Supplementary information for:

Toxicity in tumor cells, DNA binding mode and resistance to decomposition by sulfur nucleophiles of new dinuclear bifunctional transPt^{II} complexes containing long alkane linkers

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EXPERIMENTAL

Starting materials

Complexes 1,1/t,t(dien)-n (Fig. 1) were synthesized and characterized using standard synthetic methods. In brief, the two primary amines of diethylenetriamine were protected with t-butyloxycarbonyl and then reacted with the appropriate dibromoalkane to yield the protected ligands. The protecting groups were removed by treatment with HCl to yield the hydrochloride salts of the ligands. The ligands were characterized by ^{1}H and ^{13}C NMR and by ESIMS. The platinum complexes were prepared by reacting tetraiodoplatinate (obtained by reacting K_2PtCl_4 with 8 equivalents of KI in water) with the ligands in water in the presence of 6 equivalent of base. {[Pt(dien)I]$_2$-alkane}was isolated from the reaction and was converted to {[Pt(dien)Cl]$_2$-alkane} by reaction in the dark with 2 equivalents of $AgNO_3$ followed by treatment with HCl. The complexes were characterized by ^{1}H and ^{195}Pt NMR, ESIMS and elemental analysis. Complex 1,1/t,t-6 was synthesized and characterized as described previously [1]. [PtCl(dien)]Cl) was a generous gift of Prof. G. Natile from the University of Bari. The purity of platinum complexes was higher than 95% as established by combustion analysis carried out with a Hewlett-Packard 185 C, H, and N analyzer. The stock solutions of the platinum complexes were prepared in $NaClO_4$ (10 mM) and kept in the dark at 4°C. Calf thymus (CT) DNA (42 % G + C, mean molecular mass approximately 20,000 kDa) was prepared and characterized as described previously [2, 3]. Plasmid pSP73KB (2455 bp) was isolated according to standard procedure. Restriction endonucleases were purchased from New England Biolabs. EtBr was from Merck (Darmstadt, Germany). Agarose was from FMC BioProducts (Rockland, ME, USA). Radioactive products were from MP Biomedicals (Irvine, CA, USA). MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] was from Calbiochem (Darmstadt, Germany). RPMI 1640 medium, fetal bovine serum (FBS), trypsin/EDTA and DMEM medium were from PAA (Pasching, Austria). Gentamycin was from Serva (Heidelberg, Germany). Glutathione (GSH) was purchased from Sigma (Prague, Czech Republic).

Cell lines
Human ovarian carcinoma A2780 (cisplatin sensitive), A2780cisR (resistant to cisplatin) were obtained from Prof. Keppler from University of Vienna, Austria. The acquired resistance of A2780cisR cells was maintained by supplementing the medium with cisplatin (1 μM) every second passage. A2780 and A2780cisR cells were cultured in RPMI 1640 medium.

Cytotoxicity assay
The cells were seeded in 96-well tissue cultured plates at a density of 10 000 cells/well. After overnight incubation, the cells were treated with the compounds tested. After 72 h of incubation 10 μL of MTT (2.5 mg ml^{-1}) was added to each well and incubated for 4 h in culture conditions. At the end of the incubation period the medium was removed and the formazan product was dissolved in DMSO (100 μL). The cell viability was evaluated by measurement of the absorbance at 570 nm, using an Absorbance Reader SUNRISE TECAN SCHOELLER. IC_{50} values (compound concentration that produces 50% of cell death) were calculated from curves constructed by plotting cell survival (%) versus drug concentration (μM). All experiments were made in triplicate. The reading values were converted to the percentage of control (% cell survival).

DNA platination in cell free media
If not stated otherwise, CT or plasmid DNAs were incubated with platinum complexes in $NaClO_4$ (10 mM) at 37°C in the dark. After 24 h, the samples were exhaustively dialyzed first against 1 M NaCl and then against the medium required for subsequent biochemical or biophysical analysis. Aliquots of these samples were used to determine the value of r_b (r_b is defined as the number of molecules of the platinum complex bound per nucleotide residue) by FAAS.

Transcription mapping of DNA adducts
A double-stranded DNA template was prepared by digesting the pSP73KB plasmid (2455 bp) with *Nde*I restriction endonuclease. The resulting fragment was incubated with the platinum complex in $NaClO_4$ (10 mM) for 24 h at 37 °C in the dark. At the end of the incubation, the samples were precipitated by ethanol and dissolved in TE buffer [Tris-Cl (10 mM, pH 7.4) plus EDTA (1 mM)]. The level of platination (r_b) in an aliquots of these samples was checked by FAAS. In this way, the analyses of DNA transcription were performed in the absence of unbound (free) platinum complexes. Transcription of the linearized plasmid with DNA-dependent T7 RNA polymerase and electrophoretic analysis of transcripts were performed according to the protocols recommended by Promega (Promega Protocols and Applications, 43-46 (1989/90) and previously described in detail [4].

Interstrand (intramolecular) cross-linking assay
Platinum complexes were incubated for 24 h with 40 μg of pSP73KB DNA after it had been linearized by *Eco*RI and 5‘- end labeled with T4 polynucleotide kinase with [γ-^{32}P]ATP. The number of interstrand CLs was analyzed by electrophoresis under denaturing conditions on alkaline agarose gel (1%). After the electrophoresis had been completed, the intensities of the bands corresponding to single strands of DNA and interstrand cross-linked duplex were quantified. The frequency of interstrand CLs, was calculated as %ICL/Pt = XL/4910 x r_b (pSP73KB plasmid contained 4910 nucleotide residues), where %ICL/Pt is the number of interstrand CLs per adduct multiplied by 100 and XL is the number of interstrand CLs per molecule of the linearized DNA duplex and was calculated assuming a Poisson distribution of

the interstrand CLs as XL = -ln A, where A is the fraction of molecules running as a band corresponding to the non-cross-linked DNA.

Ethidium bromide fluorescence

Fluorescence measurements of CT DNA modified by platinum complexes in the presence of EtBr were performed at an excitation wavelength of 546 nm, and the emitted fluorescence was analyzed at 590 nm. The fluorescence intensity was measured at 25°C in NaCl (0.4 M) to avoid secondary binding of EtBr to DNA [5, 6]. The concentrations were 0.01 mg mL^{-1} for DNA and 0.04 mg mL^{-1} for EtBr, which corresponded to the saturation of all sites of EtBr in DNA [6].

Circular dichroism spectroscopy

Isothermal circular dichroism (CD) spectra of CT DNA were recorded using a Jasco J-720 spectropolarimeter equipped with a thermoelectrically controlled cell holder. The cell path length was 10 mm. CD spectra were collected using a Jasco J-720 spectropolarimeter. CD spectra of CT DNA at a concentration of 0.032 mg mL^{-1} ($1x10^{-4}$ M in nucleotides) modified by platinum complexes at were recorded at 25 °C in $NaClO_4$ (10 mM) with Tris-HCl (10 mM, pH 7.0) in the range of 220-325 nm.

DNA melting

The melting curves of CT DNAs were recorded by measuring the absorbance at 260 nm. The melting curves were recorded in a medium containing $NaClO_4$ (0.1 M) with Tris-HCl (1 mM)/EDTA (0.1 mM, pH 7.4). The value of the melting temperature (t_m) was determined as the temperature corresponding to a maximum of the first derivative profile of the melting curves. The t_m values could be determined with an accuracy of ± 0.3°C.

Reactions with reduced form of GSH

Reactions of GSH with platinum complexes were investigated by monitoring UV absorption at 260 nm of solutions containing the platinum complex and GSH exactly as described in the previous work [7-9]; the absorbance at 260 nm reflects the presence of platinum-sulfur and disulfide bonds. The platinum compounds were mixed with GSH at 37 °C in the medium of $NaClO_4$ (100 mM) plus4,6mM NaCl, pH 6.0) in the dark in a nitrogen atmosphere. Reactions were initiated by mixing the platinum complex with the buffer followed by immediate addition of GSH. The kinetic data were fitted by non-linear regression (GraphPad Prism) to one-phase and two-phase exponential association. The decision as to which fit was more appropriate for each dependence was made by comparing the fits of the two equations by using an F-test (GrahPad Prism).

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