Protein Expression

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Trans-Platinum/Thiazole Complex Interferes with Sp1 Zinc-Finger Protein**

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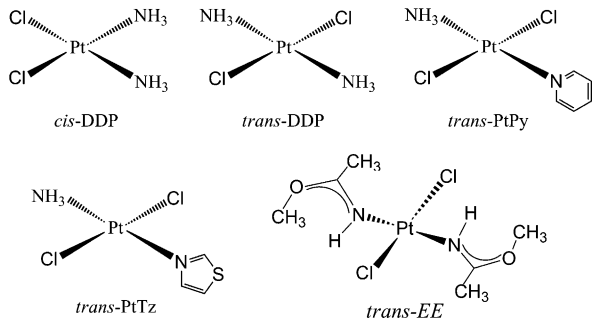
The human transcription factor Sp1 is a C2H2 zinc-finger protein that is involved in the regulation of a wide variety of genes, including housekeeping genes and tumor-developing genes.[1] The overexpression of Sp1 results in the dysfunction of cellular processes and is associated with tumor development, growth, and metastasis.[2] For instance, the survivin protein, which is one of the critical inhibitors of apoptosis proteins (IAPs), is regulated by Sp1.[3] Recent studies showed that the human telomerase reverse transcriptase (hTERT) is also regulated by Sp1,[4] and the overexpression of telomerase is found in most malignant tumors.[5] These observations highlight the importance of Sp1 in tumor development, and suggest the potential of therapeutically targeting Sp1.[1b,6]

Increasing evidence indicates that the proteins regulated by Sp1 are also involved in the mechanism of platinum-based drugs. The expression of survivin is strongly associated with drug resistance of cisplatin.[7] The copper transporter protein Ctr1 facilitates the uptake of cisplatin into cells, and a low level of Ctr1 was detected in cisplatin-resistant cells.[8] The DNA-dependent protein–kinase catalytic subunit (DNA–PKcs), a key enzyme that is involved in repairing DNA double-strand breaks, protects cells from the toxic effects of cisplatin.[9] In addition, the vacuolar ATPase is often involved in the acquired cisplatin resistance.[10] It is notable that all these proteins are regulated by the transcription factor Sp1.

Although it is widely accepted that DNA is the ultimate target of platinum antitumor drugs, only about 1 % of cellular platinum binds to nuclear DNA.[11] Many different cellular processes are involved in the mechanism of platinum drugs.[12] Proteins were proven to play important roles in determining the cytotoxicity and drug resistance of platinum agents.[13] Additionally, proteomics studies revealed that the expression of a large number of cellular proteins is influenced by platinum drugs.[13–14] The response of protein expression to the treatment with platinum drugs highly suggests the interaction of transcription factors with platinum complexes.

Platinum(II) compounds have a strong tendency to bind to sulfur-containing proteins, such as metallothionein.[15] Sp1 contains three typical C2H2 zinc-finger motifs, in which the cysteine residues are the potential binding sites of platinum. In addition to the classical *cis*-platinum drugs, several platinum complexes with *trans* geometry, which violate the classical structure–activity relationship of platinum compounds, were found to be active against tumor cells.[16] The activity of nonclassical platinum complexes to cisplatin-resistant cancer cells suggests different mechanisms in comparison to cisplatin. Indeed, more protein adducts were detected in cells treated by *trans*-Pt complexes than by *cis*-Pt complexes.[17]

In this study, we investigated the reactions of Sp1 zinc-finger (zf) domains with platinum complexes featuring different ligands and coordination geometries. For comparison with cisplatin, the clinically inactive transplatin complex and three antitumor-active *trans*-coordinated complexes were used (Scheme 1). The reactions were performed on the whole zinc-finger domain (Sp1, aa 530–623, containing three zinc-finger domains), and on the second zinc-finger domain (Sp1-zf2, aa 565–595; Scheme S2). Results showed that *trans*-



Scheme 1. Structures of platinum complexes

amine(thiazole)-dichloroplatinum (*trans*-$[PtCl_2(NH_3)(Tz)]$, *trans*-PtTz) exhibited significantly higher reactivity to Sp1 than other compounds. The binding of *trans*-$[PtCl_2(NH_3)(Tz)]$ disrupts the structure and function of Sp1 both in vitro and in vivo; in contrast, cisplatin does not have a detectable effect on Sp1.

The zinc-bound Sp1 proved to be stable in air (see the Supporting Information, Text S2 and Figure S2), and the reactions were performed in the absence of reducing agents in order to avoid additional reactions. The binding of platinum complexes to the Sp1 zinc-finger domain results in the release of zinc ions, thus the reaction can be monitored by a zinc-release assay using the zinc dye 4-(2-pyridylazo) resorcinol (PAR). The Zn^{2+} ions released from Sp1 can be quantitatively measured using UV/Vis spectra at 500 nm, based on the

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formation of the $[Zn(PAR)_2]$ complex. Results showed that *trans*-PtTz has clearly a higher affinity to Sp1-zf2 than other platinum complexes (Figure 1 A). The control experiment confirmed that PAR does not induce the Zn^{2+} ion release from Sp1-zf2 in the absence of platinum complexes.

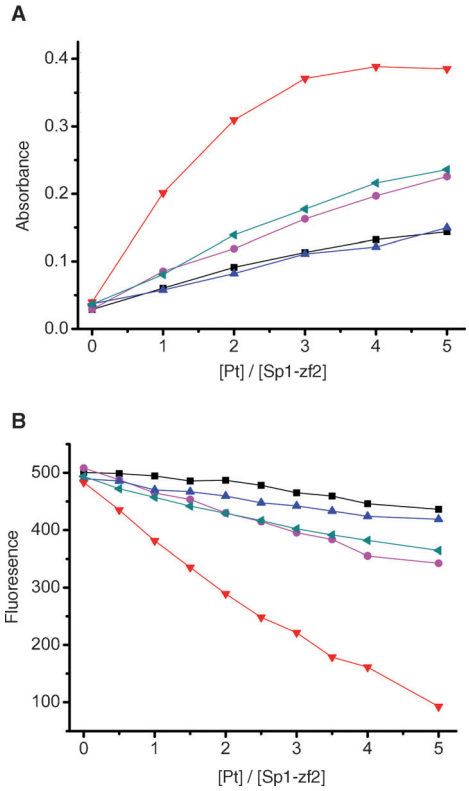

Figure 1. Reactivity of platinum complexes to zinc-bound Sp1-zf2. A) UV absorption (at 500 nm) of the zinc ion release from Sp1-zf2 triggered by platinum complexes. The reactions were performed on 30 μM Sp1-zf2 with different ratios of [Pt]/[protein] for 40 h at 37 °C in 50 mM HEPES (pH 6.8), 50 μM PAR. B) Fluorescence of Sp1-zf2 quenched by platinum complexes at different [Pt]/[Sp1-zf2] ratios. The reactions were performed in 5 μM Sp1-zf2, 10 mM HEPES at pH 6.8. The fluorescence was measured after 48 h incubation at 37 °C. Platinum complexes: *cis*-DDP (black, ■), *trans-DDP* (magenta, ●), *trans-EE* (blue, ▲), *trans*-PtTz (red, ▼), *trans*-PtPy (green, ◀).

Fluorescence measurements were also carried out to investigate the reactions. The fluorescence from the tryptophan residue in Sp1-zf2 is dependent on the protein folding,[18] so that the structure alteration caused by the zinc release can be detected. *Trans*-PtTz has the highest affinity to Sp1 (Figure 1 B), which is consistent with the zinc-release assay.

The products of the platination of Sp1-zf2 by *trans*-PtTz were characterized by ESI-MS analysis. Results showed that each zinc-finger domain can bind two or three platinum complexes, forming adducts $[(Sp1\text{-}zf2)+2\,Pt(NH_3)(Tz)]$ and $[(Sp1\text{-}zf2)+3\,Pt(NH_3)(Tz)]$ (Figure 2). During the binding reaction of the $Pt(NH_3)(Tz)$ scaffold to Sp1, the two chloro ligands in *trans*-PtTz were substituted, whereas the two carrier ligands (NH_3 and thiazole) remained at their position in the complex. As the *trans*-PtTz acts as a bifunctional complex to Sp1 binding, the four-coordination zinc-binding

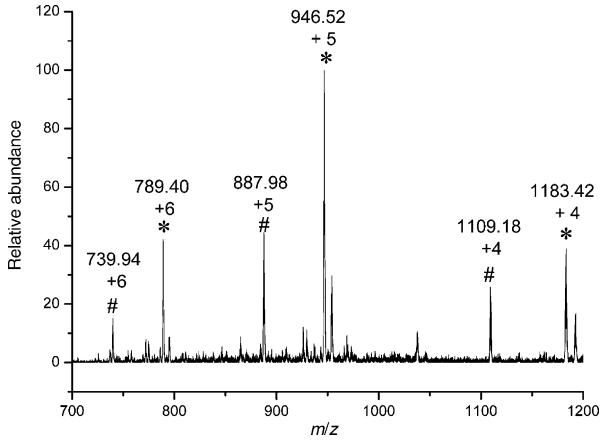

Figure 2. ESI-MS spectrum of the products from the reaction of Sp1-zf2 with *trans*-PtTz. The reaction was carried out with 50 μM Zn-Sp1-zf2 and 100 μM *trans*-PtTz at 37 °C for 30 h in 50 mM HEPES buffer (pH 6.8). The sample was purified by HPLC prior to ESI-MS analysis. #: Bisplatinated adduct $[(Sp1\text{-}zf2)+2\,Pt(NH_3)(Tz)]$, *: triplatinated adduct $[(Sp1\text{-}zf2)+3\,Pt(NH_3)(Tz)]$.

motif (C2H2) of Sp1-zf2 can provide binding sites for two platinum complexes. The third platinum may bind nonspecifically with relatively lower binding affinity.

Monitoring the reaction between *trans*-PtTz and Sp1-zf2 showed that the zinc ion is released from the protein in a time-dependent manner, with a half-life ($t_{1/2}$) of approximately 1.8 h (Figure 3 A). A similar result was obtained by HPLC analysis, showing $t_{1/2} \approx 1.5$ h (Figure S3). The reaction was also performed on the three-domain protein Sp1, which demonstrated considerably higher reactivity than the single-domain Sp1-zf2. The zinc-release assay gave a $t_{1/2} \approx 11$ min in the reaction of Sp1, which is about 9 times faster than the reaction of Sp1-zf2 (Figure 3 B). This observation suggests that the zinc release is cooperative on different zinc-finger domains. A similar result was obtained from UV absorption at 285 nm, based on the formation of Pt–S bonds ($t_{1/2} \approx 16$ min; Figure S4). These data indicate that, even with zinc binding, Sp1 readily reacts with *trans*-PtTz.

We also investigated the reactions of metallothionein (MT) and glutathione (GSH) with *trans*-PtTz in order to compare the reactivity of Sp1 with other cellular thiol-rich molecules. The kinetic results showed that the reaction rate of GSH ($t_{1/2} \approx 15$ min) was very similar to that of Sp1 ($t_{1/2} \approx 16$ min), while MT reacted relatively faster ($t_{1/2} \approx 6$ min; Figure S5). As MT and GSH are the most abundant cellular thiol-rich molecules and highly reactive toward platinum drugs, the kinetic study in this work suggests that Sp1 is kinetically competitive with other intracellular molecules in the reaction with *trans*-PtTz (Table 1).

Sp1 has a classical ββα-zinc-finger structure, and the folding is dependent on the zinc coordination.[19] To verify the disruption of the Sp1 structure upon *trans*-PtTz binding, circular dichroism (CD) and NMR spectra were recorded. CD spectra showed a well-folded structure of the zinc-bound Sp1-zf2, characterized by the large negative ellipticity at 208 nm and a shoulder at 228 nm, and positive ellipticity at 190 nm (Figure 4). Adding EDTA to Sp1-zf2 shifted the negative

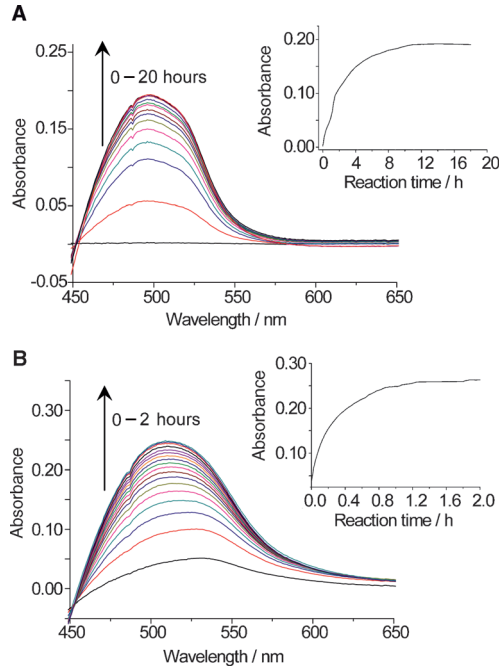

Figure 3. Time-dependent zinc-release assay on 30 μM Sp1-zf2 (A) or 25 μM Sp1 (B). Reactions were performed in 50 mM HEPES buffer (pH 6.8) and 100 mM $NaNO_3$ at 37 °C with protein/Pt ratio of 1:2 for Sp1-zf2 and 1:6 for Sp1, respectively. Spectra were recorded every two minutes, and 17 representative spectra were selected in each figure. Insets show the plot of absorbance at 500 nm (A500) versus reaction time.

Table 1: Half-lives for the reactions of *trans*-PtTz with proteins.

	Sp1-zf2	Sp1	GSH	MT
$t_{1/2}$	1.8 h[a] 1.5 h[b]	11 min[a] 16 min[c]	15 min[c]	6 min[c]

[a] Zinc-release assay. [b] HPLC measurement. [c] UV detection of the Pt–S bond formation. Reactions were performed at 37 °C and pH 6.8. For other conditions, see captions of Figures 3, and S3–S5 in the Supporting Information.

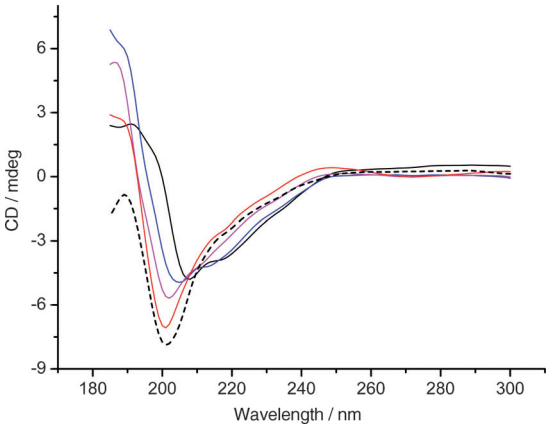

Figure 4. *trans*-PtTz disrupts the secondary structure of Sp1-zf2. CD spectra of 50 μM Sp1-zf2 were recorded after the reaction with *trans*-PtTz in 10 mM phosphate buffer (pH 6.8) for 30 h incubation at 37 °C. Ratio of [Pt]/[protein]: 0:1 (black), 1:1 (blue), 2:1 (magenta), 3:1 (red). The dashed line shows the spectrum of apo-Sp1-zf2 after addition of EDTA to remove zinc from the protein.

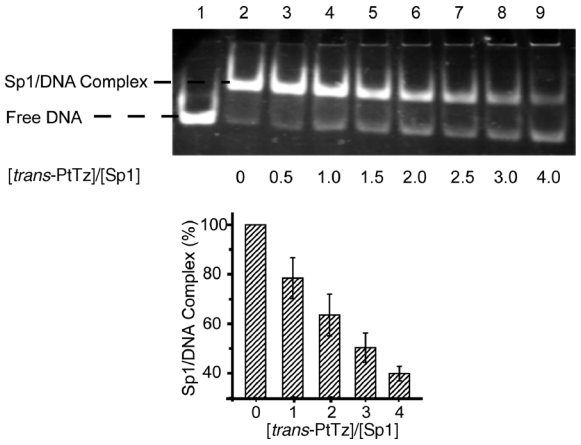

Figure 5. *trans*-PtTz inhibits the binding of Sp1 to hCtr1 promoter DNA. Gel-mobility shift assay was performed on 40 μM Sp1 with 10 μM hCtr1 promoter DNA. The reaction of *trans*-PtTz was carried out in 20 mM Tris-HNO_3 buffer (pH 8.0) containing 100 mM $NaNO_3$. A) Lane 1: DNA control; Lanes 2–9: DNA/Sp1 complex was incubated with different molar ratio to *trans*-PtTz (0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, and 4.0, respectively) for 12 h at 25 °C. B) Relative abundance of Sp1/DNA complex at different molar ratios to *trans*-PtTz.

peak from 208 nm to 200 nm, thus indicating that the removal of zinc disrupted the secondary structure of Sp1-zf2. A similar effect was observed upon the reaction with *trans*-PtTz, which also shifted the negative peak to 200 nm. This observation confirmed that the binding of *trans*-PtTz disrupted the secondary structure of Sp1. In contrast, *cis*-DDP had a negligible effect on the secondary structure of Sp1-zf2 (Figure S6); this result is consistent with the reactivity of platinum complexes to Sp1. In addition, the conformational change of Sp1 resulting from *trans*-PtTz binding was further confirmed by 2D ^{1}H-^{15}N HSQC NMR spectra recorded on ^{15}N-labeled Sp1-zf2 samples (Figure S7).

Because Sp1 is a transcription factor that regulates the expression of a wide variety of genes, we further investigated the effect of *trans*-PtTz on Sp1 binding to DNA. DNA binding was analyzed by an electrophoretic mobility shift assay (EMSA). A 20-mer DNA sequence from Ctr1 promoter recognized by Sp1 was used as a target probe in the EMSA assay (Figure 5). The result showed that Sp1 can strongly bind to DNA in the absence of a platinum complex. Binding of *trans*-PtTz to Sp1 clearly disrupts the DNA-binding property of Sp1. In contrast, *cis*-DDP has no detectable effect on Sp1 binding to DNA (Figure S8). This observation indicated that the structure perturbation that resulted from *trans*-PtTz binding strongly interferes with the function of Sp1 in DNA recognition.

The Sp1 transcription factor is a nuclear protein and executes gene regulation in the nucleus. The stable zinc-finger structure of Sp1 is required for trafficking of the protein from cytoplasm to nucleus.[20] To investigate the effect of *trans*-PtTz on the cellular localization of Sp1, we transiently expressed a fusion-protein-containing Sp1 and green-fluorescence pro-

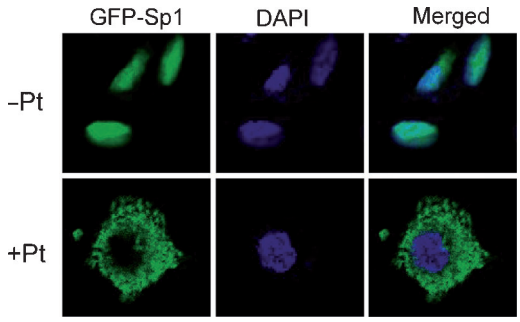

Figure 6. Effect of *trans*-PtTz on the subcellular localization of GFP-Sp1 in A549 cells. Cells were grown 6 h after transfection, and then new media were changed (with or without 10 μM *trans*-PtTz) every 12 h. After 30 h, cells were analyzed by confocal laser scanning microscopy. −Pt: control experiment in the absence of platinum complex; +Pt: cells were treated with 10 μM *trans*-PtTz.

tein (GFP-Sp1) in A549 lung cancer cells. Thus, the subcellular localization of Sp1 can be detected by confocal laser scanning microscopy.[20] Results showed that, in the absence of *trans*-PtTz, GFP-Sp1 was localized to the nucleus (Figure 6, −Pt), whereas the treatment of *trans*-PtTz led to Sp1 being localized mainly in the cytoplasm (Figure 6, +Pt). This observation clearly indicated that *trans*-PtTz prevents the nuclear trafficking of Sp1.

The reactivity of platinum complexes can be influenced by various factors, such as coordination geometry and ligand properties. Furthermore, the tryptophan residue in proteins can induce high affinity to platinum complexes containing aromatic ligands because of π–π interactions,[21] which can further enhance the platinum coordination.[22] However, we proved that the high reactivity of *trans*-PtTz does not result from the π interactions of thiazole (see the Supporting Information, Text S3 and Figure S9)

Given the significantly different reactivities of *trans*-PtPy and *trans*-PtTz, the thiazole ligand must play an important role in the reaction with Sp1. In comparison with pyridine complex, thiazole has a noncoordinating sulfur atom, which contains a lone pair of electrons, and could alter the polarity of the aromatic ring. In order to verify the contribution of the sulfur atom of the thiazole to the reactivity with Sp1, we synthesized the imidazole (Im) complex *trans*-[$PtCl_2$-(NH_3)(Im)] (*trans*-PtIm), which also has a noncoordinating heteroatom, a nitrogen atom, in imidazole. The two complexes showed nearly equal reactivity to the protein (Figure S10). This result suggests that the polar heteroatoms in the aromatic ligand of platinum complexes can strongly enhance the platination of Sp1 zinc-finger protein. Although more experiments and theoretical calculations are needed to identify the detailed mechanism leading to the selectivity, results in the present work indicated that the ligand can indeed modulate the reactivity of platinum complexes.

It is well-known that DNA is the ultimate target of platinum antitumor drugs. The binding of *trans*-PtTz to DNA has also been detected, although the binding mode is different from that of cisplatin.[23] However, the majority of cellular platinum does not reach the DNA in the nucleus, and the different antitumor activities of various platinum complexes cannot be simply explained by their DNA-binding modes. Proteins have been increasingly recognized recently in the antitumor mechanism of platinum drugs.[24] The platination of proteins interferes with their functions and influences a series of cell signal pathways involved in tumor development, growth, and metastasis.[25] Thus, in addition to the DNA binding, protein interaction could also contribute to the antitumor mechanism of platinum drugs; and the different reactivity probably results in the different antitumor properties of various platinum complexes.

Transcriptional gene expression is a central control in governing many cellular processes. The dysregulation of gene expression is associated with various diseases, including cancers, cardiovascular diseases,[26] and metabolic syndrome.[27] Interfering with zinc-finger motifs can effectively down-regulate the expression of some oncogenes.[4,28] Therefore, transcription factors are potential targets of therapeutic agents. Herein we demonstrated that *trans*-PtTz, a nonclassical platinum antitumor agent, possesses high binding affinity to Sp1. Because Sp1 regulates the expression of several oncogenes and is proposed as a potential drug target,[29] binding of *trans*-PtTz to Sp1 very likely contributes to its antitumor properties, although DNA binding eventually triggers apoptosis.

A large number of kinetic studies have been reported on cisplatin, because the reaction rate is also a crucial aspect in determining the activity of platinum drugs.[30] For platinum compounds, binding of thiol-rich molecules, such as metallothionein (MT) and glutathione (GSH), is kinetically more favorable compared with binding of DNA. It was proven that MT detoxifies cisplatin.[31] In order to target the nuclear DNA, cisplatin must traverse these cytosolic molecules before reaching the nuclear DNA. Therefore, the kinetic property of platinum compounds is important for the drug activity. The kinetic study in this work showed that, in the reaction of *trans*-PtTz, Sp1 is competitive with MT and GSH, the high-affinity platinophiles in cells. This result showed that the binding of *trans*-PtTz to Sp1 is kinetically reasonable in cells.

In conclusion, the antitumor-active *trans*-platinum complex, *trans*-PtTz demonstrated high reactivity to zinc-finger protein Sp1. The platination of Sp1 disrupts the secondary structure of the protein and interferes with the DNA-binding function of Sp1. An in vivo assay indicated that the binding of *trans*-PtTz prevents the Sp1 trafficking from the cytoplasm into the nucleus. This result indicated that binding of *trans*-PtTz can disrupt the function of Sp1, thus consequently interfering with the expression of corresponding proteins. A study on different *trans*-platinum complexes demonstrated that their reactivity is markedly dependent on the property of the ligands. These results identified a novel approach for the inhibition of Sp1, and provide more insight into the mechanism of nonclassical platinum-based antitumor agents.

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