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A comparative study on interactions of cisplatin and ruthenium arene anticancer complexes with metallothionein using MALDI-TOF-MS

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A B S T R A C T

Metallothioneins (MTs) are a group of low molecular weight (6–7 KDa) proteins featured by high cysteine content which allows the proteins to bind to a diverse range of metals. MTs, of which the gene transcription can be induced by a variety of stimuli including hormones, cytokines and metal ions, have important biological functions such as storage, trafficking and homeostasis of metal ions, detoxification of heavy metals, resistance to ionizing radiation, scavenging hydroxyl radical, etc. Importantly, it has been reported that MTs play a role in oncogenesis and cancer prognosis, and are implicated in resistance of cancer cells to anticancer metallodrug cisplatin. In this work, we present a comparative study on interactions between MTs and cisplatin and ruthenium arene anticancer complexes using MALDI-TOF-MS. The results show that cisplatin coordinates to MT-I and MT-II at either pH 3.0 or 7.4, and exhibits a higher affinity to MT-II than to MT-I. The MT–Pt complexes increase significantly in content with decrease in pH values of solutions, indicative that cisplatin competes with zinc for coordination to cysteine residues on MTs and interferes with the binding of zinc to the proteins. While the ruthenium arene anticancer complexes $[(\eta^6\text{-arene})Ru(en)Cl]PF_6$ (arene = benzene or biphenyl, en = ethylenediamine) hardly bind to MTs at acidic pH and coordinate to MTs at a much lower level than cisplatin at neutral pH, which may account for the less toxicity and lack of cross-resistance to cisplatin for this class of ruthenium anticancer complexes. With the MT–Pt complexes as model protein complexes, DDT, the widely used unfolding thiol-containing agent in proteomic research was shown to reduce the coordination of platinum to MTs significantly, implying that DDT used as unfolding reagent at high concentration may cause dissociation of bound metallodrug and should be applied with care.

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1. Introduction

Organometallic ruthenium(II) complexes $[(\eta^6\text{-arene})Ru(YZ)(X)][PF_6]$, where X is a halide and YZ is a chelating diamine such as ethylenediamine (en), have shown potential anticancer activity both in vitro and in vivo, including cytotoxic activity towards cisplatin-resistant cell lines [1–4]. The arene ligand occupies three coordination sites in these pseudo-octahedral complexes and greatly stabilizes Ru in its +2 oxidation state [5]. For the group of chlorido ethylenediamine complexes $[(\eta^6\text{-arene})Ru(en)Cl]PF_6$, the cytotoxicity increases with the size of coordinated arene, and the activity of biphenyl complexes against human ovarian cancer cell line A2780 is comparable to that of carboplatin, and tetrahydroanthracene complexes approach that of cisplatin [1–3,6,7]. As for cisplatin, DNA is a potential target for the Ru(II) arene complexes, most of which bind selectively to N7 of guanine [6–9]. However, the previous results showed that organometallic ruthenium complexes $[(\eta^6\text{-arene})Ru(en)Cl]PF_6$ (arene = *p*-cymene or biphenyl) are reactive towards thiols in cysteine [10], glutathione [11,12] and human albumin [13] and that the Ru-coordination can induce the oxidation of thiolates to sulfenates or sulfinates [11–13]. The oxidation of coordinated glutathione in the thiolato complex $[(\eta^6\text{-bip})Ru(en)(GS)]^+$ appears to provide a facile route for displacement of S-bound glutathione by G-N7 of DNA, and could play a significant role in their biological activity [12]. And these findings intrigue our further investigations on the interactions of the ruthenium(II) arene anticancer complexes with other thiol-containing proteins.

Metallothioneins (MTs) are a group of low molecular weight non-enzymatic polypeptides characterized by distinctive amino

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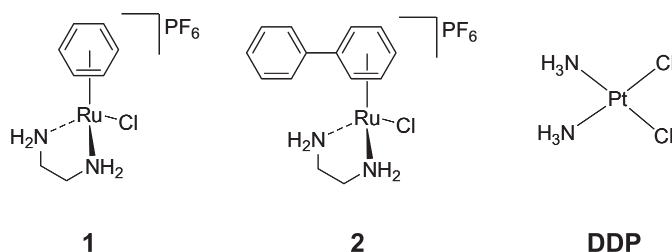

Chart 1. Chemical structures of [(η^6-beneze)Ru(en)Cl]PF_6 (**1**), [(η^6-biphenyl)Ru(en)Cl]PF_6 (**2**) and cisplatin (DDP).

acid composition (no aromatic amino acids, high cysteine content (30% in mammals)) and a high content of sulfur and metals in the form of metal thiolate clusters [14,15]. Four isoforms of mammalian MTs have been identified (MT-I–IV), of which MT-I and MT-II are the best characterized MT proteins. Under physiological conditions, mammalian MTs mostly contain 7 zinc ions which coordinate to cysteine residues to form two metal thiolate clusters located in the C-terminal α-domain (Zn_4S_{11}-cluster) and N-terminal β-domain (Zn_3S_9-cluster), respectively [16]. The metal thiolate clusters make the proteins highly stable. However, in acidic conditions, the bound metals will be dissociated, and MTs proteins become unstable and rapidly degraded [15].

The biosynthesis of MTs is induced by a wide range of stimuli, for instance, metal ions, hormones, glucocorticoides, reactive oxidative species (ROS), even stress. The low molecular weight MT proteins have been shown to be involved in a diversity range of biological functions, including storage, transportation and homeostasis of metal ions, detoxification of heavy metals, scavenging ROS, immune defense responses, angiogenesis, cell cycle progression, and cell differentiation [16,17]. MTs have also been shown to be involved in the development of resistance to anticancer drug cisplatin, one of the most widely used chemotherapeutic metallodrugs [16,18–23]. The increase in cellular content of metallothionein was thought as a possible biomarker of resistance to cisplatin treatment [22–30]. Therefore, the interactions between MTs and platinum-based anticancer drugs, in particularly cisplatin, have been extensively studied during the past decades [22,31–36]. It has been found that cisplatin reacted slower with MT-II than its inactive isoform transplatin, and that all the ligands in *cis*-Pt(II) compounds including cisplatin were replaced by cysteine thiolates of MT-II, while *trans*-Pt(II) compounds, for instance transplatin retained their N-donor ligands. The interactions of MT-II with an antimetastatic organometallic ruthenium compound [Ru(η^6-*p*-cymene)Cl_2(pta)] (RAPTA-C) was also investigated using ESI MS and ICP AES. The coordination of RAPTA-C to MT-II also required the displacement of an equivalent amount of zinc, indicative that Cys residues on MTs are the binding sites for this ruthenium complex [37].

In spite of the monofunctional (arene)Ru(en) anticancer complexes have been shown to have adequate affinity for thiols in peptides [11,12] and proteins [38], the interactions of this class of ruthenium arene anticancer complexes with MTs as well as the consequent pharmacological significance remain unexplored. In this present work, with cisplatin as a reference, the interactions between ruthenium arene anticancer complexes [(η^6-arene)Ru(en)Cl]PF_6 (arene = benzene (**1**) or biphenyl (**2**), Chart 1) and rabbit liver metallothionein I and II have been investigated by means of matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF-MS). Meanwhile, using the cisplatin-MT complexes as model proteins, the effect of dithiotheitol (DDT), the most widely used thiol-containing unfolding reagent for proteomic research, on metal–sulfur coordination has been also studied.

2. Experimental

2.1. Materials

[(η^6-benzene)Ru(en)Cl]PF_6 (**1**, en = ethylenediamine) and [(η^6-biphenyl)Ru(en)Cl]PF_6 (**2**) were synthesized as described in the literature [2,3]. Rabbit liver metallothionein I and II were purchased from Yuanye Biology Company (Shanghai) without further purification prior to use, Cisplatin from Jinke Chemicals (Shengyang, China), dithiothreitol (DTT) from Pierce, trifluoroacetic acid (TFA) from Acros, and Tris–HCl from Sigma.

2.2. MALDI-TOF-MS

MALDI-TOF mass spectrometry analysis was performed on an Autoflex III mass spectrometer (Bruker Daltonics). The instrument was equipped with a delayed ion-extraction device and a pulsed nitrogen laser operated at 337 nm. The analysis was performed under positive ion linear mode with an accelerating voltage of 19 kV and a delayed extraction for 100 ns. Typically, 300 scans were averaged. And the MALDI uses a ground steel sample target with 384 spots. The matrix used in these experiments was 20 mg/mL 2,5-dihydroxybenzoic acid (DHB), in a solution containing 1:1 acetonitrile and 1% H_3PO_4.

2.3. Sample preparation

The protein concentration of metallothionein stock solution was determined by measuring the absorbance of the dilution in 0.1% trifluroacetic acid (TFA) (pH 1.6) at 220 nm (the extinct coefficient of rabbit apo-MT at 220 nm is 48200 mol^{-1} cm^{-1} [39]). A aliquot of aqueous solution of ruthenium arene complex **1** (4 mM), **2** (4 mM) or cisplatin (1 mM) was mixed with MT-I or II in 10 mM Tris–HCl solution, of which the pH were adjusted using 0.1% TFA to 7.4 or 3, making the final molar ratio of metal compounds to MT (10 μM) be 1:2, 1:5 or 1:10, and the resulting mixture was incubated at 310 K for 48, 72 or 96 h prior to MALDI-TOF-MS analysis.

3. Results

3.1. MS characterization of metallothionein I and II

Mammalian metallothioneins (MTs) are low molecular weight proteins composed of 60–62 amino acids with molecular mass of ca. 6–7 KDa. MTs fall into at least four subgroups, namely MT-I, MT-II, MT-III, and MT-IV. The MT-I and MT-II are the most widely expressed isoforms in different tissues and have received the most attention. MT-I and MT-II differ by a single negative charge, thus they can be separated by anion exchange chromatography [40]. These two isoforms contain several sub-isoforms, which are designated by a lower-case letter, e.g. MT-Ia. In this present work, we firstly identified the sub-isoforms within the commercially obtained rabbit liver MT-I and MT-II, of which the amino acid sequence have a high degree of identity with those of human MTs [40], using MALDI-TOF-MS (Fig. 1). It can be seen that both MT-I and MT-II contain four isoforms, and that MT-Ie and MT-Id were the main components within MT-I, while the content of four MT-2 sub-isoforms does not differ from each another significantly.

3.2. Reactions of cisplatin with metallothioneins

Despite the interactions of cisplatin with MTs have been studied previously under various conditions, for comparison purpose, in this work the interactions of MT-I and MT-II with cisplatin under the given conditions was firstly characterized by MALDI-TOF-MS.

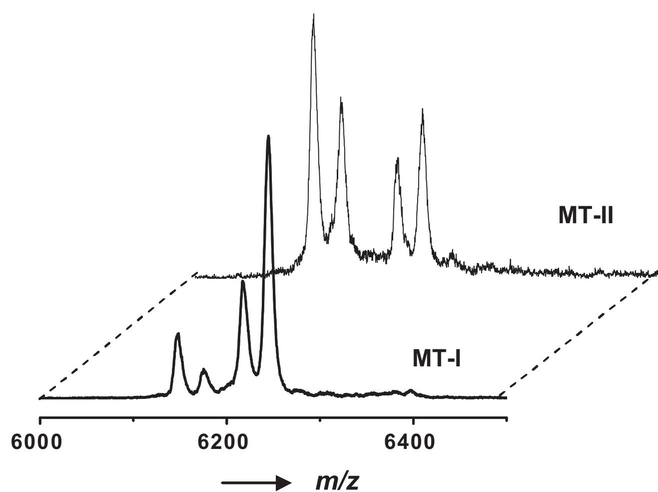

Fig. 1. Mass spectra of rabbit liver MT-I and MT-II in acidic solution (pH 3).

MT-II (10 μM) in 10 mM Tris–HCl (pH 7.4) was incubated with cisplatin (0, 20 or 50 μM) for 48 h at 310 K, the reaction mixtures were then analyzed by MALDI-TOF MS. The results (Fig. 2) show that incubation of MT-II with two mol equiv. cisplatin gave rise to five pairs of platinated MT-II complexes, which were assignable to MT-IIa–Pt_n and MT-IIc–Pt_n (n = 1–5, Table S1 in the Supplemental Information). Further increases of cisplatin concentration to 5- (Fig. 2) and 10-fold excess (data not shown) over MT-II, neither did the relative contents of MT-II–Pt adduct increase compared with that of the unbound MT-II, nor did the coordination pattern of platinum to MT-II change. However, when the reaction took place at pH 3.0, no unbound MT-II was detected, and the highest number of platinum bound to MT-II increased up to 8 (Fig. 3).

Although MT-I and MT-II share a high degree of sequence identity, their reactivity towards cisplatin was quite different due to the structure variance. At pH 7.4, the MS data (Fig. 4) showed that cisplatin hardly reacted with MT-I, only a small amount of mono- and di-platinated MT-Ie complexes were detectable by MS even in the presence of 5-fold excess of cisplatin. At pH 3.0, the reaction of MT-I with 5 mol equiv. cisplatin gave rise to four groups of platinated MT-I complexes assignable to MT-I–Pt_n (n = 1–4, Table S2 in the Supplemental Information), while significant amount of MT-I remained intact. And the results also indicate

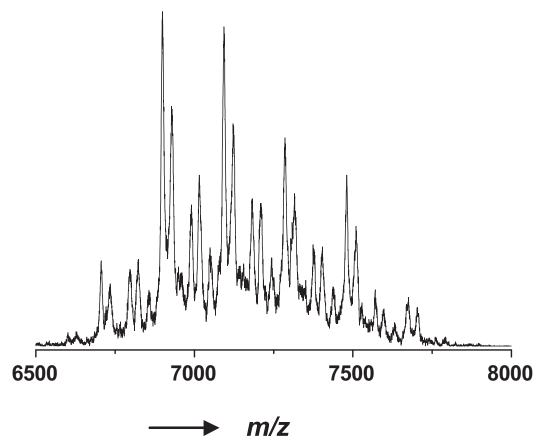

Fig. 3. Mass spectra for the reaction mixture of MT-II with 5 mol equiv. cisplatin in aqueous solution (pH 3.0) at 310 K for 48 h.

that the four isofoms of MT-I do not differ from each another at the term of reactivity towards cisplatin (Table S2).

3.3. *Reactions of ruthenium arene anticancer complexes with metallothioneins*

According to previous research, ruthenium anticancer complexes and cisplatin have different binding profile towards proteins. Cisplatin prefers to coordinate to Met and Cys residues, while the ruthenium complexes mainly bind to His and Met residues [38], the cysteine residue of glutathione is known to bind readily to ruthenium arene complexes though [11,12]. Under pH 7.4, 10 μM MT-II incubated with 5 mol equiv. of ruthenium arene anticancer complex [(η^6-benzene)RuCl(en)]PF_6 (**1**) at 310 K for 48 h afforded small amount of two ruthenated MT-II adducts, which are assignable to mono- and di-ruthenated MT-IIa with one and two $\{(\eta^6\text{-benzene})Ru\}^{2+}$ fragments bound to the protein, respectively (Fig. 5a, Table S1). Whereas only mono-ruthenated MT-IIa and MT-IIc with a bound $\{(\eta^6\text{-biphenyl})Ru\}^{2+}$ unit was observed in the mass spectrum for the reaction mixture of MT-II with 5-fold excess of complex [(η^6-biphenyl)RuCl(en)]PF_6 (**2**) at pH 7.4 (Fig. 5b and Table S1). Interestingly, when the reactions of MT-II with the two ruthenium arene complexes took place at pH 3.0, no ruthenated MT-II complexes was detectable by MS, however, the molecular

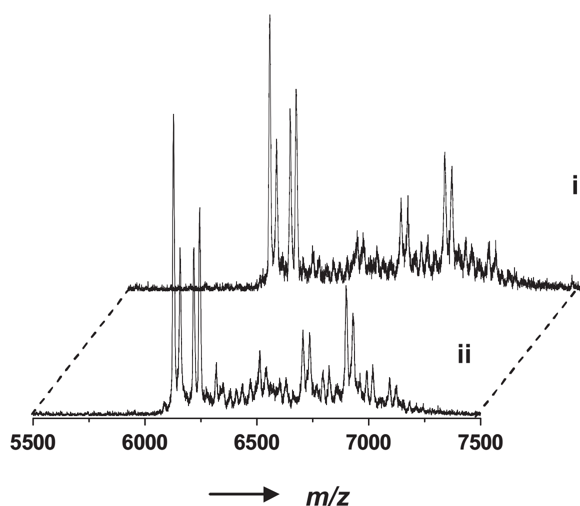

Fig. 2. Mass spectra for the reaction mixtures of MT-II with (i) 2, or (ii) 5 mol equiv. cisplatin in aqueous solution (pH 7.4) at 310 K for 48 h.

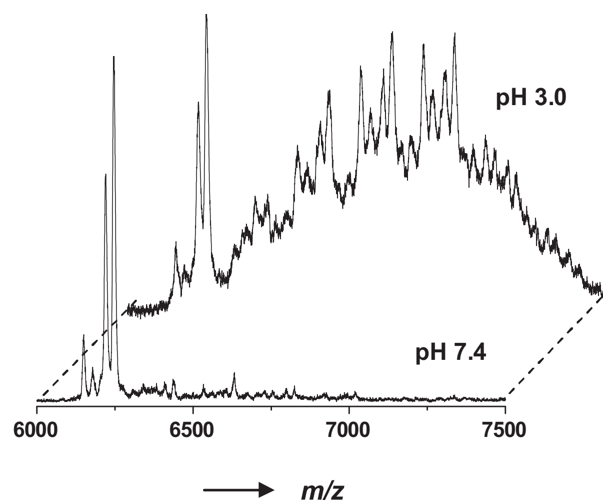

Fig. 4. Mass spectra for the reaction mixture of MT-I with 5 mol equiv. cisplatin in aqueous solution at 310 K for 48 h under pH 3.0 or 7.4.

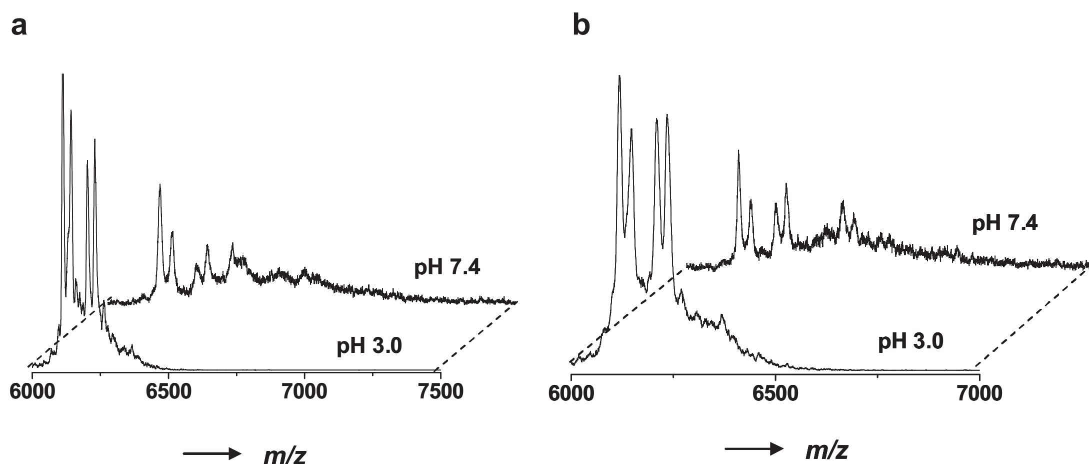

Fig. 5. Mass spectra for the reaction mixtures of MT-II with 5 mol equiv. complexes **1** (a) and **2** (b) in aqueous solution at 310 K for 48 h under pH 3.0 or 7.4.

weight of MT-IIs was found to decrease 8 or 16 Da compared with that of native apo-MT-II proteins (Fig. 5 and Table S1), which are contributed to the formation of four or eight intra-molecular disulfide bonds in the proteins in acidic solution [41].

Ruthenium arene complexes **1** and **2** show less discrimination between MT-I and MT-II compared with cisplatin, however, their affinity towards MTs is much lower than that of cisplatin. At pH 7.4, small amount of mono-ruthenated MT-Ie and Id adducts with one or two naked Ru^{2+} ions, $\{(\eta^6\text{-benzene})Ru\}^{2+}$, $\{(\eta^6\text{-biphenyl})Ru\}^{2+}$ or $\{Ru(en)\}^{2+}$ fragments were detected from the reaction mixture of MT-I with 5 mol equiv. of complex **1** or **2** incubated at 310 K for 48 h (Fig. 6 and Table S2). While at pH 3.0, minor ruthenated products MT-Ie-{Ru(en)}, MT-Ie-$\{Ru\}_2$ and MT-Ie-$\{(\eta^6\text{-benzene})Ru_2\}$ was observed by MS only for the reaction mixture of MT-I with 5-fold excess of complex **1** incubated at 310 K for 48 h. Unlike apo-MT-II, in acidic solution (pH 3.0) no intra-molecular disulfide bonds formed within apo-MT-I in the presence of both complex **1** and **2** (Table S2).

3.4. Effect of DDT on the coordination of cisplatin with MTs

Mass spectrometry based bottom-up method was the most commonly used way for characterizing the binding sites of anticancer metallodrugs on proteins. In this approach DTT was often used to cleave disulfide bonds prior to enzymatic, e.g. tryptic digestion. However, the metal–ligand coordination of metallodrug–protein complexes may be affected by the nucleophile dithiothreitol (DTT), and the thiols of DTT may displace protein residues such as cysteine, methionine and histidine residues to bind to metallodrugs. Therefore, in this work we used the Pt–MT complex, in which platinum ions most likely coordinate to thiols of cysteine residues, formed by reaction of MT-I with cisplatin at pH 3.0 as model metallated proteins to investigate whether the application of high concentration DTT would lead to the disruption of metallodrug–protein coordination. The mass spectra of the reaction mixture of MT-I and cisplatin at a molar ratio of MT-I/Pt = 1:5 incubated at 310 K for 48 h under pH 3.0 is shown in Fig. 7a. It can be seen that the ratio of the intensity of apo-MT-I and the most abundant Pt–MT-I complex was about 1:1. After addition of DTT to the reaction mixture for 5 min, this intensity ratio decreased to 1:0.6 (Fig. 7b), and as the time extends the relative intensity of the Pt–MT-II complexes decreased further (Fig. 7c and d)

4. Discussion

Cisplatin (cis-$[Pt(NH_3)_2Cl_2]$) (DDP) has been one of the most widely used anticancer drugs for the past decades, and is particularly effective for treating solid tumors such as ovarian, testicular, bladder, head and neck cancers [42]. In vivo, cisplatin is converted to its active form by aquation, being highly reactive towards biomolecules such as DNA and proteins [42]. Cisplatin-DNA adduct formation leads to final apoptotic cell death, thought to be the major mechanism for cisplatin antitumor activity [43,44]. However, the clinical use of cisplatin are hampered by severe toxic side effects such as nephrotoxicity and hearing problems and the development of acquired drug resistance, which is contributed to multi-factors including changes in intracellular accumulation of the drug, increased production of intracellular thiols to prevent toxicity, increased capability of cells to repair cisplatin-DNA damage, and a failure to initiate apoptosis in the presence of platinated DNA [22]. Many investigations confirmed that metallothioneins is involved in the development of acquired resistance to cisplatin, and that increased MT expression levels lead to increased cisplatin resistance.

In the present work, MS data showed that cisplatin has a higher reactivity towards MT-I and MT-II, the most abundant mammalian metallothionein isoforms, at acidic solution than at neutral solution. The reason may be that metallothioneins was less compact in acidic condition due to dissociation of bound zinc ions which lead to more thiols accessible for cisplatin coordination. These results are consistent with previous reports that platinum coordination with MTs requires the displacement of an equivalent amount of zinc [34]. The rabbit MT-I and MT-II exhibit a high degree of sequence identity, however, their reactivity towards cisplatin was very different. MT-I hardly react with cisplatin after 92 h of incubation at 310 K under neutral pH, while about one third of MT-II formed metallated adducts with cisplatin after 48 h incubation under the same conditions, indicating that the reactivity of MT-II towards cisplatin is much higher than that of MT-I, which may be attributed to the different configuration of MT-I and MT-II. As the coordination with MT-III [34,45], all ligands of cisplatin were displaced, most likely, by thiols of cysteine residues subject to the coordination to MT-I and MT-II.

In vivo, both platinum and ruthenium anticancer complexes would react with peptides and proteins which were rich in methionine, histidine and cysteine residues. However, many studies suggested that platinum and ruthenium complexes showed different binding affinity and coordination pattern on targeted proteins [45]. We showed here that the ruthenium arene complexes **1** and **2** are much less reactive to MTs, and less discriminatory between MT-I and MT-II than cisplatin. Similar to cisplatin, the ruthenium arene complexes partially or fully lost the chelating ligand en or/and arene ligands upon the coordination to thiols of which the sulfur

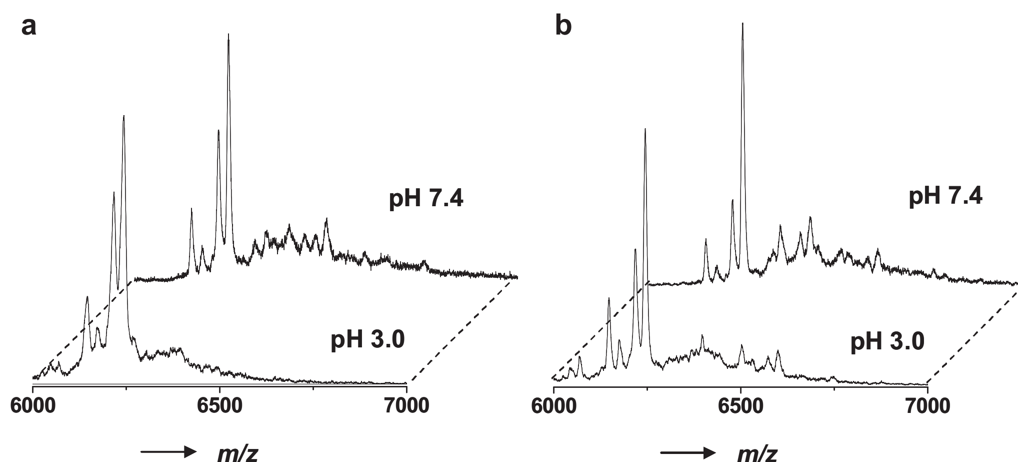

Fig. 6. Mass spectra for the reaction mixtures of MT-I with 5 mol equiv. complexes **1** (a) and **2** (b) in aqueous solution at 310 K for 48 h under pH 3.0 or 7.4.

atom may impose trans effect leading to weakening of the Ru–N bonds and the C–Ru bonds [10]. Surprisingly, unlike cispaltin, in acidic solution the ruthenium complexes hardly reacted with MT-I and MT-II, in which case the observed mass-to-charge ratio of apo-MT-II decreased by 8 or 16, indicative for the formation of four or eight intra-molecular disulfide bonds in acidic condition [41] and for involvement of ruthenium(II) in the oxidation of thiol groups of the thiol-rich apo-MT proteins [11].

In order to identify the binding sites of drug molecules on proteins, in particular large molecular weight proteins, it is necessary to treat protein complexes with reducing agents to cleave disulfide bonds prior to enzymatic digestion. DDT containing 2 thiol groups is one of the most commonly used reductant/unfolding agents which may displace the cysteine, methionine or histidine residues from metallated protein complexes. In the present work, the Pt–MT-I complexes were used as model metallated proteins

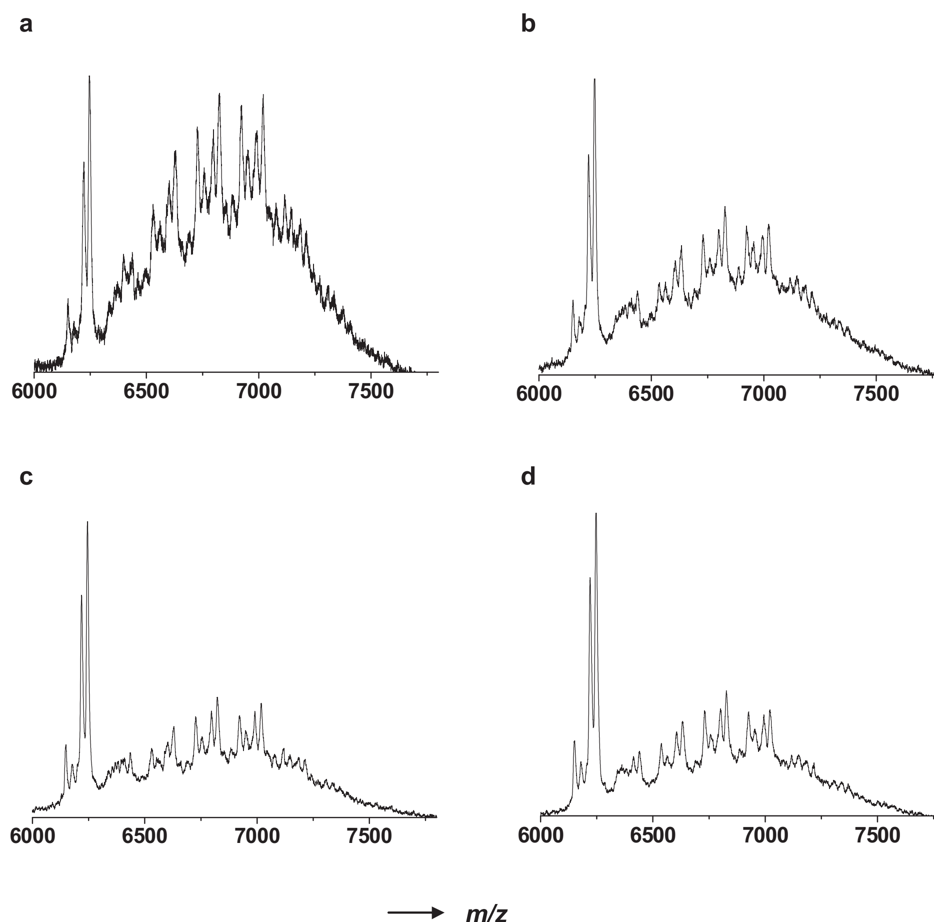

Fig. 7. Mass spectra for (a) Pt–MT-I complex, which was formed by reaction of 10 (M MT-I with 5 mol equiv. cisplatin in aqueous solution (pH 3.0) at 310 K for 48 h, and the reaction mixture of this protein complex with 200-fold excess DDT incubated at 310 K for (b) 5 min, (c) 30 min, or (d) 60 min.

to find out whether DDT could disrupt the Pt–thiol coordination of the platinated proteins. The results showed that high concentration DDT indeed destroy Pt–protein coordination to some extent within 5 min reaction, indicating that DTT used as unfolding reagent at high concentration may cause dissociation of metallodrug bound to proteins and should be applied with care.

In conclusion, the novel ruthenium arene anticancer complexes have been shown by MALDI-TOF-MS analysis to be much less reactive to thiol-rich metallotioneins which may overexpress in the cancer tissues associate with increased cisplatin resistance, than cisplatin. This finding may be helpful to a better understanding on the distinct pharmacological profile of ruthenium arene anticancer complexes, such as less toxic and no cross-resistant to cisplatin.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijms.2010.12.003.

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