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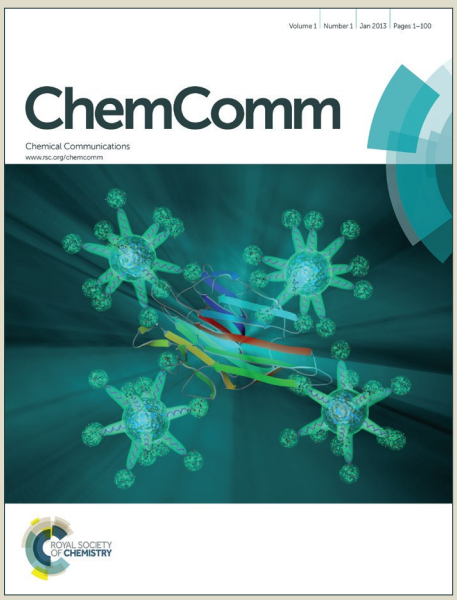

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The Role of Carrier Ligands of Platinum(II) Anticancer Complexes in the Protein Recognition of Pt-DNA Adducts

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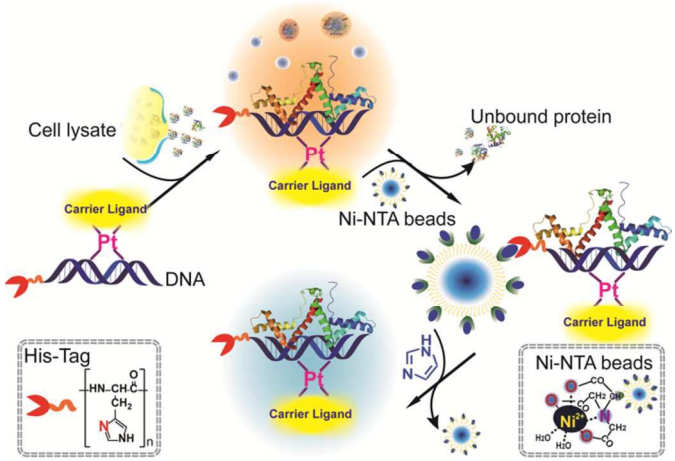
In order to systematically investigate the influence of carrier ligand on the interaction of Pt-DNA adducts with damage recognition proteins, a series of DNA probes containing 1,2-GG platinum compound crosslinks using cisplatin, oxaliplatin, (S,S-DACH)$PtCl_2$ and (cis-1,4-DACH)$PtCl_2$ (kiteplatin) has been constructed. These complexes share similar DNA binding properties although they exhibit quite different cytotoxicity. It is revealed that HMGB1 (High-mobility group protein B1) was the most commonly found protein that recognizes all Pt(II)-DNA probes and prefers cisplatin-DNA probe more than the others. Interestingly, an important component of replication protein A complex, RPA2, was found to bind to kiteplatin much more tightly than other proteins. These results may be important for the interpretation of the roles of carrier ligands in platinum(II)-based anticancer complexes.

Platinum drugs have been widely used to treat numerous patients with a variety of cancers alone or in combination with other anticancer agents.[1] Substantial evidences have been achieved over the past decades that DNA is the main target of platinum drugs which form coordination bond with the N7 of purine bases (mainly guanine) of DNA.[2,3] Generally speaking, platinum drugs bind to DNA and forms both bifunctional intrastrand and interstrand DNA cross-links,[4] which induce profound conformational changes in DNA.[5] The Pt-DNA adducts consequentially initiate a series of cellular responses by interfering a variety of different nuclear bio-functions, leading to remarkable cellular alternations or programmed cell death.[6]



Scheme 1. A pull-down experiment is designed with cell extracts using DNA probes conjugated with poly-His peptides. Cell lysate is incubated with DNA probes as a protein pool. Protein-DNA interaction complexes are isolated from cell lysate with Ni-NTA beads. The proteins captured are digested into peptide fragments and identified using mass spectrometry.

As newer generation of platinum drugs, carboplatin and oxaliplatin exhibit a wider spectrum of anticancer activities and lower toxicities than cisplatin.[7] More importantly, oxaliplatin and other diaminocyclohexane (dach) containing Pt compounds are capable of overcoming cisplatin resistance in several types of cancer,[8] even though they share similar DNA binding mode with cisplatin. It is therefore hypothesized that the carrier ligands may trigger different cellular DNA damage responses (DDR), as well as affecting the direct interactions of the drugs with protein targets.[9] Due to the complexity of platinum-induced DNA damage

repair[10] and recognition processes, evidences to support the hypotheses are currently poor and highly demanded.[9]

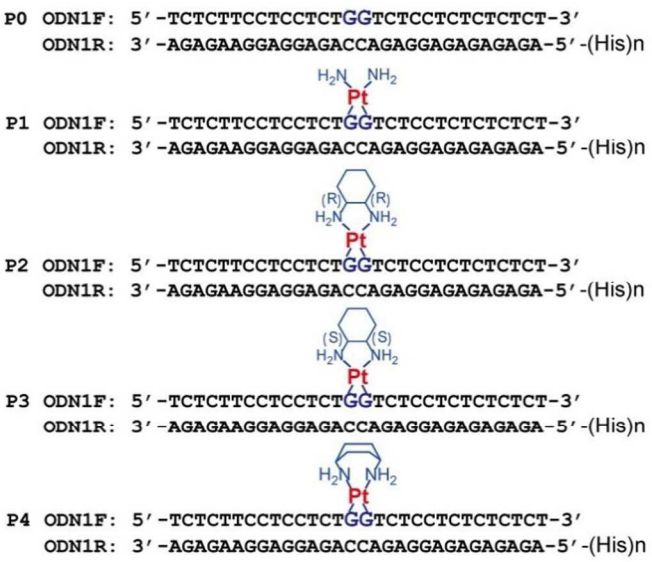


Figure 1. DNA probes without and with different Pt-DNA adducts.

Since the basal proteomic profile in the recognition of Pt-DNA adducts is highly associated with drug sensitivity/resistance of cancer cells, a variety of proteins have been identified as binding factors to Pt-DNA lesions, including DNA architectural binding proteins, DNA damage recognition proteins, DNA replication proteins, DNA repair proteins, etc.[10-12] However, a systematic methodology which can be applied for the identification of proteins bound to different Pt-DNA adducts is currently lacking.[13] To extend our previous study,[14] we constructed a series of DNA probes shown in Figure 1. Each probe contains a site specific Pt-DNA crosslink which acts as a bait for cellular proteins (Scheme 1). As demonstrated previously,[14] this method is very effective in capturing the binding proteins of Pt-DNA adducts with low background of non-specific binding. Colon cancer cell lysate is used as native protein pool for pull down experiments. Being resistant to cisplatin and sensitive to oxaliplatin, this cancer cell could provide valuable proteomic information about differences in toxicity between these two drugs.[15]

As shown in Figure 1, four probes were constructed with the same 1,2-GG Pt-DNA crosslinking but different carrier ligands (**P1** – **P4**), and P0 as the control probe.[9, 16] **P1** is the probe based on cisplatin, the parental compound contains the simplest ammines as carrier ligand. **P2** is derived from oxaliplatin which contains the 1,2-diaminocyclohexane (1,2-DACH) ligand in R,R form, and **P3** from the S,S-(DACH)$PtCl_2$ complex.[17] Notably, kitaplatin, a platinum compound formed by another geometric isomer of DACH carrier ligand, has been recently re-investigated and found to be particularly cytotoxic against most cisplatin-resistant cell lines and colorectal cancer resistant to oxaliplatin.[18, 19] For example, the IC_{50} value for (cis-1,4-DACH)$PtCl_2$ against colorectal cancer cell line SW480 is 2.12 μM, which is far below that of (R,R-1,2-DACH)$PtCl_2$ (11.12 μM) and cisplatin (7.67 μM). It is even better than the value of oxaliplatin (4.2 μM).[20] Therefore, **P4** is also constructed and studied in this work. All platinum complexes used in this work are synthesized and characterized following literature procedures and are described in supplementary materials (Figures S2-S4). Probes **P0** – **P4** are fully characterised with MALDI-TOF MS and thermal stability assay (Figures S5-S7). Larger melting temperature (T_m) decrease corresponds to the larger bending angle caused by Pt-GG crosslink. The T_m of **P2** - **P4** are basically identical while that **P1** is slightly lower (Figure S7). Compared to control probe **P0**, the introduction of Pt-DNA lesions into DNA probe destabilizes their double helix structure according to thermal stability data. Based on the 2-dimensional gel electrophoresis (2-DE) separation combined with high resolution MALDI-TOF MS, we were able to identify the significant differential protein spots. A list of proteins captured by each probe is summarized in table 1. Corresponding 2-DE images could be found in Figures S8-S11.

Compound	Carrier ligand	Proteins Identified[a]
Cisplatin	NH_3	Ku80(P13010[b]); Ku70(P12956); Splicing factor 3A subunit 3, SF3A3(Q12874) ; T-complex protein 1 subunit epsilon, TCPE(P48643); T-complex protein 1 subunit theta, TCPQ(P50990); HMGB1(P09429, 2 PTM isomers); HMGB2(P26583, 3 isomers)
Oxaliplatin	1(R),2(R)-DACH	Ku70(P12956); T-complex protein 1 subunit epsilon, TCPE(P48643); T-complex protein 1 subunit theta, TCPQ(P50990); HMGB1(P09429, 2 PTM isomers)
S,S-(DACH)$PtCl_2$	1(S),2(S)-DACH	Ku80(P13010); Ku70(P12956); T-complex protein 1 subunit epsilon, TCPE(P48643); T-complex protein 1 subunit theta, TCPQ(P50990); HMGB1(P09429, 2 PTM isomers)

Table 1. Proteins discovered with probes of DNA-Pt adduct containing different carrier ligands.

1,4-(DACH)PtCl$_2$	1,4-DACH	ATP-dependent RNA helicase(Q92499), DDX1; Heat shock cognate 71 kDa protein(P11142), hsp70; Heterogeneous nuclear ribonucleoprotein D0, hnRNP D0(Q14103); Replication protein A 32 kDa subunit, RPA2(P15927); HMGB1(P09429,3 PTM isomers)

[a] Detailed information on protein identification is listed in supporting information; [b] Accession number of UniProt Knowledgebase.

Proteins which captured by probes **P1** – **P4** are identified and listed in Table 1. As can be seen from the list, HMGB1 were recruited universally by all four probes. However, some other proteins such as TCPE and TCPQ were identified only by some of the probes. Considering the existence of protein complexes in recognizing the Pt-DNA adducts, some proteins could also be recruited by another Pt-DNA binding protein rather than by binding to DNA directly. It is important to note that some proteins are unique for certain probe. For example, Ku70 and Ku80 which are well known as a pair of proteins responsible for DNA double strand break repair,[21] were captured by the **P1** – **P3**. They are also generally accepted as recognition proteins of DNA damage caused by platinum drugs.[22] However, Ku70 protein captured by Pt-probes exhibits entirely different modification status compared to **P0**. As shown in Figure S8, there were at least 5 different isoforms of Ku70 in **P0** and only one of them was found to bind to cisplatin-DNA adduct (Figure S8, **P1**). Further assessment of total Ku70 through western blotting assay suggests that Ku70 could not discriminate oligonucleotides with or without Pt lesion. This result provides the first evidence that only modification status of Ku70 contributes to its selective binding to Pt-DNA adducts. Similar result was obtained for Ku70 recognized by **P2** and **P3** (Figures S9 and S10).

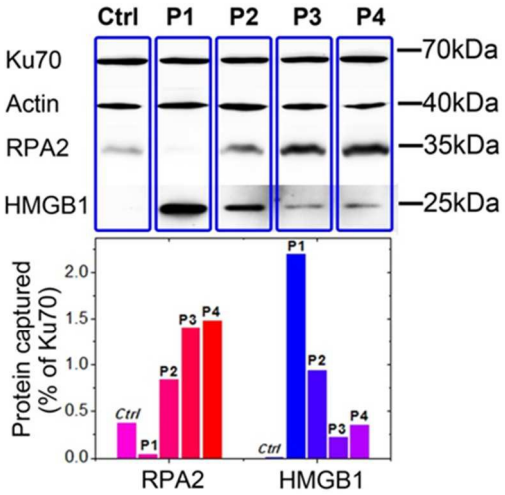


Figure 2. Pull down assay of HMGB1 and RPA2 proteins with different probes. Equal amount probes are used to capture their binding proteins from the identical portion of cell lysate. Proteins captured are resolved on SDS-PAGE and transferred to PVDF membrane for immunoblotting detection. Ku70 and actin are used as internal reference.

The only difference between probes **P2** and **P3** is the chirality of carrier ligand 1,2-cyclohexanediamine (DACH). As a result, proteins captured by these two probes are essentially identical. Compared to probe **P1**, fewer proteins were captured by these two probes. Interestingly, proteins captured by **P4** are very different from those captured by **P1**, **P2** and **P3**. Newly captured proteins include ATP-dependent RNA helicase (Q92499, DDX1), heat shock cognate 71 kDa protein (P11142), heterogeneous nuclear ribonucleoprotein D0 (Q14103, HnRNP D0) and replication protein A 32 kDa subunit (P15927), besides three isomers of HMGB1 which are universally captured by all the probes. DDX1 is a helicase facilitating template-guided repair of transcriptionally active regions of the genome.[23] This protein also acts as a co-activator to enhance some transcriptional activation processes.[24] P11142 acts as a repressor of transcriptional activation on several genes.[25] Q14103, also known as AUF1, binds to single or double strand DNA and functions as a transcription factor.[26] It also controls cell proliferation through specific degradation of mRNA of some cell cycle related proteins.[27] Among the proteins identified for **P4**, RPA2 is the most notable one besides HMGB1 isomers. Although RPA2 has been suggested as a Pt-DNA damage recognition protein for a long time,[28] this appears to be the first unambiguous evidence proving its specificity toward Pt-DNA lesion. As shown in Figure S11, protein spot 4 which is identified as RPA2 could only been recruited by **P4** probe but not by **P0**. Therefore, RPA2 may bind specifically to Pt-DNA adduct containing 1,4-DACH as a carrier ligand.

The above results indicate that the conformation of carrier ligands of platinum complexes could fine tune the interactions between Pt-DNA adduct and its recognition proteins. To elucidate this hypothesis, probes **P1** - **P4** were used to evaluate the relative binding affinity to cellular proteins. Ku70 and actin were used as internal binding markers because of their equal binding affinity to DNA with or without Pt lesions. HMGB1 and RPA2 were assessed in this assay. As shown in Figure 2, changing carrier ligand from simple ammine to 1,2-DACH and further into cis-1,4-DACH, the binding affinity of HMGB1 with Pt-DNA adducts decreased dramatically following the order from **P1** to **P4**. Meanwhile, the binding affinity of RPA2 to Pt-DNA increased significantly from **P1** to **P4**. Such a reversed order of binding affinity of Pt-DNA adducts towards HMGB1 and RPA2 could potentially correlated to their different anticancer activity therefore could provide hints for future rational design of carrier ligands in Pt(II) anticancer complexes. It has been known for a long time that HMGB1 is able to discriminate Pt-DNA adducts formed with cisplatin and oxaliplatin,[29] although the biological consequences of this binding difference remain to be clarified. According to NMR solution structural analysis, the oxaliplatin-GG adduct bends DNA by 31° in the direction of the major groove, thereby the relatively narrow minor groove is not quite fit for HMG box binding. While cisplatin-GG adducts bend the DNA by 60–80◦, the much wider minor groove is easier for HMG box to fit in.[30] This bending angle analysis is also consistent with the result drew from thermal stability assay.

As part of the heterotrimeric replication protein A complex, RPA2 is very important for DNA damage recognition and DNA repair proteins recruitment.[31, 32] In the cellular response to DNA damage,

the RPA complex controls DNA repair and DNA damage checkpoint activation.[33] This protein is a key member of several well-known DNA repair systems such as NER and MMR which are believed to be the primary machineries to process platinum drug induced DNA damage.[34]

Conclusions

In summary, we have developed a systematic method to explore the impact of carrier ligand of Pt complexes on the recruitment of DNA-binding proteins. Though little alteration of DNA double helix is caused by different carrier ligand, protein recognition of Pt-DNA adducts is greatly affected. There are important proteins identified as binding factors of Pt-DNA adducts which include SF3A3，DDX1，hsp70, hnRNP D0, RPA2 etc. Importantly, Ku70, a well-known DNA damage recognition protein, could discriminate DNA with or without Pt lesion only if it is modified into one of its isoforms. Another important discovery revealed in this work is the correlation between the carrier ligand and protein recognition of Pt-DNA adducts. The bulkiness, hydrophobicity and steric configuration of carrier ligand dramatically affect the binding affinity of HMGB1 and RPA2 to Pt-DNA adducts in a reversed manner. The discrimination of different Pt-DNA adducts by DNA damage recognition proteins could potentially result in different drug resistance and cytotoxicity. These results may also provide important implications on the understandings of platinum anti-cancer agents *in vivo*. It potentially provides us with novel leads to improve existing therapeutic drugs in terms of enhancing drug efficiency and overcoming drug resistance.

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