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Interaction between Platinum Complexes and the C-Terminal Motif of Human Copper Transporter 1

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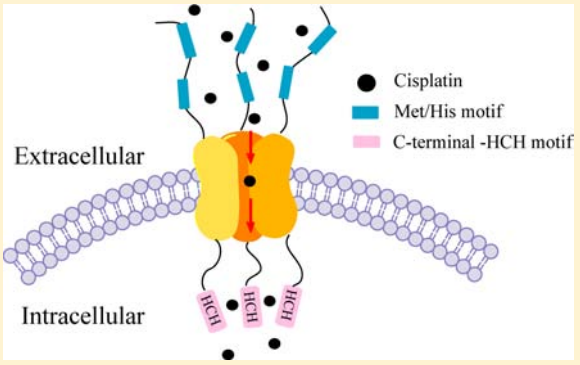
Supporting Information

ABSTRACT: Human copper transporter 1 (hCTR1) facilitates the cellular uptake of cisplatin, and the extracellular N-terminal domain has been proven to coordinate to platinum drugs. It has been reported that the intracellular C-terminal motif is crucial for the function of hCTR1 in cisplatin influx. In this work, we conduct reactions of the intracellular motif with platinum drugs. The octapeptide from the C-terminal domain of hCTR1 is used, and the reactions are investigated using ultraviolet, high-performance liquid chromatography, electrospray ionization mass spectrometry, and nuclear magnetic resonance spectroscopy. Results show that the C8 peptide is highly reactive to cisplatin and oxaliplatin, and the -HCH sequence is the most favorable binding site of platinum agents. Cisplatin first binds to the cysteine residues in the reaction with the C8 peptide. The ammine ligand, even trans to a thiol ligand, can remain coordinated in platination adducts for a >12 h reaction. Intramolecular platinum migration was observed in the C8 peptide, and the ammine ligands remain coordinated to platinum during this process. This result indicates that hCTR1 can transfer cisplatin in the active form through a *trans* chelation process. These findings provide insight into the mechanism of the C-terminus of hCTR1 in the transfer of platinum drugs from the trimeric pore of hCTR1 to the cytoplasm.



INTRODUCTION

Cisplatin and its analogues, carboplatin and oxaliplatin, have been widely used in anticancer chemotherapy.[1] The cellular uptake mechanism of platinum drugs is still unclear.[2] Cisplatin was previously believed to enter cells through passive diffusion, after which the drug is activated by hydrolysis and subsequently binds to DNA and triggers apoptosis.[3] In recent years, however, a growing body of evidence has demonstrated that the uptake of cisplatin is facilitated by a copper transporter protein (CTR1).[4−6]

CTR1 is the major copper influx transporter in mammalian cells. Human CTR1 (hCTR1) contains 190 amino acid residues; it consists of three transmembrane domains, an extracellular N-terminus, and an intracellular C-terminus.[7] A cryoelectron microscopy study demonstrates that hCTR1 constructs a channel-like architecture by the formation of a symmetrical trimer. Cu(I) is proposed to be transported through the channel from the N-terminal domain, via metal binding residues in the transmembrane domains (TM), to the C-terminal domain inside cells. *trans* chelation through the coordination to sulfur-containing residues (Met and Cys) or nitrogen-containing residues (His) plays important roles in copper transfer.[7,8] The Met-rich motifs and His-rich motifs in the N-terminal domain of hCTR1 could bind the copper ions directly and are required for the cellular uptake of Cu(I).[9−12] Therefore, the N-terminal domain is considered as a "gate" or "concentrator" for copper ion uptake.

Residues in transmembrane domains, such as Met154, Met150, and His139 in TM2, could be involved in the *trans* chelation of copper from the N-terminal domain. Moreover, the three metal coordination residues, His188, Cys189, and His190, in the C-terminus could be responsible for the delivery of copper into cells. It has been proven that the C189S mutation of hCTR1 lowers the extent of accumulation of copper in cells, and truncation of the C-terminus completely abolishes the uptake of copper through hCTR1.[12]

hCTR1 also has been proven to facilitate the cellular uptake of cisplatin.[4−6] Deletion of the gene encoding CTR1 results in the reduced extent of intracellular accumulation of cisplatin and leads to drug resistance.[4] It has also been demonstrated that yCtr1Δ causes impaired accumulation of cisplatin analogues, carboplatin and oxaliplatin.[6] However, the molecular details of platinum drug uptake through hCTR1 are not well understood. The N-terminus of hCTR1 has been shown to be important for the transport of platinum drugs. Several studies of the reactions between the N-terminal metal binding motifs and platinum complexes demonstrate that platinum binds to the Met-rich

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motifs readily.[13−16] Cisplatin shows fast binding to the Met-rich motifs of hCTR1 [$k_{cs(app)} = 7.94 \times 10^{-5}$ s^{-1}] with the formation of monofunctional species, and such binding was proposed to facilitate the activation of the drug.[16] A recent study revealed that the C-terminus was also required for hCTR1-mediated cisplatin transport.[12] Mutation of Cys189 partially reduced the extent of intracellular accumulation of cisplatin, whereas deletion of the C-terminus abolished the uptake of cisplatin through hCTR1. However, little is known about the molecular mechanism of the C-terminus in the regulation of hCTR1-mediated uptake of platinum drugs.

In this study, we have investigated the reactions of platinum complexes with the C-terminal octapeptide (C8, VDITEHCH) of hCTR1, corresponding to the sequence of residues 183−190 in the protein. The reactions were investigated using ultraviolet (UV), high-performance liquid chromatography (HPLC), electrospray ionization mass spectrometry (ESI-MS), and nuclear magnetic resonance (NMR) spectroscopy. Results show that the octapeptide reacts with platinum complexes reasonably fast and tends to form bis-platination adducts. Cysteine has been confirmed to be the favored binding site based on the results from two-dimensional (2D) heteronuclear single-quantum coherence (HSQC) and tandem mass spectra. Both the HCH and VDI motifs are involved in the platination. Interestingly, although the *trans* effect of sulfur from cysteine coordination is present, the ligand ammine can remain intact on the platinum coordination for a long period of time. The NMR signal of the ammine *trans* to thiol can be detected after reaction for 12 h. Moreover, the two ammine groups were retained in the platinum migration process.

EXPERIMENTAL SECTION

Peptide Synthesis and Purification. The octapeptide (C8, VDITEHCH) was synthesized by solid-phase 9-fluorenylmethoxycarbonyl (Fmoc) chemistry using a peptide synthesizer (CS Bio Co.). The purity of the octapeptide was analyzed using HPLC (>95%), and the identity was confirmed by ESI-MS (calc, m/z 953.41; measured, m/z 953.32) (Figure S1 of the Supporting Information).

Sample Preparation. The peptide and platinum complexes (cisplatin and oxaliplatin) were dissolved in ultrapure water. The reactions were performed in 50 mM phosphate buffer (pH 7.0). For ESI-MS analyses, no salt was added to the samples and the pH was adjusted with $NH_3 \cdot H_2O$. The pH was measured on a Mettler Toledo LE438 instrument equipped with a Hot-Line pH probe (Sentron).

Stability of the C8 Peptide in Air. To analyze the thiol stability of the C8 peptide in air, we prepared 80 μL of 1 mM peptide in 50 mM phosphate buffer (pH 7.0) and incubated the sample in a closed 1.5 mL Eppendorf tube at 37 °C. The thiol content was measured using 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB). A 110 μM DTNB solution was prepared in 100 mM Tris buffer (pH 8.0); 920 μL of 110 μM DTNB was added to the 80 μL of C8 peptide, and the UV absorption was measured immediately on an Agilent 8453 spectrophotometer.

HPLC. HPLC was performed on an Agilent 1200 system equipped with a Kromasil C18 column (250 mm × 4.6 mm, 5 mm) with UV detection at 220 nm. A linear gradient was used (eluent A, 0.1% CF_3COOH in H_2O; eluent B, 100% CH_3OH). To obtain platination adducts for MS analyses, we performed the reaction with 0.5 mM C8 peptide with 0.5 or 1.5 mM cisplatin at 37 °C. The platination adducts were collected and lyophilized immediately for further ESI-MS experiments.

ESI-MS. ESI-MS experiments were conducted on an LTQ ion trap mass spectrometer (Thermo Fisher, San Jose, CA) equipped with a nano-ESI source. Mass spectra were obtained at a capillary voltage of 32 V with a spray voltage of 1.8 kV and a capillary temperature of 200 °C. The ion trap analyzer was operated in a m/z 200−2000 range in positive mode. The lyophilized samples were dissolved in a water/methanol (1:1) solution containing 0.1% formic acid. Samples were directly injected at a flow rate of 3 μL/min. To monitor the reaction via ESI-MS, we incubated a solution containing 0.5 mM C8 peptide and 1.5 mM cisplatin at 37 °C for 0−6 h before MS measurements. Data were analyzed using XCalibur (version 2.0, Thermo Finnigan).

NMR Spectroscopy. NMR experiments were performed on either a Bruker Avance 300 MHz spectrometer or a Varian Inova 700 MHz spectrometer. For one-dimensional (1D) ^1H spectra, samples were dissolved in D_2O and the residual HDO signal was suppressed by a presaturation pulse sequence. The ^1H chemical shifts were referenced to 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS) as an internal reference. 2D $^1H-^{15}N$ HSQC NMR spectra of 0.8 mM ^{15}N-labeled cisplatin were recorded in the reaction with 0.8 mM C8 peptide. Samples were prepared in 50 mM phosphate buffer (pH 7.0) containing 10% D_2O. The reaction was monitored by NMR spectroscopy at 25 °C. Data were processed and analyzed using Topspin (version 1.3).

RESULTS AND DISCUSSION

Stability of the C8 Peptide (VDITEHCH). Reducing agents were not used because they influence the reaction of platinum compounds.[17] Therefore, the stability of C8 has to be verified because this peptide contains a redox active cysteine residue. The thiol content of the C8 peptide was measured by Ellman's assay using DTNB. The oxidation of thiol groups by DTNB forms disulfide bonds; meanwhile, thionitrobenzoic acid (TNB) is generated in the reaction. TNB absorbs at 412 nm; thus, the reaction can be monitored by UV spectroscopy.[18] The stability of the C8 peptide in air was analyzed in 50 mM phosphate buffer (pH 7.0) at 37 °C (Figure 1). The result

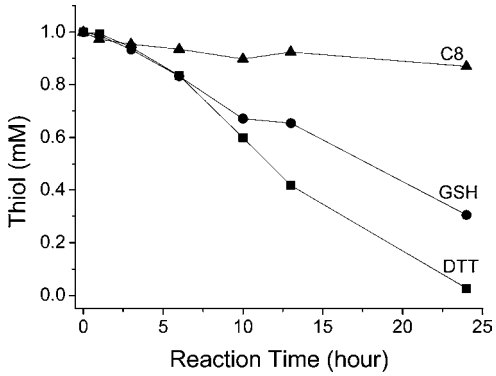

Figure 1. Oxidation of thiol groups of C8, GSH, and DTT in air. The measurements were taken on 80 μL samples of 1 mM C8, DTT, or GSH in 50 mM phosphate buffer (pH 7.0) in a closed 1.5 mL Eppendorf tube at 37 °C.

shows that the thiol content of the C8 peptide decreased very little during the 24 h measurement, while the control experiments show significant oxidation of dithiothreitol (DTT) and glutathione (GSH). This observation indicates that the C8 peptide is rather stable in air. The stable thiol in C8 is probably due to the HCH sequence, in which the thiol could form an H-bond to the neighbor histidine residue.[19] Therefore, the reactions of C8 were conducted in the absence of reducing agents in this work.

Reactions of the C8 Peptide with Cisplatin Monitored by UV and NMR Spectroscopy. The cysteine residue has a high affinity for platinum, and the formation of the S−Pt bond shows absorption at 280 nm.[20] To determine whether the cysteine residue in C8 is involved in platinum binding, the

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reaction between C8 and cisplatin was monitored by UV spectroscopy. The reaction was performed on 0.5 mM C8 peptide at 37 °C over the course of 14 h. The increase in absorption at 280 nm indicates the formation of the platination adducts with sulfur coordination (Figure 2). The reaction was

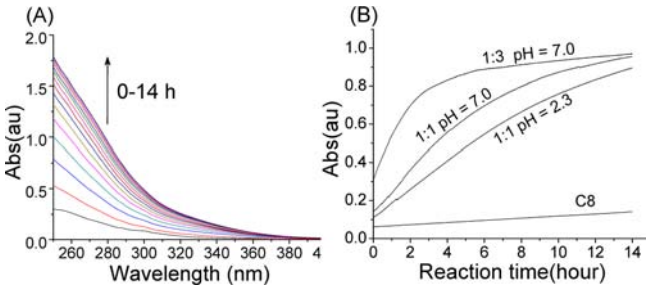

Figure 2. Reaction of the C8 peptide with cisplatin. (A) UV spectra of 0.5 mM C8 with equimolar cisplatin at pH 7.0 and 37 °C. (B) Time-dependent absorbance at 280 nm in the reactions of 0.5 mM C8 with equimolar and 3-fold molar cisplatin at 37 °C. The pH values (2.3 and 7.0) are labeled on each curve.

also studied at different pH values since the potential coordination sites (cysteine and histidine) are (de)protonable. Results show that the reaction is slower at pH 2.3 ($t_{1/2}$ = 5.3 h) than at pH 7.0 ($t_{1/2}$ = 3.3 h). The slower reaction at acidic condition implies the coordination of protonable residues at pH 2.3. This observation is consistent with the platination of cysteine residues that the thiol groups has higher nucleophilic ability in the neutral condition than in the acidic condition.[21]

The reaction was also monitored using 2D $^{1}H-^{15}N$ HSQC NMR spectroscopy on ^{15}N-labeled cisplatin {*cis*-$[PtCl_2(^{15}NH_3)_2]$} in the presence of equimolar C8 (Figure 3). Because the ^{15}N chemical shifts of NH_3 in platinum complexes are dependent on the ligand at the *trans* position, the composition of the platination adducts can be estimated.[22] The free cisplatin at −67.1 (^{15}N) and 4.04 (^{1}H) ppm is in agreement with the literature data.[16] A pair of cross-peaks at −65.8 (^{15}N) and 4.12 (^{1}H) ppm, and −82.7 (^{15}N) and 3.80 (^{1}H) ppm, were assigned to the monoaquation of cisplatin. It was clearly observed that the intensities of these cross-peaks decreased with time and new cross-peaks appeared in the reaction with the C8 peptide. The appearance of a cross-peak at −39.4 (^{15}N) and 3.51 (^{1}H) ppm relative to $^{15}NH_3$ *trans* to the sulfur atom after incubation for 1.5 h indicates that cisplatin first binds to the cysteine residue in the C8 peptide. With the reaction progressing, more signals appeared in the region of −35 to −45 ppm for ^{15}N chemical shifts, where the NH_3 is *trans* to a sulfur atom. This observation indicates that the cysteine residue is the major platinum binding site in C8. Interestingly, these signals were very intense after reaction for 12 h, suggesting that these adducts are rather stable, although the ammine group is *trans* to a thiol ligand. On the other hand, the formation of multiple platination adducts could be due to the presence of other potential binding sites, such as the two adjacent histidine residues. Although the histidine residue is not directly reactive to cisplatin,[23] platinum could transfer from the sulfur-containing residue to histidine in peptides.[24−26]

To ascertain the histidine coordination, the reaction between cisplatin and the C8 peptide was investigated by 1D ^{1}H NMR. The spectra were recorded in D_2O; thus, all exchangeable protons were not observed. Only two imidazole protons, $C^{\delta}H$ and $C^{\varepsilon}H$, can be detected in the aromatic region (Figure 4). Results show that proton signals become broadened during the reaction at pH 7.0, which strongly suggests the platination of imidazole. No obvious signals from platination adducts were observed, probably because of multiple products under the neutral condition (see below). In comparison, much less peak

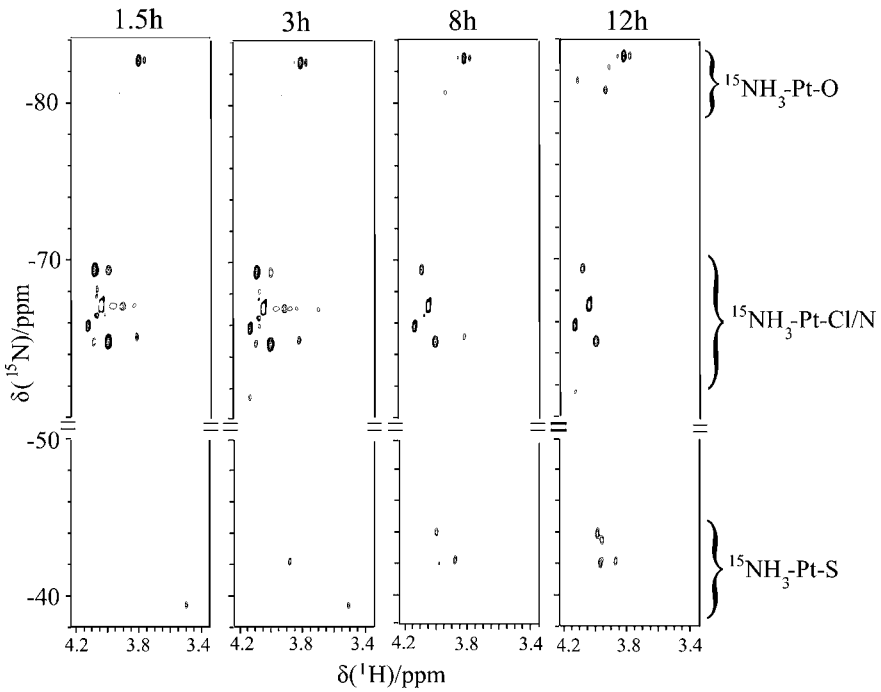

Figure 3. 2D $^{1}H-^{15}N$ HSQC NMR spectra of ^{15}N-labeled cisplatin {*cis*-$[PtCl_2(^{15}NH_3)_2]$} in the reaction with the C8 peptide. The reaction was conducted with 0.8 mM cisplatin with equimolar C8 peptide in 50 mM phosphate buffer (pH 7.0) at 25 °C. The reaction time is annotated on each spectrum.

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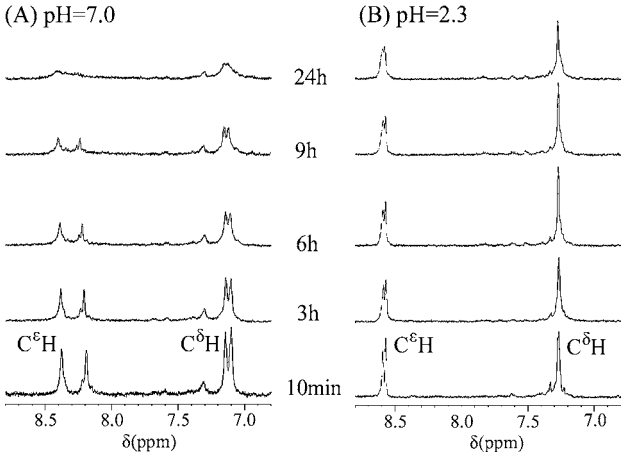

Figure 4. Aromatic regions of 1D ^{1}H NMR spectra of the C8 peptide in the reaction with cisplatin. The reaction was performed with 2 mM C8 with equimolar cisplatin in D_2O at 37 °C. NMR spectra were recorded at 25 °C at (A) pH 7.0 with 50 mM phosphate buffer and (B) pH 2.3.

broadening was observed in the reaction at pH 2.3. Although the reaction is slower at low pH, UV spectra show that a similar extent of cysteine coordination can be reached after incubation for 24 h. Hence, the smaller decrease in the magnitude of the signal of histidine at low pH demonstrates that the reactivity of imidazole is largely reduced under the acidic condition. This result indicates that protonation inhibits the reaction of imidazole to cisplatin. Although histidine is kinetically less preferable than cysteine for platinum binding, these two histidines may assist the cisplatin transport, which is consistent with the result that mutation of the cysteine residue partially preserved the ability of hCTR1 to take up cisplatin.[12]

The cysteine residue has been reported to play an important role in cisplatin influx, and mutation of this residue resulted in a decreased extent of intracellular accumulation of cisplatin.[12] Our combined UV and NMR data demonstrate that this residue in the C8 peptide is very reactive to cisplatin and is the major platination site. This result suggests that the C-terminus of hCTR1 is capable of participating in the *trans* chelation process of cisplatin through the coordination of the cysteine residue. Additionally, two histidines in the HCH motif were also found to be involved in cisplatin binding, which could be associated with the hCTR1-mediated uptake of cisplatin. One may argue the difference between this C8 peptide and the specific conformation of the C-terminus in the hCTR1 trimer. It is worth noting that the molecular details of transporting Cu^I and cisplatin via hCTR1 can be different, although they might share the same binding sites. Copper is proposed to pass through the trimer pore via a chain of three-coordinate copper exchange reactions.[7] However, cisplatin is expected to bind only one or two atoms on hCTR1 to maintain the two amine ligands, so that the drug can be transported through hCTR1 by sulfur−sulfur exchange in the formation of *cis*-$[PtCl(NH_3)_2]^+$.[16] Thus, the C8 peptide used here provides a favorable model of the C-terminus of hCTR1 for the transport of cisplatin into cells. These results suggest that the C-terminal HCH motif is crucial for the sequential *trans* chelation of the platinum complex.

Identification of Platination Adducts and Binding Sites through ESI-MS Spectroscopy. To characterize the platination adducts of C8, the products were analyzed by ESI-MS. The reaction was performed with 0.5 mM C8 peptide with a 3-fold molar excess of cisplatin at pH 7.0 and 37 °C. The samples at different reaction times (0.5, 2, 4, and 6 h) were measured by ESI-MS (Figure 5). In the initial phase of the

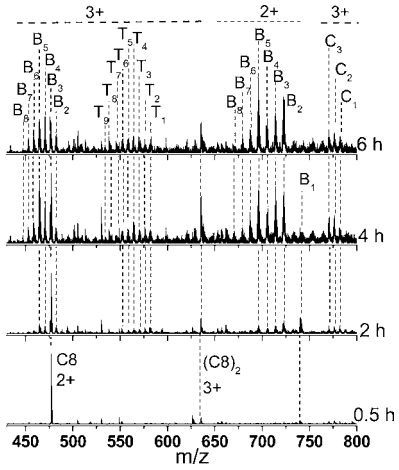

Figure 5. Time-dependent ESI-MS spectra of C8 in the reaction with cisplatin. The reaction was conducted with 0.5 mM C8 and 1.5 mM cisplatin at 37 °C and pH 7.0. The reaction time is annotated on each spectrum. Symbols B, T, and C denote the bis-platinated species, tris-platinated species, and platinum-induced peptide dimers, respectively.

reaction, a bis-platinated adduct $[C8+2Pt(NH_3)_2Cl]$ (B_1) was generated at m/z 741.10 (2+). The calculated isotopic distribution confirmed this composition (Figure S1 of the Supporting Information). The composition of B_1 indicates that the C8 peptide can bind quickly to two $[Pt(NH_3)_2Cl]^+$ units. With a longer time reaction, the ligands of cisplatin were substituted in a stepwise manner, and more products were generated in the reaction. A number of bis-platinated adducts were detected at 6 h. The major products were $[C8+Pt(NH_3)_2+Pt(NH_3)_2Cl]$ (B_2), $[C8+Pt(NH_3)+Pt(NH_3)_2Cl]$ (B_3), $[C8+2Pt(NH_3)_2]$ (B_4), $[C8+Pt(NH_3)+Pt(NH_3)_2]$ (B_5), and $[C8+2Pt(NH_3)]$ (B_6). The detailed assignments of these adducts, as well as several minor adducts (B_7 and B_8), are listed in Table 1. Additionally, several triplatinated adducts (T_1−T_9) and platinum-linked dipeptides (C_1−C_3) were observed (Table 1). The large multiformity of products is probably the reason that the adduct signals cannot be observed on NMR spectra in Figure 4.

We have attempted to separate the platination adducts using HPLC for further ESI-MS analyses. Because of the large number of products, samples could not be separated as each single adduct by HPLC (Figure S2 of the Supporting Information). Nevertheless, the platinated adducts can be separated from the free C8 peptide and cisplatin. These adducts were collected as a mixture for ESI-MS analyses. Similar to the measurements on the reaction system, a large number of adducts were observed in the MS spectra. In this case, the binding of the phosphate group was observed because the reaction was performed in phosphate buffer (Figure S3 of the Supporting Information).

Although the UV absorption and NMR spectra have indicated that cysteine and histidine residues are involved in the reaction to cisplatin, we further analyzed the binding sites using tandem mass spectra. Tandem MS is capable of identifying binding sites by the fragmentation of selected ions. The MS/MS experiment was performed on the sample

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Table 1. Analysis of ESI-MS Peaks Detected in Figure 4

peak	composition	formula	charge	observed m/z	calcd m/z
B_1	$[C8+2Pt(NH_3)_2Cl]$	$C_{39}H_{72}N_{16}O_{14}SCl_2Pt_2$	+2	741.10	741.20
B_2	$[C8+Pt(NH_3)_2+Pt(NH_3)_2Cl]$	$C_{39}H_{71}N_{16}O_{14}SClPt_2$	+2	722.20	722.71
			+3	482.40	481.80
B_3	$[C8+Pt(NH_3)+Pt(NH_3)_2Cl]$	$C_{39}H_{68}N_{15}O_{14}SClPt_2$	+2	713.69	714.19
			+3	476.10	476.13
B_4	$[C8+2Pt(NH_3)_2]$	$C_{39}H_{70}N_{16}O_{14}SPt_2$	+2	704.21	704.21
			+3	470.70	469.48
B_5	$[C8+Pt(NH_3)+Pt(NH_3)_2]$	$C_{39}H_{67}N_{15}O_{14}SPt_2$	+2	695.70	695.70
			+3	464.66	463.80
B_6	$[C8+2Pt(NH_3)]$	$C_{39}H_{64}N_{14}O_{14}SPt_2$	+2	687.19	687.19
			+3	458.72	458.12
B_7	$[C8+Pt+Pt(NH_3)]$	$C_{39}H_{61}N_{13}O_{14}SPt_2$	+2	678.67	678.67
			+3	453.10	452.45
B_8	$[C8+2Pt]$	$C_{39}H_{58}N_{12}O_{14}SPt_2$	+2	670.65	670.16
			+3	447.14	446.77
T_1	$[C8+3Pt(NH_3)_2Cl]$	$C_{39}H_{78}N_{18}O_{14}SCl_3Pt_3$	+3	582.64	582.13
T_2	$[C8+Pt(NH_3)Cl+2Pt(NH_3)_2Cl]$	$C_{39}H_{75}N_{17}O_{14}SCl_3Pt_3$	+3	576.26	576.45
T_3	$[C8+Pt(NH_3)_2+2Pt(NH_3)_2Cl]$	$C_{39}H_{77}N_{18}O_{14}SCl_2Pt_3$	+3	570.36	569.80
T_4	$[C8+Pt(NH_3)+2Pt(NH_3)_2Cl]$	$C_{39}H_{74}N_{17}O_{14}SCl_2Pt_3$	+3	564.62	564.12
T_5	$[C8+2Pt(NH_3)_2+Pt(NH_3)_2Cl]$	$C_{39}H_{76}N_{18}O_{14}SClPt_3$	+3	558.06	557.80
T_6	$[C8+2Pt(NH_3)_2+Pt(NH_3)Cl]$	$C_{39}H_{73}N_{17}O_{14}SClPt_3$	+3	552.76	552.13
T_7	$[C8+3Pt(NH_3)_2]$	$C_{39}H_{75}N_{18}O_{14}SPt_3$	+3	545.66	545.48
T_8	$[C8+Pt(NH_3)+2Pt(NH_3)_2]$	$C_{39}H_{72}N_{17}O_{14}SPt_3$	+3	540.38	539.80
T_9	$[C8+2Pt(NH_3)+Pt(NH_3)_2]$	$C_{39}H_{69}N_{16}O_{14}SPt_3$	+3	534.00	534.13
C_1	$[2C8+Pt(NH_3)+Pt(NH_3)_2]$	$C_{78}H_{128}N_{27}O_{28}S_2Pt_2$	+3	782.44	781.94
C_2	$[2C8+2Pt(NH_3)]$	$C_{78}H_{125}N_{26}O_{28}S_2Pt_2$	+3	776.14	776.26
C_3	$2[C8+Pt+Pt(NH_3)]$	$C_{78}H_{122}N_{25}O_{28}S_2Pt_2$	+3	770.46	770.59

from the reaction of C8 with equimolar cisplatin in 50 mM phosphate buffer (pH 7.0). The $[C8+Pt(NH_3)_2(PO_4)+Pt(NH_3)_2]^{3+}$ ion (m/z 502.36) in the mass spectra was selected as a precursor ion for collision-induced dissociation experiments. The MS/MS spectrum and the fragmentation results are shown in Figure 6. The fragments were identified by the characteristic isotope distribution of the peptide and platinum. The annotation of fragment ions (b and y) follows the Biemann nomenclature.[27] The asterisks (b* and y*) denote platinated fragments, and the double asterisks denote (b_n** and y_n**) bis-platinated fragments.

The platination sites can be directly read by comparing the b_n ions with the b_n* ions. In the y ions, the bis-platinated fragments were observed on y_3**–y_7** (Figure 6). The smallest y_3** fragment suggests that the sequence of H^6–H^8 is involved in the coordination of two $[Pt(NH_3)_2]$ units. In the b ions, the observed b_3** and b_4** ions and b_6** and b_7** ions indicate that the sequence of $V^1D^2I^3$ also binds to two $[Pt(NH_3)_2]$ units. Because the valine and isoleucine residues do not have metal binding sites on their side chains, only two potential coordination sites are present in this VDI motif, the N-terminal NH_2 group and the carboxyl group of Asp2. Although the coordination of the Val1 amine can occur in the C8 peptide, this residue is less likely to be a binding site in the protein where the amine forms a peptide bond.

ESI-MS data demonstrate that the C8 peptide tends to bind two cisplatins and both HCH and VDI motifs are involved in platinum binding. In our previous NMR studies, the cysteine residue was confirmed to be the preferred platinum binding site. This result is not surprising because Pt^{2+} is considered a soft acid and should favor bonding with cysteine according to the hard–soft acid–base principle. In contrast, the nitrogen- and oxygen-containing groups are kinetically much less reactive. It has been proven that the N donors are more easily platinated through an intramolecular substitution from an adjacent S ligand.[24−26] Therefore, the fast formation of the bis-platinated adduct of the C8 peptide implies that one platinum transfers

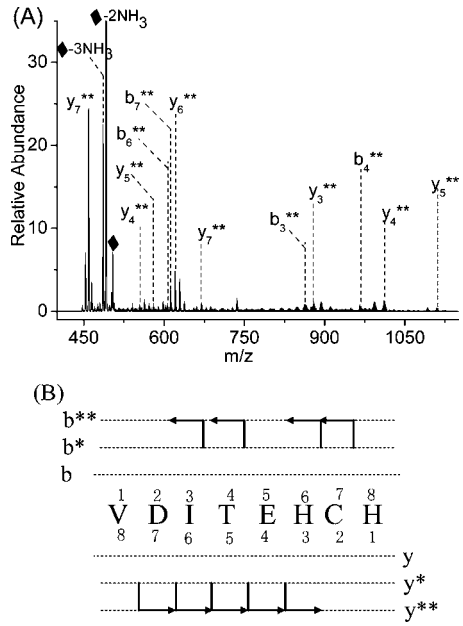

Figure 6. (A) MS/MS spectrum of $[C8+Pt(NH_3)_2(PO_4)+Pt(NH_3)_2]^{3+}$. The unlabeled diamond denotes the precursor ion at m/z 502.36. The peaks labeled ◆-2NH_3 and ◆-3NH_3 denote the release of two and three ammine ligands, respectively, from the precursor ion. (B) Fragment ions observed in the spectrum.

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from the cysteine S atom to thermodynamically preferred nitrogen-containing ligands during the reaction. A similar platinum migration has been widely observed.[24−26] As bis-platinated adducts formed quickly at the high Pt:C8 ratio, we also performed the reaction of the C8 peptide with equimolar cisplatin. The ESI-MS results show that monoplatinated adduct was detectable in the initial stage of the reaction, confirming the platinum migration in the later stage (Figure S4 of the Supporting Information). The fast platinum migration must contribute to the effective uptake of cisplatin, as cells respond to the cisplatin treatment quickly. The downregulation of Ctr1 expression by cisplatin was observed in cultured human ovarian carcinoma cells within 1 min of exposure to cisplatin.[28] Furthermore, because the intact ammine groups of cisplatin are essential for drug activity upon DNA damage, it is interesting to note that the two ammine ligands remain coordinated to platinum during the platinum migration process. It has been reported that cisplatin as well as carboplatin and nedaplatin can bind to the N-terminus of hCTR1 in its active form.[29] Given the NMR and ESI-MS results in this work, a conceivable mechanism of transfer of cisplatin from the trimer pore of hCTR1 to the cytoplasm can be proposed. The cysteine residue in the C-terminus first binds and receives cisplatin from the pore; subsequently, cisplatin migrates to other residues in the C-terminus or directly enters the cytoplasm through *trans* chelation reactions between the C-terminus and intracellular molecules with a high affinity for cisplatin, such as the copper chaperone Atox1.

Interactions between the C8 Peptide and Oxaliplatin. While sufficient evidence supports the idea that hCTR1 facilitates the uptake of cisplatin, the question of whether hCTR1 is also involved in the transport of oxaliplatin is still controversial.[30,31] Herein, we also investigated the interaction between the C8 peptide and oxaliplatin. The reaction of equimolar C8 with oxaliplatin was monitored by HPLC under neutral conditions at 37 °C (Figure S5 of the Supporting Information). The half-life of the reaction is obtained on the basis of the time-dependent decay of the oxaliplatin peak on the HPLC profiles. The $t_{1/2}$ of the reaction of oxaliplatin (~3.6 h) is similar to that of cisplatin.

The products were also analyzed using ESI-MS. C8 (0.5 mM) was incubated with equimolar or a 3-fold molar excess of oxaliplatin at pH 7.0 for 10 h. The products were separated by HPLC and collected for ESI-MS analysis. Figure 7 shows the spectrum of products from the reaction of Pt and C8 in a 1:3 ratio. The major product at m/z 784.26 (2+) was assigned to $[C8+2Pt(DACH)]^{2+}$. The result shows that, similar to the reaction of cisplatin, the bis-platinated adduct is the major product in the reaction of oxaliplatin. This observation suggests that the C8 peptide tends to bind to two molecules of platinum complexes. Different from the case for cisplatin, no carrier ligand (DACH) release was observed in the reaction of oxaliplatin, and many fewer products were generated. The same products were formed in the reaction of equimolar oxaliplatin (Figure S6 of the Supporting Information). Several other products, including $[C8+2Pt(DACH)+2H_2O]^{2+}$, $[2C8+2Pt(DACH)]^{3+}$, and $[2C8+3Pt(DACH)]^{3+}$, were also observed in the reaction.

To identify the platinum binding sites, we selected the major adduct $[C8+2Pt(DACH)]^{2+}$ at m/z 784.26 as the precursor ion for the MS/MS analysis. With the chelation of the DACH ligand, each $[Pt(DACH)]^{2+}$ unit has two sites of coordination to the peptide; therefore, four binding sites are present on the

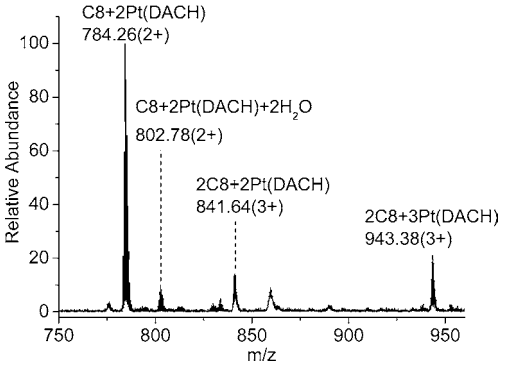

Figure 7. ESI-MS spectrum of products from the reaction of oxaliplatin. C8 (0.5 mM) was incubated with 1.5 mM oxaliplatin in 50 mM phosphate buffer (pH 7.0) at 37 °C for 10 h. The products were separated by HPLC, and the elution corresponding to the 15.1−15.6 min time point was collected for ESI-MS analysis.

C8 peptide. The spectrum and the fragmentation scheme are shown in Figure 8. The smallest y_2** ion and the largest b_6 ion

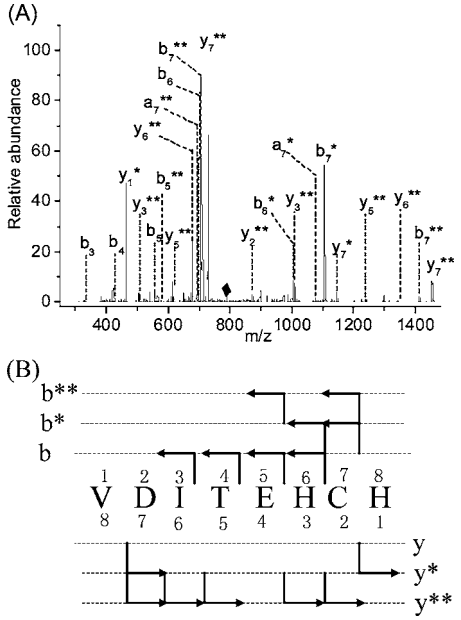

Figure 8. MS/MS spectrum of the oxaliplatin adduct. (A) Spectrum of the product $[C8+2Pt(DACH)]^{2+}$. The precursor ion at m/z 784.26 is denoted with a diamond. (B) Fragment ions observed in the spectrum.

suggest that Cys7 and His8 bind to two [Pt(DACH)] units. The observed y_1* ion confirms His8 as a binding site. Thus, both Cys7 and His8 are considered to be binding sites. In addition, the observed b_5** ion indicates that the V^1−E^5 sequence binds to two [Pt(DACH)] units. Three potential binding sites are present in this sequence, the two carboxylate groups in Asp2 and Glu5 and the N-terminal NH_2 group. It has been proven that Asp2 and the N-terminal NH_2 group are the binding sites of cisplatin; thus, these two sites could also be involved in the coordination of oxaliplatin.

The reaction of C8 with oxaliplatin was also analyzed via NMR spectroscopy (Figure S7 of the Supporting Information). The result shows that the magnitudes of the signals of $C^{\delta}H$ and $C^{\varepsilon}H$ decrease during the reaction; meanwhile, several sets of new peaks appear. This result clearly indicates the coordination of the imidazole nitrogen. Product signals are rather weak as

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multiple adducts are produced. This observation also explains the 1D NMR result from the reaction of cisplatin, in which signals were not detectable because many more products were generated. Taken together, the reaction of the C8 peptide with oxaliplatin is very similar to that with cisplatin.

CONCLUSIONS

In summary, the reactions of platinum drugs with the C-terminal peptide of the intracellular domain of hCTR1 have been studied in this work. Our results demonstrate that this peptide has a high reactivity to cisplatin and oxaliplatin and tends to bind to two molecules of platinum complexes. UV and NMR data reveal that the cysteine residue is the preferred platinum binding site, and histidine residues are also involved in the platination. Tandem MS spectra confirm that three residues at the C-terminus (-HCH) are the most favorable platination sites. In the cisplatin adducts of the peptide, two ammine ligands can remain coordinated for >12 h even for the NH_3 *trans* to a thiol group, although the release of ammine occurs after a long reaction. Platinum migration was also observed with the two ammine groups retained in this process. Our combined data suggest that the C-terminus is capable of transferring cisplatin in the active form. This observation highlights the biological relevance of hCTR1 in the transport of platinum drugs through a *trans* chelation process.

ASSOCIATED CONTENT

Supporting Information

HPLC, ESI-MS, and NMR data. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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