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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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Supporting information

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1. Experiment procedures

1.1 Chemical sources and instruments

The 5'- amino-modified oligonucleotides were purchased from Invitrogen; cisplatin and potassium tetrachloroplatinate were provided by Shandong Boyuan Pharmaceutical Corporation, Shandong province in China; GMBS was purchased from SpeedChemical Corporation. The peptide was synthesised using standard Fmoc-

based chemistry by the Genscript Corporation, Nanjing City, China. The Ni-NTA agarose beads and Histrap HP affinity column were purchased from GE Healthcare. The 2-DE experiments were performed on the Ettan IPGphor 3 system, made by GE Healthcare. The antibodies uesd for western blotting were purchased from Abcam. Ziptip C18 micro-column was purchased from Millipore. All of the other solvents were HPLC-grade and were used as received.

1.2 Cell culture

We purchased the human colon carcinoma cell line sw480 from American Type Culture Collection. The cells were maintained at 5% CO_2 at 37 °C in RPMI-1640 with 10% FBS. The cells were treated with variable concentrations of platinum complexes.

1.3 Cell lysis

The cells were harvested by first digesting with trypsin, followed by washing twice with PBS at 4 °C after low-speed centrifugation, the cell pellet was flashfrozen in liquid nitrogen. The cell lysis buffer formulation was as follows: 20 mM Tris, pH 7.4, 1 mM Na_3VO_4, 250 mM NaCl, 0.5% NP-40, 2 mM NaF, 1 mM PMSF Protease Inhibitor Cocktail (Sigma), 1 mM DTT. We used a Dounce homogeniser, with 15 strokes every 10 min on ice to homogenise the cells. Cell homogenisation was performed by centrifugation at 12000 rpm for 10 min at 4 ºC. Supernatants were transferred to clean tubes and used as the cell extract for the next step.

1.4 Compounds synthesis

[$PtCl_2$(cis-1,4-DACH)] was prepared as described previously. [$PtCl_2$(1(R),2(R)-DACH)] and [$PtCl_2$(1(S),2(S)-DACH)] was prepared according to the method of Dhara. Briefly, $K_2[PtCl_4]$ (0.726 g, 1.75 mmol) was dissolved in a minimum amount of water (15mL) resulting red solution. 1(R), 2(R)-diaminocyclohexane (200 mg, 1.75 mmol) was dissolved in methanol in equal volume and was in turn dropped into the former red solution, which caused the immediate formation of a yellow precipitate. The suspension was stirred for 3 h. The yellow precipitate, [$PtCl_2$(1(R),2(R)-DACH)], was then collected by filtration, washed with water, ethanol, and diethyl ether, dried under vacuum, and analyzed by ESI-MS and NMR(DMSO). [$PtCl_2$(1(S),2(S)-DACH)] was prepared in the same way as 1(R),2(R)-diaminocyclohexane. The ESI-MS and NMR (H, DMSO) spectrometric propertities were consistant with the data reported in the literature.

1.5 Site-specific DNA platination and purification

To prepare the diaqua platinum complex derivatives, we mixed 1.97 equiv. of $AgNO_3$ with cis-$[Pt(NH_3)_2Cl_2]$, $[PtCl_2$(1(R),2(R)-DACH)], $[PtCl_2$(1(S),2(S)-DACH)] and $[PtCl_2$(cis-1,4-DACH)] in water at 37 °C in the dark. After 5 h, the reaction mixture was filtered to remove the AgCl. The filtrate was incubated at 37 °C for an additional 2 h and was filtered again, and stored at -20 °C in the dark. The activated cisplatin solution was mixed with deoxyoligonucleotides in 10 mM HEPES (pH 6.0) at 37 °C in the dark for 8 h. The platinated oligonucleotides were purified using 30% 8M urea denature PAGE. Platinated DNA band was sliced from gel. DNA extraction from gel was performed with 20mM TE buffer under mild condition overnight. All of the Pt-DNA adducts were fully characterised by MALDI-TOF.

1.6 Oligonucleotide-peptide conjugation

Oligonucleotides (ODNs) with a 5'-or 3' aminolinker were synthesised by Invitrogen and supplied in lyophilised powder. We dissolved this lyophilised powder in ultrapure water and determined its concentration. Approximately 280 μM ODNs was allowed to react with 20 equiv. GMBS dissolved in acetonitrile (usually 50 mM) in PBS (pH=7.4) at 30 °C for 40 min. The mixture was desalted using Sephadex G-25 (GE Healthcare) to remove excess GMBS. The fraction was lyophilised and dissolved in ultrapure water. The final conjugation step was carried out with 80 μM modified ODNs in PBS (pH=7.0). Next, 0.5 equiv. peptide was mixed with ODNs every 15 min at 30 °C; this step was repeated 6-7 times. After buffer exchange (20mM NH_4Ac) through G-25, the excess peptide was removed. We used a syringe to load samples on a Histrap HP affinity column (GE Healthcare). The column was washed with 90 mM imidazole and then eluted with 500 mM imidazole. The fraction (peptide-oligo conjugate, POC) was purified using a G25 column to remove imidazole, and the fraction was then lyophilised.

1.7 Annealing of double strand DNA probe

Platinated ODNs were hybridised with 1 equiv. POC in an annealing buffer including 20 mM Tris (pH 8.0), 100 mM NaCl, 50 mM KCl and 5 mM $MgCl_2$. Double-strand ODN-annealing was conducted by first heating the mixture to 95 °C and then cooling slowly to 4°C (approximately 2 h). In the required control experiments, double-strand ODNs without platinum compound modifications were prepared in the same way.

1.8 Thermal stability assay

The probes were divided into 10 μL aliquots and mixed with 1:1000 diluted SYBR green I dye solution, making a final composite of this solution was 20 μM Tris (pH 7.4), 50 mM NaCl, 5 mM $MgCl_2$. The Experiments were performed on CFX96 Touch™ Real-Time PCR Detection System, with the temperature increasing from 30 °C to 95 °C at step of 0.5 °C per minute. The fluorescent signal was recorded with the increasing temperature. The derivatives (-d(RFU)/dT) of the curve gave the dsDNA melting temperature.

1.9 Affinity isolation of proteins

For large scale pull down, approximately 50 μl Ni-NTA Sepharose beads were incubated with 50-100 μg dsODNs (with or without incorporated cisplatin) at 4 °C for 30 min in binding buffer (20 mM Tris (pH 7.4), 150 mM NaCl). This incubation allowed the dsODNs to conjugate to the sepharose beads. Freshly prepared whole cell extracts (4-10 mg protein, 80 mM imidazole, pH 7.4) were added, and this mixture was rotated gently at 4 °C for 4 h. The supernatant was removed by centrifuging at 5000 rpm for 1 min. The beads were washed at least 5 times with PBS containing 80 mM imidazole. After that, SDS (200 μl, 2%) was added to the beads, followed by heating to 95 °C, as much supernatant was collected as possible.

For pull down assay, 20μl beads are used loading probe about 7.2μg, this conjugate was incubated with cell lysate at a concentration of 10mg/ml(200μl).

1.10 Sample preparation for 2-dimensional electrophoresis

Proteins isolated from cell extract in 2% SDS were precipitated to remove surfactant contaminants with a 2-DE protein clean up kit (GE Healthcare). The protein pellet was air-dried and dissolved in 2-DE rehydration buffer (6 M urea, 2 M thiourea, 2% CHAPS and approximately 0.1% bromophenol blue). Appropriate DTT and IPG buffer (GE Healthcare) concentrations were added to the solution immediately before dry strip rehydration.

1.11 2-Dimensional electrophoresis

DTT (18 mM for IPG3-10, 50 mM for IPG4-7) and IPG buffer (IPG4-7 or 3-10) were added to the sample solution at appropriate concentrations. The IPG dry strip (7 cm) for the first dimension isoelectric focusing (IEF) was passively rehydrated in sample solution for at least 10 h. The rehydrated strip was then isoelectric focused using the following protocol: 2 h at 100 V, 1 h at 500 V, 1 h gradient increase to 1000 V, 1 h

gradient to 4000 V, and a hold at 4000 V for a total of 16000 Vhr. Then, the IPG strips were reduced using a 1% DTT solution and alkylated using 2.5% iodoacetamide for 15 min. The SDS-PAGE was performed on a 12% SDS resolving gel and stained with Coomassie Brilliant Blue R250. The gels were scanned with a gel scanner (GE Healthcare)

1.12 Peptide mass fingerprinting

The gel spots were removed from the gel using a MiniTip. The proteins were digested with trypsin in gel. The peptides were extracted from the gel pieces first by 0.1% formic acid, followed by extraction buffer (60% acetonitrile, 35% H_2O, 5% formic acid); sonication was used twice in this process. All of the solutions that were generated in the peptide extraction were combined and concentrated for protein identification. Peptide fingerprint mapping was performed on an Bruker AutoFlex II MALDI-TOF/TOF instrument. The peptide mass lists were searched against the UniProt human protein database with Mascot distiller.

1.13 Western blotting

Proteins that were resolved by 2-DE and SDS-PAGE were transferred to PVDF (Millipore, 0.22 μm) in Towbin buffer containing 0.033% SDS. PVDF membranes were blocked for 1 h at ambient temperature in blocking buffer (5% skim milk/0.1% Tween-20/PBS). The primary antibodies in appropriate dilutions were incubated with the membranes at 4°C overnight. The blots were washed with PBST (0.1% Tween-20/PBS) and incubated with peroxidase-conjugated secondary antibody in washing buffer for 1 h. After washing with PBST, the blots were visualised by enhanced chemiluminescence kit from Millipore.

2. Results

2.1 Compounds characterization

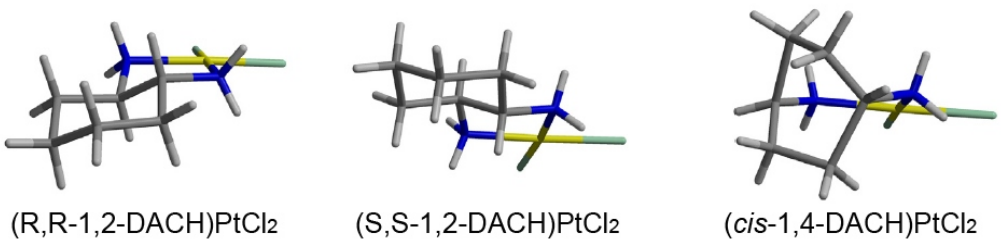

Figure S1. The spatial geometry of the cyclohexyl moiety of P2, P3 and P4

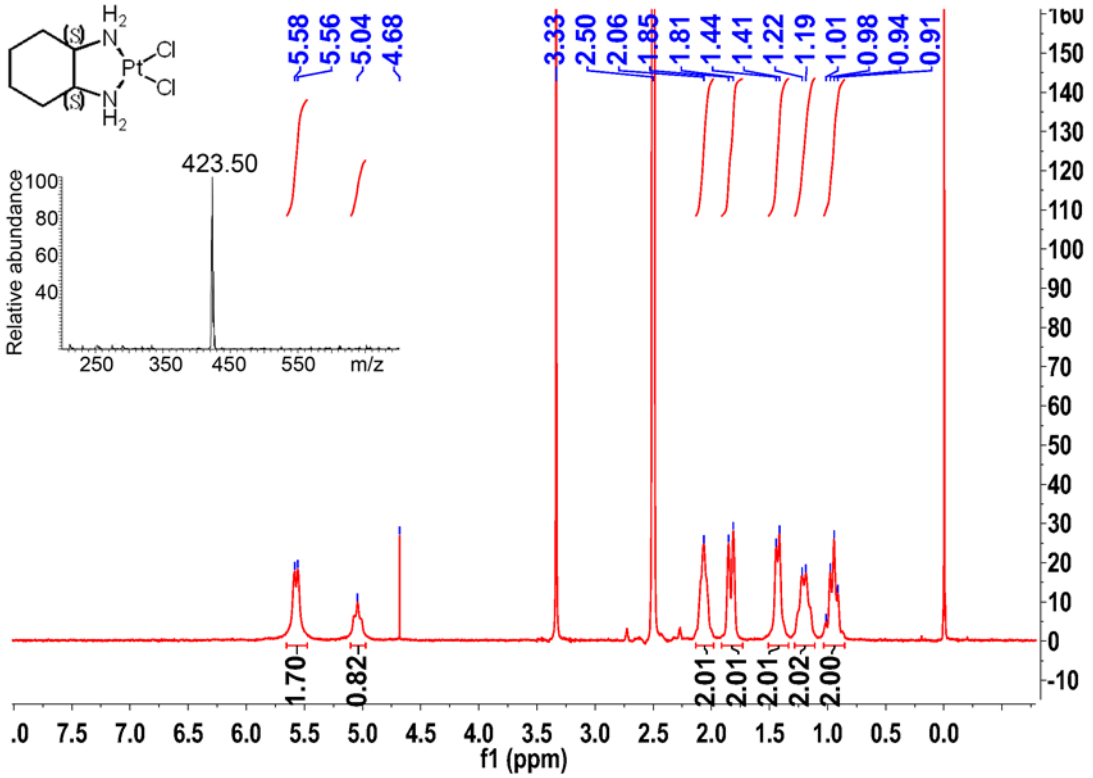

Figure S2. H-NMR spectrum and ESI-MS mass spectrum of (S,S-1,2-DACH)$PtCl_2$

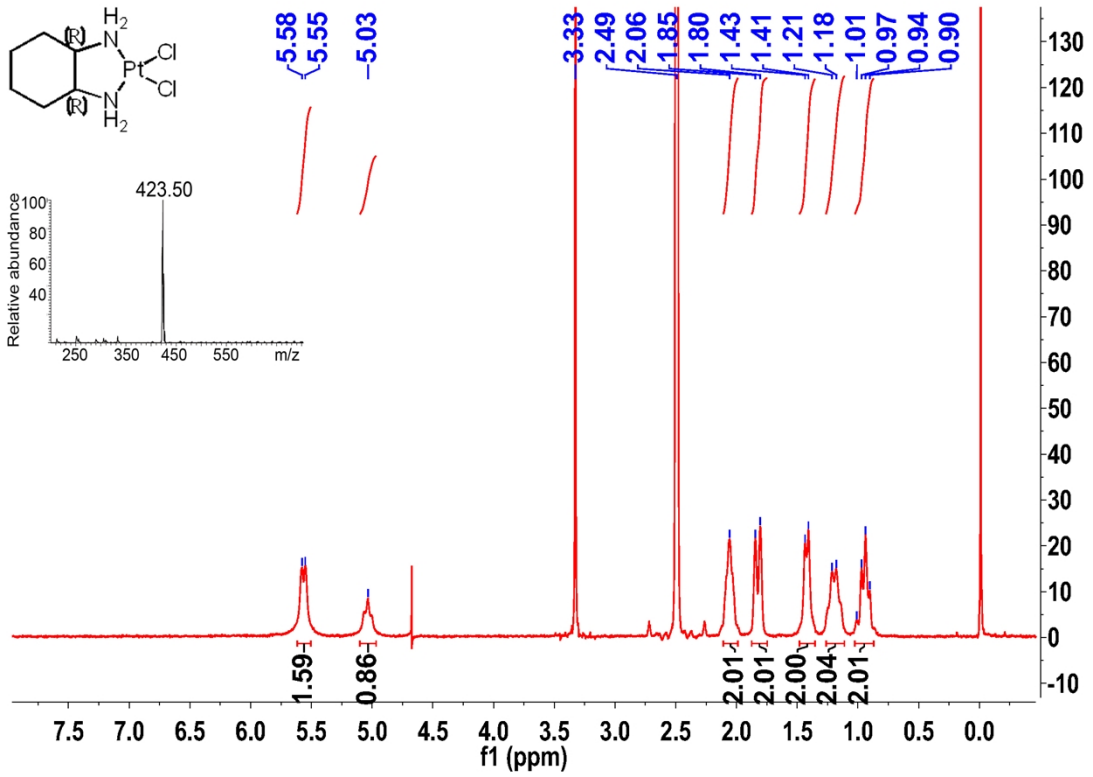

Figure S3. H-NMR spectrum and ESI-MS mass spectrum of (R,R-1,2-DACH)$PtCl_2$

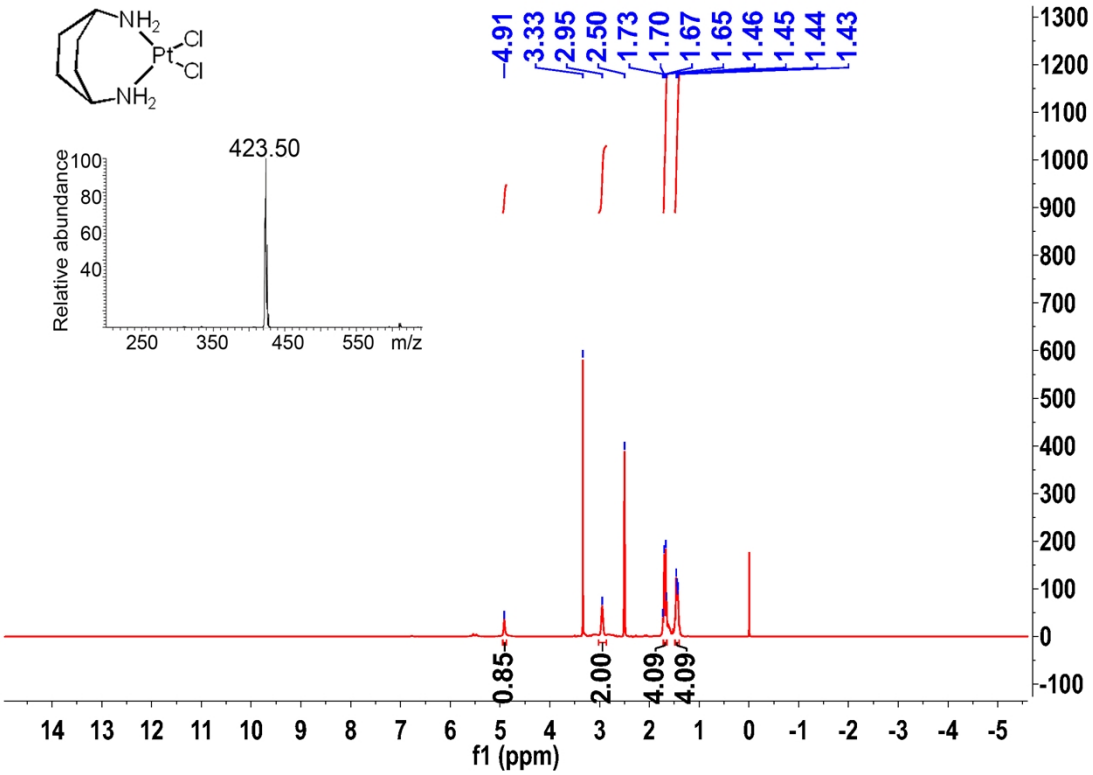

Figure S4. H-NMR spectrum and ESI-MS mass spectrum of (*cis*-1,4-DACH)$PtCl_2$

2.2 Probe characterization

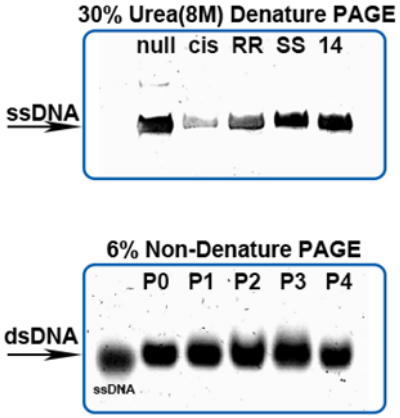

Figure S5. Denature PAGE of ssDNA (Pt-DNA adducts) and non-denature PAGE of dsDNA (Probes).

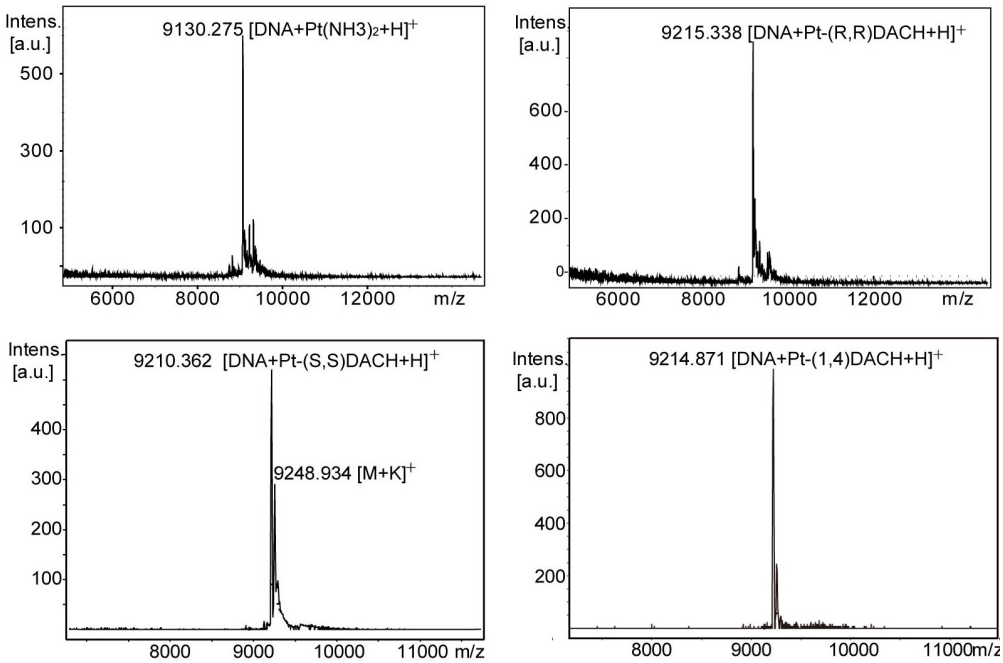

Figure S6. MALDI-TOF mass spectrum of DNA adducts with cisplatin, oxaliplatin and its enantiomer [$PtCl_2$(S,S-DACH)], and [$PtCl_2$(cis-1,4-DACH)].

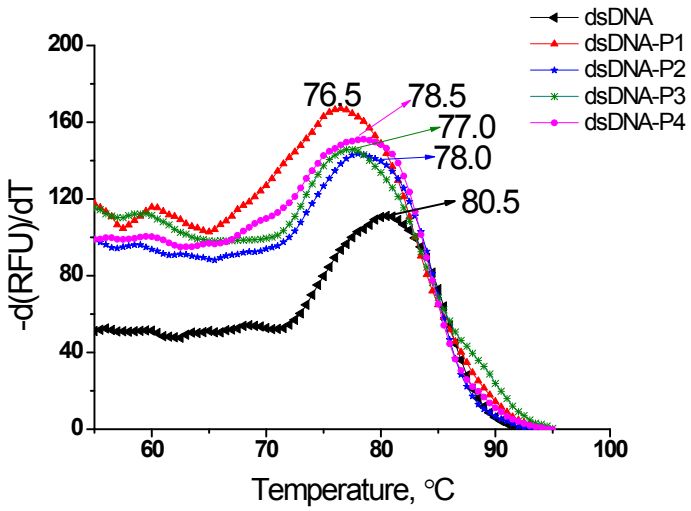

Figure S7. Thermal stability of dsDNA probes P1-P4. Melting temperatures are marked with colored arrows.

2.3 Two dimensional electrophoresis gel image and protein identification

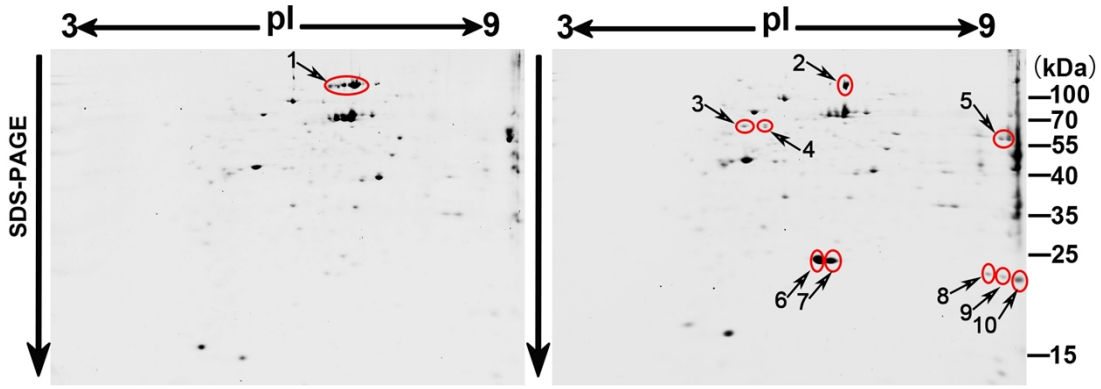

Figure S8. Native isolation of binding proteins in SW480 cell extract using DNA probe P1 with (B) or without (A) cisplatin crosslink. Proteins were separated on strips with pH range 3-9. Differential spots which were remarkably enriched on gel (B) were marked in red circles.

Table S1. Proteins identified by probe P1 from Cell Extraction under native condition.

No.	Protein ID	Nominal mass(Mr)	Caculated pI	Mascot Score
1	Mixture : XRCC6/XRCC5 or Ku70/Ku80	70084/83222	6.23/5.55	171/94
2	Mixture : XRCC6/XRCC5 or Ku70/Ku80	70084/83222	6.23/5.55	277/90
3	SF3A3	59154	5.27	78
4	Mixture : TCPE/TCPQ	60089/60153	5.45/5.42	102/99
5	GLYM	56414	8.76	307
6	HMGB1	25049	5.62	67
7	HMGB1	25049	5.62	88
8	HMGB2	24190	7.62	66
9	HMGB2	24190	7.62	131
10	HMGB2	24190	7.62	169

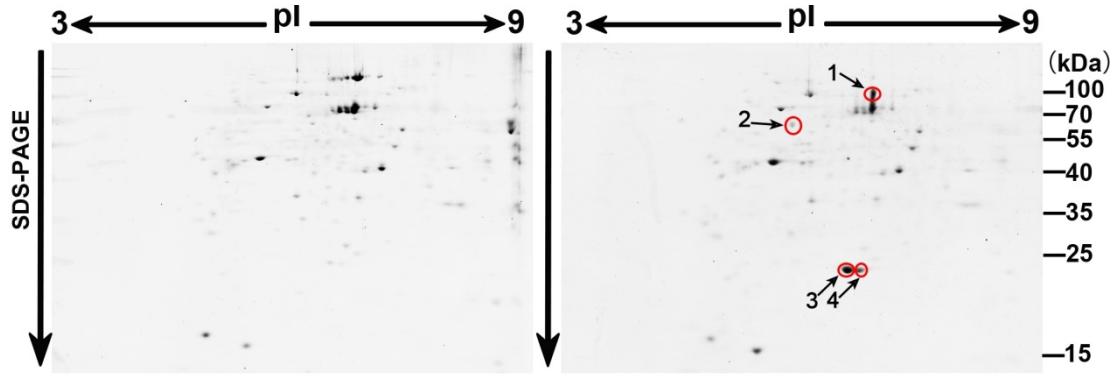

Figure S9. Native isolation of binding proteins in SW480 cell extract using DNA probe P2 with (right) or without (left) platinum compound crosslink. Proteins were separated on strips with pH range 3-9. Differential spots which were remarkably enriched on gel (right) were marked in red circles.

Table S2. Proteins identified by probe P2 from Cell Extraction under native condition.

No.	Protein ID	Nominal mass(Mr)	Caculated pI	Mascot Score
1	XRCC6 or Ku70	70084	6.23	157
2	TCPE/TCPQ	60089/60153	5.45/5.42	102/99
3	HMGB1	25049	5.62	70
4	HMGB1	25049	5.62	97

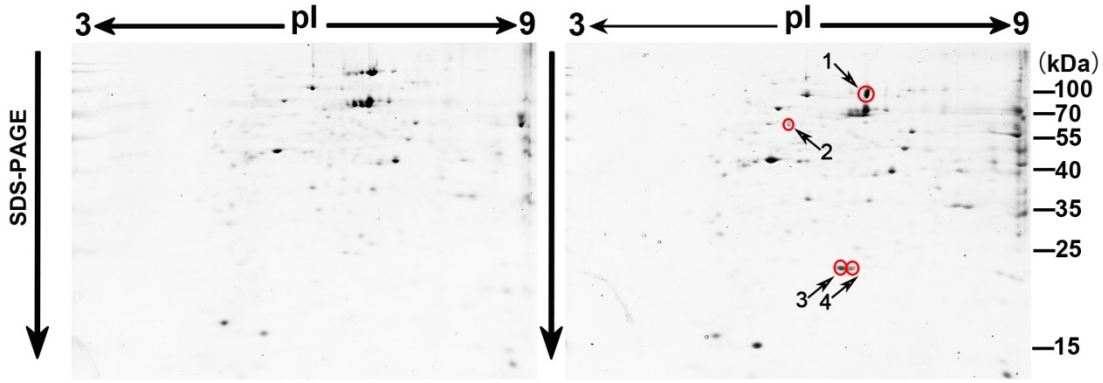

Figure S10. Native isolation of binding proteins in SW480 cell extract using DNA probe P3 with (right) or without (left) platinum compound crosslink. Proteins were separated on strips with pH range 3-9. Differential spots which were remarkably enriched on gel (right) were marked in red circles.

Table S3. Proteins identified by probe P3 from Cell Extraction under native condition.

No.	Protein ID	Nominal mass(Mr)	Caculated pI	Mascot Score
1	Mixture : XRCC6/XRCC5 or Ku70/Ku80	70084/83222	6.23/5.55	209/90
2	TCPE/TCPQ	60089/60153	5.45/5.42	148/175
3	HMGB1	25049	5.62	96
4	HMGB1	25049	5.62	107

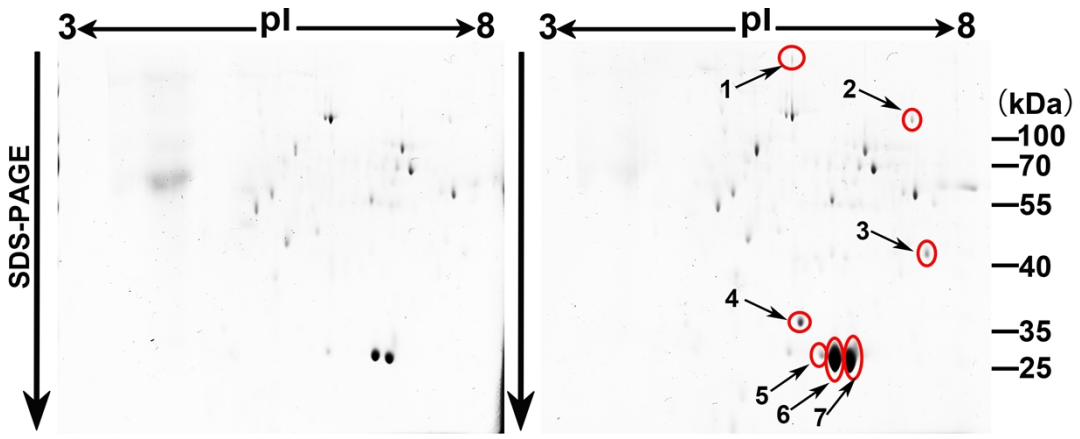

Figure S11. Native isolation of binding proteins in SW480 cell extract using DNA probe P4 with(right) or without (left) platinum compound crosslink. Proteins were separated on strips with pH range 3-9. Differential spots which were remarkably enriched on gel (right) were marked in red circles.

Table S4. Proteins identified by probe P4 from Cell Extraction under native condition.

No.	Protein ID	Nominal mass(Mr)	Caculated pI	Mascot Score
1	ATP-dependent RNA helicase, DDX1	83349	6.81	135
2	Heat shock cognate 71 kDa protein	71082	5.37	95
3	Heterogeneous nuclear ribonucleoprotein D0	38581	7.62	82
4	Replication protein A 32 kDa subunit, RFA2	29342	5.75	67
5	HMGB1	25049	5.62	78
6	HMGB1	25049	5.62	126
7	HMGB1	25049	5.62	156