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Mass Spectrometric Proteomics Reveals that Nuclear Protein Positive Cofactor PC4 Selectively Binds to Cross-Linked DNA by a *trans*-Platinum Anticancer Complex

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Supporting Information

ABSTRACT: An MS-based proteomic strategy combined with chemically functionalized gold nanoparticles as affinity probes was developed and validated by successful identification and quantification of HMGB1, which is well characterized to interact selectively with 1,2-cross-linked DNA by cisplatin, from whole cell lysates. The subsequent application of this method to identify proteins responding to 1,3-cross-linked DNA by a *trans*-platinum anticancer complex, *trans*-PtTz (Tz = thiazole), revealed that the human nuclear protein positive cofactor PC4 selectively binds to the damaged DNA, implying that PC4 may play a role in cellular response to DNA damage by *trans*-PtTz.

The DNA-targeting platinum complex cisplatin is one of the most successful drugs for clinical treatment of solid tumors, and can cross-link DNA, leading to a dramatic distortion in the duplex structure which serves as a recognition signal for nucleic acid repair and other cellular processes.[1,2] The high anticancer efficiency of cisplatin and the clinical inactivity of its *trans* isomer (transplatin) have been considered a paradigm for the classical structure−activity relationships of platinum drugs. However, several new analogues of transplatin, which contain a planar amine (L) ligand in the general structure of *trans*-$[PtCl_2(NH_3)(L)]$, have recently shown a different spectrum of cytostatic activity including activity against cisplatin-resistant tumor cells.[3−6] *trans*-$[PtCl_2(NH_3)$(thiazole)] (*trans*-PtTz) (Figure 1) represents an example of such compounds. The detailed mechanism by which cells recognize and process platinum−DNA adducts is of great interest for better understanding of the mechanisms of activity and inactivity of platinum compounds. Much effort has been made to isolate and identify specific proteins that mediate the cellular responses to DNA lesions by cisplatin. In 1992, an affinity precipitation technique using cisplatin-damaged DNA cellulose was developed to characterize proteins that respond to cisplatin-induced DNA damage.[7] Recently, poly[ADP-ribose]-polymerase 1 (PARP-1) and high-mobility group protein B (HMGB) have been identified to interact with cisplatin-damaged DNA based on a photoaffinity labeling strategy in which the photoreactive benzophenone tethered to cisplatin can cross-link the target proteins upon UV irradiation.[8] On the other hand, although a few reports have shown that the multiple DNA lesion by *trans*-PtTz, including 1,3-intrastrand, interstrand cross-linking and monofunctional binding, may reasonably contribute to the unusual cytotoxicity profile of this *trans*-platinum complex,[9,10] the details of the structural consequences of these DNA damages still remain largely unknown.[2]

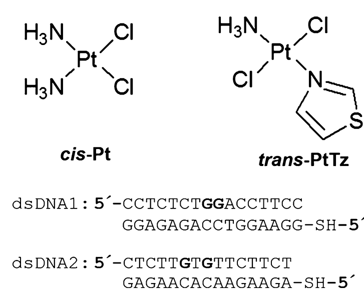

Figure 1. Chemical structures of platinum anticancer complexes cisplatin (*cis*-Pt) and *trans*-$[PtCl_2(NH_3)$(thiazole)] (*trans*-PtTz), and the sequences of the synthetic oligodeoxyribonucleotides dsDNA1 and dsDNA2 which were used to prepare *cis*-Pt and *trans*-PtTz cross-linked DNA motifs, respectively. The boldface letters in the duplexes indicate platinated bases.

In the present work, therefore, we develop an MS-based proteomic approach in combination with using chemically functionalized gold nanoparticles (AuNPs) as affinity probes to capture the large protein complexes *in vitro* for identification of the cellular proteins responding to damaged DNA by cisplatin and *trans*-PtTz. AuNPs have excellent water-solubility, large surface area, and are easily surface-functionalized and highly dispersed. AuNPs coupled to MS-based proteomics have been successfully used to isolate and quantify a transcriptional activation complex of the estrogen response element (ERE).[11]

The proof of concept was first conducted by identifying the proteins interacting with cisplatin-damaged DNA. AuNPs with an average diameter of 13 nm[12] were multifunctionalized with the well-known 1,2-cisplatin cross-linked DNA motif dsDNA1[13] (Figure 1) and thiolated polyethylene glycol (SH-PEG), which could inhibit nonspecific binding of proteins on

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the AuNP surface, to prepare the positive affinity probe, designated as Pos1 (AuNP−cisPt(DNA1)).[14] Then, *in vitro* studies with a complex whole-cell extract of human breast cancer cell line MCF-7 were carried out to verify the ability of the positive probe Pos1 to capture the well-established binding partner HMGB1 of cisplatin-cross-linked DNA.[15] To ensure that HMGB1 was captured through a specific interaction with the damaged DNA, AuNPs coated by SH-PEG alone were used as a control (Neg1 = AuNP−PEG) under the same conditions. The Pos1 probe and Neg1 control were individually incubated with the MCF-7 lysates (experimental details are given in the Supporting Information [SI]), and the captured proteins were boiled with the loading buffer and directly loaded onto a 12% SDS polyacrylamide gel for Western blotting assay. As shown in Figure 2, the results indicate that the Pos1 probe binds to

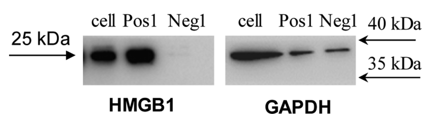

Figure 2. Western-blotting data for HMGB1 and GAPDH captured by Pos1 probe and Neg1 control. "Cell" represents whole cell lysate.

HMGB1 with high affinity and selectivity. By contrast, nonspecific binding to proteins such as GAPDH was observed for both Pos1 and Neg1 due to electrostatic interactions between positively charged proteins and the negatively charged AuNPs.

The following proteomic study starts by a one-step affinity separation based on the affinity probe Pos1, and the proteins bound to Pos1 were pulled off by boiling, digested by trypsin, and then identified by tandem mass spectrometry coupled to nano-HPLC (Figure 3). The characterization of the protein

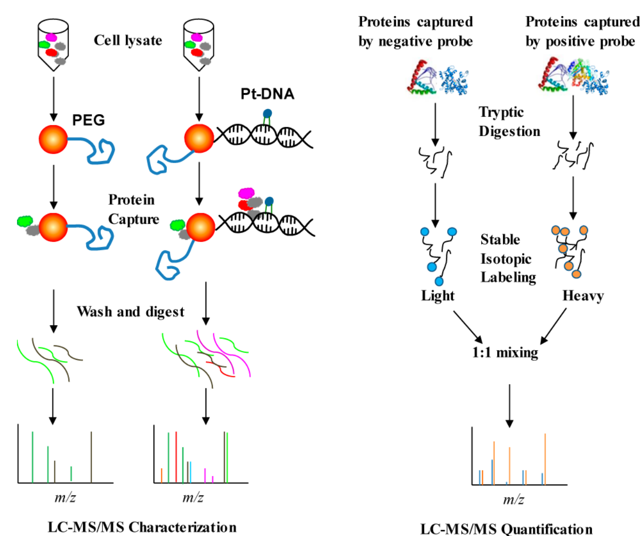

Figure 3. Schematic illustration of protein capture by functionalized gold nanoparticles in combination with the MS-based proteomics for proteins responding to platinated DNA.

complex isolated by the Pos1 probe from the whole cell extract of MCF-7 resulted in the identification of 33 proteins (Table S2 in the SI). The MS/MS spectrum of tryptic peptides from HMGB1 is shown in Figure 4a. To exclude nonspecific binding proteins to the cisplatin-damaged DNA probe, we developed a quantitative proteomic approach in combination with the use of

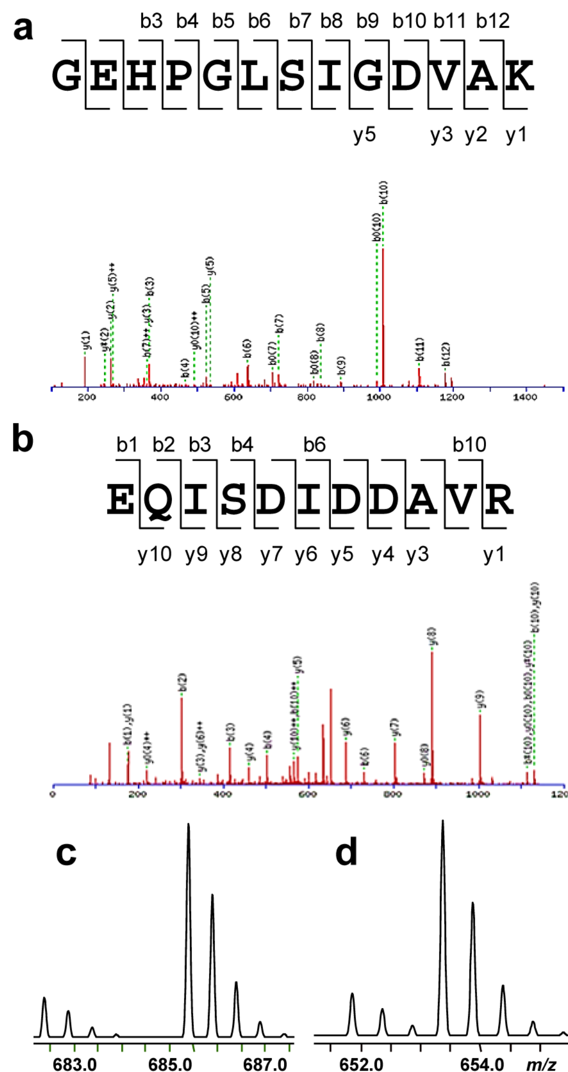

Figure 4. (a,b) Representative MS/MS spectra of tryptic peptides of (a) HMGB1 captured by positive probe Pos1 and (b) PC4 captured by positive probe Pos2 from MCF-7 lysates; (c,d) Quantitative MS spectra for peptides arising from the 1:1 mixtures (v/v) of light- and heavy-labeled tryptic digests of (c) HMGB1 captured by Neg2 and Pos1, and (d) PC4 captured by Neg3 and Pos2 probes, respectively, from the same amount of MCF-7 lysates.

the negative control (Neg1) and stable isotopic acetyl labeling of tryptic peptides by *N*-acetoxy-H_3-succinimide (H3-NAS) or *N*-acetoxy-D_3-succinimide (D3-NAS) (Figure 3). The differentially enriched proteins by Pos1 with a heavy-to-light (H_{pos1}/L_{neg1}) ratio of >2 were postulated as responding proteins to cisplatin-damaged dsDNA1 (Table 1). HMGB1 was detected with a large ratio of "heavy-to-light" (9.48), which was expected as its well-established affinity to the 1,2-intrastrand Pt-DNA cross-links.[15,16]

Since native double-stranded oligonucleotides can also bind to various cellular proteins, such as histone and other nuclear proteins, it is necessary to exclude the nonspecific DNA binding partners. In this regard, the native double-stranded DNA fragment dsDNA1 (Figure 1) was used to functionalize AuNPs with SH-PEG to construct the negative control Neg2 (AuNP−dsDNA1). Peptides derived from the pulldowns by Pos1 and Neg2 were "heavy" and "light" labeled, respectively. As shown in Table 1, among the 10 proteins which have higher affinity to

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Table 1. Proteins Identified to Bind to Platinated DNA *in Vitro* with the H/L Ratio >2, where H Is the Intensity of One or More Peptides from a Protein Captured by Positive AuNP Probes, and L Is the Intensity of the Same Peptide(s) from the Protein Captured by Negative Controls

Swiss-Prot accession No.	protein name in abbr.[a]	$H_{pos1}{}^{b}/L_{neg}$ (n = 3)		Swiss-Prot accession No.	protein name in abbr.[a]	$H_{pos2}{}^{b}/L_{neg}$ (n = 3)	
		Neg1[b]	Neg2[b]			Neg1[b]	Neg3[b]
P53999	PC4	10.63 ± 2.05	0.85 ± 0.17	P47914	RL29	7.12 ± 2.44	0.44 ± 0.14
P09429	HMGB1	9.48 ± 2.41	5.90 ± 0.52	P53999	PC4	30.45 ± 5.60	6.32 ± 0.91
P09874	PARP1	8.90 ± 0.62	0.87 ± 0.11	Q5JNZ5	RS26L	15.45 ± 8.61	1.03 ± 0.41
Q13765	NACA	7.26 ± 1.08	0.83 ± 0.22	Q07020	RL18	14.09 ± 3.07	1.20 ± 0.53
P61978	HNRPK	6.80 ± 3.09	1.15 ± 0.36	P27635	RL10	10.41 ± 2.05	1.07 ± 0.34
Q96QV6	H2A1A	3.08 ± 0.36	1.00 ± 0.45	P84098	RL19	10.44 ± 3.09	1.18 ± 0.21
Q02543	RL18A	5.79 ± 3.37	0.99 ± 0.35	P36578	RL4	8.84 ± 2.08	0.93 ± 0.08
P62979	RS27A	2.66 ± 0.45	1.35 ± 0.70	P62910	RL32	6.42 ± 2.51	1.41 ± 0.66
Q9BW65	RL14	2.40 ± 0.60	1.30 ± 0.22	P62266	RS23	6.13 ± 3.67	1.01 ± 0.22
Q32P51	RA1L2	3.49 ± 0.81	1.13 ± 0.27	Q13765	NACA	5.31 ± 2.24	0.96 ± 0.15

[a]All abbreviations used are given in the SI. [b]Pos1: AuNP−cisPt(DNA1); Pos2: AuNP−transPtTz (DNA2); Neg1: AuNP−PEG; Neg2: AuNP−DNA1; Neg3: AuNP−DNA2.

the Pos1 probe than to the Neg1 control (Table 1), only HMGB1 has a heavy-to-light ratio of ~6 (Figure 4c), consistent with HMGB1 preferentially binding to 1,2-cisplatin cross-linked DNA over native DNA.[2,13,17]

Next, the validated MS-based proteomic strategy was applied to identify the cellular proteins in response to DNA damage induced by *trans*-PtTz. It has been reported that the modification of DNA by the cytotoxic transplatinum complex *trans*-PtTz afforded monofunctional, bifunctional intra- and interstranded adducts in roughly equal proportions.[10] Herein, we chose a consensus DNA fragment dsDNA2 (Figure 1) to prepare the 1,3-intrastranded *trans*-PtTz-cross-linked DNA motif. The top strand of dsDNA2, which contains a GTG sequence, reacted with *trans*-PtTz,[18] forming the 1,3-cross-linked single strand as evidenced by the MS analysis and DMS probing (Figures S1 and S2 in the SI). Then, AuNPs were functionalized with the 1,3-cross-linked DNA motif formed by the annealing of the platinated -GTG- strand with its complementary strand (Figure 1), preparing the positive affinity probe Pos2 (AuNP−transPtTz(DNA2)).

The Pos2 probe was first incubated with MCF-7 cell lysates, and then proteins bound to the damaged dsDNA2 were digested, followed by nanoLC−MS/MS characterization. As listed in Table S2 in the SI, 25 proteins were identified from the pulldowns by Pos2. Among them, we found some DNA binding proteins and DNA-damage responding proteins, such as nascent polypeptide-associated complex subunit alpha (NACA) and activated RNA polymerase II transcriptional coactivator p15 (PC4). The MS/MS spectrum of a tryptic peptide derived from PC4 is shown in Figure 4b.

Quantitative experiments were performed to differentiate those nonspecific bindings to the PEG-functionalized AuNPs (Neg1), especially proteins with high abundance, which will present in a ratio close to 1:1 in the "light" and "heavy" samples containing the peptides derived from the pulldowns by Pos2 and Neg1 labeled by the "heavy" and "light" NAS tags, respectively. Among the 25 proteins identified from the pulldowns by Pos2, 10 proteins (Table 1) have a heavy-to-light ratio of >2 (the raw MS data for calculation of the heavy-to-light ratios are given in Table S3 in the SI), indicating that they have higher affinity to *trans*-PtTz-damaged DNA than to the AuNP−PEG control. For example, the activated RNA polymerase II transcriptional coactivator p15, also known as nuclear protein positive cofactor PC4, was detected with a large ratio (30.45) of "heavy" to "light". Another DNA binding protein NACA, which can bind with DNA and stabilize the interactions of JUN homodimers with target gene promoters, was also identified in a high "heavy"-to-"light" ratio (5:1). These suggest that both PC4 and NACA are strong candidates interacting with *trans*-PtTz-cross-linked DNA.

To exclude nonspecific binding partners from the proteins specifically interacting with duplex oligonucleotides platinated by *trans*-PtTz, a similar quantitative analysis was performed with use of the AuNPs modified with the native duplex oligonucleotide dsDNA2 as a control (Neg3 = AuNP−DNA2). Peptides derived from the pulldowns by Pos2 and Neg3 were "heavy" and "light" labeled, respectively. As is shown in Table 1, among the 10 proteins which have higher affinity to the Pos2 probe than to the Neg1 control, 9 proteins have a "heavy"-to-"light" ratio close to 1:1, whereas the nuclear protein positive cofactor PC4 prefers to bind to the damaged DNA2 rather than to the native one, as evidenced by the heavy-to-light ratio of >6 (Table 1, Figure 4d). Endogenous PC4 is a nuclear DNA-binding protein that stimulates activator-dependent class II gene transcription *in vitro*,[19] and has been found to accumulate at DNA damage sites introduced by either chemical agents or laser micro-irradiation.[20] Thus, the specific interaction between *trans*-PtTz-damaged DNA and PC4 discovered herein may be related to the unique anticancer activity of *trans*-PtTz since the interaction of PC4 with unwound double-stranded DNA inhibits transcription.[19] Further pinpointing the function of the recognition and interaction between PC4 and 1,3-*trans*-PtTz cross-linked DNA will be helpful to understand the unique mechanism of action of this kind of *trans*-platinum anticancer complex.

It is worthy to point out that the sensitivity of the MS identification and quantification was improved significantly when we first applied microscale HPLC to fractionalize the tryptic digest of the pull-downs by functionalized AuNP probes, and then separated the obtained fractions one by one by nano-HPLC prior to MS analysis. Typically, as listed in Table S4 and S5 in the SI, the number of proteins unambiguously identified by analyzing pull-downs by Pos2 and Neg3 from the same amount of cell lysate mentioned above increases from 25 to 98, among which more low abundance proteins were identified. Interestingly, except for PC4, the replication protein A (RFA1)

was also identified by differentiation analysis to be a specific protein bound to cross-linked DNA by *trans*-PtTz.

In summary, our studies demonstrate herein for the first time that DNA-conjugated gold nanoparticles have great potential to capture proteins recognizing DNA lesions by anticancer drugs *in vitro* for subsequent MS characterization and quantification analysis of the proteins of interest. The use of AuNPs, which possess excellent water-solubility and large surface area and are highly dispersed, allows the protein binding and capturing to be performed in homogeneous solution with the need of only a small amount of cell lysates. Sensitive identification of proteins responding to platinum-damaged DNA in the most physiologically relevant environment could be achieved as confirmed by the successful identification of well-known protein HMGB1 in response to platination of DNA by cisplain. The discovery of the nuclear protein positive cofactor PC4 specifically interacting with cross-linked DNA by *trans*-PtTz will shed light on elucidating the anticancer mechanism of this active *trans*-platinum complex. The subsequent biological consequences after the specific recognition of the 1,3-*trans*-PtTz cross-linked DNA by PC4 deserve our further study. We anticipate not only a broad application of this proteomic strategy in other biological systems but also a deeper understanding of the activity of platinum and other DNA-targeting anticancer drugs.

■ ASSOCIATED CONTENT

Supporting Information

Experimental procedures, MS and MS/MS 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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