Reaction of Zn_7Metallothionein with *cis*- and *trans*-[Pt(N-donor)$_2$Cl$_2$] Anticancer Complexes: *trans*-Pt^{II} Complexes Retain Their N-Donor Ligands

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Intrinsic and acquired resistance are major drawbacks of platinum-based cancer therapy. The protein superfamily of cysteine- and Zn^{II}-rich proteins, metallothioneins (MT), efficiently inactivate these antitumor drugs because of the strong reactivity of platinum compounds with S-donor molecules. In this study the reactions of human Zn_7MT-2 with twelve *cis*/*trans*-[Pt(N-donor)$_2$Cl$_2$] compounds and [Pt(dien)Cl]Cl, including new generation drugs, were investigated and the products characterized. A comparison of reaction kinetics revealed that *trans*-Pt^{II} compounds react faster with Zn_7MT-2 than *cis*-Pt^{II} compounds. The characterization of the products showed that while all ligands in *cis*-Pt^{II} compounds were replaced by cysteine thiolates, *trans*-Pt^{II} compounds retained their N-donor ligands, thus remaining in a potentially active form. These results provide an increased understanding of the role of MT in the acquired resistance to platinum-based anticancer drugs.

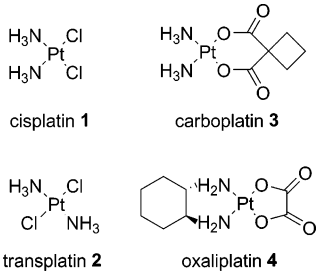
Introduction

Platinum coordination complexes are effective chemotherapeutic agents for the treatment of testicular cancer and are used in combination therapy for a variety of other tumors including ovarian, cervical, bladder, lung, and those of the head and neck.[1] Since the discovery of the antitumor efficiency of *cis*-[Pt(NH$_3$)$_2$Cl$_2$] **1** (cisplatin) almost 40 years ago, only a few platinum antitumor drugs such as *cis*-diammine(1,1-cyclobutanedicarboxylato)platinum(II) **3** (carboplatin) and (*R*,*R*)-1,2-diaminocyclohexaneoxalatoplatinum(II) **4** (oxaliplatin) have been introduced in the clinic (Chart 1).[1] These classical Pt^{II} antitumor drugs act mainly through binding to guanine (G)[a] bases of nuclear DNA causing its modification which directs a cell into apoptosis or necrosis.[1−3] Studies of the mechanism of the antitumor effects of these compounds have revealed that, in particular, 1,2-intrastrand d(GpG) cross-links, through cisplatin coordination to two adjacent *N*7-G, distort the DNA structure such that translation and excision repair are strongly inhibited.[2,4,5]

The occurrence of intrinsic resistance in some tumors and resistance acquired after initial treatment are the major drawbacks of these chemotherapeutics. Potential responses leading to the resistance include changes in intracellular accumulation of the drug, increased production of intracellular thiols to prevent toxicity, increased capability of cells to repair cisplatin−DNA damage,[6] and a failure to initiate apoptosis in the presence of platinated DNA.[7] Because of the strong reactivity of platinum compounds toward S-donor molecules and the formation of very stable Pt^{II}−S bonds, intracellular thiols, through their competition with DNA, confer resistance to antitumor platinum drugs.[1,8−11] The major intracellular thiols involved in the drug resistance are glutathione (GSH) and metallothionein (MT).[11,12] From the four MTs expressed in humans (designated MT-1 through MT-4), MT-1 and MT-2 occur ubiquitously in high amounts in mammalian cells. In contrast to MT-3/-4, the biosynthesis of MT-1/-2 is inducible by a variety of compounds including hormones, cytokines, and metal ions.[13] Both inducible MTs (MT-1/-2) play a role in resistance to antitumor platinum drugs. Although GSH deactivates **1** and in certain cancer cell lines its cellular concentration increases upon exposure to Pt^{II}-drugs,[12] MT thiols react faster with **1** compared to GSH.[14] Moreover, in cancer cells, basal MT levels are often significantly increased resulting in an even stronger Pt^{II} scavenging effect.[12,15,16] In addition, repeated administration of **1** to tumor-infected mice results in an ∼ 50-fold increase of MT expression.[16,17] Likewise, MT levels were also found to be significantly increased in patients not responding to cisplatin **1** and carboplatin **3** treatment.[18] Overall, these results suggest a significant contribution of MT to acquired Pt^{II}-drug resistance.

The extensively studied mammalian MTs (MT-1/-2) are low molecular mass Cys- and Zn^{II}-rich proteins composed of a single

Chart 1. Structures of Classical Antitumor Platinum Drugs[a]



[a] Transplatin **2** is the clinically ineffective isomer of cisplatin **1**.

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[a] Abbreviations: DACH, (*R*,*R*)-1,2-diaminecyclohexane; dien, diethylentriamine; G, guanine; GSH, glutathione; HEPES, 4-(2-hydroxyethyl)-piperazine-1-ethanesulfonic acid; L, N-donor ligand (carrier ligand); MT, metallothionein; MTF-1, metal-responsive transcription factor 1; ESI, electrospray ionization; PAR, 4-(2-pyridylazo)resorcinol; TOF, time-of-flight; X, leaving ligand (Cl$^-$/OH$^-$/H$_2$O).

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polypeptide chain of 61−62 amino acids, out of which 20 are Cys. The Cys thiolates are involved in the binding of seven divalent metal ions (Zn^{II}) forming two independent metal−thiolate clusters in which each metal is tetrahedrally coordinated by both bridging and terminal thiolate ligands.[19,20] Despite the high thermodynamic stability of these metal−thiolate complexes, they are kinetically very labile, i.e., the thiolate ligands allow both rapid metalation and demetalation. The displacement of Zn^{II} in the Zn_7MT structure by Pt^{II} *in vitro* and *in vivo* has been reported.[21−23]

After the initial discovery of **1** as an anticancer agent, its geometrical isomer *trans*-$[Pt(NH_3)_2Cl_2]$ **2** (transplatin) (Chart 1) was found to be inactive in cancer cells.[1] The transplatin−DNA adducts are much less effective arrest sites for DNA and RNA polymerases than those of **1**.[5] This was presumed to account for its inability to induce apoptosis, which led to the hypothesis that *trans*-Pt^{II} complexes may not be considered as anticancer drugs.[24] However, recent reports showed that certain new generation *trans*-Pt^{II} compounds are active *in vitro* and *in vivo*.[25−27] Moreover, on the basis of experiments with cisplatin-resistant human cancer cells treated with new generation *trans*-compounds such as *trans*-bis((*E*)-1-imino-1-methoxyethane)-dichloridoplatinum(II) **6** (*transEE*), it has been suggested that these compounds are able to overcome resistance to **1**.[27] In addition, new *cis*-Pt^{II} complexes with promising anticancer activities have been developed.[28,29] Taken together, the discovery of acquired resistance accompanying Pt^{II} treatment has markedly increased interest in molecular mechanisms of the interaction of Pt^{II}-drugs with sulfur-containing molecules of biological significance.

In this study, reactions between human Zn_7MT-2 and twelve *cis*- and *trans*-$[PtL^1L^2Cl_2]$ (L = N-donor ligand) compounds **1**−**3**, **5**−**13**, and monofunctional [Pt(dien)Cl]Cl (dien = diethylentriamine), including a number of new generation Pt^{II} drugs with potential clinical use, were investigated. The studies were conducted under native-like conditions regarding the pH, ionic strength, and temperature, and at a Pt/MT ratio of 2:1 relevant to *in vivo* studies.[21] The results are discussed in relation to available cell biological studies.

Results

Characterization of Platinated MT-2 Species. Incubations of recombinant human Zn_7MT-2 with Pt^{II}-compounds were carried out at pH, temperature, and ionic strength values comparable to physiological conditions. Because of the low intracellular Cl^- concentration (4−23 mM),[30] $[PtL^1L^2Cl_2]$ complexes upon entering the cell can hydrolyze to $[PtL^1L^2(Cl)(X)]^{a+}$ (X = OH^-, H_2O; a = 0, 1) and further to $[PtL^1L^2X_2]^{a+}$ (X = OH^-, H_2O; a = 0, 1, 2).[1] To mimic this situation, Pt^{II}-compounds were dissolved in H_2O at low concentrations (0.5 mM) and allowed to hydrolyze for at least 1 day at ambient temperature. The exchange of the leaving ligand is an important step to activate the compounds for their reactions with *N*7-G.[31] However, in the previous studies the reaction kinetics of rat and rabbit Zn_7MT-2 with **1** *versus* *cis*-$[Pt(NH_3)_2X_2]^{a+}$ (X = OH^-, H_2O; a = 0, 1, 2) or **2** *versus* *trans*-$[Pt(NH_3)_2X_2]^{a+}$ (X = OH^-, H_2O; a = 0, 1, 2) were found to be largely independent of the leaving ligand.[22,32] This finding is in agreement with the only marginally altered reactivity of the low-molecular weight S-donor compounds cysteine and GSH toward **1**, **2**, and $[Pt(dien)Cl]^+$ upon replacement of Cl^- by H_2O/OH^-.[31,33] Therefore, the degree of hydrolysis of the studied Pt^{II} complexes should only marginally influence their reactivity with Cys ligands of Zn_7MT-2. To mimic the physiological ionic strength and pH, experiments were carried out in the presence of 100 mM ClO_4^-, which does not coordinate Pt^{II}, and 10 mM HEPES/NaOH, pH 7.4.

Prior to characterization of the various MT-2 metalloforms by nanoESI-MS, a volatile buffer system (pH 7.0) was established using Zn_7MT-2. To avoid secondary reactions with Pt^{II} centers during the sample analysis, buffers free of NH_3 and primary and secondary amines were tested. A small desalting column was used to transfer a sample of Zn_7MT-2 into 10 mM 4-methylmorpholine/HCOOH, 4-methylmorpholine/HOAc, 4-ethylmorpholine/HCOOH, or 4-ethylmorpholine/HOAc, all at pH 7.0. Prior to nanoESI-MS analysis, samples were rapidly diluted into either 50:50 CH_3CN/H_2O or 50:50 CH_3OH/H_2O to maintain the pH at ∼ 7 and immediately injected. It turned out that in all buffer systems tested Zn_7MT-2 was the only species detected. However, 4-ethylmorpholine/HOAc and the subsequent dilution into 50:50 CH_3CN/H_2O was finally chosen for further analysis because it resulted in the best signal-to-noise ratio.

It has been reported that $K_2[PtCl_4]$, **1**, or **2** administration to rabbits yielded the species $Pt_{\sim 2}Zn_{\sim 5}$MT.[21] Therefore, to mimic the metal composition *in vivo*, in all our experiments 2 mole equivalents of Pt^{II} complexes were added to Zn_7MT-2. In the first experiments the protein was incubated with 2 mole equivalents of **1** or **2** in 10 mM HEPES/NaOH, pH 7.4, 100 mM $NaClO_4$ at 37 °C, and the products were characterized. To exclude Cys oxidation by air oxygen during the prolonged sample incubation, experiments were performed under anaerobic conditions. Subsequently, samples were transferred into 10 mM 4-ethylmorpholine/HOAc, pH 7.0, and analyzed by nanoESI-MS as described above. Since the molecular mass of 3 Zn (196.11 g mol^{-1}) is, considering the accuracy of the method, very close to the molecular mass of 1 Pt (195.09 g mol^{-1}), the mass spectra were simultaneously recorded also at pH ∼ 3. Under these conditions Zn^{II}, but not Pt^{II}, is released from the protein. Deconvoluted mass spectra are shown in Figure 1. The mass spectrum at pH ∼ 3, obtained upon the reaction with **1** resulted in four Pt^{II}-containing MT-2 species with mass peaks of 6233.2, 6427.1, 6619.5, and 6813.8 Da which correspond to $Pt_{(1-4)}$MT-2 complexes (Figure 1a). In comparison, the spectrum recorded at neutral pH reveals mass peaks of 6615.2, 6743.8, 6871.2, and 7068.0 Da, which correspond well to Zn_6PtMT-2, Zn_5Pt_2MT-2, Zn_4Pt_3MT-2, and Zn_4Pt_4MT-2, respectively (Figure 1b). In contrast, the mass spectrum of Zn_7MT-2 upon its reaction with **2**, obtained at pH ∼ 3, revealed three mass peaks of 6267.2, 6493.4, and 6717.6 Da (Figure 1c). These mass peaks correspond to MT-2 species containing one and two Pt^{II} bound with their molecular masses increased by Δm = 34.0, 66.3, and 98.1 Da, respectively. The mass differences are interpreted as being due to the presence of two NH_3 ligands per Pt^{II}, i.e., $[Pt(NH_3)_2]$MT-2, $[Pt(NH_3)_2]_2$MT-2, and $[Pt(NH_3)_2]_3$MT-2. The mass peaks of the reaction products with **2** obtained at neutral pH show similar mass differences corresponding to $Zn_5[Pt(NH_3)_2]_2$MT-2 and $Zn_5[Pt(NH_3)_2]_3$MT-2 (Figure 1d). Previously, on the basis of spectroscopic and kinetic measurements, it has been suggested that in the reaction of Zn_7MT-2 with **2** all Pt^{II} ligands including the NH_3 carrier ligands would be replaced.[22,32] However, the data presented here clearly demonstrate that both NH_3 ligands are retained.

To find out whether the retention of the N-donor ligands is restricted to **2** only, the studies were expanded to a number of other *cis*- and *trans*-Pt^{II} compounds depicted in Chart 2 and to [Pt(dien)Cl]Cl. The masses and the species formed are summarized in Table 1 for *cis*-$[PtL^1L^2Cl_2]$ and $[Pt(dien)Cl]^+$ and in Table 2 for *trans*-$[PtL^1L^2Cl_2]$. It may be noted that the

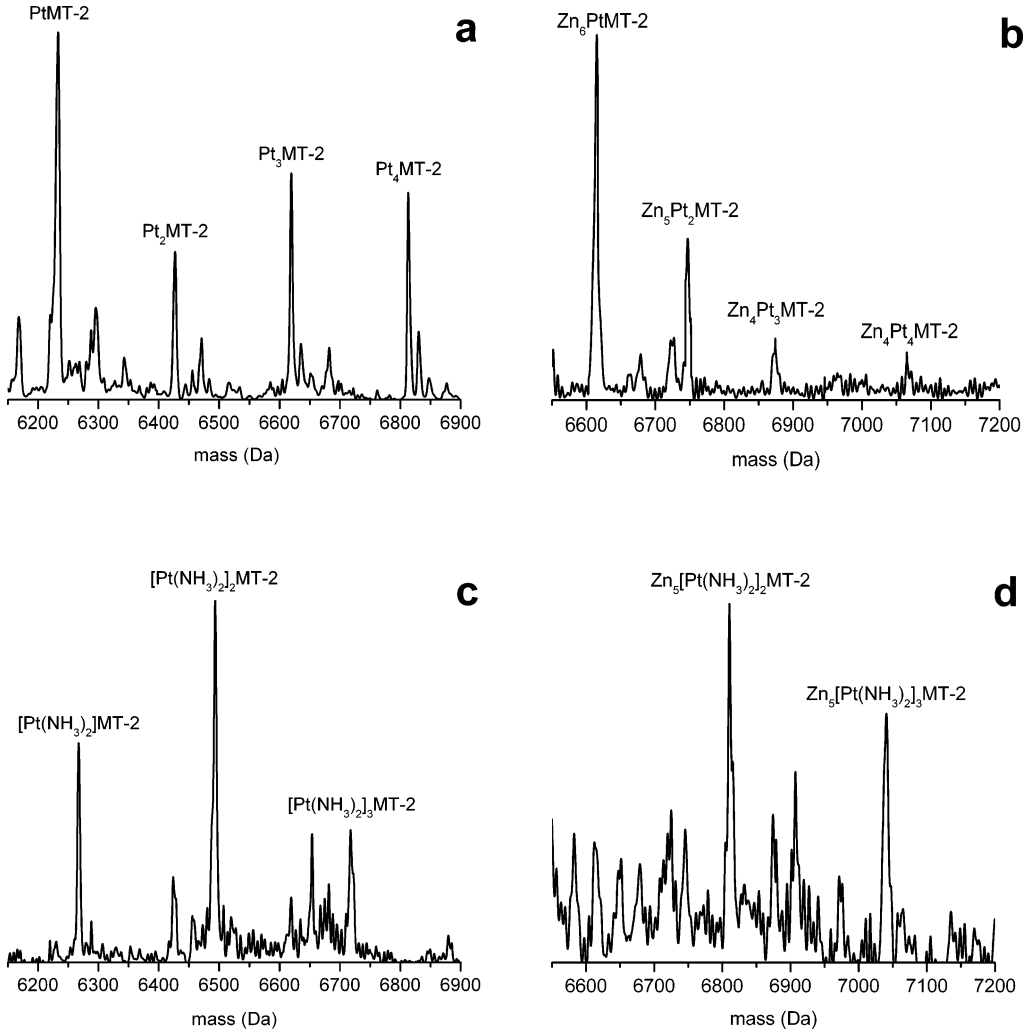
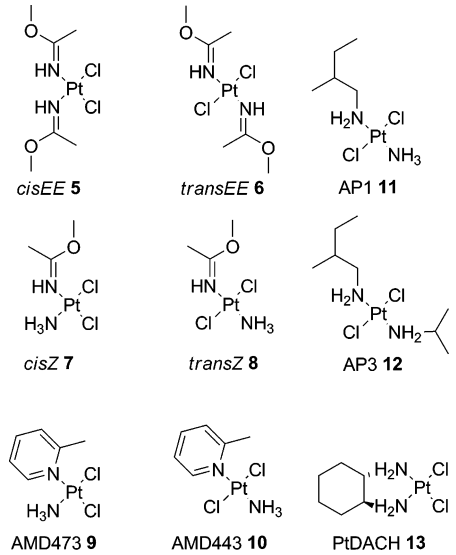


Figure 1. Representative deconvoluted nanoESI-MS spectra of recombinant human Zn_7MT-2 upon the reaction with 2 mol equiv of (a, b) cisplatin **1** and (c, d) transplatin **2** in 10 mM HEPES/NaOH, pH 7.4, 100 mM $NaClO_4$ for 120 h at 37 °C. Prior to analysis samples were rapidly diluted (a, c) into 50:50:0.2 (v/v/v) CH_3CN/H_2O/HOAc (final pH ∼ 3) or (b, d) into 50:50 (v/v) CH_3CN/H_2O (final pH ∼ 7).

Chart 2. Platinum Compounds Used In This Study in Addition to **1**, **2**, and **3**



protonation state of MT-2 molecules in the mass spectrometry experiments, in particular at pH ∼ 7, is uncertain. This accounts for small deviations between the measured and calculated mass values. The results reveal that the reaction of Zn_7MT-2 with *cis*-Pt^{II} compounds results in Pt_xZn_yMT-2 complexes in which the leaving groups and carrier ligands L are replaced by protein thiolates (Table 1). **3** and, to an even lesser extent, *cis*-(bis(*E*)-1-imino-1-methoxyethane)dichloridoplatinum(II) **5** (*cisEE*) were found to be the only exceptions. In these cases a small population of MT-2 species containing NH_3 or (*E*)-1-imino-1-methoxyethane, respectively, could also be detected. In marked contrast, all *trans*-Pt^{II} compounds including **2** retained their two N-donor ligands (Table 2). However, also in this case a few Pt_xZn_yMT-2 complexes (**5** and *trans*-((*Z*)-1-imino-1-methoxyethane)amminedichloridoplatinum(II) **8** (*transZ*)) showed small populations of species in which a partial loss of one or, to an even lesser extent, two carrier ligands L occurred. As $[PtL^1L^2]_x$-MT-2 are the major species formed, we infer that the loss of L is a slow process. In addition, the tendency to retain Zn^{II} is higher for *trans*- compared to *cis*-compounds (Table 2). A different number of thiolate ligands required for the coordination of *trans*- and *cis*-Pt^{II} compounds may explain this effect. The metal ions in both clusters of Zn_7MT-2 are coordinated by both bridging and terminal thiolate ligands. We hypothesize that the binding of *trans*-Pt^{II} compounds to more accessible terminal thiolates may cause an expansion of the original cluster structure without major perturbation. Overall, the examination of a large number of *cis*- and *trans*-$[PtL^1L^2Cl_2]$ with very different carrier ligands L strongly supports the general principle that in the

Table 1. NanoESI-MS Characterization of the Products of the Reaction between Human Zn_7MT-2 and 2 Mol Equiv of *cis*-$[PtL^1L^2X_2]^{a+}$ (X = Cl^-, OH^-, H_2O; a = 0, 1, 2) or $[Pt(dien)X]^{a+}$ (a = 0, 1) at pH ∼ 3[a] and pH ∼ 7[b]

	pH ∼ 3[a]			pH ∼ 7[b]		
cis-Pt^{II} compound	m_{exp}[c] (Da)	m_{calc}[d] (Da)	cation(s) in complex with MT-2	m_{exp}[c] (Da)	m_{calc}[d] (Da)	cation(s) in complex with MT-2
1, cisplatin	6041.4[e]		-	6484.2[f]		Zn^{II}_7
	6233.2	6236.5	Pt^{II}	6615.2	6613.9	$Zn^{II}_6Pt^{II}$
	6427.1	6431.6	Pt^{II}_2	6743.8	6743.6	$Zn^{II}_5Pt^{II}_2$
	6619.5	6626.7	Pt^{II}_3	6871.2	6873.4	$Zn^{II}_4Pt^{II}_3$
	6813.8	6821.8	Pt^{II}_4	7068.0	7068.5	$Zn^{II}_4Pt^{II}_4$
7, *cisZ*	6041.8[e]		-	6484.4[f]		Zn^{II}_7
	6234.0	6236.9	Pt^{II}	6614.2	6614.1	$Zn^{II}_6Pt^{II}$
				6676.2	6679.5	$Zn^{II}_7Pt^{II}$
	6426.4	6432.0	Pt^{II}_2	6742.2	6743.8	$Zn^{II}_5Pt^{II}_2$
	6619.4	6627.1	Pt^{II}_3	6874.2	6873.6	$Zn^{II}_4Pt^{II}_3$
	6814.0	6822.2	Pt^{II}_4			
5, *cisEE*	6041.6[e]		-	6485.2[f]		Zn^{II}_7
	6233.8	6236.7	Pt^{II}	6534.4	6549.6	$Zn^{II}_5Pt^{II}$
	6307.8	6309.8	$[Pt^{II}E]$[g]			
				6615.4	6614.9	$Zn^{II}_6Pt^{II}$
	6425.8	6431.8	Pt^{II}_2	6874.4	6875.4	$Zn^{II}_7Pt^{II}_2$
	6619.8	6626.9	Pt^{II}_3	7070.7	7070.5	$Zn^{II}_7Pt^{II}_3$
	6813.2	6822.0	Pt^{II}_4			
9, AMD473	6041.6[e]		-	6484.8[f]		Zn^{II}_7
	6233.5	6236.7	Pt^{II}	6616.2	6614.5	$Zn^{II}_6Pt^{II}$
	6295.4	6302.1	$Zn^{II}Pt^{II}$			
	6426.5	6431.8	Pt^{II}_2	6744.4	6744.2	$Zn^{II}_5Pt^{II}_2$
	6617.4	6626.9	Pt^{II}_3	6874.8	6874.0	$Zn^{II}_4Pt^{II}_3$
	6814.0	6822.0	Pt^{II}_4	7069.2	7069.1	$Zn^{II}_4Pt^{II}_4$
$[Pt(dien)Cl]^+$	6041.1[e]		-	6485.3[f]		Zn^{II}_7
	6230.2	6236.2	Pt^{II}	6615.0	6615.0	$Zn^{II}_6Pt^{II}$
				6679.1	6680.4	$Zn^{II}_7Pt^{II}$
	6428.5	6431.3	Pt^{II}_2	6744.1	6744.7	$Zn^{II}_5Pt^{II}_2$
				6874.4	6875.5	$Zn^{II}_7Pt^{II}_2$
	6620.0	6626.4	Pt^{II}_3	7068.2	7070.6	$Zn^{II}_7Pt^{II}_3$
3, carboplatin	6041.4[e]		-	6483.8[f]		Zn^{II}_7
	6231.6	6236.5	Pt^{II}	6545.4	6548.2	$Zn^{II}_5Pt^{II}$
				6613.2	6613.5	$Zn^{II}_6Pt^{II}$
	6456.0	6448.6	$Pt^{II}_2(NH_3)$			
	6472.2	6465.7	$Pt^{II}_2(NH_3)_2$	6723.8	6712.0	$Zn^{II}_4Pt^{II}_2(NH_3)_2$

[a] Samples were diluted into 50:50:0.2 CH_3CN/H_2O/HOAc prior to analysis. [b] Samples were diluted into 50:50 CH_3CN/H_2O prior to analysis. [c] Mass peak obtained in nanoESI-MS experiment. [d] Calculated mass corresponding to m_{exp}. The mass of apo-MT-2 or Zn_7MT-2, respectively, obtained in this experiment was used as a basis for m_{calc}. [e] Mass obtained for apo-MT-2; calculated: 6042.2 Da. [f] Mass obtained for Zn_7MT-2; calculated: 6499.8 Da. The experimental mass differences of 14.6−16.0 Da originate from an unpredictable protonation state of the protein at pH ∼ 7. [g] *E* = (*E*)-1-imino-1-methoxyethane.

MT-2 adducts the N-donor ligands of *trans*-Pt^{II} complexes are retained, in contrast to their *cis*-isomers.

Stoichiometry of Pt^{II}MT-2 Adducts. Mass spectrometry is not a quantitative method; indeed, in comparison to apo-MT-2 or Zn^{II}MT-2, Pt^{II}MT-2 species were hard to ionize, and, in addition, the ionization was highly variable among the different complexes studied. To obtain more accurate Pt/MT-2 ratios, Zn_7-MT-2 was incubated with 2 mole equivalents of **1** or **2** for 120 h under the same conditions as described above. Subsequently, MT-2 was quantified by amino acid analysis and the Pt concentration determined by atomic absorption spectrometry. A Pt/MT-2 ratio of 1.96 was found for **1** and of 1.92 for **2** indicating that in the complex with MT-2 all added Pt^{II} is bound.

The number of Cys thiolates involved in Pt^{II} binding can also be derived from the absorption spectra. The reported absorption spectrum of $Pt^{II}_{(1-10)}$MT species is characterized by a broad, unresolved absorption band with a broad shoulder at 250 nm and tailing to 400 nm. These features have been assigned to two closely spaced Mσ^*←Lπ (i.e., Pt^{II}←CysS) LMCT transitions.[23] A similar absorption profile was also obtained in our studies. Since at ∼ 250 nm the contribution of Zn^{II}←CysS and Pt^{II}←CysS LMCT transitions occurs, the absorption at 285 nm was used to derive the number of CysS−Pt^{II} bonds involved in the Pt^{II} coordination. The choice of this wavelength is supported by previous studies in which a nearly linear dependence of the absorbance increase *versus* the number of Pt^{II} bound was found at ∼ 280 nm, but not at 250 nm.[23] Considering the binding of two Pt^{II} equivalents to MT-2 the molar extinction coefficient at 285 nm per Pt^{II} bound was 11400 M^{-1} cm^{-1} for **1** and 5020 M^{-1} cm^{-1} for **2**. This suggests that the number of CysS−Pt^{II} bonds is double in the case of *cis*- compared to *trans*-complexes which is in agreement with the corresponding MS analysis. Consequently, a molar extinction coefficient of $\epsilon_{285nm}^{cisplatin} = 2850$ M^{-1} cm^{-1} per CysS−Pt^{II} bond for **1** and that of $\epsilon_{285nm}^{transplatin} = 2510$ M^{-1} cm^{-1} for **2** can be estimated. In the following kinetic studies the average $\epsilon_{285nm} = 2680$ M^{-1} cm^{-1} was used. However, a small alteration of this value may be expected due to variation of coordinating ligands.

Kinetics of the Reaction of Zn_7MT-2 with 2 Mol Equiv of Pt^{II} Complexes. The previously mentioned restriction to maintain a Pt/MT ratio of 2, in agreement with that found in biological studies,[21] implies that pseudo-first-order conditions cannot be fulfilled. Therefore, to allow the comparison of reaction velocities throughout the study, the time-courses of Pt^{II} binding to Zn_7MT-2 were recorded under identical conditions. The formation of Pt^{II}−S bonds in MT-2 with time was followed

Table 2. NanoESI-MS Characterization of the Products of the Reaction between Human Zn_7MT-2 and 2 Mol Equiv of *trans*-[PtL^{1}L^{2}X$_2$]$^{a+}$ (X = Cl$^-$, OH$^-$, H$_2$O; a = 0, 1, 2) at pH ∼ 3[a] and pH ∼ 7[b]

	pH ∼ 3[a]			pH ∼ 7[b]		
trans-Pt^{II} compound	m_{exp}[c] (Da)	m_{calc}[d] (Da)	cation(s) in complex with MT-2[e]	m_{exp}[c] (Da)	m_{calc}[d] (Da)	cation(s) in complex with MT-2[e]
2, transplatin	6041.2[f]		-	6485.2[g]		Zn^{II}_7
	6267.2	6270.4	$[Pt(NH_3)_2]^{II}$			
	6493.4	6499.5	$[Pt(NH_3)_2]^{II}_2$	6810.4	6812.8	$Zn^{II}_5[Pt(NH_3)_2]^{II}_2$
	6717.6	6728.7	$[Pt(NH_3)_2]^{II}_3$	7040.6	7042.0	$Zn^{II}_5[Pt(NH_3)_2]^{II}_3$
8, *transZ*	6041.8[f]		-	6484.4[g]		Zn^{II}_7
	6232.0	6236.9	Pt^{II}			
	6322.8	6327.0	$[Pt(NH_3)Z]^{II}$	6616.6	6638.9	$Zn^{II}_5[Pt(NH_3)Z]^{II}$
				6722.6	6704.3	$Zn^{II}_6[Pt(NH_3)Z]^{II}$
	6425.6	6432.0	Pt^{II}_2			
	6507.6	6522.1	$[Pt(NH_3)Z]^{II}Pt^{II}$	6773.0	6785.0	$Zn^{II}_4[Pt(NH_3)Z]^{II}Pt^{II}$
	6619.2	6612.3	$[Pt(NH_3)Z]^{II}_2$			
6, *transEE*	6041.8[f]		-	6485.4[g]		Zn^{II}_7
	6380.0	6383.1	$[PtE_2]^{II}$	6822.2	6826.7	$Zn^{II}_7[PtE_2]^{II}$
	6718.5	6724.4	$[PtE_2]^{II}_2$	7036.0	7037.3	$Zn^{II}_5[PtE_2]^{II}_2$
	7047.0	7050.3	$[PtE_2]^{II}_2Pt^{II}Zn^{II}_2$			
	7054.9	7058.0	$[PtE_2]^{II}_2[PtE]^{II}Zn^{II}$			
	7059.7	7065.7	$[PtE_2]^{II}_3$	7376.4	7378.6	$Zn^{II}_5[PtE_2]^{II}_3$
10, AMD443	6041.2[f]		-	6484.7[g]		Zn^{II}_7
	6343.8	6346.5	$[Pt(NH_3)(2\text{-pic})]^{II}$	6722.6	6724.6	$Zn^{II}_6[Pt(NH_3)(2\text{-pic})]^{II}$
				6785.2	6790.0	$Zn^{II}_7[Pt(NH_3)(2\text{-pic})]^{II}$
	6646.5	6651.7	$[Pt(NH_3)(2\text{-pic})]^{II}_2$	6962.4	6964.5	$Zn^{II}_5[Pt(NH_3)(2\text{-pic})]^{II}_2$
				7029.7	7029.9	$Zn^{II}_6[Pt(NH_3)(2\text{-pic})]^{II}_2$
	6950.6	6957.0	$[Pt(NH_3)(2\text{-pic})]^{II}_3$	7269.0	7269.8	$Zn^{II}_5[Pt(NH_3)(2\text{-pic})]^{II}_3$
				7509.1	7509.7	$Zn^{II}_4[Pt(NH_3)(2\text{-pic})]^{II}_4$
				7812.6	7814.9	$Zn^{II}_4[Pt(NH_3)(2\text{-pic})]^{II}_5$
11, AP1	6041.2[f]		-	6485.8[g]		Zn^{II}_7
	6337.6	6340.5	$[Pt(NH_3)(2\text{-MeBuA})]^{II}$	6722.2	6719.8	$Zn^{II}_6[Pt(NH_3)(2\text{-MeBuA})]^{II}$
	6633.4	6639.8	$[Pt(NH_3)(2\text{-MeBuA})]^{II}_2$	6951.4	6953.7	$Zn^{II}_5[Pt(NH_3)(2\text{-MeBuA})]^{II}_2$
				7015.8	7019.1	$Zn^{II}_6[Pt(NH_3)(2\text{-MeBuA})]^{II}_2$
				7253.2	7253.0	$Zn^{II}_5[Pt(NH_3)(2\text{-MeBuA})]^{II}_3$
12, AP3	6041.1[f]		-	6485.8[g]		Zn^{II}_7
	6379.0	6381.5	$[Pt(2\text{-MeBuA})(i\text{PrA})]^{II}$	6693.0	6695.5	$Zn^{II}_5[Pt(2\text{-MeBuA})(i\text{PrA})]^{II}$
				6761.2	6760.8	$Zn^{II}_6[Pt(2\text{-MeBuA})(i\text{PrA})]^{II}$
				6826.4	6826.2	$Zn^{II}_7[Pt(2\text{-MeBuA})(i\text{PrA})]^{II}$
	6718.2	6721.9	$[Pt(2\text{-MeBuA})(i\text{PrA})]^{II}_2$	6969.6	6970.5	$Zn^{II}_4[Pt(2\text{-MeBuA})(i\text{PrA})]^{II}_2$
				7035.8	7035.9	$Zn^{II}_5[Pt(2\text{-MeBuA})(i\text{PrA})]^{II}_2$
	7054.0	7062.3	$[Pt(2\text{-MeBuA})(i\text{PrA})]^{II}_3$	7309.8	7310.9	$Zn^{II}_4[Pt(2\text{-MeBuA})(i\text{PrA})]^{II}_3$
				7376.0	7376.3	$Zn^{II}_5[Pt(2\text{-MeBuA})(i\text{PrA})]^{II}_3$

[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 peak obtained in nanoESI-MS experiment. [d] Calculated mass corresponding to m_{exp}. The mass of apo-MT-2 or Zn_7MT-2, respectively, obtained in this experiment was used as a basis for m_{calc}. [e] Abbreviations used: 2-pic = 2-picoline (2-methylpyridine); 2-MeBuA = (*S*,*R*)-2-methylbutylamine; *i*PrA = *iso*-propylamine; *E* = (*E*)-1-imino-1-methoxyethane; *Z* = (*Z*)-1-imino-1-methoxyethane. [f] Mass obtained for apo-MT-2; calculated: 6042.2 Da. [g] Mass obtained for Zn_7MT-2; calculated: 6499.8 Da. The experimental mass differences of 14.0−15.4 Da originate from an unpredictable protonation state of the protein at pH ∼ 7.

at 285 nm, and the obtained values were normalized per Pt^{II}−S bond using the ϵ_{285nm} determined as described above. The reaction of thiolates with Pt^{II} is a very slow process,[8,21,34] therefore the reaction kinetics were followed for up to 120 h to allow almost complete reaction. Figure 2a and b show that *trans*-[PtL^{1}L^{2}Cl$_2$] complexes generally reacted faster compared to their *cis*-isomers. A similar difference in reaction kinetics between **1** and **2** has been noted previously for their reactions with Zn_7MT-2 from rat liver.[32] Our results indicate that, with a few exceptions, the reaction with 2 mole equivalents of the *cis*-[PtL^{1}L^{2}Cl$_2$] complexes resulted in up to ∼ 8 Pt^{II}−S bonds per MT-2 whereas in the case of the *trans*-[PtL^{1}L^{2}Cl$_2$] complexes up to ∼ 4 Pt^{II}−S bonds per MT-2 were formed.

In agreement with literature reports,[8,35] we suggest that the reaction of *cis*-[PtL^{1}L^{2}Cl$_2$] with the Cys of MT-2 is a stepwise process (Scheme 1a). The reaction with *trans*-[PtL^{1}L^{2}Cl$_2$] is best approximated by Scheme 1b. However, it should be noted that the individual steps of these reaction sequences could not be derived from the absorption traces recorded as a function of time. The fact that the reactions follow higher order kinetics is revealed by Figure 2a and 2b where for some compounds no simple hyperbolic dependence was seen. Evidence for the complexity of reaction kinetics comes from the nanoESI-MS data in which the formation of different reaction products was demonstrated (Figure 1, Tables 1 and 2).

From the initial slopes of the kinetic traces, apparent initial rates k_{obs} were derived and compared (Figure 2c). Overall, the data show that in case of the *cis*/*trans*-pairs such as **1**/**2**, **5**/**6**, *cis*-((*Z*)-1-imino-1-methoxyethane)amminedichloridoplatinum(II) **7** (*cisZ*)/*trans*-((*Z*)-1-imino-1-methoxyethane)-amminedichloridoplatinum(II) **8** (*transZ*), and *cis*-(2-methylpyridine)-amminedichlorido-platinum(II) **9** (AMD473)/*trans*-(2-methylpyridine)amminedichloridoplatinum(II) **10** (AMD443), the *trans*-compounds react 1.4 to 3 times faster with Zn_7MT-2 than the corresponding *cis*-isomers. The value obtained for **1** of 8.5 ± 1.8 × 10^{-4} min^{-1} (Figure 2c) is of the same order of magnitude as that reported previously for the initial rate k_{obs} = 4.27 ± 0.12 × 10^{-4} min^{-1} in the reaction of MT-1 with **1** at the same temperature.[14] The observed differences in k_{obs} may be accounted for by different buffer conditions, the MT form used

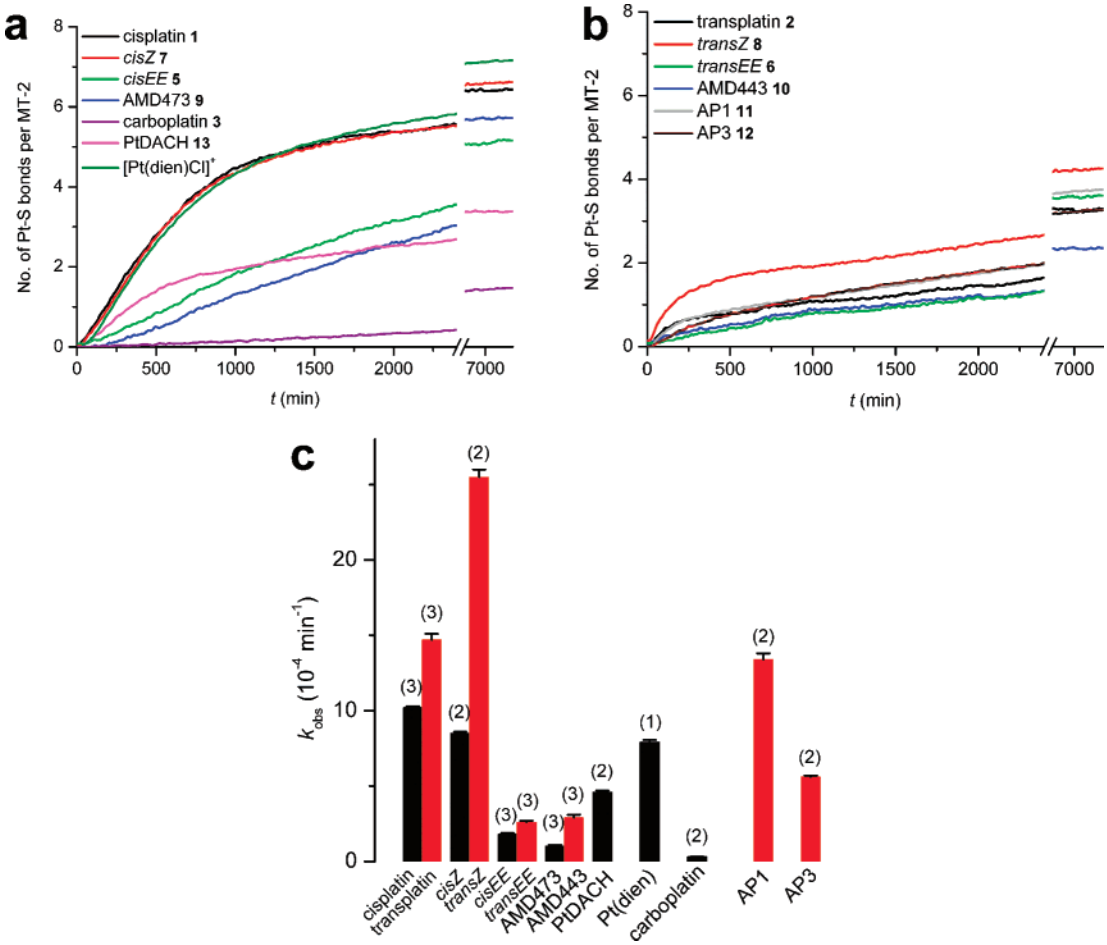
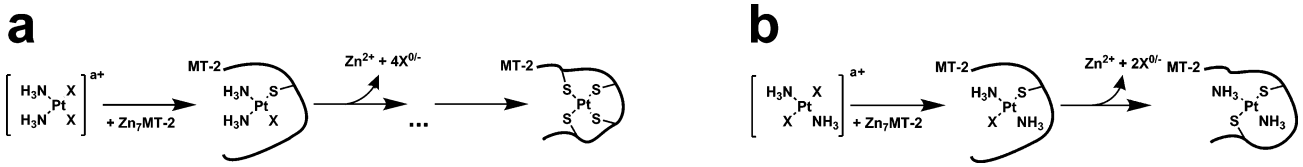


Figure 2. Kinetics of the binding of 20 μM (a) *cis*- or (b) *trans*-$[PtL^1L^2X_2]^{a+}$-compounds (X = Cl^-, OH^-, H_2O; a = 0, 1, 2) to 10 μM human Zn_7MT-2 in 10 mM HEPES/NaOH, pH 7.4, 100 mM $NaClO_4$ at 37 °C. Complex formation with time was monitored through absorption change at 285 nm. The absorption traces were normalized to the number of Pt^{II}−S bonds per MT-2. (c) Comparison of the initial apparent reaction velocities k_{obs} of the binding of Pt^{II}-compounds to human Zn_7MT-2 *cis*- (black) and *trans*-$[PtL^1L^2X_2]^{a+}$ (a = 0, 1, 2) (red). Values in parentheses refer to the numbers of individual experiments.

Scheme 1. Reaction of Human Zn_7MT-2 with (a) *cis*-$[Pt(N\text{-donor})_2X_2]^{a+}$ in Comparison to (b) *trans*-$[Pt(N\text{-donor})_2X_2]^{a+}$ [a]



[a] X = Cl^-, OH^-, or H_2O; a = 0, 1, 2.

(Cd/Zn−MT-1 from rabbit liver), and the protein and cisplatin concentrations (2.1 μM Zn/Cd−MT-1; 198 μM **1**).[14]

Time Dependent nanoESI-MS of the Reaction of Cisplatin with Zn_7MT-2. To address the question as to whether the loss of all ligands from **1** is fast in the reaction with Zn_7MT-2 and occurs already during the initial binding step or it proceeds through slowly forming intermediates, nanoESI-MS spectra were recorded after 5 min and 5, 10, and 20 h. For rapid injection, samples were desalted with C_{18}-ZipTips and eluted in 50:50: 0.01 CH_3OH/H_2O/HOAc (pH ∼ 5). The resulting mass spectra of +5 and +6 ions are shown in Figure 3. Because of the required experimental conditions (pH ∼ 5), the Zn^{II} ions in the protein are labilized and partially released giving rise to a number of species in which Pt^{II} and a varying number of Zn^{II} ions are present. In the spectra, the prominent mass peak of Zn_4MT-2 reflects the greater stability of the four-metal cluster in MT-2 at this pH value. The closer inspection of the MS spectra revealed that a similar pattern of small peaks already develops after 5 min of sample incubation (for peak assignment see caption of Figure 3). However, none of the mass spectra gave an indication for species carrying NH_3 ligands. This suggests that although the formation of the final products with **1** is a slow process, once the first Pt^{II}−S bond is created the formation of the final $Pt^{II}(SCys)_4$ complex is rapid.

Kinetics of Zn^{II} Release from Zn_7MT-2 upon Pt^{II} Binding. The binding of Pt^{II} compounds to Zn_7MT-2 is accompanied by Zn^{II} release from the protein. Therefore, in parallel to the kinetics of formation of Cys−Pt^{II} bonds, the kinetics of Zn^{II} release was also examined. Pt^{II} binding experiments were repeated in the presence of the Zn^{II}-chelating dye 4-(2-pyridylazo)resorcinol (PAR) monitoring the absorption of $Zn(PAR)_2$ complex at 500 nm.[36,37] Prior to these experiments the absence of a complex between PAR and Pt^{II} was established. The change in PAR absorption with time is summarized in Figure 4a and 4b. The results show that saturation is reached with about two Zn^{II} released per two Pt^{II} added for all compounds tested. This

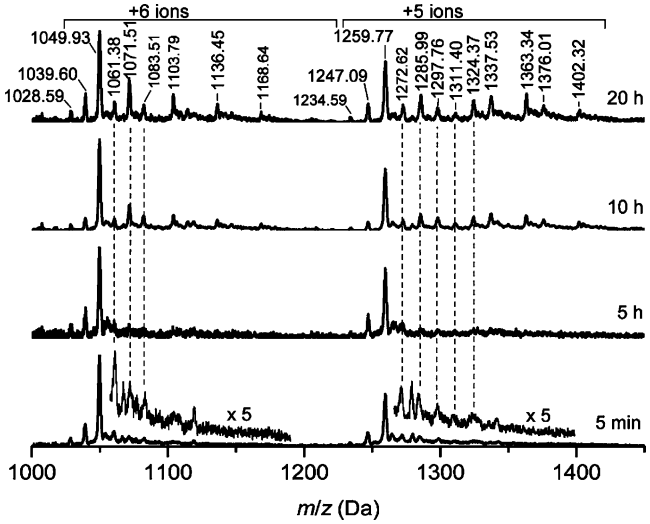


Figure 3. Time dependent nanoESI-MS (+5 and +6 ions) of human Zn_7MT-2 incubated with 2 mol equiv of cisplatin **1** in 10 mM HEPES/NaOH, pH 7.4, 100 mM $NaClO_4$ at 37 °C. Aliquots of the sample were measured after 5 min and 5, 10, and 20 h. *m*/*z* values are denoted only for the spectrum after 20 h. The mass peaks were assigned to MT-2 species containing Zn^{II} and Pt^{II} as follows: *m*/*z* = 1028.59 (+6), 1234.59 (+5): Zn_2; 1039.60 (+6), 1247.09 (+5): Zn_3; 1049.93 (+6), 1259.77 (+5): Zn_4; 1061.38 (+6), 1272.62 (+5): Zn_2Pt_1; 1071.51 (+6), 1285.99 (+5): Zn_3Pt_1; 1083.51 (+6), 1297.76 (+5): Zn_4Pt_1; 1311.40 (+5): Zn_5Pt_1; 1103.79 (+6), 1324.37 (+5): Zn_3Pt_2; 1337.53 (+5): Zn_4Pt_2; 1136.45 (+6), 1363.34 (+5): Zn_3Pt_3; 1376.01 (+5): Zn_4Pt_3; 1168.64 (+6), 1402.32 (+5): Zn_3Pt_4.

indicates that Pt^{II} binding leads to displacement of the corresponding number of mole equivalents of Zn^{II} from the protein. The initial rates of this process $k_{obs}^{Zn(II)}$ are compared in Figure 4c. A comparison with k_{obs} for the CysS−Pt^{II} bond formation reveals that the Zn^{II} release is in most cases roughly twice as fast. This is likely to be because of the strong Zn^{II} labilization in the cluster induced by the breaking of even a single CysS−Zn^{II} bond upon initial Pt^{II} binding. Therefore, it would appear that while binding Pt^{II} is a multistep process, the release of Zn^{II} is probably accomplished in parallel to the first or second CysS−Pt^{II} bond formation. However, for some pairs of *cis*/*trans*-[PtL^{1}L^{2}Cl$_2$] isomers (**5**/**6** and **7**/**8**) the *cis*-compounds cause faster Zn^{II} release compared to their *trans*-counterpart (see below). Overall, these data suggest that MT-2 binds similar amounts of *cis*/*trans*-isomer pairs in a process paralleled by the loss of equal amounts of Zn^{II}.

Discussion

The present study provides insights into the reaction of *cis*- and *trans*-Pt^{II} compounds with human Zn_7MT-2 and the products formed. Previously, it has been suggested that carrier and leaving ligands are both displaced by MT thiols upon the reaction with cisplatin **1** and transplatin **2**.[22,32] The data reported herein show, for the first time, that the N-donor ligands of *cis*-Pt^{II} compounds are indeed lost upon the reaction with MT-2, but that the majority of *trans*-Pt^{II} compounds retain their N-donor ligands (Scheme 1). The observed different binding modes of *cis*- and *trans*-Pt^{II} compounds may explain the reported differences in luminescence[38] and circular dichroism spectra[39] of MT complexes with cisplatin **1** compared to those with transplatin **2**.

Previously, it has been reported that other proteins like serum albumin,[40] ubiquitin,[41−43] myoglobin,[42,43] transferrin,[44] and cytochrome *c*[45] are able to coordinate cisplatin **1** and transplatin **2** mainly through Met, Cys, or His, and in some instances also through a Thr residue. However, in all these proteins, the Pt^{II} compounds were bound monofunctionally and retained their NH_3 ligands.[43−46] In the MT-2 molecule, besides the 20 Cys, a single thioether (Met-1) located in the β-domain is also present (Scheme 2). It has been shown that in the reaction of $K_2[PtCl_4]$ with Cd_7MT-1, Met-1 can be involved together with Cys thiolates in Pt^{II} coordination.[47] As seen in Tables 1 and 2, the reaction of Zn_7MT-2 with any of the Pt^{II} compounds **1**−**3**, **5**−**12**, and [Pt(dien)Cl]Cl does not show a high degree of specificity. In fact, several different products were formed simultaneously, indicating that the binding of Pt^{II} to MT-2 occurs in a noncooperative manner. In numerous studies dealing with the reactivity of Me^{II}_7MT it has been reported that the reactivity of β-domain thiolates with various electrophiles is generally higher compared to the α-domain thiolates.[19] That the binding of *trans*- and *cis*-Pt^{II} complexes to the β-domain also occurred in our case is supported by the fact that among the Pt^{II}-containing MT-2 species found in nanoESI-MS at pH ∼ 7 (Tables 1 and 2), a maximum loss of three Zn^{II} was detected. The preferential binding of **1** to the β-domain has been demonstrated by ^{111}Cd NMR studies of rat $^{111}Cd_7$MT-2.[36] In the Me^{II}_7MT structures, the three-metal cluster (i.e., $Me^{II}_3(CysS)_9$) in the β-domain, which can be approximated by a cyclohexane ring, is structurally less constrained and more flexible compared to the adamantane-like four-metal cluster (i.e., $Me^{II}_4(CysS)_{11}$) in the α-domain (Scheme 2).[19,20] However, in view of the kinetically stable Pt^{II}−S bond,[8] the initial selectivity of *cis*- and *trans*-Pt^{II} compounds for a specific domain will not depend on the cluster structure, but rather on the accessibility of thiolate ligands to electrophilic attack.

Whereas Zn^{II} in MT-2 is tetrahedrally coordinated (Scheme 2),[19,20] Pt^{II} chemistry is characterized by square-planar coordination. The coordination chemistry of Pt^{II} is very much dictated by the kinetically driven *trans* effect which decreases in the following order:[48]

$$SR_2 > SO_3^{2-} > SC(NH_2)_2 \sim NO_2^- \sim SCN^- > Br^- > Cl^- > \text{pyridine} > NH_3 > OH^- \sim H_2O$$

In view of this effect, the data for *cis*- and *trans*-Pt^{II} compounds can be best illustrated by two different reactions shown in Scheme 1a and 1b. The presented data are also in good agreement with density functional theory (DFT) calculations showing that the *trans* effect of protein sulfur is very strong and largely independent of its nature, i.e., Met, CysH, or Cys^-.[8] The *trans* effect labilization of N-donors *trans* to S accounts for the generation of $Pt^{II}(SCys)_4$ complexes formed upon the reaction of MT-2 with *cis*-Pt^{II} compounds. Although cisplatin binding to MT-2 is a rather slow process, none of the time dependent mass spectra gave an indication for species carrying NH_3 ligands (Figure 3). This indicates that following the initial formation of a Pt^{II}−S bond, the replacement of all *cis*-Pt^{II} ligands by the protein thiolates is fast.

The kinetic measurements showed that *trans*-Pt^{II} compounds react faster with Zn_7MT-2 compared to their *cis*-isomers (Figure 2). A different extent of solvolysis of Pt^{II} complexes plays an important role in their reactivity with DNA. However, in studies of the reaction of low-molecular weight S-donor compounds with $[PtL^1L^2X_2]^{a+}$ complexes (X = Cl^-, OH^-, H_2O; a = 0, 1, 2), the rate of reaction was found to be largely independent of the nature of X.[31,33] Also in this case faster reaction kinetics with **2** *versus* **1** were observed.[31,49] Similar, solvolysis independent reaction kinetics have also been found in the reaction of Zn_7MT-2 from rat and rabbit liver with **2** and **1**.[22,32] Overall,

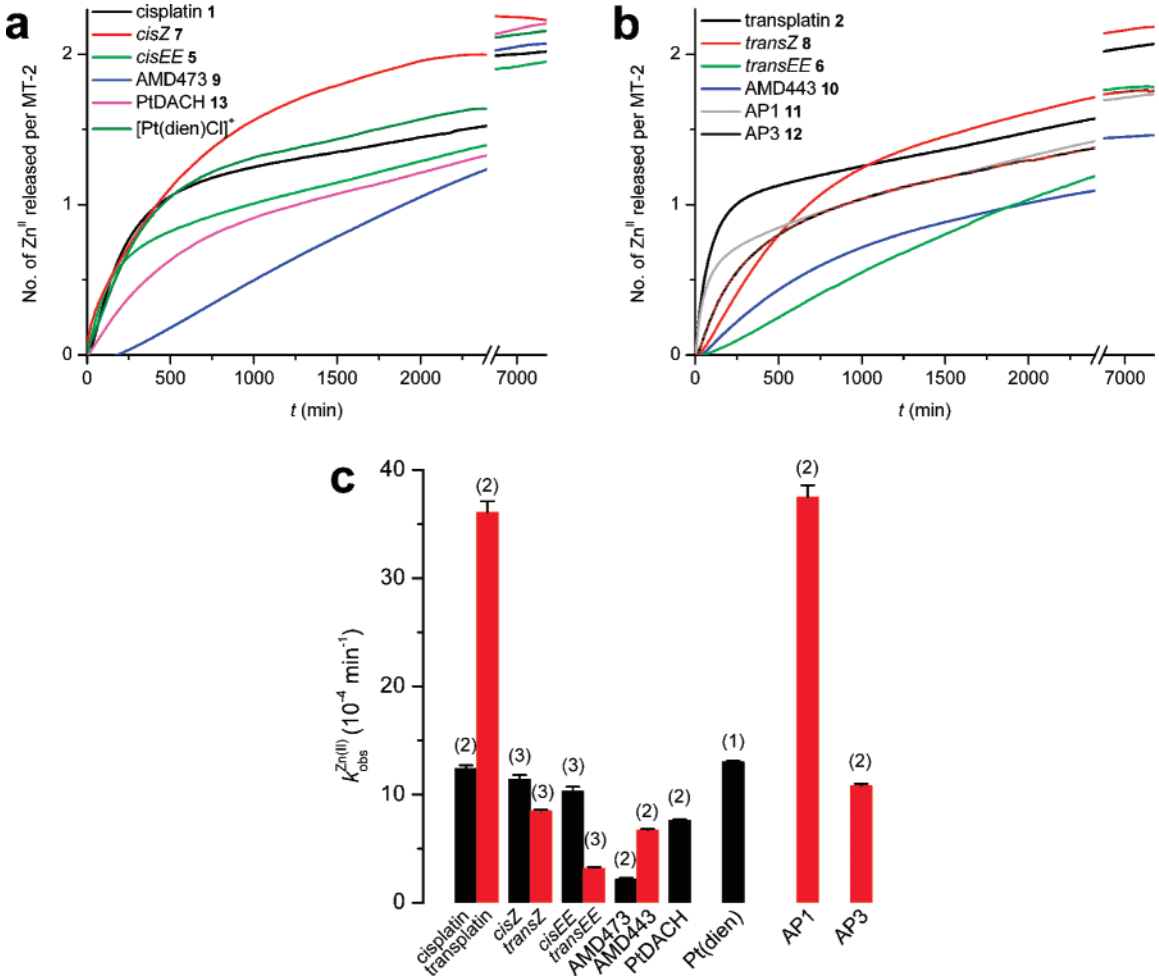
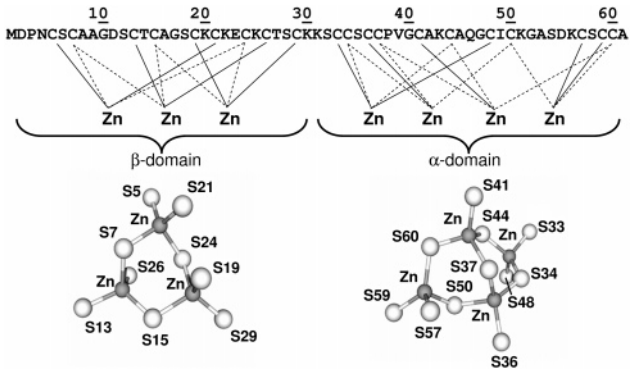


Figure 4. Time dependent release of Zn^{II} from 10 μM human Zn_7MT-2 in 10 mM HEPES/NaOH, pH 7.4, 100 mM $NaClO_4$ at 37 °C upon reaction with 20 μM (a) *cis*- or (b) *trans*-$[PtL^1L^2X_2]^{a+}$-compounds (X = Cl^-, OH^-, H_2O; a = 0, 1, 2). 4-(2-Pyridylazo)resorcinol (PAR) was used as a Zn^{II}-chelating dye. The absorption change with time was followed at 500 nm. Values were normalized to the number of Zn^{II} released per MT-2. (c) Comparison of the apparent $k_{obs}^{Zn(II)}$ of Zn^{II} release from Zn_7MT-2 upon reaction with *cis*- (black) and *trans*-$[PtL^1L^2X_2]^{a+}$ (a = 0, 1, 2) (red). Values in parentheses refer to the number of individual experiments.

Scheme 2. Amino Acid Sequence, Zn^{II}−SCys Connectivities, and Structures of the Zn_4S_{11} Cluster (α-Domain) and the Zn_3S_9 Cluster (β-Domain) in Human Zn_7MT-2[64 a]



[a] Terminal coordinative Zn−S bonds are indicated by full lines, bridging Zn−S−Zn bonds by dashed lines.

these studies suggest that *trans*-Pt^{II} compounds would be scavenged faster by MT also *in vivo*.

One of the major aims of the current research is the development of new Pt^{II} anticancer drugs showing a slow or no reaction with cellular components other than DNA, in particular with thiol-containing compounds. Although GSH is the most abundant intracellular thiol, its reactivity with Pt^{II} compounds is ∼ 50-times lower than that observed with MT.[14] This makes MT a highly effective intracellular scavenger of Pt^{II} drugs which may explain the dramatic resistance increase in cancer cells upon its overexpression.[16] The data presented imply principles that may aid the rational design of Pt^{II} complexes overcoming MT-related drug resistance. Steric hindrance appears to be a major factor that influences the nucleophilic attack of MT-2 thiolates on the Pt^{II} center. This is illustrated by the lower k_{obs} and $k_{obs}^{Zn(II)}$ for *trans*-(isopropylamine)((*S*,*R*)-2-methylbutylamine)dichloridoplatinum(II) **12** (AP3) and **6** compared to the very similar *trans*-((*S*,*R*)-2-methylbutylamine)amminedichloridoplatinum(II) **11** (AP1) and **8**, respectively (Figure 2c and 4c). The other example is the low reactivity of sterically hindered **9**,[28] which may, in part, account for its success in biological tests.[50] Besides steric hindrance, the electronic effect of the aromatic carrier ligands through the modulation of Pt^{II} electrophilicity may also influence the reactivity of thiolate ligands. However, other factors like specific interactions between N-donor ligands and protein side-chains may play an unpredictable role in the nucleophilic attack on the Pt^{II} center.

Thus far, we have discussed the thiolate reactivity of Zn_7MT-2 with Pt^{II} complexes in relation to the development of drug resistance. Our data clearly show that carboplatin **3**, already

used in the clinic, and the promising drugs **9** and **6** exhibited a very low reactivity with Zn_7MT-2. Besides the reactivity with protein thiolates, the rate of Zn^{II} release represents another factor contributing to cellular resistance. For both **9** and **6**, the lowest $k_{obs}^{Zn(II)}$ was determined (Figure 4c). The low rate of Zn^{II} release from Zn_7MT-2 may play an important role in acquired resistance because of the positive regulation of *mt2* resistance gene by free Zn^{II}. In this case, Zn^{II} binding to the metal-responsive transcription factor (MTF-1) leads to the induction of MT-2 biosynthesis.[51] This would suggest that compounds like **2** and **11** that show the highest $k_{obs}^{Zn(II)}$ could act as efficient activators of MT-2 transcription, leading to a highest overexpression of this protein. In general, our studies revealed that the *trans*-compounds released Zn^{II} faster compared to their *cis*-counterparts (Figure 4c). This observation is in line with the determined faster formation of $Pt^{II}-S$ bonds with the *trans*-compounds (Figure 2c). However, the rate of Zn^{II} release was found to be inverted for two isomer pairs of the iminoether Pt^{II}-complexes **5/6** and **7/8**. In the absence of structural data for these Pt^{II}-protein complexes, we hypothesize that both steric hindrance and stabilizing interactions between the retained ligands in the *trans*-Pt^{II} compounds and a cluster may be responsible for this effect.

Besides the release of Zn^{II} from Zn_7MT-2, the release of N-donor ligands from *cis*-Pt^{II} compounds is another factor that should be considered in drug design. NH_3, although toxic at high concentrations, is a metabolite of transamination reactions in the organism and as such detoxified via the urea cycle.[52] The fate of the other N-donor ligands is so far unknown. However, primary and secondary amines can be a subject to the endogenous formation of carcinogenic *N*-nitrosamines.[53] Overall, the data suggest that the biological effects of the carrier ligands L require more attention in case of *cis*- compared to the *trans*-[PtL^{1}L^{2}Cl$_2$] compounds.

The known high affinity of Pt^{II} compounds for sulfur atoms and the great abundance of sulfur-containing biomolecules in the cytosol and nucleus of the cell raise the question whether Pt−sulfur interactions could serve as a drug reservoir affording an additional pathway toward platination of DNA.[10] The studies using [Pt(dien)(L-methionine-*S*)]$^+$, [Pt(dien)(*S*-methyl-GSH-*S*)]$^{2+}$, [Pt(dien)(GSH-*S*)]$^+$, and *cis*-[Pt(NH$_3$)$_2$(*S*-methyl-GSH-*S*)$_2$]$^{2+}$ showed that a transfer to 5′-GMP-*N7* and d(GpG) was possible from the thioethers, but not from the [Pt(dien)(GSH-*S*)]$^+$.[54] From these studies it has been concluded that the Pt^{II}-transfer from S-donor compounds to DNA may depend highly on the nature of individual ligands involved.[10] Recently, the reactions of the 1:1 complex formed between **1** and L-methionine or *N*-acetyl-L-methionine with synthetic single- and double-stranded oligodeoxyribonucleotides and natural, high molecular mass DNA were investigated. The results showed that both L-methionine and *N*-acetyl-L-methionine decreased the extent of reaction of **1** with base residues in natural, high molecular mass DNA. Therefore, it has been concluded that it appears unlikely that **1** bound to any Met residue could serve as a drug reservoir ready to platinate DNA.[54] In the MT structure, besides terminal thiolates, bridging thiolates, possessing a different nucleophilicity, are also involved in metal binding. *trans*-Pt^{II} compounds in complex with MT-2 preserve their carrier ligands. Since MT-2 is present not only in the cytosol but also in the nucleus,[55] we have examined the possibility of Pt^{II} transfer from *trans*-Pt^{II}MT complexes to plasmid DNA, single- and double-stranded oligodeoxyribonucleotides, and GMP. However, similar to the previous studies, no evidence for Pt^{II} transfer was obtained.

In conclusion, our study showed that whereas MT replaces all ligands in *cis*-Pt^{II} N-donor complexes, it conserves the carrier ligands of *trans*-Pt^{II} complexes. Moreover, it clearly demonstrates that steric hindrance represents the major factor responsible for the different reactivities of Pt^{II} drugs with Zn_7MT-2. Besides the steric hindrance, a lower reactivity of drugs can also be accomplished by modulating the electrophilicity of the Pt^{II} center. In addition, the observed differences in the rate of Zn^{II} release from Zn_7MT-2 by the Pt^{II} compounds studied may represent another mechanism that could modulate the anticancer activity of Pt^{II} drugs through free-Zn^{II} pathways. Overall, these data on the reactivity of Zn_7MT-2 with a number of *cis*- and *trans*-[Pt(N-donor)$_2$Cl$_2$] complexes substantially increase our understanding of the role of MT in the acquired resistance to anticancer platinum-based drugs.

Experimental Section

Protein Expression and Purification. A cDNA 5′-cc ATG GAT CCC AAC TGC TCC TGC GCC GCC GGT GAC TCC TGC ACC TGC GCC GGT TCC TGC AAA TGC AAA GAG TGC AAA TGC ACT TCG TGC AAG AAA AGC TGC TGC TCC TGC TGC CCT GTG GGC TGT GCC AAG TGT GCC CAA GGC TGC ATC TGC AAA GGG GCG TCG GAC AAG TGC AGC TGC TGC GCC TGA gga tcc-3′ encoding for human MT-2[56] (upper case letters) flanked by *Nco* I and *Bam*H I restriction sites (underlined) was synthetically prepared and verified by Primm srl (Milan, Italy). The DNA was inserted into pET-3d expression plasmid (Novagen) and the resulting plasmid pEThMT2^{Amp} transformed into competent[57] *Escherichia coli* cells, strain BL21(DE3)pLysS^{Cam} (Novagen). MT-2 was expressed and purified essentially as described for human MT-3.[58,59] Anion exchange chromatography was performed on a 25 mL DEAE Sepharose FF (Amersham Biosciences) column (26 × 47 mm) equilibrated in 15 mM Tris/HCl, pH 8.6. The protein was loaded at 2 mL min^{-1}. Unbound protein was removed by washing with 50 mL of 15 mM Tris/HCl, pH 8.6, at 4 mL min^{-1}. Afterward, a linear salt gradient from 0 to 198 mM NaCl was applied. MT-2 eluted at ∼ 50 mM NaCl. The correctness of the protein expression was verified by nanoESI-MS ([MT-2 + H]$^+$: 6042.2 ± 0.1 Da; calculated: 6042.2 Da). The protein was further assessed by SDS-PAGE.[60] Fully Zn^{II}-loaded MT-2 was prepared by reconstitution.[61] Zn/MT-2 ratios were determined by measuring the Zn content by flame atomic absorption spectrometry (SpectrAA-110, Varian Inc.) upon sample dilution in 15 mM HNO_3[58] and that of the protein spectrophotometrically in 100 mM HCl (ϵ_{220nm} = 46200 M^{-1} cm^{-1}).[62] In all cases, a Zn/MT-2 ratio of 6.8 ± 0.3 was obtained. The amount of thiols was quantified using 2,2′-dithiodipyridine.[62]

Pt^{II} Compounds Used. *cis*-Diamminedichloridoplatinum(II) **1** (cisplatin), *trans*-diamminedichloridoplatinum(II) **2** (transplatin), *cis*-diammine(1,1-cyclobutanedicarboxylato)platinum(II) **3** (carboplatin), and diethylentriaminechloridoplatinum(II) chloride ([Pt(dien)Cl]Cl) were purchased from Sigma. The Pt^{II} compounds *cis*-(bis(*E*)-1-imino-1-methoxyethane)dichloridoplatinum(II) **5** (*cisEE*), *trans*-(bis(*E*)-1-imino-1-methoxyethane)dichloridoplatinum(II) **6** (*transEE*), *cis*-((*Z*)-1-imino-1-methoxyethane)amminedichloridoplatinum(II) **7** (*cisZ*), *trans*-((*Z*)-1-imino-1-methoxyethane)amminedichloridoplatinum(II) **8** (*transZ*), *cis*-(2-methylpyridine)amminedichloridoplatinum(II) **9** (AMD473), *trans*-(2-methylpyridine)amminedichloridoplatinum(II) **10** (AMD443), *trans*-((*S*,*R*)-2-methylbutylamine)-amminedichloridoplatinum(II) **11** (AP1), *trans*-(isopropylamine)-((*S*,*R*)-2-methylbutylamine)dichloridoplatinum(II) **12** (AP3), and (*R*,*R*)-1,2-diaminocyclohexanedichloridoplatinum(II) **13** (PtDACH) were synthesized as described.[26,27,63] Prior to use, aqueous solutions (500 μM) of all Pt^{II} compounds (300 μM for **2**) 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.

Platinum Quantification by Atomic Absorption Spectrometry. The determination of Pt^{II} concentrations in the stock solutions was performed in triplicate using a SpectrAA-110 flame atomic

absorption spectrophotometer (Varian AG, Switzerland). Prior to metal analysis the samples were diluted into 25 mM $LaCl_3$, 100 mM HCl. Pt-standards of 0.25, 0.5, 1.0, 2.0, 3.0, 4.0, and 5.0 ppm were prepared from a commercially available 1000 ppm standard (Fluka) using the same matrix solution.

Characterization of Pt^{II} Containing MT-2 Species by Mass Spectrometry. Samples of Zn_7MT-2 were incubated with Pt^{II}-compounds **1**−**3**, **5**−**12**, and [Pt(dien)Cl]Cl as described below. Afterward, unbound Pt^{II} compound was removed and the solvent simultaneously exchanged by passing 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). NanoESI-MS analysis of the protein-containing solution was performed on a Q-TOF Ultima API mass spectrometer (Micromass, UK). Solutions were infused through a fused silica capillary (ID 75 μm) at a flow rate of 0.5 μL min^{-1}. Electrospray Pico Tips (ID 30 μm) were obtained from New Objective (Woburn, MA). Mass spectra were acquired by scanning an *m*/*z* range from 600 to 2500 with scan duration of 1 s and an interscan delay of 0.1 s. The spray voltage was set to 2.1 kV, cone voltage to 50 V, RF lens 1 energy to 50 V, and collision to 15. Mass spectra were deconvoluted using the MaxEnt 1 software (Micromass, UK).

Time-dependent measurements of the reaction of Zn_7MT-2 with cisplatin were essentially performed as described above. Aliquots of the sample were taken after 5 min and 5, 10, and 20 h and 0.01% (v/v) HOAc was added. Before injection, samples were desalted using C_{18}-ZipTips (Millipore) from which the protein was eluted with 10 μL of 50:50:0.01 (v/v/v) CH_3OH/H_2O/HOAc (pH ∼ 5).

Calibration of Pt^{II}MT-2 Absorption. Zn_7MT-2 was incubated with 2 mole equivalents of **1** or **2** in 1 mL volumes (see above). After 120 h at 37 °C, the excess of *cis*/*trans*-$[Pt(NH_3)_2(H_2O)_2]^{II}$ was removed by a 5 mL HiTrap Desalting column (Amersham Biosciences) in 10 mM NaH_2PO_4/NaOH, pH 7.4, and the samples were concentrated with Microcon-3 ultrafiltration devices (Millipore). The absorption at 285 nm was recorded and the amount of Pt determined by flame atomic absorption spectrometry. The concentration of MT-2 was determined by amino acid analysis after the protein hydrolysis in 6 M HCl at 110 °C for 22 h. Hydrolyzed samples were dissolved in 80 μL 50 mM HCl containing 50 μM norvaline and 50 μM sarcosine as internal standards. Upon centrifugation, 1-μL samples were injected into an Amino Quant amino acid analyzer (Agilent). The MT-2 concentration was determined by using the amino acids Gly, Ala, Val, and Ile.

Binding Kinetics of Pt^{II} Compounds to MT-2 Thiols. The binding of Pt^{II} compounds **1**−**3**, **5**−**13**, and [Pt(dien)Cl]Cl to Zn_7-MT-2 was monitored by absorption spectroscopy. Zn_7MT-2 in 10 mM HEPES/NaOH, pH 7.4, 100 mM $NaClO_4$, and solutions of various Pt^{II} compounds were rendered oxygen-free by three freeze-pump cycles on the vacuum line and placed into a nitrogen-purged glove box in the dark for up to a week 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 sealed. The time courses of the reactions were followed spectrophotometrically (Ultrospec 2000, Amersham Biosciences) at 285 nm for 120 h at 37 °C. Data were analyzed, including a normalization per Pt^{II}−S bond using $\epsilon_{285\,nm} = 2680\ M^{-1}\ cm^{-1}$ (see Results), and fitted using the Origin 7.0 software package (OriginLab Corp.).

Kinetics of Zn^{II} Release from Zn_7MT-2 upon Pt^{II} Binding. To monitor Zn^{II} release, experiments were performed and analyzed as described above for the Pt^{II} binding to Zn_7MT-2 thiols, except that 10 μL of 10 mM 4-(2-pyridylazo)resorcinol (PAR) dissolved in 100 mM NaOH was also added. As a measure for Zn^{II} release, changes in the absorption were monitored at 500 nm ($\epsilon_{500nm} = 65000\ M^{-1}\ cm^{-1}$).[37]

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