Interaction of Metallothionein-2 with Platinum-Modified 5′-Guanosine Monophosphate and DNA[†]

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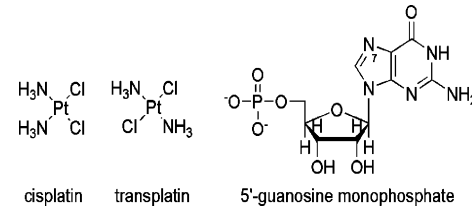
Received July 3, 2008; Revised Manuscript Received August 14, 2008

ABSTRACT: Human metallothioneins (MTs), a family of cysteine- and metal-rich metalloproteins, play an important role in the acquired resistance to platinum drugs. MTs occur in the cytosol and the nucleus of the cells and sequester platinum drugs through interaction with their zinc−thiolate clusters. Herein, we investigate the ability of human Zn_7MT-2 to form DNA−Pt−MT cross-links using the cisplatin- and transplatin-modified plasmid DNA pSP73. Immunochemical analysis of MT-2 showed that the monofunctional platinum−DNA adducts formed DNA−*cis*/*trans*-Pt−MT cross-links and that platinated MT-2 was released from the DNA−*trans*-Pt−MT cross-links with time. The DNA−*cis*/*trans*-Pt−MT cross-links were also formed in the presence of 2 mM glutathione, a strong S-donor ligand. Independently, we used 5′-guanosine monophosphate (5′-GMP) platinated at the N7 position as a model of monofunctional platinum−DNA adducts. Comparison of reaction kinetics revealed that the formation of ternary complexes between Zn_7MT-2 and *cis*-Pt−GMP was faster than that of the *trans* isomer. The analysis of the reaction products with time showed that while the formation of ternary GMP−*trans*-Pt−MT complex(es) is accompanied by 5′-GMP release, a stable ternary GMP−*cis*-Pt−MT complex is formed. In the latter complex, a fast initial formation of two Pt−S bonds was followed by a slow formation of an additional Pt−S bond yielding an unusual Pt(II)S_3N coordination with N7-GMP as the only N-donor ligand. The ejection of negligible zinc from the zinc−thiolate clusters implies the initial formation of Zn−(μ-SCys)−Pt bridges involving the terminal thiolate ligands. The biological implications of these studies are discussed.

Cisplatin {*cis*-[Pt$(NH_3)_2Cl_2$]} and a few related platinum complexes, such as carboplatin and oxaliplatin, belong to the most widely used group of anticancer therapeutic agents (*1, 2*). These classical Pt(II) antitumor drugs act mainly through binding to guanine (G) bases of nuclear DNA, causing its modification which directs a cell into apoptosis or necrosis. 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 N7-G bases distort the DNA structure such that translation and nucleotide excision repair (NER)[1] are strongly inhibited (*1, 3, 4*). The geometric isomer of cisplatin, transplatin {*trans*-[Pt$(NH_3)_2Cl_2$]} (Chart 1) is clinically ineffective (*4*).

Chart 1



The occurrence of intrinsic resistance in some tumors and that acquired after initial treatment are the major drawbacks of these chemotherapeutics. The mechanisms underlying the resistance of tumors to cisplatin are multifactorial. Potential responses leading to the resistance include changes in intracellular accumulation of the drug, enhanced cellular detoxification by the intracellular thiols glutathione (GSH) and metallothionein (MT), increased capability of cells to repair cisplatin−DNA damage, and a failure to initiate apoptosis in the presence of platinated DNA (*5*). Due to the strong reactivity of platinum compounds toward S-donor molecules and the formation of kinetically very stable Pt(II)−S bonds, intracellular thiols through their competition with DNA confer resistance to antitumor platinum drugs (*6, 7*). GSH deactivates cisplatin, and in certain cancer cell lines, its cellular concentration increases after their exposure to Pt(II) drugs (*6*). However, compared to GSH, the thiolate ligands of MT react with cisplatin faster (*8*). From the four MT isoforms expressed in humans (designated MT-1−MT-4), MT-1 and MT-2 occur ubiquitously in large amounts in

[†] This work was financially supported by Swiss National Science Foundation Grant 3100A0-111884/1, the 'Krebsliga Zürich', and the 'Hermann-Klaus Stiftung' (all to M.V.).

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[1] Abbreviations: AAS, atomic absorption spectroscopy; *cis*-Pt−GMP, *cis*-[Pt$(NH_3)_2$X(GMP)], where X is H_2O, HO^-, or Cl^-; *cis*-Pt, cisplatin or *cis*-dichlorodiammineplatinum(II); ESI-MS, electrospray ionization mass spectrometry; DMSO, dimethyl sulfoxide; GMP, 5′-guanosine monophosphate; GSH, glutathione; MT, metallothionein; NER, nucleotide excision repair; PAR, 4-(2-pyridylazo)resorcinol; r_b, number of platinum atoms bound per nucleotide; *trans*-Pt, transplatin or *trans*-dichlorodiammineplatinum(II); *trans*-Pt−GMP, *trans*-[Pt$(NH_3)_2$-X(GMP)], where X is H_2O, HO^-, or Cl^-.

10.1021/bi801253x CCC: $40.75
Published on Web 09/20/2008

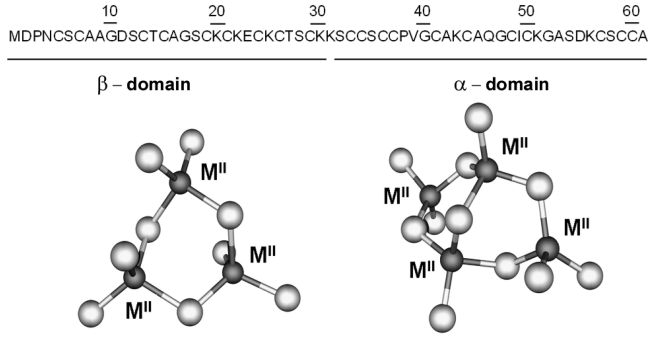

FIGURE 1: Amino acid sequence and structures of the $M^{II}_4S_{11}$ cluster (α-domain) and the $M^{II}_3S_9$ cluster (*β*-domain) in human M^{II}_7MT-2 (*59*).

mammalian cells. In contrast to MT-3 and -4, the biosynthesis of MT-1 and -2 can be induced by a variety of compounds, including hormones, cytokines, and metal ions, including cisplatin (*9*−*12*). The present knowledge suggests that MTs can have different functions in a number of biological processes. These include homeostasis and transport of physiologically essential metals (Zn and Cu), detoxification of toxic metals (Cd), protection against oxidative stress, maintenance of intracellular redox balance, regulation of cell proliferation and apoptosis, a neuroprotective role, and regulation of neuronal outgrowth (*9, 10, 12*−*14*). It has been shown that overexpression of MT-1 and -2 confers resistance to platinum anticancer drugs in many cancer cell lines (*15*). Moreover, the studies of MT-1 and -2 knockout cells revealed a higher sensitivity to the treatment with cisplatin (*16*). The increased level of expression of MT-1 and -2 and the recent finding of high concentrations of MT-3 in many cancers have been considered to be a bad prognostic factor in anticancer treatment (*17, 18*). These results suggest a significant contribution of MTs to the acquired Pt(II) drug resistance. Although MT-1 and -2 are cytosolic proteins, in normal development and cancer they are also found in the nucleus. The presence of MT-1 and -2 in the nucleus is believed to be important for the protection of DNA from oxidative damage and chemotherapeutic drugs (*19, 20*). The classical anticancer compound cisplatin may also form DNA−Pt−protein cross-links which has been shown, for instance, for chromosomal proteins, histones, cytokeratins, and other DNA-binding proteins (*21*−*24*). These adducts inhibit DNA polymerization and NER more effectively than DNA adducts not linked to proteins. The proposed reasons are increased bulkiness and different effects of such complexes on DNA geometry. DNA cross-links with proteins can be formed not only with monofunctional cisplatin adducts but also with 1,2- or 1,3-intrastrand DNA cross-links (*25*). Transplatin is also able to induce cross-links with proteins, but they are less persistent and repaired faster in vivo (*24*−*26*).

Mammalian MTs are small metalloproteins composed of a single polypeptide chain of 61−68 amino acids, of which 20 are cysteines. The cysteine thiolates are involved in the binding of seven divalent metal ions forming two independent metal−thiolate clusters in which each metal is tetrahedrally coordinated by both bridging and terminal thiolate ligands (Figure 1). A $M^{II}_3CysS_9$ cluster is located in the *β*-domain and a $M^{II}_4CysS_{11}$ cluster in the α-domain of the protein (*14, 27*). Although naturally occurring MTs contain Zn(II) ions, these can be displaced by metal ions in vivo that have a higher affinity for thiolates such as Cd(II), Hg(II), and Pt(II). Molecular mechanisms of the interaction of Pt(II) with MT-1 and -2 were the subject of a wide range of studies, including our previous work (*28*−*31*). There is evidence indicating that both *cis*- and *trans*-Pt(II) compounds bind preferentially to the *β*-domain of the protein and that equimolar amounts of Zn(II) are released during this process. Furthermore, whereas all ligands in the MT-2 complexes with *cis*-Pt(II) compounds are replaced with cysteine thiolates, in the complexes with *trans*-Pt(II) compounds their N-donor ligands are retained, preserving *trans*-Pt(II) in a potentially active form (*28*−*31*).

The aim of this work was to understand the role of human Zn_7MT-2 present in the nucleus in acquired resistance against Pt(II) drugs by studying its interaction with the cisplatin- and transplatin-modified plasmid DNA pSP73 and 5′-guanosine monophosphate (5′-GMP) used as a model compound. The studies were carried out under physiological-like conditions with regard to pH, ionic strength, and temperature. The results reveal that Zn_7MT-2 forms DNA−Pt−protein cross-links even in the presence of an excess of GSH.

EXPERIMENTAL PROCEDURES

Protein Expression and Purification. The construct of human MT-2 in the *Escherichia coli* BL21(DE3) $pLysS^{Cam}$ strain was prepared using the pET-3d expression vector as described previously (*31*). The deletion mutant of MT-2 devoid of Met^1 ($MT\text{-}2^{mut}$) (Figure 1) was prepared by introducing a Gly^2Ser^3 linker after the Met^1 by PCR, using the following primers: 5′-TAT ATT ACC ATG GGC AGC GAT CCC AAC TGC TCC TGC GC-3′ (sense) and 5′-TAT ATT AGG ATC CTC AGG CGC AGC AGC-3′ (antisense). The PCR product was digested with NcoI and BamHI restriction enzymes and ligated into a pET-3d vector. This change in the protein sequence should enable the processing of Met^1 by *E. coli* methionine aminopeptidase (*32*). However, on the basis of ESI-MS analysis of the apoprotein, ∼about 30% of the expressed MT-2 mutant still contained unprocessed Met. Since changes in the expression conditions and *E. coli* strains were unsuccessful, we have chemically modified the residual methionine sulfur in the reaction with DMSO to methionine sulfoxide (*33*). Prior to the reaction of Met^1 with DMSO, cysteine sulfurs of the apoprotein were protected with 2-thiopyridine by incubation of 10 *μ*M MT-2 with a 2-fold molar excess of 2,2-dithiopyridine in 0.1 M NaAc (pH 4.0) and 1 mM EDTA. The modification of all 20 cysteines was confirmed through the absorption spectroscopy of 2,2-dithiopyridine (*34*). The DMSO modification of Met^1 to sulfoxide was carried out in 1 M HCl and 0.1 M DMSO at 22 °C for 30 min (*33*). The 2-thiopyridine modification of cysteines was reversed by rebuffering the protein into 100 mM potassium phosphate (pH 5.5), 20 mM TCEP, and 2 M guanidinum-HCl with stirring (2 h) under an argon atmosphere. Subsequently, the pH was adjusted to 2.0 and the apoprotein purified by gel filtration chromatography (Superdex 75 column) in 10 mM HCl and reconstituted with zinc as described previously (*31, 35*). The ESI-MS analyses revealed the presence of ∼10% unmodified Met (data not shown). This form was used in the studies and is designated as $Zn_7MT\text{-}2^{mut}$. The purity of the proteins was checked with 15% SDS−PAGE gels. The concentration of

apoMT-2 was determined spectroscopically at 220 nm (ε = 48200 M^{-1} cm^{-1}) in 100 mM HCl and that of Zn(II) by atomic absorption (*34*). In all cases, a Zn(II):MT-2 or Zn(II):MT-2^{mut} ratio of 6.8 ± 0.3 was obtained. In MT-2 and MT-2^{mut}, the thiol:protein ratio was 20 ± 1 (*34*).

Pt(II) Compounds. Aqueous solutions (500 μM) of *cis*-diamminedichloroplatinum(II) (cisplatin) and *trans*-diamminedichloroplatinum(II) (transplatin) were kept in the dark at 4 °C for at least 2 days to allow their hydrolysis. The Pt(II) solutions were freshly prepared after 1−2 months. Pt(II) concentrations were determined by flameless or flame atomic absorption spectroscopy (Varian SpectraAA-110 and AA240FS) as required.

Preparation and Purification of cis- and trans-Pt−GMP. *cis*- and *trans*-[$Pt(NH_3)_2X(GMP)$], where X is Cl^-, H_2O, or HO^- (further abbreviated as *cis*- and *trans*-Pt−GMP, respectively; a total charge of the complexes is ignored for clarity), were prepared by incubation of 10 mol equiv of cisplatin or transplatin with 5′-GMP (Sigma-Aldrich Chemie) in 10 mM $NaClO_4$ for 24 h at 37 °C and subsequently purified by reversed phase HPLC (*36*). The separation was performed on the MAG1, Biospher PSI 100 C_{18}, 4.6 mm × 150 mm HPLC column (Labio a.s.) using a linear gradient of MeOH (1 to 60%) in 0.1 M NH_4CH_3COO (pH 5.4). *cis*- and *trans*-Pt−GMP elute at 2−3% MeOH and the *cis*- and *trans*-Pt−$(GMP)_2$ adducts at 5% MeOH, and free cisplatin (transplatin) elutes in the void volume. The isolated *cis*- and *trans*-Pt−GMP were lyophilized and redissolved in H_2O. The correctness of these platinated products was verified by ESI-MS in a 50:50:0.2 (v/v/v) CH_3CN/H_2O/HOAc mixture (final pH of ∼3) using the MS conditions described in the characterization of human MT-2 (*31*). Mass spectra were deconvoluted using MaxEnt 1 (Micromass). The ESI-MS characterization of platinum−GMP adducts revealed that the mass peaks corresponding to the aquated forms of *cis*- and *trans*-Pt−GMP (calculated mass of 609.3 Da, observed mass of 609.0 Da) were prevailing and a smaller part was chlorinated (∼30%) (calculated mass of 650.7 Da, observed mass of 651.2 Da) (Supporting Information). The quantification of GMP and platinated GMP was performed using an extinction coefficient ε_{260} of 10250 M^{-1} cm^{-1} (see Results).

Kinetics of the Reaction of Pt(II)−GMP with the Thiols of Zn_7MT-2. Zn_7MT-2 (10 μM) was mixed with *cis*- or *trans*-Pt−GMP in a molar ratio of 1:2 in 10 mM HEPES (pH 7.4) and 100 mM $NaClO_4$. The formation of Pt−S bonds was monitored at 285 nm (ε = 2680 M^{-1} cm^{-1}) (*31*) over 120 h in the sealed thermostated cuvette at 37 °C. After incubation for 1, 24, 72, and 120 h, the reaction mixture was concentrated using Microcon YM-3 concentrators (molecular mass cutoff of 3 kDa) (Millipore), and the concentrations of Pt(II), GMP, and Zn(II) in the high- and low-molecular mass fractions were determined. The high-molecular mass fraction contained MT-2 and bound platinated GMP. The low-molecular mass fraction contained the released Zn(II) from the protein, not bound platinated GMP, and the released nonplatinated 5′-GMP. The concentration of Zn(II) bound to the protein was determined by atomic absorption spectroscopy. The concentration of Zn(II) released from Zn_7MT-2 was determined spectrophotometrically at 500 nm through its complex with the dye 4-(2-pyridylazo)resorcinol (PAR) (Fluka AG). PAR (100 μM) dissolved in 100 mM NaOH was added to the sample and the absorption of the Zn(II)PAR_2 complex determined at 500 nm (ε = 65000 M^{-1} cm^{-1}) (*31*). The concentrations of both GMP and platinated GMP adducts were determined via absorption at 260 nm. To separate the residual not bound 5′-GMP and platinated GMP from the protein, the protein fraction was washed (three times) with the incubation buffer and the UV−vis spectrum recorded between 220 and 350 nm.

^{1}H NMR of the Products of the Reaction of cis- and trans-Pt−GMP with Zn_7MT-2. Zn_7MT-2 (100 μM) buffered in 10 mM d_{11}-HEPES (pH 7.4), 100 mM $NaClO_4$, and 10% D_2O was incubated in a nitrogen atmosphere with 2 mol equiv of *cis*- or *trans*-Pt−GMP at 37 °C. The aliquots withdrawn after 24 and 120 h were separated using Microcon YM-3 concentrators. The ^{1}H NMR spectra of the low-molecular mass fractions were acquired at 20 °C on a Bruker Avance 600 MHz spectrometer equipped with a cryogenic Z-gradient TXI probe. Acquisition parameters for the ^{1}H NMR experiments were as follows: sweep width, 12 kHz (20 ppm); acquisition pulse, 9.2 μs; relaxation delay, 1 s; total acquisition time, ∼4 min (256 transients). Water suppression was achieved by application of the presaturation pulse. The NMR data were processed and analyzed with XWINNMR version 3.2 (Bruker BioSpin). The ^{1}H chemical shifts are referenced to the resonance of sodium 2,2-dimethyl-2-silapentane-5-sulfonate.

Interaction of the Platinated pSP73 Plasmid with Zn_7MT-2. pSP73 plasmid (200 μg/mL) (Fermentas GMBH) having 2464 bp was incubated with cisplatin or transplatin for 24 h in 10 mM $NaClO_4$ at 37 °C in darkness to r_b values of 0.05. The plasmid was separated from free platinum on a GFC300 gel filtration column (Macherey-Nagel) in 5 mM HEPES (pH 7.4) and 50 mM $NaClO_4$. Subsequently, the pSP73 plasmid was concentrated with Microcon YM-30 concentrators (molecular mass cutoff of 30 kDa) (Millipore), and the r_b value was verified by atomic absorption spectroscopy. Then, 260 μL of 20 μM Zn_7MT-2 was mixed with the 34 μg (560 μg/mL) of platinated pSP73 plasmid at a Pt(II):protein ratio 1:1. After incubation for 24 and 72 h, the incubation mixture was separated on a GFC300 size exclusion column in 5 mM HEPES (pH 7.4) and 50 mM $NaClO_4$. The DNA fraction was pooled, concentrated to volumes of <20 μL, and applied to the nitrocellulose paper. DNA has been fixed by baking the nitrocellulose paper at 85 °C for 40 min (*37*). The DNA fixation was evaluated by staining with SYBR Gold dye (Molecular Probes Inc.). The immunochemical detection of the protein in dots was as described for MT-3 (*38*), the only exception being that in our case the primary polyclonal antibody (anti-hMT-2) was used. The sensitivity of Western blotting in our experiments was 0.5 ng of protein. In independent experiments, the monofunctional *cis*- and *trans*-Pt−DNA adducts were blocked or labilized by adding 10 mM thiourea to the platinated pSP73 plasmid at 30 °C for 10 min (*39*). The plasmid was subsequently purified for incubation with Zn_7MT-2 (vide supra).

RESULTS

Absorption Spectroscopy of Platinated 5′-GMP. 5′-GMP platinated at the N7 position with cisplatin and transplatin was chosen in this study as a model of Pt(II)−DNA monofunctional adducts. In our previous studies on the interaction of Zn_7MT-2 with a number of *cis*/*trans*-[Pt(N-

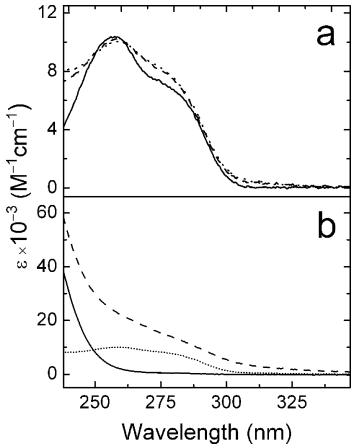

FIGURE 2: UV−vis absorption spectra of (a) 5′-GMP (—), *cis*-Pt−GMP (•••), and *trans*-Pt−GMP (---) in 10 mM HEPES (pH 7.4) and 100 mM $NaClO_4$ and (b) Zn_7MT-2 (—), *cis*-Pt−GMP (•••), and Zn_7MT-2 (---) after incubation for 24 h with *cis*-Pt−GMP in 10 mM HEPES (pH 7.4) and 100 mM $NaClO_4$ at 37 °C under a N_2 atmosphere.

donor)$_2$Cl$_2$] compounds, a molar extinction coefficient for the Pt−S bond of 2680 M^{-1} cm^{-1} at 285 nm was determined (*31*). To allow for a similar quantification of Pt−S bonds involved in the binding of platinated GMP to Zn_7MT-2, the spectroscopic properties of platinated GMP adducts were compared with those of 5′-GMP, which may be released in the binding process. The molar extinction coefficients of platinated GMP adducts (Pt:GMP ratio of 1:1) at 260 nm were determined using the concentration of platinated GMP derived from Pt quantification. The absorption spectra of both *cis*- and *trans*-Pt−GMP adducts and 5′-GMP show at 260 nm an identical molar extinction coefficient of 10250 M^{-1} cm^{-1} (Figure 2a). Platination of 5′-GMP resulted in only a minor contribution to its absorption at 285 nm ($\Delta\varepsilon = 300$ M^{-1} cm^{-1}). This contribution was neglected in the estimation of the Pt−S bonds formed with these complexes as a function of time. For illustration, the absorption features introduced by the binding of *cis*-Pt−GMP to the thiolate ligands of MT-2, normalized per protein concentration, are shown in Figure 2b.

Kinetics of the Reaction of Zn_7MT-2 with 2 mol equiv of cis- and trans-Pt−GMP. Incubations of human Zn_7MT-2 with 2 mol equiv of *cis*- or *trans*-Pt−GMP were carried out in the presence of 100 mM $NaClO_4$, which does not coordinate Pt(II), and 10 mM HEPES (pH 7.4) at 37 °C. We chose a Pt−GMP:MT ratio of 2:1 as it enables a comparison with the results obtained in our previous studies in which the interaction of 2 mol equiv of *cis*/*trans*-[Pt(N-donor)$_2$Cl$_2$] compounds with Zn_7MT-2 was studied (*31*). It may be noted that administration of cisplatin or transplatin to rabbits yielded the species $Pt_{\sim 2}Zn_{\sim 5}$MT (*40*). Because of the low intracellular Cl^- concentration (4−23 mM) (*41*), platinum-based drugs upon entering the cell can hydrolyze, resulting in replacement of Cl^- with H_2O or OH^-. The exchange of the leaving ligand is an important step in activating the compound for its reaction with N7-G (*42*). However, in the previous studies, the reaction kinetics of rat and rabbit Zn_7MT-2 with cisplatin and transplatin were found to be largely independent of the leaving ligand (*29, 43*). This finding is in agreement with the only marginally altered reactivity of the low-molecular mass S-donor compounds

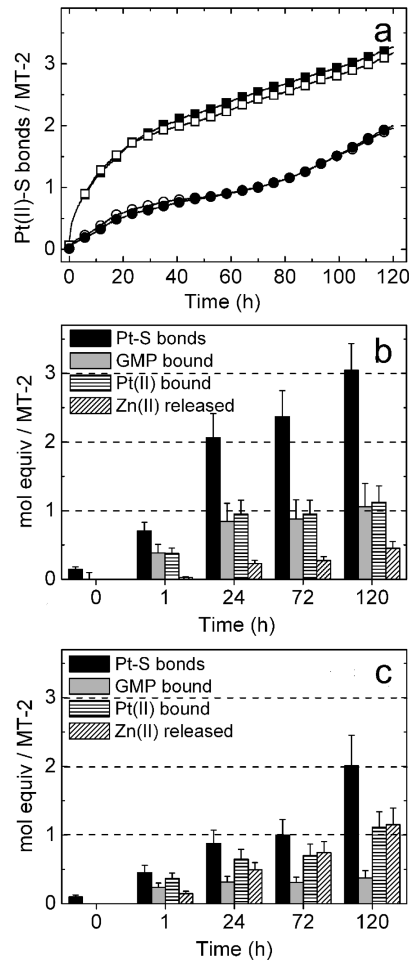

FIGURE 3: (a) Kinetics of the binding of 20 μM *cis*-Pt−GMP (■) or *trans*-Pt−GMP (●) to 10 μM human Zn_7MT-2 and that of *cis*-Pt−GMP (□) or *trans*-Pt−GMP (○) to Zn_7MT-2^{mut} in 10 mM HEPES (pH 7.4) and 100 mM $NaClO_4$ at 37 °C. Complex formation with time was monitored through absorption changes at 285 nm. The absorption traces were normalized to the number of Pt−S bonds per MT-2. (b and c) Product analysis of the reaction between Zn_7MT-2 and (b) *cis*-Pt−GMP or (c) *trans*-Pt−GMP as a function of time normalized per MT-2.

cysteine and GSH toward cisplatin, transplatin, and [Pt(dien)Cl]$^+$ upon replacement of Cl^- with H_2O or OH^- (*42, 44*). Therefore, the degree of hydrolysis of the studied platinated GMP adducts should only marginally influence their reactivity with cysteine thiolates of Zn_7MT-2. This conclusion is supported by the identity of the reaction kinetics of Zn_7MT-2 with both platinated GMP adducts obtained in the presence and absence of 20 mM NaCl in the incubation mixture (data not shown).

The used Pt−GMP:Zn_7MT-2 ratio of 2 implies that pseudo-first-order conditions cannot be fulfilled. Therefore, to allow the comparison of reaction velocities, the time courses of Pt−GMP binding to Zn_7MT-2 were recorded under identical conditions. The formation of Pt−S bonds in MT-2 with time was followed at 285 nm, and the obtained values were normalized per Pt−S bond using an ε of 2680 M^{-1} cm^{-1} (*31*). The reaction of thiolates with Pt(II) is a very slow process (*40, 45, 46*); therefore, the reaction kinetics were followed for up to 120 h (Figure 3a). To prevent Cys oxidation by air oxygen during the prolonged sample incubation, experiments were performed under anaerobic conditions. The kinetics of formation of the Pt−S bond in

the reaction between Zn_7MT-2 and *cis*-Pt−GMP was biphasic with a rapid 24 h phase followed by a slow linear absorption increase. However, the kinetics of the reaction with *trans*-Pt−GMP was slower and showed a rapid phase after ~70 h. From the initial slopes of the kinetic traces (Figure 3a), apparent initial rates (k_{obs}) were derived. A comparison of the values for *cis*-Pt−GMP of $(1.1 \pm 0.2) \times 10^{-4}$ min^{-1} with that for *trans*-Pt−GMP of $(0.3 \pm 0.1) \times 10^{-4}$ min^{-1} reveals that *cis*-Pt−GMP reacted with Zn_7MT-2 ~4 times faster. The results show, moreover, that the incubation of Zn_7MT-2 with 2 mol equiv of *cis*-Pt−GMP for 24 h resulted in the formation of approximately two Pt−S bonds per MT-2 and approximately three Pt−S bonds after 120 h. In contrast, in the case of *trans*-Pt−GMP, the formation of approximately one Pt−S bond per MT-2 occurred only after 72 h. In the following faster kinetic phase, approximately two Pt−S bonds per MT-2 were formed after 120 h. In Zn_7MT-2, only Cys residues are involved in metal binding. However, the participation of the thioether of Met[1] in the binding of Pt(II) in fully metal loaded Pt_7MT has been inferred (*47*). To examine whether N-terminal thioether Met[1] is involved in the binding of Pt−GMP adducts, we carried out similar kinetic studies using the Met[1] deletion mutant Zn_7MT-2^{mut}. Closely similar kinetic traces of wild-type Zn_7MT-2 and Zn_7MT-2^{mut} indicate that the thioether of Met[1] is not involved in the binding of *cis*- and *trans*-Pt−GMP (Figure 3a). Overall, reaction kinetics show that with both Pt−GMP adducts the reaction is not completed in 120 h.

Characterization of the Ternary GMP−Pt−Protein Complexes. Attempts to characterize the products by ESI-MS failed. Therefore, to examine the products formed in the course of the reaction of Zn_7MT-2 with 2 mol equiv of *cis*- and *trans*-Pt−GMP, aliquots were withdrawn from the incubation mixture after 1, 24, 72, and 120 h and the high- and low-molecular mass components separated by ultrafiltration using a 3 kDa membrane. The monomeric nature of the protein was confirmed by gel filtration chromatography of the incubation mixtures after a 120 h incubation. The separated high-molecular mass fraction containing the protein and its conjugate was analyzed for Pt by atomic absorption and for the Pt−S bonds by absorption spectroscopy. In the low-molecular mass fraction, the concentration of Pt reflects the unbound *cis*- or *trans*-Pt−GMP adducts, the absorption at 260 nm the concentrations of platinated and free 5′-GMP, and the determined Zn(II) concentration the zinc released in the reaction. The concentrations of GMP bound to MT-2 were obtained by subtracting the concentrations of platinated and free 5′-GMP in the low-molecular mass fraction from the initial *cis*-/*trans*-Pt−GMP concentration. The results normalized per MT-2 concentration are shown in panels b and c of Figure 3.

The results for *cis*-Pt−GMP show that although 2 mol equiv of *cis*-Pt−GMP was added, only ~1 equiv was bound to the protein (Figure 3b). The preservation of the 1:1 molar ratio between MT-2 and Pt(II) after incubation for 24 and 120 h suggests that the formation of the ternary GMP−*cis*-Pt−protein complex was completed already after 24 h. Evidence that no 5′-GMP was released from this complex was obtained from the ^{1}H NMR spectrum of the low-molecular mass fraction recorded after a 120 h sample incubation, where no H8 resonance of free 5′-GMP was detected. In our previous studies, the binding of cisplatin to

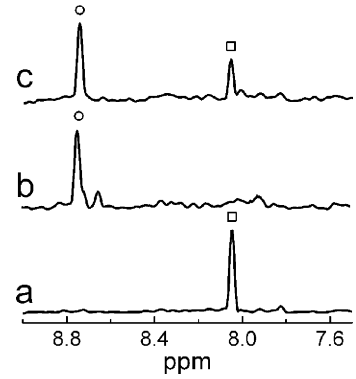

FIGURE 4: ^{1}H NMR spectra of (a) 5′-GMP and (b) *trans*-Pt−GMP in 10 mM d_{11}-HEPES (pH 7.4) and 100 mM $NaClO_4$ at 25 °C. (c) Low-molecular mass fraction obtained upon incubation of *trans*-Pt−GMP with Zn_7MT-2 for 24 h in 10 mM d_{11}-HEPES (pH 7.4) and 100 mM $NaClO_4$ at 37 °C. The H8 resonances of free GMP (□) and platinated GMP (○) are shown.

MT-2 resulted in the release of an equimolar concentration of Zn(II) from the protein (*31*). In contrast, the binding of 1 equiv of *cis*-Pt−GMP to Zn_7MT-2 released only ~0.2 mol equiv of Zn(II) in 24 h and 0.45 mol equiv in 120 h.

Similar analyses of the products formed in the reaction of Zn_7MT-2 with 2 mol equiv of *trans*-Pt-GMP as a function of time are shown in Figure 3c. Although in this case also the ternary GMP−*trans*-Pt−protein complexes are formed, the changes in the molar ratios among the determined individual components of the samples with time suggest that no single product was formed and that the reaction is rather complex. Both the decreasing ratio of Pt(II) to 5′-GMP in the protein fraction and the increasing number of thiolate ligands involved in the Pt(II) coordination with time suggest that due to the strong *trans* effect of sulfur 5′-GMP is released. Evidence for its release from the ternary GMP−*trans*-Pt−protein complex was obtained from the ^{1}H NMR studies carried out with the low-molecular mass fraction isolated after the sample incubation for 24 h. The ^{1}H NMR spectra of free 5′-GMP, *trans*-Pt−GMP, and the isolated low-molecular mass fraction are shown in Figure 4. The NMR spectrum presented in Figure 4a shows, besides the H8 resonance of *trans*-Pt-GMP at 8.7 ppm, the H8 resonance of free 5′-GMP at 8.1 ppm (*48*). Thus, different complexes are formed upon the reaction of Zn_7MT-2 with *trans*-Pt−GMP as a function of time.

Interaction of Zn_7MT-2 with Platinated DNA. We have extended our studies to plasmid circular DNA that is a widely used as a model in DNA platination studies. We used the immunochemical detection of the protein in analyzing the DNA−Pt−MT cross-links formed with the rather bulky and negatively charged Zn_7MT-2 (overall charge of −2). The pSP73 plasmid platinated with cisplatin and tranplatin to an r_b of 0.05 was incubated with Zn_7MT-2 at an equimolar ratio to bound Pt(II) (20 μM) for 24 and 72 h. The incubation mixture was separated by size exclusion chromatography and the high-molecular mass DNA fraction analyzed by Western blotting of the protein in dots (Figure 5a). The DNA−Pt−protein cross-links between cisplatin- and transplatin-modified DNA and Zn_7MT-2 were detected after incubation for 24 h with an increasing intensity of protein staining after 72 h (Figure 5a). Thiourea traps the monofunctional adduct of cisplatin with DNA in a stable adduct and labilizes the monofunctional adducts of DNA with transplatin (*49, 50*). The absence of

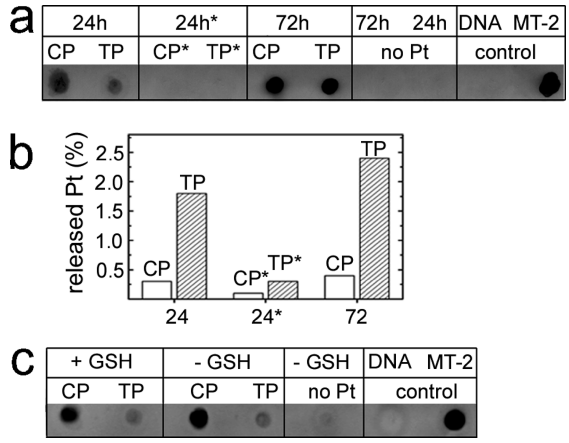

FIGURE 5: (a) Immunostaining of the protein after incubation of Zn_7MT-2 with the pSP73 plasmid (DNA) platinated with cisplatin (CP) or transplatin (TP) for 24 and 72 h to an r_b of 0.05. As a control, pSP73 plasmid (DNA), Zn_7MT-2 (MT-2), and nonplatinated pSP73 plasmid incubated with Zn_7MT-2 (no Pt) are presented. (b) Percent of platinum bound to free MT-2 upon the 24 and 72 h incubation of platinated DNA with Zn_7MT-2. Asterisks in panels a and b denote the samples in which the monofunctional DNA adducts were trapped by thiourea prior to incubation with Zn_7MT-2. (c) Immunostaining of the protein after incubation of the plasmid DNA pSP73 platinated with cisplatin (CP) or transplatin (TP) with Zn_7MT-2 for 24 h in the presence (+GSH) and absence (−GSH) of 2 mM glutathione. DNA was separated from unbound MT-2 by size exclusion chromatography and applied to nitrocellulose paper.

protein staining in the platinated DNA samples treated with thiourea indicated that monofunctional DNA adducts are involved in the formation of DNA−Pt−protein cross-links (Figure 5a). Furthermore, the absence of protein staining after the incubation of the DNA−Pt−MT cross-link with the strong nucleophile KCN confirmed the presence of platinum-mediated cross-links (data not shown) (*23*). A comparison of the platinum content of MT-2 after incubation with the cisplatin- or transplatin-modified plasmid for 24 and 72 h showed an increased level of removal of platinum from DNA platinated with transplatin, but not with cisplatin (Figure 5b). This suggests that transplatin is slowly removed, forming (*trans*-Pt)Zn_6MT-2. The absence of protein staining upon the incubation of Zn_7MT-2 with unplatinated DNA and the cross-reactivity of the primary and secondary antibody with platinated DNA confirmed the formation of the DNA−Pt−protein cross-links.

In the nucleus, besides MT-1 and -2, a high concentration of the intracellular thiol glutathione (GSH) is present. This nucleophile could compete with MT for the formation of cross-links with DNA. Therefore, we incubated the platinated plasmid with 20 μM Zn_7MT-2 in the presence of 2 mM GSH for 24 h. Western blot analyses revealed that the DNA−Pt−MT cross-links were also formed in the presence of GSH (Figure 5c).

Interaction of trans-Pt_2Zn_5MT-2 Complexes with GMP, Oligonucleotides, and DNA. The known high affinity of Pt(II) compounds for sulfur atoms and the great abundance of sulfur-containing biomolecules in cytosol and nucleus of the cell raised the question of whether Pt−sulfur interactions could serve as a drug reservoir affording an additional pathway toward platination of DNA (*7*). The studies using $[Pt(dien)(L\text{-methionine-}S)]^+$, $[Pt(dien)(S\text{-methyl-GSH-}S)]^{2+}$, $[Pt(dien)(GSH\text{-}S)]^+$, and *cis*-$[Pt(NH_3)_2(S\text{-methyl-GSH-}S)_2]^{2+}$ showed that a transfer to 5′-GMP-N7 and d(GpG) was possible from the thioethers, but not from $[Pt(dien)(GSH\text{-}S)]^+$ (*51*). In the MT structure, all cysteine residues are involved in metal binding, forming Zn−S bonds in which the sulfur nucleophilicity toward Pt(II) complexes is not known. Previously, we have shown that while in the reaction of Zn_7MT-2 with 2 mol equiv of cisplatin all ligands in (*cis*-$Pt)_2Zn_5$MT-2 are replaced with cysteine thiolates, in a similar reaction with transplatin the ammine ligands in (*trans*-$Pt)_2Zn_5$MT-2 are retained, rendering the complex potentially active (*31*). Therefore, we addressed the question of the formation of ternary complexes between (*trans*-$Pt)_2Zn_5$MT-2 and 5′-GMP, oligonucleotides, and DNA or platinum transfer to these species. We incubated (*trans*-$Pt)_2Zn_5$MT-2 with 100 mol equiv of 5′-GMP or synthetically prepared oligonucleotide 5′-CCTCGCTCTC-3′ in 10 mM Hepes/NaOH (pH 7.4) and 100 mM $NaClO_4$ at 37 °C for 72 h. The low- and high-molecular mass components of the incubation mixture were separated by gel filtration chromatography and analyzed. However, the identity of the absorption spectra of (*trans*-$Pt)_2Zn_5$MT-2 in the presence and absence of 5′-GMP or the oligonucleotide suggests that ternary complexes were not formed. Moreover, the absence of platinated adducts of 5′-GMP and the oligonucleotide in the ESI-MS spectra indicates that no platinum transfer occurred. In addition, the atomic absorption analyses of Pt in the isolated pSP73 plasmid, when it was incubated with increasing concentrations of (*trans*-$Pt)_2Zn_5$MT-2, revealed no plasmid platination. Thus, no evidence for transplatin transfer or the formation of ternary complexes was obtained (data not shown).

DISCUSSION

This investigation demonstrates that under physiological-like conditions with regard to pH, ionic strength, and temperature, human Zn_7MT-2 is capable of forming ternary complexes with *cis*- and *trans*-Pt−GMP adducts and with DNA modified with cisplatin and transplatin. The interaction of Zn_7MT-2 with the Pt−GMP adducts and platinated DNA occurs through the sulfur atom of Zn−S bonds present in the zinc−thiolate clusters. Although a stronger kinetic preference of CysS over the N7-G toward Pt(II) complexes is well-documented (*52*), that of sulfur in the Zn−S bonds is unknown. In view of the formation of ternary complexes and DNA−Pt−protein cross-links, we assessed the possibility of the formation of similar species in the reaction of 5′-GMP, oligonucleotide, and nonplatinated DNA with (*trans*-$Pt)_2Zn_5$MT-2, a species in which the ammine ligands are retained. The absence of ternary complexes and platinum transfer signifies a higher affinity of sulfur in a Zn−S bond over the N7-G base.

The kinetic measurements of apparent initial rates showed that *cis*-Pt−GMP reacts with Zn_7MT-2 faster than *trans*-Pt−GMP (Figure 3a). However, in our previous studies, carried out under identical conditions, the transplatin reacted faster with Zn_7MT-2 than cisplatin (*31*). Since the reaction of Pt(II) complexes with sulfur compounds was found to be largely independent of their solvolysis (*29, 43*), we attribute this effect to differences in the steric hindrance between the *cis*- and *trans*-Pt−GMP isoforms. Furthermore, the close similarity of the kinetic profiles of wild-type Zn_7MT-2 and Zn_7MT-2^{mut} indicates that the single thioether of Met^1 is not

involved in the interaction with platinated nucleotides (Figure 3a), suggesting a higher nucleophilicity of sulfur in the Zn−S bond.

Analysis of the interaction of Zn_7MT-2 with 2 mol equiv of *trans*-Pt−GMP revealed the formation of multiple species. The release of GMP in the course of the reaction indicates that besides the ternary GMP−*trans*-Pt−protein complex (*trans*-Pt)$_x$Zn$_{7-x}$MT-2 was formed. Previously, we have shown that in the reaction of Zn_7MT-2 with transplatin two leaving ligands are replaced with thiolates and that an equimolar concentration of Zn(II) was released. Thus, the formation of an increasing amount of (*trans*-Pt)$_x$Zn$_{7-x}$MT-2 with time is presumably responsible for a rapid kinetic phase of formation of the Pt−S bond and the increased level of Zn(II) ejection occurring after 70 h (Figure 3a). In marked contrast, in a similar reaction with *cis*-Pt−GMP, only 1 mol equiv was bound, forming a ternary GMP−*cis*-Pt−MT complex (Figure 3b). This may suggest that the binding of the bulky Zn_7MT-2 to *cis*-Pt−GMP occurs at a specific site. In the initial GMP−*cis*-Pt−MT complex, two Pt−S bonds were formed after 24 h. In this case, the substitution of a leaving ligand with sulfur labilizes the Pt(II)−ammine bond in the *trans* position. The concomitant ejection of only ~0.2 mol equiv of Zn(II) from Zn_7MT-2 implies that the formation of the GMP−*cis*-Pt−MT complex causes only a minor perturbation of the Zn_7MT-2 structure. The three-dimensional (3D) structure of mammalian M^{II}_7MT-1/-2 reveals a dumbbell-like molecule with uniformly sized and almost spherical C-terminal α-domain and N-terminal β-domain pair with a diameter of 15−20 Å. Each domain contains at its center the respective four and three metal−thiolate clusters, i.e., $M^{II}_4Cys_{11}$ and $M^{II}_3Cys_9$ (*27*). With the exception of a triad of solvent-exposed sulfur atoms (two terminal and one bridging) located on the bottom of a cleft in both domains, the remaining sulfur atoms are buried in the protein structure (*53*). Because of the kinetically stable Pt−S bond, the selectivity of *cis*-Pt-GMP will not depend on the cluster structure but rather on the accessibility and reactivity of thiolate ligands. We suggest therefore that the formation of the ternary GMP−*cis*-Pt−MT complex with *cis*-Pt−GMP may preferentially occur at one of these sites. As a result, the initial binding of *cis*-Pt−GMP to originally terminal thiolates may just expand the cluster core without a major perturbation of the protein structure. The formation of a MT−(μ-SCys)$_2$−*cis*-Pt(NH$_3$)−GMP dithiolate-bridged species resembles that found in the reaction between zinc finger synthetic analogues and platinum complexes (*54*). In this study, the analogy between alkylation and platination of the Zn−thiolate bond in zinc fingers has been suggested. The high reactivity of metal−thiolate clusters in MTs with electrophiles, including alkylation agents and platinum drugs, has been demonstrated (*27*). Thus, akin to zinc fingers, the Zn−S bonds in the zinc−thiolate clusters of Zn_7MT-2 act as the nucleophile, forming either dimetallic Zn−(μ-SCys)$_2$−*cis*-Pt(NH$_3$)−GMP or multimetallic Zn$_2$−(μ-SCys)$_2$−*cis*-Pt(NH$_3$)−GMP species.

The initial formation of two Pt−S bonds in the GMP−*cis*-Pt−MT complex, which occurs after incubation for only 1 h (Figure 3c), was followed by a slow kinetic phase in which an additional sulfur ligand coordinates the Pt(II) center. In this ternary GMP−*cis*-Pt−MT complex, three cysteine thiolates and the N7 atom of 5′-GMP participate in Pt(II) coordination. To the best of our knowledge, the structure and formation of such a complex have not yet been reported. Both the inertness of the Pt−NH$_3$ bond to nucleophilic attack and the fact that the *trans* effect of N7-G is unlikely to be responsible for the labilization of the ammine cannot explain this effect. In the absence of structural information about the ternary GMP−*cis*-Pt−MT complex, the underlying molecular mechanism leading to the formation of this species remains unclear. We hypothesize that specific structural features of the protein cavity with the closely spaced cysteine triads together with additional noncovalent interactions may play an important role in this reaction. Nevertheless, in the formed ternary GMP−*cis*-Pt−MT complex, the *trans* effect of the thiolate sulfur should lead to the release of 5′-GMP. However, no NMR evidence for 5′-GMP release after 120 h was obtained. On the other hand, this reaction may be rather slow and not seen on the time scale of our experiments, due to the decreasing electrophilicity of the Pt(II) center by π-donor thiolate ligands and/or the absence of a suitable nucleophile for 5′-GMP substitution. The interactions of Zn_7MT-2 with *cis*- or *trans*-Pt−GMP are schematically summarized in Figure 6.

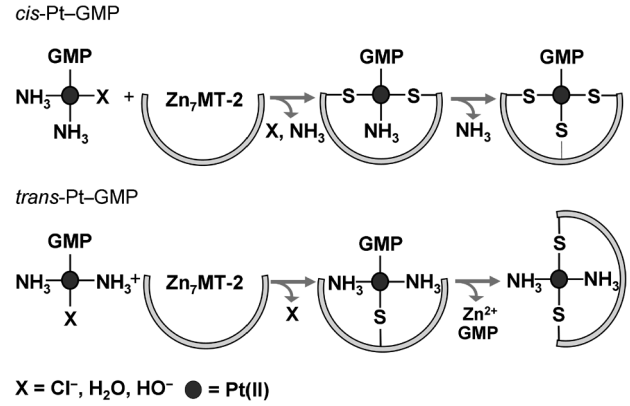

FIGURE 6: Scheme of the reaction of Zn_7MT-2 with *cis*- and *trans*-Pt−GMP.

We show that the negatively charged Zn_7MT-2 forms covalent cross-links with monofunctional adducts of plasmid DNA platinated with cisplatin and transplatin to an r_b value of 0.05. As Zn_7MT-2 does not interact with nonplatinated DNA, the DNA−MT cross-links are induced by DNA platination. In general, cisplatin forms in DNA ~90% intrastrand cross-links (1,2-GG or 1,2-AG), and to a lesser degree 1,3-GXG intrastrand and interstrand cross-links, and only a minor part remains bound monofunctionally (~1%) (*4*). At a low r_b, the DNA modification with transplatin results in monofunctional adducts which transform slowly ($t_{1/2}$ > 24 h) into bifunctional lesions that are mainly interstrand cross-links. However, this transformation is substantially faster at a high r_b, as the closure of the transplatin monofunctional adducts is strongly affected by the presence of other adducts (*55*). This implies that under our conditions the amount of monofunctional DNA adducts formed with transplatin is presumably also small. We observed larger amounts of DNA−MT cross-links with cisplatin-modified DNA than with transplatin-modified DNA. This effect is due in part to the already observed lower reactivity of Zn_7MT-2 with *trans*-Pt−GMP and to the demonstrated removal of transplatin from DNA by Zn_7MT-2. In this regard, it should be noted that in a similar reaction

of platinated DNA with the rather small GSH a higher reactivity was reported with transplatin than with cisplatin (*50, 56*).

Interactions of cisplatin with sulfur molecules are thought to be responsible for a variety of biological effects such as inactivation of Pt(II) complexes, development of cellular resistance to platinum, and toxic side effects such as nephrotoxicity (*2*). The major intracellular thiols involved in drug resistance are GSH and MT, which are both present in the cytoplasm and the nucleus. So far, the only known ternary complex between platinated DNA and sulfur atoms of biomolecules was that of GSH (*49, 50*). Here we demonstrated that Zn_7MT-2 can successfully compete for the monofunctional DNA adducts with GSH present at a physiologically relevant (2 mM) concentration. This effect is attributed to the substantially higher nucleophilicity of sulfur in the Zn−S bond compared to CysSH in GSH. The formation of cisplatin-modified DNA−MT cross-links traps monofunctional adducts, thus preventing to an unknown extent their closure.

In conclusion, although the role of MT-1 and -2 in cancer is not fully understood, their high level of overexpression, at least in some forms of malignant human tumors, is correlated with resistance to therapy and poor prognosis (*17, 57*). The results presented here together with our previous studies on the interaction of human Zn_7MT-2 with *cis*- and *trans*-[Pt(N-donor)$_2$Cl$_2$] compounds afford a better understanding of the role of MTs in the acquired resistance to platinum-based anticancer drugs at the molecular level. The studies show that Zn_7MT-2, besides the sequestration of Pt(II) complexes in the nucleus, can also successfully compete with GSH in the formation of DNA−MT cross-links, thereby modulating DNA repair and gene transcription. The sequestration of Pt(II) drugs results in ejection of Zn(II) from Zn_7MT-2, which represents a so far unrecognized factor contributing to cellular resistance. Both MT-1 and -2 isoforms, which are primarily involved in drug resistance, are inducible proteins. The transcriptional induction of *MT-1* and *-2* genes is mediated by zinc binding to metal regulatory transcription factor-1 (MTF-1) (*11*). The activation of MTF-1 by zinc ejected from MTs by toxic metals and oxidative stress has been shown (*58*). This mechanism should also be responsible for the reported overexpression of MT-1 and -2 in cancer treatment. GSH is another thiol known to function in detoxification of chemotherapeutic drugs. The heterodimeric enzyme γ-glutamyl-cysteine synthase (γGCS) is a key regulatory enzyme of GSH synthesis. It has been found that MTF-1, besides regulating the expression of MT-1 and -2, also regulates the expression of the γGCS heavy chain (*11*). Thus, targeting free Zn(II) and/or MTF-1 may represent a new approach to overcoming acquired resistance to platinum-based anticancer drugs.

ACKNOWLEDGMENT

We are grateful to Dr. Juan Hidalgo (Universidad Autonoma, Barcelona, Spain) for kindly providing antibodies against human MT-2, Dr. Markus Knipp for fruitful discussions of initial experiments and Alexander Batyuk (University of Zürich, Zürich, Switzerland) for constructing the mutant genes of human MT-2. We also thank Dr. Serge Chesnov (Functional Genomic Center, Zürich, Switzerland) for recording nano-ESI-MS spectra and Yaroslav Nikolaev (ETH, Zürich, Switzerland) for ^{1}H NMR measurements.

SUPPORTING INFORMATION AVAILABLE

ESI-MS characterization of platinum−GMP adducts formed with cisplatin and transplatin. This material is available free of charge via the Internet at http://pubs.acs.org.

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BI801253X