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ORIGINAL PAPER

Reaction of human metallothionein-3 with cisplatin and transplatin

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Abstract Human metallothioneins, small cysteine- and metal-rich proteins, play an important role in the acquired resistance to platinum-based anticancer drugs. These proteins contain a $M(II)_4(CysS)_{11}$ cluster and a $M(II)_3(CysS)_9$ cluster localized in the α-domain and the β-domain, respectively. The noninducible isoform metallothionein-3 (Zn_7MT-3) is mainly expressed in the brain, but was found overexpressed in a number of cancer tissues. Since the structural properties of this isoform substantially differ from those of the ubiquitously occurring Zn_7MT-1/Zn_7MT-2 isoforms, the reactions of *cis*-diamminedichloridoplatinum(II) (cisplatin) and *trans*-diamminedichloridoplatinum(II) (transplatin) with human Zn_7MT-3 were investigated and the products characterized. A comparison of the reaction kinetics revealed that transplatin reacts with cysteine ligands of Zn_7MT-3 faster than cisplatin. In both binding processes, stoichiometric amounts of Zn(II) were released from the protein. Marked differences between the reaction rates of cisplatin and transplatin binding to Zn_7MT-3 and the formation of the Pt–S bonds suggest that the binding of both Pt(II) compounds is a complex process, involving at least two subsequent binding steps. The electrospray ionization mass spectrometry characterization of the products showed that whereas all ligands in cisplatin were replaced by cysteine thiolates, transplatin retained its carrier ammine ligands. The ^{113}Cd NMR studies of $Pt_1{}^{113}Cd_6$MT-3 revealed that cisplatin binds preferentially to the β-domain of the protein. The rates of reaction of cisplatin and transplatin with Zn_7MT-3 were much faster than those of cisplatin and transplatin with Zn_7MT-2. The biological consequences of a substantially higher reactivity of cisplatin toward Zn_7MT-3 than Zn_7MT-2 in the acquired resistance to platinum-based drugs are discussed.

Keywords Metallothionein-3 · Cisplatin · Transplatin · NMR · Mass spectrometry

Abbreviations

Cisplatin	*cis*-Diamminedichloridoplatinum(II)
GSH	Glutathione
MT	Metallothionein
Transplatin	*trans*-Diamminedichloridoplatinum(II)
Zincon	2-Carboxy-2'-hydroxy-5'-sulfoformazylbenzene sodium salt

Introduction

cis-Diamminedichloridoplatinum(II) (cisplatin) is one of the most potent antitumor agents known, displaying clinical activity against a wide variety of solid tumors, such as ovarian, testicular, and bladder tumors. Cisplatin is also used in combination with radiotherapy or surgical treatment [1]. The cytotoxic mode of action is mediated by its binding mainly to two adjacent purine bases of nuclear DNA, forming primarily intrastrand cross-links. This DNA modification distorts the DNA structure such that translation and excision repair is strongly inhibited, directing cells into apoptosis or necrosis [2, 3].

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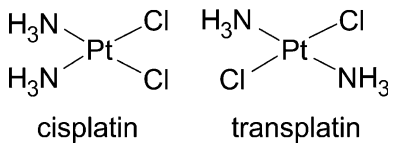

A geometrical isomer of cisplatin, *trans*-diamminedichloridoplatinum(II) (transplatin), is clinically inactive. This is believed to be due mainly to its inability to form similar DNA adducts as cisplatin [4]. The resistance of cancer cells to Pt(II) drugs can be either intrinsic or acquired following Pt(II) administration [1]. The acquired resistance after initial treatment is the major drawback of these chemotherapeutics. Because of the strong reactivity of Pt(II) compounds toward sulfur-donor molecules and the formation of very stable Pt–S bonds, intracellular thiols through their competition with DNA confer resistance to antitumor platinum drugs. The abundant intracellular thiols involved in the drug resistance are glutathione (GSH) and metallothionein (MT) [1, 5, 6]. The four MT isoforms expressed in humans (designated MT-1 through MT-4) consist of 61–68 amino acid residues, of which 20 are conserved cysteines. The structural studies revealed that MTs bind seven Zn(II) or Cd(II) through cysteine thiolates forming two metal–thiolate clusters: a $M(II)_3S_9$ cluster located in the β-domain and a $M(II)_4S_{11}$ cluster in the α-domain of the protein [7]. The ubiquitously occurring MT-1 and MT-2 isoforms are inducible by a variety of compounds, including hormones, cytokines, and metal ions, including Pt(II) drugs. These proteins are involved in the zinc and copper homeostasis, heavy metal detoxification, immune system function, and protection from apoptosis [8–11]. Although GSH effectively deactivates cisplatin and in the certain cancer cell lines its cellular concentration increases upon exposure to Pt(II) drugs, thiols of MTs react faster with cisplatin compared with GSH [12, 13]. In cancer cells basal MT-1/MT-2 expression levels are often significantly increased, resulting in an even stronger Pt(II) scavenging effect [14, 15]. The overexpression of MT-1/MT-2 isoforms in response to Pt(II) drug administration is responsible for most of the cases of acquired resistance to cisplatin [15–17]. In our previous studies we showed that the rates of Zn_7MT-2 reaction with *cis*-[Pt(N-donor)$_2$Cl$_2$] and *trans*-[Pt(N-donor)$_2$Cl$_2$] compounds depend on the nature of the coordinated ligands and that *trans*-[Pt(N-donor)$_2$Cl$_2$] compounds react faster. In this reaction, owing to the high affinity of Pt(II) for cysteine thiolates, exceeding that of Zn(II) 10^7 times, Zn(II) is substituted by Pt(II) [18, 19]. The characterization of the products showed that whereas all ligands in *cis*-Pt(II) compounds were replaced by cysteine thiolates, *trans*-Pt(II) compounds retained their nitrogen-donor ligands.

The MT-3 isoform (Zn_7MT-3), also termed "neuronal growth inhibitory factor," occurs intra- and extracellularly and shows neuroinhibitory activity in vitro that distinguishes it from the widely expressed Zn_7MT-1 and Zn_7MT-2 isoforms. MT-3 shows a brain-specific expression, mainly in glutamatergic neurons [20–23]. This isoform is unresponsive to the inducers of MT-1 and MT-2 expression mentioned above [24]. However, MT-3 was found overexpressed in a number of cancers, such as prostatic adenocarcinoma, esophageal cancer, and breast cancer, where its presence positively correlates with the poor survival prognosis after platinum chemotherapy [22, 25–27]. Furthermore, since MT-3 is absent in normal bladder tissues but is present in large amounts in bladder cancer, the use of this isoform as a marker of this disease has been suggested [28]. The changes in the primary structure of MT-3, which include a unique C(6)PCP(9) motif in the β-domain and an acidic hexapeptide insert in the α-domain (Fig. 1), translate into widely different structural properties of Zn_7MT-3 compared with Zn_7MT-1/Zn_7MT-2 [7, 29]. Thus, the Zn_7MT-3 structure is highly dynamic and the thiolate ligands possess a substantially higher nucleophilicity, indicated by their reactivity toward a number of compounds, including 5,5′-dithiobis(2-nitrobenzoic acid) and *S*-nitrosothiols [30–32]. However, the reactivity of Zn_7MT-3 toward cisplatin and the products formed is not known.

In the present work, the reactions of Zn_7MT-3 with cisplatin and transplatin were investigated and the products characterized by electrospray ionization mass spectrometry (ESI-MS) and ^{113}Cd NMR. The studies were conducted under nativelike conditions regarding pH, ionic strength, and a Pt(II) to Zn_7MT ratio of 2:1 relevant to in vivo studies [18, 33]. The results obtained were compared with those obtained with Zn_7MT-2. The biological implications of these studies for the acquired resistance to platinum-based drugs are discussed.

Materials and methods

Expression and purification of MTs

Recombinant human MT-3 and MT-2 were expressed in *Escherichia coli* as Cd(II) proteins and purified essentially as described in [18, 34]. The correctness of the protein

Fig. 1 Alignment of amino acid sequences of human metallothionein-2 (*MT-2*) and metallothionein-3 (*MT-3*) isoforms used in this study. Cysteine residues are highlighted in *gray*

expression was verified by ESI-MS at low pH. Single mass peaks of 6,925.7 Da (calculated 6,927.1 Da) and 6,041.0 Da (calculated 6,042.1 Da) corresponding to metal-free MT-3 and MT-2, respectively, were obtained. The protein purity was further assessed by sodium dodecyl sulfate polyacrylamide gel electrophoresis [35]. The concentration of reduced thiols was determined by 2,2′-dithiodipyridine assay [36]. In all cases, a cysteine to protein ratio of 20.0 ± 0.5 was obtained. Fully Zn(II) loaded or Cd(II) loaded MTs were prepared by reconstitution as described in [37]. Metal-to-protein ratios were determined by measuring the metal content by flame atomic absorption spectrometry (SpectrAA-110, Varian) in 15 mM HNO_3 [38] and that of the protein spectrophotometrically in 0.1 M HCl ($\varepsilon_{220} = 53{,}000\ M^{-1}\ cm^{-1}$ for MT-3 and 48,200 $M^{-1}\ cm^{-1}$ for MT-2) [36]. In all cases, a metal-to-protein ratio of 7.0 ± 0.3 was obtained.

Platinum quantification by atomic absorption spectrometry

Cisplatin and transplatin were purchased from Sigma (USA). Prior to use, aqueous solutions (300–500 μM) of both compounds were kept in the dark at room temperature for at least 1 day and further at 4 °C. The Pt(II) solutions were freshly prepared after 1–2 months. The Pt(II) concentrations in the stock solutions and experimental samples were determined by flameless atomic absorption using a Varian AA240FS fast sequential atomic absorption spectrometer (Varian, Australia) equipped with a GTA120 graphite tube atomizer and a PSD120 programmable sample dispenser. Prior to analysis, the samples were diluted in15 mM HNO_3.

Characterization of Pt,ZnMT species by ESI-MS

Samples of 10 μM Zn_7MT-2/Zn_7MT-3 or Cd_7MT-2/Cd_7MT-3 were incubated in a nitrogen-purged glove box with 2 mol equiv of cisplatin and transplatin in 10 mM *N*-(2-hydroxyethyl)piperazine-*N*′-ethanesulfonic acid (HEPES)/NaOH pH 7.4, 100 mM $NaClO_4$ for 120 h. Subsequently, unbound Pt(II) compounds were removed and the solvent was simultaneously exchanged by passing it through a 5-mL HiTrap desalting column (Amersham Biosciences) equilibrated in 10 mM 4-ethylmorpholine/HOAc pH 7.0. Immediately before analysis, 4 μL of the eluate was rapidly diluted with 20 μL of 50:50:0.2 (v/v/v) CH_3CN/H_2O/HOAc (final pH ∼ 3) or 50:50 (v/v) CH_3CN/H_2O (final pH ∼ 7). ESI-MS analysis of the protein-containing solution was performed using a Q-TOF Ultima API mass spectrometer (Micromass, UK) essentially as described for MT-2 in our previous work [18]. Solutions were infused through a fused silica capillary (inner diameter 75 μm) at a flow rate of 0.5 μL min^{-1}. Electrospray Pico Tips (inner diameter 30 μm) were obtained from New Objective (Woburn, MA, USA). Mass spectra were acquired by scanning an *m*/*z* range from 600 to 2,500 with a scan duration of 1 s and an interscan delay of 0.1 s. The spray voltage was set to 2.1 kV, the cone voltage to 50 V, the RF lens to 1, the energy to 50 V, and collision to 15. Mass spectra were deconvoluted using the MaxEnt 1 software program (Micromass, UK).

Kinetics of cisplatin and transplatin binding to Zn_7MT-2/Zn_7MT-3

The binding of cisplatin and transplatin to Zn_7MT-2 and Zn_7MT-3 was monitored by absorption spectroscopy following the Pt–S ligand to metal charge transfer band at 285 nm [39]. Prior to measurements, the proteins in 10 mM HEPES/NaOH pH 7.4, 100 mM $NaClO_4$, and water solutions of cisplatin or transplatin were rendered oxygen-free by three freeze–pump cycles on the vacuum line and placed into a nitrogen-purged glove box in the dark at 4 °C. An aliquot of 960 μL of the 10 μM protein solution was quickly mixed with 40 μL of 500 μM Pt(II) compound in a quartz cuvette and the cuvette was sealed. The time courses of the reactions were followed spectrophotometrically (Ultrospec 2000, Amersham Biosciences) at 285 nm at 37 °C for 120 h. The data were normalized per Pt–S bond using $\varepsilon_{285\ nm}$ of 2,680 $M^{-1}\ cm^{-1}$ and fitted using the Origin 7.0 software package [18].

Quantification of platinum bound to MT-3

The proteins were mixed and incubated with the Pt(II) compounds using the conditions described for the kinetics of platinum binding. Aliquots of 200 μL were withdrawn after 0, 2, 5, 18, and 120 h of sample incubation. The aliquots were applied to Microcon YM-3 (Millipore, USA) concentrators and the low molecular weight fraction was separated and the concentration of unbound Pt(II) was determined by atomic absorption. The concentration of Pt(II) bound to the protein was calculated by subtracting the determined Pt(II) concentration from its initial concentration in the sample. No evidence for the interaction of free Pt(II) compounds or platinated MTs with Microcon membranes was obtained in control experiments.

Kinetics of Zn(II) release from Zn_7MT-3 upon Pt(II) binding

To monitor Zn(II) release from Zn_7MT-2/Zn_7MT-3, experiments were carried out using the conditions described for the Pt(II) binding to the thiolates of Zn_7MT-2/Zn_7MT-3 and in the presence of 10 μL of 10 mM 2-carboxy-2′-hydroxy-5′-sulfoformazylbenzene sodium salt (zincon), dissolved in 10 mM NaOH. The concentration of

released Zn(II) was monitored through changes in the absorption of the Zn(II)–zincon complex at 620 nm ($\varepsilon_{620\ nm} = 23{,}200\ M^{-1}\ cm^{-1}$) with time [40].

^{113}Cd NMR of $Pt_1{}^{113}Cd_6$MT-3

^{113}Cd NMR spectra (110.9 MHz) were recorded with a Bruker DRX-500 spectrometer using inverse-gated broad-band proton decoupling to account for possible negative ^{113}Cd,^{1}H nuclear Overhauser effects. Data were sampled over a 62,500-Hz spectral width using a 0.5-s acquisition time and 0.3-s relaxation time. The ^{113}Cd chemical shifts are given in parts per million relative to external standard 0.1 M $Cd(ClO_4)_2$. The samples were prepared in 15 mM potassium phosphate/sodium phosphate pH 7.3, containing 10% $^{2}H_2O$ for the field-frequency lock, and were measured in 5-mm NMR tubes. The average protein concentration for ^{113}Cd NMR was 2.5–3 mM. The gel filtration column (Superdex 75 10/300 GL, Amersham, Sweden) was used to analyze for a protein oligomerization after NMR measurements.

Results

Characterization of platinated MT-3 species formed with cisplatin and transplatin

In vivo studies have shown that the administration of cisplatin or transplatin to rabbits results in the formation of $Pt_{\sim 2}Zn_{\sim 5}$MT species in liver and kidneys [33]. Therefore, in our studies the binding of 2 mol equiv of cisplatin or transplatin to Zn_7MT-3 was investigated. Owing to the low intracellular Cl^- concentration (4–23 mM), the *cis*-$[Pt(NH_3)_2Cl_2]$ complex will hydrolyze after entering the cell to form *cis*-$[Pt(NH_3)_2(Cl)(X)]^{a+}$/*trans*-$[Pt(NH_3)_2(Cl)(X)]^{a+}$ (X is OH^-, H_2O; $a = 0, 1$) and, to a smaller extent, *cis*–$[Pt(NH_3)_2X_2]^{a+}$/*trans*-$[Pt(NH_3)_2X_2]^{a+}$ (X is OH^-, H_2O; $a = 0, 1, 2$) species [41, 42]. To mimic this situation, cisplatin and transplatin were dissolved in H_2O and allowed to hydrolyze for at least 1 day at ambient temperature [18]. The exchange of the leaving ligands is essential for the reaction of Pt(II) compounds with DNA. However, no influence of the aquation/anation degree on the reactivity of Pt(II) compounds toward MTs and other thiols such as GSH or cysteine was observed previously [43–45]. To characterize the final products of the reaction of human Zn_7MT-3 with cisplatin and transplatin, the previously developed ESI-MS method was used [18]. The protein was incubated with both Pt(II) compounds for 120 h in 10 mM HEPES/NaOH, pH 7.4, 100 mM $NaClO_4$, at 37 °C. These buffer conditions were used in all kinetic studies. To exclude cysteine oxidation by air oxygen during the prolonged sample incubation, experiments were performed under anaerobic conditions. After incubation, the protein was transferred into 10 mM 4-ethylmorpholine/HOAc, pH ~ 7 buffer and analyzed by ESI-MS (Fig. 2). Similar ESI-MS spectra, showing the same pattern of masses with substantially lower intensity, were also obtained after 5-h sample incubation (data not shown). This observation is in good agreement with our previous time-dependent ESI-MS studies of the interaction of Pt(II) complexes with human Zn_7MT-2 [18].

Since the sum of the molecular masses of three zinc atoms (196.11 g mol^{-1}) is, considering the accuracy of the method, very close to the molecular mass of one platinum atom (195.09 g mol^{-1}), the mass spectra were simultaneously recorded also at pH ~ 3 (Fig. 2a, b). Under these conditions Zn(II), but not Pt(II) is released from the protein. The deconvoluted ESI-MS spectrum of PtMT-3 complexes with cisplatin obtained at pH ~ 3 reveals mass peaks of 7,118.6, 7,311.8, and 7,505.0 Da, which we assigned to the MT-3 species containing one, two and three Pt(II) bound to the protein, respectively (Fig. 2a). The ESI-MS spectrum of the protein species formed with transplatin shows mass peaks of 7,152.8, 7,380.5, and 7,607.0 Da. The increased mass differences of $\Delta m = 34.2$, 68.7, and 98.0 Da for one, two, and three Pt(II) bound are interpreted as being due to the presence of two NH_3 ligand per Pt(II), i.e., $[Pt(NH_3)_2]$MT-3, $[Pt(NH_3)_2]_2$MT-3, and $[Pt(NH_3)_2]_3$MT-3 (Fig. 2b). Thus, transplatin preserves its ammine ligands in complex with MT-3. A comparison of the relative intensities of the mass peaks at low pH reveals that the PtMT-3 complexes containing one and two Pt(II) are mainly formed (Table 1).

The ESI-MS spectra obtained at pH ~ 7 were more heterogeneous (Fig. 2c, d). In addition to the species of the Pt_xZn_{7-x}MT-3 ($x = 1 - 3$) type, a minor population of Pt_xZn_{8-x}MT-3 ($x = 1 - 4$) complexes was also formed (Table 1). The formation of similar complexes with cisplatin and transplatin was also seen in our previous studies of Zn_7MT-2 [18].

Kinetics of cisplatin and transplatin binding to thiols of Zn_7MT-3

The reactivity of cisplatin and transplatin toward the cysteines of Zn_7MT-3 was also investigated. As the platinum-to-MT ratio of 2 was applied in our studies, the pseudo-first-order conditions cannot be fulfilled. Therefore, to allow the comparison of the apparent initial rates of the reactions involving cisplatin and transplatin, the experimental conditions were kept identical. The formation of Pt–S bonds in MT-3 with time was followed by absorption spectroscopy for 120 h to allow almost complete reaction. The results obtained were normalized per Pt–S bond using the extinction coefficient of 2,680 $M^{-1}\ cm^{-1}$ at 285 nm [18].

The kinetic traces of the reaction of Zn_7MT-3 with 2 mol equiv of cisplatin or transplatin show a biphasic

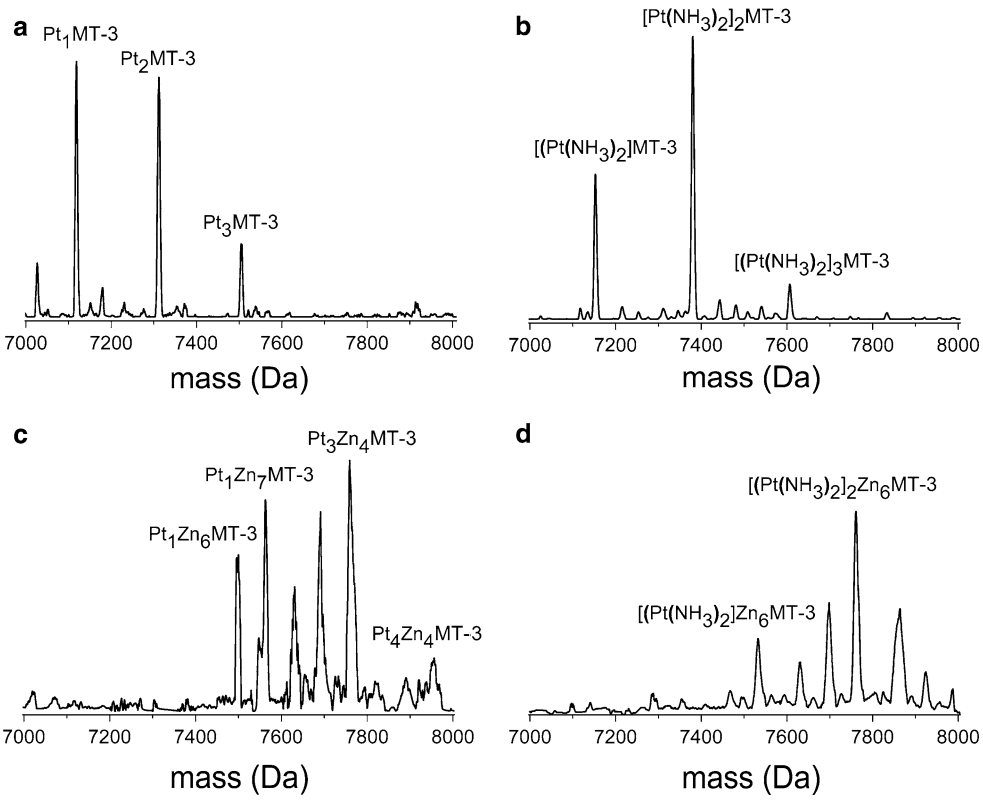

Fig. 2 Deconvoluted electrospray ionization mass spectrometry spectra of Zn_7MT-3 upon the reaction with 2 mol equiv of **a**, **c** cisplatin or **b**, **d** transplatin in 10 mM *N*-(2-hydroxyethyl)piperazine-*N*′-ethanesulfonic acid (HEPES)/NaOH pH 7.4, 100 mM $NaClO_4$ for 120 h at 37 °C. The masses of the observed species are given in Table 1. Prior to analysis, samples were rapidly diluted in **a**, **b** 50:50:0.2 (v/v/v) CH_3CN/H_2O/HOAc (final pH ∼ 3) and in **c**, **d** 50:50 (v/v) CH_3CN/H_2O (final pH ∼ 7)

Table 1 Nano electrospray ionization mass spectrometry characterization of the products of the reaction between human Zn_7MT-3 and 2 mol equiv of cisplatin or transplatin at pH ∼ 3 and pH ∼ 7

Pt(II) compound	pH ∼ 3[a]			pH ∼ 7[b]		
	m_{exp}[c] (Da)	m_{calc}[d] (Da)	Cation(s) in complex with MT-3	m_{ep}[c] (Da)	m_{calc}[d] (Da)	Cation(s) in complex with MT-3
Cisplatin	6,926.3[e]	6,927.0	–	7,369.7[f]	7,370.8	Zn_7
	7,118.6	7,120.0	Pt	7,498.4	7,500.4	Pt Zn_6
				7,563.2	7,565.8	$PtZn_7$
	7,311.8	7,313.0	Pt_2	7,629.2	7,630.0	Pt_2Zn_5
				7,692.1	7,695.4	Pt_2Zn_6
	7,505.0	7,506.0	Pt_3	7,758.5	7,759.6	Pt_3Zn_4
				7,952.0	7,954.6	Pt_4Zn_4
Transplatin	6,926.0[e]	6,927.0	–	7,370.0[f]	7,370.8	Zn_7
	7,152.8	7,154.1	$[Pt(NH_3)_2]$	7,532.9	7,534.5	$[Pt(NH_3)_2]Zn_6$
	7,380.5	7,381.2	$[Pt(NH_3)_2]_2$	7,694.9	7,698.2	$[Pt(NH_3)_2]_2Zn_5$
				7,761.5	7,763.6	$[Pt(NH_3)_2]_2Zn_6$
	7,607.0	7,608.3	$[Pt(NH_3)_2]_3$	7,864.1	7,861.9	$[Pt(NH_3)_2]_3Zn_4$

The experimental mass differences of up to 4 Da originate from an unpredictable protonation state of the protein at pH ∼ 7

MT-3 metallothionein-3

[a] Samples were diluted in 50:50:0.2 CH_3CN/H_2O/HOAc prior to analysis

[b] Samples were diluted in 50:50 CH_3CN/H_2O prior to analysis

[c] Mass peaks obtained in nano electrospray ionization mass spectrometry experiments

[d] Calculated mass for the complexes of MT-3 with the cations indicated

[e] Mass obtained for apo-MT-3 in the control experiment; calculated: 6,927.0 Da

[f] Mass obtained for Zn_7MT-3 in the control experiment; calculated: 7,370.8 Da

profile characterized by a fast initial phase followed by a slow second phase (Fig. 3a). The atomic absorption data revealed that the final products of the reaction were complexes with an overall stoichiometry of Pt_2Zn_5MT-3. In these species up to approximately seven Pt–S bonds were formed with cisplatin and approximately 3.5 Pt–S bonds with transplatin. The higher number of Pt–S bonds formed with cisplatin suggests that both ammine ligands were replaced, resulting in a $Pt(CysS)_4$ coordination. In contrast, the presence of nearly 3.5 Pt–S bonds with transplatin suggests that a $Pt(NH_3)_2(CysS)_2$ species is formed. These results are in agreement with the ESI-MS data presented above. From the initial slopes of the kinetic traces, the apparent initial rates k_{obs} were derived and compared (Fig. 3b). The value of 22.6×10^{-4} min^{-1} obtained for the reaction of MT-3 with transplatin is higher than that determined for cisplatin ($k_{obs} = 20.2 \times 10^{-4}$ min^{-1}). Thus, the reaction of both Pt(II) compounds with Zn_7MT-3 is about twice as fast as with Zn_7MT-2 [18].

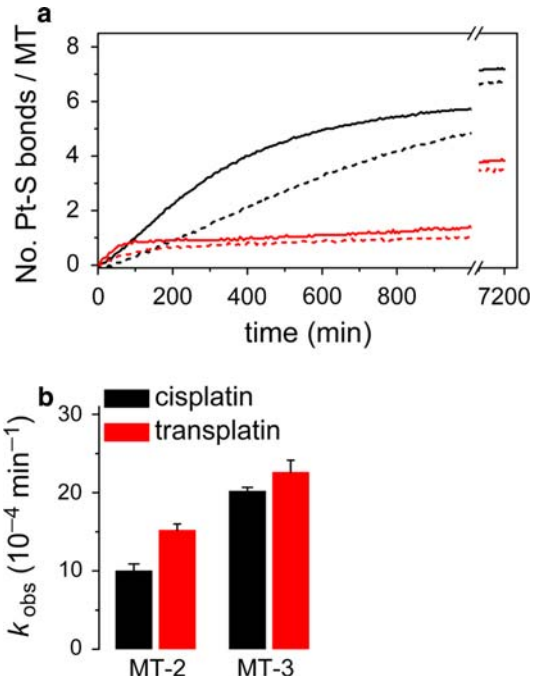

Fig. 3 **a** Kinetics of the binding of 20 μM cisplatin (*black lines*) and transplatin (*red lines*) to cysteine thiolates of 10 μM human Zn_7MT-3 (*solid lines*) and Zn_7MT-2 (*dotted lines*) in 10 mM HEPES/NaOH pH 7.4, 100 mM $NaClO_4$ at 37 °C. Complex formation with time was monitored through absorption changes at 285 nm. The absorption traces were normalized to the number of Pt–S bonds ($\varepsilon_{285\ nm} = 2{,}680$ M^{-1} cm^{-1}) [18]. **b** Comparison of the initial apparent reaction velocities k_{obs} of the cisplatin or transplatin binding to Zn_7MT-2 and Zn_7MT-3. The experiments were performed in triplicate

Kinetics of cisplatin and transplatin binding to Zn_7MT-3

To investigate the rates of cisplatin and transplatin reaction with Zn_7MT-3 and, for comparison also with Zn_7MT-2, we determined free Pt(II) concentrations in the reaction mixture as a function of time under identical experimental conditions. The proteins were anaerobically incubated with 2 mol equiv of Pt(II) compounds in 10 mM HEPES/NaOH pH 7.4, 100 mM $NaClO_4$ at 37 °C for up to 120 h. At different time intervals, aliquots of the incubation mixture were applied to Microcon YM-3 concentrators (molecular mass cutoff 3 kDa) and the low molecular mass components were separated. The concentration of free Pt(II) present in the filtrate was determined by atomic absorption. The amount of cisplatin and transplatin bound to Zn_7MT-2/Zn_7MT-3 was obtained by subtracting the free Pt(II) concentration from the initial Pt(II) concentration. These values, normalized per protein concentration, are shown in Fig. 4.

The kinetics presented in Fig. 4 show a biphasic profile of Pt(II) binding to Zn_7MT-3. A comparison of the rapid initial kinetic phases of the reaction showed that transplatin reacts with Zn_7MT-3 about 1.6 times faster than cisplatin. After 5-h incubation, a slow binding phase sets in with only minor concentrations of free Pt(II) compounds after 120-h incubation. Specifically, from the 2 mol equiv of cisplatin and transplatin initially added to Zn_7MT-3 about 1.98 mol equiv was bound to the protein. Compared with Zn_7MT-3, Zn_7MT-2 reacts with both cisplatin and transplatin on average about 1.5 times slower during the first 5 h of reaction (Fig. 4).

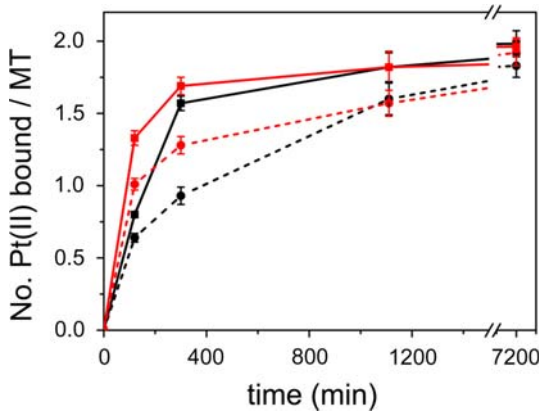

Fig. 4 Binding of 20 μM cisplatin (*black lines*) or transplatin (*red lines*) to 10 μM Zn_7MT-3 (*solid lines*) or Zn_7MT-2 (*dotted lines*) in 10 mM HEPES (pH 7.4), 100 mM $NaClO_4$, 37 °C. The concentration of bound Pt(II) was obtained by subtracting the free Pt(II) concentration from the initial Pt(II) concentration added to the protein. Values were normalized to the number of Pt(II) bound to MT-2/MT-3

Kinetics of Zn(II) release from Zn_7MT-3 upon cisplatin and transplatin binding

The release of Zn(II) upon Pt(II) binding to Zn_7MTs is a direct consequence of the competition between these metals for cysteine thiolates. The kinetics of Zn(II) release was examined using the conditions described for the reaction of cisplatin and transplatin with cysteine thiolates and in the presence of the Zn(II)-chelating dye zincon. The 1:1 Zn(II)–zincon complex formed, which is characterized by an absorption maximum at 620 nm ($\varepsilon_{620\ nm} = 23{,}200\ M^{-1}\ cm^{-1}$) [40, 46], was used to monitor Zn(II) release. Prior to these experiments, the absence of a complex between zincon and Pt(II) was established. The results show that around 1.8 mol equiv of Zn(II) was released from Zn_7MT-3 after 120-h incubation with 2 mol equiv of cisplatin and transplatin (Fig. 5a). This indicates that Pt(II) binding leads to displacement of the corresponding number of mole equivalents of Zn(II) from the protein. As judged from the initial slopes of the fast reaction phase, transplatin with $k_{obs}^{Zn(II)}$ of $54.3 \times 10^{-4}\ min^{-1}$ releases Zn(II) from Zn_7MT-3 more rapidly than cisplatin ($k_{obs}^{Zn(II)}$ of $32.2 \times 10^{-4}\ min^{-1}$).

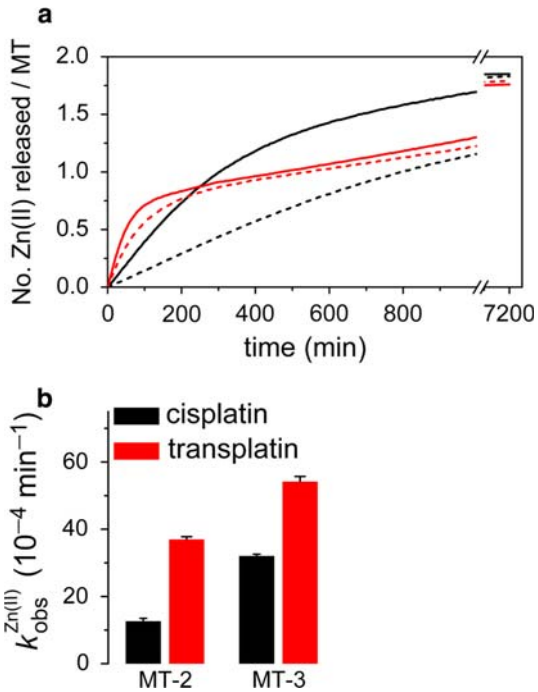

Fig. 5 **a** Time-dependent release of Zn(II) from 10 μM Zn_7MT-3 (*solid lines*) and Zn_7MT-2 (*dotted lines*) in 10 mM HEPES/NaOH pH 7.4, 100 mM $NaClO_4$, 100 μM zincon at 37 °C upon reaction with 20 μM cisplatin (*black lines*) or transplatin (*red lines*). The absorption change with time was followed was followed at 620 nm. Values were normalized to the number of Zn(II) released per MT. **b** Comparison of the initial apparent reaction velocities $k_{obs}^{Zn(II)}$ of Zn(II) release from human Zn_7MT-2, and Zn_7MT-3 upon reaction with cisplatin and transplatin. The experiments were performed in triplicate

Moreover, the apparent rates of release of Zn(II) from Zn_7MT-3 are about twice as fast as those from Zn_7MT-2 (Fig. 5b). A comparison of k_{obs} for the Pt–S bond formation (Fig. 3b) and Zn(II) release (Fig. 5b) reveals that the Zn(II) release from Zn_7MT-3 and Zn_7MT-2 is roughly twice as fast. This likely reflects the strong labilization of Zn(II) in the cluster structure induced by the breaking of even a single Zn–S bond. Although in all kinetic studies a simple hyperbolic dependence was obtained, the reactions are more complex and follow higher-order kinetics. Evidence comes from the ESI-MS data in which the formation of different products was seen (Fig. 2).

^{113}Cd NMR characterization of $Pt_1{}^{113}Cd_6$MT-3

To reveal whether the α-domain or the β-domain of MT-3 is preferentially reacting with cisplatin, we applied ^{113}Cd NMR spectroscopy. Previously, a close similarity between the structures of Zn(II)- and Cd(II)-containing metalloforms of M_7MT-2 was demonstrated [7]. Prior to NMR measurements, the additional ESI-MS experiments established that in the reaction of Cd_7MT-3 with 1 mol equiv of cisplatin heterogeneous Pt_xCd_{7-x}MT-3 ($x = 1 - 3$) complexes lacking ammine ligands, seen also with Pt,ZnMT-3, were formed (data not shown). For ^{113}Cd NMR measurements $^{113}Cd_7$MT-3 was incubated with 1 mol equiv of cisplatin in 10 mM HEPES/NaOH pH 7.4, 100 mM $NaClO_4$ for 120 h. The $Pt_1{}^{113}Cd_6$MT-3 sample was then concentrated to about 3 mM and brought into 15 mM potassium phosphate/sodium phosphate buffer pH 7.3. The ^{113}Cd NMR spectra of $Pt_1{}^{113}Cd_6$MT-3 and $^{113}Cd_7$MT-3 are shown in Fig. 6.

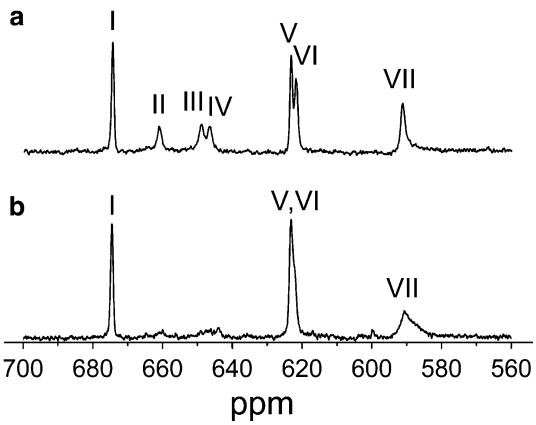

Fig. 6 ^{113}C NMR (110.9 MHz) spectra of **a** 2.7 mM $^{113}Cd_7$MT-3 and **b** 3 mM $Pt_1{}^{113}Cd_6$MT-3 in 15 mM potassium phosphate/sodium phosphate buffer pH 7.3 at 50 °C. *I*, *V*, *VI*, and *VII* correspond to the resonances of the four-metal cluster in the α-domain, and *II*, *III*, and *IV* correspond to the resonances of the three-metal cluster in the β-domain [47, 51]

The ^{113}Cd NMR spectrum of $^{113}Cd_7$MT-3 presented in Fig. 6a compares well with that published. As previously shown, the strong ^{113}Cd resonances I, V, VI, and VII originate from the Cd_4 cluster and the weak resonances II, III, and IV originate from the Cd_3 cluster [47, 48]. The characteristic features of this spectrum are a large apparent line width of all resonances (150–350 Hz), the absence of homonuclear ^{113}Cd–^{113}Cd couplings, and a markedly reduced and temperature-independent intensity (20%) of the resonances of the Cd_3 cluster. These features have been interpreted in terms of dynamic processes acting on two different NMR time scales: fast exchange processes among conformational cluster substates occurring in both clusters and additional slow exchange processes among configurational cluster substates in the Cd_3 cluster [48]. The latter exchange processes allowed only the structure of the α-domain of $^{113}Cd_7$MT-3 to be determined by NMR [47].

In the spectrum of $Pt_1{}^{113}Cd_6$MT-3, the three-metal-cluster resonances are virtually absent (Fig. 6). The perturbation of the four-metal-cluster resonances by Pt(II) binding was only minor. This was characterized by a broadening of the ^{113}Cd resonance VII and a small low-field shift of the ^{113}Cd resonance VI from 622 to 623 ppm, resulting in its overlap with resonance V (Table 2).

This suggests that Pt(II) is preferentially bound to the more dynamic three-metal cluster. Furthermore, no other ^{113}Cd resonances were detected between 900 and 350 ppm. The monomeric nature of the $Pt_1{}^{113}Cd_6$MT-3 sample after NMR measurements (72 h) was confirmed by analytical gel filtration in which the presence of about 10–15% of dimers was detected.

Table 2 Effect of $^{113}Cd_7$MT-3 platination on the chemical shifts and the relative intensities of ^{113}Cd resonances in $Pt_1{}^{113}Cd_6$MT-3

Peak	$^{113}Cd_7$MT-3		$Pt_1{}^{113}Cd_6$MT-3	
	Chemical shift (ppm)	Relative intensity	Chemical shift (ppm)	Relative intensity
I	675	1[a]	675	1[a]
II	661	0.49	661	–
III	649	0.51	649	–
IV	646	0.49	646	–
V	623	0.91	623	1.93
VI	621	0.86		
VII	591	0.93	590	0.97

The 110.9-MHz ^{113}Cd NMR spectra were recorded in 15 mM potassium phosphate/sodium phosphate buffer, pH 7.3 at 50 °C

The numbers are relative to the integrated intensity of peak I

Discussion

The present study provides insights into the reaction of human Zn_7MT-3 with cisplatin and transplatin and the products formed. The fate of the Pt(II) complexes in the cells is kinetically controlled and will depend on the accessibility of cysteine thiolates for electrophilic attack. In mammalian MTs both metal–thiolate clusters are buried in the protein structure. When compared with the structures of the Zn_7MT-1/Zn_7MT–2 isoforms, the structure of Zn_7MT-3 is more flexible and both metal–thiolate clusters show reduced metal affinity. These structural properties are responsible for the higher reactivity of nucleophilic sulfhydryl groups in Zn_7MT-3 with electrophiles such as 5,5′-dithiobis(2-nitrobenzoic acid) [31]. In this regard, the three-metal cluster in the β-domain has been shown to be more reactive than the four-metal cluster in the α-domain. In our ^{113}Cd NMR studies of $Pt_1{}^{113}Cd_6$MT-3, a similar preferential reactivity of the Cd_3 cluster with cisplatin was also seen. The virtual absence of the ^{113}Cd resonances of the Pt(II)-modified Cd_3 cluster in the β-domain presumably reflects a low population of $(Pt,Cd)_3$ complexes with varying Pt(II) distributions. This conclusion is supported by ESI-MS analysis of the product of the reaction of Cd_7MT-3 with 1 mol equiv of cisplatin, showing the formation of complexes with platinum-to-MT ratios between 1 and 3. The results obtained differ from the results of the similar ^{111}Cd NMR studies of rabbit $^{111}Cd_7$MT-2 where a cooperative cisplatin binding to the three-metal cluster was reported [49]. A number of studies showed that both protein domains in MT-3 interact with each other [50, 51]. Thus, a small perturbation of the Cd_4-cluster resonances, including a broadening of the cadmium VII resonance and a small low-field shift of the cadmium VI resonance, most likely originates from the changes in the cross-talk between both protein domains brought about by Pt(II) binding to the β-domain.

The characterization of the final products of the reaction of Zn_7MT-3 with 2 mol equiv of cisplatin or transplatin resulted in a species with overall stoichiometry of Pt_2Zn_5MT-3. In these species an average of seven Pt–S bonds were formed with cisplatin and 3.5 Pt–S bonds with transplatin. These results and the ESI-MS data indicate that whereas in the reaction of Zn_7MT-3 with cisplatin both carrier and leaving ligands are lost, the carrier NH_3 ligands in transplatin were retained. The *trans*-effect labilization of the carrier NH_3 ligands in cisplatin *trans* to CysS accounts for the generation of $Pt(II)(SCys)_4$ complexes in Pt_2Zn_5MT-3. The kinetic profiles of cisplatin and transplatin binding to Zn_7MT-3 reveal that about 1.7 mol equiv of both Pt(II) compounds is bound to MT-3 already after 5 h. In marked contrast, at the same time point only about half of the total Pt–S bonds developed and 1 mol equiv of

Zn(II) was released. These results suggest that the binding of Pt(II) complexes to the three-metal cluster in the β-domain is a complex process, involving at least two subsequent kinetic phases. This process could be interpreted as initial binding of Pt(II) complexes to a reduced number of thiolate groups (one or two). The subsequent saturation of Pt(II) coordination sites by additional thiolate ligands would then introduce substantial changes in the domain structure accompanied by the release of the second mole equivalent of Zn(II). Furthermore, as MT-3 contains 11 carboxylate donor ligands, representing a kinetically attractive first protein coordinating site for cisplatin and transplatin at pH 7.4, their possible involvement in the initial binding step cannot be ruled out. In recent studies of the binding of cisplatin to cellular DNA-binding proteins similar platinum coordination sites have been identified [52].

While the expression of the MT-1/MT-2 isoforms is induced by toxic Cd(II) and Pt(II), the MT-3 expression is unresponsive to these inducers. The exposure to these metal ions results in their scavenging in the cytosol by Zn_7MTs and Zn(II) release. As the expression of MT-1/MT-2 is regulated by zinc responsive transcription factor 1 (MTF-1), the released Zn(II), through activation of this factor, leads to overexpression of MT-1/MT-2 [53, 54]. Activated MTF-1 also regulates the expression of other proteins such as γ-glutamylcysteine synthetase, zinc transporter 1, and α-fetoprotein. The enzyme γ-glutamylcysteine synthetase is involved in the biosynthesis of GSH, another sulfur-containing compound involved in the Pt(II) resistance. As MTF-1 is not involved in the regulation of MT-3 expression, the induction of this MT isoform by free Zn(II) will not take place [55]. However, in recent studies the *MT-3* gene was identified as hypoxia-inducible in several human tissues [56–58]. Therefore, we suggest that overexpression of MT-3 observed in several cancers is a result of the hypoxic conditions, due to insufficient vascularization of the fast-proliferating tissue.

The MT-3 isoform has an increasing significance as a diagnostic marker of bladder [28] and esophageal [27] cancers and its presence in these and several other cancers tightly correlates with the poor chemotherapy outcome [25, 26, 59]. The degree of MT-based acquired resistance to Pt(II) drugs may depend on the specific expression pattern of MT isoforms. The data presented suggest that in frequent situations of the overexpression of several MT isoforms in the cancer tissues [17], the reaction of administered Pt(II) drugs with MT-3 would be kinetically preferred. The released Zn(II) in this process and its binding to MTF-1 would then result in the upregulation of the MT-1/MT-2 expression.

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