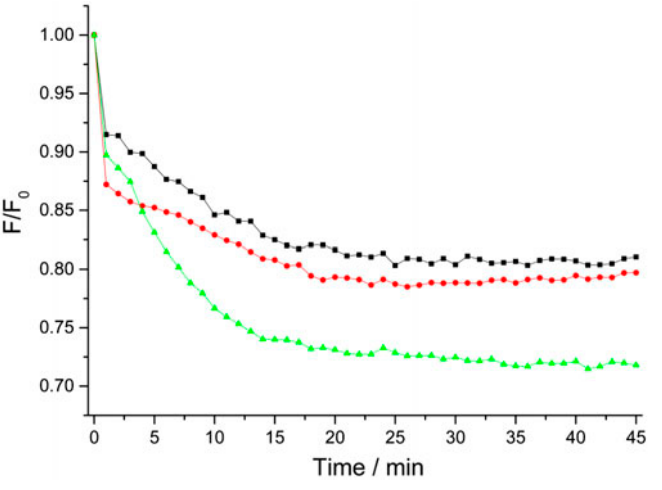


Figure 2. Time-dependent quenching of holo-Tf fluorescence by the reduced NAMI-A. Experimental conditions: [holo-Tf] = 2.0 μM, [NAMI-A] = 40 μM; [L-Cys] = 40 (▲), 30 (●) or 20 (■) μM, Tris/HCl pH 7.4; [NaCl] = 0.1 M; [$NaHCO_3$] = 25 mM, T = 37 °C; λ_{ex} = 295 nm; λ_{em} = 338 nm.

prior to addition of holo-Tf. Emission spectra were collected after 30 min incubation of the reduced NAMI-A with holo-Tf to make sure that the reaction was completed. Ru complex addition caused the maximum emission decrease (see figure 3) without a distinct shift as observed for the NAMI-A complex. This can suggest that the DMSO ligand which is not released upon hydrolysis of the reduced NAMI-A complex [15] can preserve the similar

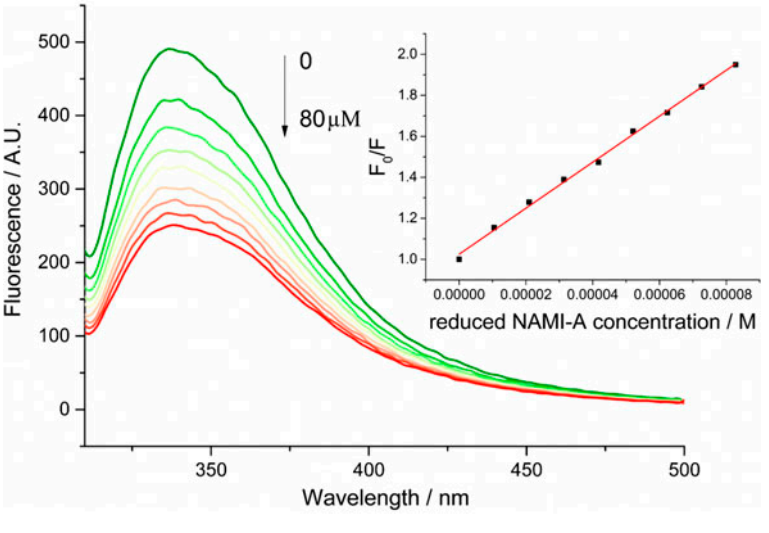


Figure 3. Emission spectra of holo-Tf in the presence of increasing NAMI-A concentrations. Inset: Stern–Volmer plot showing the quenching properties of the reduced NAMI-A. Experimental conditions: [holo-Tf] = 2.0 μM, [reduced NAMI-A] = 0–80 μM; Tris/HCl pH 7.4; [NaCl] = 0.1 M; [$NaHCO_3$] = 25 mM; T = 37 °C; λ_{ex} = 295 nm; λ_{em} = 338 nm. As reductant Cys was used at equimolar concentration.

microenvironment in the vicinity of tryptophan residues or the binding site differs from that of Ru(III) derivative. Ru(II) complexes are more kinetically labile for ligand-substitution processes in comparison with their Ru(III) analogs; therefore, one can expect that the coordination bonds to amino acid residues of holo-Tf will be formed more readily for the reduced NAMI-A, while the non-covalent interaction can predominate for nonreduced form. It has been proposed that NAMI-A can rapidly bind to HSA via hydrophobic interactions of the axial heterocyclic ligand of the complexes with the protein [17].

Fluorescence quenching data were analyzed according to the Stern–Volmer equation [34]:

$$\frac{F_0}{F} = 1 + k_q \tau_0 \cdot [Q] = 1 + K_{SV} \cdot [Q] \quad (1)$$

where F_0 and F are the fluorescence intensities of holo-Tf in the absence and presence of quencher (Q, reduced NAMI-A), respectively; k_q is the bimolecular quenching constant; τ_0 is the average lifetime of the holo-Tf fluorescence in the absence of quencher, [Q] is the concentration of quencher, K_{SV} is the Stern–Volmer constant. As shown in the inset of figure 3, the plot of F_0/F *versus* [Q] was linear to almost 80-fold excess of Ru(II) complex over holo-Tf. Based on equation (1), the calculated value of K_{SV} was 11, 200 ± 200 M^{-1} at 37 °C. The calculated k_q is ca. 6×10^{12} M^{-1} s^{-1} ($\tau_0 \sim 2 \times 10^{-9}$ s^{-1}) [13]. Such high value of the bimolecular quenching constant points out the static quenching and allows for consideration of the Stern–Volmer constant as an association constant for the formation of holo-Tf–Ru(II)-complex(es) adducts [13, 28, 35]. The association constant for the reduced NAMI-A is only slightly higher than for the parent complex (10, 300 ± 100 M^{-1} [13]) pointing out that reduction has a marginal effect on binding affinity and both forms equally can compete for binding to transferrin.

3.2. *Ru distribution in serum models*

To get information on NAMI-A behavior once injected into the blood stream, Ru distribution between Tf and HSA was analyzed in three types of serum models whose composition is presented in figure 1. Each serum model consisted of HSA and the mixture of Tf (30% of holo and 70% of apo forms) at concentrations typically found in human serum [11, 36], dissolved in physiological type buffer (pH 7.4). In Serum model 0, the mixture of biological reducing agents such as ascorbate, Cys, and GSH at concentrations found in circulation [22, 25, 37] was added to mimic the reducing environment of the blood stream. Both Cys and GSH can act as a reducing agent and can form coordination bonds with ruthenium [38]. Therefore, to clarify if the observed interaction with proteins arises only from the reduction process and/or involve coordination of the reductant to ruthenium ion, the Serum model 0*, possessing only ascorbate as a reducing agent, was also included. Serum models 1 and 2 additionally comprised amino acids (His, Glu, Gly, cystine), inorganic and organic sodium salts (chloride, carbonate, phosphate, sulfate, oxalate) as well as lactic and citric acids. Serum model 2 was moreover enriched with metal ions: calcium, magnesium, and zinc. The choice and the quantity of the components were based on the composition proposed by Harris [26].

To each serum model, NAMI-A at 20-fold excess over Tf (0.7 mM) was added and the reaction mixture was kept for 24 h at 37 °C. Subsequently, the mixture was injected into an anion exchange MonoQ 5/50 GL column to separate unbound Ru species from those bound to Tf and HSA. The obtained chromatograms are presented in figure 4.

The protein fractions were collected and analyzed for Ru content using ICP-MS method while protein concentration was determined using either ELISA test for Tf or spectrophotometric method for HSA. The Ru fraction found in ruthenated protein adducts is summarized in table 1. By comparing the concentration of NAMI-A complex and the reductants, assuming that the reducing agent/s undergo reaction and reduction takes place fast enough to be completed prior to binding to proteins, only ca. 25% of ruthenium species can occur in their reduced form. Such Ru(II)/Ru(III) species mixture has slightly lower ability to form stable adduct with Tf than non-reduced NAMI-A derivatives while the interaction with HSA is increased (compare data for Serum model 0* in table 1). There is only marginal influence of S-donor reducing agents (GSH, Cys) in comparison to ascorbate used as a sole reductant (Serum model 0* *versus* 0) on Ru content in both proteins pointing out that major impact on protein binding is more related to reduction of Ru complex than formation of coordination bond/s with reductant.

There is a pronounced suppression of protein binding properties of NAMI-A derivatives just upon addition of ascorbate (50 μM) and a small quantity of GSH (3.4 μM) to Serum model 1 or 2 (see table 1). The increased level of the reducing agent in the presence of various components (like mono/bidentate inorganic or organic salts) can result in formation of mixed valent or polyoxo ruthenium species [14, 15] which might be inactive in the formation of adducts with proteins.

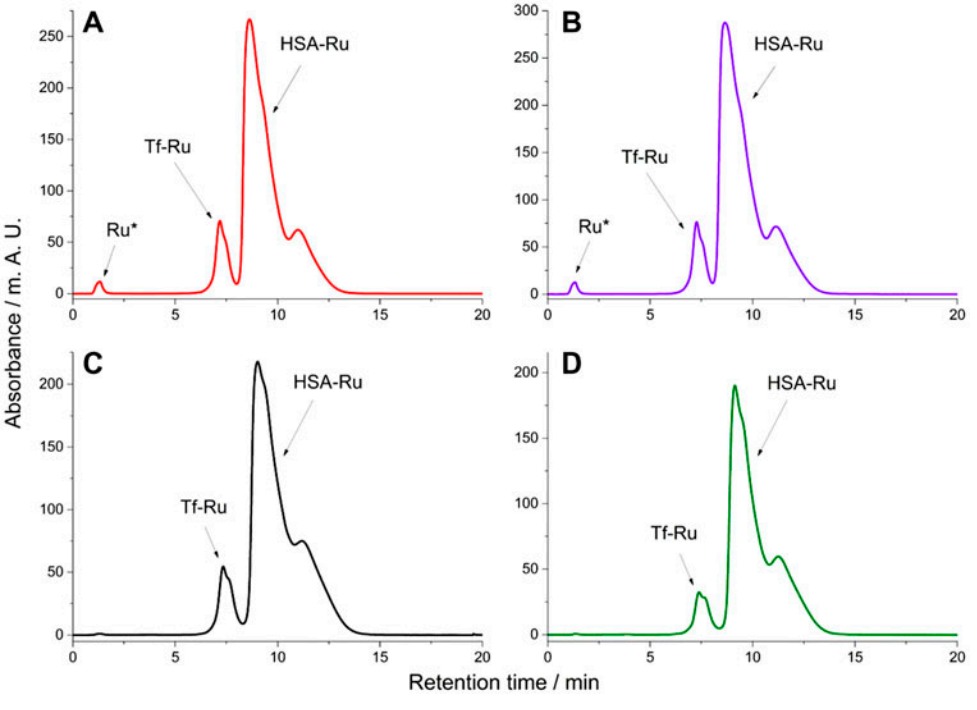


Figure 4. Chromatographic separation of serum models incubated with NAMI-A for 24 h at 37 °C. Serum model: 0 (A), 0* (B), 1 (C), 2 (D) (compositions are described in figure 1). [NAMI-A] = 0.7 mM. Ru* denotes the peak from solvent front comprising unbound Ru species. The elution conditions are described in the experimental part.

Table 1. Ru fraction (mol/mol(protein)) in ruthenated Tf or HSA produced after incubation of NAMI-A with various models of serum (the composition is depicted in figure 1) for 24 h at 37 °C. [NAMI-A] = 0.7 mM.

Model	[Reductant]/concentration (μM)	Ru/Tf (mol/mol)	Ru/HSA (mol/mol)
Serum model 0*	Ascorbate/88	0.39 ± 0.01	0.67 ± 0.02
	–	0.52 ± 0.07*	0.51 ± 0.05*
Serum model 0	Ascorbate/50 GSH/3.4 Cys/33	0.35 ± 0.02	0.60 ± 0.03
Serum model 1	Ascorbate/50 GSH/3.4 Cys/33	0.70 ± 0.05	0.91 ± 0.04
	Cys/33	2.0 ± 0.2*	1.2 ± 0.2*
Serum model 2	Ascorbate/50 GSH/3.4 Cys/33	0.60 ± 0.05	0.65 ± 0.01
	Cys/33	2.9 ± 0.5*	1.1 ± 0.1*
Serum model 1 with elevated GSH	Ascorbate/50 GSH/1200 Cys/33	1.6 ± 0.2	0.80 ± 0.09
Serum model 2 with elevated GSH	Ascorbate/50 GSH/1050 Cys/33	0.86 ± 0.04	0.69 ± 0.01

*Data taken from Ref. [13].

3.3. *Influence of elevated glutathione concentration*

To check the hypothesis of redox activation of NAMI-A, the increased GSH level (0.105 mM) was applied for fast and efficient reduction of Ru(III) complex (0.7 mM). The chosen concentration of GSH corresponds to 1.5 equivalent of NAMI-A. As shown in figure 5 at applied reagent concentrations the reduction of NAMI-A was completed within a few minutes at 37 °C. When the reaction was carried out in the presence of protein mixtures

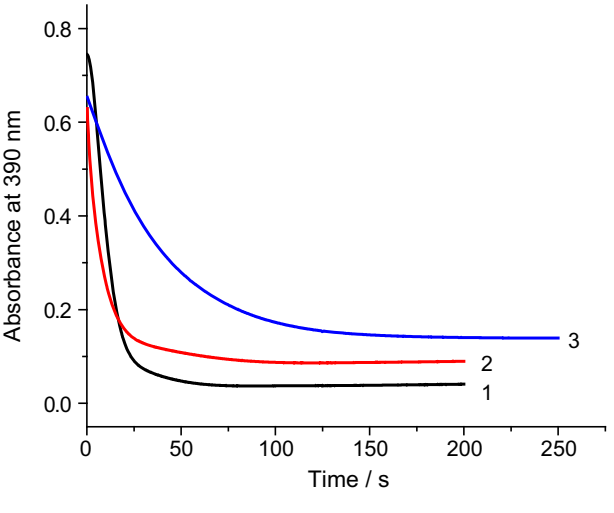


Figure 5. Absorbance at 390 nm as a function of time recorded for reduction of NAMI-A by GSH in the absence (1) and presence (2) of proteins in comparison with hydrolysis (3). Experimental conditions: [NAMI-A] = 0.7 mM, [GSH] = 0.105 mM, [HSA] = 630 μM, [apo-Tf] = 24.5 μM, [holo-Tf] = 10.5 μM; Tris/HCl pH 7.4; [NaCl] = 0.1 M; [$NaHCO_3$] = 25 mM, T = 37 °C.

at Ru protein ratio corresponding to the studied serum models (see figure 1), the reduction of Ru(III) by GSH was slower and a contribution from the hydrolysis process cannot be excluded (compare figure 1). Therefore, the addition of GSH can partially reduce NAMI-A and also facilitate the hydrolysis. It was already shown that most of the hydrolytic derivatives of NAMI-A have a higher redox potential and therefore are expected to be even more easily reduced by GSH [15]. To evaluate the effect of the increased level of GSH on binding of Ru complex to HSA and Tf, serum models 1 and 2 were enriched in higher concentrations of GSH. After incubation of these serum models with NAMI-A (0.7 mM) for 24 h at 37 °C the reaction mixture was separated on a MonoQ column (see figure S1, see online supplemental material at http://dx.doi.org/10.1080/00958972.2015.1067692) and the fractions of ruthenated transferrin and albumin were collected. Subsequently, the content of Ru and proteins was measured as described in a previous section. The obtained results presented in table 1 indicate that higher concentration of GSH does not improve Ru–protein adduct formation in comparison with non-reducing conditions (Serum models 1 and 2). However, protein-bound fraction of Ru was higher in the presence of higher reductant concentration. The reason of slightly higher affinity of Ru in the presence of elevated level of GSH than at low-reducing conditions can arise from the prevention of formation of non-active polyoxo-mixed valent Ru species by complete reduction of NAMI-A to Ru(II) species. Such high level of the GSH can also lead to reduction of some 19 intra-chain disulfide bonds in the transferrin structure [39] that can result in protein unfolding [40] and exposing of other binding sites. Additionally, it can reduce Fe(III) to Fe(II) in holo-Tf promoting its release from protein. The changes in transferrin upon addition of high levels of GSH are not so advanced to influence the chromatographic separation (see figure S1). The observed differences between Serum models 1 and 2, both with elevated GSH, can result from interaction of GSH with Mg [41] and other divalent metals like Zn and Ca [42] present in Serum model 2.

4. Conclusion

The present studies have shown that the reducing environment of blood can have a distinct effect on binding of NAMI-A derivatives to albumin and transferrin. In particular, ruthenation of transferrin was very sensitive to the presence of Ru(II) species in solution. Generally, the non-reduced species bind with higher extent to transferrin, however mode of binding can be different; non-coordinating *versus* coordinating bonds for Ru(III) and Ru(II) species were suggested, respectively. One interesting issue for investigation would be comparison of the potency to release of ruthenium from Ru(III)–protein adducts with those of Ru(II)–protein adducts. The performed experiments have demonstrated that even small changes in the composition of serum models can have a pronounced effect on binding ruthenium complexes to proteins. Therefore, it is important to conduct experiments in media closely resembling that found in the blood stream. Knowledge of the possible reactivity of ruthenium complexes with proteins is essential for understanding their mode of action and can have strong impact on their overall pharmacological and toxicological profile.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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