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Review

Metallomics and mass spectrometry for drug development: ICP-MS and MALDI TOF MS for assessment of protein-drug interactions

Romana MASNIKOSA[1*], Ana RILAK-SIMOVIĆ[2], Suzana VELIČKOVIĆ[1]

[1]*University of Belgrade, Vinča Institute of Nuclear Sciences, Department of Physical Chemistry, Belgrade, Serbia*
[2]*University of Kragujevac, Faculty of Science, Department of Chemistry, Kragujevac, Serbia*

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Summary. Ever since the discovery of the anticancer drug cisplatin, the field of inorganic medicinal chemistry has been expanding. A plethora of metal-based compounds (Pt, Ru, Au, Pd, Cu) containing organic or inorganic ligands (NH_3, Cl, DMSO, η^6-arene, imidazole, indazole, terpyridine, phosphoadamantane) has been developed with the aim to find new and improved anticancer therapeutics, which would have less side-effects, and to overcome tumour resistance. The medicinal inorganic chemistry community must determine: how these compounds enter living cells, how they distribute inside cells, what are their intracellular targets, how they behave in circulation (free vs protein-bound fraction), what are their target tissues, and what biomolecules are responsible for their transport to biological targets. All of these questions have been recently addressed with the aid of different mass spectrometry (MS) techniques. Inductively coupled plasma MS is a highly sensitive method for determining the metal content in biological samples. Matrix-assisted laser desorption/ionisation and electrospray MS are used to characterise adducts between metallodrugs and proteins. MS methods provide information on binding kinetics, binding parameters, the nature of the adducts and target sequences in proteins. This review deals with the most interesting results obtained by the most influential mass spectrometrists working in the field of inorganic biochemistry.

Keywords: ICP MS, mass spectrometry, metallodrugs, protein.

Ever since the discovery that cisplatin (Fig. 1) can be used to cure cancer, the field of inorganic medicinal chemistry has been significantly expanding. A plethora of new complexes consisting of transition metals (Pt, Ru, Au, Pd, Os, Ir, Ag, Cu) coordinated to various organic and inorganic ligands (e.g. NH_3, Cl, DMSO, η^6-arene, imidazole, indazole, terpyridine, bipyridine, polypiridine, phosphoadamantane etc.) has been developed (Gianferrara et al. 2009; Rilak-Simović et al. 2019). These metallocomplexes were synthesized with one goal, to determine if they could be used as anticancer drugs: although some of them showed antirheumatic and/or antimicrobial properties. The first experimental challenge is to assess the fate of the metallodrugs in the cell. We usually start with *in vitro* cytotoxicity tests on different cancer and normal cell lines, in comparison to drugs currently in use, such as cisplatin, oxaliplatin or carboplatin (Rilak-Simović et al. 2019). The distribution and fate of metallodrugs in the cell is another experimental challenge. Moreover, for potential compounds there are other questions for the medicinal inorganic chemistry community to answer: how do the compounds cross the plasma membrane and enter living cells, how are they distributed inside the cell, what are their intracellular targets (proteins, genomic DNA, RNA, membranes, cytoskeleton), how do they behave in circulation (free *vs* protein-bound fraction), how are they distributed within a whole living organism, what are their target tissues, what biomolecules are responsible for their transport and delivery to biological targets, etc. (Holtkamp and Hartinger 2018; Rilak-Simović et al. 2019).

E-mail: romana@vin.bg.ac.rs

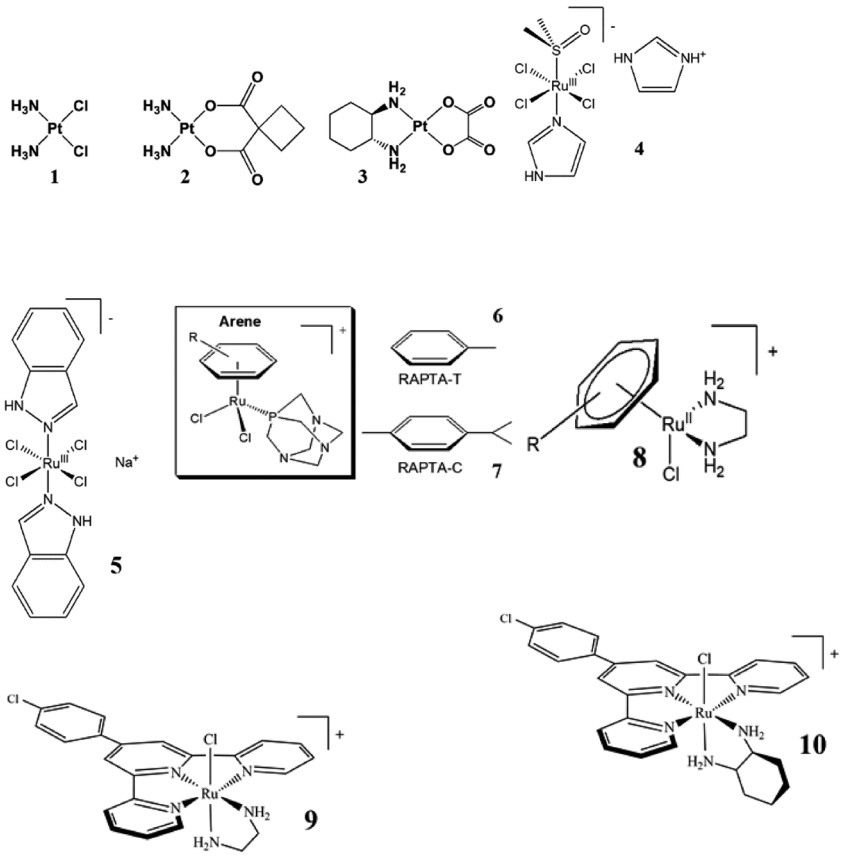

Fig. 1. Structures of some representative Pt, Ru(II) and Ru(III) complexes that exhibit anticancer activities: cisplatin (1), carboplatin (2), oxaliplatin (3), NAMI-A (4), KP1339 (5), RAPTA-T (6), RAPTA-C (7), organometallic compounds of the general formula [(η^6-arene) Ru(en)Cl]$^+$ (8), [Ru(Cl-tpy)(en)Cl]$^+$ and [Ru(Cl-tpy)(dach)Cl]$^+$ (9).

In the review presented herein, we offer some answers to the aforementioned questions, from an analytical (methodological) point of view. Cells are extremely complex systems, containing myriads of biomolecules, which most often act in a concerted manner and cellular pathways are closely intertwined. Analytical approaches to study cellular events on a global scale are still relatively new. It is far more complicated to assess the biological activity of metallocomplexes in the cellular milieu than to establish their chemical targets in the test tube (Komor and Barton 2013). That is why many of the aforementioned questions have been addressed through studies of model systems, the simplest being binary interactions in the test tube. Hence, we mix a metallocomplex with a biomolecule of interest (amino acid, nucleotide, peptide, oligonucleotide, protein, DNA) in a physiological-like buffer and, then, study the resulting adducts (for example by monitoring their formation).

Thus, the question of what is the final target of a particular metallodrug is reduced to one of two model systems: i) incubate a complex with the biomolecule of interest (or a mixture of biomolecules) or ii) incubate a complex with a biological system (cells in culture, serum, blood) and, then, quantitate the amount of drug bound (Brouwers et al. 2008; Hartinger et al. 2013). Metallomics is the study of metal species in biological systems including their interactions with biomolecules (Hartinger et al. 2013). Cellular targets of metallodrugs are: proteins, genomic DNA, RNA and membranes. Considering that only 1% of cisplatin, which has been administered to a tumour patient, ends up actually bound to genomic DNA, there is a need for an extremely sensitive technique to quantitate metal content in biomacromolecules. Inductively coupled plasma mass spectrometry (ICP MS) can quantitate metal content in biomolecules, cell cultures, cell extracts, isolated organelles, subcellular fractions (membranes, cytoskeleton, cytosol), tissues and organs (Brouwers et al. 2008). ICP MS has been widely used to monitor the cellular uptake of transition metal-based drugs, *via* measuring the metal content inside cells and organelles (Komor and Barton 2013). In brief, the biological sample is first introduced into a spray chamber, where it is nebulised into an

aerosol. These droplets are then transferred into ICP, where all molecules are atomised and ionised, leading to atomic singly charged ions (Me^+), which are then pushed towards an MS unit that is used to identify and quantitate the metal of interest. All other information concerning the sample is lost, i.e. the metal ion is the only survivor of the atomisation (Brouwers et al. 2008). ICP MS can be used as an ultrasensitive metal-selective detector in combination with speciation techniques such as liquid chromatography (LC) or capillary electrophoresis (CE) (Timerbaev 2014). IUPAC recommends that the term *speciation* is used for species distribution, and *speciation analysis* to indicate analytics for identifying and/or measuring the quantities of one or more chemical forms in a sample. This is because metallo-complexes in water solutions frequently undergo reactions such as hydrolysis, redox changes, ligand coordination and/or geometric isomerisation, so they appear as different chemical entities (Wenzel and Casini 2017).

Over the years, the most widely used techniques to study interactions between biomolecules and metallocomplexes have been: spectroscopy (fluorescence, UV-Vis, NMR and CD) and calorimetry. The most serious drawbacks of these methods is that they consume milligram quantities of samples (the majority of proteins are either expensive or must be isolated from tissues in minute quantities). Many of these methods reveal the kinetics/thermodynamics of binding in terms of affinity constants, number of binding sites, binding energy and types of interactions. However, expertise is required to calculate these parameters. MS, on the other hand, consumes only femtomolar amounts of protein and is rapidly expanding into an irreplaceable tool for the study of intermolecular interactions. The only obstacle to the widespread use of MS in bioinorganic chemistry is the price of MS instrumentation.

In the present review, we present work from leading groups in the inorganic biochemistry community that have developed major MS techniques for assessment of biomolecular interactions for the most active transition metal complexes. This review is more illustrative than comprehensive in its content, because of the large volume of literature data available on this subject. Of the many hundreds of metallocomplexes that have been tested for anticancer properties, we have focused our attention on select platinum-based anticancer drugs in use (cisplatin, oxaliplatin and carboplatin) and several Ru(III) compounds: for example NAMI-A, [imidazolium trans-[tetrachloro(dimethylsulfoxide)(1H-imidazole)ruthenate(III)]], which showed considerable antimetastatic acitivity and was the first ruthenium-based drug to enter clinical trials); Keppler's compound KP1019, which proved to be active against metastatic tumours and cisplatin-resistant tumours, particularly colorectal cancers. The sodium salt of KP1019, aka KP1339, is far more water-soluble and as such more convenient for intravenous applications than KP1019. Dyson's Ru(II) arene compounds: RAPTA-T: $[Ru(\eta^6\text{-toluene})(pta)Cl_2]$ (pta = 1,3,5-triaza-7-phosphaadamantane) and RAPTA-C: $[Ru(\eta^6\text{-}p\text{-cymene})(pta)Cl_2]$, the former of which exhibited antimetastatic potency, whereas the latter exhibited broad acting antitumour efficacy with intrinsic angiostatic activity; Sadler's half-sandwich type Ru(II) arene complexes, such as $[Ru(\eta^6\text{-}p\text{-arene})Cl(en)]^+$, (arene is either cymene or biphenyl and en is ethylenediamine), which was shown to reduce the growth of primary tumours (Rilak-Simović et al. 2019). Finally, we include some Ru(II) polypyridyl compounds that have been developed and synthesized by our group, namely [Ru(Cl-tpy)(en)Cl]Cl, [Ru(Cl-tpy)(dach)Cl]Cl and [Ru(Cl-tpy)(bpy)Cl]Cl, where Cl-tpy = 4'-(4-chlorophenyl)-2,2':6',2"-terpyridine, en = 1,2-diaminoethane, dach = 1,2-diaminocyclohexane, bpy = 2,2'-bipyridine (Rilak et al. 2014).

MS is a powerful tool for studying non-covalent interactions, due to its low sample consumption, high sensitivity and label-free nature (Chen et al. 2016). In order to decide what type of MS will give the most useful information on interactions between biomolecules and metallocomplexes, it is necessary to determine: i) the size of the biomolecule; ii) how much sample is available; iii) the type of binding and iv) interaction strength. The basic procedure involves mixing a solution containing a biomolecule with a metallodrug in an MS-compatible (and also physiological-like) buffer, leave them to incubate for a desired period of time (usually at 37 °C) and apply the incubation mixture (sample) into a mass spectrometer. Depending on the purpose, one can apply the sample directly into the instrument (so-called injection) or perform some separation process prior to the injection (LC or CE). The separation is not necessary in the case of a binary mixture of biomolecule and metallodrug, but is often used prior to analysis of complex systems such as protein mixtures, cell extracts, serum etc. Biomolecule-metallodrug adduct(s) are first ionised in the ion source of the MS instrument; the molecular ions and their potential fragments are then extracted from the source in order to be pushed toward the detector. To produce gas-phase ions out of biomacromolecules that can be resolved in the MS analyser, we use soft ionisation techniques, to prevent extensive fragmentation of analytes. These are: MALDI (matrix-assisted laser desorption/ionisation) and ESI (electrospray ionisation) ionisations (Wenzel and Casini 2017). In MALDI, a laser is directed against samples on a solid plate producing analyte ions. Samples are prepared by mixing analyte solutions with highly concentrated solutions of small organic molecules called a matrix. The matrix is often acidic and, in combination with a MALDI laser, may induce cleavage

of metal-biomolecule associations (especially non-covalent interactions). Ligands attached to a metal centre are often shed during sample preparation or ionisation. Non-specific aggregates between analytes and matrix molecules are frequently seen in MALDI MS spectra. These are the main reasons why MALDI MS is not often used for the study of biomolecular adducts (Wenzel and Casini 2017). Transfer of ionic species from solution into the gas phase by ESI involves three steps: (1) dispersal of a fine spray of charged droplets, followed by (2) solvent evaporation and (3) ion ejection from the highly charged droplets. The charged droplets pass down a pressure gradient and potential gradient toward the analyser region of the mass spectrometer (Ho et al. 2003). A mass analyser between the ion source and the detector analyses, separates and filters ions according to their size-to-charge ratio (m/z). Different analysers use different physical principles to separate ions. Sector instruments possess sector field mass analysers, which apply static electric and/or magnetic fields to bend ion trajectories according to their m/z as they pass through the analyzer. The analyser can be used to select a narrow range of m/z or to scan through a range of m/z to catalog the ions present (usually quadrupole, Q). TOF (time-of-flight) analysers separate the ions depending on their velocity during their path through the tube connecting the ion source and the detector; and ion velocity depends on its m/z. The time that it takes for an analyte to reach a detector at a known distance, TOF, is measured. Heavier particles reach lower speeds. Fourier-transform ion cyclotron resonance mass spectrometer (FT-ICR MS) is equipped with an analyzer that determines the m/z of ions based on their cyclotron frequency in a fixed magnetic field. MALDI is routinely coupled to TOF analysers (MALDI TOF MS), which possess an unlimited mass range (De Hoffmann et al. 1996). Thus, it would be possible to even detect biomolecular adducts with sizes in the megadaltons, if it was not for the detectors, which suffer from very low sensitivity in the high mass range (Wenzel and Casini 2017). The good news about MALDI MS is that it produces simple spectra (compared to ESI MS), since singly charged ions are almost exclusively seen. On the other hand, the detection of intact adducts without fragmentation is rare when analysing non-covalent interactions between proteins and metallodrugs. There are two methodological approaches to do so, either the experimental and instrumental parameters are tuned to find conditions under which the adduct is stable (e.g. non-acidic matrices and collection of first-shot spectra) or the interacting species are "artificially" stabilised by chemical cross-linking (Chen et al. 2016). There is sparse literature data on the use of MALDI MS to elucidate the binding of metals to proteins, under carefully chosen conditions (Nišavić et al. 2016). Otherwise structural information is lost under the laser. ESI MS, on the other hand, has been extensively used to study binding of various metallodrugs to proteins and to characterise the composition of non-covalent associations. Transition metals show characteristic isotopic distributions, which facilitate their identification in mass spectra. The interaction is readily identified by a specific increase in mass corresponding to the nature of the formed adducts (Ho et al. 2003). In summary, ESI MS enables elucidation of both covalent and non-covalent interactions and can be easily hyphenated to chromatographic methods (LC MS), whereas MALDI MS detects only covalent or pseudo-covalent (coordinative) bonds and cannot be directly coupled to chromatography (Wenzel and Casini 2017).

It is of crucial importance that metallation of a protein does not trigger changes in its native three-dimensional (3D) structure, which is a prerequisite for biological function (e.g. transport of a drug to its targets, binding to DNA or enzyme activity). ESI MS can also provide information on the integrity of a proteins higher order structure, i.e. "nativeness". Namely, proteins with intact tertiary structure are compact, with a solvent-exposed surface area in solution that is smaller than that of unfolded proteins. This fact leads to a dramatic difference in the mass spectra of protein ions for folded vs. unfolded proteins (Kaltashov et al. 2012).

When assessing binding sites for metallodrugs on a protein, two MS approaches can be applied: a bottom-up and a top-down approach. The former involves enzymatic cleavage of the adducts prior to MS identification of peptide fragments. In top-down (or native MS), mass spectra of the preformed adducts are acquired directly, usually under physiological-like conditions (Boeri-Erba and Petosa 2015). Through the bottom up approach, one can analyse large proteins and highly complex samples with the aid of fragmentation and tandem MS (aka MS^2 or MS/MS), but for the metallated adducts this can become pretty complicated, because of artefacts (e.g. coordination of the metal to another species during fragmentation or separation) (Wenzel and Casini 2017). It is recommended to use the top-down approach for labile metallation sites in proteins (Hartinger et al. 2013).

To reveal the binding site for a metallocomplex on a particular protein requires elucidation of the amino acid sequence containing the metallated amino acid(s) in order to identify them. To do this tandem MS must be employed. Hybrid MS instruments possess two analysers (e.g. Q-TOF or QqQ) connected by a collision cell. In brief, after the first MS scan we chose the metallated peptides (bottom-up scheme) or intact metallated proteins (top-down scheme) and then subject them to fragmentation in a collision cell. The fragments leaving the cell are also scanned (the second MS scan). From the fragmentation pattern of the particular peptides we can resolve the metallated sequence (Hartinger et al. 2013).

Few types of fragmentation can be used, depending on the type of the instrument. CID or collisional-induced dissociation is a high-energy fragmentation and, as such, prone to destroy labile interactions; however this comes standard with many commercial instruments (De Hoffmann et al. 1996).

In ECD or electron-capture dissociation, unique fragments are observed. With ECD we can effectively fragment whole macromolecules (Zubarev et al. 1998). ECD is primarily used in FT-ICR MS. ECD MS fragments can preserve posttranslational modifications (PTMs) such as carboxylation, phosphorylation and glycosylation. ETD, electron-transfer dissociation, is a method of fragmenting multiply-charged gaseous macromolecules in a mass spectrometer by transferring electrons to them. ETD is used extensively with proteins and peptides for sequence analysis. Transferring an electron causes peptide backbone cleavage while leaving labile PTMs. This makes the technique important for top-down proteomics (Elviri 2012).

Mass spectrometric analysis of peptide metallodrug adducts was often used to model protein-metal coordination and as a proof-of-principle for the versatility of MS. Met proved to be a high-affinity binder of Pt-based drugs as confirmed by ESI MS of an adduct of octapeptide MTGMKGMS (Mets7) and cisplatin. The major MS peak originated from [Mets7+Pt^{2+}] adduct, suggesting that cisplatin lost all of its original ligands upon binding (Arnesano et al. 2007). FT-ICR MS using both CID and ECD was performed on cisplatin adducts with model peptides such as: substance P (11 amino acids), angiotensin II (7 amino acids) and bombesin (14 amino acids). Although ciplatin preferably attached to the sulphur atom of Met in substance P, in angiotensin II and bombesin it preferred His (Wenzel and Casini 2017). The binding of [Ru(η^6-biphenyl)(en)Cl]$^+$ to angiotensin and bombesin using ultra-high resolution FT-ICR MS with the aid of CID and ECD was studied. The primary binding sites were Met and His (which was expected), but Phe also could be a potential binding partner for the Ru(II) arene complex. The π-π stacking between arene moieties was suggested as mode of binding (Wills et al. 2014).

In odrer to evaluate the molecular interactions of metallodrugs with proteins by MS in conditions reflecting physiological ones, soft ionisation techniques are required, to ensure conservation of the interactions and adducts formed (Wenzel and Casini 2017). To this end, MALDI MS is not quite an appropriate technique, since there is a risk that matrix interactions with the adducts will disrupt metal-protein bonds. In addition, compared to ESI MS, MALDI MS lacks sensitivity, so by using the latter one cannot obtain structural data at a satisfying level. A pioneer study that used MS to assess protein-metallocomplex interactions was an investigation of cisplatin binding to ubiquitin (Ub). Ub was chosen as a model protein to evaluate the utility of ESI MS for this type of study (Gibson and Costello 1999). Why ubiqutin? Because it is a small protein (76 amino acids), cheap and commercially available in pure state. Furthermore, Ub possesses no redox states and no disulphide bridges, which simplifies sample preparation. When treated with cisplatin, Ub formed mainly bidentate adducts: Ub-Pt$(NH_3)_2$, but there were also monodentate (Ub-Pt$(NH_3)_2$Cl) and (Ub-Pt$(NH_3)_2(H_2O)$) and tridentate adducts (Ub-Pt(NH_3)) (Gibson and Costello 1999; Peleg-Shulman et al. 2002). One group reported the comparison of different ionisation techniques, *i.e.* MALDI *vs* nESI MS, for the analysis of interactions between Ub and Pt drugs. Cisplatin, transplatin and oxaliplatin formed mainly monoadducts with Ub, but of significantly different composition. Cisplatin formed mainly bifunctional Ub–[Pt$(NH_3)_2$] adducts, while with transplatin the most abundant adduct was a monofunctional Ub–[Pt$(NH_3)_2$Cl]. Oxaliplatin formed exclusively bifunctional species such as Ub–[Pt(cyclohexane-1,2-diamine)]. Although both MALDI and ESI provided comparable results, the higher resolution of the nESI-QTOF MS enabled unambiguous characterisation of the adducts. In contrast to the ESI mass spectra, MALDI suffers from a greater extent of fragmentation, as demonstrated for the Ub–platinum conjugates (Hartinger et al. 2007). In 2008, a proof-of-principle study on the application of top-down ESI FT-ICR MS for characterisation of the primary binding sites of Pt-based drugs on Ub was published. Through employment of different fragmentations in MS2 mode (CID, HCD, ETD, IRMPD), binding sites for cisplatin and oxaliplatin were found in the N-terminal Ub fragments (Met1), whereas transplatin was attached to 19Pro-Ser-Asp-Thr-Ile-Glu24. Only ETD resulted in precise mapping of both Met1 and His68 as preferential oxaliplatin-binding sites on Ub (Hartinger et al. 2008). Casini and collaborators published several papers on the use of FT-ICR, high resolution LTQ-Orbitrap, Q-TOF and IT instruments (all ESI MS), to characterise interactions of Ub with organometallic RAPTA compounds. Only mono- and in some cases bis-adducts and tris-adducts were detected. The Ru(II) centre still contained its arene ligand, and sometimes, even the pta ligand (Wenzel and Casini 2017). Another widely used model protein for studying interactions with metallocomplexes is cytochrome c (cyt c), a redox active, small (12 kD, 104 amino acids), heme-containing protein. It contains two Met, 4 His and two Cys. A bottom-up approach with the aid of both MS^2 and MS^3 mode, on FT-ICR MS, was used to show that Met65 and Met80 on cyt c participate in binding cisplatin. In addition to cisplatin, NAMI-A and RAPTA complexes were tested for interactions with cyt c using ESI and LTQ-Orbitrap MS techniques (Wenzel and Casini 2017). Both techniques revealed monoadducts of the type cyt c-Ru(η^6-*p*-cymene) or,

to a lesser extent, cyt c-Ru(η^6-*p*-cymene)(pta). Noteable, a competition MS study was conducted where RAPTA-C and cisplatin were incubated with a mixture of three proteins (Ub, cyt c and superoxide dismutase/SOD), and resulting adducts were examined by LTQ-Orbitrap ESI MS. Cisplatin proved to be less reactive toward the tested proteins and less selective, whereas RAPTA-C exerted a high affinity for Ub and cyt c, but not for SOD. Moreover, cisplatin and RAPTA-C displayed affinities for the same amino acid residues on the proteins (Casini et al. 2009).

A potential metallo-therapeutic would preferably be administered intravenously, which means that its interaction with serum proteins is of crucial importance. Investigation of such interactions with the most abundant serum proteins is an important step in the pharmacological characterisation of each novel candidate drug (Groessl et al. 2010). This includes assessment of parameters such as binding stoichiometry, binding constant and the number of specific binding sites. Apart from being drug delivery vehicles, serum proteins may be involved in their inactivation. The most abundant serum proteins are human serum albumin (HSA), immunoglobulin G and serum transferrin (Tf). HSA consists of 585 amino acids and accounts for about 60% of the total protein in blood serum. The ability of HSA to bind metallodrugs affects its distribution in the body, rate of metabolism and excretion. In plasma taken from cancer patients treated with KP1019, most of the drug was bound to HSA. IgG is the second most abundant serum protein, but its interactions with metallodrugs have rarely been studied. Tf is a 679 amino acid glycoprotein involved in iron transport in human blood. Despite the long-standing hypothesis that Tf serves as a mediator that can deliver Ru anticancer compounds selectively into tumour cells *via* cell surface receptors for Tf (the number of which is increased in tumours), only a few reports have convincingly shown that this actually happens (Rilak-Simović et al. 2019).

Comprehensive MS analysis of metallodrug interactions with HSA (or Tf) is very complex, and involves LC (or CE) separation of metallated from non-metallated adducts before trypsin hydrolysis and MS. A solvent-accessible residue, Cys-35 in HSA has been suggested as a cisplatin binding partner. Five cisplatin-specific binding sites on hTf were identified: Met256, Glu265, Tyr314, Glu385, Thr457. These residues belong either to peripheral helices or to flexible loops within the binding pockets. Cisplatin bound to purified hTf at a single platination site (Thr457) within the Fe(III) binding site of the C-terminal lobe of Tf (Wenzel and Casini 2017). The literature data on Ru compounds binding to serum proteins are somewhat controversial. In some reports, Tf appeared to be a preferred binding partner for KP1019, with approximately two Ru atoms per Tf molecule, and attachment involving the Fe(III) binding site (Pongratz et al. 2004). On the other hand, formation of KP1019 adducts with holoTf did not require displacement of Fe(III) from their two binding sites, implying binding of Ru on sites different from those for Fe(III). KP1019 was able to attach to His side chains on HSA, and adduct formation triggered conformational changes with the loss of helical stability in the protein and local perturbations in the domain IIA binding pocket. NAMI-A bound to HSA at a ruthenium:protein ratio of 2:1, as deduced from ICP AES experiments (Trynda-Lemiesz et al. 2000a, 2000b). Using SEC-ICP-MS, NAMI-A was detected on both Tf and HSA (95%) following 24 h incubation, but with more rapid and greater reactivity toward albumin (Wenzel and Casini 2017). RAPTA-T bound to both holo- and apo-Tf and to HSA, as shown by SEC-ICP MS. Results from ESI MS show attachment of RAPTA-T to a His residue that is not involved in Fe(III) binding (Groessl et al. 2010).

We elucidated the binding sites for our novel Ru(II)-Cl-tpy complexes (Fig. 1) on albumin, applying a bottom-up approach and LC coupled to MALDI TOF MS^2. The LC step separated ruthenated from non-ruthenated peptides that were obtained by tryptic hydrolysis of the adducts, enabling MS analysis of only ruthenated peptides and simplifying fragment spectra. Based on our experimental evidence, we proposed a model involving chloride release from the first coordination sphere around the Ru(II) centre, followed by replacement with a water molecule and further coordination to a His residue (Nišavić et al. 2016). Most recently, ruthenation of HSA, upon binding to the Cl-tpy complexes, was studied using a novel, integrated approach: results from nano-LC/nano-ESI MS analysis of these complexes were combined with those from molecular docking. Six target sequences were identified on HSA for the en- and dach-containing complexes, and only four sequences for the bipy-containing complex, probably due to its inability to form hydrogen bonds with the protein side chains. One of these sequences, identified by MALDI MS^2 to be 146HPYFYAPELLFFAK159, is located at the lower entrance to subdomain IB, and consists of three alpha helices that form a hydrophobic groove (based on molecular docking). The Ru(II) centre of each complex was bound to His146, which was also involved in interactions with KP1019 (Nišavić et al. 2018a). Using a top-down approach on a high-resolution ESI Q-TOF instrument, we have shown that one molecule of albumin can bind up to nine $[Ru(Cl\text{-}tpy)(en)Cl]^+$ non-covalently (Nišavić et al. 2018b).

In this review, we highlight the importance of studying interactions between transport proteins from human circulation and newly synthesized metallocomplexes using MS.

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